# PHYTOECDYSTEROID ACCUMULATION IN PLANTS AND BIOACTIVITIES IN ANIMAL MODELS

BY

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DISSERTATION

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## ABSTRACT

Phytoecdysteroids are plant-produced polyhydroxylated steroidal compounds that have valued bioactivities including plant defense against herbivorous insects, and growth and performance-enhancing properties in mammals. In this program of research, phytoecdysteroid accumulation was investigated in *Ajuga turkestanica* (Regel) Briq., a medicinal herb indigenous to Central Asia, and in spinach (*Spinacia oleracea* L.), a valuable crop produced worldwide, and purported bioactivities were examined in *in vitro* and *in vivo* mouse models. Results showed that *A. turkestanica* hairy root cultures were a sustainable alternative source of valued phytochemicals compared to wild-harvesting (Chapter 2). Extracts from both wild-harvested *A. turkestanica* shoots and hairy root cultures enhanced protein synthesis in murine cell cultures. In spinach, significant variations in phytoecdysteroid accumulation in seeds and shoots was demonstrated, which may help further investigations of plant defense properties and biosynthetic regulation (Chapter 3). A review of strategies to investigate anabolic effects of phytoecdysteroids lead to the evaluation of a continuous infusion of 20-hydroxyecdysone, the predominant phytoecdysteroid, on body tissue composition and skeletal muscle gene expression (Chapters 4 and 5) in mice. The mass of the triceps brachii muscles was significantly increased, however, no differences between treatment groups were observed in the other parameters measured. Ingenuity Pathways Analysis identified genes with the most evidence for differential expression from microarray gene expression data, which included genes involved in cellular growth and proliferation and cell-to-cell signaling and interaction. These results provide leads and resources for future research on phytoecdysteroids, which have demonstrated potential in improving agriculture and human health.

*To my family*

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# CHAPTER 1

# INTRODUCTION

Central Asian cultures have long-standing traditions in the use of herbs as medicines and supplements (Michaels, 2003). The NIH Fogarty Center-funded International Cooperative Biodiversity Groups (ICBG) Central Asia Program: *Building New Pharmaceutical Capabilities in Central Asia*, was developed to explore natural products pharmaceutical capabilities and to promote biodiversity conservation and sustainable economic growth in Central Asia. *Ajuga turkestanica* (Regel) Briq., a wild medicinal plant indigenous to Central Asia, was prioritized for botanical investigation because it is valued for its purported adaptogenic properties. "Adaptogenic" refers to the ability of a compound to enhance physical strength and stamina as well as increase nonspecific resistance of an organism without interference with normal biological functions (Ramazanov, 2005; Brekhman and Dardymov, 1969). The bioactive properties of *A. turkestanica* are attributed to the high level of phytoecdysteroids that accumulate in the plant tissues. *Spinacea oleracea* L. (spinach) is an important commercial crop that also produces phytoecdysteroids, however, phytoecdysteroid concentrations are too low to be pharmacologically relevant in humans through typical dietary consumption (Gorelick-Feldman et al., 2008). The focus of this research was the investigation of phytoecdysteroid production in plants and biological activities in mammals.

## *Central Asia*

In 1991, the five republics of Central Asia, Uzbekistan, Kyrgyzstan, Tajikistan, Kazakhstan and Turkmenistan, became sovereign and independent nations and were formed into

the Commonwealth of Independent States (CIS) having full control of their natural resources (Olcott, 1992). Prior to their sovereignty, the Central Asian republics were geographically remote and, as members of the former Soviet Union, inaccessible for natural products bioprospecting by Western scientists. During the Soviet regime, which spanned 70-years, traditional healing practices were banned, which diminished the dissemination of cultural medicinal practices (Neumann et al., 2004). Due to physical and intellectual isolation, and a lack of the requisite analytical instrumentation and infrastructure, the bioactive potentials and phytochemical constituents in traditional Central Asian endemic or indigenous plant species were not realized. Scientific investigation of phytotherapies is absolutely necessary in nations that seek to implement indigenous plant-based pharmaceuticals into their health sector (McKee et al., 1998). Additionally, the diverse and extreme environmental conditions of these inland countries (lacking the climatic buffer of adjacent oceans) promote evolution of stress-coping mechanisms by plants in the form of unique chemical compounds and compositions (Brown *et al*., 2002) making the Central Asian region a great prospect for the discovery of novel phytochemicals and bioactive properties. The medicinal herb *A. turkestanica* was targeted as an ethnobotanic lead by our Central Asian partners in the ICBG program. *A. turkestanica* accumulates high levels of plant secondary compounds known as phytoecdysteroids, which are the purported bioactive constituents and the focus of this research.

*Phytoecdysteroids*

Ecdysteroids are polyhydroxylated steroidal compounds biosynthesized by animals (zooecdysteroids) and plants (phytoecdysteroids). They were first discovered by Butenandt and Karlson, who isolated ecdysone from silkworm pupae (Butenandt and Karlson, 1954). The first

representative of this class was named ecdysone, modified from the Greek word *ecdusis*, meaning "to put off outer skin" (Baltaev, 2000). In insects, ecdysteroids act as molting hormones and control cell proliferation, growth and developmental cycles (Sláma et al., 1996). Currently, over 400 ecdysteroid analogues have been isolated and identified (Lafont et al., 2010).

Phytoecdysteroids belong to the terpene class of secondary metabolites and are biosynthesized through the mevalonic acid pathway in which mevalonic acid, cholesterol and acetyl-CoA are direct precursors (Báthori and Pongrácz, 2005). The chemical structure of an ecdysteroid is characterized by a cyclopentano-perhydro-phenanthrene skeleton with an alkyl side chain at C17, containing a 7-en-6-one chromophore and several hydroxyl groups (Figure 1); the variations in number and location of hydroxyl groups on the steroid backbone account for the diversity of ecdysteroids in nature (Báthori et al., 2003). 20-Hydroxyecdysone (20E, also commonly reported as ecdysterone, β-ecdysterone and β-ecdysone) is the most prevalent and abundant phytoecdysteroid produced by plants, and a major component in phytoecdysteroid-containing herbal extracts (Baltaev, 2000; Báthori et al., 2008; Báthori and Kalász, 2001).

The physiological function of phytoecdysteroids in plants has not been established, however, a plant defense function for phytoecdysteroids is widely accepted. Long-term stability and rapid accumulation of phytoecdysteroids in spinach after mechanical damage of root tissue and application of methyl jasmonate, a plant-defense signal compound, are strong indicators of a plant defense function (Schmelz et al., 2000; Schmelz et al., 1999; Schmelz et al., 1998). Additionally, levels of phytoecdysteroid accumulation in certain plant tissues, such as young developing leaves and floral structures, are high enough to cause premature molting and death in non-adapted insects and nematodes after ingestion (Dinan, 1992; Adler and Grebenok, 1995; Soriano et al., 2004). A plant defense function would have significant implications for

improving crop protection through enhancement of phytoecdysteroid levels by means of breeding or genetic engineering.

Phytohormonal roles have also been evaluated, however, studies have not demonstrated compelling evidence of these effects; 20E was tested in a series of phytohormone bioassays and demonstrated only minor gibberrellin-like activity (Macháčková et al., 1995; Hendrix and Jones, 1972).

*Accumulation and biosynthesis of phytoecdysteroids in plants*

*Ajuga turkestanica* (Regel) Briq. *A. turkestanica*, a medicinal perennial plant from the family Lamiaceae (mint) and indigenous to Uzbekistan, provides a rich source of phytoecdysteroids (Abdukadirov et al., 2004). The aerial portion of the plant is dried and then steeped in hot water and the broth is ingested to alleviate bodily ailments such as heart disease, and muscle and stomach aches (Mamatkhanov et al., 1998; personal communication, Dr. B. Islamov, Samarkand State University). The phytoecdysteroid content of *A. turkestanica* includes 20E, turkesterone, cyasterone, cyasterone 22-acetate, ajugalactone, ajugasterone B, α-ecdysone and ecdysone 2,3-monoacetonide along with iridoids and neo-clerodane diterpenes (Usmanov et al., 1971, 1973, 1975, 1978; Baltaev, 2000; Ramazanov, 2005; Grace et. al., 2008). Air-dried leaves from *A. turkestanica* have been reported to contain 0.02% 20E and roots have been reported to contain 0.045% 20E and 0.052% turkesterone (Lev et al., 1990).

Plant tissue culture strategies for phytoecdysteroid production in *A. turkestanica* have been investigated, including the development of callus cultures initiated from ovaries by Lev et al. (1990) and cell suspension and hairy root cultures initiated from leaves by Cheng et al., (2008). Callus cultures initiated from ovaries of *A. turkestanica* have achieved yields of 0.12%

20E and 0.036% turkesterone on the weight of air-dried raw material (Lev et al., 1990). In later callus cultivation studies, the yield of 20E was increased from 0.1% to 0.2% and turkesterone decreased from 0.032% to 0.004% when treated with an 8 mM dose of the mutagen *N*-nitroso-*N*-methylurea (Zakirova et al., 2000; Zakirova and Yakubova, 2002). Although callus cultures of a related plant, *Ajuga reptans*, did not produce phytoecdysteroids, hairy roots of *A. reptans* transformed with *Agrobacterium rhizogenes* accumulated up to 0.12% 20E on a dry weight basis; 4 times higher than the content of the plant's roots *in vivo* (Tomas et al., 1992; Matsumoto and Tanaka, 1991).

A direct comparison of hairy root cultures and cell suspension cultures of *A. turkestanica* showed that hairy root cultures accumulated a greater diversity of phytoecdysteroids compared to cell suspension cultures (Cheng et al., 2008). Addition of an elicitor, methyl jasmonate, to cell suspension culture media increased phytoecdysteroid accumulation (Cheng et al., 2008). As a follow up to the above study, the goals of the current (PhD) program study were to evaluate the effects of phytoecdysteroid precursors or methyl jasmonate on phytoecdysteroid production in hairy root cultures of *A. turkestanica*, and to evaluate the influence of timing (duration of maintenance of cultures) on productivity. As precursor addition, elicitation and increased maturity of plant tissues have previously resulted in greater phytoecdysteroid accumulation in spinach, *A. reptans*, and other tissues of *A. turkestanica*, it was hypothesized that these treatments may enhance phytoecdysteroid accumulation in hairy root cultures of *A. turkestanica.* *A. turkestanica* hairy root cultures may be a sustainable alternative to wild harvesting plants for phytoecdysteroids.

*Spinacea oleracea* L. Spinach (*S. oleracea*) is a cool-season annual crop that also accumulates phytoecdysteroids and has been a model plant for the study of phytoecdysteroid

biosynthesis (Al-Khayri, 1997; Schmelz et al., 2000; Schmelz et al., 1999; Schmelz et al., 1998). Whereas *A. turkestanica* accumulates several phytoecdysteroids, spinach predominantly accumulates only two phytoecdysteroids, 20E and polypodine B, which allows for targeted analysis of biosynthetic regulation without the background levels of multiple phytoecdysteroids present in perennial species (Grebenok et al., 1991; Grebenok and Adler, 1993). Compared to *A. turkestanica,* the total phytoecdysteroid content of spinach is over 100-fold less (5 mg $g^{-1}$ and 40 μg $g^{-1}$ dry weight, respectively), which would require mammals to consume copious quantities to obtain pharmacologically-relevant levels (Gorelick-Feldman et al., 2008). However, spinach accumulates phytoecdysteroids at levels that are physiologically effective for the deterrence of non-adapted insect species, and therefore the presence of phytoecdysteroids in spinach may provide a natural means of crop protection (Kubo and Klocke, 1983; Grebenok et al., 1991).

With the onset of germination, phytoecdysteroid levels stored in seeds initially decrease on a per fresh weight basis. As the plant develops, phytoecdysteroids are transported from the seeds to the cotyledons and then from the cotyledons to the first true leaves, and from each leaf set to the newly developing leaves, dynamically cycling and accumulating in the most apical leaves as the plant matures (Grebenok et al., 1991; Grebenok and Adler, 1991). Using radiolabelled precursors in excised leaf assays, Bakrim et al. (2008) demonstrated that younger leaves were not able to biosynthesize the final steps to produce 20E and instead, acted as sinks for phytoecdysteroids biosynthesized by older leaf sets. Additionally, 20E biosynthesis is regulated by its own negative feedback inhibition, requiring export of de novo synthesized phytoecdysteroids to continue biosynthesis (Bakim et al., 2008; Adler and Grebenok, 1999). Elevated levels of phytoecdysteroid intermediates, phytoecdysteroid polyphophate conjugates and end products were correlated with inhibition of mevalonic acid incorporation into lathosterol

and 20E, and endogenous phytoecdysteroid production (Grebenok et al., 1994; Grebenok et al., 1996; Bakrim et al., 2008). The inhibition of carbon flux into lathosterol and 20E suggested that regulation occurs through allocation of carbon resources early in the phytoecdysteroid pathway, prior to specific hydroxylation steps.

Microsomal fractions, prepared by differential centrifugations of spinach leaf homogenates, demonstrated specific C2- and C20-hydroxylation steps in the final biosynthetic steps that produce 20E. The hydroxylation was dependent on NADPH and molecular oxygen, and was inhibited by carbon monoxide, suggesting the involvement of cytochrome P450 hydroxylase/monooxygenase enzymes (Grebenok et al., 1996; Bakrim et al., 2009). However, C2-hydroxylase is a constitutive enzyme in spinach, active in young apical leaves, which are unable to biosynthsize 20E. Thus, C2-hydroxylase was not identified as the rate limiting step or the enzyme targeted by feedback inhibition (Bakrim et al., 2009). The major regulating enzymes may be further upstream of the monooxygenases, prior to the final hydroxylation steps in the phytoecdysteroid biosynthetic pathway, perhaps with phytosterol biosynthesis.

Phytosterols are involved in the phytoecdysteroid biosynthetic process and are plant membrane components that provide rigidity and stability to the cell walls through adjustment of the sterol to phospholipid ratio (Moreau et al., 2002). The predominant sterols in spinach are avenasterol, spinasterol, and 22-dihydrospinasterol (Grebenok and Adler, 1993). A coordinated shift in the ratio of phytoecdysteroid levels to total sterols with the growth and development of spinach was demonstrated (Grebenok et al., 1991). In an excised leaf assay using radiolabeled substrates, [2-$^{14}$C] mevalonic acid was incorporated into lathosterol prior to incorporation into 20E and other phytoecdysteroids (Grebenok and Adler, 1993). Results have shown that mevalonic acid leads to the production of sterols (lathosterol was specifically identified) then the

following sequence of phytoecdysteroids: ecdysone, 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone and then 20E (Figure 2; Bakrim et al., 2008).

Previously, investigators have measured seed extracts to assess phytoecdysteroid presence and chemotaxonomic distribution among plants species (Dinan et al., 1998; Dinan et al., 2001a,b). Dinan et al. (2001b) evaluated the occurrence and levels of phytoecdysteroids in seeds, leaves, flowers, stems and roots of 180 randomly selected plant species. Phytoecdysteroids were found in leaves and flowers of more species than in their seeds, however, the highest levels of phytoecdysteroids were detected only for species which were also positive for phytoecdysteroids in the seeds (Dinan et al., 2001b). Among plant species, there is not a consistent pattern of phytoecdysteroid distribution between tissues; the highest levels may be found in roots (*Incarvillea forestii*) or stems (*Pittosporum tenuifolium*) or seeds (*Hieracium murorum*) (Dinan et al., 2001b). However, the presence or absence of phytoecdysteroids is related to the taxonomic classification of the species and the specific phytoecdysteroid composition is species-dependent, demonstrating chemotaxonomic value (Dinan et al., 1998; Dinan et al., 2001a).

Although the potential for breeding of high-phytoecdysteroid accumulating varieties for plant protection is often discussed, researchers have not investigated phytoecdysteroid levels between several varieties of the spinach species, to the best of our knowledge. Spinach was selected for this research because it has been a model for the analysis of phytoecdysteroid biosynthesis and is a valuable commercial crop. The goals of this study were to screen various accessions of spinach from several geographic locations with different degrees of insect resistance, to evaluate variation in phytoecdysteroid accumulation and determine whether seed content could predict phytoecdysteroid levels in the edible foliage. Variation in

phytoecdysteroid accumulation between accessions may facilitate further research on the regulation phytoecdysteroid biosynthesis. Spinach genotypes with inherently high levels of phytoecdysteroids may have enhanced resistance to herbivory and be a natural means of conferring crop protection.

*Biological activities of phytoecdysteroids in mammals*

The multitude of biological effects attributed to phytoecdysteroids has led to their classification as adaptogenic compounds. In the former Soviet Union, adaptogens (also referred to as resistogens) were valued for their ergogenic capacity (i.e. the ability to increase physical or mental output by eliminating fatigue) for aiding military personnel and athletes during international competitions (Mamedov, 2005). In the 1950s, Soviet researchers heavily studied ginsengs and other plants with adaptogenic capabilities, leading to their introduction as therapeutic botanicals between 1955 and 1964 (Baranov, 1982). Research in the former Soviet Union on adaptogenic plants focused on *Eleutherococcus senticosus* (Siberian ginseng), *Rhodiola rosea*, *Leuzea carthamoides* (which contains a rich source of phytoecdysteroids), *Schizandra chinensis* and *Panax ginseng* (Brekhman, 1980). Dr. I. Brekhman defined adaptogens as substances that must (a) be innocuous and cause minimal disorders in the physical functions of an organism, (b) be nonspecific, i.e. it should increase resistance to adverse influences of a wide range of factors of physical, chemical and biological nature, and (c) possess normalizing action irrespective of the direction of the foregoing pathologic changes (Brekhman and Dardymov, 1969). To determine the mechanisms of adaptogenic plant extracts, multiple targets need to be investigated in order to produce a more holistic assessment of complex pharmacological systems (Panossian et al., 1999; Rege et al., 1999).

The enhancement of strength, stamina and lean muscle mass in animals and humans are ranked high among the valued bioactive properties and commercial uses of phytoecdysteroids and their mixtures (Syrov, 1984; Báthori et al., 2008). Studies have reported that phytoecdysteroid-containing commercial preparations (e.g. Ecdysten, Prime Plus and Leveton) contribute to the increased lean muscle mass and decreased fat tissue in athletes (Gadzhieva et al., 1995, Báthori and Pongrácz, 2005). Ecdysten was approved by the Pharmaceutical Committee of the Russian Federation for medicinal use as a general tonic preparation (Saatov et al., 1999; Báthori and Pongrácz, 2005). The pharmacological effects of phytoecdysteroids have been compared to those of anabolic steroids. Advantages of phytoecdysteroids over anabolic steroids include low toxicity in vertebrates (per orum $LD_{50} > 9$ g/kg; intraperitoneal injection $LD_{50}$ 6.4 g/kg body weight), and absence of hormonal side-effects, as they do not bind vertebrate nuclear receptors (Ogawa et al., 1974; Báthori et al., 2008).

Growth promoting effects have been reported for mice, sheep, pigs and Japanese quail after ingestion of phytoecdysteroids, implying a potentially valuable application towards improving livestock production (Stopka et al., 1999; Krátky et al., 1997; Purser and Baker, 1994; Koudela et al., 1995; Sláma et al., 1996). Although there are numerous reports on the growth promoting effects of 20E in mammals, a detailed look at the studies show that 20E does not consistently demonstrate an increase in body weight. Toth et al. (2008) reported an increase in body weight with daily subcutaneous injections of 20E (5 mg/kg) for 8 days in rats. Stopka et al. (1999) found that daily intraperitoneal injections of 1 mg of 20E (approximately 100 mg/kg (juveniles, 10 g); 28.5 mg/kg (adults, 35 g) enhanced growth in female juvenile mice, but not male juvenile mice, and enhanced growth in both male and female adult mice. Others have indicated no difference in body weight with 20E treatment with daily veinous injections of 20E

(1 mg/kg) for 3 weeks in mice (Gao et al., 2008). Daily oral administration of 20E (200-2000 mg/kg) for 35 days in adult male and female rats had no effect on body weight, organ weight or skeletal muscle (Ogawa et al., 1974). Given these mixed results, the anabolic effects of phytoecdysteroids remain inconclusive and warrant further investigation.

In addition to anabolic potential, phytoecdysteroids are attributed with numerous other pharmacological properties in animals, including humans. They have been shown to stimulate carbohydrate metabolism and reduce hyperglycemic response in rats and mice and decrease weight gain in diet-induced obese mice, which have significant implications for reducing diabetes symptoms in humans (Yoshida et al., 1971; Uchiyama and Yoshida, 1974; Kizelsztein et al., 2009). Phytoecdysteroids have also been used to restore renal dysfunction and for treatment of cardiovascular disease (Syrov and Khushbaktova, 2001; Bathori and Pongracz, 2005). Recently 20E has been shown to stimulate protein synthesis in C2C12 murine myotubes and to increase skeletal muscle fiber size in rats, which demonstrates potential as a therapeutic agent to alleviate skeletal muscle atrophy (Gorelick-Feldman et al., 2008; Tóth et al., 2008).

Given the abundance of original research publications and reviews on the adaptogenic properties of phytoecdysteroids, *in vivo* treatments may be expected to improve animal physiology and performance, although in some cases this has not proven to be the case (Ogawa et al. 1974). With the growth of genomics and the elucidation of the full sequence of the mouse genome and a large number of gene functions (Duggan et al., 1999), a mouse global gene expression microarray could elucidate possible mechanisms of action behind the purported biological effects of the predominant phytoecdysteroid, 20E.

Overall, this research analyzed phytoecdysteroid production in plants and their biological activities in animal models. The objectives of this research were to 1) Enhance phytoecdysteroid accumulation in *in vitro* cultures of *A. turkestanica,* to develop an efficient and sustainable system as a source of phytoecdysteroids (Chapter 2), 2) Examine the relationship between phytoecdysteroid accumulation in seeds and shoots of different accessions of *S. oleracea* to determine whether there are variations between accessions and evaluate whether seed levels could predict levels in edible foliage (Chapter 3), 3) Review the origin of the Soviet concept of adaptogens and evaluate modern techniques to elucidate specific adaptogenic effects in *in vitro* and *in vivo* animal models (Chapter 4), and 4) Determine the anabolic potential and gene expression effects of the predominate phytoecdysteroid, 20E, *in vivo* (Chapter 5).

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**Figures**

Figure 1.1 Structure of 20-hydroxyecdysone with numbered carbons

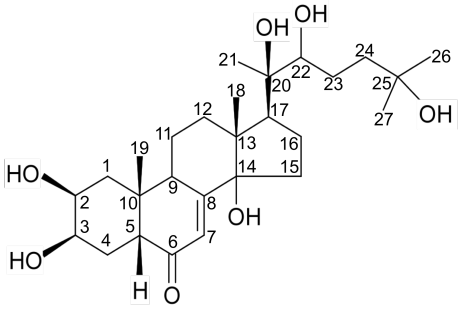
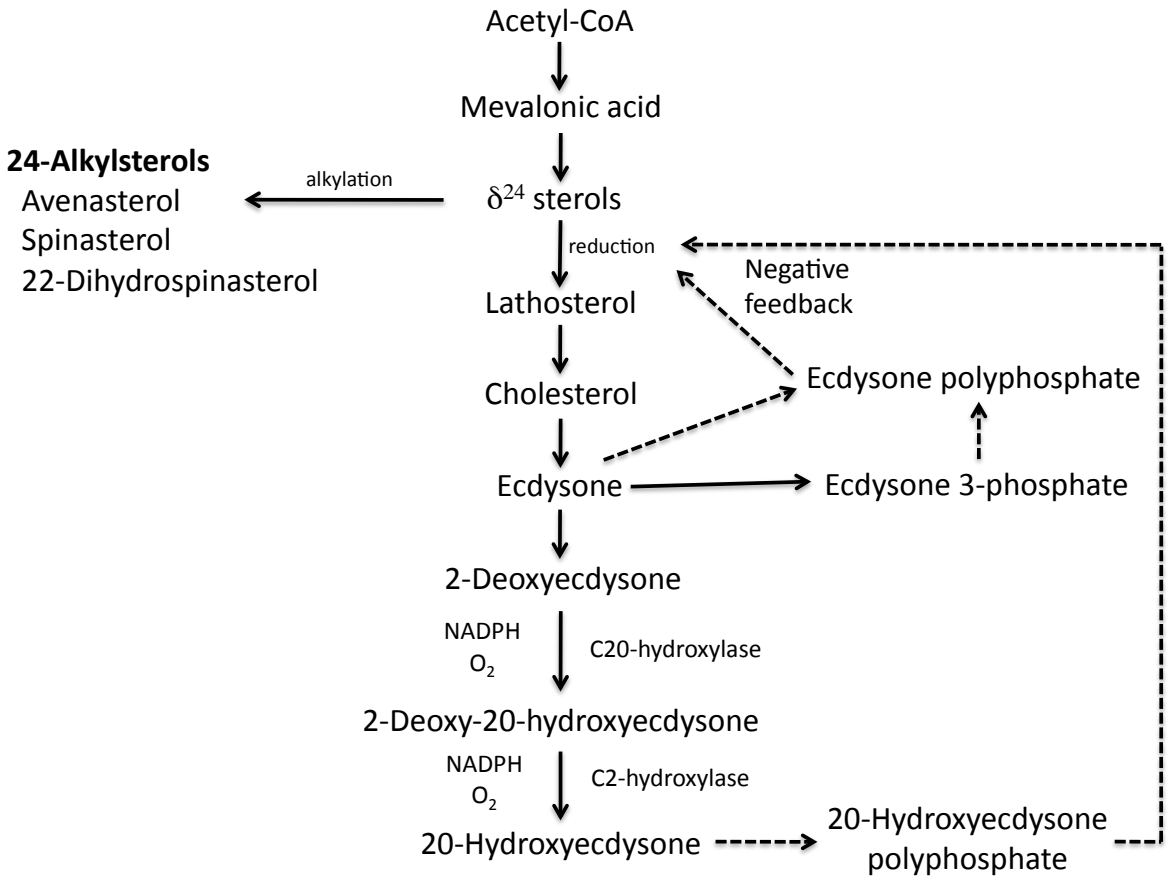



Figure 1.2 Phytoecdysteroid biosynthetic pathway (compiled from Adler and Grebenok, 1999 and Bakrim et al., 2008)



# CHAPTER 2

# IN VITRO PRODUCTION OF METABOLISM-ENHANCING PHYTOECDYSTEROIDS FROM *AJUGA TURKESTANICA*[*]


## Abstract

In order to develop a sustainable source of metabolism-enhancing phytoecdysteroids, cell suspension and hairy root cultures were established from shoot cultures of wild-harvested *Ajuga turkestanica* (Regel) Briq., a medicinal plant indigenous to Uzbekistan. Precursors of phytoecdysteroids (acetate, mevalonic acid or cholesterol) or methyl jasmonate (an elicitor) were added to subculture media to increase phytoecdysteroid accumulation. In cell suspension cultures, 20-hydroxyecdysone (20E) content increased 3- or 2-fold with the addition of 125 or 250 μM methyl jasmonate, respectively, compared to unelicited cultures. Precursor addition, however, did not provoke phytoecdysteroid accumulation. In hairy root cultures, addition of sodium acetate, mevalonic acid, and methyl jasmonate, but not cholesterol, increased phytoecdysteroid content compared to unelicited cultures. Hairy root cultures treated with 150 mg $L^{-1}$ sodium acetate, or 15 or 150 mg $L^{-1}$ mevalonic acid, increased 20E content approximately 2-fold to 19.9, 20.4 or 21.7 μg $mg^{-1}$, respectively, compared to control (10.5 μg $mg^{-1}$). Older hairy root cultures, extracted after the seventh or sixteenth subculture cycle, also showed


[*] Portions of this chapter have been published previously. With kind permission from Springer Science+Business Media: *Plant Cell, Tissue and Organ Culture*. *In Vitro* Production of Metabolism-Enhancing Phytoecdysteroids from *Ajuga turkestanica,* volume 93, 2008, pages 73-83, Cheng DM[1], Yousef GG[1], Grace MH[1], Rogers RB[1], Gorelick-Feldman J[2], Raskin I[2], and Lila, Mary Ann[1]; [1] Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana, IL 61801. [2] Biotech Center, Cook College, Rutgers University, New Brunswick, NJ 08901. Figures 2.1-2.3a, 2.5-2.8 © Cheng DM 2006. *In Vitro* Production of Metabolism-Enhancing Phytochemicals from Three Central Asian Plant Species. Master's Thesis, University of Illinois Urbana Champaign, 108 pages.

increases phytoecdysteroid content, with or without the addition of precursors, compared to control cultures maintained for a shorter duration of four subculture cycles. In addition, doses of 10 or 20 μg $mL^{-1}$ hairy root extract increased protein synthesis by 25.7% or 31.1%, respectively, in a C2C12 mouse skeletal cell line. These results suggest that sustainable production of metabolically active phytoecdysteroid can be achieved through hairy root culture systems.

