EFFECTS OF BIOACTIVE COMPOUNDS FROM DIFFERENT POTATO GENOTYPES ON PROSTATE CANCER DEVELOPMENT IN ATHYMIC NUDE MICE

A Thesis

by

SARAH DIANE TURNER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2012

Major Subject: Plant Breeding

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ABSTRACT

Effects of Bioactive Compounds from Different Potato Genotypes on Prostate Cancer Development in Athymic Nude Mice. (May 2012)

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Chair of Advisory Committee: Dr. J. Creighton Miller, Jr.

Phytochemicals are widely noted for their role in chemoprevention. Potato (*Solanum tuberosum* L.) is the third most important food crop worldwide and is considered a significant source of antioxidants, providing an ideal delivery system for beneficial compounds. The anti-proliferative and pro-apoptotic properties of potato bioactive compounds have been reported *in vitro* on human prostate cancer cell lines. However, i*n vivo* studies are limited, and more information is needed to determine the chemopreventive properties of potato in the diet.

The objective of this study was to evaluate the effects of potato bioactives on prostate cancer *in vivo* using a mouse model. Athymic nude mice received xenografts of human prostate cancer cells (PC-3) and were administered extracts of potato bioactives from either the white flesh *Solanum bulbocastanum* (PI243510) or CO112F2-2P/P (purple-flesh Colorado selection), while control mice received water. Neither potato extract provided a significant reduction in tumor growth nor reduced levels of the pro-angiogenic protein VEGF, but the *S. bulbocastanum* extract reduced expression of

metastasis associated protein 1 (MTA1) in tumors, and both potato extracts reduced MTA1 expression in lungs, suggesting the need for further research on the potential chemopreventive or chemotherapeutic properties of potato bioactives.

DEDICATION

This thesis is dedicated to the mice used in this study and to the potato, my favorite vegetable.

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1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is the third most important food crop worldwide and the leading vegetable crop in the United States, with a per capita consumption of ~117lbs in 2010 (1). Potatoes represent up to 32% of the vegetable consumption in the US (2), providing an ideal system for the delivery of health promoting phytochemicals via the diet. Phytochemicals are widely noted for their role in the prevention of chronic illnesses and are well documented as chemopreventive agents (2-10). Thus, increasing the amount of phytochemicals in potato, such as phenolics, carotenoids, and glycoalkaloids, may offer a practical means of delivering health promoting compounds and changing the market perception of both fresh and processed potato products (9,11-15).

Prior investigations have indicated that potato extracts exhibit cytotoxic activity against prostate cancer cell cultures (2,9,10). In Western countries, prostate adenocarcinoma is the second leading cause of cancer-related death in males (8,16,17), with one in 36 males dying of related complications (17). For 2010, it is estimated that there were 217,730 new cases of prostate cancer and 32,050 prostate-cancer-related deaths (16). Dietary prevention is a viable mechanism for reducing the incidence of prostate cancer, based on the late onset and delayed progression of the disease (10). Onset is around 50 years of age, with progression occurring in approximately 10 to 20 years (16).

This thesis follows the style of *Nutrition and Cancer*.

This study was designed to further evaluate the anti-prostate cancer activity of potato, which would improve the marketability of potato as part of a healthy diet. The objective was to evaluate the anti-prostate-cancer activity of potato bioactives in a tumor xenograft model. This experiment also compared two genotypes of potato, a purple-flesh, Colorado breeding line (CO112F2-2P/P), which was selected for high phenolic content and antioxidant activity (18), and a white-flesh accession of *Solanum bulbocastanum* (PI243510), a wild relative of the cultivated potato with a diverse glycoalkaloid profile (19). This study examined the effects of potato bioactives on tumor growth, factors related to cancer progression, and body weight as a measurement of overall health. Information gathered can be utilized to promote selection of potato cultivars with a higher content of beneficial compounds.

1.1 Bioactive Compounds in Potato

Phytochemicals, many of which function as antioxidants and have anticarcinogenic activity (2,3,5-10,13,20), represent a diverse group of secondary metabolites that are produced in response to biotic and abiotic stresses (21). The major classes of phytochemicals are carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (4). Potato germplasm primarily contains phenolics, carotenoids, and alkaloids, in addition to other antioxidants such as glutathione, which maintains the quality and levels of other antioxidants, vitamin C, selenium, α-lipoic acid, and α-tocopherol (5,14,21,22). Andre et al. (15) reported that potatoes rank third in the daily intake of phenolics and antioxidants from fruits and vegetables, providing an

average daily polyphenol intake of 64 mg per capita in the US (14). Potatoes are also a good source of other nutrients, such as proteins, carbohydrates, potassium, phosphorus, magnesium and vitamins B6 and B3 (15).

Phenolics

Phenolic compounds are the most abundant phytochemicals in potato (15) and include phenolic acids, flavonoids, stibenes, coumarins, and tannins (4,6,23,24). In potatoes, the major phenolics are phenolic acids and flavonoids. Phenolics also exist in free and bound states, of which the latter remains bioavailable in the colon, providing more opportunities for health promoting activity (6). Most fresh vegetables are only capable of releasing about one fourth of their phenolics in the colon, while up to half of potato bioactives can potentially be absorbed (6).

The phenolic content of potatoes is highly characterized. Phenolic acids, mostly chlorogenic acid, constitute 45-90% of the total phenolic content in potatoes (15,21). Up to 50% of the phenolic content is found in the skin and surrounding tissues, decreasing towards the center of the tuber (21). Tuber skin primarily contains chlorogenic, protocatechuic, and caffeic acids (18), but vannilic, *p*-coumaric, ferulic, sinapic, salicylic, and many unidentified phenolic acids are also present (23). In tuber flesh, the major phenolic acid is chlorogenic acid, but low concentrations of caffeic, protocatechuic, *p*-coumaric, ferulic, gallic, and sinapic acids are also present (23).

Flavonoids are categorized as flavonols, flavones, flavanones, anthocyanidins, and isoflavonoids. The flavonols kaempferol, quercetin, and myricetin are found in tuber flesh, but are not major contributors to the phenolic activity of potatoes (23). Trace amounts of catechin, epicatechin, and eriodictyol are also found in tuber flesh (23). Moderate amounts of flavanones, in particular eriodictyol and naringenin, are also found in tuber skin (23). Phenolic acids do not impact the color of tuber skin and flesh, while anthocyanins are responsible for red-, blue-, and purple-flesh colors (23,25). Comparatively, red- or purple-flesh potatoes have approximately two to threefold the antioxidant content found in white-flesh potatoes (26-28), levels which are competitive with brussels sprouts and spinach (26). The primary glycosides in red potatoes are pelargonidin and peonidin, while purple potatoes contain glycosides of pelargonidin, malvidin, delphinidin, peonidin, and petunidin (14,28).

