

**A Study on Biochemical and Nutraceutical Potential of  
Some Underutilized Apple Varieties of Kashmir**

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(MSBS/2018/08)



**Division of Basic Sciences and Humanities**  
**Faculty of Horticulture**  
**Sher-e-Kashmir University of Agricultural Sciences &  
Technology of Kashmir**

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**A Study on Biochemical and Nutraceutical Potential of  
Some Underutilized Apple Varieties of Kashmir**

**Ajaz Bashir**  
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**Thesis**

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*“A father’s affection is higher than the mountains and the  
mother’s love is deeper than the ocean”*

*DEDICATE MY THESIS TO*

*My Respected PARENTS*

*Mr. BASHIR AHMAD DAR*

*Mrs. FAHMIDA AKHTER*

*&*

*MY Beloved SISTERS*

*Miss Roohi Jehan*

*Miss Shazia Bashir*



**Sher-e-Kashmir**  
**University of Agricultural Sciences & Technology of Kashmir**  
**Faculty of Horticulture,**  
**Division of Basic Sciences and Humanities**

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This is to certify that the thesis entitled, “**A Study on Biochemical and Nutraceutical Potential of Some Underutilized Apple Varieties of Kashmir**” submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science in Biochemistry**, to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Mr. Ajaz Bashir (Regd. No. MSBS/2018/08)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or information received during the course of investigation has duly been acknowledged.

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**ABSTRACT**

The present investigation entitled “A Study on Biochemical and Nutraceutical Potential of Some Underutilized Apple Varieties of Kashmir” was carried out in the Biochemistry and Molecular Biotechnology Lab, Division of Basic Sciences and Humanities, SKUAST-K Shalimar, Transcriptomics Laboratory, Division of Plant Biotechnology, SKUAST Kashmir and RCRQA, Division of Entomology, FOH Shalimar, SKUAST-K during the year 2020-21. Methanolic and aqueous extracts of 4 genotypes of apple including two domestic and two wild (crabapples), were examined for their biochemical and nutraceutical parameters like total phenolic content, total flavonoid content, total carotenoid content, Quercetin, total anthocyanins, total antioxidant activity and anticancerous potential. The experiment was laid out in completely randomized design with three replications. Results of the current study revealed that among the 4 different genotypes, Ambri possessed the highest total phenolic content (137.83mg/100g) as well as total flavonoid content( 113.31 mg/100g) respectively. While as the highest total antioxidant activity (1,319.08  $\mu$ M FRAP/g) and the highest anthocyanin content (13.290 mg/100g) was detected in red coloured crab apple genotype. The highest amount of total carotenoid content was found in yellow

coloured crab apple (0.051 mg/g). Among the two selected genotypes viz. red coloured crab apple and Ambri, the highest amount of quercetin as determined by HPLC was found in red coloured crab apple (9.86 µg/g) as compared to Ambri apple (7.43 µg/g). Aqueous extract of indigenous local Ambri genotype evaluated for its anticancerous potential using colony proliferation assay demonstrated increased cell proliferation effect against CH-2 cancer cells in a dose-dependent manner instead of cell inhibition. Therefore, this preliminary anticancerous-based assay warrants further extensive investigations. All of the four genotypes subjected to nutrient analysis revealed that Ambri had the highest amount of moisture (83%), TSS (13.51g/100g) and crude fiber (1.61g/100g). The vitamin C content of Ambri (3.78g/100g) was higher than golden delicious (3.40g/100g). The overall results of this study revealed that the selected four apple genotypes are rich in one or other potent phytochemical and can be better exploited for nutraceutical purposes against oxidative stress related diseases as well as used for biofortification purposes.

**Keywords:** Apple, Nutrients, Nutraceuticals, Antioxidants, Anticancerous potential.

Signature of Student

Dated: \_\_\_\_\_

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*Ajaz Bashir*

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## Chapter-1

### INTRODUCTION

Apple (*Malus domestica* Borkh) belonging to genus *malus* and family Rosaceae is the most widely grown temperate fruit tree in the world and propagated in both northern and southern hemispheres of the world for its high economic value. The genus *Malus* has five sections comprising 122 species and subspecies (Chadda and Awasti, 2004). Apple tree originated in Southeastern Europe and the Tien Shan mountains of Kazakhstan in Asia (Gastier, 2000) where widespread forests of wild apple trees are seen even today. Natural varieties of cultivated apple belong to *Malus pumila* M., while its hybrid varieties come from *Malus x domestica* B. Taxonomically apple tree is dicotyledonous that belongs to the family Rosaceae with subfamily Maloideae (former Pomoideae) having basic chromosome number 17.

The major apple-producing regions in India are J and K (specifically Kashmir), Himachal Pradesh, Uttar Pradesh, Kumaon, Assam and Nilgiri Hills. Jammu and Kashmir is the leading apple-producing area in India which annually produces about 60% of the total production in India. Approximately 330 varieties of apples are reported to have been under cultivation in Kashmir valley in 1972 but only a dozen among them are propagated at present on a commercial scale (Rajoriya *et al.*, 2016). Three cultivars, Ambri, Chambura and Maharaji, are indigenous to Kashmir, whereas Golden Delicious is known to have originated in West Virginia, the United States in 1910 by the hybridization between Grimes Golden and Golden Reinette.

Jammu & Kashmir has always been popular for its indigenous apple variety Ambri. This variety has been utilized in breeding programmes extensively, as a result of which a few hybrids namely Lal Ambri (Red Delicious × Ambri), Sunehari (Ambri × Golden Delicious) and ASP-49 (Red Delicious × Ambri) were released as varieties. Ambri having thick fruit skin and longer shelf life had been

grown here long before Western introductions and continues to be better due to its crisp, sweetish flesh and distinct aroma thus representing a superior dessert variety. Ambri fruits are medium-sized oblong to conical in shape and blushed red in color. They ripen from late October to November and thus are seen in the market from November to March when most other fruits disappear.

The crab apple *Malus baccata* (Lin.) is another species of apple belonging to the genus *Malus* (Rosaceae). In India, they are cultivated as ornamentals as they have attractive flowers and fruits. Their examples include *Malus floribunda* (Flowering Crab), *M. baccata* (Siberian Crab) and *M. angustifolia* (narrow leaved Crab Apple). It is smaller in size, cold-field edible fruit and has traditional medicinal values. The crab apples are extensively spread all through the world and the European and South American parks of Europe, usually used for ornamental purposes.

“An apple a day keeps the doctor away” is a saying that has been heard quite often. This saying has been proved good as the apple fruit has been found to be the second richest source of certain phytochemicals including quercetin, catechin, phloridzin and chlorogenic acid. All of these phytochemicals are very strong antioxidants and reduce the risk of some cancers, cardiovascular diseases, asthma and diabetes. These are also responsible for inhibiting cancer cell proliferation, reducing lipid oxidation and lowering cholesterol, thus preventing oxidative stress and delay aging. The phytochemical composition of apples varies from variety to variety with some changes in phytochemicals taking place during the maturation and ripening of the fruit. While storage has a very minuscule effect on apple phytochemicals, processing has a great effect on apple phytochemicals (Boyer and Liu, 2004). Apples are sub-acid foods that have a rich pectin content acting as a detoxifier of the body through the supply of an inordinate amount of galacturonic acid. This also helps in removing harmful waste from the bloodstream and thus can lower a diabetic’s insulin requirements up to or even more than 35%. Apples are also useful for both depression and weight loss and are

considered a negative calorie food because it takes more calories to digest them than they provide to the body. They release certain chemicals into the body which is helpful in the synthesis of glutamic acid and thus controls the destruction of various nerve cells (Boyer and Liu, 2004). The apple is a rich source of nutrients and its nutrient composition, on an average, is water 84.7%, sodium (0.3 mg/100g), carbohydrates (13.9 g), potassium (145 mg/100g), proteins (0.4 g), lipid (0.3 g), ash (0.3 g), fibre (0.8 g), vitamin C (8 mg/100gm), iron (480 µg/100g), calcium (7 mg/100g), magnesium (6 mg/100gm), Phosphorus (12 mg) and Iodine (2 µg) (Hussain, 2001).

Li *et al* (2014) reported that like commercial apples, crabapples possess similar compositions like nutrients and phenolic compounds. Fruits of crab apple (*M. baccata* L.) have been reported to be comprised of long-chain alcohols like a-sitosterol, campesterol and ursolic acid along with their D-glucosides (Mulabagal *et al.*, 2007). They are rich in phenolic compounds including anthocyanins (Sharma and Nath, 2016).

Since the last decade or so, the commercial cultivation of Ambri in Kashmir valley has hit rock-bottom due to the introduction of far superior apple cultivars like Red delicious. Crab apples, on the other hand, owing to their smaller size and low market value, are not cultivated commercially. Keeping such facts in view, the above-mentioned varieties of apple, although classified as underutilized fruits because of low commercial value and little planting region, can still be more useful for nutritional and medicinal purposes. In this direction, it is thus necessary to identify the biochemical components present in them and also explore the nutraceutical potential they might hold.

Therefore, the study entitled as “A Study on Biochemical and Nutraceutical Potential of Some Underutilized Apple Varieties of Kashmir” was conducted to generate basic information on some important biochemical components as well as nutraceutical, antioxidant and anti-cancerous potential of

the selected underutilized varieties of apple found in Kashmir with the following objectives:

- To study the nutritive composition of some underutilized apple genotypes of Kashmir.
- To chemoprofile and evaluate the antioxidant/anticancerous potential of selected underutilized apple genotypes of Kashmir.

## Chapter-2

### REVIEW OF LITERATURE

In this chapter, the literature about the present study entitled “**A Study on Biochemical and Nutraceutical Potential of Some Underutilised Apple Varieties of Kashmir**” is reviewed in light of available experimental pieces of evidence

#### 2.1 Nutrient and phytochemical analysis

Petkova *et al.* (2020) performed the phytochemical characterization of ripened crab apple fruits for the evaluation of their nutritional and antioxidant potentials. Assays of moisture, ash, protein, lipid, carbohydrate content, titratable acidity, pH, total phenolic compounds and natural pigments were done. Cellulose carbohydrate was found in the highest content (6% DW), followed by sugars (sucrose, glucose and fructose) and 1.8 % DW uronic acids in the analyzed fruits. In these characterized fruits, the total chlorophyll and carotenoid content were 6.51 and 4.80 µg/g FW, respectively. While the highest amount of total phenolic compounds (2.67 mg GAE/g FW) was found in 95 % ethanol extract from fruits, the total flavonoid content was relatively low (0.1 mg QE/g FW). The *in vitro* antioxidant activities of crab apple were demonstrated by DPPH assay (17.27 mM TE/g FW) and FRAP assay (14.34 mM TE/g FW) respectively. The study concluded that *Malus baccata* fruits were a rich source of dietary fibers and phenolic compounds with substantial antioxidant potential that could be used in human nutrition.