*Keywords:* 20-Hydroxyecdysone, Cell suspension culture, Cyasterone, Hairy root, Methyl jasmonate, Mevalonic acid, Turkesterone

*Abbreviations:*

| | |
|---|---|
| 20E | 20-Hydroxyecdysone |
| CC | Column chromatography |
| $CD_3OD$ | Deuterated methanol |
| DAD | Diode array detector |
| DMEM | Dulbecco's modified eagle's media |
| DPM | Decays per minute |
| DW | Dry weight |
| ESI-MS | Electrospray ionization-mass spectroscopy |
| FW | Fresh weight |
| HPLC | High performance liquid chromatography |
| NMR | Nuclear magnetic resonance |
| TLC | Thin-layer chromatography |
| TMS | Tetramethylsilane |

## Introduction

*Ajuga turkestanica* (Regel) Briq. is a perennial plant from the family Lamiaceae and is indigenous to Uzbekistan. Traditionally, shoots and leaves are dried and steeped in hot water and the broth is ingested to treat bodily ailments such as heart disease, and muscle and stomach aches (Mamatkhanov et al., 1998; Abdukadirov et al., 2004; personal communication, Dr. B. Islamov, Samarkand State University). *A. turkestanica* produces a rich array of bioactive phytochemicals including the phytoecdysteroids turkesterone, 20-hydroxyecdysone (20E; also reported as ecdysterone), cyasterone, cyasterone 22-acetate, ajugalactone, ajugasterone B, α-ecdysone and ecdysone 2,3-monoacetonide along with the iridoids harpegide and harpegide 8-acetate (Usmanov et al., 1971, 1973, 1975, 1978; Baltaev, 2000; Ramazanov, 2005). Phytoecdysteroids are products of terpene biosynthesis and their structures vary according to the number, the locations and the positions of hydroxyl substituents on a steroid backbone (Báthori and Pongrácz, 2005). Air-dried tissue from *A. turkestanica* leaves has been reported to contain 0.02% 20E and roots contain 0.045% 20E and 0.052% turkesterone (Lev et. al., 1990).

Phytoecdysteroids have various biological effects in animal models. Growth promoting effects have been reported for sheep and Japanese quail after ingestion (Koudela et al., 1995; Purser and Baker, 1994; Slama et al., 1996). Phytoecdysteroids have also been shown to stimulate carbohydrate metabolism and reduce hyperglycemic response in rats, which has significant implications for reducing diabetes symptoms in humans (Yoshida et al., 1971). Intraperitoneal injections of 20E and turkesterone from *A. turkestanica* have been shown to increase the mass of liver and certain muscles in rats (Syrov, 1984; Báthori and Pongrácz, 2005).

Additionally, stimulation of protein synthesis in mouse C2C12 myotubes was demonstrated for individual phytoecdysteroids and *A. turkestanica* wild-harvested shoot extracts (Gorelick-Feldman et al., 2008).

Callus cultures initiated from ovaries of *A. turkestanica* have achieved yields of 0.12% 20E and 0.036% turkesterone on the weight of air-dried raw material (Lev et al., 1990). In later callus cultivation studies, the yield of 20E was increased from 0.1% to 0.2% and turkesterone decreased from 0.032% to 0.004% when treated with an 8 mM dose of the mutagen *N*-nitroso-*N*-methylurea (Zakirova et al., 2000; Zakirova and Yakubova, 2002). Although callus cultures of a related plant, *Ajuga reptans,* did not produce phytoecdysteroids, hairy roots transformed with *Agrobacterium rhizogenes* accumulated 20E up to 0.12% on a dry weight basis; four times higher than the content of the plant's roots *in vivo* (Tomas et al., 1992; Matsumoto and Tanaka, 1991). Hairy root cultures have not been reported for *A. turkestanica.*

Biosynthesis of phytoecdysteroids occurs through terpene synthesis and the mevalonic acid pathway of which acetyl-CoA, mevalonic acid and cholesterol are direct precursors (Báthori and Pongrácz, 2005; Adler and Grebenok, 1999; Seo et al., 1988). Methyl jasmonate, a plant signaling compound induced by insect damage, triggered an increase in phytoecdysteroid synthesis in spinach, *Spinacia oleracea* L. (Schmelz et al., 1999). As such, these compounds have potential as elicitors to effectively enhance phytoecdysteroid production of *A. turkestanica in vitro* cultures.

The objectives of this research were to initiate cell suspension cultures and hairy root cultures of *A. turkestanica* and to develop strategies to stimulate the production of phytoecdysteroids, particularly 20E, turkesterone and cyasterone. These phytoecdysteroids were targeted for elicitation as they were previously reported to be the most potent bioactives

produced by *A. turkestanica* (Báthori and Pongrácz, 2005; Baltaev, 2000). Additionally, as *A. turkestanica* wild-harvested shoot extracts and isolated phytoecdysteroids had previously demonstrated bioactivity, *A. turkestanica* hairy root extracts were evaluated for protein synthesis bioactivity *in vitro*.

## Materials and Methods

### *Plant Material*

Shoots and leaves of *A. turkestanica* were harvested in the mountainous regions of Uzbekistan in July 2004, air dried and transported to the laboratory at the University of Illinois Urbana-Champaign (UIUC; ICBG Central Asia, voucher UPL_00057, ILLS, MO). Plant material was frozen at -80 ºC prior to lyophilization and the dried plant material was ground in 100 g batches for 10 minutes each into a coarse powder using a Turbo-twister blender (Hamilton Beach/Proctor-Silex, Inc., Southern Pines, NC). In addition, some of the shoot tips of *A. turkestanica* were surface disinfested at Tashkent Agrarian University, Uzbekistan (Dr. I. Belolipov, personal communication) by agitating in 0.6% sodium hypochlorite solution and one drop of Tween 20 $L^{-1}$ for 15 minutes, followed by a 30 s immersion in 70% ethanol and 3 rinses with sterile double distilled water. Shoot tips were trimmed under sterile conditions and explanted into 30 mL of agar solidified MS media (Murashige and Skoog, 1962) without growth regulators. These cultures were transported to the USA and surviving shoot tips were used as a source of sterile explants for initiation of shoot cultures *in vitro*.

### *Extraction and Isolation of Phytoecdysteroids*

Dried ground tissue of *A. turkestanica* (500 g) was exhaustively extracted with 3 L of

methanol three times. The first suspension was allowed to soak overnight followed by two more immersions in 3 L over a 48 hour period. Methanol extracts were sonicated for 10 minutes before vacuum filtration through Whatman #4 filter paper (Whatman International Ltd., England). The majority of the methanol was removed by rotary evaporation under reduced pressure at 40 $^{o}$C and the concentrate was frozen at -20 ºC and lyophilized. The dried methanol extract was suspended in 200 mL water and extracted with petroleum ether (500 mL x 3) and the petroleum ether fraction was gravity filtered through Whatman #4. The combined petroleum ether was dried over anhydrous $Na_2SO_4$ and evaporated. The remaining aqueous layer was then extracted with ethyl acetate (500 mL x 4) and the ethyl acetate extract was dried over anhydrous $Na_2SO_4$ and rotary evaporated (Fig. 2.1).

The ethyl acetate extract was chromatographed over a silica gel column via flash column chromatography as described by Still et al., 1978. Chromatographic separations were achieved using silica gel 60 230-240 mesh ASTM (Merck, Darmstadt, Germany) with a 2.5 cm column diameter and silica gel packed 30 cm in height. Elution was performed using a gradient increase of ethyl acetate in hexane to 100% ethyl acetate followed by methanol in ethyl acetate up to 10%. Fractions were analyzed by TLC using silica gel 60 $F_{254}$ 250 μm pre-coated plates (EMD Chemicals Inc., Gibbstown, NJ) with solvent systems of chloroform-methanol-water at 4:1:0.1 ratios and ethyl acetate-methanol-water at 77:13:19 ratios. The TLC plates were monitored by short wave UV lamps (254 nm) and visualized with anisaldehyde spray reagent (Stahl, 1969). Similar fractions were combined and solvents were removed by rotary evaporation. Combined fractions were column chromatographed over silica gel using a chloroform based solvent system with increasing proportions of methanol to 50% methanol in chloroform or chromatographed by preparative TLC (Fig. 2.1).

The isolated phytoecdysteroids were identified by comparing physical, chemical and spectral data ($^{1}$H and $^{13}$C NMR and ESI-MS) with reported data (Okuzumi et al., 2005; Chan et al., 2005; Darwish and Reinecke 2003). $^{1}$H NMR (500 MHz) and $^{13}$C NMR (125 MHz) spectra, all in $CD_3OD$, were recorded on a Varian Inova 500 spectrometer (Varian Instruments, Palo Alto, CA), using TMS as the internal standard. Low-resolution ESI mass spectra were recorded on a Micromass ZAB-SE Spectrometer (Waters Corporation, Beverly, MA) in the Mass Spectrometry Laboratory of the School of Chemical Sciences, UIUC.

The isolated phytoecdysteroids were used as standards for quantification at concentrations of 250, 500 and 1000 μg $mL^{-1}$ with 10 μL injection volumes. A commercial standard of 20E (Sigma, St. Louis, MO) was used to compare with compounds isolated from *A. turkestanica* extracts. Analysis was performed using an Agilant 1100 HPLC system (Agilent Technologies, Inc., Wilmington, DE) with autosampler, DAD (205 nm) and Eclipse XOB-$C_{18}$ reversed phase column 5 μM x 4.6 mm x 150 mm. The mobile phase solvents consisted of $H_2O$ (A) and acetonitrile (B). A gradient of 10%, 40%, 10% and 10% solvent B was used at 0, 25, 30, and 35 min, respectively, with 0.50 ml $min^{-1}$ flow rate. Concentrations were calculated by taking the average of two HPLC runs.

*Cell Suspension Culture*

Callus cultures were initiated from leaves of shoots maintained *in vitro* on a B5 media (Gamborg et al., 1968) containing 20 g $L^{-1}$ sucrose, 2.3 μM 2,4-dichlorophenoxyacetic acid and 6 g $L^{-1}$ agar with pH adjusted to 5.8. After three one-month growth cycles, light brown friable callus was transferred to 50 mL liquid media of the same composition in 250 mL flasks. Cultures were maintained on a shaker at 145 rpm in darkness at 28 ºC. Cells were subcultured to

fresh media every 12-14 days. All experimental sets were performed in triplicate. The rate of cell growth and optimal subculture cycle was determined by measuring the FW of cell suspensions after vacuum filtration through Whatman #4 every three days for 15 days.

For elicitation trials, approximately 2 g FW of *A. turkestanica* cells were added to 50 mL liquid media in 250 mL flasks. Sodium acetate, mevalonic acid and cholesterol were prepared and added at ranges of concentrations reported previously (Panda et al., 1992; Chowdhury and Chaturvedi, 1980; Roddick and Butcher, 1972). Cell suspension cultures were elicited on the second day of subculture with 50, 100 or 150 mg $mL^{-1}$ sodium acetate (Mallinckrodt Chemical Works, St. Louis, MO) and another set of cell suspension cultures were elicited with 50, 100 or 150 mg $mL^{-1}$ cholesterol (Sigma). Mevalonolactone was prepared with an equal molar solution of sodium bicarbonate to open the lactone ring (Moreno et al., 1993). To elicit cell cultures with mevalonic acid, 15 mg $mL^{-1}$ mevalonolactone (Sigma) with 9.6 mg $mL^{-1}$ sodium bicarbonate (Mallinckrodt Chemical Works) or 150 mg $mL^{-1}$ mevalonolactone with 96.6 mg $mL^{-1}$ sodium bicarbonate were added to cell cultures. A concentration of 0.017 mM ethanol, equivalent to that used in the highest dose of cholesterol, 150 mg $L^{-1}$, was used in the ethanol control. In other treatments, cells were also elicited with 125 or 250 μM methyl jasmonate.

Cells were harvested on day 14 and vacuum filtered on Whatman #4 filter paper and FW measured. Cells were then frozen at -80 °C, thawed and extracted 3 times with 100 mL methanol and blended at high speed for 2 minutes at room temperature using an Osterizer blender (Sunbeam Products, Inc., Boca Raton, FL). Solvent was removed by rotary evaporation and lyophilization, crude extract DW was measured, and samples were stored at -80 °C. For each cell culture sample, 20 mg dried extract dissolved in 50% aqueous ethanol, filtered though 0.45 nm nylon filters and 50 μl were injected for analysis by HPLC as described above.

*Hairy Root Cultures*

*Agrobacterium rhizogenes* K599 strain and hairy root inoculation protocol were obtained from Dr. Olga Zernova at the University of Illinois Department of Crop Sciences. *A. rhizogenes* was cultured in 20 mL LB media (Sambrook et al., 1989) and shaken at 155 rpm at 120 μmol $m^{-2}s^{-1}$ irradiance, overnight. Acetosyringone (Apollo Scientific Ltd., Stockport, UK) was filter sterilized through a 0.22 μm white GSWP nitrocellulose filter (Millipore Corporation, Bedford, MA) and added to *A. rhizogenes* cultures at the start of bacterial growth, and immediately before plant inoculation to a 200 μM concentration. *In vitro* shoots were trimmed to one inch lengths, scored with a sterile needle and injected with the *A. rhizogenes* culture prior to a 15 minute immersion in 20 mL *A. rhizogenes* culture. Inoculated shoot segments were blotted dry to remove excess bacteria and placed in sterile petri dishes with moist filter paper. After two days of co-cultivation with bacteria under continuous fluorescent illumination with an intensity of 120 μmol $m^{-2}s^{-1}$, shoot segments were transferred to petri dishes on media supplemented with 1.2 mM carbenicillin (Agri-Bio Inc., Miami, FL). MS media to generate hairy root cultures was supplemented with 0.1 g myoinositol, 30 g sucrose, 1 mL FeEDTA, B5 vitamins, and 2 g $L^{-1}$ Gelrite (Scott Laboratories, Fiskeville, RI) with pH adjusted to 5.8. Once roots reached 2-4 cm in length, shoots were removed and roots were placed in the dark. Roots were subcultured every two weeks and after 4-5 subculture cycles without bacterial contamination, roots were moved onto media without carbenicillin. Roots were transferred to agar-solidified MS media 30 g $L^{-1}$ sucrose, 2.2 μM 6-benzylaminopurine, 2.5 μM indolebutyric acid and 6 g $L^{-1}$ agar with pH adjusted to 5.8 for 4 weeks before transferring to liquid media of the same composition. Roots were cultured in 250 mL flasks containing 50 mL of media, shaken in the dark at 155 rpm and elicited and extracted by the same method as described above. Hairy roots were cultured for

three subcycles prior to experimentation and extraction was done after the fourth, seventh, and sixteenth subcycle as noted.

*Cucumopine Detection*

For the detection of cucumopine, approximately 2.0 g of *in vitro* leaf, root or hairy root tissue was macerated in 10 mL distilled water with a mortar and pestle. Extracts were vacuum filtered through Whatman #4 paper and rotary evaporated. Twenty microliters of supernatant extract were spotted on Whatman 3MM paper. The spots were allowed to dry, and the papers were wetted with the formic acid-acetic acid running buffer (pH 1.8) and subjected to high voltage paper electrophoresis at 270 V for 90 min. The electrophoretograms were dried in a stream of warm air (Savka et al., 2002).

Cucumopine and its degradation products were visualized with Pauly reagent by spraying the dry electrophoretograms lightly in a solution containing equal parts of sulfanylic acid (1.0% in 1 N HCl) and sodium nitrite (5.0% in water) (Easley, 1965). Papers were allowed to dry and then sprayed with aqueous 15% sodium carbonate. Cucumopine products appeared as reddish spots as the paper was sprayed with sodium carbonate. Spots were identified as cucumopine by comparing their electrophoretic mobilities and staining properties with those of authentic standards. Cucumopine was synthesized from L-histidine and α-ketoglutaric acid (Davioud et al., 1988).

*PCR Analysis*

*In vitro* shoot, root or hairy root tissues (30-100 mg) were harvested and genomic DNAs were extracted by a cetyltrimethylammonium bromide (CTAB) method adjusted from Murray

and Thompson (1980). Extraction buffer consisted of 2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (all from Sigma) and 0.2 M β-mercaptoethanol (Fisher Scientific, Fair Lawn, NJ). *A. rhizogenes* DNA was extracted using GenELute Plasmid Miniprep Kit (Sigma).

For the detection of the *rolC* gene, PCR was performed using the following oligonucleotide primers: rolC forward: 5'-ATGGCGGAATTTGACCTATGTGCT-3' and rolC reverse: 5'-TCACTCCATTCCAAATTTGCATT-3' to produce a 532-bp fraction. The PCR reaction mixture consisted of 1 μl (100-200 ng) of plant DNA, 0.8 μl of 50 mM $MgCl_2$, 0.3 μl of Taq DNA polymerase (5 U $\mu l^{-1}$, Invitrogen, Carlsbad, CA), 3.0 μl of 10X Taq buffer (Invitrogen), 1.0 μl of 200 μM dNTPs (Sigma), 0.5 μl each of 40 μM primers and 21.9 μl of sterile distilled water. Samples were heated to 95 °C for 10 min, followed by 35 cycles at 96 °C (45 s), 50 °C (45 s), 72 °C (90 s) and then 72 °C for 90 s with an Applied Biosystems 2720 Thermal Cycler (Version 2.08, Foster City, CA).

*Protein Synthesis Assay*

The effect of *A. turkestanica* hairy root extract (after four subcycles) on protein synthesis was tested on a mouse skeletal cell line, C2C12, (ATCC CRL-1772). Cells were seeded between 3 and 10 passages at a density of $10^5$ cells $cm^{-2}$ onto 24 well tissue culture plates. Cells were grown for 48 h in 5% $CO_2$ at 37 °C on low glucose DMEM supplemented with 10% Fetal Bovine Serum, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 6 mM glutamine, 1 mM pyruvate, 100 U $mL^{-1}$ penicillin and 100 μg $mL^{-1}$ streptomycin (Gibco, New York, NY). After cells reached 80% confluency, media was replaced with differentiation media (DMEM with 2% horse serum). After 5 days, myoblasts fused into multinucleated myotubes and were washed with sodium free DMEM. Increasing concentrations of *A. turkestanica* hairy root

extract (1, 5, 10, 20 or 40 μg $mL^{-1}$), 1μM 20E or vehicle (0.1% ethanol) were added to cells in serum-free media containing 5μCi $mL^{-1}$ [$^3$H] leucine. Cells were incubated for 24 h before protein measurement.

Protein synthesis was determined by measuring amino acid incorporation as described by Montgomery et al. (2002). Briefly, cells were washed with cold phosphate buffered saline, followed by the addition of 5% trichloroacetic acid. Trichloroacetic acid was removed after 30 min at 4 °C and the precipitate was dissolved in 0.5M NaOH (500 μL). The dissolved precipitate (400 μL) was added to scintillation vials with 5 mL of scintillation fluid (Ready safe, Beckman Coulter, Fullerton, CA). The data were expressed as decays per minute (DPM) per mg total protein as mean ± SEM of 4 experiments, each done in triplicate.

*Statistical Analysis*

Statistical significance was determined by one-way analysis of variance (ANOVA) using the GLM procedures with adjustments for multiple comparisons with Dunnett's test using SAS version 9.1 for Windows (SAS Institute Inc., Cary, NC, USA). Experimental results are a mean of six replicates from two independent trials. Differences referred to as significant had a p-value $< 0.05$. For the protein synthesis assay, statistical significance was determined using the Student's *t* test ($p < 0.05$).

**Results**

*Isolation of Phytoecdysteroids from In Vivo A. turkestanica*

Methanolic extraction of 500 g dried *A. turkestanica* leaf and stem tissue yielded 62.4 g extract. The dried methanolic extract was suspended in 200 mL water and extracted with

petroleum ether to remove chlorophylls and other lipophilic molecules. The petroleum ether extract (18.5 g) was dried and set aside. The remaining aqueous layer was extracted with ethyl acetate to separate the phytoecdysteroids from hydrophilic compounds such as sugars and flavonoids (Fig. 2.1). TLC plates revealed that the major compounds were in fractions 35-48 of the ethyl acetate extract (11.9 g).

From the ethyl acetate extract, fractions 35-39 (1.8 g) were combined and eluted with 10% methanol to obtain cyasterone 22-acetate (220 mg, subfraction 19) and cyasterone (110 mg, subfraction 22) (Fig. 2.1). Both compounds gave pink coloration with anisaldehyde spray reagent on TLC plates with $R_f$ = 0.63 and $R_f$ = 0.57, respectively, on silica gel TLC plates with $CHCl_3$-MeOH-$H_2O$, 4:1:0.1 mobile phase. HPLC-MS-ESI gave molecular ion peaks of $[M+1]^+$ *m/z* 563.4 and 521.4 corresponding to $C_{31}H_{46}O_9$ and $C_{29}H_{44}O_8$ for cyasterone 22-acetate and cyasterone, respectively.

Fractions 40-47 (1.9 g) of the ethyl acetate extract were combined and chromatographed by vacuum liquid chromatography (VLC) (Fig. 2.1). 20E (120 mg) eluted in 14% methanol in chloroform, had an $R_f$ 0.43 on the same TLC system as described above, and developed a green colored spot with anisaldehyde spray reagent. These results were consistent with the commercial standard. HPLC-MS-ESI gave molecular ion peaks of $[M+1]^+$ *m/z* 481.4 corresponding to $C_{27}H_{44}O_7$.

Turkesterone (15 mg) was isolated from fraction 48 of the ethyl acetate extract by preparative TLC with $R_f$ 0.26 and developed a green colored spot with anisaldehyde spray reagent (Fig. 2.1). HPLC-MS-ESI showed a molecular ion peak of $[M+Na]^+$ *m/z* 519.3 corresponding to $C_{27}H_{44}O_8$. HPLC-MS-ESI and NMR data are in agreement with literature

reported for the isolated ecdysteroids (Okuzumi *et al*., 2005; Chan *et al*., 2005; Darwish and Reinecke, 2003).

Peak identities were recorded for the identified phytoecdysteroids: 20E ($R_t$ 10.8 min), turkesterone ($R_t$ 8.1 min), cyasterone ($R_t$ 12.5 min) and cyasterone 22-acetate ($R_t$ 14.3 min). Leaf and stem tissue contained 37.0 μg $mg^{-1}$ DW 20E, 20.4 μg $mg^{-1}$ DW turkesterone, 12.6 μg $mg^{-1}$ cyasterone and 12.9 μg $mg^{-1}$ cyasterone 22-acetate, determined by HPLC.

*Cell Suspension Cultures*

Friable tan colored callus was generated over a period of 3 months from leaf explants and was transferred to liquid media to establish cell suspension cultures. The average concentration of 20E was 6.9 μg $mg^{-1}$ DW extract, in 10 to 15 month-old control cell suspension cultures of *A. turkestanica* (16-28 subcultures from inoculation with callus). Turkesterone, cyasterone and cyasterone 22-acetate were detected only in trace amounts. Cholesterol, sodium acetate or mevalonic acid additions to the media at 50, 100 or 150 mg $L^{-1}$ did not increase 20E accumulation in cell cultures in comparison to control cultures (Fig. 2.2). Addition of 125 or 250 μM methyl jasmonate provoked slight, but not statistically significant increases in 20E concentration (23.6 or 14.7 μg $mg^{-1}$ DW extract, respectively).

*Hairy Root Cultures*

Hairy roots developed 2-3 weeks after inoculation of *in vitro* shoot segments with *A. rhizogenes* and were transferred to liquid culture, as described above. Whereas cell suspension cultures accumulated only 20E, extracts from hairy roots maintained for 4 subculture cycles contained an average of 10.5 μg $mg^{-1}$ 20E, 7.5 μg $mg^{-1}$ cyasterone, 7.1 μg $mg^{-1}$ cyasterone 22-

acetate and trace amounts of turkesterone. Addition of sodium acetate, mevalonic acid and methyl jasmonate, but not cholesterol, increased phytoecdysteroid content of hairy root cultures (Fig. 2.3a). Of these treatments, cultures treated with 150 mg $L^{-1}$ sodium acetate or 15 or 150 mg $L^{-1}$ mevalonic acid were statistically significant for an increase in 20E content to 19.9, 20.4 or 21.7 μg $mg^{-1}$ DW extract, respectively. Analysis of control untreated older hairy root culture extracts (seven subculture cycles) also showed a statistically significant increase in 20E content (24.8 μg $mg^{-1}$) and an increase in turkesterone (0.9 μg $mg^{-1}$) and cyasterone (8.1 μg $mg^{-1}$) compared to control cultures maintained for a shorter time (four subculture cycles; Fig. 2.3a).