Chu et al. (6) estimated the quality and quantity of phenolics found in the ten most common vegetables in the US (broccoli, spinach, yellow onion, red pepper, carrot, cabbage, potato, lettuce, celery, and cucumber). Of the ten vegetables analyzed, potato had the highest content of bound phenolics (39.9%), a total phenolic content of 23.31 ± 5.83 mg gallic acid equivalents (GAE) /100 g FW, and a total antioxidant activity of 4.86 ± 0.2 µmol of vitamin C equiv/g FW of sample. Potato ranked eighth out of 10 for antioxidant activity and seventh out of 10 for total phenolic content, but Nzaramba et al. (7) suggested that potato ranks second when per capita consumption is considered. It is

also worth noting that the study by Chu et al. (6) study did not account for genetic variation, which plays a major role in phenolic content.

Carotenoids

Andre et al. (15) reported that degree of yellow pigmentation was strongly correlated with carotenoid content. In potatoes, the major carotenoids in tuber flesh are xanthophylls, namely lutein, neoxanthin, zeaxanthin, antheraxanthin, and violaxanthin, which are all putative antioxidants (15,23,25,26). The potentially major contributors to total carotenoid content in yellow-fleshed tubers are zeaxanthin (6-43%) and lutein (9-27%).

Glycoalkaloids

In addition to phenolic compounds, potatoes also contain glycoalkaloids, a class of secondary metabolites that cause adverse symptoms at high levels, but also exhibit beneficial effects, including anticancer activity (19,29,30). Glycoalkaloids are valued for their role as plant defense compounds, providing protection against various pathogens and herbivores, but considered are antinutrients in the human diet (8,29,30). As a result, glycoalkaloid content is strictly monitored in commercial potatoes.

Glycoalkaloids are derived from cholesterol and are characterized by an oligosaccharide, a C27 steroid, and a heterocyclic nitrogen component (19). There are two major classes of glycoalkaloids, solanidane aglycones and spirosolane aglycones (19). The

glycoalkaloids solanine and chaconine account for over 90% of the total glycoalkaloid (TGA) content in cultivated potatoes, with chaconine occurring in higher concentrations (19). However, the diversity and biosynthesis of glycoalkaloids in potato is not well characterized (19). Wild species of *Solanum* contain many novel glycoalkaloids not found in cultivated potatoes, providing a resource for genetic variability (8,19).

TGA in potatoes is estimated as the combined amounts of α-solanine and α-chaconine (mg TGA/kg) and cannot exceed 200 mg/kg of potato, at which point potatoes develop a bitter taste and consumption causes adverse symptoms (19,30-32). Mild exposure to glycoalkaloids results in gastrointestinal discomfort (nausea, vomiting, diarrhea), and may aggravate irritable bowel syndrome, while severe exposure may result in neurological responses (19,32,33). There is also evidence that glycoalkaloids have cardio-toxic and teratogenic effects (34,35). The toxicity of glycoalkaloids primarily results from disruption of cell membranes and inhibition of acetocholinesterase (AChE) (30,33,35,36). It is possible that different types of glycoalkaloids, such as those found in wild species, have different toxic effects on mammals (32). Beneficial effects of glycoalkaloids include the potential to lower cholesterol, antipyretic and anti-inflammatory activity (29), antibiosis against foodborne pathogens (37), and anticancer activity (19,38).

Genotypic Variation

Genetic variability for the expression of beneficial compounds can be exploited to produce cultivars with improved health value (3,26). Because antioxidant levels are generally only studied in cultivated potato, Andre et al. (15) and Nzaramba et al. (7,8) suggested the use of wild potato germplasm to incorporate genetic variability for phytochemical content. Potato phenotypes include varying combinations of skin and flesh colors, namely white, yellow, red, and purple.

The white or yellow flesh phenotype is thought to be determined by a single gene (26). Red and blue pigmentation result from anthocyanin content and are controlled by a sequence of single genes, which behave in a syntenic fashion and are considered part of a single genome (26). However, degree of pigmentation does not adhere to simple genetic control and may be influenced by epistatic interactions (7,11,26).

Antioxidant profiles vary largely among genotypes and antioxidant pathways appear to be under independent control (3,15). Tetraploid cultivars of potato express only acylated forms of anthocyanidin glycosides, but both acylated and non-acylated forms are found in diploid genotypes (11). High levels of carotenoids can also be found in diploid germplasm and may offer an additional source of variation (39). Thus, there is potential to augment the bioactive content of tetraploid potato cultivars by introgression from diploid genotypes (8,19).

Total glycoalkaloid content is also dependent on genotype (30,31,40) and is considered a highly heritable, quantitative trait (32). Although cultivated potato contains primarily α-chaconine and α-solanine, wild species contain many uncharacterized glycoalkaloids, which have different chemical properties. Shakya and Navarre (19) suggested that these traits could be introgressed into cultivated potato varieties to improve nutritional content, possibly by replacing solanine and chaconine with less toxic glycoalkaloids, such as tomatine.

Effects of Growing Environment, Postharvest Storage, and Cooking Method

Andre et al. (15) proposed that environmental factors may impact pathways related to phenolic composition. There is evidence that high altitude environments with long days and cool temperatures promote increases in anthocyanin and phenolic content (28). Nzaramba et al. (7) compared greenhouse and field tuber production and found that various *Solanum* species had greater antioxidant activity and phenolic content when grown under field conditions. Tubers harvested at greater maturity expressed higher levels of anthocyanins and phenolics and minimal levels of glycoalkaloids (41). In addition, Reyes et al. suggested that abiotic stresses could be applied to augment the synthesis of phenolic compounds, thereby increasing the health value of fresh produce (13,20).

Potatoes are commonly stored and cooked prior to consumption, both of which impact phytochemical levels (42). Cold storage at 4ºC over several months induced a significant

increase in anthocyanin content (12,28,42) as well as increased antioxidant activity, total phenolics, and carotenoid content relative to non-stored tubers (42), with ratios of individual compounds remaining constant (28). However, increases in anthocyanin content were not observed when tubers were stored at higher temperatures (28). The increase in phenolic content is not attributed to water loss, but to the synthesis of anthocyanins, which may be connected to increased sugar production during storage (28).