Yoon *et al.* (2020) determined selected biochemical and physical characteristics as well as yielding of fruits in apple varieties (*Malus domestica* Borkh) such as Gala Schniga, Beni Shogun (Fuji) and Ligol, M.9 rootstock growing in the Wielkopolska region, Poland. High-performance liquid chromatography (HPLC) analysis of tested apple varieties revealed differences both in the profile and contents of soluble sugars and other metabolites. The

highest total saccharide content was found in fruits of cv. Gala Schniga, while leaves and shoots of this variety showed the lowest contents. Significant variability was recorded in the red blush on fruit surfaces of the tested varieties, with Ligol fruits having the weakest blush. Altogether, these results indicate that fruits of the analyzed apple varieties differed both in terms of their biochemical composition and physical characteristics.

Liaudanskas *et al.* (2015) used the high-performance liquid chromatography method for the analysis of the ethanol extracts of apple fruits that were harvested from different cultivars. These cultivars were Aldas, Auksis, Ligol and Lodel, each grown in Lithuania. In the fruits of all these cultivars, chlorogenic acid was found to be a predominant component barring the cultivar Ligol. Epicatechin was the major compound with catechin being the minor one. The quercetin glycosides identified and quantified included hyperoside, isoquercitrin, avicularin, rutin and quercitrin with hyperoside being the major quercetin glycoside in apple fruits.

Delgado-Pelayo *et al.* (2014) studied the composition of chlorophyll and carotenoid of the peel and flesh of thirteen marketed apple varieties presenting different external coloration (green, yellow and red). All the studied varieties were found to have a common pigment profile comprised of chlorophylls *a* and *b*, lutein, violaxanthin, neoxanthin,  $\beta$ -carotene and esterified carotenoids (mainly violaxanthin and neoxanthin) as major compounds. The total pigment content of peel (58.72-1510.77  $\mu\text{g/g DW}$ ) was always higher than in the flesh (14.80-71.57  $\mu\text{g/g DW}$ ) for each studied cultivar. In general, the green cultivars showed the highest pigment content both in the peel and the flesh. These were followed in decreasing order by the peel of certain red-skinned cultivars and the flesh of the yellow cultivars. A correlation was observed between the carotenoid content and the amount of esterified xanthophylls, particularly in the flesh, which reconfirms the role of the esterification process in the accumulation of these lipophilic compounds within the chromoplasts.

Oyinlade (2014) investigated the phytochemical constituents and proximate compositions of three different types of apples in order to understand their possible mechanisms of action in addition to their role in human and animal nutrition. The three apple varieties, *Syzygium malaccense* (L.), *Syzygium cumini* and *Malus sylvestris* were obtained from different locations in Nigeria and their aqueous extracts were screened for the presence of phytochemicals and nutrients. *S. malaccense* (L.) (Malay apple) demonstrated significantly ( $p < 0.05$ ) high levels of vitamin C, moisture and fiber but low carbohydrate content when compared to *S. cumini* and *M. sylvestris* fruits. The apple type *S. malaccense* (L.) was suggested to be the most ideal fruit for the prevention and management of the digestive disorder and oxidative stress-related diseases.

Aziz *et al.* (2013) assessed the nutritional profile of two apple varieties and found that *Malus x domestica* (Discovery) has an edge over *Malus sylvestris* (Red Sentinel). The percentage of Moisture, Potassium and Ascorbic acid was slightly high in *Malus sylvestris*. On the other hand, the Ash, Brix, Fat, Pectin, Fiber, Protein and Total Sugar percentages were comparatively high in *Malus x domestica*. The concentrations of Calcium (20.79), Sodium (11.01), Phosphorus (16.4), Magnesium (14.62), Iron (7.23) and Vitamin A (0.97) (mg/100gm) in *Malus x domestica* were much higher than *Malus sylvestris* (Calcium 0.99, Sodium 8.21, Phosphorus 0.3, Magnesium 6.82, Iron 3.23 and Vitamin A 0.04 mg/100gm).

Fatih *et al.* (2010) determined the ascorbic acid, total soluble solids, total phenols, pH and total acid values of apple fruits grown in Konya, Turkey. The amounts of total polyphenols of apple fruits were determined as chlorogenic acid equivalents and ranged from 95 mg/kg to 245 mg/kg. The total acid values of apples varied between 4.3g /kg to 20.3 g/kg. Ascorbic acid contents of apple pomace were found 4.8mg/100g to 19.5 mg/100g. The mineral contents (B, Ca, Cu, Fe, K, Mg, Na, Ni, P, Se and Zn) of samples were analyzed and Ca, K, Mg, Na, P and Zn contents were established very high in all the apple samples. The Ca

levels of apple fruits ranged between 7.2 g/kg to 10.1 g/kg. These results show that apples may be useful for the evaluation of dietary information in important food crops.

Campeanu *et al.* (2009) assessed ten apple cultivars regarding their sugars, water content, proteins, acidity, vitamin C and also mineral elements (N, P, K, Ca, Mg, Fe). The results revealed the presence of a higher content of sugar ranging from 9.53 to 12.34%. The highest sugar content was found to be present in H3/73 hybrid (12.34%). Total acidity (%) ranged in between 0.127 and 0.345%. The small content of acidity present defined all cultivars to be of decent quality having a good taste besides the highest value being showed by 'Jonathan' apple variety. Vitamin C content ranged from 25.75 to 77 mg/100g with the highest vitamin C content being obtained for 'Mutzu' and 'Jonathan' cultivars. The content of mineral elements obtained were different for each variety with the values being N (0.67 - 0.111 %), P (0.15 - 0.235 %) and K (0.40 - 0.75 %). For Ca, the values ranged from 2.5 to 7.8 mg/100g while as the values of Fe ranged from 0.2 to 0.28 mg/100g.

Wu *et al.* (2006) used gas chromatography and high-performance liquid chromatography to analyse eight commercial apple cultivars for the amounts of different compounds including sugars, organic acids, amino acids, phenolic compounds and fatty acids. The most dominant sugar in the different apple cultivars was fructose which was higher in levels than glucose and sucrose with malic acid being the major organic acid. The most abundant fatty acids were C16:0, C18:0, C18:1, C18:2 and C18:3 fatty acids besides the C18 family contributing to more than 70% of the total fatty acids content. The main amino acids were serine and asparagine while chlorogenic acid and protocatechuic acid were the principal phenolic compounds. The results of this study deliver important information about the viable use of these apple cultivars for technological research as well as processing practice.

## 2.2 Antioxidant analysis

Elhakem *et al.* (2021) researched to study some bioactive compounds and activities of five apple varieties named black, apricot, jester, big Ariane and medium Ariane. The study revealed that medium Ariane had the highest total antioxidant value followed by big Ariane, apricot black and jester apples. Apricot showed the highest antioxidant activities of 93.67, 7.71 and 85.98  $\mu\text{mol TE g}^{-1}$  FW for DPPH, ABTS and FRAP respectively. The phenolic content ranged from 21.65 – 35.11 mM and the total flavonoids 36.86- 55.52 mg quercetin  $\text{g}^{-1}$ . The extracts showed high anti-inflammatory activity (0.32-5.46  $\mu\text{g mL}^{-1}$ ) with Medium Ariane, jester and apricot possessing high anti-inflammatory activity followed by big Ariane and black extracts, respectively.

Shehata *et al.* (2020) conducted a study to estimate anthocyanin content and to evaluate the antioxidant activity of apple, blueberry and plum crude extracts. The study revealed that the highest concentration of anthocyanins was obtained from plum peels (60.45 mg/100 g), followed by blueberry (22.38 mg/100 g) while apple peels had the lowest concentration (16.39 mg/100 g). The three fruits had a very good antioxidant activity with a concentration of 500  $\mu\text{g mL}^{-1}$ , the percentage of inhibition for plum peels was 99.23%, blueberry 98.85% and apple peels 98.84%. Further, it was concluded that among the three types of fruits, the highest anthocyanin content was found in the plum peels and the lowest in apple peels. It was observed that plum peels and blueberry showed higher antioxidant activity when compared to apple peels.

Honda and Moriya (2018) provided a brief overview of previous and recent research on anthocyanin biosynthesis in both the skin and flesh of apple fruits and stated that the red color of the skin and flesh of apple fruits (*Malus × domestica* Borkh.) is because of anthocyanin accumulation. They also mentioned that redder fruits are more marketable. Several environmental factors like temperature conditions and the level of sunlight irradiation influence the pigmentation in the skin of apple fruit that varies among different cultivars. As

warmer temperatures subdue anthocyanin synthesis in the skin of apple fruit, there are everincreasing risks that global warming may prove detrimental to fruit pigmentation. Higher temperatures have been proven to inhibit anthocyanin accumulation in both red-skinned and red-fleshed apple fruits. Further studies concerning the regulation of anthocyanin synthesis in the flesh of red-fleshed apple fruit are needed.

Dadwal *et al.* (2018) performed the phytochemical characterization of the pulp and seeds of Himalayan crab apple fruits (*Malus baccata*) grown at high altitude. It was reported that among various phenolic compounds, the amount of phloretin (88.39  $\mu\text{g mg}^{-1}$ ) and phloridzin (83.03  $\mu\text{g mg}^{-1}$ ) were higher. *Malus baccata* pulp portion showed higher antioxidant activity than seed portion. It was also stated through the study that as *M. baccata* fruit is loaded with a lot of phenolic and other chemical components, it can be used in food products and nutraceutical formulations.

Kumari and Dhaliwal (2017) evaluated the nutritive composition and development of food products developed from underutilized crab apple fruit i.e. fruit bar / gelatinized layers and apple jam which is highly nutritious. High amounts of vitamin C (17.13 mg/100g) and pectin (5.57 % as calcium pectate) were found to be contained in the fruit according to the study. The products developed were found acceptable in various attributes like colour, taste and consistency/texture. These parameters were retained even up to a storage interval of 9 months at ambient conditions.

