Non-enzymatic antioxidants in potato *(Solanum tuberosum* L.*)*: Determination of polyphenol and flavonoid contents in different tissues

A
Dissertation
Submitted in the partial fulfilment of the requirement for the award of degree of
Master of Science
In
Biochemistry

THAPAR INSTITUTE
OF ENGINEERING & TECHNOLOGY
(Deemed to be University)

Submitted by:
Navpreet Kaur
(Regd. No. 301607006)

Under the guidance of
Dr. N. Das
Professor

School of Chemistry & Biochemistry
Thapar Institute of Engineering & Technology, Patiala

June 2018

Candidate's declaration

I, hereby declare that the work which is being presented in this entitled "**Non-enzymatic antioxidants in potato (*Solanum tuberosum* L.)**: **Determination of polyphenol and flavonoid contents in different tissues**" in the partial fulfillment of the requirement for the reward of degree of Master in Science in Biochemistry, Thapar Institute of Engineering & Technology, Patiala, is an original record of my own research work carried out under the guidance and supervisor **Dr. N. Das**, professor, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, India. The content in the dissertation has not been submitted to any other university or institute for award of any other degree.

Date: 23 August, 2018
Place: TIET, Patiala

(Navpreet Kaur)

Certificate

This is to certify that the dissertation entitled "**Non-enzymatic antioxidants in potato (*Solanum tuberosum* L.): Determination of polyphenol and flavonoid contents in different tissues**" submitted by **Navpreet Kaur** (Regd. No. 301607006) in partial fulfillment of the requirement for the award of the degree of Master in Science in Biochemistry, to Thapar Institute of Engineering & Technology is a record of student's own work carried out by her under my guidance and supervision. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

(Dr. N. Das)
Professor
Department of Biotechnology
Thapar Institute of Engineering & Technology
Patiala

(Navpreet Kaur)

Acknowledgement

I am grateful to each and every one who has helped me throughout the entire project for its successful completion. First of all thanks to Almighty for giving me strength and support so that the project could be completed peacefully.

I find myself privileged to acknowledge my guide **Dr. N. Das, Professor**, DBT, Thapar Institute of Engineering & Technology, Patiala, Punjab for his guidance, kindness, motivation and splendid supervision during my work. I express my heartfelt thanks for his patient support and excellent advice. It was his constant encouragement, constructive criticism and ability to handle the obstacles has helped me to gain a lot from him during this period.

I would like to express my sincere thanks to **Dr. Amjad Ali**, HOD, School of Chemistry & Biochemistry, Thapar Institute of Engineering & Technology, Patiala for giving me this opportunity to do this project. A special thanks to all the faculty members for their support and invaluable suggestions throughout the project.

I owe my gratitude to **Ms. Gurpreet Kaur** and **Mr. Rajneesh Verma** for their continuous efforts and support, without whom it would have been difficult to complete the project.

I am thankful to the help rendered by the non-teaching staff and the lab attendants. I would like to thank **Mr. Babban Yadav, Mr. Lallan Yadav** and **Mr. Surinder Kumar.**

With my heart, I specially thank my parents, **Mr. Balkinder Kumar** and **Mrs. Ramna Kumari** for their affection and faith. Also I would like to thank my close friend **Rajat Kalia** for his motivation and **Jaspreet Kaur, Riya Gupta** and **Kritika Sharma** who have stood by my side during the tough times.

The whole credit goes to all the people who had their unshakeable faith in me which has always motivated me.

(Navpreet Kaur)

Table of Contents

List of Tables

List of Figures

List of abbreviation

Abbreviation	Name
ROS	Reactive oxygen species
$O_2^{.-}$	Superoxide radical
$OH^{.}$	Hydroxyl radical
$^1O_2$	Singlet Oxygen
H_2O_2	Hydrogen Peroxide
PUFA	Polyunsaturated fatty acid
LPO	Lipid peroxidation
SOD	Superoxide Dismutase
CAT	Catalase
APX	Ascorbate peroxidase
GPX	Guaiacol peroxidase
MDHAR	Monodehydroascorbate reductase
DHAR	Dehydroascorbate reductase
AA	Ascorbic Acid
GSH	Reduced glutathione
GR	Glutathione Reductase
FRAP	Ferric reducing antioxidant potential
DPPH	2,2-Diphenyl-1-picrylhydrazyl
CUPRAC	Cupric reducing antioxidant power
ORAC	Oxygen radical absorbance capacity
TPTZ	2,4,6-tripyridyl-s-triazine
CS-1	Kufri Chipsona-1
CS-2	Kufri Chipsona-2
PR	Kufri Pukhraj
KJ	Kufri Jyoti
KCM	Kufri Chandramukhi
DE	Desiree
PPE	Polyphenolic extract
GA	Gallic acid
TPC	Total Phenolic content
PBMC	Peripheral blood mononuclear cells
MTT	3-(4,5-dimethylthiazolyl-2)- 2,5-diphenyltetrazolium bromide
Na_2Co_3	Sodium bicarbonate
$NaNO_2$	Sodium Nitrite
$AlCl_3$	Aluminium Chloride
NaOH	Sodium Hydroxide
$FeCl_3$	Iron Chloride
kDa	Kilo-dalton
nm	Nanometer
mL	Millilitre
mg	Milligram
g	Gram
mM	Millimolar
M	Molar
µg	Microgram

Abstract

Potato is the fourth agronomically important food crop. Various biotic and abiotic stresses are known to affect plant growth and development, crop productivity and the quality of produce during storage. Antioxidants are the secondary metabolite of plants that help to cope up with stress conditions by scavenging the reactive oxygen species (ROS). Potato is one of the polynutrient crops having high amount of antioxidants, vitamins, starch and proteins which are beneficial for human health. In mature tuber, polyphenols, flavonoids and Vitamin C are present in considerable amount. Extent of assismilation of these vital nutrients differ in the the different cultivars varying in the genetic makeup. This study focused on the estimation and comparison of phenolics and flavonoids. Antioxidant capacity was also measured in the tubers and other tissues of different potato cultivars by ferric reducing antioxidant potential (FRAP). The phenolic content was highest in mature leaf tissue of cultivar Kufri Chipsona-2 (14241.3µg GAE. g^{-1} FW) and was lowest in the growing tubers of Kufri Jyoti (569.83µg GAE. g^{-1} FW). The flavonoid content was also found to be highest in mature leaf of Kufri Chipsona-2 (31000 µg quercitin eq. g^{-1} FW) and was lowest in Kufri Chandramukhi (616.6 µg quercitin eq. g^{-1} FW). The antioxidant capacity was observed to be highest in leaf Kufri Chipsona-2 (22685.2 µg ascorbate eq. g^{-1} FW) and was lowest in mature tissue of Kufri Jyoti (17.6 µg ascorbate eq. g^{-1} FW). The total phenolic content was observed to be increase from the 0 day of storage (DOS) to the 60 DOS. The highest increase was observed in the case of storage at room temperature. Same trend was followed in the case of flavanoid content. Polyphenolic extract of Kufri Jyoti was found to have highest cytotoxic effect on the cells.

Keywords: Potato (*Solanum tuberosum* L.) cultivars, Non-enzymatic antioxidants, Polyphenols, Flavonoids, Antioxidant capacity, Cytotoxicity

Chapter -1: Introduction

1.1 About Potato

Potato (*Solanum tuberosum* L.) is the most important non-grain food crop in many countries worldwide including Indian subcontinent. It comes on fourth position after rice, wheat and maize (Kumar et al., 2013). This crop consists of essential amino acids, vitamins and minerals and plays an important role in food nutrition. The nutritional qualities present in potatoes are ascorbic acid (up to 42 mg/100 g), potassium (up to 693.8 mg/100 g), dietary fibers (up to 3.3%) and lesser amount of protein (0.85% - 4.2%). In 17^{th} century, potato crop first come to India either through British missionaries or Portugese traders (Averyanov and Lapikova, 1989). India ranks third position in term of area and on second position in term of its production, next to China. Potato was originated in the highlands of the Peruvian Andes-mountains around 8000 years ago on the border between Bolivia and Peru (Bachem et al., 2000). The crop grows nice in cool climates, with greater temperature foliar development over tuberization (Haverkort, 1990). It is not just frost tolerant and will likely to be killed at temperature of 3 ^{0}C or below (Li, 1977). It might grow in a range of soil forms, but it is sensitive to drought stress and for this reason it is most effective be cultivated at the place where sufficient rainfall or potential to irrigate is present (Bohl and Johnson, 2010; Haverkort, 1990). Variation in tolerance to frost and drought arise inside the species. For this reason, cultivars had been chosen with higher adaptation to these stresses. The harvest of the potato crop in India is mainly done during the month of February and March. The suitable temperature for the growth of potato crop is 7 - 30 ºC. Hot temperature lead to decrease the number of tubers per plant. The crop also requires well drained fertile soil high in organic content with pH of 5.0 to 5.5. For year-round supply of potato crop to consumers and markets, they are being stored in cold storage (Marwaha et al., 2010). In order to slow down its metabolism and to prevent its degradation, they are stored to chilling temperature (usually <10 ^{o}C) that maintain accumulation of sucrose with reducing sugar glucose and fructose and slow degradation of starch. This phenomenon is known as cold-induced sweetening (Burton, 1969).

Potato is the member of *Solanaceae* family that include tomato, eggplant, chill pepper, tobacco and petunias. It grows upto 1 m and is erect, perennial and aromatic herb with tuber bearing stolon. Potato plant produces tuber from underground portion of stem and develop fibrous root system. It is annually vegetatively propagated plant. It bears flowers of white, pink, purple or blue color with yellow anther of about 2.5 cm in diameter. The fruit of potato crop grow upto 4 cm in diameter and spherical in shape but not edible. There are many varieties of potato which differ in tuber shape, size and skin color (Dennison and Loveria, 1997). On the outside of the tuber are auxiliary buds with scars of scale leaves which are called eyes (Struik, 2007). When tubers are planted, the eyes turn into stems to form the vegetative iteration.

Fig. 1 Schematic view of potato plant (Ref: google search)

1.2 Biotic and abiotic stresses in plants

Plant undergoes various environmental stresses affecting their growth and development and hence, decreasing their agricultural productivity. These environmental stresses are categorized as biotic and abiotic stresses. Biotic stresses are due to living organisms such as bacteria, nematodes, herbivores and fungi. It leads to major loss of yield due to diseases caused by pathogen (Flint et al.,1967). On the other hand, abiotic stresses consist of atmospheric or environmental factors such as flood, salinity, drought, heavy metals, radiation, extreme of temperature, etc. It is leading factor that cause the major loss worldwide. They are responsible for many other terrestrial losses like shortage of water resources, environmental pollution and salinity of soil or water. Rather than being escaped from these problems, Plant need to survive in that environment to complete their life cycle successfully by developing various mechanisms such as various transcriptional changes was done to make the plant tolerate against various diseases and their signalling pathway that play a connecting role between the stresses of environment and appropriate response to overcome these stresses (Chen et al., 1976). There are accumulation of various metabolites that are used to relieve stresses of environment such as accumulation of callose (Kim et al., 2011).

