BIOACCESSIBILITY OF SELENIUM IN WHEAT, RICE AND MUSTARD GROWN IN SELENIFEROUS SOILS AND ITS BIOACTIVITY

A thesis submitted in fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

in

Department of Biotechnology

by

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July 2016

CERTIFICATE

It is hereby certified that the thesis **"BIOACCESSIBILITY OF SELENIUM IN WHEAT, RICE AND MUSTARD GROWN IN SELENIFEROUS SOILS AND ITS BIOACTIVITY"** which is submitted by **Mr. Sumit Kumar Jaiswal (Regd. No. 900900011),** in fulfillment of the requirement for the award of the degree of ***Doctor of Philosophy*** in the Department of Biotechnology, Thapar University, Patiala, India, is a record of the candidate's own independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

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DECLARATION

I hereby declare that the work which is being presented in the thesis "**BIOACCESSIBILITY OF SELENIUM IN WHEAT, RICE AND MUSTARD GROWN IN SELENIFEROUS SOILS AND ITS BIOACTIVITY**" submitted by me for the award of the degree of ***Doctor of Philosophy*** in the Department of Biotechnology, Thapar University, Patiala, India, is true and original record of my own independent and original research work carried out under the supervision of Dr. N. Tejo Prakash, Professor, School of Energy and Environment, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

Date: 14-10-2016

(Sumit Kumar Jaiswal)

Place: Patiala

§ Dedicated To My Parents §

Acknowledgement

At the very onset and above all I must bow with all the humility before the divine power who has bestowed me with the requisite intelligence health and above all the will to carry out to consummation the stupendous task of investigation. I also thank him for bringing in contact with learned team of guides as well as loving and caring friends all of whom have contributed to my success.

I feel privileged to express my dense sense of gratitude, indebtedness to my respected and worthy supervisor ***Prof. N. Tejo Prakash,*** *Professor, School of Energy and Environment , Thapar University, Patiala, India, for their valuable guidance, keen interest, constant and vital encouragement, constructive criticism, patronage and dedication for research rendered to me during my work as well as in preparation of manuscript, which led me with perfection to achieve my goal. I extend my heartfelt thanks to them forever throughout my life.*

I would like to thank the rest of my doctoral committee, ***Dr. Dinesh Goyal*** *(Professor and Head, DBT, TU),* ***Dr. Sanjai Saxena*** *(Professor, DBT, TU) and* ***Dr. Bonamali Pal****, (Professor and Head, SCBC, TU), for their encouragement, insightful comments, and relevant mid-course suggestion.*

I deem it a pleasure to express my regards and gratitude to ***Dr. Ranjana Prakash,*** *(Associate Professor, SCBC, TU),* ***Dr. Raghunath Acharya*** *(Scientific officer 'G', RCD, BARC, India) for their kindness, support, professional guidance and valuable advice in many different ways and* ***Dr. O. P. Pandey****, Dean (Research and Sponsored Projects) for providing help and support during the course of work.*

My warm thanks are due to all faculty members of Department of Biotechnology and School of Energy and Environment, TU, Patiala, for their meticulous guidance, keen interest, invaluable suggestion and constant support during the course of my study. The personal and professional guidance that I received from them would be cherished lifelong.

I express my regards and grateful to my seniors **Dr. Saurabh Gupta, Dr. Neetu Sharma, Dr. Satnam Singh Aulakh, Dr. Poonam Bhatia** and my lab mates **Mr. Anirudh sharma, Ms. Noorpreet, Mr. Avdhesh Kumar Gangwar** and **Ms. Rachana Pandey** for providing keen interest, unfailing support, inspiration, critical observations and ingenuous suggestions for my research work.

I would like this opportunity to express my heartfelt thanks to my friends**, Dr. Nadeem Akhtar, Dr. Raghu, Dr. Anshu Bansal, Mr. Rajneesh Verma, Mr. Vineet Meshram, Ms. Neha Lohia, Ms. Neha Kapoor, Ms. Mahiti Gupta, Ms. Divya Singhal, Mr. Vagish Dwibedi and Ms. Prerna** for their invaluable smiles and friendship during the course, which has motivated and encouraged me for my research work. I also thank those who could not find a separate name but helped me directly or indirectly.

For all the sacrifices that **my dear parents** so selflessly made, words at my command are inadequate in form and spirit to convey the depth of feelings towards them for showering their blessings, flourishing, inspiration, encouraging support which brought me up to this level. Words are too less to express my love towards my younger brother **Amit** and sweet sister **Shuchi** for their loving affection throughout.

Date: 14-10-2016 **Sumit Kumar Jaiswal**

Place: Patiala

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Abstract

Selenium is an essential element for both humans and animals. Selenium enters into the body as a dietary supplement through food. In humans, low dietary Se intakes are associated with many oxidative stress diseases, which could be prevented by dietary supplementation of selenium through inorganic selenium salts or selenium rich food crops. Most of the cultivated crops possess the ability to absorb, metabolize, and store significant amounts of Se in their tissues when grown on selenium containing soil. Selenium prevents these crops from the deteriorating effect of many environmental stresses by improving many defense mechanisms (enzymatic and non-enzymatic). Present doctoral work was focused on selenium determination, its bioaccessibility and study of different bioactive properties of wheat, rice and mustard seeds grown in seleniferous soils. Selenium levels in these crops were significantly higher when compared to the control (collected from non-seleniferous regions) and the observed selenium content in selenium-rich wheat, rice and mustard seeds were 115.4 ± 0.83, 19.14 ± 0.19 and 110.0 ± 3.04 µg/g respectively whereas, in their respective controls it was 0.48 ± 0.01, below detection limit and 0.48 ± 0.02 µg/g. Selenium content was also determined in processed samples obtained from these selenium rich crops, for example, chapati (112.3 ± 1.72 µg/g), cooked rice (18.62 ± 0.35 µg/g), different wheat storage proteins (albumin, 444.9 ± 14.59 µg/g; globulin, 377.2 ± 6.57 µg/g; glutelin, 527.8 ± 4.9 µg/g and prolamin, 495.8 ± 9.58 µg/g), wheatgrass (cultivated under visible light, 148.1 ± 4.05 µg/g and UV-B light, 151.3 ± 2.46 µg/g), mustard oil (3.50 ± 0.66 µg/g) and mustard cake (143.0 ± 5.18 µg/g).

Bioaccessibility of selenium from processed and unprocessed grains were determined by simulated *in-vitro* gastrointestinal digestion. The obtained results indicated that, thermal processing of grains (chapati, 96.26% and cooked rice, 81.53%) were significantly increased the selenium bioaccissibilty when compared to their respective raw grains (wheat, 82.20% and rice, 63.37%). From wheat storage proteins, bioaccessibility of selenium was 93.66, 87.97, 76.77 and 69.68% for albumin, globulin, glutelin and prolamin respectively. Lowest selenium bioaccessibility was observed from wheatgrass samples (30-32%).

The doctoral work was also aimed to evaluate the effect of selenium and light quality (visible and UV-B) on different *in-vitro* biochemical and enzymatic assays of wheatgrass

samples. These assays include total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging, Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), iron (Fe^{2+}) Chelating capacity and lipid peroxidation (TBARS assay), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), ascorbate peroxidase (APx) and guaiacol peroxidase (GuPx). Obtained results indicated that, selenium and UV-B radiation independently enhanced the different antioxidant properties whereas, selenium and UV-B light showed synergistic affect and significantly increases antioxidant status of wheatgrass. The aim of present work was also to determine the concentration and characterization of different free (alcohol extracted) and bound forms (alkali extracted) of phenolic compounds from wheatgrass samples and also to evaluate their *in-vitro* bioaccessibility by using HPLC. Results showed that, Selenium independently and along with UV-B light, significantly increases the levels of many free and bound phenolics, and bioaccessibility of free phenolics were high when compared to bound phenolics.

Selenium rich mustard cake was used for the Isolation, quantification and characterization of isoselenocyanates. Oily residue obtained after myrosinase based enzymatic auto hydrolysis of selenium-rich mustard cake powder was subjected to GC-MS analysis and obtained results confirmed the presence of butenyl isothiocyanate (92%), allyl isothiocyanate (4.48%), phenethyl isothiocyanate (2.8%) and allyl thiocyanate (0.43%) along with very small fractions of allyl selenocyanate (0.13%) and allyl isoselenocyanate (0.17%). Mustard cake residue left after extraction of isothiocyanate/isoselenocyanates was further used for the extraction of selenium-rich storage protein. Isolated protein was subjected to quantification of selenium and total selenium content recorded was 582.3 ± 6.23 µg/g. Further the cytoprotective effect of selenium-rich mustard protein on tert-butyl hydroperoxide (TBHP) induced cytotoxicity was studied in mouse melanoma cell line (B16-F10). Protein extracted from selenium-rich mustard was observed to protect the melanoma cells from organic peroxide induced oxidative stress with significant increase in expression of GPx enzyme.

1.0 Introduction

Selenium is a rare element on the earth crust, placed at 34th position in periodic table with six naturally occurring isotopes: Se^{74}, Se^{76}, Se^{77}, Se^{78}, Se^{80} and Se^{82}. Selenium possesses both metallic and nonmetallic properties. The natural source of selenium is metal sulfide ores and is commercially produced as a byproduct from ore refining industries. The major application of selenium is in electronics as semiconductor and in photocells. It is also used in glass pigment making industries. Naturally selenium exists in four different oxidation states that include elemental selenium (0); selenide (-2); selenite (+4) and selenate (+6). Other than commercial application, selenium also has nutritional importance as an essential micronutrient for humans and animals (Rayman 2000; Hefnawy and Tortora-Perez 2010.). Selenium present in both as inorganic and organic forms. The inorganic forms include salts of selenous (H_2SeO_3) and selenic (H_2SeO_4) acid and their respective oxidized forms are represented as selenite (SeO_3^{-2}) and selenate (SeO_4^{-2}). Whereas, organic forms include selenoamino acids: selenocysteine (SeCys) and selenomethionine (SeMet), and other methylated-selenium species such as dimethylselenide and dimethyldiselenide. Although, selenium has many health benefits, it is also toxic when consumed in excess. Whereas, a vast section of world's population still has sub-optimal dietary selenium intake; therefore they are exposed to increased risk of many diseases such as cancer, heart and viral infective diseases (Combs 2001).

1.1 Geographic distribution of selenium

On earth's surface, there is uneven distribution of selenium but the typical range is found in between zero to 12.50 mg/g. Selenium is present in oxidized forms (selenite and selenate) and found naturally in volcanic rocks, coal, granite, sandstone, limestone and some crude oil deposits (Cuvardic 2003). Selenium deposited into top soils through the weathering of selenium-rich rocks, water and wind erosion, and sedimentation process.

Depending upon the level of selenium in non-accumulator plants, soils can be classified as seleniferous or non-seleniferous status. Generally, the soil with concentration of selenium as low as >0.5 µg/g (Ravikovitch and Margolin 1957; Dhillon and Dhillon 2003) is considered seleniferous as plants grown on them contain > 4 µg/g selenium, which is the maximum permissible level for human consumption. On the other hand, diet containing selenium < 0.05-

0.1 µg/g may leads to selenium deficiency disorders in humans and animals, and such soil is considered as non-seleniferous (Fordyce 2005).

Around the globe, soil selenium content is highly variable. Areas with toxic concentration of soil selenium include Western United States with maximum selenium content of 98 µg/g (Rosenfeld and Beath 1964), San Joaquin valley of California, USA (0.28-2.32 µg/g) (Severson and Gaugh 1992) and plains of China containing total selenium concentration of $\geq$ 3 µg/g in soil and 42.9 ng/g in water soluble fractions (Tan et al. 1994). Similarly in India, soil selenium is varies from 0.23-10 µg/g in the states of Punjab, Haryana and sub-Himalayan regions of West Bengal (Singh and Kumar 1976; Dhillon et al. 1992, Dhillon and Dhillon 2009; Sharma et al. 2009a; Bajaj et al. 2011). Areas with soil selenium content from 0.03-0.08 µg/g are considered as severely selenium deficient that include North Korea, Central Africa, Southeastern China, Eastern and Central Siberia, New Zealand, Finland, Denmark, Nepal and Tibet (Oldfield 1999; Cuvardic 2003).

1.2 Biological role of selenium

Although, selenium is toxic in higher doses, it plays a vital role as an essential micronutrient in humans and animals. According to World Health Organization (WHO) report; 55-65 µg/day as the average intake of selenium is necessary to ensure meeting normative requirements of healthy adults. Similarly, the upper tolerance limit for selenium has been proposed to be 400 µg/day for adults (FAO/WHO 2002). The consumption of food containing selenium $<$ 0.1 µg/g leads to selenium deficiency disorders whereas, consumption of food containing $>$ 1 µg/g selenium may results in toxicity.

Biochemically, selenium as SeCys is involved in the catalytic centre of different antioxidant enzymes like glutathione peroxidase (GPx), thioredoxin reductase, iodothyronine deiodinase and various selenoproteins such as selenoprotein P, sperm capsule slenoprotein and selenoprotein W (Brown and Arthur 2001). In recent years, laboratory experiments, epidemiological studies and clinical trials established the role of selenium in the prevention of many degenerative diseases including ageing, neurological diseases, cardiovascular diseases, thyroid function, inflammatory diseases, cancer, infertility and viral infections (Rayman 2000; Brown and Arthur 2001).

As an essential nutrient, there is a very narrow range between selenium toxicity (> 400 µg/day) and dietary selenium deficiency (< 40 µg/day). Plants are the major dietary source of selenium for humans and animals, and selenium content of plants is mainly dependent on the soil where they are cultivated. Selenium deficiency is more wide spread than its toxicity and deficiency is prominent in those areas where soil is poor in selenium such as Scandinavian countries, Russia and China (Oldfield 1999). Two endemic diseases linked to insufficient selenium intake have been elucidated in few regions of China where the soil is depleted in selenium and hence low selenium in food crops. Keshan disease is a heart disease which includes chronic cardiac insufficiency, heart enlargement and rhythm disorder in children and young women. Several studies were devoted to this disease and supplementation with selenium diminishes the incidence of Keshan disease (Chen et al. 1980). Another major disorder associated with selenium deficiency is Kashin-Beck disease, which is a chronic degenerative osteoarthritis reported to affect about millions of children in Russia, North Korea and China (Peng et al. 1992).

A too low selenium intake leads to several diseases; but an excessive intake of 900-1600 µg/day can cause adverse health effects and intake of 3200-5000 µg/day results in selenium intoxication or selenosis (Combs 2001; Whanger 2004; Letavayova et al. 2006). Selenosis occurs in areas where the soil contains high amount of selenium and the human population heavily relies upon local produce for their food. The symptoms observed in the case of selenosis are hair loss, damaged nails, abnormalities of nervous system, skin rashes, gastrointestinal problems and garlic breath odor (Levander 1987; WHO 1987; Dhillon and Dhillon 2003a; Fairweather-Tait et al. 2011). In animals, chronic selenium poisoning was observed after consuming daily dose of 2.2-3.4 milligram of selenium per kilogram of body weight and toxicity symptoms were impaired growth, poor reproduction, cracked and bleeding hoofs along with few cases of mortality (Rosenfield and Beath 1964; Dhillon and Dhillon 1991a).

When plants exposed to high concentration of selenium, they may exhibit symptoms of injury including snow-white chlorosis, withering and drying of leaves, stunted growth, decreased protein synthesis and premature death of plant (Brown and Shrift 1982; Dhillon and Dhillon 1991a; Terry et al. 2000).

1.3 Selenium metabolism in plants

Selenium is essential micronutrients for many organisms including mammals, bacteria and certain green algae (Stadtman 1990, 1996; Fu et al. 2002). In these organisms, selenium gets incorporated into proteins as SeCys and SeMet, and these proteins are called as selenoproteins. SeCys serves as integral part of these selenoproteins at their active site. Interestingly, SeCys is encoded by an opal stop codon, which when in the right m-RNA context, encodes SeCys instead. Se-requiring organisms can also contain seleno-t-RNAs (Mihara and Esaki 2002). These selenoproteins invariably have many antioxidant functions, including free radical scavenging.

Although, selenium requires for the growth of many organisms, the essentiality for the plants is unresolved and remains controversial. Plants uptake the selenium form soil in the form of selenate, selenite or as organic selenium species (Hamilton and Beath 1963, 1964). Generally, plants exhibit preference for the selenate over selenite and this seems to be due to high adsorption of selenite by hydrated oxides or selenite reduction to elementary selenium or selenide (Cary et al. 1967; Gissel-Nielsen et al. 1984; Banuelos and Meek 1990). After uptake, plant incorporates selenium into organic compounds using different metabolic pathways similar to their respective sulfur analogue. Figure 1.1 representing the schematic overview of Se metabolism in plants.

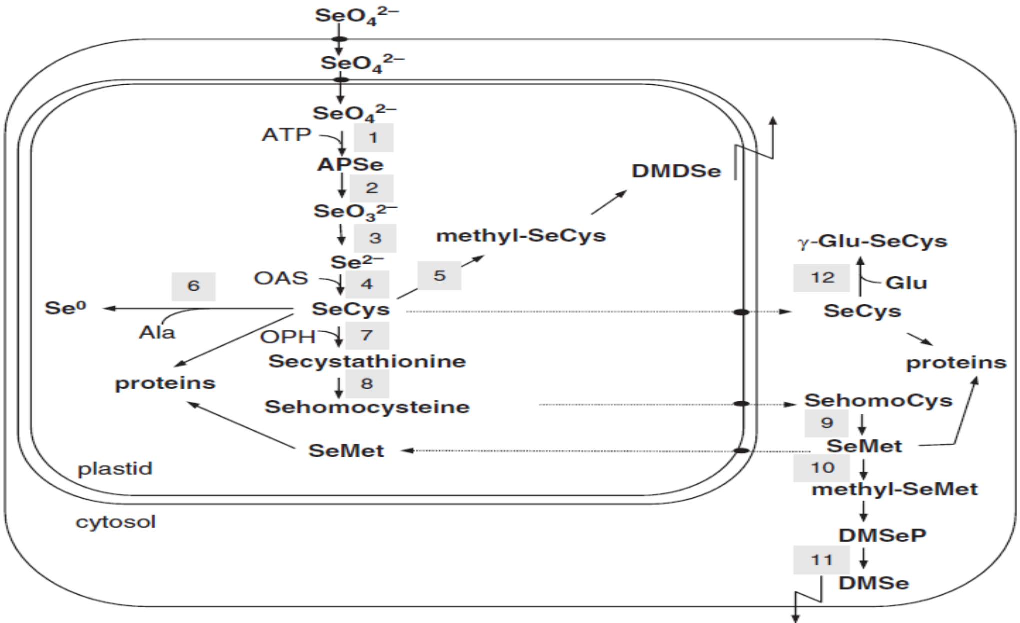


Figure 1.1: Schematic overview of Se metabolism in plants (Pilon-Smits and Quinn 2010)

Numbers in figure 1.1 denote known enzymes. (1) ATP sulfurylase, (2) adenosine phosphosulfate reductase, (3) sulfite reductase (or glutathione), (4) O-acetylserine thiol lyase, (5) SeCys methyltransferase, (6) SeCys lyase, (7) cysthathionine-γ-synthase, (8) cysthathionine-β-lyase, (9) methionine synthase, (10) methionine methyltransferase, (11) dimethylselenoproprionate lyase, (12) γ-glutamylcysteine synthetase

After assimilation, SeCys is produced as first organic form of selenium by the reduction of inorganic hydrogen selenide (H_2Se/Se^{-2}). This SeCys, either nonspecifically incorporated into proteins or get converted to SeMet which also can be misincorporated into proteins. Incorporation of SeCys and SeMet at the place of cysteine and methionine leading to the toxicity symptoms of selenium. Under high soil-selenium, SeMet can also be converted to volatile dimethylselenide (DMSe) that offers a release valve for excess selenium from the plants (Lewis et al. 1966). SeCys can also be converted into elemental selenium and methyl-SeCys which provide additional selenium detoxification mechanism to the plant (Neuhierl et al. 1999; Pilon et al. 2003). Methyl-SeCys can also act as a precursor of another forms of volatile selenium; dimethyldiselenide (DMDSe) (Terry et al. 2000; Sors et al. 2005).

Plants differ greatly in their ability to accumulate selenium from seleniferous soils and therefore could be divided into three major groups: (1) selenium-accumulators can accumulate from hundreds to several thousand micrograms of selenium g^{-1} dry weight in their tissues and these include number of plant species of *Xylorhiza*, *Oonopsis*, *Neptunia*, *Morinda*, *Stanleya* and *Astragalus* (Bosma et al. 1991; Terry et al. 2000); (2) secondary selenium-accumulators, when grown on soils of low-to-medium selenium content and accumulate selenium up to 1000 μg/g of dry weight, for example species of *Gutierrezia*, *Grindelia*, *Grayia*, *Comandra*, *Castilleja*, *Atriplex* and *Aster*; and (3) non-accumulators, this category includes mostly forage and cereal crops. These non-accumulators contain selenium less than 25 μg/g of dry weights and do not accumulate selenium above 100 μg/g when grown in seleniferous soils (Terry et al. 2000). Fast growing Brassica species like Indian mustard (*Brassica juncea*) and canola (*Brassica nupus*) are also considered as secondary selenium-accumulators that have capability to accumulate selenium up to several hundred micrograms per gram dry weight in their tissues when cultivated on soils contaminated with moderate levels of selenium (Banuelos et al. 1997; Sharma et al. 2009; Jaiswal et al. 2012b).

1.4 Selenium metabolism in humans

After gastrointestinal digestion, absorption of dietary selenium-species mainly takes place in the lower parts of the small intestine using different routes and generally metabolizes through sulfur assimilation pathways. The metabolic pathways of selenium assimilation in humans are schematically presented in figure 1.2.

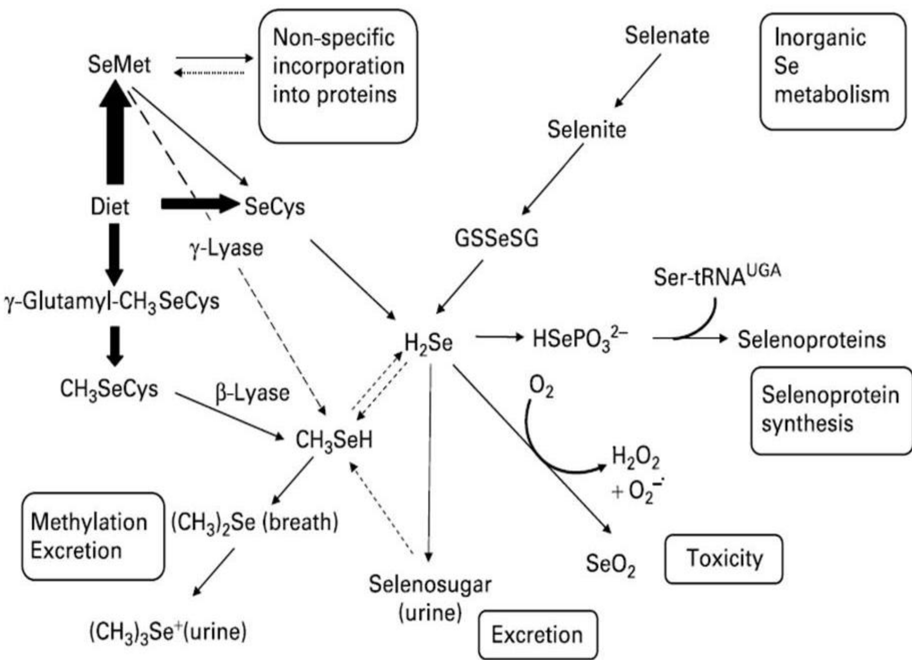


Figure 1.2: Metabolic pathways of selenium assimilation in humans (Rayman and Infante 2008)

Under normal intake and physiological conditions, organic as well as inorganic forms of selenium are readily absorbed with overall efficiency of 70-90% (Fairweather-Tait et al. 2010). After ingestion, selenate is absorbed via passive diffusion and then reduced to selenite. Exceptionally, direct absorption of selenite does not exceed 60%, whereas in the presence of reduced glutathione (GSH) as occurs in the gastrointestinal fluid, selenite reacts non-enzymatically with thiol groups of GSH to form selenodiglutathione (GS-Se-SG) which facilitate the absorption of selenite up to quantitative proportions (Gammelgaard et al. 2012). GS-Se-SG further gets decomposed into hydrogen selenide (H_2Se) by the enzyme glutathione reductase (Weekley et al. 2011). Absorption of SeMet and SeCys are mediated by transporter proteins which are used for their sulfur-containing analogues (Nickel et al. 2009). SeMet either transformed into SeCys and then H_2Se or it can be incorporated non-specifically into proteins

such as hemoglobin and serum slbumin, by randomly replacing the methionine (Suzuki and Ogra 2002). Dietary selenium-dipeptide, γ-glutamyl-methyl-SeCys is hydrolyzed by γ-glutamyl transpeptidase in gastrointestinal tract and release methyl-SeCys. γ-glutamyl-methyl-SeCys and methyl-SeCys are directly methylated by β-lyase to methyl selenol (CH_3SeH) (Suzuki et al. 2006) which excreted through breath or urine in the form of $(CH_3)_2Se$ (dimethylselenide) or $(CH_3)_3Se^+$ (trimethyl selenonium ion) respectively. These methylated selenium metabolites are generally produced during excessive selenium intake. Other than these metabolites, selenosugars are also synthesized and excreted in urine during selenium toxicity. After assimilation of dietary selenium, selenoamino acids are mostly utilized in selenoprotein synthesis.

1.5 Selenium in food crops and their biological properties

Selenium content in plants is greatly influenced by soil selenium levels where they are cultivated. The availability of selenium to plants is affected by many factors including rate of rainfall, artificial fertilization, presence of competitive ions, organic matter contents, soil texture, the redox potential, soil pH, and human activities (industrial emission, use of sewage sludge and fly ash) (Seby et al. 1997). Selenate is the most prevalent and most bioavailable form of soluble selenium for plants. Speciation study of seleniferous San Joaquin valley soils revealed that 98% soluble selenium was present as selenate followed by selenite and other organoselenium compounds (Fio and Fujii 1990).

Plant based diets are the major source of selenium in most of the countries throughout globe. Generally, food crops have relatively low tolerance to selenium toxicity but most of the crops have the capability to accumulate higher selenium in their tissues, which can be toxic for humans and animals. Vegetables have higher tendency to accumulate selenium that other plants and selenium accumulation in leaves are higher when compared to tubers. For example, Yang et al. (1983) reported that selenium content in vegetables (0.3-81.4 μg/g) was higher compared to cereals (0.3-28.5 μg/g) in seleniferous region of China. Similarly, turnip leaves were particularly high in selenium with an average of 4.57-24.89 μg/g compared to an average of 12 μg/g in tubers. Selenium concentration in grains, vegetables and forage crops varies when grown in seleniferous regions of Ireland, Columbia, Mexico, Israel, USA, China and India, and in these countries, selenium content in cereals: wheat (1.1-155 μg/g), rice (1.2-9 μg/g), corn (1-40 μg/g), barley (2-38 μg/g) and oat (2-41 μg/g); in vegetables: radish (1.4-23 μg/g), carrot (0.23-46 μg/g),

cabbage (2.3-4.5 μg/g), beans (0.2-28 μg/g), potato (0.2-1.3 μg/g), brinjal (20-51 μg/g), garlic (25-36 μg/g) and onion (0.4-36 μg/g); in forage: Egyptian clover (0.6-200 μg/g), sorghum (5-12 μg/g), sugarcane leaves (8-67 μg/g), corn leaves (0.2-9 μg/g), mustard leaves (80-160 μg/g) and cereal straws (3-58 μg/g) (Ancizar-Sordo 1947; Ravikovitch and Margolin 1957; Rosenfeld and Beath 1964; Combs and Combs 1984; Burau et al. 1988; Carlson et al. 1988; Ribang et al. 1992; Ghosh et al. 1993; Dhillon and Dhillon 1991a,b, 1997, 2003).

Although, food crops cultivated in seleniferous soils accumulate significant amount of selenium, these foods are not reachable for those human populations lived in selenium deficient regions. Thus it is important to look for an alternative source of selenium. Biofortification of crops with selenium has been suggested as one of the safest ways to enhance the selenium levels in plants which can alleviate selenium deficiency. Selenium fertilization can be applied to increase the dietary selenium intake in food crops such as cereals, pulses and oil seed crops (Gissel-Nielsen 1998; Gupta and Gupta 2002; Broadley et al. 2010). In selenium deficient countries like Finland, biofortification of food crops with selenium has been in practice since 1984, in the form of selenate, to increase soil selenium levels (Viro et al. 1988; Aro et al. 1995; Alfthan et al. 2015). After selenium fertilization, up to 10 folds increase in the selenium level of food crops was observed (Eurola et al. 1989).

Exposure of high selenium to the plants exert some toxic effects, whereas lower or moderate selenium supplementation to the plants is beneficial for growth and prevent the plants from environmental stresses. Selenium supplementation in Canola significantly enhanced pod and seed dry weight, increased photosynthesis rate, protein and carbohydrate content in leaves when compared with control (Hajiboland and Keivanfar 2012). Selenium protected ryegrass and lettuce from damaging effect of ultraviolet radiation (Hartikainen and Xue 1999). Selenium addition increased tuber yield and starch concentration in young potato leaves (Turakainen et al. 2004). In selenium deficient population, consumption of selenium-rich food crops is often recommended to overcome selenium deficiency. In particular; selenium-rich garlic, onion, broccoli, pumpkin, mushroom, cabbage and cereals like wheat have been recommended as selenium fortified food crops (Pyrzynska 2009).

Extensive studies were carried out on artificial selenium fortification to the food crops and their bioactive properties; there is limited evidence on selenium bioaccessibility and

bioactive properties of selenium-rich cereals cultivated on selenium contaminated soils of Punjab, India. Selenium bioaccessibility after gastrointestinal digestion varies with type of food matrices and cooking process. Many reports are available on bioaccessibility of nutrients from cereal crops but to the best of our knowledge, no study is available on comparative study of selenium bioccessibility from raw and cooked selenium-rich cereals cultivated in seleniferous soils. The earlier observations reported by various researchers also show that exogenous supplementation of selenium protects plants from many abiotic stress such as UV light but no report is available of effect selenium on changes in bioactive properties of selenium-rich cereal like wheat when exposed with UV-B light during germination. Oil-crop like mustard also has capability to accumulate high selenium in their seeds when grown in selenium-rich soil. Mustard plant is well known for synthesizing many organosulfur secondary metabolites, like derivatives of isothiocyanates and due to similar chemistry of sulfur with selenium, it is presumed that high-selenium mustard may have capability to synthesize selenium analogue of isothiocyanates known as isoselenocyanates, few research groups have shown the existence of isoselenocyanates but their biosynthesis pathway is yet not elucidated.

Keeping this in view, the work presented here has been carried out to understand the bioaccessibility and bioactivity studies on selenium-rich cereals and mustard crop cultivated on selenium-rich soil seleniferous sites.

2.0 Review of Literature

2.1 Selenium in soil

In environment, selenium exist in different oxidation states namely Se^0, Se^{-2}, Se^{+4} and Se^{+6} that exhibit as different chemical behavior in each of the states. Selenium distribution in soil is largely influenced by a variety of environmental factors such as combustion of fossil fuels, volcanic activities, groundwater transport, weathering of rocks and soils, soil leaching, chemical and biological redox reactions, plant and animal uptake and release, disposal of sewage sludge, use of fertilizers and selenium containing irrigation water (Cuvardic 2003; Fordyce 2005). The worldwide distribution of soil selenium is ranges from 0.01-2.0 µg/g with average value of 0.4 µg/g, but in some seleniferous areas high soil selenium concentration up to 1200 µg/g have been reported (Fordyce 2005). The selenium content of soil varies around the globe, due to the fact that Se content mainly relies on concentration of this element in native substrate, which varies from country to country. Toxic concentrations occur in arid and semiarid parts of China, in western parts of the USA and Canada, Columbia, Hawaii and Mexico. In central US, for example, there are regions in which plants contain selenium levels 10 times higher than the toxic level, while selenium levels in plants in eastern and western US are low (Kubota et al., 1967). In eastern Canada, selenium concentrations in plant dry matter are much below 0.1 µg/g (Winter and Gupta, 1979), while they are 10 times higher in western Canada. Selenium deficiency was observed in some parts of South America, North Korea, Central Africa, Southeastern China, Eastern and Central Siberia, New Zealand, Finland, Denmark, Nepal and Tibet. In these areas soil selenium content ranges from 0.03-0.08 µg/g which are considered as severely selenium deficient (Oldfield 1999; Cuvardic 2003). Soils from certain regions of Serbia have very low selenium concentrations. For example, the soils in Northern Pomoravlje have the selenium contents from 0.12 to 0.44 µg/g, with the mean value of 0.24 µg/g, in western Serbia (0.12±0.07 µg/g), in the Zeta River valley from 0.2 to 0.41 µg/g, with the mean value of 0.28 µg/g (Maksimovic et al. 1992; Cuvardic 2003). The soils can be classified as seleniferous or non-seleniferous depending upon the selenium level of non-accumulators (cultivated agricultural crops) grown on that soil. The soils containing >0.5 µg/g of selenium (Ravikovitch and Margolin 1957; Dhillon and Dhillon 2003) are considered as seleniferous because the forages grown on such soils, absorb Se more than the maximum permissible level for animal consumption.

In India, high selenium contents have been observed in various regions. These areas include parts of Northwestern region of Punjab (Dhillon and Dhillon 1991a), Haryana (Arora et al. 1975), Assam, Meghalaya (Dey et al. 1999) and West Bengal (Ghosh et al. 1993). In the sub-Himalayan West Bengal soils, an increase in selenium pasture has been located in the Jalpaiguri district due to deposition of soluble selenium compounds carried by heavy rainfall. Visible and specific toxic effects of selenium were observed in wild animal species from various locations in sates of Assam and Meghalaya states (Dey et al. 1999). Among all the locations identified, the agriculturally rich belt along border of Nawanshahr-Hoshiarpur district of Punjab has gained prominence due to extensive research and agri-extension activity. The affected villages, covering > 1000 acres are Barwa, Bhano Majra, Sikandarpur, Rakker, Simbli, Jainpur, Mahdipur and Nazarpur (Dhillon and Dhillon 1997). Selenium concentrations in these soils are relatively variable ranging from 0.32-4.55 µg/g. The toxic sites are located along the path of underground rivulets flowing from upper ranges of Shiwalik Hills, which are used for continous cropping by farmers. It is likely that materials rich in selenium might have been transported down along with the floodwater and deposited in the low-laying areas. When these sites were brought under cultivation, the top soil might have been enriched through recycling of selenium by continuous cropping. When plants are exposed to high selenium in their root medium, they may exhibit symptoms of injury including stunted growth, chlorosis, withering and drying of leaves and decreased protein synthesis at the initial phase of growth (Terry et al. 2000). These high selenium areas were initially identified on the basis of visible symptoms associated with selenium toxicity among domestic animals and humans like hair fall, softening of nails and horns, etc. Further analysis of water and soil samples along with the plants grown in this region confirmed higher selenium content in these areas. Chronic selenium poisoning in humans, animals and plants has been reported in seleniferous soils of northwest India (Dhillon and Dhillon 1997). With reference to selenium uptake by human population, the average selenium intake by both men and women in the region was more than nine times than that of the non-seleniferous areas. More than 60% of male population exceeded the maximum tolerable limit. Mean selenium content of hairs, nails, and urine of both men and women was ten times higher than in the non-endemic areas (Hira et al. 2004). Further, studies carried out by Sharma et al. (2009), examined selenium concentrations in soil and crop products (wheat husk, rice, maize and

mustard), using neutron activation analysis, and found it in the range of 2.7-6.5 µg/g and 13-670 µg/g respectively, with significantly high selenium content in these crop products.

2.2 Selenium in food

Foods are the major source of dietary selenium for both human and animals. Selenium content in food depends on several climatic, geological and geochemical factors. Selenium enters the food chain through plants and plant selenium content varies according to available soil selenium, its uptake by plant roots and species of plants (Dhillon and Dhillon 1997). Generally, selenium in plant based food varies across the world. The selenium content of fruits and vegetables is the lowest with values between 0.001-0.022 µg/g fresh weights. In eggs, fish and meat the values ranges between 0.01-0.36 µg/g fresh weights, in milk and dairy products selenium ranges from 0.001-0.17 µg/g and in cereals and cereal products it varies between 0.01-0.55 µg/g (Reilly 1996). Mushrooms (26-145 µg/g, dry weight) and Brazil (8-83 µg/g) nuts are found to accumulate extremely high levels of selenium (Thomson et al. 2008; Bhatia et al. 2013). Mushrooms contain substantial amounts of protein, in the range from 16.5% to 39% of dry matter, therefore their protein fractions exhibit high levels of organic selenium (Maseko et al. 2013). Cereals are the main staple food and dietary source of selenium across world, which are succeded by fruits, vegetables and other products. Cereals possess ten folds higher total selenium content than meat. Wheat flour and bread are important sources of Se in diet schedule of United Kingdom (Barclay et al., 1995). Different strategies such as use of Se-rich fertilizers along with supplementation of animals with Se-rich feed/fodder have been practicised in areas low in Se content. Wheat is generally considered as selenium no accumulators but there is a wide variation in wheat grain selenium level, depending upon geographical location. Depending upon geographical location there is a wide variation in wheat grain selenium levels recorded. Selenium levels in wheat grains range from as low as 0.001 µg/g in south-west Western Australia (White et al. 1981) to 30 µg/g found in highly seleniferous soil of South Dakota (University of California, 1988), but major distribution of selenium content in global wheat falls within the range of 0.02-0.6 µg/g (Alfthan and Neve 1996). Whereas, lowest selenium level in wheat was recorded in Eastern Europe and New Zealand with average value of 0.028 µg/g (Mihailovic et al. 1996), in some parts of China (0.01-0.23 µg/g) and Finland (0.01-0.015 µg/g) (Alfthan and Neve 1996; Koivistoinen and Vara 1987). Like hyperaccumulator plants, it has been reported first time that wheat, a typical non-accumulator also accumulates high selenium content in their grains, ranging

from 29-185 µg/g in Nawanshahr-Hoshiarpur region of Punjab, India (Sharma et al. 2009; Cubadda et al. 2010). Being one of the major food crops, rice is also an important source of selenium for more than half of the world's population and selenium concentration in rice rarely exceed 1.0 µg/g (Williams et al. 2009). Selenium content in rice sample collected from Nawanshahr-Hoshiarpur region of Punjab was found highest and ranges from 5-58 µg/g (Dhillon and Dhillon 2009; Sharma et al. 2009; Jaiswal et al. 2012a). Similarly, in the case of maize, selenium was recorded upto 29 µg/g (Jaiswal et al. 2012a). Onion and garlic are reported as hyper accumulators of selenium and can accumulate more than 1000 µg/g of selenium when cultivated in selenium rich soils (Ip and Lisk 1995; Sharma et al. 2007). High selenium content has also been found in plants of the *Brassica* genus (broccoli, cabbage, cauliflower, and kohlrabi) (Dumont et al. 2006).

2.3 Selenium intake and bioavailability

Around the globe, selenium intakes in diet on individual basis were estimated to be lying in the range of 3-7000 µg/day (Rayman 2002; WHO 2004; Fordyce 2005; Rayman 2008). The seleniferous regions of China has been reported to have higher levels (>4990 µg/day) of dietary selenium intake (Yang et al., 1983). The mean selenium intakes for European countries were found to be lying in the typical range of < 50 µg/day per person, (Rayman, 2004; Rayman, 2005), which is found to be quite near or lesser than the recommended nutrient intake level the (Rayman, 2008; Flynn et al., 2009). Similarly, among Asian contries such as India, the selenium intakes for men and women were estimated to be 632 µg/day and 475 µg/day respectively which are considered to be relatively high (Hira et al., 2003). In United Kingdom, the intake for men and women were found to be 55 and 43 µg/day respectively. The intake is much higher in United States with the mean value for women and men intake to be 92.6 ± 1.57 and 133.5 ± 2.42 µg/day respectively (USARS, 2008).

Bioavailability is defined as the quantity of nutrient ingested, which in turn is absorbable and utilizable through individual's diet (Van Campen and Glahn 1999). Both inorganic and organic species of selenium could be easily absorbed by the body (Suzuki et al., 1998). Among inorganic forms, selenate and selenite are less easily absorbed and selenite is found to be more toxic than selenate (Magnuson et al. 1997), however, the organic forms, (such as selenomethionine) are considered more bioavailable than the inorganic forms (selenites or

selenates). Selenomethioneine (SeMet) is the predominant form of selenium reported to be present in diet (Swanson et al. 1991). Selenium is specifically incorporated into proteins and is responsible for their enzymatic activity (Sturchlerpierrat et al. 1995). On the other hand, selenium in the form of SeMet is non-specifically incorporated into haemoglobin and plasma (Burk et al. 2001) and is retained in the tissues to larger extent than other species. Supplements mainly containing SeMet, can maintain the expression of selenoenzymes during selenium deficiency for much longer time than inorganic sources due to recycling of SeMet from protein stores (Combs 1988). An important product of selenium metabolism which is excreted via urine is trimethylselenonium ion (Levander 1972). Apart from these, there are other methylated forms of organoselenium which are produced as a result of detoxification (Pyrzynska 1998) and are excreted. Bioavailability of selenium is affected both by the chemical form as well as other dietary components of selenium.

2.4 Selenium deficiency and toxicity

A narrow range exists between selenium toxicity (>400 µg/day) and its deficiency (<40 µg/day). Selenium deficiency was observed in humans and animals living in those geographical regions where soils are characterized by low selenium content (Mistry et al. 2012). Severe selenium deficiency diseases in humans have been reported in large part of China as well as central and Eastern Siberia, which include Keshan (dilated cardiomyopathy) and Kashin-Beck disease (Osteoarthropathy) (Pedrero and Madrid 2009). Keshan disease is a juvenile cardiomyopathy that occurs primarily in young women and children (2-10 years) (Wlodarek 2006). The average intake of Se in Keshan disease endemic areas has been estimated at 10 µg/day (Tapiero et al. 2003). Kashin-Beck disease is also affect children aged between 5-13 years and it is characterized by shortened fingers and toes, rheumatoid arthritis and deformation of bone due to oxidative damage of hyaline cartilage (Lyons et al. 2003; Navarro-Alarcon and Cabrera-Vique 2008; Yao 2011). Selenium deficiency has also been linked to many clinical disorders such as viral infection, increased risk of cancer, various neurologic diseases such as Parkinson's and Alzheimer's disease, decrease in thyroid and immune functioning and male infertility (Rayman 2000). Moderate selenium deficiency may also have negative impact on human health such as increasing risk of neurological diseases, nephropathy, prostate cancer and infertility in men (Kryczyk and Zagrodzki 2013). Selenium also alleviate the health implications associated with oxidative stress and that includes cystic fibrosis (Kauf et al. 1994), arthritis

(Peretz et al. 2001), kwashiorkor (Ashour et al. 1999), systemic inflammatory response syndrome (Angstwurm et al. 1999), muscular dystrophy (Kurihara et al. 2000), diabetes (Kowluru et al. 2001), osteoarthritis (Kurz et al. 2002), and asthma (Shaheen et al. 2001; Jahnova et al. 2002).

Like in humans, selenium is also essential for animals. Deficiency of selenium in animals leads to major health consequences such as mulberry heart disease in pigs, retained placenta in ruminants, white muscle disease also known as muscular dystrophy in lambs, pancreatic fibrosis in birds and fertility disorders. Clinical signs of selenium deficiency in animals include muscle weakness, unthriftiness, reduced reproduction, appetite and growth (Levander 1986; WHO 1987, 1996; Oldfield 1999).

