Physiological and Biochemical Responses of Three *Echinacea* Species to Salinity Stress

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ABSTRACT

To determine the level of salt tolerance of the medicinal plant *Echinacea*, the physiological and biochemical characteristics of *E. purpurea*, *E. pallida* and *E. angustifolia* exposed to different NaCl levels (0, 50, 75, and 100 mM) were evaluated under hydroponic culture. Dry weights of shoots and roots were not affected by salinity; however *E. purpurea* and *E. pallida* exhibited higher survival rate than *E. angustifolia*, which also showed high salt injury index and electrolyte leakage compared to the other two species. Gas exchange (photosynthetic rate, stomatal conductance, and transpiration rate) showed a decline with increasing salt concentrations in all species with a more pronounced reduction in *E. angustifolia*. *E. purpurea* was able to retain more Na^+ in the roots than the other two species showing its capacity to regulate Na^+ translocation to shoots (Na^+ exclusion). Moreover, the activities of two major antioxidant enzymes; superoxide dismutase (SOD) and ascorbate peroxidase (APX) were increased by salinity in *E purperea*, while the activities were decreased in *E. angustifolia*. The characteristic phytochemical profiles of caffeic acid derivatives (CADs) and alkamides/ketones were obtained for the three species, and quantitative changes were determined. Cichoric acid, the major CAD in *E. purpurea*, was increased with salinity up to 75 mM NaCl. A relative increase in alkamides and CADs was recorded in *E. angustifolia*, while in *E. pallida*, the level of echinacoside and major ketones (22 and 24) decreased, suggesting that the medicinal value of this species was compromised by salt stress. First evidence of salt-induced changes in alkamides and ketones in *Echinacea* was demonstrated in this study. Activity of phenylalanine ammonia-lyase (PAL), the major enzyme involved in the biosynthesis of CADs, was increased only in the roots of *E. purpurea*, further reflecting

the differences in salt tolerance between species. It can be concluded that *Echinacea* species exhibited a limited degree of salt tolerance; however, *E. purpurea* showed a higher tolerance than *E. pallida* and *E. angustifolia.* This tolerance was mainly attributed to the increase in Na^+ exclusion capacity, antioxidant activities and PAL activity.

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CHAPTER 1. INTRODUCTION

Medicinal plants are an important source of natural products that are widely used to treat a large number of ailments in ethnomedicine and phytotherapy (Hudaib et al., 2008). It has been suggested that two thirds of plants on earth bear medicinal values (Krishnaiah et al., 2011). In addition, about 25% of the commonly-used medicines are derived from compounds isolated from plants (Mukhtar et al., 2008), and according to the World Health Organization (WHO), three-quarters of the world population rely upon herbal medicines for their health care needs (WHO, 1991). *Echinacea* is an important medicinal plant consisting of nine species, all native to North America (McGregor, 1968; McKeown, 1999). Three species of *Echinacea* (*E. purpurea*, *E. pallida* and *E. angustifolia*) are of great biological and pharmaceutical significance as their roots and aerial parts are widely used as herbal medicines and dietary supplements (Barnes et al., 2005). Although the uses ascribed to *Echinacea* preparations are so diverse, most of these uses are confined to combating viral diseases and inflammation. The efficacy of *Echinacea* preparations is attributed to their secondary metabolites namely caffeic acid derivatives, alkamides/polyacetylenes, polysaccharides, glycoproteins and essential oils (Bauer, 1999). However, caffeic acid derivatives and alkamides are considered important for exerting most of the biological activities ascribed to *Echinacea* preparations (Bauer and Wagner, 1991, Barrett, 2003). From an economical point of view, *Echinacea* preparations are currently among the best-selling herbal medicines in North America and Europe and its cultivation has been introduced to other parts of the world including Australia, South America, North Africa, and China to fulfill the increasing market demand for the pharmaceutical purposes (Yu and Kaarlas, 2004). However, the

increasing interest in expanding the cultivation of *Echinacea* requires investigating the environmental conditions that may affect the productivity and yield of bioactive substances.

During their growth, plants including *Echinacea* are vulnerable to a wide range of environmental constraints such as drought, salinity, extreme temperature, and nutrient depletion, which cause a great loss of plants (Singla-Pareek et al., 2006). Therefore, studying the interaction between environmental factors and plants is essential for understanding the physiological and biochemical bases for their tolerance and for their successful establishment. Salinity is one of the major abiotic stresses affecting vast areas of the globe and reduces the growth and productivity of the majority of plants. It has been estimated that 20% to 50% of all irrigated lands are affected by salinity and the problem is increasing progressively, particularly in arid and semi-arid environments (Pitman and Läuchli, 2002). Moreover, using saline water has significantly increased due to the scarcity of water resources and increasing population. Therefore, dealing with this problem and adopting strategies that alleviate or reduce the effects of salinity on plants constitute an important approach for improving the agriculture industry and ensuring the sustainability of food supply. Generally, salinity problem stems from the occurrence of ions particularly Na^+, Cl^-, HCO_3^- and CO_3^{-2} at higher concentrations in the soil solution, which impose both osmotic and ionic stresses on plants (Manchanda and Garg, 2008). This leads to disruption in gas exchange, ionic homeostasis, water relations, and eventually to cessation of growth and death of plants (Parida and Das, 2005). As a consequence of the disruption in cell functions, high levels of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$)

are generated and cause oxidative damage to lipids, protein and nucleic acids in the absence of efficient protective mechanisms (Gill and Tuteja, 2010).

Secondary metabolites are stress-induced compounds that accumulate in plants under different biotic and abiotic stresses (Dixon and Paiva, 1995). Caffeic acid derivatives (CADs) in *Echinacea* represent a major group of phenolic compounds that are synthesized via the phenylpropanoid pathway, which yield a wide array of compounds with physiological and ecological significance (Weisshaar and Jenkins, 1998; Lopez-Martinez et al., 2011). For example, phenolic compounds in plants can be involved in the deposition of lignin and suberin in cell walls, play a role as antioxidant agents against reactive oxygen species (ROS), and participate in the formation of phytoalexins in response to pathogen and herbivore attacks. Recent studies have shown that plant exposure to abiotic stresses can also stimulate the accumulation of secondary compounds (Sgherri et al., 2008; Lopez-Berenguer et al., 2009; Oh et al., 2009 a).

Despite the intensive research on the therapeutic and pharmacological investigations of *Echinacea* species (McKeown, 2004), little is known about the effects of salinity on physiological characteristics and the chemical composition of the secondary metabolites in *Echinacea* species. Until recently, only a few studies (Gray et al., 2003; Zheng et al., 2006 b) had reported on the phytochemical changes in response to abiotic stresses in *Echinacea*, with none on salinity. In 2008, one study has been published on *Echinacea* (Montanari et al., 2008); however, the complete picture of the phytochemical changes was still missing as this study focused only on one species (*E. angustifolia*) and investigated only the caffeic acid derivatives (CADs). Therefore, there was a need to fill the gap by studying salinity-induced changes in two of the main phytochemical groups

(alkamides and ketones) and compare the differential response of the three commonly-used *Echinacea* species. These two groups of compounds are considered the main lipophilic fractions contributing to the pharmacological efficacy of *Echinacea* as evidenced by recent studies (Boonen et al., 2012; Zhang et al., 2012).

The objectives of my research were: 1) to compare the effect of NaCl on the physiological parameters (growth, gas exchange, ion accumulation, injury and survival) of the three *Echinacea* species; 2) to investigate the differential antioxidant responses of these *Echinacea* species after exposure to NaCl and determine whether they contribute to salt tolerance (chapter 3); 3) to profile the CADs and alkamides/ketones and determine the quantitative changes induced by salinity in the three species (chapter 4); and 4) to determine the activities of enzymes related to the metabolism of phenolic compounds in salt-stressed *Echinacea* and correlate their activity with the accumulation of CADs (chapter 5). Such information will improve our understanding of salinity tolerance characteristics/mechanisms and will be beneficial in selecting species better adapted to saline conditions.

CHAPTER 2. LITERATURE REVIEW

2.1. Introduction

Medicinal plants are important economical plants as they are a source of a wide array of plant natural products, such as phenolic compounds, flavonoids, alkaloids, essential oils, etc. (Ramawat et al., 2009). Beside their uses as active ingredients in medicinal preparations or the cosmetics industry, these natural products have ecological roles in plants such as in pollination, as allelopathic agents, in defense mechanisms against biotic stresses such as pathogen and herbivore attack and also against abiotic stresses such as UV radiation and drought (Weisshaar and Jenkins 1998; Guidi et al., 2005). Therefore, accumulation of these compounds is usually encountered under both biotic and abiotic stresses to fulfill these biological functions. *Echinacea* is one of the most important medicinal plants used to treat many ailments related to common colds in folk and contemporary medicine (Barnes et al., 2005). Its range of cultivation has been expanded outside native habitats (North America) to almost every continent to fulfill the increased demand on raw materials (Yu and Kaarlas, 2004). *Echinacea*, like any other plant, is prone to different environmental conditions such as drought, salinity, extreme temperatures, high radiation and pollutants in addition to biotic factors that can alter growth and productivity. Salinity in particular is considered a major environmental factor that affects each aspect of the plant from the molecular to the whole plant level (Parida and Das, 2005). Moreover, salinity is considered a global problem related to food security as vast areas (at least 20% of all irrigated lands) are being affected by salinity worldwide (Pitman and Läuchli, 2002). Therefore, it is of high importance to understand the effects

of salinity on plants and the possible mechanisms of tolerance, which will be introduced in this review.

The aims of this review are: 1) to describe the taxonomy and uses of *Echinacea*; 2) to summarize the chemistry of *Echinacea* as well as methods of extraction and analysis of bioactive compounds from the most-common species; 3) to summarize the different components of salinity (osmotic, ionic, and oxidative stress) and highlight the potential mechanisms of tolerance associated with each type of stress; 4) to present the effects of environmental stresses on the phytochemical content and composition of *Echinacea* and other medicinal plants; and 5) to address the phenylpropanoid-related enzymes and stress tolerance.

2.2. *Echinacea*

2.2.1. Taxonomy and description

All species of the genus *Echinacea*, a member of the sunflower family (Asteraceae) are native to the U.S. and Canada and their range extends from southern Alberta, Saskatchewan, and Manitoba to the Gulf of Mexico (McGregor 1968; Flagel et al., 2008). The genus consists of either nine species and four varieties based on morphological differences (McGregor 1968) or four species and eight varieties based on random amplified polymorphic DNA (RAPD) technique (Binns et al., 2002). In the latter taxonomic revision, *E. pallida* and *E. atrorubens* were considered varieties rather than distinct species. Except for *E. pallida*, which is a tetraploid, all *Echinacea* species are diploid (Flagel et al., 2008). Recently, an alternative taxonomic approach based on metabolic profiling of lipophilic constituents has been used for revising the taxonomy of

the genus *Echinacea*, and according to the distinct lipophilic fingerprint for each species, *E. pallida* was considered a diverse allopolyploid in favor of the McGregor classification (Wu et al., 2009).

Echinacea species are herbaceous perennials with either simple or branched stems emerged from basal rosettes. Plants grow to a height of 2 to 3 feet, and the stems bear thick hairy leaves, which are 3 to 8 inches long. The leaf shape may be ovate or lanceolate, pubescent or smooth depending on the species and developmental stage (McKeown, 1999, Gualandi, 2010). The flowers appear from July to October and their color varies from rich purple to pale purple, and some can be yellow (e.g. *E. paradoxa*). The flower heads consist of disk florets, which are surrounded by the infertile ray flowers that have 2 or 3 teeth at each end. Roots are either single taproot as in *E. pallida* and *E. angustifolia* or fibrous as in *E. purpurea* (McKeown, 1999). Accordingly, morphological characters of the roots, leaves, and flowers can differentiate between *Echinacea* species (McKeown, 1999). Nine species namely *E. purpurea, E. angustifolia, E. pallida, E. paradoxa*, *E. simulata*, *E. laevigata*, *E. sanguinea*, *E. tennesseensis*, and *E. atrorubens* have been recognized (McKeown, 1999). The first three species are the most-commonly used and widespread species (Perry et al., 2001); however the other *Echinacea* species contain also bioactive substances with medicinal properties (Binns et al., 2002). In addition to their medicinal activities, *E. purpurea* and *E. paradoxa* have ornamental values and are used as cut flowers (McKeown, 1999).

2.2.2. Therapeutic uses

Echinacea has been traditionally used in North America for treating colds, coughs, toothaches, snake bites, rabies and wound infections (Barnes et al., 2005, Onica and Cachita, 2009). From the ethnobotanical point of view, *Echinacea* preparations were used as an alterative, antiseptic, diaphoretic, depurative and digestive (Barnes et al., 2005). Today, *Echinacea* is among the most frequently-utilized herbal medicines used to increase immune function and to treat upper respiratory tract infections around the world (Letchamo et al., 2002; Ramasahayam et al., 2011) with annual sales exceeding 300 million dollars (Zheng et al., 2006). Commercial *Echinacea* products may be marketed as tinctures, tablets, teas, capsules, extracts, etc. Preparations can be used externally for treating wounds, eczema, burns, and herpes simplex or internally to treat upper respiratory tract infections at the onset of cold and flu symptoms (Barrett, 2003; Barnes et al., 2005). Further studies have demonstrated that *Echinacea* extracts have phototoxic antimicrobial activity against pathogenic fungi (Binns et al., 2000), and can inhibit the activities of different viruses, such as rhinoviruses and influenza viruses including H1N1, herpes simplex virus, and certain pathogenic respiratory bacteria (Cech et al., 2010; Hudson, 2010). Furthermore, alkamides and ketones in *Echinacea* extracts have been shown to exhibit anti-inflammatory properties through inhibiting the cyclooxygenase and 5-lipoxygenase activities (Hou et al., 2010; LaLone et al., 2010; Boonen et al., 2012), inhibiting several cytochrome P450 isoforms (Modarai et al., 2010) or inhibiting prostaglandin E_2 (PGE_2) production (LaLone et al., 2007). Hepatoprotective effects are also attributed to root extract of *E. purpurea* (Hou et al., 2011). The flowers of *E. purpurea* have been shown to contain compounds with the potential to increase insulin-stimulated glucose uptake in type 2 diabetes (Christensen et al., 2009) and exhibit good

antioxidant and antimutagenic activities (Tsai et al., 2012). In addition, acetylenic compounds isolated from *Echinacea* exhibited cytotoxic activities against several human cancer cell lines (Chicca et al., 2010). Although anti-inflammatory properties of *Echinacea* are mainly attributed to the lipophilic constituents (Hou et al., 2010; Zhang et al., 2012), alkamides along with cichoric acid also contribute to the immunomodulatory properties of *Echinacea* preparations (Toselli et al., 2010; Goey et al., 2011). It can be concluded that the immunomodulatory and anti-inflammatory activities along with other properties ascribed to *Echinacea* preparations are dependent on the species and the plant part used. In addition, these diverse medicinal uses reflect the phytochemical richness of *Echinacea* species.

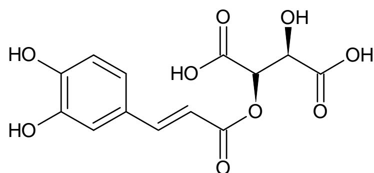
2.2.3. Phytochemistry of *Echinacea*

Several groups of compounds have been identified as putative active constituents in *Echinacea* species. These include caffeic acid derivatives, alkamides/ketones, polysaccharides, glycoproteins, and essential oils (Bauer and Wagner, 1991). It is not clear yet which group of compounds is responsible for the biological activity of *Echinacea*, but it is generally thought that the efficacy of *Echinacea* is attributed to the combined effect of various bioactive substances; however both caffeic acid derivatives and alkamides are responsible for most of the biological activities and pharmacological efficacy ascribed to *Echinacea* products (Hudaib et al., 2002; Zhai et al., 2009). Considerable variations in the content of CADs and alkamides have been found in *Echinacea* products, which may affect the physiological efficacy of *Echinacea* products (Wills and Stuart, 1999; Li et al., 2004). Therefore, standardization of *Echinacea*

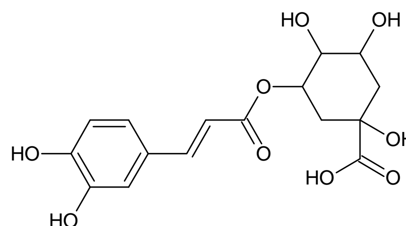
products according to their content from certain marker compounds is required to assure consistency before carrying out clinical trials (Goel et al., 2004). In this regard, cichoric acid and alkamides have been proposed as suitable marker compounds for the standardization and quality assurance of *E. purpurea* products (Bauer, 1999; Zolgharnein et al., 2010), while in *E. angustifolia*, echinacoside and cynarin are the two major phenolic compounds that are used for the standardization of commercial preparations (Wolkart, et al., 2004).

2.2.3.1. Caffeic acid derivatives (CADs)

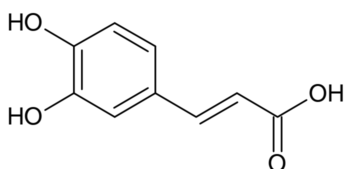
The major caffeic acid derivatives identified in *Echinacea* are caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside, cichoric acid, and caffeoyl echinacoside (Fig. 2.1). Tables 8.1-8.6 (Appendix F) depict their distribution in the three *Echinacea* species in the literature. Roots of *E. angustifolia* are characterized by cynarin, which is absent from *E. pallida* and *E. purpurea*, whereas cichoric acid is the predominant caffeic-acid compound in *E. purpurea* (Bone, 1997; Perry et al., 2001). A comprehensive study was carried out on the differential distribution of CADs in *Echinacea* species (Perry et al., 2001). While, chicoric acid was the major phenolic in roots and shoots of *E. purpurea*, echinacoside was the main phenolic in *E. angustifolia* and *E. pallida* roots. *E. angustifolia* roots were also characterized by the occurrence of cynarin. This indicates that *Echinacea* species have differential distribution of CADs in plant tissues.



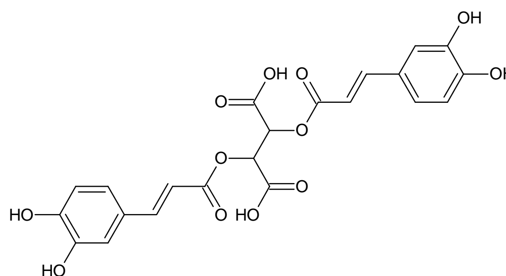
Caftaric acid (2-*O*-caffeoyl tartaric acid)



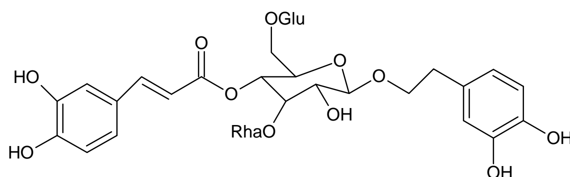
Chlorogenic acid (3-*O*-caffeoyl quinic acid)



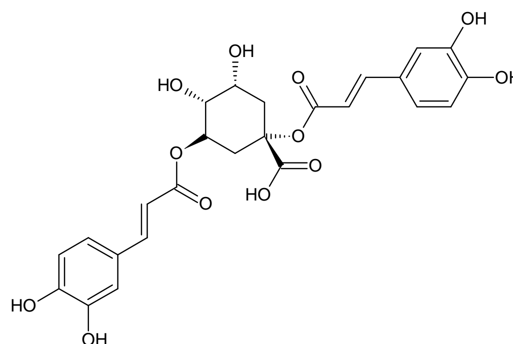
Caffeic acid (3, 4-dihydroxy-cinnamic acid)



Cichoric acid (2, 3-*O*-dicaffeoyl tartaric acid)



Echinacoside



Cynarin (1, 3-dicaffeoyl quinic acid)

Fig. 2.1. Structures of caffeic acid derivatives identified in the roots of *Echinacea* species. Adapted from Pellati et al. (2004) with permission from Elsevier Publishing.

2.2.3.2. Lipophilic compounds

The lipophilic compounds contain two groups, the alkamides and the polyacetylenes (Bone, 1997). They are widely distributed in the Compositae family members, especially in the roots (Harborne and Williams, 2004). Alkamides are amides of unsaturated fatty acids (i.e. undeca and dodecanoic acids) with olefinic and/or acetylenic bonds that determine the degree of their biological activities (Woelkart et al., 2005; Toselli et al., 2010). *E. purpurea* and *E. angustifolia* in particular contain significant amounts of alkamides, and the isomeric pair dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide (8/9) is the major type of alkamides in both species (He et al., 1998). However, the majority of alkamides present in *E. angustifolia* are monoene ones, while the alkamides found in *E. purpurea* contain the 2, 4 diene moiety. On the other hand, the lipophilic fraction of *E. pallida* is predominated by the occurrence of polyacetylenes, which are absent in the other *Echinacea* species. Therefore, it is possible to discriminate between *Echinacea* species based on their lipophilic chemical profiles (Wu et al., 2004).

About 20 alkamides were isolated and identified in *Echinacea* species more than 20 years ago (Bauer and Remiger, 1989; Bauer, 1999). Their chemical structure can be found in Fig 2.2**.** In 2005, Chen and collaborators have isolated and identified two new alkamides. The first alkamide was identified as dodeca-2*Z*, 4*E*,10*Z*-trien-8-ynoic acid isobutylamide isolated from the roots of *E. angustifolia,* and the second alkamide was identified as dodeca-2*Z*, 4*E*-diene-8,10-diynoic acid isobutylamide isolated from the roots of *E. purpurea* and *E. pallida* (Chen et al., 2005).

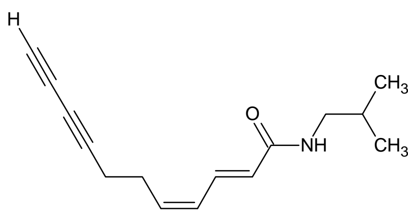
Although a lot of information is available regarding the pharmaceutical and medicinal uses of alkamides, little is known about their physiological and ecological significance in

plants. For example, certain alkamides; undeca-2E-ene-8, 10-diynoic acid isobutylamide and dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide were found to be responsible for the allelopathic activity in *E. angustifolia* root (Piechowski et al., 2006). Alkamides isolated from the roots of *Heliopsis longipes* have been found to influence the architecture of the root system and regulate cell division and differential processes in *Arabidopsis thaliana* (López-Bucio et al., 2006). Moreover, alkamides may be implemented in the response to biotic and abiotic stresses; however their role during stress is unknown, and need to be determined. The differential quantitative distribution among *Echinacea* species is presented in tables 8.7-8.13 (Appendix F).

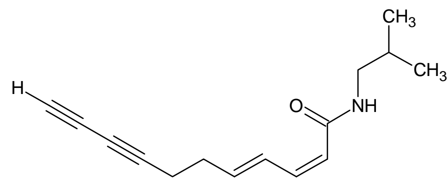
2.2.3.3. Other phytochemical constituents

2.2.3.3.1. Polysaccharides

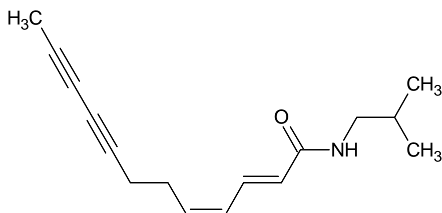
Unlike alkamides and CADs, the characterization of polysaccharides and glycoproteins is less studied, and information regarding their level and composition in *Echinacea* species is limited (Hall III, 2003). Generally, fructans, pectic polysaccharides and arabinogalactans are the three major categories of polysaccharides (Hall III, 2003). Both aerial parts and roots can be a significant source of polysaccharides in *Echinacea*. For example, two polysaccharides with molecular weight up to 50,000 Da have been isolated from the aerial parts of *E. purpurea*, and both demonstrated immunostimulatory properties (Harborne and Williams, 2004). On the other hand, arabinogalactans and arabinogalactan-containing proteins (AGPs) with immunomodulatory effects were isolated from the roots of *E. purpurea* (Classen et al., 2000). A recent study has characterized the polysaccharide fractions isolated from different parts



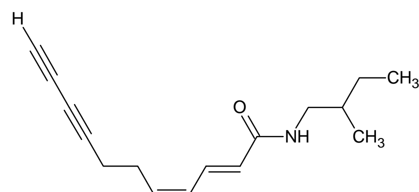
(1) Undeca-2*E*, 4*Z*-diene-8, 10-diynoic acid isobutylamide



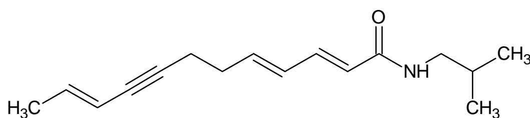
(2) Undeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide



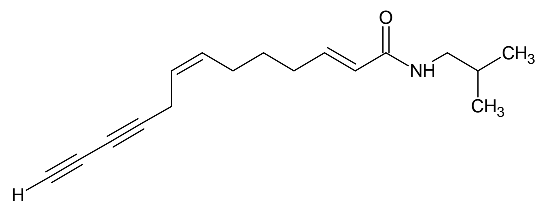
(3) Dodeca-2*E*, 4*Z* -diene-8, 10-diynoic acid isobutylamide



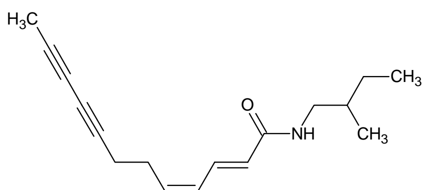
(4) Undeca-2*E*, 4*Z*-diene-8, 10-diynoic acid 2-methylbutylamide



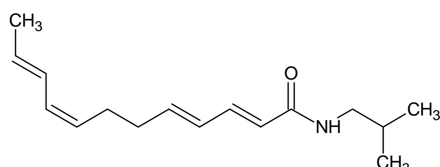
(5) Dodeca-2*E*, 4*E,* 10*E*-triene-8-ynoic acid isobutylamide



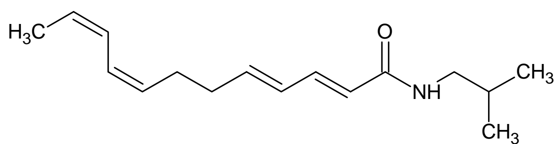
(6) Trideca-2*E*, 7*Z* -diene-10, 12-diynoic acid isobutylamide



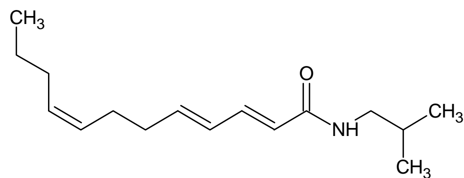
(7) Dodeca-2*E*, 4*Z*-diene-8, 10-diynoic acid 2-methylbutylamide



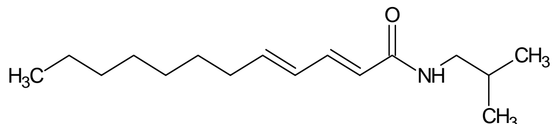
(8) Dodeca-2*E*, 4*E*, 8*Z*, 10*E*-tetraenoic acid isobutylamide



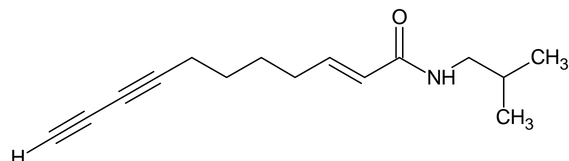
(9) Dodeca-2*E*, 4*E*, 8*Z*, 10*Z*-tetraenoic acid isobutylamide



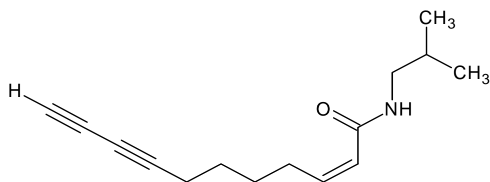
(10) Dodeca-2*E*, 4*E*, 8*Z*-trienoic acid isobutylamide



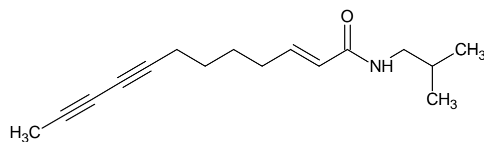
(11) Dodeca-2*E*, 4*E*-dienoic acid isobutylamide



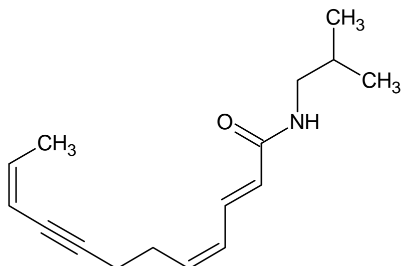
(12) Undeca-2*E*-ene-8, 10-diynoic acid isobutylamide



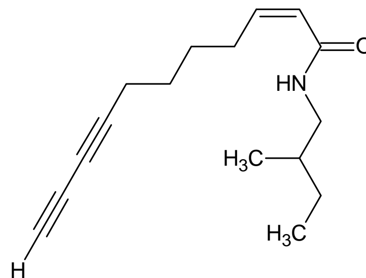
(13) Undeca-2*Z*-ene-8, 10-diynoic acid isobutylamide



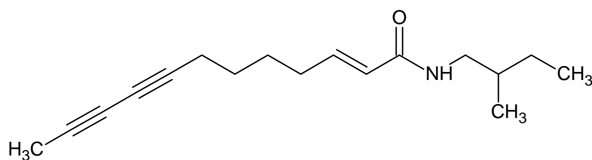
(14) Dodeca-2*E*-ene-8, 10-diynoic acid isobutylamide



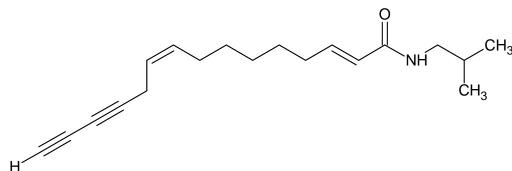
(15) Dodeca-2*E*, 4*Z*, 10Z-trien-8-ynoic acid isobutylamide



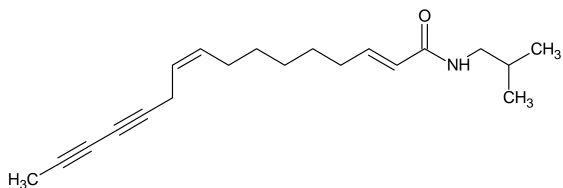
(16) Undeca-2*Z* -ene-8, 10-diynoic acid 2-methylbutylamide



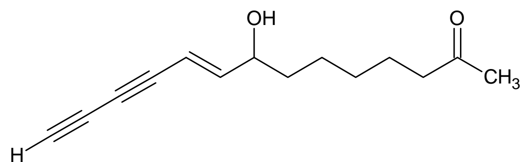
(17) Dodeca-2*E* -ene-8, 10-diynoic acid 2- methylbutylamide



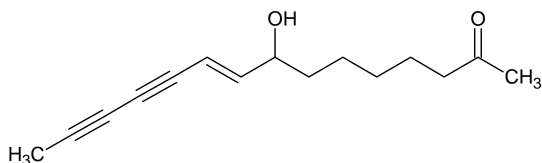
(18) Pentadeca-2*E*, 9*Z* -diene-12, 14-diynoic acid isobutylamide



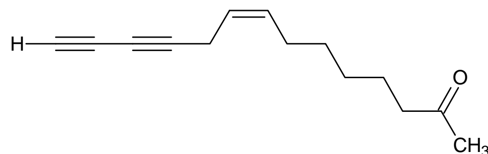
(19) Hexadeca-2*E*, 9*Z* -diene-12, 14-diynoic acid isobutylamide



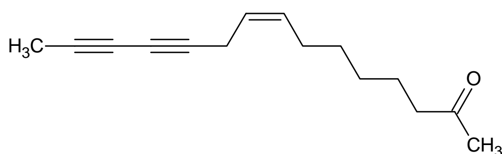
(20) 8-Hydroxytetradeca-9*E* -ene-11, 13-diyn-2-one



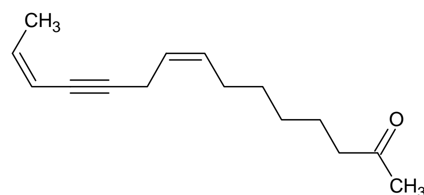
(21) 8-Hydroxypentadeca-9*E* -ene-11, 13-diyn-2-one



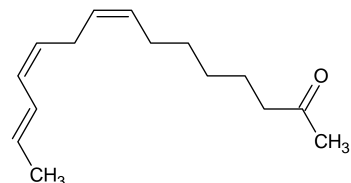
(22) Tetradeca-8*Z* -ene-11, 13-diyn-2-one



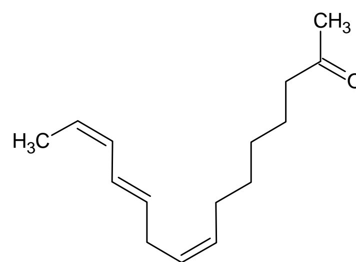
(23) Pentadeca-8*Z* -ene-11, 13-diyn-2-one



(24) Pentadeca-8*Z*, 13*Z* -dien-11-yn-2-one



(25 a) Pentadeca-8*Z*, 11*Z*, 13*E* -trien-2-one



(25 b) Pentadeca-8*Z*, 11*E*, 13*Z* -trien-2-one

Fig. 2.2. Alkamides and ketones identified in the roots of *Echinacea* species. Adapted from Binns et al. (2002). Copyright ©2002, American Chemical Society.

(stems, leaves, flower buds, flowerheads, and roots) of *E. purpurea* (Barsett et al., 2012). These polysaccharides comprised galacturonic acid, rhamnose, galactose, and arabinose with glycosidic linkages compatible with rhamnogalacturonan, arabinogalactan I and arabinogalactan II, and showed similar structures in different plant parts with minor differences in the arabinan component (Barsett et al., 2012). In addition to the polysaccharide-rich species (*E. purpurea*), *E. angustifolia* radix was found also to contain two polysaccharides; a low molecular weight 4500 Da polysaccharide that corresponds to inulin, and a high molecular weight (128 000 Da) that corresponds to a high metoxy pectin compound (Cozzolino et al., 2006).

2.2.3.3.2. Glycoproteins

Comprising a protein moiety and a sugar, glycoproteins with molecular weights of 17, 21 and 30 kDa were isolated from the roots of *E. purpurea* and *E. angustifolia* (Harborne and Williams, 2004). Aspartate, glycine, glutamine and alanine were the predominant amino acids, while arabinose, galactose, and glucosamine were the major sugars. Arabinogalactan-proteins (AGPs) were isolated from the high molecular weight fraction of the aqueous extract of *E. purpurea* roots. Amino acid composition and structure of the polysaccharide moiety was characterized (Bossy et al., 2009). Similarly in the roots of *E. pallida*, two major glycoproteins; arabinogalactan-protein (AGP) and an arabinan were isolated and their structures were elucidated by NMR spectroscopy (Thude and Classen, 2005).

2.2.3.3.3. Essential oils

Echinacea species differ in their essential oil content; while *E. pallida* roots had the highest essential oil content; the lowest one was obtained from *E. purpurea* (Letchamo et al., 2002). Among different organs, roots had the highest essential oil content in all species with terpenes and sesquiterpenes as the major constituents (Harborne and Williams, 2004). The differential distribution of the volatile compounds in different parts of the three commonly-used *Echinacea* species was studies more than 10 years ago (Mazza and Cottrell, 1999). Aldehydes were the principal volatile compounds in the root tissues, while terpenoids including α- and β-pinene, β-myrcene, ocimene, limonene, camphene, and terpinene were more abundant in flower tissues. Germacrene D was found to be the principal sesquiterpene hydrocarbon constituting (57.8%) in the aerial parts of *E. purpurea* (Hudaib et al., 2002). In a similar and more comprehensive study, germacrene-D was identified as the main compound in the essential oil of *E. purpurea*, *E. pallida* and *E. angustifolia* (Mirjalili et al., 2006). However, in another study, germacrene-D percentage was only 4.8% in flower heads of *E. purpurea* (Holla et al., 2005). Recently, 30 compounds were identified in *E. purpurea* challenged with CMV virus and phytoplasma, and significant increases were observed in three compounds (limonene, *cis*-verbenol and verbenone) due to infection (Pellati et al., 2011). These results suggest that not only species and organ, but also some abiotic and biotic factors contribute to the variability in essential oil content and composition in *Echinacea* species.

2.2.4. Analysis of *Echinacea* bioactive compounds

As most of the biological activities of *Echinacea* are ascribed to both hydrophilic and lipophilic compounds, the next section will focus on the extraction and analysis of these compounds (CADs, alkamides and ketones) in the three species.

2.2.4.1. Extraction

The bioactive constituents from the roots and aerial parts of *Echinacea* have been extracted using different techniques including maceration (Spelman et al., 2009), sonication (Luo et al., 2003, Li et al., 2004), solid-phase extraction (SPE) (Glowniak et al. 1996), supercritical fluid extraction (Sun et al 2002 ; Li et al., 2011), microwave-assisted solvent extraction (MASE) (Hudaib et al., 2003), and accelerated solvent extraction (ASE), which represents the most recent approach for extracting active constituents from *Echinacea* preparations (Hudson et al., 2005). Many advantages have been ascribed to ASE including high recovery percentage, time saving and less solvent consumption. Despite its high efficiency in extracting various active constituents from complex matrices, ASE has been used for the extraction of hydrophilic and lipophilic constituents from *Echinacea* species in quite few studies (Hudson et al., 2005). Recovery of the active ingredients from *Echinacea* material is influenced by different factors including extraction method, the type of solvent, its concentration, and composition and extraction temperature. For example, the highest recovery percentage of CADs obtained from freeze-dried flowers of *E. purpurea* was attained using 50% aqueous ethanol at an extraction temperature of 65°C (Tsai et al., 2012). A complete recovery of CADs from root samples of *Echinacea* was achieved by using methanol in the range of 60-80% (Li et al., 2004; Pellati et al., 2005). Similarly, ultrasonic extraction with methanol 70% was found to be the most efficient method for recovering cichoric acid, echinacoside and the

alkamides in the root samples of *Echinacea* (Bergeron et al., 2000). For recovering the lipophilic fractions, microwave-assisted solvent extraction (MASE) technique gave the highest recovery percentage compared to two conventional extraction methods; Soxhlet and ultrasonic extraction especially at 70% methanol (Hudaib et al., 2003). On the other side, using methanol 70% or ethanol 60% allowed for simultaneous extraction of phenolic compounds and alkamides in the same sample (Bergeron et al., 2000). Therefore, attention should be paid to the extraction procedure as different factors can affect the recovery percentage and the stability of bioactive compounds.

2.2.4.2. Analysis of hydrophilic and lipophilic compounds

2.2.4.2.1. Thin-layer chromatography (TLC)

Thin-layer chromatography analysis was able to discriminate between *Echinacea* species based on the differences in the lipophilic spectrum (Bauer and Remiger, 1989; Gilroy et al., 2003). The lipophilic fraction of *E. purpurea* and *E. angustifolia* roots was rich in alkamides, while *E. pallida* profile was dominated by ketones and almost void of alkamides (Bauer and Remiger, 1989); the same finding that was obtained in more advanced methods like HPLC and GC. Although different mobile phase systems have been used for the separation of lipophilic constituents from *Echinacea* radix, toluene/ethylacetate (70/30, v/v) mixture gave good separation and this method was claimed to be superior to the HPLC method due its high selectivity (Schicke et al., 2004). Differentiation between *Echinacea* species based on the TLC analysis of lipophilic constituents in the oil seeds was also achieved (Oomah et al., 2006). Although, mostly used for qualitative analysis, a validated TLC method was used for quantitative analysis

of cichoric acid in *E. purpurea* using image analysis, and this method was convenient, efficient, and moderately accurate for the quantitative analysis (Tie-xin and Hong, 2008).

2.2.4.2.2. High performance liquid chromatography (HPLC) and gas liquid chromatography-mass spectrometry (GC-MS)

HPLC is the most commonly-used method for the analysis of the two major classes of compounds, hydrophilic and lipophilic compounds separately or in combination in *Echinacea* species. Some studies focused only on the analysis of phenolic compounds in *Echinacea* species. For example, 12 caffeoyl conjugates were isolated from *E. pallida*, and their distribution in roots, leaves, and flowers was investigated by HPLC more than 20 years ago (Cheminat et al., 1988). More recently, major phenolic compounds, such as caftaric acid, chlorogenic acid, echinacoside, cynarin, and cichric acid were analysed using a validated HPLC method in the roots and aerial parts of three species of *Echinacea*, thus providing a significantly improved tool for ensuring the quality of *Echinacea*-derived botanicals (Brown et al., 2010). A new, rapid method utilizing liquid chromatography (LC-MS) has been developed and validated for the quantitation of cichoric acid. This method allowed for the separation of this marker compound in a fairly short time (3 min) as compared to up to 60 min in conventional HPLC methods (Chen, 2006). The distribution of CADs (caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside and cichoric acid) in nine *Echinacea* species were best studied using a fast and reliable HPLC method utilizing a short C_{18} column and optimum mobile phase gradient (Pellati et al., 2004, 2005). Not only qualitative differences between *Echinacea* species were identified but also quantitative differences were determined. Cichoric acid followed by caftaric acid were the two major CADs in root

extracts of *E. purpurea.*While *E. purpurea* showed the highest caffeic acid derivatives content (23.23 mg g^{-1}), *E. angustifolia* had the lowest content (10.49 mg g^{-1}) (Pellati et al., 2004). The variations in CAD contents among various commercial preparations of *E. purpurea* were detected by using RP-HPLC method with photodiode array detection and electrospray ionization mass spectrometry (Li et al., 2004).

Other studies focused only on separation and characterization of alkamides in *Echinacea* (Bauer and Remiger, 1989; Hudaib et al., 2002; LaLone et al., 2007; Wu et al., 2009). For example, HPLC coupled with electrospray mass spectrometry was used for the analysis of alkamides in roots and achenes of *E. purpurea* (He at al., 1998). About 12 alkamides were identified, but only the isomeric pair 8/9 was quantified in the root extract. The most comprehensive profile of alkamides (24 compounds) identified so far in *Echinacea* was obtained by ultrafast liquid chromatography (UFLC) coupled with diode array detection and electrospray ionization mass spectrometry (Mudge et al., 2011). In a similar study, a high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS) method was used for profiling the lipophilic compounds in *E. purpurea* root extracts, but in this study a less comprehensive profile (15 alkamides) was identified and quantified (Spelman et al., 2009). Liquid chromatography electrospray ionization ion-trap mass spectrometry was used as an efficient method for identification and quantification of alkamides not only in plant material, but also in human plasma after administration of *E. angustifolia* (Woelkart et al., 2005) and *E. purpurea* (Goey et al., 2011). In addition to alkamides, polyacetylenes and polyenes, which are the most abundant lipophilic constituents in *E. pallida* were characterized by using RP-HPLC method, and based on this analysis, the structure of three compounds, namely 8-hydroxy-

tetradec-(9*E*)-ene-11,13-diyn-2-one, tetradec-(8*Z*)-ene-11,13-diyn-2-one and pentadec-(8*Z*)-en-2-one, was described for the first time (Pellati et al., 2007). In a similar study, the structure elucidation of two polyacetylenes and three polyenes was described in *E. pallida* based on multiple analytical methods (Pellati et al., 2006).

Gas chromatography-mass spectrometry (GC-MS) was also an efficient method for identification and quantification of the lipophilic compounds in *Echinacea* extracted from roots of the three commonly-used *Echinacea* (*E. purpurea, E. pallida,* and *E. angustifolia*) and based on their lipophilic profiles, discrimination between the three species was possible (Lienert et al., 1998). Similarly, in a more recent study, gas chromatography/mass spectrometry and data mining allowed for classification between the three most commonly used *Echinacea* species based on their phytochemical profiles (Hou et al., 2010). Consistent with this, Mazza and Cottrell, (1999) found that the three major *Echinacea* species have differential distribution of their volatile components in the roots, stems, leaves, and flowers, which was determined by GC/MS analysis (Mazza and Cottrell, 1999). Volatile compounds extracted from *E. purpurea* were analysed by GC-MS, and the outcome was the identification of 17 compounds, 11 of which were alkamides (Hudaib et al., 2002). Gas chromatography-mass spectrometry (GC-MS) was also an efficient method for identification and quantification of ketones and unsaturated hydrocarbons in *Echinacea* (Li et al., 2011).

Simultaneous determination of CADs and alkamides in *Echinacea* extracts was reported to be advantageous over the separate analysis of each type of compounds as it reduces both the total time of separation and sample volume required for analysis

(Laasonen et al., 2002; Cech et al., 2006). HPLC or HPLC with electrospray ionization mass spectrometry are the most common methods for performing the simultaneous analysis of both hydrophilic and lipophilic compounds, in which phenolic constituents are resolved in the first 25 min followed by the lipophilic compounds (Gocan et al., 2003; Luo et al., 2003; Molgaard et al., 2003; Cech et al., 2006). However, all these studies focused on one of the major species, *E. purpurea*, and only two studies focused on comparing the phytochemical profiles in the three species in a single run (Sloley et al., 2001, Laasonen et al., 2002). Obtaining the distinctive phytochemical profile from both hydrophilic and lipophilic compounds in each *Echinacea* species is very important for proving the authenticity of each species. In addition, this may have implications related to quality assurance of *Echinacea* products, as biological activities of *Echinacea* extracts are attributed to a wide spectrum of active ingredients (Vimalanathan et al., 2009).