Stanivuković *et al.* (2017) conducted the assessment of the quality of old apple varieties which could be utilised for the preservation and use of these germplasms in the countries of Bosnia and Herzegovina. Based on good characteristics of sensory analysis, ten old varieties of apples were selected and characterised. The parameters including fruit weight, fruit length and width, stalk length and width, fruit flesh firmness; soluble solids content and total dry matter content of the fruit juice were taken into consideration. Apart from these, other

parameters considered were pH, titratable acidity (TA), vitamin C, total phenolics, total flavonoids and antioxidant potential evaluated by DPPH radical scavenging assay. For sensory, pomological and biochemical characterization (in two consecutive years), separate statistical analysis was done through analysis of variance. Among the selected apple varieties, the maximum weight, width and height along with low content of titratable acidity was showed by ‘Vinjugara’, which proves it to be a preferred variety for consumption. The variety ‘Đedovača’ showed the highest content of phenols (1711.8 mg GAE/100 g of fruit weight), which happens to be in complete relationship with free radical scavenging activity (11.1 mg of fresh fruit/ml). As far as nutritional characteristics are concerned, ‘Kolačara’ and ‘Gospoinjača’ varieties were found to be decent, especially regarding the content of phenols, flavonoids and free radical scavenging ability as well as suitable storage characteristics, °Brix content and firmness. Based on the characteristics that were looked-for, both pomological and biochemical, these varieties were endorsed to be included in the programs of breeding for the germplasm preservation of the apples in these areas.

Stojiljkovic *et al.* (2016) conducted a study on the characterization of wild apple fruits of Serbia aimed at exploring their probable use as the source of the substances with antioxidant properties and production of nutraceuticals and cosmeceuticals, by adopting different extraction methods and solvents. Different techniques like maceration, percolation, soxhlet and ultrasonic extraction using polar solvents (70% ethanol, 45% and 80% propylene–glycol and distilled water) and non-polar solvents (sunflower and olive oil) were put to use for making extracts. Total phenolic content (TPC) and total flavonoid content (TFC) were determined. Also the determination of total tannins (TT) and total anthocyanins (TA) was carried out. Estimation of the antioxidant activity of the extracts was carried out by three different assays: DPPH (1, 1-diphenyl-2-picryl-hydrazyl) test, FRAP (Ferric Reducing Antioxidant Power) test and test with linoleic acid test respectively. The correlation between antioxidant properties and polyphenolic

compound content were found to be positive. Polyphenolic compound content and antioxidant activity depended on the extraction method and solvents used. The highest amount of polyphenolic compounds were contained in the extracts obtained by ultrasonic extraction (using ethanol and distilled water as solvents) and these extracts also demonstrated the best antioxidant activity according to the study. The study concluded that wild apple fruit is a potent source of antioxidant substances usable for food, dermo-cosmetic and cosmetic industry.

Jelodaruan *et al.* (2015) evaluated the antioxidant potential of fruits of 4 cultivars of *Malus domestica* cultivated in the Kashan, Qamar area. This was done using two antioxidant assays 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and  $\beta$ -carotene/linoleic acid tests that are complementary to each other. The results of this study were compared with the synthetic standard antioxidant butylated hydroxytoluene (BHT). In both DPPH  $\beta$ -carotene/linoleic acid tests, samples from Hossain cultivar showed moderate antioxidant activity with  $63.92 \pm 0.42$  and  $6.02 \pm 0.03$  inhibition percentages, respectively. Other samples showed weak activity. Very little phenolic compounds were showed by the Folin-Ciocalteu's phenol test for these fruits.

Tian *et al.* (2015) analyzed leaf coloration, anthocyanin levels and the expression levels of anthocyanin biosynthetic and regulatory genes in three crab apple cultivars (Royalty, Prairifire and Flame) following various temperature treatments. The study indicated that those low temperatures (LTs) promoted anthocyanin accumulation in 'Royalty' and 'Prairifire', leading to red leaves, but not in 'Flame', which accumulated abundant colourless flavanols and retained green-colored leaves. Quantitative reverse transcriptable PCR (RT-PCR) analysis indicated that the expression of several anthocyanin biosynthetic genes was induced by LTs, as were members of the R2R3-MYB, basic helix-loop-helix (BHLH) and WD40 transcription factor families. The results obtained propose that anthocyanin biosynthesis is differentially regulated in the three cultivars by LTs via the expression of members of this anthocyanin regulatory complex.

Wang *et al.* (2014) evaluated four red fleshed varieties of apple for their phenolic composition and strong antioxidant capacity. Among these four varieties, three belonged to Xinjiang, P.R. China and one was from the USA. Two commercial white-fleshed varieties were also included for comparison. The contents of phenolics (total phenolics, flavonoids, flavanols and anthocyanins) and antioxidant activity were significantly higher in the flesh of red-fleshed varieties than white-fleshed varieties according to the samples studied. In addition to this, flavonoid profiles *were also* different for the red and white-fleshed varieties. ‘Roberts Crab’ had the highest amounts of total phenolics, flavonoids and anthocyanins, as well as the strongest antioxidant activity among the red-fleshed varieties. ‘Xiahongrou’ had the least levels of total phenolics and flavonoids and the weakest activity. Individual phenolic compounds varied significantly within the red-fleshed varieties as shown by HPLC analysis. The red-fleshed apples were thus endorsed by the study to be the promising source of antioxidants for human nutrition.

Francini and Sebastiani (2013) summarized the information on the occurrence of phenolic compounds in apple (*Malus x domestica* Borkh.) fruit and juice, with special reference to their health-related properties.

Mitić *et al.* (2013) measured the total phenolic, total flavonoid content and antioxidant activity from whole fruits of 15 apple cultivars cultivated in Serbia. Polyphenolic content for the whole apple was in the range of 72.80 – 217.37 mg GAE/100 g fresh weight. The apple extracts had different TAA in relation to the method applied and the different TAA of apples can be ascribed to their TP and TF content. A rippling correlation between TP and TAA was observed using the FRAP method.

Manzoor *et al.* (2012) studied the differences in phenolics, antioxidant activity and mineral contents of five apple cultivars from Pakistan in their peel and pulp. Red Delicious, Golden Delicious, Kashmiri Ambri, Kala Kulu and Sky Spur were the studied cultivars. The yield (mean extract) of antioxidant

components on a dry weight basis {obtained with 80:20 methanol-water (v/v)} for peel was found to be 22.1 g/100 g and 14.2 g/100 g for pulp. The amount of total phenolics ranged from 1, 907.5–2, 587.9 mg gallic acid equivalent/100 g DW and total flavonoids ranged from 1, 214.3–1, 816.4 mg catechin equivalent/100 g DW in peel while they ranged from 1, 185.2–1, 475.5 mg GAE/100 g DW and 711.8–999.3 mg CE/100 g DW respectively in pulp. The values of inhibition of linoleic acid peroxidation ranged from 71.7–84.9 in the peel and the DPPH scavenging activity varied from 66.6–80.8% there. The values of these two were found to be 43.9–52.8 and 42.9–51.1% in pulp respectively. For tested fruit part extracts, reducing power at a concentration of 12.5 mg/mL ranged from 2.54–2.89 for peel and 1.37–1.73 for pulp respectively. Additionally, both peel and pulp showed K to be the mineral present in the highest amounts followed by Mg, Ca, Fe, Na and Zn. The general results of the study demonstrated that there were major differences shown by the peel and pulp of the studied apple cultivars as the peel had superior antioxidant capacity and mineral concentration than the pulp.

Ampomah-Dwamena *et al.* (2012) studied apple genotypes with varying flesh and skin colour for the metabolic and gene expression analysis of carotenoid pathway in order to understand the control of carotenoid concentrations in them. Major variation both in carotenoid concentrations and compound profile was reported between tissues and genotypes, with carotenes and xanthophylls being found only in fruit accumulating high carotenoid concentrations.

Lee (2012) reported significant variation of nutrients and bioactive chemicals among different apple cultivars. He also highly highlighted apples to be an excellent source of bioactive phenolic phytochemicals that may provide special nutraceutical benefits compared to other common fruits.

Lachman *et al.* (2006) analyzed fifteen apple varieties for their total polyphenol content (TP) by two methods – by Folin-Ciocalteu reagent (FC) and by EBC method with carboxymethylcellulose/sodiummethylendiamintetraacetate

(CMC/EDTA) and their antiradical activity (ARA) by DPPH method using stable free radical, 1-diphenyl-2-picrylhydrazyl (DPPH ·). TP and ARA were determined in freshly expressed apple juice and apples and obtained results were statistically evaluated. The differences between varieties were significant. The correlation between TP content determined by the FC method determining all free aromatic hydroxyls and EBC method determining mainly ortho-aromatic hydroxyls was found to be  $r = 0.73$ . The highest differences among the analyzed varieties were found for ARA values in both, juice and apples and for TP content determined by FC in apples. High polyphenol content was found in Jonagold, Jonalord, Melodie and Melrose varieties both, in apples and juices. On the contrary low TP, contents were estimated in Gloster and Rosana varieties. The highest ARA levels were found in Rajka, Bohemia and Melrose varieties, compared to low ARA levels found in apple fruits of Šampion and Topaz varieties.

### **2.3 Anticancerous potential**

Han *et al.* (2019) evaluated different parameters like phenolic profile, total polyphenols, antioxidant activity and anti-proliferative activity of Fuji apples and crabapple against several human cancer cells. The results of this study showed that the extracts of crabapple possess ample phenols and higher total polyphenols (from 4.46 to 46.63 mg GAE ·g<sup>-1</sup> DW) compared to 'Fuji' apples and presented higher antioxidant activity also. All the extracts demonstrated inhibitory effects on the proliferation of different cancer cells with crabapple extracts performing significantly better. The values varied for various cancer cell lines, for example 48.34 µg mL<sup>-1</sup> to 974.81 µg ·mL<sup>-1</sup> for SW480 colon cancer cells, 64.67–1466.35 µg mL<sup>-1</sup> for BGC-803 stomach cancer cells, 78.88–910.64 µg ·mL<sup>-1</sup> for CaEs-17 esophageal cancer cells. In addition to this, the red crabapples performed better by showing better a antioxidant activity and anti-proliferative activity as compared to yellow fruits. These results revealed that crabapples, especially red crabapples, have great potential asssss healthy food, owing to the possession of

rich phenolic compounds along with high antioxidant and anti-proliferative activities against cancer cells.

Tu *et al.* (2016) reported the anticancerous molecular mechanism(s) of various components of apple showing growth-inhibitory effects against various cancer cell types. Moreover, an attempt was made to delineate the direction of future studies that could lead to the development of apple components as a potent chemopreventive/ chemotherapeutic agent against cancer.