1.3 Reactive oxygen species (ROS): site of production and their effects

Plant have to go through various stresses like soil salinity by excess of sodium, potassium and chloride ion, air pollution by gases like O_2, SO_2, CO, NO_2 and drought by water deficit conditions, heavy metals like aluminium, cadmium, lead, copper, gold and by pathogen attack and extreme temperature. These stresses affect on their growth, development and response to stimuli (Morelli et al., 2003). It was estimated that consumption of around 1-2 % oxygen lead to formation of ROS. Reactive oxygen species (ROS) are toxic by-products of aerobic metabolism such as respiration and photosynthesis. But after some years, it was considered important for signalling in plant especially for biotic and abiotic environment signalling (Foyer and Noctor, 2005). They are produced in normal as well as in stress condition. ROS comprises of free radicals such as $O_2^{\cdot -}$ (Superoxide radical), $^{\cdot}OH$ (Hydroxyl radical) and non-radicals like $^1O_2$ (Singlet Oxygen), H_2O_2 (Hydrogen Peroxide). The site of generation for ROS is mitochondria, peroxisomes and chloroplast but there are also secondary sites such as cell wall, endoplasmic reticulum, cell membrane, apoplast. Chloroplast and peroxisomes are the major source of producer in presence of light whereas mitochondria are the major producer during dark. The production and elimination of ROS should be balanced because they are very deadly and cause damage to proteins, lipids and DNA (Apel and Hirt, 2004; Foyer and Noctor, 2005).

Lipids are the important component to adapt in stressful environment because during stressful condition the level of ROS increased than normal condition and it affects the phospholipid which consists of double bond of carbon atoms and ester linkage between glycerol and fatty acid. Polyunsaturated fatty acid (PUFA) is the main component for the destruction of lipid by ROS. $^1O_2$ and $OH^{\cdot}$ are the free radicals that attack the PUFA like linoleic and linolenic acid out of which $OH^{\cdot}$ is most causing free radical because it proceed the chain reaction of lipid peroxidation (LPO) that form free radicals. Lipid peroxidation (LPO) enhances the membrane fluidity that leak important substance which enter the cell through special pathway. Then it cause damage to membrane receptors, ion-channels and membrane-localised enzymes (Smirnoff, 2000).

Proteins oxidation mainly occurred when ROS produced during stressful conditions. Protein has to undergo two main mechanisms during these stresses which may be direct and indirect. During direct modification, various chemical modification occur like disulphide bond formation, nitrosylation, carboxylation and glutathionylation out of which protein carboxylation is considered as a marker for protein evaluation (Moller et al., 2007). During indirect modification, protein interact with the product of lipid peroxidation (LPO) which result in ROS production that lead to site-specific modification of amino acid like Arginine, Lysine, Proline, Threronine that increased susceptibility to proteolytic degradation when it crosses threshold level. Cysteine and Methionine are more prone to damaged by radicals but amino acid containing thiol and sulphur are more vulnerable.

Chloroplastic and mitochondrial DNA are attacked by ROS because of lack of histones and associated proteins but that of plant DNA is fully protected. Free radicals like hydroxyl cause several damage like oxidation of deoxyribose sugar backbone by extracting H-atom, modifications of nucleotide base, breaking in either side of DNA, cross-linking of DNA and proteins (Halliwell, 2006). The attack of ROS to deoxyribose causes it to form single strands break in DNA (Evans et al., 2004). Critical cellular processes like replication and transcription will not proceed if damage is not being repaired on time because it's very lethal and non-repairable.

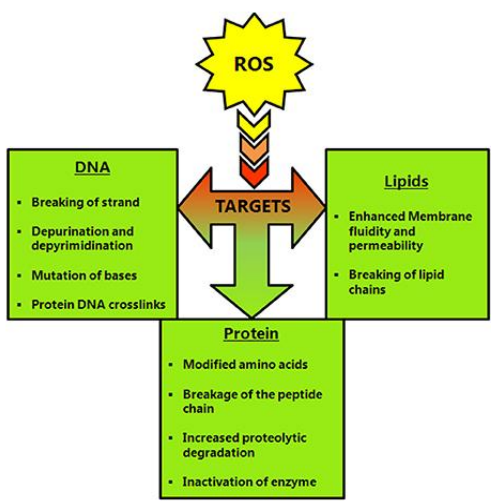

Fig. 2: Targets of ROS Ref: Das and Roychoudhury, 2004

1.4 Antioxidative defence mechanism in plants

Natural antioxidants or photochemical antioxidants are the potential source of plants and it is also secondary metabolite of plants (Ghasemzadeh et al., 2012). Flavonoids, polyphenols, ascorbic acid, folic acid, etc. are the antioxidant for the growth of plants. These antioxidants are very useful to decline the food disintegration and enlarge the shelf life of food in industry (Ghasemzadeh et al., 2012; Fresco et al., 2006).

Low concentration of antioxidant prevent the oxidation of substance but its high consumption by fruits and vegetables significantly reduces the risk of many diseases like cancer in human and liver damage that was suspected by synthetic antioxidants therefore the search of natural antioxidants considered as important. These agents are low toxic, safe and accepted for use in diet (Fresco et al., 2006).

To overcome from above diseases and stress-induced damages, plants have antioxidants machinery with two arms:

i) Enzymatic components like monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and dehydroascorbate reductase (DHAR).

ii) Non-enzymatic components like polyphenols, flavonoids, reduced glutathione (GSH), carotenoids, ascorbic acid (AA), α- tocopherols and proline.

1.5 Various non-enzymatic systems to overcome ROS in plants

By modification in cellular processes like cell elongation, senescence, mitosis and cell death, there are various non-enzymatic enzymes comprising of polyphenols, flavonoids, reduced glutathione (GSH), ascorbic acid (AA), carotenoids, α- tocopherols and proline play important role in growth and development of plant (Pinto and Gara, 2004).

1.5.1 Ascorbic Acid (AA)

AA is one of the most extensively and abundant used antioxidant compounds as it has powerful weapon to donate electron to both enzymatic and non-enzymatic reaction. Majority of AA is generated in plant mitochondria and rest in cytosol, apoplast thus is considered as first line of defence against ROS attack (Barnes et al., 2002). It protects the membrane from oxidative damage and also from metal binding enzymes.

1.5.2 Reduced Glutathione (GSH)

Glutathione is low molecular weight thiol tripeptide and found abundantly in all cellular compartments like cytosol, endoplasmic reticulum, mitochondria, chloroplast, vacuoles, peroxisomes and apoplast. It is also involved in various regulation of cell like cell growth, cell differentiation, senescence and cell death, detoxification of xenobiotics, synthesis of protein and nucleotides and expression of stress responsive genes (Mullineaux and Rauseh, 2005). GSH help in scavenging free radicals and therefore protect different biomolecules. It has two forms i.e. GSH and GSSH and it should be balanced to maintain the redox state of cell.

1.5.3 α- tocopherol

The α-tocopherol has highest antioxidant capacity than any other isomer and it is form by photosynthetic organism thus it is present in all green tissues of plants. It belongs to lipophilic family and help in scavenging of ROS and lipid radicals (Czytko et al., 2005).

1.5.4 Carotenoids

Like α-tocopherol, it also belongs to lipophilic family and resides in plastids of both photosynthetic and non-photosynthetic tissues of plant. It is also present in micro-organisms in addition to plants. It absorbs the light at wavelength 450-570 nm and transfers that energy to chlorophyll molecules. Carotenoid exhibits their antioxidant capacity by scavenging free radicals and inhibits lipid peroxidation.

1.5.5 Polyphenols

Polyphenols are considered as plant metabolites or secondary metabolite and largely being studied because of its many benefits on human health. Their presence affects many of the qualities of plant like its taste, color and texture. They have potential source to protect from different types of cancer and from different types of biotic and abiotic stresses like UV radiations, wounds and herbivore (Andreu A.B, 2018). It also play important role in protection from cardiovascular diseases and diabetes. From various in vivo and in vitro assays, it is defined as polyphenols act as antioxidant, anti-cancer, anti-microbial and anti-inflammatory compounds. Potato represents the level of antioxidant according to the level of polyphenols and carotenoids present. In comparsion to non-pigemented potatoes, pigmented one have high level of polyphenols (Akyol et al., 2016).

1.5.6 Flavonoids

In plant kingdom, flavonoids are widely present in leaves, floral organ and pollen grains of plants. Their presence affects many of qualities of plants like flavour, colour of fruits and vegetables. On the basis of structures, they are divided into four classes like flavonols, flavones, isoflavones and anthocyanins. Its main role is to scavange ROS produced by damaged caused to photosynthetic apparatus. They have distinct role in plants like pigmentation in seeds, fruits and flowers that involved in germination of pollen, plant fertility and defence against plant pathogens (Fini et al., 2011). Outer envelope of choloroplastic membrane is damaged by singlet oxygen and flavanoids play important role to scavenge it (Agati et al., 2012).

Chapter-2: Review of Literature

2.1 Reactive oxygen species (ROS)

Reactive oxygen species are unavoidable products of various aerobic cellular processes such as respiration and photosynthesis and occur in various organelles of the cell such as peroxisomes, mitochondria and chloroplasts. They are produced as a result of various biotic and abiotic stresses and are also produced in normal condition (Apel and Hirt, 2004). There are various type of free radicals such as superoxide radical ($O_2^{\cdot -}$), Hydrogen Peroxide (H_2O_2), Hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$). The production and scavenging of free radicals should be balanced in body instead it lead to various degenerative disease in the body such as cancer, diabetes, cirrhosis, arthritis and many more (Fresco et al., 2006). The imbalance also affects lipids by peroxidation and break the lipid chain that enhanced the membrane fluidity and permeability, proteins by causing change in amino acid sequence and breaking of peptide bond that lead to inactivation of enzymes and DNA by breaking the strands and causing depurination or depyrimidination that causes mutation of bonds. To overcome these stresses and disease, there are various enzymatic and non-enzymatic enzymes that act as antioxidant machinery to protect the plant from different types of reactive oxygen species (Das and Roychoudhury, 2014).

2.2 Polyphenols

Phenolic compounds are secondary metabolites in plant of molecular weight ranging from 500-3000 kDa and possess common structures based on aromatic ring with one or more hydroxyl substituent's (Beckman C, 2000). On the basis of chemical structure, polyphenols can be divided into flavonoids, phenolic acid, tannins, stlbenes, courmarins and lignin (Lemos et al., 2015). Among polyphenols, phenolic acid is important that get concerned to protect the plants from various categories of stresses such as thermal stresses, biotic stresses and injuries and their intolerance to ozone and UV rays. It also provides protection from various diseases such as cancer, cardiovascular and diabetes (Andreu A.B, 2018). Therefore, polyphenols act as antioxidant, anti-cancer, anti-microbial and anti-inflammatory compounds.