As significant increases in phytoecdysteroid accumulation in hairy root cultures were demonstrated with the addition of sodium acetate or mevalonic acid, as well as with increased number of subculture cycles, these methods were examined in combination. Sodium acetate or mevalonic acid was added to the media of hairy root cultures maintained for sixteen subculture cycles. Hairy root cultures extracted after the sixteenth subculture cycle showed a statistically significant increase in 20E content (28.7 μg $mg^{-1}$ DW extract) compared to cultures extracted after the fourth subculture cycle (Fig. 2.3b). However, addition of precursors to media at the sixteenth subculture cycle did not demonstrate significant increases in phytoecdysteroid content as compared to untreated cultures of the same age or cultures maintained for seven subculture cycles. Increased phytoecdysteroid content was not due to biomass accumulation as there were no statistical differences between fresh masses of hairy roots between all treatments. Total phytoecdysteroid contents were 1.5 and 1.6 μg $g^{-1}$ of the fresh weight of untreated hairy roots subcultured for the durations of seven and sixteen cycles, respectively. These concentrations were significantly different compared to hairy roots maintained for four subculture cycles (0.7 μg $g^{-1}$ FW). 20E yields were significantly greater in hairy roots with a greater number of

subculture cycles (seven and sixteen subculture cycles) and in hairy roots with sixteen subculture cycles treated with 15 mg $L^{-1}$ sodium acetate compared to cultures maintained four subculture cycles (Fig 2.4a). Yield of total phytoecdysteroid from hairy root cultures maintained four subculture cycles treated with 150 mg $L^{-1}$ mevalonic acid were significantly increased compared to untreated cultures also maintained four subculture cycles (Fig. 2.4b).

*Hairy Root Confirmation*

Hairy root transformation was confirmed by detection of cucumopine, an opine produced by *A. rhizogenes* strain K599 after T-DNA insertion into the host plant. *A. turkestanica* hairy root extracts produced reddish spots that corresponded with cucumopine standard when visualized with Pauly regent (Fig 2.5). Water extracts from untransformed *A. turkestanica* shoot and root tissue did not produce a corresponding spot. Natural, untransformed shoot cultures rarely produced roots, and those few untransformed roots were less productive in terms of biomass accumulation.

DNA extracted from hairy roots showed a 532-bp fragment amplified by PCR, corresponding to that of the *rolC* gene of *A. rhizogenes*. Plasmid DNA extracted from *A. rhizogenes* also produced a 532-bp fragment whereas genomic DNA extracted from *in vitro* shoot and root did not produce a DNA band in the agarose gel (Fig. 2.6).

*In Vivo and In Vitro Shoot Tissue*

Wild-harvested shoot extracts from Uzbekistan contained 37.0 μg $mg^{-1}$ 20E, 20.4 μg $mg^{-1}$ turkesterone, 12.6 μg $mg^{-1}$ cyasterone and 12.9 μg $mg^{-1}$ cyasterone 22-acetate. *In vitro* shoot extracts had an average 20E content equivalent to wild-harvested shoots, but the content of the other phytoecdysteroids were lower: 37.0 μg $mg^{-1}$ 20E, 10.7 μg $mg^{-1}$ turkesterone, 1.7 μg $mg^{-1}$

cyasterone and 2.2 μg $mg^{-1}$ cyasterone 2-acetate. The total phytoecdysteroid content on the dry weight of wild-harvested and *in vitro* shoots was 10.3 mg $g^{-1}$ and 12.7 mg $g^{-1}$ respectively.

HPLC chromatograms illustrate the differences in phytochemical profiles of cell culture extracts, hairy root extracts, and wild-harvested shoot extracts (Fig. 2.7). In all three types of plant tissue extracts, 20E was a major component, up to 37.0 μg $mg^{-1}$ in shoots, both wild-harvested *in vivo* and *in vitro*. Cell suspension cultures accumulated primarily 20E; hairy roots accumulated 20E, cyasterone and cyasterone 22-acetate, and wild-harvested *in vivo* and *in vitro* shoots accumulated 20E and turkesterone in greater amounts than the other phytoecdysteroids quantified in this study. HPLC chromatograms of hairy root and shoot extracts also revealed unidentified compounds with absorption at 242 nm.

*Protein Synthesis Assay*

*A. turkestanica* hairy root extracts containing 1.6 μg $mg^{-1}$ turkesterone, 20.7 μg $mg^{-1}$ 20E, 5.8 μg $mg^{-1}$ cyasterone and 6.8 μg $mg^{-1}$ cyasterone 22-acetate, increased protein synthesis in C2C12 myotubes in a dose dependant manner after 24 h of treatment (Fig. 2.8). Hairy root extracts at doses of 10 and 20 μg $mL^{-1}$ provoked statistically significant increases in protein synthesis by 25.7% and 31.1%, respectively. 20E at 1 μM also increased protein synthesis by 29.0% above the control.

**Discussion**

The greatest concentrations of 20E in the extract of each of the *A. turkestanica* tissue types were 23.6 μg $mg^{-1}$ for cell suspension cultures elicited with 125 μM methyl jasmonate, 24.8 μg $mg^{-1}$ for older (7 subculture cycles; approximately 14 weeks old) hairy roots and 37.0 μg

$mg^{-1}$ from both wild-harvested and *in vitro* shoots (Fig 2.3a). While cell suspension cultures produced predominantly 20E, hairy root cultures also produced turkesterone, cyasterone and cyasterone 22-acetate. The data supports previous studies which have show that undifferentiated plant cell cultures often lose their ability to accumulate certain secondary products (Tomas et al., 1992; Zakirova and Yakubova, 2002). Hairy root cultures, on the other hand, demonstrated potential as a sustainable and enhanceable production system for phytoecdysteroids, providing yields of total phytoecdysteroids up to 317 μg $mL^{-1}$ media and 233 μg $mL^{-1}$ media for 20E (Fig 2.4).

In addition to producing a wider range of phytochemicals than cell suspension cultures, hairy root cultures typically have greater genetic stability. Cytological assessment of hairy root cultures from eight different species ranging from 6 to 17 months in culture showed chromosomal stability, whereas cell suspensions of two species demonstrated variable chromosome numbers (Aird et al., 1988). Hairy root cultures of *Hyoscyamus muticus* biosynthesized hyoscyamine and maintained the same biosynthetic capacity for 15 years (Kim et al., 2002). Callus and cell suspension cultures derived from ovaries of *A. turkestanica* have previously been developed (Lev et al., 1990; Zakirova and Malikova, 2001). Multiyear cultivations of cell suspension cultures of *A. turkestanica* were analyzed by Zakirova and Yakubova (2002), who reported that phytoecdysteroid accumulation decreased over time and only trace quantities of 20E and turkesterone were detected after 4 years of culture. The change in biosynthetic capacity was explained as an effect of chromosomal changes and the rapidly growing population of nonproductive cells.

Phytoecdysteroids are stable compounds that may accumulate in organized plant tissues with increasing age and biomass. In experiments with hydroponically grown spinach (*Spinacia*

*oleracea)*, the stability of 20E and dominant phytoecdysterols synthesized from a pulse of [2-$^{14}$C]mevalonic acid ([2-$^{14}$C]MeVA) was examined over 8 and 28 days (Schmelz et al., 2000). Levels of [2-$^{14}$C]MeVA incorporation in root and shoot pools of 20E did not change significantly over time and there was no evidence of turnover of 20E during a span of 28 days. Similarly, levels of phytoecdysteroids increased with greater number of subculture cycles for *A. turkestanica* hairy roots which showed statistically significant increases in total phytoecdysteroid accumulation after the seventh (1.5 μg $g^{-1}$ FW) and sixteenth (1.6 μg $g^{-1}$ FW) subculture cycle compared to the fourth subculture cycle (0.7 μg $g^{-1}$ FW). Differences were not due to biomass accumulation, but may have been due to maturation and adaptation of tissues *in vitro,* to tissues that produce phytoecdysteroids more effectively. For example, only mature leaves of spinach were able to biosynthesize phytoecdysteroids, which were transported to young newly developing leaves where they accumulated (Bakrim et al., 2008).

Methyl jasmonate is a widely used elicitor in plant cell culture (Mirijalili and Linden, 1996; Ketchum et al*.,* 1999). Accumulation of 20E and other phytoecdysterols in spinach was induced by methyl jasmonate signaling and tissue damage (Schmelz et al., 1999). Our results also illustrated an increase in phytoecdysteroid accumulation in cell suspension and hairy root cultures of *A. turkestanica* with methyl jasmonate elicitation. Additionally, methyl jasmonate was added concurrently with individual precursors to the media of hairy root cultures, however there was no increase in phytoecdysteroid accumulation.

Mevalonic acid, cholesterol and acetyl-CoA are direct precursors to phytoecdysteroid biosynthesis as demonstrated by radiolabel experiments (Adler and Grebenok, 1999; Nagakari et al., 1994; Heftmann et al., 1968; Sauer et al., 1968; Joly et al., 1969; De Souza et al., 1970). Addition of mevalonic acid and sodium acetate enhanced phytoecdysteroid accumulation in

hairy root cultures, but not in cell suspension cultures of *A. turkestanica*. Differences in metabolite responses could be due to cellular uptake of chemical compounds (Yeoman and Yeoman, 1996). Also, addition of cholesterol did not increase phytoecdysteroid accumulation in either cell suspension or hairy root cultures. $^{13}C$ NMR analysis of *A. reptans* hairy root cultures revealed that [2-$^{13}C$]acetate and [26,27-$^{13}C_2$]cholesterol were incorporated in 20E, and acetate, but not cholesterol, was incorporated into cyasterone and 29-norcyasterone (Nagakari et al., 1994). These results illustrate metabolite specificity with phytoecdysteroid biosynthesis.

20E and turkesterone stimulated carbohydrate metabolism and protein synthesis resulting in increases in muscle mass in rats (Yoshida et al., 1971; Syrov, 1984; Bathori and Pongracz, 2005, Baltaev, 2000). In our experiments, *A. turkestanica* hairy root extracts stimulated increased protein synthesis in a mouse skeletal cell line. Protein synthesis increased 29.0% or 31.1% over control after treatment with 1μM 20E or 20 μg $mL^{-1}$ hairy root extract, respectively. In a related study, Gorelick-Feldman et al. (2008) showed that 8 μg $mL^{-1}$ wild harvested *A. turkestanica* extract and 8 μg $mL^{-1}$ spinach extract increased protein synthesis by 16% and 18% above the control, respectively.

In this study, a sustainable *A. turkestanica* hairy root culture line was established which demonstrated enhanced levels of phytoecdysteroid accumulation with elicitation, precursor addition and increased subculture cycles. In addition, *A. turkestanica* hairy root culture extracts have demonstrated an ability to stimulate protein synthesis bioactivity in a murine cell line. Further studies will concentrate on improving phytoecdysteroid production and investigation of therapeutic properties of hairy root cultures, such as muscle enhancement *in vivo*.

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## Figures

Figure 2.1 Scheme for extraction of *in vivo A. turkestanica* shoots (leaves and stems). (C.C.: column chromatography, F.:fraction, Sf.:subfraction, MeOH:methanol and EtOAc:ethyl acetate.)

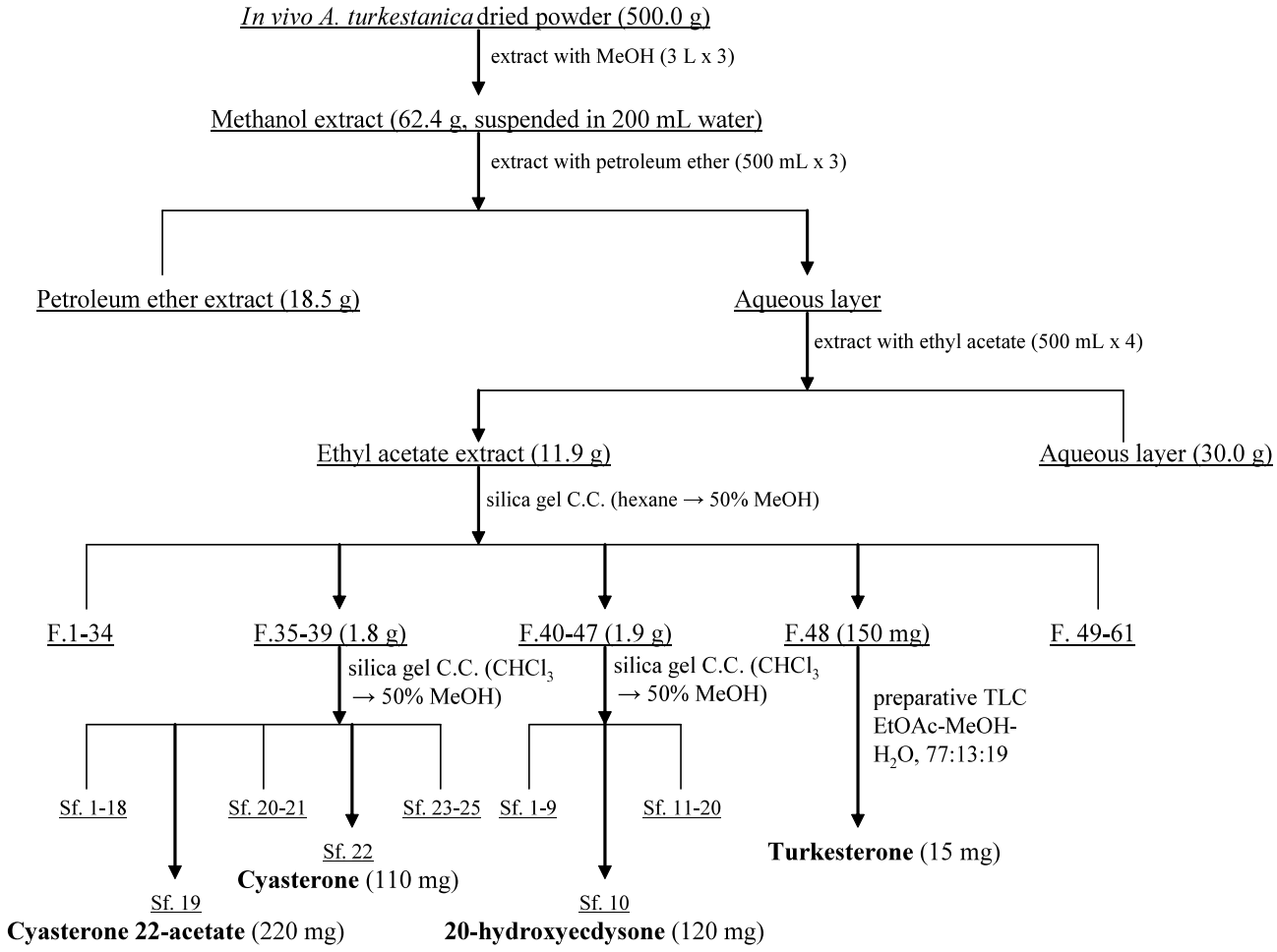


Figure 2.2 Concentration of 20-hydroxyecdysone in *A. turkestanica* cell suspension cultures treated with 150 mg $L^{-1}$ cholesterol (Cho150), 150 mg $L^{-1}$ sodium acetate (NaA150), 15 and 150 mg $L^{-1}$ mevalonic acid (MVA15, MVA150), or 125 and 250 μM methyl jasmonate (MJA125, MJA250).

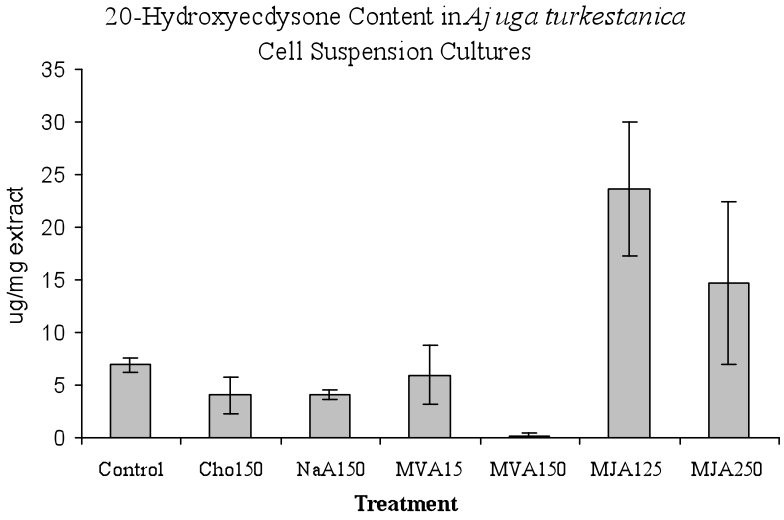


Figure 2.3a Phytoecdysteroid accumulation of *A. turkestanica* hairy root cultures (4 four subculture cycles; approximately eight wks old) treated with 15 and 150 mg $L^{-1}$ cholesterol (Cho15, Cho150), 150 mg $L^{-1}$ sodium acetate (NaA15, NaA150), 15 and 150 mg $L^{-1}$ mevalonic acid (MVA15, MVA150), or 125 and 250 μM methyl jasmonate (MJA125, MJA250). An additional control treatment was extracted after an extended time in subculture (seven subculture cycles) to determine the effect on culture age on phytoecdysteroid content. * Indicates statistical significance ($p < 0.05$).

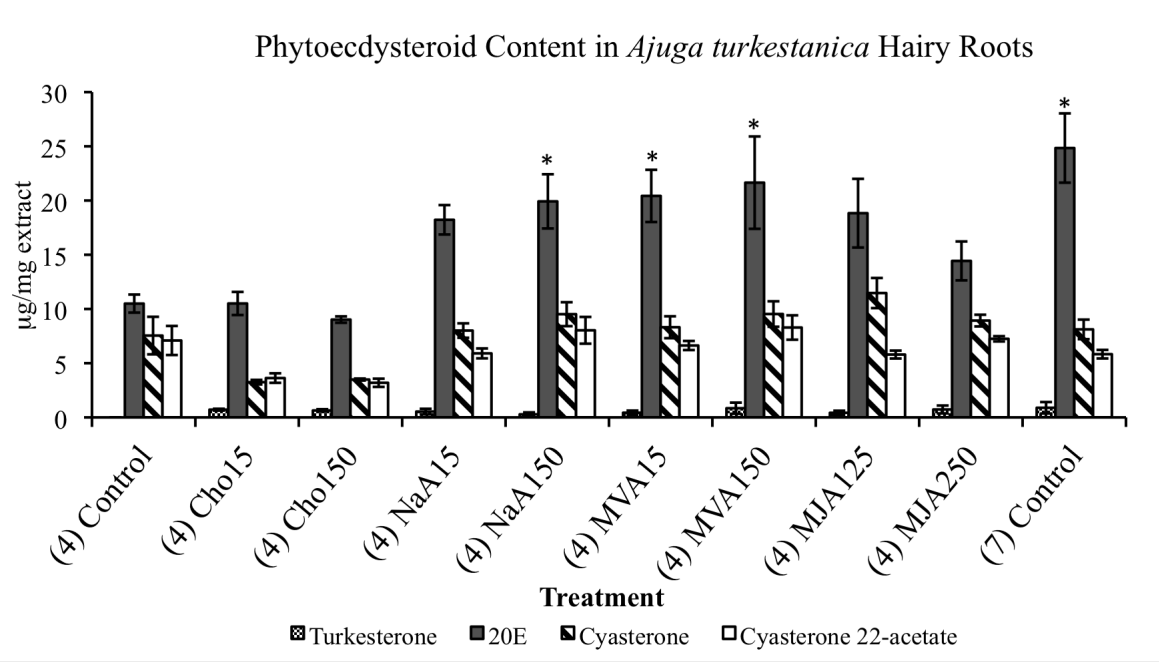


Figure 2.3b Phytoecdysteroid accumulation of *A. turkestanica* hairy root cultures after four, seven and sixteen subculture cycles. Hairy roots maintained for sixteen subculture cycles were treated with 15 or 150 mg $L^{-1}$ sodium acetate (NaA15, NaA150), and 15 or 150 mg $L^{-1}$ mevalonic acid (MVA15, MVA150). * Indicates mean 20E content is significantly different from control hairy root cultures maintained for four subculture cycles ($p < 0.05$).

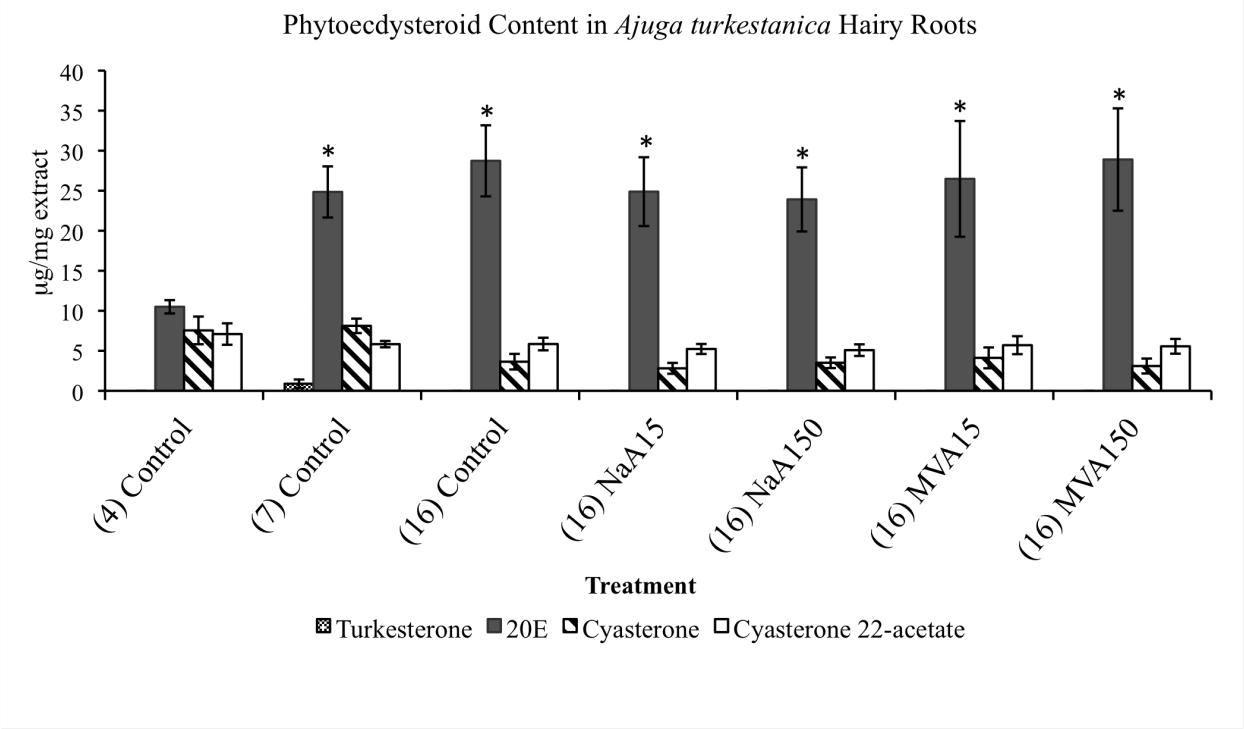


Figure 2.4 Yield of (a) 20-hydroxyecdysone (20E) and (b) total phytoecdysteroids per liter media for hairy root cultures and *in vitro* shoot cultures of *Ajuga turkestanica*. Hairy roots were maintained for four, seven or sixteen subculture cycles and treated with 15 or 150 mg $L^{-1}$ sodium acetate (NaA15, NaA150), 15 or 150 mg $L^{-1}$ mevalonic acid (MVA15, MVA150) or 125 μM methyl jasmonate (MJA125). * Indicates mean yields significantly different from control hairy root cultures maintained for four subculture cycles ($p < 0.05$).

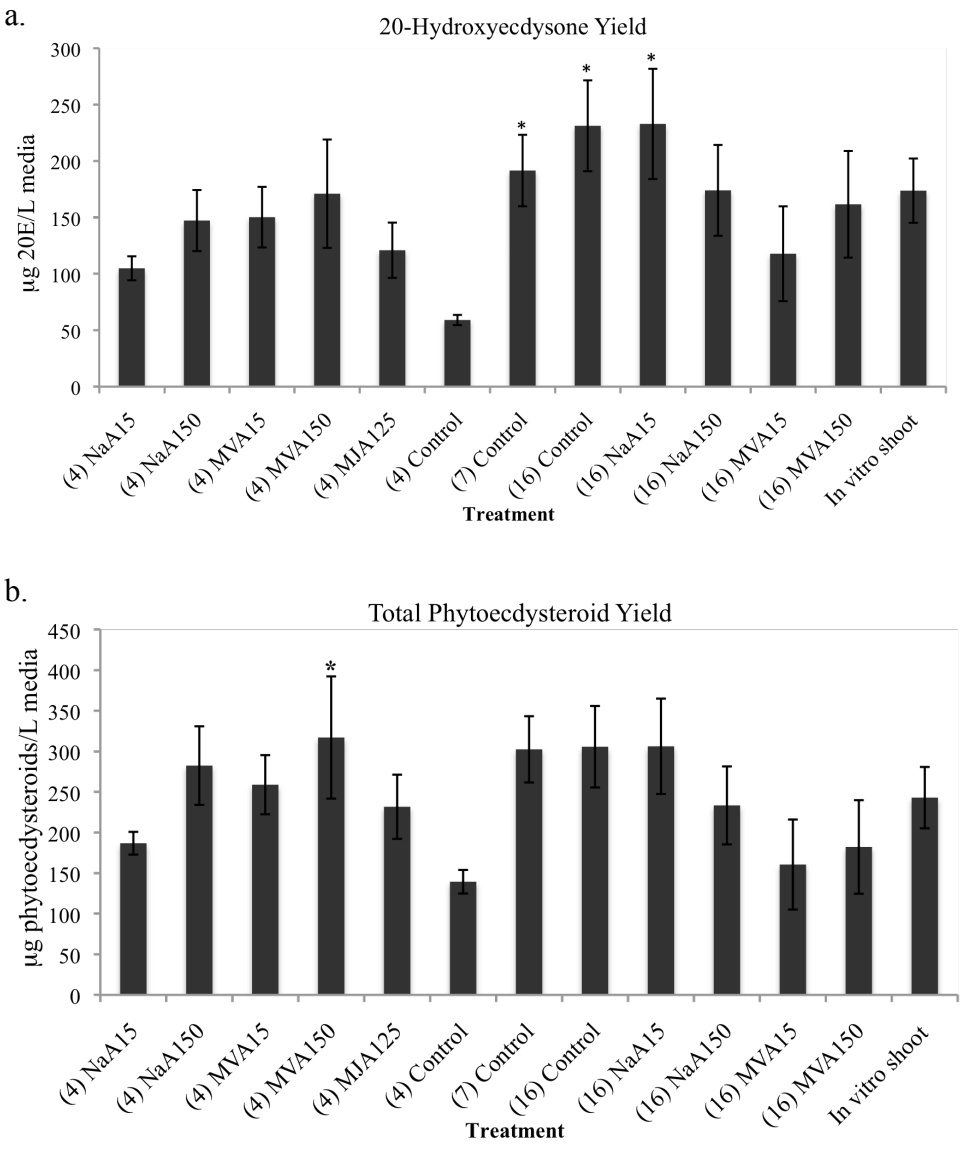


Figure 2.5 Detection of cucumopine in hairy root extracts of *A. turkestanica* transformed by *A. rhizogenes* K599 following paper electrophoresis. C, Cucumopine standard; HR, *A. turkestanica* hairy root; S, *A. turkestanica* shoot.