Various cooking methods, such as baking, boiling, microwaving, and frying, also influence phytochemical levels, with red- and purple-flesh potatoes maintaining equal to or greater than 75% of their anthocyanin content (26). Some studies suggest that cooking preserves or increases phytonutrient content (28,42). In a study by Blessington et al. (42), baked, boiled, fried, and microwaved potato samples were compared to raw tubers. All treatments had increased amounts of chlorogenic and vanillic acids. Baked, fried, and microwaved samples had greater levels of caffeic and p-coumaric acids, and microwaved samples had an increased content of epicatechin. Of the cooking methods used in this study, boiling resulted in the lowest phenolic content. Lachman et al. (28) reported similar results and suggested that retention of anthocyanins is more efficient in baked, microwaved, or steamed potatoes relative to boiled or uncooked tubers. However, Brown et al. (27) reported that boiling increased antioxidant activity. The increase in phenolic content during the cooking process was attributed to increased extractability of compounds and the conversion of complex polyphenols into more soluble forms (28,42).

In contrast, Perla et al. (22) concluded that boiling, microwaving, and baking significantly reduced phenolics and that losses were minimized by boiling. Variability in conclusions is likely due to different cooking conditions, such as duration and temperature. In relation to glycoalkaloid content, various cooking methods had little to no effect (31,32,34), unless potatoes were peeled, in which case glycoalkaloid content was reduced (30).

Acrylamide is a toxic, potentially carcinogenic compound produced in fried potato products (43). Acrylamide is produced by the Maillard reaction, in which the carbonyl group of reducing sugars reacts with the nucleophilic amino group found in amino acids, peptides, and proteins. Zhu et al. (43) analyzed 16 potato varieties to determine the relationship between acrylamide content, concentration of reducing sugars, and phenolic content. The tuber concentrations of fructose, glucose, and asparagine were positively correlated with acrylamide production. In addition, high content of phenolic compounds was connected to lower levels of acrylamide (43). Antioxidants are also known to reduce acrylamide levels by interacting with the Maillard reaction. Based on these results, Zhu et al. (43) suggested that acrylamide levels may be reduced by breeding for lower concentrations of reducing sugars and asparagine and higher concentrations of phenolics and other antioxidants.

1.2 Prostate Cancer

Prostate adenocarcinoma (PCa) is the second leading cause of cancer-related death in males in Western countries, largely in relation to diet (8,16,17). For 2010, it is estimated that there were 217,730 new cases of prostate cancer and 32,050 prostate-cancer-related deaths (16). One in 36 males die of related complications (17) and the major cause of mortality is skeletal metastasis (44,45). Prostate cancer develops later in life at around 50 years of age, with progression occurring in approximately 10-20 years (16).

Carcinogenesis is often characterized in terms of three major stages: initiation, promotion, and progression (46,47). Thus, potential treatments for PCa often target the prevention of initiation, the inhibition of promotion, and the treatment of premalignant stages (46). Initiation is a rapid and irreversible process involving mutagenesis and DNA damage by reactive species (46), with no observable effects on morphology at the cellular or tissue level (47). Promotion is a prolonged, reversible process (46,47) that reduces the sensitivity of tissues to growth restraints via wounding or inflammation of normal tissue (47), which results in the development of a nonmalignant tumor (46,47). Promotion relies on increased blood flow (47), and therefore inhibition of blood vessel formation, or angiogenesis, is a common target for intervention (48). Progression occurs when a tumor develops invasive or metastatic potential (46,47). At this stage, cells are able to enter the blood stream and colonize additional tissues (44).

The initiation, promotion, and progression of PCa is a complex process involving more than one genetic event and various molecular processes, many of which are not well understood (44,45). Initiation of PCa involves the production of mutated cells in the epithelial layers of the prostate (45). Promotion is characterized by hyperproliferation of cells, resulting in prostatic intraepithelial neoplasia (PIN), which is the precursor for the development of a benign tumor, which is classified as high-grade PIN (HGPIN) (45). Early stages of PCa are androgen dependent (44,45), thus androgen deprivation therapy, or chemical castration, is a viable method of treatment and results in tumor regression (45). Many cases relapse and, as PCa develops, cells experience additional mutations and can become hormone refractory (44,45). These cells are insensitive or resistant to chemical castration and non-hormonal treatments for androgen resistant PCa have limited efficacy (44). Progression of PCa often involves lymphatic metastasis to regional nodes and the skeletal system (44).

There are many molecular pathways involved in the various stages of PCa development (45), and thus many opportunities for intervention, which can be accomplished by prevention of mutagenesis, antiproliferative activity, induction of apoptosis (programmed cell death), and inhibition of angiogenesis and metastasis (10,44). These processes can be regulated by activating or deactivating cell cycle proteins, inflammatory proteins, enzymes, and cytokines, as well as transcription factors and growth factors (9,10,44,49). Angiogenesis is commonly targeted by reducing levels of vascular endothelial growth factor (VEGF), a protein which is associated with promotion

of cell proliferation, survival, and metastasis in many cancers (48). Metastasis associated protein 1 (MTA1) has also been linked to angiogenesis and metastasis, with evidence that high levels of MTA1 expression are correlated with poor prognosis in prostate cancer patients (50). Studies have shown that MTA1 inhibition can decrease malignancy in many cancer cells, such as melanoma and breast cancer cells (50). MTA1 has also been linked to the upregulation of VEGF (50). Kai et al. (50) demonstrated that MTA1 deficient xenografts had reduced angiogenesis, smaller blood vessels, and reduced VEGF levels relative to MTA1-positive counterparts.

Mouse models are commonly used to study the different stages of carcinogenesis and the efficacy of anticancer compounds (45). To study prostate cancer, the ideal mouse model would develop PIN, HGPIN, local invasive adenocarcinoma, and invasive cancer and/or androgen insensitivity, with eventual metastasis to the lymph nodes and skeleton (45). The xenograft mouse model involves the implantation of human cancer cells into immunodeficient mice, allowing a quick evaluation of a specific treatment, but does not characterize prostate cancer progression in humans (45). There are also genetically engineered mouse models (GEMMs), which include transgenic models in which prostate-specific promoters are used to express various oncogenes, and traditional and non-traditional knockout models. GEMMs are not an ideal model as mutations may influence other physiological processes and are present throughout mouse development, whereas humans develop cancer via spontaneous mutation (45). Mice are also 3000 times smaller and have a lifespan 30-50 times shorter than humans, thus there are

differences in the uptake and effects of potential cancer treatments (45). Valkenburg and Williams (45) suggested that a variety of models may be necessary to study the many aspects of prostate cancer.

