Biochemical studies on the non-enzymatic antioxidants in the potato (*Solanum tuberosum* L.) cultivars, and cytotoxic activity of polyphenol extract on animal cell lines

A
Dissertation
Submitted in the partial fulfilment of the requirement for the award of degree of
Master of Technology
In
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Submitted by:
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Candidate's Declaration

I, hereby declare that the work which is being presented in this entitled "**Biochemical studies on the non-enzymatic antioxidants in the potato (*Solanum tuberosum L.*) Cultivars, and cytotoxic activity of polyphenol extract on animal cell lines**" in the partial fulfilment of the requirement for the reward of the degree of Master of Technology in Biotechnology, 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.

Ritika Ghai

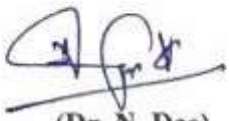
Date: 26-08-2019

Place: Patiala

(Ritika Ghai)

Certificate

This is to certify that the dissertation entitled "**Biochemical studies on the non-enzymatic antioxidants in the potato (*Solanum tuberosum L.*) Cultivars, and cytotoxic activity of polyphenol extract on animal cell lines** "submitted by **Ritika Ghai** (Regd. No. 601704004) in the partial fulfilment of the requirement for the reward of the degree of Master of Technology in Biotechnology, 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.



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Ritika Ghai

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List of abbreviations

Abbreviation	Name
AA	Ascorbic Acid
$AlCl_3$	Aluminium Chloride
APX	Ascorbate peroxidase
AS	Kufri Ashoka
BSA	Bovine Serum Albumin
CAT	Catalase
CS-1	Kufri Chipsona-1
CS-2	Kufri Chipsona-2
DE	Desiree
DHAR	Dehydroascorbate reductase
DNPH	Dinitrophenyl hydrazine
FRAP	Ferric reducing antioxidant potential
$FeCl_3$	Iron Chloride
g	Gram
GA	Gallic acid
GPX	Guaiacol peroxidase
GR	Glutathione Reductase
GSH	Reduced glutathione
H_2O_2	Hydrogen Peroxide
HPO_3	Metaphosphoric acid
kDa	Kilo-dalton
KJ	Kufri Jyoti
LPO	Lipid peroxidation
MDHAR	Monodehydroascorbate reductase
mg	Milligram
mL	millilitre
mM	Millimolar
MTT	3-(4,5-dimethylthiazolyl-2)- 2,5-diphenyltetrazolium bromide
Na_2CO_3	Sodium bicarbonate
nm	Nanometre
$NaNO_2$	Sodium Nitrite
NaOH	Sodium Hydroxide
$OH^{\bullet}$	Hydroxyl radical
$^1O_2$	Singlet Oxygen
PPE	Polyphenolic extract
PR	Kufri Pukraj
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SOD	Superoxide Dismutase
$SC(NH_2)_2$	Thiourea
TPC	Total Phenolic content
TPTZ	2,4,6-tripyridyl-s-triazine

Abstract

During aerobic metabolism in plants, the cellular compartments like chloroplast, mitochondria and peroxisomes generate a number of reactive oxygen species (ROS). Earlier it was regarded as the toxic by-products of aerobic metabolism. Currently, it is believed that ROS plays dual role; at low concentration they are involved in cell signalling and at high concentration they cause damage to various biomolecules, cellular structures and even cell death. As revealed by the numerous reports, a wide variety of antioxidants are associated with active defense under stress conditions as they can potentially scavenge the reactive oxygen species (ROS) to less harmful products. Various enzymatic and non-enzymatic antioxidants are involved overcoming the effects of ROS. The objectives of the study were a) estimation and comparison of ascorbic acid, phenolic and flavonoid contents; antioxidative capacity; sugar content; total protein content in different organs of potato cultivars at different stages of growth under field condition and also in the mature tubers under different storage conditions; b) To study the cytotoxic effects of polyphenolic extracts on animal cell lines namely, MCF-7 and RAW 264.7. It was observed that the ascorbate content was highest in mature leaves and lowest in mature tubers. The ascorbate content was found to be gradually decreased during storage (up to 60 days) under different experimental temperatures. Phenolic and flavonoid contents and overall antioxidative capacities were found to be at significantly higher level in the growing potato organs in comparison to the matured ones. Prolonged storage at room temperature, in particular, for a period of 60 days led to increase of total phenolic and flavonoid contents significantly in the harvested mature tubers. As expected, total protein content was found to be at higher in the young leaves as compared to the other organs. Protein content was found to be gradually decreased in the tubers during storage under different conditions. On the contrary, soluble sugar contents (both reducing and total sugars) were significantly increased during storage at low temperatures. This explains why potatoes becomes sweeter during storage. Apart from the select potato cultivars, all these assays were also done on some potato varieties collected from the local markets termed as 'Market selection' which included MS1 (purple coloured skin), MS2 (red coloured skin), MS3 (Agra variety), MS4 (Pahadi aaloo), MS5 (locally grown). MS1 and MS2 were found to contain higher levels of polyphenol, flavonoid and ascorbate contents. Polyphenolic extracts were to be effective in reducing the viability of the cell lines namely, MCF-7 (human breast cancer cell lines) and RAW 264.7 (Murine macrophage cell line).

Keywords: Potato (*Solanum tuberosum* L.) cultivars, Non-enzymatic antioxidants, Ascorbic acid, Polyphenols & Flavonoids, Antioxidant capacity, Sugars, Protein, Cell lines

CHAPTER-1: INTRODUCTION

1.1 About ROS

Reactive oxygen species (ROS) are the by-product of aerobic metabolism. They are partially reduced or excited forms of atmospheric oxygen (O_2). ROS are free radicals possessing the ability to cause various deleterious effects to cell leading cell injury and even cell death. Various studies have proven that there reactive molecules are also important in cell signaling pathways and metabolism. Nowadays, ROS are considered to have dual functions. At basal level; they influence some physiological processes through bio-signaling (a few is regarded as secondary messengers) and at higher level, they cause oxidative damage to various cellular components like nucleic acids (DNA and RNA) lipids, proteins, plasma membranes (Foyer and Noctor et al., 2005) leads to cell injury and cell death. ROS are generated in various cell compartments sites such as mitochondria chloroplast, peroxisomes, apoplast, and endoplasmic reticulum ROS are necessary for the progression of several basic biological processes, including cellular proliferation and differentiation. At basal levels ROS is not harmful and is removed by concomitant action of antioxidant machinery of the cells. Drought, salinity, metal toxicity, UV radiation are the various environmental factors which leads to ROS generation in plants. Oxidative stress results in physiological or programmed pathway for cell death triggered by ROS. Types of ROS, their cellular production sites, targets, and scavenging mechanism mediated by branches of the antioxidant systems, highlights the potential role of antioxidants in stress tolerance and cellular survival. To develop genetically engineered stress-tolerant plants, strategies are used using a knowledge of ROS action and their regulation on antioxidants (Das and Roy Choudhury 2014).

1.2 Types of ROS

Various types of ROS include the following:

Superoxide radical ($O_2^{\cdot -}$): Superoxide radical or superoxide anion is the first radical produced in the stress condition. It is generated in photo system one (PS-1) during non-cyclic electron transport chain in chloroplast and is scavenged by enzymatic antioxidant i.e. superoxide dismutase It reacts with double bond- containing compounds such as iron-sulphur proteins via the Fe- center. They are mainly present in compartments like chloroplasts and mitochondria and can migrate up to a distance of 30 nm and have short half-life($t_{1/2)}$ of 1-4μs (Halliwell, 2006).

Hydroxyl radical ($OH^{\cdot}$): It is a highly reactive ROS with short half-life ($t_{1/2}$) of 1µs and migration distance of 1 nm. It rapidly reacts with DNA and proteins and scavenged by non – enzymatic antioxidants like flavonoid and proline. It is produced in cells organelles like the chloroplasts and Mitochondria by lipid peroxidation (LPO), protein damage and membrane destruction (Pinto et al., 2003).

Hydrogen peroxide (H_2O_2): H_2O_2 is moderately reactive in nature and has half- life ($t_{1/2}$) 1ms and migration distance of 1µm. It is produced under normal as well as under stress conditions. It causes cellular damages by oxidizing proteins. Catalases and various peroxidase are the enzymatic scavengers of hydrogen peroxide. It is generated in mitochondria, chloroplasts, membranes and peroxisomes (Sharma et al., 2012).

Singlet Oxygen ($^1O_2$): Singlet oxygen oxidizes the proteins, polyunsaturated fatty acids (PUFAs) and DNA and it reacts with Glycine (G) residue and attacks with amino residues like Trp, His, Tyr, Met, and residues and it get scavenged by non-enzymatic antioxidants like carotenoids and α- tocopherol and has half-life ($t_{1/2}$)1-4 µs and migration distance of 30 nm and it is present in membranes, chloroplasts and mitochondria (Hatz et al., 2007).

1.3 Targets of ROS

Effects of ROS leads to the mutation in the genetic material DNA, by depurination, depyrimidination, change in the base pair, thus effecting the crosslinking of the strands of DNA. Whereas in the case of proteins (the building blocks of the cell) changes in the specific amino acids, disrupts the function of proteins, leading to truncated form. Hence disrupting the function of the protein.

Lipids enhance the membrane fluidity and permeability; any change leading to breakage or disruption of lipid chain; leads to the leakage of cell membrane

1.4 Sites of ROS production:

ROS is produced in all major cell organelles such as mitochondria, chloroplast, plasma membrane, endoplasmic reticulum, peroxisomes and cell wall. In the presence of light Chloroplasts and peroxisomes are the major sites of ROS production; whereas in dark mitochondria becomes the main site of ROS production. (Choudhary et al., 2013). ROS are generally termed as consequence of electron transport activities in mitochondria, plasma membrane and chloroplast and generated during various metabolic pathways occurring inside the cellular compartment as shown in Fig.1.

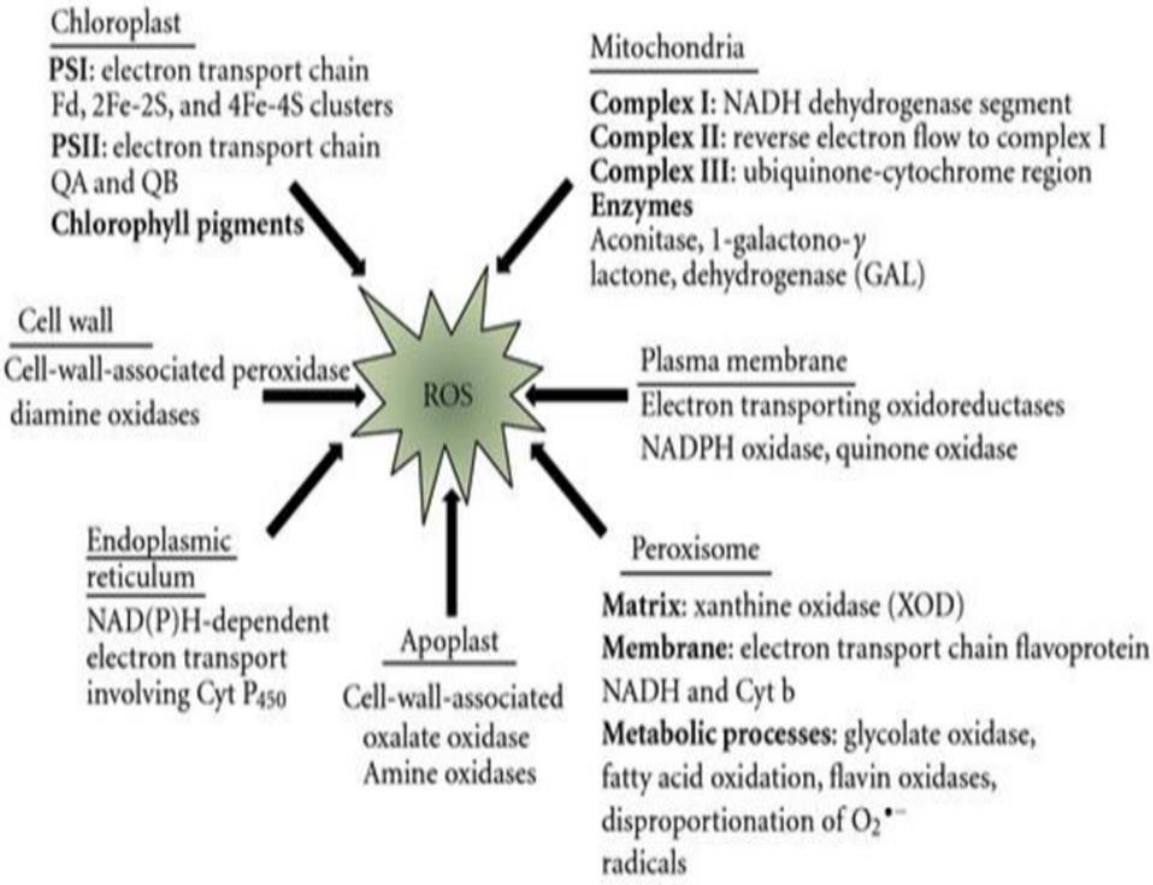


Fig. 1 Sites of ROS Production (Sharma et al., 2012)

1.5 ROS defense machinery

The cell has developed some pathways to mitigate the effect of ROS and to save cell organelles from damage. It is an antioxidant machinery that scavenge ROS and mitigate the damages caused by them. The antioxidants machinery consists of enzymatic components and non-enzymatic antioxidants that works in a coordination (Das and Roychoudhury 2014).

Enzymatic Antioxidants:

Superoxide dismutases (SOD): SOD (E.C.1.15.1.1) forms the first line of defense against ROS induced damages. It requires cofactors such as manganese, copper or zinc for its antioxidant activity. It converts two superoxide ions into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). It is also up- regulated by abiotic stress conditions (Boguszewska et al., 2010).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow 2H_2O_2 + O_2 \quad (1)$$

Catalase (CAT): CAT (E.C.1.11.1.6) is an important antioxidant enzyme. It is ubiquitous tetrameric enzyme containing heme group and catalyzes the dismutation of H_2O_2 to form water and molecular oxygen. It is highly specific for H_2O_2 and shows weak activity against organic peroxides (Percy et al., 1984). It works when the H_2O_2 level are high above the basal level. This plays a crucial role in maintaining the function of the cell. It is found in other subcellular compartments such as chloroplast, mitochondria, cytosol, and peroxisomes (Mhamdi et al., 2010).

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (2)$$

Guaiacol peroxidase (GPX): GPX (E.C.1.11.1.7) heme-containing enzyme consists of 40–50 kDa. It is a key enzyme in extracellular removal of hydrogen peroxide and degradation of hydrogen peroxide both at normal metabolism as well in stress. (Asada, 1999).

$$H_2O_2 + GSH \rightarrow H_2O + GSSG \quad (3)$$

Ascorbate Peroxidase (APX): APX (E.C.1.1.11.1) reduces hydrogen peroxide into water and DHA using ascorbic acid (AA) as a reducing agent. It is an efficient scavenger under various stress conditions found in cytosol, mitochondria, peroxisomes, and chloroplast (Sharma et al., 2004).

$$H_2O_2 + AA \rightarrow 2H_2O + DHA \quad (4)$$

Monodehydroascorbate reductase (MDHAR): MDHAR (E.C.1.6.5.4) regenerates ascorbic acid from MDHA which is short lived using NADPH as a reducing agent. It is present in Mitochondria, cytoplasm, and chloroplast (Mittler et al., 2002).

$$MDHA + NADPH \rightarrow AA + NADP^+ \quad (5)$$

Dehydroascorbate reductase (DHAR): DHAR (M.C.1.8.5.1) uses reduced Glutathione (GSH) as an electron donor and it reduces dehydroascorbate (DHA) to ascorbic acid. It is present in Mitochondria, cytoplasm, and chloroplast and also found in roots, shoots, green and etiolated shoots (Chen and Gallie, 2006).

$$DHA + 2GSH \rightarrow AA + GSSG \quad (6)$$

Non Enzymatic Antioxidant machinery include the following:

Ascorbic acid: Ascorbic acid (AA) called as vitamin C considered as a most abundant and powerful antioxidant. It can donate electrons thus making it the first line of defence against ROS. Ascorbate is present in both cytosol and apoplast. It detoxifies H_2O_2 via action of ascorbate peroxidase. It is also a major component of human nutrition and it is a water soluble antioxidant in humans and electron donor in nature (Upadayatty et al., 2009). It acts as a coenzyme and used as a reducing agent in various metabolic pathways and helps in maintaining normal immune system and play a great role in maintaining bones and connective tissue. Ascorbic acid biosynthesis occurs through D-mannose/ L- galactose which further gets oxidised to form precursor of ascorbic acid i.e. L- galactono-γ- lactone (Wheeler et al., 1988). Primary component in Ascorbic acid synthesis is galacturonic acid (Agius et al., 2003).

Polyphenols: Polyphenols are considered as secondary metabolites of plant possess various health benefits to humans and it presence affects taste, colour and texture of plant product and act as antioxidant, anti-cancer, anti-microbial, anti-inflammatory agents. Pigmented potato have high level of antioxidant as compared to non-pigmented potatoes. It lowers the risks of certain diseases such as cancer, diabetes, and cardiovascular diseases (Akyol et al., 2016).

Flavonoids: Flavonoids are phenolic compound which inhibit lipid peroxidation and lipoxygenase activity. It is present in leaves, pollen grains and floral organs. They are divided into four classes like flavonols, flavones, iso-flavones and anthocyanins on the basis of their structures. Main role of flavonoid is to scavenge damaged caused by ROS to photosynthetic apparatus. They play role in various processes in plants like pigmentation in seeds, fruits and flowers. They are also involved in germination of pollen, plant fertility and defence against plant pathogens (Fini et al., 2011).

α -Tocopherol: It is also called as vitamin E has highest antioxidant capacity. It helps in scavenging of ROS and lipid radicals and belongs to lipophilic family. It detoxifies products of membrane LPO (Czytko et al., 2005).

Carotenoid: It belongs to family of the lipophilic antioxidants like α -tocopherol and present in plastids of both photosynthetic and non-photosynthetic plant tissues. Absorbs 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 (Agati et al., 2012).

Proline: Proline is basically an amino acid with imidiazole ring. It is an osmolyte and regarded as powerful antioxidant. It efficiently scavenges hydroxyl radical and singlet oxygen and prevent damages due to LPO. Due to reduced degradation or enhanced synthesis proline accumulates in large amounts in plants (Verbruggen and Hermans, 2008).

1.6 About Potato Plant

Potato (*Solanum tuberosum L.*) is the important staple and non- grain food crop found in many countries including Indian subcontinents. It is the fourth largest food crop in the world after rice, wheat and maize and India ranks second position in term of production, and third with regard to area of cultivation (Bachem et al ., 2000).It requires sufficient water and sensitive to drought stresses. Pigmented potatoes as compared to white, yellow have shown to contain higher levels of antioxidants. It grows well at 7-30 °C and harvesting occur during February to March and it grows well in fertile soil with pH 5.0-5.5. Different vitamins are also present in potatoes, among them vitamin C (ascorbic acid) is most abundant and powerful antioxidant, which needs to be studied extensively. Good source of vitamin C and minerals such as potassium, phosphorous and magnesium and contains adequate dietary antioxidants. Tuber contains ~80% water and ~20% is dry matter out of which 60 to 80% of dry matter is starch. Protein content is similar to cereals and has very low fat content. It is available throughout the year to the customers as they are being stored in cold storage under appropriate temperature conditions so that nutritional quality and freshness should be preserved (Marwaha et al., 2010) (usually <10°C) that helps in slow degradation of starch and maintain accumulation of sucrose with reducing sugar glucose and fructose. This process is known as cold-induced sweetening (Burton, 1969). Member of *Solanaceae* family and it also include various plant like tobacco, chilli pepper, eggplant, tomato and petunias. It is an aromatic herb with tuber having stolon and perennial, erect and grows upto 1m.This plant produce every year and it develops into fibrous root system and produce tuber from underground portion of the stem. Plant bears flowers of various colours like white, pink, purple and blue colour and yellow anther having 2.5cm in diameter. Various varieties of plant having different morphological characteristics like shape, size, and skin colour (Dennison and Loveria, 1997). Eyes which are present outside the tuber with scars of scale leaves are the auxiliary buds. These eyes turns into stem to form the vegetative iteration when tubers are planted (Struik et al., 2007).

1.7 Animal cell lines

Polyphenols, the plant metabolite playing role in ROS, also have health benefits. Several studies have reported that polyphenol being an antioxidant might lower the risk of developing some health risks like diabetes, heart diseases and cancer (Andre C et al., 2007). MCF-7 is a breast cancer cell line was established in 1973 by Dr. Soule and colleagues at the Michigan cancer foundation. It was isolated from the pleural effusion of 69 year old woman, with a disease in a metastatic state. It is commonly a breast cancer cell line that has been used and propagated by multiple group to study the effect of various antioxidants in it with time, more data bank has been created for the care of breast cancer patients. It is ER positive progesterone receptor (PR) positive cell line belonging to the luminal. It is a molecular sub type, poorly aggressive and non- invasive cell line with low metastatic potential. Hence it is used widely in different types of studies (Comsa, S et al., 2015).Raw cell lines are very fast growing cell lines which mean it is easy to propagate and has high efficiency for a DNA to transfect and it is sensitive to RNA interference and which supports murine noroviruses replication. These cell line was derived from a tumour and has monocyte/ macrophage –like cells which is induced by Abelson murine leukaemia virus and capable for performing pinocytosis and phagocytosis. These cells are able to kill the target cell by antibody dependent cell cytotoxicity (ADCC). It was reported that phenotype and functional characteristics was remain stable from passage no.10 to passage no.30. Our current study mainly focus on to check the cytotoxic activity of polyphenol extract on animal cell lines i.e. MCF-7 and RAW264.7 (Taciak, B et al., 2018).