Qin *et al.* (2015) conducted an MTT cancer cell growth inhibition assay of dihydrochalcone compounds isolated from crab apple and demonstrated that phloretin and their derivatives showed significantly positive anticancer activities against several human cancer cell lines, including the A549 human lung cancer cell line, Bel 7402 liver cancer cell line, HepG2 human ileocecal cancer cell line and HT-29 human colon cancer cell line. Phloretin and its derivatives from crab apple had significant effects on all cancer cell lines suggesting its potential applications as an anticancer agent.

## **Chapter -3**

### **MATERIALS AND METHODS**

The present investigation entitled “**A Study on Biochemical and Nutraceutical Potential of Some Underutilized Apple Varieties of Kashmir**” was carried out during the year 2019-2020 in the Biochemistry and Molecular Biotechnology Laboratory, Division of Basic Sciences and Humanities, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Science and Technology of Kashmir.

#### **3.1 Experimental site and plant material:**

In the current investigation, the four different apple fruit samples were collected from diverse locations of Kashmir. Ambri fruit sample was collected from Sharatpora Shopian while the Golden Delicious fruit sample was collected from the local market of Shalimar. The crab apple fruit samples were collected from KVK Shopian. the fruit samples were subjected to analysis on fresh weight as well as on a dry weight basis.

#### **3.2 Preparation of aqueous and methanolic extract:**

1 gram of each fresh sample was extracted in 15ml of distilled water and filtered using Whatman filter paper 42. The extraction was repeated three times for each sample. The pooled extracts were pooled and stored in cryo vials at -20°C till further analysis. The same procedure was followed for the preparation of methanolic extracts by using methanol instead of distilled water.

#### **3.3 Nutrient analysis**

##### **3.3.1 Determination of Moisture content**

The moisture content of ten different genotypes of Apple fruits was determined by adopting a laboratory oven method (AOAC, 1985). For this, 100g of leaf sample (in triplicate) of each genotype was weighed accurately and oven-



**Plate 1: Dried apple fruit sample (Red Crab Apple and Ambri)**



**Plate 2: Lab work**

dried to constant weight at 45°C for 4hrs. The loss of weight of dried plant sample was then determined to calculate the moisture content as follows:

$$\text{Moisture content (\%)} = \frac{\text{initial weight(g)} - \text{final weight(g)}}{\text{initial weight(g)}} \times 100$$

### 3.3.2 Determination of Total free soluble sugars

The total free soluble sugar content of each Apple fruit sample from each genotype was determined using the anthrone method reported by Dubious *et al.* (1951). 0.1 ml of the extract was combined with 4 ml of the anthrone reagent, heated for 10 minutes in a boiling water bath and further cooled to room temperature under running tap water. Absorbance was measured in a UV-VIS spectrophotometer at 625 nm against a reagent blank. The total free soluble sugar content in each sample was then estimated from a standard curve prepared with the known concentration of glucose and expressed as mg equivalents of glucose per 100 g of plant sample.

### 3.3.3 Determination of Crude fiber

In the current investigation, the crude fiber content of Apple fruit samples of each genotype was determined by employing protocol and adopted by Thimmaiah (1999). For this, 1g of leaf sample was first subjected to acid hydrolysis with 2.5N HCl followed by alkali digestion with 0.1 N NaOH. The residue so obtained was then washed with double distilled water and ignited in a muffle furnace at 600 °C for 6 hours. At high temperatures, the organic matter in the residue got oxidized and inorganic residue or ash was left behind. The difference in the weight before and after ashing of each sample was then calculated to estimate the crude fiber content in the sample using formulae.

$$\text{Crude fiber (\%)} = \frac{W1 - W2}{w} \times 100$$

Where,

W = weight of sample(g)

W1 = Residue (crude fiber + minerals)

W2 = ash remained (g)

### 3.3.4 Determination of Vitamin C content

The ascorbic acid content of apple fruit samples of each genotype was estimated by the volumetric method as described by Sadasivam and Theymoli (1987). For determining vitamin C content in apple fruit samples, 0.5 g of fruit sample was ground in 10 ml of 4% oxalic acid using mortar and pestle centrifuged at 8000 rpm and the supernatant was preserved. The extraction was repeated three times and the final volume of extract was made up to 100ml. The appearance of pink color indicated the end point which persisted for few minutes. To 5 ml of this supernatant, each leaf extract 10ml of 4% oxalic acid was added and titrated against the dye solution, dichlorophenol indophenols (V2 ml). The amount of ascorbic acid (mg/g) in each test sample was then calculated by using the following formula:

$$\text{Ascorbic acid (mg/100g)} = \frac{0.5\text{mg}}{V1 \text{ ml}} \times \frac{V2\text{ml}}{15\text{ml}} \times \frac{100\text{ml}}{\text{weight of the sample}} \times 100$$

### 3.3.5 Determination of Minerals (Ca, Mg and K)

For mineral estimation, 2g of each apple fruit sample of each genotype was taken in a flask and to it 20 ml of the mixture of nitric acid and perchloric acid in the ratio of 9:4 (di-acid) was added. The flask was then kept undisturbed overnight and the next day, it was placed on a hot plate at 115-118°C for digestion till a watery transparent aliquot was obtained. The digested samples were then filtered and diluted with double distilled water to make the final volume up to 50 ml, which was then ultimately used directly for estimation of minerals.

The calcium and magnesium of each fruit sample were determined by atomic absorption spectrophotometry (Agilent technologies, USA) while potassium was estimated by the flame photometric method as reported by Piper (1966).

### **3.4 Qualitative Analysis of Phytochemicals**

Qualitative screening of different phytochemicals in aqueous and methanolic extracts of four different Apple genotypes was carried out with the following standardized protocols:

#### **3.4.1 Test for saponins (Frothing test)**

In this, 1 ml of each extract was mixed with 2 ml of distilled water in a test tube. The test tube was stoppered and shaken vigorously for 5 min and was allowed to stand for 1 min. The appearance of honeycomb froth in the test solution confirmed the presence of saponins in the selected extract.

#### **3.4.2 Test for Sterols (Salkowski's Test)**

In this, 2 ml chloroform and 2ml concentrated H<sub>2</sub>SO<sub>4</sub> were added to 2 ml extract. The formation of a reddish-brown colored ring indicates the presence of sterols in the test sample extract.

#### **3.4.3 Test for Cardiac Glycosides (Liebermann's Test)**

For this 2 ml of glacial acetic acid and 2 ml of chloroform were added to 2 ml of extract taken in a test tube. The mixture was then cooled for 1min and 1ml of H<sub>2</sub>SO<sub>4</sub> was added to each test tube. The appearance of green color shows the presence of cardiac glycosides in the test sample extract.

#### **3.4.4 Test for Terpenoids**

In this 100µl of the extract was added to 1ml of chloroform followed by the addition of 1ml of sulphuric acid to each tube. The formation of a reddish-brown precipitate indicates the presence of terpenoids in the test extract.

#### **3.4.5 Test for Alkaloids**

For this, 2ml of each test extract was mixed with 2ml of hydrochloric acid. A few drops of Mayer's reagent were added to it. The development of green color or white precipitate indicates the presence of alkaloids in the test solution.

Mayer's reagent test solution: Mercuric chloride (1.358 g) dissolved in 60 mL of water and Bi potassium iodide (5 g) dissolved in 10 mL of water. The two solutions (A and B) were mixed and finally made up to 100 ml with water.

### **3.5 Quantitative analysis of antioxidants**

To check various antioxidant constituents in aqueous and methanolic extracts of four selected apple genotypes, various analytical-based procedures were employed and for each sample, three replications were studied.

#### **3.5.1 Determination of Flavonoids**

The total flavonoid content of in aqueous and methanolic extracts of four different apple genotypes was determined according to the modified method of Lalliansawna *et al* (2013). Briefly, 1.5 ml of each fruit extract was combined with 75  $\mu$ l of 5% NaNO<sub>2</sub> solution and incubated for 6 min then 150  $\mu$ l of 10% AlCl<sub>3</sub>.6H<sub>2</sub>O was added to the mixture and further incubated at room temperature for 6 minutes. The procedure was followed by the addition of 0.5 ml of 1M NaOH to each tube and the total volume of the solution made up to 2.5 ml with the addition of de-ionized water. The resulting solution was mixed well and immediately, the absorbance was measured at 510 nm, against reagent blank. A standard calibration curve was prepared using Quercetin as standard. The total flavonoid content of each sample was calculated and was expressed as the mg equivalents of quercetin (QE) per 100g fruit sample.

#### **3.5.2 Determination of Carotenoids**

The carotenoids content in the aqueous and methanolic extracts of fruit of four different apple genotypes was estimated using the procedure as reported by Mahadevan A and R. Srihar (1986). For this, 20ml of each extract was partitioned thrice with 10ml of petroleum ether using a separatory funnel, evaporated to dryness at 35°C using a rotator evaporator and residue so obtained was dissolved in 10 ml of ethanol followed by 30 minutes in a shaking water bath at 37°C using 60% alcoholic KOH cation. This was followed by the addition of 15ml of

DDH<sub>2</sub>O and the saponified extract was transferred into a separating funnel (Packed with glass wool and calcium carbonate) containing 15ml of petroleum ether and mixed gently which take up the carotenoid pigments into the petroleum ether layer. The lower aqueous phase was transferred to another separating funnel and petroleum ether extract containing the carotenoid pigments to an amber-colored bottle. Extraction of the aqueous phase was repeated thrice with petroleum ether until it was colorless. The aqueous layer was discarded. To the petroleum ether extract, a small quantity of sodium sulfate was added to remove turbidity. The final volume of the petroleum ether extract was noted and the absorbance was recorded at 450nm against reagent blank in a spectrophotometer.

The total carotenoid content of each apple genotype was then calculated according to the following formulae:

$$\text{Carotenoids (microgram)} = P \times 4 \times V \times 100 \times W$$

P= Optical density of the sample

V= Volume of the sample

W=Weight of the sample

### 3.5.3 Determination of Anthocyanins

In aqueous and methanolic fruit extracts of four different genotypes of apple genotypes, anthocyanin content was determined according to the method of Rangana (1997). Briefly, 10 ml of each extract was partitioned thrice with 10 ml of acidified ethanol (95% ethanol containing 1.5 N HCL) and to this acidified ethanol was added until the final volume is made to 100ml and finally, the extract was kept overnight in the refrigerator at 4°C. The extract was then filtered through Whatman filter paper No. 42. and the optical density was measured at 535 nm. The contents of anthocyanin (mg/100g) in each apple genotype was then calculated according to the following equation:

$$\text{Total optical density (O.D.)} = \frac{\text{OD} \times \text{Volume made}}{\text{weight of sample}} \times 100$$

$$\text{Anthocyanin (mg/100g)} = \frac{\text{total optical density (OD)}}{98.2} \times 100$$

### **3.5.4 Determination of Total phenolic content**

In aqueous and methanolic fruit extracts of fruits of four different Apple genotypes, total phenolic content was determined by a modified method of Malick and Singh (1980). To determine phenol content, 500µl of the extract was first combined with 2.5ml of double distilled water and immediately 0.5ml of Folin-Ciocalteu reagent was added. After 3 minutes of the incubation period, 2ml of 2% sodium carbonate was added to each sample, vortexed and boiled in a water bath for exactly one minute. The absorbance of each sample was measured at 630nm against the reagent blank. A standard curve was established using gallic acid as standard and the concentration of phenol in the sample was determined accordingly.