2.2.4.2.3. Other methods

A liquid chromatography-particle beam/mass spectrometry (LC-PB/MS) method with electron impact (EI) and glow discharge (GD) ionization sources was able to identify and quantify the major CADs including caftaric acid, chlorogenic acid, caffeic acid, cichoric acid in *Echinacea* (Castro et al., 2010). However, CADs were separated in a relatively long time (40 min) as compared to other methods. The major phenolic compound, cichoric acid, was analysed in *E. purpurea* tissues using a validated capillary electrophoresis, EC (Mancek and Kreft, 2005). Compared to HPLC method, EC is more environmentally-friendly and has lower operating costs. For lipophilic compounds analysis, a technique called high-speed countercurrent chromatography (HSCCC) was used for the isolation of alkamides from the roots of *E. angustifolia* and a new alkamide;

dodeca-2E, 4E-dienoic acid 2-methylbutylamide was reported for the first time using this method (Lopes-Lutz et al., 2011). As this method relies on the partition of a compound between two immiscible solvents without using a stationary phase, this eliminated the irreversible loss of samples and yielded alkamides with high purity (92-99%) compared to silica gel-based column chromatography (Lopes-Lutz et al., 2011). Alkamides in *Echinacea* species were also characterized using ^{1}H-NMR, and based on the metabolic fingerprint of each species, the distinction between the three pharmaceutical species; *Echinacea purpurea*, *E. pallida*, and *E. angustifolia* was possible (Frederich et al., 2010). The distinctive phytochemical profile of both alkamides and CADs in *E. purpurea* was obtained using a micellar electrokinetic chromatography (MEKC) method giving a complete separation in less than 10 min (Gotti et al., 2002), which is considered a significantly short time compared to the regular HPLC analysis time (50 min) reported for *Echinacea* (Sloley et al., 2001; Molgaard et al., 2003).

2.2.5. Factors affecting the content and composition of bioactive substances in *Echinacea*

There are many factors that influence the level of bioactive substances in *Echinacea* either before harvest or post harvest which may affect the quality and the pharmacological efficacy of *Echinacea*-derived preparations. These factors include genotype differences, age and developmental stage of plants, environmental conditions, seasonal variations, growing location of plants, and agricultural practices (Binns et al., 2002; Letchamo et al., 2002; Zheng et al., 2006). These factors show how complex the

production of secondary metabolites is, particularly when multiple factors interact together to exert their effects on the accumulation of these bioactive substances.

2.2.5.1. Species.

Each *Echinacea* species has its own chemical profile of the hydrophilic and lipophilic compounds (Bauer, 1991). Cichoric acid predominated in *E. purpurea* root extracts, whereas cynarin and dodeca-2E, 4E, 8Z, 10Z/E- tetraenoic acid isobutylamide were the major chemicals characteristic to *E. angustifolia* root extracts. Echinacoside and 6-*O*-caffeoylechinacoside predominated in the extract of *E. pallida* roots (Sloley et al., 2001). In another study (Pellati et al., 2005), where the differential chemical profile of caffeic acid derivatives was observed in at least five *Echinacea* species, the highest contents of caffeic acid derivatives were found in *E. paradoxa* and *E. purpurea*, followed by *E. angustifolia, E. simulata* and *E. pallida.* For the lipophilic fraction, different distribution of alkamides in *Echinacea* species was observed (Wu et al., 2004). Among the nine species studied, six species including *E. angustifolia, E. sanguinea,* and *E. simulata* showed abundant presence of alkamides, which confirm that the distribution of lipophilic and hydrophilic compounds in *Echinacea* is species-dependent. Furthermore, among the three medicinally-used species, *E. purpurea* and *E. angustifolia* contain significant amounts of alkamides, while the chemical profile of *E. pallida* does not show any alkamides (Binns et al., 2002, Wu et al., 2004). However, a few reports mentioned the occurrence of alkamides in *E. pallida* (Sloley et al., 2001; Binns et al., 2002). Differential distribution of phytochemicals among *Echinacea* species was observed not only in roots, but also in aerial parts. For example, in flower heads and leaves, *E.*

purpurea accumulated more cichoric acid and caftaric acid, while *E. atrorubens* var. *paradoxa* accumulated more echinacoside (Chen et al., 2009).

2.2.5.2. Plant organ

Large variation in the content of lipophilic and hydrophilic compounds has been found between plant organs. Letchamo et al. (2002) compared the relative distribution of cichoric acid and isobutylamides in different organs of *E. purpurea, E. angustifolia* and *E. pallida*. Lingulate florets showed the highest concentration of cichoric acid, while the endosperm and seed coat had none. In a similar study, the highest amount (18.9%) of cichoric acid in *E. purpurea* was obtained from the aerial parts including stems, leaves, and flowerheads, while the roots contained the lowest amount (8.95%). On the contrary, the roots contained the highest amount (14.4%) of total alkamides, while the young tops contained the least (0.77%) amount (Qu et al., 2005). Recently, the differential distribution of CADs was also demonstrated in *E. angustifolia* (Maggini et al., 2012). While, root tissues contained high levels of echinacoside, cynarin and chlorogenic acid, leaves were void of these metabolites. Meantime, inflorescences (stem and capitulum) contained lower amounts of caftaric acid and echinacoside than roots (Maggini et al., 2012). Both roots and flowerheads of *E. purpurea* are rich in CADs and alkamides and can be used medicinally, while only roots are considered the optimal pharmaceutical part in *E. pallida* and *E. angustifolia.* However, distribution differs between fine and older roots; while the root crown had higher cichoric acid content than the fine or thick roots in *E. purpurea* (Callan et al., 2005), in *E. angustifolia*, the bark and secondary roots contained the highest levels of CADs and isobutylamides (Kabganian et al., 2003).

2.2.5.3. Age and phenological stage

Seemannova and collaborators (2006) studied the effect of plant age on the biomass and phytochemical content of *E. purpurea*. Alkamide content accumulated progressively in the roots of *Echinacea* plants with maturity, reaching a maximum content at the fruiting stage. Similarly, alkamides 8/9 were increased with time over a period of 4 years in field-grown *Echinacea* (Romero et al., 2010). While, Gray et al. (2003) found that age is the predominant factor in determining the phytochemical content of *E. purpurea*, Kreft (2005) indicated that age had no significant impact on the contents of caftaric acid and cichoric acid in *E. purpurea* grown in Slovenia. In addition to age, the quality of *E. purpurea* raw material is affected by the developmental stage and harvesting time. For example, Wang et al. (2002) found that the maximum cichoric acid content in the above ground parts of *E. purpurea* was in the blooming stage of mid-July, while in the roots, the maximum cichoric acid content of *E. purpurea* was obtained in the seedling stage. Consistent with these results, the highest cichoric acid concentration in *E. purpurea* was determined in immature flowers (Callan et al., 2005). In addition to the variation in cichoric acid, other less abundant CADs, such as caftaric acid, chlorogenic acid, and caffeic acid show quantitative changes at different developmental stages, and tend to accumulate at the highest levels in the middle stage of full blossoming (Liu et al., 2007). Field-grown *E. purpurea* plants grown in Taiwan in autumn produced more caffeic acid derivatives, particularly caftaric acid and cichoric acid, in the leaves and flowers than plants grown in spring (Chen et al., 2008), whereas, Perry at al. (2001) found that cichoric acid content in *E. purpurea* roots and tops grown in New Zealand was higher in summer than autumn. This contradictory response could be due to the

differences in agricultural practices or environmental conditions in both studies. In addition to CADs, alkamides were also found to be influenced by developmental stage. For example, alkamides were more abundant in the mature flower developmental stage (Mistrikova and Vaverkova, 2009). The ratio between individual alkamides was also found to be affected by the developmental stage. For example, the ratio of amide 3 to its isomer amide 5 was 1:9 in 3-month-old plants; while it was about 2:3 in 6-month-old plants indicating that isomeration of alkamides may be regulated by developmental stage (Wu et al., 2004).

2.2.5.4. Exposure to biotic and abiotic stresses

Environmental factors, such as drought, salinity, nutrient depletion, geographical location may also affect the content and composition of phytochemicals in *Echinacea* (Letchamo et al., 2002); therefore, impacting the quality or therapeutic efficacy of *Echinacea*-derived products. The majority of the studies conducted were mainly on *E. purpurea* with few studies on the other two medicinally-used species (*E. pallida* and *E. angustifolia*). *E. purpurea* plants subjected to a brief drought stress produced roots with significantly greater cichoric acid concentration than well-watered plants (Gray et al., 2003). However, total alkamides and chlorogenic acid contents were not affected. This may suggest a role of cichoric acid under drought stress, most likely by enhancing the scavenging capacity against reactive oxygen species (ROS) formation. On the other hand, *E. purpurea* and *E. angustifolia* grown in a hydroponic culture showed no changes in the phytochemicals as a result of exposure to periodic water stress (Zheng et al., 2006 b), which indicates that response of plants grown hydroponically may differ from plants

grown in the field. Furthermore, accumulation of bioactive substances in *E. purpurea* was found to be largely influenced by growing location (Letchamo et al., 2002; Kreft, 2005). Different *E. purpurea* root samples harvested from different locations in Australia contained different ranges of cichoric acid and alkamides (Wills and Stuart, 1999).

The first study to investigate the response of *E. purpurea* to biotic stress was conducted 10 years ago (Hudaib et al., 2002 b). When plants were challenged with the cucumber mosaic virus (CMV), this led to increased accumulation of the alkamides in the roots (Hudaib et al., 2002 b). Moreover, CMV infection caused significant changes in the content and composition of the essential oil extracted from the aerial parts of *E. purpurea* (Hudaib et al., 2002 a). More recently, CADs, alkamides, and essential oils were studied in *E. purpurea* in response to virus and phytoplasma infections (Pellati et al., 2011). Both pathogens significantly reduced the content of cichoric acid, while the major lipophilic compound (alkamide 8/9) was only decreased in virus-infected plants. Moreover, the relative proportion of the isomers 8/9 changed in infected plants. From these results, it can be concluded that exposure of *Echinacea* to abiotic and biotic stresses may affect the accumulation of secondary metabolites. However, how the accumulation of secondary metabolites is beneficial to the plants under stress conditions is not fully known. There is a need for more studies that dissect the role of these compounds in salt-stressed plants.

2.2.5.5. Agricultural practices and method of cultivation

Some agricultural practices, such as plant density, fertilization, application of stress factors (elicitors), and mycorrhizal colonization influence the phytochemical content, particularly CADs, in *Echinacea*. For example, the accumulation of phenolic

compounds including cichoric acid, caftaric acid, and chlorogenic acid was largely increased in the roots and shoots of *E. purpurea* in response to foliar application of plant stress mediators (salicylic acid and its derivatives) and metal elicitor (titanium (IV) ascorbate) (Kuzel et al., 2009) as well as root colonization with arbuscular mycorrhizal (AM) fungus (Araim et al., 2009). Cultivation of *E. purpurea* at very dense populations (over 15 plants m^{-2}) under field conditions reduced cichoric acid accumulation (Callan et al., 2005). Consistent with these results, echinacoside, the major caffeoyl conjugate obtained from the roots of *E. angustifolia* grown hydroponically at a high density of 122 plants m^{-2}, was below the minimum quality concentration, which suggests that high-density greenhouse hydroponic production may not ensure sufficient accumulation of marker compounds in *E. angustifolia* (Maggini et al., 2012).

Accumulation of secondary metabolites in *Echinacea* is also influenced by the method of cultivation (field versus hydroponic production). Some previous studies support the idea that plants may accumulate more secondary metabolites under hydroponic culture compared to field cultivation. For example, *E. purpurea* grown hydroponically for 8 months produced more cichoric acid than the corresponding field-grown plants harvested after three years (Letchamo et al., 2002). Similarly, *E. purpurea* and *E. angustifolia* grown under controlled conditions contained similar or higher amounts of caftaric acid, cynarin, echinacoside, and cichoric acid than wild harvested or field-cultivated plants (Zheng et al., 2006 b). The high accumulation of *Echinacea* phytochemicals under hydroponic culture may be due to the high availability of nutrients in the growing medium or the retention of fine roots that are known to contain significant amounts of active ingredients. The composition of the nutrient solution in hydroponic

culture can further alter the rate of production of secondary metabolites. For instance, chlorogenic acid, echinacoside and caffeic acid contents were increased in *E. angustifolia* in nitrate-fed plants (Montanari et al., 2008). However, Zheng et al. (2006 b) found that NO_3^-/NH_4^+ ratio had no effect on the content of CADs in *E. purpurea* and *E. angustifolia.*

2.2.5.6. Storage, processing and extraction method

Drying methods and storage conditions of plant material were found to affect caffeic acid derivatives and total phenolic contents in *E. purpurea* (Kim et al., 2000; Lin et al., 2011). For example, increasing drying temperature in the range of 40-70 °C decreased cichoric acid concentration in *E. purpurea* tissues particularly the aerial parts, but did not affect the alkamides content (Stuart and Wills, 2003). However, Hevia and collaborators (2002) found that drying temperature has a significant effect on both cichoric acid and alkamides. Cichoric acid, the main phenolic in *E. purpurea,* was best retained when the plant material was dried by microwave or steam heating (Zhang et al., 2011). Similarly, echinacoside and cynarin, the two major phenolics in *E. angustifolia* roots, were influenced by postharvest processing (Maggini et al., 2010). The method of extraction, type of solvent, and its concentration were found to influence the content of active substances, and therefore the quality of *Echinacea* preparations (Tsai et al., 2012).

2.3. Salinity stress: Effects and mechanisms of tolerance

2.3.1. Introduction

Salinity is a major adverse environmental factor affecting the yield and productivity of plants worldwide (Parida and Das, 2005). Saline conditions arise from the presence of ions, such as Na^+ and Cl^- in the soil; however Ca^{2+}, Mg^{2+}, and K^+ cations as well as SO_4^{2-}, HCO_3^- and CO_3^{2-} anions contribute to salinization to a lesser extent (Mechanda and Garg, 2008). A saline soil is defined as a soil with an electrical conductivity of more than 4 dS m^{-1}, an exchangeable sodium percentage (ESP) of < 15 and a pH < 8.5 (Khan et al., 2001). Salinity can be classified as primary salinity or secondary salinity (Munns, 2005). Accumulation of salts as a result of weathering of rocks and the deposition of oceanic salts is the cause of primary salinity, while secondary salinity results from human activities, such as artificial irrigation, using fertilizers, and replacement of perennial crops with the annual ones (Mechanda and Garg, 2008). According to FAO (2005) more than 6% of the world total land area and 20% of all irrigated land (45 million ha) are affected by salinity, and this percentage is expected to increase to 50% by the 2050 (Wang et al., 2003). This imposes athreat to the global food production with respect to the increasing population and the limitation in soil and water resources.

Plants are classified as glycophytes or halophytes according to their ability to grow in presence of high salt concentrations (Sairam and Tyagi, 2004). The majority of plants cannot withstand elevated salt concentrations and are referred to as glycophytes; however differences in salt tolerance levels among glycophytes are observed. On the other hand, halophytes are the native flora of saline environments that have the capacity to tolerate extreme salinity levels, because of their morphological, biochemical and anatomical features and avoidance mechanisms (Hasegawa et al., 2000). For example, the

optimum growth for some halophytes, such as *Salicornia dolichostachya*, was obtained at 300 mM NaCl (Katschnig et al., 2012). However, distinction between the two groups is challenging, because the threeshold for salt tolerance is not known and some plant species fall between the two categories.

Salinity exerts its negative effects on plants through imposing osmotic, ionic and oxidative stresses on plants (Parida and Das, 2005). Osmotic stress results from high salt concentration in the growing medium, while ionic stress results from accumulation of specific ions like Na^+ and $Cl^−$ and altered K^+/Na^+ and $Cl^−/NO_3^−$ ratios in plant tissues leading to ionic toxicity and ion imbalance (Blumwald, 2000). Moreover, oxidative stress is likely to result from increased generation of reactive oxygen species (ROS) as a result of disturbance in the dissipation of the light-induced high energy state under osmotic and ionic stresses (Turkan and Demiral, 2009). Reduction in the growth of salt-stressed plants can be explained by the two-phase model (Munns, 2005; Munns and Tester, 2008). In the first phase, immediately after exposure to salts, plants suffer from osmotic stress leading to reduction in water uptake, reduction in cell expansion and initial reduction of growth, with little variation among genotypes in this phase. In the second phase, with the extended exposure to salts, Na^+ and $Cl^−$ accumulate in the plants and enter the chloroplast causing biochemical limitations to the photosynthetic activity and carbon assimilation, and thus further contributing to growth limitation. If the accumulation of salts in the transpiring leaves exceeds the rate of ion compartmentalization into the vacuoles, a severe reduction in growth or even death of plants occurs. Although distinguishing between the two-phase effects of salinity is feasible in most plants, separating the two phases in salt-sensitive genotypes is quite challenging particularly when high salt

concentrations are used (Munns and Tester, 2008). Both osmotic and ionic components of salinity can lead to reduction in plant growth. Yet, the proportional contribution of each component is difficult to predict, therefore polyethylene glycol (PEG), a commonly-known osmoticum agent has been used in numerous reports to study the iso-osmotic effect of NaCl (Munns, 2002; Silva et al., 2010; Sucre and Suarez, 2011). It can be concluded that the degree of growth suppression in salt-stressed plants is affected by various factors including intensity of salt concentrations, duration of exposure, genotypes, plant organs, and how the stress was applied to plants. Although salt tolerance is usually evaluated based on the degree of reduction in growth, maintaining low growth rate may be an adaptive response of plants to salt stress by reducing the transpiring surface, which limits Na^+ and Cl^- uptake and water loss.

Salinity tolerance in plants requires the coordinated activation of multiple genes encoding compatible solutes, ion channels and carriers, antioxidant enzymes, and components of signal transduction pathways including regulatory transcription factors, which implies the complexity of salt tolerance (Tuteja, 2007; Munns and Tester, 2008; Turkan and Demiral, 2009). Collectively, salt tolerance mechanisms fall into three categories: tolerance to osmotic stress, exclusion of Na^+ and Cl^- from leaves, and tolerance of tissues to high levels of Na^+ and Cl^-. However, the relative importance of these three mechanisms varies according to the genotypes, duration of exposure, salt concentration, and also the surrounding environmental conditions (Munns and Tester, 2008). Understanding physiological and biochemical responses and mechanisms of tolerance at the whole-plant, organelle, and molecular levels will be an essential step

towards identifying genotypes with enhanced salt tolerance, and ultimately contributing to resolving the salinity problem.

2.3.2. Perception and signal transduction of salinity

The perception of salinity by plants is achieved through both osmotic and ionic (Na^+) signals (Manchanda and Garg, 2008; Turkan and Demiral, 2009). For osmotic stress, membrane-associated stretch-activated channels, calcium channels, and transmembrane protein kinases, such as histidine kinases may be the potential sensors of signals, which subsequently activate complex signalling cascades leading to the generation of secondary messengers, such as Ca^{2+} and ROS (Tuteja, 2007; Manchanda and Garg 2008; Turkan and Demiral 2009). Cells in the roots have the ability to sense the changes in the osmotic potential of the surrounding solution and respond by the *de novo* synthesis of the phytohormone ABA. In addition to its role in regulating growth and development, ABA plays an important role in the response of plants to the surrounding environment and stress conditions (Chaves et al., 2009). Abscisic acid is involved in the long-distance signalling between the roots and shoots in salt-stressed plants and alters the expression of osmotic responsive genes (Tuteja 2007; Munns and Tester, 2008). Moreover, ABA is involved in the regulation of stomata aperture via altering the flux of ions into the guard cells in plants exposed to osmotic stress (Munns and Tester, 2008). Although the exact signalling networks for detecting high Na^+ ions in the soil are not fully understood, plasma membrane proteins, ion transporters and Na^+ sensitive enzymes could be the potential sensors for the excess amount of Na^+ ions in intracellular and extracellular sites (Turkan and Demiral, 2009). The discovery of SOS (Salt-Overly-Sensitive) pathway in *Arabidopsis* led to the identification of the plasma membrane

Na^+/H^+ antiporter (SOS1), which is a potential Na^+ sensor and is also involved in the efflux of Na^+ outside the cells (Turkan and Demiral, 2009). Mitogen activated protein kinases (MAPK) are important classes of protein kinases that are implicated in signal transduction pathways associated with the exposure to different types of stresses (Tuteja, 2007). As members of MAPK are activated by both biotic and abiotic stresses, it is suggested that MAPK cascades regulate the cross-talk in biotic and abiotic stress signalling, and both types of stresses may diverge at this point (Chinnusamy et al., 2004).

2.3.3. Osmotic stress

Some of the early responses upon exposure to salinity stress are the changes in water relations and gas exchange (Parida and Das, 2005). Accumulation of high salt concentrations outside the roots imposes osmotic stress on plants that lead to reduction in water movement from the roots, and subsequently reduce the cell turgor pressure and reduce relative water content (RWC) (Manchanda and Garg, 2008). This reduction in turgor pressure causes a parallel inhibition in leaf cell expansion and reduction in growth rate (Grenway and Munns, 1980). However, growth suppression might occur even with maintaining turgor pressure and cell extension as it was found in tomato (*Lycopersicon esculentum*), suggesting that turgor is not the only parameter controlling growth process (Romero-Aranda et al., 2001). The decrease in the cell wall extensibility could also limit the plant growth (Volkmar et al., 1998).

Plants exposed to water deficit or salinity exhibit a leaf turgor decline and tend to close their stomata in order to reduce the loss of water (Chaves et al., 2009). This subsequently causes a reduction in the internal CO_2 concentration and its incorporation in

the photosynthesis process. However, partial closure of stomata under mild water deficit conditions may be an adaptive response to help plants reduce transpiration rate and canopy water loss and increase the water use efficiency, which is the carbon gained in photosynthesis in exchange for water used in transpiration (Chaves et al., 2009). As photosynthetic activities are related to plant growth and productivity, the photosynthetic changes under salinity stress has gained great interest (Paramychianakis and Chartzoulakis, 2005). In general, plants exposed to salinity up to 100 mM NaCl exhibit lower rates of photosynthesis compared to non-stressed plants, indicating that chloroplast function in the leaves might be adversely affected by salt treatments. The inhibition of photosynthesis that occurs under low to moderate salinity stress is thought to be mainly attributed to diffusion limitations (both stomatal and mesophyll conductance); while biochemical limitations to photosynthesis occur at higher salinity levels when the accumulation of Na^+ and Cl^- in plant tissues increases to toxic levels (Paramychianakis and Chartzoulakis, 2005). These biochemical limitations involve the inhibition of the carboxylating enzyme, ribulose 1, 5-biphosphate carboxylase oxygenase (rubisco) or a decrease in the amount of ATP produced by photophosphorylation (Reddy et al., 2004). Different species exhibit different degrees of stomatal, mesophyll or biochemical limitations when challenged with water deficit conditions (Chaves et al., 2009). Nevertheless, to which degree stomatal and biochemical limitations contribute to the reduction in photosynthetic activity during salinity stress is not known.

Changes in photosynthetic regime can be considered a mechanism for reducing osmotic stress (Vinocur and Altman, 2005). For example, the facultative halophyte *Mesembryanthemum crystallinum* switches from C3 photosynthetic regime to

crassulacean acid metabolism (CAM) to enhance the water use efficiency and increase the efficiency of photosynthesis by suppressing photorespiration and reducing oxidative damage under saline conditions. This can be attained by closing the stomata at day time and opening them at night to reduce water loss by transpiration (Cushman, 2001).

In response to the disruption in water relations and the increased water loss, plants increase the accumulation of compatible solutes in the cytoplasm to mediate osmotic adjustment and to balance the osmotic potential of salts being sequestered in the vacuole (Turkan and Demiral, 2009). However, the response of halophytes may be different from glycophytes; while halophytes can use both ions and osmolytes, glycophytes use only compatible solutes for osmotic adjustment (Manchanda and Garg, 2008). N-containing solutes (proline and glycine betaine), sugars (fructose, glucose, sucrose, and raffinose), polyols (mannitol and sorbitol), and cyclic polyhydric alcohols (myoinositol and pinitol) are considered the main types of compatible solutes with turgor maintenance function (Munns, 2005). These osmolytes lower the osmotic potential to allow for the influx of water into the cell in response to a water potential gradient and maintain its turgidity (Sairam and Tyagi, 2004). These compounds are also required for maintaining the osmotic balance between the cytosol and the vacuole after the sequestration of high amounts of Na^+ and Cl^- ions into the vacuole (Turkan and Demiral, 2009). In addition to their role in osmotic adjustment, these solutes may interact with cellular macromolecules such as proteins and enzymes and stabilize their structures and functions (Misra and Gupta, 2005). They are also involved in the scavenging of free radicals, and regulating cellular redox status of the cell (Sairam and Tyagi, 2004; Parida and Das, 2005). The advantage of such compounds is that they do not interfere with the metabolic functions in

the cell (Hasegawa et al., 2000; Sairam and Tyagi, 2004). Although accumulation of proline was associated with salinity tolerance in plants in the majority of studies (Ashraf and Harris, 2004), other reports found that salt sensitive genotypes accumulated more proline than salt tolerant genotypes (Lutts et al., 2002), implying that its accumulation could be an indicator for stress rather than an adaptation to alleviate the stress. Moreover, synthesis of these osmolytes is metabolically expensive and requires high amounts of carbon resources that could otherwise be used for growth-related metabolic processes (Greenway and Munns, 1980; Volkmar et al., 1998). Clarifying such controversies requires further investigations at the molecular and whole plant levels.

Induction of protective proteins and N-containing compounds that counteract dehydration is a common response to salinity stress (Ashraf and Harris, 2004). This response includes both quantitative and qualitative changes in protein synthesis and regulation (Parida and Das, 2005). There are several proteins that are induced and are involved in osmotic adjustment. These include osmotin, and late embryogenesis abundant (LEA) proteins, which stabilize proteins and membranes against denaturation (Ashraf and Harris, 2004; Parida and Das, 2005). Pyrroline-5-carboxylate synthetase and betaine aldehyde dehydrogenase, which are responsible for proline and glycine-betaine synthesis, respectively, are also considered osmotic adjustment proteins (Sairam and Tyagi, 2004). Aquaporins are also involved in osmotic homeostasis by regulating water transport within the plant. They form membrane channels specified to the flux of water across biological membranes, thus maintaining the turgor in salt-affected cells (Shao et al., 2008). A new category of plant growth regulators; the polyamines involved in different physiological processes during development, are also playing a role in plant response to environmental

stresses including salinity (Ashraf and Harris, 2004). Although their mode of action is not fully understood, it has been suggested that these compounds could enhance the endogenous antioxidant capacity and minimize cellular membrane damage (Ashraf and Harris, 2004). Polyamines can also improve K^+ homeostasis and transport across cell membranes, enhance the K^+/Na^+ ratio in the cytoplasm and contribute to cellular osmotic adjustment (Shabala and Cuin, 2007). One potential mode of action of polyamines may be through blocking the nonselective cation channels (NSCC), thus reducing the salt-induced K^+ efflux from cells (Shabala and Cuin, 2007).

2.3.4. Ionic stress

Ionic stress is a characterized feature of salt-stressed plants due to the over accumulation of ions, particularly Na^+ and Cl^-, in plant tissues. While halophytes can tolerate high Na^+ concentration up to 500 mM in cytoplasm without deleterious effects on plant metabolism (Matoh et al., 1986), glycophytes are sensitive to high levels of ions in cytoplasm and cannot withstand more than 100 mM Na^+ in cells (Mahajan et al., 2008; Munns and Tester, 2008). However there is a wide variation in the threshold level that causes toxicity in cytoplasm among genotypes depending on the stress intensity and time of exposure. It is believed that the toxic effects of NaCl are primarily attributed to Na^+ ions; however in some woody species, such as *Citrus* spp. and *Vitis* spp., Cl^- is the major ion causing toxicity, and salt tolerance is associated with controlling its transport (White and Broadley, 2001; Teakle and Tyerman, 2010). In other species, both Na^+ and Cl^- are causing foliar injury. For most species, Na^+ seems to reach a toxic level in cytoplasm before Cl^-, and salt tolerance is correlated with the ability to restrict the Na^+ transport to leaves. Therefore, Na^+ transport has attracted more attention than Cl^- in the literature

(Munns and Tester, 2008). In addition to injury, high concentrations of ions in the cytoplasm affect cell functions through altering biochemical reactions and inhibiting enzymes (Tuteja, 2007). Interestingly, enzymes in halophytes are salt sensitive like enzymes in glycophytes. In the absence of an efficient sequestration mechanism, ions may build-up in the cell wall, thus affecting the permeability of membranes and causing leakage of solutes. As a result, dehydration of the cell occurs and the cell dies of salt toxicity or dehydration (Munns, 2005).

To prevent ion build-up and toxicity in cytoplasm, plants are equipped with various mechanisms related to ionic homeostasis. One of these mechanisms, is the secretion of extra salts via cellular structures called salt glands and bladders in the leaves (Parida and Das, 2005; Shabala and Cuin, 2007). This allows for the regulation of internal ion concentrations and maintains low Na^+ in the cytosol, and therefore, reduces the disturbance in ionic homeostasis. However, this mechanism is restricted to halophytes.

Two other mechanisms that regulate the level of Na^+ in the cell, and therefore, influence the salt tolerance capacity of plants, have been proposed (Munns, 2002). The first mechanism is minimizing the entry of Na^+ into cells (salt exclusion) and the second one is reducing Na^+ and Cl^- concentrations in the cytoplasm below the toxic levels by their sequestration into the vacuoles (Munns, 2005; Manchanda and Garg, 2008). Salt exclusion involves the retention of ions in the roots and limiting their transport to the leaves in order to reduce injury and necrosis symptoms. Furthermore, the selective uptakes of K^+ by root cells, as well as the removal of Na^+ from the xylem and preferential loading of K^+, are considered major mechanisms for excluding salts from leaves (Munns,

2002). In this regard, halophytes are more efficient in excluding salts from entering the cells than glycophytes (Munns, 2002). Nevertheless, some glycophytes can regulate ionic movement by selectively limiting the uptake of Na^+ from the soil solution and reducing its transport to the shoots (Cheeseman, 1988). The entry of Na^+ inside the cell is usually a passive transport process, while the Na^+ exclusion outside the cell and its compartmentalization into the vacuole are active transport processes, since Na^+ ions have to be transported against their electrochemical gradient (Blumwald et al., 2000). Various channels and carriers are involved in ionic homeostasis in the cell (Fig. 2.3). Influx of Na^+ is mediated through nonselective cation channels (NSCCs), which play an important role in salinity tolerance in plants (Demidchik and Maathuis, 2007; Kronzucher and Britto, 2011). These channels have high selectivity for monovalent cations like Na^+ and NH_4^+ and to a lesser extent to divalent cations like Ca^{2+} and Mg^{2+} down the electrochemical gradient. Other channels, such as the K^+ inward-rectifying channels (KUP/HAK/KT) can selectively mediate the influx of K^+ over Na^+, thus maintaining high K^+ content in the cytosol and adjusting K^+/Na^+ ratio (Tuteja, 2007). However, the similarity of the ionic radius between Na^+ and K^+ make it difficult for transport proteins to discriminate between the two ions, and this can consequently cause Na^+ toxicity (Blumwald, 2000). Active transport of ions (Na^+) is mediated through plasma membrane antiporters and symporters that can transport ions (Na^+) against the electrochemical potential gradient across the membranes (Zhu, 2003; Munns, 2005). One of the important antiporters involved in the efflux of excess Na^+ ions from the cytosol into the external medium is the plasma membrane Na^+/H^+ antiporter (SOS1), which was discovered through the salt overlay sensitive (SOS) pathway in *Arabidopsis* (Liu and Zhu, 1998;

Zhu, 2001; Tuteja 2007). Another component of this pathway is the SOS2-SOS3 complex that can inhibit the activity of a low-affinity Na^+ transporter (HKT1) to restrict the entry of Na^+ ions into the cytosol, and thus contributing to the regulation of Na^+ homeostasis. It is worthnoting that the SOS pathway is not only confined to glycophytes, which cannot tolerate salts above 100 mM, but it is also functional in halophytes that can tolerate more than 250 mM (Mahajan et al., 2008). Despite the advancement of new techniques to dissect the role of transporters in salt tolerance, the mechanism is still unknown (Yamaguchi and Blumwald, 2005). Unlike Na^+ transport, mechanisms of Cl^- transport, including xylem loading, intracellular compartmentation into vacuoles and efflux of Cl^- from roots, are not well studied in plants and further research is needed for understanding these mechanisms (Brumos et al., 2010; Teakle and Tyerman, 2010).

The presence of physical barriers, such as Casparian bands and suberin lamellae of the endodermis and the exodermis in the roots, may regulate the transport of ions to the shoots by blocking the uncontrolled movement through the apoplastic pathway (Krishnamurthy et al., 2009; Davies, 2011). Therefore, salt tolerance of plants may increase as a result of the presence and strengthening of these physical barriers (i.e. endodermal Casparian bands) (Davies, 2011). Casparian barriers were found to show histological changes in response to NaCl salinity in some woody species; *Prosopis strombulifera* (Reinoso et al., 2004) and *Cornus sericea* (Davis, 2011). In the latter study, a modified cortical layer functioning as an additional barrier to ion movement was formed and both the endodermis and the exodermis matured closer to the root tip, which suggest their involvement in limiting Na^+ transport to shoots.

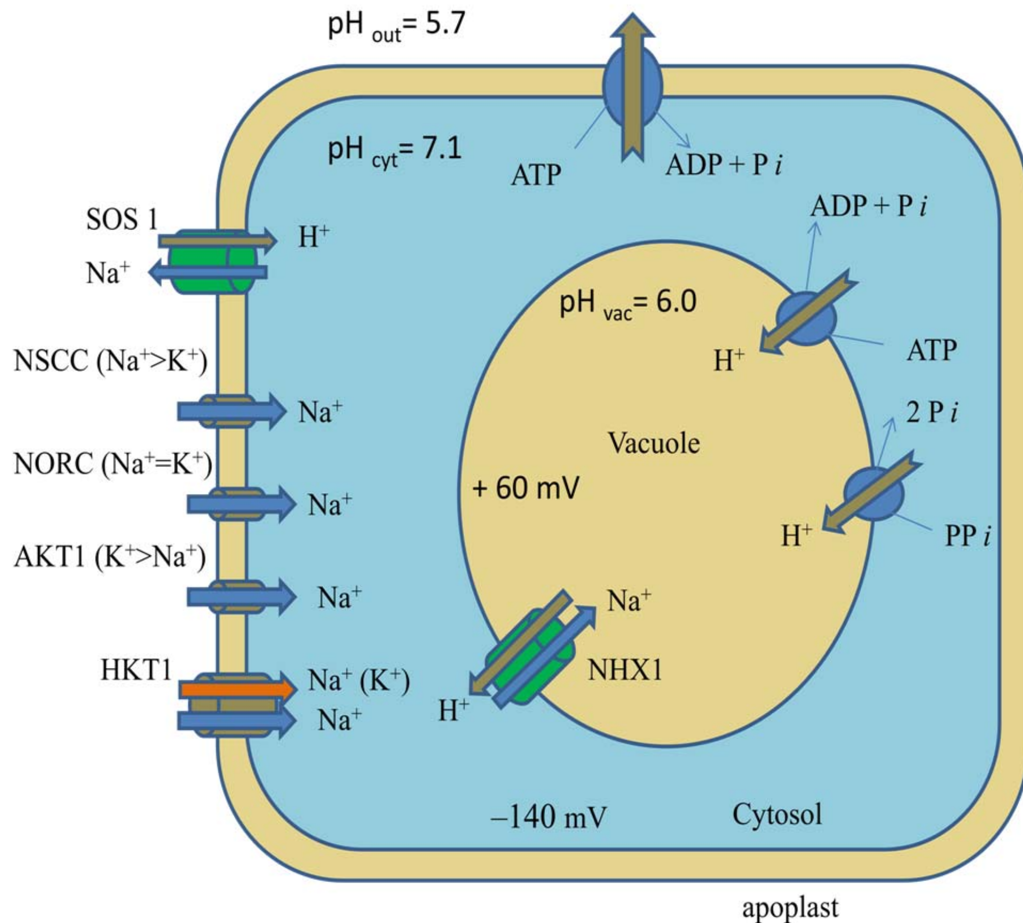


Fig. 2.3. Regulation of Na^+ transport and maintenance of K^+/Na^+ balance in plant cells through the activation of various pumps and channels. H^+-ATPase in plasma membrane and vacuolar membrane as well as H^+-PPiase in the vacuolar membrane are the major pumps that generate electrochemical gradient for H^+ across the cell and vacuolar membranes. Different channels (AKT1, NORC, NSCC) or carriers (i.e. the high affinity K^+ transporter, histidine kinase, HKT1) mediate the influx of Na^+ into the cell, then Na^+ is sequestered into the vacuole via a vacuolar Na^+/H^+ antiporter (NHX1) or translocated out of the cell by the action of a plasma membrane Na^+/H^+ antiporter (SOS1). Copied from Yamaguchi and Blumwald, (2005) with permission from Elsevier Publishing.

Ion sequestration into the vacuole is the other mechanism employed by plants to avoid ion toxicity in the cytoplasm (Zhu, 2003; Munns, 2005). This mechanism maintains appropriate K^+ and Ca^{2+} concentrations required for different metabolic processes (Mansour et al., 2003). Glycophytes and halophytes differ in their ability to sequester Na^+ and Cl^- into the vacuole; while halophytes can use ions in the vacuole as osmoticum to maintain an osmotic potential and turgor pressure, glycophytes have limited capacity for ion sequestration (Blumwald et al., 2000). The sequestration process is mediated by Na^+/H^+ antiporters embedded in tonoplast membranes (Mansour et al., 2003; Turkan and Demiral, 2009). Through the salt overlay sensitive (SOS) pathway (Fig. 2.4), a serine/threonine protein kinase (SOS2) can phosphorylate and activate the vacuolar Na^+/H^+ antiporter (NHX) to facilitate the sequestration of excess Na^+ ions into the vacuole, and thus further contribute to ion homeostasis (Qiu et al., 2002).

Some previous studies have shown that salt tolerance is enhanced in plants as a result of enhanced activities or overexpression of H^+-ATPases of plasma and tonoplast membranes, and H^+-PPase of the tonoplast membrane (Otoch et al., 2001; Manchanda and Garg, 2008). Consequently, the coordinated regulation of these transport proteins contributes to the overall ability of plants to tolerate salts in their growing medium (Mansour et al., 2003). However, it is unlikely that a single gene or a gene product can solely confer salt tolerance, because salinity tolerance is a quantitative trait that involves the activation of different genes. Unlike Na^+ transport, little is known about compartmentalization of Cl^- into the vacuole.

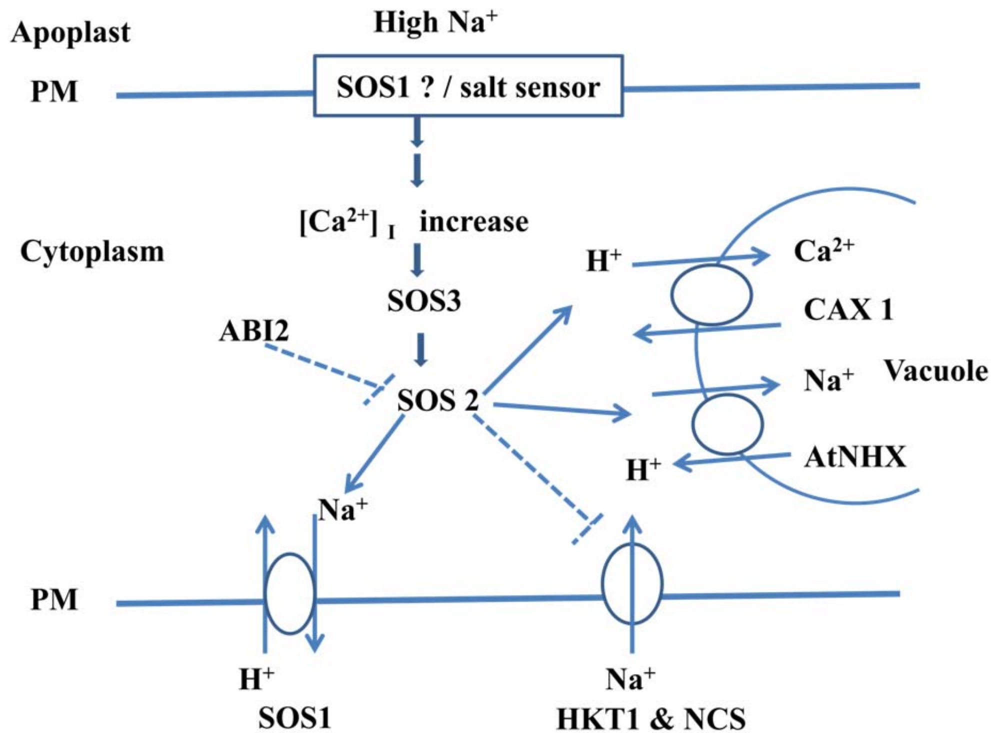


Fig. 2.4. Schematic representation of the regulation of ion homeostasis in *Arabidopsis* through the SOS signalling pathway. The extracellular salt stress is perceived by unknown salt sensor at the plasma membrane, which triggers an increase in cytosolic Ca^{2+}. The calcium signal is sensed by SOS3 which activates SOS2. The activated SOS3-SOS2 protein kinase complex phosphorylates SOS1, the plasma membrane Na^+/H^+ antiporter, resulting in Na^+ efflux. SOS2, independently of SOS3, also activates Na^+/H^+ (AtNHX) exchanger and Ca^{2+}/H^+ (CAX1) antiporter on the vacuolar membrane. The protein phosphatase ABI2 can negatively regulate SOS2 or SOS1. Activities of Na^+ influx transporters (HKT1 and NCS) are down-regulated by the SOS pathway, thus reducing Na^+ content in cytoplasm. Copied from Zhang et al. (2004) with permission from the American Society of Plant Biologists.

In addition to ion toxicity, high salt concentrations, particularly Na^+ and Cl^- in the soil solution, can cause ion imbalance due to interference with the uptake and translocation of major elements, which result in nutrient deficiencies (Grenway and Munns, 1980). For example, Na^+ may compete with K^+ and Ca^{2+} in the absorption sites, while Cl^- can compete with NO_3^- uptake, leading to decreased K^+, Ca^{2+} and NO_3^- content, and increased Na^+ and Cl^- content (Hu and Schmidhalter, 2005). High Na^+ concentration also has an inhibitory effect on the low and high-affinity K^+ transporters, which leads to a decrease in K^+ uptake (Shabala and Cuin, 2007). In addition, increased Na^+ accumulation inside the tissues alter the K^+/Na^+ ratio, and compromise the role of K^+ in activating various enzymes in the cytoplasm (Maathuis and Amtmann, 1999; Blumwald, 2000; Kronzucher and Britto, 2011). More than 50 enzymes related to metabolic processes in the cytoplasm are activated by K^+ (Britto and Kronzucher, 2008). Therefore, maintaining a high K^+/Na^+ ratio in the cytoplasm is crucial for cell function and is considered an important variable in determining the degree of salt tolerance in different plant species (Shabala and Cuin, 2007). It was also found that addition of supplemental Ca^{2+} to the growth medium can alleviate some of the negative effects of salinity on plants (Renault, 2005; Shabala and Cuin, 2007). Such amelioration was associated with reduction in Na^+ uptake induced by Ca^{2+} as well as inhibition in Na^+-induced K^+ efflux from cytoplasm, thus assisting in maintaining a high K^+/Na^+ ratio required for various physiological functions (Shabala and Cuin, 2007).

2.3.5. Oxidative stress

Various environmental conditions including salinity, drought, high light intensity, nutrient deficiency, and extreme temperature generate reactive oxygen species (ROS),

such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), that cause oxidative stress in plant tissues (Gill and Tuteja, 2010). In the case of salt-stressed plants, formation of these free radicals is induced by closure of stomata and the reduction in the consumption of NADPH by the Calvin cycle. As a result, accumulation of these reductants exceeds the rate of their utilization in the biochemical reactions due to reduced growth rate (Turkan and Demiral, 2009). During the photosynthetic electron transport process, when ferredoxin is over reduced, electrons may be transferred from PSI to oxygen to form superoxide radicals ($O_2^{\bullet -}$) through the Mehler reaction that initiates the formation of other harmful free radicals through a series of reactions (Turkan and Demiral, 2009). The production sites of these ROS can be the cytoplasm, the chloroplasts, the mitochondria, the peroxisomes and other cellular compartments (Parida and Das, 2005). Although ROS have the potential to cause oxidative damage to lipids, proteins, membranes and nucleic acids in cells, sustaining ROS at the required level is important for cell signalling in response to pathogen attack, programmed cell death, and different environmental stimuli (Mittler et al., 2004; Miller et al., 2010). This points out the importance of achieving a balance between the rate of ROS production and the rate of their scavenging in the plant cell. Information regarding ROS regulatory pathways and how different branches cross talk to determine the level of ROS in the cell is not fully understood (Turkan and Demiral, 2009).

To ameliorate or reduce the oxidative damage by ROS, plants have evolved efficient protective mechanisms composed from both enzymatic and non-enzymatic systems (Parida and Das, 2005). The enzymatic antioxidant system includes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase

(GR) and a variety of general peroxidases (POX), which counteract the detrimental effects of ROS (Ashraf, 2009; Gill and Tuteja, 2010). Many of the antioxidant enzymes work in a harmonized way through the ascorbate–glutathione cycle (Fig. 2.5). The detoxification process starts with the activity of SOD, which catalyzes the first step in the ROS scavenging system by dismutating $O^{2-\cdot}$ to H_2O_2 and O_2. The H_2O_2 produced is then scavenged to water and molecular O_2 by CAT or POX through the ascorbate-glutathione cycle (Asada, 1992). Regeneration of ascorbate in the cycle involves the activity of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), in addition to electron donors, such as NADPH. Although catalases are virtually absent from chloroplasts, detoxification of H_2O_2 is carried out by the action of APX activity (Dionisio-Sese and Tobita, 1998).