Although selenium is essential for humans, it leads to toxicity when consumed in large doses. Chronic selenium toxicity is manifested by selenosis symptoms, which includes skin rash, gastrointestinal upset, fragility of fingernails, hair loss, infertility, neural disorders and garlic odour in breath (Li et al. 2012; Nazemi et al. 2012). Other toxic symptoms involved the disturbance in synthesis of endocrine hormones such as thyroid hormones, insulin like growth factor (IGF-I), growth hormone, decreased triiodothyronine [T3] concentration, loss of natural killer cells and the serious liver damage (Navarro-Alarcon and Cabrera-Vique 2008). Selenium toxicity is associated with production of free radicals and affinity of selenium towards thiol groups of amino acid, that change the integrity of proteins responsible for DNA repair (Letavayová et al. 2008). In China, selenosis symptoms were observed in the individuals who consumed excessive selenium at a dose exceeding 850 µg/day (Mistry et al. 2012). Acute selenium poisoning was reported in Venezuela after the consumption of *Lecythis ollaria* nuts containing high selenium above 5 µg/g (Fordyce 2005). Similarly in India, in parts of the state of Punjab (Northwestern region); observations on human population has also shown signs of selenium toxicity with chronic selenosis mainly through consumption of selenium hyperaccmulated crop products produced in Se-rich agricultural fields (Hira et al., 2003).

In animals, chronic selenium toxicity occurs only when they consume forage containing selenium 5-40 µg/g over weeks or months. Toxicity symptoms include prostration, abdominal pain, labored respiration, diarrhea, abnormal posture and movement, reduced reproductive performance, liver cirrhosis, lameness, anemia, erosion of the joints and bones, sloughing of the

hooves, emaciation, lack of vitality, dullness, respiratory failure and death (Levander 1986; WHO 1987, 1996; Dhillon and Dhillon 1991)

2.5 Metabolic importance of selenium

Selenium reported as essential micronutrients of humans, animals as well as some microorganisms. Many studies have described nutritional importance of selenium with various biological functions (Mugesh et al. 2001; Birringer et al. 2002) such as thyroid metabolism, antioxidant defense and immune functions. After absorption in vertebrates, selenium is incorporated into amino acids (SeMet and SeCys) or become an integral part of selenoproteins and some antioxidant enzymes such as glutathione peroxidase (GPx), thioredoxin reductase (TRxR), and iodothyronine deiodinase (DIO), these seleno-enzymes protect cells from free radical induced oxidative damage. (Daniels 1996; Kieliszek and Błażejak 2016). Approximately, 25 different types of selenoproteins have been reported in human where selenium is present as SeCys at the active site of protein. The function of numerous selenoproteins is still not clearly understood. GPx is well known to catalyze the reduction of H_2O_2 and organic peroxides (ROOH), and plays a protective role against oxidative damage of lipid membrane. The SeCys residue located at the active site of GPx is participated directly in electron donation to reduce the peroxides (Jovanovic et al. 2013; Ruseva et al. 2013). Selenium is also an integral component of enzyme DIO, which plays a key role in regulation of thyroid hormones and catalyzes the conversion of thyroxin (T4) to triiodothyronine (T3). During selenium deficiency, deiodonization of T4 is disrupted, resulting in thyroid gland disorder (Roman et al. 2014; Shini et al. 2015). TRxR is a seleno-enzyme, which catalyzes the reduction of oxidized thioredoxin. Thioredoxins have strong capability to donate electron during thioredoxin peroxidase and ribonucleotide reductase mediated reduction reactions. TRxR also reduces H_2O_2, lipid peroxides, vitamin K, dehydroascorbic acid and oxidized glutathione (GS-SG) (Tamura and Stadtman 1996). Other than seleno-enzymes, selenium also present as an essential component of many active selenoproteins. Selenoprotein P (SEPP1) performs many physiological functions and is effectively involved in intercellular transport and storage of selenium. SEPP1 protects the cellular system from free radical induced damage and also exhibits the ability of heavy metal chelation through the formation of non toxic metal-selenium complexes (Rayman 2012). Similarly, selenoprotein W involved in muscle metabolism and prevents from excessive oxidation reactions (Holben and Smith 1999), selenoprotein S probably controls redox status

inside the cell and selenoprotein R involved in antioxidant reactions. Selenium also occurs as an active component of enzyme selenophosphate synthetase that catalyzes the formation of selenophosphate and binding of SeCys to selenoproteins. Selenium also plays a significant role in the regulation of immune function by stimulating the production of antibodies (IgG and IgM) and increases the activity of macrophages and T cells (Kieliszek and Błażejak 2016). Selenium is reported for inhibiting the progression of HIV infection and has protective properties against hepatitis A, B, C and E (Rayman 2000; Khan et al. 2012; Kamwesiga et al. 2015). Many clinical studies have shown the protective role of selenium against occurrence of colorectal, lung and prostate cancer (Brozmanova et al. 2010).

Numerous studies have demonstrated that supplementation of selenium benefits in the treatments of health disorders. Many *in-vivo* animal model studies and *in-vitro* cell culture studies demonstrated the effect of selenium on cancer prevention along with their potential mechanism of actions. The proposed mechanisms include the apoptosis and cell cycle regulation, through antioxidant action of different selenoproteins particularly GPx1, GPx4, TRxR and SEPP1 (Rayman 2009; Zhuo and Diamond 2009), immune system modulation, alteration of DNA damage and repair mechanisms, carcinogen detoxification, inhibition of histone deacetylase and angiogenesis modulation (Lu and Jiang 2005; Jackson and Combs 2008; Selenius et al. 2010; Pinto et al. 2011).

2.6 Bioactive properties of selenium in plants

In plants, metabolic importance of selenium is still not clear, it exert both toxic and beneficial effect when exposed in dose dependent manner. Phytotoxic effect of selenium in plants varies species to species, for the agricultural crops the toxic level of selenium are generally above 5 µg/g (Reilly 1996). When plant exposed to high concentration of selenium, various toxicity symptoms were observed including chlorosis, stunted growth, decreased protein synthesis, withering and drying of leaves and premature death of plant (Dhillon and Dhillon 1991; Terry et al. 2000). Selenium toxicity might be due to replacement of sulfur with selenium in amino acids which can disrupt enzyme functions and biological reactions inside the cell. Selenium toxicity in crop plants reduces growth and productivity, however, level of toxicity varies for plant species. In ryegrass (*Lolium perenne*) plants 10 µg/g soil selenium induced oxidative stress and resulting loss of yield (Hertikainen et al. 2000), whereas, in the case of rice 2

µg/g soil selenium content caused 10% decrease in yield. In buckwheat (*Fagopyrum esculentum*) plants, high selenium supplementation reduced the seed production and primary branching (Breznik et al, 2004). Chlorosis and reduction in growth were observed in wheat (*Triticum aestivum*) plants when grown in soil content (0.2 µg/g selenium as seleante) (Tripathi and Misra 1974). In rapeseed (*Brassica napus*) plants, a significant decrease in total dry matter yield was observed when soil was supplemented with 0-4 µg/g selenium as selenite and selenate (Sharma et al. 2010). High selenium exposure decreased total chlorophyll content in mung bean (*Phaseolus aureus*) seedlings (Padmaja et al. 1989).

Although high selenium content may exert threat to the plants, its lower to optimal concentration poses some positive effect. Turakainen et al. (2004) reported that small amount of selenium increased antioxidant activity and thus enhanced quality and production of edible plants. Selenium promoted the plant growth by enhancing the starch accumulation in chloroplast (Pennanen et al. 2002). Supplementation of selenium with nitrogen promoted the quality and tuber yield of potato plants when compared to control (Yassen et al. 2011). Selenium stimulated the growth and yield in Indian mustard when cultivated in 0.5 µg/g selenium in soil (Singh et al. 1980), similarly, selenium supplementation in soil was associated with a 43% increased in seed production of *Brassica rapa* (Lyons et al. 2009). In hydroponics culture condition, 5.0 µg/ml selenium had adverse effect whereas, 1.0 µg/ml selenium promoted the growth of wheat seedlings (Peng et al. 2001). In cucumber plants, very low soil selenium (0.026 µg/g) decreased lipid peroxidation by enhancing the activity of GPx (Luo et al. 2000). Foliar spray of selenium (1.5 µg/ml) on pumpkins showed that selenium along with ambient UV-B radiation increased the yield than those that received either treatment independently (Germ et al. 2005). Djanaguiraman et al. (2004) reported that foliar spray of selenium (50 µg/ml) on soybean plants increased yield because of prevention of chlorophyll loss. In garlic, selenium supplementation stimulated the metabolism by enhancing the concentration of RNA, DNA, soluble sugars and amino acids (Duan and Fu 1997). Foliar spray of selenium in sweet basil enhanced the content of anthocyanin and phenolic compounds in comparison to control (Hawrylak-Nowak 2008). Selenium also protected crops from fungal pathogens (Wu et al. 2014). Thus, it is quite clear that the effect of selenium, whether inhibiting or stimulatory, depends upon the selenium content in plants and the plant species.

Basically, the protective and beneficial role of selenium in organisms is due to their antioxidative behaviour by forming the active center of GPx and TRxR enzymes. These enzymes are known to play important roles in reducing certain oxidized molecules in animals (Liu et al. 2010). Recently, role of selenium as antioxidant was also observed in many plants. Xue et al. (2001) reported that 0.1 μg/g soil selenium stimulated the growth of senescing lettuce seedlings by enhancing superoxide dismutase (SOD) activity and by preventing reduction of tocopherol concentration. Ekelund and Danilov (2001) reported that selenium effectively repaired photosynthetic apparatus after the damage caused by UV radiation. In *Spirulina platensis*, selenium supplementation (≤150 μg/ml) induced the expression of guaiacol-dependent peroxidases (POD), catalase (CAT), SOD and guaiacol peroxidase (GuPx) activities, which indicated that these antioxidant enzymes protect the cells from high selenium stress (Chen et al. 2008). Selenium can play a protective role against many environmental stresses by enhancing the antioxidant defense system. For example, selenium has been found to counteract the detrimental effects of several environmental stresses, such as high temperature (Djanaguiraman et al. 2010), and desiccation (Pukacka et al. 2011), cold stress (Chu et al. 2010), senescence (Hartikainen et al. 2000), water stress (Wang 2011), salt stress (Hasanuzzaman et al. 2011; Kaur and Nayyar 2015), UV-B (Yao et al. 2010a, 2010b), drought (Hasanuzzaman & Fujita 2011) and heavy metal stress (Kumar et al. 2012). These stresses cause accumulation of ROS in plants, which are also believed to act as signals for the activation of stress response and defense pathways (Mittler 2002). These defense mechanisms involved the components of enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include GPx, GuPx, SOD, CAT, glutathione reducatse (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and ascorbate peroxidase (APX), and non-enzymatic antioxidants are reduced glutathione, ascorbic acid, tocopherols, carotenoids and many flavonoids and phenolic acids (Gill and Tuteja 2010).

Selenium can efficiently be delivered to the humans via the soil-plant-animal system (Rayman, 2000; Combs, 2001). Among the edible crops, the notable sources of selenium are wheat, pulses and certain vegetable crops (Sharma et al., 2007; Cubadda et al., 2009). Selenium-rich wheat, maize and mustard grown on selenium rich soil in India, were found to be potential sources of selenium (Jaiswal et al., 2012a; Jaiswal et al., 2012b). Cruciferous edible crops such as broccoli contain low levels of Se (0.1-0.3 μg /g DW), but when cultivated in the presence of adequate Se, many have got the capability to accumulate Se at much higher magnitudes than

normal (Finley et al., 2000; Banuelos, 2002). Therefore, selenium enrichment of these crops generates a scope for production of functional foods for selenium deficient human population with high economic values.

2.7 Lacunae

The review of literature indicates selenium is now recognized as an essential micronutrient both in humans and animals. The optimum bioactivity of selenium is determined by its bioaccessibility after digestion. In-vitro (simulated) gastrointestinal (GI) digestion method is a useful tool which provides an idea about possible interactions between nutrients and food components, type of food preparation and processing practices. There is limited evidence on in-vitro bioaccessible studies on selenium using selenium-enriched raw grains. Whereas, to the best of our knowledge, no reports are available on selenium bioaccessibility studies from processed or cooked grains. Limited work is done on isolation of selenium containing storage proteins from selenium enriched cereal grain, and on bioaccessibility of selenium from these protein fractions. In literature, most of the environmental stress studies have been carried out on plants with exogenous supplementation of inorganic selenium (selenate or selenite), in addition, there are no published findings on bioactive properties of high selenium-rich wheat grains cultivated under UV-B light.

With the growing importance on enhancing the bioavailability of selenium, edible crops that accumulate selenium are being envisaged as natural sources of selenium supplements/neutraceuticals for both animals and human beings in areas of selenium-deficiency. The doctoral work was thus, carried out to study the bioaccessibility and bioactivity studies on selenium-rich crops cultivated on seleniferous region of Punjab, India with following objectives:

1. Quantification of selenium in cereals from seleniferous and non-seleniferous soils
2. Evaluation of the factors associated with bioaccessibility of selenium
3. Evaluation of antioxidant and bioactive properties of cereals under in vitro conditions

3.0 Materials and Methods

3.1 Sample collection

The selenium rich soil and post harvested crop produce such as grains of wheat (*Triticum aestivum*, cv. PBW343), paddy (*Oryza sativa*, cv. PR124) and seeds of Indian mustard (*Brassica juncea*, cv. SEJ-2) were collected from Se contaminated soils at a site near the village of Jainpur geographically located at 31° 13′ N, 76° 21′ E, in the Nawanshahar-Hoshiarpur region, Punjab (India). Produce from non-seleniferous crops produce were collected from Patiala district (30° 34′ N, 76° 38′ E), which is located about 75 miles away from the seleniferous region. Collected samples were manually cleaned, washed with water and air-dried for 2 days at room temperature.

3.2 Sample preparation

3.2.1 Raw and baked wheat

Whole wheat grains were grounded using electric grinder and further passed through a 0.5 mm sieve to obtain homogenous fine powder (whole meal wheat flour). Grounded wheat flour was used for making unleavened whole-cereal flat Indian breads (chapati or roti). Few pieces of ready to eat chapati were dried at 60° C in a hot air oven for 12 h and further grounded in fine powder.

3.2.2 Raw and cooked rice

Outer hull of paddy was removed using mortar and pestle but the bran layer and germ were retained. De-husked rice grains were grounded into fine powder using electric grinder. Around 25 g of rice grains were boiled in a pressure cooker through conventional method. Cooked rice meal was then dried at 60° C in hot air oven and further grounded into fine powder.

3.2.3 Wheatgrass

Adequate quantity of wheat grains collected from both seleniferous and non-seleniferous regions were soaked overnight in water. The soaked grains were spread separately in plastic trays filled with sterile soil. Trays were covered with wet bloating paper to maintain the moisture. Both wheat samples were sowed in duplicate and irrigated with half strength of Hoagland solution. All trays were incubated in dark for 2 days at 20° C. After germination, one set of tray was placed under visible light (source: 2×40 W fluorescent lamp) whereas another set was

transferred under narrow band (285-310 nm) UV-B light (source: 4×20 W ultraviolet lamp). All trays were exposed to light with photoperiods of 16 h light/8 h dark at 25° C and seeds were allowed to germinate for 8 days. Wheatgrass (14 – 16 cm long shoots of germinated seeds) were harvested, washed and stored at -80° C for further use. Approximately 50 g of fresh wheatgrass samples were air dried at 25° C for 48 h, grounded into fine powder and stored at -20° C for further biochemical analysis.

3.2.4 Mustard seed, oil and oil cake

Approximately 10 kg of collected selenium rich mustard seeds were used for oil extraction through a screw oil expeller. Extracted oil was stored in a light weight, food grade plastic container and residue (mustard meal/cake) was collected and stored in an air tight plastic bag. Non-seleniferous mustard seeds, oil and cake were purchased from local market and used as control. Chunks of cakes were dried overnight at 40° C, grinded into fine powder and stored in plastic bag.

3.3 Estimation of total selenium

3.3.1 Estimation of selenium using fluorescence spectrometer

Whole meal wheat flour, chapati powder, raw and cooked rice flour, mustard seed, oil and oil cake powder were subjected to selenium analysis using fluorescence spectrometric method (Levesque and Vendette, 1971). 0.1 g of each sample was taken in a 50 ml microwave digestion vessel and all the samples were digested in a closed vessel microwave digestion system (MARS 6, USA). Digestion was performed in 2 steps, first step involved, digestion of samples with mixture of 5 ml HNO_3 and $HClO_4$ (3:1) for 30 min at 200° C and in second step 5 ml, 6 N HCl used as reducing acid, for 10 min at 150° C. Microwave power was set at 600 W during digestion. After cooling all digests were quantitatively transferred into a volumetric flask and final volume of digest was made up to 50 ml with 0.1 N HCl. 1.0 ml of digest was taken in a test tube containing 200 µl of formic acid (12.5 M) and 200 µl of stabilizing solution (0.04 M solution of Na_2EDTA in 10 % hydroxylamine hydrochloride). pH of this solution was adjusted to 1.8 with 4.0 N NH_4OH and placed in a water bath at 50° C for 10 min. 2.0 ml of DAN (2,3-diaminonaphthalene) solution (0.1 % in 0.1 N HCl) was added to the above reaction mixture, shaken thoroughly and kept in water bath at 50° C for 30 min. After cooling, 3.0 ml of cyclohexane were added to each tube, vigorously mixed for extraction of piazselenol (complex

of Se^{+4} and DAN) and allowed the two layers to separate. The emission spectrum of upper organic phase containing piazselenol was measured using fluorescence spectrometer (Perkin Elmer LS-45, UK) at excitation and emission wavelength of 360 and 520 nm respectively. Selenium quantification in each sample was carried out by relative method using emission spectrum of National Institute of Standards and Technology (NIST) certified Se-ICP standard solution (SRM-1349) (2-10 ng/ml). All the samples were analyzed in triplicate and results are expressed as mean ± standard deviation.

3.3.2 Estimation of selenium in mustard samples using instrumental neutron activation analysis (INAA)

Total selenium in mustard seed, oil and cake were determined using INAA. Approximately 50 mg of mustard seeds and powdered cake were packed in thin aluminium foil. As liquid samples can not be irradiated directly in INAA, 50 mg of either selenium rich or control oil were carefully fused with 20 mg of pure amorphous silica powder placed on aluminium foil strip. All the samples were packed in a square geometry with dimension of 1 cm. Additionally, for analytical comparison, elemental standard of selenium was prepared using Se-ICP standard solution (SRM-1349) containing known amount of selenium (5-25 μg) fused in pure amorphous silica powder. Two certified reference materials (CRMs) namely RM 8436 (durum wheat flour) from NIST and SL-1 (lake sediment) from the International Atomic Energy Agency (IAEA) were analyzed to evaluate the accuracy of the INAA method. Fifty milligrams of amorphous silica powder were also used as a negative control.

The samples, reference materials, selenium standards and silica blank, sealed appropriately, were introduced into Harwell cans and co-irradiated in the self-server position of the CIRUS reactor (Bhabha Atomic Research Centre, Mumbai, India) for 7 h duration with a neutron flux of $\sim10^{13}$ cm^{-2} s^{-1}. The samples were allowed to cool for about 9 days, and then the radioactive assays of the samples were carried out by high energy resolution, Compton-suppressed high-purity germanium (HPGe) detector for 1–10 h depending on Se concentration in samples. The HPGe detector is coupled to a PC-based multi channel analyzer (8 k MCA) and had fixed sample-to-detector geometry. The detector system had a resolution of 1.9 keV at 1332 keV of Co^{60}. With the help of detector, activity of several radionuclides can be measured

simultaneously. The complex gamma ray spectra was analysed by peak fitting software PHAST, developed at Bhabha Atomic Research Centre, Mumbai, India (Mukopadhyay, 2001).

After irradiation of the samples, most of the elements become radioactive. In the case of selenium, Se^{74} (stable isotope, natural abundance 0.87%) on interaction with neutrons, gets converted to a radioactive isotope Se^{75} (half-life 120 days) emitting 4 gamma rays of different energy levels (121, 136, 264 and 279 keV). Using the mass of the selenium in the standard (M_{std}), count rates (counts per second, CPS) of standard (CPS_{std}) and sample (CPS_{samp}), mass of the selenium present in the sample (M_{samp}) was determined by the following equation:

$$M_{samp} = M_{std} \times \left(\frac{CPS_{samp}}{CPS_{std}} \times \frac{D_{std}}{D_{samp}} \right)$$

Where D is the decay factor ($D = exp(-\lambda t_d)$), λ is the decay constant of the radioisotope produced in neutron activation and t_d is the decay time (time in between end of irradiation and start of counting). The M_{samp} (µg or mg) was converted to the concentration (µg g^{-1} or mg kg^{-1}) by dividing by the sample mass (g or kg).

3.4 Studies on bioaccessibility of selenium in various matrices

Bioaccessibility of selenium was measured in raw and processed grains (wheat and rice); different protein fractions from wheat flour and wheatgrass matrices using *in vitro* gastro-intestinal (GI) digestion as described by Kulkarni et al. (2007) with some modifications.

3.4.1 Sample preparation

In case of wheat flour different protein fractions were extracted from defatted sample based on their solubility at 25° C in distilled water (for albumin), 5% NaCl (for globulin), 0.1 M NaOH (for glutelin fraction) and 70% ethanol (for prolamin) following the procedure given by Ju et al. (2001) with minor modifications. In brief, a 10 g of defatted wheat flour were reconstituted in 50 ml of distilled water and mixed on stirrer for 4 h [step 1]. Albumin extract was separated by centrifugation at 5000 rpm, 4° C for 30 min [step 2]. After spinning, 5-6 drops of 1N HCl was added and kept overnight at 4°C [step 3], followed by centrifugation at 13000 rpm at 4^oC for 20 min [step 4]. The supernatant was discarded and pellet (albumin) was dehydrated using acetone and stored at 4° C for further use [step 5]. The residue left after separation of albumin was re-suspended in 50 ml, 5% NaCl followed by processing through

steps 1 and 2. The supernatant collected after centrifugation was processed through steps 3-5 to extract globulin. The residue was treated with 50 ml of 0.1M NaOH followed by steps 1 and 2. The supernatant was used for extraction (Steps 3-5) of glutelin and the residue was re-suspended in 70% ethanol and followed by stirring and centrifugation (steps 3-5) to separate prolamin. All protein fractions were grounded in fine powder and subjected to their selenium estimation (section 3.3.1).

3.4.2 Procedure for *in-vitro* GI digestion

Digestive enzymes, pepsin extrapure (1:3000) and pancreatin extrapure 3× (amylase 100 U/mg, lipase 8 U/mg, protease 100 U/mg) were used for *in vitro* GI digestion. Accurately weighed amounts (1.0 g) of selenium-rich raw and cooked wheat and rice flour, wheatgrass powders and 50 mg of different protein fractions obtained earlier were transferred to 50 ml clean conical flasks, each containing 10 ml of gastric juice solution (6% w/v pepsin in 0.15 M NaCl, adjusted to pH = 1.8 with HCl). Initially, the mixture was shaken vigorously for 1-2 min. The flask were then degassed, sealed tightly with a parafilm, and placed in shaker-incubator set at 37° C with gentle shaking (50 rpm) for 3 h. All flasks were then adjusted to pH 7.0 with saturated solution of $NaHCO_3$. After adding 10 ml of intestinal juice solution (mixture of 2% w/v pancreatin, 0.2% bile salt in 0.15 M NaCl), flasks were degassed and further incubated for 4 h at 37° C with gentle shaking. The digests were then centrifuged at 8000 rpm at 4° C for 10 min and filtered. Each filtered solution was labeled and stored in airtight container at 4° C until analysis for selenium content.

3.4.3 Quantification of bioaccessible selenium

For selenium quantification in GI digest, clear supernatant was taken for analysis. 1.0 ml of digests was transferred into microwave digestion vessels and selenium was estimated by fluorescence spectrometer (section 3.3.1). From the concentration of selenium in GI digest, the percentage of selenium bioaccessibility (%B) from each sample was calculated using following formula:

$$\% B = 100 \times \frac{[GI_{Se}]}{[T_{Se}]}$$

Where $[GI_{Se}]$ = concentration of selenium in GI digests and $[T_{Se}]$ = total selenium content in samples.

3.5 Studies on selenium induced properties in wheatgrass

As essential trace element, selenium plays a significant role during environmental stress. This section is focused on to investigate (i) *In vitro* bioactive properties of wheatgrass (ii) *In vitro* antioxidant enzyme assays of wheatgrass (iii) Characterization of free and cell wall bound phenolic compounds present in wheatgrass and their bioaccessibility by HPLC.

3.5.1 Bioactive properties of wheatgrass - *In-vitro* assays

This section is focused on in-vitro bioactive properties of wheatgrass alcoholic extract, which includes total phenolic content, total flavonoid content, DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay, TEAC (trolox equivalent antioxidant capacity) assay, FRAP (ferric reducing antioxidant power) assay, metal chelating assay, lipid peroxidation by TBARS (thiobarbituric acid reactive substances) assay and estimation of chlorophyll contents.

3.5.1.1 Preparation of extract

Dried wheatgrass powder (0.5 g) were mixed with 80 % ethanol in mortar-pestle and extracted for 10 min. Supernatant was collected after centrifugation at 4° C, 5000 rpm for 10 min. Extraction was repeated 3 times and supernatant was pooled together. Ethanol was removed using rotary evaporator and concentrated extracts were dried under the blow of nitrogen gas. Dried extracts were resuspended in methanol with final concentration of 1 mg/ml and stored at -20° C until further analysis.

3.5.1.2 Determination of total phenolic content (TPC)

Total phenolic content in various wheatgrass extracts were determined by using Folin-Ciocalteu (FC) reagent as described by Sriplang et al (2007) with minor modifications. Briefly, 100 µl of wheatgrass extract (1 mg/ml) was mixed with 1.5 ml deionized water, followed by addition of 100 µl FC reagent. After incubation for 10 min at room temperature, 200 µl of $NaCO_3$ (5% w/v) solution was added and mixture was allowed to stand for 1 h at room temperature. Absorbance of solution was recorded at 760 nm. Gallic acid (10-100 µg/ml) was used as standard and total phenolic content was expressed as microgram of gallic acid equivalent (GAE) per milligram of extract. Solution without extract was used as blank.

3.5.1.3 Determination of total flavonoid content

Total flavonoid content in wheatgrass extracts was determined according to Luximon-Ramma et al (2002) with slight modifications. In brief, 200 µl of extract (1 mg/ml) was mixed with 800 µl of deionized water and 60 µl of $NaNO_2$ (5% w/v). Solution was allowed to stand for 5 min at room temperature. 60 µl of $AlCl_3$ (10% w/v) was added to above mixture and further incubated for 1 min. After incubation, 400 µl of NaOH (1 N) was added and total volume was adjusted to 2 ml with deionized water. Quercetin (50-250 µg/ml) was used as standard. Absorbance was recorded at 510 nm using spectrometer and concentration of flavonoids was expressed as microgram quercetin equivalent per milligram of extract.

3.5.1.4 DPPH radical scavenging assay

Ability of wheatgrass to scavenge DPPH radicals was determined according to Kitts et al. (2000) with minor modifications. 50 µl of different wheatgrass extracts (1 mg/ml) were added with 1.0 ml solution of DPPH (100 mM in methanol) and mixed thoroughly. Mixture was incubated for 30 min at room temperature in dark and absorbance was measured at 517 nm. Quercetin (10-50 µg/ml) was used as standard and DPPH radical scavenging capacity was expressed as microgram of quercetin equivalents per milligram of extract. Pure methanol was used as blank and working DPPH solution served as control.

3.5.1.5 Trolox equivalent antioxidant capacity (TEAC) assay

The Trolox equivalent antioxidant capacity (TEAC) method is based on the ability of an antioxidant to scavenge the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical, relative to that of the standard antioxidant Trolox. TEAC assay performed according to Re et al. (1999) with minor modifications. The $ABTS^{\cdot+}$ radical was generated by mixing of 7 mM ABTS (in 0.1 M phosphate buffer saline, pH= 7.4) and 2.45 mM potassium persulphate in equal volume and mixture was allowed to stand in dark at room temperature for 16 h to produce a dark green color solution. Working solution of $ABTS^{\cdot+}$ radical was prepared by diluting the above solution with phosphate buffer saline to an absorbance of 0.9 to 1.0 at 734 nm. For the study of radical scavenging study, an aliquot of 10 µl of extract (1 mg/ml) was added with 1.0 ml of diluted $ABTS^{\cdot+}$ solution. The mixture was incubated for 6 min and decrease in absorbance was recorded at 734 nm. Phosphate buffer saline was used as blank and working $ABTS^{\cdot+}$

solution was taken as control. Trolox was used as standard (0-1 mg/ml) and radical scavenging capacity was expressed as microgram of Trolox equivalent per milligram of extract.

3.5.1.6 Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to Benzie and Strain (1996) with minor modifications. FRAP reagent was prepared by mixing of sodium acetate buffer (0.3 M, pH= 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (in 40 mM HCl) and 20 mM $FeCl_3$ in the ratio of 10:1:1. To the freshly prepared FRAP reagent (1.0 ml) an aliquot of 10 µl of wheatgrass extract (1 mg/ml) was added and mixed thoroughly. The solution was allowed to stand for 30 min at room temperature in dark. Increase in absorbance was measured at 595 nm using spectrophotometer. FRAP working solution with deionized water was used as blank. Ascorbic acid (0-100 µg/ml) as standard and FRAP was expressed as microgram ascorbic acid equivalent per milligram of extract.

3.5.1.7 Iron (Fe^{2+}) Chelating Capacity

The ferrous ion chelating activity of wheatgrass extracts was performed according to the method described by Dinis et al. (1994). Briefly 50 µl of extract (1 mg/ml) was mixed with 5 µl of ferrous sulphate ($FeSO_4.7H_2O$, 2 mM) and incubated for 5 min. Reaction was started by adding 100 µl ferrozine (Sigma Aldrich, 5 mM) and total volume was adjusted to 1.0 ml with deionized water. The mixture was mixed thoroughly and absorbance was recorded at 562 nm. EDTA (0-1 mg/ml) was used as positive control whereas solution without extract was used as negative control. The chelating capacities were expressed as microgram EDTA equivalents per milligram of extract.

3.5.1.8 Determination of lipid peroxidation (TBARS assay)

Lipid peroxidation in various wheatgrass extracts were measured by using the method proposed by Minotti and Aust (1987) with minor modifications. Approximately, 100 mg of frozen wheatgrass leaves (stored at -80° C) were grounded into fine powder using liquid nitrogen. Lipid peroxides were extracted with 2 ml of metaphosphoric acid (5% w/v) and 50 µl of butylated hydroxytoluene (0.2% w/v in ethanol). Homogenates were centrifuged at 10,000 rpm for 10 min at 4° C. 100 µl supernatant was mixed with 250 µl of thiobarbituric acid (1% w/v in 50 mM NaOH), 50 µl of butylated hydroxytoluene (0.2% w/v in ethanol), 250 µl of HCl (2 N)

and 400 µl deionized water. The chromogen was formed by incubating the reaction mixtures at 95° C for 30 min in a water bath. Reaction was stopped by cooling in an ice bath and chromogen was extracted with 3 ml n-butanol. The upper organic phase was separated and emission spectrum was recorded by fluorescence spectrometer at excitation wavelength of 532 nm and emission at 550 nm. Control sample was prepared by replacing thiobarbituric acid with 50 mM NaOH, whereas blank sample was prepared by replacing the sample with 5% metaphosphoric acid. Malondialdehyde tetrabutyl ammonium salt was used as standard for determination of total thiobarbituric acid reactive substances (TBARS) content.

3.5.1.9 Estimation of chlorophyll content in wheatgrass

Total chlorophyll was extracted from wheatgrass powder using mixture of acetone and triss buffer (0.1 M, pH=7.8) in the ratio of 80:20. Briefly 50 mg of wheatgrass powder was taken in 2 ml microfuge tube. 1.0 ml of extraction solvent was added to the tube and mixed thoroughly. Tubes were transferred to ultrasonic water bath for the extraction of pigments. After 10 min tubes were centrifuged at 10,000 rpm for 5 min and supernatant was collected in fresh tube. Extraction procedure repeated 4-5 times and supernatant were pooled together. Final volume of extracts were adjusted to 50 ml with extraction solvent and stored at -20° C until analysis. Chlorophyll *a*, chlorophyll *b* and total chlorophyll was quantified spectrophotometrically using the following equations given by Porra (2002)

$$\text{Chlorophyll } a \ (\mu g/ml) = 12.25\ (A_{663}) - 2.55\ (A_{647})$$

$$\text{Chlorophyll } b \ (\mu g/ml) = 20.31\ (A_{647}) - 4.91\ (A_{663})$$

$$\text{Total chlorophyll } (\mu g/ml) = 17.76\ (A_{647}) + 7.34\ (A_{663})$$

Where A_{647} and A_{663} are the absorbance of extracts at 647 and 663 nm respectively.

3.5.2 Bioactive properties of wheatgrass - Antioxidant enzyme assay

In order to access the role of selenium against pro-oxidant activity induced by visible and UV-B light radiations, level of some antioxidant enzymes were monitored in wheatgrass. This section includes preparation of enzyme extracts and their spectroscopic assays.

3.5.2.1 Preparation of enzyme extracts

Frozen wheatgrass leaves (2.0 g) were crushed into fine powder under liquid nitrogen and the homogenized in 10 ml of potassium phosphate buffer (50 mM, pH 7.0) containing 1mM Na_2EDTA, 5 mM sodium ascorbate, 1 mM PMSF (Phenylmethylsulfonyl fluoride), 5% (w/v) insoluble PVPP (Polyvinylpolypyrrolidone) and 0.05% (w/v) Triton X-100. The homogenates were centrifuged at 4° C; 15,000 rpm for 10 min and supernatants were divided into aliquots and stored at -20° C for further analysis. Protein levels in enzyme extracts were estimated by Bradford method (Bradford 1976) using bovine serum albumin as standard.

3.5.2.2 Catalase (CAT) activity

Catalase activity was assayed spectrophotometrically according to Miyagawa et al. (2000) with minor modifications. The assay solution (3.0 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 10.5 mM H_2O_2 and enzyme extract containing 20 µg of protein. The reaction was started by adding H_2O_2 and catalase activity was calculated by measuring the rate of H_2O_2 disappearance at 240 nm. The reaction was run for 2 min at 25° C after addition of H_2O_2. One catalase unit is defined as the amount of enzyme necessary to decompose 1 µmole min^{-1} H_2O_2, taking molar extinction coefficient ($\Delta\varepsilon$) at 240 nm as 43.6 M^{-1} cm^{-1}.

3.5.2.3 Ascorbate peroxidase (APx) activity

Ascorbate peroxidase activity was determined according to Nakano and Asada (1981) with minor modifications. Assay solution (3.0 ml) consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM Na_2EDTA, 0.5 mM sodium ascorbate, 0.25 mM H_2O_2 and enzyme extract containing 50 µg of protein. The reaction was run for 2 min at 25° C after the addition of H_2O_2. Ascorbate peroxidase activity was calculated by measuring the rate of reduction in concentration of sodium ascorbate at 290 nm ($\Delta\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). One APx unit is defined as amount of enzyme necessary to oxidize 1 µmol min^{-1} ascorbate.

3.5.2.4 Glutathione reductase (GR) activity

Glutathione reductase activity was assayed according to Halliwell and Foyer (1973) with some changes. The assay solution (3.0 ml) contained 50 mM Tris-HCl (pH 7.8), 3.5 mM $MgCl_2$, 33 µM NADPH, 0.5 mM oxidized glutathione (GSSG) and enzyme extract containing 50 µg of proteins. The reaction was run at 25° C for 5 min after addition of NADPH. The activity of GR

was calculated by measuring the rate of decrease in absorbance at 340 nm ($\Delta\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) due to oxidation of NADPH. One GR unit is defined as the amount of enzyme that oxidizes 1.0 µmol min^{-1} NADPH.

3.5.2.5 Glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was determined according to the method given by Weydert and Cullen (2010). 1.0 ml of disposable polystyrene cuvette containing 900 µl of GPx assay solution (50 mM potassium phosphate buffer (pH 7.0), 1.0 mM Na_2EDTA, 1.0 mM NaN_3, 1.0 U/ml glutathione reductase, 1.0 mM reduced glutathione (GSH) and 0.2 mM NADPH) and enzyme extract containing 50 µg of protein was mixed together and incubated for 5 min at 25° C. Reaction was started after addition of 10 µl of H_2O_2 (2.5 mM) followed by measuring the change in absorbance at 340 nm for 5 min. Glutathione peroxidase enzyme (0.02-0.1 U/ml) was used as standard to calculate the GPx activities in samples.

3.5.2.6 Guaiacol peroxidase (GuPx) activity

Guaiacol peroxidase was assayed as method given by Taliana et al (1999) with minor modifications. The assay solution (3.0 ml) containing 50 mM potassium phosphate buffer (pH 7.0), 2.7 mM guaiacol, 2 mM H_2O_2 and enzyme extract containing 5 µg of protein. The reaction was started by adding enzyme extract and the oxidation of guaiacol was determined by increase in absorbance at 470 nm ($\Delta\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). One GuPx unit is defined as the amount of enzyme that produces 1 µmol min^{-1} oxidized guaiacol.

3.5.2.7 Superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined according to Beyer and Fridovich (1987) with minor changes. The SOD activity is based on the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) in presence of riboflavin. The SOD assay solution was prepared by mixing 81 ml of 50 mM potassium phosphate buffer (pH 7.8), 3.0 ml of NBT salt (1.44 mg/ml), 4.5 ml of L-methionine (30 mg/ml) and 2.25 ml of Triton X-100. All above reagents were mixed thoroughly on magnetic stirrer to avoid foam formation. 1.0 ml aliquot of assay solution was taken into disposable polystyrene cuvette followed by addition of 50 µl of enzyme extract and 10 µl of riboflavin (44 µg/ml). The solution was mixed and cuvettes were transferred into a light box containing 40 W fluorescent lamp. The cuvettes were illuminated for 12 min at 25° C. A

control solution in which enzyme was replaced by 50 μl of buffer was run in parallel and absorbance of formazan formed was read at 560 nm. One SOD unit was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%.

3.5.3 Characterization of free and bound phenolic compounds, and their bioaccessibility from wheatgrass

3.5.3.1 Extraction of free phenolic compounds

Free or soluble phenolics from wheatgrass matrices were extracted as described by Hung et al (2011). Approximately, 1.0 g of wheatgrass powder was added to a 15 ml centrifuge tube containing 10 ml of 80% ethanol. The content was mixed thoroughly, flushed with nitrogen and tube was transferred to an ultrasonic water bath for 30 min at 30° C. After centrifugation at 5000 rpm for 10 min, the supernatant was collected. The phenolics were re-extracted twice, filtered through a 0.2 μm syringe filter and concentrated using rotary evaporator. Dried extracts were resuspended in methanol with final concentration of 1 mg/ml, purged with nitrogen and stored at -20° C until further analysis.

3.5.3.2 Extraction of cell wall bound phenolics

Residue left after centrifugation in above step (3.5.3.1), was used for the extraction of bound phenolics by alkaline hydrolysis according to the method given by Kardas and Durucasu (2014). Residue was transferred to a 50 ml centrifuge tube containing 20 ml, 4 N NaOH. The mixture was flushed with nitrogen to generate an inert environment. The tube was stirred for 6 h at 30° C. After alkaline hydrolysis, the solution was acidified with ice cold 6 N HCl. The tube was centrifuged at 5000 rpm for 10 min and liberated phenolic acids were extracted from supernatant with ethyl acetate. Extraction was repeated thrice and ethyl acetate was evaporated to dryness using rotary evaporator. Dried extracts were resuspended in methanol with final concentration of 1 mg/ml, purged with nitrogen and stored at -20° C until further analysis.

3.5.3.3 HPLC analysis of phenolics

Both free (3.5.3.1) and bound (3.5.3.2) phenolic extracts were filtered through a 0.2 μm syringe filter and analyzed on HPLC system (Shimadzu Corporation, Japan) equipped with a 20 μl loop injection valve and connected with photo diode array (PDA) detector at 280 nm. The analytical column was Enable C18G (250×4.6 mm, 5 μm) connected with C18 guard column.

The mobile phase was 2% glacial acetic acid in water (solvent A) and methanol (solvent B). The programmed run was 45 min using a constant solvent flow rate of 1.0 ml/min with following gradient: 10% B to 40% B in 10 min, 40% B to 80% B in 20 min, 80% B to 100% B in 30 min and hold of 100% B up to 45 min. The individual phenolic compound was quantified against corresponding calibration curves drawn after co-elution of following phenolic standards (5-25 μg/ml): catechin, chlorogenic acid, p-hydroxybenzoic acid, epicatechin, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, o-coumaric acid, ellagic acid, quercetin, luteolin, kaempferol and butylated hydroxylanisole (BHA).

3.5.3.4 Bioaccessibility of phenolic compounds from wheatgrass

Bioaccessibility of different phenolic species from wheatgrass was determined by *in-vitro* gastro-intestinal digestion followed by HPLC analysis. Selenium rich wheatgrass grown under UV-B light were taken as a sample. Digestive enzymes were prepared according to the method described in section 3.4.2. Approximately, 2 g of wheatgrass powder was taken in 250 ml conical flask containing 30 ml of gastric juice solution. Flask was sealed tightly with parafilm and incubated for 3 h, 50 rpm at 37° C. Flask was then adjusted to pH 7.0 with saturated solution of $NaHCO_3$ followed by addition of 20 ml intestinal juice solution. Flask was further incubated for 4 h, 50 rpm at 37° C. The digest was then centrifuged at 8000 rpm at 4° C for 10 min and filtered. pH of filtrate was adjusted to 1-2 with 1 N HCl and phenolic compounds released after enzymatic hydrolysis were extracted with ethyl acetate. Ethyl acetate was removed using rotary evaporator and concentrated extract was dried under the blow of nitrogen gas. Dried extracts were resuspended in methanol with final concentration of 1 mg/ml and HPLC analysis was carried out as described before (section 3.5.3.3).

3.6 Isoselenocyanates and Se-rich proteins form selenium-rich mustard cake

Present section is focused on (i) extraction and characterization of isoselenocyanate species and (ii) isolation of selenium-rich storage protein and their bioactive properties from selenium-rich mustard cake.

3.6.1 Isolation of isoselenocyanates from selenium-rich mustard cake

Isothiocyanates (ITCs) are the organosulfur compounds responsible for the pungent odor of mustard oil. When mustard seed are broken during oil extraction, the enzyme myrosinase is

released and acts on a sulfur containing secondary metabolites called glucosinolates to give ITCs. During biosynthesis of glucosinolates, sulfur atom is donated by cysteine amino acid. Mustard plants accumulate high selenium in their seeds mainly in the form of selenoamino acids (selenocysteine and selenomethionine) when cultivated in selenium contaminated soil. In high-selenium mustard seeds it is presumed that Se-glucosinolates and isoselenocyanates (ISeCs) would be synthesized due to similar biochemical properties of sulfur with selenium.

In present work, myrosinase based enzymatic auto hydrolysis of glucosinolates/Se-glucosinolates was carried out for the synthesis of ITCs/ISeCs from selenium-rich mustard cake obtained after oil extraction. A 100 g of defatted Se-rich mustard cake powder was taken in 250 ml conical flask containing 50 ml of extraction buffer (10 mM, pH 7.5 potassium phosphate buffer, 1.0 mM Sodium-EDTA and 5% (v/v) glycerol). The powder was mixed thoroughly and flask was incubated at 37° C for 6 h. For the extraction of isothiocyanates/isoselenocyanates (ITCs/ISeCs), 100 ml of acetonitrile was added to flask and shaken vigorously for 30 min. Extraction was repeated 3 times and acetonitrile was pooled together after filtration. Acetonitrile was dried over anhydrous sodium sulfate and evaporated using rotary evaporator. Oily residue left after evaporation was re-suspended into 10 ml, 25% (v/v in water) acetonitrile and loaded on to SEP-PAK C_{18} cartridge (Waters Association, Ireland) prewashed with methanol and distilled water. Cartridge was washed with 25% acetonitrile and ITCs/ISeCs were eluted with 10 ml volume of acetonitrile. Solvent was removed using rotary evaporator and oily residue containing crude ITCs/ISeCs was stored at -20° C for further use. Mustard cake residue left after extraction of ITCs/ISeCs, was air dried and stored for further use.