1.3 Anticancer Properties of Potato Bioactives

Many epidemiological studies have elucidated the role of fruits and vegetables in reduced risk of chronic diseases, including many types of cancer (2,3,5-10,13,20). Results indicate that the most effective control of cancer development and progression is prevention rather than treatment (6,17). Phytochemicals offer a means of prevention by reducing oxidative stress (3,6,11), with five plus servings of fruits and vegetables reducing the risk of developing many cancer types by approximately half (2). Prevention is best accomplished by a combination of phytochemicals obtained from many sources (6), which coincides with the conclusion that it is more practical to consume plant products than individual compounds (7).

Oxidative stress occurs in a cell via free radical damage, which can result from increased levels of reactive oxygen species (ROS) within the cell and/or a loss of cell antioxidant capacity (6). ROS are well documented as the causal agents of mutations, DNA breakage, DNA cross-linking, and chromosomal breakage and rearrangement, all of which can induce cancer development if left unrepaired (6). In the diet, the synergistic activity of antioxidants quenches free radicals, relieving oxidative stress (2,6). Phytochemicals aid in cancer prevention by averting DNA adduct formation,

neutralizing carcinogens, ameliorating inflammation, obstructing angiogenesis, and exerting cytotoxic effects on cancer cells (2,6,7,26).

Phenolics, particularly anthocyanins, are linked to anticarcinogenic activity, such as apoptosis, inhibition of cell proliferation, and reduction of angiogenesis (3,9). Boivin et al. (2) assessed the anticancer properties of extracts from common fruits and vegetables on multiple human cancer cell lines. The use of whole extracts allowed an evaluation of the synergistic activity of many bioactives, as well as the ability to better represent human consumption. This study demonstrated that potato phytochemicals inhibit the proliferation of breast and prostate cancer cell lines up to 50%.

Reddivari et al. (9,10) analyzed specialty potato extracts and their fractions for antiproliferative activity on androgen-dependent (LNCaP) and independent (PC-3) prostate cancer cell lines. The anthocyanin fraction induced caspase-dependent and independent apoptosis, with no evidence of necrosis. Of the lines evaluated, the CO112F2-2P/P cultivar ranked best in total phenolic content, antioxidant activity, and antiproliferative activity (9,10,18). Further investigation revealed that the potato bioactives α-chaconine and gallic acid were most effective at reducing LNCaP and PC-3 cell proliferation (10).

Although potentially toxic, glycoalkaloids promote apoptosis and reduce proliferation in human liver, cervical, lymphoma, stomach, and prostate cancer cell lines (8,19,38).

There is also a diverse array of glycoalkaloids, many of which are unidentified (19). Further characterization may allow the selection of potato cultivars with a higher content of health promoting glycoalkaloids (19). Nzaramba et al. (8) evaluated accessions of *Solanum jamesii* for antioxidant activity, phenolic content, glycoalkaloids, and antiproliferative activity on human colon and prostate cancer cells. The extracts inhibited cell proliferation in both cell lines without cytotoxicity, and antiproliferative activity varied based on genotype. There was no significant correlation between glycoalkaloid content and antiproliferative activity, but the pro-apoptotic activity of α-chaconine has been reported in human colon cancer cells (38). In addition, the antiproliferative activity of α-chaconine and α-tomatine on liver cancer cells was similar to the anticancer drug adriamycin (29).

2. MATERIALS AND METHODS

2.1 Phytochemical Extraction and Phenolic Content

Potato genotypes were selected based on prior screening for total phenolics, antioxidant activity, and unique metabolic profile (9,10,18). The potato breeding line CO112F2-2P/P (purple-flesh) was selected based on high phenolic content, antioxidant activity, and antiproliferative activity in prostate cancer cell cultures (9,10,18). This line was obtained from Dr. David Holm (Colorado State University, San Luis Valley Research Center). An accession of *S. bulbocastanum* (white-flesh, PI243510) was selected as it has a diverse glycoalkaloid profile (19), but relatively low amounts of solanine and chaconine, which are the glycoalkaloids known to cause toxicity in cultivated potatoes. This accession was provided by Dr. Roy Navarre (USDA-ARS, Prosser, Washington). Tuber material was diced into cubes and lyophilized.

Phenolics were extracted using methanol acidified with formic acid (1 ml formic acid/L) (pH < 3.0) to maintain antioxidant stability (21). Lyophilized tuber tissue was homogenized with acidified methanol at a ratio of 1 g/5 ml. Samples were centrifuged in a JA-17 rotor (Beckman Coulter, Indianapolis, IN) at 31,000 g for 15 min, after which the supernatant was collected. The extraction was repeated on the pellet and the supernatant was collected. The extract was sterile filtered using a 0.22 µm polyethersulfone (PES) membrane. Following filtration, the extract was lyophilized to a powder and redissolved in cold, sterile water.

Total phenolic content of the extract was determined using the Folin-Ciocalteu colorimetric method (51). Extracts (150 µl) were diluted with 2400 µl of nanopure water and allowed to react with 150 µl of 0.25N Folin-Ciocalteu reagent for 3 min, at which point 300 µl of 1N Na_2CO_3 was added and allowed to react at room temperature in the dark for 2 h (18). Phenolic content was determined by absorbance at 725 nm and is reported as mg chlorogenic acid equivalents (CAE) per ml (18). The extracts were concentrated by rotovarporization to a final content of 3.75 mg CAE/ml. The control treatment was lyophilized extraction solvent resuspended in sterile water.

2.2 Cell Culturing

The androgen receptor (AR)-negative human prostate carcinoma cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in a 5% CO_2 jacketed incubator in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 2.38 g/L *N*-2-hydroxyethylpiperazine-*N'*-2-2ethanesulfonic acid (HEPES), 1.5 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate, 4.5 g/L glucose, 100 mL/L fetal bovine serum (FBS), 10 mL/L antibiotic antimycotic solution (Sigma-Aldrich), and 0.2 g/L bovine serum albumin (BSA). Cells were passaged at 80% confluence.