Polyphenols are found both in plant and animal. In plants, polyphenol is present in various tissues such as leaf tissues, bark layers, flowers and fruit and plays a predominant role in plant life such as prevents the microbial infection, release and suppression of growth hormones such as auxin, to provide plant pigmentation and ripening of plants. So their presence affects taste, colour and texture of plants (Alasalvar et al., 2001). In animals, polyphenol present in arthropods such as insects and crustaceans that result in epicuticle hardening. Polyphenols are also present in large quantities in green tea, red wine, fruits and vegetables, coffee, olives, chocolate and many more. There are many food sources that are rich in particular polyphenol like citrus food consist of flavonones, soya consist of isoflavones and is present around 58-82% of total antioxidant activity than other cultivars in addition to fruit and vegetables like tomatoes, carrots or onions (Reddivari et al., 2007). Since high level antioxidants are found in fruits and vegetables which are beneficial for human health (Kulen et al., 2013).

There are many factors that affect the loss of phenolic compounds such as stage of maturity, presence of water or moisture during cooking and cooking temperature and time. The two most important poyphenolic acid present in potato are cholorogenic acid that constitute around 80% of gallic acid, cinnamic acid, ferulic acid, vanillic acid, syringic acid, sinapic acid, para-coumaric acid and salicyclic acid (Lewis et al., 1998; Navarre et al., 2011; Reddivari et al., 2007). In comparison to high altitude, chlorogenic acid is found more in warm location with regular period of drought. In potato, calcium enhances the amount of chlorogenic acid and caffeic acid (Ngadze et al., 2014). The polyphenol are present both in peel and flesh. The amount of antioxidant level varies according to the pigmentation and also according to flesh and skin color. Pigmented potatoes have higher level of antioxidant in comparison to the non-pigmented one and in case of flesh and skin color, red and purple flesh potatoes are known to have high phenolic content as compared to those of yellow and white flesh potatoes (Lachman et al., 2008). Madiwale et al., 2011 demonstrated positive correlation between polyphenol in-take and inhibition in proliferation of breast and colon cancer. Decrease in blood glucose level by the use of freeze dried potato was reported by Scheiber and Saldana, 2009. Andre et al., 2007 reported that polyphenolic content of potato spectate the cell cytotoxic activities.

2.3 Flavonoids

Flavonoids are considered as one of the important natural product, belonging to class of secondary metabolites having polyphenolic structure. It structure consists of a 15 carbon skeleton, which have two phenyl rings and heterocyclic ring. On the basis of structure feature of

C-ring, flavonoids are classified into subgroups such as flavones, flavanones, flavanonols, flavonols, anthocyanins and chalcones. Flavonoid protects the plants and animals from various stresses like biotic and abiotic stresses, UV filters and from various diseases such as cancer, Alzheimer's disease, atherosclerosis, etc. Therefore, flavonoids are associated with a broad spectrum of health-promoting effects because of antioxidant, anti-mutagenic anti-carcinogenic and anti-inflammatory properties.

Flavonoids are abundantly found in food and beverages of plants such as fruits, vegetables, cocoa, tea and wine, hence they are known as dietary flavonoids. They play a crucial role in metabolic activity in plants, animals and in bacteria. In plant, flavonoids act as one of the common phenolic compounds and their presence influence flavor and color of vegetables and fruits (Andre et al., 2007). It can be easily extracted from plants as they are found in various tissues and are used by plants for their growth and defence against pathogen. They are responsible for color and aroma of flowers, growth and development of seedling and in fruits to attract pollination and also function as signalling molecules, allopathic compounds and anti-microbial defensive compounds. They are involved in frost hardiness, drought resistance and protect plant from heat acclimatization.

In potato, catechin is one of the abundant flavonoid ranging from 0-204 mg/100 g of dry weight (Mader et al., 2009). Flavonols such as quercitin and kaempferol rutinose are also present in addition to rutin (Schieber and Saldana, 2009). Anthocyanin is a sub-class of pigmented potatoes and due to it, flavonoid content is double in purple and red fleshed potatoes in comparison to white fleshed potatoes (Burgos et al., 2013). The most common type of anthocyanin present in potatoes are petunidin, malvidin, delphindin and peonidin in purple tubers and pelargonidin in red ones (Brown et al., 2005). Cyanidin, petanin and Aglycones are also present in addition to anthocyanin. It is indicated that heating cause change in content of anthocyanin but did not cause any change to phenolic acid.

2.4 Antioxidant Capacity

Antioxidant capacity is described by the capacity of redox reactions in biological system to tramp free radicals consisting of the integrative effects of all antioxidants. An antioxidant is a molecule that hinders the oxidation of other molecules. Oxidation is chemical reactions that can form free radical, thus damaging occurred. Antioxidant such as thioester and vitamin C terminate these types of reactions.

The antioxidant capacity is a relevant method for exploring the relationship between the antioxidant and pathologus induced by oxidative stress. There are many methods for the determination of antioxidant capacities based on the different principles such as:

i) FRAP (Ferric reducing antioxidant potential)

ii) DPPH (2,2-Diphenyl-1-picrylhydrazyl)

iii) CUPRAC (Cupric reducing antioxidant power)

iv) ORAC (Oxygen radical absorbance capacity)

Among this, FRAP and DPPH method are rapid, simple and accurate to calculate the antioxidant and scavenging activity. Ghasemzadeh et al., 2012 estimated polyphenolic content and their antioxidant capacity in leaf extract of sweet potato (*Ipomoa batatas*). Julia et al., 2018 also studied the antioxidant activity by DPPH in Andean potato cultivar.

2.4.1 FRAP assay

Ferric reducing antioxidant power (FRAP) assay is a greatly utilized system that uses antioxidants as reductant in a redox-linked colorimetric response where Fe^{3+} is reduced to Fe^{2+}. Ferric (Fe^{3+}) TPTZ complex which is colorless complex is reduced to ferrous (Fe^{2+}) TPTZ complex which a blue colored complex by action of electron donating antioxidants at low pH factors (Benzie et al., 1996). This reaction was observed by studied the change in absorbance at 593 nm. The FRAP assay is cheap, reagents are easy to arrange, results are totally reproducible and the approach is straight-forward and rapid. On the basis of reduction of Fe^{3+} to Fe^{2+}, the level of antioxidant activity was measured.

2.5 Objectives of the present study

Reactive oxygen species (ROS) play pivotal role during plant growth and development in terms of signaling. However, under stress situations an imbalance between ROS production and scavenging lead to damaging consequences where various antioxidants play crucial role in cell defense and rescue. This study focused on mainly non-enzymatic antioxidants namely, polyphenols and flavonoids along with assessing antioxidant capacity in different potato tissues including developing, mature and stored tubers. Keeping in view with the aforesaid points, the following objectives were framed:

- To grow and harvest different tissues of the potato cultivars grown under field condition
- Estimation of polyphenols , flavonoids along with assessment of antioxidant capacity
- To study the cytotoxic effects of polyphenols on the cell line

Chapter-3: Materials and Methods

3.1 Maintenance of potato germplasm

For the experiment study, different cultivars such as Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Pukhraj (PR), Kufri Jyoti (KJ), Kufri Chandramukhi (KCM) and Desiree (De) were maintained in growth room, laboratory 4, Thapar Institute of Engineering & Technology, Patiala. After completion of acclimatization process, they were transferred to the field in starting of November which is a best suited period for the growth with optimal temperature and light condition. As potato is a cold weather crop, so optimum conditions were taken care of while cultivating above cultivars. They were harvested in different growing stage like in January, February and March for the experiment purpose.

Other materials: A number of chemicals and biochemical, biological items required in the experiment were bought from different companies in India. Laboratories like Sigma – Aldrich India Pvt. Ltd and high media laboratories Mumbai were selected for purchasing the chemicals. All the other requirements like various glasswares were obtained from Borosil Pvt. Ltd and plastic wares were purchased from Tarsons Pvt. Ltd.

3.2 Procurement of different plant samples

During growth under field condition, harvesting was done at different time periods. Various tissues of potato such as tubers, leaves and stems were collected and thoroughly washed with water to remove dirt. After being dried, different tissues were frozen in liquid nitrogen to arrest their metabolic stages, and stored at -80 ºC for further experiments.

3.3 Preparation of crude extracts from different potato tissues

Tuber: Extract from tuber tissue for the study were made according to Andreau A.B, 2018. The tubers were first collected, washed and dried and then freezed in liquid nitrogen and stored at -80 ºC. Then approx. 1 g of tuber was crushed to fine powder with the help of liquid nitrogen in mortar pestle. Next, it was extracted with 20 mL of 100% methanol and was kept overnight with constant agitation (100 rpm) at 4 ºC. Then followed by centrifugation at 12,000 rpm at 4 ºC for 20 min to let the pellet settle down. The supernatant was further clarified by filteration and was placed in rotary evaporator in order to concentrate it. Then the concentrate was finally resuspended in 1 mL of 100% methanol (Andreau A.B, 2018). Potato polyphenolic extracts (PPEs) were stored at -20 ºC and were further used for the experiment studies.

Leaf and stem: Leaves and stems were collected, washed, dried and freezed in liquid nitrogen to arrest their metabolic stage. For estimation, 1 g of sample was crushed in liquid nitrogen, then was extracted in 10 mL of 80% methanol. Then it was allowed to swirl for 60 min at room temperature. Extracts were then filtered and stored at -20 ºC for analysis.

3.4 Determination of total phenolic content from different potato tissues

Tuber: The total phenolic content was determined by Folin-Ciocalteu colorimetric method according to Campos et al., 2006 and Andreau A.B, 2018 with minor modification. Briefly, 150 μL of PPE were diluted to a final volume 0.5 mL with HPLC grade methanol, followed by addition of 7.5 mL of autoclaved water. Thus making the total volume of 8 mL. Then 0.5 mL of Folin-Ciocalteu reagent was added, diluted in water (1:7) followed by incubation for 3 min. Then 1 mL of sodium carbonate (0.5 M) was added and allowed to react for 10 min in dark. Finally absorbance was measured at 725 nm in visible light with help of UV-visible spectrophotometer-Shimadzu. Gallic acid in methanol was used as a standard and total phenolic content was estimated as μg of GA equivalent per one gram of potato tuber fresh weight (μg of GA equiv. / gfw). Each reaction was set in triplicate in three independent experiments.

Leaf and stem: The total phenolic content was estimated in leaf and stem tissues by the Folin-Ciocalteu method according to Ghasemzadeh et al., 2012. For the estimation of TPC, 1 mL of plant extract from total 10 mL was taken and further diluted with 10 mL of deionized water followed by the addition of 1 mL of Folin-Ciocalteu reagent. It was mixed well and incubated for five minutes. After incubation, 2 mL of 20% sodium carbonate was added to the mixture and mixed well. The solution was now incubated in total darkness for reaction to take place. After the incubation, absorbance was measured at 750 nm using visible light with the help of UV-visible spectrophotometer-Shimadzu. Gallic acid was used as a standard and total phenolic content was estimated as μg of GA equivalent per one gram of potato tuber fresh weight (μg of GA equiv. / gfw). Each reaction was set in triplicate in three independent experiments.