3.6.2 Estimation of selenium from crude ITCs/ISeCs fraction

For selenium quantification, a 20 mg of sample was transferred into microwave digestion vessel and selenium of estimated by fluorescence spectrometer (section 3.3.1).

3.6.3 Cyclocondensation assay based quantification of ITCs/ISeCs in crude oily residue

Total ITCs/ISeCs content in isolated oily residue was quantified by the method given by Jiao et al. (1998) with some modifications. This method is based on cyclic condensation reaction of ITCs/ISeCs with a dithiol reagent for quantification of total ITCs/ISeCs content. In brief, 25 µl of diluted oily residue (2 mg/ml in methanol) was mixed with 1.0 ml of 1.2-benzenedithiol (10 mM, in methanol) and 1.0 ml of phosphate buffer (100 mM, pH 8.5) in a 5 ml screw capped vial

with teflon septum. The reaction mixture was mixed thoroughly and purged with nitrogen gas. Vial was incubated at 65° C for 2 h and centrifuged at 8000 rpm for 10 min. The single cyclo-condensation product (1,3-benzenedithiole-2-thione) formed was analyzed by HPLC system (Shimadzu Corporation, Japan) equipped with a 20 µl loop injection valve and connected with photo diode array (PDA) detector at 365 nm. The analytical column was Enable C18G (250×4.6 mm, 5 µm) connected with C18 guard column. The isocratic mobile phase consisted of 80% methanol and 20% water at a flow rate of 1.0 ml/min. Cyclocondensation product of butyl isothiocyanate was used as standard (5-25 µg/ml) to draw a calibration curve.

3.6.4 Speciation of ITCs/ISCs in oily residue using GC-MS

After isolation, crude oily residue was subjected to gas chromatography-mass spectrometry (GC-MS) analysis for identification of ITCs/ISeCs species using gas chromatograph (45X-GC, Bruker) coupled with a mass selective detector (MS-SCION-45P, Bruker) equipped with a 30m × 0.25mm × 0.25 µm SLB-5MS column. The mass detector was operated in electron-impact ionization mode (EI+) at 70 eV with ion source temperature at 280° C. Scan mass range was 20 to 500 *m/z*. Helium was used as carrier gas with flow rate of 1.0 ml/min. The injector temperature was set at 250° C. The GC oven temperature program was: 50° C for 3 min, 50 to 150° C at a rate of 5° C/min and subsequently held isothermally for 5 min, 150 to 280° C at a rate of 10° C/min and finally held isothermally for 5 min. One micro liter diluted sample (100 µg/ml, in acetonitrile) was injected automatically with 1:50 GC split ratio. Identification of ITCs/ISCs components was confirmed by comparing their mass spectra with those contained in the National Institute for Standards and Technology (NIST, Search Version 2.0).

3.6.5 Extraction of protein from Se-rich mustard cake

Mustard cake residue left after extraction of ITCs/ISeCs, was used for isolation of storage protein. Approximately 50 g of dried residue was taken in a 250 ml conical flask containing 100 ml of distilled water. The flask was stirred for 3 h followed by centrifugation at 8000 rpm for 10 min. The extraction was repeated twice and supernatant was pooled together. A 4 volume of chilled acetone (-20° C) was added to the supernatant and incubated overnight at -20° C for precipitation of proteins. After centrifugation protein pellet was collected and washed again with chilled acetone. Pellet was dried and grounded into fine powder for further use. Total selenium

content in isolated protein was determined by fluorescence spectrometer as described previously (section 3.3.1).

3.6.6 Cytoprotective effect of Se-rich mustard protein

After extraction of Se-rich mustard protein, their cytoprotective effect was performed against tert-Butyl hydroperoxide (TBHP) on mouse melanoma cell lines (B16-F10). Before the treatment, 500 mg Se-rich mustard protein powder was subjected to *in-vitro* gastro-intestinal (GI) digestion (section 3.4.2) to increase the bioaccessibility of selenium. After digestion, GI digest was incubated at 90° C in water bath for 1 h to inactivate the digestive enzymes (pepsin and pancreatin). The digest was centrifuged and selenium concentration was estimated by fluorescence spectrometer (section 3.3.1). Sodium selenite was taken as positive control, whereas undigested Se-rich mustard protein and GI digested non-seleniferous mustard protein (isolated as described in section 3.6.5) were taken as negative control. Sample for undigested Se-rich mustard protein was prepared by dissolving 50 mg of protein powder into 10 ml of PBS, followed by selenium determination.

B16-F10 cells were grown in DMEM nutrient medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin. Before selenium treatment, a total of 1.0×10^4 cells was seeded in collagen precoated 96 well tissue culture plate. The plate was then incubated in 5% CO_2 containing humidified air at 37° C. After 70% confluence, growth media was replaced with a fresh medium containing 1µM of selenium from different sources and further incubated for 24 h. Each well was washed twice with PBS and a fresh medium was added containing 2.0 mM TBHP, followed by additional 12 h incubation. Details of sample treatment in each column of 96 well plate was as follow: column (A) only growth medium (B) 2.0 mM TBHP (C) 1µM selenium as sodium selenite (D) Pretreatment with 1µM selenium as sodium selenite + 2.0 mM TBHP (E) GI digested non-seleniferous mustard protein (F) Pretreatment with GI digested non-seleniferous mustard protein + 2.0 mM TBHP (G) 1µM selenium as undigested Se-rich mustard protein (H) Pretreatment with 1µM selenium as undigested Se-rich mustard protein + 2.0 mM TBHP (I) 1µM selenium as GI digested Se-rich mustard protein (J) Pretreatment with 1µM selenium as GI digested Se-rich mustard protein + 2.0 mM TBHP.

TBHP induced cytotoxicity was assessed by observation of cell viability and cell morphological changes. Cell viability was determined by the mitochondrial dependent reduction

of MTT (3-(4,5-Dimethylthiazolyi-2)-2,5-Diphenyltetrazolium Bromide) into intracellular purple formazan. Growth medium from each well was removed (except last well of each column) and 20 µl of MTT reagent (5 mg/ml, in PBS) was added. Plate was incubated for 4 h in culture incubator. When the purple precipitate was clearly visible under the microscope, 100 µl of dimethyl sulfoxide (DMSO) was added to all wells and absorbance of each well was measured at 570 nm using microtiter plate reader. Last well of each column was used for the staining and microscopic imaging of the cells. Dead and live cells were differentiated by staining with trypan blue and neutral red respectively. Before staining, growth medium was removed from wells and washed twice with PBS. A 100 µl of neutral red dye (40 µg/ml, in DMEM) was added to each well. Plate was incubated for 1 h at appropriate culture conditions. Wells were washed twice with PBS, followed by addition of 20 µl trypan blue solution (0.4% w/v) per well. Plate was incubated for another 10 min. Each well was washed once with PBS and a 100 µl of PBS was added to each well before microscopic imaging.

3.6.7 Assay of antioxidant enzyme

Cytoprotective effect of Se-rich mustard protein against TBHP is due to increase in the expression of selenium dependent antioxidant enzyme called glutathione peroxidase (GPx). Present section is focused on selenium dependent assessment on levels of GPx in mouse melanoma cells. A total of 1.0×10^6 cells was seeded in collagen precoated 90 mm tissue culture plates containing DMEM growth medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin. The plates were then incubated in 5% CO_2 containing humidified air at 37° C. After 70% confluence, growth media was replaced with a fresh medium containing 1µM of selenium from different sources as described in section 3.6.6 (treatment A-J) and plates were incubated for 24 h. Plates were washed twice with PBS and fresh medium was added containing 0.5 mM TBHP followed by additional 12 h incubation. In this particular set of experiments, 0.5mM TBHP was taken keeping in view to obtaining viable cells but under stress, as compared to the earlier section where 2mM TBHP was observed to exhibit 100% cytotoxicity. Cells were washed twice with PBS free of Ca^{2+} and Mg^{2+} and harvested by scraping. Scraped cells were resuspended into 1.0 ml of 50 mM phosphate buffer (pH 7.8), sonicated on ice for 2 min (pulse time 9 sec)and centrifuged at 15,000 rpm, 4° C for 30 min. Cell homogenates were transferred into 1.5 ml microfuge tube and stored at -20° C until

assayed. Total protein concentration in cell homogenates was estimated by Bradford method (Bradford 1976) using bovine serum albumin as standard.

Glutathione peroxidase activity determined according to method given by Weydert and Cullen (2010). 1.0 ml of disposable polystyrene cuvette containing 900 µl of GPx assay solution (50 mM potassium phosphate buffer (pH 7.0), 1.0 mM Na_2EDTA, 1.0 mM NaN_3, 1.0 U/ml glutathione reductase, 1.0 mM reduced glutathione (GSH) and 0.2 mM NADPH) and cell homogenate containing 100 µg of protein was mixed together and incubated for 5 min at 37° C. Reaction was started after addition of 10 µl of H_2O_2 (2.5 mM) followed by measuring the change in absorbance at 340 nm for 5 min. Glutathione peroxidase enzyme (0.02-0.1 U/ml) was used as standard to calculate the GPx activities (U/mg) in samples.

Statistical analysis

Estimations were carried in triplicate, where ever applicable, and were expressed as mean ± standard deviation (SD) values. The comparison between seleniferous and non-seleniferous samples were drawn using student‘t’ test ($P < 0.05$) on Graph pad prism software 5.0.

4.0 Results and Discussion

4.1 Estimation of total selenium

Total selenium content in soil and different crop matrices were analyzed by fluorescence spectrometer and instrumental neutron activation analysis (INAA) are presented in table 4.1 and 4.2. Selenium concentrations in those samples collected from seleniferous region were significantly higher ($P < 0.001$) over their respective controls (collected from non-seleniferous region). Where as levels of selenium uptake among selenium-rich cereals were significantly different ($P < 0.001$) from each other.

Table 4.1: Total selenium content in selenium-rich and non-seleniferous samples analyzed by fluorescence spectrometer* (n=6; mean±SD)

Sample	Total Selenium (µg/g)	
	Seleniferous (Se)	**Non-seleniferous (NSe)**
Soil	6.23 ± 0.17	0.35 ± 0.04
Wheat grain	115.4 ± 0.83	0.48 ± 0.01
Baked Indian wheat bread (Chapati)	112.3 ± 1.72	0.43 ± 0.01
Rice grain	19.14 ± 0.19	BDL
Cooked rice	18.62 ± 0.35	BDL
Wheatgrass (exposed to visible light)	148.1 ± 4.05	0.67 ± 0.05
Wheatgrass (exposed to UV-B light)	151.3 ± 2.46	0.75 ± 0.04

*: detection limit (2 ng/ml); BDL- below detection limit

There was no significant decrease in the selenium levels even after processing of grains. Baking of wheat flour (as Indian bread/chapati) and conventional cooking of rice showed negligible changes in selenium concentrations i.e.112.3 ± 1.72 and 18.62 ± 0.35 µg/g respectively.

Further selenium-rich wheatgrass cultivated under visible and UV light accumulated significantly higher amount of selenium ($P < 0.001$) when compared to their respective control.

Whereas no significant difference in selenium content were observed in wheatgrass samples cultivated under visible and UV light.

As a selenium accumulator, mustard plant, cultivated on selenium impacted soils, also accumulated high levels of selenium in their seeds. Table 4.2 represents levels of selenium in both non-seleniferous and selenium-rich mustard samples analyzed by INAA and fluorescence spectrometer.

Table 4.2: Total selenium content in selenium-rich and non-seleniferous mustard samples (n=3; mean±SD)

Sample	Total Selenium (µg/g)	
	INAA*	**Fluorescence spectrometer**
Mustard seed (Se)	110.0 ± 3.04	105.4 ± 1.54
Mustard oil (Se)	3.50 ± 0.66	2.60 ± 0.29
Mustard cake (Se)	143.0 ± 5.18	138.90 ± 1.6
Mustard seed (NSe)	0.48 ± 0.02	0.87 ± 0.10
Mustard oil (NSe)	BDL	BDL
Mustard cake (NSe)	0.72 ± 0.14	1.18 ± 0.06
CRM**	**Certified value (µg/g)**	**Obtained value (µg/g)**
IAEA SL-1	2.85	2.47 ± 0.4
NIST-8436	1.23 ± 0.09	1.26 ± 0.03

*: detection limit (10 ng/g); **: Used for validating INAA results; BDL- below detection limit; NSe- non-seleniferous; Se- seleniferous

A typical gamma-ray spectrum generated for mustard-based matrix, using data obtained from HPGe detector of the gamma ray spectrometer with Compton-suppressed detection system, is presented in figure 4.1. The figure indicates gamma rays (121, 136, 264.7 and 279 keV) that typically signify the presence of radioactive selenium isotope (Se^{75}). The accuracy of INAA method for Se determination, as assessed through certified reference material (CRM) analysis was found satisfactory. The values obtained for IAEA SL-1 and NIST 8436 respectively were

2.47 ± 0.4 µg/g and 1.26 ± 0.03 µg/g, which were comparable with the certified values, i.e., 2.85 µg/g and 1.23 ± 0.09 µg/g, respectively.

Total selenium content analyzed by INAA in the control (NSe) and selenium-rich mustard seeds were 0.48 ± 0.02 µg/g and 110.0 ± 3.04 µg/g respectively, whereas in mustard cake it was found to be 0.72 ± 0.14 µg/g and 143.0 ± 5.18 µg/g respectively. A very small fraction of selenium was also detected in selenium-rich mustard oil (3.50 ± 0.66 µg/g) but it was below detection limit in control mustard oil. A significant difference ($P < 0.001$) in selenium levels were observed in all mustard samples when selenium-rich sample compared with their respective controls.

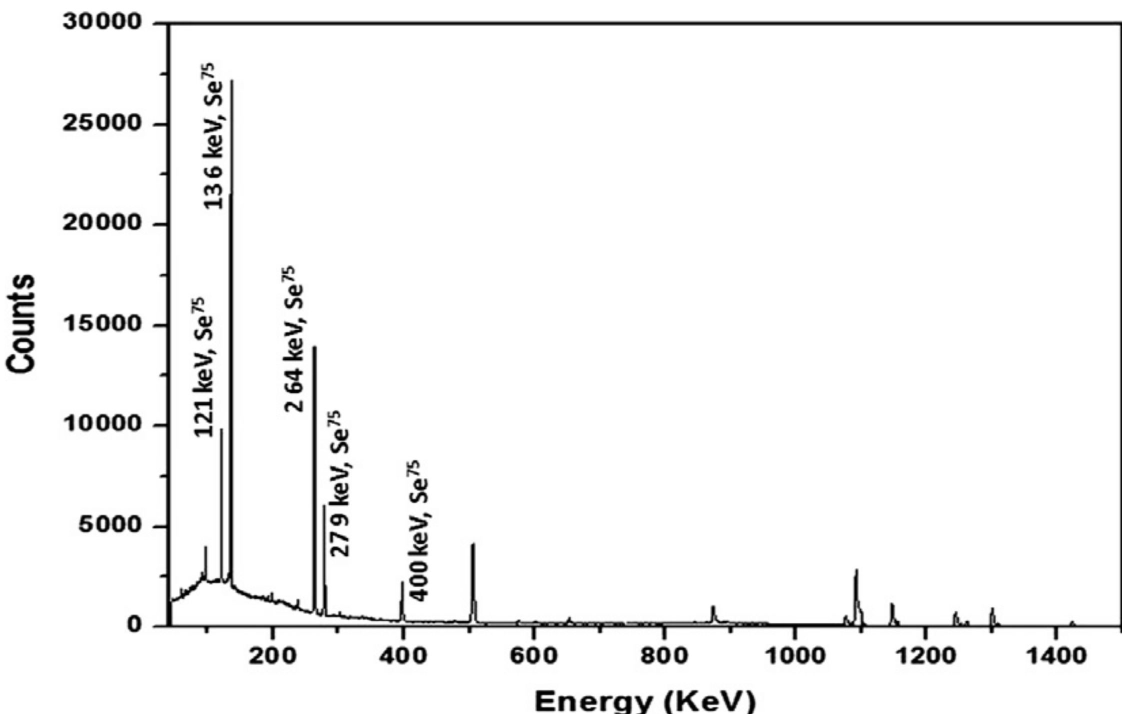


Figure 4.1: Typical gamma ray spectrum of selenium as acquired after irradiation, using high purity germanium-bismuth germanate (HPGe-BGO) Compton suppressed detection system

INAA is an isotope-specific nuclear analytical technique capable of simultaneous multi-element determination in diverse matrices. This technique is capable of determining as many as 70 elements of the periodic table. The technique involves irradiation of the samples with neutrons in a nuclear reactor, leading to the formation of radionuclides, whose radioactivity is measured, preferably using a high-resolution gamma ray spectrometer. It has many advantages; it

is nondestructive, has high analytical sensitivity, high selectivity, no spectral interference, good detection limits (mg/kg to µg/kg), negligible matrix effect and can analyze many samples in one irradiation. Like other analytical techniques, INAA also have some limitations. One major disadvantage is requirement of a high flux neutron source to obtain significant sensitivity therefore this technique is not easily available. For radioactive selenium species (Se^{75}, half-life 120 days) cooling time is around 10 days that facilitate the decay of short-lived radioactive nuclei and radioactive assay is carried out by HPGe detector require 1-10 h depending upon selenium concentration in samples.

Unlike INAA, fluorescence spectrometer requires less time (1-2 days) for complete selenium analysis, but this technique involved many steps viz. dissolution of sample with oxidizing acids, reduction of Se^{+6} to Se^{+4}, formation of Se-DAN complex (piazselenol), extaction of Se-DAN complex and fluorescence analysis. Due to multi step process, certain loss of selenium is expected. The most serious concern is the interference of ferric ions during complex formation with DAN; therefore it is necessary to chelate ferric ions with EDTA before addition of DAN solution. Despite many disadvantages, both INAA and fluorescence spectrometer have better sensitivity for selenium, wherein the minimum detection limit for fluorescence spectrometer was found to be 2 ng/ml (Levesque and Vendette 1971) where as detection limit of INAA for selenium in biological samples was as low as 10 ng/g (Di Piero et al. 2008). In present work, there was no significant difference ($P > 0.05$) observed in the selenium content of mustard samples analyzed by INAA and fluorescence spectrometer (table 4.2).

Most of the cultivated plants possess the ability to absorb, metabolize and store significant amount of selenium in their tissues when grown on Se-contaminated soils. Selenium phyto-accessibility is dependent on diverse variety of soil and climatic conditions such as soil pH, redox conditions, organic matter, competing ionic species, microbial activity, level of rainfall during the growing season etc. (Dhillon and Dhillon 2003; Spadoni et al. 2007). Absorption of selenium is highest at lower pH value and absorption of selenite ion (SeO_3^{-2}) decreases at pH above 6.0, whereas absorption of selenate ion (SeO_4^{-2}) decreases over the entire pH range (2.5-10).

Selenium content in soil varies from low to toxic ranges; low in the range from 0.03-0.08 µg/g and toxic (seleniferous) levels can extend up to ≤ 1200 µg/g (NSA-NRC 1976; Fordyce

2005). In India, particularly in the Nawanshahr-Hoshiarpur region of Punjab, a large agricultural land (> 1000 hectares) is severely affected with high levels of selenium ranges from 0.25 to 4.55 µg/g with a mean value of 3.63 µg/g (Dhillon and Dhillon, 2003). Further studies carried out by Sharma et al. (2009), examined selenium concentration in soils using INAA and found the ranges of 2.7-6.5 µg/g, selenium in the crop produce (wheat, rice, maize and mustard) were lying in the range of 13-670 µg/g. Level of selenium in water is an important factor for determining its suitability for different purposes. According to water quality guidelines for selenium of the United States Environmental Protection Agency (NAS–NAE, 1973), the maximum permissible level (MPL) for water used for irrigation is 20 µg/L and the maximum contamination level (MCL) of selenium in water for drinking purposes is 10 µg/L. In the Nawanshahr-Hoshiarpur region of Punjab, the ground water used for irrigation purpose is the only source of selenium and was found as high as 479 µg/L with a mean value of 170 µg/L (Srivastava et al. 2006), which is significantly above permissible level for drinking and irrigation purpose.

Selenium uptake by plants is influenced by concentration of anions such as SO_4^{-2} and Cl^- present in saline soil. Transport of SeO_4^{-2} ion by roots in many crop plants is mediated by cell membrane carrier common for SO_4^{-2} and other anions, due to this antagonism, SO_4^{-2} and Cl^- ions inhibit the selenium uptake to a greater extent (Grieve et al. 2001). Study conducted by Dhillon and Dhillon (2000) at the same study site, indicated that addition of gypsum as a source of sulfur in the crop field significantly reduced selenium accumulation by plants. Reduction in selenium accumulation were 49% in wheat straw, 64% in wheat grain, 51% in rice straw and 63% in rice grain with an application of gypsum at 0.8 ton per hectare.

Due to similar chemistry of selenium with sulfur, plants transport and metabolize selenium as similar to sulfur assimilation pathways. In the case of higher plants, selenium has not been shown to be essential element but to be a beneficial nutrient. It act as antioxidant and inhibit lipid peroxidation; promotes the growth of plant under UV induced oxidative stress; increases starch accumulation in chloroplast; shows positive effect on carbohydrate accumulation in potatoes and protects the plant under drought stress by increasing water uptake capacity of the root system (Hartikainen et al. 2000; Germ et al. 2007; Pennanen et al. 2002; Turakainen et al. 2004; Kuznetsov et al. 2003).

In plants, selenium metabolizes via sulfur assimilation pathways, which involves biosynthesis of selenomethionine (SeMet) and selenocysteine (SeCys), which are nonspecifically incorporated into proteins at the place of methionine and cysteine respectively (Terry et al 2002). Plants that have tendency to accumulate high levels of selenium (1000-10,000 µg/g dry weight) are categorized as selenium hyperaccumulators and these plants are belong to families of Asteraceae, Brassicaceae, Chenopodiaceae, Lecythidaceae, Fabaceae, Rubiaceae and Schrophulariaceae. These plants accumulate selenium, 100 fold higher than those of surrounding vegetation without showing any toxicity symptoms (Pilon-Smits and Quinn 2010). These selenium hyperaccumulator plants primarily biosynthesize non-protein selenoamino acids, such as methyl-selenocysteine (MeSeCys) and γ-glutamyl-methyl-selenocystein (γ-Glu-MeSeCys), which prevent the damaging effect on plant functions resulting from incorporation of SeCys and SeMet in proteins (Cubadda et al. 2010).

There is a wide variation in wheat grain selenium level, depending upon geographical location. Reported selenium levels in wheat grains range from as low as 0.001 µg/g in south-west Western Australia (White et al. 1981) to 30 µg/g found in highly seleniferous soil of South Dakota (University of California, 1988), but major distribution of selenium content in global wheat falls within the range of 0.02-0.6 µg/g (Alfthan and Neve 1996). In USA and Canada, levels of selenium were found relatively high, usually in the range of 0.2-0.6 µg/g (Reilly 1996) whereas suboptimal selenium level in wheat was found in Eastern Europe and New Zealand with average value of 0.028 µg/g (Mihailovic et al. 1996), in some parts of China (0.01-0.23 µg/g) and Finland (0.01-0.015 µg/g) (Alfthan and Neve 1996; Koivistoinen and Vara 1987). Like other hyperaccumulator plants, it has been reported first time that wheat also accumulates high selenium content in their grains, ranging from 29-185 µg/g in Nawanshahr-Hoshiarpur region of Punjab, India (Sharma et al. 2009; Cubadda et al. 2010). Present study also confirmed that selenium content in wheat (115.4 ± 0.83 µg/g) is higher than those reported in selenium-impacted region of Enshi, China or from other selenium-rich areas (Aureli et al. 2012).

For humans, wheat is one of the most important sources of selenium (Comb 2001). In wheat, most of the selenium is protein bound in the form of SeMet, which accounts for 80-90 % of total seleno-compounds detected in wheat (Stadlober et al. 2001; Kirby et al. 2008; Diaz et al. 2003; Warburton et al. 2007). Other than SeMet, this high-selenium wheat also contains MeSeCys, selenocystine ($SeCys_2$), SeO_4^{-2} and selenosugars (monosaccharides and disaccharides)

(Cubadda et al. 2010, Aureli et al. 2012). The sectional studies of high-selenium wheat grain showed that selenium concentration was found highest as SeMet in embryo followed by endosperm and then seed coat (Lyons et al. 2005). Wheat grain contains up to 15% storage proteins which also serve as important energy reserve for seedlings.

During germination of cereals, thioredoxin h (trx h), a small redox protein plays a vital role. It is involved in the regulation of redox environment of the cell through reducing protein disulfide bonds. During seed germination, trx h increases the susceptibility of storage proteins to proteolysis by breaking the intramolecular disulfide bonds of prolamins and glutelins to make them metabolically active (Guo et al. 2013). After proteolysis, free amino acids are released from their protein complex and further utilized for synthesis of new proteins, which are eventually available for use by developing embryo. In high-selenium wheat, major part of selenium is localized into embryo and endosperm as SeMet and during germination, SeMet gets utilized in a similar way as methionine. In present the study, presence of high selenium in wheatgrass (148-151 µg/g) of dry weight confirmed that, selenium present in seed was metabolized and translocated to the leaves during development.

Rice is non-accumulators of selenium and selenium concentration in grains rarely exceed 1.0 µg/g. Sun et al. (2010) investigated the distribution and translocation of selenium from soil to grain in a selenium rich environment and found that total selenium concentration in the rice fractions were in the following order: straw > bran > whole grain > polished rice > husk. These results clearly indicated that selenium in soil has a linear relationship with different fractions as well. According to Williams et al. (2009), X-ray fluorescence (µ-XRF) imaging of selenium rich rice grain showed that inorganic selenium were 10 times higher in husk and bran than those of endosperm. SeMet, $SeCys_2$ and MeSeCys were all detected in husk, with SeMet being dominant. In comparison to husk, MeSeCys was the only organic selenium species detected in bran and contributed to 47% of total selenium. Inorganic selenium was mostly absent in endosperm tissue, 94.5% of the detected species were organic selenium, and of that 59% were identified as MeSeCys. Being one of the major food crops, rice is also an important source of selenium for more than half of the world's population. Worldwide survey of selenium concentration in white polished rice was conducted by Williams et al. (2009) to determine variation in global rice grain selenium concentration and range of selenium was found as follow: China (0.002-1.37 µg/g), Egypt (0.006-0.087 µg/g), France (0.053-0.241 µg/g), Ghana (0.021-0.254 µg/g), India (0.035-

0.371 µg/g), Italy (0.032-0.158 µg/g), Japan (0.026-0.109 µg/g), Philippines (0.056-0.241 µg/g), Spain (0.006-0.104 µg/g), Thailand (0.006-0.487 µg/g) and USA (0.006-0.406 µg/g). In present study, selenium content in rice sample collected from Nawanshahr-Hoshiarpur region of Punjab was found highest (19.14 µg/g) in comparison to the previously reported values (5-16.2 µg/g) by Dhillon and Dhillon (2009) and Sharma et al. (2009).

Seeds of *Brassica juncea* (Indian mustard) are a good source of essential nutrients. According to the USDA National Nutrient Database, 100 g mustard seed contain approximately 28 g carbohydrate, 26 g protein, 36 g total fat, 12 g dietary fibre and 0% cholesterol. They also contain trace amount of all types of vitamins, electrolytes (Na and K) and essential minerals, like Ca, Cu, Fe, Mg, Mn, Se and Zn. Due to its high nutritional value, mustard cake is prominently used as a supplement for animal feed. When this species is grown on selenium contaminated soil, it accumulates significantly high amounts of selenium in its seeds (670 µg/g), due to its excellent hyperaccumulating potential (Sharma et al. 2009). Indian mustard plants accumulate large amounts of organic selenium as noted in the present study and also supported by other observations, indicating rapid translocation of selenium from root to shoot, especially in the presence of selenium as selenate (de Souza et al. 1998). Once selenium is taken up by plant roots in a particular chemical form, whether organic or inorganic, it undergoes certain metabolic changes that determine the final product as well as its translocation and accumulation in different plant tissues (Zayed et al. 1998). A study on comparison of selenium levels in different plant organs of hyperaccumulator plants, including those of *Brassica* species, suggest that selenium is actively transported through the plant in favourable growth conditions (Galeas et al. 2007). As the leaves mature, their Se levels decline which could be a result of dilution as the leaves expand, and also due to export to reproductive tissues, such as seeds, which contain some of the highest levels in the entire plant (Galeas et al. 2007). These observations support the findings in the present study that Se levels were significantly high in seeds.

In the perspective of biological application, species belonging to Brassicaceae normally produce a high concentration of glucosinolates and form isothiocyanates, as a by-product after glucosinolate hydrolysis (Angus et al. 1994; Brown and Morra, 1996; de Souza et al. 2002; Fenwick et al. 1983). In theory, the selenium-containing phytochemicals produced in selenium hyperaccumulating *Brassica juncea*, might include selenium analogues of isothiocyanates (isoselenocyanates) that actually possess more anti-carcinogenic potential than the normal sulfur-

containing products of glucosinolates. In present work, detection of selenium in selenium-rich mustard oil was expected due to the presence of isoselenocyanate species formed after hydrolysis of seleno-glucosinolates (Jaiswal et al. 2012b). The observation were further confirmed by Ouerdane et al. (2013), wherein presence of several novel selenium metabolites such as selenoglucosinolates, selenosinapine, selenoacylated choline, selenocarbohydrates and their metabolites selenourea, isoselenocyanates were reported in selenium-rich mustard seeds from the same study site. Recent findings on synthesis and activity of selenium analogues of isothiocyanates, namely isoselenocyanates, showed improved therapeutic efficacy of these compounds towards killing cultured melanoma cells or inhibiting tumour development in animals when administered systemically (Schlobe et al. 2002).

4.2 Quantification of bioaccessible selenium

Present section was aimed to study the bioaccessibility of selenium in selenium-rich matrices subjected to *in-vitro* gastrointestinal (GI) digestion. The clear extract obtained after GI digestion were analyzed for total soluble selenium content released from matrices using fluorescence spectrometer and percentage of selenium bioaccessibility from different samples are presented in table 4.3 and 4.4.

Table 4.3 Bioaccessibility of selenium from different food matrices (n=3; mean±SD)

Sample	Selenium (µg/g)		% Bioaccessibility
	Total	Released after GI digestion	
Wheat grain	115.4 ± 0.83	94.52 ± 1.04	82.20 ± 0.91
Baked Indian wheat bread (Chapati)	112.3 ± 1.72	108.2 ± 1.08	96.26 ± 0.96
Rice grain	19.1 ± 0.19	12.13 ± 0.30	63.37 ± 1.60
Cooked rice	18.6 ± 0.35	15.28 ± 0.41	81.53 ± 2.21
Wheatgrass (exposed to visible light)	148.1 ± 4.05	23.97 ± 0.38	32.37 ± 0.52
Wheatgrass (exposed to UV-B light)	151.3 ± 2.46	23.35 ± 0.21	30.86 ± 0.28

The percent bioaccessibility of selenium from wheat is significantly higher ($P < 0.001$) when compared to rice and wheatgrass samples. Similarly, processing of the grains (cooking/baking) also increased the selenium bioaccessibility significantly ($P < 0.01$) when compared to their respective unprocessed/raw samples. Although wheatgrass contains higher selenium, the observed selenium bioaccessibility from these samples were significantly low ($P < 0.001$) when compared to wheat grain, whereas there were no significant difference ($P > 0.05$) observed in selenium bioaccessibility amongst wheatgrass samples cultivated in visible and UV light.

It is well known that, selenium, after absorption by the plants metabolizes via sulfur assimilation pathways and it nonspecifically incorporated into proteins as SeMet and SeCys. Therefore, present section is also focused on the determination of selenium bioaccessibility in different protein fractions isolated from selenium-rich wheat grain. There were four major storage protein fractions isolated on the basis of their solubility in different solvents i.e. albumin (water), globulin (5% NaCl), glutelin (0.1 M NaOH) and prolamin (70% ethanol); and their respective selenium levels were 444.9 ± 14.59 µg/g, 377.2 ± 6.57 µg/g, 527.8 ± 4.9 µg/g and 495.8 ± 9.58 µg/g (table 4.4), with significantly difference ($P < 0.001$) in levels between each other. Extent of selenium bioaccessibility from these fractions varied from 69 to 96% according to the nature of protein. There were significant difference ($P < 0.001$) observed even in selenium bioaccessibility of these fractions when compared to each other, except, in the case of albumin and globulin ($P > 0.05$).

Table 4.4: Bioaccessibility of selenium from different wheat protein fractions (n=3)

Sample	Selenium (µg/g)		% Bioaccessibility
	Total	Released after GI digestion	
Albumin	444.9 ± 14.59	416.8 ± 15.16	93.66 ± 3.40
Globulin	377.2 ± 6.57	331.6 ± 10.59	87.97 ± 2.81
Glutelin	527.8 ± 4.9	405.4 ± 11.7	76.77 ± 2.21
Prolamin	495.8 ± 9.58	307.3 ± 5.13	69.68 ± 1.16

Generally, bioaccessibility is defined as fraction of nutrients released from the food matrix after digestion in the lumen of the intestine and that can possibly be absorbed through intestinal mucosa, whereas bioavailability is defined as, part of nutrients effectively absorbed through the intestinal epithelium and that enters to the systemic blood stream (Thiry et al. 2013).

Bioavailability of selenium has direct relationship with its bioaccessibility. Bioavailability of selenium varies between different types of food. Organic selenium species (SeMet and SeCys) have high bioavailability (more than 90%), when compared to inorganic selenate and selenite which exceeds maximum of up to 50% (Thomson 2004). A study conducted by Meltzer et al. (1993), in humans showed that diet containing selenium-enriched wheat increases serum selenium significantly, whereas no significant increase was observed after consumption of selenium-enriched fish. Fox et al. (2005) found that selenium absorption was significantly higher from wheat (81%) and garlic (78%) when compared to fish (56%).

In-vitro (simulated) GI method is a useful tool which provides an idea about possible interactions between nutrients and food components, type of food preparation and processing practices, effect of gastric and intestinal factors (pH and enzymes), nature of food matrix and extent of digestibility for a nutrients to be absorbed. In-vitro methods are faster, less expensive, offer better control on experimental variables and to certain extent useful for primary screening, ranking or categorizing tool (Sandberg 2006).

In-vitro GI digestion is a simulated system which includes two-step digestions (gastric and intestinal digestion). In gastric digestion, pepsin (from porcine stomach) is used as digestive enzyme at pH 2 (to simulate gastric pH of an adult). Acidification of enzyme solution to pH 2 is important, because at pH $\geq$ 5, pepsin begins to denature itself and thus will lose its activity. During intestinal digestion, pH of sample solution is adjusted up to pH 7 prior to the addition of pancreatin, which is a cocktail of lipase, ribonuclease, pancreatic amylase and proteolytic enzyme trypsin. Bile salt can be used as emulsifiers.

For humans, cereals are the most important sources of selenium and most of the selenium is protein bound as SeMet. Selenium bioaccessibility from these cereals depends upon nature of storage proteins, processing of cereals, enzyme action and digestibility of food matrices. In present work, selenium bioaccessibility for the cereals under study are presented in tables 4.3 and 4.4, and high bioaccessibility was found in wheat samples when compared to rice and

wheatgrass, similarly among the protein fractions, selenium from albumin showed maximum bioaccessibility. The major factor which affects the selenium bioaccessibility and bioavailability is susceptibility of selenium rich storage proteins to proteolytic breakdown of peptide bonds to liberate the amino acids needed by the body. Albumin and globulin are low molecular weight proteins with reduced disulfide bonds (Kruger and Reed 1988), whereas glutelin and prolamin are high molecular weight storage proteins with intra/inter-chain disulfide linkage and showing biochemical polymerization (Alias and Linden 1991). Due to polymerization and disulfide linkage, both glutelin and prolamin fractions are less susceptible for proteolytic breakdown and thus there is less bioaccessibility of selenium (table 4.4) when compared to albumin and globulin. Total selenium content in different protein fractions of wheat presented in table 4.4 were in the order of glutelin > prolamin > albumin > globulin, an observation that was different from our previous observation (Jaiswal et al. 2015), where wheat grains were collected in different season and from different crop field and order of total selenium content was albumin > glutelin > globulin > prolamin.

Bioaccessibility of selenium from rice is not well documented in literature. The distribution study of selenium in protein fractions from selenium-rich rice carried out by Fang et al. (2010) showed that majority of selenium in rice was found as organic selenium, whereas only 2.48% of total selenium was determined as inorganic selenium. As per the observation nearly 54% of the total selenium was present in four different protein factions (albumin, globulin, glutelin and prolamin) and out of which glutelin fraction was account for 80% of total protein and 31.3% of total selenium in rice; however 33.8% of total selenium was sequestered in the starch bound proteins.

In present work, bioaccessibility of selenium from rice is lower when compared to wheat; this is due to the higher accumulation of selenium in glutelin fraction and starch bound proteins. However, bioaccessibility of selenium in rice is also determined by the activity of pancreatic amylase.

Bioaccessibility of selenium form selenium-rich wheatgrass samples were observed only up to 32% when compared to wheat and rice flour (table 4.3). During germination of selenium-rich wheat, free selenoamino acids (SeMet and SeCys) are released after proteolysis and further utilized for synthesis of new proteins and translocated to the leaves during development. Most of

the selenium in wheatgrass is protein bound and localized into cellular proteins, but due to cellulose based matrix, bioaccessibility of selenium from wheatgrass decreases significantly. Although cellulose is indigestible by humans, it does form a part of the human diet in the form of plant foods. Although wheatgrass rich in fiber content, it also contain some essential elements namely K, Mn, Zn, Fe and Na, and their bioaccessibility also ranges from 37–57% (Kulkarni et al. 2007).

A similar selenium bioaccessible study carried out previously using only raw rice and maize flour by our research group (Jaiswal et al. 2012a), showed that selenium bioaccessibility after *in-vitro* gastrointestinal (GI) digestion was higher when compared to only *in-vitro* gastric (GA) digestion in both the investigated samples. In addition, the concentration of selenium in terms of its bioaccessible levels was significantly higher in rice when compared to maize. This is due to the fact that the materials undergo pancreatic digestion in the GI tract at neutral pH resulting in higher accessibility of the bioavailable forms of the element. Pancreatin, which is a major enzymatic component, added in the GI, is a mixture of many enzymes that breaks complex nutrients into simple molecules, making various bioavailable forms of selenium more bioaccessible. The percentage of extracted Se in GA (52% in case of rice and 32% in case of maize) and GI (65% in case of rice and 51% of maize) was observed to be notably lesser than the reported literature (Reyes et al. 2006; Rayman et al. 2008) This might possibly be due to the type of matrix analyzed, as the high extraction of Se reported by researchers were with reference to yeast. Secondly, the variation in the relatively low active units of amylase in the pancreatin used in the present study might also be the reason for low levels of Se extraction in GA and GI hydrolysates.

Thermal processing (cooking/baking) of wheat and rice flour also increases the selenium bioaccessibility when compared to the raw grains. During heat treatment process, most of the proteins get denatured and partial gelatinization of starch takes place which make them susceptible for proteolytic degradation. Jurkovic and Colic (1993), evaluated the effect of thermal processing on the nutritive value of wheat germ protein and found that roasted wheat germs at 130-150° C for 20 min, has higher digestibility, protein efficiency ratio and improved net protein utilization than the raw wheat germ. It was also proved that roasting destroyed the digestive enzyme inhibitors.

The bioaccessibility of selenium from numerous foods and food sources including buckwheat bran (Reeves et al., 2005), wheat (Mutanen et al. 1987), selenium-enriched yeast (Smith and Picciano 1987), and broccoli (Finley 1998) have been studied using slope rate assay methods of selenium species under *in-vivo* conditions. However, majority of the studies are associated with low selenium food sources, with limited studies on naturally enriched sources such as cereals. As the dominant selenium form in rice and maize is assumed to be SeMet, similar to that in wheat (Cubadda et al. 2010), it is important to understand that the intake of this form of selenium is multi-fold when compared to normal selenium intake.

The selenium concentrations of the samples investigated in this study appear to be the highest ever recorded in cereal grains for human consumption, similar to wheat reported earlier by our group (Cubadda et al., 2010; Sharma et al., 2009). These levels of intake might be leading to chronic toxic effects of selenium accumulation in human through selenium-rich food grains and livestock fed on wheat and rice straw as fodder.

4.3 Studies on selenium induced properties in wheatgrass

4.3.1 Bioactive properties of wheatgrass - *In-vitro* biochemical assays

The aim of present section was to evaluate the effect of selenium and light quality (visible and UV-B) on different *in-vitro* bioactive properties of four different wheatgrass samples. These properties includes total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging, Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), iron (Fe^{2+}) Chelating capacity and lipid peroxidation (TBARS assay). Table 4.5 represents summarized results of above bioactive properties.

4.3.1.1 Total phenolic content (TPC)

Phenolics are the antioxidant compounds found in plants having ability to scavenge free radicals through oxidation reactions. TPC of alcoholic extract of different wheatgrass was evaluated using Folin-Ciocalteu reagent and this method involves the reduction of phosphotungstate-phosphomolybdenum complex by phenolic functional group resulting in color change to blue (Prior et al. 2005), the intensity of which depends on the concentration of phenolic compounds in sample.

Table 4.5: ***In-vitro*** **bioactive properties of different wheatgrass samples (n=4; mean±SD)**

In-vitro assays	Wheatgrass samples			
	NSe-visible	Se-visible	NSeUV-B	SeUV-B
Total phenolic content (TPC) *	25.71 ± 0.76	30.71 ± 0.74	36.50 ± 0.74	51.09 ± 0.72
Total flavonoid content (TFC) **	112.6 ± 4.10	116.2 ± 3.57	133.4 ± 1.80	195.86 ± 3.40
DPPH radical scavenging #	18.58 ± 0.25	26.05 ± 0.76	39.61 ± 0.32	55.86 ± 0.31
Trolox equivalent antioxidant capacity (TEAC) # #	325.0 ± 9.70	434.7 ± 3.36	502.2 ±14.55	758.6 ± 7.73
Ferric reducing antioxidant power (FRAP) $	38.22 ± 1.88	47.02 ± 0.65	65.72 ± 1.88	107.1 ± 2.99
Iron (Fe^{2+}) chelating capacity $$	287.0 ± 3.31	310.4 ± 4.37	526.7 ± 4.37	816.7 ± 3.73
Lipid peroxidation Δ	253.7 ± 6.10	202.6 ± 8.49	310.8 ± 2.65	262.3 ± 5.33

*: µg gallic acid equivalent/mg of extract; **: µg quercetin equivalent/mg of extract; #: µg quercetin equivalent/mg of extract; **# #**: µg Trolox equivalent/mg of extract; **$**: µg ascorbic acid equivalent/mg of extract; **$$**: µg EDTA equivalent/mg of extract; Δ: nM MDA equivalent/g of fresh weight

In present work, TPC of different wheatgrass extracts are represented in table 4.5 and results expressed as µg gallic acid equivalent per mg of extract (µg GAE/mg of extract). The TPC of all wheatgrass samples were significantly different ($P < 0.001$) from each other and were in the order of SeUV-B > NSeUV-B > Se-visible > NSe-visible. Non-seleniferous wheatgrass cultivated under visible light (NSe-visible) was considered as control. Figure 4.2 represents the comparative TPC among wheatgrass and values in parentheses represented the fold increase in TPC of other wheatgrass samples with respect to control.

Phenolic compounds are secondary metabolites, produced by all plants and characterized by presence of at least one aromatic ring with one or more hydroxyl groups. They are mainly synthesized from cinnamic acid through shikimate pathway and phenylpropanoid metabolism (Dixon and Paiva 1995).