Various studies have shown a correlation between the antioxidant enzyme activities and salt tolerance in different glycophytes, such as *Oryza sativa* (Dionisio-Sese and Tobita 1998; Demiral and Turkan, 2005), *Calendula officinalis* (Chaparzadeh et al., 2004), and *Catharanthus roseus* (Misra and Gupta, 2006) and halophytes, such as *Crithmum maritimum* (Ben Amor et al., 2005). Compared to sensitive genotypes, salt tolerant genotypes usually exhibit reduced lipid peroxidation and oxidative stress due to elevated antioxidant enzyme activities (Sreenivasulu et al., 2000; Mittova et al., 2002). Moreover, transgenic plants overexpressing antioxidant enzymes, such as SOD, APX, and GR have been shown to exhibit enhanced tolerance to salt-induced oxidative stress (Vinocur and Altman, 2005; Turkan and Demiral, 2009; Miller et al., 2010). However at high salinities (above 100 mM), some antioxidant enzyme activities were decreased in both salt sensitive and salt tolerant *Oryza sativa* genotypes

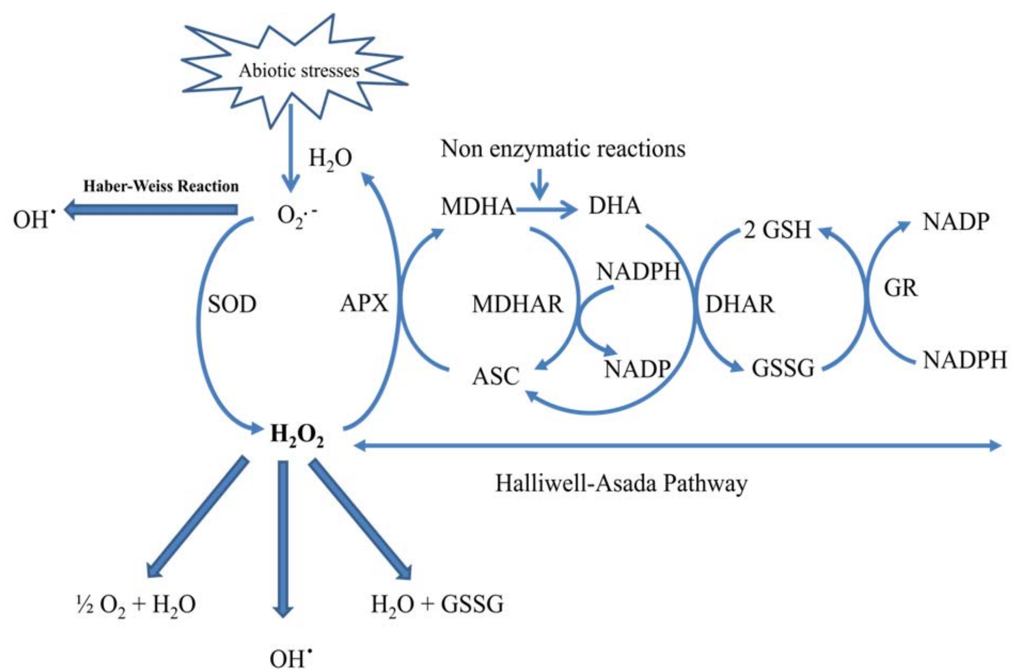


Fig. 2.5. Scavenging of reactive oxygen species (ROS) through the antioxidant enzyme system including the Halliwell-Asada cycle in plant cells. SOD, superoxide dismutase; APX, ascorbate peroxidase; ASC, ascorbate; DHA, dehydroascorbate; DHAR; dehydroascorbate reductase; GR, glutathione reductase; MDHA, mono-dehydroascorbate; MDHAR, mono-dehydroascorbate reductase; GSH, reduced glutathione; GSSG, oxidized glutathione. Copied from Gill and Tuteja, (2010) with permission from Elsevier Publishing.

(Dionisio-Sese and Tobita, 1998). Moreover, activity of ascorbate peroxidase (APX), a major antioxidant enzyme in cells, was found to increase in leaves of salt-sensitive wheat (*Triticum aestivum*) compared to salt tolerant genotype (Mühling and Läuchli, 2003). Therefore, it can be concluded that the pattern of antioxidant enzyme activities is highly influenced by salt concentration, genotype, and duration of exposure, but the majority of studies support the idea that particular antioxidant enzymes are relevant to salinity tolerance.

The other group of antioxidants in the cell is the non-enzymatic components composed of ascorbate (AsA) and reduced glutathione (GSH) that have the ability to scavenge $O_2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2 (Gill and Tuteja, 2010). Protecting the thiol group of protein during oxidative stress is an important physiological function ascribed to GSH. Moreover, GSH can generate ascorbic acid, which is another potent antioxidant in the cell through the ascorbate-glutathione cycle (Blokhina et al., 2003; Miller et al., 2010). In addition, GSH can regulate the redox status of the cell under normal and stress conditions. Considered as essential lipophilic components of biological membranes, tocopherols have also the ability to scavenge free radicals, thus preventing cellular lipid peroxidation (DellaPenna and Pogson, 2006). These compounds have been suggested to stabilize the membranes and regulate their fluidity as well as the permeability to different ions and molecules. Carotenoids, such as β-carotenes, lycopenes, and xanthophylls also posses antioxidant activities based on their capacity for quenching singlet oxygen. There are more than 600 types of carotenoids that are synthesized naturally as pigments in plants, microorganisms, but not in animals, thus consuming vegetables and fruits

containing carotenoids is important to prevent chronic diseases including cancer and cardiovascular diseases (Paiva and Russell, 1999).

Phenolic compounds are abundant non-enzymatic molecules in tissues of some plants with higher ROS scavenging activity than tocopherols and ascorbate (Blokhina et al., 2003). Different phenolic compound derivatives; such as flavonoids, isoflavonoids, hydroxycinnamates, tannins, and lignin are expected to show differential scavenging capacity, and flavonoids in particular have the highest activity. Antioxidative properties of polyphenol compounds arise from their ideal structural chemistry (i.e. large number of OH group) and from their high reactivity as electron donors, their ability to stabilize and delocalize the unpaired electron, as well as their ability to chelate transition metal ions (Blokhina et al., 2003). It has been suggested that phenolic compounds reduce the fluidity of membranes and therefore limit the diffusion rate of ROS and the incidence of oxidative damage (Arora et al., 2000). However, this mechanism is not fully understood, and further investigations are required to clarify this process.

2.4. Secondary metabolites and salinity stress

Biosynthesis of secondary metabolites in medicinal plants is not only controlled genetically, but it is also affected by other environmental factors including salinity (Baghalian et al., 2008). Although numerous studies on medicinal plants investigated the phytochemical changes in response to drought, one of the major environmental abiotic stresses (Mohemed et al., 2001; Gray et al., 2003; Kirakosyan et al., 2004; Chung et al., 2006), until a few years ago, a limited number of studies has been conducted to assess the effects of salinity on medicinal plants (Table 2.1). In the last four years, an increasing

Table 2.1. Quantitative variations in the accumulation of some secondary metabolites in various plant species in response to salt concentrations.

NaCl (mM)	Plant species	Compounds	Response	Reference
25-50	*Cynnara cardunculus*	Phenolic compounds	+	Hanen et al., 2008
150	*Cynnara cardunculus*	Phenolic compounds	–	Hanen et al., 2008
15-30	*Capsicum annuum*	Phenolic compounds	+	Navarro et al., 2006
Up to 100	*Mentha pulgium*	Phenolic compounds	+	Oueslati et al., 2010
Up to 116	*Lycopersicon esculentum*	Phenolic compounds	+	Krauss et al., 2006
51.7	*Carthamus tinctorius*	Phenolic compounds	+	Karray-Bouraoui et al. 2011
50	*Echinacea angustifolia*	Phenolic compounds (cihoric acid and chlorogenic acid)	+	Montanari et al., 2008
50-1000	*Lactuca sativa*	Phenolic compounds	–	Kim et al., 2008
25	*Salvia sclarea*	Essential oil	+	Ben Taarit et al., 2011
75	*Salvia sclarea*	Essential oil	–	Ben Taarit et al., 2011
25-50	*Coriandrum sativum*	Essential oil (linalool and camphor)	+	Neffati and Marzouk, 2008
Above 50	*Coriandrum sativum*	Essential oil (linalool and camphor)	–	Neffati and Marzouk, 2008
75	*Salvia officinalis*	Essential oil	+	Ben Taarit et al., 2009
100	*Salvia officinalis*	Essential oil	–	Ben Taarit et al., 2009
168-336	*Matricaria chamomilla*	Essential oil	–	Razmjoo et al., 2008
10-50	*Raphanus sativa* (sprouts)	total phenolic, glucosinolate and glucoraphasatin	–	Yuan et al., 2010
77	*Thymus vulgaris*	Essential oil	+	Ezz El-Din et al., 2009
46-184	*Matricaria recutita*	Essential oil and apigenin (flavonoid)	no change	Baghalian et al., 2008
60	*Nigella sativa*	Vanillic acid	–	Bourgou et al., 2010 b
60	*Nigella sativa*	Quercitin, apigenin, *trans* cinnamic acid	+	Bourgou et al., 2010 b
Up to 160	*Artemisia annua*	Artemisinin	+	Qureshi et al., 2005
Up to 100	*Artemisia annua*	Artemisinin	+	Qian et al., 2007
150	*Catharanthus roseus*	Vincristine alkaloid	+	Osman et al., 2007
150	*Solanum nigrum*	Solasodine	+	Bhat et al., 2008
153.8	*Datura innoxia*	alkaloids	+	Brachet and Cosson, 1986
80	*Catharanthus roseus*	Alkaloid ajmalicine	+	Jaleel et al., 2008
100	*Digitalis purpurea*	Cardenolide	+	Morales et al., 1993
40-80	*Brassica oleracea* var. *Italica*	Glucosinolates	+	Lopez-Berenguer et al., 2009

+, increase; –, decrease

number of studies has focussed on this topic, showing the needs for this type of research (Table 2.1). Salt concentration below 100 mM NaCl has a stimulatory effect on the level of secondary metabolites in the majority of plants studied. On the other hand, higher levels of salts diminished the accumulation of secondary metabolites with some exceptions. For example, alkaloids content was significantly increased in *Catharanthus roseus* at 150 mM NaCl (Osman et al., 2007). These examples demonstrate that salt level is the predominant factor influencing the level of secondary metabolites; however other factors such as genotype differences and duration of stress and interaction with other stresses can further contribute to the quantitative changes of bioactive substances in medicinal plants. Furthermore, the composition of bioactive compounds can be influenced by salinity. For example, *cis*-piperitol and elmol levels were increased in the essential oil of *Cymbopogon schoenanthus,* while α-eudesmol was decreased under salinity (Khadhri et al., 2011). In salt-stressed black cumin (*Nigella sativa*), a major change in the composition of essential oil was observed, and the levels of δ terpinene/*p*-cymene, the two major volatile compounds, were increased (Bourgou et al., 2010 a). Interaction between salts could also affect the accumulation of secondary metabolites. For instance, addition of $CaCl_2$ to NaCl increased the content of indole alkaloids in shoots and roots of *Catharanthus roseus* when compared to NaCl-treated or untreated plants (Jaleel et al., 2007). Similarly, the addition of $CaCl_2$ was found to enhance the accumulation of phenolic diterpene and carnosic acid in the leaves of rosemary, *Rosmarinus officinalis* (Tounekti et al., 2011). This increase could be due to the direct beneficial effect of Ca^{2+} on the biosynthesis of these compounds or to the enhanced K^+/Na^+ ratio in the cytoplasm (discussed earlier).

It was found that the differential accumulation of secondary metabolites can be an indicator for determining salt tolerance degree of genotypes. For example, the salt tolerant lettuce (*Lactuca sativa*) cultivar contained higher levels of carotenoids and phenolics than the salt sensitive one when exposed to 100 mM NaCl (Mahmoudi et al., 2010). Similarly, genotypes with enhanced polyphenol accumulation showed better performance in response to salinity stress as compared to the cultivars with lower content as was found in *Cakile maritima* (Ksouri et al., 2007), and *Zea mays* (Hichem et al., 2009). However some salt sensitive genotypes may contain higher phenolic amounts than salt tolerant ones as was found in *Allium sativum* (Siddiqui et al., 1996). One possible reason for the increased accumulation of secondary metabolites under salinity and potentially other stresses (i.e high sunlight radiation) is the increase in the allocation of newly assimilates towards the synthesis of polyphenols and flavonoids that possess antioxidant properties (Remorini et al., 2009). Other secondary metabolites, such as glycosinolates, have been shown to be involved in osmotic adjustment by decreasing water potential, and thus allowing the entry of water into cells in salt-affected plants (Qasim et al., 2003). This suggests that the increase in the accumulation of secondary metabolites during salinity stress is a part of the plant adaptation and tolerance mechanisms. More research is needed to dissect the role of plant secondary metabolites under salt stress.

In addition to salinity-induced changes in content and composition, nutritional quality of fruits and vegetables has been shown to be influenced by salinity. For example, salinity increased the quality of strawberry fruit by increasing amino acids, such as proline, asparagine, and glutamine (Keutgen and Pawelzik, 2008) and improved the

quality attributes (dry matter, soluble solid content and acidity) of pepper, *Capsicum annuum* (Marin et al., 2009). In cherry tomatoes, *Solanum lycopersicum*, nutritional value and vitamin C, vitamin E, chlorogenic acid and dihydrolipoic acid contents were also enhanced using diluted seawater for irrigation (Sgherri et al., 2008). Moreno et al. (2008) and Lopez-Berenguer et al. (2009) found that moderate salt stress improved the nutritional quality of the edible florets of broccoli (*Brassica oleracea* var. *Italica*) due to the increased accumulation of the glucosinolates and phenolic compounds. Exposing radish (*Raphanus sativa*) sprouts to salinity (100 mM NaCl) has been proposed as an efficient way to enhance the nutritional value and contents of health-promoting substances (phenolic compounds and glucosinolates) (Yuan et al., 2010). On the contrary, fruit quality and sensory attributes of salt sensitive species like strawberry (*Fragaria* × *ananassa*) were decreased by application of salt stress (Keutgen and Pawelzik, 2007).

2.5. Phenylpropanoid-related enzymes and stress tolerance

2.5.1. Phenylalanine ammonia-lyase (PAL) activity

Since phenolic compounds are implicated in plant resistance to different biotic and abiotic stresses (Dixon and Paiva, 1995), the study of their biosynthesis and regulation has attracted much attention. Plant tolerance to adverse environmental conditions may be regulated through the metabolism of phenolic compounds as well as the activities of related enzymes. For instance, accumulation of specific phenolic compounds (i.e. 3-deoxyanthocyanidins) was used as a good marker for the resistance of *Sorghum* varieties to biotic and abiotic stresses (Dicko et al., 2005). In addition, PAL activity was found to be the limiting factor in regulating the phenolic compound

accumulation in *Astragalus membranaceus* exposed to low temperature (Pan et al., 2008). PAL (EC 4.3.1.5) catalyzes the first step in the phenylpropanoid pathway in plants; the deamination of phenylalanine to produce *trans*-cinnamic acid, which is the precursor of many phenolics including hydroxycinnamates, flavonoids, coumarins, and lignins with various biological functions in response to biotic and abiotic stresses (Hahlbrock and Scheel, 1989; Winkel-Shirley, 2001; Yan et al., 2006). Activation of PAL in response to stress is considered part of the defence mechanisms in cells (Dixon and Paiva, 1995). Indeed, many studies showed increased activity of PAL under abiotic stresses and in most cases this increase in activity was correlated with the accumulation of phenolic compounds. Examples for this correlation were found in various species such as tomato (*Lycopersicon esculentum*) and watermelon (*Citrullus lanatus*) plants exposed to heat stress (Rivero et al., 2001); wheat (*Triticum aestivum*) exposed to Al treatment (Zakir Hossain et al., 2006); chamomile (*Matricaria chamomilla*) challenged with nitrogen deficiency (Kovacik et al., 2007), and lettuce (*Lactuca sativa*) exposed to water stress (Oh et al., 2009 a). This indicates that PAL may be the key enzyme regulating the biosynthesis of the major phytochemical constituents in these plants. On the other hand, no correlation between the accumulation of phenolic compounds and the PAL activity was found in other species such as *Camellia sinensis* exposed to high levels of Ni (Basak et al., 2001); Romaine lettuce (*Lactuca sativa*) treated with jasmonic acid (JA) and salicylic acid (SA) (Campos-Vargas and Saltveit, 2002) and *Salvia miltiorrhiza* root culture treated with elicitor (yeast extract) (Yan et al., 2006). The lack of correlation between phenolic accumulation and PAL activity may be due to the increased accumulation of *trans* cinnamic acid (*t*-CA), the end product of PAL in the pathway,

which may result in a feedback inhibition for PAL, thus determination of the increase in free phenylalanine would be an important indicator for PAL inhibition (Kovacik et al., 2011). These results also suggest that the synthesis of phenolic compounds is a complex process and the activities of enzymes downstream of PAL may be more relevant to the regulation of the synthesis of these compounds.

Some previous studies have shown that the PAL activity can be a reflection of the response to environmental stresses. In this regard, the activity of PAL was used as an indicator for discriminating between genotypes for their drought tolerance. For example, drought sensitive tomato (*Lycopersicon esculentum*) cultivars showed more reduction in PAL and phenolic compounds than the drought-tolerant cultivars (Sanchez-Rodriguez et al., 2011). Similarly, the more salt-tolerant maize (*Zea mays*) inbred exhibited higher PAL activity and phenolic compound accumulation than the salt sensitive inbred (Gholizadeh and Kohnehrouz, 2010). Nevertheless, PAL activity was increased in both resistant and susceptible barely (*Hordeum spontaneum nigrum*) cultivars challenged with fungal pathogen, indicating that the response was not a reflection of cultivar resistance (Shiraishi et al., 1995). These results suggest that the response of PAL activity under stress conditions is highly dependent on the species, plant part, time of exposure, the intensity of the stress and the physiological stage of the plant.

2.5.2. Polyphenol oxidase (PPO) and other peroxidases

Polyphenol oxidase (PPO, 1.10.3.2) is a ubiquitous enzyme in plants, animals, fungi, and bacteria responsible for the oxidation of the indigenous simple phenolic substrates, such as chlorogenic acid and caffeic acid into *o*-quinones. This enzyme plays

a key role in defence mechanisms against biotic factors (Mayer, 2006). PPO is located mainly in the thylakoid membranes in the chloroplast, while its phenolic substrates are located primarily in the vacuoles. Following wounding or pathogen attack, the enzyme is activated once in contact with substrates leading to the formation of quinines and brown pigments as a defense mechanism (Bhonwong et al., 2009). These quinones are toxic to the invading organisms and reduce the availability of dietary proteins to insect herbivores leading to their starvation (Lagrimini, 1991; Pourcel., 2007). Overexpression of the PPO gene in tomato plants enhanced the resistance to *Pseudomonas syringae* (Li and Steffens, 2002), highlighting the linkage between PPO and defense mechanisms to biotic stress. Furthermore, it has been shown that PPO is involved in the response to abiotic stress (Mayer, 2006).

PPO activity has been shown to be a suitable biochemical marker for resistance to biotic stresses like pathogens. For instance, when different tomato (*Lycopersicon esculentum*) cultivars were evaluated after been challenged with bacterial wilt pathogen, resistant cultivars showed increased defence-related enzymes (PPO and PAL) activities compared to susceptible ones (Vanitha et al., 2009). Nevertheless, a contradictory result was obtained by Shimizu and Mazzafera (2007) where the inoculation of soybean (*Glycine max*) roots with the nematode *Meloidogyne javanica* lead to increased PPO activity in the susceptible variety but not in the resistant one, suggesting that the contribution of PPO to resistance is dependent on the type of stress and plant species. On the other hand, under abiotic stress, information regarding using PPO activity as a marker for tolerance was not consistent. For example, an increase in PPO activity was observed in salt sensitive rice (*Oryza sativa*) cultivars compared to salt tolerant ones (Mittal and

Dubey, 1991), while, Aghaleh and Niknam (2009) found increased PPO activity in both salt tolerant and salt sensitive soybean (*Glycine max*) cultivars.

In addition to PPO, substrate-specific peroxidases (chlorogenic acid peroxidase [CGAPX] and caffeic acid peroxidase [CAPX] have been shown to be involved in the metabolism of phenolic compounds in *Panax ginseng* cell cultures exposed to copper stress (Ali et al., 2006) and elevated CO_2 levels (Ali et al., 2005). How the increased activities of these enzymes enhanced tolerance to elevated copper and CO_2 was not explained, but it was suggested that they may be related the biosynthesis of phenolic compounds with high antioxidant capacity. In barley (*Hordeum vulgare*) plants exposed to heavy metals (Cd, Cu, Ni, and Pb), CGAPX was shown to be involved in tolerance to metals by enhancing the lignifications of the meristematic zone and root tip to prevent the uncontrolled flux of heavy metals (Halušková et al., 2010). However, Ramiro and collaborators (2006) studied the role of phenolic compounds and peroxidase enzymes in the defence mechanisms of coffee plants against leaf miner (*Leucoptera coffeella*), and concluded that phenolic compounds, as well as the activity of peroxidase (POX) and polyphenoloxidase (PPO), do not play a crucial role in the defence mechanisms against leaf miner in coffee plants. In *Echinacea*, polyphenol oxidase activity is responsible for the degradation of echinacoside and cynarin in *E. angustifolia* products (Wolkart et al., 2004) as well as cichoric acid in *E. purpurea* preparations (Nusslein et al., 2000). However, little is known about the *in vivo* role of peroxidases in *Echinacea* under salinity stress.

2.6. Conclusion

The literature reviewed indicates that salinity has adverse effects on most of the physiological and biochemical attributes of plants, but also induces mechanisms of tolerance, by which plants can reduce these constraints and adapt to saline environments. Such mechanisms include the synthesis of compatible solutes, induction of antioxidant activities, activation of specific and transport proteins, ion sequestration, ion exclusion, and accumulation of secondary metabolites, which reflects the complexity of salt tolerance mechanisms in plants. Despite the plethora of salt tolerance studies, most notably on crops, more research is needed for evaluating salt tolerance in the economically-important medicinal plants such as *Echinacea*. Studies dissecting and comparing the physiological and phytochemical changes in genotypes would be extremely valuable not only in understanding the basis for salinity tolerance differences but also for the possibility of utilizing salt tolerant species in saline affected areas.

CHAPTER 3. DIFFERENTIAL PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF THREE *ECHINACEA* SPECIES TO SALINITY STRESS*

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3.1. Abstract

The physiological and biochemical responses of the three *Echinacea* species, *Echinacea purpurea*, *Echinacea pallida* and *Echinacea angustifolia* were evaluated in response to NaCl salinity (0, 50, 75 and 100 mM) under hydroponic cultivation. Growth, injury index, gas exchange, ion accumulation, pigment content, electrolyte leakage (EL) in addition to antioxidant enzyme activities were measured after two weeks of salt treatments. Salinity did not alter the root or shoot biomass of the three *Echinacea* species studied. However, the survival rate varied among the species, with the highest rate (96.8%) in *E. purpurea* and the lowest one (70.7%) in *E. angustifolia.* In parallel, *E. angustifolia* plants showed high injury index and EL values compared to the other two species, which indicates its higher sensitivity to salinity-induced membrane damage even at the lowest salt concentration tested. This species showed a decrease in stomatal conductance, photosynthetic and transpiration rates at all salt concentrations; while, in *E. purpurea* and *E. pallida*, the photosynthetic rate was reduced only at 75 and 100 mM NaCl. The decline in the leaf gas exchange was highly correlated with Na^+ and Cl^- contents in all *Echinacea* species. Although Na^+ and Cl^- concentrations increased in both roots and shoots with increasing external salt concentrations, the accumulation of Na^+

ions in *E. angustifolia* shoots was higher than in the other two species, resulting in a significant increase in Na^+/K^+ ratio. Furthermore, *E. purpurea* retained more Na^+ ions in the roots compared to the other species suggesting higher efficiency in excluding Na^+ from the shoots. *E. purpurea* showed increased superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities at all salt concentrations. On the other hand, salinity induced a reduction in catalase (CAT) activity with no change in glutathione reductase (GR) activity in any of the species. Our results indicate that the *Echinacea* species studied showed a limited salt tolerance. However, *E. purpurea* and to a lesser extent, *E. pallida* were more salt tolerant than *E. angustifolia*. The relative tolerance of *E. purpurea* was associated with the higher Na^+ exclusion capacity and the increased antioxidant activities particularly SOD and APX.

Keywords: Echinacea; Salt stress; Gas exchange; Ion content; Electrolyte leakage; Antioxidant enzymes

3.2. Introduction

Salinity is considered one of the major environmental stresses worldwide that depresses the growth and yield of the majority of glycophytic plants (Parida and Das, 2005). Salinity induces both osmotic and ionic stresses resulting in the disruption of many physiological and biochemical processes including water relations, gas exchange, ionic homeostasis, and mineral nutrition (Parida and Das, 2005; Munns and Tester, 2008). As a result of limited CO_2 fixation, reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) can be overproduced in the chloroplasts and other organelles leading to the disruption in cellular

metabolism through membrane lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acids (Parida and Das, 2005). To protect their cells from the oxidative damage induced by ROS, plants activate both enzymatic and non-enzymatic antioxidant mechanisms (Gill and Tuteja, 2010). There is a close relationship between salt tolerance and maintaining an efficient antioxidant capacity as was demonstrated previously in *Oryza sativa* (Demiral and Türkan, 2005), and *Cakile maritima* (Ben Amor et al., 2006). However, salt tolerance is a complex trait that requires modulation of both osmotic and ion homeostasis in addition to the activation of the antioxidant machinery (Parida and Das, 2005).

It has been predicted that up to 50% of the world's irrigated lands could be adversely affected by salinity by the year 2050 (Wang et al., 2003). This represents a serious threat to food production and environmental sustainability especially in arid and semi-arid regions (Paranychianakis and Chartzoulakis, 2005). Moreover, scarcity of water resources together with overpopulation and the increasing water demand for the industrial purposes necessitates the use of saline or low-quality water in agriculture (Chartzoulakis, 2005). Different studies have shown that saline water could be practically used for the irrigation of a variety of horticultural crops such as *Capsicum annuum* (Niu et al., 2010) and *Olea europaea* (Stefanoudaki et al., 2009) without a large reduction in the growth. As genotypes differ in their response to salinity, selecting plants that will survive and maintain economic yield under saline conditions is a potential solution for minimizing the adverse effects of salinity (Ashraf and Harris, 2004). In this regard, different halophyte plants, such as *Crithmum maritimum* and *Cakile maritima* (Ben Amor et al., 2005, 2006) as well as *Trachyspermum ammi* (Ashraf and Orooj, 2006), have been

utilized for cultivation in salt-affected areas. On the other hand, studies have been carried out to examine the feasibility of utilizing different glycophytic plants, such as *Solanum lycopersicum* (Sgherri et al., 2008), *Calendula officinalis* (Fornes et al., 2007), and *Matricaria recutita* (Baghalian et al., 2008) for cultivation under a range of saline conditions.

Echinacea, commonly known as coneflower, is a member of the Asteraceae family comprising of nine species and four varieties, all of which are native to North America (McGregor, 1968). With its large bright deep rose-colored flower heads, *Echinacea purpurea* is commonly grown as cut flowers and landscape perennial (McKeown, 1999). In addition, the roots and aerial parts of three of the species, *E. purpurea*, *E. pallida* and *E. angustifolia* are used commercially as herbal medicines for boosting the immune system and treating common cold (Bauer and Wagner, 1991). Therefore, to fulfill the increasing demand, expansion of *Echinacea* cultivation is required worldwide (Li, 1998). *Echinacea* has been reported to tolerate a wide range of climate and soil environmental conditions (Letchamo et al., 2002) including drought (Chapman and Auge, 1994). However, only few studies have been conducted on the salinity tolerance of the economically important *Echinacea* species. Two studies have compared the salinity tolerance of *E. purpurea* with several other herbaceous perennial plants, and reported either an intermediate tolerance (Zollinger et al., 2007) or a low tolerance (Niu and Rodriguez, 2006) depending on the environmental conditions (temperature and light intensity) in which the plants were grown. In these two studies, plants were exposed to a mixture of NaCl and $CaCl_2$, making it difficult to determine the effect of NaCl on *Echinacea* as calcium has been shown to alleviate some of the adverse

effects of salinity on some plants (Cramer et al., 1985). For the other two species of interest, *E. pallida* and *E. angustifolia*, there is no information in the literature regarding the physiological attributes that may determine their level of salt tolerance.

Considering the magnitude of salinity worldwide and the economical importance of *Echinacea*, the present study was undertaken to evaluate the differential physiological performance of the three major *Echinacea* species under NaCl salinity based on growth, salt injury index, gas exchange, electrolyte leakage (EL), elemental and pigment contents. Moreover, the differential antioxidant activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were studied to determine their contribution to salinity tolerance in *Echinacea* species. Understanding the physiological and biochemical responses of *Echinacea* plants to salinity will be crucial for selecting plants that could grow on soil irrigated with saline water and thus extremely valuable to the horticulture industry.

3.3. Materials and methods

3.3.1. Plant material and hydroponic culture

Seeds of *E. purpurea* (L.) Moench, *E. pallida* (Nutt.) Nutt., and *E. angustifolia* DC were obtained from Richters (Goodwood, Ontario, Canada). Seeds were germinated first in Petri Dishes on filter paper at room temperature (25 ± 5 ^oC) for 2 weeks to overcome difficulties reported for the germination of some *Echinacea* species (Sari et al., 2001). Germinating seeds were initially planted into seedling trays containing a mixture of sand:top soil:peatmoss (2:1:1, v:v:v), and then transferred to 1 L plugs containing the same soil mixture after three months. Plants were grown under the

following greenhouse conditions; 18/6 h photoperiod, average day/night temperature 25/18 ^{o}C and natural light supplemented with sodium lamps (P.L. light systems, Beamsville, ON, Canada). Plants were watered every 2-3 days and fertilized every 3 weeks with a nutrient solution (5 g L^{-1}) containing N:P:K (20:20:20) with micronutrients. At the age of six months, a total of 96 plants from each species were transferred to an aerated half strength modified Hoagland nutrient solution in a hydroponic set-up (Renault et al., 2001). Six plants from each species were placed in 10-L containers, which were distributed in a completely random block design in four replicates. Plants were grown for 4 weeks in the hydroponic system under the above-mentioned conditions for acclimation. Sodium chloride was added to the nutrient solution to reach the concentrations of 0, 50, 75 and 100 mM NaCl, which were equivalent to electrical conductivities of 1.04, 5.50, 7.89, and 9.96 dS m^{-1}, respectively. To avoid osmotic shock, NaCl was added to the nutrient solution in a stepwise fashion by 25 mM increment twice a day until reaching the required concentration. The nutrient solution was aerated continuously by an air pump, and was replaced twice a week to prevent nutrient deficiency. This nutrient solution was composed of 0.5 mM KH_2PO_4; 2.5 mM KNO_3; 2.5 mM $Ca(NO_3)_2$; 1 mM $MgSO_4$; 0.023 mM H_3BO_3; 0.005 mM $MnCl_2$; 0.0004 mM $ZnSO_4$; 0.0002 mM $CuSO_4$; 0.00007 mM H_2MoO_4 in addition to 0.007 mM Fe-EDTA. Plants were harvested after two weeks of treatment, washed three times with distilled water, and separated into shoots and roots. Fresh weights and the number of leaves were immediately recorded, and plants were lyophilized for determining the dry weights. Water content (WC) of shoots and roots was measured using the following formula WC (%) = (FW - DW) x 100/FW.

3.3.2. Gas exchange

Photosynthetic rate, stomatal conductance and transpiration rate were measured after two weeks of exposure to salt treatments. The uppermost fully expanded leaves were selected for measuring gas exchange using an infrared gas analyzer (IRGA, LI-6400, LI-COR, Inc., Lincoln, Nebraska, USA) between 10 am and 3 pm. Gas exchange parameters were measured under photosynthetically active radiation of 400 μmol m^{-2} s^{-1}, atmospheric CO_2 of 400 μmol mol^{-1}, relative humidity of 50%, and temperature of 22 ºC. Areas of fully expanded leaves were measured by a leaf area meter (Model 3000, LI-COR Inc., Nebraska, USA).

3.3.3. Photosynthetic pigments

To determine chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), and carotenoids, 3 - 5 discs (0.8 cm diameter) were cut from the uppermost fully expanded leaves randomly selected from three plants per replicate and subjected to freeze drying process. Discs were homogenized with 2 mL of acetone (80%) and washed two times with an additional 2 mL acetone. The absorbance of the pooled extracts was measured using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, England) at 480, 645, and 663 nm. Quantifications of Chl *a*, Chl *b* and carotenoids in the extracts were determined using MacKinney equations (Sestak et al., 1971):

Chl *a* (mg g^{-1} DW) = (12.72 × A663) - (2.58 × A645)

Chl *b* (mg g^{-1} DW) = (22.87 × A645) - (4.67 × A663)

Carotenoids (mg g^{-1} DW) = (0.114 × A663) + A480 - (0.638 × A645)

3.3.4. Electrolyte leakage (EL)

Electrolyte leakage, an indicator for the extent of membrane damage, was determined in the uppermost fully expanded leaves with no injury symptoms in *Echinacea* species. Three leaf discs (0.9 cm diameter) were collected from 3 plants per replicates for each species, rinsed with deionized water and incubated with10 mL fresh deionized water on a shaker for 5 h. The initial electrical conductivity of this solution (EC1) was measured using a conductivity meter (Fisher Scientific, ON, Canada). To measure the total electrolytes released from leaf tissues, discs were frozen in liquid N_2 and thawed (5 times, 3 min.), then placed in the water solution for an additional 5 h and the conductivity was recorded (EC2). Electrolyte leakage (%) was calculated using the following formula: EL (%) = (EC1/EC2) x 100.

3.3.5. Elemental analysis

Lyophilized shoots and roots were ground separately using a coffee grinder. For chloride determination, ground tissues (1 g from 3 combined plants per replicate) were extracted with 0.5 M HNO_3 for 30 min at room temperature (Rieger and Litvin, 1998), and chloride ion was measured with a chloride selective electrode (Accumet, Fisher Scientific, Canada). Elements, Na^+, K^+, Ca^{2+} were analyzed in the shoot and root dry tissues using an inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian Vista-PRO RL) after digestion with concentrated HNO_3.

3.3.6. Salt injury index

Injury index was determined by evaluating the visual appearance of the leaves using a five point scale according to the severity of necrotic tissues and number of injured

plants as previously described (Zhen et al., 2010). Level 1 represented plants that had no necrotic areas; level 2 (1-25% necrotic areas); level 3 (26-50% necrotic areas); level 4 (51-75% necrotic areas); and level 5 (76-100% necrotic areas or dead plant). The salt injury index was calculated according to the following equation:

Salt injury index =∑ (level of injury x number of plants)/total number of plants.

3.3.7. Extraction of antioxidant enzymes

Total protein and antioxidant enzymes were extracted from frozen leaf and root tissues based on the method of Agarwal et al. (2005). One gram was crushed into a fine powder with liquid nitrogen using pestle and mortar, and then homogenized with 10 mL of 100 mM K- phosphate extraction buffer (pH 7.0) containing 1 mM $EDTA.Na_2$, 1% polyvinylpyrrolidone (PVP), and 5 mM ascorbic acid (AsA). The homogenate was centrifuged at 19,000 × *g* for 20 min at 4ºC, and the supernatant was divided into separate aliquots and stored at -20 ºC until used for enzyme assays. The activities of SOD, CAT, APX, and GR are presented as the mean of three replicates ± SE. Protein content was determined in the crude extract according to the Bradford, (1976) method using bovine serum albumin as a standard.

3.3.8. Antioxidant enzyme assays

Superoxide dismutase (EC 1.15.1.1) activity was determined spectrophotometrically at 560 nm according to the method described by Dionisio-Sese and Tobita, (1998). The activity was assayed in a 3 mL reaction mixture containing 50 mM K- phosphate buffer (pH 7.8), 13 mM $_{L}$-methionine, 75 µM nitoblue tetrazolium (NBT), 0.1mM EDTA, 2 µM riboflavin, and different volumes of the enzyme extract (0-

100 µl). To initiate the reaction, test tubes were exposed to 300 µmol m^{-2} s^{-1} light intensity in aluminum foil-wrapped box. The reaction was stopped by switching the light off after 20 min of incubation. Test tubes developed a gradient color proportional to the volume of enzyme extract being the darkest in test tubes with no enzyme extract due to a maximum reduction of NBT. The other set of test tubes was kept in dark and served as blank. One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition in the photochemical reduction of NBT. Catalase (EC 1.11.1.6) activity was assayed based on the method of Aebi, (1984) by monitoring the disappearance of H_2O_2. The reaction was initiated by adding 50 µl of enzyme extract to 1.5 mL of reaction mixture containing 50 mM K- phosphate buffer (pH 7.0), and 15 mM freshly prepared H_2O_2. The decrease in absorbance was measured at 240 nm for 1 min, and the degradation of 1 µmol H_2O_2 per min was defined as one unit of CAT. Ascorbate peroxidase (EC 1.11.1.11) activity was determined according to the method described by Nakano and Asada, (1981). The assay was initiated by adding 100 µL enzyme extract to 1.6 mL of reaction mixture containing 100 mM K- phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM EDTA.Na_2, and 1.2 mM H_2O_2. The decrease in the absorbance of AsA at 290 nm was monitored every 10 s for 2 min. One unit of APX was defined as the amount of enzyme required for the oxidation of 1 µmol ascorbate per min. Glutathione reductase (EC 1.6.4.2) activity was assayed in 1 mL reaction mixture containing 100 mM K-phosphate buffer (pH 7.0), 1 mM EDTA.Na_2, 0.2 mM NADPH, and 1 mM oxidized glutathione (GSSG) according to a modified method of Fryer et al. (1998). The reaction was initiated by the addition of 70 µL enzyme extract, and the decrease in the absorbance due to NADPH oxidation to NADP was monitored at 340 nm for 2 min at 10 s intervals.

One unit of GR activity was defined as the amount of enzyme that oxidizes 1 µmol NADPH per min at ambient temperature. For CAT, APX and GR, the activities were calculated using the extinction coefficients of H_2O_2 (39.4 mM^{-1} cm^{-1}), ascorbate (2.8 mM^{-1} cm^{-1}) and NADPH (6.22 mM^{-1} cm^{-1}), respectively.

3.3.9. Data analysis

All data were subjected to a two-way ANOVA analysis, and Tukey's HSD test was used to determine the significant differences between means of treatments at a probability level of ≤ 0.05. Pearson correlation analysis was used to study the relationship between different physiological parameters.

3.4. Results

3.4.1. Growth, survival and injury

E. angustifolia showed more leaf injury and reduction in survival rate than *E. purpurea* and *E. pallida* (Table 3.1). *E. angustifolia* also showed a significant reduction in shoot water content at 75 and 100 mM NaCl, while it was not affected in *E. purpurea* and *E. pallida* by salt treatments. *E. purpurea* was the species with the highest root water content in all treatments (Table 3.1). Although the salt treatments did not affect the shoot or root dry weights in any of the three *Echinacea* species studied, the highest salt concentration (100 mM NaCl) significantly reduced the leaf area of *E. purpurea* and *E. pallida* compared to control plants.

3.4.2. Gas exchange and photosynthetic pigments

All salt concentrations reduced the photosynthetic rate in *E. pallida* and *E. angustifolia* (Fig. 3.1), while in *E. purpurea*, the photosynthetic rate was reduced at the moderate and high salt concentrations (75 and 100 mM NaCl). Similar to the photosynthetic rate trend, stomatal conductance and the transpiration rate were decreased at all salt concentrations in *E. angustifolia,* and at 75 and 100 mM NaCl in *E. purpurea*. On the other hand, in *E. pallida*, stomatal conductance was significantly reduced only at 100 mM NaCl with no change in transpiration rate. The highest salt concentration decreased Chl *a*, Chl *b*, and carotenoid contents in *E. purpurea* and *E. angustifolia*, while there were no significant changes in *E. pallida* plants (Table 3.2).

3.4.3. Electrolyte leakage

Electrolyte leakage was increased in all species at 75 and 100 mM NaCl (Fig. 3.2); whereas at 50 mM NaCl, *E. angustifolia* was the only species that showed a four-fold increase in electrolyte leakage compared to the respective control plants.

3.4.4. Elemental analysis

Sodium concentration was increased in the shoots and roots of all *Echinacea* species with increasing salinity concentrations (Tables 3.3 and 3.4). The shoot Na^+ concentration in *E. angustifolia* exposed to 50 and 75 mM NaCl was 2 and 3 times higher than the one in *E. pallida*, and *E. purpurea*, respectively. At 100 mM NaCl, the amount of Na^+ was similar in *E. pallida* and *E. angustifolia*, while it remained 2 times lower in *E. purpurea*. On the contrary, Na^+ content in the roots was about 2 times higher in *E. purpurea* than in *E. pallida* and *E. angustifolia* at all salt concentrations (Table 3.4). As a

result, *E. purpurea* was able to maintain a higher root Na^+/shoot Na^+ ratio than the other two species. Potassium concentration showed no change in shoots or roots of salt-stressed *Echinacea* species, with the exception of *E. purpurea* roots exposed to 100 mM NaCl, where there was a 42% reduction (Table 3.3). *E. angustifolia* was the only species to exhibit a significant reduction in Ca^{2+} concentration in the shoots at all salt concentrations. In contrast, Ca^{2+} content was decreased in the roots of *E. purpurea* and *E. pallida* by the highest salt concentration with no change in *E. angustifolia* (Table 3.4). Salinity caused a significant increase in the Cl^- concentration in both shoots and roots in all *Echinacea* species, with the exception of *E. pallida* exposed to 50 mM NaCl. The concentration of Cl^- was 7 to 8 times higher in the shoots than the roots of all *Echinacea* species exposed to NaCl (Tables 3.3 and 3.4). Overall, *E. angustifolia* and *E. pallida* accumulated the highest and the lowest amount of Cl^-, respectively. In comparison to control plants, the Na^+/K^+ ratio increased in the shoots and roots of salt-stressed *Echinacea* species particularly at 75 and 100 mM NaCl as a result of increased Na^+ concentration. In the shoots, this ratio correlated with salt injury index ($r = 0.919$, $p \leq 0.0001$), EL ($r = 0.879$, $p \leq 0.0001$), photosynthetic rate ($r = -0.695$, $p \leq 0.012$), stomatal conductance ($r = -0.712$, $p \leq 0.009$), and transpiration rate ($r = -0.708$, $p \leq 0.01$). Overall, *E. angustifolia* had the highest shoot Na^+/K^+ ratio, while *E. purpurea* exhibited the lowest ratio at all salt concentrations.

3.4.5. Changes in the antioxidant enzyme activities

Activities of SOD, APX, CAT, and GR in the shoots of the three *Echinacea* species are presented in Table 3.5. Catalase activity was decreased in the shoots of all species studied at both 75 and 100 mM NaCl. At the lowest salt concentration, *E. pallida*

was the only species to show a reduction in CAT activity. *E. purpurea* exposed to different salt concentrations showed a significant increase in SOD activity, while there was a decrease in activity in *E. angustifolia* and no effect in *E. pallida*. Ascorbate peroxidase activity was increased by all salt concentrations in the shoots of *E. purpurea*, whereas the activity was not changed as a result of salinity in *E. pallida.* In *E. angustifolia,* APX activity was reduced only at the highest salt concentration (100 mM NaCl). None of the salt concentrations had an influence on the activity of GR in any of *Echinacea* species (Table 3.5).

3.5. Discussion

Our study showed that photosynthetic activity in *Echinacea* was affected by salinity, and the extent of the reduction was dependent on the intensity of the salts with only small variations at 50 mM NaCl between the species. Zollinger et al. (2007) also found a similar reduction in photosynthetic rate and stomatal conductance in *E. purpurea* irrigated with increasing concentrations of salinity up to 5 g L^{-1}. Reduction in photosynthetic rate under salinity can be attributed to both stomatal and non stomatal limitations (Paranychianakis and Chartzoulakis, 2005). In this study, we found a strong negative correlation between stomatal conductance and both Na^+ ($r = -0.734$, $p \leq 0.007$) and Cl^- ($r = -0.811$, $p \leq 0.001$) concentrations in the shoots of the *Echinacea* species, which indicates that *Echinacea* plants tend to close their stomata as a result of specific-ion accumulation. This reduction in stomatal conductance led subsequently to a reduction in photosynthetic rate as indicated by the high correlation between stomatal conductance and photosynthetic rate ($r = 0.975$, $p \leq 0.005$). However, biochemical limitations, such as inhibition of Rubisco activity and ATP synthesis have been proposed as potential causes

for the reduction in the photosynthetic activity (Reddy et al., 2004). In fact, the relative contribution of stomatal and non stomatal factors to the decline in photosynthetic activity is still a matter of debate (Paranychianakis and Chartzoulakis, 2005). The significant negative correlation between photosynthetic rate and both shoot Na^+ concentration (r = -0.717, $p \leq 0.009$), and shoot Cl^- concentration (r = -0.833, $p \leq 0.001$) suggests that the accumulation of ions was another cause for the reduction in photosynthetic rate of *Echinacea.* In the absence of a sufficient sequestration mechanism into the vacuole, toxic concentrations of Na^+ and Cl^- in the cytosol can have detrimental effects on photosynthetic processes through inhibiting the enzyme activities of Calvin cycle (Parida and Das, 2005). The highest salt concentration (100 mM NaCl) reduced Chl *a*, Chl *b* and carotenoid contents in *E. purpurea* and *E. angustifolia*, and overall in both species, this reduction was correlated with shoot Na^+ content rather than Cl^-, suggesting that Na^+ was the major ion causing pigment reduction. Nevertheless, in other plant species like *Vicia faba*, the decline in the leaf Chl was strictly attributed to Cl^- accumulation in the leaves (Tavakkoli et al., 2010).