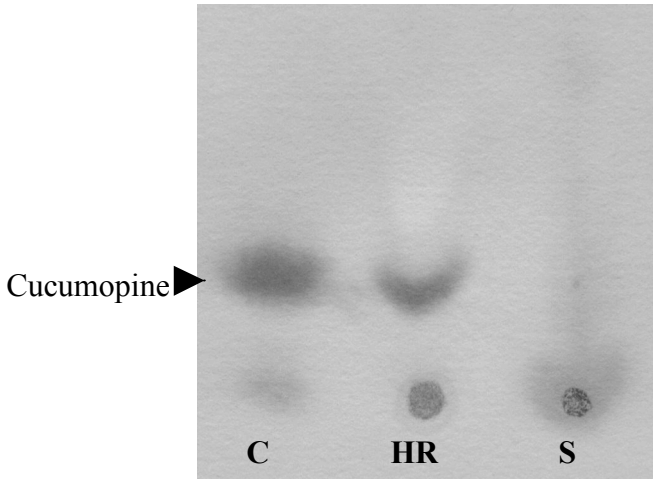



Figure 2.6 Polymerase chain reaction analysis of hairy root DNA amplifying at 532-bp fragment of the *rolC* gene. 1, *A. rhizogenes* plasmid; 2, hairy root DNA; 3, hairy root DNA; 4, root DNA; 5, shoot DNA; 6, negative control (no DNA).

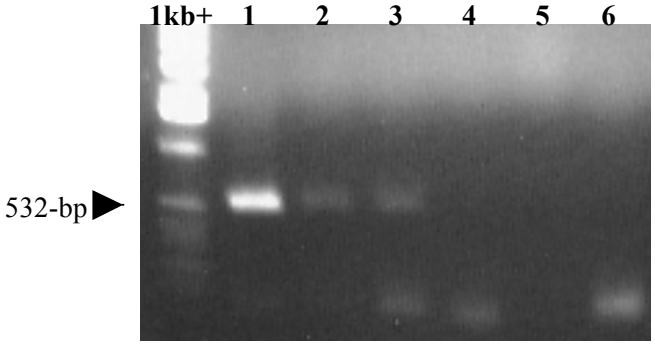


Figure 2.7 HPLC chromatogram of *A. turkestanica* (a) cell suspension culture, (b) hairy root culture and (c) wild-harvested shoot extracts. Peaks correspond to: 1, turkesterone; 2, 20-hydroxyecdysone; 3, cyasterone; 4, cyasterone 22-acetate.

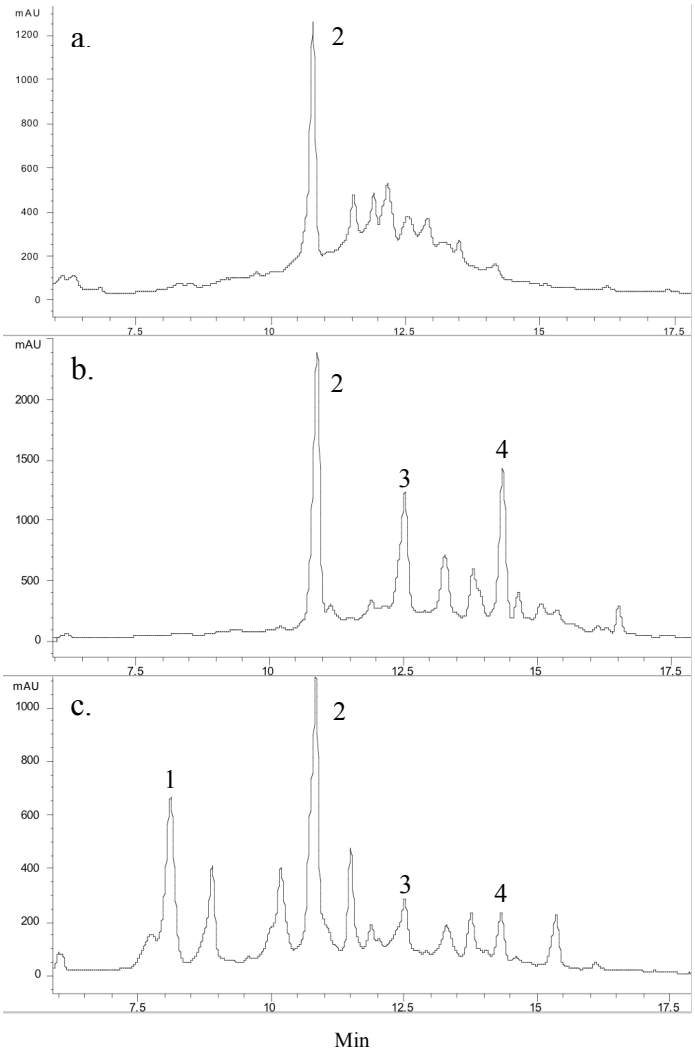
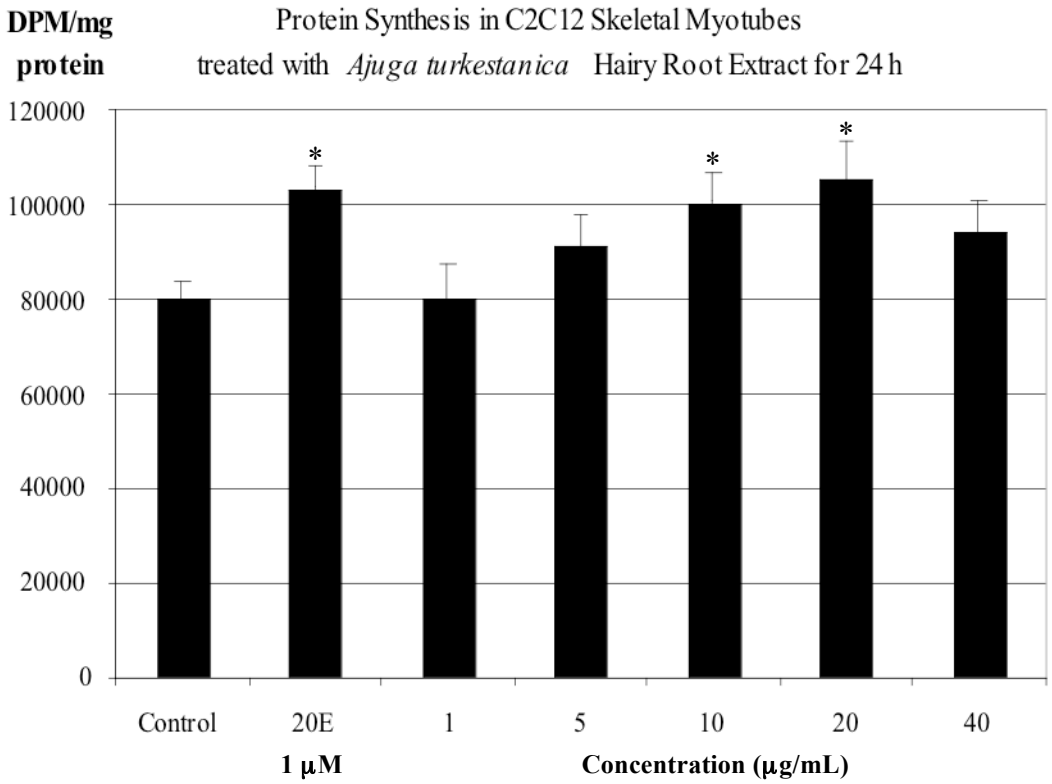


Figure 2.8 [$^3$H] Leucine incorporation in C2C12 myotubes treated with *A. turkestanica* hairy root extract and 20-hydroxyecdysone. Differentiated myotubes were treated for 24 h with increasing concentrations of *A. turkestanica* hairy root extract, 20-hydroxyecdysone (20E) or vehicle. Decays per minute (DPM) were normalized by total protein. The data represent the mean values ± standard error of four samples, each performed in triplicate. * Indicates $p < 0.05$ compared with control (Student's *t* test).



# CHAPTER 3

# VARIATION OF PHYTOECDYSTEROID ACCUMULATION IN SEEDS AND SHOOTS OF *SPINACIA OLERACEA* L. ACCESSIONS


## Abstract

Spinach (*Spinacea oleracea* L.) is a valuable agricultural crop that accumulates phytoecdysteroids, polyhydroxylated steroidal compounds, which may play a role in plant defense and have purported health benefits for human consumers. Thus, improved agricultural production of spinach as well as delivery of more health-protective produce to the marketplace may be achieved through the development of high-phytoecdysteroid accumulating varieties. In this study, phytoecdysteroid accumulation was measured in seeds and shoots of fifteen spinach accessions to determine whether phytecdysteroid levels vary with spinach varieties and whether whether seed content could reliably predict relative levels in the edible foliage. Additionally, spinach phytosterols, precursors to phytoecdysteroids, were identified to determine potential points of regulation in phytoecdysteroid biosynthesis. Significant variations in phytoecdysteroid levels between accessions were observed ($p < 0.05$). The significant variation in phytoecdysteroid content between spinach accessions suggests that there is potential for genetic manipulation, perhaps at the level of phytosterol biosynthesis, and that traditional breeding strategies or genetic engineering may increase phytoecdysteroid levels in spinach.


## Introduction

Spinach (*Spinacea oleracea* L.) is a valuable agricultural crop that accumulates phytoecdysteroids, and a model plant for the study of phytoecdysteroid biosynthesis (Grebenok

et al., 1991). The main phytoecdysteroids that accumulate in spinach are 20-hydroxyecdysone (20E) and polypodine B (Grebenok et al., 1991; Grebenok et al., 1994). Phytoecdysteroids are polyhydroxylated triterpenoids biosynthesized from phytosterols through the mevalonic acid pathway (Adler and Grebenok, 1999). Although the role of phytoecdysteroids in plants has not been established, due in part to their dynamic and polar characteristics, they have been hypothesized to function as a long-distance transport water-soluble form of non-polar phytosterols and as plant defense compounds against non-adapted insects (Grebenok and Adler, 1993; Schmelz et al., 1999; Schmelz et al., 2000).

In spinach, phytoecdysteroids are actively biosynthesized in older leaf sets, transported to newly developing apical parts of the plant, including flowers, seeds and young leaves and accumulate at levels (upwards of 100 μg/g fresh weight leaves) that are physiologically-capable of deterring non-adapted insects (Bakrim et al., 2008; Adler and Grebenok, 1995; Kubo and Klocke, 1983; Grebenok et al., 1991). Ingestion of phytoecdysteroids by insects and nematodes caused premature molting and death, as the analogous structures produced by insects, ecdysteroids, are arthropod molting hormones (Soriano et al., 2004; Nakagawa and Henrich, 2009). The levels of dietary phytoecdysteroids that cause physiological effects in insect range from 0.03 to 100 mg phytoecdysteroid/kg fresh weight, dependant on the particular insect species, stage of development and specific physiological response measured (Adler and Grebenok, 1999; Jones and Firn, 1978; Soriano et al., 2004). Additionally, after mechanical damage, or application of the plant-defense signaling compound methyl jasmonate, to spinach roots, the roots responded with a rapid increase in phytoecdysteroid production (Schmelz et al., 1998). In subsequent trials by Schmelz et al. (2002), spinach roots with increased phytoecdysteroids levels (25 to 50 μg/g wet mass) deterred *Bradysia impatiens* (dark winged

fungus gnat) feeding and had lower levels of damage compared to untreated control roots with lower levels of phytoecdysteroids. Taken together, these studies make a firm case for phytoecdysteroids to play a role in plant defense. Thus, improved agricultural yields may be gained through the development or identification of high-phytoecdysteroid accumulating spinach genotypes that may confer an enhanced resistance to non-adapted invertebrates.

Phytoecdysteroids have also been attributed with various pharmacological properties in mammals including enhanced physical performance and stimulation of growth (Bathori et al., 2008). The purported bioactive components in medicinal plants such as *Ajuga turkestanica* and *Rhaponticum carthamoides* are phyotoecdysteroids, which accumulate in high levels (up to 0.5% and 1.2% 20E of dried aerial tissue, respectively) in these perennial species (Syrov 2008; Gorelick-Feldman et al., 2008; Kokoska, 2009). Phytoecdysteroid concentrations in spinach, on the other hand, are generally below the purported pharmacologically active levels achieved through normal dietary consumption by mammals; the average phytoecdysteroid content in spinach foliage (40 μg/g dry weight) was reported to be over a 100-fold less compared to *A. turkestanica* (5 mg/g dried aerial portion) (Gorelick-Feldman et al., 2008). However, health benefits from dietary consumption of phytoecdysteroids may potentially be achieved through the development of high-phytoecdysteroid accumulating spinach cultivars.

Phytosterols, precursors to phytoecdysteroids, also contribute to total phytosterol consumption to confer health benefits such as lowering serum cholesterol levels and protection against certain cancers (Piironen et al., 2003; Jones and AbuMweis, 2009). Phytosterols, in general, provide membrane stability and rigidity in plants (Moreau et al., 2002). Levels of total phytosterols increased or decreased in coordination with phytoecdysteroid levels during the growth and development of spinach (Grebenok et al., 1991). In an excised leaf assay, [2-$^{14}$C]

mevalonic acid was incorporated into lathosterol prior to incorporation into 20E and other phytoecdysteroids (Grebenok and Adler, 1993). Additionally, inhibition of endogenous phytoecdysteroid production by elevated levels of phytoecdysteroid intermediates and end products, prevented mevalonic acid incorporation into lathosterol (Grebenok et al., 1994; Grebenok et al., 1996; Bakrim et al., 2008). Inhibition of carbon flux into lathosterol suggests that phytoecdysteroid regulation may also occur prior to the final hydroxylation steps, impacting carbon allocation early in the phytoecdysteroid pathway, such as during phytosterol biosynthesis.

Phytoecdysteroid content has been evaluated among numerous species within plant genera to investigate their chemotaxonomic applications, however, to the best of our knowledge, levels of phytoecdysteroid accumulation among different varieties within the same species have not been investigated. Among plant species, the production and accumulation of phytoecdysteroids is not consistent, allowing the presence and levels of certain phytoecdysteroids to serve as chemotaxonomic markers (Dinan et al., 2001c; Zibareva et al., 2003). However, within a plant species the phytoecdysteroid content in seeds may provide a gauge of the foliar content for the plant (Dinan et al., 2001b). For *Chenopodium* species, if phytoecdysteroids were detected in the seed, they were consistently detected in the germinated plant foliage (Dinan, 1992). In an evaluation of 180 randomly selected plant species, phytoecdysteroids were more readily detected in leaves than in seeds, however, the highest levels of phytoecdysteroids were detected only for species which were also positive for phytoecdysteroids in the seeds (Dinan et al., 2001b). Similar relationships between secondary compound accumulation in seed and vegetative tissues have been established for aliphatic glucosinolates, which were correlated in 35 different *Arabidopsis* ecotypes (Kliebenstein et al., 2001).

The aims of this study were to measure phytoecdysteroid accumulation in seeds and shoots of various spinach accessions and evaluate whether or not seed content could reliably predict relative levels in the edible foliage. Establishing a correlation between levels of phytoecdysteroid in the seed, and corresponding levels in spinach foliage would expedite the screening of germplasm for higher phytoecdysteroid content, and subsequent breeding trials. Identification of differences in phytoecdysteroid content between genotypes could be used to determine whether greater levels of phytoecdysteroid accumulation result in enhanced resistance to herbivory and to further investigate genetic regulation of phytoecdysteroid biosynthesis. In addition, a metabolic profile of phytoecdysteroid precursors, phytosterols, could help to elucidate a network of potential regulatory points of *de novo* phytoecdysteroid biosynthesis.

## Materials and Methods

### *Plant material*

Fifteen *S. oleracea* accessions (Table 3.1) were obtained through the USDA, ARS, National Genetic Resources Program (www.ars-grin.gov). Seeds were surface sterilized by immersing for 15-20 min in 10% sodium hypochlorite, followed by a 70% ethanol rinse and immersion in a plant preservative mixture (PPM; Plant Cell Technology, Washington DC) for 4-8 hours. Water purified by filtering dd$H_2O$ through a Barnstead NANOpure II ultrafiltration system (>18 mega-ohms) was used for all experiments. Seeds were germinated on sterile moist filter paper in Petri dishes before transferring to culture vessels (Magenta® GA, Phytotechnology Labs, Shawnee Mission, KS) containing 45 mL MS media (Murashige and Skoog, 1962) supplemented with 0.1 g $L^{-1}$ myoinositol, 30 g $L^{-1}$ sucrose and rose vitamins (Rogers and Smith, 1992). Plants were grown *in vitro* at 25 °C on a short-day light cycle (8L:16D) with 120 μmol

$m^{-2}$ $s^{-1}$ irradiance from cool white fluorescent lights. Spinach shoots, whole aerial portion of the plantlets, (5-9 individual plants) from each accession were harvested when the sixth true leaf developed, 21-36 days after germination (Table 3.1), and frozen at -80 ºC prior to lyophilization. Dried plant tissue was ground to a powder with a glass rod in a 20 mL vial and a 25 mg subsample was extracted as described below.

*Extraction*

Spinach seeds from each accession were sampled by measuring approximately 100 mg of seed, which were ground with a mortar and pestle. A 25 mg subsample of ground seed was extracted in 1 mL of methanol for an hour at 55 ºC, and the process was repeated two more times for each sample. Methanolic extracts were pooled (3 mL) and water (1.3 ml) was added and partitioned with 2 mL hexane. The upper hexane layer was removed and the extract was dried down by rotary evaporation and stored at -20 °C (Dinan et al., 2001a). All samples were re-dissolved in 775 μL of 70% methanol, filtered through 0.45 nm nylon filters and 30 μL was injected for HPLC analysis.

*Phytoecdysteroid analysis*

A commercial standard of 20E (Bosche Scientific, New Brunswick, NJ) was dissolved in 70% methanol and used for quantification by HPLC at concentrations of 250, 125 and 62.5 μg $mL^{-1}$ with 5 μL injection volumes. Phytoecdysteroid content was measured as 20E equivalents due to co-elution of 20E and polypodine B. Analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies, Inc., Wilmington, DE) with autosampler, DAD (242 nm) and Kromasil 100-5$C_{18}$ normal-phase column (250 x 5 μM x 4.6 mm; Eka chemicals, Brewster,

NY). The mobile phase solvents consisted of 0.1% trifluoroacetic acid (TFA, Acros Organics, Fair Lawn, NJ) in water (A) and 0.1% TFA in 90% acetonitrile (B). The system was eluted with solvent A from 0-30 min, then 70% solvent B from 30-40 min, followed by 100% solvent B from 40-50 min and 100% solvent A from 50-60 min with 0.5 mL $min^{-1}$ flow rate.

*Sterol extraction*

Phytosterols were extracted from spinach as described by Piironen et al., (2002) using a method that measured total phytosterol content, which includes free phytosterols and bound phytosterol conjugates. Dried spinach shoots were pooled into three groups (approximately 140 mg), containing five individual plants per group, and extracted with 20 mL of hexane-diethyl ether (1:1) by moderately shaking for 10 min using a vortex (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY). The organic layer was separated by centrifuging for 10 min at 2600 rpm then transferred to a round-bottom flask and evaporated to dryness in a rotary evaporator at 40 °C. For saponification, 8 mL of absolute ethanol was added to the dry residue and transferred to a 50 mL Falcon tube. Next, 0.5 mL of saturated aqueous KOH solution was added and vortexed for 10 s prior to placing the tube in a shaking water bath (80 – 85 °C) for 30 min. After the sample was cooled, 12 mL of water and 20 mL of cyclohexane were added followed by shaking for 10 min to extract unsaponifiable lipids. An aliquot of 15 mL of the organic layer was transferred to a round-bottom flask, rotary evaporated, then re-dissolved in 1 mL of chloroform. A C18 cartridge (Waters Corporation, Milford, MA) was activated by 5 mL of methanol and 5 mL of water. The chloroform solution was eluted by gravity flow for a couple of minutes and pressed through with a syringe to purify the unsaponifiable fraction. The sterol fraction was eluted with 15 mL of methanol-chloroform (5:95) and rotary evaporated to dryness.

*Sterol sample processing for GC-MS*

Sterol extraction samples were prepared and analyzed in triplicate by the Metabolomics Center in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana Champaign and were derivatized in two steps as follows: 60 min at 50 ºC with 80 µl of methoxyamine hydrochloride in pyridine (20 mg $ml^{-1}$; Sigma, St. Louis, MO) followed by 60 min treatment at 50 ºC with 80 µl N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Thermo Fisher Scientific Inc., Pittsburgh, PA). Sample volume of 5 µL was injected in splitless mode. The GC-MS system consisted of an Agilent 7890A (Agilent Inc, Palo Alto, CA) gas chromatograph, an Agilent 5975C mass selective detector and Agilent 7683B autosampler. Gas chromatography was performed on a 60 m HP-5MS column with 0.25 mm inner diameter and 0.25 µm film thickness (Agilent) with an injection temperature of 250 ºC, the interface set to 250 ºC, and the ion source adjusted to 230 ºC. The helium carrier gas was set at a constant flow rate of 1.5 ml $min^{-1}$. The temperature program was 5-min isothermal heating at 70 ºC, followed by an oven temperature increase of 5 ºC $min^{-1}$ to 310 ºC and a final 20 min at 310 ºC. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in the m/z 50-800 scanning range.

The spectra of all chromatogram peaks were compared with electron impact mass spectrum libraries: NIST08 (NIST, Gaithersburg, MD, USA), WILEY08 (Palisade Corporation, Ithaca, NY), and the custom library. The chromatograms and mass spectra were evaluated using the MSD ChemStation (Agilent) and AMDIS (NIST, Gaithersburg, MD) programs. The retention time and mass spectra were implemented within the AMDIS method formats. To allow comparison between samples, all data were normalized to the internal standard, lathosterol (Sigma, St. Louis, MO), which does not accumulate in spinach shoots, at 1 mg $ml^{-1}$ in each

chromatogram. Relative concentrations were calculated as the ratio of the target peak area divided by the lathosterol peak area, over the dry weight of each pooled sample.

*Statistical analysis*

Statistically significant differences in mean phytoecdysteroid content between seeds or shoots of spinach accessions were determined by analysis of variance (ANOVA) using Proc GLM and Fisher's LSD using SAS version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Pearson's correlation coefficients between mean seed and shoot phytoecdysteroid accumulation were calculated by the Proc CORR function. A p-value $< 0.05$ was considered statistically significant.

**Results and Discussion**

Spinach seeds were obtained from the USDA germplasm repository and are representative varieties from a variety of geographic locations including Turkey, Hungary, the Netherlands and the USA. Both genetic diversity and the plant's ecological environment may condition phytoecdysteroid levels (Volodin et al., 2002). There were over 300 accessions of *S. oleracea* in the germplasm repository, and 15 accessions were selected for this study based on rankings of insect resistant varieties (Mou, 2008), as well as seed size, and flowering and bolting times available on the ARS GRIN database (Table 3.1). Accumulation of phytoecdysteroids, measured as 20E equivalents in seeds and shoots of *S. oleracea,* varied significantly between accessions on a per seed weight, per seed and per dry weight shoot basis (Table 3.2). Levels of phytoecdysteroids from spinach grown *in vitro* ranged from 0.7-1.2 mg/g dry weight shoot, 37.6 - 77.4 μg/g fresh weight shoot, and 19.9 - 44.1 μg per plant. In seeds, the phytoecdysteroid

content ranged from 3.2 - 9.6 μg per seed and 0.5 - 1.1 μg/mg seed. Accessions PI 531456 ("Popey") and PI 606707 ("America") had the highest and lowest phytoecdysteroid content per shoot, respectively, and PI 92513 ("Bloomsdale Long-Standing") and PI 606707 ("America"), had the highest and lowest phytoecdysteroid content per dry weight of shoots, respectively. Accession PI 92513 also had the highest phytoecdysteroid content per gram seed and per seed. These values are within the range previously reported for spinach seeds and foliage (Grebenok et al., 1991; Dinan, 1995). The significant differences in phytoecdysteroid accumulation between varieties illustrate the potential for further manipulation of phytoecdysteroid levels. The selection of high or low-accumulating varieties would be of use for breeding research or studying regulation of biosynthesis.

A significant moderate correlation was found between phytoecdysteroid accumulation per weight of seeds and phytoecdysteroid accumulation per dry weight of shoots ($r = 0.52$, $p = 0.04$, $n = 15$) and between phytoecdysteroid accumulation per seed and phytoecdysteroid accumulation per dry weight of shoots ($r = 0.58$, $p = 0.02$, $n = 15$) among the fifteen spinach accessions (Figures 3.1a and b). However, the significant correlation was driven by an outlier and when this data point was removed, the Pearson's correlation coefficients were no longer significant. These results do not suggest that estimation of phytoecdysteroid levels in shoots may be achieved by measuring levels in the seeds.