Chapter- 2: Review of Literature

2.1 Reactive Oxygen Species

ROS plays the dual role of being the ineludible derivate of aerobic metabolism such as respiration and photosynthesis occurring in various organelles of the cell like peroxisomes, mitochondria and chloroplasts on one side and serving as a marker during stressful conditions on other side (Apel and Hirt, 2004). Different type of free radicals such as superoxide anion radical ($O2^{-}$), Hydrogen Peroxide (H_2O_2), Hydroxyl radical (OH.), and Singlet oxygen ($^1O_2$) which plays a significant role in ROS metabolism. Scavenging mechanism conciliated by enzymatic and non- enzymatic antioxidants leads to abiotic stress tolerance, cellular survival and thus could help to develop the strategies related to genetically engineer stress tolerant plant. Several issues during multiple environmental stresses regulation and their interaction with ROS and calcium signalling remain unanswered. So in future advanced molecular techniques is useful for better understanding of ROS metabolism like markers with Ca^{2+}. So various non-enzymatic antioxidants were studied till date and also have many applications in day to day life (Das and Roychoudhury, 2014).

2.2 Introduction to ascorbic acid

Vitamin C (L-Ascorbic acid) is a major component of human nutrition. It is a white, crystalline solid and consists of two asymmetric carbon atoms, C-4 and C-5. Plants and several animals can synthesize their own ascorbic acid but humans cannot. It is an important metabolite which plays a crucial role against the stress in plants. It occurs in both reduced form (ascorbic acid) and oxidized form (dehydroascorbic acid) (Kapur et al., 2012). It is a vital antioxidant in detoxification of various reactive oxygen species. Hence it acts as a good scavenger of free radicals and other oxygen derived species by oxidising them. Being a powerful antioxidant, ascorbic acid not only scavenges free radicals but also regenerates vitamin E from its oxidized form and prevents lipid peroxidation. Rich sources of vitamin C include various citrus fruits like kiwi, mango and vegetables like tomatoes, potatoes and broccoli. It is a food additive and its deficiency causes scurvy.

2.3 Ascorbic acid biosynthesis and mode of action

Ascorbic acid biosynthesis in plants takes place through D- mannose /L- galactose pathway i.e. majorly known as Smirnoff- Wheeler pathway (Wheeler et al., 1998; Agius et al., 2003). It converts the D- mannose first into GDP- D-mannose and then into GDP- L-galactose and then

further oxidation of L- galactose results in the formation of precursor to ascorbic acid that is L– galactono-γ – lactone (Wheeler et al., 1998). Apart from this pathway, with the help of galacturonic acid some of the ascorbic acid biosynthesis takes place. Oxidation of ascorbic acid takes place via two consecutive steps. First of all, it gets oxidised to form monodehydroascorbate (MDHA) which further gets converted either immediately into ascorbate or breaks into ascorbic acid and dehydroascorbate (DHA). In other words, we can say that ascorbic acid on losing one electron, forms monodehydroascorbate (MDHA) which is highly unstable in nature. This MDHA before further getting reduced, breaks down into Ascorbic acid and dehydroascorbate (DHA). Also it has been studied that with the help of several reductases, MDHA and DHA can be reduced back to Ascorbic acid. To prevent the oxidative damage ascorbic acid ultimately reacts with various free radicals like hydrogen peroxide, hydroxyl radical and superoxide radical as shown in Fig.2.

Fig. 2 Biosynthesis of L- ascorbic acid in plants (Hemavathi et al., 2009)

2.4 Ascorbic acid as an antioxidant

Ascorbic acid is an important antioxidant which protects the plants against the oxidative damage caused by various free radicals like superoxide anion, hydrogen peroxide and hydroxyl radical. It helps in maintaining an antioxidant α- tocopherol in reduced state (Packer et al., 1979). Increased oxidation and insufficient regeneration makes the ascorbic acid pool highly oxidised during stress conditions.

2.5 Polyphenols

Polyphenol is one of the secondary metabolites of the plants, consisting of an aromatic ring with one or more hydroxyl substituents. The weight of polyphenols ranges from 500-4000 Da (Beckman C, 2000). On the basis of their chemical constituents and structures, they are divided into different type's likes flavonoids, phenolic acid, tannins, stilbenes, coumarins, and lignins (Lemos et al., 2015). Polyphenols affects the various attributes of plant fruit organs such as colour, texture and taste (Akyol et al., 2016). It is found in both plants and animals. In plants it is found in tissues such as leaf tissues, fruits, flowers, and bark layers and play important role in various plant metabolism such as prevention against the microbial infection, suppression and release of growth hormones such as auxin which helps in ripening of fruits and vegetables and plant pigmentation (Alasalvar et al., 2001). In animals, it is present in arthropods such as crustaceans and insects which results in epicuticle hardening. Polyphenols are presents in citrus fruits which consists of flavonones, and present in vegetables such as tomatoes, onions, carrots and potatoes (Reddivari et al., 2007). The potatoes contains highest polyphenolic content as compared with other vegetables. High levels of antioxidants present in fruits and vegetables are beneficial for human health (Kulen et al., 2013).

There are many factors such as stage of maturity, presence of water or moisture during cooking and cooking temperature and time that affect the loss of phenolic compounds. Polyphenolic 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). Chlorogenic acid is found more in warm location with regular period of drought in comparison to high altitude and also enhances the amount of calcium in potatoes (Ngadze et al., 2014). Polyphenolic content of potato spectate the cell cytotoxic activities has been reported (Andre et al., 2007). Positive correlation between polyphenol in-take and inhibition in proliferation of breast and colon

cancer (Madiwale et al., 2011). Blood glucose level was decrease by the use of freeze dried potato. High bioactive compounds are located in the potato skin (Scheiber and Saldana, 2009).

2.6 Flavonoids

Flavonoids belongs to class of secondary metabolites having polyphenolic structure consisting of a 15 carbon skeleton, which have two phenyl rings and heterocyclic ring. They are the most important phenolic compounds in plants. Flavonoids consists of six subclasses namely flavones, flavan-3-ols, flavonols, anthocyanidins, flavanones, isoflavones and can also be found as aglycones. Most flavonoids are attached to sugars compounds (glycosides). Most abundant flavonoids present in potato is catechin, ranges to 204mg/100g dry weight. Quercetin and kaempferol rutinose are the flavonols are also present in potato tubers. More than 30 mg per 100 g fresh weight were flavonoids in white fleshed potatoes and due to presence of anthocyanins flavonoids level is doubled in purple and red colour fleshed potatoes (Burgos et al., 2013). Various types 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 reported that heating cause change in content of anthocyanin but did not effect to phenolic acid. Their presence influence the flavour and colours which is present mostly in fruits and vegetables. In plants they protect photosynthetic apparatus from damage in plants by scavenging ROS due to the excess excitation energy (Fini et al., 2011). It associated with a broad spectrum of health-promoting effects because of antioxidant, anti-mutagenic, anti-carcinogenic and anti-inflammatory properties.

2.7 Antioxidant Capacity

Antioxidant capacity is defined as the ability to assess the antioxidant status of various biological samples and evaluate their antioxidant response against the free radicals produced in the particular disease (Rubio et al., 2016). It is measured based on the abilities of different substances to scavenge $ABTS^{\cdot +}$radical cation compared with standard antioxidant (Miller et al., 1996).

FRAP assay: Ferric reducing antioxidant power (FRAP) assay is a widely used system where Fe^{3+} is reduced to Fe^{2+} in a redox-linked colorimetric response uses antioxidants as reductant. Ferric (Fe^{3+}) TPTZ complex which is colourless 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). Absorbance of the sample was taken at 593nm.The level of antioxidant activity was measured on the basis of reduction of Fe^{3+} to Fe^{2+}.

2.8 Role of sugars in plant defence against oxidative stress

Sugars are the primary products of plants and highly sensitive to stresses and regulates the translation of carbohydrates from the source to sink tissues. Sucrose along with other hexoses plays a dual function in gene regulation .Sugar signalling pathways effects the plant metabolism by interacting with stress pathways (Rosa et al., 2009). Link between the ROS production and photosynthetic metabolism is very important which leads to anti oxidative defense mechanism; this balance prevents cellular damage (Couee et al., 2005).Thus soluble sugar plays a crucial role in stress induced signals by acting like hormones which act as primary messengers. So there role is crucial in plant development. To estimate total soluble sugars in different stages of plant growth, could be a step forward in understanding ROS metabolism. Hence; in this study estimation of sugars was done along with non-enzymatic antioxidants.

2.9 MCF-7 and Raw cell lines

MCF-7 which is a breast cancer cell line it shows that anti-cancerous extracts activity has ability to inhibit the growth of the cells and it also induce apoptosis in MCF-7 cancer cells through intrinsic and extrinsic pathways. Polyphenolic tuber extract had an important effect on cell proliferation. It was reported that raw cell lines did not have any cytotoxic effects and helps in increased the cell viability. Higher the concentration of raw cell lines high amount of extract which could reduce the viability of macrophages cells. It is commonly a breast cancer cell line that has been used and propagated by multiple group to study the effect of various antioxidants in it with time, more data bank has been created for the care of breast cancer patients. It is ER positive progesterone receptor (PR) positive cell line belonging to the luminal. It is a molecular sub type, poorly aggressive and non- invasive cell line with low metastatic potential. Hence it is used widely in different types of studies (Comsa, S et al., 2015)

2.10 Studies on ascorbic acid and polyphenol till date

Ascorbic acid: Ball et al., 1994 studied the biological and chemical nature of the water-soluble vitamins, in human nutrition. Verma, et al., 1996 studied the solid-phase extraction clean-up for determining dehydroascorbic acid and ascorbic acid by titration with 2, 6-dichlorophenolindophenol. Friedman et al., 1997 studied the Dietary role, and biochemistry of Potato Polyphenols. Sataya et al., 1998 found the Photometric methods for the determination

of vitamin C. Nyyssönen et al., 2000 found the ascorbic acid, in modern chromatographic analysis of vitamins. Xia et al., 2003 studied the Fluorimetric determination of ascorbic acid with o-phenylenediamine. Agius et al., 2003, found that engineering increased vitamin C levels in plants by overexpression of D galacturonic acid reductase. Casella et al., 2006 studied the rapid enzymatic method for vitamin C assay in fruits and vegetables using peroxidase. . Khan et al., 2006 studied the simple UV-spectrophotometric method for the determination of vitamin C content in various fruits and vegetables.

Polyphenols: Geleijnse et al., 2008 studied cardiovascular health and the flavonoids. Eiten miller et al., 2008 studied the Vitamin analysis for the food sciences and health. Hale, et al., 2008 studied the interspecific variability for phenolic content and antioxidant capacity among Solanum species Hemavathi et al., 2009, investigated that enhanced ascorbic acid accumulation in transgenic potato confers tolerance to various abiotic stresses. Madiwale et al., 2011 studied the Storage elevates antioxidant activity and phenolic content but suppresses antiproliferative and pro-0apoptotic properties of coloured-flesh potatoes against human colon cancer cell line. Albishi et al., 2013 studied the antioxidant capacity and phenolic content of selected potato varieties and their processing by-products. Tejeda et al., 2014 studied the Relating genes in the biosynthesis of the polyphenol composition of Andean coloured potato collection. Galani et al., 2016 studied the effect of storage temperature on total phenolics, Vitamin C, UPLC phenolic acid profile and antioxidant capacity of eleven potato varieties.

2.11 Origin of the problem

Ascorbic acid (vitamin C) and polyphenols along with flavonoid are effective antioxidant against oxidative stress. Despite knowing the fact that during harvest, a considerable amount of vitamin C and polyphenols is present in the potatoes, very less is known about decrease in ascorbic acid content at low temperatures conditions (Kumar et al., 2013). It is not known clearly the level of ascorbic acid during period of tuber growth and development and subsequently storage. Storage of potato tubers at low temperature affects the metabolism and alter the phytochemical properties. We need to determine the changes of the levels of various antioxidants during storage of tubers. No comprehensive report available with regard to above aspects in the Indian potato varieties. Keeping in view, this study focused mainly on non-enzymatic antioxidants namely ascorbic acid and polyphenol.

2.12 Objectives of the study

As discussed earlier, under stresses an imbalance between production and scavenging of ROS lead to deleterious consequences where various antioxidants play crucial role in cell defense. This study focused mainly on non-enzymatic antioxidants namely, ascorbic acid, polyphenols and flavonoids. Antioxidative capacities were measured in different potato organs. Experiments were carried out in view of the following objectives:

- To grow disease-free potato cultivars under field condition and harvest of different organs
- Estimation and comparison of ascorbic acid, phenolic and flavonoid contents, antioxidative capacity, sugar and total protein contents in different organs and also under storage conditions
- To see the cytotoxic effects of polyphenolic extracts on animal cell lines namely, MCF-7 and RAW 264.7.

Chapter-3: Material & Methods

3.1 Plant materials

For the purpose of the above said objectives of the study, six different varieties potato cultivars earlier procured from CPRI Shimla namely, Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Pukhraj (PR), Kufri Jyoti (KJ), Kufri Ashoka (AS) and Desiree (De) were used. They are maintained in growth room of Thapar Institute of Engineering & Technology, Patiala in laboratory 4. After acclimatization these potato plantlets were transferred into field in the month of November which is the best suited temperature and short day photoperiod for potato growth. Harvesting of crops was done at various time intervals i.e. in month of January, February, and March, at different stages of growth i.e. young, growing and mature.

Different potato varieties were purchased from local market outside the Thapar Campus namely as MS1 (purple coloured skin), MS2 (red coloured skin), MS3 (Agra variety), MS4 (Pahadi aaloo), MS5 (locally grown).

Other materials: Various chemical and biochemical reagents and biological items required in the experiment were purchased from different companies in India. For purchasing the chemicals Laboratories like Sigma -Aldrich India Pvt. Ltd and high media laboratories Mumbai were selected. Glass wares and plastic wares were obtained from Borosil Pvt. Ltd and Tarsons Pvt. Ltd.

3.2 Harvesting of plant materials

Harvesting of different organs were done during growth under field condition, at different time intervals. Various organs of potato such as tubers, leaves and stems were collected and thoroughly washed with water to remove dirt. After drying under shade different organs were frozen in liquid nitrogen to arrest them at their metabolic state, and stored at -80 ^{o}C for further experiments.

3.3 Preparation of the enzyme extracts:

Sample preparation was done according to Kapur et al, 2012. 2.5 grams of sample were taken, crushed and homegenized with 12.5 mL of metaphosphoric acid-acetic acid and was transferred into a 30 mL centrifuge tubes after filteration. It was centrifuged at 4000 rpm for 15 minutes and supernatant was used for spectrophotometric determination of vitamin C content in different varieties of potato organs.

Chemical reagents

Metaphosphoric acid-acetic acid: 15 grams of solid metaphosphoric acid were dissolved in 40 mL of glacial acetic acid and 450 mL of distilled water in a 500 mL of flask. The solution was filtered so that impurities were removed.

2, 4-dintrophenylhydrazine solution and thiourea solution: 2, 4 – dintrophenylhydrazine is a chemical compound with molecular weight 198.14 Da and is a hydrazine derivative and is potential mutagenic agent also. 2 g of 2, 4 – dintrophenylhydrazine and 4 grams of thiourea were dissolved in 100 mL of 4.5 M H_2SO_4.

Bromine water: To carry out the experiment, it was prepared by adding the fumes of liquid bromine directly to water using a fume hood and proper protective clothing, mask and gloves. For estimation of vitamin C, 3% bromine water was prepared in lab by taking the protective measures and was kept inside the dark bottle.

85% sulphuric acid: It is miscible with water and alcohol with the generation of heat. Sulphuric acid is stable under recommended storage conditions. It also emits toxic fumes when heated to decomposition. 85% sulphuric acid was prepared and was kept in refrigerator to obtain chilled sulphuric acid

Iodine solution: In our experiment, 100 mL of 0.05 M iodine solution was prepared using potassium iodide in minimum amount of water.

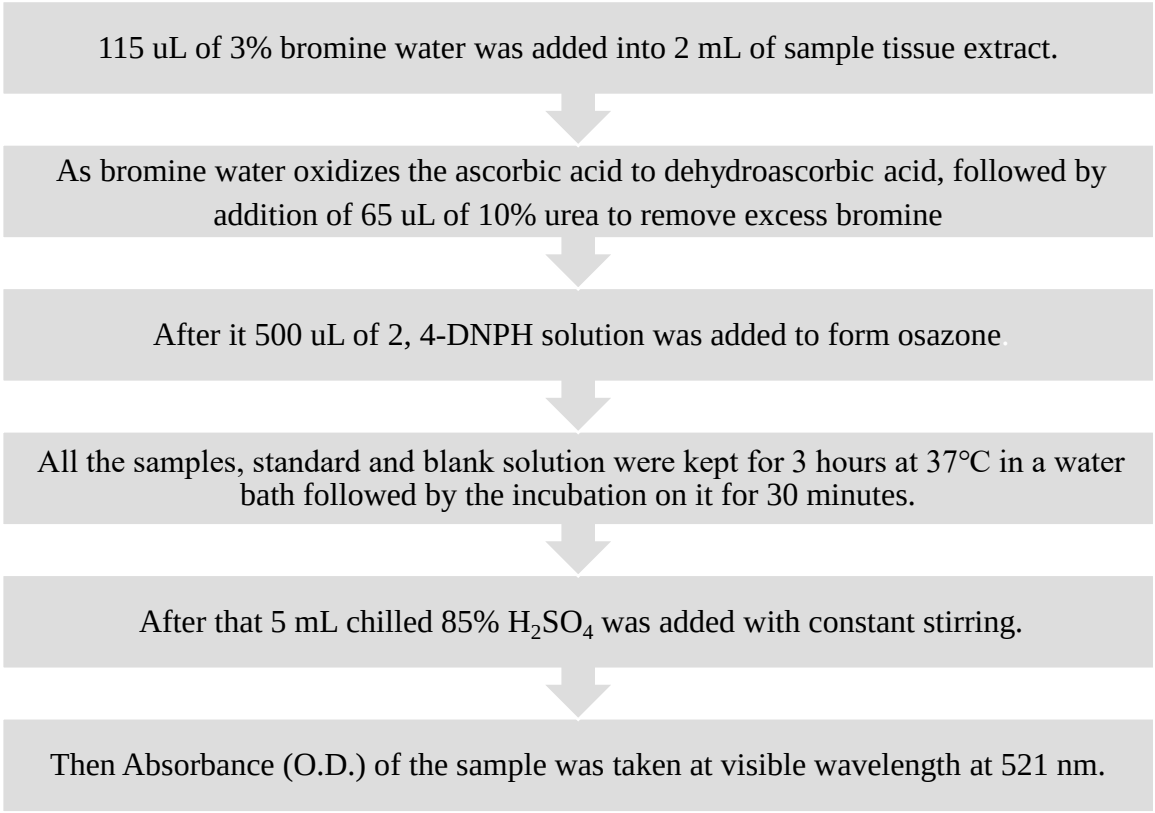
Starch Indicator: 1% starch solution was used as an indicator in redox titration in determination of vitamin C in potatoes. 1 gram of starch was dissolved in 5 mL of water. The mixture was stirred thoroughly and the volume was made about 100 mL by adding distilled water. The solution formed was boiled for few minutes, cooled and then filtered before use.