3.5 Determination of total flavonoid content in different potato tissues

For determination of total flavonoid, earlier reported spectrophotometric method by Ghasemzadeh et al., 2012 was followed.

Sample in case of tuber tissues- for tuber tissue,100 µL of PPE sample was diluted by adding 900 uL methanol. In order to make volume 1 mL of extract stem and leaf tissues, similar experiment was done. The extract from respective tissue were diluted with 4 mL of sterilized and deionized water in 50 mL of flask followed by addition of 300 µL 5% $NaNO_2$ (Sodium nitrite) and five min of incubation. After incubation, 100 µL of 10% $AlCl_3$ (w/w) was added and incubated in darkness for six min for allowing the mixture to form complex with $AlCl_3$ (Aluminium Chloride). After six min, 2 mL of 1 M NaOH was added and the absorbance was taken at far UV range i.e. 430 nm using visible light with help of UV-visible spectrophotometer- Shimadzu. Here, Quercitin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromo-4-one) was used as a standard and total flavonoid content was estimated as µg of quercitin equivalent per one gram of fresh weight of tissue (µg of quercitin equiv. / gfw). Each reaction was set in triplicate in three independent experiments.

3.6 Determination of antioxidant capacity in different potato tissues

Antioxidant capacity in tissues was measured by Ferric reducing antioxidant potential (FRAP) assay according to Benzie and Strain, 1996 method.

Stock solution for estimating the FRAP assay:

i) Acetate buffer- 300 mM of pH 4.5

ii) TPTZ (2, 4, 6-tripyridyl-s-triazine) solution -100 mM in 40 mM HCl

iii) Iron chloride ($FeCl_3$)- 20 mM in deionized water

For making the reaction mixture, 25 mL of acetic acid buffer pH 4.5, 2.5 mL of TPTZ in 40 mM HCl were mixed, followed by addition of 2.5 mL of $FeCl_3$ to the above stock. For the analysis, 150 µL of plant extract were added to 2850 µL of the working solution of FRAP mixture and was incubated at room temperature in complete darkness for 30 min. Methanol was used as blank. The absorbance was taken at 593 nm using visible light with the help of UV-visible spectrophotometer-Shimadzu. Vitamin C was used as a standard for the determination of FRAP assay and antioxidant activity was estimated as μg of ascorbate equivalent per gram of fresh weight of plant extract. Each reaction was set in triplicate in three independent experiments.

3.7 Cytotoxicity of polyphenolic extracts

Peripheral blood mononuclear cells (PBMC) were isolated by ficoll density gradient method. 8 mL of whole blood was carefully layered onto equal volume of Histopaque®-1077 from the edge of the tube and centrifuged at 400 xg for 30 min at room temperature in swinging bucket rotor (Thermo Scientific Biofuge Stratos). This density based centrifugation technique fractionates blood into red blood cells, plasma and peripheral blood mononuclear cells (PBMC). The upper plasma layer was discarded with a micropipette after centrifugation and opaque interface (buffy coat) containing PBMC was transferred in a sterile 15 mL conical centrifuge tube. The cells were washed twice in 10 mL of isotonic phosphate buffered saline solution (PBS) and centrifuged at 250 xg for 10 min. Finally, the cell pellet was re-suspended in 1 mL of complete media (RPMI-1640supplemented with 10% foetal bovine serum, 100 μg/mL streptomycin, 100 IU/mL penicillin and 10 mM HEPES) (Marquardt et al., 2012)

Cells were counted on haemocytometer by trypan blue exclusion assay. Briefly, 10 μL of cell sample was diluted with 0.4% Trypan Blue solution in the ratio 1:5 or 1:10 in a vial and incubated for 1-2 min. The preparation was loaded on a haemocytometer and examined immediately under a microscope at 40X magnification (Nikon Eclipse E100-LED). The numbers of unstained viable cells (non-viable cells take up dye and appear blue) were counted in all the four corner squares. Number of viable cells in the original cell suspension was calculated from the following formula:

$$\text{Number of viable cells/mL} = \text{Total no of unstained cells x dilution factor x } 10^4$$

Freshly isolated healthy PBMC were cultured with individual extracts in order to test the cytotoxic potential of extracts at varying concentration. Extract induced cytotoxicity was measured by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) based colorimetric assay (doLivramento et al., 2013). MTT assay measures the cell viability based on the formation of purple formazan crystals by the reduction of the tetrazolium salt MTT (yellow colored) by mitochondrial succinate dehydrogenase of metabolically active cells. To measure proliferation, blank and Unstimulated cells were used. Cells were treated with extracts of different concentrations. Positive control was a set stimulated with 10 μg/mL of concanavalin A, whereas the negative control was unstimulated cells. At the end of 2nd day, the plate was incubated at 37 °C at 5% CO_2 after the addition of MTT (0.5 mg/mL). After an incubation of 4 h, 170 μL of the media was removed from each well without disturbing the purple colored crystals. After solubilizing formazan crystals in 100 μL DMSO, using the microplate reader (Tecan Austria) absorbance of each well was measured at 570 nm, using 630 nm as the reference wavelength.

Chapter-4: Results and Discussion

4.1Plant growth and harvesting of the tissues

For study, different potato varieties were harvested at Thapar Instititute of Engineering & Technology, Patiala, and various tissues of it like leaves, stems and tubers were stored at ambient conditions. They were stored separately in different plastic bag and at specific temperature for further analysis.

Fig. 3 Steps of plant grown in laboratory to field's conditions :(a) Culture maintained in MS medium in lab (b) Acclimatization and hardening of plantlets (c) field growth (d) Collection of plants

Fig. 4 Collection of (a) young and (b) mature tissues (c) developing tubers (d) growing tubers (e) mature tubers

4.2 Standard curve for estimation of phenolic, flavonoid content and antioxidant activity

Phenolics, flavonoids and antioxidant activity was measured from different tissues such as leaf, stem and tubers according to Andreau A.B., 2018 and Ghasemzadeh et al., 2012 method as reported in literature. The tissues were extracted in methanol.

I. Standard curve for the estimation of total phenolic content- For the estimation of any compound or material, we need a standard or control.

   For the estimation of total phenolics, gallic acid (as reported in literature) was used as standard. The stock of 500 µg was and different concentration from 50 to 500 µg was used to made standard curve according to Andreau A.B, 2018 and Ghasemzadeh et al., 2012 as shown in Table 1.

Table 1 Prepartion of the standard curve of gallic acid (absorbance measured at 725 nm)

Tube No.	Concentration (µg)	A_{725} value
1	Blank	0
2	50	0.087
3	100	0.124
4	150	0.360
5	200	0.451
6	250	0.601
7	300	0.783
8	350	0.976
9	400	1.112
10	450	1.226
11	500	1.336

Test tubes with different concentrations of gallic acid from 50-500 µg for poyphenol estimation is shown in Fig. 5 and curve for gallic acid in Fig. 6.

Test tubes: 1 2 3 4 5 6 7 8 9

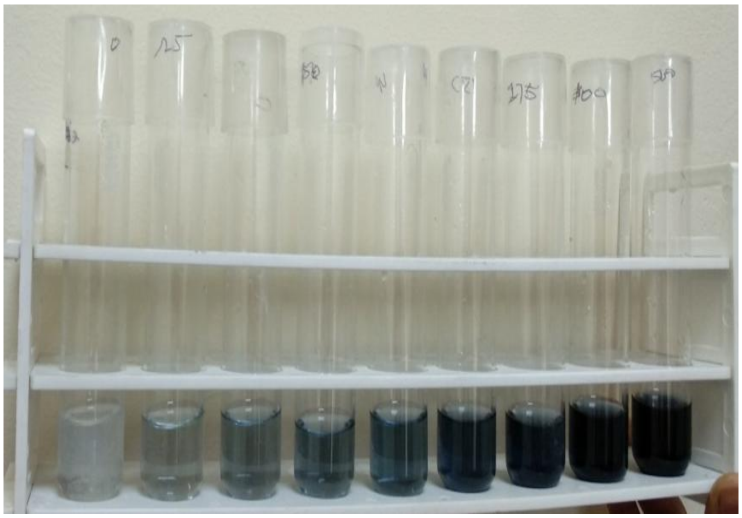

Fig. 5 Colour gradient developed by the different concentrations of gallic acid

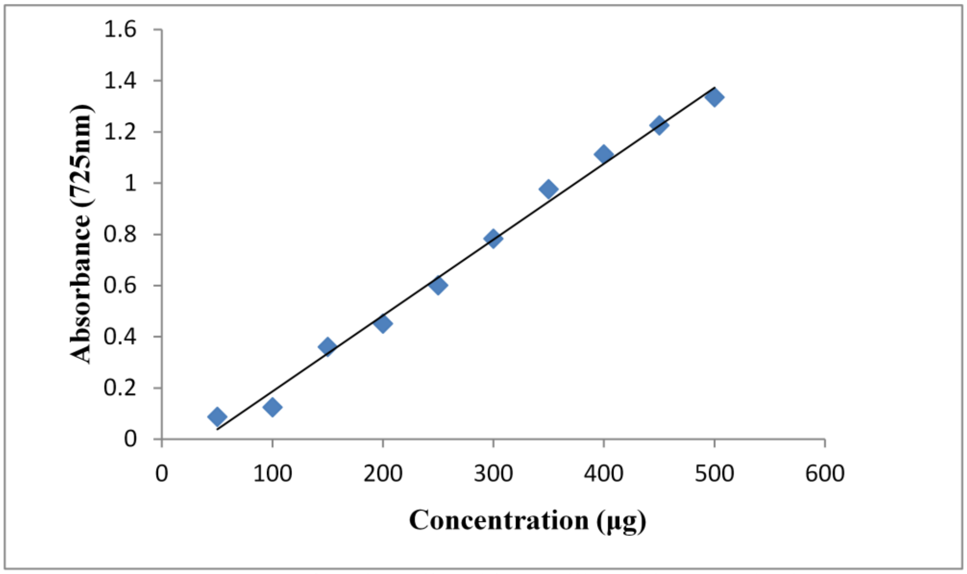

Fig. 6 Curve for phenolic taking gallic acid as standard

II. Standard curve for the estimation of total flavonoid content- Quercitin was used as standard and standard curve was made by using different concentration of 1000 µg stock of quercitin as shown in Table 2. Ghasemzadeh et al., 2012 method was followed to perform it and absorbance was taken at 430 nm. It was estimated in µg of quercitin equivalent per one gram of potato tissue fresh weight.