### **3.5.5 Determination of Quercetin**

High-performance Liquid chromatography (HPLC) was performed for quantitative estimation of quercetin in fruit samples of four different apple genotypes using an Agilent 1260 liquid chromatography equipped with a variable wavelength detector. The analytical column was a reverse-phase Supleco Discovery C18, 250mm x 4.6mm with a packing material of 5µm particle size. The composition of the mobile phase was 0.05% trifluoroacetic acid in acetonitrile at a flow rate of 0.8 min/min. Standard quercetin was chromatographed separately. Retention time and spectra of standards were compared with that of samples. The amount of quercetin in the Apple fruit sample was measured from peak areas obtained at 340 nm, computed automatically using Agilent chemstation 4.0 measuring peak areas and linear regression and results expressed in terms of µg/g of leaf sample on a dry weight basis.

### **3.6 Determination of total anti-oxidant activity:**

Antioxidant activity in aqueous and methanolic fruit extracts of four different apple genotypes was determined by ferric reducing antioxidant (FRAP) assay as reported by Benzie and the strain (1999). The FRAP reagent is composed of 10mM 2, 4, 6-tripyridyl -S-triazine (TPTZ), 40mM HCL and 20mM ferric chloride prepared in 300mM sodium acetate buffer (pH 3.6) in the ratio of 1:1:10 (v/v). In this 500  $\mu$ l of each aqueous leaf extract was added to 3ml of FRAP reagent mixed properly and immediately absorbance '0 mins' was recorded at 593nm. After incubation for 4 min at room temperature, absorbance '4 mins' was again recorded at 593 nm. A standard graph was plotted using ammonium ferrous sulfate as standard and accordingly the total antioxidant potential of each sample was determined and expressed as  $\mu$ M FRAPg<sup>-1</sup> on the sample on the dry weight.

### **3.7 Determination of anti-cancerous potential of Ambri (aqueous) extract**

#### **3.7.1 Cell Culture Experiments and Stable Transfection**

C4-2 cancer cell lines were provided by Prof. Zhou Wang, University of Pittsburgh, USA under MTA between Transcriptomics Laboratory, Division of Plant Biotechnology, SKUAST Kashmir with MD Anderson Cancer Centre, Texas, USA. Cell lines were routinely maintained in RPMI medium supplemented with 10% FBS, 1% L-glutamine and 100  $\mu$ g/ml penicillin-streptomycin in 5% CO<sub>2</sub> incubator at 37 °C.

#### **3.7.2 Colony-forming unit assay (CFU)**

The Colony Forming Unit assay was utilized to determine the effect of Ambri aqueous extract on the cell regrowth and proliferation of prostate cancer cells. C4-2 cell lines were seeded in 12 well plates before treatment with extracts. Cell lines at 70–75% confluency were washed with PBS and treated with different concentrations of Ambri aqueous extract (6.25, 12.5, 25, 50 and 100  $\mu$ g/ml) prepared in distilled water and DMSO control and incubated for 48 Hrs. After incubation, an equal number of cells (counted with hemocytometer) from treated

and control wells were seeded in 10 cm dishes to form colonies for at least 7 to 10 days. After removing the media gently from dishes, sufficient 100% methanol was added to the plates to cover the cells completely and then incubated for 20 mins. Methanol was removed and cells were rinsed carefully with water. A crystal violet solution was sufficiently added to cover the cells and then incubated for 5 mins at room temperature. After incubation, the cells were washed with water to remove excess dye. Then the plates were kept inverted on tissue paper to dry. Plates were imaged and colonies over 0.5 mm were counted using ImageJ software (NIH, USA). Colony formation assay was carried out in three replications and repeated three times. Data was analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc. La Jolla, CA).

## Chapter-4

### EXPERIMENTAL FINDINGS

The biochemical and nutraceutical potential of the various underutilized apple genotypes is imperative for their efficient utilization as nutritious food as well as for their effective conservation. Therefore, the present investigation entitled “**A Study on Biochemical and Nutraceutical Potential of Some Underutilized Apple Varieties of Kashmir**” was carried out during the year 2019-2020 in the Biochemistry and Molecular Biotechnology Laboratory, Division of Basic Sciences and Humanities, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Science and Technology of Kashmir.

In the study, the four different apple fruit samples were collected from diverse locations of Kashmir. Ambri fruit sample was collected from Sharatpora Shopian while the Golden Delicious fruit sample was collected from the local market of Shalimar. The crab apple fruit samples were collected from KVK Shopian. These four fruit samples were then subjected to biochemical investigations.

#### 4.1 Nutrient analysis

##### 4.1.1 Moisture (%)

As shown in Table 1, the moisture content of fresh fruit samples of four selected apple genotypes varied significantly from as low as 76.30% to as high as 83%. Among the selected genotypes, Ambri was found to possess significantly higher moisture content (83%) followed by Golden Delicious (82%), Red Crab Apple (77.80%) and Yellow Crab Apple (76.30%) genotype, respectively. The results indicate that these apple fruit genotypes are a good source of dietary water.

**Table 1: Total Moisture Content in 4 Apple Genotypes**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple	77.80 <sup>c</sup>
Yellow Crab Apple	76.30 <sup>d</sup>
Ambri	83.00 <sup>a</sup>
Golden Delicious	82.00 <sup>b</sup>
<b>C.D (p≤ 0.05)</b>	<b>0.674</b>

**4.1.2 Total soluble sugars (g/100g DW)**

The total soluble sugar content of 4 selected apple genotypes varied significantly from 7.33 g/100g DW to 13.51 g/100g DW (Table 2). The highest total soluble sugar content was found in Ambri (13.51 g/100g) genotype, followed by Golden Delicious (11.86g/100g), Yellow Crab Apple (7.50g/100g) and Red Crab Apple (7.33 g/100g) genotype, respectively.

**Table 2: Total soluble sugars content in four apple genotypes (g/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple	7.33 <sup>c</sup>
Yellow Crab Apple	7.50 <sup>c</sup>
Ambri	13.51 <sup>a</sup>
Golden Delicious	11.86 <sup>b</sup>
<b>C.D (p≤ 0.05)</b>	<b>0.404</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.1.3 Vitamin C (mg/100g DW)

In the current investigation, it was found that the vitamin C content of 4 genotypes of apples varied significantly from as high as 4.37mg/100g DW to as low as 3.40mg/100g DW (Table 3). Among the selected 4 genotypes, Yellow Crab Apple exhibited the maximum vitamin C content (4.37 mg/100g DW) followed by Red Crab Apple (4.32mg/100g), Ambri (3.78mg/100g) and Golden Delicious (3.40mg/100g) genotype, respectively.

**Table 3: Total vitamin C content in four apple genotypes (g/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple	4.32 <sup>a</sup>
Yellow Crab Apple	<b>4.37<sup>a</sup></b>
Ambri	3.78 <sup>b</sup>
Golden Delicious	<b>3.40<sup>c</sup></b>
<b>C.D (p≤ 0.05)</b>	<b>0.061</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.1.4 Crude fiber (g/100g DW)

The total crude fiber content in fruits of 4 selected apple genotypes varied significantly and was found to lie in the range of 0.52 g/100g DW to 1.61 g/100g DW (Table 4). The maximum crude fiber content was found to be present in Ambri (1.61 g/100g) genotype, followed by Golden Delicious (1.51g/100g), Red Crab Apple (0.72 g/100g) and Yellow Crab Apple(0.52g/100g) genotype, respectively.

**Table 4: Crude fiber content in four apple genotypes (g/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple	0.72 <sup>c</sup>
Yellow Crab Apple	<b>0.52<sup>d</sup></b>
Ambri	<b>1.61<sup>a</sup></b>
Golden Delicious	1.51 <sup>b</sup>
<b>C.D (p≤ 0.05)</b>	<b>0.079</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.1.5 Minerals

##### 4.1.5.1 Calcium (ppm)

In the current study a significant variation was observed with respect to the calcium content in the fruits of 4 selected apple genotypes, which ranged from 70.80 ppm to 74.20 ppm (Table 5). Among the apple genotypes, Red Crab Apple (74.80ppm) demonstrated the highest level of calcium content followed by Yellow Crab Apple (73.20ppm), Golden Delicious (71.30ppm) and Ambri (70.80ppm) genotype, respectively.

**Table 5: Calcium content in four apple genotypes (mg/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple	74.20 <sup>a</sup>
Yellow Crab Apple	73.20 <sup>b</sup>
Ambri	70.80 <sup>c</sup>
Golden Delicious	71.30 <sup>d</sup>
<b>CD (p≤ 0.05)</b>	<b>0.548</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

##### 4.1.5.2 Potassium (mg/100g DW)

The potassium content in the fruits of selected apple genotypes ranged from 4660 ppm to 8604 ppm (Table 6). The Yellow Crab Apple (8604 ppm) genotype possessed the maximum potassium content, followed by Red Crab Apple (7895 ppm), Golden Delicious (6445 ppm) and Ambri (4660 ppm) genotype, respectively.

**Table 6: Potassium content in four apple genotypes (mg/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red CrabApple	7, 895.00 <sup>b</sup>
Yellow Crab Apple	8, 604.00 <sup>a</sup>
Ambri	4, 660.00 <sup>d</sup>
Golden Delicious	6, 445.00 <sup>c</sup>
<b>CD (p≤ 0.05)</b>	<b>134.73</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.1.5.3 Magnesium (mg/100g)

The magnesium content in four selected apple genotypes was found to range from 123.30ppm to 143.10ppm (Table 7). Among the four selected genotypes, the highest magnesium content was found in the Yellow Crab Apple (143.10 ppm) genotype, followed by Red Crab Apple (138.10 ppm) Golden Delicious (128.30 ppm) and Ambri (123.30 ppm) genotype, respectively.