3.4 Assay of Vitamin C in different potato organs

In this study three methods were adopted for vitamin C assay and their sensitivity and safety aspects were also analysed.

i) Spectrophotometric
ii) Titrimetric
iii) HPLC

i) Spectrophotometric assay of vitamin C



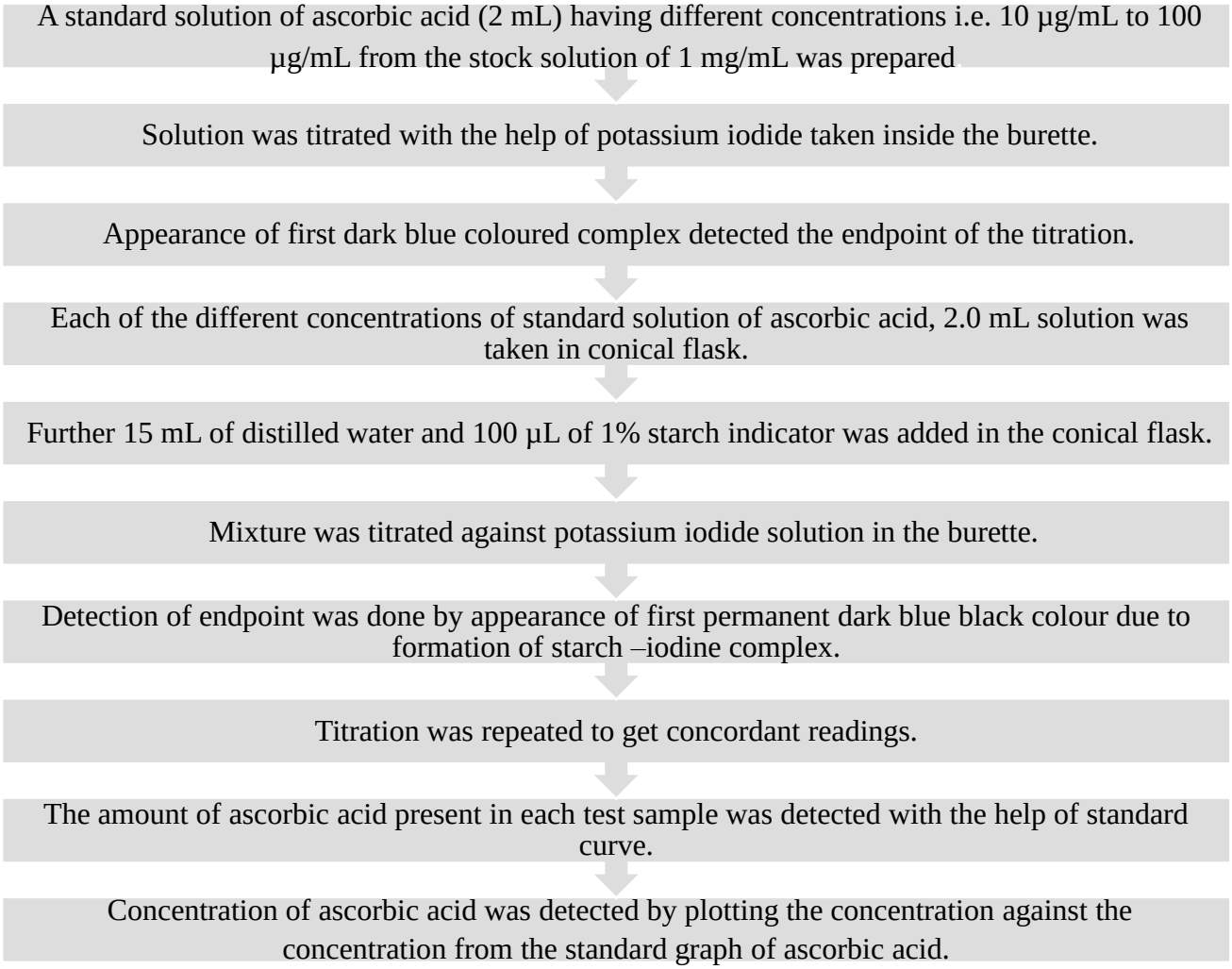
Reaction

By addition of bromine water ascorbic acid is oxidized to dehydroascorbic acid. Osazone formation take place when L-dehydroascorbic acid reacts with 2, 4-DNPH which forms red coloured solution when treated with 85% H_2SO_4. A plot was made which is used to determine the concentration of ascorbic acid in the sample.

ii) Titrimetric assay of vitamin C

Principle: Vitamin C estimation can be done by redox (oxidation- reduction) titration. The samples to be titrated are allowed to react with iodine solution and starch solution is used as an indicator in such titration. Initially iodine on reaction with iodide, forms triodide which further oxidises vitamin C. Vitamin C on oxidation by triodide gets converted into dehydroascorbic acid. The endpoint of this titration is indicated by blue black coloured product. Triodide converts all the vitamin c present in sample to iodide ion, hence no coloured product is produced. But the excess of triodide left after the oxidation of vitamin C, reacts with starch and produces the blue black coloured product. Hence the endpoint is detected by the formation of blue black coloured product.

Methodology



Reactions involved: The estimation of vitamin C was done using redox titration method. The redox titration was carried out with the help of iodine solution .Starch solution was used as an indicator in this titration. During titration, iodine was added in burette. Ascorbic acid was oxidized to dehydroascorbic acid by the addition of iodine solution. On the other hand, iodine was reduced to iodide ions. As long as ascorbic acid was present, the iodine was immediately reduced to iodide ions. But when all ascorbic acid was oxidized completely, the excess iodine left behind was free to react. This free iodine was responsible for the formation of blue- black coloured complex. This complex was result of reaction between the free iodine and the starch solution. The formation of this coloured complex completed the titration as the endpoint was detected by the first permanent appearance of this blue black coloured complex.

iii) Assay of vitamin C by HPLC

Principle: High performance liquid chromatography in which there is a stationary phase which is non-polar i.e. hydrophobic in nature and polar mobile phase is used which is a mixture of two solvents and it works on the principle of hydrophobic interactions which means more the non- polar material is, longer it will be retained.

Methodology

Mature tubers extract was prepared according to Kapur et al.,2012.

20-25 µL supernatent was taken and analysed by technique i.e. is HPLC using dC18 column (4.6mm×250.5µm) with having mobile phase 100% methanol and 0.2% TFA at 1mL/min.

After that eluants were estimated at 250 nm and analyses were carried out.

3.5 Extraction and assay polyphenols in the potato organs

*Tubers***:** All extracts from tubers were prepared according to Andreau A.B, 2018. Briefly tubers were first collected, washed and dried and then freeze dried in liquid nitrogen and stored at -80 ºC. Then approx. 1.0 g of fresh weight tuber was taken and 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 constantly agitated (100 rpm) at 4 ºC overnight followed by centrifugation at 6000rpm at 4 ºC for 20 min to let the pellet settle down was done. The supernatant was further clarified by filtration and concentrated it using rotary evaporator. Then the concentrate was finally resuspended in 1.0 mL of 30% methanol (Andreau et al., 2018) and then this concentrate, Potato polyphenolic extracts (PPEs) was stored at -20 ºC for further use.

Leaf and stem: Stems and leaves after removal of dirt, were washed and freeze dried under liquid nitrogen at -80℃.One gram of the sample was crushed in mortar pestle and extracted with 80% (50mL) methanol. After that extract was swirled for 1hr at room temperature using

an orbital shaker and then above extract was filtered through filter paper and stored at -20℃ for further use.

Estimation of Total Phenol content (TPC): The Total phenolic content (TPC) was estimated spectrophotometrically as described Ghasemzadeh et al., 2010.

Firstly 1.0 mL extract was diluted with 10 mL of distilled water followed by addition of 1.0 mL Folin-ciocalteu phenol reagent.

After the incubation for 5 min, 20% sodium carbonate (2.0 mL) was added to the reaction mixture.

The solution was kept in total darkness and absorbance of the sample was taken at visible wavelength of 750 nm using a U-V spectrophotometer Shimadzu

Gallic acid (GA) was used as standard for total phenolic content determination and units was expressed in terms of (mg of gallic acid /g FWt)

***Estimation of flavonoids*:** The total flavonoid content was also estimated spectrophotometrically according to Ghasemzadeh et al., 2010.

Firstly 100 µL extract of each organ was diluted with 400 µL water followed by addition of 30 µL of 5% $NaNO_2$ and 5min incubation at room temperature.

30 µL $Alcl_3$ was added, followed by incubation of 5 min and addition of 200 uL of 1.0M NaOH. Absorbance of the reaction were measured at a wavelength of 430 nm.

Quercitin (2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4H-chromo-4-one) was used as a standard for total flavonoids contents and units was expressed as (mg of quercetin equiv/g FWt).

Determination of antioxidant activity by Ferric reducing antioxidant potential: Antioxidant activity was measured by Ferric reducing antioxidant potential (FRAP) assay according to Benzie and Strain, 1996 method.

Stock solution for estimating the FRAP assay:

- **Acetate buffer**
  300 mM of pH 4.5
- **TPTZ (2, 4, 6-tripyridyl-s- triazine) solution**
  100 mM in 40 mM HCl
- **Iron chloride ($FeCl_3$)**
  20 mM in deionized water

Master mix for FRAP assay was prepared by adding 25 mL of 300mM acetate buffer and 10 mM TPTZ in 40 mM HCL and then 2.5 mL $FeCl_3$ were mixed.

For the reaction 150µL of organ extract added to 2850µL master mix of FRAP solution.

The sample were then kept in total darkness for 30 minutes and followed by measuring the absorbance at 593 nm under U-V spectrophotometer Shimadzu.

3.6 Extraction and estimation of soluble sugars

From the freshly harvested potato organs i.e. (leaves, stem and tuber) collected at various stages of growth as mentioned above and mature harvested tubers stored at different temperatures (room temperature, 4℃, -20℃). 200mg of sample was taken and crushed using mortar and pestle into fine paste and then 1 mL of 80% alcohol was added to it and then the slurry into microfuge tube and then again rinsed with 1 mL of 80% alcohol and transferred the slurry was transferred into same tube. The sample was incubated in water bath at 80℃ for 45 minutes and

followed by the centrifugation at 10,000 rpm for 10 minutes and supernatant containing the soluble extract was collected in a beaker and then again the pellet was re-extracted with 80% alcohol and supernatant was collected in the same beaker. For complete evaporation of the solvent the supernatant was kept at 65℃ for two hours. After that residual matter in a beaker was solubilized thoroughly in 1.5 mL of sterile water and soluble extract was filtered and transferred into clean and fresh microfuge tubes. Extracts were stored at -20℃ and was used for calculation of total sugars by Anthrone method and reducing sugar by Nelson-Somogyi's method

Total soluble sugars by Anthrone method: *Total* soluble sugar was used for both reducing as well as non-reducing sugars. In the presence of conc. Sulphuric acid sugars get dehydrated and produce 5-hydroxymethylfurfural (from hexoses) and furfural (from pentose) which then react with anthrone to produce a coloured compound and absorbance of the sample was recorded at 630nm. Pentose's, hexoses, heptose and their derivatives produce coloured substance and trioses, tetroses and amino sugars do not yield any coloured product. Varying concentrations of aliquots from (10, 20,40,60,80,100 µg/mL) and D- glucose stock (1mg/mL) were made along with blank. The volume of each sample made up to 1.0 mL with distilled water and followed by 5.0 mL anthrone reagent (200mg of anthrone was dissolved in 100 mL of ice cold 95% H_2SO_4). All the test tube were kept in boiling water for 10 minutes using marbles so that on top to prevent the evaporation loss. Absorbance of sample was taken at 630 nm after cooling the samples at room temperature.

Reducing sugars by Nelson-Somogyi's method

Principle

When the alkaline copper tartrate is heated the copper from Cu^{2+}gets reduced to cuprous oxide. The reduction of molybdic acid to molybdenum takes place when cuprous oxide is treated with arsenomolybdic acid. The blue colour formation take place and it is compared with standard and the intensity of colour is estimated at 620nm. The colour is stable for at least 18 hours.

$$CuSO_4.5H_2O + \text{reducing sugar} \rightarrow Cu_2O + \text{sugar (oxidized)}$$

$$Cu_2O + \text{arsenomolybdate reagent} \rightarrow \text{blue coloured complex} + CO_2$$

Procedure

Different aliquots of each soluble sugar extract of varying concentration were prepared in a clean test tube and standard was prepared by taking different aliquots of varying concentration (10, 20,40,60,80,100,120,140 µg/mL) from D-glucose (stock 1 mg mL^{-1}) along with blank.

Sample volume is made up to 2.0 mL by addition of water followed by addition of 1 mL of alkaline copper tartrate reagent is added [Alkaline copper tartrate: Solution A- 2.5 g of anhydrous Na_2CO_3, 2 g $NaHCO_3$, 2.5 g sodium potassium tartrate and 20 g anhydrous Na_2SO_4 dissolved in 80 mL of water and make the final volume 100mL with water. Solution B- 15 g of $CuSO_4$ was dissolved in a small volume of water and having drop of conc. H_2SO_4 and final volume is made up to 100 mL with water. 96 mL of solution A+ 4 mL of solution B were mixed well before use]. All the test tube were kept in boiling water bath for 10 minutes. After cooling the test tubes to room temperature 1.0 mL of arsenomolybdic acid reagent was added in each test tube [Arsenomolybdate reagent: 2.5 g of ammonium molybdate was dissolved in 45 ml of water and then add 2.5 mL of con H_2SO_4 and 0.3 g of disodium hydrogen aresenate was dissolved in 25 mL of water. Above solution were mixed and incubated for more than 24 hrs at 37℃]. The volume of each sample was made up to 10 mL by adding distilled water. It was followed by 10 minute at room temperature. Finally the absorbance of the sample was taken at visible wavelength of 620 nm after vortexing.

3.7 Protein estimation of different potato organs by Lowry method

According to Dunn et al, 1992 Lowry method was used for estimation of protein in different plant organs i.e. leaves, stems, and tubers. This method is sensitive at very low concentration of proteins. Disadvantage of Lowry method is the narrow pH range within which it is accurate. Amino acids derivatives such as certain buffers, drugs, lipids, sugars, nucleic acids, and sulfhydryl reagents interferes with Lowry procedures.

- Reagent-A

  2 % Na_2CO_3 in 0.1 NaOH
- Reagent-B

  2 % $CuSO_4.5H_2O$
- Reagent –C

  2 % Sodium Potassium tartrate
- Reagent –D (prepare fresh every day)

  0.25 mL of Reagent B was mixed into 0.25 mL of Reagent C, and then 49.5 mL of Reagent A was added.
- Reagent-E

  Folin-Ciocalteau reagent is diluted with water to 1 N.
- BSA (1mg/mL)

- Sterile water
- Spectrophotometer

Sample Preparation

200mg of potato sample was taken and crushed into a fine paste using mortar pestle and then homogenised by adding 5mL of 0.1M phosphate buffer solution of pH 7.0. Followed by the addition of a pinch of PVP and then centrifugation at 2500rpm at 4℃ for about 20 minutes. After that sample was filtered and store at -20℃ for further use.

Methodology

100µL of sample was taken of all the six cultivars of potato plant i.e. Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Pukhraj (PR), Kufri Jyoti (KJ), Kufri Ashoka (AS) and Desiree (De) and add 900 µL water was added to it. The test tube with 1 ml distilled water was used as blank. After that 5.0 mL reagent I was added and sample was incubated for 10 minutes. Followed by addition of 0.5 mL of reagent II and then incubate for 30 minutes. In darkness measure the absorbance at 660nm and plot the standard graph.

3.8 Effects of polyphenolic extracts of mature tubers on different cell lines

Materials

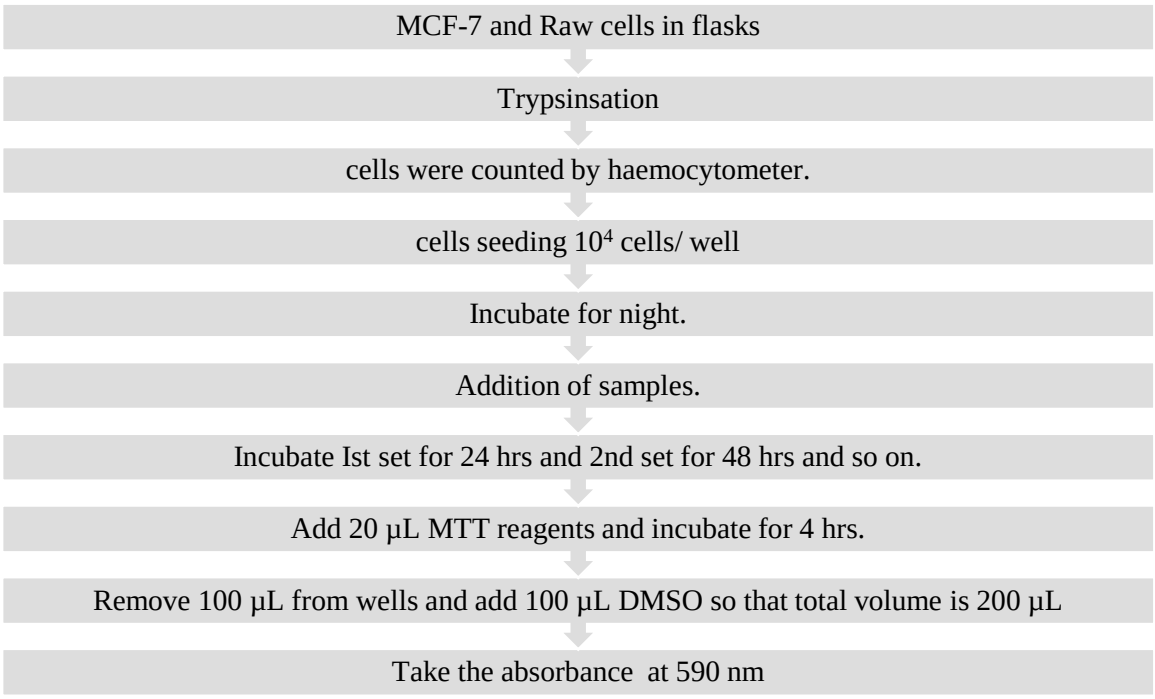
- Polyphenolic tuber extracts
- Cell lines(RAW & MCF-7)
- Dulbecco's modified Eagle's medium (DMEM)
- MTT reagent (5.0 mg/mL)
- DMSO (Merck, Germany)
- Micro plate reader (Tecan infinite, Austria)

Cell Culture maintenance

Cell lines were procured from National Centre for cell Sciences (NCCS), Pune, India. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 IU/mL penicillin, 100µg/mL streptomycin, 2.5µg/mL amphotericin in an atmosphere of 5% CO_2 and 95% humidity at 37℃.

Methodology:

MTT assay: Using the MTT assay the effect of polyphenolic extract on the viability of cancer cells was estimated. A colorimetric assay measures the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. Reduced insoluble coloured (dark purple) formazan product forms when MTT enters the cells and passes into mitochondria. These products measured spectrophotometrically when formazan can be solubilized with an organic solvents according to Sugata et al., 2015.



Based on above method, 200 µL of RAW264.7 (5×10^4, 1×10^5 and 2×10^5), MCF-7 (5×10^4) cells/ml was cultured in 96-well microculture plate (Tarsons, India) for overnight. The cells were treated with polyphenolic extracts (0.1, 0.5, 1, 2 and 3 mg/ml for RAW 264.7 and 1, 2, 3, 4, 5 mg/ml for MCF-7) for 24 hrs, 48 hrs, 72 hrs at 37°C and 5% CO_2 as shown in Fig.26 &27. Subsequently, cells were washed with PBS and 20 µL of MTT dye (5mg/mL) was added. After four hours, 100 µL of DMSO was added to dissolve the formazan crystals and the absorbance was taken at 590 nm. Cell viability was calculated as:

$$\text{Cell viability (\%)} = (A_{\text{sample}}/A_{\text{control}}) \times 100\%$$

4.1 Plant growth and harvesting of the potato organs

Different potato varieties were grown in the field of TIET, Patiala, and various organs like leaves, stems and tubers were harvested at different stages of growth as shown in Fig 3 & 4.

Fig. 3 Steps of plant grown in lab to field conditions; **A** Culture maintained in MS medium in lab; **B** Acclimatization and hardening of plantlets; **C** field growth; **D** Collection of plants

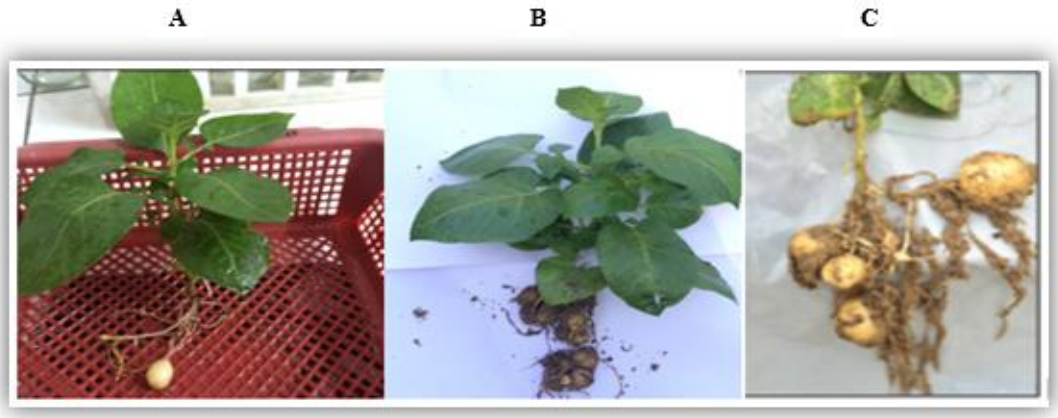


Fig. 4 A Plant at early stage (Stem, Leaf and Tuber collected in January, **B** Plants at mid-maturation (Stem, Leaf and Tuber collected in early March) and **C** Mature plants Mature plants (Stem, Leaf and Tuber collected in late March)

4.2 Estimation of ascorbate by spectrophotometric method

For the estimation of ascorbate content from the above mentioned potato cultivars, different organs like tubers, stems and leaves were harvested from the field grown plants. The respective organs were crushed in liquid nitrogen and homogenized in the extracting solution (metaphosphoric acid) and centrifuged to get supernatant which was used as extract for determination of ascorbic acid.

The standard curve for ascorbic acid estimation was made using the mean values of different concentrations (10-100 µg/ mL) from standard ascorbate of SRL Company and the absorbance was measured at 521 nm as shown in Fig.5 &6 and Table 1.