Table 2: Preparation of the standard curve of quercitin (absorbance measured at 430nm)

Test tubes	Concentration (µg)	A_{430} value
1	Blank	0
2	100	0.121
3	200	0.305
4	300	0.421
5	400	0.513
6	500	0.670
7	600	0.730
8	700	0.870
9	800	0.982
10	900	1.121
11	1000	1.300

Test tubes with different concentration of quercitin from 100 to 1000 µg for flavonoid estimation is shown in Fig. 7 and curve for quercitin in Fig. 8.

Test tubes: 1 2 3 4 5 6 7 8 9

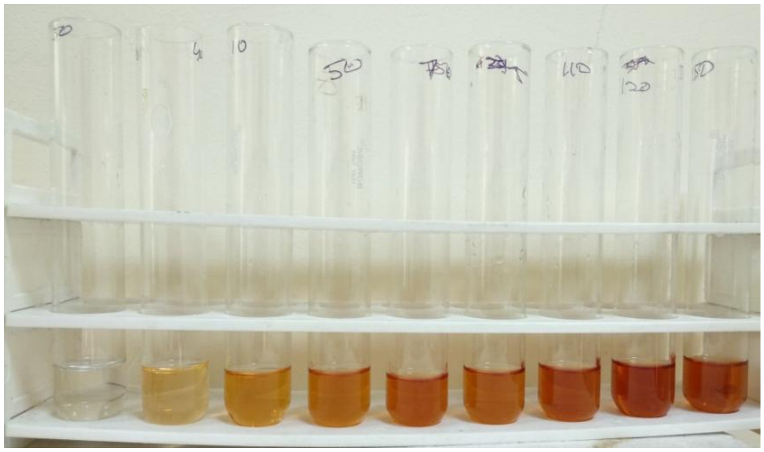

Fig. 7 Colour gradient developed by the different concentrations of quercitin

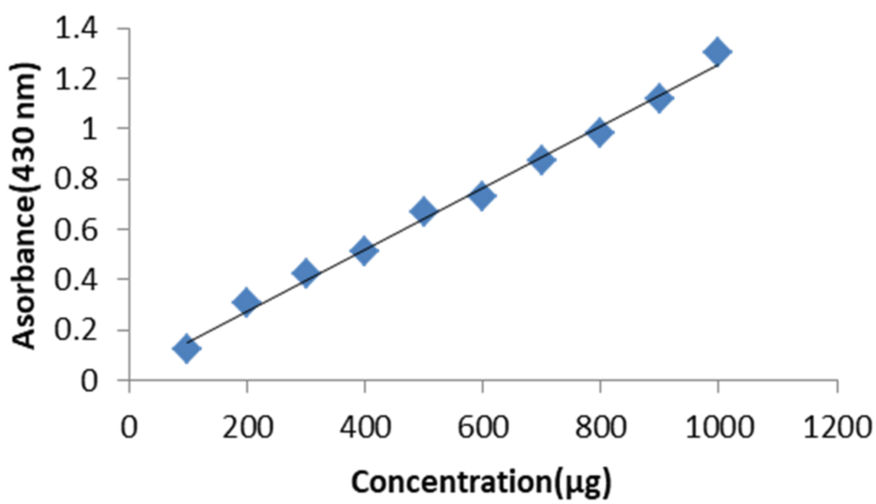

Fig. 8 Curve for flavonoid taking quercitin as standard

III. Standard curve for the estimation of antioxidant capacity by FRAP using different concentrations from 1 mg/ml stock of ascorbate according to Benzie and Strain, 1996 method as shown is Table 3. The absorbance was taken at 593 nm and was estimated in µg of ascorbate equivalent per one gram of potato tissue fresh weight.

Table 3 Preparation of the standard curve of ascorbate (absorbance measured at 593 nm)

Test tubes	Concentration (µg)	A_{593} value
1	Blank	0
2	10	0.164
3	20	0.300
4	30	0.420
5	40	0.561
6	50	0.711
7	60	0.866
8	70	0.977
9	80	1.042
10	90	1.111
11	100	1.297
12	110	1.312
13	120	1.589

Test tubes with different concentrations of ascorbate from 10 to 120 µg for estimation of antioxidant activity is shown in Fig. 9 and curve for ascorbate in Fig. 10.

Test tubes: 1 2 3 4 5 6 7 8 9

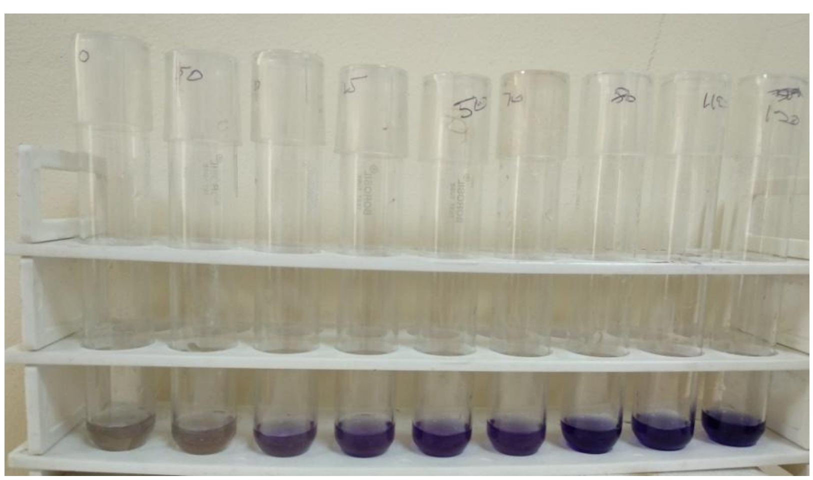

Fig. 9 Colour gradient developed by the different concentrations of ascorbate

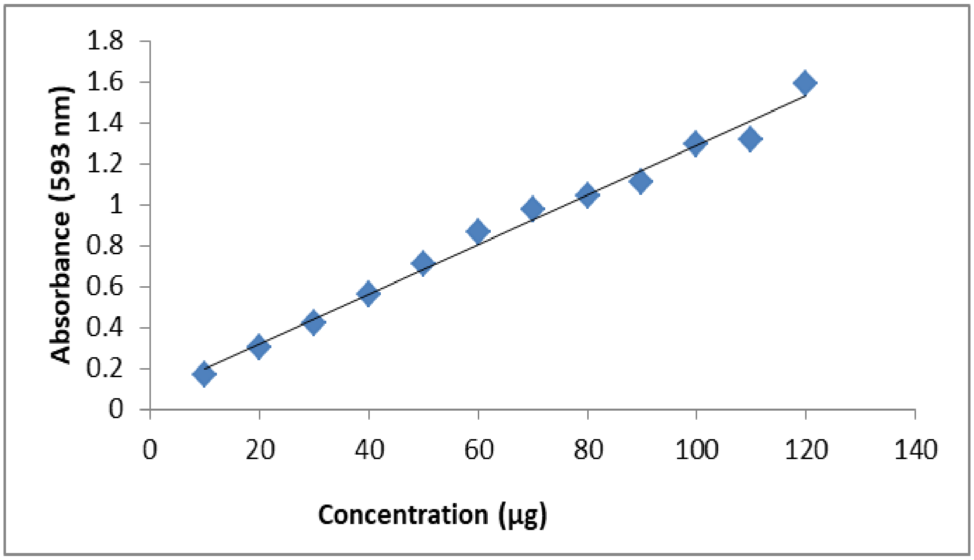

Fig. 10 Curve for FRAP (antioxidant capacity) taking ascorbate as standard

4.3 Estimation of phenolic content, flavonoid content and antioxidant activity in different potato cultivars

Phenolic content in tuber

From Table 4, it was observed that total phenolic content was highest in KJ (1483.6 µg GAE. g^{-1} FW) during developing stage, CS-1 (817.6 3µg GAE. g^{-1} FW) during growing stage and KJ (1308.9 µg GAE. g^{-1} FW) during mature stage. It was lowest in KJ (569.8 µg GAE. g^{-1} FW) during growing stage, PR (813.6 µg GAE. g^{-1} FW) during developing stage and (741.7 µg GAE. g^{-1} FW) mature stage. From the data, it was observed that phenolic content decrease during growing stage after passing from developing stage but again got increased during mature stage except CS-1. The values show some fluctuations and it might be due to the different antioxidant capacity at different stages of growth.

Table 4 Estimation of total phenolic content from the tuber extract of potato (expressed in µg of gallic acid equivalent per gram of fresh weight of tuber tissue)

Cultivars	Developing tubers	Growing tubers	Mature tubers
CS-1	1183.6	817.6	770.7
CS-2	997.0	571.9	969.5
PR	813.6	603.2	741.7
KJ	1483.6	569.8	1308.9
KCM	1250.3	739.5	1011.9
DE	1293.6	757.3	824.3

Flavonoid content in tuber

Flavanoid content depends upon the color and pigmentation of the tissues. Highest flavonoid content was observed in the developing tuber of cultivar DE (3850 µg quercitin eq. g^{-1} FW), followed by cultivar KJ (3300 µg quercitin eq. g^{-1} FW) which decreased with the growth of tuber significantly as observed from Table 5. The flavonoid content decreased abruptly in the growing tuber and then showed increase in the mature tuber, hence followed a parabolic pattern.

Table 5 Estimation of total flavonoid content from the tuber extract of potato (expressed in µg of quercitin equivalent per gram of fresh weight of tuber tissue)

Cultivars	Developing tuber	Growing tuber	Mature tuber
CS-1	2300.0	1280.3	1458.3
CS-2	1900.0	1383.3	1466.6
PR	1766.0	1050.0	1500.0
KJ	3300.0	1183.3	1600.0
KCM	2441.6	1191.6	2033.3
DE	3850.0	1383.3	1566.6

Polyphenolic content in leaf and stem extract

The highest polyphenol content was observed in the leaf tissue, which almost doubled from young to mature stage. It was a linear graph with the growth. Highest polyphenolic content was observed in the mature leaf of cultivar CS-2 (14241.3 µg GAE. g^{-1} FW) followed by CS-1

(11308.0 µg GAE. g^{-1} FW) as evident from the Table 6. The highest polyphenolic compounds were reported in leaf tissue. Initially the phenolic content was lowest in KJ (4294 µg gallic acid eq. g^{-1} FW) at young stage and the highest in CS-2 (14241.3 µg gallic acid eq. g^{-1} FW) at mature stage in the tissues respectively.

In case of stem, the phenolic content was low in comparison to leaf tissue , but was in significant amount ranging from 3771.0 µg gallic acid eq. g^{-1} FW in young stem of KJ to 1234.0 µg gallic acid eq. g^{-1} FW) in young stem of CS-1. Except cultivar KJ where the phenolic content decreased in mature stem and was constant respectively. However, in the remaining cultivars it was almost doubled from young to mature stage.