**Table 7: Magnesium content in four apple genotypes (mg/100g DW)**

Genotypes	Mean
Red Crab Apple	138.1 <sup>b</sup>
Yellow Crab Apple	<b>143.10<sup>a</sup></b>
Ambri	123.30 <sup>d</sup>
Golden Delicious	128.30 <sup>c</sup>
<b>CD (p≤ 0.05)</b>	<b>0.714</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.2 Qualitative analysis of phytochemicals

The presence and absence of phytochemicals like alkaloids, sterols, terpenoids, saponins and cardiac glycosides in four apple genotypes was found as shown

Genotypes	Alkaloids	Sterols	Terpenoids	Saponins	Cardiac Glycosides
Red Crab Apple(Aq)	+	-	+	+	-
Yellow Crab Apple(Aq)	+	-	+	+	-
Ambri(Aq)	+	-	+	+	-
Golden Delicious(Aq)	+	-	+	+	-
Red Crab Apple(M)	+	+	+	+	-
Yellow Crab Apple(M)	+	+	+	+	-
Ambri(M)	+	+	+	+	-
Golden Delicious(M)	+	+	+	+	-

Alkaloids, terpenoids and saponins were detected in both aqueous as well as methanolic extracts of each genotype, while cardiac glycosides were absent in each extract and sterols were detected in the methanolic extracts of each genotype only.

#### 4.3 Quantitative analysis of antioxidants

##### 4.3.1 Total phenols (mg/100g DW)

It was observed that there existed a significant variation in the total phenol content of fruit extracts from 4 genotypes that ranged from 102.15 mg/100g DW to 137.83 mg/100g DW (Table 8). Among the selected apple genotypes, the maximum total phenol content was found in the aqueous extract of Ambri(137.83 mg/100g), followed by methanolic extract of Ambri (133.16 mg/100g), aqueous extract of Red Crab Apple (117.07 mg/100g), aqueous extract of Yellow Crab Apple (116.13 mg/100g), methanolic extract of Yellow Crab Apple (114.25 mg/100g), aqueous extract of Golden Delicious (109.28 mg/100g), methanolic extract of Red Crab Apple (108.06 mg/100g) and methanolic extract of Golden Delicious (102.15 mg/100g), respectively.

**Table 8: Total phenol content in extracts of apple genotypes (mg/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple(Aq)	117.07 <sup>c</sup>
Yellow Crab Apple(Aq)	116.13 <sup>d</sup>
Ambri(Aq)	137.83 <sup>a</sup>
Golden Delicious(Aq)	109.28 <sup>f</sup>
Red Crab Apple(M)	108.06 <sup>g</sup>
Yellow Crab Apple(M)	114.25 <sup>e</sup>
Ambri(M)	133.16 <sup>b</sup>
Golden Delicious(M)	102.15 <sup>h</sup>
<b>C.D (p≤ 0.05)</b>	<b>0.669</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.3.2 Total flavonoids (mg/100g DW)

The total flavonoid content in the fruit extracts of apple genotypes was found to vary considerably and ranged from 61.28 mg/100g DW to 113.31 mg/100g DW as shown in (Table 9). Among these genotypes, the aqueous extract of Ambri possessed the maximum flavonoid content (113.31 mg/100g) followed by methanolic extract of Ambri(112.25 mg/100g), methanolic extract of Red Crab Apple (96.86 mg/100g), methanolic extract of Yellow Crab Apple (94.33 mg/100g), methanolic extract of Golden Delicious (67.67 mg/100g), aqueous extract of Golden Delicious (66.06 mg/100g), aqueous extract of Yellow Crab Apple (64.13 mg/100g) and aqueous extract of Red Crab Apple (61.28 mg/100g), respectively.

**Table 9: Total flavonoid content in the fruit extracts of leaves of apple genotypes (mg/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple(Aq)	61.28 <sup>h</sup>
Yellow Crab Apple(Aq)	64.13 <sup>g</sup>
Ambri(Aq)	113.31 <sup>a</sup>
Golden Delicious(Aq)	66.06 <sup>f</sup>
Red Crab Apple(M)	96.86 <sup>c</sup>
Yellow Crab Apple(M)	94.33 <sup>d</sup>
Ambri(M)	112.25 <sup>b</sup>
Golden Delicious(M)	67.67 <sup>e</sup>
<b>C.D (p≤ 0.05)</b>	<b>0.798</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.3.3 Total carotenoids (mg/100g DW)

The 4 apple genotypes varied significantly with respect to total carotenoid content and ranged from 0.039 mg/g to 0.051 mg/g (Table 10). The maximum carotenoid content was found in the methanolic extract of Yellow Crab Apple (0.051 mg/g) genotype followed by the methanolic extract of Golden Delicious (0.043 mg/g), methanolic extract of Red Crab Apple (0.042 mg/g) and methanolic extract of Ambri(0.039 mg/g), respectively.

**Table 10: Total carotenoid content in methanolic extracts of apple genotypes (mg/100g DW)**

Genotypes	Mean
Red Crab Apple(Aq)	ND
Yellow Crab Apple(Aq)	ND
Ambri(Aq)	ND
Golden Delicious(Aq)	ND
Red Crab Apple(M)	0.042 <sup>b</sup>
Yellow Crab Apple(M)	0.051 <sup>a</sup>
Ambri(M)	0.039 <sup>b</sup>
Golden Delicious(M)	0.043 <sup>b</sup>
<b>C.D (p≤ 0.05)</b>	<b>0.006</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.3.4 Total anthocyanins (mg/100g DW)

The anthocyanin content in the fruits of the selected apple genotypes also varied significantly and ranged from 2.88 mg/100g to 13.29 mg/100g (Table 11). Among the selected genotypes, the methanolic extract of Red Crab Apple possessed the maximum anthocyanin content (13.29 mg/100g) followed by methanolic extract of Yellow Crab Apple (11.27 mg/100g), methanolic extract of

Ambri (10.28 mg/100g), methanolic extract of Golden Delicious (9.8 mg/100g), aqueous extract of Red Crab Apple (3.9 mg/100g), aqueous extract of Yellow Crab Apple (3.31 mg/100g), aqueous extract of Ambri (3.02 mg/100g) and aqueous extract of Golden Delicious (2.88 mg/100g), respectively.

**Table 11: Total anthocyanin content in fruit extracts of apple genotypes (mg/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple(Aq)	3.90 <sup>d</sup>
Yellow Crab Apple(Aq)	3.31 <sup>de</sup>
Ambri(Aq)	3.02 <sup>e</sup>
Golden Delicious(Aq)	2.88 <sup>e</sup>
Red Crab Apple(M)	13.290 <sup>a</sup>
Yellow Crab Apple(M)	11.270 <sup>b</sup>
Ambri(M)	10.280 <sup>c</sup>
Golden Delicious(M)	9.800 <sup>c</sup>
<b>C.D (p≤ 0.05)</b>	<b>0.706</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.3.5 Total Quercetin (µg/g)

Two genotypes of apple, Ambri and Red Crab Apple were subjected to HPLC based determination of quercetin.

Red Crab Apple showed a higher amount of quercetin (9.86 mg/100g) as compared to the amount present in Ambri (7.43 mg/100g).

**Table 12: Total quercetin content in fruit extracts of apple genotypes (mg/100g DW)**

<b>Genotypes</b>	<b>Mean</b>
Red Crab Apple	9.867 <sup>a</sup>
Yellow Crab Apple	ND <sup>*</sup>
Ambri	7.433 <sup>c</sup>
Golden Delicious	ND <sup>*</sup>
<b>CD (p≤ 0.05)</b>	<b>0.304</b>

\*Not Detected

#### 4.4 Total anti-oxidant activity ( $\mu\text{M FRAP} / 100\text{g DW}$ )

The total antioxidant activity in the fruit samples of selected apple genotypes ranged from 371.76  $\mu\text{M FRAP/g DW}$  to 1319.08  $\mu\text{M FRAP/g DW}$  (Table 13). The maximum anti-oxidant activity was found to be present in methanolic extract of Red Crab Apple (1319.08  $\mu\text{M FRAP/g}$ ) genotype followed by methanolic extract of Yellow Crab Apple (1042.74  $\mu\text{M FRAP/g}$ ), aqueous extract of Yellow Crab Apple (820.86  $\mu\text{M FRAP}/100\text{g}$ ), aqueous extract of Red Crab Apple (737.27  $\mu\text{M FRAP}/100\text{g}$ ), aqueous extract of Golden Delicious (593.17  $\mu\text{M FRAP}/100\text{g}$ ), methanolic extract of Ambri (527.73  $\mu\text{M FRAP}/ 100\text{g}$ ), methanolic extract of Golden Delicious (471.36  $\mu\text{M FRAP}/100\text{g}$ ), aqueous extract of Ambri (371.76  $\mu\text{M FRAP}/100\text{g}$ ), respectively.

**Table 13: Total antioxidant activity in extracts of apple genotypes ( $\mu\text{M FRAP}/100\text{g DW}$ )**

Genotypes	Mean
Red Crab Apple(Aq)	737.27 <sup>d</sup>
Yellow Crab Apple(Aq)	820.86 <sup>c</sup>
Ambri(Aq)	371.76 <sup>h</sup>
Golden Delicious(Aq)	593.17 <sup>e</sup>
Red Crab Apple(M)	1,319.08 <sup>a</sup>
Yellow Crab Apple(M)	1,042.74 <sup>b</sup>
Ambri(M)	527.73 <sup>f</sup>
Golden Delicious(M)	471.36 <sup>g</sup>
<b>C.D (<math>p \leq 0.05</math>)</b>	<b>6.824</b>

Mean values with different superscripts are statistically significant at 5% level of significance.

#### 4.5 Anticancerous potential of Ambri aqueous extract

In order to check the anticancerous activity, an aqueous extract of the Ambri genotype was subjected to a CFU assay. Surprisingly the results showed that the extract has no anticancer activity against the CH-2 cancer cell lines. Instead, it was found that by increasing the concentration of the extract there was a dose-dependent increase in the number of CFUs.