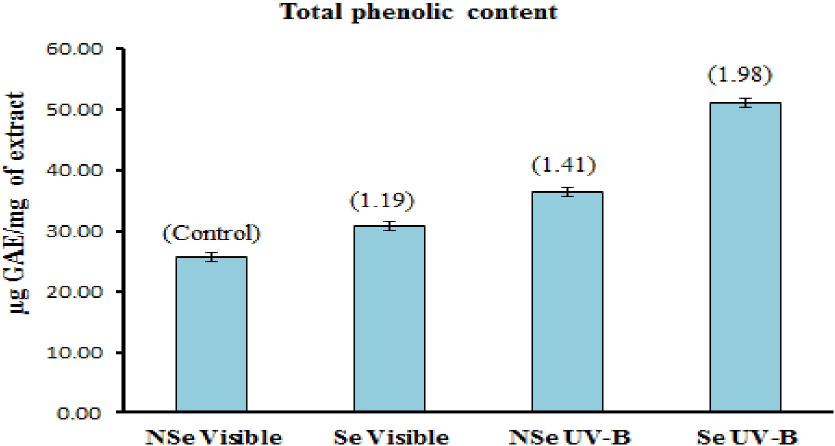


Figure 4.2: Total phenolic content in different wheatgrass samples and values in parentheses showing fold increase in TPC with respect to control (n=4; mean±SD)

Phenolic compounds are divided in several groups (for example simple phenols, flavonoids, benzoic acids and phenylpropanoids) based on the number of constitutive carbon atoms in conjugation with the structure of basic phenolic skeleton (Michalak 2006). Antioxidant capacity of these phenolic compounds is dependent upon the degree of hydroxylation (number of -OH groups) and extent of conjugation in polyphenols (Pulido et al. 2000).

Plant phenolics may be divided in two classes; (i) ubiquitous phenolics that are synthesized during the normal development of plant like growth, reproduction and pigmentation, and (ii) induced phenolics that are synthesized by plants in response to physical injury, infection or environmental stresses such as UV-irradiation, heavy metal stress, temperature etc. (Lattanzio et al. 2006).

Increasing flux of UV-B (290-320 nm) radiation is reaching the earth's surface as a consequence of stratospheric ozone depletion. Resultant damage in higher plants by UV-B radiation can be classified as (i) DNA damage by the formation of cyclobutane dimers or indirect physical damage by free radical formation, (ii) membrane damage by lipid peroxodation, (iii) damage to photosynthetic apparatus by change in the routes of electron paths in photosystem II (PS II), thylakoid ultrastructural damage, chlorophyll reduction and damage to Rubisco, and (iv) photo-reduction of growth hormones. Each of these types of damage is directly or indirectly related to the formation of reactive oxygen species (ROS) under UV-B stress (Shanker 2006).

Plants protect themselves from harmful radiation by synthesizing phenolic compounds as secondary metabolites. Solar UV-B radiation is known to stimulate the enzyme phenylalanine ammonia lyase, chalcone synthase and other branch-point enzymes of phenylpropanoid pathway (Rozema et al. 2002). These enzymes catalyze the transformation of phenylalanine to trans-cinnamic acid, which further leads to the formation of other complex phenolic compounds such as flavonoids, tannins and lignin. Under UV-B induced stress in higher plants, flavonoids and other phenylpropanoid derivatives such as sinapic acid esters accumulate in large quantities in the vacuole of epidermal cells which attenuate the UV component of sunlight. These secondary metabolites protect the plants by specifically absorbing the solar radiation in the wavelength region from 280 to 340 nm but with minimal effect on the visible region of the spectrum. Study conducted by Landry et al. (1995) on *Arabidopsis thaliana* mutants that are not able to accumulate flavonoids/sinapic esters were found to be more sensitive to UV-B radiation than the wild type and exhibited the highest level of lipid and protein oxidation.

In higher plants, although selenium has not yet been classified as an essential element, its role has been considered to be beneficial. The question of the essentiality of selenium for plants is unresolved and remains controversial. According to Hamilton (2004), the role of selenium depends mainly on its concentration and has three levels of biological activity in plants; (i) trace concentrations are required for normal growth and development, (ii) moderate concentrations can be stored to maintain homeostatic functions and (iii) elevated concentrations can results in toxic effects. Studies on lettuce (*Lactuca sativa*) and ryegrass (*Lolium perenne*) showed that, selenium reduces biomass at concentration of 1.0 and >10 mg/kg respectively, but it has beneficial effect at low concentration (0.1 mg/kg) in soil (Hartikainen et al. 2000; Xue et al. 2001). Different researchers have demonstrated that selenium not only promotes development and growth of plants but also increase antioxidant capacity and resistance of plants when subjected to various stresses (Hartikainen and Xue 1999; Djanaguiraman et al. 2005; Peng et al. 2002). Studies conducted by Xue et al. (2001) and Djanaguiraman et al. (2005) showed that effect of selenium in the form of selenate enhances the antioxidant activity in lettuce and soy, and reduces the oxidative damage. During the senescence of lettuce, the addition of selenium increases the antioxidant capacity by preventing the reduction of tocopherol concentration and by enhancing superoxide dismutase (SOD) activity.

An experiment conducted by Saffaryazdi et al. (2012) on spinach plants for determination of impact of selenium supplementation on TPC showed that selenium (as sodium selenite) significantly increases TPC in shoots and roots. According to Xu and Hu (2004), phenolic content in green tea leaves was greatly increased by foliar application of selenium-enriched fertilizer. Walaa et al. (2010) indicated that selenium treatment caused as significant increase in phenylalanine ammonia lyase activity in cucumber seedlings. A similar result has been observed by Ardebili et al. (2015) on basil plant (*Ocimum basilicum*) where exogenously applied selenium significantly stimulated the phenylalanine ammonia lyase activity and thus increased soluble phenol content as compared with control. Rise in phenylalanine content have been reported in selenium fertilized potato plants (Munshi et al. 1990; Jazek et al. 2011). Phenylalanine is a main component required for the production of different phenylpropanoids and their synthesis is triggered as one of the defense mechanisms under a variety of biotic and abiotic stressors (Dixon and Paiva 1995). Yao et al. (2010) demonstrated that selenium significantly enhances the antioxidant ability and reduces lipid peroxidation in aerial parts of wheat seedlings when exposed to UV-B radiation. This indicated that selenium minimizes the effect of oxidative stress by reducing the active oxygen content. Although UV-B radiation generally decreases the activities of antioxidant enzymes, in combination with selenium, it increases the activity of catalase (CAT), SOD and glutathione-S-transferase (GST) in different plants (Hasanuzzaman et al. 2010). This suggests that selenium may increase the antioxidative capacity of plants by multiple systems that act alone or may act synergistically with UV-B radiation.

Selenium is not essential for the growth of plants but due to its antioxidant properties, it exerts positive effect on the development of plants especially grown under stressful environment (heavy metal toxicity, drought, high temperature stress, UV-B, salinity etc.) (Feng et al. 2013; Iqbal et al. 2015). In literature, most of the studies have been carried out on plants with exogenous supplementation of inorganic selenium (selenate or selenite). At higher dose, toxicity of selenium can be attributed to its pro-oxidant effect as well as to metabolic disturbance (Hartikainen et al. 2000). When plants are exposed to high concentration of selenium, they may exhibit symptoms of injury including stunted growth, withering and drying of leaves, chlorosis, decreased protein synthesis and premature death of the plant (Terry et al. 2000). In the seleniferous areas of Punjab, India and wheat plants exhibiting varying degree of toxicity symptoms contained selenium ranging from 107 to 262 µg/g in shoots and from 29 to 66 µg/g in

roots (Dhillon and Dhillon 1991). In present experimental conditions, there were no toxic symptoms observed in wheatgrass samples cultivated from grains naturally accumulated with selenium, even though there was significant high selenium levels (151µg/g) in shoots. This is presumably due to metabolism of protein bound selenium and the translocation of selenium either in the form of selenoamino acids or other organo-selenium compounds, during germination of selenium-rich grains. Unlike inorganic selenium species (selenate or selenite), organic forms of selenium is less toxic and do not exert any stress to the plant.

Figure 4.2 clearly indicated that both selenium and UV-B radiation independently enhanced the total phenolic content by 1.19 and 1.41 fold respectively. Selenium and UV-B light showed synergistic effect and total phenolic content was increased significantly (1.98 fold) w.r.t control and this suggests that selenium and UV-B radiation increase the TPC of wheatgrass by inducing phenylpropanoid metabolism.

4.3.1.2 Total flavonoid content (TFC)

Flavonoids are the chemically diverse group of secondary metabolites which occur widely in plants. Flavonoids can be divided into subgroups including flavonols, flavanols, flavones, flavanones, anthocyanidins, chalcones, dihydro-chalcones and dihydro-flavonols. Total flavonoid content in different wheatgrass extracts were determined by colorimetric method using aluminum chloride ($AlCl_3$), which forms acid stable yellow complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols (Popova et al. 2004). In the present work, TFC of different wheatgrass extracts are represented in table 4.5 and results are expressed as µg quercetin equivalent per mg of extract (µg QE/mg of extract). There were no significant differences ($P > 0.05$) observed between TFC of wheatgrass samples exposed to visible light (NSe-visible and Se-visible), whereas wheatgrass cultivated under UV-B light (NSeUV-B and SeUV-B) showed a significant increase ($P < 0.05$) in TFC when compared to NSe-visible and Se-visible. There was also a significant increase ($P < 0.001$) in TFC content in SeUV-B sample when compared to NSeUV-B. Figure 4.3 represents the comparative TFC among wheatgrass samples and values presented in parentheses indicated the fold increase in TFC of Se-visible, NSeUV-B and SeUV-B with respect to control (NSe-visible) and their respective fold increase in TFC were 1.03, 1.18 and 1.73.

As described in previous section (4.3.1.1), UV-B radiation induces accumulation of a range of secondary metabolites like flavonoids and sinapic acid esters, which protect the plants by specifically absorbing in the wavelength region from 280 to 340 nm. These flavonoids also have free radical scavenging capacity which might offer additional protection to the plants (Rice-Evans et al. 1997). Biosynthesis of UV-screening flavonoids is regulated at transcription level and is under the control of UV-B photoreceptor (UVR8), that exists as a homodimer that instantly monomerises upon UV-B absorption via specific intrinsic tryptophans, acting as UV-B chromophores. The UVR8 monomer interacts with an E_3 ubiquitin ligase which initiate a molecular signaling pathway. This signaling output leads to UVR8-dependent responses including UV-B induced photomorphogenesis and the accumulation of UV-B absorbing flavonoids (Tilbrook et al. 2013). Analysis of UV induced accumulation of anthocyanins revealed that the photoreceptor has its maximum activity at 290 nm, which works either alone or in association with phytochrome (Jansena et al. 1998). UV-B boosts transcript levels for 4-coumarate:coA ligase, phenylalanine ammonia lyase, dihydroflavonol-4-reductase and chalcone flavones isomerase. Chalcone synthase catalyses the committal step in flavonoid biosynthesis that can be activated by UV-B and a variety of other environmental and physiological stimuli (Jansena et al. 1998). UV-B induced chalcone synthase expression is mainly in epidermal cells, where the flavonoids are localized.

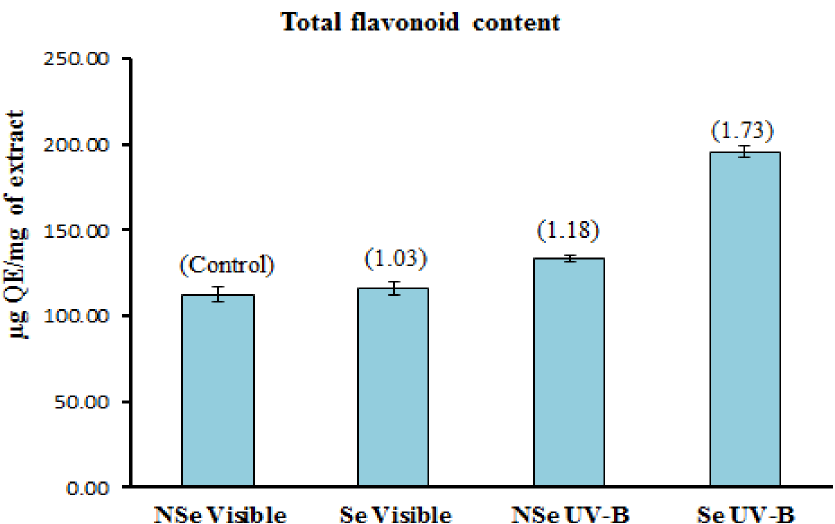


Figure 4.3: Total flavonoid content in different wheatgrass samples and values in parentheses showing fold increase in TFC with respect to control (n=4; mean±SD)

The effect of selenium on flavonoid biosynthesis is not well described. According to Tian et al. (2016), irrespective to UV light, selenate and selenite treatment significantly increases the flavonoid content in broccoli sprouts. Ozbolt et al. (2008) demonstrated that highest concentration of flavonoids was found in the leaves of buckwheat plants when seeds were treated with selenite under ambient and enhanced levels of UV-B radiation. Xue and Hartikainen (2000) observed that selenium reduces the lipid peroxidation irrespective of light conditions, even though the antioxidative effect was relatively more pronounced in the plants subjected to the short wavelength light. Although UV radiation generally diminished the activities of antioxidant enzymes, in combination with selenium, it increases the activity of CAT, SOD, GST, and glutathione peroxidase (GPx) in different plants. This suggests that selenium may increase the antioxidant capacity of plants by multiple systems that act independently or synergistically (Hasanuzzaman et al. 2010).

In present work, there were no significant differences observed in TFC among non-seleniferous and selenium rich wheatgrass samples cultivated under visible light because these plants were obviously well protected from stress and there was no need to synthesize and accumulate large amounts of protective flavonoids. Low levels of flavonoid content were observed in NSeUV-B which might be due to UV-B induced oxidative stress and associated damage of enzymes involved in flavonoid biosynthesis. In contrast, in case of SeUV-B, selenium protects these enzymes from oxidative damage.

4.3.1.3 DPPH radical scavenging capacity

Free radicals are inevitably produced in biological systems and also encountered exogenously. These free radicals are responsible for various degenerative disorders, like carcinogenesis, mutagenesis, cardiovascular disturbances and ageing (Singh and Singh 2008). Antioxidants are the compounds which neutralize the free radical effects by intervening at any step of the free radical mediated oxidative process, viz., initiation, progression and termination (Cui et al. 2004). Thus, it is important to understand the antioxidant content and their efficacy in food for protection against oxidative damage. DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging method generally known as antioxidant assay. This assay offers the first approach for evaluating the antioxidant potential of a compound or an extract from biological sources. The compound of interest or extract is mixed with DPPH solution and absorbance is recorded after a

defined period. This method was developed by Blois (1958) with the viewpoint to determine the antioxidant activity by using a stable free radical DPPH. DPPH is characterized by delocalization of the spare electron over the molecule which gives rise to the deep violet color, with an absorption in methanol solution at 517 nm. On mixing DPPH solution with a antioxidant compound (that can donate a hydrogen atom), it gives rise to the reduced form of DPPH with the loss of violet color and reduction in the color intensity that is directly proportional to the strength of antioxidant capacity.

DPPH radical scavenging capacity of different wheatgrass extracts are presented in table 4.5 and results are expressed as µg quercetin equivalent per mg of extract (µg QE/mg of extract). Free radical scavenging capacity of these wheatgrass extracts were significantly different ($P < 0.001$) from each other and scavenging capacities (as µg QE/mg of extract) were in the order of SeUV-B > NSeUV-B > Se-visible > NSe-visible. Similarly, the percentage DPPH radical scavenging capacities of NSe-visible, Se-visible, NSeUV-B and SeUV-B were 15%, 22%, 36% and 51% respectively. Figure 4.4 represents the comparative DPPH radical scavenging capacity among wheatgrass samples and values represented in parentheses indicated the fold increase in scavenging capacity with respect to control (NSe-visible) and respective fold increase in scavenging capacity of Se-Visible, NSeUV-B and SeUV-B were 1.46, 2.31 and 3.32.

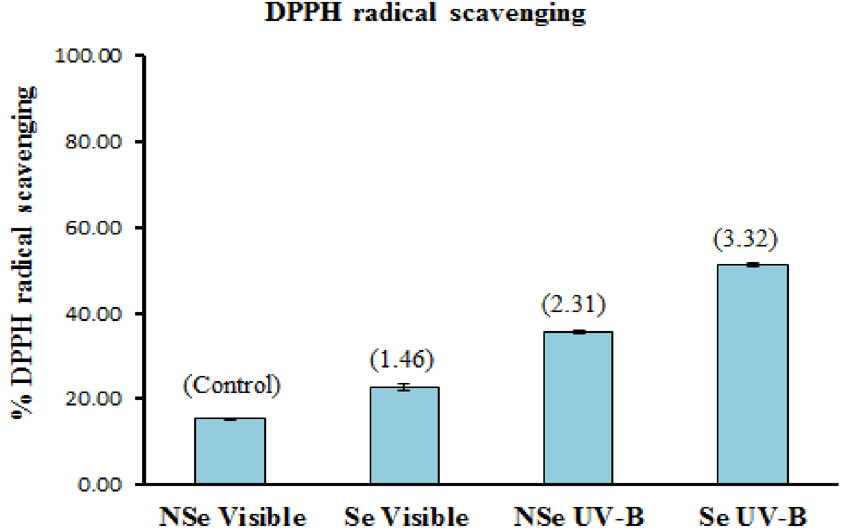


Figure 4.4: Percentage DPPH radical scavenging capacity of different wheatgrass samples and values in parentheses showing fold increase in scavenging capacity with respect to control (n=4; mean±SD)

In plants, various abiotic stresses lead to the over production of ROS, which are highly reactive, toxic and cause damage to biomolecules like proteins, lipids, carbohydrates and nucleic acids, that ultimately results in oxidative stress. These ROS comprises both, free radicals ($O_2^{\bullet -}$, superoxide; $HO_2^{\bullet}$, perhydroxyl; $OH^{\bullet}$, hydroxyl; and $RO^{\bullet}$, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen). In plant cell, photosystem I and II (PS I and II) of chloroplast are the major sites for the production of $^1O_2$ and $O_2^{\bullet -}$, whereas in mitochondria, electron transport chain (ETC) is the major site for the generation of $O_2^{\bullet -}$. Plants protect themselves by antioxidant defense machinery which includes both enzymatic (superoxide dismutase, catalase, glutathione reductase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione peroxidase, guaicol peroxidase and glutathione-S-transferase) and non-enzymatic (ascorbic acid, glutathione, alkaloids, phenolic compounds, non-protein amino acids and α-tocopherols) antioxidant defense systems, that control a cascade of uncontrolled oxidation and protect plant cell from oxidative damage by scavenging ROS (Gill and Tuteja 2010).

As described in previous sections (4.3.1.1 and 4.3.1.2), both selenium and UV-B radiation induce phenolic and flavonoid content in wheatgrass and these compounds serve as non-enzymatic defense mechanism in plants, responsible for the quenching of different free radical species. Higher scavenging capacity in NSeUV-B and SeUV-B coincides with higher phenolic and flavonoid contents. These phenolics and flavonoids serve as antioxidants and their potential is determined by reactivity as hydrogen or electron donating agents (which relates to its reduction potential) and ability of resulting antioxidant-derived radical to stabilize and delocalize the unpaired electrons (Rice-Evans et al. 1997).

DPPH assay is widely used in scavenging of antioxidant potential of different plant extracts. However, interpretations of results are complicated when the compounds such as carotenoids have spectra that overlap with DPPH at 515-517 nm (Noruma et al. 1997). DPPH is stable nitrogen radical and does not have any similarity with highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidant compounds that react quickly with peroxide radical may react slowly or may even be inert to DPPH due to steric inaccessibility. DPPH also can be decolorized by hydrogen transfer as well as by reducing agents, which contribute to inaccurate interpretations of antioxidant capacity. Therefore, to further establish the antioxidant potential of wheatgrass samples, other assays were also used.

4.3.1.4 Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay was first reported by Miller et al. (1993), which was based on the scavenging ability of antioxidants to the long-life radical cation $ABTS^{\bullet+}$. In this assay, radical cation is generated by oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) using peroxyl radicals, which gives intense blue-green color with absorption maxima at 734 nm. Antioxidant capacity of test compounds is measured as their ability to decrease the color intensity by reacting directly with the $ABTS^{\bullet+}$ radical and results of test compounds are expressed relative to Trolox. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water soluble analog of vitamin E. Due to difficulties in analyzing individual antioxidant components of a complex mixture (plant extracts), Trolox equivalent is used as a bench mark for the antioxidant capacity of such a mixture.

TEAC of different wheatgrass extracts are presented in table 4.1 and results are expressed as µg Trolox equivalent per mg of extract. TEAC of all wheatgrass extracts were found significantly different ($P < 0.001$) from each other and as expected, wheatgrass samples rich in high selenium and exposed to UV-B radiation possessed significantly high TEAC and order of TEAC for all wheatgrass extracts were SeUV-B > NSeUV-B > Se-visible > NSe-visible. The respective percentage ABTS radical scavenging capacity of wheatgrass extracts were 22% (NSe-visible), 31% (Se-visible), 36% (NSeUV-B) and 57% (SeUV-B) (figure 4.5). Values represented in parentheses indicated the fold increase in ABTS radical scavenging capacity of wheatgrass extracts with respect to control (NSe-visible) and respective fold increase in scavenging capacity of Se-Visible, NSeUV-B and SeUV-B were 1.40, 1.65 and 2.60.

Both selenium and exposure of UV-B light induces production of antioxidant compounds (phenolics and flavonoids) by inducing phenylpropanoid and chalcone synthase activity, which results in significant increase in TEAC in wheatgrass samples exposed to selenium and UV-B radiation.

Due to operational simplicity in the method, TEAC assay is widely used for studying antioxidant capacity. This method has been used to determine hydrophilic and lipophilic antioxidant activity of plant foods, beverages, fruits and vegetables, fat soluble vitamins, oils etc. (Alcoea et al. 2001; Gill 2000; Pellegrini et al. 2003; Proteggente et al. 2002; Cano et al. 2000). $ABTS^{\bullet+}$ radical reacts rapidly with antioxidants (within 30 min) and can be used over a wide pH

range to study effect of pH on antioxidant mechanisms (Lemanska et al. 2001). $ABTS^{\bullet+}$ can be also used in multiple media (aqueous and organic olvents) to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (Awika et al. 2003). Thermodynamically, an antioxidant compound can reduce $ABTS^{\bullet+}$ if it has redox potential lower than that of ABTS (0.68 V) and many phenolic and flavonoid compounds have low redox potentials and can thus react with $ABTS^{\bullet+}$ (Prior et al. 2005).

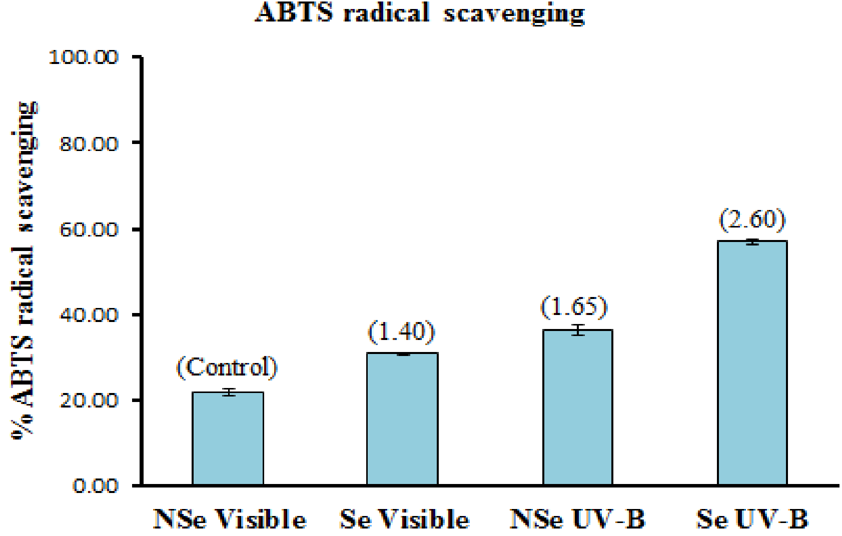


Figure 4.5: Percentage ABTS radical scavenging capacity of different wheatgrass samples and values in parentheses showing fold increase in radical scavenging capacity with respect to control (n=4; mean±SD)

4.3.1.5 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was developed by Benzie and Stain (1996) to measure reducing power in plasma. Subsequently, the assay has also been extensively used for the antioxidant determination in plants (Benzie and Szeto 1999; Ou et al. 2002; Gil 2000; Pellegrini et al. 2003; Proteggente et al. 2002). In this assay, reduction of ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyl triazine (Fe^{2+}-TPTZ) form, having an intense blue color, can be monitored by measuring the change in absorption at 595 nm.

FRAP of different wheatgrass extracts are presented in table 4.1 and results expressed as µg ascorbic acid equivalent per mg of extract (µg AAE/mg of extract). FRAP of wheatgrass extracts were significantly different ($P < 0.001$) from each other and respective values were in the order of SeUV-B > NSeUV-B > Se-visible > NSe-visible. FRAP is directly proportional to

the concentration of antioxidants present into the test samples and from the current results it is clear that both selenium and UV-B radiation increases FRAP by inducing antioxidant components in wheatgrass. Figure 4.6 represents the comparative FRAP of wheatgrass samples and values in parentheses indicating the fold increase in FRAP values with respect to control, which followed the similar trends as observed in TPC, where selenium and UB-B radiation significantly increases the phenolic content in wheatgrass samples.

The oxidizing strength of any free radical is determined by its one-electron reduction potential (E°), with higher E° values corresponding to more potent oxidants. The E° values of many oxygen and nitrogen free radicals range from 0.92-2.31 V (Buettner 1993). The ability of a compound to act as an antioxidant/free radical scavenger is also partly related to its standard one-electron redox potential, which is a measure of the reactivity of an antioxidant as electron or hydrogen donor under standard conditions. The E° of many phenols and flavonoids range from 0.1-0.87 V (Simic et al. 2007; Frei and Higdon 2003) and a lower redox potentials of these phenols and flavonoids with respect to free radicals, indicate that less energy is required for electron or hydrogen donation and is only one factor in determining antioxidant activity (Frei and Higdon 2003).

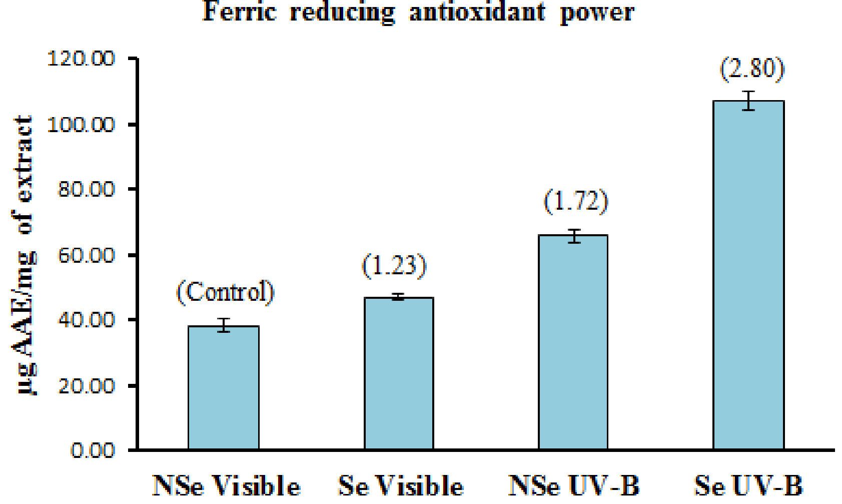


Figure 4.6: Ferric reducing antioxidant power (FRAP) of different wheatgrass samples and values in parentheses showing fold increase in FRAP with respect to control (n=4; mean±SD)

The redox potential of Fe^{3+}-TPTZ is 0.7 V. The antioxidant compounds with redox potential of < 0.7 V maintain redox status in cells or tissue. FRAP mechanism is totally based on electron transfer, therefore FRAP assay cannot detect compounds that act by radical quenching (hydrogen transfer), particularly thiols and proteins (Ou et al. 2002). Although FRAP assay is simple, speedy, inexpensive and robust, it actually measures only the reducing capability based upon the ferric ion, which is not relevant to antioxidant activity mechanistically and physiologically (*in-vivo*).

4.3.1.6 Iron (Fe^{2+}) chelating capacity

Most reactive oxygen species (ROS) are generated as by-products during electron transport in mitochondria and other metabolic pathways. In addition, ROS are also formed as necessary intermediates of different redox-active transition metal (iron, copper, chromium, cobalt, vanadium, cadmium, arsenic and nickel) catalyzed oxidation reactions (Konic et al. 2001). Free ferrous ions (Fe^{2+}) are one of the most effective pro-oxidants and interaction with H_2O_2 in biological systems can lead to formation of highly reactive hydroxyl radicals ($OH^{\bullet}$). Chelation of Fe^{2+} by certain compounds decreases their pro-oxidant effect by reducing their redox potentials and stabilizing the oxidized form of the iron.

Ferrozine is a ferroin compound that forms a stable magenta colored complex with free Fe^{2+} ions. In the presence of chelating agents, the availability of free Fe^{2+} decreases and determining the rate in color reduction allows estimation of the chelating activity of the co-existing chelator.

The iron chelating capacity of different wheatgrass extracts are presented in table 4.1 and chelation capacity was expressed as µg EDTA equivalent per mg of extract (µg EE/mg of extract). The observed iron chelation capacity of these wheatgrass extracts were significantly different ($P < 0.001$) from each other and strongest chelating activity was observed in SeUV-B (816.7 ± 3.73 µg EE/mg of extract) followed by NSeUV-B (526.7 ± 4.37 µg EE/mg of extract), Se-visible (310.4 ± 4.37 µg EE/mg of extract) and NSe-visible (287.0 ± 3.31 µg EE/mg of extract). Figure 4.7 presents the percent iron chelation capacity of wheatgrass extracts, showing highest chelation capacity in case of SeUV-B (65.5%) and lowest in NSe-visible (23.3%). Values indicated in parentheses showing the respective fold increase in chelation capacity when compared to control.

Transition metals play a significant role in the generation of ROS in living organisms. Iron exists in two distinct oxidation states, Fe^{2+} and Fe^{3+} ions. Fe^{3+} is relatively inactive form of iron, however it can be reduced to active Fe^{2+}, depending on the conditions during digestion and absorption, particularly at low pH of stomach and in the presence of reducing agents such as ascorbic acid (Strlic et al. 2002). Fe^{2+} oxidized back through Fenton reactions with production of hydroxyl radicals; or Haber-Weise cycle reactions with superoxide anions (Kehrer 2000; Wong and Kitts 2001). The production of these radicals can lead to protein modification, lipid peroxidation and DNA damage. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes (Finefrock et al. 2003).

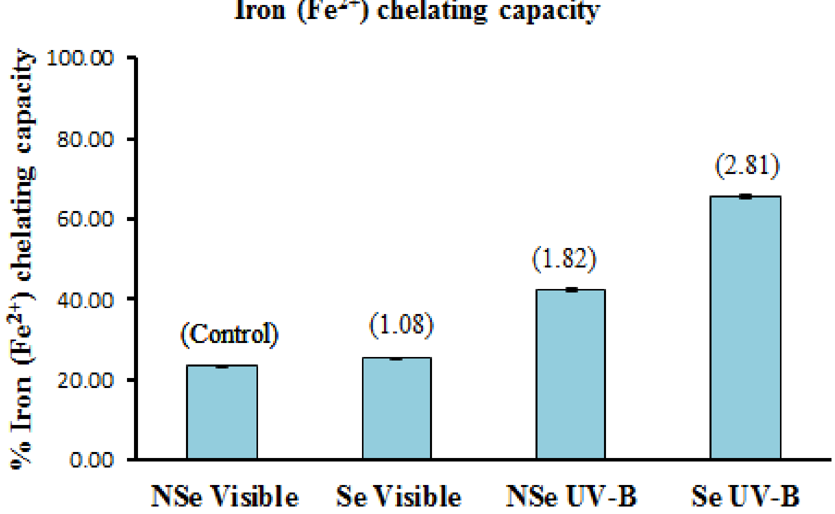


Figure 4.7: Percentage iron (Fe^{2+}) chelating capacity of different wheatgrass samples and values in parentheses showing fold increase in chelation capacity with respect to control (n=4; mean±SD)

The majority of studies deal with metal chelation activity of phenolic and polyphenolic compounds. Phenolic acids bearing catechol or galloyl groups (caffeic acid, gallic acid, protocatechuic acid and chlorogenic acid) shows significant iron chelation capacity when compared to those without these functional groups (ferulic acid, syringic acid and vanellic acid) (Andjelkovic et al. 2006). Other than phenolic acids, flavonoids also serve as potent chelators of bioavailable iron. According to Moran et al. (1997) general chelating capacity of phenolic and flavonoid compounds is probably related to the high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule.

In present study, chelation power of wheatgrass extracts was analyzed and it was observed that SeUV-B sample exhibit better capacity to chelate ferrous ions in comparison to other wheatgrass fractions. As the production of phenolics and flavonoids in wheatgrass induced by selenium and UV-B, presumably responsible for increased iron chelation capacity. Therefore, chelation of metal ions by natural phytochemicals from wheatgrass can prove to be of therapeutic importance, as chelation therapy making uses of synthetic compounds have certain side effects as well (Sudan et al. 2014).

4.3.1.7 Lipid peroxidation (TBARS assay)

The extent of lipid peroxidation in fresh wheatgrass samples were measured by TBARS assay. Thiobarbituric acid reactive species (TBARS) are formed as by-products of lipid peroxidation that can be detected by using thiobarbituric acid (TBA) as a reagent. TBARS assay measures MDA (malondialdehyde) present in the sample. MDA is the by-product formed via decomposition of certain primary and secondary lipid peroxidation products. MDA forms adduct with TBA and produces pink colored fluorescent complex on boiling, with excitation wavelength at 532 nm and emission at 550 nm. In present work, the level of lipid peroxidation in different wheatgrass samples are presented in table 4.1 and extent of lipid peroxidation was expressed as nM malondialdehyde equivalent per gram of fresh weight (nM MDAE/g of fresh weight). Statistical analysis indicates that UV-B light significantly increases ($P < 0.05$) the TBARS content in NSeUV-B when compared to the other sample. Whereas, due to antioxidant role of selenium, TBARS levels in Se-visible and SeUV-B samples significantly ($P < 0.05$) decreased when compared to NSe-visible and NSeUV-B. However, there was no significant ($P > 0.05$) difference observed in TBARS content between NSe-visible and SeUV-B samples. In figure 4.8, parentheses showing fold change in TBARS levels in wheatgrass samples when compared to control (NSe-Visible). As discussed in previous sections, selenium protects the plants from detrimental effect of UV-B light by inducing different enzymatic (CAT, SOD and GST) and non-enzymatic antioxidants (phenolics and flavonoids), and these components supposed to protects wheatgrass from UV-B induced damage.

Exposure of plant tissue to UV-B radiation accelerates the level of ROS and enhanced production of ROS can cause oxidative damage to nucleic acids, proteins and lipids. Phospho and glycolipids are main components of plant cell membranes containing unsaturated fatty acids,

which are easily destroyed by UV-B radiation in presence of oxygen (Kramer et al. 1991; Panagopoulos et al. 1990). During UV-B exposure, generated hydroxyl radicals or singlet oxygen can react with the methylene groups of lipid and form conjugated dienes, lipid peroxide radicals and hydro-peroxides (Hollosy 2002). The peroxy radicals can abstract hydrogen from other polyunsaturated fatty acids, leading to a chain reaction of peroxidation of membrane lipids and further breakdown of their structure and function (Hollosy 2002).

Figure 4.8: TBARS contents of different wheatgrass samples and values in parentheses showing fold change in TBARS content with respect to control (n=4; mean±SD)

The possible role of selenium-induced tolerance/resistance of plants to environmental stresses has not been fully clarified. The environmental stresses result in the accumulation of ROS in plants and can pose a threat to plant development. These ROS also act as signals for the activation of the stress response and defense pathways (Mittler 2002), via regulation of antioxidants. Selenium can control the production and quenching of ROS (either directly or indirectly). In buckwheat plant, exogenous applied selenium mitigated the negative effect of UV-B radiation on effective quantum yield of photosystem II (PS II) by improving light harvesting mechanism (Breznik et al. 2005). Cartes et al. (2012) observed that aluminum induced oxidative stress in ryegrass roots were alleviated by selenite addition mainly through enhancing the spontaneous dismutation of superoxide radical and H_2O_2. Mroczek-Zdyrska and Wojcik (2011) found that low dose of selenium (1.5 μM) as sodium selenite decreased the level of superoxide

radical induced by lead toxicity in roots of *Vicia faba*. Selenium, as sodium selenate, further decreased the level of H_2O_2 and superoxide radical observed in sorghum plant when exposed to high temperature (Djanaguiraman et al. 2010). In wheat seedling, selenium supplementation as sodium selenite reduced the level of superoxide radicals when exposed to UV-B radiation (Yao et al. 2011) and cold stress (Chu et al. 2010).

In conclusion, UV-B light alone significantly increases TBARS content in wheatgrass, but the combined treatment with UV-B and selenium reduces the damage caused by UV-B alone in wheatgrass to certain extent by increasing antioxidant content.

4.3.1.8 Chlorophyll content

Present section is focused on quantification of chlorophyll content in different wheatgrass samples exposed to visible and UV-B light. Figure 4.9 presents the content of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*), total chlorophyll and ratio of chlorophyll *a* and *b* (chl *a/b*) in wheatgrass and expressed as milligram per gram of dry weight (mg/g of DW). The results indicated that UV-B light significantly ($P < 0.05$) decreased chl *a*, chl *b* and total chlorophyll content when compared to the wheatgrass cultivated in visible light. However, when compared to NSe-visible, selenium increases the levels chl *a* ($P < 0.05$), chl *b* ($P < 0.05$) and total chlorophyll ($P < 0.05$) in Se-visible. UV-B light showed a significant decrease ($P < 0.001$) in chl *a* and increase ($P < 0.001$) in chl *b* in NSeUV-B samples when compared to SeUV-B, however, total chlorophyll contents were not affected by UV-B, when compared among NSeUV-B and SeUV-B.

Normally the ratio of chl *a* to chl *b* in well illuminated chloroplast with visible light ranges from 3 to 3.4 and from 2.4 to 2.7 in shade (Lichtenthaler et al. 2007). In present work, UV-B radiation significantly decreased ($P < 0.001$) chl *a/b* values in NSeUV-B when compared to NSe-visible, Se-visible and SeUV-B, and their respective average chl *a/b* values were 1.84, 3.30, 3.14 and 2.92. Decreased chl *a/b* values below 2.4 in NSeUV-B indicated that, due to the detrimental effect, UV-B light significantly alters the photosynthetic pigment ratio in chloroplast, whereas, due to the antioxidant potential, selenium protects the wheatgrass from UV-B light with reference to in pigment ratio.

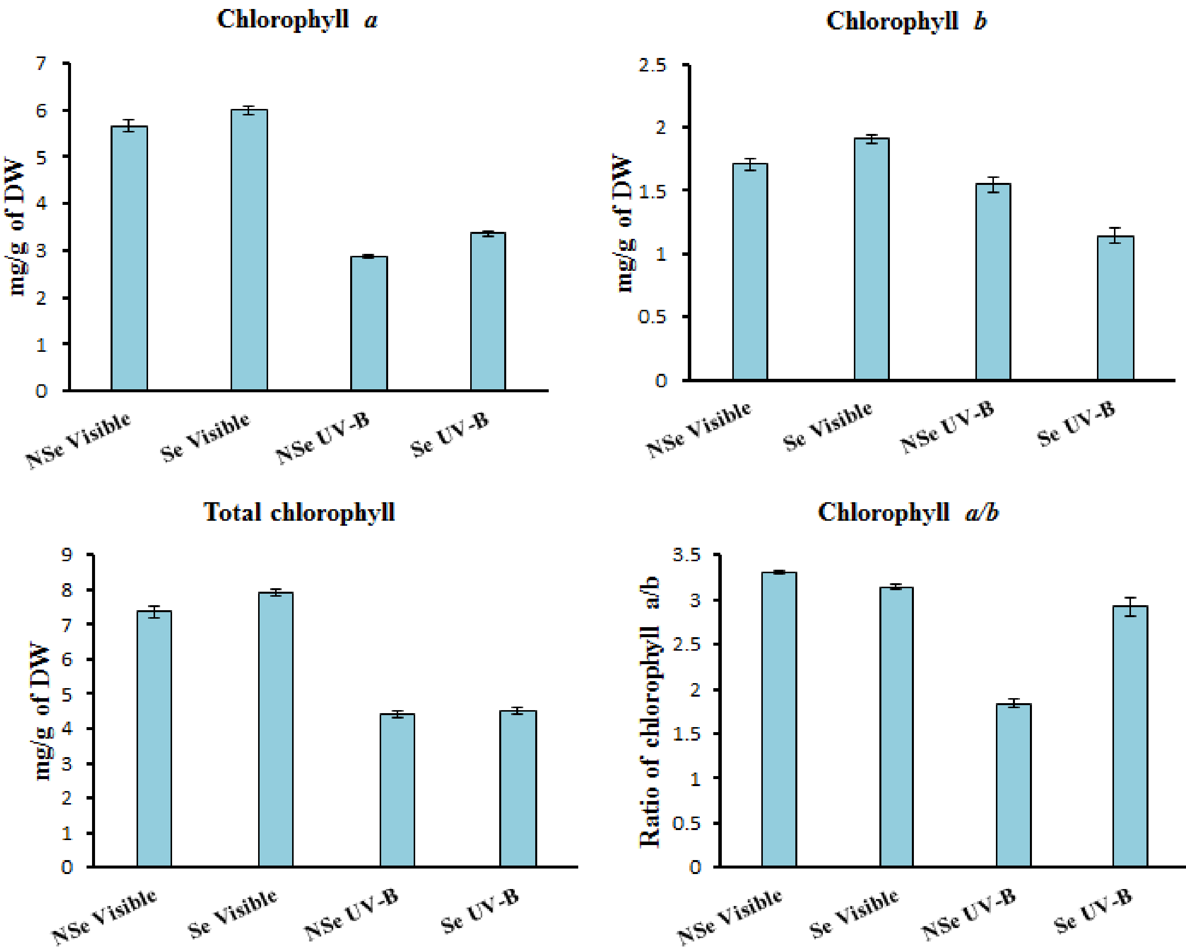


Figure 4.9: Chlorophyll contents of different wheatgrass samples (n=3; mean±SD)

During photosynthesis, chlorophyll plays central role in harvesting light energy and its conversion to chemical energy. The present experimental results showed that UV-B radiation causes significant reduction in chlorophyll content of wheatgrass leaves affecting the plant photosynthetic efficiency. UV-B radiation has significant photobiological influence on development and growth of plant (Jordan 1996; Jayakumar et al. 2003), as well as on the light reaction of photosynthesis (McNamara and Hill 2000). Direct absorption of UV-B light by thylakoid membrane components; damage the membrane organization (Kulandaivelu and Noorudeen 1983) that may include reduction in chlorophyll, light harvesting chlorophyll binding proteins of PSII, D_1 protein, Rubisco levels (Strid et al. 1990) and the expression of photosynthetic genes (Mackerness et al. 1999). UV-B has a very strong impact on the content of photosynthesis dependent pigments, activity of photosynthetic enzymes and photosynthetic efficiency (Frohnmeyer and Staiger 2003; Teramura 1983). Reduction in total chlorophyll

content due to UV-B exposure might be associated with the inhibition of aminolevulinic acid and protochlorophyllides levels, which are responsible for chlorophyll synthesis (Stobart et al. 1985; Boddi et al. 1995). Further, depending on the particular crop species, UV-B radiation either increases or decreases the chlorophyll content (Larsson et al, 1998; Sun and Payn 1999; Barsig and Malz 2000). Our results suggest that total chlorophyll levels decreased after UV-B exposure and these results agreed with those of Strid et al. (1990) and Choi and Roh (2003), who showed significant decrease in total chlorophyll content in pea and jackbean leaves after UV-B exposure. Strid and Porra (1992) proposed that UV-B radiation leads to the destruction of chlorophyll by influencing the genetic regulation of the chlorophyll-binding protein.