2.3 *In vivo* PC-3 Tumor Xenograft Model

Forty male athymic nude-*Foxn1*^{nu} mice were obtained from Harlan Laboratories (Indianapolis, IN) and handled in accordance with the Animal Use Protocol #2010-39 approved by the Texas A&M Institutional Animal Care and Use Committee (IACUC). The mice were housed and handled in sterile conditions at the Lab Animal Resources & Research (LARR) facility at Texas A&M University and monitored by Comparative Medicine Program (CMP) staff. Upon arrival, mice were given an acclimatization period of one week.

PC-3 cells were combined with Matrigel (BD Biosciences, Franklin Lake, NJ) in a 1:1 (v/v) ratio. Thirty mice received subcutaneous injections (½" 27 gauge needle) of 1.5 x 10^6 PC-3 cells in each rear dorsal flank (50,52,53). Two weeks after cell injection, mice developed palpable tumors and were randomized by tumor volume and body weight. Mice were treated with extracts from either *S. bulbocastanum* (n = 10) or CO112F2-2P/P (n = 10) or a control (n = 9). Nine mice did not receive xenografts in order to observe the effects of potato bioactives on body weight in healthy mice. Mice without tumors received extracts from either *S. bulbocastanum* (n = 4) or CO112F2-2P/P (n = 5). Treatments (26 mg CAE/kg) were administered once every two days by oral gavage using a 20 G stainless steel curved needle. Tumor length, width, and height were measured once every four days using a Vernier caliper. An ellipsoid model, [π/6(l*w*h)], was used to estimate tumor volume (mm^3) (53,54). Final tumor weight was

taken at the end of the study following surgical removal of the tumors. Body weight (g) was measured once every four days to track significant weight loss or gain.

Mice were sacrificed if health significantly deteriorated or at the experimental endpoint, after which they were immediately placed on ice for tumor, liver, kidney, and lung collection. All tissues were placed into liquid nitrogen for protein analysis (50,53). The tumors were extracted and weighed. Liver and kidney tissues were analyzed for symptoms of toxicity, such as pallid color.

2.4 Statistics

One-way analysis of variance (ANOVA) was used to compare means. Significant differences were determined by Fisher's LSD at 5% significance. Data are presented as means ± SE.

2.5 Western Blot Analysis

Tumor and lung tissues were analyzed via Western blot for protein expression related to angiogenesis and metastasis, namely vascular endothelial growth factor (VEGF) (Rockland Immunochemicals, Gilbertsville, PA) and metastasis-associated protein 1 (MTA1) (Santa Cruz Biotechnology, Santa Cruz, CA) (50). Proteins were extracted into a high salt buffer [50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton-X-100] containing 1% protease inhibitor cocktail (Sigma-Aldrich) and 1% phosphatase inhibitor cocktail 2&3 (Sigma-Aldrich) and quantified

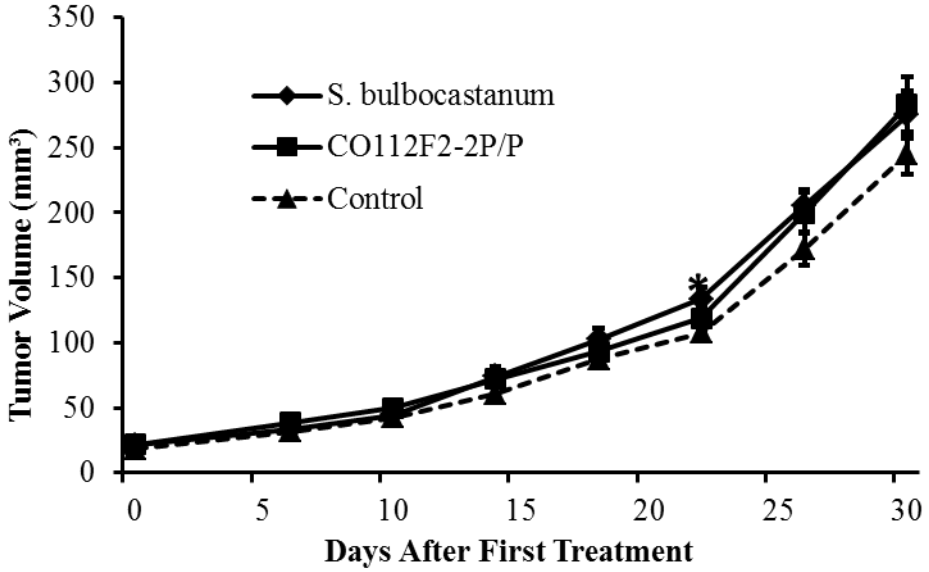
using Bradford reagent. Protein lysates were boiled with β-mercaptoethanol for 5 min at 100°C. Proteins were separated using 7.5% (high molecular weight proteins) or 12% (low molecular weight proteins) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 30 V for 12 h. Proteins were transferred by wet blotting onto a 0.2 μ polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) by wet electroblotting in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol for 1.5 h at 0.90 Å. Membranes were blocked for 30 min with 3% milk TBST [10 mM Tris-HCl, 150 mM NaCl (pH 8.0), 0.05% Tween, 3% non-fat dry milk] and incubated with primary antibody in either fresh 3% milk TBST or 3% BSA TBST overnight at 4°C with gentle shaking. Primary antibody concentrations were 1:200 for MTA1, 1:1000 for VEGF, and 1:5000 for β-actin, which was used as a loading control. Membranes were then washed with distilled water for 10 min (3x) and incubated with the secondary antibody, anti-IgG_1 (1:5000) or anti-rabbit (1:5000), in 3% milk TBST overnight at 4°C. Membranes were washed with distilled water for 10 min (3x), incubated with chemiluminescence substrate for 1 min, and exposed using Kodak image station 4000 mm Pro (Carestreamhealth, Woodbridge, CT) (9,10,55).

3. RESULTS

3.1 Tumor Growth Rate and Size

Effects of potato bioactives on tumor growth were evaluated by measuring tumor volume over the course of the study. Mice were given xenografts of androgen receptor (AR)-negative PC-3 cells and were randomized into treatment groups by tumor volume (mm^3) with means ± 1.22 SD of each other. Treatments (0.75 mg CAE/dose) of extracts from either *S. bulbocastanum* or CO112F2-2P/P were administered every other day, with water serving as a control. Tumor volume was measured every four days, as illustrated in **Figure 3.1A**. The results show that the *S. bulbocastanum* and CO112F2-2P/P extracts did not significantly reduce tumor volume relative to the control. On day 22, the *S. bulbocastanum* treated mice had a significantly greater tumor volume than the control, but final tumor volume was not significantly different among treatments. To account for variability within treatment groups, growth rate was also evaluated by comparing final and initial tumor volume (**Figure 3.1B**). Growth rate was calculated using the formula [(final tumor volume-initial tumor volume)/initial tumor volume]. Tumor weights (g) were taken at the endpoint of the study as an additional measurement of tumor development (**Figure 3.2**). Again, there were no significant differences observed among treatments.