Among the fifteen *S. oleracea* accessions selected for screening were three genotypes that demonstrated leafminer resistance and two genotypes that demonstrated high susceptibility (Mou, 2008). Leafminer (*Liriomyza* spp.) is a major agricultural pest around the world, damaging vegetable crops such as spinach (Mou, 2008; Parrella, 1987). As phytoecdysteroids demonstrated strong anti-feedant effects and disrupted insect development when ingested, higher

inherent levels of 20E may be expected in genotypes that demonstrate enhanced insect resistance (Adler and Grebenok 1999; Jones and Firn, 1978; Robbins et al., 1970; Singh and Russell, 1980; Mele et al., 1992). In cage and field tests, *S. oleracea* accessions PI 274065, PI 174385 and PI 169673 had the fewest mines or lowest sting density produced by leafminers and PI 175312 and PI 433208 had high sting density in field trials (Mou, 2008). However, the level of 20E in these genotypes did not reflect the hypothesis that high levels of phytoecdysteroids may account for greater leafminer resistance. An explanation could be that the leafminers in the Mou (2008) study were adapted to phytoecdysteroids and would not deter the leafminer species or that the phytoecdysteroid content in these varieties were below anti-feedant levels.

The greatest level of phytoecdysteroid accumulation was in accession PI 92513 and therefore this accession was considered to be a suitable candidate for investigation of phytoecdysteroid precursors. Phytosterols and their relative levels are presented in Table 3.3. Spinasterol was the predominant phytosterol, making up an average of 79.8% of total phytosterols identified, which was consistent with previous studies (Grebenok and Adler, 1993; Piironen et al., 2003). Other common phytosterols accumulated a lower levels, including 5-dihydroergosterol (6.4%), 22-dihydrospinasterol (4.6%), stigmasterol (2.4%), cholesterol (2.2%). Although, the formation of lathosterol was previously identified as an intermediate in 20E biosynthesis using radiolabeled [2-$^{14}$C] mevalonic acid (Grebenok and Adler, 1993), lathosterol was not detected in this study. However, Grebenok and Adler (1993) also reported that lathosterol did not accumulate and instead was subsequently metabolized, which likely explains why lathosterol was not identified in this analysis.

Cycloartenol (0.8% of total phytosterols) and lanosterol (0.6% of total phytosterols) were detected in spinach. In higher plants, there are two biosynthetic pathways for phytosterol

biosynthesis, via cycloartenol or via lanosterol (Benveniste et al., 2004). Lanosterol synthase genes were only recently identified in dicotyledonous plants and the expression of the lanosterol synthase gene was reportedly induced by jasmonates, suggesting that secondary metabolites produced through the lanosterol pathway may contribute to plant defense (Suzuki et al., 2006; Ohyama et al., 2009). The influence of lanosterol and other phytosterols on phytoecdysteroid production is an interesting avenue of investigation. The phytosterol biosynthetic pathway has been extensively researched due to their importance in plants and benefits to human health (Moreau et al, 2002). Thus, molecular tools, such as phytosterol biosynthesis inhibitors and gene over-expression or knockout constructs, are accessible means of evaluating phytosterol influence on phytoecdyteroid biosynthesis.

In this study, phytoecdysteroids were detected in all seeds and shoots of *S. oleracea* accessions selected for this study. Significant variation in phytoecdysteroid content between spinach accessions suggests potential for molecular biotechnology or conventional breeding to enhance levels of phytoecdysteroid accumulation. Additionally, manipulation of phytosterol biosynthesis may help to elucidate regulation of phytoecdysteroid biosynthesis. Furthermore, the ability to manipulate phytoecdysteroid levels may be used to determine their effectiveness on insect deterrence and potential to improve plant fitness and yield.

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**Tables and Figures**

Table 3.1 Accessions of *Spinacia oleracea* and their corresponding names, geographic origins, average seed weight +/- SEM, days to harvest +/- SEM (day of germinated seed transplanted to media until the development of six true leaves), percentages of plants that flowered after 28 days with 16 hours of light, and selection criteria traits.

| Accession[#] | Plant name[#] | Origin[#] | Seed wt (mg) | Days to harvest | Percent flowering[#] | Traits |
|---|---|---|---|---|---|---|
| Ames 20169 | Hu shi yuan ye bo cai | China | 9.1 +/- 0.5 | 21 +/- 0 | 73 | |
| NSL 6084 | Giant thick leaved/Nobel | US, California | 6.4 +/- 0.7 | 24 +/- 3 | 22 | |
| NSL 6092 | Viking | US, New York | 13.0 +/- 0.7 | 32 +/- 3 | 33 | Very late to flower[#] |
| NSL 92513 | Bloomsdale long standing | US, Oregon | 9.2 +/- 0.7 | 25 +/- 2 | 7 | |
| PI 169673 | Harlan 1788 | Turkey, Aydin | 6.7 +/- 0.1 | 30 +/- 1 | 22 | Fewest mines[^] |
| PI 173130 | Harlan 8557 | Turkey, Malatya | 8.2 +/- 0.5 | 30 +/- 4 | 93 | |
| PI 174385 | CGN 9504 | Turkey, Diyarbakir | 7.7 +/- 0.7 | 27 +/- 2 | 45 | Fewest mines[^] |
| PI 175312 | Palak | India | 8.9 +/- 1.3 | 27 +/- 3 | 59 | High sting density & number of mines[^] |
| PI 179589 | Giant spinach | Belgium | 7.0 +/- 0.1 | 36 +/- 4 | 75 | |
| PI 274065 | Wisemona | England/Germany | 8.3 +/- 0.4 | 34 +/- 4 | 0 | Lowest sting density[^] |
| PI 433208 | Xiao Ye | China | 6.0 +/- 1.1 | 28 +/- 2 | 47 | High sting density & number of mines[^] |
| PI 445782 | Shami | Syria | 9.3 +/- 0.6 | 26 +/- 2 | 100 | |
| PI 499372 | Ispolinskij | Former Soviet Union | 6.8 +/- 0.5 | 24 +/- 2 | 38 | |
| PI 531456 | Popey | Hungary | 9.8 +/- 0.2 | 26 +/- 2 | 28 | |
| PI 606707 | America | Netherlands | 5.7 +/- 0.4 | 35 +/- 2 | 0 | Matures later than other Bloomsdale varieties & extremely slow bolting[#] |

[#]ARS National Plant Germplasm System Germplasm Resources Information Network

[^] Mou, 2008

Table 3.2 Phytoecdysteroid content (20-hydroxyecdysone equivalent) in shoots and seeds of fifteen accessions of *Spinacia oleracea* reported as mean +/- SEM followed by letters indicating least significant difference groupings ($p < 0.05$). Letters that are the same within each column are not significantly different between accessions.

| | **Phytoecdysteroid Content** | | | |
|---|---|---|---|---|
| ***S. oleracea*** | **Shoots** | | **Seeds** | |
| **Accession** | µg/shoot | µg/mg DW shoot | µg/seed | µg/mg seed |
| Ames 20169 | 20.9 +/- 3.6 c | 0.7 +/- 0.2 b | 5.2 +/- 0.7 bcd | 0.6 +/- 0.0 bc |
| NSL 6084 | 40.3 +/- 4.0 ab | 0.9 +/- 0.1 ab | 3.2 +/- 0.3 d | 0.5 +/- 0.0 c |
| NSL 6092 | 28.4 +/- 3.7 bc | 0.8 +/- 0.1 ab | 6.0 +/- 0.5 b | 0.5 +/- 0.0 c |
| NSL 92513 | 33.7 +/- 5.6 abc | 1.2 +/- 0.2 a | 9.6 +/- 2.3 a | 1.1 +/- 0.3 a |
| PI 169673 | 38.5 +/- 5.2 ab | 0.9 +/- 0.0 ab | 3.4 +/- 0.4 d | 0.5 +/- 0.1 c |
| PI 173130 | 34.2 +/- 6.0 abc | 0.8 +/- 0.1 b | 4.3 +/- 0.3 bcd | 0.5 +/- 0.1 bc |
| PI 174385 | 31.9 +/- 5.0 abc | 0.8 +/- 0.0 b | 4.2 +/- 0.7 bcd | 0.5 +/- 0.0 bc |
| PI 175312 | 22.6 +/- 3.6 c | 0.8 +/- 0.1 b | 4.5 +/- 0.4 bcd | 0.5 +/- 0.1 bc |
| PI 179589 | 31.3 +/- 4.3 abc | 0.9 +/- 0.1 ab | 3.7 +/- 0.2 cd | 0.5 +/- 0.0 bc |
| PI 274065 | 21.8 +/- 3.9 c | 1.0 +/- 0.2 ab | 5.1 +/- 0.5 bcd | 0.6 +/- 0.0 bc |
| PI 433208 | 38.7 +/- 1.8 ab | 0.9 +/- 0.1 ab | 4.2 +/- 0.4 bcd | 0.6 +/- 0.1 bc |
| PI 445782 | 20.4 +/- 5.5 c | 0.9 +/- 0.2 ab | 4.6 +/- 0.2 bcd | 0.5 +/- 0.0 bc |
| PI 499372 | 31.4 +/- 3.2 abc | 0.8 +/- 0.1 b | 3.7 +/- 0.1 cd | 0.5 +/- 0.1 bc |
| PI 531456 | 44.1 +/- 9.3 a | 0.9 +/- 0.1 ab | 5.5 +/- 0.4 bc | 0.6 +/- 0.0 bc |
| PI 606707 | 19.9 +/- 1.1 c | 0.7 +/- 0.1 b | 4.5 +/- 0.5 bcd | 0.8 +/- 0.0 b |

Figure 3.1 Scatterplots representing the relationship between mean phytoecdysteroid accumulation between the seeds and shoots. The 95% (---) and 99% (—) confidence interval is drawn for reference. Significant correlations were determined using Pearson's correlation procedure. (A) Scatterplot of µg phytoecdysteroid per mg seed by µg phytoecdysteroid per mg dry weight (DW) shoot ($r = 0.52$, $p = 0.04$, $n = 15$). (B) Scatterplot of µg phytoecdysteroid per seed by µg phytoecdysteroid per mg DW shoot ($r = 0.58$, $p = 0.02$, $n = 15$).

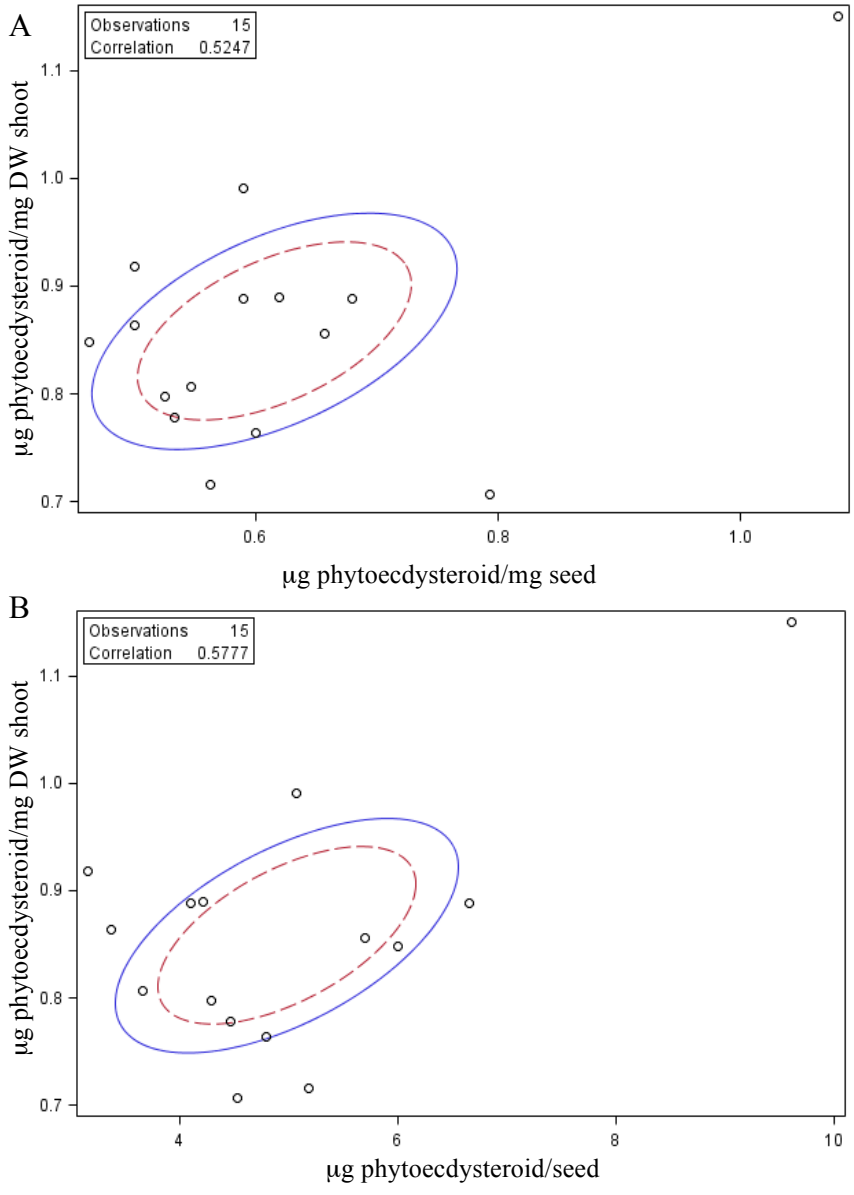

Table 3.3 Spinach phytosterol content reported as relative concentration of phytosterols per gram dry weight spinach shoots and the standard error for each phytosterol. Concentrations are the average of three pooled samples standardized relative to lathosterol. The percentages of each phytosterol to total phytosterols are also presented.

| Phytosterol | Relative concentration /g dry weight | **%** |
|---|---|---|
| (24Z)-3-Hydroxystigmasta-7,24(28)-diene | 116 +/- 15 | 1.7 |
| 22-Dihydrospinasterol | 313 +/- 34 | 4.6 |
| 24-Methylenecycloartanol | 12 +/- 2 | 0.2 |
| 3-Hydroxycholest-8(14)-ene | 14 +/- 1 | 0.2 |
| 3-Hydroxyergost-8(14)-ene | 26 +/- 1 | 0.4 |
| 5-Dihydroergosterol | 426 +/- 44 | 6.3 |
| Cholesterol | 151 +/- 11 | 2.2 |
| Cycloartenol | 57 +/- 5 | 0.8 |
| Lanosterol | 43 +/- 1 | 0.6 |
| Sitosterol | 43 +/- 3 | 0.6 |
| Spinasterol | 5394 +/- 220 | 79.8 |
| Stigmasterol | 160 +/- 10 | 2.4 |
| Total | | 100 |

# CHAPTER 4

# PHYTOCHEMICALS AND HUMAN HEALTH: BEYOND CHRONIC DISEASE PREVENTION*


## Abstract

Adaptogen is a term for phytochemicals and phytochemical mixtures from natural plant extracts that exert nonspecific, beneficial responses to stress in the body, and have a normalizing influence on body metabolism and physiology (homeostasis). Adaptogens have been used to improve performance, maintain human health and prevent chronic diseases, however, rigorous science has yet to provide conclusive evidence of potent bioactivities. In this review, the multi-target therapeutics of adaptogens, analogous to long-standing practices in traditional medicine, are contrasted with the single-target approach of modern pharmaceuticals. Considerations and caveats of methods of phytochemical administration and physiological evaluation in animal models are discussed, highlighting the need to minimize uncontrolled stresses. Focus is directed towards evaluating purported performance-enhancing capabilities of adaptogens and advantages and disadvantages of various exercise modalities are also discussed. The evaluation of the multifaceted biological properties attributed to adaptogens has been a challenge, however, recent scientific advances such as in "omic" technologies (genomic, proteomic and metabolomic), provide comprehensive, sensitive, and reproducible data that will help to elucidate multifarious biological interactions. This review evaluates the concept of adaptogen and corresponding strategies that gauge their multi-targeted therapeutic capabilities.


* This is a review chapter examining the concept adaptogens and the evaluation of adaptogenic compounds in animal models.

## Introduction

The adaptogenic benefits of phytochemicals are the recognized abilities of certain natural extracts to enhance performance, relieve stress, depression, or fatigue, and boost non-specific resistance in the human consumer. Adaptogenic plants have long been an ingredient in folk and traditional medicines, however their mechanisms of action have seldom been pinpointed, nor has science, until very recently, had the capacity and the fine precision analytical instrumentation needed to elucidate their metabolism-balancing activities. Because adaptogens can have diffuse effects on the body, and are assumed to interact with multiple human therapeutic targets, their pharmacodynamic activities can be challenging to quantify, and there have been difficulties attributing adaptogenic activity to discrete phytochemical groups. A range of modern *in vitro* and *in vivo* bioassays has been developed in order to gauge adaptogenic properties, and a few caveats concerning various approaches are discussed. The diversity of phenotypic effects that can be impacted by adaptogens mandates a comprehensive, multi-faceted approach to successfully gauge adaptogenic benefits *in vivo*.

Adaptogen is a term used to describe a natural product which provides restorative or rejuvenating benefits to the body of animals (including humans) who ingest it. The term adaptogen was originally coined in 1947, but the functional definition was first recorded in 1968 by a Soviet scientist, Dr. I. I. Brekhman: Adaptogens are non-toxic substances that produce nonspecific, positive responses to stress in the body, and have a normalizing influence on body metabolism and physiology (homeostasis). In addition to use as normalizing treatments, adaptogens have been used as performance enhancers. Numerous studies have evaluated these performance-enhancing properties through measures of improved endurance, strength and recovery time, maximum oxygen uptake, muscle mass accretion and reduction in body fat mass

(Bucci, 2000). Exercise is a form of stress on the body's mobile functions, and adaptogens are valued as a means to counter the negative ramifications of extreme physical exertion. Human and animal studies have induced stresses such as noise, high-altitude and mental stress (achieved by requiring subjects to perform complex psychomotor tests), and have monitored the ability of natural adaptogens to alleviate negative impacts on performance (Davydov and Krikorian 2000).

While a vast array of plants and plant-derived extracts have been identified and used to combat chronic disease conditions, there are comparatively few medicinal plants that can be cited in the adaptogen category. Interactions between different phytochemical groups that co-occur within a plant are expected to result in the adaptogenic benefits to the consumer, and as previously noted, the diffuse nature of the bioactive effects confound assignments of activity to certain phytochemical constituents. Adaptogenic compounds are routinely used against a plethora of diverse disorders that, by the gauges of modern medicine, seem to have no pathophysiological connections (Govindarajan et al., 2005). For these reasons, adaptogens have defied standardized classification into bioactive groups, however, a simple classification breaking adaptogens into three major groups (Triterpenes, Phenylpropanes, and Oxylipins) has been proposed by Panossian (2003) and others. The triterpenes include phytochemicals produced through the mevalonate pathway, including phytoecdysteroids, phytosterols, and saponins. The shikimate pathway produces the phenylpropane group of adaptogenic compounds, which includes lignans and flavonoids. Finally, the oxylipins are produced via the acetate pathway; the hydroxylated fatty acids fall into this group. Many adaptogens contain polysaccharides that together with the components above, stimulate immune responses. Clearly, adaptogens include quite divergent categories of compounds, and as stated previously, it is

expected that potentiating interactions (either additive or synergistic) between them may account for the potency of the plant extract.

A few examples of recognized adaptogenic species illustrate the potentially multifaceted chemical contributions to activity. *Panax ginseng* is one of the most studied and well-known adaptogenic plants from Chinese, Korean, Japanese, and Soviet origins. It has been prized as a tonic to invigorate weak bodies and help the restoration of homeostasis. The most intensively studied active phytochemicals in ginseng are ginsenosides, a unique class of steroid-like compounds. Active constituents of *P. ginseng* include not only the ginsenosides, but also polysaccharides, peptides, polyacetylenic alcohols, and fatty acids (Attele et al., 1999). The unique mixture of phytochemicals act through different biological mechanisms, conferring an overall adaptogenic response. Current *in vivo* and *in vitro* studies have shown ginseng's beneficial effects in a wide range of pathological conditions such as cardiovascular diseases, cancer, immune deficiency, reduction in age-related deficits, central nervous system (CNS) disorders, and neurodegenerative diseases (Radad et al., 2006). Another adaptogenic species, *Eleutherococcus senticosus*, has the common name Siberian ginseng although it is not botanically a true ginseng. Extracts of *E. senticosus* include phenylpropanoids, lignans, saponins, coumarins, the triterpene betulinic acid, and vitamins and provitamins (Davydov and Krikorian, 2000). Although *E. senticosus* and *P. ginseng* are both potent adaptogens and are in the Araliaceae family, they accumulate different classes of phytochemicals, and promote adaptogenic effects through different physiological mechanisms. Traditionally-valued adaptogens can also come from outside the plant kingdom, for example, the Tiaga (hard bracket mushroom fungus) was harvested as an immune-booster by Native Americans when they observed restorative effects on injured or sick animals that sought it out, and ingested it. In this

case, the polysaccharides (β-1,3-D-glucans) are purported to be the most active ingredients, but fibers and other components are assumed to modify the bioactivity. Cordyceps, a fungus that grows on caterpillar larvae, is one of the most valued adaptogens in traditional Chinese medicines, and considered an equal to ginseng as a restorative tonic (Huang, 1999).

Even though adaptogens are a relatively recent category, research into adaptogenic properties and the elucidation of the plant or fungal hosts that produce adaptogens are a strong current focus in the medical arena. Consumer demand in the marketplace for adaptogenic products, especially those that can naturally bolster endurance or improve sports performance, is robust. In modern pharmacology, higher therapeutic value is typically assigned to the most potent and target-specific drugs, yet the non-specific nature of adaptogens makes them ill-suited to ranking via these standard industry criteria. In contrast to the high throughput screens used to evaluate either synthetic drugs or even some natural products that combat chronic disease, evaluation methods used to gauge the bioactive potency and efficacy of adaptogens must be adjusted to accommodate nonspecific or multi-target therapeutics. Robust evaluation of adaptogens is complicated by the lack of well-accepted, reliable bioassays or clinical stress models (Rege et al., 1999). Performance evaluations require substantiated scientific criteria, and adaptogens can best be evaluated through standardization of the (multiple) bioactives involved in the effect, and validation of structure-activity relationships (Yuan and Lin, 2000; Chopra and Doiphode, 2002). The pharmacodynamic actions of adaptogens can be quite different than those of stimulant drugs, or even of some phytochemicals which are recognized antagonists to specific chronic diseases such as cancer or diabetes. Many adaptogens demonstrate antioxidant capacity *in vitro* and *in vivo*, but that is not the sole proposed mechanism of action. Since adaptogens promote optimal homeostasis (by *toning down* hyperfunctioning systems, and *upregulating*

hypofunctioning systems), checks and balances and more complex modus operandi are expected to be involved. To determine the mechanisms of adaptogenic plant extracts, multiple targets need to be investigated in order to produce a more holistic assessment of complex pharmacological systems (Panossian et al., 1999; Rege et al., 1999). Research is needed to fill in the gap of information from dose response, to evaluation of efficacy based on the triangulation of performance, tissue and molecular expression information.

Are adaptogens, as their classic definition implies, really 'non-specific'? Or, has science just not yet identified the varied active mechanisms and therapeutic targets? These questions bear further scrutiny, and allude to the need for complex, multifaceted strategies for thorough evaluation of this group of natural compounds.

**Adaptogens in Traditional Medicine**

Although the term adaptogen is relatively new and has only recently gained popularity in herbal medicine, parallel concepts of adaptogenic herbs have long been in use in Ayurvedic medicine and Traditional Chinese Medicine (TCM). In both Ayurvedic medicine and TCM, the focus is placed on individual patients, rather than on diseases, and treatment of disease symptoms takes place via the restoration of balance of the body. In TCM, the balance of the opposing energies, yin and yang, is key to maintaining homeostasis. The balance of yin and yang can be physically conceptualized into the interaction between the four bodily humors (qi, blood, moisture and essence) and internal organ systems (Patwardhan et al., 2005; Yuan and Lin, 2000). Similarly, in Ayuvedic medicine, biological systems are governed by the interactions between the five elements of ether, air, fire, water and earth. The interplay between them is organized into three forces, known as doshas, which are responsible for the physiological and

psychological balance and therefore health of the individual (Chopra and Doiphode, 2002). The aim of Ayurvedic medicine is to balance the integrated body, mind, and spirit to help prevent illness and promote wellness (nccam.nih). Specifically, the *rasayana* herbs of Ayurvedic medicine coincide with the adaptogenic concept. *Rasayana* plants are said to prevent aging, re-establish youth, strengthen life and brain power and prevent disease (Rege et al., 1999). A prime example is Ashwagandha root (*Withania somnifera*), which has demonstrated anti-inflammatory, anticonvulsive, antitumor, immunosuppressive and antioxidant properties mainly attributed to the steroidal compounds, withanolides (Ganzera et al., 2003; Bhattacharya and Muruganandam, 2003). Both Ayurvedic medicine and TCM feature treatments that alleviate individual patient symptoms, emphasizing restoration and maintenance of balance.

In contrast, the Soviet concept of adaptogen was developed to distinguish a new group of chemical substances that conferred "a state of nonspecifically increased resistance" in an organism. As previously noted, the term adaptogen and its definition were originally recorded by Soviet scientists (Mamedov, 2005). Specifically, the concept was used by Lazarev to describe the effectiveness of dibazol (2-benzyl-benzimedazol) on damaged regions of the nervous system and increasing nonspecific resistance to adverse influences (Brekhman and Dardymov, 1969). Brekhman further defined adaptogens as substances that must (a) be innocuous and cause minimal disorders in the physical functions of an organism, (b) be nonspecific, i.e. it should increase resistance to adverse influences of a wide range of factors of physical, chemical and biological nature, and (c) possess normalizing action irrespective of the direction of the foregoing pathologic changes. Research in the former Soviet Union on adaptogenic plants focused on *E. senticosus* (Siberian ginseng), *Rhodiola rosea*, *Leuzea carthamoides, Schizandra chinensis* and *P. ginseng* (Brekhman, 1980). In the former Soviet

Union, adaptogens were valued for their ergogenic capacity (i.e. the ability to increase physical or mental output by eliminating fatigue); for aiding military personnel and athletes during international competitions (Mamedov, 2005). Most of the original studies on Siberian ginseng were conducted in the former Soviet Union, and results are difficult to interpret due to a lack of published details (Bucci, 2000). In a comparison of eight subsequent investigations on Siberian ginseng and endurance performance, three were deemed to have severe methodological flaws, and the remaining five did not demonstrate any performance benefit with administration of the Siberian ginseng preparation (Goulet and Dionne, 2005).