Chlorophyll *a* alone exists in the core complexes of photosystems and their composition and organization are highly conserved, whereas, chl *a* and *b* are the parts of peripheral antenna complexes that help optimal utilization of available light. It is well described that chl *a/b* ratio is higher in high-light growth conditions when compared to low-light, which is accompanied by larger size of antenna complexes in low-light conditions, therefore, regulation of chl *b* synthesis is important for the plants during adaptation under various light intensities (Tanaka et al. 1998).

In present study, the chl *a/b* ratio significantly decreased in NSeUV-B when compared to SeUV-B, this may have been a result of faster breakdown or decreased synthesis of chl *a* compared to chl *b*, even though the level of latter also increased. Increase in chl *b* level in NSeUV-B might be due to stress response of plant against UV-B light. The observations are comparable with those of Choi and Roh (2003), where UV-B radiation decreased chl *a/b* ratio by decreasing chl *a* and increasing chl *b* levels in jackbean leaves when compared to control. Relative to NSeUV-B, UV-B light did not reduce chl *a/b* ratio in SeUV-B very significantly ($P = 0.03$) when compared to NSe-visible and Se-visible. This may suggest that due to the antioxidant nature of selenium, chloroplast is protected from damaging effect of UV-B. In present study, selenium significantly protects the chl *a* and maintained chl *a/b* ratio in SeUV-B sample when compared to NSeUV-B. Other than UV-B induced stress, selenium also protects chloroplast and chlorophyll content in wheat exposed to 2,4-dichlorophenoxyacetic acid (2,4-D) (Filek et al. 2009), cucumber seedling under salt stress (Hawrylak-Nowak 2009), white clove under water-stress (Wang 2011) and mungbean under arsenic stress (malik et al. 2012). Through proteomic analysis, Wang et al. (2012) revealed that low doses of selenium enhanced photosynthesis in rice seedlings, and its application significantly increased the photosynthetic rate, stomatal

conductance and transpiration rate in sorghum (Djanaguiraman et al. 2010). The restoration of photosynthesis in stressed plants after selenium application may be closely related to the decreased ROS levels by activation of antioxidant enzymes or by inducing secondary metabolites like phenolics and flavonoids.

In conclusion, UV-B light significantly decreases chl a, chl b, total chlorophyll and also changes the chl *a/b* ratio in wheatgrass, whereas selenium in combination with UV-B minimizes the damage caused by UV-B alone to certain extent by increasing antioxidant content.

4.3.2 Bioactive properties of wheatgrass – *In-vitro* Antioxidant enzyme assay

The aim of present section was to evaluate the effect of selenium (naturally enriched) and quality of incident light (visible and UV-B) in wheatgrass on the expression of different antioxidant enzymes, viz., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), ascorbate peroxidase (APx) and guaiacol peroxidase (GuPx). The activities of said enzymes are presented as unit per milligram of protein (U/mg of protein).

4.3.2.1 Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) is the most effective intracellular metalloenzyme that acts as antioxidant and is found in all subcellular organelles prone to free radical mediated oxidative stress. In present study, SOD levels in different wheatgrass samples are presented in figure 4.10. SOD activities are found significantly different ($P < 0.001$) from each other and vary in the order of SeUV-B > NSeUV-B > Se-visible >NSe-visible.

Within the cell, SOD serves as first line of defense against superoxide radicals ($O_2^{\bullet -}$) produced by partial reduction of O_2 at any location where an electron transport chain is present, for example chloroplast, mitochondria, glyoxysomes, microsomes, peroxysomes, apoplast and cytosol (Alscher et al. 2002). SOD converts $O_2^{\bullet -}$ radicals into less harmful H_2O_2 and O_2 through dismutation. $O_2^{\bullet -}$ can reduce Fe^{3+} to Fe^{2+}, which can then further trigger the formation of more reactive ROS like hydroxyl radicals ($OH^{\bullet}$) (Halliwell 2006). The SOD removes $O_2^{\bullet -}$ by catalyzing its dismutation and hence decreases the risk of $OH^{\bullet}$ formation via metal catalyzed Fenton and Haber-Weise type reactions (Gill and Tuteja 2010).

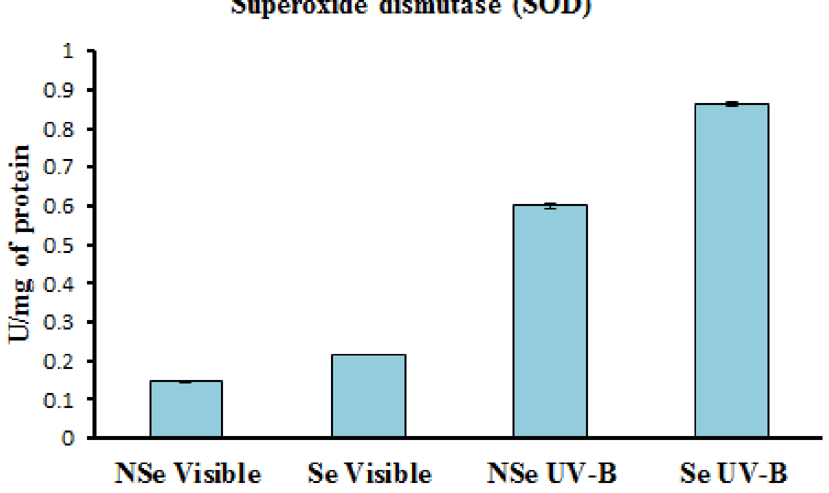


Figure 4.10: SOD activities of different wheatgrass samples (n=3; mean±SD)

Environmental stress such as UV-B radiation leads to the enhanced generation of ROS in plants due to disruption of cellular homeostasis. In cellular organelles, electron transport system (ETS) activity is responsible for the production of $O_2^{\bullet -}$. According to Germ et al. (2005), ambient solar radiation showed a higher mitochondrial ETS activity in plants when compared with those grown under UV-B free solar radiation. Increased numbers of mitochondria were observed in strawberry leaves exposed to ultraviolet radiation (Heijari et al. 2006) and that could be responsible for increased ETS activity and $O_2^{\bullet -}$ production inside the cell. Yao et al. (2011) reported that UV-B light significantly increased the rate of $O_2^{\bullet -}$ production in wheat seedlings over the control.

In present work, activity of SOD significantly increased in wheatgrass as a response of exposure to UV-B radiation, but the combined treatment with UV-B and selenium enhanced SOD activity to further extent. Similar results were obtained by Yao et al. (2011), where selenium induced SOD activity was examined in UV-B treated wheat seedlings. Selenium addition also increases SOD activity in senescing ryegrass and in lettuce plant (Hartikainen et al. 2000; Xue et al. 2001). Several authors have also reported enhanced SOD activity after selenium supplementation in many stressed plants, for example in light-stressed potato (Seppanen et al. 2003), aluminum-stressed ryegrass (Cartes et al. 2010), selenium-stressed *Pteris vittata* (Feng and Wei 2012), water-deficient *Trifolium repens* (Wang 2011), senescing soybean (Djanaguiraman et al. 2005), cadmium-stressed marine red algae (Kumar et al. 2012) and high-

temperature-stressed sorghum (Djanaguiraman et al. 2010). The mechanism of UV-B induced ROS generation and indirect evidence of antioxidant activity of selenium in the plants has not been studied till date.

4.3.2.2 Catalase (CAT) activity

Hydrogen peroxide, generated after dismutation of $O_2^{\bullet-}$, also acts as a moderately reactive species and excess of H_2O_2 in cells leads to the occurrence of oxidative stress. H_2O_2 is also produced due to the reduction of $O_2^{\bullet-}$ by reducing agents present inside the cell such as ascorbate, thiols, ferredoxins etc. (Asada and Takahashi 1987). To prevent the oxidative damage in plants, CAT is frequently used by cells that rapidly decompose H_2O_2 into less reactive O_2 and H_2O molecules (Gaetani et al. 1996). Therefore, CAT plays an important role in plant defense, ageing and senescence. In present work, CAT activity of different wheatgrass samples is presented in figure 4.11 and enzyme activities are found significantly different ($P < 0.001$) from each other. Similar to SOD, the trend in activity of CAT in wheatgrass samples was in the order of SeUV-B > NSeUV-B > Se-visible >NSe-visible.

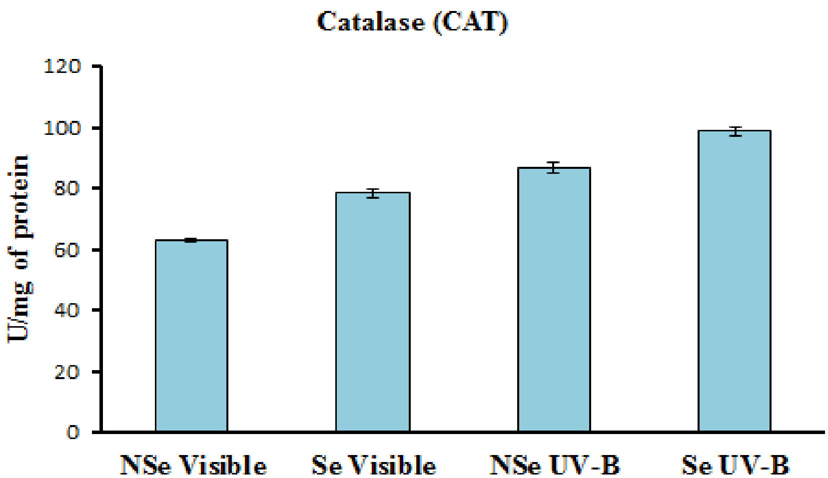


Figure 4.11: CAT activities of different wheatgrass samples (n=3; mean±SD)

In eukaryotic cells, most of the H_2O_2 is produced in peroxisomes, mitochondria and chloroplast by β-oxidation of fatty acids, purine catabolism and photorespiration (Gill and Tuteja 2010). CAT plays an important role in removal of H_2O_2 because of which variable response of CAT activity has been observed under different environmental stress. In support to our observations, CAT activity was reported to be increased due to UV-B exposure in tomato (Balakumar et al. 1997), sunflower (Hagh et al. 2012), wheat seedlings (Yao et al. 2011) and

cassia seedlings (Agarwal 2007). Reduced CAT activity has been observed under metal stress (cadmium) in *Glycine max* (Balestrasse et al. 2001), *Capsicum annuum* (Leon et al. 2002), *Phragmites australis* (Iannelli et al. 2011) and *Arabidopsis thaliana* (Cho and Seo 2005). Whereas, CAT activity increased in mustard (Mobin and Khan 2007), rice (Hsu and Kao 2004), wheat (Khan et al. 2007), chickpea (Hasan et al. 2008) and blackgram (Singh et al. 2008) when exposed to cadmium. Eyidogan and Oz (2005) reported a significant increase in CAT activity under salt treatment in chickpea.

In present study, selenium significantly induces CAT activity in wheatgrass samples cultivated under visible and UV-B light. Current observation is supported by Yao et al. (2011), where selenium induces CAT activity independently and in combination with UV-B light in wheat seedlings. The reason behind increase in CAT activity after selenium supplementation is not clear. However, there might be an indirect influence, wherein increase in SOD activity converts $O_2^{\bullet -}$ radicals into H_2O_2 followed by subsequent stimulation of CAT expression.

Other than with UV-B stress, selenium also induces the CAT activity in plants under different environmental stress. Selenium application enhances CAT activity in cadmium-stressed rape seedlings (Filek et al. 2008; Hasanuzzaman et al. 2012), salinity and drought stressed rape seedlings (Hasanuzzaman et al. 2011; Hasanuzzaman and Fujita 2011), high-temperature-stressed sorghum (Djanaguiraman et al. 2010), cold-stressed wheat seedlings (Chu et al. 2010), senescing soybean (Djanaguiraman et al. 2004) and arsenic-stressed mungbean (Malik et al. 2012). These observations suggest that selenium induced up-regulation in CAT activity could protect the plant from oxidative damage by lowering the levels of H_2O_2.

Thus, selenium observably reduces oxidative stress in wheatgrass by inducing both enzymatic (SOD and CAT) and non enzymatic (phenolics and flavonoids) antioxidants, which protect the plant by $O_2^{\bullet -}$ and H_2O_2 induced oxidative stress.

4.3.2.3 Glutathione peroxidase (GPx), ascorbate peroxidase (APx), guaiacol peroxidase (GuPx) and glutathione reductase (GR) activity

Like CAT, accumulation of H_2O_2 is also prevented in the cell by proxidase related enzymes viz., glutathione peroxidase (GPx), ascorbate peroxidase (APx), guaiacol peroxidase (GuPx). Glutathione reductase (GR) is important for maintaining the reduced glutathione (GSH) pool of cell during reduction of H_2O_2 mediated by different peroxidases (Gill and Tuteja 2010).

The enzyme activities of GPx, APx, GuPx and GR in wheatgrass samples are presented in figure 4.12 and activities of all these enzymes were found significantly different ($P < 0.001$) when compared among wheatgrass samples. Expression of all above mentioned enzymes were in the order of NSeUV-B > SeUV-B > NSe-visible > Se-visible.

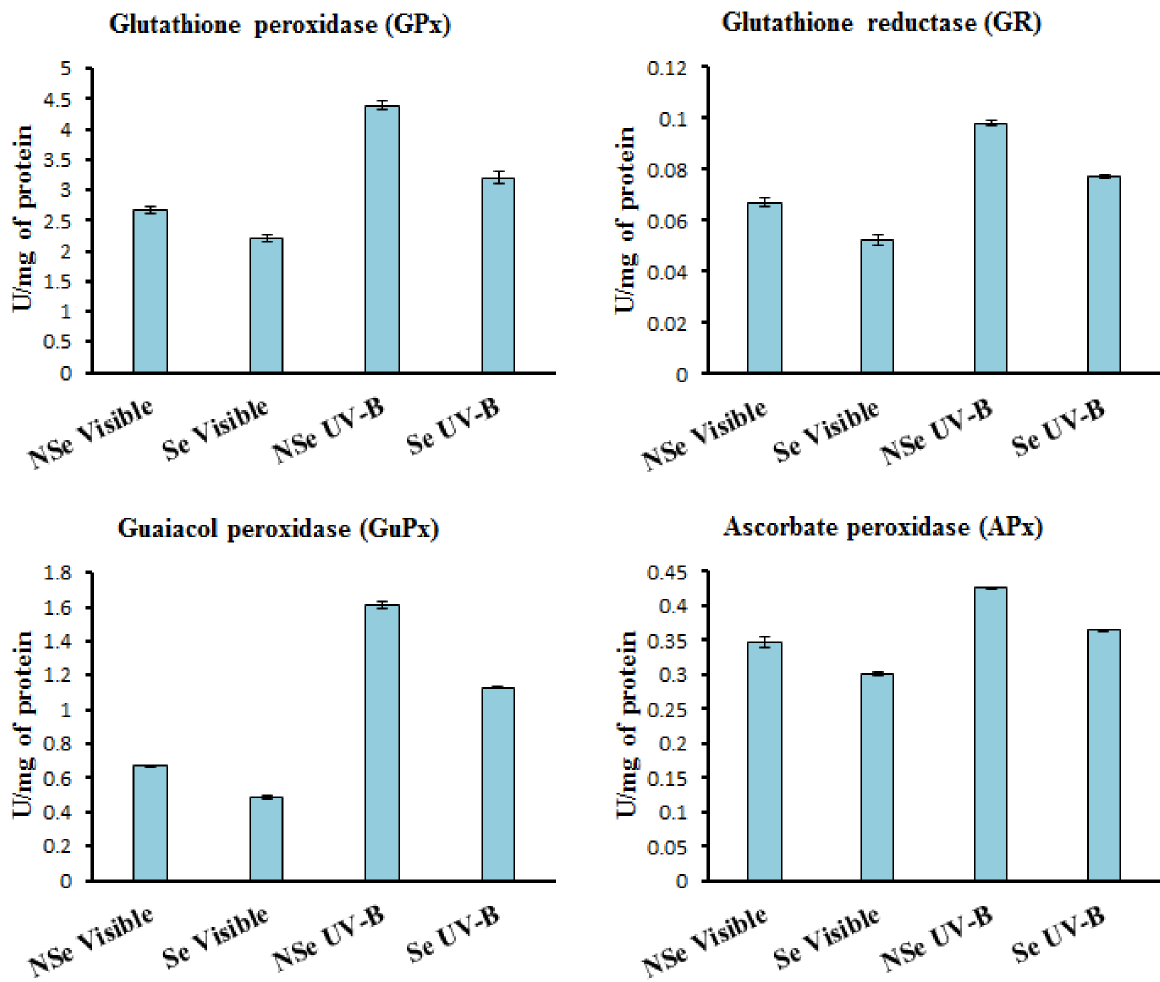


Figure 4.12: GPx, APx, GuPx and GR activities of different wheatgrass samples (n=3; mean±SD)

H_2O_2 has relatively longer half-life when compared to superoxide ($O_2^{\bullet -}$) and highly permeable across the membrane (Quan et al. 2008). Accumulation of H_2O_2 inside the cell may lead to the production of highly reactive $OH^{\bullet}$ radical. As described by Czegeny et al. (2014), UV-B radiation increases cellular H_2O_2 concentration and also induces photo-conversion of H_2O_2 into $OH^{\bullet}$ in plant leaves. Therefore, it is necessary for the plant cell to reduce H_2O_2 by plant cell through multi-level antioxidant mechanisms which involve both enzymatic and non-

enzymatic antioxidants. Other than cellular organelles, H_2O_2 is also produced in the extracellular matrix, plasma membrane and cytoplasm (Slesak et al. 2007). The enhanced production of H_2O_2/ROS during environmental stresses causes damage of nucleic acid, lipid peroxidation, enzyme inhibition, oxidation of proteins, activation of programmed cell death pathway and ultimately leading to cell death (Sharma et al. 2012). Elimination of H_2O_2 is directly connected with the less production of other ROS and steady state levels of cellular H_2O_2 is determined by the redox status of the cell (Karpinski et al. 2003; Mateo et al. 2006).

Peroxidase enzyme such as GPx reduces H_2O_2 along with other organic and lipid hydroperoxides with the help of GSH and therefore prevents the plant cells from oxidative damage (Noctor et al. 2002). Unlike CAT (mainly localized in peroxisomes), GPx is present in cytosol, endoplamic reticulum, mitochondria and chloroplast as reported in *Arabidopsis* (Millar et al. 2003) and generally more efficient with high substrate affinity (Shanker 2006). In the present work, enhanced GPx activities were observed in wheatgrass samples treated with UV-B radiation when compared to their respective control. Current observations are also supported by those of many workers, wherein UV-B and other environmental stresses were noted to enhance GPx activity in plants (Depege et al. 1998; Hartikainen and Xue 1999; Li et al. 2000; Leon et al. 2002; Avsian-Kretchmer et al. 2004; Sreenivasulu et al. 2004; Gapinska et al. 2008).

Like CAT and GPx, APx also plays an essential role in the control of intracellular H_2O_2/ROS levels. It uses two molecules of ascorbic acid (AsA) for reduction of H_2O_2 into water (Sharma et al.2012). Expression of APx is regulated by change in redox signals (concentration of AsA and GSH) and H_2O_2 content inside cell (Patterson and Poulos 1995). APx found in those subcellular locations which produces and scavenges H_2O_2 and based on amino acid sequences, five distinct isoenzymes of APx have been reported in cytosol, mitochondria, thylakoid, peroxysome and stroma (Nakano and Asada 1987; Jimenez et al. 1997; Madhusudhan et al. 2003; Sharma and Dubey 2004). In plants, APx is one of the most widely distributed antioxidants and its isoforms have much higher affinity for H_2O_2 than CAT that makes APx more efficient H_2O_2 scavengers under oxidative stress (Wang et al. 1999). Like other antioxidant enzymes, APx activity is also enhanced by abiotic stresses such as drought, nickel and aluminum stress in rice seedlings (Sharma and Dubey 2005, 2007; Maheshwari and Dubey 2009), UV-B stress in *Picea asperata* seedlings (Han et al. 2009), chilling and salt stress in tomato (Wang et al. 2005), UV-B and ozone induced stress in *Arabidopsis thaliana* (Rao et al. 1996), cadmium

stress in *Ceratophyllum demersum*, mustard and wheat (Arvind and Prasad 2003; Mobin and Khan 2007; Khan et al. 2007).

GuPx is a heme containing antioxidant enzyme that preferentially oxidizes aromatic electron donor such as pyrogallol and guaiacol by consuming H_2O_2 and widely accepted as stress enzyme. Inside the plant cell, GuPx is localized in vacuole, cell wall and in cytosol (Asada 1992), and plays an important role in biosynthetic processes including decomposition of indol-3-acetic acid (IAA), biosynthesis of lignin and ethylene, wound healing and defense against biotic and abiotic stresses (Sharma et al. 2012). Activity of GuPx in plants is also induced by various environmental stresses such as cadmium, drought, lead and salinity stress in rice seedlings (Shah et al. 2001; Verma and Dubey 2003; Sharma and Dubey 2005; Mishra et al. 2013), UV-B induced stress in *Picea asperata* and *Arabidopsis thaliana* (Rao et al. 1996; Han et al. 2009), drought stress in maize (Moussa and Abdel-Aziz 2008) and cadmium stress in spruce needles (Radotic et al. 2000).

During enzymatic and non-enzymatic antioxidant process, GSH participates in oxidation-reduction cycles and serves as electron donor during the reduction of ROS. For example, during ascorbate-glutathione cycle, GSH reduces the oxidized form of ascorbic acid (dehydroascorbate, produced during APx mediated reduction of H_2O_2) with the help of enzyme dehydroascorbate reductase (DHAR), similarly, GSH also serve as electron donor during the GPx mediated reduction of H_2O_2/ROS. After donating an electron, glutathione itself becomes oxidized and forms glutathione disulfide (GSSG). GR is a NADPH dependent enzyme that catalyzes the reduction of GSSG into its reduced form (GSH) and thus important for maintaining GSH pool inside the cell (Reddy and Raghavendra 2006; Chalapathi Rao and Reddy 2008). Although GR is located in mitochondria, chloroplast, cytosol, and peroxisomes, around 80% of GR activity is accounted for by chloroplast only (Edwards et al. 1990). GR activity is also influenced by environmental stress as reported by several authors in studies on drought, nickel and aluminum stress in rice seedlings (Sharma and Dubey 2005, 2007; Maheshwari and Dubey 2009), salt stress in pea (Hernandez et al. 2001), ozone stress in *Arabidopsis thaliana* (Yoshid et al. 2006) and chilling stress in maize (Fryer et al. 1998).

In present study, UV-B radiation significantly increased ($P < 0.001$) peroxidases (GPx, APx and GuPx) and GR enzyme activities in NSeUV-B and SeUV-B wheatgrass when

compared to their respective control (NSe-visible and Se-visible), whereas, in comparison between seleniferous and nonseleniferous, a significant decrease ($P < 0.001$) in peroxidases and GR activity were observed in selenium-rich samples. The results suggest that UV-B radiation triggers the peroxidases and GR activity directly by inducing the formation of H_2O_2/ROS which are responsible for increase in TBARS content (figure 4.8). Increased H_2O_2/ROS/TBARS content is/are supposed to act as secondary messenger(s) in response to environmental stresses which further triggers the signal transduction cascade of multi-level defense mechanisms in plants. Similar to present observations, stress induced activation of antioxidant enzymes is also supported by many workers (Cakmak and Horst 1991; Mittal and Dubey 1991; Fryer et al. 1998; Han et al. 2009; Sgherri et al. 2000; Sharma and Dubey 2005; Valderrama et al. 2006; Zhang et al. 2008; Sayfzadeh and Rashidi 2011; Mishra et al. 2013). As described previously, UV-B radiation induces enzymatic (SOD and CAT) and non-enzymatic (phenolics and flavonoids) activity in wheatgrass samples wherein the activities were significantly low in nonseleniferous samples when compared to those that are selenium-rich. These results indicate that increase in activities of SOD, CAT and nonenzymatic antioxidants were not sufficient to protect the plant from UV-B induced damage, which is confirmed by increased TBARS content. Both, SOD and CAT play an important role in reduction of $O_2^{\bullet-}$ and H_2O_2, whereas due to enhanced production of these ROS and insufficient expression of SOD and CAT, other peroxidase enzymes may serve as alternative routes for ROS quenching.

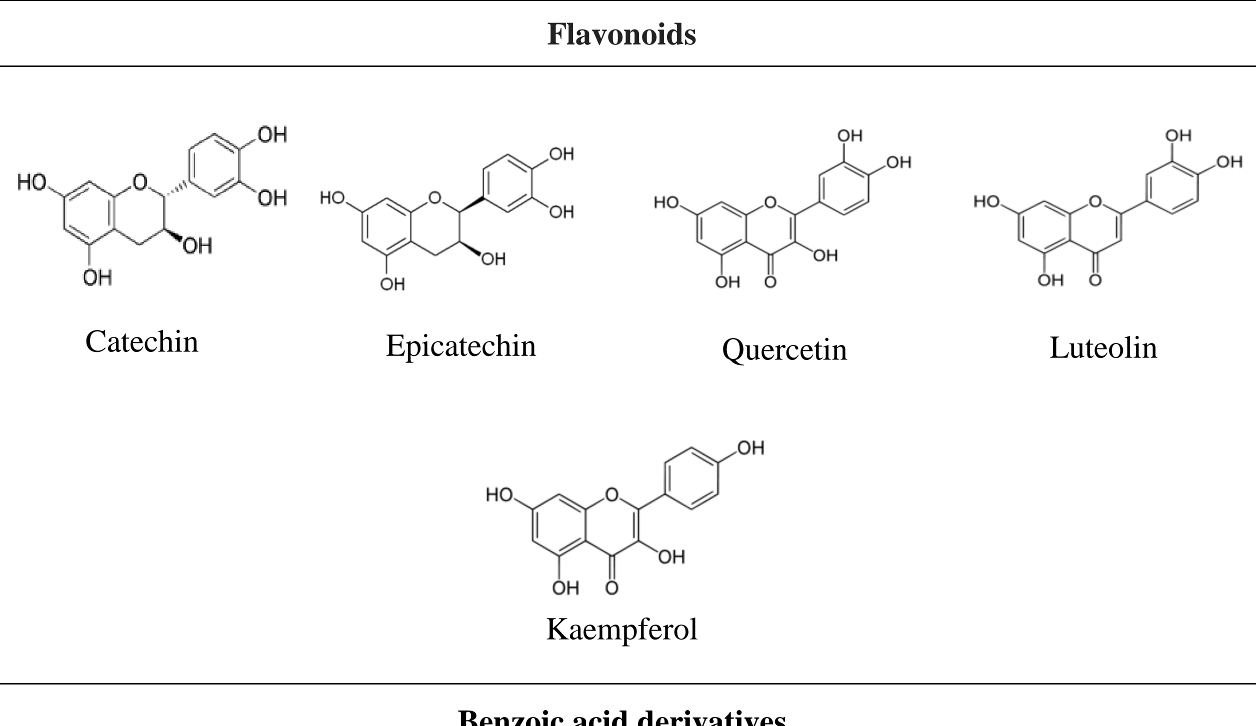
In present work, a significant decrease in peroxidases and GR enzyme activities in selenium-rich wheatgrass samples w.r.t. their controls suggest that, selenium supplementation is efficiently protects the plant from $O_2^{\bullet-}$/ H_2O_2 mediated oxidative stress by effectively inducing both enzymatic (SOD and CAT) and non-enzymatic antioxidants. Reduced TBARS content in selenium-rich samples indicate enhanced ROS scavenging and therefore required reduced expression of peroxidases enzymes. Irrespective of present study, many workers have reported that along with SOD and CAT, selenium also induces other peroxidase enzymes under different environmental stresses (Hartikainen et al. 2000; Djanaguiraman et al. 2004; Filek et al. 2008; Chu et al. 2010; Cartes et al. 2010; Yao et al. 2011; Hasanuzzaman et al. 2011, 2012; Abbas 2012).

4.3.3 Characterization of free and bound phenolic compounds, and their *in-vitro* bioaccessibility from wheatgrass

The aim of present section was to determine the concentration and characterization of different free (alcohol extracted) and bound forms (alkali extracted) of phenolic compounds from wheatgrass samples and also to evaluate their *in-vitro* bioaccessibility by using HPLC. Fifteen phenolic compounds viz., catechin, chlorogenic acid, p-hydroxybenzoic acid, epicatechin, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, o-coumaric acid, ellagic acid, quercetin, luteolin, kaempferol and butylated hydroxylanisol (BHA) were used as analytical standards (5-25 µg/ml, R^2= 0.997) and these phenolics are classified in table 4.6 according to their molecular structures.

Table 4.6: Classification of standard phenolic compounds and their molecular structures

Flavonoids



Catechin | Epicatechin | Quercetin | Luteolin

Kaempferol

Benzoic acid derivatives

p-hydroxybenzoic acid | Vanillic acid | Syringic acid

Cinnamic acid derivatives			
o-coumaric acid	p-coumaric acid	Ferulic acid	Sinapic acid

Ester of caffeic and quinic acid	Dilactone of hexahydroxydiphenic acid
Chlorogenic acid	Ellagic acid

Butylated hydroxylanisole (BHA)

Extensive studies have been carried out on the quantification of free and bound phenolics in different plant-derived foods like fruits, vegetables, beverages and different cereals (Mattila and Kumpulainen 2002; Stratil et al. 2007; Liyana-Pathirana and Shahidi 2006; Mattila et al. 2005) but very limited reports are available on wheatgrass (Benincasa et al. 2015; Kardas and Durucasu 2014; Moheb et al. 2011). To the best of our knowledge, no work has been carried out on the profile of free, bound and bioaccessible phenolic compounds in selenium-rich wheatgrass, especially those induced by UV-B light.

Individual phenolic compounds in wheatgrass extracts were quantified against corresponding calibration curves drawn after co-elution of standard phenolics. A typical

chromatographic profile of standard, free and bound phenolics are presented in figure 4.13, 4.14 and 4.15 respectively.

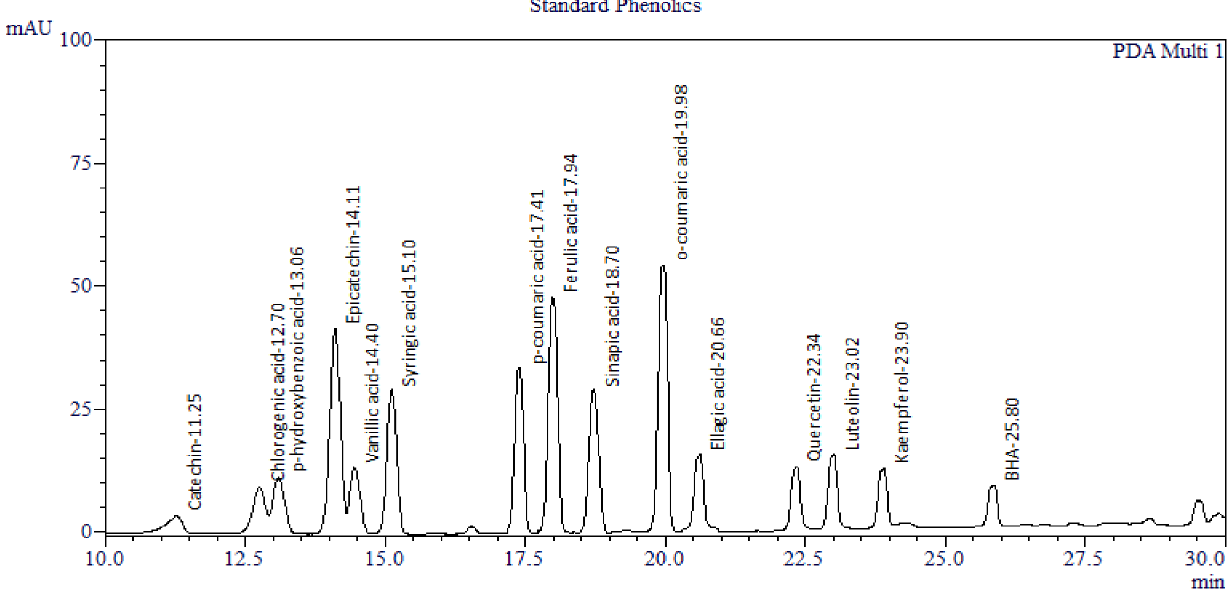


Figure 4.13: A typical HPLC chromatogram of standard phenolic compounds

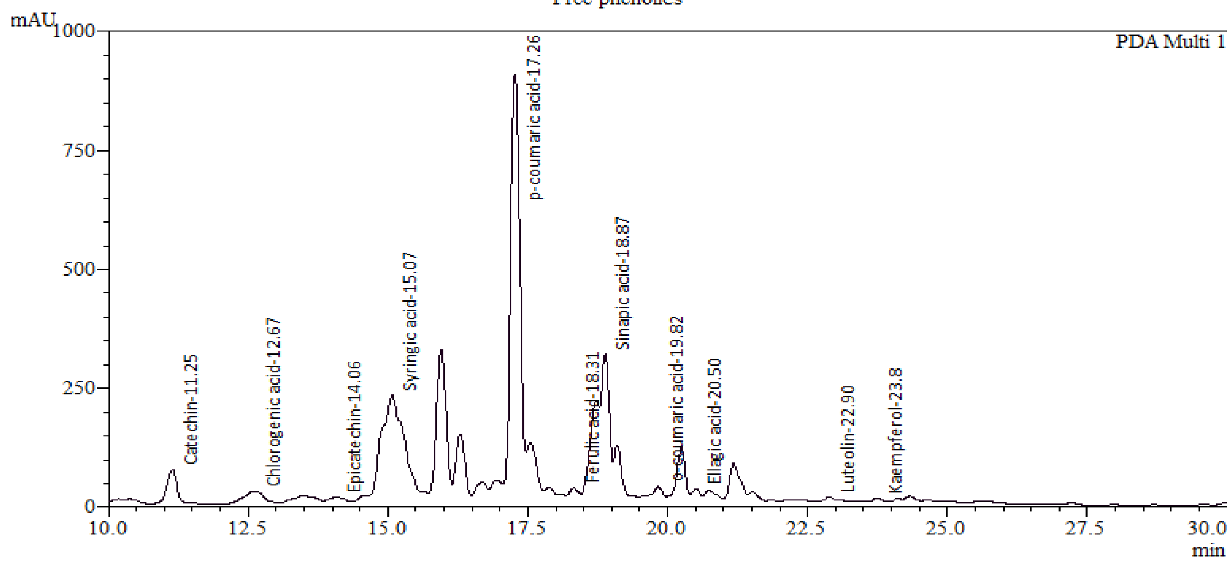


Figure 4.14: A typical HPLC chromatogram of free phenolics from wheatgrass

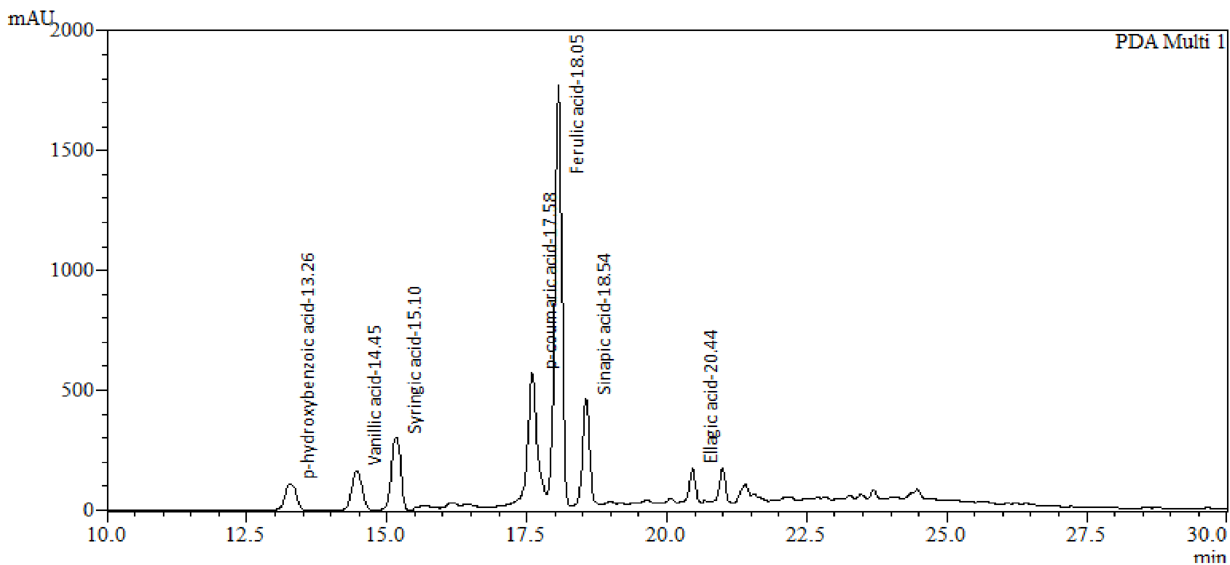


Figure 4.15: A typical HPLC chromatogram of bound phenolics from wheatgrass

Table 4.7 and 4.8 represent the concentrations of different free and bound phenolics respectively in wheatgrass samples and results are expressed as µg/ml of extract. The results indicated that total bound phenolics were predominant over free phenolics in each wheatgrass sample. It was also found that both selenium and UV-B radiation significantly ($P<0.05$) enhanced concentration of many phenolic compounds when compared to the control (NSe-visible) In addition, combined effect of selenium with UV-B radiation showed increased expression of phenolics over their individual effects. Among wheatgrass samples, the concentration of total phenolics (free and bound) were in the order of Se-UV-B > NSe-UV-B > Se-visible > NSe-visible.

As described previously (section 4.3.1.1), plants protect themselves from harmful UV-radiations by synthesizing phenolic compounds as secondary metabolites and UV-B radiation known to stimulate the enzymes responsible for the synthesis of many phenolics and flavonoids (Rozema et al. 2002). Under UV-B stress, flavonoids and other phenylpropanoid derivatives (derivatives of benzoic and cinnamic acid) accumulate in large quantities in vacuole of epidermal cells (Mazza et al. 2000). Like UV-B radiation, selenium also induces the synthesis of different phenylpropanoids in plants by stimulating the activity of enzyme phenylalanine ammonia lyase (Walla et al. 2010; Ardebili et al. 2015).

Table 4.7: Concentration of free phenolic species in different wheatgrass samples (n=4)

	Content of free phenolic compounds in wheatgrass (µg/ml of extract)			
	NSe-visible	**Se-visible**	**NSeUV-B**	**SeUV-B**
Catechin	26.14±0.55[a]	32.57±0.42[b]	46.45±1.68[c]	77.88±3.82[d]
Chlorogenic acid	7.87±0.49[a]	12.55±0.76[b]	19.17±0.68[c]	29.37±0.92[d]
Epicatechin	1.08±0.04[a]	1.61±0.05[b]	2.33±0.10[c]	2.70±0.12[d]
Syringic acid	22.31±0.69[a]	33.46±1.25[b]	37.75±2.01[c]	48.06±1.59[d]
p-coumaric acid	35.06±2.38[a]	43.26±2.34[a]	87.82±2.59 [c]	127.7±3.72[d]
Ferulic acid	3.61±0.16[a]	4.77±0.08[b]	7.52±0.21[c]	7.09±0.17[c]
Sinapic acid	24.05±2.47[a]	31.93±2.28[a]	36.70±1.93[b]	51.77±2.54[c]
o-coumaric acid	3.26±0.18[a]	3.01±0.06[a]	4.52±0.15[b]	7.79±0.17[c]
Ellagic acid	12.92±0.17[a]	16.66±0.38[b]	20.25±0.14[c]	38.40±0.49[d]
Luteolin	5.35±0.32[a]	6.83±0.41[a]	8.79±0.68[b]	12.26±0.31[c]
Kaempferol	7.77±0.17[a]	9.14±0.11[b]	10.25±0.27[c]	14.84±0.29[d]
Total	149.4	199.6	281.6	417.9

Different letters in rows indicate statistically significant differences at P<0.05 (t-test)

Table 4.8: Concentration of bound phenolic species in different wheatgrass samples (n=4)

	Content of bound phenolic compounds in wheatgrass (µg/ml of extract)			
	NSe-visible	**Se-visible**	**NSeUV-B**	**SeUV-B**
p-hydroxybenzoic acid	20.82±0.58[a]	30.73±1.97[b]	29.75±0.87[b]	30.05±1.87[b]
Vanillic acid	28.71±1.44[a]	45.47±1.63[b]	47.46±1.81[b]	53.79±2.38[c]
Syringic acid	24.29±1.37[a]	31.26±1.71[b]	41.41±1.16[c]	52.59±3.33[d]
p-coumaric acid	42.82±1.44[a]	52.58±1.80[b]	70.55±1.79[c]	85.25±1.78[d]
Ferulic acid	169.6±3.02[a]	169.9±3.83[a]	178.2±3.94[a]	191.2±4.31[b]
Sinapic acid	36.30±3.14[a]	50.8±4.33[b]	56.40±4.17[b]	73.77±4.28[c]
Ellagic acid	35.47±1.91[a]	42.36±1.87[b]	56.22±2.37[c]	57.55±1.95[c]
Total	358.1	423.2	479.9	544.2

Different letters in rows indicate statistically significant differences at P<0.05 (t-test)

From present study, it was found that wheatgrass contains both free and bound phenolic compounds that include different flavonoids (catechin, epicatechin, luteolin and kaempferol), derivatives of benzoic acid (syringic acid, vanillic acid and p-hydroxybenzoic acid), cinnamic acid derivatives (p-coumaric acid, o-coumaric acid, ferulic acid and sinapic acid), ester of caffeic and quinic acid (chlorogenic acid) and dilactone of hexahydroxydiphenic acid (ellagic acid). Free phenolics are solvent extractable and can be extracted into hot water or aqueous/organic solvent mixtures whereas, bound phenolics are ester-linked to cell-wall polymers and can be extracted through alkaline hydrolysis.

In present work, flavonoids and esterified phenolic (chlorogenic acid) are extracted only as free phenolics whereas; some phenolic acids (derivatives of benzoic and cinnamic acid) are found common in their free and bound forms (table 4.7 and 4.8). As flavonoids, catechin, epicatechin, luteolin and kaempferol were detected and their concentrations were found relatively higher in UV-B treated selenium-rich wheatgrass (SeUV-B). Among the free phenolics, concentration of p-coumaric acid was found higher in all wheatgrass samples where as ferulic acid was found to be higher amongst bound forms. In comparison with bound form of p-coumaric acid, it's free form was found in higher concentration ($P<0.001$) in UV-B exposed wheatgrass samples (NSeUV-B and SeUV-B). Other phenolic acids like syringic, ferulic, sinapic and ellagic acid were also found in their free and bound forms but unlike p-coumaric acid, their concentrations were observed higher in bound forms. Benzoic acid derivatives like p-hydroxybenzoic acid and vanillic acid were detected only in their bound forms.

Increase in phenolics/flavonoids in UV-B treated wheatgrass confirm that UV-radiation significantly increases the concentration of UV absorbing secondary metabolites and thus protect the plants form UV induced oxidative stress. These phenolic compounds have distinctive absorption characteristics. For example, benzoic acid derivatives show absorption maxima in the range of 250-290 nm; cinnamic acid derivatives have principal maxima in the range of 290-330 nm whereas, flavonoids exibit absorption bands in the range of 250-350 nm (Lattanzio et al. 2006). In present work, after exposure of UV-B (290-320 nm) radiation on wheatgrass, level of flavonoid and cinnamic acid derivatives were induces significantly and thus protects the plants by absorbing harmful UV-B radiation. Studies have shown that phenolic compounds especially flavonoids and cinnamic acid derivatives located in the epidermal cells, vacuole and cuticle of leaves are important in protecting terrestrial plants against UV-B radiation whereas, role of cell-

wall bound phenolics in UV-B protection is not clearly understood (Rozema et al. 1997; Landry et al. 1995; Sheahan 1996; lavola et al. 1997; Burchard et al. 2000; Stephanou and Manetas 1997). Furthermore, phenolic acid esters like chlorogenic acid also act as active antioxidant and provide additional protection from UV-B radiation (Larson 1988).