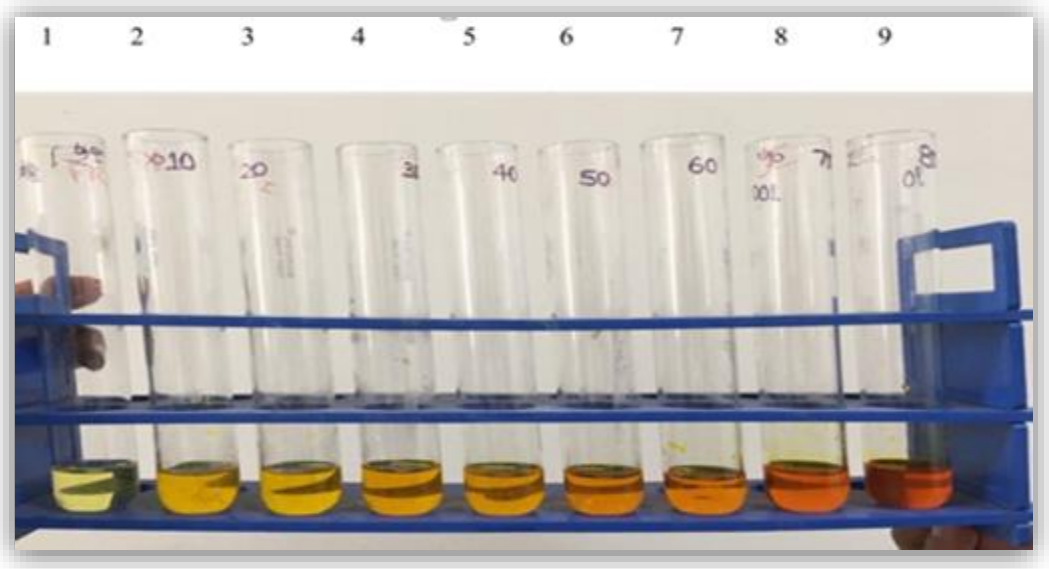


Fig. 5 Colour gradient developed at different concentrations of ascorbic acid

Table 1: Measurement of absorbance at 521 nm

Ascorbic acid (µg)	**A_{521} Value**
Blank	0
10	0.12
20	0.24
30	0.31
40	0.43
50	0.523
60	0.629
70	0.702
80	0.809
90	0.912
100	1.141
110	1.29

A_{521} value is taken as mean of three independent experiments done in triplets

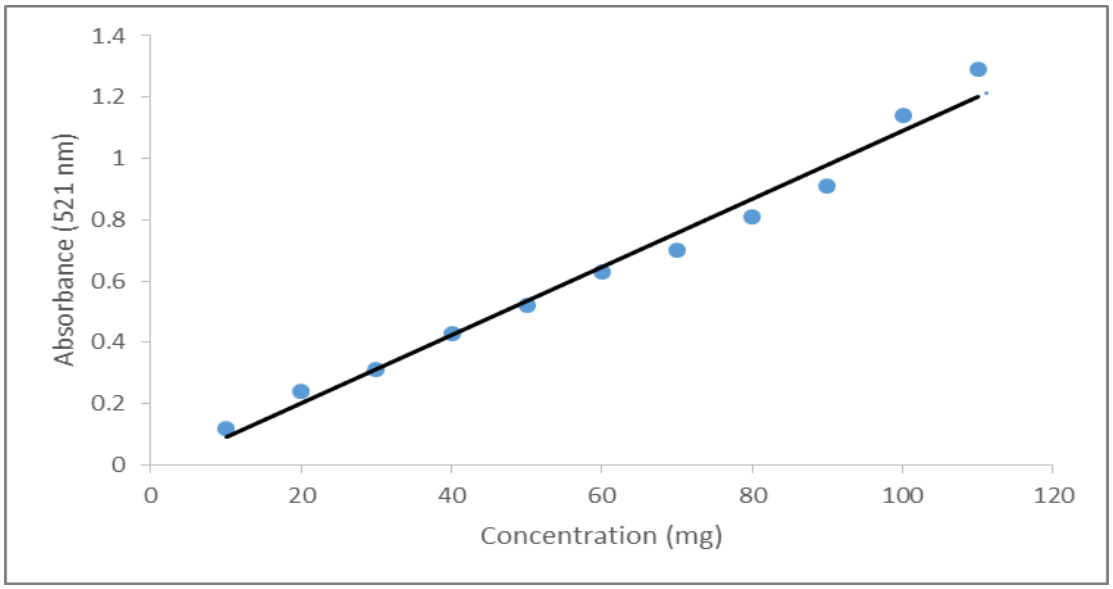


Fig. 6 Ascorbic acid Standard curve

4.2.1 Ascorbate estimation in different potato organs

Estimation of ascorbate was done with the help of UV-visible spectrophotometer at 521 nm. The amount of ascorbic acid in each sample was expressed in mg/gram of FW of tissue.

Tubers: The inter-varietal difference in ascorbate content was observed at various stages of tubers growth i.e. developing, growing and mature the ascorbate content were observed. The bell shaped pattern was observed i.e. low in early growing (small), increase in growing (medium) and decrease in mature (large) stage except in cultivar CS-1. Ascorbate content was found highest in KJ (0.6542 mg/gFW) and lowest in mature cultivars of KJ (0.1240 mg/gFW) as shown in Table 2.

Table 2: Estimation of ascorbate (mg/g F.W.) in different stages of tuber development.

Cultivar	Early growing	Growing	Mature
CS -1	0.2224	0.3147	0.5090
CS -2	0.2364	0.4577	0.3063
PR	0.2448	0.4986	0.1524
DE	0.2601	0.5114	0.2340
KJ	0.1911	**0.6542**	**0.1240**
AS	0.1801	0.2999	0.2222

*Stem***:** In case of stem organs the ascorbate content was estimated at early and mature stages. .The mature cultivar of PR has highest amount of ascorbate content i.e. 0.5408 mg/ g FW. On the other hand, the ascorbic acid content was found to be lowest i.e. 0.1474 mg/ g FW in young cultivar of CS-1 as shown in Table 3.

Table 3: Estimation of ascorbate (mg/g F.W.) in young, growing and mature stem samples

Cultivar	Young	Growing	Mature
CS -1	**0.1474**	0.2222	0.4144
CS -2	0.3704	0.3874	0.3984
PR	0.3294	0.4896	**0.5408**
DE	0.2444	0.3546	0.5294
KJ	0.2414	0.2985	0.3904
AS	0.1864	0.2156	0.3299

*Leaves***:** Ascorbate estimation from the leaf organs was also done at young and mature stage. The mature cultivar of CS-1 has highest amount of ascorbic acid content i.e. 3.5224 mg/ g FW was observed On the other hand, the ascorbic acid content was found to be lowest i.e. 0.985mg/ g FW in young cultivar of DE as shown in Table.4. High ascorbic acid content in leaves show high antioxidative potential in the leaf organ might be due to dual role of source and sink organs. Whereas high ascorbate content in mature leaves might be a harbinger of more oxidative stress as they have to go undergo senescence process also.

Table 4: Estimation of ascorbic acid (mg/g F.W.) in young. Growing and mature leaf samples

Cultivar	Young	Growing	Mature
CS -1	2.131	2.5896	**3.5224**
CS -2	1.9096	2.2312	2.9892
PR	1.5842	1.6545	1.8124
DE	**0.985**	1.2120	1.4262
KJ	1.9742	2.3456	2.7292
AS	1.3142	1.5892	1.8894

4.2.2 Estimation of ascorbate in mature tubers by titration method

The amount of ascorbate present in large tubers was also estimated with the help of standard curve plotted using a standard solution of ascorbic acid at different concentrations i.e. 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL up to 100 µg/mL from the stock solution of 1.0 mg/mL. Concentration of ascorbate was detected by plotting the concentration against the concentration from the standard graph of ascorbate as shown in table.5 and Fig.7

Table 5: Ascorbate at different concentration (10-100 µg/mL)

Ascorbic acid (µg)	Iodine (mL)
Blank	0
10	0.32
20	0.51
30	0.86
40	1.23
50	1.64
60	2.35
70	2.49
80	2.84
90	3.21
100	3.72

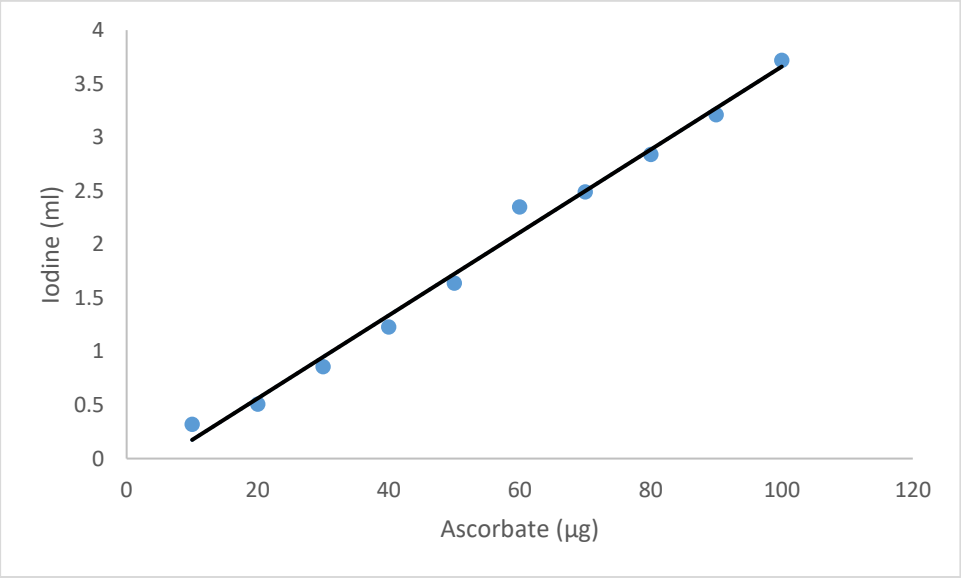


Fig. 7 Ascorbic acid Standard curve

Table 6: Estimation of ascorbate (mg/g) F.W in large tuber samples

Cultivar	Amount of ascorbate
CS -1	0.331
CS -2	0.274
PR	0.228
DE	0.211
KJ	0.249
AS	0.181

4.2.3 Ascorbic acid estimation by HPLC:

From the given below diagram in Fig.8 we calculate that retention time of ascorbic acid is 8.5 minutes and from this graph we validate that in Fig.9 and Fig.9.1 the retention time of CS-1 cultivar and DE cultivar of ascorbic acid has same retention time.

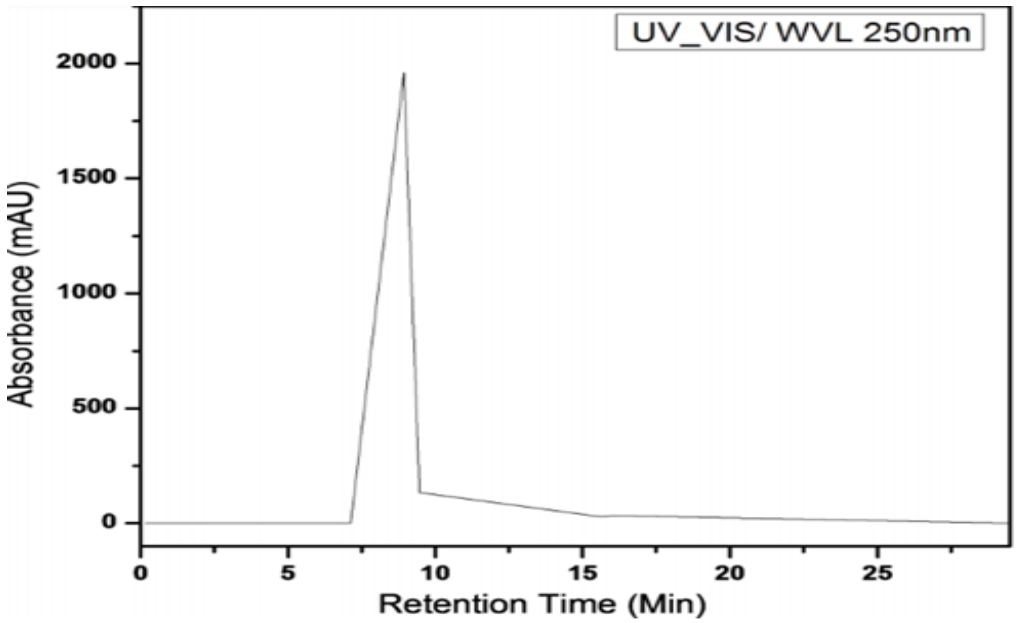


Fig. 8 Standard graph of ascorbate by HPLC

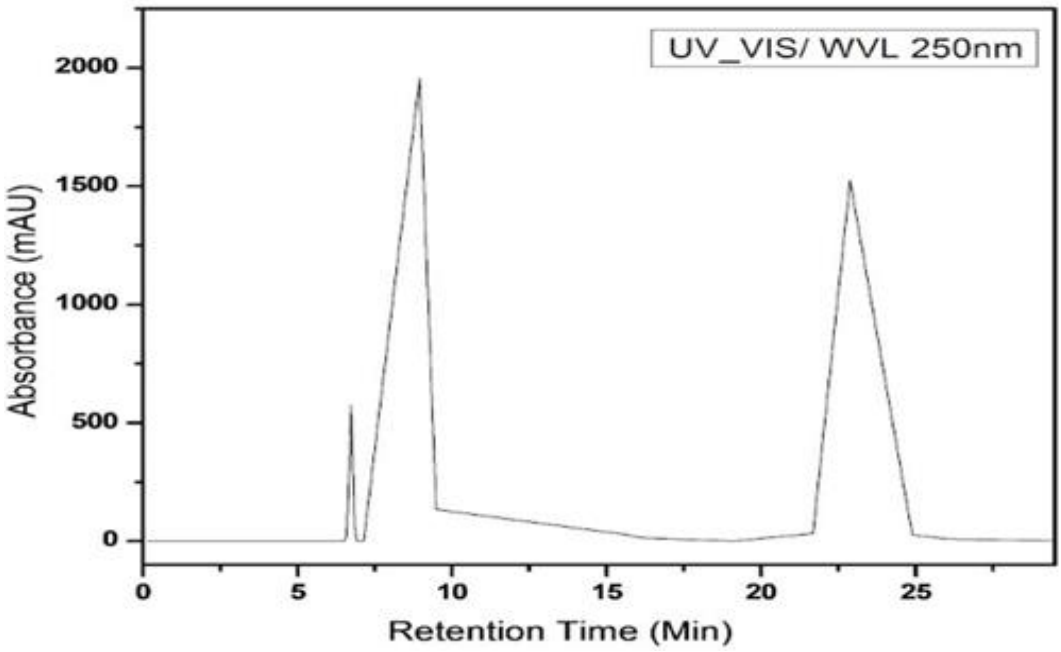


Fig. 9 Standard graph of CS-1

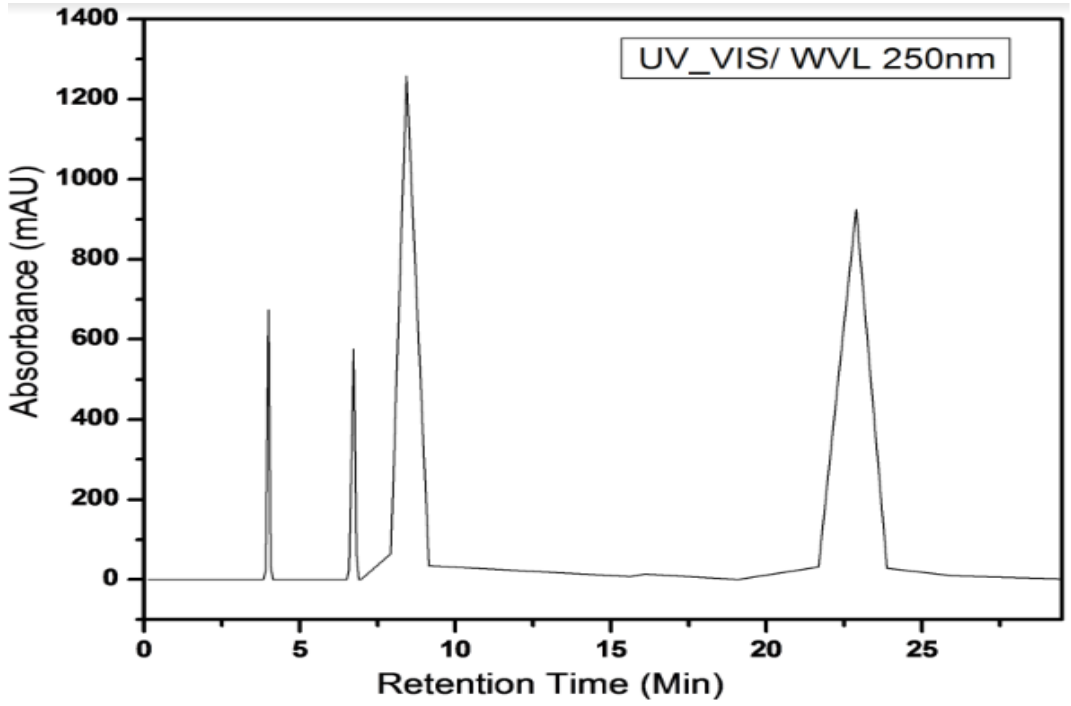


Fig. 9.1 Standard graph of DE

Tubers stored for 15 days: The inter-varietal differences in ascorbate content in tubers stored at three different temperatures i.e. room temperature, 4ºC and -20º ºC were observed. The high amount of ascorbic acid content was found to be at 4℃ in CS-2 (0.5392 mg/g FW) and lowest in DE i.e. 0.2082 mg/g FW as shown in table 7.

Table 7: Estimation of ascorbate (mg/g F.W.) in tuber samples stored at different temperature for 15 days

Cultivar	Room Temp	4℃	-20℃
CS -1	0.5084	0.5190	0.5272
CS -2	0.3794	**0.5392**	0.4600
PR	0.2242	0.2942	0.2844
DE	**0.2082**	0.3154	0.3722
KJ	0.2802	0.3942	0.3292
AS	0.2579	0.2552	0.4952

Tubers stored for 30 days: Tuber stored for 30 days at three different temperature i.e. room temperature, 4℃ and -20℃. Except CS-1 there is significant change in every cultivars. But in case of AS and DE from room temperature to -20℃ there is double in increase in the ascorbic acid content which shows high antioxidant potential as shown in Table 8.

Table 8: Estimation of ascorbate (mg/g F.W.) in tuber samples stored at different temperature for 30 days

Cultivar	Room Temp	4℃	-20℃
CS -1	0.5030	0.5294	0.5759
CS -2	0.4002	0.6922	0.5266
PR	0.2394	0.3374	0.3333
DE	0.1750	0.3652	0.4409
KJ	0.3980	0.4622	0.5722
AS	0.3140	0.3478	0.7802

Tubers stored for 45 days: Ascorbate content was estimated at three different temperature and at -20℃ the ascorbate content in KJ i.e. 0.6192 mg/ gFW found to be highest and lowest was also found in KJ cultivar at room temperature which is 0.2242 mg/ gFw. In DE and KJ the ascorbate content from room temperature to -20℃ there is almost double increase. But in CS-1 and AS the ascorbate content were decrease at 4℃ as compared to room temperature as shown in table 9.

Table 9: Estimation of ascorbate (mg/g F.W.) in tuber samples stored at different temperature for 45 days

Cultivar	Room Temp	4℃	-20℃
CS -1	0.3948	0.2981	0.5024
CS -2	0.2824	0.4477	0.4726
PR	0.2594	0.2628	0.4299
DE	0.2370	0.2886	0.5372
KJ	**0.2242**	0.3702	**0.6192**
AS	0.3542	0.3032	0.4372

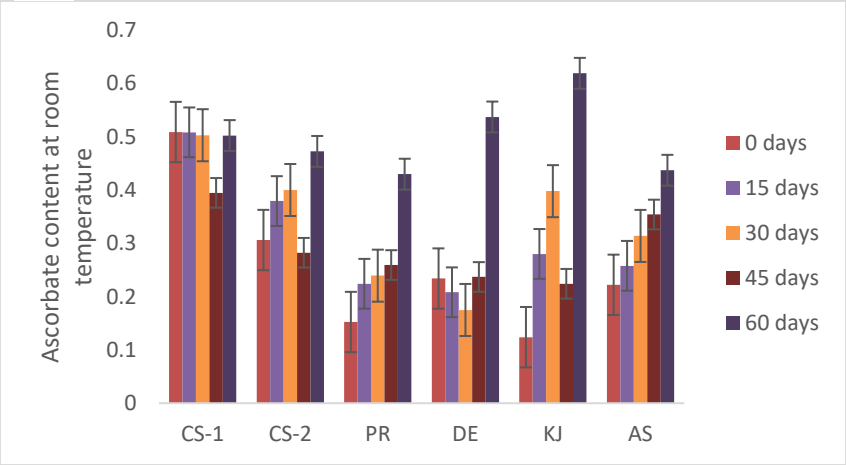
Tubers stored for 60 days: During 60 day's storage in CS-1 and CS-2 there is double increase in ascorbate content from room temperature to -20℃. The high amount of ascorbate content was found to be at -20℃ in DE (0.3172 mg/g FW) and lowest in CS-2 i.e. 0.1139 mg/g FW at room temperature. There is some fluctuation in the amount was also observed as shown in Table 10.