A



B

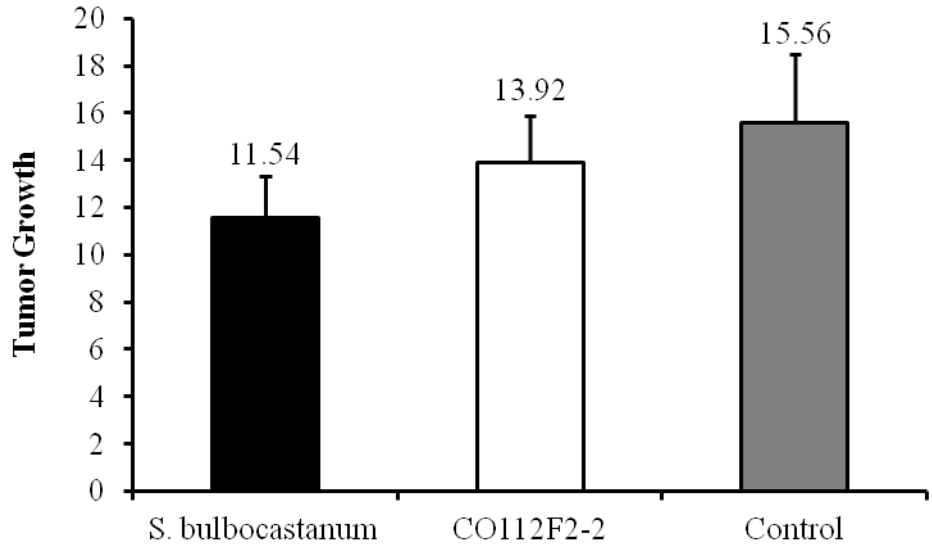


Figure 3.1. Tumor volume (mm³) was estimated using an ellipsoid model [π/6(l*w*h)] over the duration of the study (A) and as a comparison of final and initial tumor volume (B). All data are presented as means ± SE. No significant differences were detected among treatment groups (Fisher's LSD at $p<0.05$), with the exception of day 22 (*) in Figure A, where the tumor volume of the *S. bulbocastanum* treated mice was significantly greater than that of the control ($p=0.0438$).

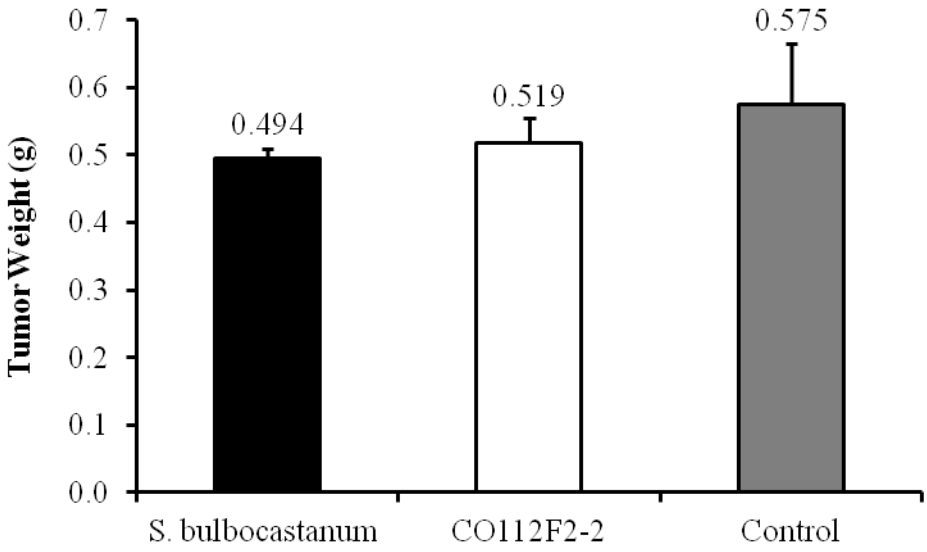


Figure 3.2. Potato extracts did not significantly reduce tumor weight (Fisher's LSD at $p<0.05$). Tumor weight (g) is expressed as means ± SE.

3.2 MTA1 Expression in Tumors and Lungs

Metastasis associated protein 1 (MTA1) supports cancer cell survival, angiogenesis, and metastasis (50). In prostate cancer cells, MTA1 silencing has been reported to promote apoptosis and reduce levels of vascular endothelial growth factor (VEGF) (50). Mice treated with the *S. bulbocastanum* extract had reduced MTA1 levels relative to the control and CO112F2-2P/P treatments (**Figure 3.3A**).

Although rare, lung metastases have been reported in subcutaneous xenograft models of prostate cancer (56-58). Lung tissues were tested for MTA1 expression as an indicator of metastasis (**Figure 3.3B**). MTA1 was not detectable in the lungs of mice treated with the *S. bulbocastanum* extract and was only found in one of the mice treated with the CO112F2-2P/P extract, whereas three mice treated with the control had detectable MTA1 levels.

3.3 VEGF Expression in Tumors

Vascular endothelial growth factor is a putative regulator of angiogenesis and thus a common target for chemotherapy (44). High levels of VEGF are associated with cancer cell proliferation, survival, and metastasis (44,48). VEGF expression in mice treated with potato extracts was similar to that of mice treated with the control (**Figure 3.4**), suggesting that the potato extracts did not directly target angiogenesis.