Although there were no significant changes in shoot or root DW in response to all salt treatments in *Echinacea* species, based on the reduction pattern of gas exchange parameters, it is most likely that fresh and dry matter accumulation would be adversely affected after a prolonged exposure to salts. In support of this idea, shoot and root dry weights of *E. purpurea* irrigated with a saline solution containing NaCl and $CaCl_2$ up to 5 g L^{-1} for 8 weeks were reduced compared to control plants (Zollinger et al., 2007). It has been shown that leaf area is more sensitive than shoot dry weight in some glycophytes exposed to salinity stress (Chartzoulakis, 2005). This was confirmed in our study as we

observed a decrease in leaf area at the highest salt concentration (100 mM NaCl) in *E. purpurea* and *E. pallida* with no noticeable reduction in plant dry biomass. Reduction in leaf area has been attributed to the decrease in cell division and leaf surface expansion, which occurs at the early stages of exposure to salt stress (Parida and Das, 2005). Nevertheless, reduction in leaf area can be considered an adaptive mechanism for reducing water loss via transpiration from plants (Torrecillas et al., 2003).

Leaf necrosis is considered one of the early symptoms of NaCl injury (Yin et al., 2010). Previous studies have shown the association between salt injury index (SI) and the degree of salt tolerance in *Malus domestica* (Yin et al., 2010) and *Cucumis sativus* (Zhen et al., 2010). In the current study, *E. purpurea* and *E. pallida* showed the lowest degree of injury, while *E. angustifolia* showed the highest degree of injury suggesting that the latter species has a higher degree of sensitivity to salinity. The strong correlation between salt injury index and Na^+ accumulation in *E. purpurea* ($r = 0.975$, $p \leq 0.025$), *E. pallida* ($r = 0.995$, $p \leq 0.005$), and *E. angustifolia* ($r = 0.981$, $p \leq 0.019$) suggests that Na^+ was the major toxic ion causing leaf necrosis in all the three species. However, Cl^- seems to further contribute to the necrosis only in two of the three species, *E. pallida* and *E. angustifolia*, as shown by the significant relationship between Cl^- accumulation and salt injury index ($r = 0.973$, $p \leq 0.027$ and $r = 0.969$, $p \leq 0.031$, respectively). Overall, the concentrations of Na^+ and Cl^- in the shoots and roots of *Echinacea* species are comparable to the values reported in some other species, such as *Ocimum basilicum* (Tarchoune et al., 2011) grown under hydroponic culture and exposed to NaCl salinity for two weeks.

Characteristics such as survival, yield, and the extent of leaf damage are commonly used as criteria for evaluating salt tolerance among different plant genotypes (Ashraf and Harris, 2004). Nevertheless, ion exclusion from shoots has been proposed as a physiological indicator for salinity tolerance in glycophytes (Munns and Tester, 2008). In the current study, Na^+ concentration was increased in both shoots and roots of the three *Echinacea* species studied with increasing salt concentrations in the nutrient solution. Our study shows that *E. purpurea* contained less Na^+ in the shoots than the other two species. Moreover, the ratio of Na^+ in the roots to Na^+ in the shoots was significantly higher in *E. purpurea* than *E. pallida* and *E. angustifolia* at all salt concentrations (Table 3.4). This may imply that *E. purpurea* possesses a distinctive mechanism for excluding Na^+ ions from the shoots presumably by its sequestration into the root vacuoles or limiting its translocation to the shoots (Greenway and Munns, 1980). Retrieval of Na^+ from the root xylem before it reaches the shoots by high-affinity K^+ transporters (KHT) is another possible mechanism for ion exclusion (Munns and Tester, 2008). Similar results were found in *L. esculentum* (Juan et al., 2005), *O. europaea* (Chartzoulakis, 2005), and *C. annuum* (Niu et al., 2010), where the most salt tolerant cultivars were able to control Na^+ accumulation in the shoots. Although the Na^+ content was quite similar in the roots of both species, there was twice as much Na^+ in the shoots of *E. angustifolia* than *E. pallida* at 50 and 75 mM NaCl, which may specify the differences between the two species regarding Na^+ partitioning in shoots and roots. It is widely accepted that maintaining a low Na^+/K^+ ratio in the cytoplasm is associated with the salt tolerance of plants (Munns and Tester, 2008; Li et al., 2010). The high Na^+/K^+ ratio in the shoots of *E. angustifolia* at all salt concentrations supports the idea that this species is more sensitive

to NaCl salinity than the other two species. It is worthnoting that none of the *Echinacea* species studied seems to possess a regulatory mechanism for excluding Cl^- from entering shoots as contrary to *Medicago sativa*, in which salt tolerance was associated with the ability to regulate Cl^- transport to the shoots (Sibole et al., 2003). Excessive amount of Na^+ and Cl^- in the growing medium can also cause competition with other elements, such as K^+, Ca^{2+}, and NO_3^- leading to ion imbalance and/or ion deficiency (Hu and Schmidhalter, 2005). This was illustrated in our study, where Ca^{2+} content was reduced in *E. angustifolia* shoots exposed to salinity. Furthermore, the negative correlation between Ca^{2+} content and EL ($r = -0.991$, $p \leq 0.009$) suggests that Ca^{2+} could have been displaced by Na^+ from the membrane, increasing the leakage of solutes (Cramer et al., 1985).

Oxidative damage to membranes has been reportedly measured by determination of malonyl dialdehyde (MDA) or other ketone fragments (Gill and Tuteja, 2010); however EL can be considered an appropriate index for the stability of membrane and the degree of injury under stress (Li et al., 2010). Maintaining lower EL rate has been associated with salinity tolerance in different plant species (Dionisio-Sese and Tobita, 1998; Ben Amor et al., 2006). In our study, differential response was observed at the low salt concentration (50 mM NaCl), where the relatively salt sensitive species (*E. angustifolia*) showed increased EL compared to the other two species. At the higher salt concentrations (75 and 100 mM NaCl), all species were affected to the same degree, displaying their glycophytic characters. The correlation analysis indicated a significant relationship between EL and both Na^+ ($r = 0.876$, $p \leq 0.001$) and Cl^- ($r = 0.974$, $p \leq 0.001$) contents in the shoots of all the three *Echinacea* species. This may suggest that Na^+ and Cl^- ions were most likely responsible for the disruption in the membrane

integrity and leakage of solutes (Tavakkoli et al., 2010). Excessive salts can stimulate the production of ROS that have the ability to cause oxidative damage to lipid membranes, proteins and nucleic acids (Parida and Das, 2005). It has been mentioned that limited CO_2 fixation encountered under salinity leads to a decrease in carbon reduction by Calvin cycle and subsequently to the generation of $O_2^{\bullet -}$ and other more aggressive oxygen radicals through series of biochemical reactions (Demiral and Türkan, 2005). In the current study, the photosynthetic rate and stomatal conductance were decreased in salt-stressed *Echinacea* species leading likely to the overproduction of ROS and imposing oxidative stress. The equilibrium between the ROS production rate and their scavenging rate by enzymatic and nonenzymatic antioxidants will determine the degree of damage induced by ROS (Gill and Tuteja, 2010). SOD in particular, is the most effective intracellular enzyme mediating the removal of $O_2^{\bullet -}$ radicals to H_2O_2 (Gill and Tuteja, 2010). The enhanced activity of this enzyme in *E. purpurea* and the concomitant decreased activity in *E. angustifolia* implies that the first species had more efficient capacity for the removal of $O_2^{\bullet -}$ radicals, thus reducing its oxidative damage and cellular toxicity than *E. angustifolia*. Similar results have shown that increased SOD activity reduced the oxidative stress and improved salinity tolerance in *C. maritima* (Ben Amor, 2006). H_2O_2 can then be scavenged by CAT and peroxidases to minimize the oxidative stress. APX plays a key role in scavenging H_2O_2 through ascorbate-glutathione cycle in the cytosol and chloroplast, and its enhanced activity has been associated with the tolerance to oxidative stress in various plant species (Chaparzadeh et al., 2004; Ben Amor et al., 2006). In the current study, *E. purpurea* seems to be the only *Echinacea* species capable of quenching H_2O_2 by activating APX, while the activity was diminished in *E.*

angustifolia at 100 mM NaCl, suggesting that the latter species was prone to excessive oxidative stress resulting in increased membrane permeability and EL (Fig. 3.2). On the other hand, we found a decrease in CAT activity in the leaves of all *Echinacea* species in response to salinity. The negative correlation between CAT activity and both Na^+ (r = -0.622, $p \leq 0.031$) and Cl^- (r = -0.622, $p \leq 0.031$) contents suggests that the accumulation of these ions in the leaves could have inhibited the enzyme activity; a result similar to what was found in O. *sativa* (Lee et al., 2001). It is worthnoting that salt-stressed *E. pallida* plants were able to maintain the same levels of SOD and APX activities as the control plants, which may have led to improved protection against ROS compared to *E. angustifolia*. No significant changes were recorded for GR activity in any of the *Echinacea* species exposed to salinity stress; however in other plant species, such as *Vigna radiata* (Nazar et al., 2011) and one accession of *C. maritima* (Ben Amor et al., 2006), the GR activity was enhanced by salinity, which indicates that enzyme activity is species-dependent.

In conclusion, salinity induced several physiological and biochemical changes in *Echinacea* species including reduction in the gas exchange, and pigment contents, as well as increase in the Na^+ and Cl^- accumulation, salt injury index, and electrolyte leakage. This suggests a limited level of salt tolerance in the three *Echinacea* studied as it has been reported previously in one of the *Echinacea* species; *E. purpurea* (Niu and Rodriguez, 2006; Zollinger et al., 2007). However, this study demonstrated some physiological differences in salt tolerance between *Echinacea* species. *E. purpurea,* the species with the highest ornamental value, had a relative salt tolerance up to 50 mM NaCl followed by *E. pallida*, whereas *E. angustifolia* was the most sensitive species. It can be concluded that

the relative salt tolerance of *E. purpurea* was associated with the superior capacity for Na^+ exclusion and increased activities of antioxidant enzymes particularly SOD and APX.

Table 3.1. Growth parameters, survival and salt injury index in *E. purpurea*, *E. pallida* and *E. angustifolia* treated with 0, 50, 75, and 100 mM NaCl for two weeks.

	NaCl (mM)	*E. purpurea*	*E. pallida*	*E. angustifolia*
Shoot DW (g)	0	9.57 ± 0.68[a]	5.98 ± 0.47[bc]	0.98 ± 0.16[d]
	50	9.41 ± 0.64[a]	5.25 ± 0.73[bc]	0.95 ± 0.17[d]
	75	9.51 ± 0.73[a]	4.43 ± 0.19[c]	0.86 ± 0.13[d]
	100	7.25 ± 1.10[ab]	3.82 ± 0.85[c]	0.89 ± 0.05[d]
Root DW (g)	0	4.02 ± 0.21[a]	4.31 ± 0.18[a]	1.32 ± 0.17[b]
	50	4.87 ± 0.33[a]	3.93 ± 0.12[a]	1.22 ± 0.18[b]
	75	4.69 ± 0.30[a]	4.20 ± 0.34[a]	1.02 ± 0.27[b]
	100	3.77 ± 0.55[a]	4.25 ± 0.12[a]	1.06 ± 0.17[b]
Leaf area (cm^2 $plant^{-1}$)	0	102 ± 3[a]	53.2 ± 6.3[cd]	13.3 ± 1.8[f]
	50	102 ± 3[a]	40.1 ± 3.2[de]	11.5 ± 1.4[f]
	75	88 ± 8[ab]	37.2 ± 5.1[de]	9.92 ± 3.7[f]
	100	71± 12[bc]	29.3 ± 5.3[ef]	8.13 ± 1.9[f]
Shoot WC (%)	0	83.0 ± 0.5[a]	84.8 ± 0.4[a]	79.9 ± 0.5[a]
	50	80.7 ± 0.2[a]	84.2 ± 0.3[a]	74.6 ± 2.7[ab]
	75	77.6 ± 0.7[ab]	83.4 ± 0.7[a]	66.1 ± 5.9[bc]
	100	75.4 ± 1.5[ab]	80.8 ± 1.3[a]	61.6 ± 6.7[c]
Root WC (%)	0	86.7 ± 0.5[a]	81.8 ± 0.6[bc]	77.8 ± 1.3[cd]
	50	86.6 ± 0.3[a]	79.0 ± 1.2[cd]	76.5 ± 0.9[d]
	75	86.0 ± 0.3[ab]	78.5 ± 0.9[cd]	80.1 ± 0.8[cd]
	100	86.6 ± 0.3[a]	77.6 ± 1.7[cd]	80.0 ± 1.8[cd]
Survival (%)	0	100 ± 0 [a]	100 ± 0 [a]	95.8 ± 4.2[a]
	50	100 ± 0 [a]	100 ± 0 [a]	70.7 ± 4.2[bc]
	75	100 ± 0 [a]	95.8 ± 4.2[a]	62.4 ± 4.1[c]
	100	87.5 ± 4.2[ab]	83.3 ± 6.8[ab]	54.1 ± 4.1[c]
Salt injury index	0	1.0 ± 0.0[d]	1.0 ± 0.0[d]	1.04 ± 0.04[d]
	50	1.25 ± 0.08[d]	1.42 ± 0.05[cd]	2.38 ± 0.27[b]
	75	1.88 ± 0.14[bcd]	1.59 ± 0.17[bcd]	3.75 ± 0.36[a]
	100	2.42 ± 0.14[b]	2.17 ± 0.21[bc]	3.92 ± 0.29[a]

Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when followed by different letters within the same parameter.

Table 3.2. Pigment content (mg g^{-1} DW) in the leaves of *E. purpurea*, *E. pallida* and *E. angustifolia* treated with 0, 50, 75, and 100 mM NaCl for two weeks.

	NaCl(mM)	*E. purpurea*	*E. pallida*	*E. angustifolia*
Chl *a*	0	3.54 ± 0.07^{a}	2.44 ± 0.13^{bcd}	2.25 ± 0.15^{bcd}
	50	2.95 ± 0.13^{ab}	1.93 ± 0.10^{cde}	1.68 ± 0.13^{de}
	75	2.71 ± 0.22^{abc}	1.96 ± 0.27^{cde}	1.78 ± 0.12^{cde}
	100	2.44 ± 0.32^{bcd}	2.02 ± 0.37^{cde}	1.32 ± 0.19^{e}
Chl *b*	0	1.13 ± 0.04^{a}	0.76 ± 0.04^{bc}	0.81 ± 0.06^{bc}
	50	0.87 ± 0.07^{ab}	0.66 ± 0.07^{bcd}	0.61 ± 0.08^{bcd}
	75	0.83 ± 0.09^{bc}	0.67 ± 0.06^{bcd}	0.54 ± 0.03^{cd}
	100	0.77 ± 0.14^{bc}	0.71 ± 0.12^{bcd}	0.40 ± 0.05^{d}
Carotenoids	0	0.149 ± 0.003^{a}	0.106 ± 0.005^{bcd}	0.106 ± 0.009^{bcd}
	50	0.127 ± 0.005^{ab}	0.095 ± 0.005^{bcde}	0.086 ± 0.007^{cde}
	75	0.116 ± 0.009^{abc}	0.096 ± 0.006^{bcde}	0.077 ± 0.009^{de}
	100	0.11 ± 0.01^{bcd}	0.10 ± 0.02^{bcde}	0.067 ± 0.01^{e}

Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when followed by different letters within the same parameter.

Table 3.3. Shoot elemental content (g Kg^{-1} DW) and Na^+/K^+ ratio of *E. purpurea*, *E. pallida* and *E. angustifolia* treated with 0, 50, 75, and 100 mM NaCl for two weeks.

Element	Species	NaCl (mM)			
		0	50	75	100
Na^+	*E. pur*	0.38 ± 0.16^{f}	8.1 ± 0.7^{ef}	16.1 ± 2.6^{cde}	20.3 ± 1.9^{cde}
	E. pal	0.30 ± 0.07^{f}	14.6 ± 0.9^{de}	22.1 ± 1.6^{cd}	37.2 ± 2.0^{ab}
	E. ang	0.95 ± 0.46^{f}	27.1 ± 1.4^{bc}	43.8 ± 6.1^{a}	40.8 ± 3.9^{a}
K^+	*E. pur*	28.0 ± 0.5^{a}	29.6 ± 0.8^{a}	30.5 ± 1.7^{a}	28.4 ± 1.8^{a}
	E. pal	34.8 ± 1.8^{a}	35.6 ± 2.2^{a}	27.8 ± 3.0^{a}	28.9 ± 2.2^{a}
	E. ang	26.9 ± 1.7^{a}	30.0 ± 1.8^{a}	27.9 ± 1.8^{a}	27.3 ± 1.2^{a}
Ca^{2+}	*E. pur*	25.4 ± 2.0^{ab}	21.9 ± 1.1^{b}	22.2 ± 1.7^{b}	22.2 ± 1.0^{b}
	E. pal	28.2 ± 1.0^{ab}	22.7 ± 0.9^{ab}	20.9 ± 0.5^{b}	21.1 ± 2.3^{b}
	E. ang	30.3 ± 0.8^{a}	22.4 ± 1.9^{b}	20.4 ± 1.1^{b}	20.6 ± 2.6^{b}
Cl^-	*E. pur*	8.2 ± 1.8^{d}	38.4 ± 6.0^{bc}	49.6 ± 9.7^{abc}	55.9 ± 8.1^{ab}
	E. pal	5.2 ± 1.1^{d}	25.1 ± 1.9^{cd}	44.9 ± 8.5^{abc}	61.7 ± 6.9^{ab}
	E. ang	6.6 ± 1.0^{d}	46.2 ± 2.5^{abc}	65.6 ± 3.7^{a}	61.9 ± 4.1^{ab}
Na^+/K^+	*E. pur*	0.01 ± 0.01^{f}	0.27 ± 0.03^{ef}	0.54 ± 0.10^{cde}	0.72 ± 0.09^{cd}
	E. pal	0.01 ± 0.003^{f}	0.42 ± 0.05^{def}	0.82 ± 0.08^{cd}	1.30 ± 0.06^{ab}
	E. ang	0.04 ± 0.02^{f}	0.91 ± 0.04^{bc}	1.58 ± 0.21^{a}	1.50 ± 0.13^{a}

Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when followed by different letters within the same parameter. *E. pur* (*E. purpurea*); *E. pal* (*E. pallida*); *E. ang* (*E. angustifolia*).

Table 3.4. Root elemental content (g Kg^{-1} DW), Na^+/K^+ ratio and Na^+root/ Na^+shoot of *E. purpurea*, *E. pallida* and *E. angustifolia* treated with 0, 50, 75, and 100 mM NaCl for two weeks.

Element	Species	NaCl (mM)			
		0	50	75	100
Na^+	*E. pur*	0.12 ± 0.03^{f}	13.8 ± 0.6^{b}	16.3 ± 1.9^{b}	23.5 ± 0.6^{a}
	E. pal	0.10 ± 0.01^{f}	7.40 ± 0.82^{de}	7.66 ± 0.66^{de}	9.76 ±1.66^{cde}
	E. ang	0.22 ± 0.13^{f}	6.24 ± 0.57^{e}	7.82 ± 0.53^{d}	11.1 ± 1.5^{cd}
K^+	*E. pur*	18.4 ± 0.2^{a}	14.5 ± 1.5^{ab}	14.2 ± 1.2^{ab}	10.7 ± 1.0^{bc}
	E. pal	14.7 ± 1.1^{ab}	11.5 ± 1.0^{bc}	10.7 ± 0.2^{bc}	10.7 ± 0.4^{bc}
	E. ang	10.5 ± 1.7^{bc}	9.0 ± 0.7^{c}	9.0 ± 0.7^{c}	8.4 ± 0.5^{c}
Ca^{2+}	*E. pur*	5.79 ± 0.32^{a}	5.50 ± 0.38^{ab}	4.78 ± 0.16^{ab}	4.48 ± 0.14^{bc}
	E. pal	3.40 ± 0.08^{cde}	2.83 ± 0.32^{def}	2.38 ± 0.12^{ef}	2.26 ± 0.27^{f}
	E. ang	3.59 ± 0.23^{cd}	2.92 ± 0.06^{def}	2.94 ± 0.27^{def}	2.64 ± 0.06^{def}
Cl^-	*E. pur*	1.11 ± 0.30^{def}	5.72 ± 0.99^{abc}	5.21 ± 0.6^{abc}	7.55 ± 1.6^{ab}
	E. pal	0.26 ± 0.044^{f}	3.90 ± 0.54^{cde}	5.60 ± 0.69^{abc}	7.84 ± 0.87^{ab}
	E. ang	0.53 ± 0.10^{ef}	4.66 ± 0.55^{bcd}	6.89 ± 0.78^{abc}	8.31 ± 0.4^{a}
Na^+/K^+	*E. pur*	0.006 ± 0.002^{d}	0.99 ± 0.15^{bc}	1.16 ± 0.13^{bc}	2.26 ± 0.25^{a}
	E. pal	0.007 ± 0.001^{d}	0.66 ± 0.10^{cd}	0.72 ± 0.08^{bc}	0.91 ± 0.16^{bc}
	E. ang	0.023 ± 0.013^{d}	0.69 ± 0.01^{c}	0.87 ± 0.03^{bc}	1.36 ± 0.28^{b}
Na^+root/	*E. pur*	0.40 ± 0.09^{c}	1.76 ± 0.25^{a}	1.08 ± 0.17^{b}	1.18 ± 0.10^{b}
Na^+shoot	*E. pal*	0.41 ± 0.08^{c}	0.53 ± 0.09^{c}	0.35 ± 0.01^{c}	0.26 ± 0.04^{c}
	E. ang	0.29 ± 0.12^{c}	0.23 ± 0.01^{c}	0.19 ± 0.03^{c}	0.29 ± 0.07^{c}

Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when followed by different letters within the same parameter. *E. pur* (*E. purpurea*); *E. pal* (*E. pallida*); *E. ang* (*E. angustifolia*).

Table 3.5. Activities of CAT, SOD, APX and GR in the leaves of *E. purpurea*, *E. pallida* and *E. angustifolia* treated with 0, 50, 75, and 100 mM NaCl for two weeks.

NaCl (mM)	*E. purpurea*	*E. pallida*	*E. angustifolia*
		CAT (μmol min^{-1} mg^{-1}protein)	
0	102.5 ± 8.9^{abc}	116.5 ± 11.9^{ab}	131.1 ± 3.6^{a}
50	128.9 ± 12.6^{a}	76.9 ± 5.3^{cde}	134.4 ± 4.9^{a}
75	$89.0 \pm 5.2bc^{d}$	56.9 ± 4.4^{de}	76.0 ± 6.5^{cde}
100	57.7 ± 4.9^{de}	48.2 ± 4.2^{e}	58.4 ± 5.2^{de}
		SOD (unit mg^{-1} protein)	
0	37.6 ± 2.2^{bcd}	44.4 ± 2.9^{bc}	46.6 ± 3.0^{b}
50	72.7 ± 2.3^{a}	40.1 ± 3.1^{bcd}	28.4 ± 3.7^{de}
75	84.4 ± 4.0^{a}	38.3 ± 2.5^{bcd}	29.6 ± 1.0^{de}
100	76.1 ± 3.8^{a}	31.6 ± 1.4^{cde}	21.1 ± 0.6^{e}
		APX(μmol min^{-1} mg^{-1} protein)	
0	0.316 ± 0.028^{de}	0.344 ± 0.049^{cde}	0.468 ± 0.027^{bcd}
50	0.589 ± 0.033^{abc}	0.449 ± 0.024^{bcd}	0.384 ± 0.035^{cde}
75	0.692 ± 0.128^{ab}	0.329 ± 0.044^{de}	0.307 ± 0.025^{de}
100	0.734 ± 0.015^{a}	0.400 ± 0.038^{cde}	0.177 ± 0.032^{e}
		GR (μmol min^{-1} mg^{-1} protein)	
0	0.146 ± 0.026^{ab}	0.114 ± 0.015^{ab}	0.088 ± 0.008^{b}
50	0.151 ± 0.009^{ab}	0.169 ± 0.027^{ab}	0.166 ± 0.014^{ab}
75	0.170 ± 0.021^{ab}	0.192 ± 0.025^{a}	0.136 ± 0.007^{ab}
100	0.149 ± 0.020^{ab}	0.123 ± 0.001^{ab}	0.140 ± 0.012^{ab}

Values are the mean of 3 replicates ± S.E. Means are considered different at $p \leq 0.05$ when followed by different letters within the same enzyme.

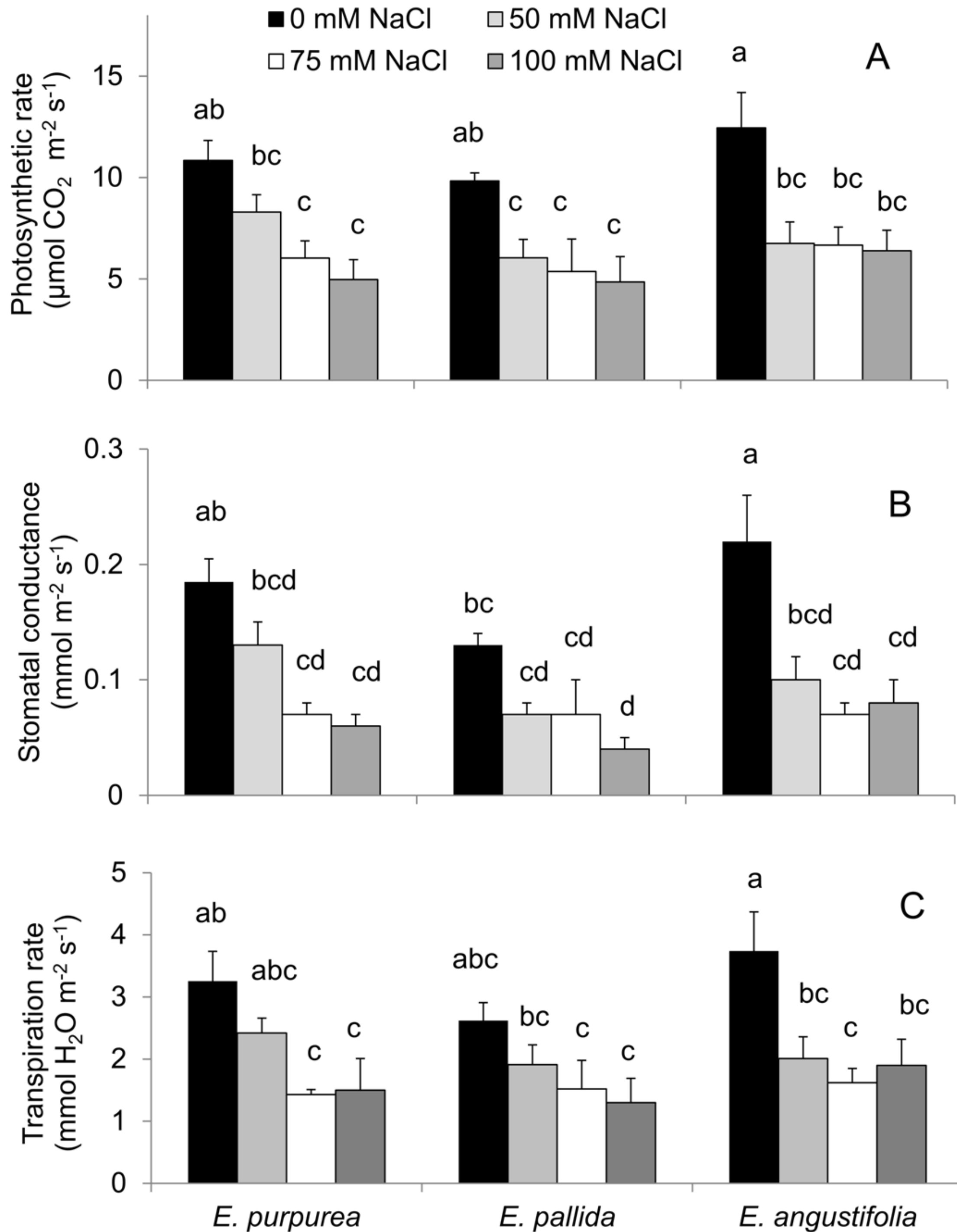

Fig. 3.1. Photosynthetic rate (A), stomatal conductance (B), and transpiration rate (C) of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when followed by different letters within the parameter measured.

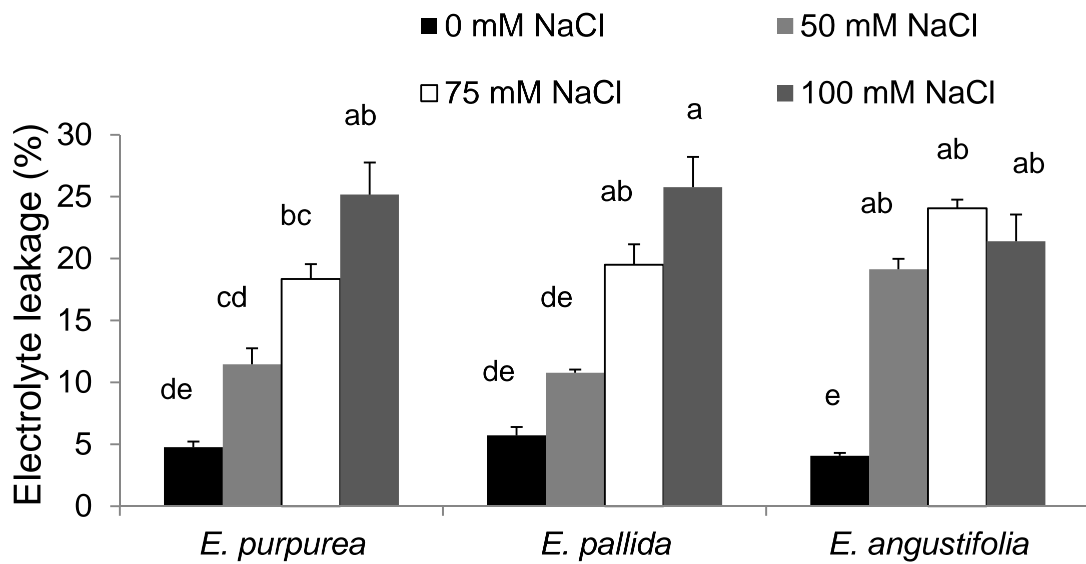

Fig 3.2. Electrolyte leakage (%) of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for two weeks. Values are the mean of 4 replicates ± S.E. Means are considered different at $p \leq 0.05$ when followed by different letters within the parameter measured.

CHAPTER 4. SALINITY-INDUCED CHANGES IN CAFFEIC ACID DERIVATIVES, ALKAMIDES AND KETONES IN THREE *ECHINACEA* SPECIES*

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4.1. Abstract

A hydroponic study was carried out to investigate the changes in caffeic acid derivatives, alkamides and ketones in three *Echinacea* species in response to salinity stress (0, 50, 75 and 100 mM NaCl). These hydrophilic and lipophilic compounds were extracted from the roots by Accelerated Solvent Extractor and analyzed simultaneously by RP-HPLC. The characteristic phytochemical profile of caffeic acid derivatives, alkamides and/or ketones was not affected by salinity. However, significant changes in their relative amount were found depending on the species and salinity intensity. Two of the *Echinacea* species studied, *Echinacea purpurea* and *Echinacea angustifolia* exposed to salinity up to 75 mM NaCl for two weeks, had higher amount of caftaric acid, cynarin, and cichoric acid (*E. purpurea*) as well as chlorogenic acid, cynarin, echinacoside, caffeic acid and alkamides 12, 13, 14/15, 16, 17, 8/9, 18 (*E. angustifolia*) than the respective control plants. In contrast, in *Echinacea pallida*, caftaric acid, echinacoside, and major ketones 22, 24, 25 and pentadeca-8*Z*, 11*Z*-dien-2-one levels were reduced at 50 and/or 75 mM NaCl. The highest concentration of salt (100 mM NaCl) reduced the level of cynarin, cichoric acid, cichoric acid derivative and alkamides 1, 3, 6, 7, 8/9 in *E.*

purpurea and caftaric acid, alkamide 2, ketones 24, 25 in *E. pallida*, as well as alkamides 1, 2, 11 in *E. angustifolia*. Our results suggest that the quality of the raw material obtained from both *E. purpurea* and *E. angustifolia* exposed to salinity up to 75 mM NaCl would be higher than the one obtained from *E. pallida* as the plants retained most of their phytochemical marker compounds.

Keywords: *Echinacea*; NaCl salinity; Caffeic acid derivatives; Alkamides; Ketones

4.2. Introduction

The genus *Echinacea*, from the Asteraceae family, is a small genus of plants consisting of nine known species, all native to North America (McGregor, 1968; McKeown, 1999). Preparations from three of these species, *Echinacea purpurea* (L.) Moench, *Echinacea pallida* (Nutt.) Nutt., and *Echinacea angustifolia* DC, are widely used as herbal medicines and food supplements for boosting the non-specific immune system and for treating common cold, wounds, and upper respiratory tract infections (Bauer and Wagner, 1991). A wide array of chemical compounds has been identified in *Echinacea* species as putative active constituents including caffeic acid derivatives, alkamides/polyacetylenes, polysaccharides, glycoproteins and essential oils (Bauer, 1999). However, most of the biological activities and immunomodulatory properties of *Echinacea* have been attributed to caffeic acid derivatives and alkamides (Pietta et al., 2004). Among the caffeic acid derivatives, cichoric acid is the major phenolic compound in *E. purpurea*, while echinacoside is the main caffeoyl conjugate in *E. pallida* and *E. angustifolia* (Perry et al., 2001). Alkamides (amides of fatty acids) are the major lipophilic constituents in both *E. purpurea* and *E. angustifolia*, whereas polyacetylenes

(ketones) are mainly found in *E. pallida* (Bauer, 1999). Although not one single compound is responsible for *Echinacea* efficacy, it is well accepted that echinacoside, cichoric acid and alkamides are considered suitable marker compounds for the standardization of most *Echinacea*-derived products (Hall, 2003; Wölkart et al., 2004).

Commercial preparations from the roots and aerial parts of *Echinacea* are currently among the best-selling over-the-counter herbal medicines in North America and Europe with an annual retail sale exceeding $300 million in the U.S. alone (Yu and Kaarlas, 2004). The large portion of *E. purpurea* raw materials is produced from cultivated sources, while *E. pallida* and *E. angustifolia* raw materials are primarily collected from the wild (Letchamo et al., 2002). To meet the increasing demand for the pharmaceutical industry, expansion in the cultivation of the three commonly used *Echinacea* species has been steadily increased throughout Australia, South America, North Africa, and China (Yu and Kaarlas, 2004). However, it has been shown that various environmental factors such as geographical location (Letchamo et al., 2002) and drought (Gray et al., 2003) can contribute to the variability in the phytochemical content of *Echinacea*, thus affecting the quality of the raw materials.

Salinity is one of the major environmental factors that reduce the productivity of many crops worldwide as a result of imposing osmotic stress, ionic toxicity, nutritional imbalance and oxidative stress conditions (Parida and Das, 2005). It is estimated that 20% of world arable land is adversely affected by salinity (Flowers and Yeo, 1995). Furthermore, diluted saline water has been successfully used to irrigate some plants (Sgherri et al., 2008). An increase in phenolic compounds in response to salinity stress has been reported in some plants such as *Cakile maritima* (Ksouri et al., 2007), *Capsicum*

annuum (Navarro et al., 2006), and *Morus alba* (Agastian et al., 2000). In addition, it has been suggested that applying mild environmental stresses, such as drought, could be used as an efficient strategy for enhancing the accumulation of some phenolic compounds and improving the product quality in *Echinacea purpurea* (Gray et al., 2003), *Crataegus* spp (Kirakosyan et al., 2004), *Hypericum brasiliense* (de Abreu and Mazzafera, 2005) and *Hypericum perforatum* (Zobayed et al., 2007). Controlled irrigation with diluted sea water has also been shown to increase the level of chlorogenic acid in *Solanum lycopersicum* (Sgherri et al., 2008). Nevertheless, much less is known about the variations in the hydrophilic and lipophilic active constituents in *Echinacea* species under saline conditions; only one species, *E. angustifolia* has been studied. Montanari et al. (2008) focussed on the hydrophilic compounds (caffeic acid derivatives) and found an increase in chlorogenic acid and cichoric acid contents in the roots of salt-treated plants. To the best of our knowledge, the response of alkamides and ketones to salinity stress has not been investigated yet.

The current study was conducted to investigate the effects of salinity on both the hydrophilic and lipophilic active constituents in the three *Echinacea* species. We hypothesized that salinity stress will enhance the production of some secondary compounds depending on the salt concentration used. Specifically, we determined the qualitative and quantitative changes in the caffeic acid derivatives, the alkamides and ketones in response to NaCl salinity. Such putative changes may have significant relevance to the quality and biological activities of the preparations derived from salt-stressed *Echinacea* species.

4.3. Materials and methods

4.3.1. Plant material and hydroponic set-up

Seeds of *E. purpurea*, *E. pallida* and *E. angustifolia* were purchased from Richters (Goodwood, ON, Canada). Due to the difficulties reported for seed germination of *E. angustifolia* and *E. pallida* (Sari et al., 2001), seeds from the 3 species were germinated first in Petri Dishes on filter paper at room temperature for 2 weeks, and then transplanted into seedling trays filled with a mixture of sand:top soil:peatmoss (2:1:1, v:v:v) for growth under the following greenhouse conditions; 18/6 h light and dark regime supplemented by 400W high-pressure sodium lamps (P.L. light systems, Beamsville, ON, Canada) and average temperature $25^{o}C \pm 5$. Plants were watered every 2-3 days and fertilized with a nutrient solution ($5\ g\ L^{-1}$) containing N:P:K (20:20:20) with micronutrients every 3 weeks. Three-month-old seedlings were transferred into 1 L plugs containing the same soil mixture for an additional 3 month period to allow for high biomass and phytochemical accumulation in the roots (El-Gengaihi et al., 1998). Following this period of growth, a total of 96 plants from each species were transplanted to an aerated modified half strength Hoagland nutrient solution in a hydroponic set-up (Hoagland and Arnon, 1950; Renault et al., 2001). The nutrient solution was composed of 0.5 mM KH_2PO_4, 2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 23 µM H_3BO_3, 5 µM $MnCl_2$, 0.4 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.07 µM H_2MoO_4 in addition to 7 µM Fe-EDTA. For each species, 6 plants per treatment were placed in one container (10 L). These containers were distributed in a complete random block design with a total of four replicates. After acclimatizing the plants for 4 weeks in the hydroponic system, the nutrient solution was supplemented with 0, 50, 75 and 100 mM NaCl to reach electrical

conductivity equivalent to 1.04, 5.50, 7.89, and 9.96 dS m^{-1}, respectively. To avoid osmotic shock, the salt solution was added progressively (25 mM every 12 h). The nutrient solution was replaced twice a week to prevent nutrient depletion. Two plants per replicates, from each species in each treatment were harvested after two weeks of treatment, washed, immediately dipped in liquid nitrogen and stored at -80 ^{o}C until used for the freeze-drying process. Root dry weights were recorded.

4.3.2. Sample preparation and extraction of hydrophilic and lipophilic compounds

Freeze-dried roots of two harvested plants were combined and ground into powder using a commercial coffee grinder, then stored at -20 ^{o}C. For the recovery of both hydrophilic and lipophilic fractions from the roots of *Echinacea* species, one-gram sample was extracted with 70% methanol in water (70:30, v:v) using an Accelerated Solvent Extractor (ASE 200, Dionex, CA, USA) according to the method described by Hudson et al. (2005). The extraction procedures were accomplished in 3 static cycles according to the following conditions: 11 mL cell size, 5 min for each static cycle, 70% flush volume, 100 s purge time, and 40°C solvent temperature. The volume of the extract was made up to 25 mL using methanol 70% to give a concentration equivalent to 0.04 g root mL^{-1}. Two mL aliquots were filtered with 0.45 µm PTFE filters (Fisher Scientific, Canada) and stored at -20 ^{o}C. Subsamples of 120 µL were transferred to vial inserts prior to HPLC analysis.

4.3.3. Chemicals and standards

Phenolic standards (caftaric acid, chlorogenic acid, cynarin, echinacoside and cichoric acid) as well as alkamide standards (dodeca-2*E*, 4*E*-dienoic acid isobutylamide,

alkamide 11; undeca-2*E* -ene-8, 10-diynoic acid isobutylamide, alkamide 12; dodeca-2*E*-ene-8,10-diynoic acid isobutylamide, alkamide 14 were purchased from Chromadex (Santa Ana, CA, USA). Caffeic acid and *o*-phosphoric acid (85%) were from Sigma-Aldrich (ON, Canada). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (ON, Canada). Water was purified using a Milli-Q Plus 185 system from Millipore (Milford, MA, USA).

4.3.4. High-performance liquid chromatography (HPLC-DAD) analysis

Simultaneous analysis of the hydrophilic and lipophilic constituents in the root extracts of *Echinacea* species was based on the method reported by Luo et al. (2003). This HPLC method has the advantage to reduce the time and sample size required for quality control analysis (Luo et al., 2003). A Waters 2695 HPLC module (Waters, Milford, MA) equipped with an autosampler, vacuum degasser, pump, and a Waters 996 photodiode array detector (DAD) was used for chromatographic analysis. Separation of compounds was performed on a reverse-phase 5 µm 250-4 LiChrospher®100 RP-18 column (LiChroCART®4-4, Germany) attached to a 5 µm LiChrospher®100 RP-18 guard column (LiChroCART®4-4, Germany) to maintain the integrity of the analytical column. The mobile phase consisted of eluent A: Millipore water acidified with 0.1 % *o*-phosphoric acid (85%), and eluent B: acetonitrile. A linear gradient elution with attenuated flow rate was run over 50 min as follows: 0-13 min, 10-22% B; 13-14 min, 22-40% B; 14.5-39 min, 40-80% B; 39-42 min, 80-100% B; 42-50 min, 100-10% B. The flow rate was 1.5 mL min^{-1} from 0 to 14.5 min and 1 mL min $^{-1}$ from 15 to 50 min. Three successive injections were run for each sample or standard material, with 20 µL in each injection. Chromatograms were displayed and integrated using Millenium Software,

version 3.2. Detection and quantification of caffeic acid derivatives and alkamides/ketones were done using the UV maxplot absorbance in the range 200-400 nm.

4.3.5. Identification of caffeic acid derivatives (CADs), alkamides, and ketones

The hydrophilic components were eluted in the first 20 min of the chromatogram, followed by the lipophilic components (between 20 and 50 min), which reflects their degree of polarity on the reversed phase column. Identification of the extracted compounds was based on comparing their retention times and UV spectra (Figs. 8.8-8.14 in Appendix D) with those of the standards, in parallel with the previously published data (Bauer et al., 1987; Bauer and Remiger, 1989; Sloley et al., 2001; Supplementary Table 4.1). Root extracts were spiked with known amounts of reference standards to further confirm the identity of phenolic compounds and the three alkamides, whose standards were commercially available. We identified most of the alkamides and ketones in *Echinacea* species using HPLC fractionation followed by GC-MS analysis. In preparative HPLC, 50 μL of the methanolic extract were injected and analyzed using the same gradient described above. Individual eluted peaks collected manually from 2 to 5 HPLC runs were pooled together fractionwise and kept overnight at -20 °C. The upper phase (acetonitrile) was transferred to new glass vials and evaporated under a stream of gaseous N_2 until dryness. The residue was suspended in 80 μL of methylene chloride (CH_2Cl_2), vortexed gently, and then 1 μL was subjected to GC-MS analysis in splitless mode. A Varian CP-3800 Gas Chromatograph coupled with a Varian 320-TQ Mass Spectrometer operating in electron impact (EI) mode and ionization voltage 70 eV was used. Separation of lipophilic compounds was carried out on a capillary column VF-5 ms (30 m, 0.25 mm, 0.25 μm) using a gradient program with initial oven temperature at 80°C,

held for 4 min, then ramped up to 270 °C at a rate of 20 °C min^{-1} and maintained for 20 min. Helium was the carrier gas with a constant flow rate of 3 mL min^{-1}, and a total running time 33.5 min. Data were integrated using Varian MS WorkStation Software 6.91, and mass spectra were acquired in the range of 50-400 amu with 3 min collection delay in full scan mode (scan time 0.228 sec). Obtained mass spectra of major alkamides and ketones (Fig. S4.2 and Figs 8.15 and 8.16 in Appendix E) were compared with the NIST mass spectral database and with those previously published in the literature (Bauer et al., 1988b, 1989; Pellati et al., 2006, 2007).

4.3.6. Quantitative determination of hydrophilic and lipophilic compounds

A stock standard solution (10 mg mL^{-1}) was prepared with methanol from each reference standard (caftaric acid, chlorogenic acid, cynarin, echinacoside, cichoric acid, alkamide 11, alkamide 12, and alkamide 14). A series of successive dilutions using the same solvent was made to reach the required range of concentrations for each standard compound. Standard curves were established by plotting different concentrations of the standard material against their peak areas, and the obtained linear regression equation (Figs. 8.1-8.5 in Appendix A) was used to quantify the corresponding caffeic acid derivative or alkamide in the sample root extract. The linearity of the calibration curves ($R^2 > 0.9962$) was achieved in the range of 20-400 µg mL^{-1} for caftaric acid, chlorogenic acid, cynarin, and cichoric acid; from 50 to 500 µg mL^{-1} for echinacoside, and from 10 to 100 µg mL^{-1} for caffeic acid, alkamides 11, 12, and 14. Phenolic compounds with no available external standards were quantified as chlorogenic acid equivalents. Similarly, alkamides and ketones with no standards were quantified using the response factor of alkamide 11 and expressed as alkamide 11 equivalents (mg g^{-1} root DW). Yield of

selected marker compounds was determined by multiplying the content (mg g^{-1} DW) by the dry root biomass (g) per plant.