Table 6 Estimation of polyphenolic content in leaf and stem extract of potato (expressed in µg of gallic acid equivalent per gram of fresh weight of tissue)

Cultivars	Young		Mature	
	Leaf	Stem	Leaf	Stem
CS-1	5500.6	1234.0	11308.0	2207.3
CS-2	4580.6	1667.3	14241.3	1687.3
PR	5914.0	1707.3	10241.3	2214.0
KJ	4294.0	3771.0	11308.0	1900.6
KCM	4467.3	1567.3	6601.3	2067.3
DE	6034.0	1500.6	10134.6	1927.3

Flavonoid content in leaf and stem extract

In case of leaf, it was observed that the flavonoid content increase tremendously from young stage to mature stage except in cultivar KCM and DE where it increased almost double from the young stage. From the data in Table 7, it was observed to be highest in DE (6266.6 µg quercitin eq. g^{-1} FW) and lowest in CS-1(3766.6 µg quercitin eq. g^{-1} FW) at young stage. And at mature stage, it was highest in CS-2 (31000.0 µg quercitin eq. g^{-1} FW) and lowest in DE (8233.3 µg quercitin eq. g^{-1} FW).

In case of stem, the flavonoid content increases in all cultivars except KJ. It was observed to be lowest in CS-1(807.3 µg quercitin eq. g^{-1} FW) and highest in KJ (2283.3 µg quercitin eq. g^{-1} FW) at young stage but at mature stage, it was highest in PR (5266.6 µg quercitin eq. g^{-1} FW) and lowest in KCM (616.6 µg quercitin eq. g^{-1} FW).

Table 7 Estimation of flavonoid content in leaf and stem extract of potato (expressed in µg of quercitin equivalent per gram of fresh weight of tissue)

Cultivars	Young		Mature	
	Leaf	Stem	Leaf	stem
CS-1	3766.6	807.3	23666.6	3850.0
CS-2	4800.0	1816.6	31000.0	3483.3
PR	4600.0	966.6	21000.0	5266.6
KJ	4166.0	2283.3	23666.0	2100.0
KCM	3950.0	940.0	11900.0	616.66
DE	6266.6	933.3	8233.3	800.0

Antioxidant activity in tubers at different development stages

From the data in Table 8, it was observed that antioxidant capacity first increases in growing stage after passing from developing stage but its content again decreased in mature stage like CS-1, CS-2, KJ and DE. In rest of cultivars such as PR and KCM, its content increases as it move from developing stage to growing stage and at last to mature stage. It was lowest in KJ (138.0 µg ascorbate eq. g^{-1} FW) at developing stage, PR (612.8 µg ascorbate eq. g^{-1} FW) at growing stage and CS-2 (473.4 µg ascorbate eq. g^{-1} FW) at mature stage and it was highest in KCM (264.0 µg ascorbate eq. g^{-1} FW) at developing stage, CS-1(1584.9 µg ascorbate eq. g^{-1} FW) at growing stage and KJ (1722.6 µg ascorbate eq. g^{-1} FW) at mature stage. It is following bell shaped pattern. Its increased almost upto seven times from developing tuber to growing tuber and at matured stage it was reduced except in cultivar PR and KJ where some increase in the level was observed. It might be due to different antioxidant capacity of different cultivars. Thus, the trend was marked with some fluctuations.

Table 8 Estimation of antioxidant capacity from the tuber extract of potato (expressed in µg of ascorbate equivalent per gram of fresh weight of tuber tissue)

Cultivar	Developing tuber	Growing tuber	Mature tuber
CS-1	241.5	1584.9	874.2
CS-2	245.9	1137.4	473.4
PR	158.1	612.8	792.2
KJ	138.0	1439	1722.6
KCM	264.0	1048.6	630.8
DE	142.0	1248.6	707.9

Antioxidant capacity in leaf and stem

From the data in Table 9, it was observed that the antioxidant activity increases too much higher concentration from young leaf to mature except DE in which its content decreases. Its content was highest in DE (5276.0 µg ascorbate eq. g^{-1} FW) at young stage and in CS-2 (22685.2 µg ascorbate eq. g^{-1} FW) at mature stage but it was lowest in KJ (3343.3 µg ascorbate eq. g^{-1} FW) at young stage and in DE (4963.9 µg ascorbate eq. g^{-1} FW) at mature stage.

In case of stem, the antioxidant capacity decreases from young to mature in all cultivars except CS-1. It was highest in KJ (2651.4 µg ascorbate eq. g^{-1} FW) at young stage and CS-1 (751.3 µg ascorbate eq. g^{-1} FW) at mature stage but was lowest in CS-1 (690.8 µg ascorbate eq. g^{-1} FW) at young stage and KJ (17.6 µg ascorbate eq. g^{-1} FW) at mature stage.

Table 9 Estimation of antioxidant capacity from leaf and stem tissues of potato (expressed in µg of ascorbate equivalent per gram of fresh weight of tissue)

Cultivars	Young		Mature	
	Leaf	Stem	Leaf	Stem
CS-1	3711.3	690.8	20062.2	751.3
CS-2	3815.7	921.5	22685.2	196.6
PR	4699.8	1207.1	15226.2	295.5
KJ	3343.4	2651.4	5177.0	17.6
KCM	3733.3	938.0	17849.2	443.7
DE	5276.5	1245.5	4963.9	141.7

Total phenolic content in the tubers under different storage conditions

In the case of tuber tissue, initially increase in the TPC content and then decrease was observed. The significant overall increase in total phenolic compound was observed from 0 to 60 DOS. The TPC content ranged from 600 to 1988 µg GA equiv. / gfw at room temperature and from 500 to 2421.1 µg GA equiv./ gfw at 4 ºC and from 609.9 to 1628.3 µg GA equiv./ gfw at -20 ºC from 0 to 60 days of storage. The TPC fluctuated during the whole storage period but until the 60 DOS, but it was higher during 0 DOS as observed from Fig. 11. The TPC content increased to 1.5 fold at room temperature, 1.6- 2.4 fold at 4 ºC and was almost constant at -20 ºC. This study is also in concordance with Galani et al., 2017 and Akyol et al., 2016.

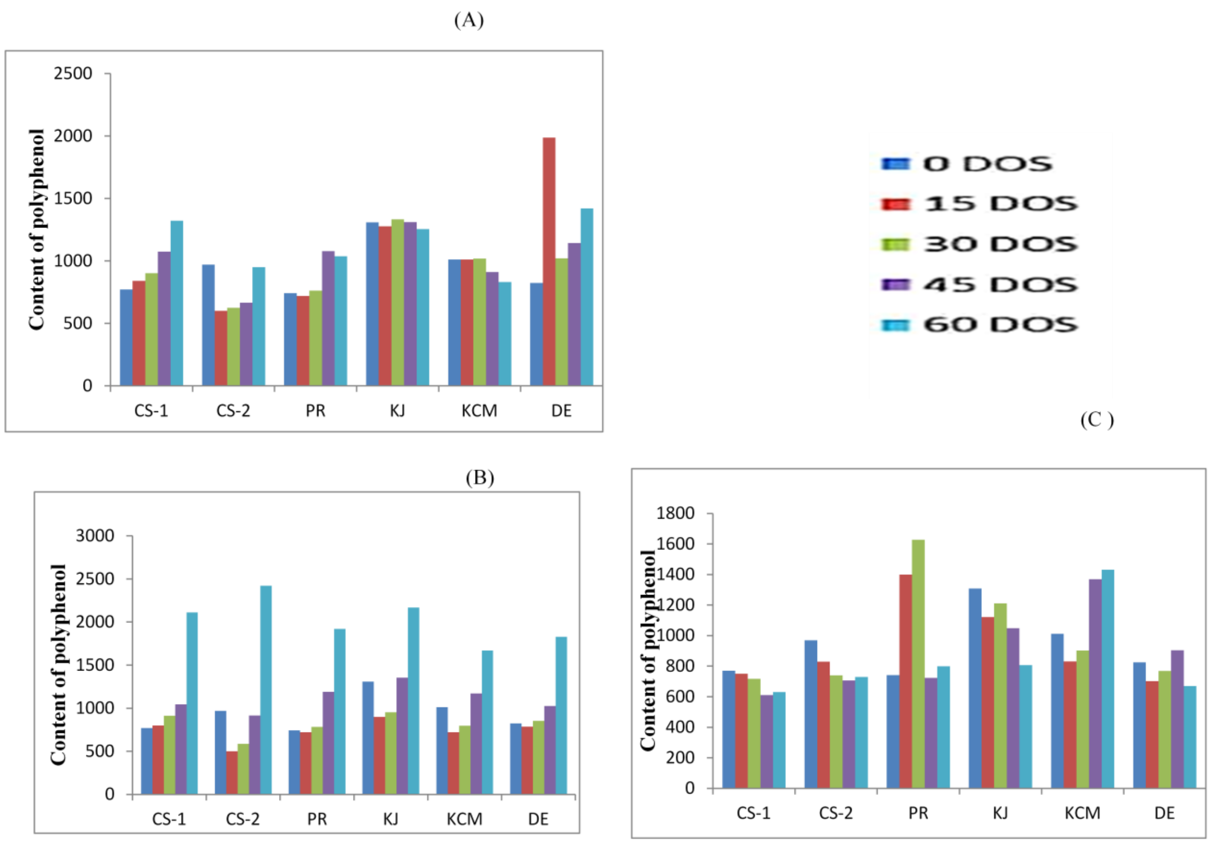

Fig. 11 Phenolic content at different storage temperature (A) at room temperature (B) at 4ºC (C) at -20ºC

Total flavonoid content in the tuber under different storage conditions

Flavonoids content first increased and then decreased similar to phenolic content during storage period. It was observed to be overall increase in flavonoid content from day 0 to day 60. From the data in Fig. 12, the flavonoid content ranged from 1216.0 µg quercitin eq. g^{-1} FW to 3175.0 µg quercitin eq. g^{-1} FW at room temperature and from 1233.3 µg quercitin eq. g^{-1} FW to 3566.6 µg quercitin eq. g^{-1} FW at 4 ºC and from 908.3 µg quercitin eq. g^{-1} FW to 3133.3 µg quercitin eq. g^{-1} FW at -20 ºC from day 0 to day 60. This study is in concordance with Galani *et al*., 2017.