Under normal growth condition of plants, free forms of phenolic acids are present at much lower level when compared with their bound forms (Zhou et al. 2004; Mattila and Kumpulainen 2002). In present work, similar results were obtained wherein, concentrations of phenolic acids viz., ferulic sinapic and ellagic acid were found significantly higher ($P<0.05$) in their bound forms when compared to corresponding free forms. Bound form of p-coumaric acid was found significantly higher ($P<0.05$) when compared with free p-coumaric acid only in wheatgrass cultivated under visible light (NSe-visible and Se-visible). Whereas, contrasting results were observed in UV-B treated samples (NSeUV-B and SeUV-B) wherein, levels of free p-coumaric acid were significantly high ($P<0.001$). Present results suggested that along with flavonoids, cinnamic acid derivatives especially those of free forms of p-coumaric acid expressed significantly in UV-B treated wheatgrass and thus protect the plants from UV-B induced stresses.

Major phenolic compounds identified in wheatgrass after alkaline hydrolysis were recently reported by Kardas and Durucasu (2014) wherein, gallic acid, caffeic acid, benzoic acid, quercetin and BHA were also identified as additional phenolics and the ferulic acid was contributed to 65% of the total detected phenolics. Similar work has been carried out by Benincasa et al. (2015) wherein, phenolic compounds viz., p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid and ferulic acid were identified in wheat grain, wheat sprouts and wheatgrass. Among all bound phenolics, p-coumaric acid accounted for about 50% in grains and its concentration decreased after germination in sprouts followed by wheatgrass. Moheb et al. (2011) investigated changes in wheatgrass phenolic compounds during cold acclimation using a combination of HPLC-ESI-MS techniques and in methanolic extract of wheatgrass, a total of 40 phenolic and flavonoid compounds were identified that consisted mainly eight simple phenolic derivatives, two coumarin derivatives, ten hydroxycinnamoyl amides and twenty flavonoid derivatives.

Present section was also focused on the determination of bioaccessibility of different phenolic compounds from wheatgrass after *in-vitro* gastrointestinal digestion. A typical chromatographic profile of bioaccessible phenolics is represents in figure 4.16. Table 4.9 represents the percentage bioaccessibility of different phenolics from UV-B treated selenium-rich wheatgrass (SeUV-B). Percentage bioaccessibility of individual phenolic was calculated from their bioaccessible fraction and their total concentration (free + bound).

Table 4.9: Bioaccessibility of different phenolic compounds from wheatgrass (SeUV-B), (n=4)

	Phenolic content (µg/ml of extract)		% Bioaccessibility
	Total (Free + Bound)	Bioaccessible fraction	
Catechin	77.88	5.94±0.63	7.62±0.80
Chlorogenic acid	29.37	15.64±1.10	53.24±3.76
Vanillic acid	53.79	4.88±0.30	9.06±0.55
Syringic acid	100.65	7.86±0.49	7.80±0.48
p-coumaric acid	213.01	11.51±0.22	5.40±0.10
Ferulic acid	198.29	7.03±0.84	3.54±0.42
Sinapic acid	125.54	33.69±1.71	26.83±1.36
o-coumaric acid	7.79	1.69±0.16	21.65±2.05
Ellagic acid	95.95	16.04±1.66	16.71±1.72
Luteolin	12.26	8.27±0.59	67.45±4.84
Kaempferol	14.84	6.66±0.34	44.90±2.29

Results indicated that percentage bioaccessibilities of flavonoids like luteolin (67.45±4.84 %) and kaempferol (44.90±2.29 %) were higher when compared to phenolic acids (benzoic and cinnamic acid) derivatives. Among flavonoids, even though the total concentration of catechin was found maximum, their bioaccessibility was less when compared to luteloin and kaempferol. Phenolic acid ester like chlorogenic acid also showed higher bioaccessibility (53%) when compared to phenolic acid derivatives.

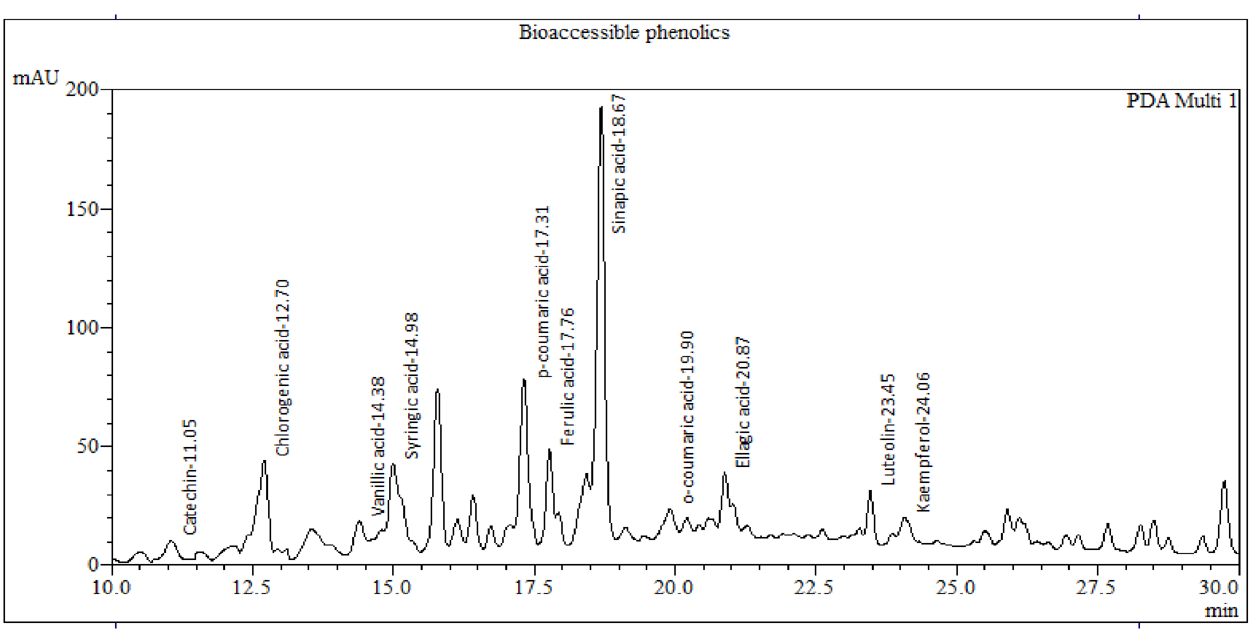


Figure 4.16: A typical HPLC chromatogram of bioaccessible phenolics from wheatgrass

Although phenolic acid derivatives were found as major constituents of wheatgrass, their observed *in-vitro* bioaccessibility is lower than flavonoids and phenolic acid esters, and order of their percentage bioaccessibility was as follows: sinapic acid > o-coumaric acid > ellagic acid > vanillic acid > syringic acid > p-coumaric acid > ferulic acid.

In general, plant derived foods such as fruits, vegetables, cereals, spices and nuts are the primary source of phytochemicals in the human diet. These phytochemicals are plant secondary metabolites, especially phenolic compounds that generally act as antioxidants. In fruits and vegetables, most of the phenolic compounds are present in soluble or free conjugate forms of glucosides (Vinson et al. 1998, 2001) whereas, in cereals, phenolic compounds exist mostly as insoluble bound forms associated with cell-wall polysaccharides (Mekeehen et al. 1999; Bunzel et al. 2001). Among phenolic compounds, benzoic and cinnamic acid derivatives are universally present in plant based foods (Herrmann 1989). In cereals, ferulic and p-coumaric acid are the major bound hydroxycinnamic acid derivatives (Smith and Hartley 1983; Zhou et al. 2004). The commonly existing ferulic and p-coumaric acid are predominantly esterified to hemicelluloses via covalent linkage to arabinofuranose in the heteroxylans (Mueller-Harvey et al. 1986). Generally, digestion of cell-wall materials by endogenous enzymes in the small intestine is difficult and thus results in reduced bioaccessibility of cell-wall bound phenolics (Liyana-Pathirana and Shahidi 2005). Present work also indicates similar observations wherein,

bioaccessibility of bound phenolic acids were very low, although they were present in higher concentration. Concentrations of syringic acid, p-coumaric acid and catechin were found higher in their free forms but their bioaccessibilities were less than 10%. These compounds can be extracted only with hot water, alcohol or organic solvents (Robbins 2003) and not gastric or intestinal juice; hence such compounds mostly survive gastrointestinal digestion to reach the colon.

Liyana-Pathirana and Shahidi (2005) reported that simulated gastrointestinal pH treatment significantly enhanced the *in-vitro* antioxidant activity of wheat samples and lower pH had improved extractability of the phenolic compounds from wheat by solubilizing them from cell-wall polymers. According to Baublis et al. (2000), pH conditions of gastrointestinal tract causes a dramatic increase in antioxidant activity of aqueous extracts of wheat based diet. Kroon et al. (1997) reported that significant amount (95%) of esterified feruloyl groups solubilized from plant fiber by the enzymatic hydrolysis of microbial ferulic acid esterage and xylanase activities present in large intestine. These results indicated that colonic fermentation of fiber rich foods may leads to the release of some bound phenolics and hence exert their unique health benefits in the colon after absorption.

Consumption of plant based diet rich in phenols/polyphenols offered some protection against development of oxidative stress related diseases like diabetes, cancer, cardiovascular diseases, osteoporosis and neurodegenerative disease (Graf et al. 2005; Arts and Hollman 2005). These phenolics and flavonoids have the capability of scavenging various free radicals such as $O_2^{\bullet -}$ and $OH^{\bullet}$ and can significantly reduce the oxidative stress for living cells (Namiki 1990; Rybka et al. 1993). Along with these phytochemicals, wheatgrass also contains significant amount of proteins, vitamins and minerals (Shih and Lai 2006; Hong and Lai 2006; Lin et al. 2006). Regular ingestion of wheatgrass juice improves the digestive system, detoxifies the blood and promotes general well being (Ben-Arye et al. 2002; Devogel et al. 2005; Ferruzia and Blakslee 2007). Consumption of wheatgrass also has shown potential antioxidant, anti-inflammatory and antiaging properties (Arya and Kumar 2011). Wheatgrass juice serves as an effective alternative of blood transfusion in thalassemia patient and reduce blood transfusion requirement by up to 400% with no adverse effect (Marawaha et al. 2004). Wheatgrass also has been used as nutritional alternative to chemotherapy for primary peritoneal cancer (Guisseppi 2005). Dry powder of wheatgrass is rich in fibers and consumption of dietary fibers has been

related to reducing the risk of colorectal cancers, diabetes, diverticulitis and heart disease (Craig et al. 1988; Topping and Clifton 2001).

Like other nutrients, selenium is also an essential micronutrient for humans and animals which involved as selenocysteine (SeCys) in functioning at the catalytic centre of several selenoproteins, such as glutathione peroxidases, thioredoxin reductase and iodothyronine-deiodinases (Rayman, 2002). Therefore, consumption of selenium-rich wheatgrass as a dietary supplement can be serves as natural source of dietary selenium with additional benefits of natural antioxidants.

4.4 Isolation, quantification and characterization of isoselenocyanates (ISeCs) from Selenium-rich mustard cake

In general, pungent smell of mustard oil is due to the presence of naturally occurring isothiocyanates (ITCs) produced by myrosinase enzyme mediated hydrolysis of sulfur containing secondary metabolites called glucosinolates that are mostly found in almost all plants of order Brassicales. Glucosinolates are synthesized from certain amino acids where sulfur atom is donated by cysteine via glutathione (Sonderby et al. 2010).

Mustard plant accumulates high selenium content in their seeds (up to 670 µg/g) when grown in selenium contaminated soil (Sharma et al.2009). Due to similar biochemical properties of sulfur with selenium, sulfur atom is replaced by selenium from sulfur containing amino acids viz., cysteine and methionine; results in the formation of selenocystiene (SeCys) and selenomethionine (SeMet). Therefore, it is presumed that Se-glucosinolates would be synthesized when mustard is grown in selenium contaminated soil, which after enzymatic hydrolysis produces isoselenocyanates (ISeCs), (Ouerdane et al. 2013).

In present section, oily residue obtained after myrosinase based enzymatic auto hydrolysis of selenium-rich mustard cake powder was analyzed for their total selenium content, total ITCs/ISeCs content and characterization of ITCs/ISeCs species. The obtained total selenium content in oily residue determined by fluorescence spectrometer was 358.8±2.79 µg/g, which confirmed the presence of ISeCs or other selenium containing metabolites in selenium-rich mustard. Total ITCs/ISeCs content in oily residue analyzed after cyclocondensation based assay was found 114.3±2.01 mg/g.

4.4.1 GC-MS based speciation of ITCs/ISeCs in oily residue isolated from selenium-rich mustard cake

Table 4.10 summarized the identified ITCs/ISeCs species along with their percentage relative abundance in oily residue. Percentage relative abundance of each species was determined from GC-chromatogram (figure 4.17) after analyzing the peak area of individual compound with total peak area of identified ITCs/ISeCs species. GC-MS analysis provided details retention time (Rt) and *m/z* fragmentation patterns of individual compounds. Fragmentation patterns of detected allyl thiocyanate, allyl isothiocyanate, butenyl isothiocyanate and phenethyl isothiocyanate matched with mass spectra of those contained in National Institute for Standards and Technology (NIST, Search Version 2.0) and with previously published data (Meija et al. 2002; Al-Gendy and Lockwood 2003). To the best of our knowledge, there were no records available for the fragmentation patterns of allyl selenocyanate and allyl isoselenocyanate. Presence of these seleno-compounds in oily residue is presumed due to high selenium content (358.8±2.79 μg/g).

Table 4.10: ITCs/ISCs detected in crude oily residue isolated form selenium-rich mustard cake

Compound	Retention time (min)	*m/z*	Percentage relative abundance
Allyl thiocyanate	6.41	99	0.43%
Allyl selenocyanate	6.48	147	0.13%
Allyl isothiocyanate	6.71	99	4.48%
Allyl isoselenocyanate	7.07	147	0.16%
Butenyl isothiocyanate	9.73	113	92%
Phenethyl isothiocyanate	23.58	163	2.8%

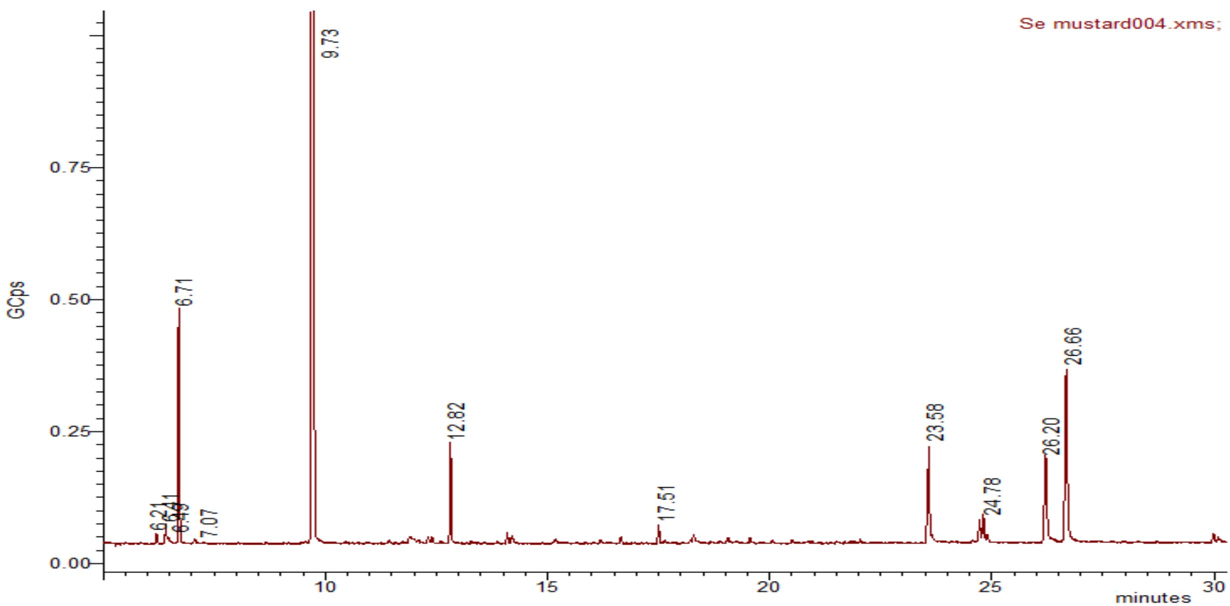


Figure 4.17: Gas chromatographic profile of crude oily residue isolated form selenium-rich mustard cake

Following mass spectra represents the fragmentation patterns of individual compounds along with their fragmented ion intensities:

***Allyl thiocyanate*:** Figure 4.18, Rt.6.41 min, *m/z* (% relative intensity); 38 (12%), 39 (100%), 41 (96%), 45 (10%), 58 (9%), 72 (45%) and 99 (M^+, 46%).

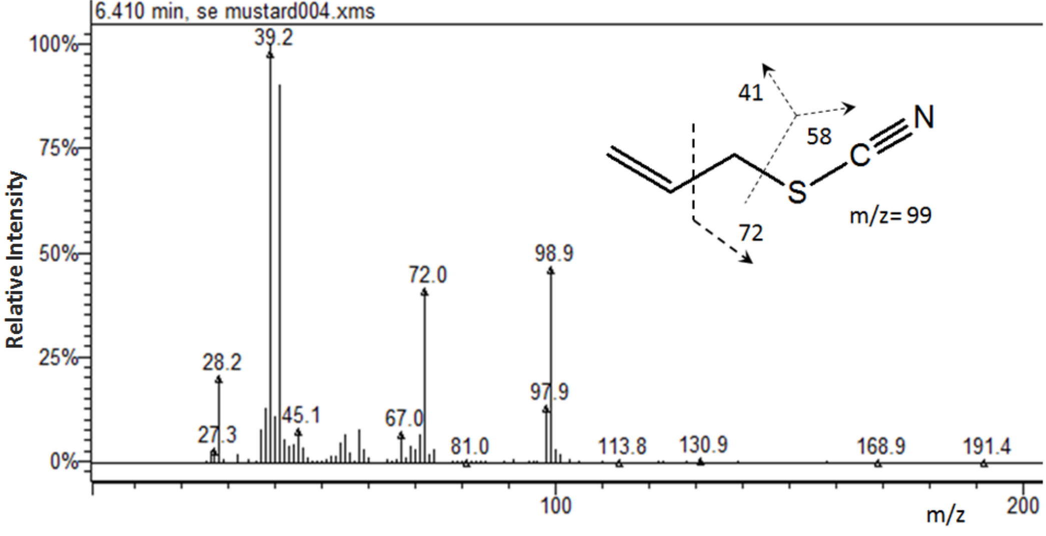


Figure 4.18: Mass spectrum of allyl thiocyanate

***Allyl selenocyanate*:** Figure 4.19, Rt. 6.49 min, *m/z* (% relative intensity); 39 (48%), 41 (36%), 45 (15%), 55 (100%), 106 (40%), 120 (24%) and 147 (M^+, 19%).

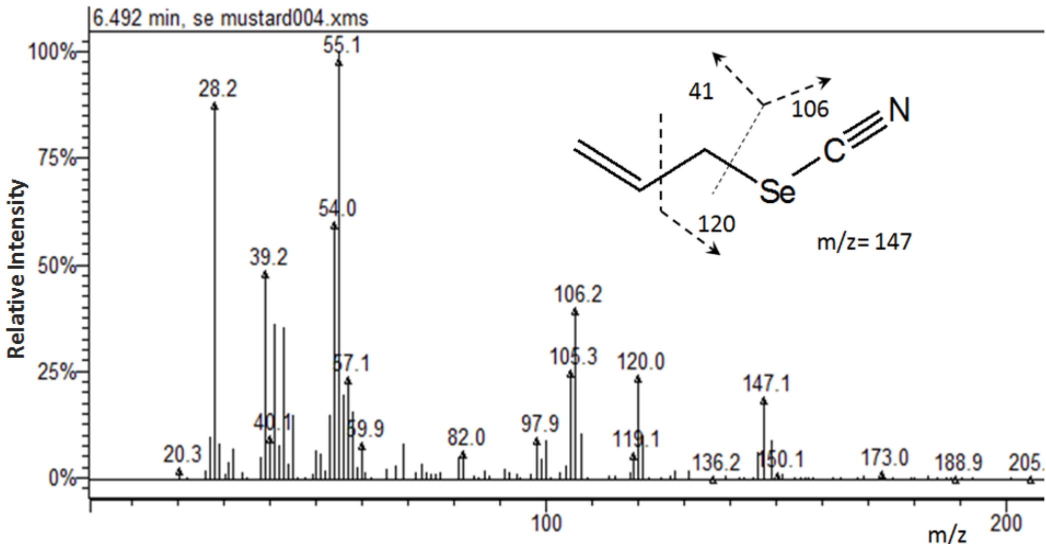


Figure 4.19: Mass spectrum of allyl selenocyanate

***Allyl isothiocyanate*:** Figure 4.20, Rt. 6.71 min, *m/z* (% relative intensity); 39 (69%), 41 (60%), 45 (8%), 67 (6%), 72 (50%), 98 (11%) and 99 (M^+, 100%).

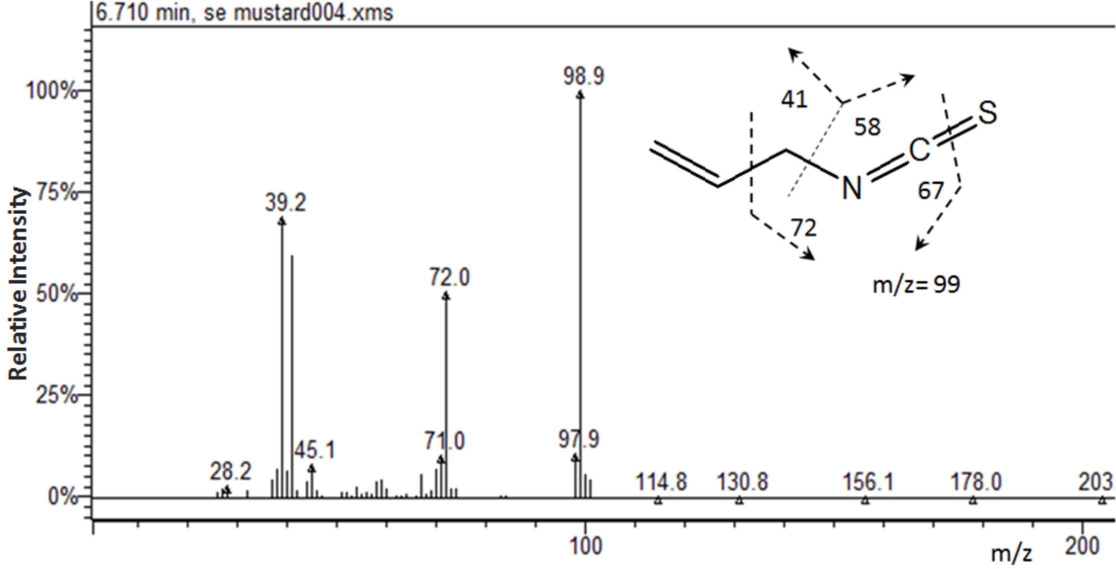


Figure 4.20: Mass spectrum of allyl isothiocyanate

***Allyl isoselenocyanate*:** Figure 4.21, Rt. 7.07 min, *m/z* (% relative intensity); 39 (8%), 41 (53%), 51 (11%), 67 (24%), 91 (100%), 92 (26%), 105 (19%), 106 (42%), 120 (19%) and 147 (M^+, 18%).

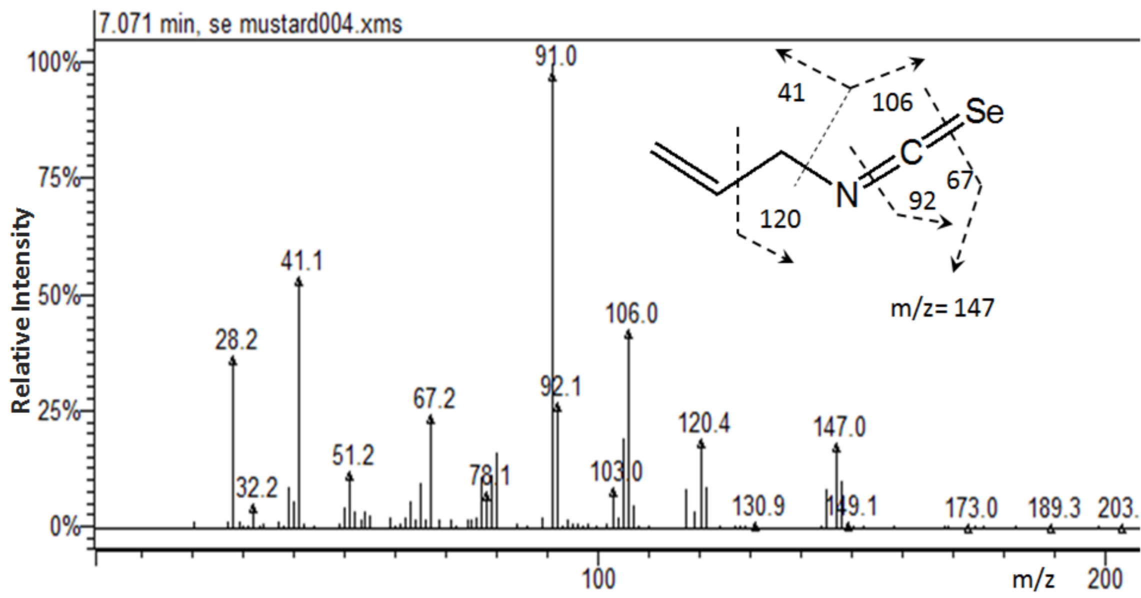


Figure 4.21: Mass spectrum of allyl isoselenocyanate

Butenyl isothiocyanate**:** Figure 4.22, Rt. 9.73 min, *m/z* (% relative intensity); 39 (17%), 41 (5%), 55 (23%), 58 (3%), 72 (100%), 85 (24%) and 113 (M^+, 48%).

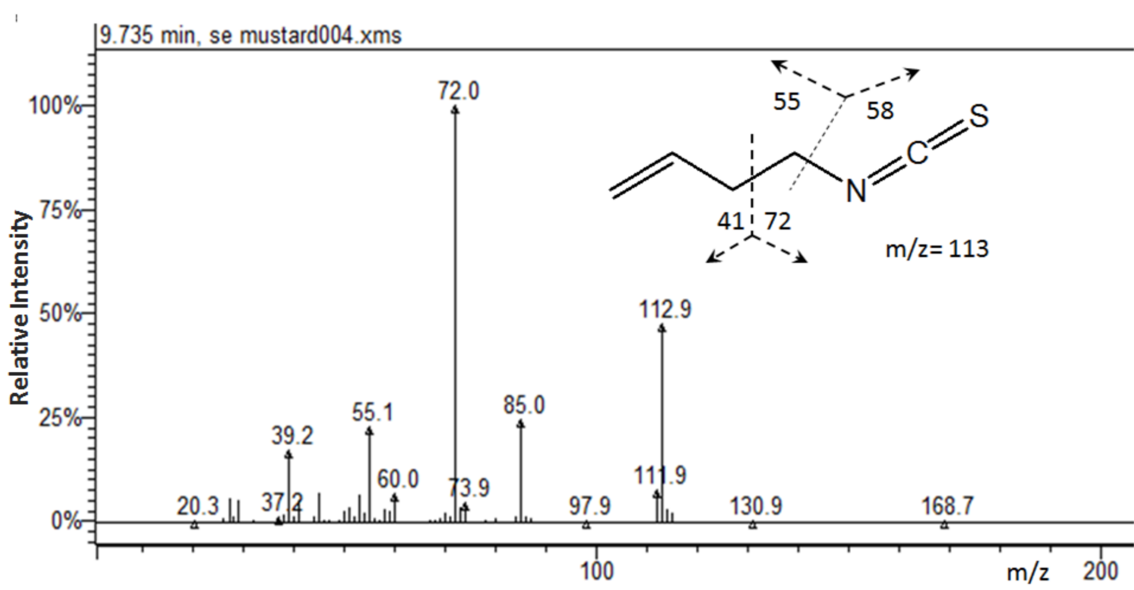


Figure 4.22: Mass spectrum of butenyl isothiocyanate

Phenethyl isothiocyanate**:** Figure 4.23, Rt. 23.58 min, *m/z* (% relative intensity); 44 (8%), 51 (8%), 65 (15%), 77 (10%), 91 (100%), 105 (30%) and 163 (M^+, 10%).

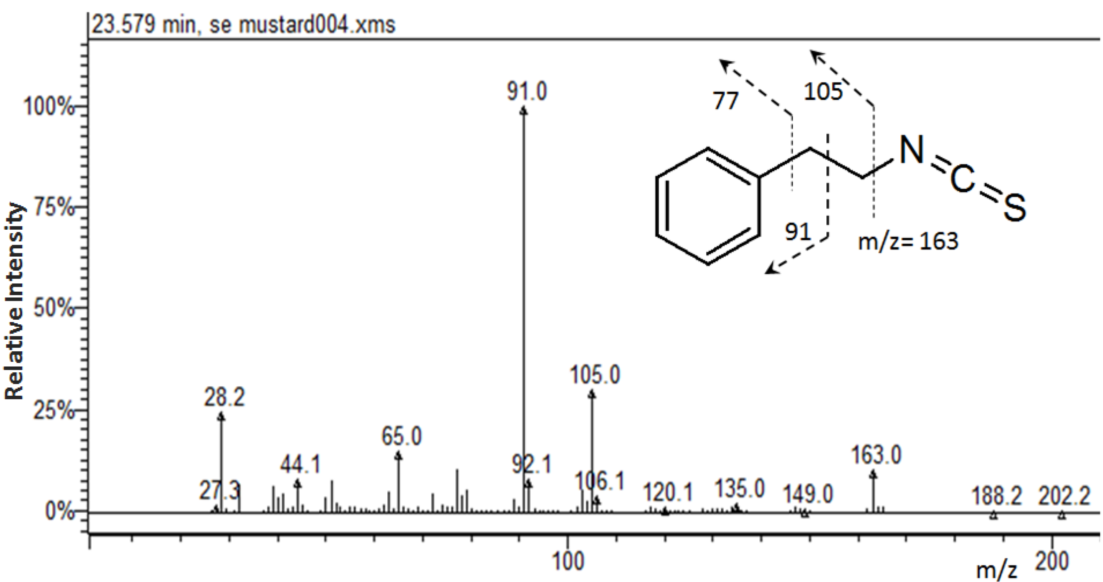


Figure 4.23: Mass spectrum of phenethyl isothiocyanate

Although the mustard cake used in present work contains high selenium (143.0±5.18 µg/g), a very small fraction of selenocyanate/isoselenocyanate (0.13-0.16 %) were observed when compared to their sulfur analogues. The major identified sulfur containing species were butenyl isothiocyanate (92%) followed by allyl isothiocyanate (4.48%), phenethyl isothiocyanate (2.8%) and allyl thiocyanate (0.43%). The above result was different from previous reports where allyl isothiocyanate was found as major species in mustard seed (Yu et al. 2003; Tsao et al. 2002).

A number of thiocyanates, isothiocyanates and methylthioalkyl nitriles are reported previously in many *Brassica* plants (Matich et al. 2012; Verkerk et al. 2009; Sonderby et al. 2001). These sulfur containing secondary metabolites are biosynthetic hydrolysis products of glucosinolates which are associated with herbivores resistance for the plants (Halkier and Gershenzon 2006; Mumm et al. 2008). Myrosinase or thioglucoside glucohydrolase mediated hydrolysis of glucosinolates produces glucose and an unstable aglycone that spontaneously rearranges to an isothiocyanates. Alternatively, formation of nitriles and thiocyanates from glucosinolates are produced by the action of epithiospecifier proteins present in plants (Burow et al. 2007; Kissen and Bones 2009). Trace amount of selenium-containing glucosinolates (selenoglucosinolates) were previously reported in the *Brassica* family plants like *Stanleya pinnata* and *Nasturtium officinale* (Bertelsen et al. 1988; Weilanek et al. 2005). Theoretically,

the glucosinolates in *Brassica* plants might incorporate up to three atoms of selenium: in the β-thioglucose, the modified amino acid (selenomethionine or methylselenocysteine) side chain or in the sulfate. Thus, depending where selenium is incorporated in the glucosinolate molecule, hydrolysis by myrosinase may produce a number of selenium containing products.

There are very limited reports available on detection and characterization of selenium metabolites especially glucisinolate derivatives in *Brassica* plants. Matich et al. (2012) investigated the incorporation of selenium into broccoli, cauliflower and rape plants when fertilized with sodium selenate. LC-MS/MS analysis was used to locate the position of selenium atom in the selenoglucosinolate that indicates preferential incorporation of selenium via selenomethionine into the methylselenyl moiety rather than into the sulfate or β-thioglucose groups. Ouerdane et al. (2013), for the first time, reported the presence of isoselenocyanate functional group containing secondary metabolites in seeds of black mustard grown on selenium-rich soil. In addition with these compounds, they have also identified more than 30 low-molecular weight selenium metabolites that include selenoglucosinolates, selenoamino acids, selenosinapine, selenosugars and selenourea derivatives.

Generally, sulfur containing secondary metabolites present in *Brassica* plants exhibit many bioactive properties. Regular consumption of *Brassica* vegetables such as turnip, kale, cauliflower, cabbage, Brussels sprouts and broccoli is associated with reduced incidence of cancer (van Popped et al. 1999). In recent years, research has been focused on the biological activities of ITCs. Kinae et al. (2000) reported that allyl isothiocyanate exhibit anti-mutagenic and superoxide scavenging potency. ITCs found in *Brassica* plants are powerful anti-microbial agents (Isshiki et al. 1992), inhibit the platelet aggregation by inhibiting arachidonic acid pathway (Kumagai et al. 1994) and anti-asthmatic (Dorsch et al. 1985). Many species of ITCs also have capability to prevent the initiation and progression of carcinogenesis. ITCs present in broccoli and watercress suppress the invasive potential of human MDA-MB-231 breast cancer cells in *in-vitro* condition (Rose et al. 2005). Dietary ITCs obtained from *Brassica* vegetables appeared to reduce the risk of lung cancer (London et al. 2000), colorectal cancer (van Poppel et al. 1999) and prostate cancer (Giovannucci et al. 2003). Bhattacharya et al. (2010) reported that mustard seed powder appeared to be more robust for inhibiting bladder cancer growth and muscle invasion than that of pure ally isothiocyanate in rat. Other than anti-cancerous activities, ITCs have been reported for their anti-carcinogenic activities against a wide variety of chemical

carcinogens including azo dyes, polycyclic aromatic hydrocarbons, fluorenyl acetamide, ethionine and several nitrosamines (Wu et al. 2009). Benzyl isothiocyanate is reported for preventing benz (a) pyrene induced carcinogenesis in mouse forestomach (Wattenberg 1987). Phenethyl isothiocyanate, benzyl isothiocyanate, and phenyl isothiocyanate were tested for their abilities to inhibit tobacco-specific nitrosamine induced lung tumorigenesis (Morse et al. 1989a; 1989b; 1991; 1993).

As previously reported, ITCs prevent the initiation and progression of carcinogenesis, while synthetic ISeCs are reported to be more potent inhibitors of cancer cell and tumor growth than their sulfur analogues. Ip and Ganther (1992) reported that organoselenium compounds like selenocystamine, Se-methylselenocysteine and selenobetaine are much more active in cancer prevention than their sulfur analogues. Sharma et al. (2008) reported that synthetic phenylalkyl isoselenocyanates induces apoptosis in breast, prostate, melanoma, glioblastoma, sarcoma and colon cancer cell lines more effectively when compared to respective phenylalkyl isothiocyanates. Synthetic ISeCs species are also reported for significant decrease in Akt3 signaling pathway (prominently expressed in melanomas) in cultured melanoma cells and also decrease in tumor development by ~60% compared to corresponding ITCs (Sharma et al. 2009). According to Emmert et al. (2010) ISeC analogue of sulforaphane more effectively enhanced Nrf2-dependent induction of glutathione in mouse embryonic fibroblast.

It is not clear, how replacement of sulfur with selenium in an isothiocyanate functional group would change its reactivity and or target specificity, as both the elements have similar electronegativity. However, it is clear from many examples, where selenium in place of sulfur greatly changes a protein’s reactivity. For example, in active sites of selenoproteins the ionized selenol of selenocysteine possess higher redox sensitivity at physiological pH (Copeland 2005). In proteins, oxidized selenomethionine can be repair themselves non enzymatically while oxidized methionine requires methionine sulfoxide reductase (Emmert et al. 2010).

In literature, existence of ISeC species in Brassica plants yet not fully confirmed. Ouerdane et al. (2013) reported the presence of ISeC functional group in selenium-rich mustard after LC-MS analysis. In present work, GC-MS analysis of oily residue obtained from selenium-rich mustard cake also suggests the initial confirmation for the presence of ISeC, whereas further studies on biosynthesis of ISeCs needs to be explored. In conclusion, mustard cultivated in selenium-rich

soil of Punjab, India, can be a good source of dietary ITCs, ISeCs and other low-molecular weight selenium metabolites.

4.5 Cytoprotective effect of selenium-rich mustard protein

Mustard cake residue left after extraction of ITCs/ISeCs was further used for the extraction of selenium-rich storage protein using water as solvent. Isolated protein was subjected to quantification of selenium using fluorescence spectrometer and total selenium content recorded was 582.3 ± 6.23 µg/g. Present section is aimed to study the cytoprotective effect of selenium-rich mustard protein on tert-butyl hydroperoxide (TBHP) induced cytotoxicity in mouse melanoma cell line (B16-F10). Cytoprotective effect of selenium-rich protein against TBHP was monitored by microscopic imaging and MTT assay. To increase the bioaccessibility of selenium to the cells, isolated mustard protein was digested with proteolytic enzymes pepsin and pancreatine as described previously following *in-vitro* gastrointestinal (GI) digestion method (section 3.4.2). Sodium selenite was taken as positive control whereas; undigested selenium-rich mustard protein and GI-digested non-Se mustard protein were taken as negative control. Before the exposure of TBHP, melanoma cells were pretreated with 1µM of selenium for 24 h. Percentage viability of cells were determined by MTT assay and microscopic imaging was carried out for differentiation in between live and dead cell using neutral red and trypan blue as cell staining dyes. All the treatments were performed in 96 well tissue culture plate and detail of treatments in each column of 96 well plate are as follow:

A. Only culture medium (control)

B. 2.0 mM TBHP

C. 1µM selenium as sodium selenite

D. Pretreatment with 1µM selenium as sodium selenite + 2 mM TBHP

E. GI-digested non-Se mustard protein

F. Pretreatment with GI-digested non-Se mustard protein + 2 mM TBHP

G. 1µM selenium as undigested selenium-rich mustard protein

H. Pretreatment with 1µM selenium as undigested selenium-rich mustard protein + 2 mM TBHP

I. 1µM selenium as GI-digested selenium-rich mustard protein

J. Pretreatment with 1µM selenium as GI-digested selenium-rich mustard protein + 2 mM TBHP

Figure 4.24 showing the layout of 96 well plate for above treatments (A-J) and MTT assay, the last well of each column was used for staining the cells and figure 4.25A-J representing morphological differentiation between live and dead cells along with their percentage viability.