4.3.7. Data analysis

Quantitative levels of hydrophilic and lipophilic compounds were analyzed by a general linear model (GLM) using SPSS program (version 16, SPSS Inc., Chicago, IL, USA). One-way ANOVA and Duncan's test were used to compare the significance between the means of treatments. Each value represents the mean of three HPLC successive injections in four replicates. Means with different letters are significantly ($p < 0.05$) different.

4.4. Results

4.4.1. Caffeic acid derivatives

The chromatographic fingerprint of the CADs in each species was similar in control and salt-treated plants (data not shown). Five phenolic compounds: caftaric acid, chlorogenic acid, cynarin, cichoric acid and a cichoric acid derivative (2-caffeoyl-3-feruloyl tartaric acid) were identified in the methanolic extract of *E. purpurea* (Supplementary Fig. 4.1A and Table 4.1). Cichoric acid was quantified as the principal CAD in the methanolic extract, and its level was increased at 75 mM NaCl, while it was reduced at 100 mM NaCl (Table 4.1). Cichoric acid derivative was the second most abundant CAD, followed by caftaric acid, while chlorogenic acid and cynarin were only minor constituents. Caftaric acid content was increased at all salt concentrations. Chlorogenic acid content did not change in plants treated with different salt concentrations, while cynarin and the cichoric acid derivative were reduced at 100 mM

NaCl; resulting in a 18.7% decrease in total CADs content as compared to control plants. Caftaric acid, echinacoside, cichoric acid and 6-*O*-caffeoylechinacoside were identified in the methanolic extract of *E. pallida* roots (Supplementary Fig. 4.1B and Table 4.1). Echinacoside constituted more than half of the total CADs content in *E. pallida* control plants, followed by 6-*O*-caffeoylechinacoside, a characteristic caffeic acid glycoside in *E. pallida* (Table 4.1). Echinacoside content was reduced by 75 mM NaCl, while caftaric acid, the second major phenolic in *E. pallida*, was reduced at all salt concentrations. On the other hand, increases were recorded for cichoric acid and 6-*O*-caffeoylechinacoside at 100 mM NaCl. Overall, in spite of some relative changes in individual CADs, the total phenolic content was not affected by different NaCl concentrations in this species (Table 4.1). In the root extract of *E. angustifolia*, five caffeoyl conjugates namely chlorogenic acid, cynarin, echinacoside, caffeic acid, and cichoric acid were identified (Supplementary Fig. 4.1C and Table 4.1). Cynarin and echinacoside were the two major phenolic compounds. On the other hand, cichoric acid was detected in trace amounts and was not quantifiable in *E. angustifolia.* Salinity-induced increases in cynarin and echinacoside were observed in plants treated with 50 and 75 mM NaCl, respectively (Table 4.1). Caffeic acid content increased by more than 50% at all salt concentrations, while chlorogenic acid content was only enhanced at 50 mM NaCl. The total CADs content increased by 29.8 and 19.4% at 50 and 75 mM NaCl treatments, respectively.

4.4.2. Alkamides and ketones

The chromatographic fingerprint of the lipophilic compounds in each species was similar in control and salt-treated plants (data not shown). In *E. purpurea* root extract, we identified 8 alkamides (Supplementary Fig. 4.1A, and 4.2, Table 4.1) including alkamide

1, alkamide 2, alkamide 3, alkamide 4, alkamide 6, alkamide 7, alkamides 8/9, and dodeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide (Chen alkamide), which was identified as a new alkamide by Chen et al. (2005). The isomeric pair 8/9 was quantitatively determined as the major type of alkamides (Table 4.2). Alkamide 2 and alkamide 3 were the second major alkamides found in the root extract of *E. purpurea*. Other lipophilic constituents (alkamide 1, alkamide 6, alkamide 7 and Chen alkamide) were quantified as minor components (< 1.0 mg g^{-1} root DW). The major isomer mixture of alkamides 8/9 was not altered by low or moderate salt concentrations (50 or 75 mM NaCl), but decreased at 100 mM NaCl (Table 4.2). This high level of salinity caused a significant decrease in most of the identified alkamides, with the exception of alkamide 2, resulting in a decrease in the total amount of alkamides. In *E. pallida* methanolic extract, we identified four polyacetylenic compounds (ketone 20, ketone 21, ketone 22, and ketone 23) and four polyenes (ketone 24, ketone 25, 8-hydroxypentadeca-9*E*, 13*Z*-dien-11-yn-2-one, and pentadeca-8*Z*, 11*Z*-dien-2-one), in addition to pentadeca-8Z-en-2-one and 3 types of alkamides (alkamide 2, alkamide 3 and Chen alkamide) presented in Supplementary Table 4.1 and Figs. 4.1B and 4.2. Although salinity stress did not affect the total alkamide/ketone content in *E. pallida* (Table 4.2); relative changes occurred in individual compounds. Among the lipophilic compounds quantified, ketone 22 and ketone 24 were the two major compounds constituting 19.1 and 17.7% from the total lipophilic fraction, respectively in the control plants (Table 4.2). The level of these two compounds was reduced by salinity stress, with the exception of 100 mM NaCl for ketone 22. Alkamide 2 followed a similar pattern to the major lipophilic compounds, while ketone 20, ketone 21 and pentadeca-8*Z*-en-2-one (peak 40) increased substantially

at most salt concentrations with the exception of 75 mM NaCl for peak 40. The lipophilic fraction of *E. angustifolia* is characterized by the occurrence of the monoene-type isobutylamides (Bauer et al., 1989). In our study, 13 alkamides most of which with a monoene structure were identified in the root extract of *E. angustifolia* (Supplementary Figs. S4.1C and S4.2, Table 4.1). The lipophilic fraction was predominated by the tetraene alkamides 8/9 which constituted 51.4% of the total lipophilic constituents (Table 4.2). The level of this main constituent showed an increase at 50 mM NaCl. Other alkamides, such as 10, 14/15, 13, 12, 11, and 18 comprised 3.9-10.8% of the total lipophilic content, whereas alkamides 16, 17, and 19 were quantified as minor constituents (≤ 1.0%). Alkamides 1 and 2 with a 2, 4-diene moiety characterized for *E. purpurea*, were also detected as minor constituents in the root extract of *E. angustifolia*. While the levels of alkamides 12, 13, 14/15, 16, 17, and 18 were increased at the lowest level of salinity (50 mM NaCl); the higher salt concentrations (75 and/or100 mM NaCl) reduced alkamide 1, alkamide 2, and alkamide 11 contents. Overall, the total amount of alkamides was not significantly ($p < 0.05$) affected by any of the tested salt treatments in *E. angustifolia* (Table 4.2).

4.4.3. Root biomass and yield of marker compounds

There was no significant effect of NaCl on the root biomass of the three *Echinacea* species (Table 4.3). In *E. purpurea,* cynarin yield was significantly increased at 50 mM NaCl compared to control plants. Similarly, cichoric acid yield showed an increase at 75 mM NaCl. This species was also able to maintain alkamides 8/9 yield at 50 and 75 mM NaCl at a similar level to control plants. However, at the highest salt concentration (100 mM NaCl), alkamides 8/9 yield was decreased (Table 4.3). In *E.*

pallida, although cichoric acid yield was higher at moderate and high salt concentrations than control plants, the yield of echinacoside, the major caffeic acid glucoside, showed a decrease at low and moderate salt concentrations. There was no change in the yield of the main compounds, cynarin, echinacoside and alkamides 8/9 in *E. angustifolia*.

4.5. Discussion

We identified CADs, alkamides and ketones characteristic to the three *Echinacea* species studied (Binns et al., 2002; Letchamo et al., 2002; Gray et al., 2003; Pellati et al., 2004). Three alkamides namely alkamide 2, alkamide 3, and Chen alkamide that are known to be characteristic to *E. purpurea* (Bauer et al., 1988b) were identified in *E. pallida*. These results confirm the occurrence of alkamides as minor constituents in the root extract of *E. pallida*, a finding that has been reported previously in a limited number of studies (Binns et al., 2002; Kraus et al., 2006).

Our results indicate that although salinity did not affect the profile of the CADs, alkamides or ketones, relative changes in the level of some of these hydrophilic and lipophilic compounds occurred in the roots of three *Echinacea* species. The response was dependent on the level of salinity, a result which agrees with the work of López - Berenguer et al., (2009) on *Brassica oleracea* var. *italica*. In our experiment, low and/or moderate salt concentrations (50 and/or 75 mM NaCl) enhanced the contents of chlorogenic acid, cynarin, echinacoside, and their metabolic precursor caffeic acid in *E. angustifolia,* resulting in a significant increase in the total amount of CADs. An increase in the level of caftaric acid, cynarin, and cichoric acid was also recorded in *E. purpurea*. These results show similar trend to other studies by Gray et al. (2003) in which cichoric

acid was enhanced by drought stress and by Kuzel et al. (2009) where the application of a plant stress hormone (salicylic acid) caused a substantial increase in cichoric acid, caftaric acid and chlorogenic acid in *E. purpurea* roots. Montanari et al. (2008) also found enhanced accumulation of cichoric acid and chlorogenic acid in the roots of salt-stressed *E. angustifolia*, and attributed that increase to the increased phenylalanine ammonia-lyase (PAL) activity, which is involved in the biosynthesis of phenylpropanoid metabolites. Phenolic compounds are derived from the phenylpropanoid pathway, which can be activated under a wide range of biotic and abiotic stresses (Dixon and Paiva, 1995), including salinity (Ksouri et al., 2007). High accumulation of these phenolic compounds in plant tissues has been shown to be involved not only in defence mechanisms against pathogens and herbivory (Dixon and Paiva, 1995), but also in the adaptation to abiotic stresses, such as drought (de Abreu and Mazzafera, 2005), chilling, heat, high light intensity (Oh et al., 2009), and UV radiation (Moglia et al., 2008). Some phenolic compounds, like flavonoids have antioxidant function (Hernández et al., 2009); they can stabilize membranes by decreasing membrane fluidity, thus limiting the diffusion of free radicals across the lipid bilayer and reducing lipid peroxidation (Arora et al., 2000). Moreover, phenolic compounds have the ability to scavenge reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), which are overproduced under elevated salt concentration (Ksouri et al., 2007). Among the caffeic acid phenols in *Echinacea*, cichoric acid had the highest antioxidant activity in different in vitro assays (Dalby-Brown et al., 2005). In the current study, *E. purpurea* and *E. pallida* may have preferentially increased the accumulation of cichoric acid to limit the oxidative damage induced by ROS. This result agrees with a previous

study by Montanari et al. (2008) who suggested that the variation in the content of phenolic compounds can confer protection against oxidative damage in salt-stressed *E. angustifolia*. Further evidence of the roles of phenolic compounds as antioxidants is illustrated by a recent study on *Achillea collina* where the level of chlorogenic acid was enhanced at high altitude to protect the plant against climatic stresses including cold and high light stresses (Giorgi et al., 2010). Divergence of assimilates from the biosynthesis of primary metabolites to the biosynthesis of secondary metabolites (Kirakosyan et al., 2004) could have also contributed to the enhanced accumulation of some phenolic compounds at low or moderate salt concentrations in *E. purpurea* and *E. angustifolia*.

At the highest salt concentration (100 mM NaCl), the increase in CADs was observed only in a limited number of compounds, caftaric acid in *E. purpurea*, cichoric acid in *E. pallida* and caffeic acid in *E. angustifolia*; while the levels of cynarin, cichoric acid, cichoric acid derivative, and the total phenolic content in *E. purpurea* and caftaric acid in *E. pallida* were decreased. These decreases could be attributed to a decline in the activity of some enzymes involved in the biosynthetic pathways of caffeic acid phenolics (Chung et al., 2006). Cinnamate 4-hydroxylase, for instance, is an enzyme that has been shown to be associated with the endoplasmic reticulum membranes (Dixon and Paiva, 1995), and it is likely to get inhibited if specific ions (Na^+ and Cl^-) accumulate in the cytosol in the absence of an efficient sequestration mechanism into the vacuole. In the three *Echinacea* species, we observed a high level of variation in the response of the plants to 100 mM NaCl. At this NaCl concentration, although the plants used for the extraction of the active constituents did not show any injury, some leaves of the remaining plants started to show leaf chlorosis. These results suggest that nutrient

imbalance could have occurred and altered the synthesis of some secondary compounds. The key role of nutrient availability is further illustrated in our study by the high level of cichoric acid in control plants compared to the one reported by Gray et al. (2003) in field-grown *E. purpurea*, where the level of nutrients is less likely to be optimum. Further studies are required to understand the effects of the interaction between the soil nutrient status and salinity on the production of the active constituents of *Echinacea.*

Most of the alkamides were enhanced in *E. angustifolia* exposed to 50 mM NaCl, which suggests that they may be involved in a biochemical function associated with the response to abiotic factors (López-Bucio et al., 2006). Alkamides have been recently classified as a new group of regulatory compounds in plants (Ortiz-Castro et al., 2010). For instance, they are involved in signal transduction pathways as endogenous signalling molecules able to regulate growth and development in *Arabidopsis thaliana* (López-Bucio et al., 2007). This group of compounds seems to be able to interact with hormones to control root and shoot development; as well as interact with cytokinins to induce cell division and reprogram differentiated cells in *Arabidopsis* shoots (Ortiz-Castro et al., 2010). However, their potential role during salinity stress remains unknown. In contrast, in *E. purpurea*, there was no relative change in most of the alkamides up to 75 mM NaCl (with the exception of Chen alkamide); a trend similar to what was observed previously in *E. purpurea* exposed to a brief water stress (Gray et al., 2003).

Our results indicate that high level of salinity (100 mM NaCl) reduced the content of some of the alkamides, particularly the ones with 2, 4-diene moiety (i.e. 1, 3, Chen alkamide, 7, 8/9 in *E. purpurea,* 2 in *E. pallida* and 1, 2, 11 in *E. angustifolia*). This implies that this type of alkamides is more susceptible to salinity-induced degradation

than the monoene-type alkamides. Similar finding has been observed in *E. purpurea* under different storage conditions, where the structure of alkamides influenced their stability in the extracts (Liu and Murphy, 2007).

Although present in relatively small amounts and mainly in *E. pallida*, polyacetylenes have different biological activities including phototoxic properties against microorganisms (Binns et al., 2000) and cytotoxic activities against different types of cancer cell lines (Pellati et al., 2006). Moreover, naturally occurring polyacetylenes have ecological significance as fungicides, phytoalexins, insecticides and allelopathic agents (Minto and Blacklock, 2008). Various studies on the Apiaceae family have shown that the levels of these polyacetylenic constituents can be influenced by environmental conditions, such as drought and waterlogging (Lund and White, 1990), geographical origin (Kidmose et al., 2004) as well as the soil type and fertility (Søltoft et al., 2010). The current study demonstrated for the first time that salinity is a further environmental factor that influences the accumulation of polyacetylenes and other ketones in *Echinacea*, although their biological function under salinity stress remains to be determined.

Understanding the biogenesis of alkamide metabolites is still in its infancy; however, it is known that these compounds are derived from the ligation of alkyl amines with unsaturated fatty acids (Minto and Blacklock, 2008). The majority of ketones are also derived from fatty acid precursors (Minto and Blacklock, 2008). Such fatty acids have been reported to exhibit quantitative changes in some species (e.g. *Salvia officinalis*) under salinity stress (Ben Taarit et al., 2010). Therefore, a reduction in the polyunsaturated fatty acid supply at high salt concentration could have contributed to the decrease in some of the alkamide and ketone contents in *Echinacea*. Moreover, we

cannot rule out the possibility of the conversion of ketones 24 and 25 to ketones 20 and 21 via oxidation, a process that has been observed in *E. pallida* roots under storage conditions (Bauer et al., 1988a).

The quality of *Echinacea* raw material is determined not only by the concentration of bioactive substances but also by the plant dry biomass (Gray et al., 2003), therefore the yield of marker compounds per plant from the three *Echinacea* was determined (Table 4.3). Cichoric acid and alkamides 8/9 are believed to be responsible for the immunostimulatory properties and biological activities ascribed to *Echinacea* preparations (Hall, 2003). Cynarin and echinacoside are also important compounds frequently used for the standardization of *Echinacea* products (Wölkart et al., 2004). Our results suggest that the roots obtained from *E. purpurea* exposed to 50 and 75 mM NaCl had the same degree of quality as the control plants or even higher at 75 mM NaCl as a result of the increase in cichoric acid (31%). However, at the highest salt concentration (100 mM NaCl), the yield of alkamides 8/9 was lower (39% reduction) than the control, presumably impacting the quality of the raw material at that concentration. In *E. pallida*, cichoric acid yield was increased by 75 and 100 mM NaCl, however this species is not a significant source of cichoric acid (yield is about 22-fold lower than that of *E. purpurea*). Furthermore in this species, there was a decrease in the echinacoside yield at 50 and 75 mM NaCl, thus reducing the quality of raw material. On the other hand, it seems that the quality of raw material of *E. angustifolia* was not affected by salinity as plants retained the same amount of marker compounds as the control plants. These overall results are likely to reflect various levels of salt tolerance in the *Echinacea* species as it has been

suggested that the changes in phenolic compounds in response to salinity stress vary based on the salinity tolerance of the species (López -Berenguer et al., 2009).

In conclusion, this study shows the first evidence of salt-induced changes in both alkamides and ketones in *Echinacea*. In *E. angustifolia* exposed to 50 mM NaCl, a relative increase in most of the alkamides was observed in parallel to an increase in phenolic compounds, whereas the yield of the marker compounds was not changed. *Echinacea purpurea* retained most of the alkamides and increased both the level and yield of chicoric acid, cynarin and caftaric acid at 50 and/or 75 mM NaCl suggesting relatively high quality preparations from this plant species. In contrast, in *E. pallida*, echinacoside, cafteric acid and the major ketones were reduced by 50 or 75 mM NaCl, thus likely compromising the raw material quality in spite of the increase in one of the marker compound, cichoric acid. Future work will focus on long term exposure to salinity, in field studies, to further validate the phytochemical changes in *E. purpurea* and *E. angustifolia.* Moreover, the putative role of alkamides as signalling molecules will be investigated in salt-stressed *Echinacea* species.

Table 4.1. Caffeic acid derivatives content (mg g^{-1} root DW) of *Echinacea* species in response to 0, 50, 75, and 100 mM NaCl. Each value represents the mean of 4 replicates ± S.E.

Caffeic acid derivatives	NaCl (mM) 0	50	75	100
		E. purpurea		
Caftaric acid	6.99 ± 0.37 ^{c*}	8.62 ± 0.35 ^{b}	10.0 ± 1.14 ^{a}	8.20 ± 0.62 ^{b}
Chlorogenic acid	0.43 ± 0.06 ^{a}	0.46 ± 0.09 ^{a}	0.46 ± 0.02 ^{a}	0.48 ± 0.06 ^{a}
Cynarin	1.84 ± 0.09 ^{b}	2.26 ± 0.13 ^{a}	1.96 ± 0.39 ^{ab}	1.48 ± 0.23 ^{c}
Cichoric acid	35.0 ± 1.5 ^{b}	35.2 ± 2.74 ^{b}	39.2 ± 0.99 ^{a}	28.6 ± 2.78 ^{c}
Cichoric acid derivative	11.9 ± 0.69 ^{a}	12.5 ± 0.58 ^{a}	12.1 ± 0.93 ^{a}	6.87 ± 0.92 ^{b}
Total	56.1 ± 2.2 ^{a}	59.1 ± 3.0 ^{a}	63.8 ± 3.2 ^{a}	45.6 ± 5.1 ^{b}
		E. pallida		
Caftaric acid	4.06 ± 0.42 ^{a}	2.45 ± 0.49 ^{c}	2.52 ± 0.64 ^{c}	3.31 ± 0.31 ^{b}
Echinacoside	11.1 ± 1.48 ^{a}	9.93 ± 0.75 ^{ab}	8.64 ± 1.15 ^{b}	11.5 ± 0.24 ^{a}
Cichoric acid	1.48 ± 0.04 ^{b}	1.54 ± 0.26 ^{b}	1.93 ± 0.20 ^{ab}	2.14 ± 0.28 ^{a}
6-O-caffeoyl echinacoside	2.55 ± 0.31 ^{bc}	2.05 ± 0.32 ^{c}	2.81 ± 0.33 ^{b}	3.46 ± 0.54 ^{a}
Total	19.2 ± 1.85 ^{a}	16.0 ± 1.27 ^{a}	15.9 ± 1.60 ^{a}	20.4 ± 0.81 ^{a}
		E. angustifolia		
Chlorogenic acid	1.97 ± 0.16 ^{b}	3.10 ± 0.32 ^{a}	1.95 ± 0.25 ^{b}	1.97 ± 0.19 ^{b}
Cynarin	4.28 ± 0.21 ^{b}	5.13 ± 0.22 ^{a}	4.84 ± 0.31 ^{ab}	4.94 ± 0.55 ^{ab}
Echinacoside	4.97 ± 0.38 ^{bc}	5.84 ± 0.25 ^{ab}	6.07 ± 1.01 ^{a}	4.03 ± 0.16 ^{c}
Caffeic acid	1.16 ± 0.07 ^{b}	2.03 ± 0.15 ^{a}	1.96 ± 0.12 ^{a}	1.76 ± 0.13 ^{a}
Total	12.4 ± 0.58 ^{c}	16.1 ± 0.31 ^{a}	14.8 ± 1.01 ^{ab}	12.7± 0.29 ^{bc}

*Different letters indicate significant differences between treatments at $p \leq 0.05$.

Table 4.2. Alkamides/ketones content (mg g^{-1} root DW) of *Echinacea* species in response to 0, 50, 75, and 100 mM NaCl. Each value represents the mean of 4 replicates ± S.E.

Alkamides/ketones	NaCl (mM)			
	0	50	75	100
		E. purpurea		
Alkamide 1	$0.23 \pm 0.03^{ab*}$	0.21 ± 0.03^{bc}	0.27 ± 0.04^{a}	0.16 ± 0.03^{c}
Alkamide 2	1.2 ± 0.13^{ab}	1.04 ± 0.11^{b}	1.39 ± 0.26^{a}	0.98 ± 0.09^{b}
Alkamide 3	1.42 ± 0.12^{a}	1.24 ± 0.12^{ab}	1.26 ± 0.15^{ab}	1.06 ± 0.13^{b}
Chen alkamide	0.74 ± 0.05^{b}	0.79 ± 0.08^{b}	1.0 ± 0.19^{a}	0.52 ± 0.08^{c}
Alkamide 6	0.34 ± 0.03^{a}	0.30 ± 0.05^{a}	0.32 ± 0.04^{a}	0.22 ± 0.03^{b}
Alkamide 7	0.74 ± 0.07^{a}	0.59 ± 0.02^{b}	0.70 ± 0.09^{a}	0.47 ± 0.05^{c}
Alkamides 8/9	2.36 ± 0.24^{a}	2.15 ± 0.23^{a}	2.08 ± 0.34^{a}	1.55 ± 0.23^{b}
Total	7.04 ± 0.64^{a}	6.32 ± 0.48^{ab}	7.01 ± 0.93^{a}	4.96 ± 0.57^{b}
		E. pallida		
Ketone 20	0.25 ± 0.05^{b}	0.42 ± 0.07^{a}	0.42 ± 0.06^{a}	0.41 ± 0.07^{a}
Ketone 21	0.26 ± 0.05^{b}	0.52 ± 0.10^{a}	0.54 ± 0.10^{a}	0.45 ± 0.08^{a}
Alkamide 2	0.38 ± 0.04^{a}	0.35 ± 0.05^{a}	0.22 ± 0.04^{b}	0.25 ± 0.05^{b}
Ketone 22	0.82 ± 0.11^{a}	0.52 ± 0.01^{b}	0.48 ± 0.06^{b}	0.70 ± 0.11^{a}
Ketone 23	0.46 ± 0.08^{a}	0.51 ± 0.07^{a}	0.37 ± 0.09^{a}	0.395 ± 0.04^{a}
Ketone 24	0.76 ± 0.19^{a}	0.42 ± 0.02^{b}	0.29 ± 0.13^{b}	0.37 ± 0.08^{b}
Ketone 25	0.35 ± 0.12^{a}	0.14 ± 0.07^{b}	0.12 ± 0.07^{b}	0.14 ± 0.05^{b}
Peak 39	0.53 ± 0.13^{a}	0.46 ± 0.08^{ab}	0.36 ± 0.06^{b}	0.43 ± 0.06^{ab}
Peak 40	0.49 ± 0.07^{b}	0.60 ± 0.07^{a}	0.51 ± 0.08^{b}	0.63 ± 0.07^{a}
Total	4.29 ± 0.55^{a}	3.93 ± 0.34^{a}	3.32 ± 0.35^{a}	3.77 ± 0.30^{a}
		E. angustifolia		
Alkamide 1	0.18 ± 0.06^{a}	0.16 ± 0.02^{ab}	0.15 ± 0.06^{ab}	0.11 ± 0.02^{b}
Alkamide 12	0.73 ± 0.05^{bc}	0.92 ± 0.07^{a}	0.84 ± 0.08^{ab}	0.62 ± 0.03^{c}
Alkamide 2	0.24 ± 0.02^{a}	0.24 ± 0.02^{a}	0.16 ± 0.06^{b}	0.13 ± 0.05^{b}
Alkamide 13	0.64 ± 0.03^{b}	0.91 ± 0.14^{a}	0.64 ± 0.06^{b}	0.56 ± 0.07^{b}
Alkamides 14/15	0.47 ± 0.06^{b}	0.64 ± 0.08^{a}	0.62 ± 0.07^{a}	0.40 ± 0.02^{b}
Alkamide 16	0.09 ± 0.02^{b}	0.23 ± 0.07^{a}	0.11 ± 0.03^{b}	0.10 ± 0.04^{b}
Alkamide 17	0.08 ± 0.01^{b}	0.13 ± 0.05^{a}	0.11 ± 0.01^{ab}	0.08 ± 0.01^{b}
Alkamides 8/9	5.14 ± 0.09^{b}	6.18 ± 0.46^{a}	5.95 ± 0.38^{ab}	5.45 ± 0.28^{ab}
Alkamide 18	1.08 ± 0.05^{b}	1.37 ± 0.14^{a}	0.93 ± 0.06^{b}	0.93 ± 0.06^{b}
Alkamide 10	0.39 ± 0.13^{a}	0.43 ± 0.06^{a}	0.35 ± 0.04^{a}	0.37 ± 0.07^{a}
Alkamide 19	0.10 ± 0.02^{ab}	0.10 ± 0.02^{a}	0.09 ± 0.01^{b}	0.08 ± 0.02^{b}
Alkamide 11	0.88 ± 0.11^{a}	0.91 ± 0.13^{a}	0.60 ± 0.02^{b}	0.63 ± 0.08^{b}
Total	10.0 ± 0.52^{ab}	12.2 ± 1.08^{a}	10.54 ± 0.61^{ab}	9.46 ± 0.57^{b}

*Different letters indicate significant differences between treatments at $p \leq 0.05$.

Table 4.3. Root dry weight (DW) per plant (% of control) and marker compound yield per plant (% of control) in *Echinacea* species exposed to 0, 50, 75, and 100 mM NaCl. Each value represents the mean of 4 replicates ± S.E.

	NaCl (mM)			
	0	50	75	100
		E. purpurea		
Root DW	100 ± 5 [a*]	121 ± 8 [a]	117 ± 7 [a]	94 ± 14 [a]
Cynarin	100 ± 7 [bc]	149 ± 8 [a]	124 ± 23 [ab]	73 ± 13 [c]
Cichoric acid	100 ± 9 [bc]	121 ± 10 [ab]	131 ± 11 [a]	78 ± 16 [c]
Alkamides 8/9	100 ± 14 [a]	109 ± 10 [a]	96 ± 17 [a]	61 ± 14 [b]
		E. pallida		
Root DW	100 ± 4 [a]	91 ± 3 [a]	97 ± 8 [a]	99 ± 3 [a]
Echinacoside	100 ± 12 [a]	81 ± 5 [b]	74 ± 4 [b]	102 ± 3 [a]
Cichoric acid	100 ± 5 [b]	96 ± 18 [b]	147 ± 17 [a]	148 ± 24 [a]
		E. angustifolia		
Root DW	100 ± 13 [a]	92 ± 14 [a]	77 ± 20 [a]	80 ± 13 [a]
Cynarin	100 ± 11 [a]	114 ± 18 [a]	93 ± 26 [a]	93 ± 11 [a]
Echinacoside	100 ± 13 [ab]	116 ± 19 [a]	87 ± 9 [ab]	68 ± 11 [b]
Alkamides 8/9	100± 14 [a]	110± 15 [a]	89 ± 17 [a]	88 ± 18 [a]

[*]Different letters indicate significant differences between treatments at $p \leq 0.05$. For root biomass of the control plants (0 mM NaCl), 100 % corresponded to 4.02 g, 4.31 g and 1.32 g for *E. purpurea*, *E. pallida* and *E. angustifolia*, respectively.

Supplementary Table 4.1. Peak assignments for individual caffeic acid derivatives, alkamides and ketones in the methanolic extract of *E. purpurea*, *E. pallida* and *E. angustifolia.*

Peak no	t_R. (min)	UV λ_{max} (nm)	Compound	Reference
1	3.8	217.9, 243.8, 329	Caftaric acid	std[c]
2	4.5	217.9, 241.4, 326.6	Chlorogenic acid	std
3	6.4	217.9, 239.1, 324.2	Caffeic acid	std
4	7.6	216.8, 243.8, 321.9	Cynarin	std
5	8.1	201.5, 331.4	Echinacoside	std
6	9.5	217.9, 243.8, 329	N.I[a]	
7	12	219.1, 245, 330.2	Cichoric acid	std
8	13.9	219.1, 242.6, 329.6	N.I	
9	14.5	217.9, 243.8, 327.8	N.I	
10	15	219.1, 243.8, 324.2	N.I	
11	15.5	329	6-O-Caffeoylechinacoside	[1]
12	16	219.1, 243.8, 330.2	N.I	
13	16.2	219.1, 245, 330.2	2-Caffeoyl-3-feruloyl tartaric acid (cichoric acid derivative)	[2]
14	22.8	209.7, 263.8, 279.2	8-Hydroxytetradeca-9*E* -ene-11, 13-diyn-2-one (ketone 20)[b]	[3, 4]
15	24.3	209.7, 263.8, 279.2	8-Hydroxypentadeca-9*E* -ene-11, 13-diyn-2-one (ketone 21)	[3,4, 5]
16	25.3	260.3	Undeca-2*E*, 4*Z*-diene-8, 10-diynoic acid isobutylamide (alkamide 1)	[3]
17	25.4	192.2, 263.8	8-Hydroxypentadeca-9*E*, 13*Z*-dien-11-yn-2-one	[5, 6, 7]
18	26.1	225, 285.1, 301.7	N.I ketone	
19	26.4	210.9	Undeca-2*E*-ene-8, 10-diynoic acid isobutylamide (alkamide 12)	std, [3]
20	26.6	257.9	Undeca-2*Z*, 4*E*-diene-8,10-diynoic acid isobutylamide (alkamide 2)	[3 d e]
21	27.3	262.6	Dodeca-2*E*, 4*Z* -diene-8, 10-diynoic acid isobutylamide (alkamide 3)	[3 d]
22	27.5	210.9	Undeca-2*Z*-ene-8, 10-diynoic acid isobutylamide (alkamide 13)	[3]
23	27.6	261.5	Undeca-2*E*, 4*Z*-diene-8, 10-diynoic acid 2-methylbutylamide (alkamide 4)	[3]
24	28.7	212.1(alk 14) and 230.8, 261(alk 15)	Dodeca-2*E*-ene-8, 10-diynoic acid isobutylamide/Dodeca-2*E*, 4*Z*, 10Z-trien-8-ynoic acid isobutylamide (alkamides 14/15)	std, [3]
25	28.7	257.9	Dodeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide (Chen alkamide)	[8]
26	29.2	210.9	Trideca-2*E*, 7*Z* -diene-10, 12-diynoic acid isobutylamide (alkamide 6)	[3]
27	29.6	261.5	Dodeca-2*E*, 4*Z*-diene-8, 10-diynoic acid 2-methylbutylamide (alkamide 7)	[3]
28	29.9	214.4	Undeca-2*Z* -ene-8, 10-diynoic acid 2-methylbutylamide (alkamide 16)	[3]
29	30.9	210.9	Dodeca-2*E* -ene-8, 10-diynoic acid 2-methylbutylamide (alkamide 17)	[3]
30	31.4	200.4	Tetradeca-8*Z* -ene-11, 13-diyn-2-one (ketone 22)	[3, 4, 7]
31	31.7	231, 260.3	Dodeca-2*E*, 4*E*, 8*Z*, 10*E*/*Z*-tetraenoic acid isobutylamide (alkamides 8/9)	[3]

Supplementary Table 4.1. continued

Peak no	t_R. (min)	UV λ_{max} (nm)	Compound	Reference
32	33.7	211, 199.2	Pentadeca-2*E*, 9*Z* -diene-12, 14-diynoic acid isobutylamide (alkamide 18)	[3]
33	33.7	194.5	Pentadeca-8*Z* -ene-11, 13-diyn-2-one (ketone 23)	[3, 4, 7]
34	34.4	260.3	Dodeca-2*E*, 4*E*, 8*Z*-trienoic acid isobutylamide (alkamide 10)	[3 d]
35	35.5	225, 230	Pentadeca-8*Z*, 13*Z* -dien-11-yn-2-one (ketone 24)	[3, 4, 6, 7]
36	35.7	193.4	Hexadeca-2*E*, 9*Z* -diene-12, 14-diynoic acid isobutylamide (alkamide 19)	[3]
37	38.4	259.1	Dodeca-2*E*, 4*E*-dienoic acid isobutylamide (alkamide 11)	std, [3]
38	38.9	233.2	Pentadeca-8*Z*, 11*Z*, 13*E* -trien-2-one (ketone 25a)	[3, 7]
39	42.2	198	Pentadeca-8*Z*, 11*Z*-dien-2-one	[7]
40	44.6	199.2	Pentadeca-8Z-en-2-one	[4, 7]

[a] N.I: Not identified; [b] Nomenclature of alkamides and ketones in *Echinacea* species according to Bauer and Remiger (1989); [c] std: standard; [d] *E. purpurea*; [e] *E. angustifolia*. [1] Sloley et al. (2001); [2] Dalby-Brown et al. (2005); [3] Bauer and Remiger (1989); [4] Pellati et al. (2007); [5] Bauer et al. (1987); [6] Pellati et al. (2006); [7] Bauer et al. (1988a); [8] Chen et al. (2005).

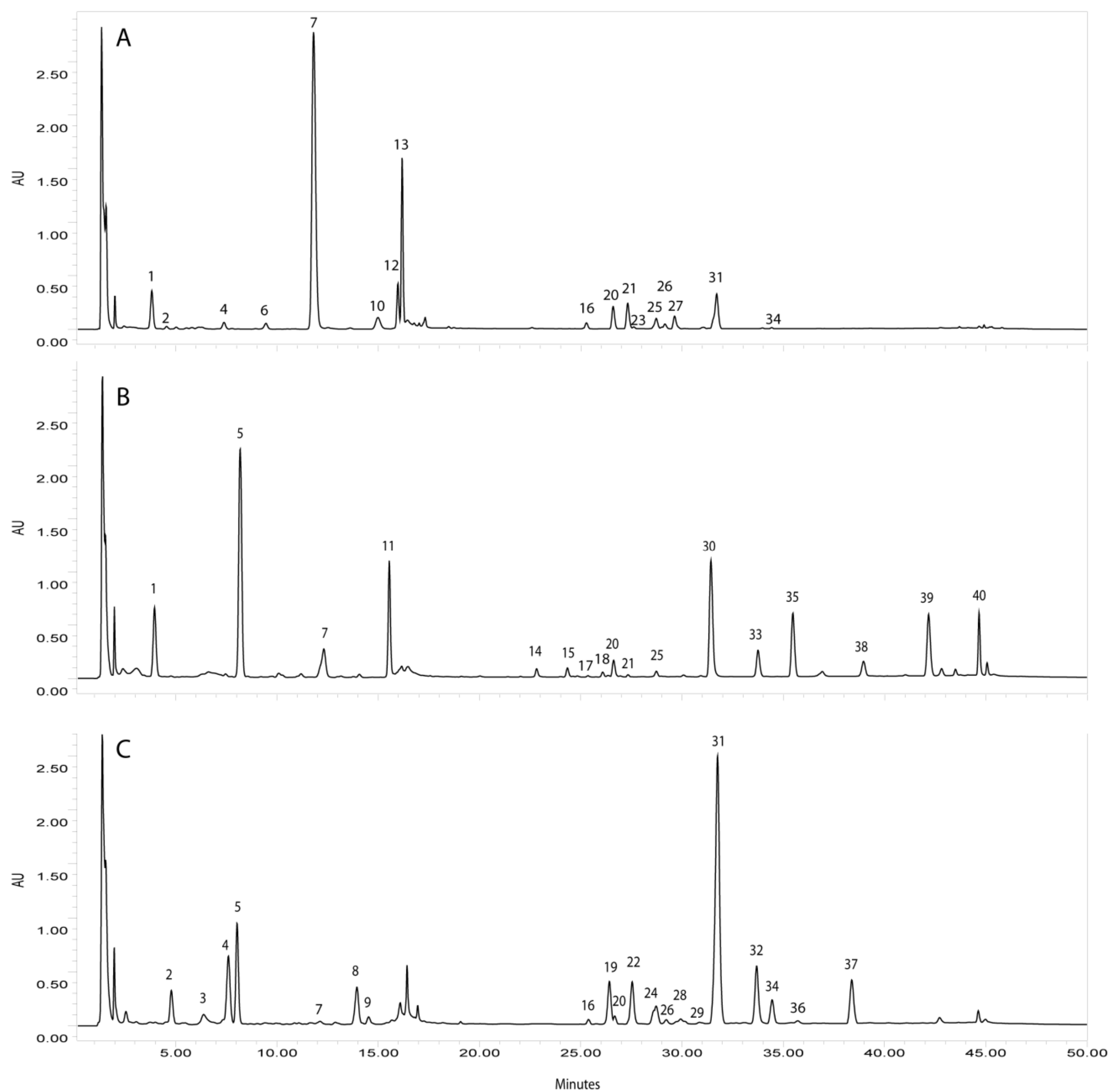

Fig. S4.1. HPLC profiles of caffeic acid derivatives and alkamides/ketones obtained from the methanolic extracts of *E. purpurea, E. pallida* and *E. angustifolia* control plants. Detection at UV maxplot 200-400 nm. For compound identification see Supplemental Table 4.1.

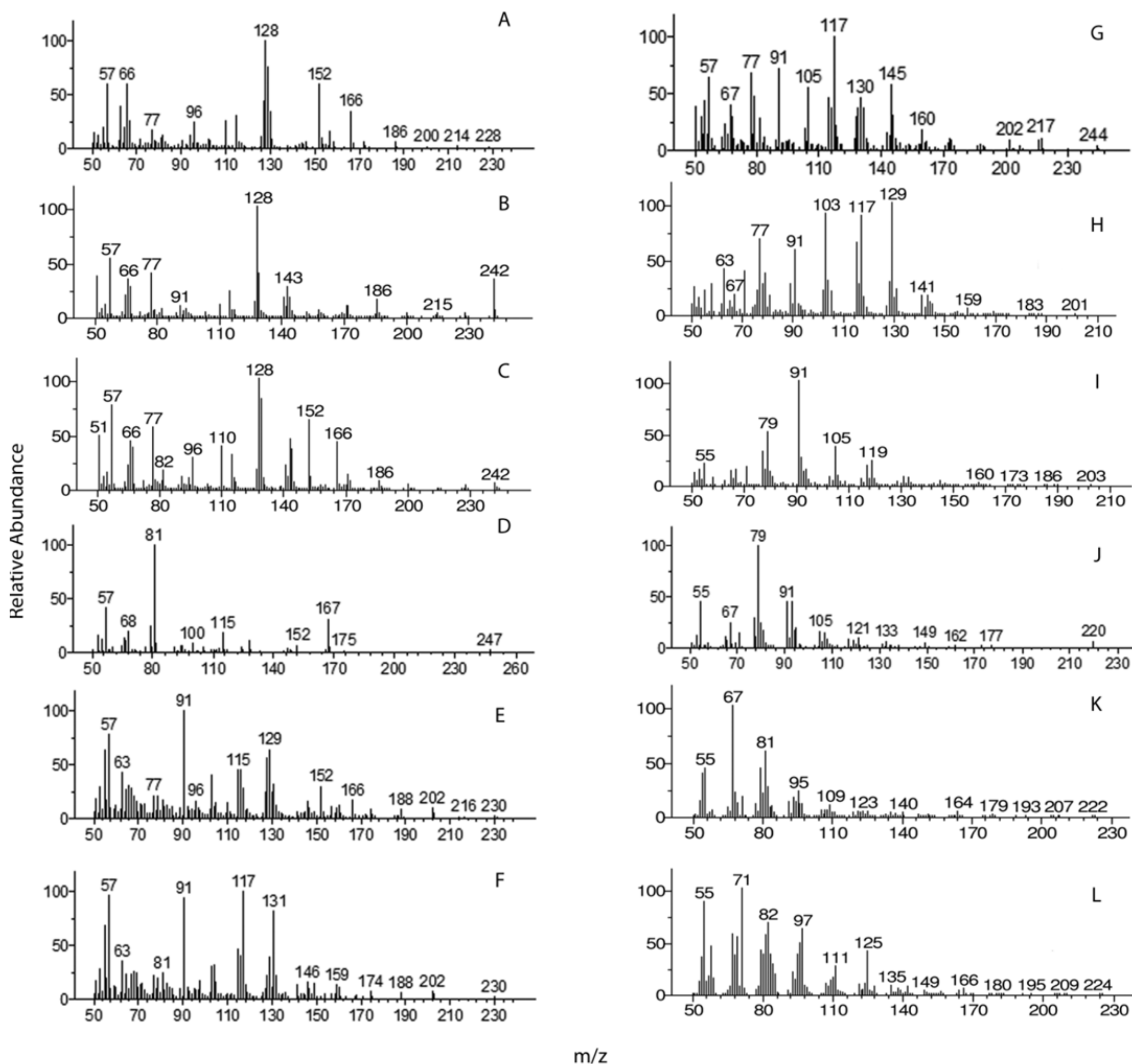

Fig. S4.2. Electron-Impact Mass Spectrometry analysis of 12 lipophilic compounds isolated from *Echinacea* species. MS Panels: (A) alkamide 2 isolated from *E. purpurea* and *E. pallida* , M^+ *m/z* 229; (B) alkamide 3 isolated from *E. purpurea* and *E. pallida*, M^+ *m/z* 243; (C) dodeca-2*Z*, 4*E*-diene-8, 10-diynoic acid isobutylamide (Chen alkamide) isolated from *E. purpurea* and *E. pallida*, M^+ *m/z* 245; (D) alkamides 8/9 isolated from *E. purpurea* and *E. angustifolia*, M^+ *m/z* 247 ; (E) alkamide 12 isolated from *E. angustifolia*, M^+ *m/z* 231; (F): alkamide 13 isolated from *E. angustifolia*, M^+ *m/z* 231; (G) alkamide 14 isolated from *E. angustifolia*, M^+ *m/z* 245; (H) ketone 22 isolated from *E. pallida*, M^+ *m/z* 202; (I) ketone 24 isolated from *E. pallida*, M^+ *m/z* 218; (J) ketone 25a isolated from *E. pallida*, M^+ *m/z* 220; (K) Pentadeca-8*Z*, 11*Z*-dien-2-one isolated from *E. pallida*, M^+ *m/z* 222; (L) Pentadeca-8Z-en-2-one isolated from *E. pallida*, M^+ *m/z* 224.

CHAPTER 5. ACTIVITIES OF ENZYMES RELATED TO PHENOLIC COMPOUNDS METABOLISM IN SALT-STRESSED *ECHINACEA**

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5.1. Abstract

Echinacea contains a rich spectrum of caffeic acid derivatives whose accumulation is influenced by the activities of enzymes related to their synthesis and oxidation. Both caffeic acid derivatives and the activities of these enzymes can be affected by abiotic stress such as salinity. The purpose of this study was to investigate the effects of salinity (0, 50, 75 and 100 mM NaCl) on the activities of enzymes related to phenolic metabolism, such as phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), guaiacol peroxidase (GPX), caffeic acid peroxidase (CAPX), and chlorogenic acid peroxidase (CGAPX) and to determine the potential correlation between the quantitative changes in phenolics and the activities of such enzymes in roots and leaves after 7 and 14 days of salt treatment. Overall, PAL activity in the roots of salt-stressed *E. purpurea* was higher than in the other two species. PAL activity was increased in the roots by all salt concentrations after 7 days. However, after 14 days, only 100 mM NaCl caused a significant increase. In the leaves, PAL activity was increased only by 75 mM NaCl. In this species, a decrease in PPO (roots and leaves after 14 days), CGAPX and GPX (roots after 7 days at 75 and/or 100 mM NaCl) activities were obtained. In *E. pallida*, increases were observed in CGAPX and GPX activities (roots and leaves after 7 days) and in PPO activities (leaves after 14 days and roots after 7 days at 75 and 100 mM

NaCl, respectively). PAL activity was also stimulated by 50 mM NaCl after 7 days in leaves. However, there was a decrease in PAL activity in roots after 7 days and in leaves after 14 days at the highest concentrations. In *E. angustifolia*, CAPX and CGAPX activities were stimulated in roots and leaves by 75 and/or 100 mM NaCl; while PPO activity was increased after 7 days (roots and leaves at 50 mM NaCl), but decreased after 14 days (roots at 100 mM NaCl). GPX activity was also decreased in leaves (after 7 days at 100 mM NaCl and after 14 days by all salt concentrations). This study provides evidence that salinity causes significant changes in enzymes related to phenolic metabolism depending on salt concentration and duration of exposure. Furthermore, it can be concluded that differences in PAL activity could reflect the salt tolerance level among *Echinacea* species.