**Screening for Adaptogenic Properties**

*In Vitro*

Only a limited number of *in vitro* (cell culture) screens have proven routinely useful for gauging adaptogenic properties; most approaches combine *in vitro* and *in vivo* bioassays in tandem. *In vitro* animal cell cultures (muscle myotubes, microglia cell lines, etc.) have been used to measure related parameters after administration of purportedly adaptogenic natural compounds. For example, Gorelick-Feldman et al. (2008) recently used cultured C2C12 mouse skeletal muscle cell lines to detect increases in protein synthesis after administration of 20-hydroxyecdysone, a phytoecdysteroid present in spinach as well as *Ajuga turkestanica*, an adaptogenic herb from Central Asia. Subsequently the adaptogenic extract was found to affect a small but significant increase in the grip strength of treated mice. Similar tests can be done using primary muscle cells biopsied from animals or humans. Simple *in vitro* tests for antioxidant capacity/reduction of lipid peroxidation have been conducted on various adaptogenic herbs like *Rhodiola* and *Shizandra*, as antioxidant capacity is the single most studied mode of action and

the most verified outside of the former Soviet Union, although it is well acknowledged that this can not be the main mode of action of adaptogens (Panossian et al., 1999). Alternative assays include measurements of glucose uptake from blood into muscle cells and tissues (accounting for the 'burst of energy' effect attributed to some adaptogens), and various anti-inflammation assays. Because inflammation plays a key role in arthritis and other conditions with relevance to strength and metabolic balance, Dey et al. (2008) demonstrated anti-inflammatory activities in a range of adaptogenic herbs using gene expression assays. Murine macrophage cell lines were selected as monocytes play a central role in inflammation, and activated monocytes induce proinflammatory genes that can be monitored *in vitro*. A great advantage of these gene expression arrays (high content screens) is that a potential mechanism of action can be evaluated before particular bioactive principles or constituents in a phytochemical mixture have been identified. Catecholamine inhibition (related to stress) and immunostimulant assays have also been applied to measurements of adaptogenic properties. *In vitro* bioassays can seek, in a simplified forum, to identify at least some of the mechanisms of action that account for the observed metabolic improvements attributed to adaptogenic compounds. Well-established screens for antioxidant capacity, central nervous system function, inflammation, and other bioactivities can be used in conjunction with more mechanistic *in vivo* screens. Multiple bioassays together can contribute to a clearer picture of mechanisms responsible for adaptogenic properties.

*In Vivo*

Animal models are invaluable tools for evaluating the potential effect that a treatment might ultimately have in human systems, but the animal model is only robust if the test subject is given the chance to respond to the *treatment – not* to possible stress (anxiety or even injury)

imposed during the treatment administration. This is particularly true when adaptogenic improvements in metabolism or performance are being gauged, as stress-induced reactions can greatly obscure and confound measured results. Stress is defined as the effect produced by external events or internal factors, which induce an alteration in an animal's biological equilibrium (Institute for Laboratory Animal Research, 1992). For adaptogenic response measurements, nonspecific and chronic stress responses must be minimized, allowing only the deliberately imposed, controlled form of stress (or treatment) to impact the test animal. Both the method of adaptogen delivery, and the subsequent performance measurement procedures, can adversely stress test animals and skew measurements of adaptogenic response.

*Treatment Delivery*

In order to assess reproducible and reliable responses to adaptogen treatment, particular care must be taken to avoid stress during delivery of the adaptogen to the animal. Routine procedures such as handling, venipuncture and orogastric gavage are acutely stressful to animals, and previous research verifies that the stress-induced responses can be quantifiable and substantial. Handling of research animals triggers stress responses such as increased concentration of corticosterone, hyperthermia and increased plasma glucose (flight or fight response). Other procedures that are considered routine in animal research, including venipuncture and orogastric gavage, can lead to elevations of heart rate, blood pressure, and glucocorticoid concentrations (Balcombe et al., 2004).

*Oral Administration.* To administer compounds by oral gavage, the animal must be restrained, and a rigid metal or plastic tube, with a rounded end to prevent tissue puncture, is inserted down the throat to dispense solution directly into the stomach. In addition to the stress

of restraint, which must be done properly to prevent injury, the animal experiences the stress of breathing interference during the procedure and the discomfort of stomach distension after the solution is dispensed. Other complications that may occur during gavage include inadvertent tracheal administration, reflux, aspiration pneumonia, esophageal impaction, trauma or perforation, hemothorax, and even death (Balcombe et al, 2004).

As an alternative to gavage, an orally-administered compound may be incorporated into animal feed (diet) or into the water. This method of administration circumvents handling stress. However, in a feeding study, the amount of material administered is more difficult to judge (animals may not eat as much chow if the treatment adversely alters taste or palatability; water or chow may be inadvertently spilled by the animal and the treatment dosage may therefore not be accurate). In addition, feed and water intake of rodents is strongly correlated with body weight. Substantial differences in intake coincident with body weight of the test animal can further confound comparisons between animals. For example, mean adjusted water intakes of various strains of mice can range from 5.7 +/- 02 mL to 11.4 +/- 0.5 mL per 30 g body weight (Bachmanovet al., 2002).

In order to gauge the effects of adaptogenic compounds, it makes sense to try to mimic the recommended methods of administration of traditional medicinal preparations, which are taken at specific doses and times, rather than throughout the day and night. One possible method, which again circumvents any handling stress, is to incorporate the adaptogenic compound into a 'treat' that a laboratory animal will preferentially consume before regular chow. The treats can be periodically delivered at the times chosen in the experimental design, and the animal will willingly ingest it. For water-soluble compounds, the treatment can be dissolved in a 5% sugar solution, and administered to rats that have been trained to drink from a syringe

(Schleimer et al., 2005). Sugar tablets or tablets with flavor additives may help mask unappealing tastes of compounds and require even less attention from the investigator. However, costs can sky rocket if the compound costs are high, as large quantities are needed to produce accurate doses using tablet press machines. Another method is to incorporate compounds into a gel form of the animal diet offered by Testdiet®. The compound can be added to a powder diet mix that contains a gelling agent and flavor additive. Mixing the powder with hot water activates the gelling agent, which can then be poured into molds and cut to desired quantities for administration to animals (Fig. 4.1). This method offers control of dose and timing with minimal handling by the investigator. Recently, during experiments to test the effects of an adaptogenic plant phytoecdysteroid from *Ajuga turkestanica* in a rodent model, we determined that the mice did not tolerate the stress imposed by routine gavage; the rodents were disadvantaged by the procedure to the extent that benefits from the administered dosage could not be gauged. As an alternative, the adaptogenic phytoecdysteroid was formulated as a gel treat, using the method described above. The mice preferentially consumed the full gel treat immediately at the time of introduction to the cage, ensuring that the fully administered dose was consumed (Fig. 4.2).

*Injection or Surgical Implantation*. When oral administration to the animal is not suitable (for example, due to issues with bioavailability, or if the animal model is unable to tolerate the stress of gavage), compounds can be directly delivered to the animal's system through injection or the use of implantable pumps. Small osmotic pumps, such as those produced by Alzet®, can be implanted into animals to allow for continuous infusion of compounds into circulation. However, this requires surgery and a minimal recovery period. The invasiveness of implants may cause stress to the animal, and inflammation, which can confound measurement of positive

adaptogenic benefits. Multiple injections would involve the same caveats. As an alternative, injectable gel matrixes and biodegradable polymers allow for sustained release of compounds into the animal's body, and eliminate the need for multiple injections (Gao et al., 1995; Kempe et al., 2008; Chitkara et al., 2006). These release agents are becoming more attractive for the controlled, time-course delivery of therapeutic agents.

*Topical Administration.* Another major non-invasive route of administration of therapeutic agents is by topical application through the skin. Topical administration also affords the advantage of bypassing first pass metabolism, however, metabolic activity of the skin should not be over-looked (Hadgraft and Guy, 1989; Calvery et al., 1946; Hadgraft and Lane, 2005). Ointments, creams, gels or patches are typically applied to the skin's surface and the drug then diffuses through the skin. Human skin is composed of four main layers: the stratum corneum, the epidermis, the dermis and subcutaneous tissue which provide a protective barrier, but also presents a barrier to the absorption of topically applied drugs and eventual release into the blood stream (Foldvari, 2000). Penetration enhancers, or absorption promoters, are chemicals that reversibly disrupt the organized structure of the skin to accelerate drug permeation (Williams and Barry, 2004). After the (sometimes serendipitous) discovery of some of these compounds, further research has uncovered the mechanisms of action (Hadgraft and Lane, 2005).

Dimethyl sulfoxide (DMSO) is frequently used as a vehicle for drug delivery due to the strong solvent and penetration properties afforded by its amphipathic structure (Pope and Oliver, 1966). DMSO dissolution of adaptogens and topical delivery to animal skins can be quite advantageous because no surgeries are involved. Experimental animals are marginally disadvantaged by handling stress, but this is a minor stress compared to invasive surgery or multiple injections. In addition, topical applications of a DMSO-based adaptogen can be

repeated at time-course intervals, allowing a more realistic assessment of effects over time on performance and muscles/tissues.

As, compounds independently diffuse through the skin in response to alterations caused by absorption promoters, other absorption promoters such as Azones, alcohols, oils, glycols, surfactants and terpenes, may be more appropriate depending on the compound of study (Kurihara-Bergstrom, 1987; Williams and Barry, 2004). And although topical application of DMSO is generally well tolerated, it is important to be aware of the systemic side effects with its use and those of other penetration enhancers (Santos et al., 2003). Development of novel matrixes for non-invasive and sustained compound delivery allow for compounds with low oral bioactivity, such as resveratrol, to be therapeutically effective with minimal confounding stress factors (Hung et al., 2008).

*Physiological Measures of Performance and Endurance*

Ergogenic aids are any training technique, mechanical device, nutritional practice, pharmacological method or psychological technique that can improve exercise performance capacity and/or enhance training adaptations (Kreider et al., 2004). Because adaptogens are often used as ergogenic aids, gauging their ability to enhance performance is a logical criteria for determining efficacy and possible applications. Exercise protocols should be designed to test the desired physiological adaptations without producing confounding nonspecific chronic activation of the stress response. Intensity, frequency, and duration, as well as perceived control and novelty of environment can also have an impact on the stress response of animals (Kregel et al., 2006). Although acute activation of the stress response is normal and an attempt of the body to maintain or restore homeostasis, it is important to minimize stress that would confound

measurements which attempt to gauge adaptogenic effects.

The three major modalities in gauging exercise performance capabilities in rodents are treadmill running, voluntary wheel running and forced swimming (often weighted). Researchers attempt to reduce activation of the stress response by repeatedly exposing the test animals to handling (and the treadmill or other apparatus) at the same time of day, and by consistently assigning the same personnel to conduct the actual training sessions. These practices greatly reduce the stress response triggered by a novel environment and activity. Mice are nocturnal, so treadmill familiarization and training can be done during their dark cycles (Kregel et al., 2006). Treadmill and voluntary wheel performance assessments can also be skewed by genetic factors. Swiss Webster and FVB/NJ mice performed best and C57BL/6J mice performed the worst in treadmill running tests, whereas Swiss Webster and C57BL/6J mice had significantly longer running durations on voluntary treadmill than other strains of mice (Lerman et al., 2002).

*Treadmill Running.* Among the three primary exercise modalities, running on a motorized treadmill allows the most control over the intensity and duration of physical performance. Some researchers consider the treadmill to be the 'gold standard' for assessing the influence of adaptogens on performance in rodent models. Additionally, in combination with treadmill running, the total amount of external work can be quantified by measuring maximum endurance capacity or maximal $O_2$ uptake ($Vo_{2\ max}$). $Vo_{2\ max}$ is an indication of the capacity of transport and utilization of $O_2$ between the lungs, cardiovascular system, and musculoskeletal system (Henderson et al., 2002). To evaluate endurance capacity, an animal is run to the point of fatigue, where $Vo_{2\ max}$ is normally defined as the point at which $Vo_2$ does not increase, even though further increases in external workload are imposed on the animal (Kregel et al., 2006). In general, $Vo_{2\ max}$ correlates with exercise endurance and is a reliable method to assess fitness.

A main disadvantage of treadmill running in gauging adaptogenic effects is the stress imposed on the animal from a forced exercise modality. Often, animals need to be stimulated to exercise to their physical capacity through the use of external stimuli such as tapping their tails or hindquarters lightly with a stick, electric shock or bursts of high-pressure air (Kregel et al., 2006). Additionally, chronic exercise training on the treadmill increases the rodent's $Vo_{2\ max}$ and leads to adaptations in both the cardiovascular and skeletal muscle systems which can confound effects of adaptogens (Kemi et al., 2002; Davies et al., 1981). However, motorized treadmill offers the examination of factors that contribute to exercise performance under well-defined experimental conditions.

*Voluntary Wheel Running.* Unlike motorized treadmill running, voluntary wheel running does not require constant surveillance by the investigator to prevent animal injury and does not impose stress responses from a forced exercise. As such, wheel running exercise studies can be performed long-term with minimal intervention by the investigator. Additionally, voluntary wheel running can be used to determine the effects of adaptogenic compounds on animal physiology and behavior by monitoring changes in duration and distance run over a given time period (Dolinsky et al., 1983; Teramoto et al., 1988). Thus, exercise-promoting effects and ergogenic aid capabilities can be investigated using this exercise modality (Avraham et al., 2001). However, a major disadvantage is the difficulty in regulating exercise duration or intensity of running and that exercise is largely determined by the animal's motivation to exercise. Another consideration with chronic use of wheel running is that substantial hypertrophy of hindlimb muscles and myocardium develops, which may confound the determination of adaptogenic effects (Henriksen et al., 1995).

*Forced Swimming.* Swim tests can be used to determine physiological, biochemical, and

molecular changes to acute and chronic exercise (Baar et al., 2002; Jones et al., 2002). A major advantage of swimming exercise is the employment of a large volume of muscle mass and uniform exercise. However, forced swimming produces psychological stress and results in survival behavior, such as floating, climbing, diving and bobbing, as the animal attempts to prevent drowning (Ferrandez and De la Fuente, 1999; Kregel, 2006). These types of behavior can confound exercise adaptations and interpretation of results due to the intermittent bouts of hypoxia. Additionally, swimming produces extensive adaptations to the cardiovascular system and is often used as a stimulus to produce adaptations rather than to determine performance (Kaplan et al., 1994). However, with training, non-continuous swimming behavior can be reduced and the effect of adaptogens on improved performance can be quantified by measuring swim duration.

*Skeletal Muscle and Gene Expression Analysis*

Examination of skeletal muscle can be performed to further substantiate the mechanisms by which adaptogenic compounds work *in vivo*. The analyses also uncover possible therapeutic applications for muscle diseases, through administration of natural adaptogenic extracts. Skeletal muscle is made up of heterogeneous specialized myofibers, or muscle cells, that generate force and movement of the body through contractile activity. The composition of myofibers in each muscle group determines their optimal specialized function. Myofibers are controlled by signaling pathways that respond to changes in metabolic and functional demands of the organism (Bassel-Duby and Olson, 2006).

Skeletal muscle hypertrophy, or the increase in muscle, in adult animals is a result of the increase in size of muscle cells, as opposed to hyperplasia, the increase in the number of muscle

cells (Glass, 2005). Adaptogenic plants such as *L. carthemoides* and *A. turkestanica* contain compounds that have resulted in increased muscle mass after oral administration to animals (Koudela et al., 1995; Hikino et al., 1968; Kratky et al., 1997). However, increase in muscle mass alone does not necessarily benefit the functional capacity of the animal. Skeletal muscle fiber type distribution and mitochondrial density are two major parameters of measure that determine muscle function and endurance capacity (Burkholder et al., 1994; Wang et al., 2004; Philippi and Sillau, 1994).

The ergogenic effects of certain adaptogenic compounds can be explained through investigation of fiber type composition. Skeletal muscles are generally classified as type I (red/oxidative/slow) or type II (white/glycolytic/fast) fibers. Type I fibers are rich in mitochondria and fatigue-resistant due to the use of mainly oxidative metabolism, which provides a stable long-lasting supply of ATP. Type I fibers also have high levels of slow isoform contractile proteins, high capillary densities and high levels of myoglobin, an oxygen-binding protein that give the fibers their red color. Type II fibers have low levels of mitochondria, are susceptible to fatigue, rely mainly on glycolytic metabolism as a major energy source and exhibit fast contractile activity (Spangenburg and Booth, 2003; Wang et al., 2004). Shifts in levels of oxidation enzymes, mitochondrial biogenesis, and fiber type-specific contractile proteins indicate muscle fiber type switching and changes in functional capacity. Adult skeletal muscle can undergo muscle type conversion in response to exercise training, genetic manipulation or pharmacological intervention (Booth and Thomason, 1991; Wang et al., 2004; Choo et al., 1992; Rajab et al., 2000; Kadi et al., 1999). Thus, adaptogenic compounds may increase stamina, strength and endurance in animals through the modulation of skeletal muscle fiber type composition.

Mitochondria are the main subcellular structures that determine the oxygen consumption and energy demand of muscle (Philippi and Sillau, 1994). One of the most important adaptations after endurance training is the increase in muscular oxidative capacity due to the increase in mitochondrial density and activities of mitochondrial enzymes (Zoll et al., 2002). Although both fiber types adjust to match functional demand, the difference in oxidative capacity between slow oxidative fibers and fast glycolytic fibers is the result of much greater mitochondrial volume density in the subsarcolemmal area of the slow oxidative fibers (Philippi and Sillau, 1994). Analysis of fiber type and mitochondrial density in skeletal tissues can identify biological changes induced by adaptogens to support their performance enhancing capabilities.

Adaptogens, given the breadth of their influence, have potential to interface with a range of gene pathways. A valuable and efficient means of obtaining a mass screen of multiple systems is with global gene expression microarrays. With the growth of genomics and the full sequence of mice DNA and most of their gene functions, a mouse global gene expression microarray provides a robust screen of adaptogenic targets *in vivo* (Duggan et al., 1999). Identification of pathways affected by adaptogens will uncover modes of actions in the molecular environment.

Global gene expression arrays provide a screen of functions and can be corroborated with studies targeting specific key pathways. Activation of gene expression appears to be instrumental in controlling the accumulation of posttranscriptional adaptations leading to structural and biochemical adaptations of the mitochondrial compartment in exercised skeletal muscle (Hoppler and Fluck, 2002). Increase in mitochondrial volume density is supported by proportional increases in the steady-state level of a number of mRNAs encoding mitochondrial proteins (Puntschart et al., 1995). The combination of gene and protein expression, skeletal

muscle composition and physical performance capacity provides a fuller illustration of the ergogenic function of adaptogens.

**Comprehensive Strategies for Gauging Adaptogenic Phytochemical Potency**

Natural adaptogenic phytochemicals – substances which increase non-specific resistance and help to normalize body functions (maintain homeostasis) are some of the most intriguing new targets for discovery and development for human health applications. The concept of homeostasis as a complex dynamic equilibrium persistently challenged by stress factors (Chrousos and Gold, 1992) inherently resists precise quantification by scientific criteria. Therefore, not surprisingly, adaptogens – natural products which are meant to ameliorate a state of threatened homeostasis – present particular challenges for science-based evaluation. As noted above, the diversity of phenotypic effects that can be impacted by adaptogens mandates a comprehensive, multi-faceted approach to successfully gauge adaptogenic benefits *in vivo.* Multiple mechanisms of action may be simultaneously in operation, multiple phytochemicals act together in harmonic synergy to provoke an adaptive response in the human body. *In vitro* mechanistic tests need to dovetail with *in vivo* evaluations, and in addition, performance measurements should ideally be supported with physiological and gene expression data on the mechanisms of action.

Tests of adaptogen efficacy should strive to mimic, as much as possible, the methods by which an adaptogenic herb or other preparation would be administered in traditional medical practice. However, costly clinical trials are generally not warranted until many parameters (dosage, timing, composition, toxicology, etc.) have been narrowed down in a series of preclinical tests. Since distress, tension, fatigue, etc. are difficult to precisely regulate,

investigators have resorted to some unusual means to simulate stress that a human subject might experience (Govindarajan et al., 2005). For example, animals injected with pathogenic bacteria (to evaluate the ability of a plant extract treatment to combat infection) have been further disadvantaged by ligating the caecum, or in other cases, animals were rendered immunosuppressed before deliberate infection (Thatte and Dahanukar, 1989). In order to evaluate the ability of an adaptogen to alleviate muscle damage, some research strategies have deliberately injected muscles with snake venom to induce muscle necrosis (Harris et al., 2000; Toth et al., 2008). For example, the phytoecdysteroid 20-hydroxyecdysone was injected into rat soleus muscle, narcotized, and treated for muscle regeneration followed by daily injections of 20-hydroxyecdysone for 7 days, to see if it could alleviate muscle damage/degeneration and therefore provide a potential treatment for muscle atrophy (Toth et al. 2008). Analysis of muscle fibers post treatment showed an increase in fiber size and myonuclear number in normal and regenerating cells given 20-hydroxyecdysone treatment, indicating its potential for use as a therapeutic treatment for muscle atrophy. Caveats in this case include that muscle necrosis, as induced in these experiments, is a damage that can be caused by injury (or venom injection) whereas muscle degeneration or atrophy can have multiple other causes including forced confinement (failure to exercise the muscles) or genetic factors. Snake venom, in addition to causing rapid muscle necrosis, also induces additional responses in a test animal such as inflammation and pain that would not be symptomatic of simple atrophy due to lack of muscle use (Dixon and Harris 1996; Teixeira et al 2003). Therefore, researchers who attempt to provoke muscle damage using this mechanism must also treat to alleviate the side effects in order to evaluate muscle regeneration. According to Lee and Bianchi (1971), the lack of good experimental or clinical stress models is one major obstacle in discovery and/or development of

adaptogenic formulae. Given that multiple therapeutic targets seem to be involved, robust evaluative assays for gauging adaptogenic properties will inevitably demand evaluation of multiple complementary bioassay screens, *in vitro* and *in vivo*, and the animal screens ideally should evaluate both performance as well as analysis of muscle and tissue composition. By linking evaluative criteria in this way, the particularly complex and multifacteted roles of adaptogenic phytochemicals can be elucidated, and subsequently understood by consumers as proactive means for human health protection.

Breakthroughs in modern metabolomics, with multiple platforms for analysis, are for the first time permitting rigorous analysis of complicated adaptogenic mixtures, and translating their benefits, from both well-known mainstream food crops as lesser-known endemic medicinal plant sources, to the public at large. Increasingly, adaptogens are an up and coming category in the industry, which much easier routes to commercialization than traditional pharmaceuticals, and burgeoning demand from consumers interested in enhanced healthspan and improved performance. There are multiple manifestations of adaptogenic compounds on the human metabolism. In order to thoroughly gauge the efficacy, science needs to center attention on the common mechanisms of action such as immunostimulation (Rege et al., 1999), as it is widely believed that the future of plants for human health will be in functional foods, personalized medicine, health *maintenance* (rather than restorative *treatment*), performance enhancement, and other means to help people cope with stresses and pressures of modern life.

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**Figures**

Figure 4.1 Gel treat containing phytoecdysteroid treatment (20E), solidified and removed from tray mold (left). Individual gel treats are cut into triangles (right) for regulated dosage 0-50 mg/kg body weight for each animal.

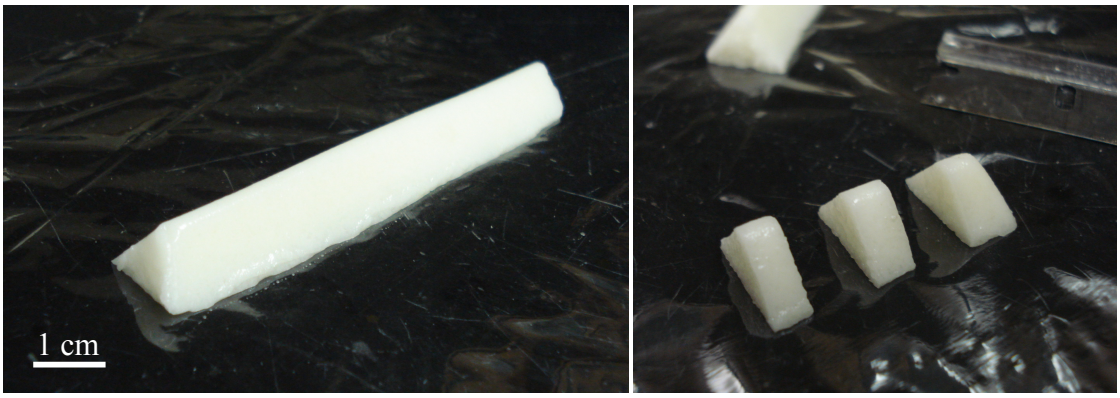
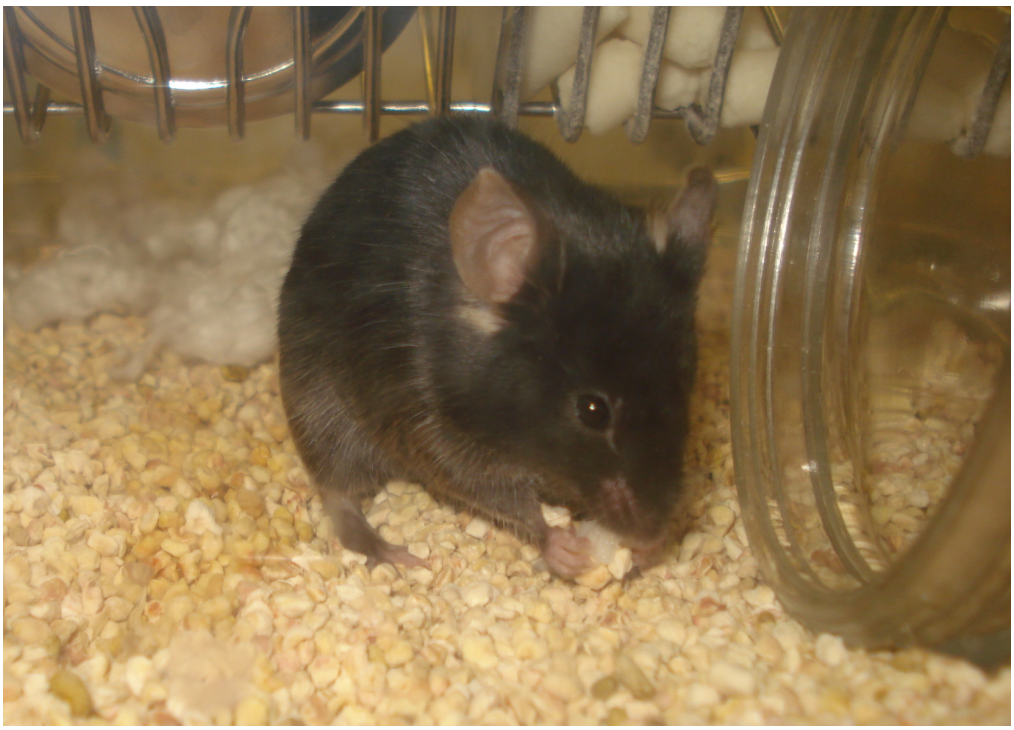



Figure 4.2 C57BL/6J mouse consuming a gel treat.