**Table 14: No of CFUs formed against various concentrations of aqueous ambri extract**

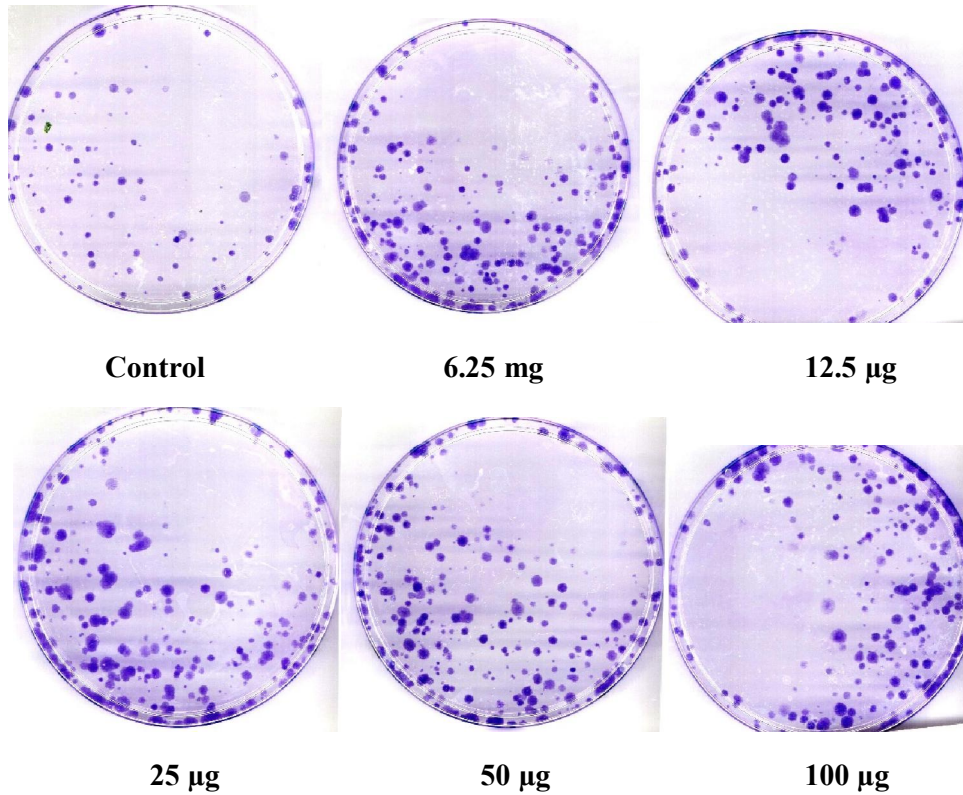
Concentration ( $\mu\text{g}$ )	Mean
Control	183.00 <sup>e</sup>
6.25	223.00 <sup>d</sup>
12.5	230.00 <sup>cd</sup>
25.0	233.50 <sup>c</sup>
50.0	247.00 <sup>b</sup>
100	274.50 <sup>a</sup>
CD ( $p \leq 0.05$ )	8.64436

#### 4.6 Correlation of phenols with the total antioxidant activity of selected genotypes of underutilized apples of Kashmir

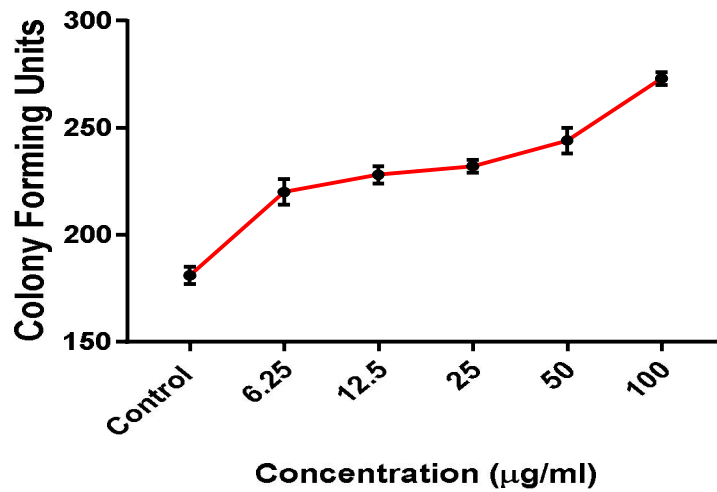
The study reveals that there exists a strong positive correlation between selected bioactive compounds with the total antioxidant activity of different apple genotypes. The correlation of total antioxidant activity (Table 15) was found to be negative with phenols ( $r^2 = -0.78$ ) while it was found to be positive with flavonoids ( $r^2 = 0.19$ ), carotenoids ( $r^2 = 0.56$ ) and anthocyanins ( $r^2 = 0.57$ ).

**Table 15: Correlation coefficient of phenols with the total antioxidant activity**

	Correlation with Antioxidant activity
Total phenolic content	-0.74
Total flavonoid content	0.19
Anthocyanin	0.57
Carotenoids (only methanolic extracts)	0.56



CFU assay determining the effect of aqueous extract of Ambri apple



Graphical representation of the no of CFUs against various concentrations of aqueous extract of ambri

Plate 3: Anti-cancer activity

## Chapter-5

### DISCUSSION

Fruits and vegetables that are consumed in large quantities may make a significant contribution to nutrient intake even though their nutrient content is not high. Among the common fruits, fresh apples are considered a food of moderate energy value, while processed apple products are either comparable to fresh apples in energy value or higher because of concentration (dehydrated) or addition of sugars during processing. In common with other fruits, apples contain insignificant amounts of protein (less than 0.2%) and lipid (less than 0.4%). Apples are not rich in vitamins and minerals like some other fruits, though they do provide a bit of vitamin C and potassium. However, apples are a good source of soluble fiber, especially pectin, which helps control insulin levels by slowing the release of sugar into the bloodstream (low glycemic index). Pectin is also known to help reduce cholesterol levels by lowering insulin secretion (Lee 2012). Today's natural approaches to wellness are becoming more popular and interest in the role of nutrition in overall health is increasing. In addition to common nutrients indicated above, there is continued interest in other phytonutrients that appear to prevent and ameliorate a variety of human diseases. Among the different phytochemicals, special interest has risen in plant polyphenols, specifically the flavonoids, whose key role appears to be in acting as antioxidants, similar to vitamin C and E (Lee 2012). Hippocrates, the ancient Greek physician who is known as the father of modern medicine, was the man who inspired this famous phrase over two thousand years ago, "an apple a day keeps the doctor away." These special traditional attributions of apples may have originated from the nutraceutical function of bioactive compounds. Currently there is a strong interest among scientists in anti-oxidative, anti-cancer, anti-neurodegenerative and anti-obesity activities of apple phenolic compounds

In recent years more interest has been paid to protect foods and human beings against oxidative damage caused by free radicals like hydroxyl, peroxyl

and Superoxide radicals. It is well known that many plant polyphenolic compounds such as phenolic acids, flavonoids, anthocyanidins and tannins possess remarkable antioxidant and anticancer activities.

In India, apples are grown at many places like Himachal Pradesh, Uttarakhand, Nilgiri Hills and Assam but at very small levels. Kashmir that produces over 1800 metric tonnes of apples and contributes about 80% of total India production, is endowed to have the richest pilgrim of different varieties of apples. Many of the varieties are found in the same season. Apples from Kashmir are exported to the different parts of the world, thus cements their place as the backbone of Kashmir's economy.

Both crabapples and cultivated apples belong to the genus *Malus* (Rosaceae family) and have similar profiles of phenolic compounds (Chen *et al.*, 2014). However, unlike apples, crabapples are typically used as ornamental plants. To our knowledge, most studies (Zhang *et al.*, 2014, 2017) have focused on the ornamental features of crabapples, including flower and leaf colors. In some parts of China, crabapples are also used as a food source. However, only a few studies have focused on other, non-ornamental functions of crabapple. Li *et al.* (2014) reported different antioxidant activities among 10 wild crabapples varieties. Li *et al.* (2016) discovered that fruit extractions of five crabapples can lower hypercholesterolemia. In a separate study (Qin *et al.*, 2015), dihydrochalcone compounds isolated from crabapple leaves were shown to have anticancer effects.

It is a well-known fact that the amount of nutrients in any crop is region-specific (Nair 2018). The general opinion is that the concentration of various bioactive compounds of apple depends on the species and cultivar of a fruit, as well as on the fruit maturity degree, cultivation methods, soil and climatic conditions and insolation [Podsędek *et al.* 2000, Sluis *et al.* 2001, Kondo *et al.* 2002, McGhie *et al.* 2005, Duda-Chodak *et al.* 2010]. Since the genotypes like crabapple and Ambri are underutilized, present investigation was an attempt to

study the variation in the phytochemical profile of these genotypes of Kashmir so as to look into their potential medicinal uses.

## **5.1 Nutrient composition**

### **5.1.1 Moisture**

Moisture percentage reflects the ability of the fruit to store water concentration in it, which is a symbol of life. The moisture content determination of fruit is a useful tool to show its ability of survival and freshness. In the current study, among the four selected apple genotypes, a great deal of variation was observed among them that ranged from 76.30% to 83%. Among the collected apple genotypes, Ambri was found to possess the maximum moisture content (83%). These results are slightly higher than the results reported by Petkova *et al.* (2020) and coincide with the results demonstrated by Mukhtar *et al.* (2010).

### **5.1.2 Total soluble sugars**

The total soluble sugars represent the most readily available form of carbohydrates present in the plant cells (Ammar *et al.*, 2013). In the current study, the total soluble sugar content of the four selected apple genotypes varied significantly ranging from 7.33 g/100g DW to 13.51 g/100g DW. Among the collected apple genotypes, Ambri was found to possess the maximum total soluble sugar content (13.51 g/100g). Our study demonstrates results that coincide with the soluble sugar content present in the apple as reported by Dar *et al.*(2015).

### **5.1.3 Crude fiber**

Fiber is an essential component of many diets. It increases food bolus and enhances digestion thereby reducing the risk of constipation. It may be used as an important agent for the management of hypercholesterolemia and related nutritional disorder. In the present investigation, the crude fiber content of apple genotypes was found to range from 0.52 g/100g to 1.61g/100g. The maximum crude fiber content (1.61 g/100g) was found in the Ambri genotype. These results

are in full agreement with the values reported by Lim and Rabeta (2013) (0.86-1.81g/100g).

#### **5.1.4 Vitamin C**

As we know vitamins are essential for the normal growth and development of multicellular organisms (Fortman *et al.*, 2013). In the present study, ascorbic acid was evaluated in selected fruits of four apple genotypes. In some fruits, ascorbic acid is the main compound contributing to their antioxidant activity and plays a very important role in regulating blood pressure, contributes to reducing cholesterol and aids in the removal of cholesterol from arterial walls and thus prevent arteriosclerosis (Khan *et al.*, 2011; Kumar *et al.*, 2014). In this study, the ascorbic acid of selected apple genotypes ranged in between 3.40 mg/100g to 4.37 mg/100g. Among the four genotypes, the maximum ascorbic acid content was found in yellow coloured crab apple genotype and thus represents the best source of vitamin C among the collected genotypes. The results are slightly lower than reported by Campeanu *et al* 2009 and coincides with the results reported by Nour *et al.* (2010) (2.6 – 18.7 mg/100g) in fifteen apple cultivars of Romania.