Table 10: Estimation of ascorbate (mg/g F.W.) in tuber samples stored at different temperature for 60 days

Cultivar	Room Temp	4℃	-20℃
CS -1	0.1572	0.2862	0.3064
CS -2	**0.1139**	0.2284	0.2729
PR	0.1527	0.1392	0.2424
DE	0.2147	0.1942	**0.3172**
KJ	0.2097	0.1722	0.2902
AS	0.2271	0.2844	0.3102

4.2.4 Ascorbate at different storage conditions of tuber

A



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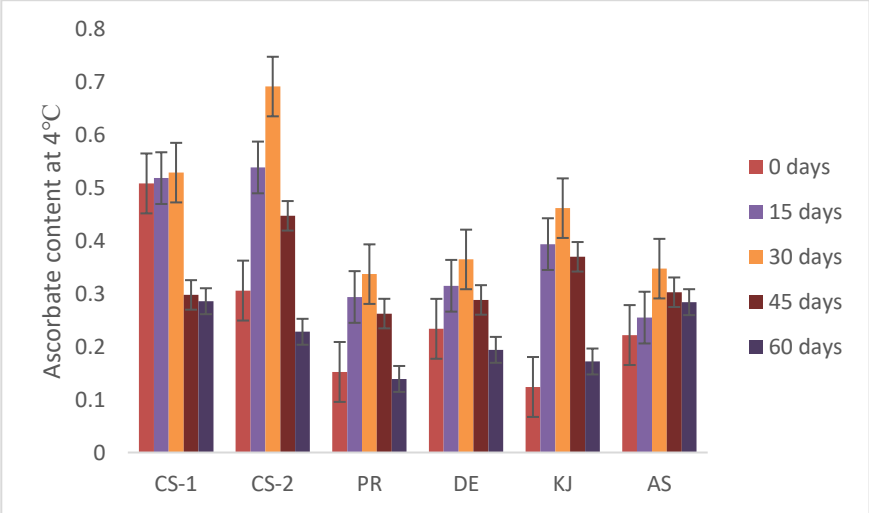


C

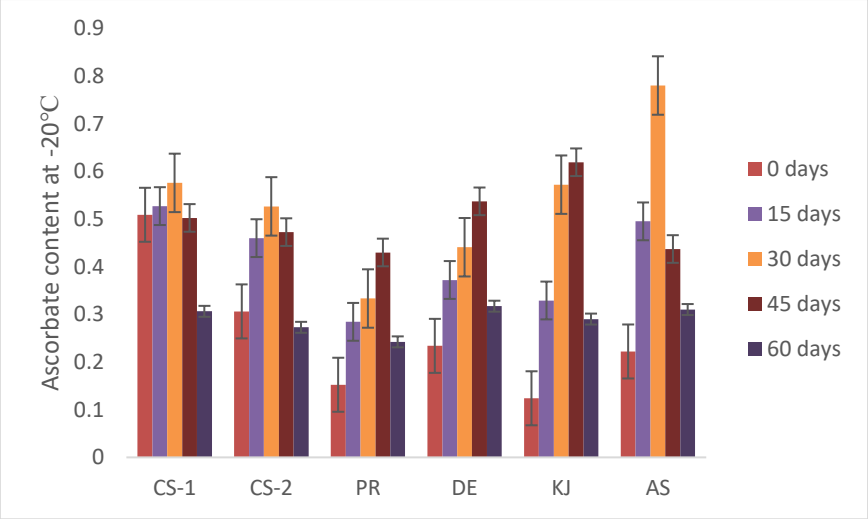


Fig. 10 Ascorbate content of different storage conditions of mature tuber (a) room temperature (b) 4°C and (c) -20°C

The ascorbate content on 0 DOS i.e.at initial day of observation was in the range, lowest (0.1240mg/ g FW) in cultivar KJ and highest (0.6542 mg/ g FW) in the cultivar KJ. After 15 days of storage the inter-varietal differences in ascorbate content in tubers stored at three different temperatures i.e. room temperature, 4ºC and -20º °C were observed. The high amount of ascorbic acid content was found to be at 4°C in CS-2 (0.5392 mg/g FW) and lowest in DE i.e. 0.2082 mg/g FW. Tuber stored for 30 days except CS-1 there is significant change in every cultivars. But in case of AS and DE from room temperature to -20°C there is double in increase in the ascorbic acid content which shows high antioxidant potential. Ascorbate content was estimated at three different temperature and at -20°C the ascorbate content in KJ i.e. 0.6192 mg/ gFW found to be highest and lowest was also found in KJ cultivar at room temperature which is 0.2242 mg/ gFW. In DE and KJ the ascorbate content from room temperature to -20°C there is almost double increase. But in CS-1 and AS the ascorbate content was found to decrease at 4°C as compared to room temperature. During 60 day's storage in CS-1 and CS-2 there is double increase in ascorbate content from room temperature to -20°C. The high amount of ascorbate content was found to be at -20°C in DE (0.3172 mg/g FW) and lowest in CS-2 i.e. 0.1139 mg/g FW at room temperature. There is some fluctuation in the amount was also observed. Decrease in ascorbate content was observed from 0 day of storage to 60 DOS. Ascorbate content was reduced 3 folds from 0 DOS to 60 DOS in case of storage at room temperature; while at 4°C, it was found to be consistent and approximately 2 fold decrease was recorded at -20°C.

4.2.5 Selection of market potatoes

Storage of the tuber of market potatoes: MS1 (Purple colour potato) has highest ascorbate content (1.2142 mg/g FW) and MS5 (Local potatoes) has lowest ascorbate content (0.4001 mg/g FW) as shown in Table 11.

Table 11: Estimation of ascorbate (mg/g F.W.) in various market selection tuber samples.

Cultivars	Ascorbic acid(content)
MS1	1.2142
MS2	0.4629
MS3	0.5296
MS4	0.5379
MS5	0.4001

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

Phenolic, flavonoids and antioxidant activity was measured from different organs 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 total phenolic, 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 Ghasemzadeh et al., 2012, Andreau A.B, 2018 and as shown in Table 12 and Fig.11&12.

Table 12: Preparation of the standard curve of Gallic acid (absorbance measured at 725 nm)

Tube No.	Concentration (µg)	A_{725} value
1	Blank	0
2	50	0.085
3	100	0.129
4	150	0.361
5	200	0.451
6	250	0.605
7	300	0.783
8	350	0.976
9	400	1.112
10	450	1.225
11	500	1.337

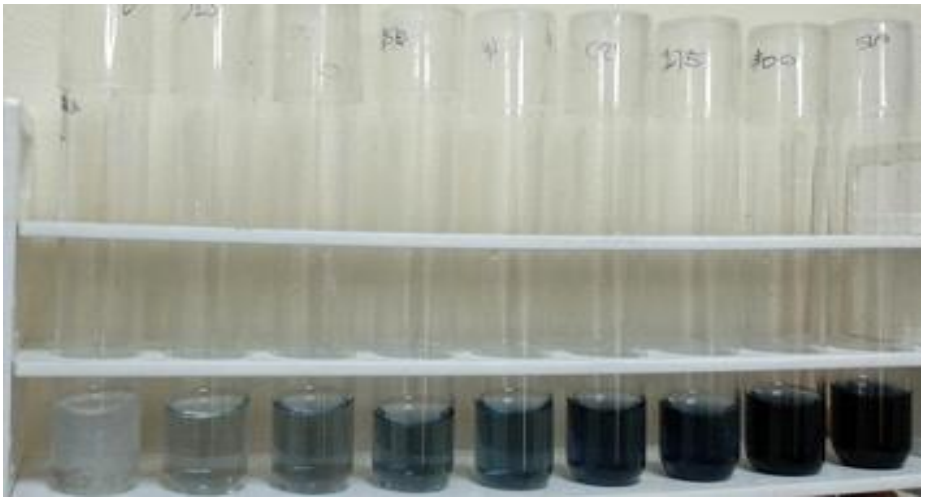


Fig. 11 Colour gradient developed at different concentrations of Gallic acid

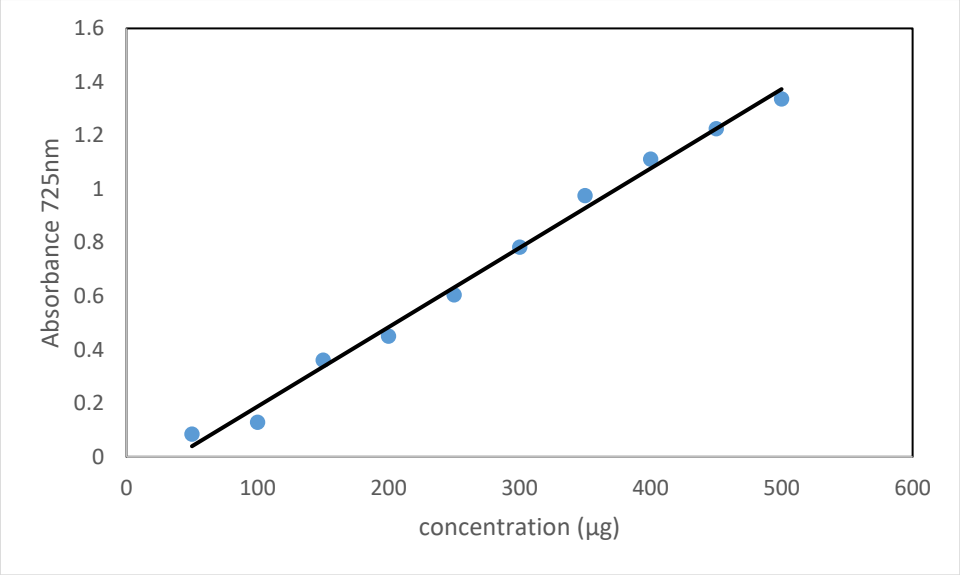


Fig. 12 Standard curve of Gallic acid

II *Standard curve for the estimation of total flavonoid content*- Quercetin was used as standard and standard curve was made by using different concentration of 1000 µg stock solution of quercetin as shown in Table. Ghasemzadeh et al., 2012 method was followed to perform it and absorbance was taken at 430 nm. It was estimated in µg of quercetin equivalent per one gram of potato tissue fresh weight as shown in Fig.13&14 and Table 13.

Table 13: Preparation of the standard curve quercetin absorbance wavelength at 430nm

Test tubes	Concentration (µg)	A_{430} value
1	Blank	0
2	100	0.124
3	200	0.305
4	300	0.421
5	400	0.513
6	500	0.674
7	600	0.730
8	700	0.878
9	800	0.985
10	900	1.128
11	1000	1.329

Test tubes with different concentration of quercetin from 100 to 1000 µg for flavonoid estimation

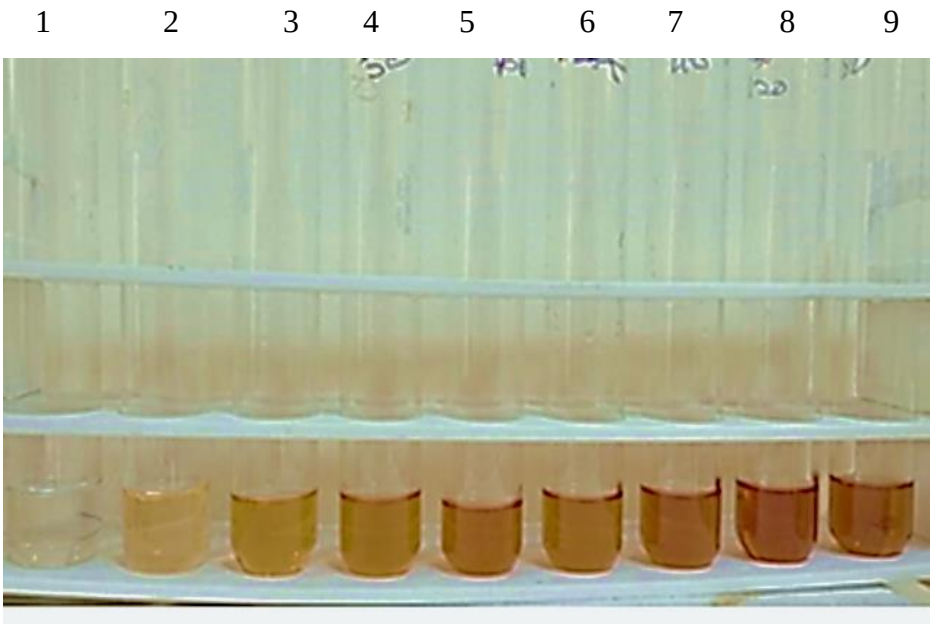


Fig. 13 Colour gradient developed at different concentrations of quercetin

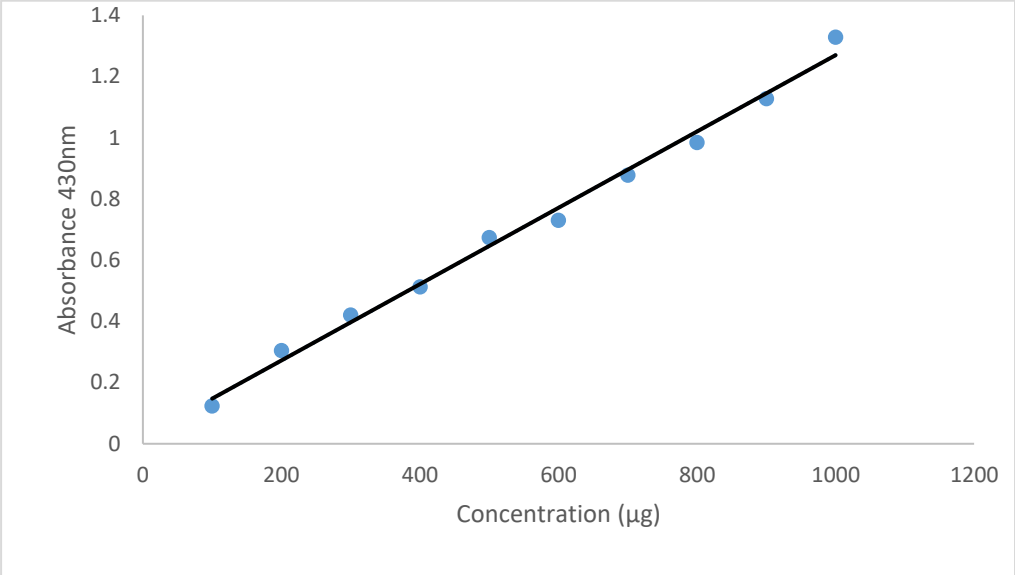


Fig. 14 Standard curve of quercetin

III. *Standard curve for the estimation of antioxidant activity by FRAP* using different concentrations from 1 mg/ml stock of ascorbate according to Ghasemzadeh et al., 2012 the absorbance was taken at 593 nm and was estimated in µg of ascorbate equivalent per one gram of potato tissue fresh weight as shown in Fig.15& 16 and Table 14.

Table 14: Preparation of the standard curve of ascorbate at 593 nm

Test tubes	Concentration (µg)	A_{593} value
1	Blank	0
2	10	0.165
3	20	0.308
4	30	0.425
5	40	0.565
6	50	0.718
7	60	0.866
8	70	0.977
9	80	1.054
10	90	1.111
11	100	1.297
12	110	1.319
13	120	1.599

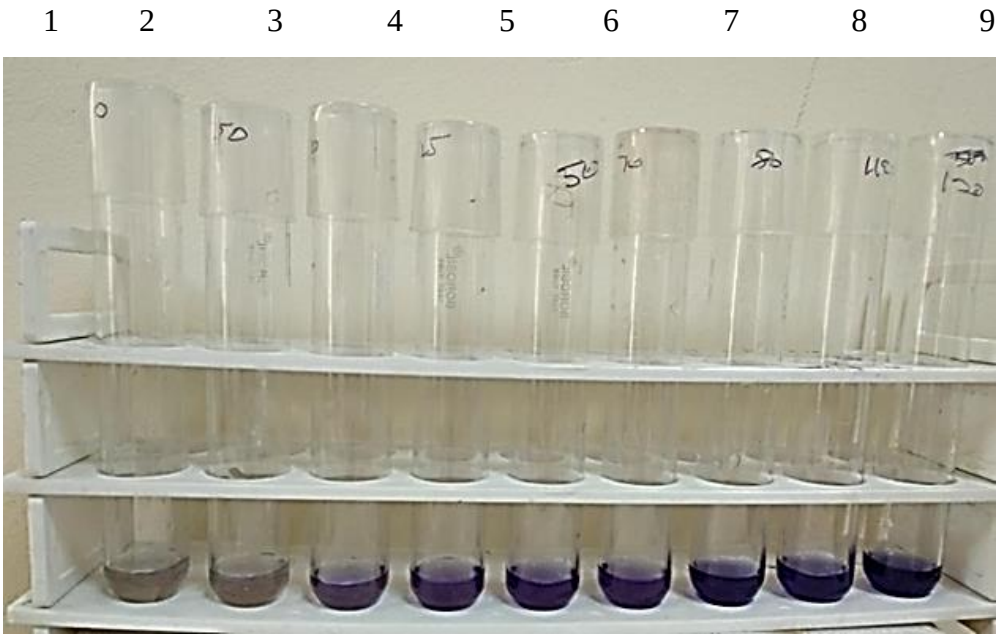


Fig. 15 Colour gradient developed at the different concentration of antioxidant capacity

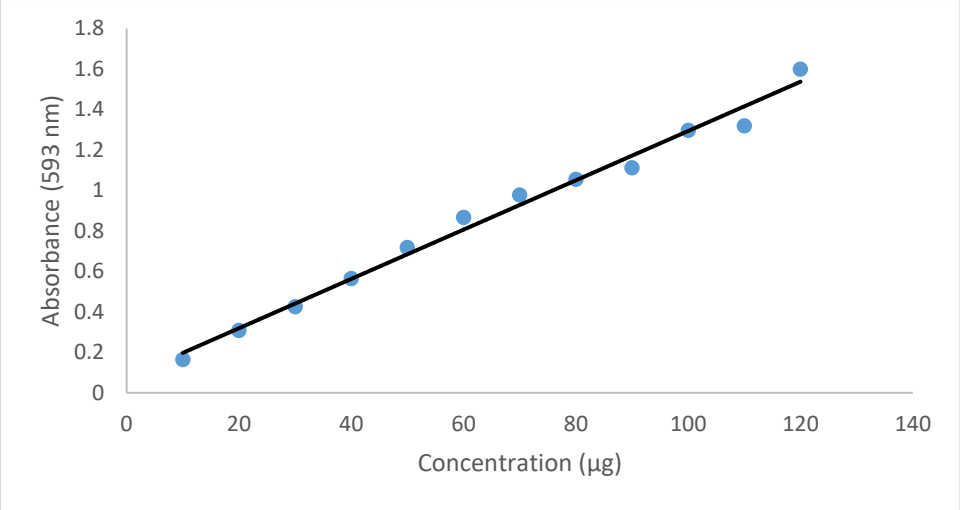


Fig. 16 Standard curve of ascorbate

IV. *Standard curve for the estimation of reducing sugar content*- Glucose was used as standard and standard curve was made by using different concentrations of 1 mg/ml stock of glucose as shown in Table 15 and Fig.17. The absorbance was taken at 620 nm. It was estimated in µg of glucose equivalent per one gram of potato tissue fresh weight.

Table 15: Preparation of the standard curve of reducing sugar at 620nm

Test tubes	Concentration (µg)	A_{620} value
1	Blank	0
2	10	0.126
3	20	0.246
4	40	0.511
5	60	0.844
6	80	1.072
7	100	1.048
8	120	1.620
9	140	2.16
10	160	2.418
11	180	2.712
12	200	2.777

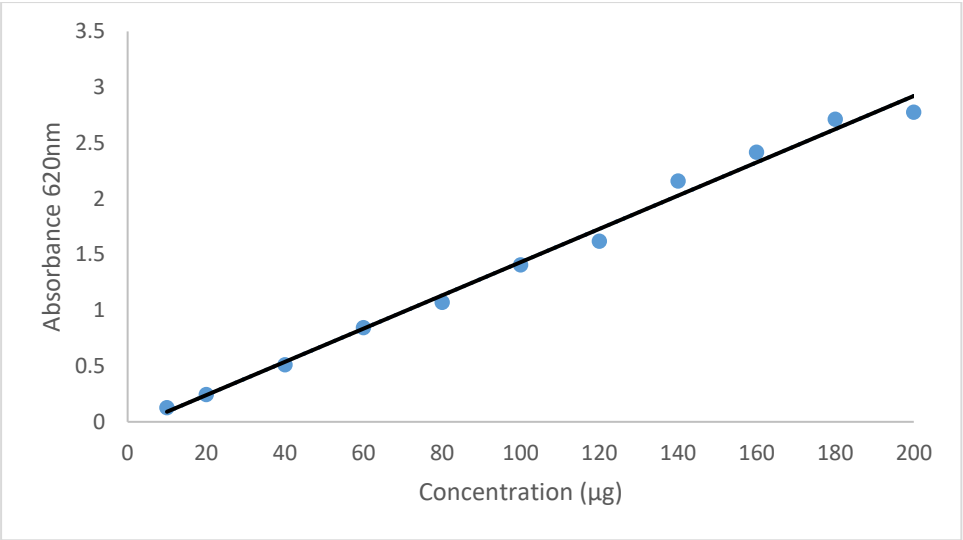


Fig. 17 Standard curve of reducing sugar

V. *Standard curve for the estimation of Total soluble sugar content*- Glucose was used as standard and standard curve plotted by using values obtained at different concentrations of 1 mg/ml stock of glucose as shown in Table 16 and Fig.18. The absorbance was taken at 630 nm. It was estimated in µg of glucose equivalent per one gram of potato tissue fresh weight.