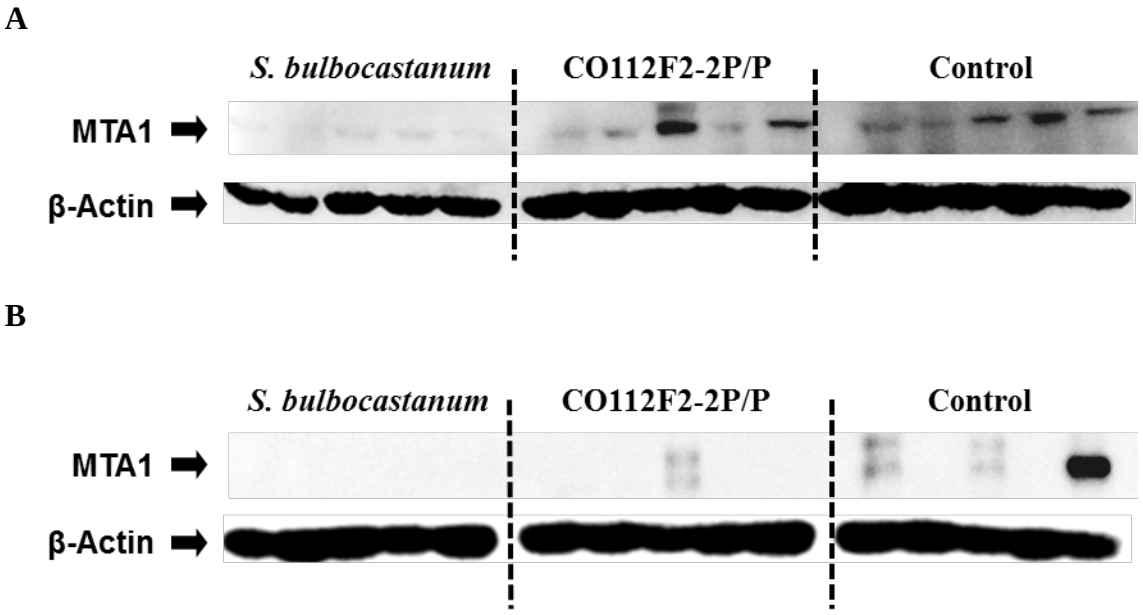


Figure 3.3. MTA1 expression was lower in the tumors of mice treated with the *S. bulbocastanum* extract, while tumors from mice treated with the CO112F2-2P/P extract had comparable expression to the control (A). Both *S. bulbocastanum* and CO112F2-2P/P extracts reduced MTA1 levels in lung tissues relative to the control, with greater reduction in mice treated with the *S. bulbocastanum* extract (B). Cell lysates were analyzed by Western blotting as described in the Materials and Methods and β-actin was used as a loading control.

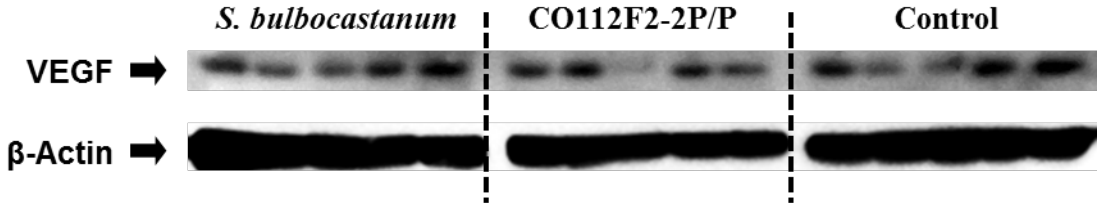


Figure 3.4. Neither potato extract reduced vascular endothelial growth factor (VEGF) levels in tumor tissue relative to the control treatment. Cell lysates were analyzed by Western blotting as described in the Materials and Methods.

3.4 Body Weight

Body weight was monitored for the duration of the study to determine the impact of the potato extracts on overall health (**Figure 3.5**). There were no significant differences within or among treatment groups, but this measurement does not account for the contribution of tumor size to body weight.

As a control, body weight was also measured in mice without tumors that received extracts from *S. bulbocastanum* (n = 4) and CO112F2-2P/P (n = 5) (**Figure 3.6**). There were no significant differences in body weight between the treatment groups. However, the final body weight of mice treated with the *S. bulbocastanum* extract was significantly greater than the initial body weight.

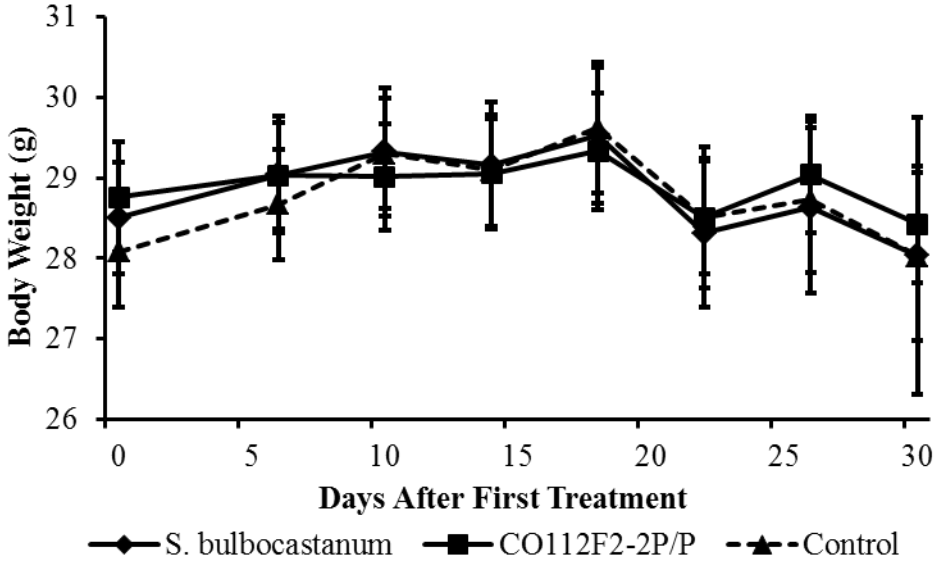


Figure 3.5. Body weight of mice with tumors. Potato extracts did not cause any significant weight loss or gain over the duration of the study (Fisher's LSD at $p<0.05$).

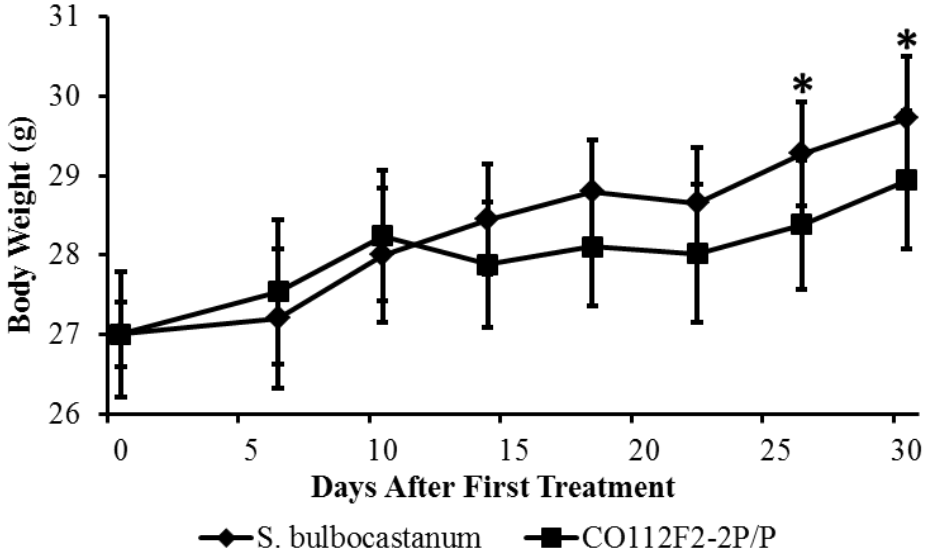


Figure 3.6. In mice without tumors, there were no significant differences in body weight between the *S. bulbocastanum* and CO112F2-2P/P treatment groups. The CO112F2-2P/P extract caused no significant weight gain or loss. The *S. bulbocastanum* extract induced a significant increase in body weight (*) relative to the initial body weight.