# CHAPTER 5

# EFFECTS OF CONTINUOUS INFUSION OF 20-HYDROXYECDYSONE IN C57BL/6 MICE


## Abstract

20-Hydroxyecdysone (20E) is the most abundant phytoecdysteroid produced by several plant families and has been attributed with numerous pharmacological properties in animals including anabolic and immune modulating effects. However, the *in vivo* bioactivity of phytoecdysteroids in mammals remains ambiguous, and moreover, the mechanism of action in mammals has yet to be determined. In this study, the physiological and gene expression effects of 20E were analyzed in C57BL/6 mice given a continuous infusion of saline or 20E (5 mg/kg/day) for five or fifteen days using subcutaneously implanted Alzet® osmotic pumps. The weights of the total body, muscle groups and organs were recorded and carcass composition was determined to evaluate changes in nutrient partitioning. There was a significant increase ($p = 0.01$) in the weight of triceps brachii in mice treated with 20E for five days (115 +/- 8 mg) compared to mice treated with saline for five days (88 +/- 3 mg), however, there were no differences in the other measured parameters. To determine potential mechanisms of action of 20E in skeletal muscle, Illumina's Mouse Whole Genome-6 v2.0 Expression BeadChips and Ingenuity Pathways Analysis (IPA) were used to identify genes with the most evidence for differential expression in the context of biological functions, canonical pathways and networks. IPA analysis provided evidence for 20E involvement in cellular movement, cell-to-cell signaling and interaction, and cellular growth and proliferation. Overall, the data suggests that 20E does

not have potent anabolic properties; however, IPA identified potential lead biological processes and pathways of 20E.

## Introduction

Phytoecdysteroids are a diverse class of plant steroids with a cyclopentano-perhydrophenanthrene skeleton, and vary in the number, position, and orientation of hydroxy substituents as well as conjugated moieties. 20-Hydroxyecdysone (20E) is the most abundant phytoecdysteroid produced by several plant families, the subject of various pharmacological studies, and the main phytoecdysteroid to which phytoecdysteroid-containing herbal preparations are standardized (Fig. 5.1; Baltaev, 2000; Báthori et al., 2008; Báthori and Kalász, 2001). Phytoecdysteroids are plant-produced analogues of ecdysteroids, insect molting hormones that control cell proliferation, growth and developmental cycles (Singh et al., 1980; Sláma et al., 1996). In plants, they serve as defense compounds against non-adapted insects and nematodes (Schmelz et al., 2000; Soriano et al., 2004).

Phytoecdysteroids have been attributed with numerous pharmacological properties in animals, including humans. Growth-promoting effects have been reported for mice (Stopka et al., 1999), rats (Syrov 2000; Syrov et al., 2001), sheep (Purser and Baker, 1994), pigs (Krátky et al., 1997) and Japanese quail (Koudela et al., 1995; Sláma et al., 1996) after ingestion of phytoecdysteroids, implying a potentially valuable application in improving livestock production. The enhancement of strength, stamina and muscle mass in mammals (known as 'adaptogenic' effects) are among the main purported bioactive properties and commercial uses of phytoecdysteroids and phytoecdysteroid-containing extracts (Báthori and Kalász, 2001). 20E stimulated protein synthesis *in vitro* in C2C12 murine myotubes as well as *in vivo* in rats given a

single daily dose of 5 mg/kg for 10 days (Gorelick-Feldman et al., 2008; Syrov 2000). Additionally, 20E increased the weight (tibialis anterior) and size (soleus and extensor digitorum longus) of skeletal muscle and stimulated muscle fiber growth in regenerating soleus muscles (Syrov 2000; Tóth et al., 2008), demonstrating the potential of 20E as a therapeutic agent for treating muscle atrophy. Pharmacological effects of phytoecdysteroids have been compared to those of anabolic steroids; however, phytoecdysteroids do not bind vertebrate steroid receptors and do not have adverse hormonal side effects (Gorelick-Feldman et al., 2008; Báthori et al., 2008). As a relatively new class of medicinally active compounds, comprehensive reviews of the purported biological effects of phytoecdysteroids have only been published within the past decade (Dinan, 2001; Lafont and Dinan, 2003; Báthori and Pongrácz, 2005; Báthori et al., 2008).

Recent interest in phytoecdysteroids as adaptogens is based on their observed pharmacological effects in mammalian models. Conversely, in the unrelated realm of gene therapy research, phytoecdysteroids have been prioritized for use as inducers in exogenous gene expression systems, specifically because they are expected to be *inert* (lack pharmacological influence and do not induce endogenous gene expression) in mammals and because of their ability to rapidly and precisely induce gene switch systems (Albanese et al., 2000; Saez et al., 2000). The objective of gene switch constructs is that inert drugs or molecules must be able to 'switch on' an inserted gene of interest (act as a gene switch system), but not otherwise interfere with host gene regulation or metabolism. Ideal inducers in gene expression systems are rapidly cleared to allow for quick and reversible induction of the transgene, are pharmacologically inert to avoid pleiotropic effects in the host, and have receptor binding specificity for exclusive expression of the transgene (Palli et al., 2005).

Phytoecdysteroid molecules have qualified as potentially compatible gene inducers (in gene switch systems) because they are rapidly cleared in mammals, bind specifically to insect ecdysteroid receptors, which are not naturally present in vertebrates (Dzhukharova et al., 1987; Riddiford et al., 2000) and have not appeared to interact with host regulatory networks to confound transgene expression (Albanese et al., 2000; Saez et al., 2000). However, if phytoecdysteroids do impart adaptogenic properties to the mammalian host, it would not be consistent with their supposedly 'inert' status in gene switch systems, because information expressed by the switched-on gene would be confounded or obscured by the physiological activity of the inducer.

Because the *in vivo* bioactivity of phytoecdysteroids in mammals remains ambiguous, it is necessary to examine the effects of phytoecdysteroids on mammalian gene expression and physiology *in vivo*. Moreover, the mechanism of action, if any, of phytoecdysteroids in mammals is yet to be determined. In this study, the physiological and gene expression effects of 20E *in vivo* were analyzed. Mice were administered a steady infusion of phytochemical during treatment using subcutaneously implanted Alzet® osmotic pumps. Total body, extracted muscle groups and organs, were weighed. Carcass composition was determined to evaluate changes in nutrient partitioning. Global gene expression changes were evaluated in skeletal muscle and potential molecular targets of 20E were identified using the Illumina BeadArray platform.

## Materials and Methods

### *Animals*

All experimental protocols were approved by the University of Illinois Institutional Animal Care and Use Committee. Adult male mice (12 weeks old) strain C57BL/6J were

purchased from Jackson Labs (Bar Harbor, ME). Animals were individually housed in plastic cages in a temperature controlled room (22 °C) with 12:12 hour light-dark cycles and given ad libitum access to food (AIN-93G) and water.

Following a one week acclimation period, mice were weighed and randomly assigned to weight balanced treatment groups (n = 9-10). Alzet® osmotic pumps (model 2002; Cupertino, CA) were subcutaneously implanted according to manufacturer specifications to provide a continuous infusion of the test compound. Pumps supplied a dose of 5 mg/kg/day of 20E dissolved in saline (0.9% NaCl) or saline alone (control), with a flow rate of 0.5 μl/hr. During surgery, animals were anesthetized with isoflurane from a gas vaporizer maintained between 2-4%. After pumps were implanted, incisions were sealed with surgical clamps and animals were given a s.c. injection of carprofen (5 mg/kg).

Mice were euthanized by $CO_2$ asphyxiation followed by cervical dislocation on the fifth and fifteenth day after implantation. Blood was drawn immediately postmortem by cardiac puncture, plasma was separated by centrifugation at 14,000 x g for 15 min at 4 °C, and samples were stored at -20 °C. The following muscle groups and organs were extracted and weighed: biceps femoris, triceps brachii, tibialis anterior, gastrocnemius, kidney, liver and heart. All tissues were immediately frozen and stored at -80 °C. Statistical significance was determined by comparing the 20E treatment group with saline control at each time point by analysis of variance (ANOVA) using SAS version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). A probability of $p < 0.05$ was considered statistically significant.

*RNA isolation*

The triceps muscles were selected for RNA isolation and microarray analysis because an

increase in mass was demonstrated in this muscle group with 20E treatment after five days. Total RNA was extracted from triceps (*ca.* 100 mg) by homogenization with 1 mL TriReagent (St. Louis, MO) for 3 min at 30Hz using a Tissuelyser II (Qiagen, Gaithersburg, MD). Total RNA was treated with DNase I and purified using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Quantity and purity of RNA was determined using the NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). Total RNA was pooled into groups containing three individual biological replicates (except for one pool of saline infused group B which had two replicates). Each pool contained an equal quantity of RNA (1 μg) from each individual animal. Three pools were analyzed per treatment using Illumina's MouseWG-6 v2.0 Expression BeadChips (San Diego, CA).

*Microarray hybridization and labeling reactions*

For Illumina microarray analysis, samples were prepared and analyzed by the W. M. Keck Center for Comparative and Functional Genomics in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. RNA quality was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). 300 ng of high quality total RNA were primed with an oligo(dT) primer bearing a T7 promoter, and reverse transcribed in the first-strand cDNA synthesis using the Illumina® TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX). Single stranded cDNA was then converted into double stranded cDNA according to the manufacturer's instructions. The double-stranded cDNA was purified and served as a template in the 14-hour *in vitro* transcription (IVT) reaction. Prior to hybridization, the synthesized biotin labeled cRNA was cleaned up using the same Amplification Kit. After the quality control assessment, 1.5 μg of cRNA from each experimental sample along

with hybridization controls were hybridized for 16 hours to the MouseWG-6 v2.0 Expression BeadChips (Illumina, Inc., San Diego, CA) in the 58 °C Illumina Hybridization Oven. Washing, staining with streptavidin-Cy3 (GE Healthcare Bio-Sciences, Piscataway, NJ), and scanning were performed according to the Illumina Whole-Genome Gene Expression Direct Hybridization Assay Guide (revision A). Two BeadChips were used in this study, each containing six arrays. The arrays were scanned using an Illumina BeadArray Reader. Each array image was visually screened to discount for signal artifacts, scratches or debris.

*Data processing and analysis*

The raw bead-level files were processed with Illumina® BeadStudio 3.1.3, Gene Expression Module v3.4.0 (Illumina), without background correction or normalization, to get one value per beadtype for each array. These beadtype values were normalized and analyzed in R (R Development Core Team) using the beadarray package (Dunning et al., 2007) from the Bioconductor Project (Gentleman et al., 2004). Background fluorescence was corrected by subtracting the mean of the negative control beadtypes; negative and zero values after background correction were set to 0.5. Quantile normalization was performed and data were log2 transformed. Differential expression was assessed using a linear model using the limma package (Smyth, 2005), which uses an empirical Bayes correction (Smyth, 2004) that helps to improve power by borrowing information across beadtypes. BeadStudio's Detection p-values were used to filter out 21,572 beadtypes that were not detected ($p > 0.05$) above the negative controls in any of the 12 samples. The remaining 23,709 beadtypes were input to Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) as the reference set. For each pairwise comparison, (20E vs. saline) at each time point, beadtypes with raw p-values <

0.01 from the limma analysis were selected for functional analysis, pathway mapping, and network generation.

## Results

*Body Tissue Analysis*

To determine whether 20E conferred an anabolic effect on skeletal muscle, muscle groups were weighed in mice infused with 20E or saline (Table 5.1). Results showed that there was a significant increase in the weight of the triceps brachii in the mice treated with 20E for five days (115 +/- 8 mg) compared to mice treated with saline for five days (88 +/- 3 mg) ($p = 0.01$). However, there were no differences in weight of the other muscle groups (tibialis anterior, biceps femoris and gastrocnemius) and also no differences in mice treated with 20E compared to saline for the longer duration of fifteen days. Additionally, results showed no significant differences in body weights, organ weights or average daily feed intake between the treatment groups. Administration of 20E by infusion was confirmed by measuring the volume of 20E solution or saline remaining in the reservoirs and plasma samples were analyzed by LCMS (data not shown).

*Microarray Analysis*

To determine potential leads for the biological action of 20E, RNA was extracted from the triceps brachii, where there was an observed increase in muscle weight with 20E treatment, for whole-genome microarray analysis. Neither the response to 20E (20E vs. saline) at five days nor the response to 20E at fifteen days had any beadtypes on the array that were significantly different after False Discovery Rate correction (Benjamini & Hochberg, 1995). This did not

mean there were no genes with differential expression due to 20E treatment, just that the model was not able to distinguish them from the expected false positives of testing 23,709 beadtypes. Instead, we decided to look at the genes with the most evidence for differential expression and see if there were any interesting candidate genes or overall trends in these lists using IPA. We used a raw p-value cutoff of 0.01, which resulted in 159 beadtypes for the response to 20E at five days and 224 beadtypes for the response to 20E at fifteen days. Beadtypes meeting the raw p-value cutoff of 0.01 were input into IPA, which mapped them to 104 network eligible genes for the response to 20E at five days and 147 network eligible genes for the response to 20E at fifteen days. The top ten genes with the greatest fold change in mRNA abundance at each time point are listed in Table 5.2. In addition to providing gene-level information, IPA determines the biological functions of the genes, categorizes them by Disease and Disorders, Molecular and Cellular Function, and Physiological System Development and Function, and tests whether each function is significantly over-represented in the selected list of genes; over-representation can indicate that a particular biological process is affected by the 20E treatment. Cell-to-cell signaling and interaction and hematological system development and function are among the top biological functions that show up in both treatments (Table 5.3).

IPA also maps genes to canonical pathways using Ingenuity's Knowledge Base and tests whether the association between the genes in the dataset and the pathway is due to random chance. The significance of association is calculated by a ratio (the number of molecules from the dataset that map to the pathway divided by the total number of molecules that map to the canonical pathway) and an associated p-value that determines the probability of association due to chance alone. Top ranking signaling pathways identified by IPA are interferon signaling (ratio: 2/30, $p = 0.03$), TGF-β signaling (ratio: 3/86, $p = 0.04$), for the response to 20E at five

days and hypoxia signaling in the cardiovascular system (ratio: 4/70, p = 0.02) for the response to 20E at fifteen days. Interferons are involved in the innate and adaptive immune responses and TGF-β signaling are involved in cellular growth and morphological functions. Significant canonical pathways and genes are provided in Table 5.4.

Finally, IPA also algorithmically generates networks to maximize the connectivity of genes from the input dataset. Networks show the interconnectedness of the genes, using the network eligible genes (raw p-value < 0.01) as starting points for generating networks and then merging smaller networks with genes form the knowledge base. Networks differ from canonical pathways in that they were generated *de novo* from the input dataset and may contain molecules from several pathways, whereas canonical pathways were generated from published literature and do not change based on the input dataset. Network scores indicate the likelihood of the focus genes being found in a network due to chance alone and are calculated by taking the negative log of the p-value. All the networks in Tables 5.5a and b have scores greater than two (p-value < 0.01), and provide a high level of confidence that the focus genes are related to the network not solely due to chance. Top network functions identified by IPA, in which 20E has demonstrated effects in other studies, include cell-to-cell signaling and interaction, hematological system development and function, and skeletal muscle development and function (Tables 5a and b; Todorov et al., 2000; Syrov et al., 1997; Gorelick-Feldman et al., 2008).

**Discussion**

In this study, physiological and gene expression effects were analyzed in mice given a continuous infusion of 20E. Total body, individual muscle groups, and organ weights were recorded, and carcass composition was determined to evaluate changes in nutrient partitioning.

Results showed a significant increase in the weight of the triceps brachii in mice treated with 20E for five days compared to mice treated with saline for five days and no differences in weight between treatment groups in the other parameters (Table 5.1). Statistical analysis of microarray beadtypes with False Discovery Rate correction did not identify any genes as differentially expressed. Consequently, IPA was used to identify genes with the most evidence for differential expression in the triceps muscles. Cell-to-cell signaling and interaction, hematological system development and function, hair and skin development and function, and cellular growth and proliferation were among the top systems identified as affected by 20E by IPA. Overall, the data suggests that 20E does not have potent anabolic properties; however, IPA identified potential lead biological processes and pathways of 20E.

Although there are numerous reports on the bioactive properties of phytoecdysteroids, especially on the growth promoting effects of 20E in mammals, studies carried out in rodents do not consistently demonstrate an increase in body weight (Gao et al., 2008; Toth et al., 2008; Stopka et al., 1999; Ogawa et al., 1974). In this study, 20E infusion (5 mg/kg/day) for five or fifteen days did not result in an increase in body weight. Toth et al. (2008) reported an increase in body weight with single daily s.c. injections of 20E (5 mg/kg) for 8 days in rats. Conversely, others have indicated no difference in body weight with 20E treatment with daily veinous injections of 20E (1 mg/kg) for 3 weeks in mice (Gao et al., 2008). The circumstantial reports and our study suggest that 20E may not have potent growth enhancement effects. However, an increase in the weight of triceps brachii was demonstrated. Other muscle specific increases in the soleus, extensor digitorum longus and tibialis anterior have been reported for rats given daily administration of 20E orally or by s.c. injection (Toth et al., 2008; Syrov, 2000). Further research involving the optimization of dose, duration and route of administration of

phytoecdysteroids, to demonstrate reproducible responses *in vivo*, would be required to show anabolic efficacy.

An osmotic pump implant provided a well-defined and continuous infusion of 20E to address issues of bioavailability and stress from daily handling and injections and allow for biological effects to develop. Elimination of 20E in mice is rapid, with a half-life of 8.15 min and approximately 90% of the compound eliminated in 30 min after caudal vein injection at 50 mg/kg (Dzhukharova et al., 1987). The anabolic drugs, salmeterol and salbutamol did not promote anabolic effects when given orally, but resulted in significant increases in muscle and body mass when infused via osmotic pump implants. Absence of anabolic effects may have been due to poor absorption when administered orally and the need for a longer duration of continuous exposure for $\beta_2$-adrenoreceptor activation (Moore et al., 1994; Choo et al., 1992). Although continuous exposure of 20E did not increase the body weight of the mice in this study, a muscle specific increase in the weight of the triceps was observed for the five day infusion of 20E but not the fifteen day infusion. Likewise, clenbuterol, a potent anabolic drug, demonstrated rapid anabolic action after two days, but a decline was noted after the about tenth day with continuous drug exposure (unpublished observations by Choo et al., 1992). It is possible that similar response occurred in this study in which there was an increase followed by a decrease in skeletal muscle weight due to adaptive changes from prolonged continuous administration.

In a preliminary study (data not shown), dystrophic *mdx* mice were selected to evaluate possible adaptogenic or normalizing effects of 20E when administered to an animal model with the specific stress of muscular dystrophy. *Mdx* mice (strain C57BL/10ScSn-*Dmd*$^{mdx}$/J) were given daily oral step-wise doses of 20E (0, 10, 30 or 50 mg/kg) as a gel-solidified AIN 93G supplement once a day for 28 days, with seven animals per treatment group, but did not exhibit

significant treatment changes in our endpoints of measure. This design allowed for voluntary consumption of the test compound and eliminated any stress from handling and forced feeding methods such as gavage. However, despite full assurance that the mice consumed recorded, defined doses of 20E, there were no significant differences in the paramenters measured (treadmill performance, weight of skeletal muscle groups, weight of fat pad, and body weight) between treated animals and untreated controls. However, perhaps the physiological endpoints of measure, selected from previous published effects, were not an accurate measure of the effects of 20E. In view of that, microarray analysis was selected for its sensitive and robust measure of changes in gene expression and the breadth of information available from the whole mouse genome.

A whole genome mouse array was used to screen for molecular targets of action and IPA was used to identify biological functions and pathways with the most evidence for differential expression in response to 20E, which included cell-to-cell signaling, and interaction and cellular growth and proliferation. *In vivo* studies using radiolabeled precursors of protein, RNA and DNA synthesis demonstrated dynamic effects of 20E on molecular processes in organs of mice (Todorov et al., 2000). *In vitro* studies using C2C12 murine myotubes suggested a phosphoinositide kinase-3 (PI3K) mediated mechanism that activates Akt, a serine/threonine-specific protein kinase, to increase protein synthesis (Gorelick-Feldman et al., 2009). Additionally, adaptogenic extracts have been suggested to exert their normalizing effects via molecular chaperone proteins (Ponassian et al, 2009). Anabolic actions of androgens occur via multiple pathways in skeletal muscle including promoting satellite cell or myoblast proliferation (Diel et al., 2008) and increasing muscle protein synthesis (Scheffield-Moore, 2000; Maclean et

al., 2008). 20E may exert influence on cell signaling mechanisms and protein interaction rather than effects on gene expression.

Therapeutic interest in phytoecdysteroids also includes applications in gene therapy where phytoecdysteriods are targeted for use as inducers in gene switch constructs because they are considered innocuous in mammals (Saez et al., 2000). Gene expression effects of 20E were tested in human embryonic kidney cells (HEK 293) using microarray and found that 20E did not affect gene expression in endogenous genes (Panguluri et al., 2007). This study did not find pronounced effects on gene expression by 20E *in vivo*, which may be of benefit gene switch systems, but given a physiological response of an increase in muscle weight, other molecular signaling responses are not completely excluded.

The potential therapeutic applications and commercial market for phytoecdysteroid-containing supplements illustrate the importance of discerning their specific biological activities, clarifying claims of anabolic potency and providing insight on *in vivo* mechanisms of action in mammals. Administration of 20E by continuous infusion did not demonstrate potent anabolic properties, however, there was a significant increase in the weight of the triceps brachii. Genes and biological pathways identified by IPA provide potential leads on the mechanism of 20E influence *in vivo*.

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**Tables and Figures**

Figure 5.1. 20-Hydroxyecdysone

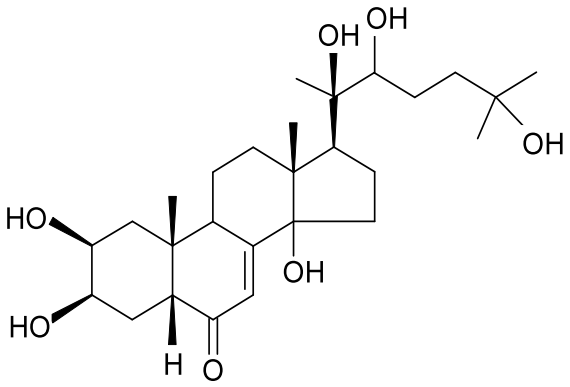


Table 5.1 Body weights, average daily feed intake, muscle group weights and organ weights of mice infused with saline (control) or 20-hydroxyecdysone (20E) for 5 days or 15 days reported as the mean +/- standard error. * Significantly different from control, $p < 0.05$.

| | Treatment | | | |
|---|---|---|---|---|
| | 5 days | | 15 days | |
| | Saline | 20E | Saline | 20E |
| **Body Weight (g)** | | | | |
| Initial | 23.1 +/- 0.5 | 23.0 +/- 0.5 | 23.3 +/- 0.6 | 23.1 +/- 0.6 |
| Final | 24.4 +/- 0.8 | 24.3 +/- 0.7 | 23.8 +/- 0.6 | 23.4 +/- 0.5 |
| **Avg daily feed intake (g)** | 3.0 +/- 0.2 | 3.2 +/- 0.1 | 3.1 +/- 0.1 | 3.1 +/- 0.1 |
| **Muscle groups (mg)** | | | | |
| Tibialis anterior | 61 +/- 4 | 63 +/- 3 | 66 +/- 5 | 62 +/- 4 |
| Gastrocnemius | 137 +/- 4 | 135 +/- 3 | 128 +/- 4 | 131 +/- 4 |
| Triceps | 88 +/- 3 | *115 +/- 8 | 94 +/- 8 | 89 +/- 5 |
| Biceps | 173 +/- 10 | 161 +/- 16 | 169 +/- 12 | 170 +/- 8 |
| **Organs (mg)** | | | | |
| Kidney | 361 +/- 18 | 355 +/- 8 | 357 +/- 15 | 386 +/- 15 |
| Heart | 142 +/- 7 | 158 +/- 18 | 141 +/- 7 | 145 +/- 10 |
| Liver | 913 +/- 89 | 953 +/- 73 | 1037 +/- 44 | 1058 +/- 49 |

Table 5.2 Genes with the greatest fold-change in mRNA abundance in triceps brachii of mice infused with 20-hydroxyecdysone for 5 days or 15 days (positive fold-change indicates increased mRNA abundance and a negative fold-change indicates a decrease in mRNA abundance).

| **5 days** | | | | |
|---|---|---|---|---|
| **Symbol** | **Gene Name** | **Fold-change** | **p-value** | **Illumina ID** |
| HOXC9 | Homeobox C9 | 42.5 | 0.005 | ILMN_2772764 |
| RECQL | RecQ protein-like (DNA helicase Q1-like) | 21.0 | 0.006 | ILMN_1249655 |
| TOR2A | Torsin family 2, member A | 20.8 | 0.007 | ILMN_2519488 |
| SPPL2A | Signal peptide peptidase-like 2A | 18.0 | 0.0003 | ILMN_2914347 |
| KCNS2 | Potassium voltage-gated channel | 17.2 | 0.010 | ILMN_2757688 |
| FBXW8 | F-box and WD repeat domain containing 8 | 16.5 | 0.002 | ILMN_2708519 |
| AOC3 | Amine oxidase, copper containing 3 | 16.4 | 0.010 | ILMN_1235989 |
| KIAA1279 | KIAA1279 | 16.1 | 0.004 | ILMN_1252989 |
| KCNC3 | Potassium voltage-gated channel | 15.3 | 0.005 | ILMN_1220038 |
| RGS3 | Regulator of G-protein signaling 3 | 14.0 | 0.008 | ILMN_1255271 |
| SEPT6 | Septin 6 | -27.0 | 0.002 | ILMN_2625867 |
| COL17A1 | Collagen, type XVII, alpha 1 | -24.5 | 0.003 | ILMN_2879995 |
| LMX1A | LIM homeobox transcription factor 1, alpha | -23.6 | 0.002 | ILMN_2823980 |
| GCNT2 | Glucosaminyl (N-acetyl) transferase 2 | -16.2 | 0.006 | ILMN_2678801 |
| DSTYK | Dual serine/threonine and tyrosine protein kinase | -15.9 | 0.001 | ILMN_2724910 |
| NPHP4 | Nephronophthisis 4 | -15.8 | 0.002 | ILMN_2641152 |
| BAHCC1 | BAH domain and coiled-coil containing 1 | -14.2 | 0.006 | ILMN_2741694 |
| CCL20 | Chemokine (C-C motif) ligand 20 | -13.7 | 0.007 | ILMN_1218692 |
| CNTNAP4 | Contactin associated protein-like 4 | -11.9 | 0.004 | ILMN_2932508 |
| FOXO3 | Forkhead box O3 | -11.7 | 0.028 | ILMN_2690574 |
| **15 days** | | | | |
| **Symbol** | **Gene Name** | **Fold-change** | **p-value** | **Illumina ID** |
| LRFN3 | Leucine rich repeat and fibronectin type III domain | 25.7 | 0.008 | ILMN_2771787 |
| TSPAN17 | Tetraspanin 17 | 23.3 | 0.0004 | ILMN_2817717 |
| RHO | Rhodopsin | 17.3 | 0.003 | ILMN_1228510 |
| STYXL1 | Serine/threonine/tyrosine interacting-like 1 | 15.5 | 0.001 | ILMN_2680229 |
| LTBP1 | Latent transforming growth factor beta binding protein 1 | 15.0 | 0.002 | ILMN_1234779 |
| USP26 | Ubiquitin specific peptidase 26 | 14.0 | 0.019 | ILMN_2960235 |
| CD86 | CD86 molecule | 11.7 | 0.010 | ILMN_1216386 |
| GANAB | Glucosidase, alpha; neutral AB | 11.2 | 0.012 | ILMN_1231453 |
| PTPRO | Protein tyrosine phosphatase, receptor type, O | 10.6 | 0.002 | ILMN_1213978 |
| RAB11B | RAB11B, member RAS oncogene family | 10.4 | 0.037 | ILMN_2500700 |
| PATE4 | Prostate and testis expressed 4 | -61.5 | 0.001 | ILMN_2797040 |
| SAA2 | Serum amyloid A2 | -35.9 | 0.096 | ILMN_2768154 |
| INA | Internexin neuronal intermediate filament protein, alpha | -27.9 | 0.002 | ILMN_2836494 |
| ITIH3 | Inter-alpha (globulin) inhibitor H3 | -23.1 | 0.179 | ILMN_1231336 |
| DAB1 | Disabled homolog 1 (Drosophila) | -20.3 | 0.003 | ILMN_2658980 |
| SSH1 | Slingshot homolog 1 (Drosophila) | -17.6 | 0.001 | ILMN_1244009 |
| BCL2A1 | BCL2-related protein A1 | -13.9 | 0.174 | ILMN_2660555 |
| CMAH | Cytidine monophosphate-N-acetylneuraminic acid hydroxylase | -13.8 | 0.006 | ILMN_2626252 |
| FNBP1 | Formin binding protein 1 | -13.7 | 0.002 | ILMN_3115777 |
| CD3G | CD3g molecule, gamma (CD3-TCR complex) | -13.3 | 0.242 | ILMN_2722784 |

Table 5.3. Top diseases and biological functions and the number of genes involved in each system identified by IPA. The p-value associated with each gene indicates the probability of the biological function or disease assigned to the dataset by chance alone. A range of p-values is provided as IPA assigned multiple genes to the biological functions and diseases.