Table 16: Preparation of the standard curve of Total soluble sugar at 630nm

Test tubes	Concentration (µg)	A_{630} value
1	Blank	0
2	10	0.106
3	20	0.292
4	40	0.356
5	60	0.689
6	80	0.879
7	100	0.970
8	120	1.179
9	140	1.225
10	160	1.551

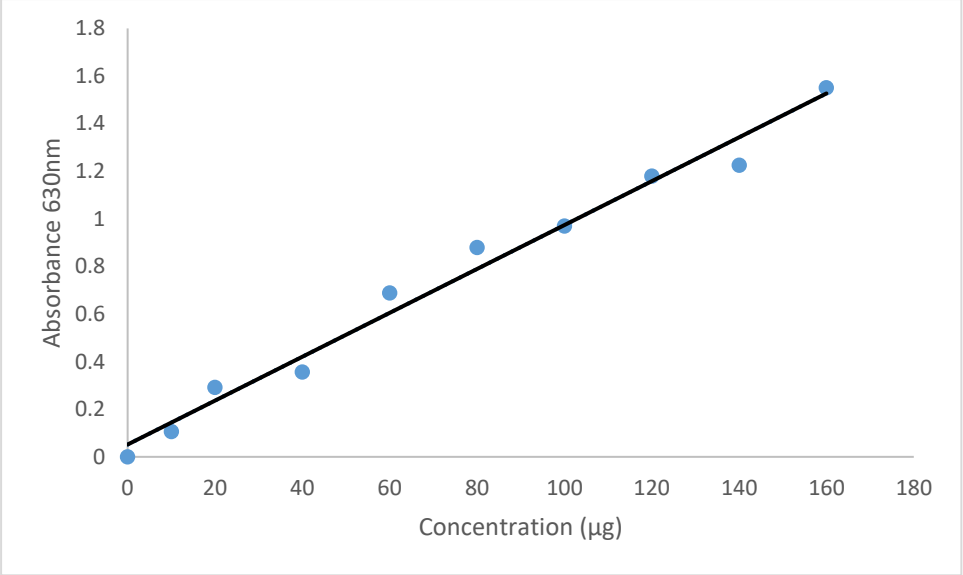


Fig. 18 Standard curve of total soluble sugar

VI. *Standard curve for the estimation of Protein content*- BSA was used as standard and standard curve was made by using different concentration of 1 mg/ml stock of glucose as shown in Table 17 and Fig.19. The absorbance of the sample was taken 660 nm. It was estimated in µg of glucose equivalent per one gram of potato tissue fresh weight.

Table 17: Preparation of the standard curve of BSA at 660nm

Test tubes	Concentration (µg)	A_{660} value
1	Blank	0
2	50	0.178
3	100	0.277
4	150	0.369
5	200	0.452
6	250	0.567
7	300	0.617
8	350	0.724
9	400	0.781
10	450	0.822
11	500	0.940

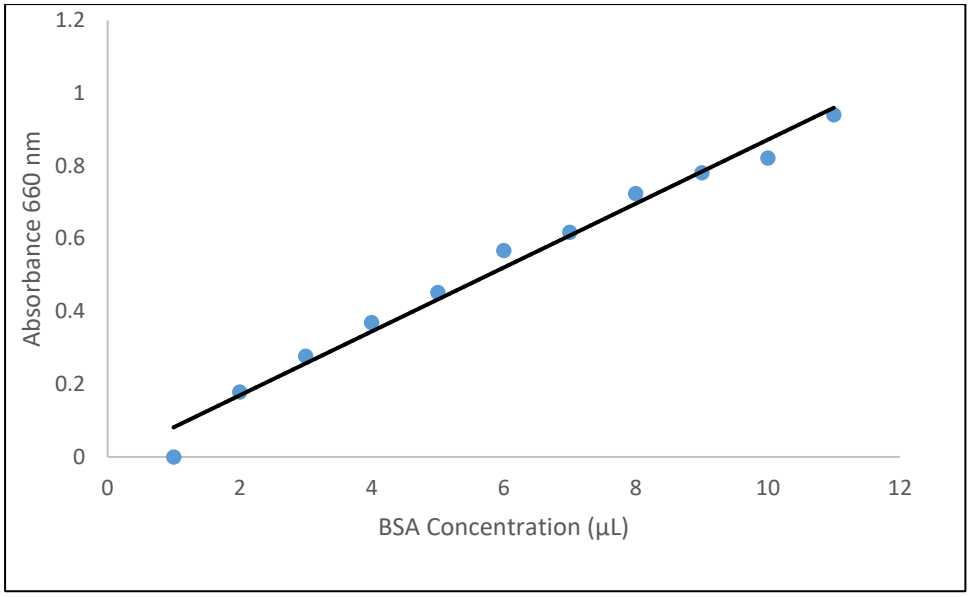


Fig. 19 Standard curve of BSA

4.3.1 Estimation of phenolic content, flavonoid content and antioxidant activity in different potato organs

Polyphenolic content in leaves

The highest polyphenolic content was observed in early growing stage of C2-2 cultivar (27.6mg GAE. g^{-1} FW) and lowest was observed in mature stage of AS cultivars (5.85 mg GAE. g^{-1} FW). Decrease in polyphenolic content was observed towards maturity as shown in Table 18. The observations are in correlation with the studies of Padda et al., 2007 where a decrease in polyphenolic content towards maturity was observed in leaves of sweet potato.

Table 18: Estimation of polyphenolic content in leaf extract of potato (expressed in mg of Gallic acid equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	23.15	19.48	11.15
CS -2	27.6	24.20	18.60
DE	17.32	13.82	8.95
KJ	19.76	11.76	10.74
PR	11.60	10.55	7.20
AS	10.88	9.84	5.85

Polyphenolic content in stems

Polyphenolic content in stem was found to increase in the mature stems. The increase ranged from 2-5 fold depending on the cultivar.The highest polyphenolic content was calculated in the mature stage of CS-1(15.74 mg GAE. g^{-1} FW) and lowest in stage PR (2.22 mg GAE. g^{-1} FW) as shown in Table 19.

Table 19: Estimation of polyphenolic content in stem extract of potato (expressed in mg of Gallic acid equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	3.01	9.65	**15.74**
CS -2	3.84	4.90	8.81
DE	3.20	5.85	9.53
KJ	4.29	4.65	9.49
PR	3.17	2.22	7.10
AS	3.8	2.89	5.20

Polyphenolic content in tubers

According to the studies done by Kirui et al., 2018 the polyphenolic content in tubers decreases towards maturity. In our studies also, approximately two folds decreased was observed towards the maturity as shown in Table 20. Highest polyphenolic content was observed in early stage of C2-2 cultivar (4.05 mg GAE. g^{-1} FW) and lowest in mature stage of CS-2 (1.70 mg GAE. g^{-1} FW).

Table 20: Estimation of polyphenolic content in potato extract of potato (expressed in mg of Gallic acid equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	2.14	1.93	1.86
CS -2	4.05	2.51	1.70
DE	3.95	2.16	2.09
KJ	2.14	2.72	3.24
PR	2.55	2.44	1.96
AS	3.15	2.24	1.83

Flavonoid content in leaf

The high amount of flavonoid content was observed in growing stage of KJ Cultivar (40.32 mg quercetin eq. g^{-1} FW) and lowest in DE and AS cultivar of mature leaf (15.05mg quercetin eq. g^{-1} FW).There is decrease in flavonoid content from early growing stage to mature stage in every stage of leaf. In mature stage it was highest in CS-2 cultivar (29.49 mg quercetin eq. g^{-1} FW) and early growing stage it was highest amount in KJ cultivar (40.32 mg quercetin eq. g^{-1} FW) as shown in Table 21.

Table 21: Estimation of flavonoid content in leaf extract of potato (expressed in mg of quercetin Equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	21.32	21.07	19.07
CS -2	40.20	32.49	29.49
DE	25.70	23.53	15.05
KJ	40.32	39.32	26.70
PR	23.82	33.8	20.90
AS	22.95	17.16	15.05

Flavonoid content in stem

Flavonoid content was observed highest in early growing cultivar of KJ i.e. (11.25 mg quercetin eq. g^{-1} FW) and lowest flavonoid content in AS (1.25 mg quercetin eq. g^{-1} FW). Surprisingly, in cultivar AS, the flavonoid content increased towards maturity as revealed in Table 22. This difference might be due to genetic makeup of the plant, which needs to be understood.

Table 22: Estimation of flavonoid content in stem extract of potato (expressed in mg of quercetin Equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	5.40	3.28	4.32
CS -2	3.35	3.87	4.45
DE	6.8	3.37	4.82
KJ	**11.25**	4.70	2.53
PR	4.24	5.65	5.99
AS	**1.25**	2.99	4.20

Flavonoid content in tuber

According to the literature reported in Kirui, G. K., et al 2018. Flavonoid content depends upon the color and pigmentation of the tissues. Highest flavonoid content was observed in the young tuber of cultivar CS-1 (7.58 mg quercetin eq. g^{-1} FW), followed by cultivar DE (6.32mg quercetin eq. g^{-1} FW) .The flavonoid content decrease abruptly in the mature tuber hence followed a parabolic pattern as shown in Table 23.

Table 23: Estimation of flavonoid content in tuber extract of potato (expressed in mg of quercetin Equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	7.78	4.78	3.58
CS -2	3.78	2.31	1.18
DE	6.32	5.56	2.32
KJ	5.45	2.49	3.98
PR	3.16	3.24	2.98
AS	5.85	3.24	2.41

Antioxidant activity in leaf

It was observed that the antioxidant activity of the leaf decreases ten folds towards maturation. It was observed to be highest in KJ (5.19 mg ascorbate eq. g^{-1} FW) at young stage on the contrary, it was lowest in PR (0.122 mg ascorbate eq. g^{-1} FW) at mature stage. The increase in antioxidant capacity represent the increase ROS level and increased tendency to combat the stress. Approx. 10 fold increase in antioxidant capacity in young leaf was reported according to Padda, M. S., et al 2007 as shown in Table 24.

Table 24: Estimation of antioxidant capacity from leaf of potato (expressed in mg of Ascorbate equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	2.04	1.68	0.208
CS -2	2.17	6.02	0.507
DE	3.69	3.15	0.491
KJ	5.19	3.72	1.92
PR	1.61	1.42	0.122
AS	2.08	1.19	0.286

Antioxidant capacity in stems

In case of stem, the antioxidant capacity decreases from young to mature in all cultivars except CS-1. It was highest in KJ (1.380 mg ascorbate eq. g^{-1} FW) at young stage and CS-1 (1.17 mg ascorbate eq. g^{-1} FW) at mature stage but was lowest in PR (0.360 mg ascorbate eq. g^{-1} FW) at young stage and CS-2 (0.093 mg ascorbate eq. g^{-1} FW) at mature stage. It might represent that fully mature stems; represent the low photo respiration activity and hence low antioxidant capacity as shown in Table 25.

Table 25: Estimation of antioxidant capacity from stem tissues of potato (expressed in mg of Ascorbate equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	0.368	0.515	1.17
CS -2	0.442	0.208	0.093
DE	0.376	0.163	0.114
KJ	1.380	0.909	0.179
PR	0.360	0.573	0.356
AS	0.229	0.106	0.097

Antioxidant capacity in tubers

It was observed that antioxidant capacity first increase in growing stage after passing from developing stage but its content again decreased in mature stage except AS. In all cultivar antioxidant content deceases as it move from developing stage to growing stage and at last to mature stage. It was lowest in PR (0.672 mg ascorbate eq. g^{-1} FW) at developing stage, KJ (1.12 mg ascorbate eq. g^{-1} FW) at growing stage and PR(0.490 mg ascorbate eq. g^{-1} FW) at mature stage and it was highest in CS-1 (4.14 mg ascorbate eq. g^{-1} FW) at developing stage, CS-2(3.43 mg ascorbate eq. g^{-1} FW) at growing stage and CS-2 (2.55 mg ascorbate eq. g^{-1} FW) at mature stage. It is following bell shaped pattern which could be due to different antioxidant capacity of different cultivars. Thus, the trend was marked with some fluctuations. According to the literature reported in Kirui, G. K., et al 2018 as shown in Table 26.

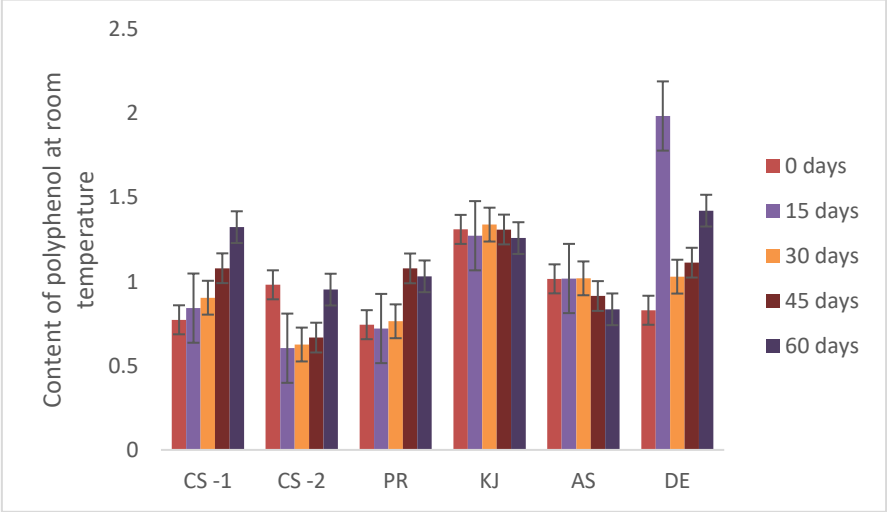
Table 26: Estimation of antioxidant capacity from tuber tissues of potato (expressed in mg of Ascorbate equivalent per gram of fresh weight of tissue)

Cultivar	Early growing	Growing	Mature
CS -1	2.919	2.686	2.20
CS -2	4.14	3.43	2.55
DE	2.42	2.30	1.45
KJ	2.04	1.789	0.780
PR	1.72	1.521	0.490
AS	0.672	1.12	1.49

4.4 Total phenolic content in the tubers under different storage conditions

At initial stages there is increase in TPC content and then decrease was observed in case of tuber sample. Overall increase in total phenolic compound was observed from 0 to 60 DOS. The TPC content ranged from 0.604 to 1.983 mg GA equiv. / gFW at room temperature and from 0.511 to 2.427 mg GA equiv. / gFW at 4 ºC and from 0.609to 1.629 mg 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. At room temperature the TPC content increased to 1.5 fold and 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 as shown in Fig.20.

A



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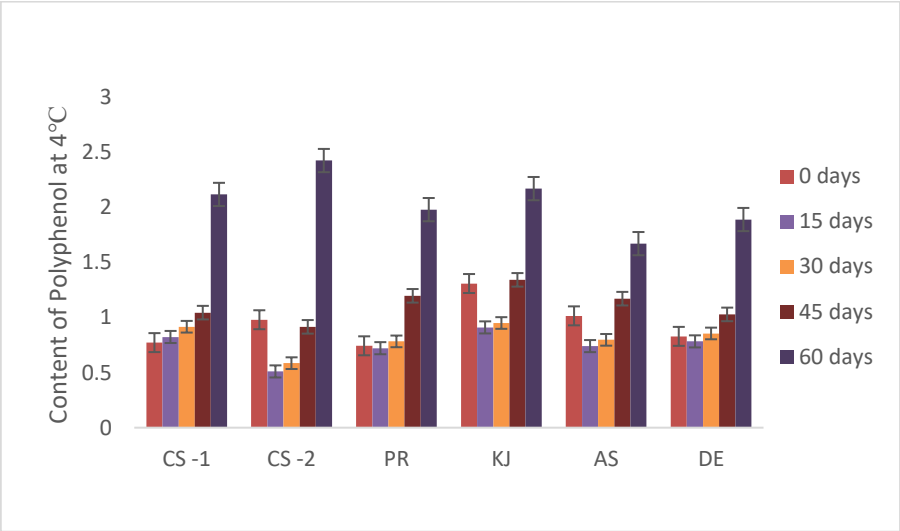

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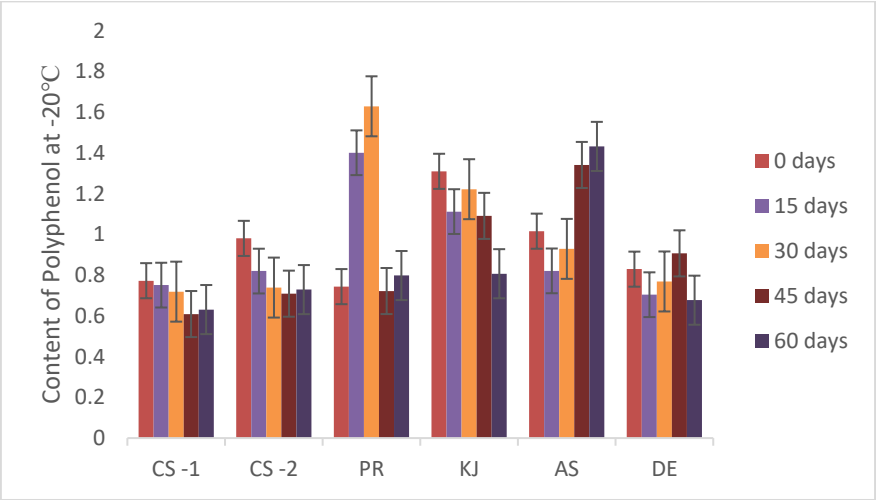
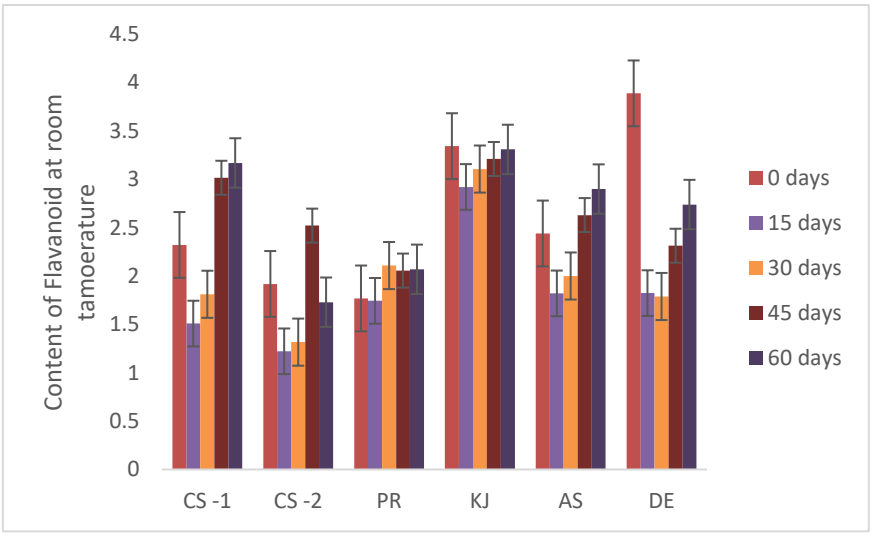


Fig. 20 Total polyphenolic content of mature tuber (a) room temperature (b) 4℃ and (c) -20℃

4.5 Total flavonoid content in the tuber under different storage conditions

At initial stages there is increase in TFC content and then decrease was observed in case of tuber sample 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 the flavonoid content ranged from 1.222 mg quercetin eq. g^{-1} FW to 3.890 mg quercetin eq. g^{-1} FW at room temperature and from 1.231 mg quercetin eq. g^{-1} FW to 3.890 mg quercetin eq. g^{-1} FW at 4 ºC and from 0.909 mg quercetin eq. g^{-1} FW to 3.147 mg quercetin eq. g^{-1} FW at -20 ºC from day 0 to day 60. This study is in concordance with Galani *et al.*, 2017 as shown in Fig.21.