4. DISCUSSION AND SUMMARY

Phytochemicals found in fruits and vegetables are associated with reduced risk of chronic diseases, including many cancers (2-10). As an affordable, nutrient dense food crop with high levels of consumption, dietary intake of potato is a potential means of chemoprevention (18). Breeding for increased content of phytochemicals in potato, such as phenolics, carotenoids, and glycoalkaloids, has the potential to increase amount of health promoting compounds in the diet, thus improving the market perception of both fresh and processed potato products (9,11-15).

Our lab has previously demonstrated that specialty potato selections contain high levels of phenolics (18) and exhibit antiproliferative and pro-apoptotic activity in prostate cancer cell cultures (9,10). We aimed to further study the anti-prostate cancer activity of potato bioactives using a PC-3 xenograft mouse model. The goals of this study were to identify the effects of potato bioactives from different genotypes on tumor growth, factors related to cancer progression, and overall health. The purple-flesh breeding line CO112F2-2P/P was selected for this study based on high anthocyanin levels, phenolic content, and antioxidant activity (9,10,18), while a white-flesh wild relative of potato, *S. bulbocastanum,* was selected based on a unique glycoalkaloid profile (19). Multiple studies have demonstrated that phytochemicals reduce tumor size in PC-3 tumor xenograft models (59-61), but no significant reduction in tumor volume or weight (**Figures 3.1 & 3.2**) was observed in this study. The lack of a significant effect on tumor

volume could be due to an insufficient dosage of bioactives (26 mg CAE/kg/dose) and/or the physiology of the PC-3 cell line. A higher dosage of phenolics could have been administered by dosing prior to PC-3 cell injection, increasing the frequency of dosage, or dosage escalation. It is also worth noting that potato bioactives may exhibit greater antiproliferative activity on earlier stages of prostate cancer that exhibit androgen dependence or sensitivity. The PC-3 cell line was chosen for the ease of tumor establishment, but there are many transgenic mouse models that may be more appropriate. One such model is the ARR_2PB-*Myc* model, in which prostate adenocarcinoma develops in accordance with the human disease, allowing evaluation of the early stages of prostate cancer (45). An additional benefit of using a transgenic model is that potato could be incorporated into the diet, which would more accurately model human consumption.

In addition to tumor size, this study looked at proteins related to cancer aggressiveness. Kai et al. (50) reported that expression of metastasis associated protein 1 (MTA1) is correlated with cancer aggressiveness, angiogenesis, inhibition of apoptosis, and metastasis. Although there were no significant differences in tumor size, mice treated with the *S. bulbocastanum* extract showed lower levels of MTA1 in tumors than the CO112F2-2P/P and control treatments, which had comparable levels of MTA1 (**Figure 3.3A**). Lung tissues were also analyzed for MTA1 expression as a measurement of metastasis, which has been reported in xenograft models (57,58). Both potato extracts reduced MTA1 levels in the lungs, with the *S. bulbocastanum* extract providing a greater

reduction than the CO112F2-2P/P extract (**Figure 3.3B**). The observed reduction of MTA1 suggests that bioactives in potato may have potential to reduce tumor growth, angiogenesis, and metastasis via downregulation of MTA1, but this conclusion requires verification in future studies. The reduction of MTA1 levels in lung tissue does not agree with the results of Li et al. (62), in which MTA1 was highly expressed in lungs of normal BALB/c mice. This difference could be due to differences in physiology between the mouse strains or differences between the MTA1 antibody used in each study.

Angiogenesis is necessary for cancer progression, and is thus a common target for many chemotherapies (48). Vascular endothelial growth factor (VEGF) levels were measured in tumor tissues as an indication of angiogenic activity. It has also been reported that MTA1 and VEGF are positively correlated (50). Despite the reduced levels of MTA1, there were no detectable differences in VEGF expression among the treatment groups (**Figure 3.4**). It is possible that the duration of the study was not long enough to observe the impact of reduced MTA1 activity on VEGF levels. It is also important to note that this study only evaluated two proteins, which do not characterize the heterogeneity and complexity of the molecular pathways involved in prostate cancer. Determination of any significant effects of potato bioactives on prostate cancer would require the analysis of many factors related to cancer development, such as growth factors, kinases, cytokines, and various cell cycle regulators.

Toxicity of potato extracts was evaluated by monitoring body weight in mice with and without tumors. There was no significant weight loss or gain in mice with tumors (**Figure 3.5**). In mice without tumors, the CO112F2-2P/P extract had no significant impact on weight, while mice receiving the *S. bulbocastanum* extract experienced a significant weight gain (**Figure 3.6**). This was an unexpected result as glycoalkaloids are commonly associated with weight loss (34). However, many glycoalkaloids are not well characterized and may impact health differently. Histological analysis of vital organs is necessary to determine any toxicity induced by the potato extracts.

In this study, potato bioactives did not significantly reduce tumor size or VEGF, but did reduce MTA1 levels. Reduction of MTA1 could have implications in the promotion and progression of prostate cancer. Bioactives from *S. bulbocastanum* were more effective at reducing MTA1 levels than bioactives from CO112F2-2P/P. The reduction of MTA1 and observed increase in body weight may result from the unique glycoalkaloid profile of this species, which could provide a source of genetic diversity for beneficial compounds in a breeding program. However, more evidence is necessary to elucidate the role of potato bioactives in prostate cancer progression. This data could be obtained by repeating this study using a higher dosage of potato bioactives or by using a transgenic mouse model for prostate cancer in which potato could be incorporated into the diet.

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