**Diseases and Biological Functions**

| 5 days | Genes | p-value |
|---|---|---|
| **Diseases and Disorders** | | |
| Cancer | 12 | 4.69E-05 - 4.60E-02 |
| Reproductive System Disease | 6 | 4.69E-05 - 3.31E-02 |
| Gastrointestinial Disease | 7 | 2.66E-04 - 3.31E-02 |
| Hepatic System Disease | 3 | 2.66E-04 - 2.66E-02 |
| Cardiovascular Disease | 9 | 5.40E-04 - 3.96E-02 |
| **Molecular and Cellular Functions** | | |
| Cellular Movement | 10 | 3.17E-04 - 4.12E-02 |
| Cell-To-Cell Signalling and Interaction | 18 | 6.29E-04 - 3.96E-02 |
| Cellular Growth and Proliferation | 10 | 1.05E-03 - 4.60E-02 |
| Carbohydrate Metabolism | 8 | 1.22E-03 - 4.60E-02 |
| Cellular Develoment | 10 | 1.56E-03 - 3.96E-02 |
| **Physiological System Development and Function** | | |
| Tumor Morphology | 4 | 4.69E-05 - 3.37E-02 |
| Cell-mediated Immune Response | 7 | 3.17E-04 - 4.12E-02 |
| Hematological System Development and Function | 16 | 3.17E-04 - 4.12E-02 |
| Immune Cell Trafficking | 12 | 3.17E-04 - 4.12E-02 |
| Nervous System Development and Function | 9 | 7.69E-04 - 4.60E-02 |
| **15 days** | **Genes** | **p-value** |
| **Diseases and Disorders** | | |
| Inflammatory Response | 12 | 2.35E-03 - 4.64E-02 |
| Neurological Disease | 33 | 2.76E-03 - 4.64E-02 |
| Cancer | 37 | 3.06E-03 - 4.64E-02 |
| Hematological Disease | 8 | 3.06E-03 - 4.64E-02 |
| Reproductive System Disease | 23 | 4.53E-03 - 4.64E-02 |
| **Molecular and Cellular Functions** | | |
| Cellular Compromise | 9 | 8.89E-05 - 4.64E-02 |
| Cell Morphology | 17 | 8.72E-04 - 4.64E-02 |
| Protein Trafficking | 2 | 1.30E-03 - 1.30E-03 |
| Cell-To-Cell Signaling and Interaction | 13 | 2.35E-03 - 4.64E-02 |
| Cellular Assembly and Organization | 19 | 2.82E-03 - 4.70E-02 |
| **Physiological System Development and Function** | | |
| Renal and Urological System Development and Function | 9 | 8.72E-04 - 3.73E-02 |
| Hematological System Development and Function | 19 | 2.35E-03 - 4.64E-02 |
| Embryonic Development | 8 | 2.40E-03 - 3.73E-02 |
| Hair and Skin Development and Function | 7 | 2.40E-03 - 3.73E-02 |
| Tissue Development | 14 | 4.62E-03 - 4.64E-02 |

Table 5.4. Top canonical pathways identified by IPA. Ratios indicate the number of genes from the data set that map to the canonical pathway divided by the total number of molecules of the canonical pathway. The p-value indicates the probability of the pathway assigned to the dataset by chance alone.

**Top Canonical Pathways**

| **5 days** | **Genes** | **Ratio** | **p-value** |
|---|---|---|---|
| Interferon Signaling | SOCS1, TYK2 | 2/30 | 0.03 |
| TGF-β Signaling | INHA, HOXC9, ACVR2A | 3/96 | 0.04 |
| Phenylalanine Metabolism | AOC3, EPX | 2/109 | 0.04 |
| Aminophosphonate Metabolism | PCYT1B, PIGO | 2/65 | 0.05 |
| **15 days** | | | |
| Methane Metabolism | EPX, PRDX2 | 2/66 | 0.02 |
| Stilbene, Coumarine and Lignin Biosynthesis | EPX, PRDX2 | 2/78 | 0.02 |
| Hypoxia Signaling in the Cardiovascular System | BIRC6, HIF1A, NOS3, UBE2L6 | 4/70 | 0.02 |
| Phototransduction Pathway | GNB3, RHO | 2/63 | 0.05 |

Table 5.5a. Top networks identified by IPA for the response to 20E infusion for 5 days. Focus genes were identified as those with raw p-values < 0.01. A score for each network was calculated by taking the negative log of the p-value and indicates the likelihood of the focus molecules being found in a network due to chance alone.

**Top Biological Networks**

| **5 days** | Score | Genes |
|---|---|---|
| **Lymphoid Tissue Structure and Development, Dermatological Diseases and Conditions, Hair and Skin Development and Function** | 45 | 22 |
| ADARB1, Akt, ARFIP2, ARPC5, ARRB1, ASXL2, CBR3, CCL20, Cofilin, DGAT1, DOK5, GCNT2, GHR, GLT8D3, HLX, IKK (complex), IL1, IL12 (complex), Interferon alpha, JAK, KIFAP3, Mapk, NFkB (complex), PDGF BB, PI3K, PRF1, Rac, RBP1, RGS3, RIPK2, SOCS1, SSH1, Tnf receptor, TRAF6, TYK2 | | |
| **Neurological Disease, Cancer, Embryonic Development** | 30 | 16 |
| GHR, HNF4A, KCNC3, KLHDC3, MIRN331, NPC2, NPNT, PGM1, PIGH, PIGP, PIGY (includes EG:84992), PIK3R3, PINK1, PKM2, RAB34, RECQL, RIOK1, SORBS1, SP1, TM7SF2, TMEM43, TNC, WBP4, XPR1 | | |
| **Cellular Movement, Nervous System Development and Function, Cancer** | 30 | 16 |
| BAHCC1, BTBD10, CACNA2D1, CASK, CELA2A, CNTNAP4, COL17A1, CXCL12, DSTYK, EPB41L1, ITGA10, ITGA8 (includes EG:8516), ITGB1, KCNS2, LAMA5, MAPRE3, MIR292, MIR133A-2, MIR133B (includes EG:723817), MIR15B (includes EG:406949), MIR199A1, MIR302A (includes EG:407028), MMP9, NPHP4, NPY2R, OLFM1, PAX3, PHF12, PUM2, PXDN, RC3H2, RGS3, SIGIRR, TSPAN3, ZDHHC5 | | |
| **Cell Death, Hematological System Development and Function, Cancer** | 24 | 14 |
| ARRB1, C11ORF82, C16ORF48, DUT (includes EG:1854), EFEMP2, EPX, GIP2, GIT2, Glutathione peroxidase, GMFB, GPX3, GPX8, IFI30, IFNG, IKIP, KIAA1279, KIF1B, KRT82, MGST2, NFATC2IP, NUP98, PRPF4B, RAE1, RIPK2, SGTA, SGTB, SMARCA4, SPATA2, SPPL2A, TBX3, TNFRSF18, TP53, TRAF2, YWHAG, ZNF638 | | |
| **Cancer, Amino Acid Metabolism, Post-Translational Modification** | 23 | 13 |
| Amino acids, BCL3, C9ORF3, CCL24, CD300A, DNAJB6, EPB49, Il12 receptor, INTS12, KALRN, KIT, MAP4K4, MET, MLXIP, N4BP2, NDEL1, PIAS3, PIBF1, PINK1, PLOD2, PPM2C, RAMP2, SHP, SLC16A3, SLC8A1, SLC8A3, SOCS1, SPSB4, STAT4, STAT6, STK17A, TGFB1, TYK2, YWHAE, | | |
| **Endocrine System Development and Function, Organ Morphology, Skeletal and Muscular System Development and Function** | 20 | 12 |
| Actin, Activins / Inhibins, ACVR2A, ACVR2B, AOC3, ARRB1, B3GAT3, ERK, EZH2, FGF4, FXN, GDF5, Histone h3, IGSF1, INHA, INHBC, INHBE, Insulin, LIMA1, MIRN326, MKNK1, MZF1, P38 MAPK, Proteasome, PRX, PTEN, RGS3, RIPK2, SBF1, SELT, SLC22A16, SOX12, TGFBR3, Type II Receptor, VEGFB (includes EG:7423) | | |
| **Cancer, Cell Death, Endocrine System Disorders** | 16 | 10 |
| BUB3, C11ORF51, CASP1, CEP57, Cofilin, CSDE1, DHX30, E2F4, ENO1, FAM38B, FBXW8, GNPAT, GSTT2, HDAC1, HNRNPA1, HOXC9, INS1, LGMN, LIMA1, LMX1A, MIR18A, MIRLET7A1, MYC, PEG3, PIGO, PMP22, PREP, PRKCSH, SLC25A12, TBX18, TMPO, TNC, TOP2A, TOR2A, YME1L1 | | |
| **Cell Cycle, Carbohydrate Metabolism, Cellular Compromise** | 12 | 8 |
| ABCA2, ABCC1, ACTB, ACTR10, ACTR1B (includes EG:10120), AFG3L1, AFG3L2, ATP8, ATP11B, ATP13A2, ATP5H (includes EG:10476), ATP5S, ATP6V1G2, ATPase, CDC42EP2, CDC42EP5, CDH1, CLPX, DDX19B, DDX3Y (includes EG:26900), DHX15, DHX16, KATNA1, KIF1B, MYH1, MYO9A, PCYT1B, PKP4, RECQL5, SEPT6, SEPT7, SEPT9, SMARCA4, TOP3B, VPS4B | | |

Table 5.5b. Top networks identified by IPA the response to 20E infusion for 15 days. Focus genes were identified as those with raw p-values < 0.01. A score for each network was calculated by taking the negative log of the p-value and indicates the likelihood of the focus molecules being found in a network due to chance alone.

**Top Biological Networks**

| **15 days** | Score | Genes |
|---|---|---|
| **Cell-To-Cell Signaling and Interaction, Cell Signaling, Neurological Disease**<br>Akt, ARRB1, ASXL2, BIRC6, Calmodulin, Caspase, CMA1, DAB1, Gpcr, GPR65, Hdac, HIF1A, Hsp70, Hsp90, Ifn, INA, INVS, LCN2, MYO5A, NFkB (complex), P2RY6, PHKB, PRDX2, Proteasome, Rb, RIPK2, S1PR2, SPIN1, TRAF6, TTN, UBE2, UBE2L6, Ubiquitin, UBR4, USP2 | 38 | 22 |
| **Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Inflammatory Response**<br>ACTN4, Alpha Actinin, AQP1, BMP6, CD86, Collagen(s), CTSB, Cytochrome c, DOCK10, DOK1, EPX, ERK, GTF2I, Ige, Jnk, LDL, LTBP1, Mek, Nfat (family), NOS3, P38 MAPK, Pdgf, PDGF BB, PF4, Pkc(s), PLEKHA1, PPP3CA, PRKD1, SSH1, TES, Tgf beta, TRIB1, USF2, Vegf, VWF | 32 | 20 |
| **Cell-To-Cell Signaling and Interaction, Skeletal and Muscular System Development and Function, Cellular Growth and Proliferation**<br>ABPA (includes EG:11354), APP, ATL3, C6ORF138, C7ORF60, CPEB2, DCP1A, F2, GNB3, GP1BB, HDAC4, HIF1A, HMG20A, HS3ST2, IL1B, IQSEC2, MARK2, MIR294, MIR214 (includes EG:406996), MIR297-2, MIRLET7B (includes EG:406884), PF4, PLXDC2, PPM1G, RAB38, RC3H2, RPL28, RTN3, SIPA1L3 (includes EG:23094), SLC38A9, SMAD4, TGFBR1, TPBG, TSR1, YWHAZ | 28 | 18 |
| **Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function**<br>2210023G05RIK, ALKBH6, ALOX5AP, C1ORF50, Ces, CES1 (includes EG:1066), CMA1, CMAH, DOK1, ELOVL1, ESD, GP5, GPBP1, GTF2B, HIF1A, HNF1A, IGJ, IL3, IL4, IL6, IL19, IL-1R, JUN, KITLG, LAMP2, MRVI1, norepinephrine, PIWIL1, PPM1G, PTPRO, STT3A, SVIL, TEC, TRAF7, USF2 | 25 | 16 |
| **Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function, Cellular Development**<br>ABL1, ABR, ACOT1 (includes EG:26897), ANGPTL4, BATF2, CCDC120, CDC42SE1, DAZAP2 (includes EG:9802), DLX5, DNM3, FAM127A, FNBP1, FOS, GNPAT, HIF1A, INS1, KALRN, LNPEP, LRBA, LRFN3, LTBP1, LTBP2, MIR125B1, PLXNB1, RAC1, RBL2, RHO, RNF208, SDPR, SFRP1, SOCS7, STAT3, TCN2, TGFB1, TNKS | 25 | 16 |
| **Cancer, Cell Cycle, Nucleic Acid Metabolism**<br>AQR, BIRC5, BIRC6, butyric acid, CCNB2, CCNF, CDCA8, COL14A1, CREM, CUL7 (includes EG:9820), FAM62B, FBXW8, FRMD6, HDAC4, HIF1A, HIST1H1C, IFI202B, IFIT3, MDM4, MEST, MYOD1, POU5F1, RASSF8, RBL2, RRM2, RRM1 (includes EG:6240), RRM2B, SERPINB2, SKP1, TGFBI, TP53, TSPAN17, WNT6, YWHAG, ZFHX3 | 24 | 16 |
| **Cancer, Cell Cycle, Cellular Growth and Proliferation**<br>ABCC1, AQP1, BCL9L, beta-estradiol, BOP1, CCDC80, CCNE2, CHKA, COPG2, CTNNB1, DHH, GCN1L1, HHIP, HRAS, IFNA2, IL2, IMPACT, KLK6, MTMR4, MYC, PDCD11, PMP22, PTCH1, PTCH2, PXDN, RAPGEF4, RBL2, RET, SDK1, SEMA3C, SFRP1, SUSD2, TGTP, USP9X, ZC3HAV1 | 24 | 15 |
| **Cellular Development, Embryonic Development, Organismal Development**<br>ABCB8, ABCF3, ACIN1, BTBD10, CCDC44, CLPX, DDOST, DERA, DHX8, DNAJC30, EAF1, ELL, HNF4A, KDELC1, KIF1B, LDB1, MREG, OSBPL11, PAAF1, PSMC1, PSMC2, PSMC3, PSMC4, PSMD1, PSMD7, PSMD8, RAD51, SMC1A, SPAST, SRPRB, SSBP4, STYXL1, SUCLA2, TFPT, TMUB2 | 21 | 13 |
| **Cancer, Reproductive System Disease, Gene Expression**<br>Actin, ATAD4, ATP6AP2, BCAT2, BUB1, CCNE2, CHKA, CRADD, CYB561, DAB1, DHRS7 (includes EG:51635), Egfr-Erbb2, ERBB2, ERCC8, F Actin, FBLN2, HIF1A, Histone h3, IL1, Insulin, Interferon alpha, Mapk, MBD1, MT1G, MYO5A, Myosin5, PI3K, RBL2, RHO, RNA polymerase II, RNASE2, SHROOM3, SUPT16H, SYNE2, WNT5B | 17 | 12 |

# CHAPTER 6

# CONCLUSION

A plethora of biological activities for phytoecdysteroids and phytoecdysteroid-containing botanical extracts have been reported, with research continuing to expand their functional utility. Among the major areas of research for phytoecdysteroids are 1.) crop protection through herbivorous insect deterrence and 2.) growth and physical performance enhancement in mammals after ingestion (Schmelz et al., 2000; Báthori et al., 2008). In this program of research, phytoecdysteroid accumulation was investigated in *Ajuga turkestanica* (Regel) Briq., a medicinal herb indigenous to Central Asia, and in spinach (*Spinacia oleracea* L.), a valuable crop produced worldwide. Additionally, purported bioactivities of phytoecdysteroids and phytoecdysteroid-containing plant extracts used in traditional and modern applications were examined in *in vitro* and *in vivo* mouse models. Overall, this research program investigated phytoecdysteroid production in plants and the advantages they may present in plants and mammals.

Specifically, hairy root cultures developed from *A. turkestanica* were shown to be a controllable and sustainable alternative to wild-harvesting plants for medicinal phytochemicals (Chapter 2). In addition, extracts from wild-harvested *A. turkestanica* shoots and *A. turkestanica* hairy root cultures enhanced protein synthesis in murine cell cultures. In spinach, significant variations in phytoecdysteroid accumulation in seeds and shoots were demonstrated, suggesting the potential for genetic engineering or convention breeding to increase phytoecdysteroid levels for further evaluation of insect deterrent properties and biosynthetic regulation (Chapter 3). A review of approaches to investigate effects of phytoecdysteroids in animal models led to the evaluation of continuous infusion of 20-hydroxyecdysone (20E), the predominant

phytoecdysteroid, on mouse body tissue composition and skeletal muscle gene expression (Chapters 4 and 5). The mass of the muscle group triceps brachii were significantly increased in mice given a continuous infusion of 20E for five days, however, no other differences were observed. Ingenuity Pathways Analysis was used to identify genes with the most evidence for differential expression from microarray gene expression data, which included genes involved in cellular growth and proliferation and cell-to-cell signaling and interaction. These results provide resources and leads for further research on phytoecdysteroids, which continue to demonstrate potential for the improvement of agricultural production and enhancement of human health.

Several *Ajuga* species are used as botanical medicines in indigenous cultures in Asia and Africa and their bioactive properties have, in part, been attributed to their high-phytoecdysteroid content (Israili, 2009). Development of *in vitro* plant production systems offers a sustainable source of valued phytochemicals and aids in the conservation of plants growing in the wild. In this study, *A. turkestanica* hairy root cultures were developed, however other medicinal plants and plant tissues are also suitable for the production of phytochemicals of interest using the plant's inherent production system. Thus, plants in culture may be optimized to produce parallel phytochemicals to their wildgrown counterparts.

In the field of botanical medicine, reproducibility of phytochemical extracts has been a continuous challenge due the complex mixtures produced by plants and batch-to-batch variations of wild-harvested plants from natural environmental fluctuations. Production of phytoecdysteroids from hairy root cultures of *A. turkestanica* demonstrated that *in vitro* plant systems are a controllable and reproducible method to produce complex phytochemical mixtures that would otherwise be impractical to synthesize individually. Conversely, *in vitro* plant production systems afford the opportunity to alter and manipulate phytochemical composition

for biosynthetic investigations. Production of phytochemicals of varying levels can be used to analyze biosynthetic pathways, through induction of regulatory enzymes that stimulate phytochemical production (Suzuki et al., 1996). *In vitro* systems developed from plant, animal, and bacterial cell cultures are tools used to determine specific biological functions.

The application of phytoecdysteroids as natural pesticides requires the evaluation of their insect deterrent properties *in situ,* that is, in combination with other phytochemicals and environmental variations, such as multiple insect species. In a field survey of various plants attacked by insect pests, *Ajuga remota*, which also accumulates a high level of phytoecdysteroids, was observed to be more resistant to insect attack compared to other plants in the same field plot (Kubo and Kloche, 1983). Extracts of *A. remota* and individual phytoecdysteroids were added to a solution on which insects feed and results demonstrated that phytoecdysteroids were part of a matrix of phytochemicals that confer protection against insect herbivory in the plants natural environment rather than the sole effective compound class (Kubo and Kloche, 1983). Thus, in certain instances, phytochemical mixtures may be equally as important in providing advantages to crop health as to human health.

Spinach has been a model plant used to uncover the phytoecdysteroid biosynthetic pathway, and as a crop consumed worldwide, would be a worthwhile plant to focus the evaluation of phytoecdysteriods in insect deterrence (Grebenok and Adler, 1993; USDA, 2007). The effectiveness of various phytoecdysteroids in disrupting insect function has been well established, and potency of individual phytoecdysteroids has been investigated using *in vitro* insect bioassays (Harmatha and Dinan, 1997). However, certain species have developed resistance to phytoecdysteroids by modifying phytoecdysteroid structures to become nontoxic (Blackford and Dinan, 1997). Development of spinach cultivars with step-wise accumulation

levels of phytoecdysteroids, in the context of a phytochemical matrix, on various insect species would determine their effectiveness in crop protection against pests and strengthen the support for a plant defense role.

The numerous biological activities attributed to dietary botanicals and phytochemicals can be more thoroughly evaluated with high-throughput "omic" technologies, to account for their diverse actions in mammalian systems. Omic refers to high-throughput genomic, proteomic and metabolomic platforms that can simultaneously screen thousands of genes, proteins and metabolites expressed in a biological system (Ulrich-Merzenich et al., 2007). As research continues to expand the list of purported bioactivities for phytoecdysteroids, which include cardioprotection against oxidation (Sahach et al., 2008), decreased weight gain in diet-induced obese mice (Kizelsztein et al., 2009) and improvement of bone health (Gao et al., 2008; Kapur et al., 2010), omic technology will continue to be a valuable tool to evaluate the multifaceted actions of phytochemical components of botanical medicines.

Omic technologies provide the capability to evaluate the chemical composition of complex mixtures of single plants or multiple plant concoctions used in traditional medicines, to efficiently and reproducibly standardize components in botanical medicines (Wang et al., 2009). Although, phytochemical mixtures are used for therapeutics in traditional practices such as traditional Chinese medicine and Ayurvedic medicine, scientific research has focused on the isolation of single bioactive compounds to which dietary botanical supplements are standardized (Yuan and Lin, 2000; Rege et al., 1999). However, completely different gene expression profiles can be induced from single compound treatment compared to a mixture that includes the target compound (Cheok et al., 2003). Additionally, gene expression does not account for the array of proteins in a biological system as the Human Genome Project revealed that there are over ten

times the number of proteins (~400,000) compared to the number of genes (~22,000) (Service, 2001; Ulrich-Merzenich et al., 2007). Thus, protein expression profiles may also uncover different modes of interactions in animal models induced by phytochemical mixtures. Metabolic activities of gut microflora alter the composition of phytochemicals post ingestion, adding further complexity and variations in biological activity (Bowey et a., 2003). Methods in the evaluation of complex biological systems of plants and animals, and their interactions, will greatly benefit from advances in omic platforms.

The term "adaptogenic" captures the broad range of protective and homeostasis-maintaining bioactivities for phytoecdysteroids. Adaptogens refers to botanicals and phytochemicals that increase the nonspecific resistance of an organism, without disruption to normal bodily functions (Brekhman and Dardymov, 1969). Although the term has been defined, the biological implications of botanicals and phytochemicals labeled as adaptogens are extensive and ambiguous. Investigations of adaptogens in omic platforms may help to categorize and define what constitutes an adaptogen according to prescribed genomic, proteomic and metabolomic expression profiles as progress continues in botanical medicine research.

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