A



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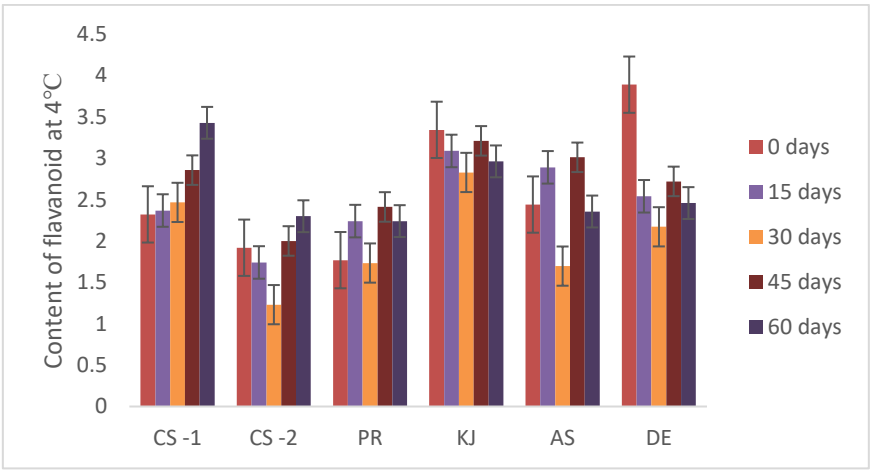

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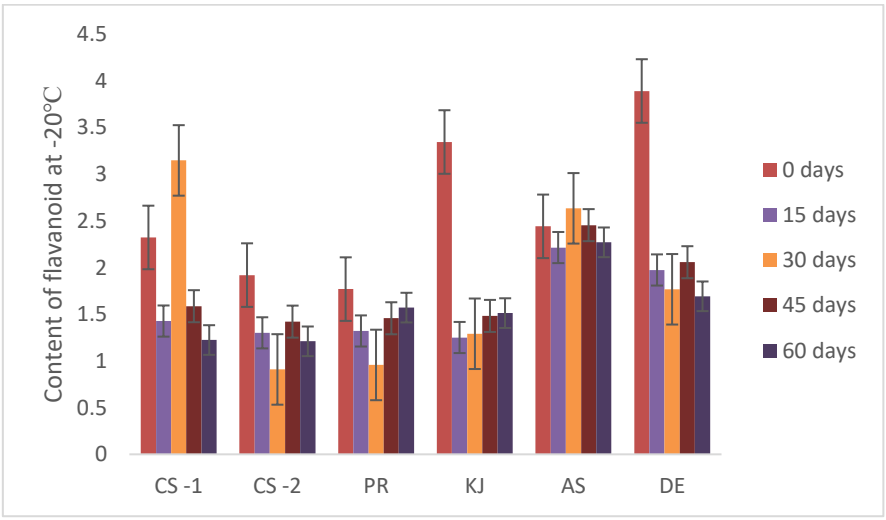
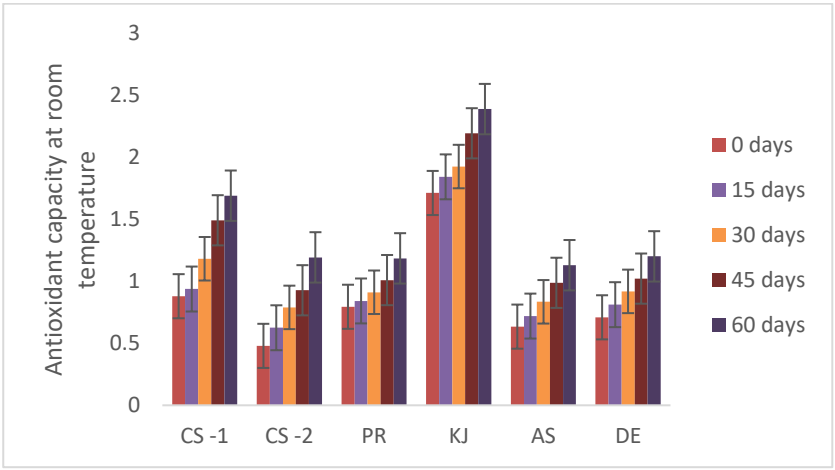


Fig. 21 Total flavonoid content of mature tuber (a) room temperature (b) 4℃ and (c) -20℃

4.6 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. The antioxidant activity ranged from 0.479 to 2.387 mg ascorbate eq. g^{-1} FW at room temperature and from 0.479 to 2.214 mg ascorbate eq. g^{-1} FW at 4 ºC and from 0.479 to 2.499 mg ascorbate eq. g^{-1} FW -20 ºC from 0 to 60 days of storage as shown in Fig.22. Our data is in concordance with the study conducted by Akyol et al., 2016

A



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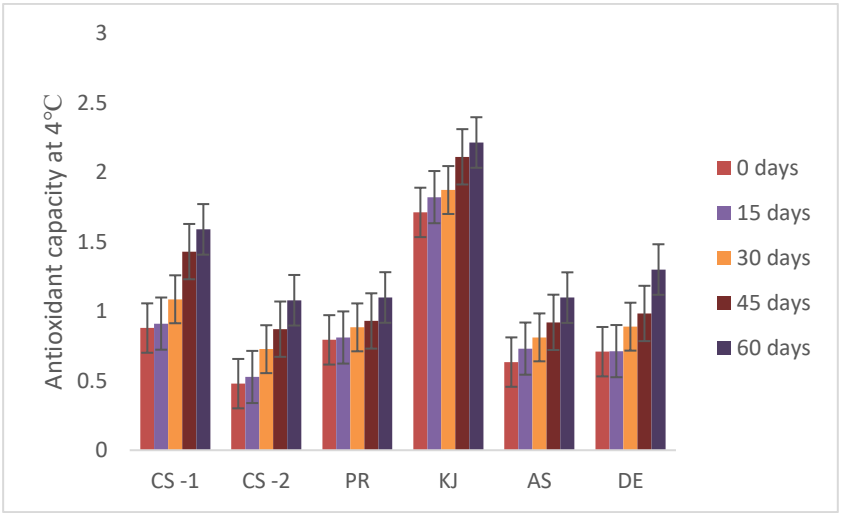

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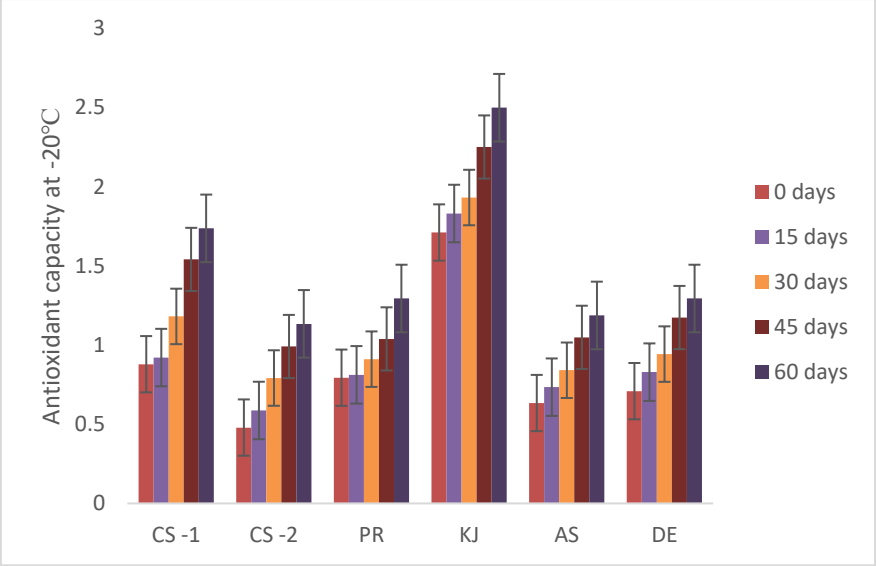

Fig. 22 Antioxidant capacity of mature tuber (a) room temperature (b) 4°C and (c) -20°C.

4.7 Total polyphenolic content in different market selection varieties:

From the data as shown in Table 27, the TPC content is found to be highest in MS4 (6.78 mg Gallic equiv. / gFW) and lowest in MS3 (3.49 mg gal equiv./ gFW).

Table 27*:* Estimation of TPC in tuber extract of potato of various market selection varieties (expressed in mg of Gallic acid equivalent per gram of fresh weight of tissue)

Cultivars	Polyphenol(Content)
MS1	6.71
MS2	5.57
MS3	3.49
MS4	6.78
MS5	4.46

4.8 Total Flavonoid content in different market selection varieties:

From the data it was concluded that the TFC content was found to be highest in MS2 (6.16 mg quercetin eq. g^{-1} FW) and lowest in MS4 (1.61 mg quercetin eq. g^{-1} FW) as shown in Table 28.

Table 28: Estimation of TFC in tuber extract of potato of various market selection varieties (expressed in mg of quercetin equivalent per gram of fresh weight of tissue)

Cultivars	Flavonoid(Content)
MS1	4.09
MS2	6.16
MS3	3.43
MS4	1.61
MS5	3.11

4.9 Antioxidant capacity in different market selection varieties:

From the data it was concluded that antioxidant activity of MS5 (1.294 mg ascorbate eq. g^{-1} FW) was significantly higher than other potato varieties of marketas shown in Table 29.

Table 29: Estimation of Antioxidant capacity in tuber extract of potato of various market selection varieties (expressed in mg of ascorbate equivalent per gram of fresh weight of tissue)

Cultivars	FRAP(Content)
MS1	0.839
MS2	1.178
MS3	0.752
MS4	0.553
MS5	1.294

4.10 Estimation of Sugars

The approximate weight of:

1. *Early growing tubers = 1g to 4g*
2. *Growing tubers = 7g to 14g*
3. *Mature tubers = 20g to 28g*

Reducing sugar content in leaf

As mature leaves slowly, becomes the sink tissues hence storage of more plant metabolites. The sugar content was also found to increase two- folds from young to mature as shown in Table 30, thus supporting the above concept. Reducing sugar content in leaf was found to be highest in mature stage of CS-2 cultivar (24.5 mg eq. g^{-1} FW) and lowest in early growing stage of AS cultivar (2.41 mg eq. g^{-1} FW).

Table 30: Estimation of reducing sugars in leaf extract of potato (expressed in mg equivalent per gram of fresh weight of tissue) by Nelson-Somogyi's Method

Cultivar	Early growing	Growing	Mature
CS -1	4.23	10.71	11.67
CS -2	18.89	20.26	24.5
DE	5.11	10.6	10.18
KJ	3.76	9.29	10.11
PR	4.96	9.79	11.60
AS	2.41	6.51	11.60

Reducing sugar content in Stem

Reducing sugar content in stem is found to be highest in mature stage of KJ cultivar (70.9 mg eq. g^{-1} FW) and lowest in early growing stage of CS-1 cultivar (7.2 mg eq. g^{-1} FW) and from developing stage to mature stage reducing sugar content in stem is increased and high amount of reducing sugar is found in developing stage of KJ Cultivar (26.92 mg eq. g^{-1} FW) and in growing stage in KJ cultivar (46.45 mg eq. g^{-1} FW). So it is concluded that KJ cultivar is the best cultivar in early growing, growing and mature stem as shown in below Table 31.

Table 31: Estimation of reducing sugars in stem extract of potato (expressed in mg equivalent per gram of fresh weight of tissue) by Nelson-Somogyi's Method

Cultivar	Early growing	Growing	Mature
CS -1	7.20	17.02	20.64
CS -2	19.01	35.29	58.39
DE	12.77	22.06	47.9
KJ	26.92	46.45	70.9
PR	9.29	14.54	42.89
AS	10.91	22.38	37.4

Reducing sugar content in tuber

Reducing sugar content in tuber is found to be highest in developing stage of KJ cultivar (32.21 mg eq. g^{-1} FW) and lowest in early growing stage of CS-1 cultivar (5.11 mg eq. g^{-1} FW).In mature stage high amount of reducing sugar is found to be in KJ (32.21 mg eq. g^{-1} FW).There is some fluctuation in data is also be found as shown in Table 32.

Table 32: Estimation of reducing sugars in tuber extract of potato (expressed in mg of equivalent per gram of fresh weight of tissue) by Nelson-Somogyi's Method

Cultivar	Early growing	Growing	Mature
CS -1	5.11	12.95	25.56
CS -2	8.30	19.86	26.40
DE	9.51	12.95	23.87
KJ	14.27	28.9	32.21
PR	6.03	13.12	20.9
AS	6.35	15.85	16.53

Reducing sugar content in different market selection varieties:

From the data it was concluded that the reducing sugar content is found to be highest in MS1 (11.95 mg eq. g^{-1} FW) and lowest in MS3 (4.66 mg eq. g^{-1} FW) as shown in Table 33.

T**able 33**: Estimation of reducing sugars in tuber extract of potato of various market selection varieties (expressed in mg of equivalent per gram of fresh weight of tissue) by Nelson-Somogyi's Method

Cultivars	Reducing sugar (Content)
MS1	11.95
MS2	5.11
MS3	4.66
MS4	11.85
MS5	11.56

Total soluble sugar in leaf

Total soluble sugar content in leaf is found to be highest in mature stage of KJ cultivar (21.68 mg eq. g^{-1} FW) and lowest in growing stage of DE cultivar (5.01 mg eq. g^{-1} FW). In developing

stage high amount of total soluble sugar is found in CS-2 cultivar (18.28 mg eq. g^{-1} FW).There is some fluctuation in data is also be found as shown in Table 34.

Table 34: Estimation of total soluble sugars in leaf extract of potato (expressed in mg equivalent per gram of fresh weight of tissue) by Anthrone Method

Cultivar	Early growing	Growing	Mature
CS -1	6.41	5.97	11.84
CS -2	18.28	6.46	12.13
DE	12.86	5.01	10.10
KJ	15.41	5.84	21.68
PR	13.50	6.33	16.34
AS	9.58	6.60	21.53

Total soluble sugar in stem

Total soluble sugar content in stem is found to be highest in mature stage of DE cultivar (22.52 mg eq. g^{-1} FW) and lowest in growing stage of CS-1 cultivar (4.21mg mg eq. g^{-1} FW) and from developing stage to mature stage total soluble sugar content in stem is increased except PR (13.85 mg eq. g^{-1} FW) and high amount of total soluble sugar is found in developing stage of PR Cultivar (15.28 mg eq. g^{-1} FW) and in growing stage in AS cultivar (15.16 mg eq. g^{-1} FW). Higher sugar content in mature stem represents the well differentiated vascular system for transport as shown in Table 35.

Table 35: Estimation of total soluble sugars in stem extract of potato (expressed in mg of equivalent per gram of fresh weight of tissue) by Anthrone Method

Cultivar	Early growing	Growing	Mature
CS -1	**4.21**	7.51	11.59
CS -2	11.43	14.31	20.81
DE	10.26	10.59	**22.52**
KJ	13.33	10.20	14.68
PR	15.28	11.87	13.85
AS	11.46	15.16	14.57

Total soluble sugar in tuber

Total soluble sugar content in tuber is found to be highest in mature stage of DE cultivar (22.52 mg eq. g^{-1} FW) and lowest in growing stage of CS-2 cultivar (9.88 mg eq. g^{-1} FW) and from developing stage to mature stage total soluble sugar content in tuber is increased and high amount of total soluble sugar is found in developing stage of DE Cultivar (20.47 mg eq. g^{-1} FW) and in growing stage in AS cultivar (18.99 mg eq. g^{-1} FW) as shown in Table 36.

Table 36*:* Estimation of total soluble sugars in tuber extract of potato (expressed in mg equivalent per gram of fresh weight of tissue) by Anthrone Method

Cultivar	Early growing	Growing	Mature
CS -1	11.48	14.98	20.26
CS -2	**9.88**	10.43	11.88
DE	20.47	17.24	**22.52**
KJ	16.73	17.23	21.35
PR	11.17	13.06	16.63
AS	10.23	18.99	13.49

Total soluble sugar content in different market selection varieties:

From the data it was concluded that the reducing sugar content is found to be highest in MS3 (11.30 mg eq. g^{-1} FW) and lowest in MS2 (3.10 mg eq. g^{-1} FW) as shown in Table 37.

Table 37: Estimation of Total soluble sugars in tuber extract of potato of various market selection varieties (expressed in mg of glucose equivalent per gram of fresh weight of tissue) by Anthrone Method

Cultivars	Total soluble sugar (Content)
MS1	10.75
MS2	3.10
MS3	11.30
MS4	9.23
MS5	10.83

Reducing sugar content in the tubers under different storage conditions

Reducing sugar content under different storage condition is increased from 15 to 60 DOS. The Reducing sugar content ranged from 9.29 to 35.20 mg eq. g^{-1} FW at room temperature and from 8.22 to 35.41 mg eq. g^{-1} FW at 4 ºC and from 10.31 to 40.18 mg eq. g^{-1} FW at -20 ºC from 0 to 60 days of storage. The reducing sugar content fluctuated during the whole storage period until the 60 DOS as shown in Fig.23.

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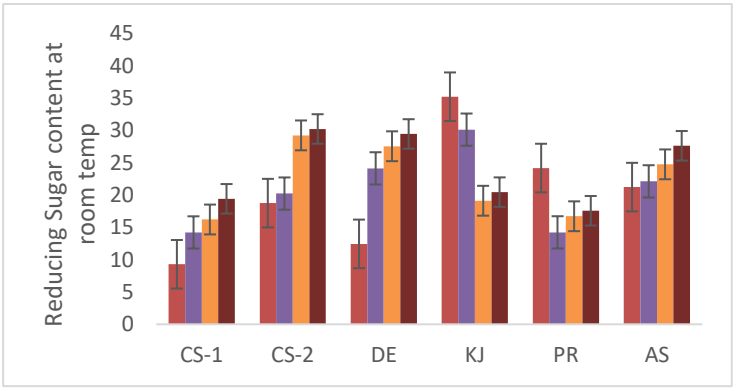

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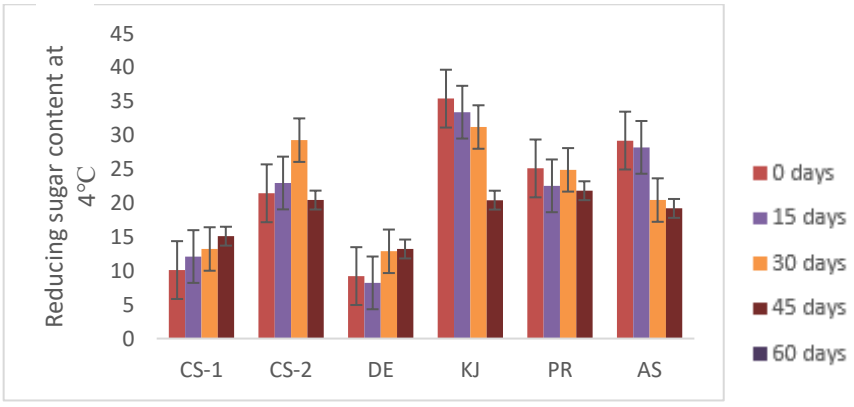

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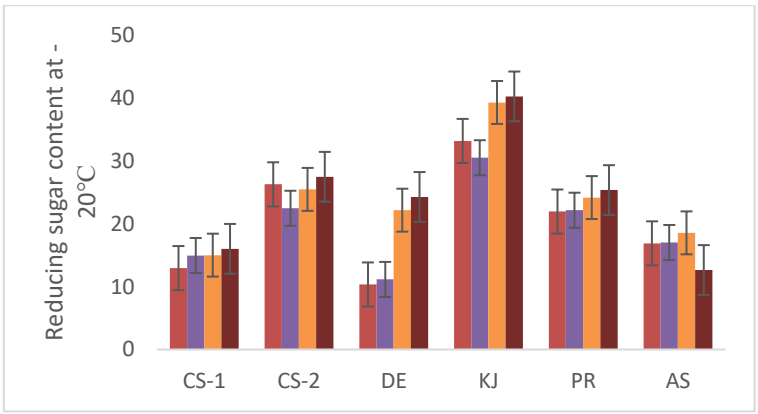

Fig. 23 Reducing sugar content of mature tuber (a) room temperature (b) 4°C and (c) -20°C

Total soluble sugar content in the tubers under different storage conditions

Total soluble sugar content under different storage condition is increased from 15 to 60 DOS. The total soluble sugar content ranged from 6.40 to 29.72 mg eq. g^{-1} FW at room temperature and from 9.21 to 29.40 mg eq. g^{-1} FW at 4 ºC and from 9.29 to 29.21 mg eq. g^{-1} FW at -20 ºC from 0 to 60 days of storage. The total soluble sugar content fluctuated during the whole storage period until the 60 DOS as shown in Fig.24.

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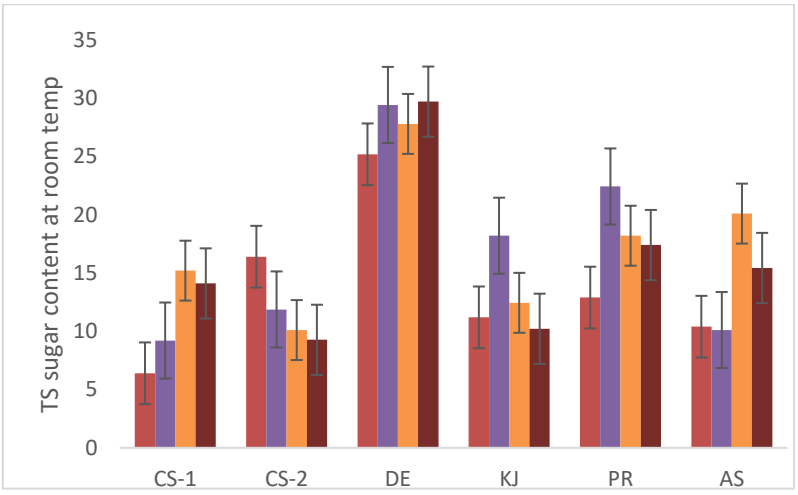

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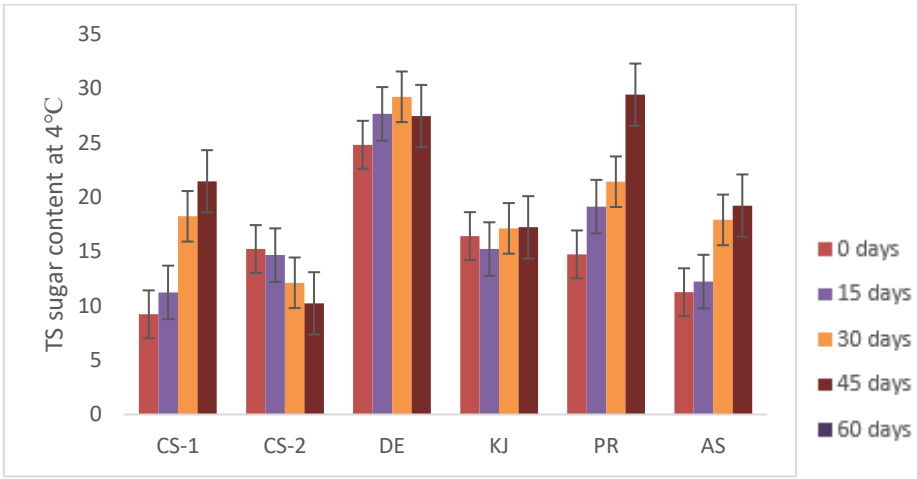

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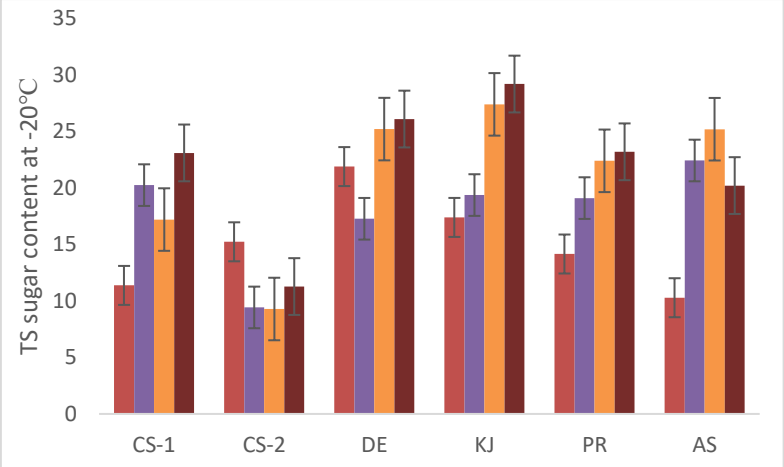


Fig. 24 Total soluble sugar content of mature tuber (a) room temperature (b) 4℃ and (c) -20℃

4.11 Estimation of Protein content by Folin-Lowry method

Protein content in leaf

We have observed inter-varietal differences in protein activity in growing and mature leaf. Protein content was found to be highest in early growing KJ cultivar and lowest in growing AS cultivar. In early growing leaf all cultivar of potato, protein content is high as compared to mature leaf. It might be due to different stress conditions in regard with genetic makeup as shown in Table 38.