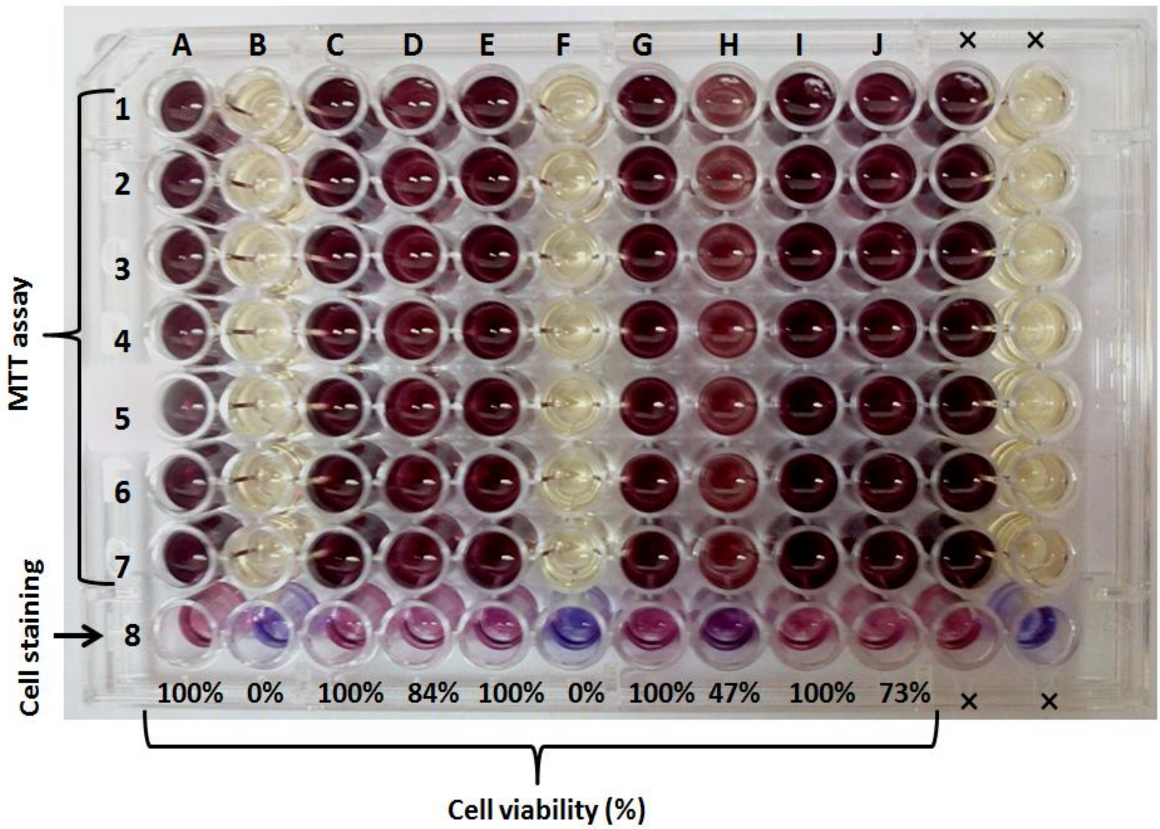


Figure 4.24: 96 well tissue culture plate showing layout of treatment details (A-J), MTT assay, cell staining and percentage cell viability

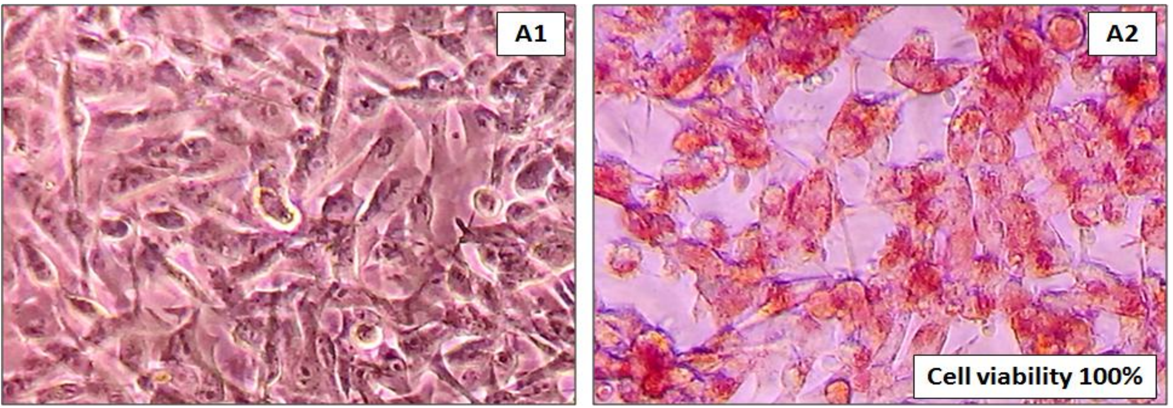


Figure 4.25A: Microscopic image of B16-F10 cells (at 400×) without treatment; (A1) unstained cells, (A2) cells were stained with trypan blue and neutral red

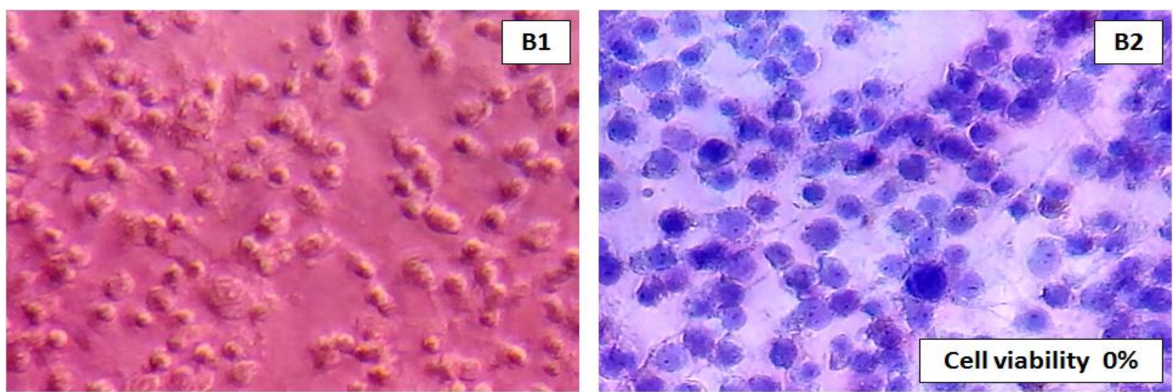


Figure 4.25B: Microscopic image of B16-F10 cells (at 400×) treated with 2 mM TBHP; (B1) unstained cells, (B2) cells were stained with trypan blue and neutral red

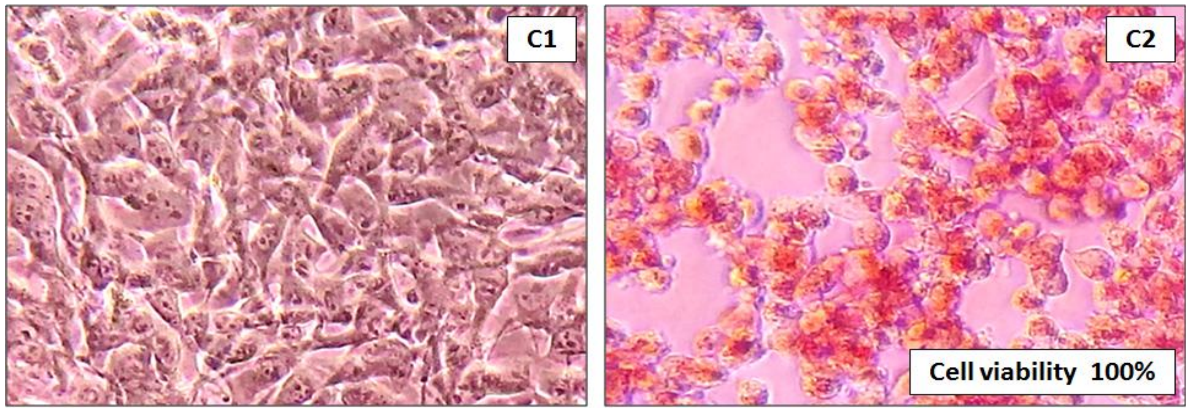


Figure 4.25C: Microscopic image of B16-F10 cells (at 400×) treated with 1 µM sodium selenite; (C1) unstained cells, (C2) cells were stained with trypan blue and neutral red

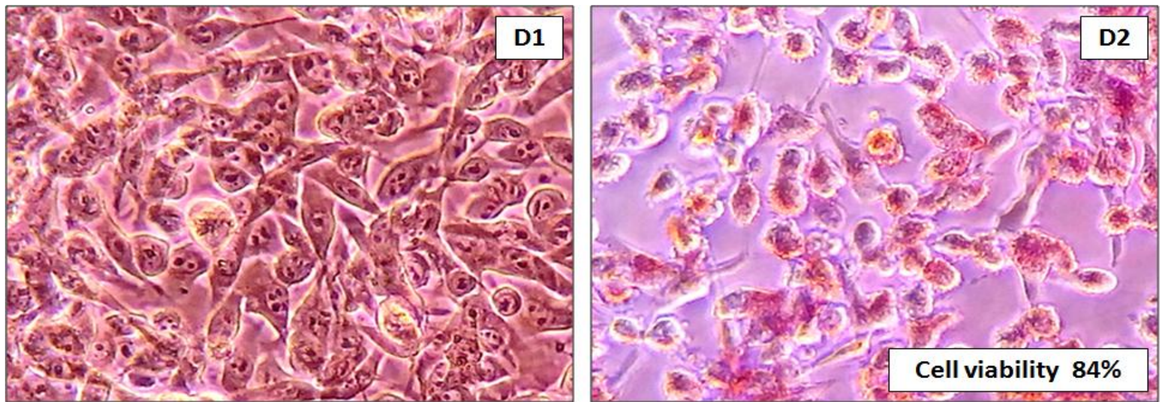


Figure 4.25D: Microscopic image of B16-F10 cells (at 400×) pretreated with 1 µM sodium selenite and then 2 mM TBHP; (D1) unstained cells, (D2) cells were stained with trypan blue and neutral red

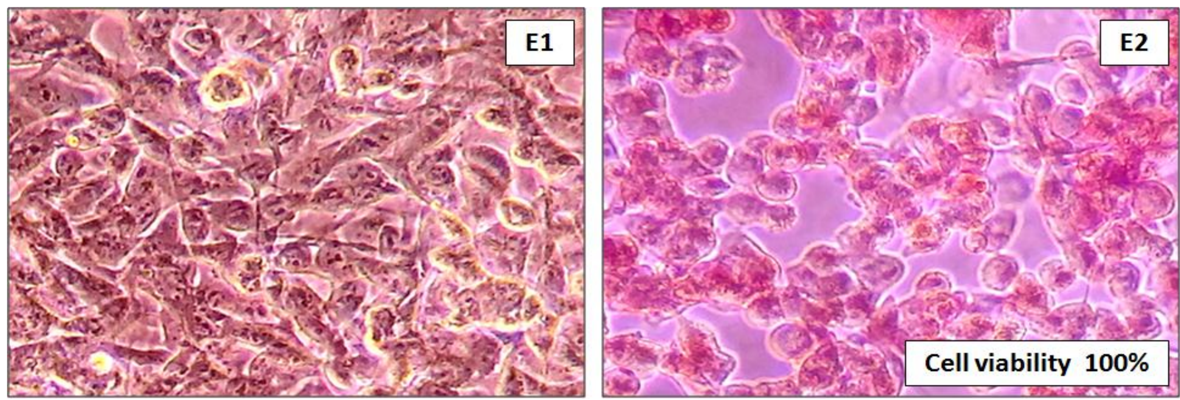


Figure 4.25E: Microscopic image of B16-F10 cells (at 400×) treated with GI digested non-Se mustard protein; (E1) unstained cells, (E2) cells were stained with trypan blue and neutral red

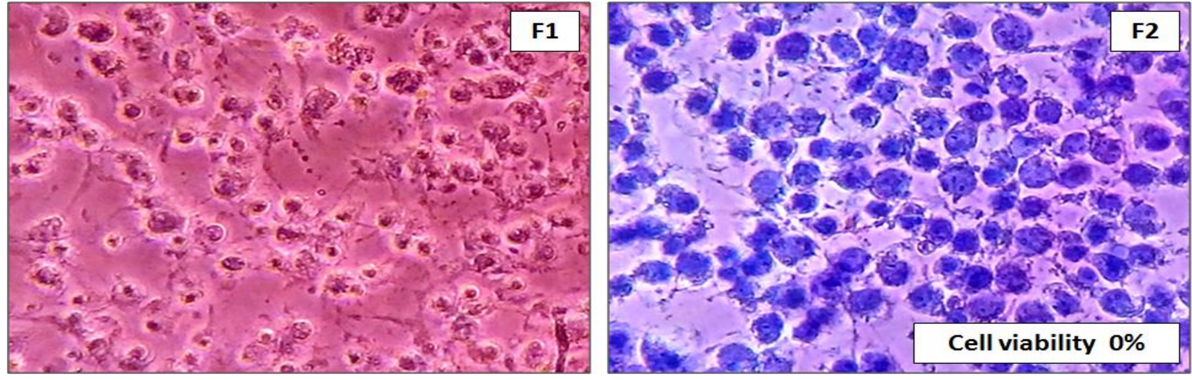


Figure 4.25F: Microscopic image of B16-F10 cells (at 400×) pretreated with GI-digested non-Se mustard protein and then 2 mM TBHP; (F1) unstained cells, (F2) cells were stained with trypan blue and neutral red

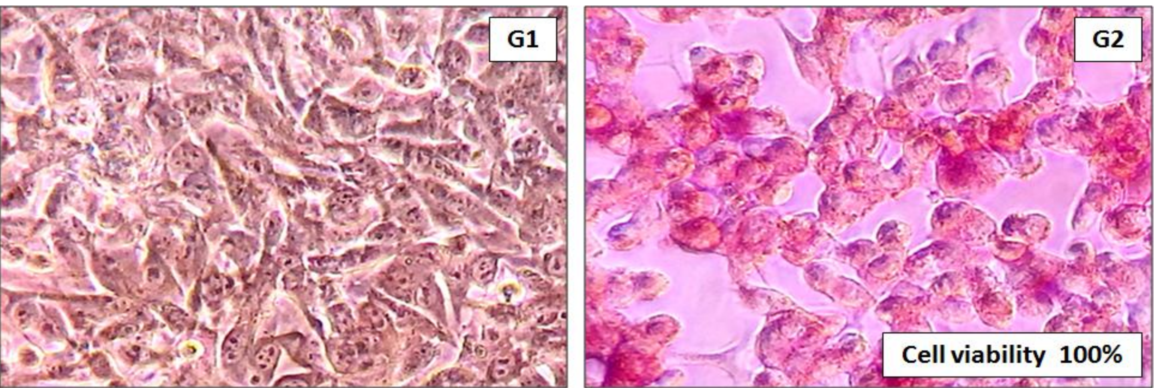


Figure 4.25G: Microscopic image of B16-F10 cells (at 400×) treated with 1 µM selenium from undigested selenium-rich mustard protein; (G1) unstained cells, (G2) cells were stained with trypan blue and neutral red

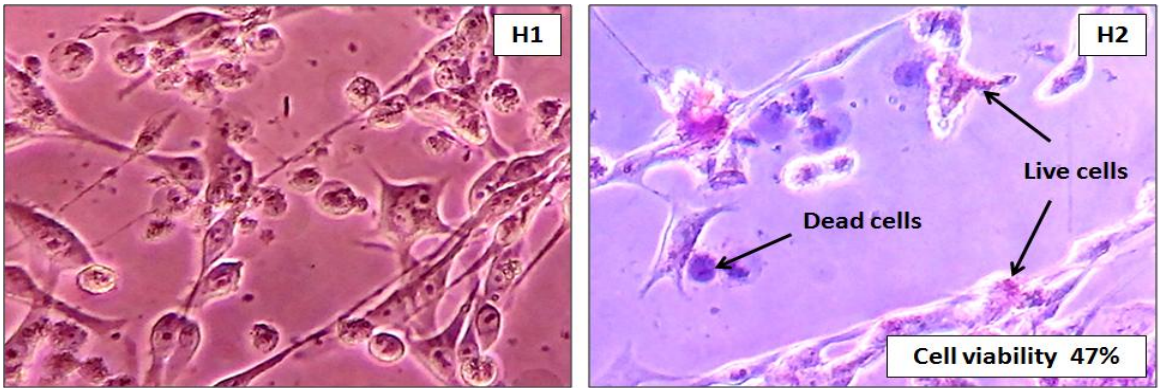


Figure 4.25H: Microscopic image of B16-F10 cells (at 400×) pretreated with 1 µM selenium from undigested selenium-rich mustard protein and 2 mM TBHP; (H1) unstained cells, (H2) cells were stained with trypan blue and neutral red

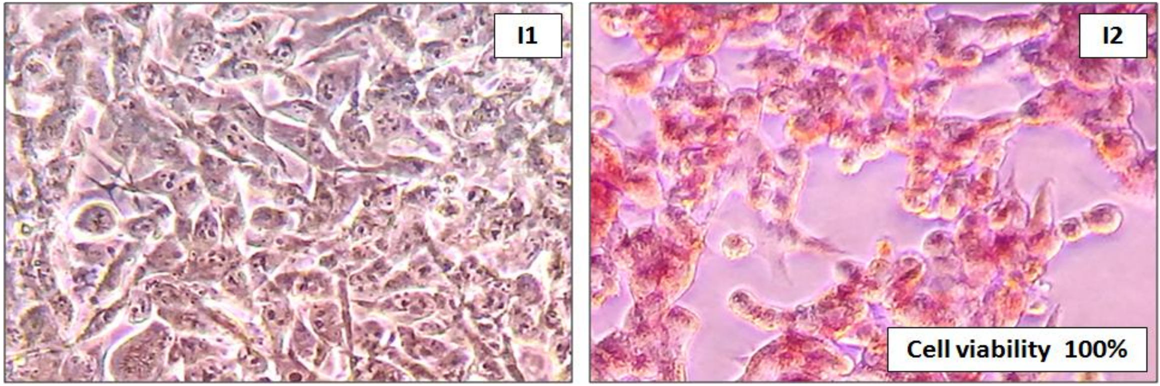


Figure 4.25I: Microscopic image of B16-F10 cells (at 400×) treated with 1 µM selenium from GI-digested selenium-rich mustard protein; (I1) unstained cells, (I2) cells were stained with trypan blue and neutral red

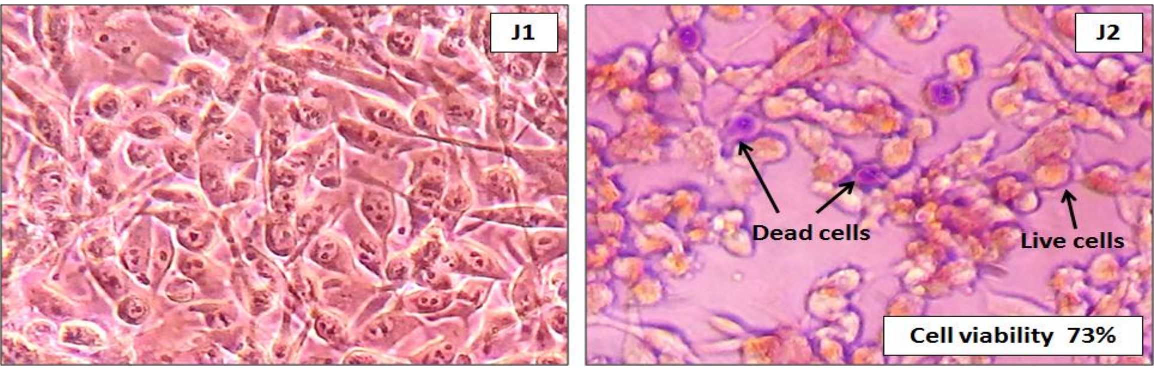


Figure 4.25J: Microscopic image of B16-F10 cells (at 400×) pretreated with 1 µM selenium from GI-digested selenium-rich mustard protein and 2 mM TBHP; (J1) unstained cells, (J2) cells were stained with trypan blue and neutral red

MTT assay results indicated that selenium protects the melanoma cells from TBHP induced cytotoxicity. Without selenium pretreatment or when the cells treated with non-Se mustard protein, TBHP induces 100% mortality of the cells (figure 4.25B and F), this indicated that non-Se mustard protein does not exert any cytoprotective effect against TBHP. No cytotoxicity was observed in the cells when treated only with selenium as sodium selenite and selenium-rich mustard protein (figure 4.25C, G and I). In present work, the cytoprotective effect of selenium was decided by nature of selenium treatments, for example when melanoma cells were treated with sodium selenite, the observed cell viability was 84%, followed by 73% in presence of GI-digested and 47% in undigested selenium-rich mustard protein. These results indicated that, selenium present in sodium selenite is more bioaccessible when compared to digested and undigested protein.

Cell viability was determined by MTT assay, which is a well known colorimetric assay. MTT is a yellow colored water soluble compound which is reduced by NADPH dependent dehydrogenase enzyme present in metabolically active cells, resulting in formation of intracellular purple formazan which can be solubilized and quantified spectroscopically. The extent of MTT reduction is proportional to number of viable or metabolically active cells. For microscopic imaging, two vital stains viz., neutral red and trypan blue was used for the differentiation in between live and dead cells respectively. In many studies, both the dyes were used for selective staining of tissues or cells in histology (Avelar-Freitas et al. 2014; Altman et al. 1993; Sarvel et al. 2006; Repetto et al. 2008). Neutral red uptake is based on the ability of live cells for incorporation and intracellular binding. It is a weak cationic dye which enters to the cell

via nonionic passive diffusion and gets accumulated into lysosomes, where it binds to phosphate and/or anionic groups of lysosomal matrix electrostatic hydrophobic bonds (Winckler 1974; Nemes et al. 1979). At physiological pH, the net charge on neutral red dye is zero which enables it to penetrate cell membrane. Inside the cell there is a proton gradient in between lysosome and cytoplasm that maintain lower pH of lysosome than that of cytoplasm. Thus, the dye become charged and retained inside the lysosome (Repetto et al. 2008) that provides orange-red appearance to the cells. When the cells die, the dye cannot be retained inside the cell due to reduced pH gradient (Filman et al. 1975). Due to passive diffusion and nonionic nature of neutral red at physiological pH, it enters into both live and dead cells, which is a major disadvantage of neutral red staining. To overcome this problem, trypan blue was used as a second dye because it is not absorbed by viable cells due to intact cell membrane whereas, trypan blue penetrates membrane of dead cells and provide blue color to cytoplasm. In present work, microscopic imaging of melanoma cells after double-dye staining is clearly differentiate between live and dead cells (figure 4.25A-J).

Present section is also focused on cytoprotective effect of selenium in mouse melanoma cells against TBHP. TBHP is an organic hydroperoxide that causes alteration on redox state of cell, lipid peroxidation, DNA damage and also has been used to induce *in-vitro* cellular oxidative stress (Chen and Chen 2011; Huang et al. 2005; Geetha et al. 2009). The cell viability results indicated that selenium protected the cells from TBHP induced cytotoxicity, wherein, sodium selenite and GI-digested selenium-rich mustard protein provided more protection than those of non-Se and undigested protein. GI-enzymes hydrolyze the dietary proteins into amino acids and small peptides, which can be easily absorbed by the cells when compared to intact proteins. Thus, the cytoprotective effect of GI-digested selenium-rich protein is higher (73%) than undigested selenium-rich protein (47%).

In mustard cake protein, methionine is found as major sulfur containing amino acid (Sarker et al. 2015) and due to similar chemistry of sulfur with selenium, it is expected that selenium-rich mustard proteins also contains SeMet along with methionine. Previous reports on biofortification of mustard with selenium shown that after enzymatic hydrolysis of seed or oil-free meal, SeMet was identified as major selenoamino acid (Seppanen et al. 2010; Banuelos et al. 2012).

TBHP is commonly used as a prototype for investigating the mechanism of oxidative stress inside the cell. Exposure of mammalian cells with TBHP induces as array of toxic events, such as cell necrosis, loss of membrane potential in mitochondria, changes in intracellular calcium ion homeostasis, breakdown of DNA strand, depletion in cellular ATP, lipid peroxidation and depletion of reduced glutathione and protein thiols (Aherne and O'Brien 2000; Klotz et al. 2003; Baker and He 1991; Choi et al. 1997; Sestili et al. 1999; Kucera et al. 2014) and these changes inside the cell finally lead to the cell death.

The protective role of selenium from free radicals is well documented in literature. The unregulated production of free radicals inside the cell has been related to various oxidative stress diseases (Brenneisen et al. 2005). Free radicals can cause the oxidation of low density lipoprotein (LDL), which is associated with atherosclerosis in heart disease (Zhang et al. 2007) whereas, presence of high selenium in blood as antioxidant selenoproteins and/or selenoenzymes like thioredoxin reductase (TrxR), glutathione peroxidase (GPx) and selenoprotein P play an important role in protecting the oxidation of LDL, regulate the level of plasma cholesterol, preventing the atherosclerosis and myocardial ischemia (Dhingra and Bansal 2006; Traulsen et al. 2004; World et al. 2006; Motghare et al. 2001). Many studies have indicated the role of selenium in reduction and prevention of cancer induced by variety of carcinogens, including radiation and chemical carcinogens. Supplementation of dietary selenium as selenized yeast, which is a major source of SeMet, reduced the incidence of skin, colon, lung and prostate cancer in human trials (Clark et al. 1996). The most possible mechanism of selenium as anticancer or chemopreventive agent is its integrated role in the antioxidant defense system to reduce the oxidative stress and limit DNA damage (Rayman 2005; McKenzie et al. 2002; Whanger 2004; El-Bayoumy 2001; Lu and Jiang 2005). Karunasinghe et al. (2004) reported an inverse relationship in between high blood serum selenium levels and DNA damage in blood leucocytes in men who had high prostate cancer risk. The effects of selenium on oxidative damage caused by different chemical oxidants were also studied on rodents. Selenite either alone or in combination with other antioxidants reduces the TBARS content and oxidation of heme protein in multiple organs of rat when exposed to TBHP, bromotrichloromethane ($CBrCl_3$) or due to spontaneous oxidative reactions (Leibowitz et al. 1990; Chen et al. 1993; Chen and Tappel 1995; De Mulder et al. 1995). Selenium has also been reported for the treatment of heavy metals (Cd, Hg, methylmercury, thallium and silver) poisoning and protective against chemical carcinogens

(3-methylcholanthrene, benzopyrene, 7,12-dimethylbenz (a) anthracene, aflatoxin, diethylnitrosamine, 2-acetylaminofluorene and 3-methyl-4-dimethylaminoazobenzene) that used to induce the mammary, liver and skin tumors (Whanger 1992). Selenium as chemopreventive agent for cancer is generally acted in a dose dependent manner. In present work, there was no cytotoxic effect (100% viability) observed in melanoma cells after treatment with 1 µM selenium, whereas, Song et al. (2009) reported that when melanoma cells were exposed with 1-100 µM of selenium as sodium selenite, a significant increase in apoptosis of cell was observed only above 50 µM of selenium. Thus, the present work suggests that, lower concentration of selenium (1 µM) only provide cytoprotective effect against organic peroxides but does not induces any cytotoxic/apoptotic effect.

4.6 Selenium induced glutathione peroxidase (GPx) enzyme in mouse melanoma cells

Glutathione peroxidase (GPx) is a selenoprotein and an essential component of cellular detoxification systems that protects the cells from free radical induced oxidative stress. In present section, GPx activity was demonstrated in selenium treated mouse melanoma cells. The treatments of selenium were similar as described in pervious section (4.5, A-J) but with 0.5 mM of TBHP. Exposure of cells with 0.5 mM TBHP was taken into consideration to obtained viable cells in stress, as compared to 2 mM TBHP where 100% mortality of cells were observed. After different set of treatments, cell homogenates were estimated for their GPx activity. Figure 4.26 presents the GPx activity in melanoma cells (expressed in U/mg of protein).

The obtained results indicated that GPx activities in selenium treated cells (treatment C, G and I) were significantly higher ($P<0.05$) w.r.t control (treatment A). The observed fold increase in GPx activities for C, G and I treatments were 2.51, 1.38 and 2.31 respectively when compared with control. No significant difference ($P>0.05$) was observed in GPx activity when cells were treated with non-Se mustard protein (treatment E). Gpx activities were also varies with the nature of selenium treatments. The cells treated with sodium selenite expressed significantly higher ($P<0.05$) GPx activity when compared to GI-digested and undigested selenium-rich mustard protein, and the order of GPx expression among these treatments were C > I > G.

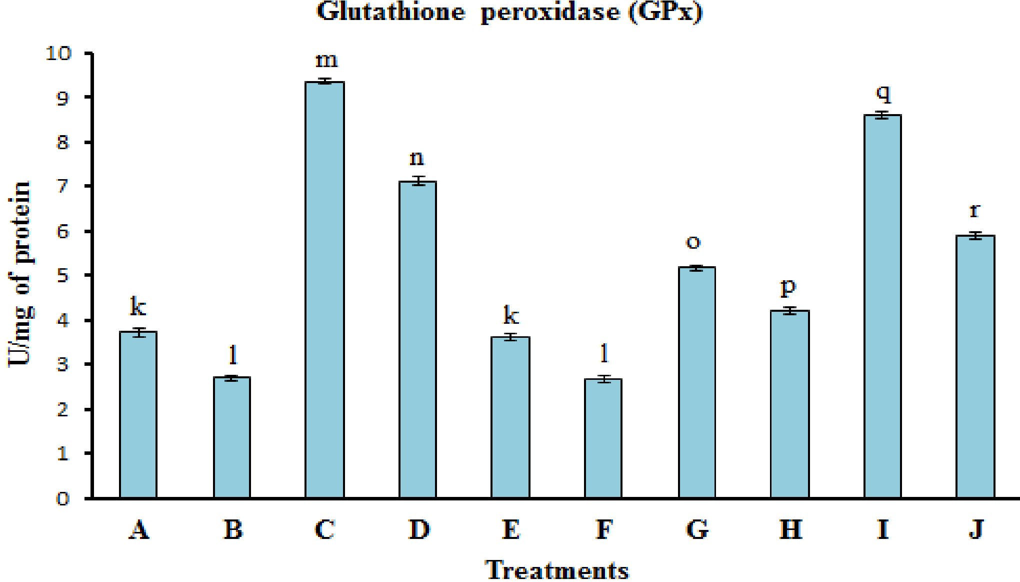


Figure 4.26: GPx activities in melanoma cells after different treatments, (n=3), different letters on top of bars indicate statistically significant differences at P<0.05 (t-test). Treatments: (A) Only culture medium (control); **(B)** 0.5 mM TBHP; **(C)** 1µM selenium as sodium selenite; **(D)** Pretreatment with 1µM selenium as sodium selenite + 0.5 mM TBHP; **(E)** GI-digested non-Se mustard protein; **(F)** Pretreatment with GI-digested non-Se mustard protein + 0.5 mM TBHP; **(G)** 1µM selenium as undigested selenium-rich mustard protein; **(H)** Pretreatment with 1µM selenium as undigested selenium-rich mustard protein + 0.5 mM TBHP; **(I)** 1µM selenium as GI-digested selenium-rich mustard protein; **(J)** Pretreatment with 1µM selenium as GI-digested selenium-rich mustard protein + 0.5 mM TBHP

Although, GPx acts as an antioxidant enzyme, in present work, its activity significantly decreased (P<0.05) with exposure of TBHP, which indicated that TBHP induces oxidative stress inside the cell and damage the cellular proteins. Selenium dependent increase in GPx activity (1.38-2.51 folds) provided as cytoprotective mechanisms to the cells when they are exposed to oxidative stress and this might be the reason for the protection of melanoma cells from higher concentration (2 mM) of TBHP as described in section 4.5.

GPx is a family of antioxidant enzyme that has the capability to scavenge and inactivate the H_2O_2 and lipid peroxides, thereby protect the cell against oxidative damage. There are eight isoenzymes of the GPx family reported in humans and out these; five GPxs (GPx1-4 and GPx6) are known to contain selenocysteine at their active site. Although, all these isoenzymes have same free radical scavenging capacity, they vary in cellular location and substrate specificity.

GPx1 is ubiquitous and found in cytoplasm of all the tissues in mammalian body, GPx2 is also cytosolic but found only in gastrointestinal tract, GPx3 is extracellular glycoprotein and occurs in plasma, GPx4 is found in mitochondria and more specific for lipid hydroperoxides produced by free radical induced damage of lipoproteins and cholesterol, GPx5 is confined to the male reproductive tissues and protect the spermatozoa from oxidative damage, GPx6 is found in Bowman's gland of the olfactory system but its function is still unknown, GPx7 and GPx8 are located in the lumen and as membrane protein of endoplasmic reticulum respectively (Arthur 2000; Brigelius-Flohe and Maiorino 2013).

GPx enzymes catalyze the reduction of hydroperoxide, including H_2O_2 and a wide variety of organic peroxides (ROOH) to the corresponding water and stable alcohols (R-OH) using cellular glutathione (GSH) as a reducing agent. The main reaction that GPx catalyzes is as follow:

$$H_2O_2 + 2\ GSH \longrightarrow 2\ H_2O + GS\text{-}SG$$

Where GSH represents monomeric reduced glutathione and GS-SG represents oxidized glutathione disulfide. The selenocysteine residue located at the active site of GPx enzyme is present in selenol form (GPx-Se-H) which participate directly in electron donation to reduce the peroxide and get oxidized as derivative of selenenic acid (GPx-Se-OH). The selenenic acid then reduced back to the selenol form by utilizing two molecules of GSH as a hydrogen donor. The simplified reaction mechanism is represented below:

$$GPx\text{-}Se\text{-}H + H_2O_2 \longrightarrow GPx\text{-}Se\text{-}OH + H_2O$$

$$GPx\text{-}Se\text{-}OH + GSH \longrightarrow GPx\text{-}Se\text{-}SG + H_2O$$

$$GPx\text{-}Se\text{-}SG + GSH \longrightarrow GS\text{-}SG + GPx\text{-}Se\text{-}H$$

To complete the cycle, GS-SG further reduced to GSH by the enzyme glutathione reductase using NADPH as hydrogen donor. From the above reaction mechanism, it is clear that selenium plays an important role at the catalytic site of enzyme and hence selenium dependent GPx significantly reduces free radical induced oxidative stress.

In present work, the growth medium used for the culture of melanoma cells are considered as selenium deficient and thus very low GPx activities were observed in control and

non-Se mustard protein treated cells whereas, selenium supplementation significantly enhanced GPx expression. Studies on selenium dependent GPx expression in mammalian cells were also carried out by many workers. Kayanoki et al. (1996) reported that under selenium deficient condition, lower dose of H_2O_2 exhibited apoptotic cell death by internucleosomal DNA fragmentation in bovine renal epithelial cell lines, when compared to selenium supplemented ones and this was due to decreased expression of GPx under selenium deficient conditions. Germain and Arneson (1977) demonstrated selenium induced GPx activity in mouse neuroblastoma cells, where supplementation of growth medium with 600 nM selenite resulted in a 30 fold increase in the enzyme activity. Baker et al. (1993) studied selenium dependent regulation of GPx in human hepatoma cell line; and reported that selenium stabilized the mRNA that coded for GPx, whereas, selenium deficiency decreased mRNA level by 60% and GPx activity by 93%.

In the case of selenium deficiency and with the resulting impaired function of GPx, the destructive H_2O_2 breaks down into even more dangerous hydroxyl radical which damage cell membranes and DNA, leading to the serious diseases. Research done on selenium proves the connection between low selenium levels and many health disorders that show low GPx levels. Selenium supplementation significantly increased plasma selenium concentration and red blood cell GPx activity in patients with chronic kidney disease (Sedighi et al. 2014). In the patients with multiple sclerosis, serum selenium level and GPx activity were found significantly low as compared to healthy population, whereas after consumption of selenium rich food, GPx activity was increased (Socha et al. 2014). Du et al. (2016) suggested that selenium intake may be beneficial in reducing Alzheimer's disease. Epidemiological study supported an inverse relationship between serum selenium concentrations and thyroid volume, risk of thyroid tissue damage and risk of goiter in people with mild iodine deficiency (Derumeaux et al. 2003; Rasmussen et al. 2011). Selenium supplementation prevented the osteoblast inflammatory stress response to bone metastatic breast cancer (Chen et al. 2009). Low selenium intake has been linked to various diseases, including cancer, cardiomyopathy, rheumatoid arthritis, atherosclerosis and viral infection including HIV-AIDS (Azoicai et al. 1997; Knekt et al. 2000; Combs 1999; Rayman 2000), where chronic inflammation forms the underlying basis of these diseases. It has been observed that, cyclooxygenase-2 (Cox-2), an inflammatory enzyme, over expressed during injury or inflammation (Smith and Langenbach 2001). Many studies have

shown the diverse molecular mechanisms involved in the anti-inflammatory properties of selenium supplementation (Vunta et al. 2008; Zamamiri-Davis et al. 2002; Gandhi et al. 2013; Prabhu et al. 2002)

In plants, selenium present mostly in the form of selenoamino acids (SeCys and SeMet) and plant based diets are the major source of selenium for humans and animals. In present work, high levels of bioaccessible selenium in mustard proteins from selenium-rich mustard cake can be a good source of selenium to enhance antioxidant activity in cellular systems.

Conclusions

- Selenium concentrations in different crop samples (wheat, rice and mustard) collected from seleniferous region were significantly higher over their respective controls.
- There was no significant loss observed in selenium in contents after processing (cooking and baking) of grains.
- Percentage bioaccessibility of selenium from wheat is significantly higher when compared to rice and wheatgrass. Similarly, the processing of the grains significantly increases the selenium bioaccessibility over their respective unprocessed/raw samples.
- Amongst the storage proteins, high levels of selenium was observed gutelin fraction followed by prolamin, albumin and globulin, whereas bioaccessibility of selenium from these protein fractions were in the order of albumin ≥ globulin > glutelin > prolamin.
- Selenium and UV-B radiation independently enhanced the different antioxidant properties whereas, selenium and UV-B light showed synergistic effect and significantly increases antioxidant status of wheatgrass.
- Selenium independently and along with UV-B light, significantly increases the levels of many free and bound phenolics.
- High selenium content was also observed in seed, cake and to certain extent in oil of mustard obtained from selenium-rich feed. Small fractions of selenocyanate/isoselenocyanate were also observed in cake.
- Protein extracted from selenium-rich mustard was observed to protect the melanoma cells from organic peroxide induced oxidative stress with significant increase in expression of GPx enzyme.

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Publications

- Jaiswal S. K., Prakash R., Acharya R., Nathaniel T. N., Reddy A. V. R., Tejo Prakash N. Bioaccessibility of selenium from Se-rich food grains of the seleniferous region of Punjab, India as analyzed by instrumental neutron activation analysis. *CyTA - Journal of Food*, **2012**, 10: 160-164.
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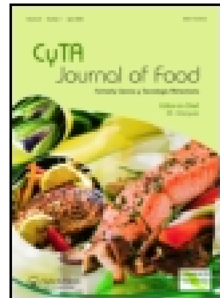

CyTA - Journal of Food
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tcyt20

Bioaccessibility of selenium from Se-rich food grains of the seleniferous region of Punjab, India as analyzed by instrumental neutron activation analysis

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Available online: 07 Feb 2012

To cite this article: Sumit K. Jaiswal, Ranjana Prakash, R. Acharya, T.N. Nathaniel, A.V.R. Reddy & N. Tejo Prakash (2012): Bioaccessibility of selenium from Se-rich food grains of the seleniferous region of Punjab, India as analyzed by instrumental neutron activation analysis, CyTA - Journal of Food, DOI:10.1080/19476337.2011.606479

To link to this article: http://dx.doi.org/10.1080/19476337.2011.606479

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CyTA – Journal of Food
2012, 1–5, i*First* article

Bioaccessibility of selenium from Se-rich food grains of the seleniferous region of Punjab, India as analyzed by instrumental neutron activation analysis

Bioaccesibilidad de selenio de cereales ricos en Se de la región selenífera de Punjab, India, analizada por análisis de activación neutrónica

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(*Received 24 March 2011; final version received 10 July 2011*)

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In the present study, selenium (Se) bioaccessibility was measured in rice and maize cultivated in seleniferous soil of India using *in vitro* gastric (GA) digestion and gastro-intestinal (GI) digestion methods. The concentration of bioaccessible Se was determined by instrumental neutron activation analysis (INAA). The total Se was observed to be about 58.0 mg kg^{-1} and 29.0 mg kg^{-1} in flours of rice and maize, respectively. Total Se content in maize flour sample after GA and GI digestions were approximately 9.5 mg kg^{-1} and 15.0 mg kg^{-1}, respectively; and in case of rice samples, the levels were about 32.0 mg kg^{-1} and 38.0 mg kg^{-1}, respectively. The results indicate that the bioaccessibility of GI digestion (51% in maize and 65% in rice) was higher compared to GA digestion (32% and 52% in maize and rice, respectively). In addition, the bioaccessible levels of Se were significantly more in case of rice compared to maize.

Keywords: selenium; cereals; *in vitro* digestion; bioaccessibility; instrumental neutron activation analysis (INAA)

En el presente estudio se midió la bioaccesibilidad del selenio en arroz y maíz cultivados en suelo selenífero en India, usando métodos de digestión gástrica *in vitro* (GA) y digestion gastrointestinal (GI). La concentración de selenio bioaccesible se determinó por análisis de activación de neutrones (INAA). El Se total observado fue 58,0 mg kg^{-1} y 29,0 mg kg^{-1} en harinas de arroz y maíz, respectivamente. El contenido total de Se en muestras de harina de maíz tras GA y GI fue de 9,5 mg kg^{-1} y 15,0 mg kg^{-1}, aproximadamente. En el caso de las muestras de arroz, los niveles fueron de alrededor de 32,0 mg kg^{-1} y 38,0 mg kg^{-1}. Los resultados muestran que la bioaccesibilidad de GI (51% en maíz y 65% en arroz) fue mayor comparada con la de GA (32% y 52% en maíz y arroz, respectivamente). Además, los niveles bioaccesibles de selenio fueron significativamente más altos para el arroz comparado con el maíz.

Palabras clave: selenio; cereales; digestión *in vitro*; bioaccesibilidad; análisis de activación de neutrones (INAA)

Introduction

Selenium (Se) is an essential element for both humans and animals. Se enters into the body as a dietary supplement through food. In humans, low dietary Se intakes are associated with Se deficiency diseases like cardiomyopathy, especially in children and women of childbearing age, which could be prevented by supplementation with sodium selenate (Virtamo & Huttunen, 1993). Se deficiencies are also linked with health disorders including oxidative stress-related conditions, reduced fertility, immune functions, and increased risk of cancers, whereas consumption of a high Se-rich diet leads to chronic selenosis and includes effect on keratinized tissues such as loss of hairs and lesions on the nails, claws, horn and skin (Kumar, 1995). Food (cereals, pulses, and vegetables) is the main source of Se. But it is also true that after digestion all mineral contents present in the diet may not be completely absorbed and utilized for physiological function of the body and storage. It depends on efficiency of the digestive system and species of the elements. For example, Se as selenomethionine (SeMet) is more easily absorbed when ingested than selenite, selenate, or selenocystine (National Academy of Science [NAS]/National Research Council [NRC], 1983). In short, bioaccessibility reflects the efficiency with which the nutrients are absorbed from the alimentary tract and it is made available for further storage and use (Fairweather-Tait, 1992).

Most of the cultivated plants possess the ability to absorb, metabolize, and store significant amounts of Se in their tissues when grown on Se-containing soil. Se phytoaccessibility is dependent on a diverse variety of soil and climatic conditions such as soil pH, redox conditions, organic matter, competing ionic species, microbial activity, level of rainfall during the growing season, etc. (Dhillon & Dhillon, 2003; Spadoni et al., 2007). Se dominantly enters the food chain through plants (Rayman, 2008). Accumulation of Se by plants is due to their ability to transform inorganic Se into a variety of organoselenium species, including bioactive compounds, which have important implications on human nutrition and health. The tolerable upper level (TUL) Se-intake for adults is 400 μg day^{-1} against the daily requirement of 40 μg (Combs, 2001; Goldhaber, 2003). In general, the apparent absorption of the organic Se compounds in

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ISSN 1947-6337 print/ISSN 1947-6345 online
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http://dx.doi.org/10.1080/19476337.2011.606479
http://www.tandfonline.com

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foods is appreciable (about 70–95%) (Combs & Combs, 1986). However, it can vary according to the digestibility of the various Se-containing food proteins and to the pattern of Se compounds present in the particular food. Bioavailability of Se is determined by either *in vivo* administration to species similar to humans, for example, murine models or *in vitro* methods by simulating digestive system (Kulkarni, Acharya, Rajurkar, & Reddy, 2007). *In vitro* methods were developed which are rapid and inexpensive (Miller, Schricker, Ramussen, & Van, 1981). The results obtained by *in vitro* methods are based on the formation of digestive products that are soluble or dialyzable (Ruby et al., 1999).

Cereals like wheat collected from the seleniferous region of Punjab, India (Nawashahr–Hoshiarpur) were earlier reported by this group to contain highest total Se (115.0 ± 2.4 mg kg^{-1}) ever recorded in wheat across the globe (Sharma et al., 2009). The wheat was observed to contain SeMet as a dominant organic form of Se (Cubadda et al., 2010).

Se content in the raw grains and the gastric (GA)/gastro-intestinal (GI) digests were estimated by instrumental neutron activation analysis (INAA). The INAA based approach has been routinely used to analyze various biological samples like medicinal leaves, cereals including wheat, rice, and maize, legumes, foodstuffs and reference materials for multielement analysis (Acharya, Nair, Reddy, & Manohar, 2002; Balaji et al., 2000a,b; Rajurkar & Pardeshi, 1997; Sharma et al., 2009). Neutron activation analysis (NAA) is an isotope specific nuclear analytical technique capable of simultaneous multielement determination in diverse matrices. This technique is capable of determining as many as 70 elements of periodic table. Elements include Na, K, Mg, Ca, V, Al, Ti, Mn, Sc, Fe, Cr, Ni, Co, Zn, Ga, Se, As, I, Mo, Zr, Sn, In, Ta, W, Hf, Au, rare earth elements (La–Lu), Th, and U. The technique involves irradiation of the samples with neutrons in a nuclear reactor, leading to the formation of radionuclide whose radioactivity is measured preferably using high-resolution gamma ray spectrometer. It has many advantageous characteristics like nondestructive, high analytical sensitivity, high selectivity, no spectral interference, good detection limit (mg/kg to μg/kg), negligible matrix effect, and many samples in one irradiation.

In the present study, we determined the bioaccessible concentrations of Se in the rice and maize collected from the seleniferous region of Punjab, India by processing the Se-rich samples by *in vitro* GA and GI digestion. Se content in the raw grains and the GA/GI digests were estimated by INAA.

Experimental

Sample collection and preparation

Harvested paddy and maize grains were collected from the seleniferous region of Punjab, India. A composite sample of grains was collected after harvest, and approximately 25 g of grains were taken, washed with distilled water and dried overnight at 40°C. Outer hull of paddy was removed using mortar and pestle but the bran layer and germ were retained and stored for further analysis. De-husked rice (semi-milled) and whole maize grains were ground into fine powder using mortar and pestle. Precautions were taken during sample preparation by cleaning the grinding apparatus with ethanol after homogenization of every sample.

Reagents

Digestive enzymes, pepsin (1:3000) extrapure and pancreatin 3X extrapure (amylase 100 U/mg, lipase 8 U/mg, protease 100 U/mg), were procured from SRL Chemicals, Mumbai. Bile salts mixture, NH_4HCO_3, HCl, and pure amorphous silica powder were purchased from SD Fine Chemicals, Mumbai. All chemicals used in the experiments were of analytical grade and used without further purification. Ashless Whatman filter papers (no. 41, 42.5 mm Ø), Whatman nitrocellulose membrane filters (0.45 μm, 25 mm Ø), and Tarsons syringe filter setup (25 mm Ø) were used. Freshly prepared gastric juice solution (6% w/v pepsin in HCl, pH 1.75) and pancreatic digestion solution (mixture of 2% w/v pancreatin and 0.2% w/v bile salts) were used in the experiments (Kulkarni et al., 2007). Saturated solution of NH_4HCO_3 was used to adjust the pH to 7. All the solutions were prepared in doubly distilled water.