Keywords: *Echinacea*; NaCl; CADs; PPO; CAPX; CGAPX; GPX.

5.2. Introduction

Many medicinal plants, vegetables and fruits are valuable sources of phytochemical ingredients that are widely used as a source of pharmaceuticals, food additives, and flavors (Zhao et al., 2005, Sultana and Anwar, 2008). Among these phytochemicals are phenolic compounds, which have health benefits as potent antioxidant agents against free radicals, and therefore, reduce the incidence of chronic and cardiovascular diseases (Perez-Vizcaino and Duarte, 2010). Attention has been primarily paid to plant-derived caffeic acid derivatives, flavonols, isoflavones, flavanones, catechins and anthocyanins (Tomas-Barberan and Espin, 2001). *Echinacea,* a popular genus used as a source of natural antioxidants has a rich spectrum of caffeic

acid derivatives, such as caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside, and cichoric acid that possess high antioxidant activities *in vitro* (Hu and Kitts, 2000; Sloley et al., 2001; Pellati et al., 2004). Caffeic acid derivatives in *Echinacea* are synthesized through the general phenylpropanoid pathway, which is a link between the primary and secondary metabolism (Winkel-Shirley, 2001; Montanari et al., 2008). This pathway yields a wide array of plant natural products including cinnamic acid derivatives (i.e. chlorogenic acid, caffeic acid), tannins, flavonoids, and coumarines with physiological and ecological significance (Guidi et al., 2005; Camacho-Cristóbal et al., 2002; Yan et al 2006; Olsen et al., 2008).

Some of the key enzymes that are involved in phenolic compounds metabolism are phenylalanine ammonia-lyase (PAL), polyphenyl oxidase (PPO), and peroxidases, such as caffeic acid peroxidase (CAPX) and chlorogenic acid peroxidase (CGAPX) (Ali et al., 2005, 2006). The entry enzyme in the biosynthesis of phenolic compounds through the general phenylpropanoid pathway is PAL, which converts $_{\mathrm{L}}$-phenylalanine into *trans*-cinnamic acid, the precursor of a wide array of plant natural products with structural and defence-related functions (Dixon and Paiva, 1995; Guidi et al., 2005; Dong et al., 2010). Some of these functions include the deposition of lignin and suberin in cell walls, regulation of membrane permeability, the scavenging of reactive oxygen species (ROS), formation of phytoalexins in response to pathogen and herbivore attack, UV-protection, and cell signalling (flavonoid nodulation factors and salicylic acid) (Weisshaar and Jenkins, 1998; Lopez-Martinez et al., 2011). In addition, these compounds have some roles in determining food quality characteristics (Tomas-Barberan and Espin, 2001). Numerous studies in the literature support the idea that phenolic compounds are stress-

induced compounds that show increased levels under biotic and abiotic stress conditions (Dixon and Paiva, 1995; Solecka, 1997; Kim et al., 2009). For example, it has been shown that PAL activity can be induced by the fungus *Phytophthora* in *Capsicum annuum* (Koj et al., 2011), drought in winter triticale (× *Triticosecale*) (Hura et al., 2007), nitrogen deficiency in *Matricaria chamomilla* (Kovacik and Backov, 2007), salinity in *Jatropha curcas* (Gao et al., 2008), and application of salicylic acid in *Salvia miltiorrhiza* suspension culture (Dong et al., 2010). These studies suggest that PAL plays a key role in the mechanisms of defense/survival to biotic and abiotic stresses by producing the precursor of phenolic compounds.

Activation of phenolics by oxidation is important for most of their ecological interactions against biotic and abiotic stresses, thus variation in the response to environmental conditions may be attributed to changes in the level of the oxidation process and activities of related enzymes (Appel, 1993). Nevertheless, from a nutritional point of view, activation of oxidizing enzymes such as PPO and peroxidases degrade the quality of horticultural crops (Tomas-Barberan and Espin, 2001). PPO is a major oxidizing enzyme and its enzymatic reaction involves the O_2-dependent oxidation of mono and *o*-diphenolic substances to *o*-quinones. These quinones are highly reactive intermediates that spontaneously react with phenols, proteins, or amino acids to form brown pigments (Yoruk and Marchall, 2003; Li-Qin et al., 2009). Moreover, quinones are known to have antimicrobial properties and direct cytotoxic activities against pathogens and plant-feeding insects (Pourcel et al., 2007). In most plant species under non-stressed conditions, PPO is a latent enzyme located mainly in the plastids apart from its substrates, mono and diphenols, which are stored in the vacuoles. As a consequence of wounding,

pathogen and herbivore attack and senescence, cellular decompartmentalization occurs enabling the contact of the enzyme with its substrates, thus converting it to an active form (Bhonwong et al., 2009). Although PPO is induced mainly in response to biotic stress, this enzyme has been shown to be involved in response to abiotic stresses including salinity (Chisari et al., 2010; Newman et al., 2011); however, the underlying mechanisms are not fully-understood (Mayer, 2006).

Caffeic acid peroxidase (CAPX) and chlorogenic acid peroxidase (CGAPX) are also phenolic oxidizing enzymes, which are implicated in different metabolic functions, such as the regulation of cell elongation, wound healing, defense against pathogens in addition to cross linking of cell wall polysaccharides and lignifications (lignin biosynthesis) (Lagrimini, 1991). In addition, peroxidases may also function as scavengers for ROS (Diaz et al., 2001). Previous studies have shown that activities of CAPX and CGAPX enzymes are induced by exposure to biotic stress (*Pseudomonas syringae*) in *Lactuca sativa* (Bestwick et al., 1998) as well as abiotic stresses (high CO_2 and Cu^{2+} levels) in *Panax ginseng* (Ali et al., 2005, 2006), and this increase in activity was correlated with stress tolerance.

Plants including *Echinacea* are prone to a wide array of environmental stresses, such as drought, salinity, UV light, high or low temperature, and biotic stress during their growth and development (Wang et al., 2003; Sanchez-Rodriguez et al., 2010). Among these stresses, salinity is considered a major constraint that imposes osmotic, ionic and oxidative stress on plants (Parida and Das, 2005). In spite of the importance of these enzymes in phenolic metabolism, studies on the effect of salinity on the activities of enzymes involved in phenolic metabolism in *Echinacea* species are very limited, and

only one study investigated PAL activity in one of *Echinacea* species; *E. angustifolia* (Montanari et al., 2008). Moreover, I am not aware, to the best of my knowledge of any studies dealing with the response of CAPX and CGAPX to salinity stress in *Echinacea* species or any other plant species. There is evidence that the accumulation of phenolic compounds in *Echinacea* species is widely affected by salinity stress (Sabra et al., 2012 b; Chapter 3). However, to what extent does the activities of some enzymes related to phenolic metabolism contribute to the quantitative changes in phenolic metabolites in *Echinacea* species is not known. I hypothesized that salinity could affect the activity of these enzymes and potentially affect the content and quality of the major phenolic compounds. The objectives of this study were to 1) investigate the activity of the phenolic compound-related enzymes PAL, PPO, CAPX, CGAPX, and GPX from root and shoot tissues of the three commercially-used *Echinacea* species exposed to different salinity levels for 7 and 14 days and 2) study the relationship between these enzymes and the accumulation of individual phenolic compounds to determine if any of these enzymes are involved in the adaptation to salinity stress.

5.3. Materials and methods

5.3.1. Plant growth and hydroponic cultivation

Echinacea species (*E. purpurea*, *E. pallida*, and *E. angustifolia*) seeds were purchased from Richters, Ontario, Canada. Seeds were kept under running tap water for 4 days in a cloth to overcome the dormancy of seeds especially for *E. angustifolia* (Macchia et al., 2001). The seeds were cultivated in 1 L plugs containing a mixture of top soil, peatmoss, and sand (1:1:2, v:v:v) for 6 months in the greenhouse under a 18/6 h

photoperiod, a temperature of 25/18 ^{o}C (day/night) and natural light supplemented with sodium lamps. Plants were transferred in a hydroponic system containing half strength modified Hoagland solution in 10 L plastic containers aerated with air pumps (Renault et al., 2001). For each species, six plants per treatment were placed in the nutrient solution in each container, and all containers were arranged in a completely random block design in three replicates. Salt treatments (50, 75 and 100 mM NaCl) were applied to the nutrient solution gradually to prevent the osmotic shock, and the solution was replaced twice a week to compensate for nutrient uptake. Leaf and root samples were collected 7 and 14 days after the beginning of salt treatments, washed three times and then rapidly frozen in liquid N_2 and stored at -80 ^{o}C for enzyme assays.

5.3.2. Extraction of guaiacol peroxidase (GPX), polyphenol oxidase (PPO), chlorogenic acid peroxidase (CGAPX), and caffeic acid peroxidase (CAPX)

One gram of frozen leaf or root tissues combined from three plants in each replicate was ground with liquid N_2 into a fine powder using a chilled mortar and pestle, and homogenized in 10 mL extraction buffer consisting of 50 mM K-phosphate buffer (pH 7.1), 1% PVP (polyvinyl-pyrrolidone), 1 mM EDTA, and 5 mM ascorbic acid. The homogenate was centrifuged at 23,000 x *g* for 20 min at 4 °C, and the supernatant was used as the extract for measuring enzyme activities and determining the protein content using the Coomassie blue dye method and BSA as a standard (Bradford, 1976). All enzyme assays were carried out in triplicate.

5.3.3. Enzyme assays

5.3.3.1. Guaiacol peroxidase (GPX, EC 1.11.1.7)

GPX activity was determined using guaiacol as a substrate as described previously (Pütter, 1974) with some modifications. The enzyme assay solution (2.5 mL) consisted of 25 mM Na-phosphate buffer (pH 7.1) containing 2 mM of guaiacol, 0.5 mM H_2O_2 and 100 µL of enzyme extract. The reaction was initiated by the addition of enzyme extract to the reaction solution. The activity was calculated from the increase in the absorbance at 470 nm for 2.5 min due to the oxidation of guaiacol to tetraguaiacol (ε = 26.6 mM^{-1} cm^{-1}). One unit of activity was defined as the formation of one µmol product min^{-1}, and the specific activity was expressed as µmol min^{-1} mg $protein^{-1}$.

5.3.3.2. Caffeic acid peroxidase (CAPX)

Activity of CAPX was determined as previously described (Ali et al., 2006) with some modifications. The assay mixture (1.0 mL total volume) consisted of McIlvaine buffer (50 mM sodium phosphate buffer and 50 mM citric acid, pH 5.5), 1.2 mM caffeic acid, 15.8 mM H_2O_2, and 100 µl enzyme extract. After 1 min incubation at room temperature, the increase in the absorbance at 410 nm as a result of caffeic acid oxidation ($\varepsilon = 1.5 \times 10^5$ M^{-1} cm^{-1}) was monitored for one min. One unit of activity was defined as the amount of enzyme that catalyzes the oxidation of one µmol caffeic acid min^{-1}.

5.3.3.3. Chlorogenic acid peroxidase (CGAPX)

CGA activity was assayed according to the method described by Ali et al. (2005) with modifications. The reaction mixture of 1.4 mL consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM chlorogenic acid, 1 mM H_2O_2 and 25 µl enzyme extract. The oxidation of chlorogenic acid was monitored at 410 nm for one min at room

temperature, and the activity was measured using the extinction coefficient of chlorogenic acid ($\varepsilon = 2.02 \times 10^5 M^{-1} cm^{-1}$).

5.3.3.4. Polyphenol oxidase (PPO, EC 1.10.3.2)

PPO activity was assayed by monitoring the increase in the absorbance at 420 nm due to catechol oxidation (Nagai and Suzuki, 2001). The reaction mixture (1.5 mL) contained 100 mM phosphate buffer (pH 7.1), 20 mM catechol, and 50 µL of the enzyme extract. The absorbance was recorded for one min at 10 sec intervals. One unit of PPO was defined as the amount of enzyme that causes an increase in the absorbance of 0.01 per min, and specific activity was expressed as unit mg protein^{-1}.

5.3.3.5. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5)

PAL was extracted from fresh tissues after 7 and 14 days of salt treatment and the activity was determined by measuring the *trans*-cinnamic acid produced from the substrate phenylalanine using HPLC method as previously described (Klaiber et al., 2005) with some modifications. Enzyme assays were measured in leaves and roots of plants treated with different salt levels in three replicates. Ten mg polyvinylpolypyrrolidone (PVPP) were added to two grams of frozen leaf or root tissues combined from three plants in each replicate, and ground with liquid nitrogen using 10 mL of 50 mM Tris-HCl buffer (pH 8.7) containing 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM *β*-mercaptoethanol, and 1.5 mM dithioerythritol (DTT). The homogenate was centrifuged at 19, 000 × g for 20 min at 4 ^{o}C, and the supernatant was used for PAL assay. The reaction mixture contained 0.8 mL of 50 mM Tris-HCl buffer (pH 8.7), 0.4 mL enzyme extract, and the reaction was initiated by the addition of 0.1 mL of 100 mM

L-phenylalanine. The mixture was incubated in a water bath at 37 ^{o}C for 30 min, and then 800 µl of methanol was added to stop the reaction. Control reaction contained buffer, enzyme extract without the substrate (L-phenylalanine). The reaction mixture was centrifuged at 12, 000 × g for 10 min for removing the debris, and the supernatant was filtered using syringe filter (0.45 µm PTFE). For the quantification of *trans*-cinnamic acid, HPLC system consisted of Waters 2695 HPLC module (Waters, Milford, MA), autosampler, vacuum degasser, pump, and a Waters 996 photodiode array detector (DAD) was used. The analytical column used for the separation of *trans*-cinnamic acid was (LiChrospher®100 RP-18 column; 250 x 4 mm; 5 um particle size, LiChroCART®4-4, Germany) and the guard column was LiChrospher®100 RP-18; LiChroCART®4-4, Germany. *Trans* cinnamic acid was eluted isocratically at a retention time of 3.9 min using *o*-phosphoric acid aqueous acetonitrile (50:50, v:v) as a mobile phase with 1 mL min^{-1} flow rate in total running time of 5 min. Detection of *trans*-cinnamic acid was measured at the maximum absorbance of 276 nm, and quantified using the calibration curve established from external standard method (Fig. 8.6 in Appendix B). Three successive injections were run for each sample, with 20 µl in each injection, and one unit of PAL activity was defined as the production of nmol *trans*-cinnamic acid per hour.

5.3.4. Data analysis

A Two-way ANOVA was conducted using JMP program (Version 6, 2006, SAS Institute). Differences between means of treatments and species were compared by Tukey's HSD test at the probability $p \leq 0.05$ level. Pearson correlation analysis between different independent variables was performed and the correlation coefficient was

computed. Simple linear regression was used to examine the relationship between salt levels and enzyme activities.

5.4. Results

5.4.1. Caffeic acid peroxidase (CAPX)

Salinity treatments did not cause any changes in the activity of CAPX in leaves of any *Echinacea* species exposed to NaCl for 7 days (Table 5.1). After 14 days, there was more than a two fold increase in CAPX activity in one of the *Echinacea* species, *E. angustifolia* exposed to 75 mM NaCl. In the roots, salinity treatments did not alter the activity of CAPX in *E. purpurea* and *E. pallida*. However, in *E. angustifolia*, 75 mM NaCl stimulated the activity by 102% and 83% after 7days and 14 days of treatment, respectively (Table 5.2). Overall, CAPX activity in leaves after 14 days was correlated (r=0.951, p=0.049) with the activity in roots. Moreover, in the roots, the pattern of activity after 7days was similar to that after 14 days (r= 0.991, p=0.009).

5.4.2. Chlorogenic acid peroxidase (CGAPX)

The activity of CGAPX did not change in the leaves of *E. purpurea* plants after exposure to salt treatments for 7 and 14 days, while significant changes were found in *E. pallida* and *E. angustifolia* (Table 5.3). In *E. pallida*, 50 mM NaCl stimulated CGAPX activity after 7 days; whereas in *E. angustifolia*, the activity of CGAPX was stimulated at moderate NaCl concentration after 7 days and at both moderate and high NaCl concentrations after 14 days. In the roots, after 7 days of treatment, the high salt concentration (100 mM NaCl) diminished the CGAPX activity in *E. purpurea,* and overall, CGAPX activity was negatively correlated with salinity levels (r= -0.93, p=0.07),

while in *E. angustifolia*, the activity increased by 94% at 75 mM NaCl (Table 5.4). After 14 days, there was no significant difference between the treatments in any of the *Echinacea* species.

5.4.3. Guaiacol peroxidase (GPX)

Echinacea purpurea plants exposed to salinity for 7 days maintained the same GPX activity in the leaves as control plants. While the activity of this enzyme was enhanced in *E. pallida* by 75 and 100 mM NaCl with a high correlation between the activity and salinity (r= 0.985, p=0.015), a reduction was observed in *E. angustifolia* at the highest salt concentration. After 14 days of treatments, *E. angustifolia* was the only species that showed significant changes in GPX activity; a reduction reaching 42.5%, 66.5%, 77.5% at 50, 75, and 100 mM NaCl, respectively was recorded (Table 5.5). In the roots, a reduction in the GPX activity after 7 days of salt exposure was associated with moderate and high salt concentrations in *E. purpurea*. On the contrary, GPX activity was enhanced at 75 and 50 mM NaCl in *E. pallida* and *E. angustifolia*, respectively. After 14 days in the roots, no significant changes in GPX activity were found in any of the *Echinacea* species (Table 5.6).

5.4.4. Polyphenol oxidase (PPO)

Echinacea purpurea plants exposed to salinity for 7 days maintained the PPO activity in both leaves and roots, while after 14 days of treatment, the activity was significantly reduced by salinity in both roots and leaves (Tables 5.7 and 5.8), and there was a positive correlation between PPO activity in leaves and roots (r= 0.979 p=0.021). In *E. pallida* exposed to salinity for 7 days, PPO activity did not change in leaves, while in the roots,

an increase was observed at 100 mM NaCl. After 14 days, a more than two fold increase was found at 75 mM NaCl in leaves. *Echinacea angustifolia* exposed to low salt concentration for 7 days showed an increase in PPO activity in both leaves and roots, and a positive correlation between the activity in leaves and the activity in roots was established (r= 0.941, p=0.059). After 14 days of exposure, the highest salt concentration (100 mM NaCl) caused a reduction in PPO activity particularly in the roots, and overall, PPO activity in *E. angustifolia* was lower than the other two species.

5.4.5. Phenylalanine ammonia-lyase (PAL)

PAL activity was measured as the production of *trans*-cinnamic acid (*t*-CA) in the roots and leaves of *Echinacea* (Fig. 5.1). In *E. purpurea*, PAL activity in the roots was enhanced by all salt concentrations after 7 days, while in the leaves, an increase in activity occurred only at 75 mM NaCl (Tables 5.9 and 5.10). After 14 days, the trend remained the same in the leaves, with a further increase in activity at 75 mM (3 fold), while in the roots the activity was stimulated (42%) at the highest salt concentration (100 mM NaCl). Contrary to the response of *E. purpurea*, which showed increase at all salt concentrations in the roots after 7 days of exposure to salts, *E. pallida* showed a substantial decrease in PAL activity in roots; however in the leaves, the activity was stimulated by two-fold at the low salt concentration (50 mM NaCl). After 14 days of salt treatments, PAL activity was reduced by nearly 45% in the leaves at 75 and 100 mM NaCl in this species. In *E. angustifolia*, root and leaf PAL activities were not changed by any of salt treatments (Tables 5.9 and 5.10). Linear regression analysis revealed that the relationship between salt levels and PAL activity was significant only in *E. pallida* leaves after 14 days of treatments (r= -0.961, p=0.039), while this relationship was not

significant in *E. purpurea* leaves and roots (r=0.684, r= 0.771, respectively) or *E. angustifolia* leaves and roots (r= 0.89, r= -0.35, respectively). Moreover, no correlation was found between the accumulation of total phenolic compounds (results from chapter (4) of the thesis) and PAL activity in any of the *Echinacea* species (Table 5.11). Overall, PAL, PPO and CAPX activities were significantly affected by the interaction between salt levels and *Echinacea* species ($p \leq 0.05$).

5.5. Discussion

The pattern of PAL activity in *Echinacea* species shows that *E. purpurea* has a constitutive higher activity in the roots than the other two species, which may be responsible for the highest constitutive phenolic content. Moreover, differences in PAL activity between the species after 7 days of treatment was demonstrated in our study as we found a reduction in PAL in *E. pallida* and an increase in *E. purpurea*, while there was no change in *E. angustifolia* in response to salt treatments. This may reflect the differences in salt tolerance among *Echinacea* species observed in chapter 3 with *E. purpurea* having the highest salt tolerance. This result supports previous studies that have shown that PAL activity is induced under various types of stresses, such as boron starvation in *Nicotiana tabacum* (Camacho-Cristobal et al., 2002), ozone (O_3) in *Vitis vinifera* (Sgarbi et al., 2003), heavy metals like Ni, Cd, Cu, and Al in *Matricaria chamomilla* (Kovacik and Backor, 2007; Kovacik et al., 2009), nitrogen depletion in *Arabidopsis thaliana* (Olsen et al., 2008), and pathogen invasion in *Pennisetum glaucum* (Nagarathna et al., 1993). All these results suggest the involvement of PAL in tolerance mechanisms to a wide range of biotic and abiotic stresses. Caffeic acid derivatives in *Echinacea* are most likely derived from the phenylpropanoid pathway (Montanari et al.,

2008), and the main regulatory enzyme in this pathway is PAL that catalyzes the conversion of phenylalnine to *trans*-cinnamic acid, which is the precursor of a wide range of secondary metabolites including cinnamic acids, lignin, flavonoids and phytoalexins (Dixon and Paiva, 1995). The PAL-induced accumulation of phenolic compounds in stressed tissues is considered important to plants as these compounds have different physiological functions including lignin deposition, defence mechanisms against herbivore and pathogen, and as antioxidant agents against ROS (Dixon and Paiva, 1995). As salinity at high concentrations increases the rate of ROS production more than their scavenging rate, oxidative damage to membranes and macromolecuoles might occur and this was represented by increased EL, an indicator for the membrane damage, in all *Echinacea* species, particularly at high salt concentration (100 mM NaCl). Nevertheless, differential antioxidant response was observed between species. For example, *E. purpurea* in particular was able to activate a set of antioxidant enzymes, such as SOD and APX to limit the oxidative damage (Chapter 3). As phenolic compounds contribute considerably to the overall antioxidant properties of plants under stress conditions, including salinity (Ksouri et al., 2008), it would be beneficial for plants to activate the phenylpropanoid pathway and the main regulatory enzyme (PAL) for yielding phenolic compounds with antioxidant capacity.

In the current study, although PAL activity in *E. purpurea* was stimulated by some salt treatments, there was a decrease in total caffeic acid derivatives (CADs) at the highest salt concentration (100 mM NaCl), and overall, there was no correlation between PAL activity and the total CADs or any of the individual compounds (Table 5.11). This lack of correlation in our findings between PAL activity and CADs in *E. purpurea* could

be due to the partition of *trans*-cinnamic acid (*t*-CA), the major precursor produced by PAL, into different biosynthetic streams (other than CADs biosynthesis) within phenolic metabolism. The downstream metabolic needs determines the *t*-CA influx rate towards the synthesis of closely related compounds, such as lignins, flavonoids, coumarines and signaling molecules (i.e. salicylic acid) with different biological functions and defense-related mechanisms (Weisshaar and Jenkins, 1998; Shadle et al., 2003; Lopez-Martinez et al., 2011). How plants preferentially induce the influx of precursors into certain biosynthetic branches under environmental conditions is an interesting research topic, which needs further investigation. The lack of correlation between PAL activity and phenolic content has also been commonly found in response to other stresses such as Ni treatment in *Camellia sinensis* (Basak et al., 2001) and *Matricaria chamomilla* (Kovacik et al., 2009), biotic and abiotic elicitors (i.e. yeast extract and Ag^+) in *Salvia miltiorrhiza* (Yan et al., 2006), cold stress in soybean (*Glycine max*) (Posmyk et al., 2005), and high CO_2 concentration in cherimoya fruit (*Annona cherimola*) (Assis et al., 2001). On the other hand, other studies on biotic/abiotic stresses have found a correlation between PAL activity and increased accumulation of phenolic compounds like in *E. purpurea* grown under continuous light (Abbasi et al., 2007), *Salvia miltiorrhiza* cell cultures treated with elicitors (Dong et al., 2010), and *Matricaria chamomilla* exposed to Al stress (Kovacik et al., 2010). This indicates that the involvement of PAL in the regulation of phenylpropanoid compound biosynthesis in plants may be dependent on plant species, type of stress, its intensity, and the duration of exposure (Lister et al., 1996). The fact that cichoric acid, the major CADs in *E. purpurea*, was positively correlated with PAL activity after 7 days but not after 14 days indicates that the accumulation of CADs is

influenced by the interaction between the PAL activity and the developmental stage of plants. This points out the importance of considering the variation in both phenolic accumulation and the enzymatic machinery in the phenylpropanoid pathway as a function of the phenological stage; a topic that should be looked at in future studies.

In *E. pallida*, the pattern of PAL activity was different between roots and leaves; while it was increased by 50 mM NaCl in leaves, it was reduced in roots by all salt concentrations. In the current study, there was a negative relationship between PAL activity in leaves after 14 days and salt treatments (r= -0.961, p= 0.039), while this relationship was not established in roots indicating that PAL activity in leaves was more sensitive to salts than in roots. This reduction in PAL activity could be attributed to the inhibitory effects of ions accumulation particularly Cl^- in leaves as there was a significant negative correlation between Cl^- content in leaves (results from chapter 3) and PAL activity after 14 days (r= -0.952, p= 0.048). On the other hand, PAL activity in leaves of *E. pallida* was stimulated after 7 days at low salt concentration, but showed a reduction after 14 days of exposure to moderate and high salt concentrations (75 and 100 mM NaCl) indicating that concentration and duration of exposure to a stress are critical factors for determining the pattern of PAL activity. The differences in PAL activity with duration have been previously reported in *Matricaria chamomilla*, where the activity was increased in plants exposed to nitrogen deficiency for 4 and 8 days, while the prolonged exposure for 12 days decreased the activity (Kovacik et al., 2007). Furthermore, variation in PAL activity in different organs was demonstrated in pearl millet, *Pennisetum glaucum*, seedlings challenged with downy mildew disease, where PAL activity was increased in shoot but decreased in mesocotyl and root (Nagarathna et al., 1 993).

In *E. angustifolia*, PAL activity was similar in leaves and roots of salt-stressed plants and control plants after 7 and 14 days of treatment. Moreover, PAL activity did not correlate with the total phenolic content or individual phenolics. However, in a previous study on *E. angustifolia*, PAL activity was increased in response to NaCl salinity, and this increase was correlated with the accumulation of chlorogenic acid and cichoric acid in the roots (Montanari et al., 2008). These differences may be due to the duration of the experiments and/or the experimental conditions, such as the age of plants (Mandal et al., 2009), salt level (Gao et al., 2008), and the composition of the nutrient solution (Zheng et al., 2006 b). For example in Montanari's experiment, plants were harvested at the age of 3-4 month after emergence as compared to 7-month old in our study; furthermore, low salt level (50 mM NaCl) was used.

PPO activity was reduced by all salt concentrations in *E. purpurea* after 14 days of treatments in both roots and leaves. One possible cause for this reduction is the relatively high accumulation of Na^+ in the roots of *E. purpurea* (Na^+ exclusion mechanism) compared with the two other species studied (Sabra et al., 2012 b, Chapter 3). Retention of high Na^+ content in roots may suppress the expression of the PPO genes (Chisari et al., 2010), and inhibit enzyme activities. As PPO is a metalloenzyme-containing copper, Na^+ ions may interfere with the copper ions at the active sites leading to its inhibition (Gawlik-Dziki et al., 2008; Chisari et al., 2010). The accumulation of Cl^- ions may further contribute to the corresponding reduction in PPO activity in leaves. In support of this idea, a negative correlation between Cl^- content in leaves of *E. purpurea* and PPO activity after 14 days was observed (r= -0.97, p=0.03). Moreover, Na^+ content in the roots correlated with PPO activity in leaves after 14 days (r= -0.947, p= 0.053). It

is worth noting that oxidation of phenolic compounds by PPO may produce ROS that can cause oxidative damage to membranes and macromolecules (Appel, 1993; Gill and Tuteja, 2010), and this ROS formation is further enhanced by salinity-induced osmotic stress and reduction in photosynthetic rate. Therefore, the reduction in PPO activity could be an adaptation of plants to reduce the rate of ROS generation produced during salinity stress. This idea was supported in a previous study on tomato (*Lycopersicon esculentum*) plants in which the suppression of PPO gene enhanced tolerance to drought stress due to the delay in the onset of oxidative damage (Thipyapong et al., 2004). Similarly, PPO activity was down-regulated in olive (*Olea europaea* L. cv. Chemlali) plants in response to saline water irrigation (7.5 dS m^{-1}), suggesting an adaptation to improve the antioxidant capacity of phenolic compounds by reducing their degradation, and subsequently maintaining an acceptable level of phenols with enhanced antioxidant capacity (Ben Ahmed et al., 2009). Moreover, it has been shown that the adaptation of tomato plants and watermelon (*Citrullus lantatus*) to thermal stress was through reduction in phenolic-oxidizing enzymes, such as PPO and peroxidase in order to maintain phenolic levels (Rivero et al., 2001). However, in *Echinacea* exposed to 100 mM NaCl, we found a simultaneous decrease in both PPO activity and the phenolic content, which indicates that reduction in PPO activity did not lead to the retention of phenolic compounds in our study at the highest salt concentration. In addition, a correlation could not be established between PPO activity and the phenolic content in *E. purpurea*, a finding that has been reported in *Coffea arabica* challenged with pathogens and insects (Melo et al., 2006) and in *Helianthus annuus* under boron (B) deficiency (Pfeffer et al., 1998). On the contrary, a positive correlation between polyphenol content

and PPO has been found in other species such as *Zea mays* inoculated with mycorrhiza (Subramanian et al., 2011), and in *Lactuca sativa* exposed to low temperature regime (Boo et al., 2011). As PPO activity was reduced in *E. purpurea* by all salt concentrations in the current study, this might suggest that PPO did not play a major role as antioxidant enzyme in this species. In fact other antioxidant enzymes particularly SOD and APX have been found to be the principal scavenging machinery in *E. purpurea* under salinity stress and contributed to its overall relative salt-tolerance (Chapter 3; Sabra et al., 2012 a). Reduction in PPO activity can be further explained through the dual nature of the enzyme in healthy and stressed plants. PPO is known to be activated in response to pathogen and herbivore attack due to the physical damage and decompartmentalization of cellular components, which enables the conversion of this enzyme from the latent form to the active form upon the contact between the enzyme located in plastids and phenolic substrates located in vacuoles (Chisari et al., 2010). Therefore, the decrease in PPO in *E. purpurea* suggest that salinity-induced injury and membrane damage may not be sufficient for homogenizing the enzyme with its substrates, as this species showed a relatively low injury index and electrolyte leakage (Chapter 3). Contrary to our findings that PPO did not contribute to the antioxidant capacity of *E. purpurea*, PPO activity was increased and assigned an antioxidant role in *Panax ginseng* exposed to Cu^{2+} stress (Ali et al., 2006), and *Catharanthus roseus* treated with salinity and the fungicide propiconazole (Jaleel et al., 2008) suggesting that the antioxidant role of PPO depends on the plant species and type of stress. In *E. angustifolia*, PPO activity was also reduced in the roots after 14 days, and this reduction may be due to the inhibitory effect of high Na^+ as supported by the negative correlation between Na^+ content and PPO activity in roots

after 14 days (r= - 0.949, p=0.051). Moreover, reduction in enzyme activity could be a result of the decrease in water availability required for the enzymatic reactions, a change that is often encountered during osmotic stress. In my experiment, water content in the shoots of *E. angustifolia* was lower than the other two species under salt treatments (Chapter 3).

On the other hand, PPO activity was increased in leaves and roots of *E. pallida* and *E. angustifolia* treated with 50 mM NaCl. This may be due to the conversion of a latent PPO to an active form (Winters et al., 2008) following the salt treatments; however the regulation of this process *in vivo* is not known. Similar increases in PPO activity have been found in different plant species, such as *Cassia angustifolia* (Agarwal and Pandey, 2004) and *Zea mays* exposed to salts (Koskeroglu and Tuna, 2010), and *Catharanthus roseus* treated with paclobutrazol and gibberelic acid (Jaleel et al., 2010). An increase in PPO activity was also observed in *Nicotiana tabacum* plants exposed to boron deficiency, and this increase corresponded with the increase in the accumulation of total soluble phenolics in leaves (Camacho-Cristobal et al., 2002). Despite the well-characterized role of PPO in response to biotic stress particularly pathogen and herbivore attack (Li and Steffens, 2002; Mayer, 2006), its role in plants under abiotic stress is not well-understood (Gawlik-Dziki et al., 2008). Regulating the redox status of phenolic compounds in the phenylpropanoid pathway is another proposed function for the PPO enzyme (Ali et al., 2006). As PPO is involved in the formation of more complex phenolics from mono and diphenols, I suggest that the formation of the phenolic glycoside (6-*O*-caffeoyl echinacoside) in *E. pallida* may be due to the oxidative activity of PPO enzyme as there

was a positive correlation (*r*=0.963, *p*=0.037) between PPO activity in roots after 7 days of salt treatment and the level of this compound.

CAPX activity did not change after salt treatments in *E. purpurea* and *E. pallida*, while in *E. angustifolia*; the activity was stimulated by moderate salt concentration (75 mM NaCl) after 7 days in roots and after 14 days in both leaves and roots. Overall, significant higher CAPX activity was observed in *E. angustifolia*, particularly in leaves, than the other two species. A previous study has shown that spring wheat (*Triticum aestivum*) genotypes with low growth rates had higher peroxidase activity than the genotypes with vigorous growth (Moore and Cubitt, 1981). Similarly, the differential response among *Echinacea* species may be due to the differences in growth rate. Although growth rate was not measured, the biomass of *E. purpurea* and *E. pallida* was 4-10 times higher than the biomass of *E. angustifolia*, which reflects their high growth rate compared to *E. angustifolia* (Chapter 3). An increase in peroxidase activity could increase the lignification and deposition of phenolic compounds in the cell wall making it mechanically rigid, and therefore, reducing growth rate (Devi and Prasad, 1996). It has also been shown that activation of oxidizing complex polyphenoloxidases (i.e. IAA oxidase) reduce the indigenous indole acetic acid (IAA) content that induces cell and cell wall growth, thus resulting in plant growth reduction (Omran, 1980). An increase in CAPX along with other phenylpropanoid enzymes, such as CGAPX, PPO, and PAL activities correlated with the increase in phenols and flavonoids contents in the suspension cultures of *Panax ginseng* exposed to high CO_2 and Cu^{2+}, indicating that these enzymes may play a role in the synthesis of phenolic compounds (Ali et al., 2005 and 2006). However in the present study, there was a negative correlation between CAPX in

the roots after 14 days and total phenolics in *E. purpurea* (r=0.941, p=0.059), which implies that this enzyme is more likely involved in phenolics oxidation rather than their synthesis. This idea is supported by a previous study on *Capsicum* spp, where peroxidases were responsible for the degradation of capsaicinoids, the major cinnamic acid ingredients responsible for the pungent taste (Contreras-Padilla and Yahia, 1998).

Similar to CAPX, CGAPX showed a differential response among *Echinacea* species, and this was dependent on the salt concentration and the duration of exposure to salts. For example, the activity was increased when the plants were exposed to moderate and/or high salt concentrations in roots and leaves after 7 days and in leaves after 14 days in *E. angustifolia*. While there was an increase in *E. pallida* leaves at 75 mM NaCl, a decrease was found in *E. purpurea* root at 100 mM NaCl only after 7 days. Previous studies have shown that CGAPX activity is highly influenced by stress conditions as it was demonstrated in *Panax ginseng* exposed to high CO_2 (Ali et al., 2005) and Cu^{2+} (Ali et al., 2006). It was found in these two studies that CGAPX along with other enzymes related to phenolic metabolism may be involved in the biosynthesis of phenolic compounds in this species; however which role could CGAPX play under salinity is not known and further investigation is needed. Chlorogenic acid (CGA) is well known to act as antioxidant in plants, and this was demonstrated in transgenic tobacco (*Nicotiana tabacum*) plants that showed symptoms of oxidative damage and increased malondialdehyde (MDA), a product of lipid peroxidation, when the accumulation of CGA was suppressed (Tamagnone et al., 1998), therefore it is expected that oxidation of CGA to quinine may reduce the antioxidant capacity of plants. Chlorogenic acid PX activity in leaves after 14 days of treatment negatively correlated (r= -0.995, p=0.005)

with the phenolic glycoside (6-*O*-caffeoyl echinacoside) content in the roots of *E. pallida* indicating that this compound may work as a potential substrate, although it is not known if this enzyme is mobile in plants and how it can be transported from leaves to roots.

Overall, salinity caused a reduction in GPX activity in *E. angustifolia* leaves after 7 days of exposure to salt treatments, and there was a negative relationship between salt levels and GPX activity (r = -0.959, p=0.041), with an even more pronounced reduction after 14 days, (r= -0.994, p=0.006), probably due to increasing salt accumulation over time in leaf tissues. This was supported by the negative correlation between GPX activity and both shoot Na^+ content (r= -0.98, p=0.02) and shoot Cl^- content (r= -0.976, p=0.024), which indicates that both ions may have inhibited the GPX activity. In the roots, GPX activity was not reduced and overall, the relationship between root enzyme activity and salt levels was not significant (r= -0.209 and r= -0.434 after 7 and 14 days, respectively), suggesting that roots may be more efficient in compartmentalizing ions in the vacuole, thus maintaining low cytoplasmic Na^+ concentration than the leaves (Blumwald et al., 2000; Munns, 2002). The differential response of GPX in shoots and roots has been observed before in salt-stressed maize (*Zea mays*) plants (de Azevedo Neto et al., 2006). In *E. pallida*, the species that showed the medium level of salt tolerance among the three studied species (Chapter 3), GPX activity was increased or maintained as the same level as the control after 7 days of exposure to salts, while in *E. purpurea,* the relatively salt-tolerant species, there was a reduction in GPX activity in roots. This suggests that GPX could be an active component of the antioxidant machinery in *E. pallida*, but not in *E. purpurea*. These results not only show that different species exhibit differential antioxidant activities in response to salinity stress, but also suggest that determination of

GPX activity may not be a biomarker or does not reflect the salt tolerance degree among *Echinacea* species. The decreased GPX activity in the salt tolerant genotypes has also been found in *Oryza sativa* plants exposed to NaCl salinity (Dionisio-Sese and Tobita, 1998) and Cd toxicity (Nahakpam and Shah, 2011). On the contrary, GPX activity was only increased in salt tolerant genotypes of *Sorghum bicolor* (Costa et al., 2005) and *Zea mays* (de Azevedo Neto et al., 2006). In addition to its role as antioxidant enzyme in plants, GPX was found to be involved in the oxidation of phenolic compounds and its activity was correlated with the resistance of *Lycopersicon esculentum* to thermal stress (Rivero et al., 2003).

It can be concluded from this study that salinity causes significant changes in some of the enzymes related to phenolic metabolism, and this effect was influenced by the salt concentration and the duration of exposure; however no clear trend was observed for some enzymes, such as CAPX and CGAPX. As there was no significant correlation between PAL and phenolic compounds, this may indicate that biosynthesis of CADs in *Echinacea* species is more complex to be solely regulated through the activation of PAL, and it is most likely that other corresponding enzymes downstream of PAL are more relevant to the biosynthesis of phenolics in *Echinacea* through different biosynthetic branches. Determination of PPO activity may not be considered a biomarker for the salt tolerance degree among *Echinacea* species as PPO activity was reduced at all salt concentrations in leaves and roots of the relatively salt tolerant species, *E. purpurea.* On the other hand, GPX may have contributed to the antioxidant machinery in one of the species, *E. pallida*, where the activity was maintained and/or increased in salt-treated plants. Lack of correlation between the enzymes (PAL, PPO, CAPX, and CGAPX)

related to phenolic metabolism and phenolic content suggests that determination of these enzymes is not an indicator for evaluating the changes in phytochemical marker compounds. Nevertheless, determination of PAL may reflect the differences among *Echinacea* species in salt tolerance, because activity was enhanced by salinity only in the species with the relative salt tolerance, *E. purpurea*. As promising as these preliminary results may be, the relationship between individual phenolic compounds and the activity of their corresponding biosynthetic enzymes is more complex and far from being understood. Studies pertaining to molecular characterization of these enzymes with relation to phytochemical accumulation in *Echinacea* are needed for more understanding of their potential physiological roles and warrant further investigation.

Table 5.1. Activities of CAPX in the leaves of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			(µmol min^{-1} mg^{-1} protein)			
0	12.5±1.73^{e}	19.0±3.08^{ab}	18.8±3.19^{c-e}	18.5±5.08^{ab}	32.1±3.48^{ab}	11.2±1.47^{b}
50	17.5±5.55^{de}	9.5±0.72^{b}	15.9±2.51^{de}	16.1±0.68^{ab}	28.7±2.83^{bc}	13.9±1.44^{ab}
75	11.4±0.91^{e}	8.5±0.39^{b}	14.4±1.09^{de}	19.5±2.13^{ab}	24.6±2.29^{b-d}	22.8±3.53^{a}
100	9.30±1.67^{e}	11.8±0.99^{ab}	14.8±0.91^{de}	13.5±1.09^{ab}	41.3±3.49^{a}	17.3±1.48^{ab}

Table 5.2. Activities of CAPX in the roots of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			(µmol min^{-1} mg^{-1} protein)			
0	22.6±1.61^{a-c}	15.6±1.92^{ab}	18.5±1.82^{a-c}	13.6±1.83^{ab}	15.7±3.89^{bc}	11.4±2.03^{b}
50	25.2±4.95^{a-c}	11.9±0.89^{b}	26.9±4.8^{ab}	13.1±1.0^{ab}	11.1±2.15^{c}	10.4±0.97^{b}
75	22.8±2.09^{a-c}	10.1±0.99^{b}	20.8±1.05^{a-c}	18.5±2.81^{ab}	31.7±3.26^{a}	20.9±2.17^{a}
100	12.0±1.09^{c}	17.7±2.09^{ab}	15.3±0.78^{bc}	15.1±1.01^{ab}	24.0±1.97^{a-c}	16.2±1.51^{ab}

Table 5.3. Activities of CGAPX in the leaves of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			(µmol min^{-1} mg^{-1} protein)			
0	340±28.9 [b-d]	373±35.0 [ab]	155±10.8 [e]	197±19.5 [c-e]	295±48.2 [b-e]	189±24.3 [de]
50	289±7.60 [b-e]	323±36.8 [a-c]	333±25.9 [b-d]	215±16.9 [c-e]	419±42.6 [ab]	255±24.2 [b-e]
75	371±25.6 [a-c]	246±18.8 [b-e]	217±19.3 [de]	179±22.8 [de]	494±36.0 [a]	374±27.0 [ab]
100	257±23.3 [c-e]	284±18.1 [a-d]	254±21.5 [c-e]	150±15.8 [e]	333±33.5 [b-d]	416±41.1 [a]

Table 5.4. Activities of CGAPX in the roots of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			(µmol min^{-1} mg^{-1} protein)			
0	675±87.8 [a]	607±50.9 [a]	437±42.3 [b-d]	421±56.0 [a-c]	275±25.0 [d]	448±30.7 [a-c]
50	633±34.8 [ab]	531±33.8 [ab]	350±25.8 [cd]	445±56.6 [a-c]	285±43.6 [d]	375±68.3 [bc]
75	476±45.2 [a-d]	486±32.5 [a-c]	369±25.3 [cd]	349±31.7 [bc]	533±39.8 [a-c]	399±26.9 [a-c]
100	441±58.8 [b-d]	593±38.4 [a]	407±51.8 [b-d]	416±29.2 [a-c]	360±27.9 [cd]	310±22.6 [c]

Table 5.5. Activities of GPX in the leaves of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			(μmol min^{-1} mg^{-1} protein)			
0	166±15.1 ^{a}	96.5±12.6 ^{ab}	74.1±8.45 ^{cd}	59.8±1.79 ^{bc}	147±15.1 ^{ab}	130±8.65 ^{a}
50	127±8.44 $^{a-d}$	144±11.0 ^{a}	121±19.1 $^{a-d}$	69.5±8.02 ^{bc}	127±17.6 $^{a-d}$	74.8±7.68 ^{bc}
75	125±14.6 $^{a-d}$	77.3±13.7 ^{bc}	159±12.6 ^{a}	67.8±11.3 ^{bc}	85.2±4.73 $^{b-d}$	43.6±9.57 ^{c}
100	146±19.8 $^{a-c}$	100±16.1 ^{ab}	167±12.2 ^{a}	44.7±4.78 ^{c}	73.4±9.43 ^{d}	29.2±2.44 ^{c}

Table 5.6. Activities of GPX in the roots of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			(μmol min^{-1} mg^{-1} protein)			
0	255±14.9 ^{a}	170±15.0 ^{a}	56.1±7.51 ^{de}	104±5.07 ^{bc}	19.2±2.42 ^{e}	45.4±5.97 ^{de}
50	240±11.3 ^{a}	157±14.3 ^{ab}	39.2±2.8 ^{de}	123±4.26 $^{a-c}$	70.3±3.17 ^{d}	6.91±0.51 ^{e}
75	187±14.8 ^{b}	134±9.44 $^{a-c}$	134±14.6 ^{bc}	104±14.7 ^{bc}	10.4±2.85 ^{e}	21.2±6.64 ^{e}
100	131±13.7 ^{c}	165±19.5 ^{a}	81.5±1.72 ^{d}	87.7±8.39 ^{cd}	13.0±3.09 ^{e}	29.8±5.03 ^{e}

Table 5.7. Activities of PPO in the leaves of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			(Unit mg^{-1} protein)			
0	38.9±4.59 [b]	100±11.6 [a]	31.1±3.56 [b]	49.4±4.87 [bc]	48.5±5.63 [b]	51.7±4.0 [bc]
50	37.3±4.78 [b]	52.0±4.48 [bc]	43.5±2.21 [b]	46.5±3.39 [bc]	79.5±5.58 [a]	38.8±4.33 [bc]
75	44.7±2.98 [b]	49.2±4.64 [bc]	39.8±5.18 [b]	103±12.0 [a]	42.8±4.5 [b]	73.8±6.80 [ab]
100	44.4±3.28 [b]	45.2±6.4 [bc]	53.6±8.5 [b]	64.5±4.16 [bc]	36.8±2.47 [b]	29.6±10.4 [c]

Table 5.8. Activities of PPO in the roots of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			(Unit mg^{-1} protein)			
0	79.0±10.1 [a]	129±12.4 [a]	44.5±11.5 [c-e]	61.0±7.3 [bc]	19.4±2.62 [ef]	88.1±9.5 [ab]
50	62.9±7.30 [a-c]	78.7±12.4 [bc]	34.6±4.48 [d-f]	81.1±10.9 [b]	50.0±4.05 [b-d]	44.7±3.69 [bc]
75	65.1±5.67 [ab]	82.0±11.9 [b]	43.6±3.11 [b-e]	64.4±2.81 [bc]	27.2±3.46 [d-f]	51.3±3.63 [bc]
100	62.3±3.59 [a-c]	84.6±7.6 [ab]	64.0±4.13 [ab]	85.1±10.2 [ab]	10.5±0.38 [f]	36.2±4.65 [c]

Table 5.9. Activities of PAL in the leaves of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			($nmol\ h^{-1}\ mg^{-1}$ protein)			
0	129±6.2 [d-f]	140±20.2 [c]	95.0±13.8 [f]	553±79.2 [a]	167±20.5 [b-f]	353±45.2 [a-c]
50	207±16.6 [a-d]	177±13.7 [c]	190±15.2 [a-e]	433±42.2 [ab]	233±34.0 [a-c]	482±73.8 [ab]
75	278±10.6 [a]	436±44.2 [ab]	159±18.0 [c-f]	299±32.1 [bc]	258±28.0 [ab]	448±70.1 [ab]
100	105±17.7 [ef]	284±43.5 [bc]	100±11.4 [ef]	305±14.3 [bc]	183±3.4 [b-f]	505±32.8 [ab]

Table 5.10. Activities of PAL in the roots of *E. purpurea*, *E. pallida*, and *E. angustifolia* exposed to 0, 50, 75, and 100 mM NaCl for 7 and 14 days.