Table 38: Estimation of protein content in leaf extract of potato (expressed in mg equivalent per gram of fresh weight of tissue) by Folin-Lowry method

Cultivar	Early growing	Growing	Mature
CS -1	12.50	10.84	10.34
CS -2	12.35	12.15	9.32
AS	12.77	**6.93**	9.32
KJ	**15.98**	8.25	8.35
PR	15.22	8.75	8.16
DE	11.32	6.23	6.29

Protein content in stem

We have observed inter-varietal differences in protein activity in growing and mature stem. Protein content was found to be highest in early growing CS-1 cultivar and lowest in mature PR cultivar. In early growing stem all cultivar of potato, protein content is high as compared

to mature stem. It might be due to different stress conditions in regard with genetic makeup as shown in Table 39.

Table 39: Estimation of protein content in stem extract of potato (expressed in mg equivalent per gram of fresh weight of tissue) by Folin-Lowry method

Cultivar	Early growing	Growing	Mature
CS -1	**5.89**	5.85	2.51
CS -2	4.76	3.04	3.32
AS	5.62	4.28	4.34
KJ	4.94	4.50	3.44
PR	3.90	3.74	**2.39**
DE	5.50	5.48	4.42

Protein content in tuber

We have observed inter-varietal differences in protein activity in growing and mature tuber. Protein content was found to be highest in early growing CS-1 cultivar and lowest in mature AS cultivar. In early growing tuber all cultivar of potato, protein content is high as compared to mature tuber. It might be due to different stress conditions in regard with genetic makeup as shown in Table 40.

Table 40: Estimation of protein content in tuber extract of potato (expressed in mg equivalent per gram of fresh weight of tissue) by Folin-Lowry method

Cultivar	Early growing	Growing	Mature
CS -1	**10.28**	4.64	3.94
CS -2	4.86	4.64	3.98
AS	8.61	4.14	**2.96**
KJ	6.27	4.48	4.21
PR	7.75	4.61	4.40
DE	6.47	4.30	4.39

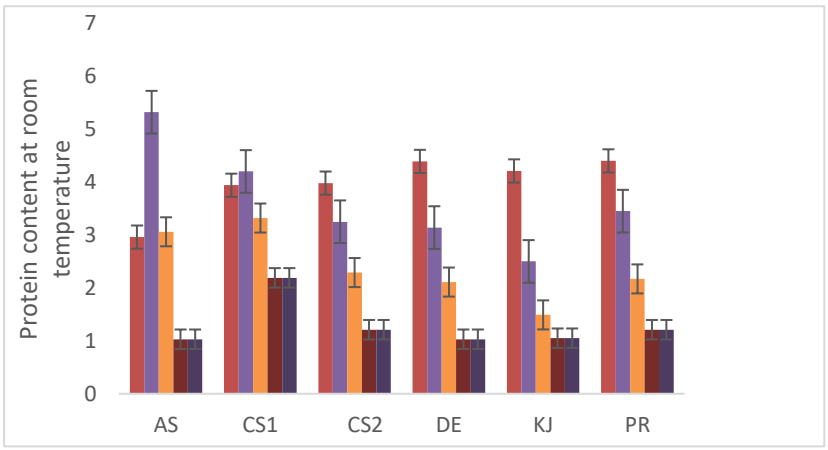
To estimate the protein content in the tubers under different storage conditions

Protein content under different storage condition is increased from 0 to 60 DOS. The total protein content ranged from 1.03 to 5.32 mg equiv. / gFW at room temperature and from 0.717

to 4.98 mg equiv. / gFW at 4 ºC and from 0.659 to 5.89 mg equiv. / gFW at -20 ºC from 0 to 60 days of storage. The protein content fluctuated during the whole storage period until the 60 DOS as shown in Fig.25.

Protein content at different storage conditions of tuber

A



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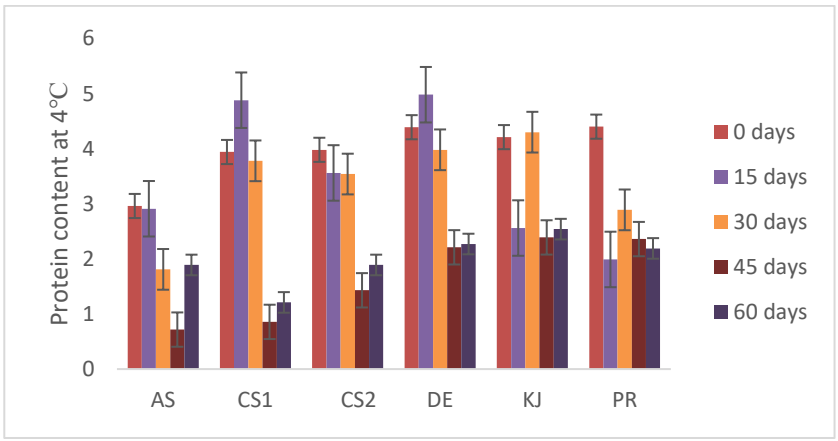

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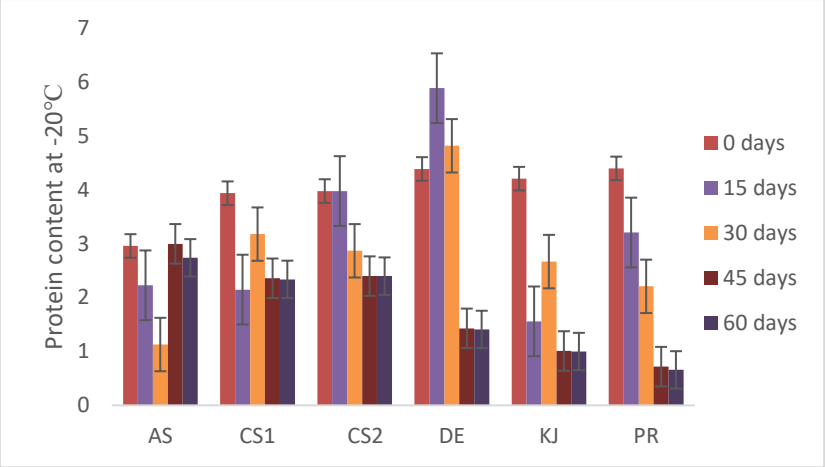


Fig. 25 Protein content of mature tuber (a) room temperature (b) 4℃ and (c) -20℃

4.12 Effect of mature extract of tuber on different cell viability

Effect of polyphenolic tuber extract on RAW 264.7 cell viability: The results showed that the cell viability of RAW 264.7 was increased at 5 $\times 10^4$ cells/mL, but at higher concentrations the cell viability was decreased at the higher concentrations. Hence, after optimizing the cytotoxic effect of the extracts of cultivar CS-1 as shown in Fig. 27 (A).After that extracts from the other cultivars were treated with optimized concentration as shown in Fig. 27(B).

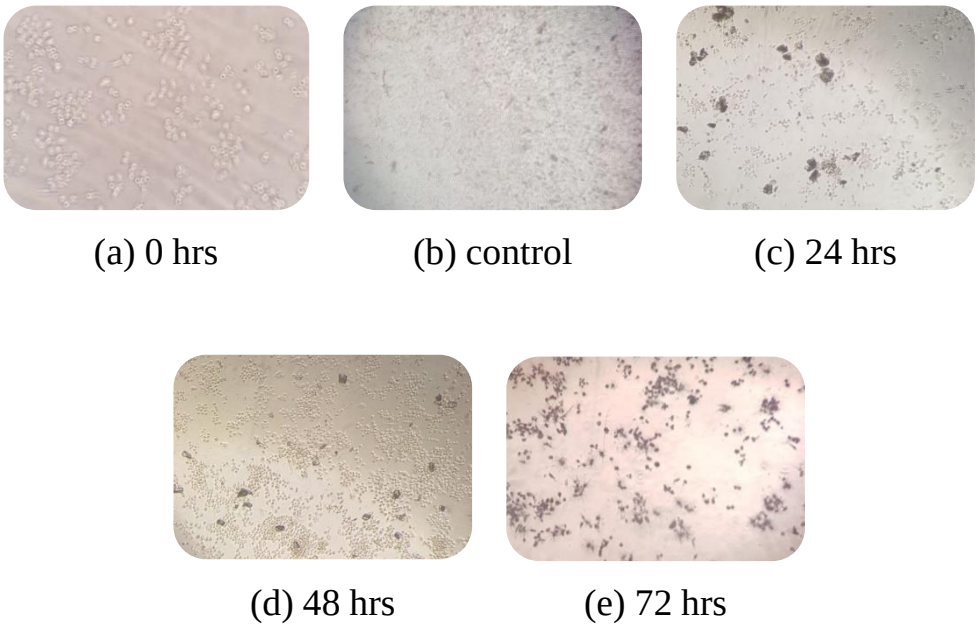
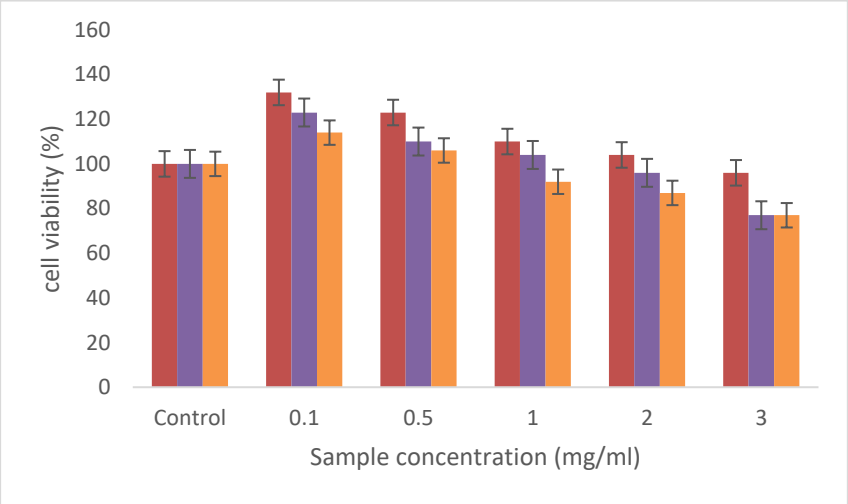


Fig. 26 Effect of polyphenolic extract on RAW 264.7 cells (a) at 0 hrs (b) Control (c) 24 hrs (d) 48 hrs (e) 72 hrs

Pure polyphenolic extracts from fruits and vegetables have exhibited cytotoxic activity towards various cancer cell lines (Wang et al., 2011 and Yang, et al., 2011) Cell proliferation was inhibited and hence the cell viability was reduced by the ability of polyphenols to interact with basic cellular mechanism and through the modal action of nitric oxide production by inhibiting inducible nitric oxides synthase. As shown in figure polyphenols inhibited cancer cells and macrophages. These results were in concordance with the previous that have reported. The anti- cancerous ability of polyphenols for RAW was observed that at high concentration cell count, cell viability was reduced to almost 80% in case of macrophages (Hawang et al., 2000). For the optimisation, the different concentrations of polyphenols were reported with different cell count of RAW. The concentration with lowest viability was considered as best and was chosen for other cultivar. Out of six cultivars used in the study DE, followed by AS and KJ showed the lowest cell viability. Cultivar DE showed the viability representing its higher antioxidant potential (Sugata et al., 2015).

A



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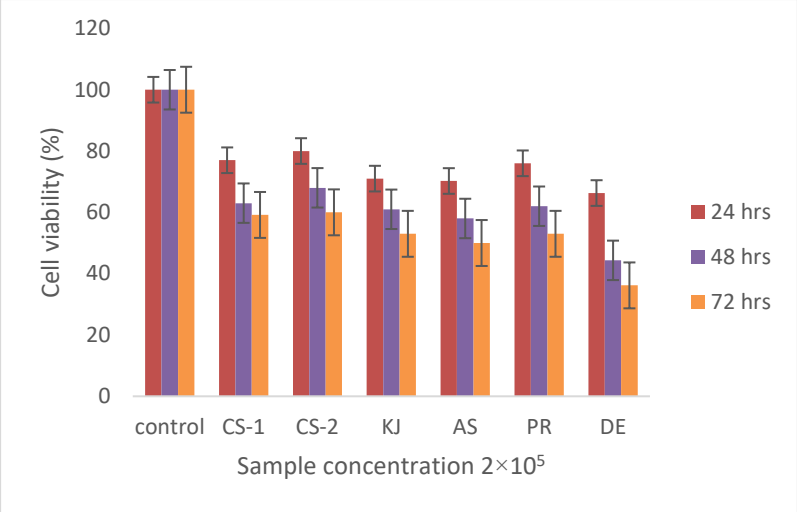

Fig. 27 (a) Cell viability of RAW 264.7 treated with polyphenolic extract at different concentration of CS-1(b) Cell viability of RAW 264.7 treated with polyphenolic extract conc. of 2×10^5 at 24 hrs, 48 hrs and 72 hrs

Effect of mature extract of tuber on MCF-7 cell line: The polyphenolic extracts showed cytotoxic effect on MCF-7 at low concentration as compared with RAW 246.7. After optimizing the best concentration of polyphenolic extracts of cultivar CS-1 for cytotoxicity on the MCF-7, the extracts from the other cultivars were treated with optimized concentration as shown in Fig. 29.

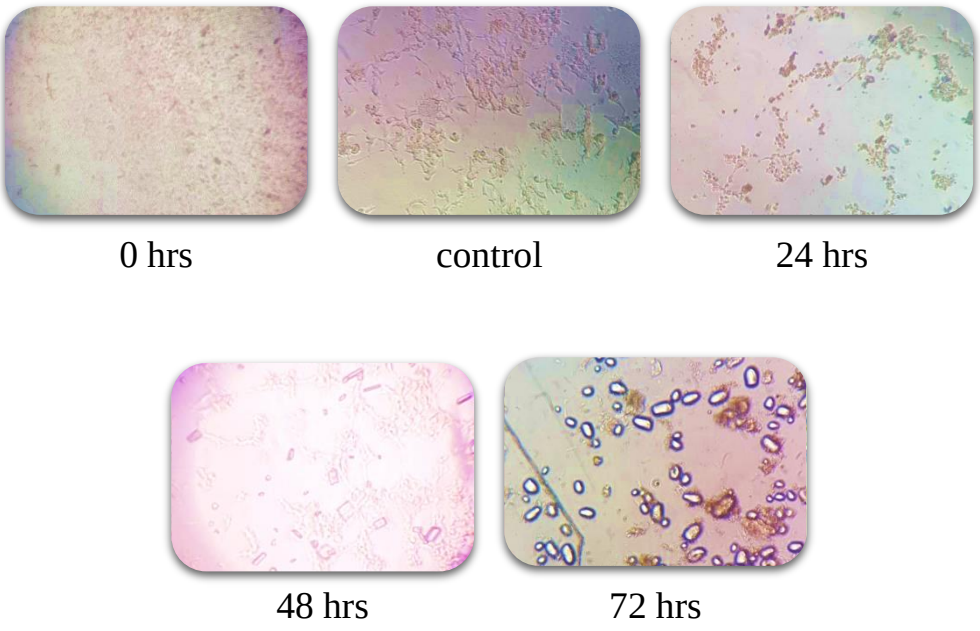

Fig. 28 Effect of polyphenolic extract on MCF-7 cells (a) at 0 hrs (b) Control (c) 24 hrs (d) 48 hrs (e) 72 hrs

To inhibit the growth of cancer cell lines, anticancer activities of polyphenolic extract were displayed in MCF-7 in concentration and time dependent manner. Polyphenolic extracts could induce apoptosis in MCF-7 through intrinsic and extrinsic pathway. PPE may have potential to be applied in food industries and nutraceuticals. Out of six cultivars used in the study DE showed the lowest cell viability and representing its higher antioxidant potential (Sugata et al., 2015).

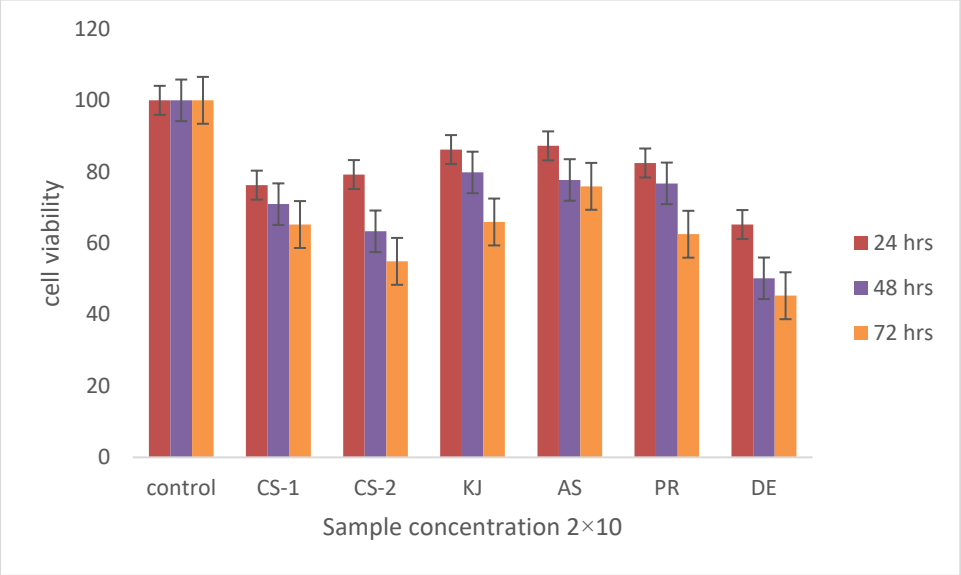

Fig. 29 Cell viability of MCF-7 treated with polyphenolic extract conc. of 2×10^5 at 24 hrs, 48 hrs and 72 hrs

Conclusions

On the basis of experimental data the following conclusions were made:

- ROS plays a dual role; at low concentration they are involved in cell signalling and at high concentration they cause damage to various biomolecules, cellular structures and even cell death.
- It was observed that the ascorbate content was highest in mature leaves, but accumulated at low level in the mature tubers.
- Estimation of ascorbate by HPLC appeared to be more sensitive; whereas spectrophotometric determination was more convenient but slightly unsafe as the process involved the use of Bromine water and concentrated acid. However, although estimation of ascorbate by titrimetric method was easy-to-execute but not sensitive.
- The ascorbate content was found to be gradually decreased during storage (up to 60 days) under different experimental temperatures.
- Phenolic and flavonoid contents and overall antioxidative capacities were found to be at significantly higher level in the growing potato organs in comparison to the matured ones.
- Prolonged storage at room temperature, in particular, for a period of 60 days led to increase of total phenolic and flavonoid contents significantly in the harvested mature tubers.
- High sugar level as noticed in the mature and growing tubers appeared to be biologically significant; it was likely that the sugars act as a signals regulating different genes, especially involved in photosynthesis, sucrose metabolism and ROS metabolism.
- High sugar content in the mature leaves reflected increasing sink capacity.
- High sugar content was also observed in the mature stem as expected probably due to the transport of sugars through phloem tissues.
- Soluble sugar contents (both reducing and total sugars) were significantly increased during storage at low temperatures.
- Total protein content was found to be at higher in the young leaves as compared to the other organs. Protein content was found to be gradually decreased in the tubers during storage under different conditions.
- MS1 and MS2 were found to contain higher levels of polyphenol, flavonoid and ascorbate contents.
- Polyphenolic extracts were to be effective in reducing the viability of the cell lines namely, MCF-7 (human breast cancer cell lines) and RAW 264.7 (Murine macrophage cell line).

Future Scope of the work

This study deals with the determination of non-enzymatic antioxidants namely ascorbic acid, polyphenols and flavonoids along with assessment of antioxidative capacity, sugar and protein contents in various organs of the Indian potato cultivars. Overall, the level of antioxidants were found to be high in the following cultivars CS-1, CS-2 and KJ, particularly in the tubers. Therefore the genetic potential of these cultivars could be employed in crop improvement.

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