Instrumentation

Radiochemistry laboratories have computer-controlled gamma ray spectrometry systems which directly provide the absolute activity of all the radionuclides present in the sample. Owing to their high energy resolution, Compton suppressed high purity geranium (HPGe) detectors have become the workhorse of any radiochemistry laboratory. An HPGe detector coupled to a PC-based multichannel analyzer (8k MCA) with a fixed sample-to-detector geometry was used for detection. The detector system had a resolution of 1.9 keV at 1332 keV of Co^{60}. With the help of these detectors, activity of several radionuclides can be measured simultaneously. The complex gamma ray spectra are analyzed by peak fitting software PHAST, developed at Bhabha Atomic Research Centre, Mumbai, India (Mukopadhyay, 2001).

Procedures

Gastric (GA) digestion

The *in vitro* GA digestion protocol was adapted from Kulkarni et al. (2007). Accurately weighed amounts (1.25 g) of rice and maize flour were transferred to 50 mL conical flasks, each containing 12.5 mL of gastric juice solution (6% w/v pepsin in HCl, pH 1.75). Initially, the mixture was shaken vigorously for 1–2 min. The flasks were then sealed tightly with a parafilm and placed in shaker-incubator set at 37°C and 150 rpm for 3 h. Each sample was digested in triplicate and these digests were then cold centrifuged (Hitachi-RX II) at 4°C for 20 min at 5000 rpm. The supernatant was filtered through Whatman nitrocellulose membrane filters (0.45 μm, 25 mm Ø). The filtered solutions were labeled and stored in an airtight container at 0–4°C for further analysis.

Gastro-intestinal (GI) digestion

For GI digestion, the pH of the each solution obtained after GA digestion were adjusted up to pH 7 by drop wise addition of a saturated solution of NH_4HCO_3. To this mixture, 10 mL of pancreatic digestion solution (mixture of 2% w/v pancreatin and 0.2% w/v bile salts) was added and shaken

vigorously for 1 min. The flasks were incubated at 37°C, 150 rpm for 4 h in shaker incubator. The digests were then centrifuged at 4°C and filtered, followed by the similar GA digest procedure. Each filtered solution was labeled and stored in airtight container at 0–4°C.

Quantification of total selenium

For total Se quantification in raw samples, approximately 100 mg of rice and maize flour were taken in triplicate and packed in thin aluminum foils. Additionally, for analytical comparison, elemental standard of Se was prepared using inductively coupled plasma spectrometry (ICP) liquid standard (Spex) containing known amount of Se (5–25 μg) fused in pure amorphous silica powder. Two reference materials, namely CRM DOLT-1 (Dogfish Liver) from National Research Council of Canada (NRCC) and SL-1 (Lake Sediment) from International Atomic Energy Agency (IAEA), were analyzed to evaluate accuracy of INAA method. The samples, the reference materials, Se standards, and silica blank were sealed appropriately and introduced into Harwell cans and co-irradiated in the self-server position of the CIRUS reactor (Bhabha Atomic Research Centre, Mumbai, India) for 7 h duration with a neutron flux of $\sim 10^{13}$ cm^{-2} s^{-1}. The samples were allowed to cool for about 9 days, and then the radioactive assays of the samples were carried out by HPGe detector for 1–10 h depending on Se concentration in samples. The peak areas were determined using peak-fit software PHAST. The peak areas were used for the calculation of the Se concentration by relative method.

Sample preparation for GA and GI digests for INAA

For Se quantification in GA and GI digests, clear supernatant of both GA and GI digestion was taken for analysis. As liquid samples are not irradiated in the nuclear reactors for INAA, 200 μ L of either GA or GI digests were carefully transferred onto a Whatman filter paper (no. 41, 42.5 mm Ø) with the help of micropipette and then air dried. This step was repeated 10 times so as to transfer a total solution of 2 mL. Each filter paper was folded three to four times to make it in square geometry and packed in thin aluminum foil. Further analysis was similar to the protocol outlined for Se quantification in grain flour using INAA.

Determination of selenium concentration

After irradiation of the samples, most of the elements become radioactive. Se^{74} (stable isotope; natural abundance, 0.87%) when interacts with reactor neutrons gets converted to a radioactive isotope Se^{75} (half life 120 days) emitting three gamma rays of different energy levels (136, 264 and 279 keV) (Figure 1). Using mass of the element in the standard ($m_{x,std}$), count rates (counts per second, cps) of standard ($cps_{x,std}$) and sample ($cps_{x,samp}$), mass of the element present in the sample ($m_{x,samp}$) was determined by the following equation:

$$m_{x,samp} = m_{x,std} \times \frac{cps_{x,samp}}{cps_{x,std}} \times \frac{D_{std}}{D_{samp}}$$

where D is the decay factor ($D = exp(-\lambda\ t_d)$), λ is the decay constant of the radioisotope produced in neutron activation, and t_d is the decay time. The $m_{x,samp}$ (μg or mg) was converted to the concentration (μg g^{-1} or mg kg^{-1}) by dividing with sample mass (g or kg).

From concentration of Se in GA and GI digests, the percentage of bioaccessibility (% B) of the Se from each grain was calculated using the following formula:

$$\%B = \frac{[GD]\ \text{or}\ [GI]}{[T]} \times 100$$

where [GD] = concentration of a Se in GA digest, [GI] = concentration of a Se in GI digest, and [T] = total Se content in the grains.

Results and discussion

A typical gamma ray spectrum generated using data obtained from HPGe detector of the gamma ray spectrometer with Compton suppressed detection system is presented in Figure 1. The figure indicates gamma rays (136, 264.7 and 279 keV) that typically signify the presence of ^{75}Se.

The Se concentrations determined in two reference materials, IAEA SL-1 and NRCC CRM DOLT-1, were 2.82 $\pm$ 0.08 mg kg^{-1} and 7.43 $\pm$ 0.18 mg kg^{-1}, respectively, as against the reported values of 2.9 mg kg^{-1} and 7.34 $\pm$ 0.42 mg kg^{-1}, respectively. The percent deviations from certified/information values are within $\pm$ 3%. The Se concentration in raw flour, GA and GI hydrolysates (digests) are given in Table 1. The uncertainties quoted in Table 1 are the standard deviations at $\pm$ 1 s confidence limits obtained from three independent sample analyses and the percentage relative standard deviations are in the range of 1.5–5%. INAA quantification of Se in cereal grains, as reported by this group (Sharma et al., 2009), was found to agree with quantification by ICP-MS in the case of wheat (Cubadda et al., 2010).

Total Se concentrations in maize and rice flour were 29.05 $\pm$ 3.5 mg kg^{-1} and 58.2 $\pm$ 5.9 mg kg^{-1}, respectively. Keeping in consideration that maize being taken as whole grain flour and rice as semi-milled (unpolished) in rural Punjab, daily consumption of 100 g of maize flour or semi-milled rice would possibly result in an approximate intake of Se from 7.5 to 15.0 fold the TUL for adults (400 μg Se day^{-1}).

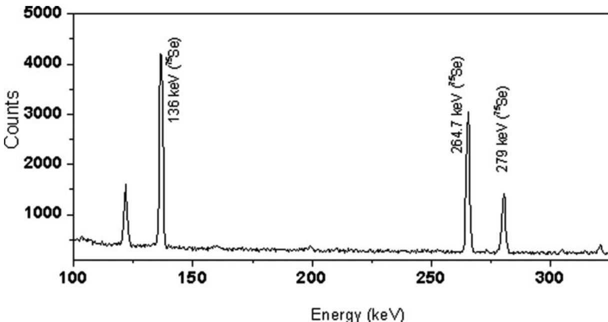

Figure 1. Typical gamma ray spectrum of selenium as acquired after irradiation, using HPGe-BGO Compton suppressed detection system.

Figura 1. Espectro típico de rayos gamma de selenio tal como se consigue después de irradiación, usando sistema de detección suprimido Compton de HPGe-BGO.

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Table 1. Selenium content in cereal flours, gastric (GA) and gastrointestinal (GI) hydroysates and their percent bioaccessibility (%B).

Tabla 1. Contenido de selenio en harinas de cereales, hidrolizados gástrico (GA) y gastrointestinal (GI) y su porcentaje de bioaccesibilidad (%B).

Sample	*n*	Se in flour (mg kg^{-1})	Se-after GA (mg kg^{-1})	Se-after GA %B	Se-after GI (mg kg^{-1})	Se-after GI %B
Maize	3	29.05 ± 3.5	09.53 ± 1.1	32	14.82 ± 2.3	51
Rice	3	58.25 ± 5.9	30.66 ± 2.8	52	38.01 ± 3.5	65

Plant foods, and especially cereal grains, are the major dietary sources of Se in most of the countries throughout the world. Mustard, mushrooms, alliums (onion and garlic), broccoli, and Brazilian nuts have the ability to accumulate Se from soil to significantly high levels (Food and Agriculture Organization [FAO]/World Health Organization[WHO], 2001). The typical levels of Se in vegetables and fruits in Portugal, New Zealand, Finland, and Mainland China range from 1–25 mg kg^{-1} (Diaz-Alarcon, Navarro-Alarcon, De La Serrana, & Lopez-Martinez, 1996; Ventura, Freitas, Pacheco, Meerten, & Wolterbeek, 2007). Presently, limited data are available on the Se levels in crop products and food grains from Indian sub-continent as estimated by nuclear analytical and other techniques. Majority of the reports indicate quantification of Se using invasive techniques such as ICP, atomic absorption spectrometry (AAS), etc. (Hira, Partal, & Dhillon, 2003).

Rice and maize are non-accumulators of Se, and Se concentration in grains rarely exceed 30 mg kg^{-1} even in seleniferous areas, such as the northern great plains of the USA (Moxon, Olson, Whitehead, Hilmoe, & White, 1943). It is worth noting that since tolerance to Se toxicity varies depending on several factors, notably soil properties and sulphate supply (Lyons, Stangoulis, & Graham, 2005; Terry, Zayed, De Souza, & Tarun, 2000) threshold, Se concentrations may not be the same in other areas with different soil conditions. However, risk characterization of dietary Se exposure based on intake calculations, using realistic consumption figures of wheat-based products, showed that the population of the seleniferous belt of the Nawanshahr–Hoshiarpur region may largely exceed the tolerable TUL for Se (Cubadda et al., 2010).

With reference to the estimates of bioaccessibility of Se, the present study indicated that the bioaccessibility obtained by GI digestion was higher than compared to GA digestion in both the investigated samples. In addition, the concentration of Se in terms of its bioaccessible levels was significantly higher in rice when compared to maize. This is due to the fact that the materials undergo pancreatic digestion in the GI tract at neutral pH resulting in higher accessibility of the bioavailable forms of the element. Pancreatin, which is a major enzymatic component, added in the GI, is a mixture of many enzymes that breaks complex nutrients into simple molecules, making various bioavailable forms of Se more bioaccessible. In the present study, the quantification of Se was not carried out in the residue or filter medium which can possibly retain some of the Se. However, the concentration of Se in the filtrates itself is significantly high drawing attention to the bioaccessible levels, despite the possible retention of Se in the residue. In addition, the percentage of extracted Se in GA (52% in case of rice and 32% in case of maize) and GI (65% in case of rice and 51% of maize) was observed to be notably lesser than the reported literature (Laura, Jorge, Juan, José, & Alfredo, 2006; Margaret, Heidi, & Mike, 2008). This might possibly be due to the type of matrix analyzed, as the high extraction of Se reported by researchers were with reference to yeast. Secondly, the variation in the relatively low active units of amylase in the pancreatin used in the present study might also be the reason for low levels of Se extraction in GA and GI hydrolysates.

The bioaccessibility of Se from numerous foods and food sources including buckwheat bran (Reeves et al., 2005),wheat (Mutanen, Koivistoinen, Morris, & Lavender, 1987), Se-enriched yeast (Smith & Picciano, 1987), and broccoli (Finley, 1998)has been studied using slope rate assay methods of Se species under *in vivo* conditions. However, majority of the studies are associated with low Se food sources, with limited studies on naturally enriched sources such as cereals. We have recently reported the bioaccessibility and the speciation of Se-rich wheat from the Seleniferous region of Punjab, India (Cubadda et al., 2010). It is to be noted that SeMet was the major Se species in the wheat grains and this compound has a lower chronic toxicity compared to other selenocompounds, such as selenocysteine ($SeCys_2$) or selenate (Se^{IV}), in animal studies (Barceloux, 1999). Although, the dominant Se form in rice and maize is assumed to be SeMet, similar to that in wheat, it is important to understand that the intake of this form of Se is multi-fold when compared to normal Se intake.

The Se concentrations of the samples investigated in this study appear to be the highest ever recorded in cereal grains for human consumption, similar to wheat reported earlier by our group (Cubadda et al., 2010; Sharma et al., 2009). These levels of intake might be leading to chronic toxic effects of Se accumulation in human through Se-rich food grains and livestock fed on rice and maize straw as fodder. As an alternative to the social, economic, and health impact caused by the selenium rich grains cultivated in the region, use of these matrices as naturally enriched Se supplements to Se deficient population, is a promising avenue worth considering. Further studies are in progress to investigate the bioaccessible species of Se in products derived from high-Se grains grown in the study area.

Acknowledgement

The authors acknowledge the research grant provided by the Board of Research in Nuclear Sciences and the lab facilities provided by reactor personnel at BARC, Mumbai.

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Food Chemistry 134 (2012) 401–404

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Short communication

Selenium content in seed, oil and oil cake of Se hyperaccumulated *Brassica juncea* (Indian mustard) cultivated in a seleniferous region of India

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ARTICLE INFO

Article history:
Received 11 November 2011
Received in revised form 6 February 2012
Accepted 22 February 2012
Available online 3 March 2012

Keywords:
Selenium
Mustard
Hyperaccumulation
Neutron activation analysis

ABSTRACT

Selenium (Se) hyperaccumulated Indian mustard (*Brassica juncea*) cultivated in a seleniferous region of India was collected and quantified for Se levels using instrumental neutron activation analysis (INAA). The seeds were subjected to oil extraction using a conventional screw extractor and Se content was estimated in seed, oil and oil cake. High uptake of selenium by the mustard seeds occurred, which was predominantly found to be retained and concentrated in the oil cake (143 ± 5.18 mg kg^{-1}) when compared to seed before extraction (110 ± 3.04 mg kg^{-1}) or oil (3.50 ± 0.66 mg L^{-1}) after extraction. In conclusion, the study envisages application of Se-rich mustard oil or cake as sources of chemotherapeutic isoselenocyanates and exploitation of their bioactivity.

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1. Introduction

We can use crop plants with an augmented capacity to accumulate minerals to aid sustainable agriculture and to improve human health through balanced mineral nutrition (Guerinot & Salt, 2001). This requires an understanding of how plants accumulate and store minerals. Hyperaccumulation is the ability of certain plants to accumulate extraordinarily high levels of metals and metalloids, even from soil containing corresponding lower concentrations (Baker & Brooks, 1989). Most plants contain only low foliar concentrations of Se, of less than 25 mg kg^{-1} dry weight, and rarely exceed 100 mg kg^{-1} dry weight even when grown on high-Se soils. They are termed non-accumulators (Bell, Parker, & Page, 1992). Certain plant species are known to accumulate Se at levels far beyond those observed in other species, even from low Se soils. Their Se levels can reach 1000–5000 mg kg^{-1} dry weight or 0.1–5.0% of plant dry weight without any signs of toxicity (Brown & Shrift, 1982; Feist & Parker, 2001). They are generally found in soils derived from Se-rich Cretaceous shale where Se concentrations can be >10 mg kg^{-1}. (Beath, Gilbert, & Eppson, 1939; Reeves & Baker, 2000). These accumulating plants can be divided into two groups: primary accumulators (hyperaccumulators) and secondary accumulators (indicator species). Hyperaccumulation of Se has been observed in plant families Asteraceae, Cruciferae, Chenopodiaceae, Lecythidaceae, Fabaceae, Rubiaceae and Schrophulariaceae. Se uptake by plants generally follows sulphate absorption mechanism, especially in Cruciferae species such as *Brassica* sp., as there is not distinct differentiation between soluble forms of selenium ions and sulphate ions (Banuelos, Mead, & Hoffman, 1993).

Selenium has been shown to be essential for life and to be toxic at levels slightly above those required for health. Indeed, dietary levels of the desired amount of Se are in a very narrow range; consumption of food containing less than 0.1 mg kg^{-1} of this element will result in its deficiency, whereas dietary levels above 1 mg kg^{-1} will lead to toxic manifestations (Wada, Kurihara, & Yamazaki, 1993). Although selenium is toxic in large doses, it is an essential micronutrient for health. Most of the *in vitro* and animal studies carried out in the last 35 years demonstrate that application or intakes of Se at supra-nutritional levels can inhibit tumorigenesis (Björkhem-Bergman et al., 2005; Ip, 1998).

Seeds of *Brassica juncea* (Indian mustard) are a good source of essential nutrients. According to the USDA National Nutrient Database, 100 g mustard seed contain approximately 28 g carbohydrate, 26 g protein, 36 g total fat, 12 g dietary fibre and 0% cholesterol. They also contain trace amount of all types of vitamins, electrolytes (Na and K) and essential minerals, like Ca, Cu, Fe, Mg, Mn, Se and Zn. Due to its high nutritional value, mustard cake (de-oiled mustard meal) is prominently used as a supplement for

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0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.02.140

animal feed. When this species is grown on Se contaminated soil, it accumulates significantly high amounts of Se in its seeds, due to its excellent hyperaccumulating potential (Banuelos et al., 1993).

In the present study we aimed to determine levels of selenium in seed, oil and cake of Indian mustard (*B. juncea*) collected from a seleniferous region of Punjab, India, using instrumental neutron activation analysis (INAA). INAA is an isotope-specific nuclear analytical technique capable of simultaneous multi-element determination in diverse matrices. This technique is capable of determining as many as 70 elements of the periodic table. The technique involves irradiation of the samples with neutrons in a nuclear reactor, leading to the formation of radionuclides, whose radioactivity is measured, preferably using a high-resolution gamma ray spectrometer. It has many advantages; it is nondestructive, has high analytical sensitivity, high selectivity, no spectral interference, good detection limits (mg kg^{-1} to μg kg^{-1}), negligible matrix effect and can analyse many samples in one irradiation. INAA has been used by our group to analyse Se in various cereals, including wheat, rice and maize (Sharma et al., 2009; Jaiswal et al., 2012). The selenium levels in these matrices, which were further validated using inductively coupled plasma-mass spectroscopy (ICP-MS), were observably the highest ever recorded in cereals for human consumption (Cubadda et al., 2010). Some of the other prominent reports on Se levels in foods and crops estimated using INAA include those in alliaceous species (Noda & Taniguchi, 1983), raw and processed meats (Diaz-Alarcon, Navarro-Alarcon, de la Serrana, & Lopez-Martinez, 1996), and fruits and vegetables (Ventura, Freitas, Pacheco, Meertan, & Wolterbeek, 2007).

2. Materials and methods

2.1. Sample collection

Seeds of Indian mustard grown in winter were collected at a site near the village of Jainpur geographically located at 32°46′ N, 74°32′ E, in the Nawanshahar-Hoshiarpur region, Punjab (India). Collected sample was manually cleaned, washed with water and air-dried for 2 days.

2.2. Extraction of oil

Approximately 10 kg of mustard seeds were used for oil extraction. Mustard seeds were subjected to a screw oil expeller. Extracted oil was stored in a lightweight, food grade plastic container and residue (mustard meal/cake) was collected and stored in a thick plastic bag. Non-seleniferous mustard seed, mustard oil and cake were used as control.

2.3. Sample preparation for INAA

For Se quantification all the samples were taken in triplicates. Approximately 50 mg of both Se-rich and normal mustard seeds were packed in thin aluminium foil. Before packing 1 g of both mustard cakes (Se rich and control) was kept at 40 °C overnight and ground to fine powder using agate mortar and pestle. Precautions were taken during sample preparation by cleaning the grinding apparatus with ethanol after homogenisation of every sample. Fifty milligrams of powdered samples were packed in aluminium foil. As liquid samples are not irradiated directly in INAA, 50 μL of either Se-rich mustard oil or normal oil were carefully fused with 20 mg of pure amorphous silica powder placed on an aluminium foil strip. All the samples were packed in a square geometry with dimensions of 1 cm.

Additionally, for analytical comparison, elemental standard of selenium was prepared using ICP liquid standard (Spex) containing known amount of Se (5–25 μg) fused in pure amorphous silica powder. Two certified reference materials (CRMs) namely RM 8436 (durum wheat flour) from National Institute of Standards and Technology (NIST) and SL-1 (lake sediment) from the International Atomic Energy Agency (IAEA) were analysed to evaluate the accuracy of the INAA method. Fifty milligrams of amorphous silica powder were also used as a negative control.

2.4. Neutron activation analysis

The samples, reference materials, Se standards and silica blank, sealed appropriately, were introduced into Harwell cans and co-irradiated in the self-server position of the CIRUS reactor (Bhabha Atomic Research Centre, Mumbai, India) for 7 h duration with a neutron flux of $\sim 10^{13}$ cm^{-2} s^{-1}. The samples were allowed to cool for about 9 days, and then the radioactive assays of the samples were carried out by high energy resolution, Compton-suppressed high-purity germanium (HPGe) detector for 1–10 h depending on Se concentration in samples. The HPGe detector is coupled to a PC-based multichannel analyser (8 k MCA) and has fixed sample-to-detector geometry. The detector system had a resolution of 1.9 keV at 1332 keV of Co^{60}. With the help of these detectors activity of several radionuclides can be measured simultaneously. The complex gamma ray spectra are analysed by peak fitting software PHAST, developed at Bhabha Atomic Research Centre, Mumbai, India (Mukopadhyay, 2001).

2.5. Determination of Se concentration

After irradiation of the samples, most of the elements become radioactive. In the case of selenium, Se^{74} (stable isotope, natural abundance 0.87%) when it interacts with reactor neutrons gets converted to a radioactive isotope Se^{75} (half-life 120 days) emitting 4 gamma rays of different energy levels (121, 136, 264 and 279 keV) (Fig. 1). Using the mass of the Se in the standard (M_{std}), count rates (counts per second, CPS) of standard (CPS_{std}) and sample (CPS_{samp}), mass of the Se present in the sample (M_{samp}) was determined by the following equation:

$$M_{samp} = M_{std} \times ([CPS_{samp} \div CPS_{std}] \times [D_{std} \div D_{samp}])$$

Where D is the decay factor ($D = exp(-\lambda t_d)$), λ is the decay constant of the radioisotope produced in neutron activation and t_d is the decay time (time in between end of irradiation and start of counting). The M_{samp} (μg or mg) was converted to the concentration (μg g^{-1} or mg kg^{-1}) by dividing by the sample mass (g or kg).

3. Results and discussion

A typical gamma-ray spectrum generated for mustard-based matrix, using data obtained from HPGe detector of the gamma ray spectrometer with Compton-suppressed detection system is presented in Fig. 1. The figure indicates gamma rays (121, 136, 264.7 and 279 keV) that typically signify the presence of Se^{75}. The accuracy of INAA method for Se determination as assessed through CRM analysis was satisfactory (Table 1). The values obtained for IAEA SL-1 and NIST 8436 respectively were 2.47 ± 0.4 mg kg^{-1} and 1.26 ± 0.03 mg kg^{-1}, which were comparable with the certified values, i.e., 2.85 mg kg^{-1} and 1.23 ± 0.09 mg kg^{-1}, respectively.

The selenium concentrations in the samples are given in Table 1. The uncertainties (variances) quoted in Table 1 are the standard deviations at ±1 s confidence limits obtained from three independent sample analyses and the % relative standard deviations are in the range of 1.5–5.0%.

Total Se concentration in the normal and Se-rich mustard seeds were 0.48 ± 0.02 mg kg^{-1} and 110 ± 3.04 mg kg^{-1}, respectively,

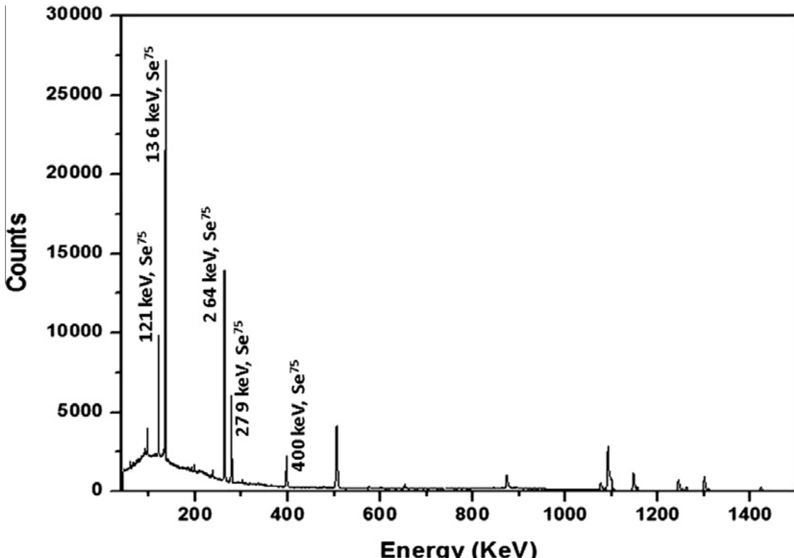

Fig. 1. Typical gamma ray spectrum of selenium as acquired after irradiation, using high purity germanium-bismuth germanate (HPGe-BGO) Compton suppressed detection system.

Table 1
Concentration of selenium in mustard seed, and oil and oil cake after extraction.

Sample	n	Se concentrations ($mg\ kg^{-1}/mg\ L^{-1*}$)	
		Normal	Se-rich
Soil	3	1.08 ± 0.23	6.5 ± 0.3
Mustard seed	3	0.48 ± 0.02	110 ± 3.04
Mustard cake	3	0.72 ± 0.14	143 ± 5.18
Mustard oil	3	BDL	3.50 ± 0.66*
Standard		Certified values	Obtained values
IAEA SL-1	2	2.85	2.47 + 0.4
NIST	2	1.23 + 0.09	1.26 + 0.03

BDL: Below detection limits.

whereas Se levels in the normal and Se rich mustard cake were found to be $0.72 \pm 0.14\ mg\ kg^{-1}$ and $143 \pm 5.18\ mg\ kg^{-1}$, respectively. Very small concentrations of Se ($3.50 \pm 0.66\ mg\ L^{-1}$) were present in the mustard oil extracted from Se-rich seeds, while levels of Se in the oil extracted from the normal mustard seeds were found to be below the detection limit of the HPGe detector. Indian mustard plants accumulate large amounts of organic selenium as noted in the present study and also supported by other observations, indicating rapid translocation of selenium from root to shoot, especially in the presence of selenium as selenate (de Souza et al., 1998; de Souza, Pickering, Walla, & Terry, 2002). Once selenium is taken up by plant roots in a particular chemical form, whether organic or inorganic, it undergoes certain metabolic changes that determine the final product as well as its translocation and accumulation in different plant tissues (Zayed, Lytle, & Terry, 1998). A study on comparison of Se levels in different plant organs of hyperaccumulator plants, including those of *Brassica* sp., suggest that Se is actively transported through the plant in favourable growth conditions (Galeas, Zhang, Freeman, Wagner, & Pilon-Smits, 2007). As the leaves mature their Se levels decline which could be a result of dilution as the leaves expand, and also due to export to reproductive tissues, such as seeds, which contain some of the highest levels in the entire plant (Galeas et al., 2007). These observations support the findings in the present study that Se levels were significantly high in seeds.

In the perspective of biological application, although species belonging to Cruciferae are not cyanogenic, they produce thiocyanates, as a by-product of glucosinolate hydrolysis (Angus, Gardner, Kirkegaard, & Desmarchelier, 1994; Brown & Morra, 1996; De Souza et al., 2002; Fenwick, Heaney, & Mullin, 1983). Brassicas normally produce a high concentration of glucosinolates, which through various studies have shown a significant inverse association with the intake of total brassica consumption, and risk of cancer at various sites (Irion, 1999; Navarro, Li, & Lampe, 2011; Verhoeven, Godbohm, van Poppel, Verhagen, & van der Brandt, 1996). In theory, the selenium-containing phytochemicals produced in selenium hyperaccumulating *B. juncea*, might include selenium analogues of isothiocyanates (isoselenocyanates), that actually possess more anti-carcinogenic potential than the normal sulphur-containing products of glucosinolates. Isothiocyanates, although providing protection against murine tumorigenesis, have low chemotherapeutic potency on melanoma cells, requiring high concentrations for therapeutic efficacy (Kassahum, Davis, Hu, Martin, & Baillie, 1997).

Recent findings on synthesis and activity of selenium analogues of isothiocyanates, namely isoselenocyanates, showed improved therapeutic efficacy of these compounds towards killing cultured melanoma cells or inhibiting tumour development in animals when administered systemically (Schlobe, Holze, Richter, & Tricker, 2002). Isoselenocyanates also inhibit melanocytic lesion development in synthetic and mouse skin, by decreasing Akt3 signalling to trigger apoptosis (Nguyen et al., 2011). In addition, mice supplemented with isoselenocyanates exhibited decreased activity of cytochrome P450 and lower DNA adducts formation (Crampsie et al., 2011).

The present study is significant in terms of demonstrating selenium hyperaccumulation by *B. juncea* cultivated in a seleniferous region of India in the context of its use as a cooking oil by local communities and the use of oil cake as fodder mix for livestock. Furthermore, with isoselenocyanates representing a promising adjunct to currently available chemopreventive agents, Se-hyperaccumulated *Brassica* sp. could provide possible natural sources of these compounds for therapeutic applications. Se elemental, speciation and nutritive analysis is now underway to test the bioactivity of Se-rich natural extracts from these matrices.

Acknowledgment

The authors acknowledge the research grant provided by Board of Research in Nuclear Sciences, Government of India to carry out the present studies.

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Acta Alimentaria, Vol. 44 (2), pp. 235–241 (2015)
DOI: 10.1556/AAlim.2014.0007

SELENIUM IN STORAGE PROTEINS OF WHEAT CULTIVATED ON SELENIUM IMPACTED SOILS OF PUNJAB, INDIA

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(Received: 23 February 2013; accepted: 14 July 2013)

Wheat, an important staple cereal crop cultivated in seleniferous region of India, noted to accumulated significantly high concentrations of Se, was examined for the distribution of selenium in various protein fractions of the grains. Amongst the protein fractions, Se was dominantly (33–37%) present in the albumin fraction in Se rich grains followed by other fractions viz., globulin (20–25%), glutelin (20–25%), and prolamin (17–20%). The observations are important in context of exploring the use of this material as functional foods in formulating Se-enriched diets for Se-deficient population.

Keywords: selenium, wheat, proteins, fraction

Selenium (Se) is an essential micronutrient for humans and animals, involved as selenocysteine (SeCys) in functioning at the catalytic centre of several selenoproteins, such as glutathione peroxidases (GPx), thioredoxin reductase (TRx), and iodothyronine-deiodinases (DIs) (Rayman, 2002). As a constituent in different selenium containing enzymes and proteins, Se performs various functions, such as protection of body tissues against oxidative stress, immune function, reproduction, and modulation of growth and development (Fairweather-Tait et al., 2011). With the growing importance and increasing evidence of the role of Se, countries such as Finland and UK (Broadley et al., 2006) have been involved in agronomic biofortification of selenium during cultivation of wheat. Studies also indicate that inter-species variations play a significant role in the genetic and agronomic fortification of Se in wheat for consumer diets (Bóna et al., 2008).

Se has no known physiological function in plants (Sors et al., 2005) and is metabolized via the S-assimilation pathway, which involves biosynthesis of selenoamino acids, i.e., SeCys and selenomethionine (SeMet), and other seleno-compounds, such as Se-methylselenocysteine (MeSeCys), g-glutamyl-Se-methylselenocysteine (GGMeSeCys), and selenocystothioneine, which, however, are non-specifically incorporated into proteins in place of cysteine and methionine, respectively (Aureli et al., 2013).

Because of the narrow range existing between Se deficiency and toxicity, and since plants can accumulate huge amounts of Se, this metalloid can be toxic to humans and livestock. Human toxicity from dietary exposure to Se is rare and it was only reported in seleniferous areas, e.g., the Enshi District (China) and Nawanshahr Region (India), where the population heavily relies upon local produce for their food (Fordyce et al., 2000; Hira et al., 2004).

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0139–3006/$ 20.00 © 2014 *Akadémiai Kiadó, Budapest*

Among the few locations in the world, seleniferous areas have been identified in certain states in India (Dhillon & Dhillon, 2003). The largest one (>1000 ha) is the seleniferous belt of the District Nawanshahr (31°13' N, 76°21' E, Punjab, India) with significantly high Se concentrations being reported in locally grown crops (Sharma et al., 2009). The quantification and speciation studies carried out by our group indicated that wheat, cultivated in this region, is able to accumulate Se at concentrations up to 146 $\mu g\ g^{-1}$ in vegetative tissues and 185 $\mu g\ g^{-1}$ in grain, with selenomethionine (SeMet) constituting dominant fraction (>75%) followed by selenite and selenocystine (Aureli et al., 2013).

Based on the observations on significantly high uptake of Se by wheat cultivated in the region, we attempted to study the distribution of Se amongst the dominant storage proteins, like albumins, glutelins, globulins, and prolamins.

1. Materials and methods

1.1. Chemicals

The analytical chemicals such as hexane (solvent grade), sodium chloride (NaCl), sodium hydroxide (NaOH), ammonium sulphate ($(NH_4)_2SO_4$), hydroxylamine hydrochloride ($NH_2OH.HCl$), nitric acid (HNO_3), perchloric acid ($HClO_4$), and hydrochloric acid (HCl) were procured from SD Fine Chemicals, Mumbai, India. Formic acid, absolute ethanol, and cyclohexane were obtained from Lobachem, Mumbai, India. Na_2EDTA and 2,3-diaminonaphthalene (DAN) were purchased from Himedia, Mumbai, India. All chemicals used in the experiments were of analytical grade and used without further purification except DAN.

1.2. Samples

Samples of wheat (*Triticum aestivum*, cv. PBW343) were collected at sites near the villages of Jainpur (high Se impact) and Barwa (moderate Se impact) geographically located at 31°13' N, 76°21' E, in the Nawanshahr-Hoshiarpur region of Punjab (India). The impact of Se in soils and crops was considered based on the report by Dhillon and Dhillon (2003). Samples from Patiala district (30°34' N, 76°38' E), which is located about 75 miles away from the seleniferous region, were collected to represent non-seleniferous region. Collected samples were manually cleaned, washed with water, and air-dried for 2 days. Sample handling was carried out in a clean room under a laminar flow chamber. Whole wheat grains were grounded into fine powder using Warner electric grinder impregnated with stainless steel blade. Precautions were taken during sample preparation by cleaning of the grinding apparatus with ethanol after homogenization of every sample. Each wholemeal wheat flour sample was passed through a 0.5 mm sieve to make homogenous fine powder. About 50 g of different wheat flour samples were transferred to 250 ml conical flask containing 100 ml hexane and defatted samples were dried under laminar hood at room temperature for 24 h.

1.3. Preparation of DAN solution

Diaminonaphthalene (DAN) solution was prepared by dissolving 0.1 g of DAN into 100 ml of 0.1 N HCl containing 0.5 g of $NH_4OH.HCl$ in an amber bottle and kept at 50 °C for 30 min in water bath. On cooling, solution was extracted twice with 20 ml cyclohexane to remove impurities. The aqueous layer was then separated and stored at 4 °C in a brown bottle.

1.4. Protein fractionation

Different protein fractions were extracted from defatted wheat flour on the basis of their solubility at 25 °C with distilled water (for albumin), 5% NaCl (for globulin), 0.1 M NaOH (for glutelin), and 70% ethanol (for prolamin) following the procedure given by Ju and co-workers (2001).

1.5. Determination of selenium

Selenium was determined in defatted wheat flour, starch residue (residue left after extraction), and all dry protein fractions using fluorescence spectrometric method (Levesque & Vendette, 1971). This method involved digestion with HNO_3 and $HClO_4$, reduction of selenium from Se^{+6} to Se^{+4} with HCl, complexing of Se^{+4} with DAN to form piazselenol and extraction of the piazselenol in cyclohexane. The cyclohexane layer was washed with 5 ml 0.1 N HCl. The emission spectrum of piazselenol complex formed during the reaction was measured using fluorescence spectrometer (Perkin Elmer LS-45, USA) at excitation and emission wavelength of 360 and 520 nm, respectively. Se quantification in each sample was carried out by relative method using emission spectrum of NIST certified Se-ICP standard solution (SRM-1349). The results are expressed as mean ± standard deviation.

2. Results and discussion

The storage protein fractions of cereals such as wheat contain four different species of proteins, that is albumins, glutelins, globulins, and prolamins, and are generally called as ‘Osborne’ fractions. Seed storage proteins are generally accumulated in significant quantities in developing seed and generally constitute the major source of nitrogen during germination and early stages of seedling growth (Higgins, 1984). In cereals, the albumins and globulins are concentrated in the seed coats, the aleurone cells, and the germ, with somewhat lower concentrations in the mealy endosperm (Sramkova et al., 2009). The albumin and globulin fraction cover about 25% of the total grain proteins (Belderok et al., 2000). Most of the physiologically active proteins are found in the albumin and globulin groups. Glutelins, along with gliadins, cover about 75% of the total protein content. They are mainly located in the mealy endosperm (Sramkova et al., 2009).

Proteins were extracted and separated from the Se-rich wheat as described earlier and the overall distribution of Se found in different protein and non-protein residual fractions is presented in Table 1.

The majority of Se was found in protein fractions when compared to the non-protein residue. There was significant difference in the selenium levels in fractions separated from the wheat of the three different sites with varying environmental selenium levels, indicating the variations in selenium accumulation pattern. Although, glutelins comprised the dominant group of proteins (65–70%) in wheat grain, the selenium content with reference to protein fraction was more prominent in albumins, which comprised 38–40% of the protein fraction followed by glutelins, globulins (25–30%), and prolamins (25–30%) (Fig. 1).

Present observations also reveal that Se was mostly stored (>77%) in wheat protein with a small portion being retained in the non-protein fraction, such as carbohydrate, lipid, and fibre complexes of wheat.

Table 1. Se content in whole grain, protein and non-proteinous residues of wheat collected from regions varying in environmental selenium levels.

Wheat source (field)	Se content (μg g^{-1})					
	Wheat flour	Non-protein residue after extraction	Protein fractions			
			Albumin	Globulin	Glutelin	Prolamin
Highly seleniferous	46.29±2.20	13.36±0.21	233.9±5.80	149.5±4.90	197.9±3.10	122.6±2.00
Moderately seleniferous	12.20±0.12	3.43±0.10	94.05±3.30	50.22±1.40	49.04±4.10	56.23±1.40
Non-seleniferous	1.86±0.20	BDL	13.67±0.70	12.27±0.40	15.68±1.50	10.21±1.60

The data is mean of three determinations ± standard deviation. BDL represents below detection limit.

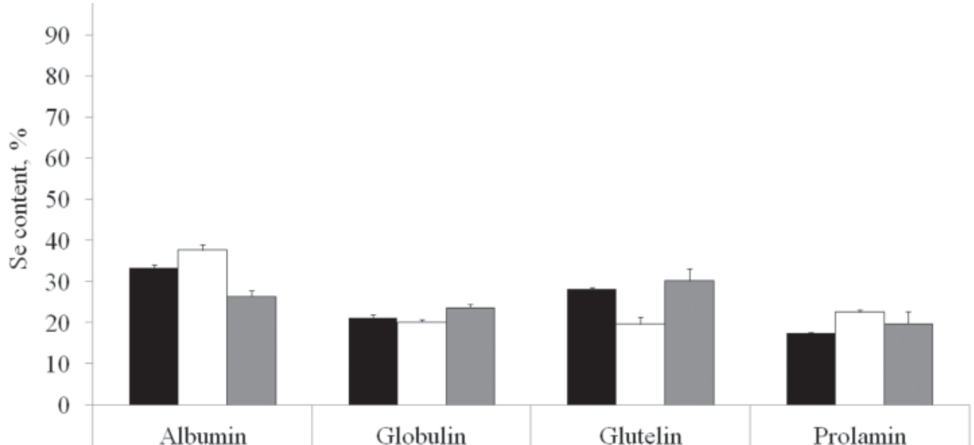

Fig. 1. Selenium content in wheat collected from three different fields with varying impact of selenium ■: Wheat from Se-rich fields; □: Wheat from moderately Se-impacted fields; and ■: Wheat from non-seleniferous fields. The data (n=3) is depicted in terms of percentage (mean±SD)

Our recent observations indicated that Se in wheat is distributed in approximately 1:2 ratio between the water-soluble fraction containing small molecular weight metabolites and the water insoluble fractions (AURELI et al., 2013). However, the speciation in water insoluble fractions has been a very difficult task (BIANGA et al., 2013). Fractionation scheme, carried out by FANG and co-workers (2010), showed that the largest Se content was found in the glutelin fraction of rice grains, which is observably different from the present study on wheat. Recent report by BIANGA and co-workers (2013) demonstrated an approach to describe family of storage proteins belonging to glutenin group that notably accumulate selenium. The study also showed evidence for the substitution of sulphur by selenium in cysteine. The Se concentration in different protein fractions seems to represent abundance of methionine (Met) and cysteine (Cys) in each fraction, since selenocysteine (SeCys) and selenomethionine (SeMet) are non-specifically incorporated into proteins in place of Cys and Met, respectively. Protein-bound SeMet is the predominant Se type in wheat grain and typically accounts for 70–85% of the Se present (STADLOBER et al., 2001). Our previous studies showed organoselenium compounds other than SeMet accounting up to 18–22% of the total

chromatographed Se (Cubadda et al., 2010). In addition, observations on bioaccessibility of Se-rich wheat grains showed SeMet having high bioavailability (>90%) during in vitro gastric and gastro-intestinal digestion as well as simulated human intestinal microbial environment (Diaz-Bone et al., 2010), indicating that high Se in wheat grain, therefore, has high bioavailability. In wheat grain, Se is mostly protein-bound and more evenly distributed throughout the kernel than other minerals, thus little Se is removed in the milling process and the concentration in flour is usually 80–90% of that in grain (Lyons et al., 2005; Cubadda et al., 2009).

Wheat produced in Western Europe contains only 1/10th of the selenium that is present in wheat grown in North America (Combs & Gray, 1998). For example, the mean value of Se in wheat sampled in UK is 27 μg Se kg^{-1} compared to 370 μg Se kg^{-1} in the USA (Wolnik et al., 1983; Adams et al., 2002). To counter the decreased intake of selenium in diet, one of the important strategies followed was to fertilize crops with selenium, as was practiced in Finland and in the UK (Broadley et al., 2006) to a certain extent. Another important paradigm that has been emerging in the recent past is the use of cereal grains cultivated in Se-rich soils that are naturally enriched with selenium and containing unique selenium compounds, as nutritional or supra-nutritional supplements (Kokarnig et al., 2011).

Cereal grains provide a significant portion of protein for the nutrition of humans and livestock. In addition to nutritional importance, cereal seed proteins also influence the utilization of the grain in food processing, especially in case of wheat, which is largely consumed after processing. Storage proteins account to about 50% of the total protein in mature cereal grains and have important impact on their nutritional quality and on their functional properties. In addition to their nutritional importance, wheat proteins also influence the utilization of the grain in food processing (Shewry & Halford, 2002). Therefore, the use of Se rich wheat for human supplementation requires a better understanding of the uptake and accumulation of Se in various protein fractions.

3. Conclusions

Keeping this in view, the present study describes the distribution profile of selenium in storage proteins, that is albumins, globulins, prolamins, and glutelins, in wheat collected from the seleniferous fields in comparison to those cultivated in non-seleniferous fields. The primary goal of the on-going studies on cereals cultivated in the said region is to explore the opportunities of using locally grown grains for fortification of low-Se grain batches or production of naturally enriched products as Se supplements for human and animal nutrition. Understanding the selenium fractionation in the storage proteins as carried out in this study opens an insight on biological mechanisms that determine their trafficking and deposition in the grain and lead to future attempts to improve the end use of these dietary sources of selenium toward nutritional supplementation and food fortification.

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The authors acknowledge the funding provided by BRNS, Government of India for the present work carried out on selenium quantification and speciation.

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