	E. purpurea		*E. pallida*		*E.angustifolia*	
NaCl (mM)	7 days	14 days	7 days	14 days	7 days	14 days
			($nmol\ h^{-1}\ mg^{-1}$ protein)			
0	203±30.2 [cd]	459±33.2 [bc]	427±7.9 [a]	378±56.0 [b-d]	198±12.0 [cd]	414±23.4 [b-d]
50	479±37.2 [a]	522±47.2 [ab]	144±10.4 [d]	280±28.0 [d]	263±26.0 [b-d]	490±38.7 [bc]
75	413±42.0 [ab]	483±47.6 [bc]	201±22.2 [cd]	337±26.9 [cd]	327±35.9 [a-c]	352±40.7 [cd]
100	459±64.6 [a]	653±31.1 [a]	139±18.8 [d]	263±31.2 [d]	200±24.2 [cd]	392±31.3 [b-d]

Table 5.11. Correlation between PAL, PPO, CAPX, and CGAPX activities and individual and total phenolic content identified in the roots of *Echinacea* species exposed to different salinity levels for 7 and 14 days under hydroponic culture.

Enzyme	Species	Duration	Marker compound Contents (mg g^{-1} DW)						
			Caftaric acid[1]	Chlorogenic acid[2]	Cynarin[3]	Echinacoside[4]	6-*O* caffeoyl echinacoside[5]	Cichoric acid[6]	Total phenolics
PAL	*E.pur.*	7days	NS	NS	NS			NS	NS
		14days	NS	NS	NS			NS	NS
	E. pal	7days	NS			NS	NS	NS	NS
		14days	NS			NS	NS	NS	NS
	E. ang	7days		NS	NS	NS			NS
		14days		NS	NS	NS			NS
PPO	*E. pur*	7days	NS	NS	NS			NS	NS
		14days	NS	NS	NS			NS	NS
	E. pal	7days	NS			NS	0.963*	NS	NS
		14days	NS			NS	NS	NS	NS
	E. ang	7days		NS	NS	NS			NS
		14days		NS	NS	NS			NS
GPX	*E. pur*	7days	NS	NS	NS			NS	NS
		14days	NS	NS	NS			NS	NS
	E. pal	7days	NS			NS	NS	NS	NS
		14days	NS			NS	-0.975*	NS	NS

Table 5.11 continued

Enzyme	Species	Duration	Marker compound Contents (mg g^{-1} DW)						
			Caftaric acid[1]	Chlorogenic acid[2]	Cynarin[3]	Echinacoside[4]	6-*O* caffeoyl echinacoside[5]	Cichoric acid[6]	Total phenolics
	E. ang	7days		0.993**	NS	NS			NS
		14days		NS	NS	NS			NS
CAPX	*E. pur*	7days	NS	NS	NS			NS	NS
		14days	NS	NS	NS			NS	-0.941*
	E. pal	7days	NS			NS	NS	NS	NS
		14days	NS			NS	NS	NS	NS
	E. ang	7days		NS	NS	NS			NS
		14days		NS	NS	NS			NS
CGAPX	*E. pur*	7days	NS	NS	NS			NS	NS
		14days	NS	NS	NS			NS	NS
	E. pal	7days	NS			NS	NS	NS	NS
		14days	NS			NS	NS	NS	NS
	E. ang	7days		NS	NS	NS			NS
		14days		NS	NS	NS			NS

E. pur (*E. purpurea*); *E. pal* (*E. pallida*); *E. ang* (*E. angustifolia*). NS, not significant at $P \leq 0.05$; *significant at $P \leq 0.05$; ** significant at $p \leq 0.01$. 1, 6 characteristic to *E. purpurea* and *E. pallida*; 2, 3, characteristic to *E. purpurea* and *E. angustifolia*; 4, characteristic to *E. pallida and E. angustifolia*; 5, characteristic to *E. pallida.*

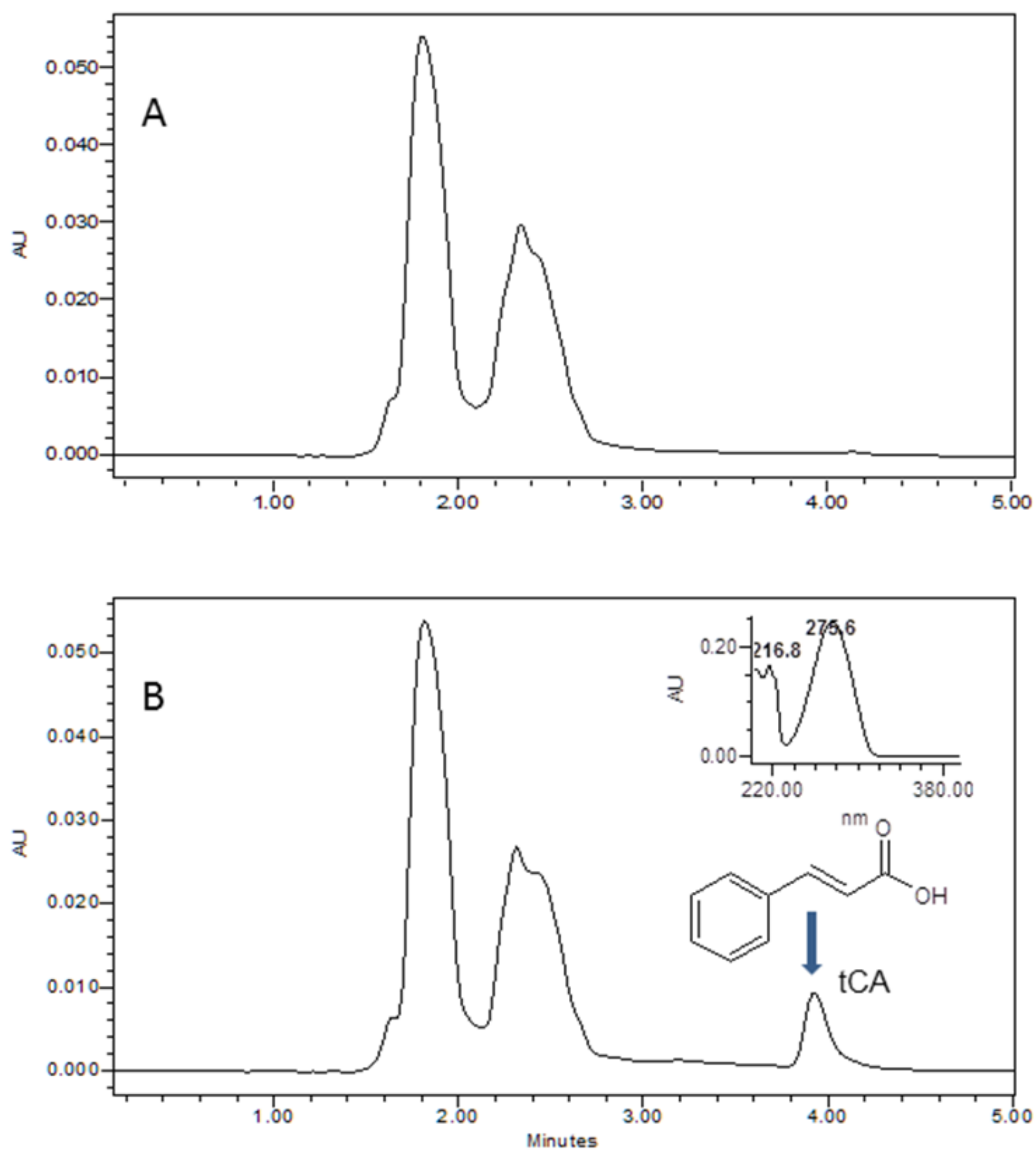

Fig. 5.1. HPLC Chromatogram showing *trans* cinnamic acid (*t*-CA) as the product of reaction after 30 min incubation of the plant extract with the substrate (phenylalanine) (B), and control reaction without the substrate (A).

CHAPTER 6. SUMMARY AND CONCLUSIONS

Salinity is an important environmental constraint that affects the growth and productivity of many plants worldwide (Parida and Das, 2005). This problem is caused by the occurrence of high concentrations of dissolved ions mainly, Na^+ and Cl^-, in the soil solution, which impose both osmotic and ionic stress and cause physiological and biochemical changes in plants (Munns and Tester, 2008). In the case of medicinal plants, previous studies have shown that changes also occur in the phytochemical constituents (Table 2.1), and such changes may be relevant to the quality of raw materials used in the pharmaceutical preparations. As secondary metabolites are known to be stress-induced compounds that accumulate in relation to biotic and abiotic stresses for their physiological and ecological functions (Weisshaar and Jenkins, 1998), I tested the hypothesis that saline conditions may enhance the accumulation of the health-promoting substances in *Echinacea*.

In addition to utilizing halophytes as well as breeding and genetic engineering approaches, selecting glycophytes that can tolerate moderate saline environments can be an important approach for reducing the adverse effects of salinity on plants. My objectives aimed at evaluating the relative salt tolerance of different *Echinacea* species based on physiological and biochemical changes. Understanding such changes can provide information about the behaviour of different species and could be an important approach in the overall management of salinity.

The physiological, biochemical, and phytochemical changes in the three medicinally-used *Echinacea* when challenged with salinity stress for two weeks were

investigated through the current thesis. In the first study (Chapter 3), the effects of various NaCl concentrations on gas exchange, elemental contents, electrolyte leakage, pigments, growth, survival, and injury were investigated. Furthermore, responses of the antioxidant enzyme activities were studied. Salinity had no effect on the growth of the three species; however, survival and injury index were higher in *E. angustifolia* than the other two species indicating its sensitivity to salts. Moreover, this species was also characterized by increased EL values, an indicator for membrane damage. Generally, photosynthetic rate, stomatal conductance and transpiration rate decreased in all species in response to salinity, particularly at 75 and 100 mM NaCl. Reduction in these characteristics was attributed mainly to the accumulation of ions (Na^+ and Cl^-) in shoots. It is possible that these ions have inhibited some enzymes in the Calvin cycle and caused biochemical limitations, thus leading to reduction in photosynthetic activity; however closure of stomata also contributed to this reduction as there was a correlation between reduction in photosynthetic rate and closure of stomata. This study also suggest that Na^+ was the major toxic ion causing leaf necrosis as there was a positive correlation between Na^+ content in shoots and salt injury index in all species. However, Cl^- ions seem to further contribute to salt injury only in *E. pallida* and *E. angustifolia*.

Proposed as a physiological indicator for salt tolerance, ion exclusion capacity was able to differentiate between *Echinacea* species. For example, *E. purpurea* accumulated less Na^+ ions in shoots and more Na^+ in roots than the other species. This suggests that this species probably possess a distinctive mechanism for controlling the uptake and/or translocation of Na^+ to shoots especially that this species has fibrous roots, while *E. pallida* and *E. angustifolia* have tap roots. How root structure affect ion

movement in plants is not clearly understood. Differences between *E. pallida* and *E. angustifolia* in the capacity of excluding ions were also observed in favor of *E. pallida*. *E. angustifolia* showed also the highest Na^+/K^+ ratio in shoots, which supports the idea that this species is more sensitive to salts than the other two species.

Antioxidant enzyme activities including SOD, APX, CAT and GR were determined in roots and leaves of *Echinacea* species after 14 days of exposure to salts (Chapter 3). Among these enzymes, the activities of SOD and APX increased in *E. purpurea*, did not change in *E. pallida* and decreased in *E. angustifolia*. This may indicate that species exhibited a differential antioxidant response which reflects their salt tolerance degree.

It can be concluded from this study that the metabolic constraints in salt-stressed *Echinacea* are mainly attributed to ionic stress particularly Na^+ ions. Generally, the *Echinacea* species studied did not tolerate the highest salt concentration (100 mM NaCl), suggesting only a limited tolerance to salinity. However differential responses were observed at the low level of salinity (50 mM NaCl) and these differences were related to Na^+ excluding mechanism, reducing solutes leakage and enhancing antioxidant activities. *Echinacea* species can be ranked as the following: *E. purpurea* > *E. pallida* > *E. angustifolia* in terms of salt tolerance.

The three species were also subjected to various salt concentrations for investigating the quantitative and qualitative phytochemical changes; caffeic acid derivatives, mainly caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside, and cichoric acid, as well as lipophilic constituents, mainly alkamides and ketones, in the

roots (Chapter 4). Both types of compounds were simultaneously extracted by ASE and analysed by HPLC. Nearly 40 compounds were differentially identified in the roots of *Echinacea* species, and the distinctive profile for each species was obtained allowing the distinction between species, a criterion required for quality control of preparations. Results showed no change in the composition of CADs, alkamides, and ketones as a result of salinity; however quantitative changes were observed. These quantitative changes are summarized in Table 6.1 and 6.2. For example, cichoric acid yield, the major CADs used for standardization of products was increased by salts up to 75 mM NaCl in *E. purpurea*, while echinacoside, a major CAD in *E. pallida*, was decreased at low and moderate salt concentrations, suggesting that the quality of the latter species was compromised.

From the physiological point of view, this increase in cichoric acid may be due to its potential role as antioxidant agent in plants as this compound possesses higher antioxidant capacity than the other CADs. However, such proposed role *in vivo* needs further investigation. In *E. angustifolia*, most of alkamides were retained suggesting that these compounds may play a role under salt stress; however their mode of action is not known. In a recent study, alkamides conferred resistance to *Botrytis cinerea* in *Arabidopsis thaliana* by activating jasmonic acid pathway and enhancing the signalling pathways (Méndez-Bravo et al., 2011). Moreover, it was suggested that alkamides have a role as signalling molecules during growth and development of *Arabidopsis* (López-Bucio et al., 2006). Nevertheless, retaining alkamides did not improve salt tolerance in *Echinacea*, and *E. angustifolia*, in particular, was more affected by salt stress than the other two species. Consequently, it could be concluded that the involvement of alkamides

in conferring resistance to a stress is dependent on the type of stress, plants species, age and the developmental stage. That is why the role of alkamides in salt stress as a function of different developmental stages seems to be an interesting research area particularly when the structures and types of alkamides are considered. In addition, in the relatively more salt tolerant species, *E. purpurea*, alkamides showed no significant changes or a decrease in their contents, which may be attributed to the differences in the nature of structures between *E. angustifolia*-containing alkamides (monoene type) and *E. purpurea*-containing alkamides (2,4-diene moiety). The latter types of alkamides are relatively more sensitive to degradation by oxidation than the monoene-moiety-type alkamides.

Quality of the raw materials obtained from *Echinacea* is a function of marker compounds content and dry biomass per plants. Cichoric acid, alkamides 8/9, and echinacoside can be considered marker compounds for the standardization of *Echinacea* products (Bauer, 1999 a; Wölkart et al., 2004). Quality of *E. purpurea* radix not only was retained under salinity at 50 mM NaCl, but also was increased at 75 mM NaCl. However, at 100 mM NaCl, quality was presumably reduced as a result of decreased content in lipophilic fraction. Taken into account the pattern of changes in marker compounds in the three species and the changes in root biomass per plant (no significant changes were observed in root biomass), it can be concluded that the quality of raw material varied between species, and quality of *E. purpurea* was higher than that of *E. pallida*. Moreover, this might be relevant to the pharmaceutical industry indicating that separation of raw materials is essential when considering making blends from different *Echinacea* species grown in saline areas. Despite the fact that the majority of marker compounds were

retained in salt-stressed *E. angustifolia*, it should be taken into consideration that this species is more sensitive to salts than the two other species as this species has low survival rate, increased EL and injury damage index. Therefore, utilizing this species in areas irrigated with saline water is not recommended for commercial production. *E. purpurea* seems to be the most appropriate *Echinacea* species to be utilized in saline irrigation with concentrations not exceeding 75 mM NaCl as it retained major marker compounds and exhibited relatively salt tolerance characteristics compared to the other two species. These findings could be implemented in the greenhouse production system of *E. purpurea*, where the exposure of plants to salinity up to 75 mM NaCl for two weeks enhances the desired marker compound contents (e.g. cichoric acid). Nevertheless, this proposed application has to be verified by testing longer exposure periods and determining the changes in the whole phytochemical spectrum (CADs, alkamides/ketones, polysaccharides and essential oils) as well as the yield.

In chapter 5, I studied the activities of some enzymes related to the metabolism of phenolic compounds after 7 and 14 days from exposure to different salt concentrations. The hypothesis was that activities of these enzymes may affect the accumulation of CADs in *Echinacea*, therefore affecting the content and quality of raw materials. No clear trend was observed regarding the effect of time of exposure on the activities of the majority of enzymes studied. However, differences between species were observed. For example, PAL activity showed an increase in *E. purpurea*, a decrease in *E. pallida* and no change in *E. angustifolia*. As *E. purpurea* is relatively more salt tolerant than the other two species, I suggest that the determination of PAL may be a reflection of the differences in salt tolerance among *Echinacea* species studied. However, this was not

applicable for the other enzymes, such as PPO, CAPX, CGAPX or GPX. This increase suggests that PAL, as a regulatory enzyme to phenylpropanoid pathway, may be involved in the response of *E. purpurea* to salinity stress. For instance, the end product of its activity is a wide array of phenolic compounds that can contribute to increasing the antioxidant capacity of plants under abiotic stress. Nevertheless, PAL activity after 14 days did not correlate with the accumulation of any of the phenolic compounds studied, which may be due to the complexity of the regulation of CADs biosynthesis in *Echinacea*. Downstream enzymes (e.g. 4-coumarate coenzyme A ligase, 4CL) in the general phenylpropanoid pathway could be more relevant to the regulation of phenolics synthesis.

It seems that salinity has complex effects on *Echinacea*. This includes physiological, biochemical and phytochemical changes. To the best of my knowledge, the current study is the first report on the changes of alkamides and ketones in *Echinacea* in response to salinity. The present study will contribute to the knowledge in the field of phytochemistry of medicinal plants (*Echinacea*) and provides further solid background about the physiology of *Echinacea* plants under saline conditions, which overall contribute to the management of salinity problem.

I have shown that salinity induce phytochemical changes in *Echinacea*. As drought stress is another constraint with large magnitude like salinity, and as both stresses impose the osmotic stress, it would be interesting to compare the effects of both stresses to determine if the phytochemical changes induced are attributed mainly to osmotic or ionic components. Such experiment can be conducted using iso-osmotic solutions.

Different questions arise from this work; what is the potential physiological or ecological role of alkamides in plants and are they involved in response to salinity stress? Recently, alkamides has been found to confer resistance to biotic stress by activating jasmonic acid biosynthesis and signalling pathways in *Arabidopsis* (Méndez-Bravo et al., 2011). As *E. angustifolia* and *E. purpurea* retained alkamides especially at low to moderate salt concentrations (50 and 75 mM NaCl), it would be of great interest to investigate their mode of action in response to salinity, and whether it is similar to the mode of action under biotic stress. As there was no correlation between PAL activity and the accumulation of CADs in *Echinacea*, studying other genes/enzymes downstream of PAL that may closely regulate the biosynthesis of CADs could be the core of an interesting study. Research on how plants regulate the influx of substrates towards the synthesis of specific marker compounds (e.g. cichoric acid), and what their potential roles under salinity stress will be the next step. To answer these questions and further understand plant functions in general, integration of multidisciplinary approaches employing biochemical and molecular tools such as gene expression and microarray studies in addition to proteomic and metabolomic analyses should be used.

Table 6.1. Summary of the response of individual caffeic acid derivatives and total phenolic content in salt-stressed *E. purpurea*, *E. pallida* and *E. angustifolia.*

Compound	*E. purpurea*			*E. pallida*			*E. angustifolia*		
	NaCl (mM)			NaCl (mM)			NaCl (mM)		
	50	75	100	50	75	100	50	75	100
Caftaric acid	+	+	+	–	–	–			
Chlorogenic acid	NS	NS	NS				+	NS	NS
Caffeic acid							+	+	+
Cynarin	+	NS	–				+	NS	NS
Echinacoside				NS	–	NS	NS	+	NS
6-*O*-caffeoyl echinacoside				NS	NS	+			
Cichoric acid	NS	+	–	NS	NS	+			
Cichoric acid derivative	NS	NS	–						
Total phenolic content	NS	NS	–	NS	NS	NS	+	+	NS

+, increase; –, decrease; NS, no significant change

Table 6.2. Summary of the response of individual alkamides and ketones and total content in salt-stressed *E. purpurea*, *E. pallida* and *E. angustifolia.*

Compound	*E. purpurea*			*E. pallida*			*E. angustifolia*		
	NaCl (mM)			NaCl (mM)			NaCl (mM)		
	50	75	100	50	75	100	50	75	100
Alk 1	NS	NS	–	–	–	–	NS	NS	–
Alk 2	NS	NS	NS	NS	–	–	NS	–	–
Alk3	NS	NS	–						
Alk 5	NS	+	–						
Alk 6	NS	NS	–						
Alk 7	–	NS	–						
Alk 8/9	NS	NS	–				+	NS	NS
Alk 10							NS	NS	NS
Alk 11							NS	–	–
Alk 12							+	NS	NS
Alk 13							+	NS	NS
Alk 14/15							+	+	NS
Alk 16							+	NS	NS
Alk 17							+	NS	NS
Alk 18							+	NS	NS
Alk 19							NS	NS	NS
Ketone 20				+	+	+			
Ketone 21				+	+	+			
Ketone 22				–	–	NS			
Ketone 23				NS	NS	NS			
Ketone 24				–	–	–			
Ketone 25				–	–	–			
Peak 39				NS	–	NS			
Peak 40				+	NS	+			
Total	NS	NS	–	NS	NS	NS	NS	NS	NS

+, increase; – , decrease; NS, no significant change; Alk, alkamide.

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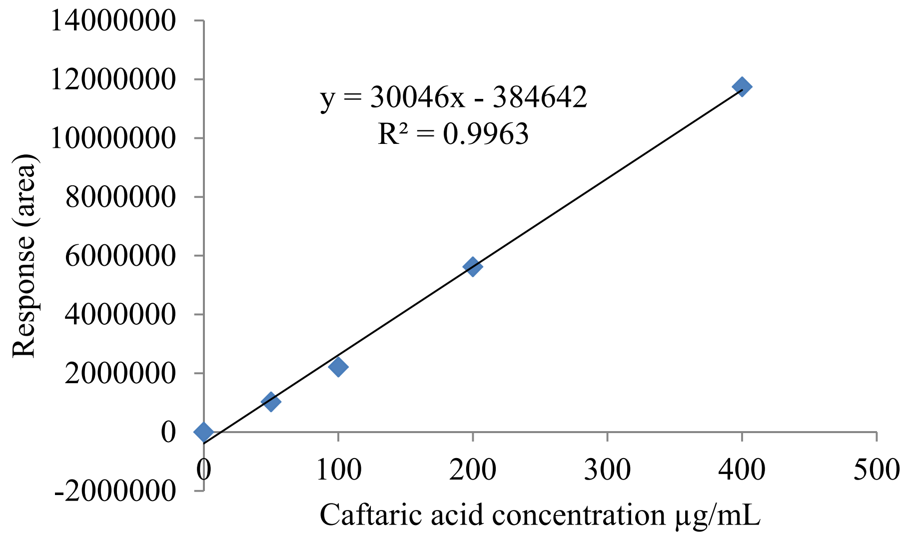
8. APPENDICES

Appendix A



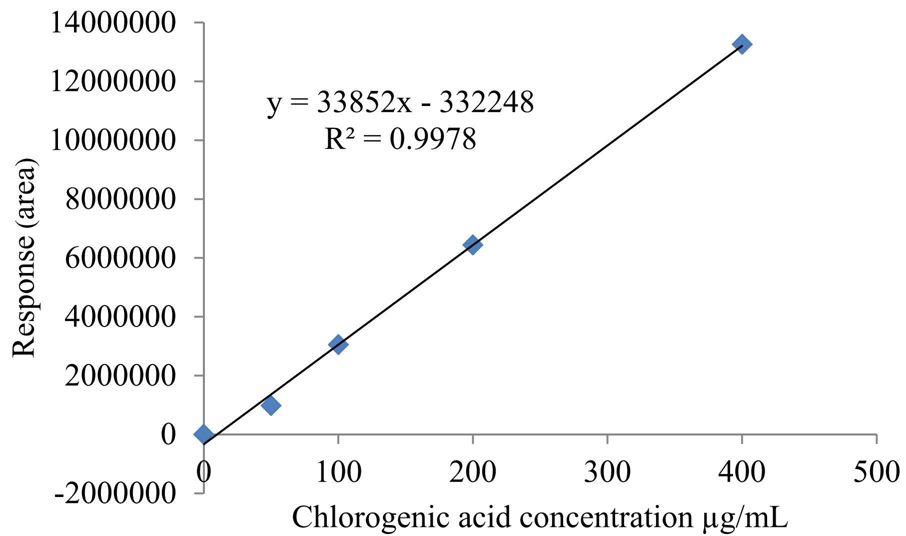
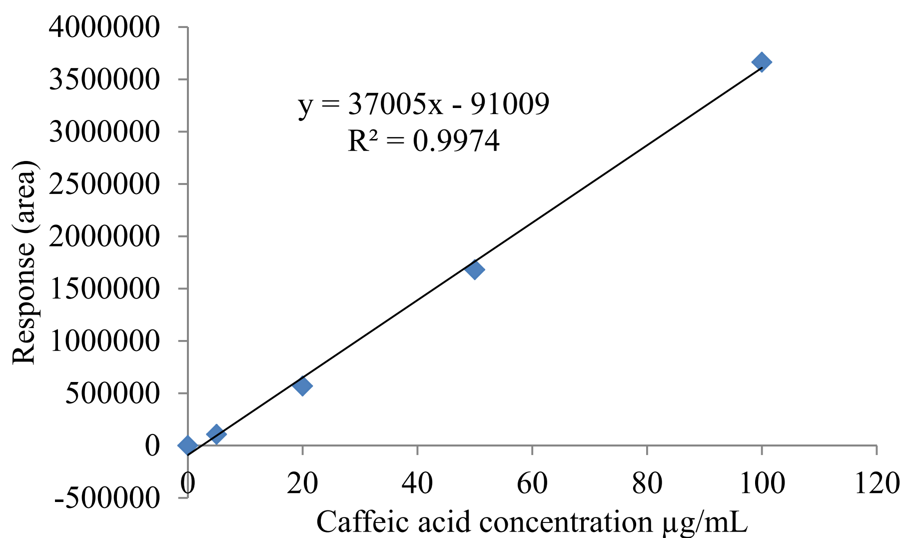


Fig. 8.1. Standard curves of caftaric acid and chlorogenic acid. The equation was used to calculate the corresponding compound content in the plant extract of *Echinacea* species. Each point represents the mean of three replicates.



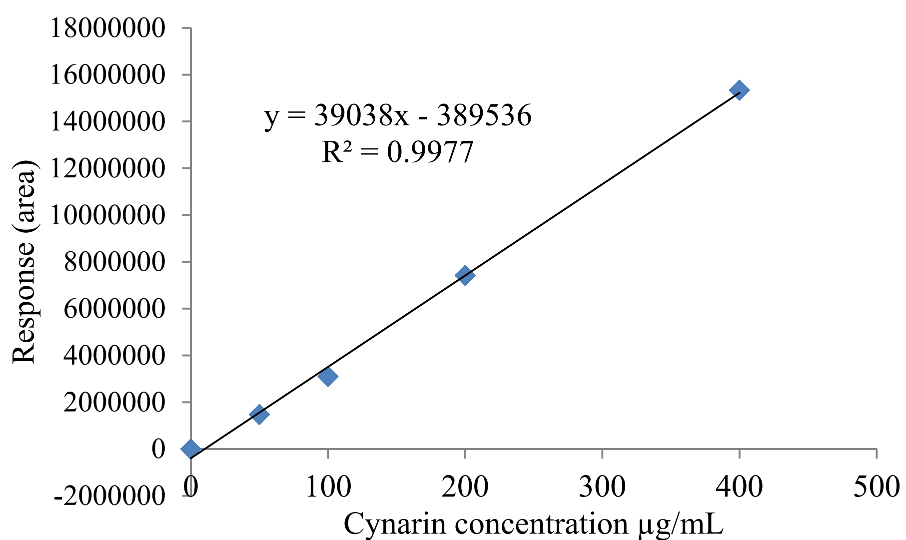
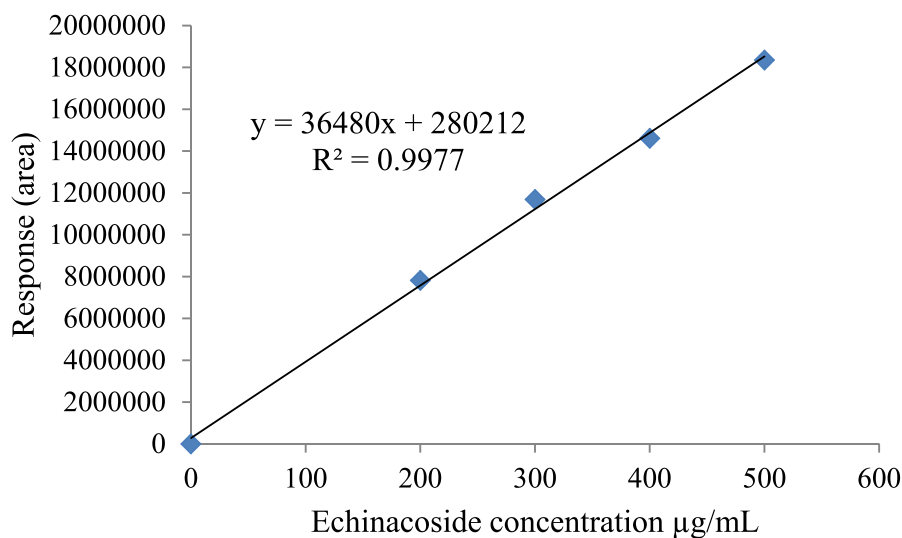


Fig. 8.2. Standard curves of caffeic acid and cynarin. The equation was used to calculate the corresponding compound content in the plant extract of *Echinacea* species. Each point represents the mean of three replicates.



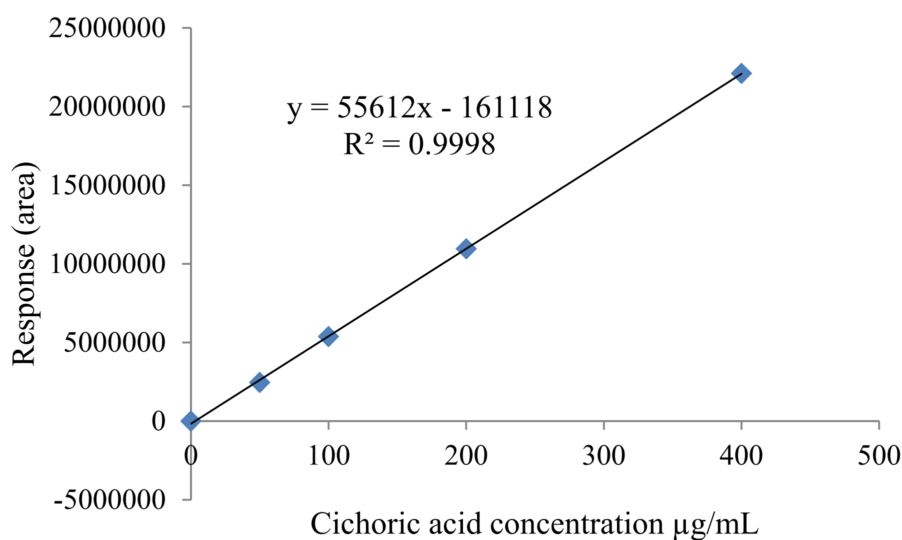
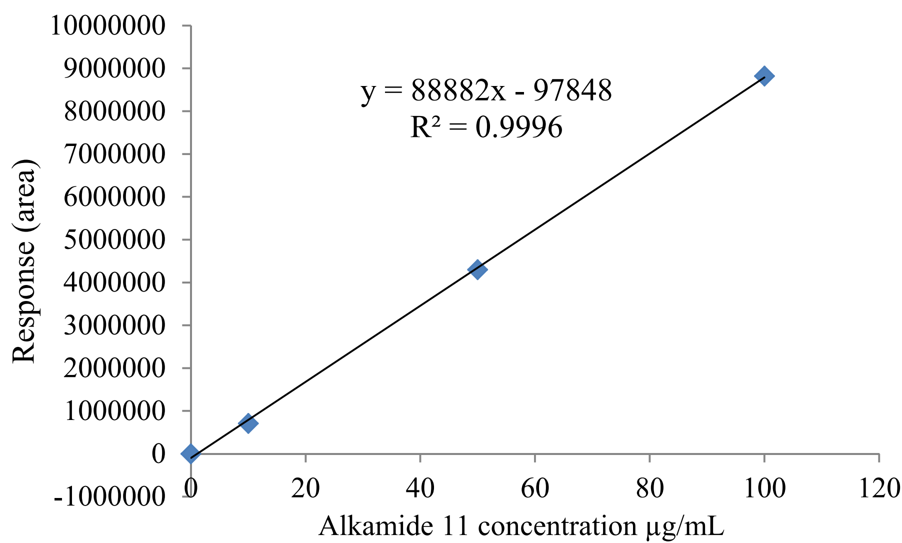


Fig. 8.3. Standard curves of echinacoside and cichoric acid. The equation was used to calculate the corresponding compound content in the plant extract of *Echinacea* species. Each point represents the mean of three replicates.



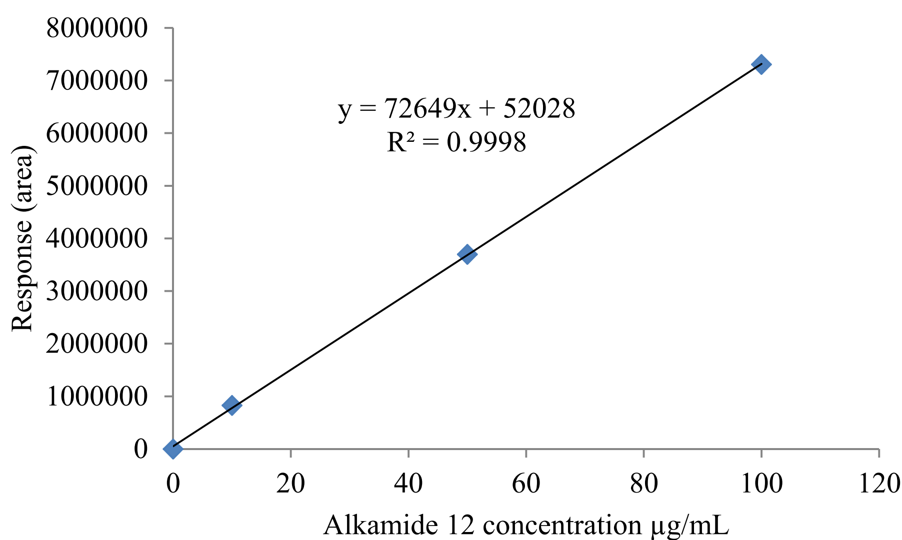


Fig. 8.4. Standard curves of alkamides 11 and 12. The equation was used to calculate the corresponding alkamide or ketone content in root extracts of *Echinacea* species. Each point represents the mean of three replicates.

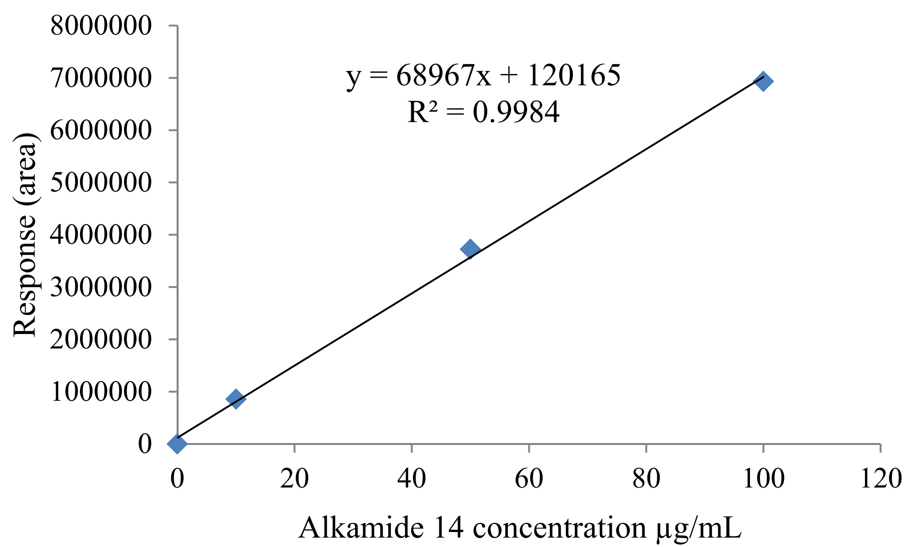


Fig. 8.5. Standard curves of alkamide 14. The equation was used to calculate the corresponding alkamide or ketone content in root extracts of *Echinacea* species. Each point represents the mean of three replicates.

Appendix B

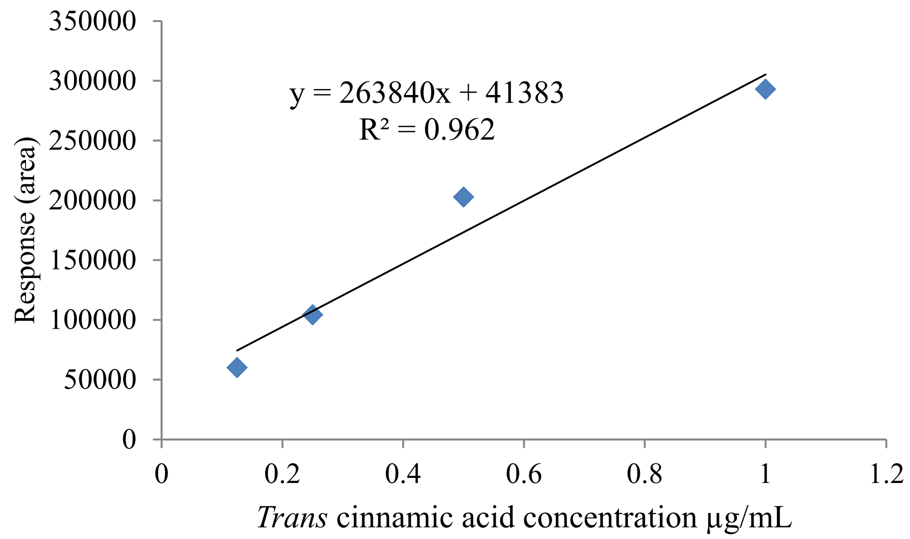


Fig. 8.6. Standard curve of *trans*-cinnamic acid (*t*-CA). The equation of the standard curve was used to calculate the content of *trans*-cinnamic acid, the product of PAL activity in the plant extract.Each point represents the mean of three replicates.

Appendix C

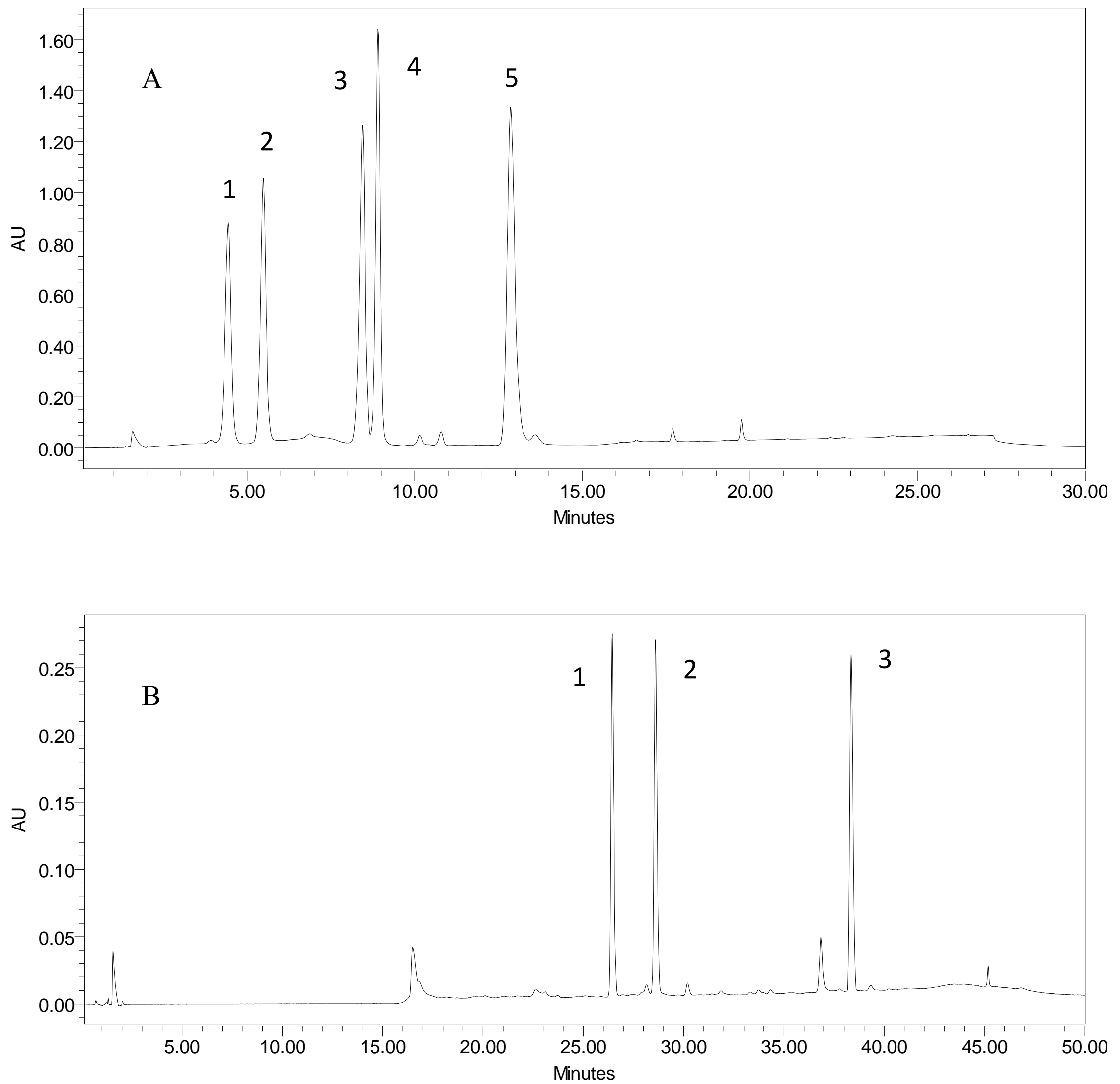
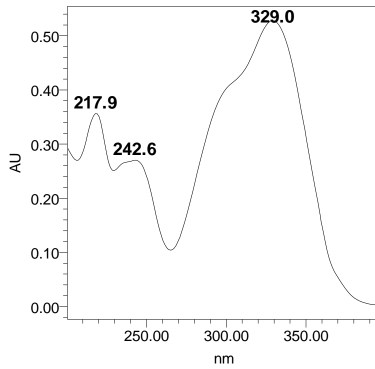
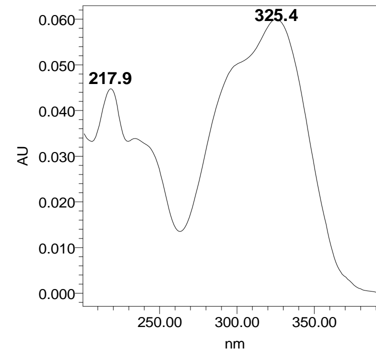


Fig 8.7. HPLC chromatogram depicting the analysis of five standards of caffeic acid derivatives (A) in the order of R*t*: 1, caftaric acid; 2, chlorogenic acid; 3, cynarin ; 4, echinacoside; 5, cichoric acid, and three standards of alkamides (B) in the order of R*t*: 1, alkamide 12; 2, alkamide 14; 3, alkamide 11.