(A)

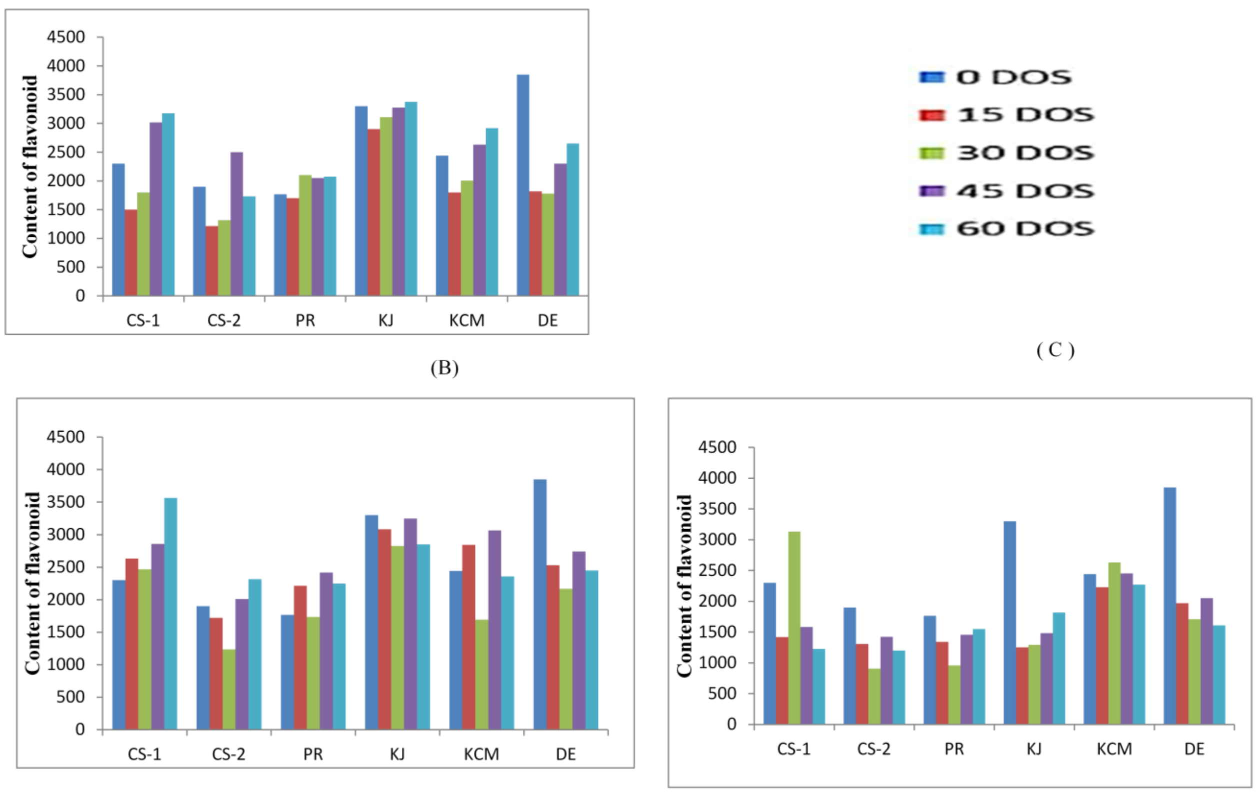

Fig.12 Flavonoid content at different storage temperature (A) at room temperature (B) at 4ºC (C) at -20ºC

Antioxidant activity by FRAP in the tuber under different storage conditions

The notable enhancement in the antioxidant capacity was only observed in the case of tubers stored at room temperature from 0 to 60 DOS, when compared with the storage at 4 ºC and -20 ºC as shown in Fig. 13. Our data is in concordance with the study conducted by Akyol et al., 2016.

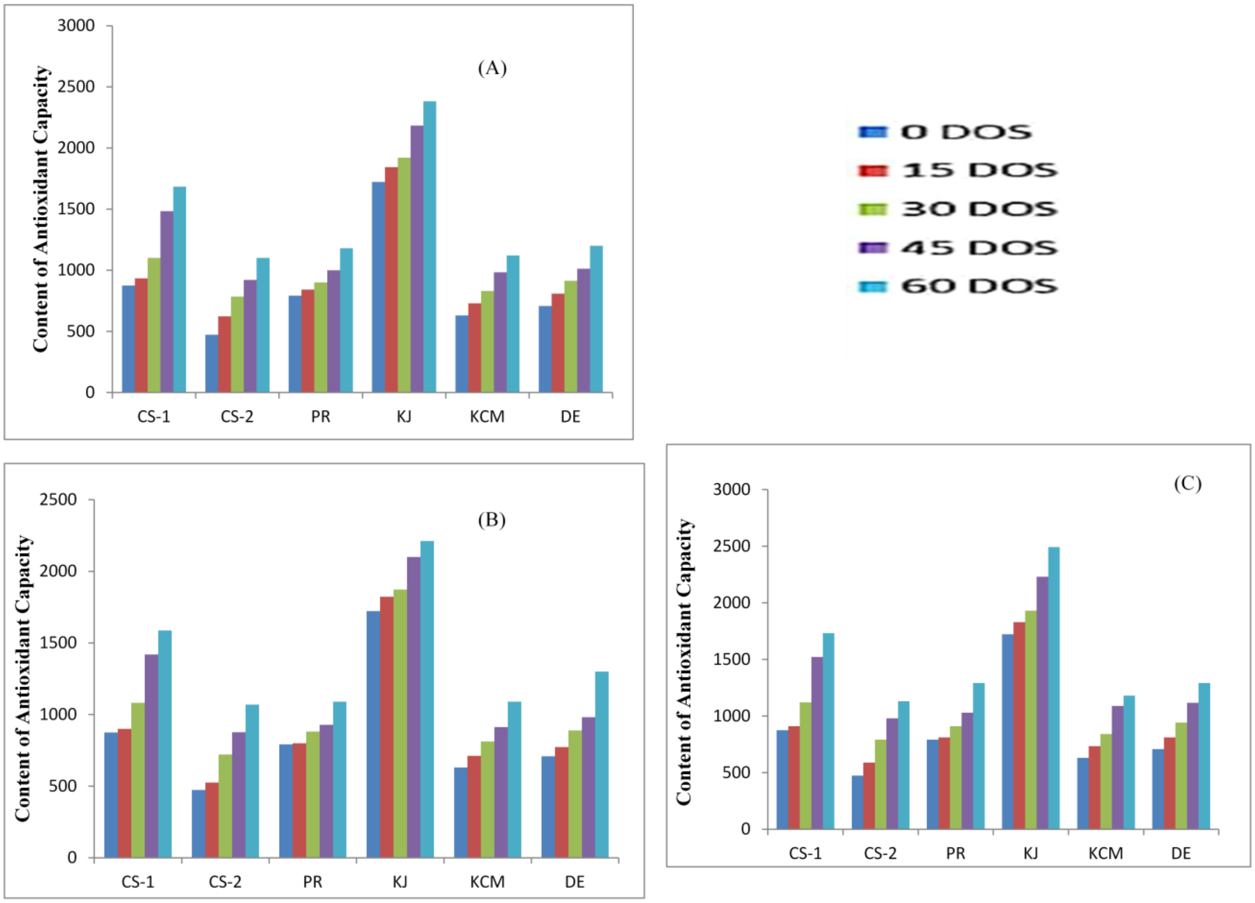

Fig. 13 Antioxidant capacity at different storage temperature (A) at room temperature (B) at $4^{\circ}C$ (C) at $-20^{\circ}C$

4.4 Cytotoxic effect of polyphenols from different potato cultivars

The cytotoxic activity of six PPEs (E1- CS-1, E2- CS-2, E3- PR, E4- KJ, E5- KCM, E6- DE) was evaluated on a PBMCs. The cells were treated with different concentrations of PPEs (0, 25, 50, 100, 200 and 400 equiv. /mL) for 24 h and then the cytoxicity was measured by the MTT assay. Fig reveals the viability rate of PBMCs treated with the extracts. All the experiments were validated by the incubation with 30% methanol in the absence of any cytotoxic effects, compared to non-treated cells. The values are calculated difference between measurement and reference measurement. The more the value, the more the purple crystals were formed. Hence, more the cells present. We took average of the readings for blank, control and positive control as readings were taken in duplicates. E3, E4 and E5 were selected for further analysis as they showed consistent results. Then, for each extract, analysis was done. Blank reading was subtracted from measured readings and the result was divided by control reading. Finally, E4 was selected based on the analysis as it showed the most consistent results. According to these results cultivar KJ showed highest cytotoxic activity at 400 µg GA equiv. / mL concentration. This is only a preliminary study to reveal the cytotoxic nature of the extracts which needs to be further investigated.

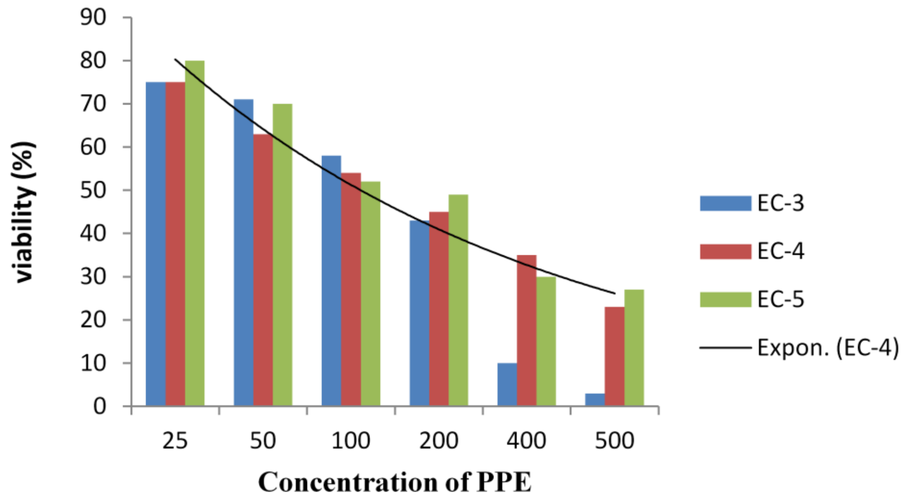

Fig. 14 Cytotoxic effects of PPE on PBMC

Concluding remarks: High dietary value and antioxidant capacity of potato tubers attract the attention of many researchers and nutritionists all over the world. Flavonoids content in the potato tuber contributes to the major nutritional aspects of human health. The salient features of this study include determination of polyphenol and flavonoid contents along with assessment of antioxidant capacity in the tubers and other potato tissues. These biochemical attributes were also studied under different storage conditions. The levels of these non-enzymatic antioxidants were found to vary between different tissues and across the cultivars. There was a steady increase of antioxidant capacity in the tubers depending on the duration of storage. The results suggest that non-enzymatic antioxidants are crucial for defense under stresses.

Highlights of the study

- Potato tubers at different stages of growth showed parabolic pattern in terms of both total phenolic content (TPC) and total flavonoid content (TFC).

- Both TPC and TFC contents were found to be increased from young to mature stage in the cases of leaf and stem.

- Antioxidant capacity showed bell shape pattern for tuber growth in most of the cultivars.

- Antioxidantive capacity of the mature leaf tissues was found to be more as compare to young and tender leaves; however, stem tissues showed reverse pattern.

- The phenolic extract of KJ was found to exhibit more cytotoxic effect.

References:

1. Agati, G., Azzarello, E., Pollastri, S., & Tattini, M. (2012). Flavonoids as antioxidants in plants: location and functional significance. *Plant Science*, *196*, 67-76.
2. Akyol, H., Riciputi, Y., Capanoglu, E., Caboni, M. F., & Verardo, V. (2016). Phenolic compounds in the potato and its byproducts: An overview. *International Journal of Molecular Sciences*, *17*(6), 835.
3. Alasalvar, C., Grigor, J. M., Zhang, D., Quantick, P. C., & Shahidi, F. (2001). Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. *Journal of Agricultural and Food Chemistry*, *49*(3), 1410-1416.
4. Andre, C. M., Ghislain, M., Bertin, P., Oufir, M., del Rosario Herrera, M., Hoffmann, L., ...& Evers, D. (2007). Andean potato cultivars (Solanum tuberosum L.) as a source of antioxidant and mineral micronutrients. *Journal of Agricultural and Food Chemistry*, *55*(2), 366-378.
5. Andreu, A. B. (2018) Chemical characterization of polyphenol extracts from Andean and indus-trial Solanum tuberosum tubers and their cytotoxic activity on human hepatocarcinoma cells.
6. Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.*, *55*, 373-399.Averyanov, A. A., & Lapikova, V. P. (1989). The interaction of sugars with the hydroxyl radical in relation to fungotoxicity of leaf excretions. *Biokhimiya*, *54*(10), 1646-1651.
7. Averyanov, A. A., & Lapikova, V. P. (1989). The interaction of sugars with the hydroxyl radical in relation to fungotoxicity of leaf excretions. *Biokhimiya*, *54*(10), 1646-1651.
8. Bachem, C., van der Hoeven, R., Lucker, J., Oomen, R., Casarini, E., Jacobsen, E., & Visser, R. (2000). Functional genomic analysis of potato tuber life-cycle. *Potato Research*, *43*(4), 297-312.
9. Barnes, J., Zheng, Y., & Lyons, T. (2002). Plant resistance to ozone: the role of ascorbate. In *Air pollution and Plant Biotechnology* (pp. 235-252). Springer, Tokyo.
10. Beckman, C. H. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants?. *Physiological and Molecular Plant Pathology*, *57*(3), 101-110.

11. Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Analytical Biochemistry*, *239*(1), 70-76.
12. Bohl, W. H., & Johnson, S. B. (Eds.). (2010). *Commercial Potato Production in North America: The Potato Association of America Handbook*. Potato Association of America.
13. Brown, C. R., Culley, D., Yang, C. P., Durst, R., & Wrolstad, R. (2005). Variation of anthocyanin and carotenoid contents and associated antioxidant values in potato breeding lines. *Journal of the American Society for Horticultural Science*, *130*(2), 174-180.
14. Burgos, G., Amoros, W., Muñoa, L., Sosa, P., Cayhualla, E., Sanchez, C., ...& Bonierbale, M. (2013). Total phenolic, total anthocyanin and phenolic acid concentrations and antioxidant activity of purple-fleshed potatoes as affected by boiling. *Journal of Food Composition and Analysis*, *30*(1), 6-12.
15. Burton, W. G. (1969). The sugar balance in some British potato varieties during storage. II. The effects of tuber age, previous storage temperature, and intermittent refrigeration upon low-temperature sweetening. *Potato Research*, *12*(2), 81-95.
16. Campos, D., Noratto, G., Chirinos, R., Arbizu, C., Roca, W., & Cisneros-Zevallos, L. (2006). Antioxidant capacity and secondary metabolites in four species of Andean tuber crops: native potato (Solanum sp.), mashua (Tropaeolum tuberosum Ruiz & Pavón), Oca (Oxalis tuberosa Molina) and ulluco (Ullucus tuberosus Caldas). *Journal of the Science of Food and Agriculture*, *86*(10), 1481-1488.
17. Chen, P. M., Burke, M. J., & Li, P. H. (1976). The frost hardiness of several Solanum species in relation to the freezing of water, melting point depression, and tissue water content. *Botanical Gazette*, *137*(4), 313-317.
18. Das, K., & Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science*, *2*, 53.
19. de Pinto, M. C., & De Gara, L. (2004). Changes in the ascorbate metabolism of apoplastic and symplastic spaces are associated with cell differentiation. *Journal of Experimental Botany*, *55*(408), 2559-2569.
20. Dennison, C., & Lovrien, R. (1997). Three phase partitioning: concentration and purification of proteins.*Protein expression and purification*, *11*(2), 149-161.

21. Evans, M. D., Dizdaroglu, M., & Cooke, M. S. (2004). Oxidative DNA damage and disease: induction, repair and significance. *Mutation Research/Reviews in Mutation Research*, *567*(1), 1-61.
22. Fini, A., Brunetti, C., Di Ferdinando, M., Ferrini, F., & Tattini, M. (2011). Stress-induced flavonoid biosynthesis and the antioxidant machinery of plants. *Plant Signaling & Behavior*, *6*(5), 709-711.
23. Flint, H. L., Boyce, B. R., & Beattie, D. J. (1967). Index of injury—a useful expression of freezing injury to plant tissues as determined by the electrolytic method. *Canadian Journal of Plant Science*, *47*(2), 229-230.
24. Foyer, C. H., & Noctor, G. (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell*, *17*(7), 1866-1875.
25. Fresco, P., Borges, F., Diniz, C., & Marques, M. P. M. (2006). New insights on the anticancer properties of dietary polyphenols. *Medicinal research reviews*, *26*(6), 747-766.
26. Galani, J. H. Y., Mankad, P. M., Shah, A. K., Patel, N. J., Acharya, R. R., & Talati, J. G. (2017). Effect of Storage Temperature on Vitamin C, Total Phenolics, UPLC Phenolic Acid Profile and Antioxidant Capacity of Eleven Potato (Solanum tuberosum) Varieties. *Horticultural Plant Journal*, *3*(2), 73-89.
27. Ghasemzadeh, A., Azarifar, M., Soroodi, O., & Jaafar, H. Z. (2012). Flavonoid compounds and their antioxidant activity in extract of some tropical plants. *Journal of Medicinal Plants Research*, *6*(13), 2639-2643.
28. Ghasemzadeh, A., Omidvar, V., & Jaafar, H. Z. (2012). Polyphenolic content and their antioxidant activity in leaf extract of sweet potato (Ipomoea batatas). *Journal of Medicinal Plants Research*, *6*(15), 2971-2976.
29. Halliwell, B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant physiology*, *141*(2), 312-322.
30. Haverkort, A. J. (1990). Ecology of potato cropping systems in relation to latitude and altitude. *Agricultural Systems*, *32*(3), 251-272.
31. Holländer-Czytko, H., Grabowski, J., Sandorf, I., Weckermann, K., & Weiler, E. W. (2005). Tocopherol content and activities of tyrosine aminotransferase and cystine lyase in Arabidopsis under stress conditions. *Journal of Plant Physiology*, *162*(7), 767-770.

32. Kim, T. H., Hauser, F., Ha, T., Xue, S., Böhmer, M., Nishimura, N., ...& Lee, S. (2011). Chemical genetics reveals negative regulation of abscisic acid signaling by a plant immune response pathway. *Current Biology*, *21*(11), 990-997.
33. Külen, O., Stushnoff, C., & Holm, D. G. (2013). Effect of cold storage on total phenolics content, antioxidant activity and vitamin C level of selected potato clones. *Journal of the Science of Food and Agriculture*, *93*(10), 2437-2444.
34. Kumar, D., Rawal, S., Singh, B. P., Chalkoo, S., & Kumar, R. (2013). Soil macronutrients influence antioxidant status in early season potatoes. *Vegetos*, *26*, 311-317.
35. Lachman, J., Hamouz, K., Sulc, M., Orsák, M., & Dvorak, P. (2008). Differences in phenolic content and antioxidant activity in yellow and purple-fleshed potatoes grown in the Czech Republic. *Plant Soil and Environment*, *54*(1), 1.
36. Lemos, M. A., Aliyu, M. M., & Hungerford, G. (2015). Influence of cooking on the levels of bioactive compounds in purple majesty potato observed via chemical and spectroscopic means. *Food Chemistry*, *173*, 462-467.
37. Lewis, C. E., Walker, J. R., Lancaster, J. E., & Sutton, K. H. (1998). Determination of anthocyanins, flavonoids and phenolic acids in potatoes. I: Coloured cultivars of Solanum tuberosum L. *Journal of the Science of Food and Agriculture*, *77*(1), 45-57.
38. Li, P. H. (1977). Frost killing temperatures of 60 tuber-bearingSolanum species. *American Potato Journal*, *54*(9), 452-456.
39. Mäder, J., Rawel, H., & Kroh, L. W. (2009). Composition of phenolic compounds and glycoalkaloids α-solanine and α-chaconine during commercial potato processing. *Journal of Agricultural and Food Chemistry*, *57*(14), 6292-6297.
40. Madiwale, G. P., Reddivari, L., Holm, D. G., & Vanamala, J. (2011). Storage elevates phenolic content and antioxidant activity but suppresses antiproliferative and pro-apoptotic properties of colored-flesh potatoes against human colon cancer cell lines. *Journal of Agricultural and Food Chemistry*, *59*(15), 8155-8166.
41. Marquardt, J. U., Galle, P. R., & Teufel, A. (2012). Hepatocellular carcinoma: molecular pathogenesis and novel targets for therapy. *Deutsche Medizinische Wochenschrift (1946)*, *137*(16), 855-860.

42. Marwaha, R. S., Pandey, S. K., Kumar, D., Singh, S. V., & Kumar, P. (2010). Potato processing scenario in India: industrial constraints, future projections, challenges ahead and remedies—a review. *Journal of Food Science and Technology*, *47*(2), 137-156.
43. Møller, I. M., Jensen, P. E., & Hansson, A. (2007). Oxidative modifications to cellular components in plants.*Annu. Rev. Plant Biol.*, *58*, 459-481.
44. Morelli, R., Russo-Volpe, S., Bruno, N., & Lo Scalzo, R. (2003). Fenton-dependent damage to carbohydrates: free radical scavenging activity of some simple sugars. *Journal of Agricultural and Food Chemistry*, *51*(25), 7418-7425.
45. Mullineaux, P. M., & Rausch, T. (2005). Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. *Photosynthesis Research*, *86*(3), 459-474.
46. Navarre, D. A., Pillai, S. S., Shakya, R., & Holden, M. J. (2011). HPLC profiling of phenolics in diverse potato genotypes. *Food Chemistry*, *127*(1), 34-41.
47. Ngadze, E., Coutinho, T. A., Icishahayo, D., & Van der Waals, J. E. (2014). Effect of calcium soil amendments on phenolic compounds and soft rot resistance in potato tubers. *Crop Protection*, *62*, 40-45.
48. Reddivari, L., Hale, A. L., & Miller, J. C. (2007). Determination of phenolic content, composition and their contribution to antioxidant activity in specialty potato selections. *American Journal of Potato Research*, *84*(4), 275.
49. Schieber, A., & Saldaña, M. D. (2009). Potato peels: a source of nutritionally and pharmacologically interesting compounds-a review.
50. Smirnoff, N. (2000). Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Current Opinion in Plant Biology*, *3*(3), 229-235.
51. Struik, P. C. (2007). Above-ground and below-ground plant development. In *Potato Biology and Biotechn.*