Appendix D



Peak 1



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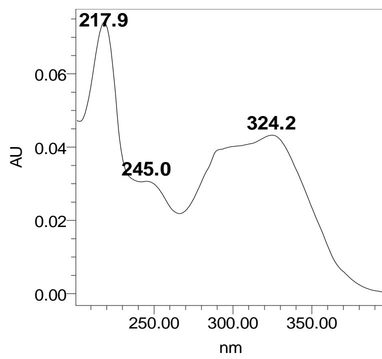

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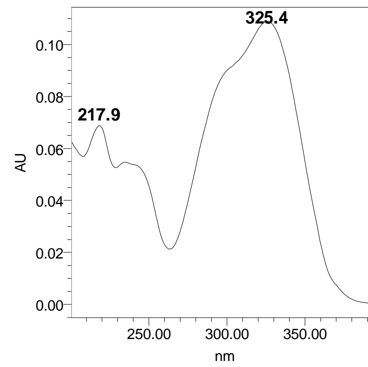

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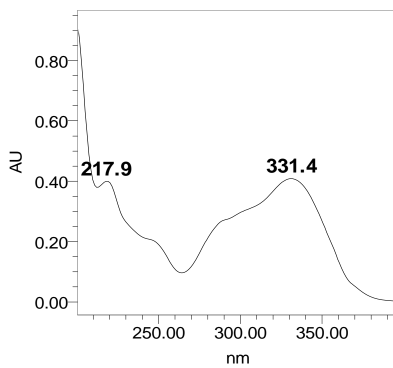

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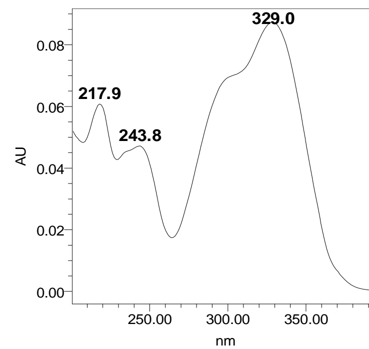
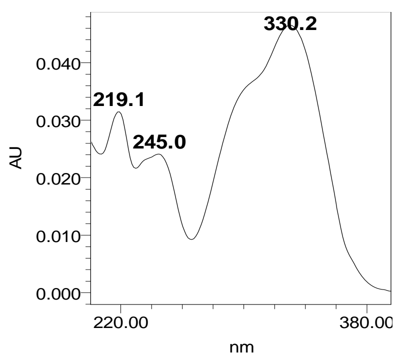
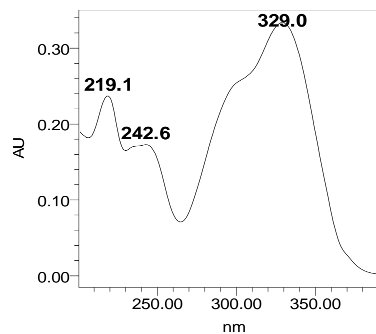


Peak 6

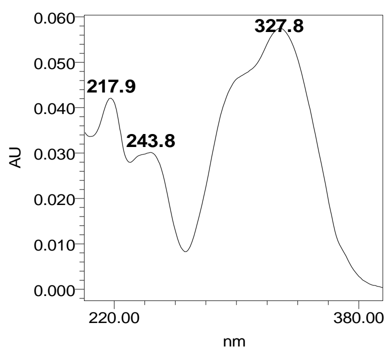
Fig 8.8. UV spectra of peaks 1-6 identified in the root extract of the three *Echinacea* species (Table S4.1)



Peak 7



Peak 8



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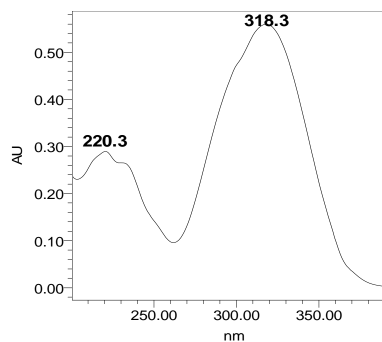
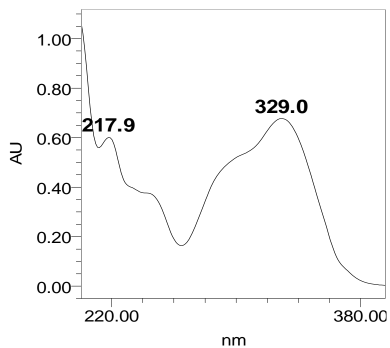
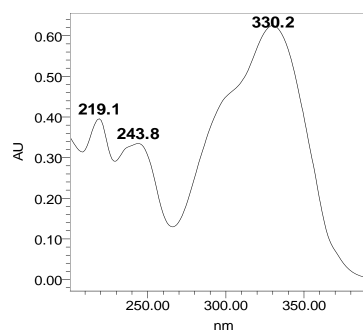


Peak 10

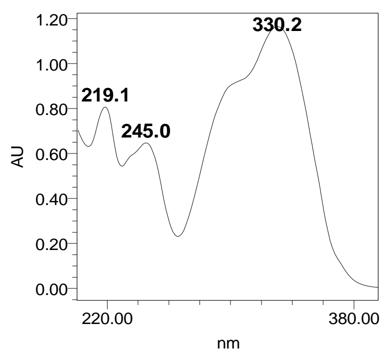


Peak 11

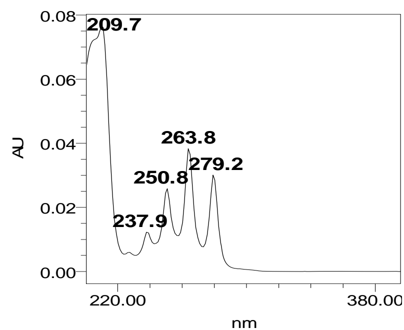


Peak 12

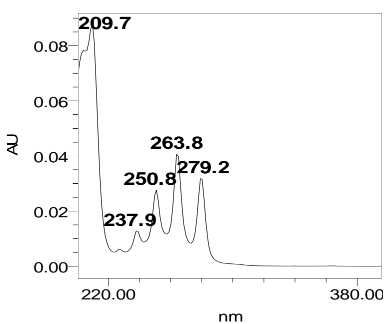
Fig 8.9 UV spectra of peaks 7-12 identified in the root extract of the three *Echinacea* species (Table S4.1)



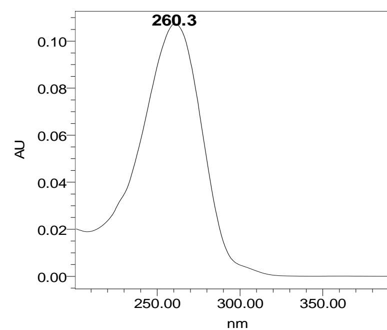
Peak 13



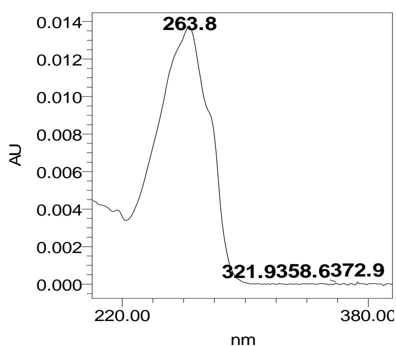
Peak 14



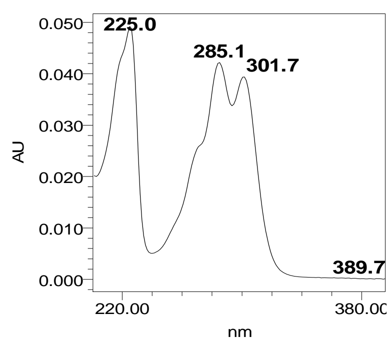
Peak 15



Peak 16

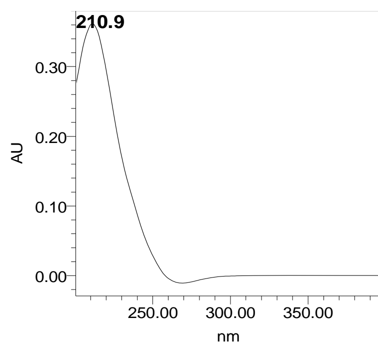


Peak 17

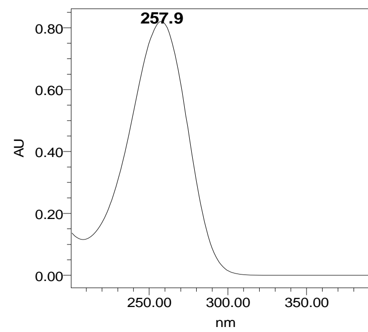


Peak 18

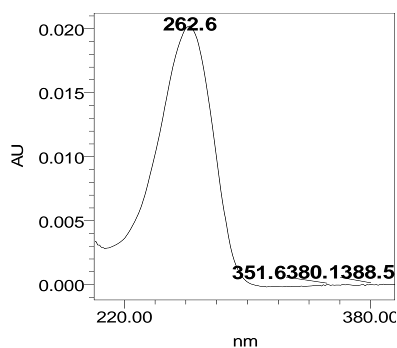
Fig 8.10. UV spectra of peaks 13-18 identified in the root extract of the three *Echinacea* species (Table S4.1)



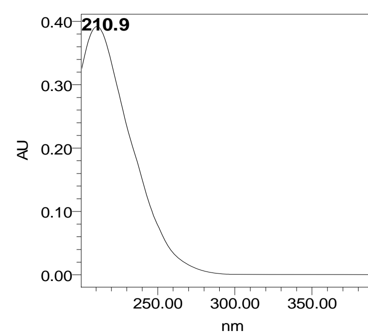
Peak 19



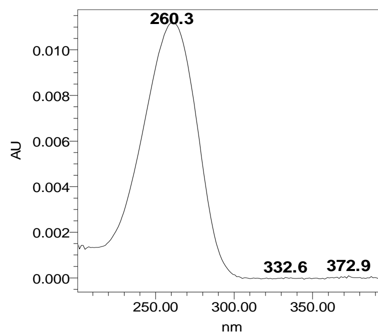
Peak 20



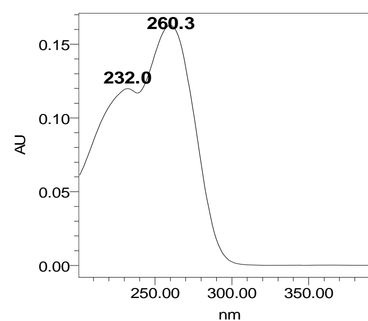
Peak 21



Peak 22

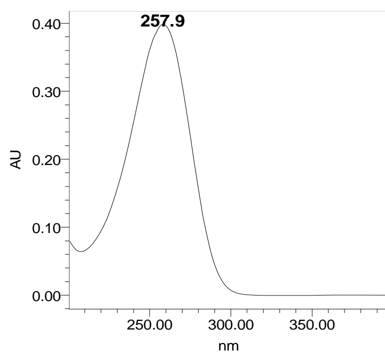


Peak 23

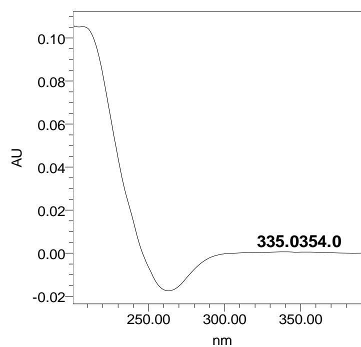


Peak 24

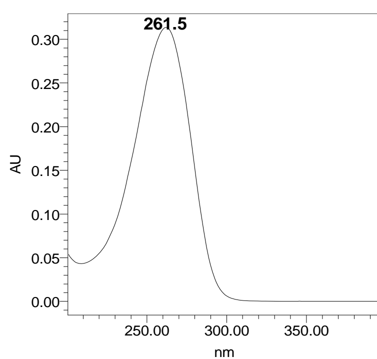
Fig 8.11. UV spectra of peaks 19-24 identified in the root extract of the three *Echinacea* species (Table S4.1)



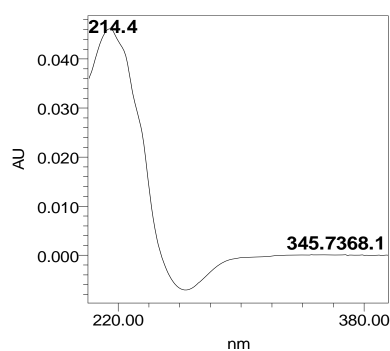
Peak 25



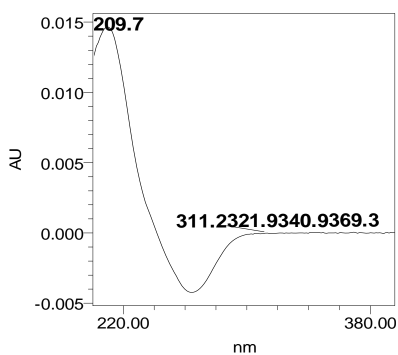
Peak 26



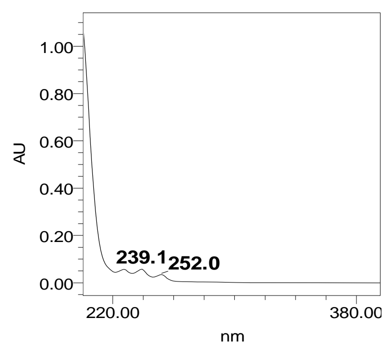
Peak 27



Peak 28

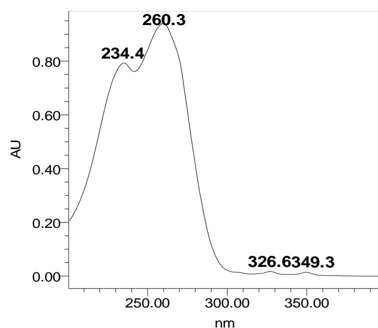


Peak 29

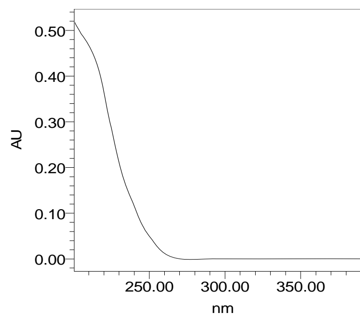


Peak 30

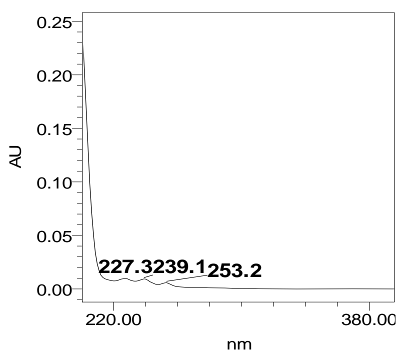
Fig 8.12. UV spectra of peaks 25-30 identified in the root extract of the three *Echinacea* species (Table S4.1)



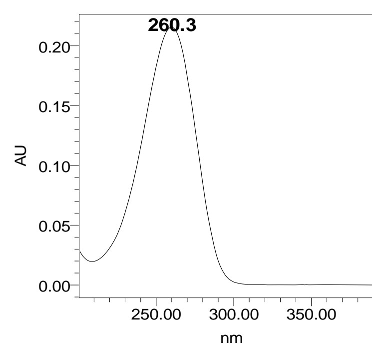
Peak 31



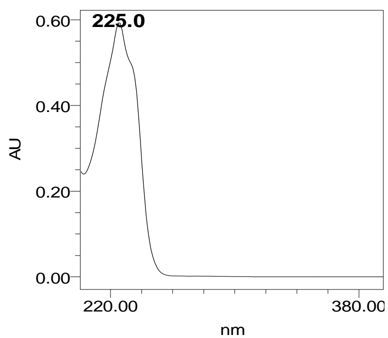
Peak 32



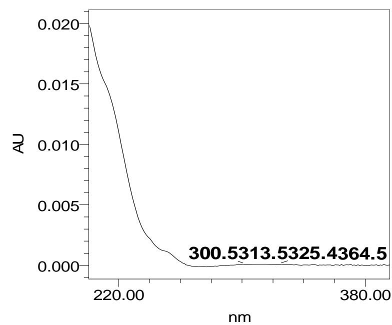
Peak 33



Peak 34

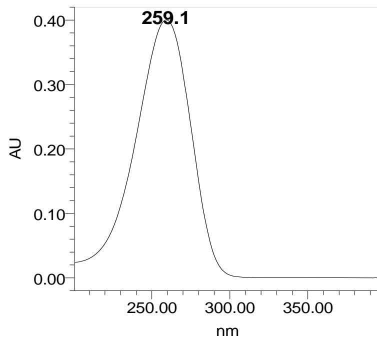


Peak 35

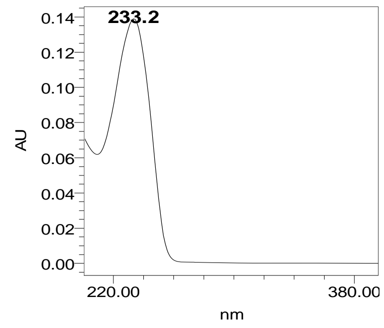


Peak 36

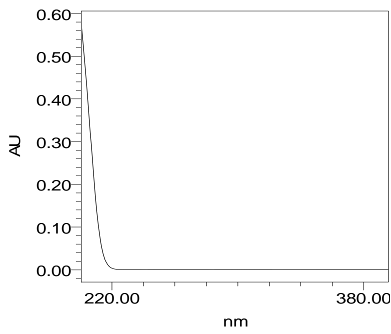
Fig 8.13. UV spectra of peaks 31-36 identified in the root extract of the three *Echinacea* species (Table S4.1)



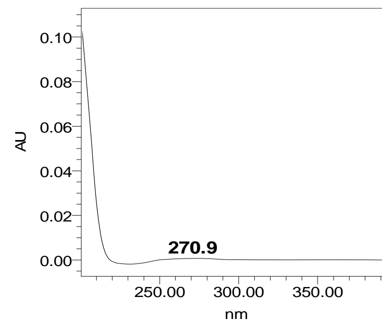
Peak 37



Peak 38



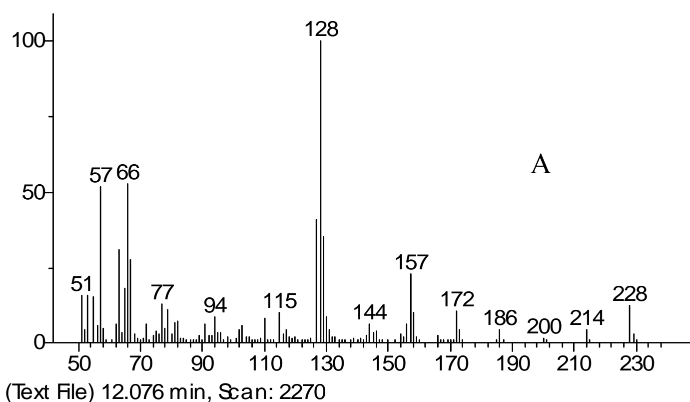
Peak 39

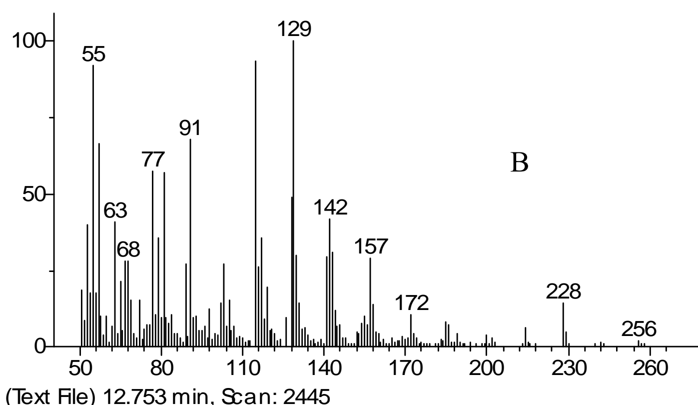


Peak 40

Fig 8.14. UV spectra of peaks 37-40 identified in the root extract of the three *Echinacea* species (Table S4.1)

Appendix E





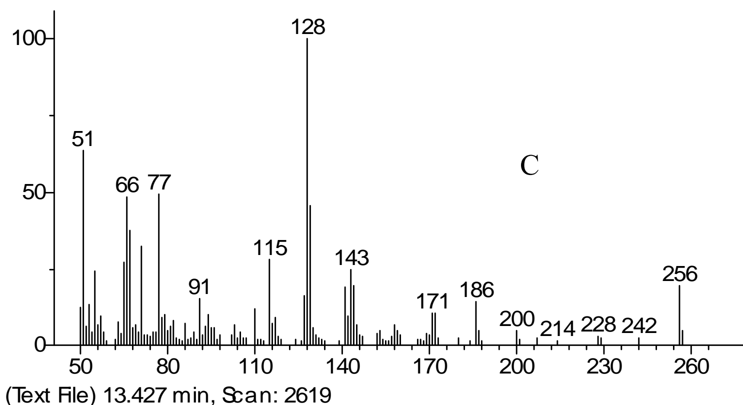


Fig 8.15. Mass spectra of alkamide 1 (A), alkamide 6 (B) and alkamide 7 (C) isolated from the roots of *E. purpurea* and analysed by GC-MS. The ten largest peaks (%) are: 128 (100); 66 (52.5); 57 (51.5); 127 (40.3); 129 (34.9); 63 (30.7); 67 (27.1); 157 (22.5); 65 (17.7); 53 (15.5) for alkamide 1; 129 (100), 115 (92.9); 55 (91.5); 91 (67.3); 57 (66.3); 77 (57.3) 81 (56.6) 128 (48.7); 142 (41.5); 63 (40.7) for alkamide 6; 128 (100); 51 (63.2); 77 (48.9); 66 (48.1); 129 (45.3); 67 (37.0); 71 (31.8); 115 (27.6); 65 (27.0); 143 (24.4) for alkamide 7. $[M]^+$ are 251 for alkamide 1; 257 for alkamide 6; 257 for alkamide 7.

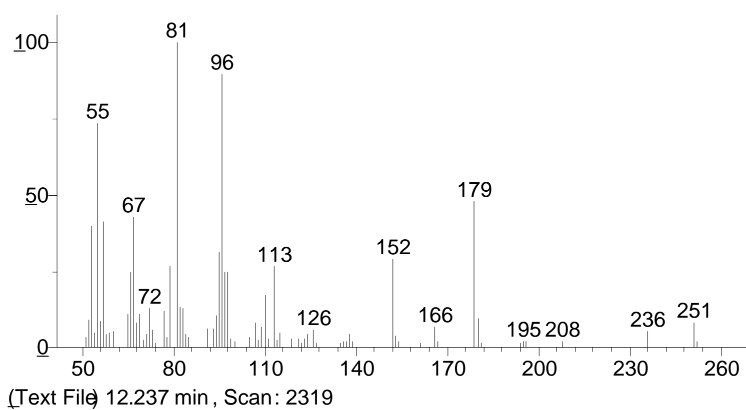


Fig 8.16. Mass spectra of alkamide 11 isolated from the roots of *E. angustifolia* and analysed by GC-MS. The ten largest peaks (%) are 81 (100); 96 (89.2); 55 (73.0); 179 (47.8); 67 (42.4); 57 (41.2); 53 (39.5); 95 (31.0); 152 (28.9); 113 (26.5) for alkamide 11. $[M]^+$ 251.

Appendix F

Table 8.1. Range of caftaric acid content in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Plant part	Extraction	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Roots	Methanol 80% (stirring)	HPLC	3.82 mg g^{-1} DW	0.78 mg g^{-1} DW	< LOQ*	Pellati et al., 2005
Roots	Ethanol 70% (shaker)	HPLC	4.1 mg g^{-1}	0.4 mg g^{-1}	< 0.1 mg g^{-1}	Perry et al., 2001
Roots	Methanol 70% (ultrasonic)	HPLC	3.5 mg g^{-1} DW			Zheng et al., 2006
Roots	Methanol and water	HPLC	3.1 mg g^{-1} DW			Kuzel et al., 2009
Roots	methanol 70% with H_3PO_4 (sonication)	HPLC	3.2 mg g^{-1}			Liu Y-C et al., 2007
Roots	Ethanol 70% (sonication)	HPLC	2.9 mg g^{-1} DW			Araim et al., 2009
Roots stored (18 months)	Ethanol 50%	HPLC, ESI-MS	0.7 mg ml^{-1}			Cech et al., 2006
Roots (3-yr old)	Methanol 80%	HPLC	3.97 mg g^{-1} DW	0.81 mg g^{-1} DW	< LOQ	Pellat et al., 2004
Roots	Ethanol 55% (ASE)	HPLC	0.781 mg ml^{-1}	0.048 mg ml^{-1}	0.02 mg ml^{-1}	Hudson et al., 2005
Hairy roots	Methanol 70%	HPLC	3.56 mg g^{-1} DW			Liu et al., 2006
Shoots	Methanol and water	HPLC	5.1 mg g^{-1} DW			Kuzel et al., 2009
Shoots	Ethanol 70% (sonication)	HPLC	11.3 mg g^{-1} DW			Araim et al., 2009
Shoots	Ethanol 70% (ASE)	HPLC	0.913 mg ml^{-1}			Vimalanathan et al., 2005
Flowers	Ethanol 70% (ASE)	HPLC	0.919 mg ml^{-1}			Vimalanathan et al., 2005
Flowers	Methanol 50%	Capillary electrophoresis	5.05 mg g^{-1} DW			Kreft, 2005
Buds	methanol 70% with H_3PO_4 (sonication)	HPLC	9.6 mg g^{-1}			Liu et al., 2007
Herb	Ethanol 70% (ASE)	HPLC	0.767 mg ml^{-1}			Vimalanathan et al., 2005
Dried powder	Methanol 60% (sonication)	HPLC	3.95 mg g^{-1}			Li et al., 2004

* LOQ, limit of quantification

Table 8.2. Range of chlorogenic acid content in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Roots(Jan,May, Aug)	Ethanol 70% (shaker)	HPLC	< 0.1 mg g^{-1}	0.3 mg g^{-1}	1.5 mg g^{-1}	Perry et al., 2001
Roots (3- yr old)	Methanol 80%	HPLC	< LOQ*	< LOQ	< LOQ	Pellat et al., 2004
Roots	Methanol 80% (stirring)	HPLC	LOQ	LOQ	0.77 mg g^{-1} DW	Pellati et al., 2005
Roots	Ethanol 55% (ASE)	HPLC	0.055 mg ml^{-1}	0.003 mg ml^{-1}	0.282 mg ml^{-1}	Hudson et al., 2005
Hairy roots	Methanol 70%	HPLC	0.93 mg g^{-1} DW			Liu et al., 2006
Roots	Methanol and water	HPLC	0.011 mg g^{-1} DW			Kuzel et al., 2009
Shoots	Ethanol 70% (ASE)	HPLC	0.045 mg ml^{-1}			Vimalanathan et al., 2005
Shoots	Ethanol 70% (sonication)	HPLC	0.3 mg g^{-1} DW			Araim et al., 2009
Shoots	Methanol and water	HPLC	0.152 mg g^{-1} DW			Kuzel et al., 2009
Flower	Ethanol 70% (ASE)	HPLC	0.208 mg ml^{-1}			Vimalanathan et al., 2005
Dried powder	Methanol 60% (sonication)	HPLC	0.29 mg g^{-1}			Li et al., 2004

* LOQ, limit of quantification

Table 8.3. Range of caffeic acid content in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Roots	Ethanol 55% (ASE)	HPLC	0.027 mg ml^{-1}	0.007 mg ml^{-1}	0.015 mg ml^{-1}	Hudson et al., 2005
Roots (3- yr old)	Methanol 80%	HPLC	< LOQ*	< LOQ	< LOQ	Pellat et al., 2004
Roots	Methanol 80% (stirring)	HPLC	LOQ	LOQ	LOQ	Pellati et al., 2005
Roots	Ethanol 70% (sonication)	HPLC	0.6 mg g^{-1} DW			Araim et al., 2009
Roots	Methanol and water	HPLC	0.08 mg g^{-1} DW			Kuzel et al., 2009
Shoots	Ethanol 70% (ASE)	HPLC	0.421 mg ml^{-1}			Vimalanathan et al., 2005
Shoots	Ethanol 70% (sonication)	HPLC	1.0 mg g^{-1} DW			Araim et al., 2009
Flowers	Ethanol 70% (ASE)	HPLC	0.160 mg ml^{-1}			Vimalanathan et al., 2005
Herb	Ethanol 70% (ASE)	HPLC	0.220 mg ml^{-1}			Vimalanathan et al., 2005

* LOQ, limit of quantification

Table 8.4. Range of cynarin content in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Roots (Jan, May, Aug)	Ethanol 70% (shaker)	HPLC	< 0.1 mg g^{-1}	< 0.1 mg g^{-1}	1.2 mg g^{-1}	Perry et al., 2001
Roots (3- yr old)	Methanol 80%	HPLC	< LOQ*	< LOQ	1.39 mg g^{-1} DW	Pellat et al., 2004
Roots	Ethanol 60% (ultrasonic)	LC-MS			0.09 mg ml^{-1}	Wolkart et al., 2004
Roots	Ethanol 55% (ASE)	HPLC	0	0	0.238 mg ml^{-1}	Hudson et al., 2005
Roots	Methanol 80% (stirring)	HPLC	LOQ	LOQ	3.44 mg g^{-1} DW	Pellati et al., 2005
Roots	Ethanol 70% (sonication)	HPLC	0.13 mg g^{-1} DW			Araim et al., 2009
Shoots	Ethanol 70% (ASE)	HPLC	0.005 mg ml^{-1}			Vimalanathan et al., 2005
Shoots	Ethanol 70% (sonication)	HPLC	0.2 mg g^{-1} DW			Araim et al., 2009
Flowers	Ethanol 70% (ASE)	HPLC	0			Vimalanathan et al., 2005
Herb	Ethanol 70% (ASE)	HPLC	0			Vimalanathan et al., 2005
Dried powder	Methanol 60% (sonication)	HPLC	0.8 mg g^{-1}			Li et al., 2004

* LOQ, limit of quantification

Table 8.5. Range of echinacoside content in the three *Echinacea* species (*E. purpurea, E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Roots (Jan)	Ethanol 70% (shaker)	HPLC	< 0.1 mg g^{-1}	3.4 mg g^{-1}	10.4 mg g^{-1}	Perry et al., 2001
Roots	Ethanol 70%	HPLC		3.7 mg g^{-1}	3.6 mg g^{-1}	Laasonen et al., 2002
Roots (3- yr old)	Methanol 80%	HPLC	< LOQ*	16.18 mg g^{-1} DW	9.10 mg g^{-1} DW	Pellat et al., 2004
Roots	Ethanol 60% (ultrasonic)	LC-MS			0.245 mg ml^{-1}	Wolkart et al., 2004
Roots	Ethanol 55% (ASE)	HPLC	0	0.618 mg ml^{-1}	1.859 mg ml^{-1}	Hudson et al., 2005
Roots	Methanol 80% (stirring)	HPLC	LOQ	12.72 mg g^{-1} DW	10.6 mg g^{-1} DW	Pellati et al., 2005
Roots	Methanol 70% (ultrasonic)	HPLC	1.1 mg g^{-1} DW			Zheng et al., 2006
Shoots	Ethanol 70% (ASE)	HPLC	0			Vimalanathan et al., 2005
Shoots	Ethanol 70% (sonication)	HPLC	0.5 mg g^{-1} DW			Araim et al., 2009
Flowers	Ethanol 70% (ASE)	HPLC	0			Vimalanathan et al., 2005
Herb	Ethanol 70% by ASE	HPLC	0			Vimalanathan et al., 2005

* LOQ, limit of quantification

Table 8.6. Range of cichoric acid content in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Root Jan, Aug, May	Ethanol 70% (shaker)	HPLC	22.7 mg g^{-1}		0.9 mg g^{-1}	Perry et al., 2001
Roots	Ethanol 70%	HPLC	9.4 mg g^{-1}	0.5 mg g^{-1}	0.1 mg g^{-1}	Laasonen et al., 2002
Roots from China	Methanol 70% with 0.1% H_3PO_4 (ultrasonic)	HPLC-ESI-MS	11.02 mg g^{-1} DW			Luo et al.,2003
Roots from USA	Methanol 70% with 0.1% H_3PO_4 (ultrasonic)	HPLC-ESI-MS	20.82 mg g^{-1} DW			Luo et al., 2003
Roots (3- yr old)	Methanol 80%	HPLC	19.27 mg g^{-1} DW	0.83 mg g^{-1} DW	< LOQ*	Pellati et al., 2004
Roots	Ethanol 80%	HPLC	13.6 mg g^{-1} DW			Dalby-Brown et al., 2005
Roots	Ethanol 55% (ASE)	HPLC	4.774 mg ml^{-1}	0.032 mg ml^{-1}	0.046 mg ml^{-1}	Hudson et al., 2005
Roots	Methanol 80% (stirring)	HPLC	18.97 mg g^{-1} DW	0.41 mg g^{-1} DW	0.27 mg g^{-1} DW	Pellati et al., 2005
Roots stored for 18 months	Ethanol 50%	HPLC, ESI-MS	0.71 mg ml^{-1}			Cech et al., 2006
Hairy roots	Methanol 70%	HPLC	19.2 mg g^{-1} DW			Liu et al., 2006
Roots	Methanol 70% (ultrasonic)	HPLC	25 mg g^{-1} DW			Zheng et al., 2006
Roots	Methanol 70% containing H_3PO_4 (sonication)	HPLC	7.7 mg g^{-1}			Liu et al., 2007
Main roots	Methanol 70% (sonication)	HPLC	8.17 mg g^{-1} DW			Thygesen et al., 2007
Rootstock	Methanol 70% (sonication)	HPLC	7.63 mg g^{-1} DW			Thygesen et al., 2007
Roots	Ethanol 70% (sonication)	HPLC	11.3 mg g^{-1} DW			Araim et al., 2009
Roots	Methanol and water	HPLC	13.9 mg g^{-1} DW			Kuzel et al 2009
Shoots	Ethanol 70% (ASE)	HPLC	6.001 mg ml^{-1}			Vimalanathan et al., 2005
Leaves	Methanol 70% (sonication)	HPLC	41.3 mg g^{-1} DW			Thygesen et al., 2007
Stems	Methanol 70% (sonication)	HPLC	8.57 mg g^{-1} DW			Thygesen et al., 2007
Shoots	Ethanol 70% (sonication)	HPLC	20.2 mg g^{-1} DW			Araim et al., 2009
Shoots	Methanol and water	HPLC	16.7 mg g^{-1} DW			Kuzel et al., 2009

Table 8.6. continued

Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Flowers	Methanol 50%	Capillary electrophoresis	10.76 mg g^{-1} DW			Kreft, 2005
Flowers	Ethanol 70% (ASE)	HPLC	7.340 mg ml^{-1}			Vimalanathan et al., 2005
Buds	70% methanol + H_3PO_4 (sonication)	HPLC	31.3 mg g^{-1}			Liu et al., 2007
Herb	Ethanol 70% (ASE)	HPLC	2.879 mg ml^{-1}			Vimalanathan et al., 2005
Dried powder	Methanol 60% (sonication)	HPLC	7.88 mg g^{-1}			Li et al.,2004

* LOQ, limit of quantification

Table 8.7. Range of alkamides 1 and 2 contents in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Compound	Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Alkamide 1	Roots (34 day-old)	Ethanol 95%	HPLC		2.0 mg g^{-1} DW		Binns et al., 2001*
	Roots	Methanol 70% (ultrasound)	HPLC	21.4 mg g^{-1}			Hudaib et al., 2002
Alkamide 2	Roots (34 day-old)	Ethanol 95%	HPLC		16.0 mg g^{-1} DW		Binns et al., 2001
	Roots	Methanol 70% (ultrasound)	HPLC	194.3 mg g^{-1}			Hudaib et al., 2002
	Roots	Ethanol 80%	HPLC	1.7 mg g^{-1} DW			Dalby-Brown et al., 2005
	Roots stored for 18 months	Ethanol 50%	HPLC, ESI-MS	2.0 mg ml^{-1}			Cech et al., 2006
	Roots, 6 month old	Ethanol 95%	HPLC	0.826 mg g^{-1} FW	0.09 mg g^{-1} FW	0.02 mg g^{-1} FW	Kraus et al., 2006
	Rootstock	Methanol 70% (sonication)	HPLC	0.002 mg g^{-1} DW			Thygesen et al., 2007
	Main Roots	Methanol 70% (sonication)	HPLC	0.004 mg g^{-1} DW			Thygesen et al., 2007

*calculated from figure

Table 8.8. Range of alkamides 3, 4, 5, and 6 contents in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Compound	Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Alkamide 3	Roots (34 day-old)	Ethanol 95%	HPLC		9.0 mg g^{-1} DW		Binns et al., 2001
	Roots	Methanol 70% (ultrasound)	HPLC	67.7 mg g^{-1}			Hudaib et al., 2002
Alamide 4	Roots (34 day-old)	Ethanol (95%)	HPLC		1.5 mg g^{-1} DW		Binns et al., 2001
	Roots	Methanol 70% (ultrasound)	HPLC	34.8 mg g^{-1}			Hudaib et al., 2002
Alkamide 5	Roots	Methanol 70% (ultrasound)	HPLC	1.33% 13.3 mg			Hudaib et al., 2002
	Roots, 6- month old	Ethanol 95%	HPLC	0.82 mg g^{-1} FW	traces	traces	Kraus et al., 2006
Alkamide 6	Roots (34 day-old)	Ethanol 95%	HPLC		0.5 mg g^{-1} DW		Binns et al., 2001
	Roots	Methanol 70% (ultrasound)	HPLC	4.0 mg g^{-1}			Hudaib et al., 2002

Table 8.9. Range of alkamides 7, 8 and 9 contents in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Compound	Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Alkamide 7	Roots (34 day-old)	Ethanol 95%	HPLC		11.5 mg g^{-1} DW		Binns et al., 2001
	Roots	Methanol 70% (ultrasound)	HPLC	46.8 mg g^{-1}			Hudaib et al., 2002
Alkamide 8	Roots	Methanol 70% (ultrasound)	HPLC	150.7 mg g^{-1}			Hudaib et al., 2002
Alkamide 9	Roots	Methanol 70% (ultrasound)	HPLC	293.7 mg g^{-1}			Hudaib et al., 2002

Table 8.10. Range of the isomer alkamides 8/9 content in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction method, and analysis method.

Compound	Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Alkamides 8/9	Roots	chloroform	HPLC	0.37 mg g^{-1} DW			He et al., 1998
	Roots (34 day-old)	Ethanol 95%	HPLC		10 mg g^{-1} DW		Binns et al., 2001
	Roots	Ethanol 70%	HPLC	0.173 mg g^{-1} DW	0.012 mg g^{-1} DW	2.229 mg g^{-1} DW	Laasonen et al., 2002
	Roots (China)	Methanol 70% with 0.1% H3PO4 (ultrasonic)	HPLC-ESI -MS	0.35 mg g^{-1} DW			Luo et al., 2003
	Roots (USA)	Methanol 70% with 0.1% H3PO4 (ultrasonic)	HPLC-ESI -MS	1.43 mg g^{-1} DW			Luo et al., 2003
	Roots	Ethanol 80%	HPLC	2.3 mg g^{-1} DW			Dalby-Brown et al., 2005
	Roots	Ethanol 55% (ASE)	HPLC	0	0	0.483 mg ml^{-1}	Hudson et al., 2005
	Roots	Ethanol 70% (sonication)	HPLC	1.8 mg g^{-1} DW			Araim et al., 2009
	Achenes	Chloroform	HPLC	0.75 mg g^{-1} DW	0.08 mg g^{-1} DW	1.06 mg g^{-1} DW	He et al., 1998
	Leaves and stems	Ethanol 70% (ASE)	HPLC	0.019 mg ml^{-1}			Vimalanathan et al., 2005
	Flowers	Ethanol 70% (ASE)	HPLC	0.039 mg ml^{-1}			Vimalanathan et al., 2005
8/9 isomers + dodeca 2E, 4E, 8E, 10Z tet acid isobutyl.	Roots	Ethanol 70%	HPLC	1.4 mg ml^{-1}			Spelman et al., 2009

Table 8.11. Range of alkamides 10, 11 and 12 contents in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Compound	Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Alkamide 10	Roots (34 day-old)	Ethanol 95%	HPLC		1.0 mg g^{-1} DW		Binns et al., 2001
	Roots	Methanol 70% (ultrasound)	HPLC	7.0 mg g^{-1}			Hudaib et al., 2002
Alkamide 11	Roots (34 day-old)	Ethanol 95%	HPLC		traces		Binns et al., 2001
	Roots	Methanol 70% (ultrasound)	HPLC	6.6 mg g^{-1}			Hudaib et al., 2002
Alkamide 12	Roots, 3 month-old	Liquid N_2, Ethanol 95%	HPLC			0.45 mg g^{-1} FW	Wu et al., 2004
	Roots, 6 month-old	Liquid N_2, Ethanol 95%	HPLC			0.08 mg g^{-1} FW	Wu et al., 2004

Table 8.12. Range of alkamides 13, 14, 15 and 16 contents in the three *Echinacea* species (*E. purpurea*, *E. pallida* and *E. angustifolia*) as influenced by plant part, extraction and analysis method.

Compound	Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E. angustifolia*	Reference
Alkamide 13	Roots (34 day-old)	Ethanol 95%	HPLC		2.5 mg g^{-1} DW		Binns et al., 2001
	Roots 3 month-old	Liquid N_2, ethanol 95%	HPLC			0.05 mg g^{-1} FW	Wu et al., 2004
	Roots 6 month-old	Liquid $N_{2,}$ ethanol 95%	HPLC			0.11 mg g^{-1} FW	Wu et al., 2004
Alkamide 14	Roots 3 month-old	Liquid N_2, ethanol 95%	HPLC			0.13 mg g^{-1} FW	Wu et al., 2004
	Roots 6 month-old	Liquid N_2, ethanol 95%	HPLC			0.04 mg g^{-1} FW	Wu et al., 2004
Alkamide 15	Roots (34 day-old)	95% ethanol	HPLC		1.5 mg g^{-1} DW		Binns et al., 2001
Alkamide 16	Roots (34 day-old)	95% ethanol	HPLC		2.0 mg g^{-1} DW		Binns et al., 2001

Table 8.13. Range of ketone contents in *E. pallida* as influenced by plant part, extraction and analysis method.

Compound	Plant part	Extraction method	Analysis method	*E. purpurea*	*E. pallida*	*E.angustifolia*	Reference
Ketene 20	Roots	*n*-hexane (Soxhlet)	HPLC		0.31 mg g^{-1} DW		Pellati et al., 2007
Ketene 21	Roots	*n*-hexane (Soxhlet)	HPLC		0.18 mg g^{-1} DW		Pellati et al., 2007
Ketene 22	Roots	*n*-hexane (Soxhlet)	HPLC		1.13 mg g^{-1} DW		Pellati et al., 2007
Ketene 23	Roots	*n*-hexane (Soxhlet)	HPLC		0.45 mg g^{-1} DW		Pellati et al., 2007
Ketene 24	Roots	*n*-hexane (Soxhlet)	HPLC		0.98 mg g^{-1} DW		Pellati et al., 2007
Ketene 25 a +b	Roots (34 day-old)	Ethanol 95%	HPLC		1.5 mg g^{-1} DW		Binns et al., 2001
Pentadec-(8Z)-en-2-one	Dried roots	*n*-hexane using Soxhlet	HPLC		0.74 mg g^{-1} DW		Pellati et al., 2007
8-hydroxy-pentada-(9E, 13Z)-dien-11-yn-2-one	Dried roots	*n*-hexane using Soxhlet	HPLC		0.23 mg g^{-1} DW		Pellati et al., 2007

*Compounds cichoric acid derivative, 6-O caffeoyl echinacoside, alk 17, 18, 19 never quantified.