#### **5.1.5 Minerals**

As far as mineral content in diet is concerned, these are regarded as important chemical constituents used by the body in many ways. The vital role of mineral elements in human, animal and plant nutrition has been well recognized for the past many decades, as their deficiencies or disturbances in nutrition can cause a variety of diseases. Therefore, in the current study, complete profiling of some essential minerals was carried out in all the four selected apple genotypes.

Potassium is a nutrient of concern for Indians and is involved in blood pressure regulation, carbohydrate metabolism, fluid balance, growth and development, heart future, muscle concentration, nervous system function, protein function. The potassium content of fruit samples evaluated in this study ranged from 4660 ppm to 8604 ppm with the Yellow Crab Apple genotype possessing the

maximum potassium content as compared to other genotypes. These observations are in complete agreement with the results reported by Nour *et al.* (2010) (82.25 – 160.85 mg/100g).

Calcium represents one of the most important minerals due to its vital role to maintain various body functions, especially controls nerve excitability. It is necessary for the maintenance of the integrity of the skeletal muscles and maintains the tone of the heart (Provina *et al.*, 2013). In the current study, the calcium content of fruit samples ranged from 70.8 ppm to 74.2 ppm, with the Red Crab Apple genotype sample exhibiting the maximum calcium content. These values are lower than those reported by Aziz *et al.*(2013) (7.87-21.99 mg/100g) in apple fruits.

Magnesium is another important mineral and is the most abundant ion present in living cells. Its plasma concentration is remarkably constant in healthy subjects. It plays an important role in the structure and function of the human body and is involved in more than 300 essential metabolic reactions with special reference to the maintenance of internal fluid and electrolyte balance (USDA, 2016). The magnesium content found in fruit samples of the selected apple genotypes ranged from 123.30 ppm to 143.10 ppm with the yellow Crab Apple genotype representing the highest content of magnesium. These results coincide well with the values reported by Aziz *et al.* (2013) (4.44-15.58 mg/100g)in apple fruits.

## **5.2 Qualitative analysis of phytochemicals**

Compelling scientific evidence suggests that overproduction of free radicals can cause oxidative damage to biomolecules (lipids, proteins, DNA) in a cell, eventually leading to many chronic diseases such as cancer, diabetes, rheumatoid arthritis and other degenerative diseases in humans (Huang, 2018). This condition can be cured through the increased dietary intake of natural

antioxidants. Investigations relating to the antioxidant power of plants, especially in the case of ignored and underutilized crops is a very active field of research.

In this direction, the current study was carried out to reveal the presence of medicinally active constituents such as alkaloids, terpenoids, saponins, sterols and cardiac glycosides in the selected apple genotypes.

As far as alkaloids are considered, they represent the important secondary metabolite group and are loaded with therapeutic properties. Alkaloids can prevent numerous degenerative diseases by fighting off free radicals or binding with the oxidative reaction catalyst. Screening of all the five apple samples showed the presence of alkaloids in both aqueous and methanolic extracts. The results of our study corroborated very well with the observations of Oyinlade (2014).

Saponins are a diverse family of secondary metabolites that are derived from plants and used in various traditional medicines worldwide because of their therapeutic properties. The role of these compounds in plants is to safeguard against potential pathogens due to their potent antimicrobial activity. In the current study, phytochemical screening on aqueous and methanolic extracts of apple showed the presence of saponins in all the genotypes.

Terpenoids, also referred to as terpenes, are the largest group of natural compounds and many of them display biological activities especially in treating human diseases. They act against inflammation, cancer, malaria and many infectious diseases (bacterial and viral) (Wang *et al.*, 2005). In this study, the presence of terpenoids was observed in both methanolic and aqueous extracts of all apple genotypes.

Food products derived from plant sterols are popularly used as a therapeutic dietary option for reducing atherosclerosis risk and cholesterol. In addition, they are also used to decrease serum LDL-cholesterol levels. The cholesterol-lowering action of plant sterols is thought to occur, at least in part,

through competition with dietary and biliary cholesterol for intestinal absorption in mixed micelles (Calpe-Berdiel *et al.*, 2010). In our current study, presences of sterols were observed in all the methanolic extracts of apple genotypes.

Cardiac glycosides belong to group steroids and have the potential to exert powerful action on the muscles of the heart. Even a small amount can exert an aidful stimulation on a diseased heart. These compounds are potentially used in the treatment of congestive heart failure (Morsy, 2017). In our current study, the presence of cardiac glycosides was not found in any of the apple extracts.

### **5.3 Quantitative analysis of antioxidants**

Among natural antioxidants, polyphenols are widely distributed in plant foods and medicinal herbs and exhibit a wide range of biological effects (Altemimi *et al.*, 2017). Phenols represent a distinct class of secondary metabolites with a wide distribution throughout the plant kingdom and are associated with colour, nutritional properties, antioxidant properties and sensory quality of plant foods. They are biologically active substances and have also been reported to possess antidiabetic, anti-cancerous, antibacterial and antiseptic properties (Islam 2016). Nowadays, analysis of these bioactive metabolites in plants and the dietary intake of phenols and their health benefits is highly desired due to their high antioxidant activities (Chaturvedi *et al.*, 2015). Therefore, in the current investigation, the fruit samples of selected genotypes of apple were evaluated for their total phenolic content that ranged from 102.15 mg/100g DW to 137.83 mg/100g DW with Ambri genotype with the highest phenol content (137.83 mg/100g DW) and its dietary inclusion may be quite beneficial and helpful to fight various oxidative stress-related disorders in humans.

Phenolic compounds include flavonoids like flavonols, flavanones, isoflavones and anthocyanins, hydroxybenzoic acid, hydroxycinnamic acid, chalcones, aurones, hydroxycouramins and hydroxystilbenes (Panche *et al.*, 2016). Among them, flavonoids are widely distributed in most plant tissues and

have been well recognized for their potent antioxidant, antidiabetic and anti-proliferative effects as well as for protecting the body from various oxidative stress-related diseases (Pulipati *et al.*, 2017). Therefore, keeping in view the diverse role of flavonoids, in the current investigation, the fruit samples of four selected genotypes of apple were evaluated for their total flavonoid content, which came out to range from 61.28 mg/100g DW to 113.31 mg/100g DW of quercetin equivalents. In the current study, the aqueous extract of Ambri represented the highest flavonoid content (113.31 mg/100g). The presence of such flavonoid-rich diet has been reported to enhance cellular antioxidant defenses and may thereby prolong healthy life (Carvalho *et al.*, 2011). These results for phenols and flavonoids were almost similar to the ones reported by Mitic *et al.* (2013) (73-217 mg/100g for phenols and 42-112mg/100g for flavonoids) in apple fruit extracts of Serbia.

People consuming diets rich in carotenoids from natural foods such as fruits and vegetables are healthier and have lower mortality from a number of chronic illnesses due to its strong antioxidant nature (Somner *et al.*, 2012). Carotenoids also act as a strong precursor of vitamin A. In the current study, the carotenoid content of the four apple genotypes was found to lie in between 0.039 to 0.051 mg/100g. Among the four apple genotypes, the maximum carotenoid content was found in methanolic extract of yellow-colored crab apple (0.051mg/100g) and have thus better chances to be exploited as potential vitamin A source as well as antioxidant-rich food. The results agree with the values reported by Delgado-pelayo *et al.* (2014) (0.0017- 0.15 mg/g) in apple fruits.

Likewise, anthocyanins due to their rich antioxidant properties have been found to possess innumerable health benefits. In the current study, the maximum anthocyanin content of the apple genotypes was found to lie in the range of 2.88 mg/100g to 13.29mg/100g. The methanolic extract of the Red Crab Apple genotype represented the best genotype with respect to total anthocyanin content and can thus be better exploited as a health-promoting food. The results of our

current study are slightly lesser than the values reported by Shehata *et al.* (2020) (16.39mg/100g) in apple peels.

Quercetin, a flavonoid found in fruits and vegetables, has unique biological properties that may improve mental/physical performance and reduce infection risk (Davis *et al.*, 2009). These properties form the basis for potential benefits to overall health and disease resistance, including anti-carcinogenic, anti-inflammatory, antiviral, antioxidant and psychostimulant activities, as well as the ability to inhibit lipid peroxidation, platelet aggregation and capillary permeability and to stimulate mitochondrial biogenesis (Aguirre *et al.*, 2011). HPLC-based estimation of quercetin showed that the quercetin concentration was higher in red coloured crab apple (9.86mg/100g) than Ambri (7.43mg/100g). the results are lower than the values reported by Patocka *et al.* (2020) (13.2mg/100g) in apple fruits.

#### **5.4 Anti-oxidant activity**

In order to check the cumulative effect of the different antioxidants present in apple genotypes, in the present study, the fruit samples of selected apple genotypes were further evaluated for their total antioxidant activities that ranged from 371.76 $\mu$ M to 1319.08 $\mu$ M FRAP/g on a dry weight basis. Among the four apple genotypes analyzed in the current study, methanolic extract of red coloured crab apple demonstrated the highest antioxidant activity (1319.08 $\mu$ M FRAP/g) and showed a positive co-relationship with flavonoid content. The results are in agreement of the values reported by Petkova *et al.* (2020) (1037-5850  $\mu$ M FRAP/g) and Mitic *et al.* (2013) (319-1003  $\mu$ M FRAP/g). Thus, it may be suggested that the increment of dietary intake of natural antioxidant-rich underutilized plants like flavonoid-rich red coloured crabapple genotype may be quite beneficial in ameliorating the cellular damage caused by oxidative stress related to various chronic diseases. This may be probably through the inhibition of initiation or propagation of oxidative chain reactions, mainly acting either as free radical scavengers or quenchers of singlet oxygen and reducing agents. (Murugan

*et al.*, 2013). The overall results of the current study are an indication that the selected apple genotypes have a great potential to be used as a rich source of nutrients as well as nutraceuticals in alleviating macro and micronutrient deficiencies and promoting overall health.

### **5.5 Anticancer potential**

Previous reports show that apple extracts display dose-dependent anti-cell proliferation activity in Caco-2 colon cancer and Hep G2 liver cancer cells. Such types of bioactivity were attributed to a higher amount of phytochemical nutrients in apple peel (Eberhardt *et al.*, 2000). Surprisingly, aqueous extract of indigenous local Ambri genotype that was evaluated for anticancerous potential using colony proliferation assay showed increased cell proliferation effect on C4-2 prostate cancer cells in a dose-dependent manner. This can be attributed to the fact that we used whole apple instead of only peel which may have decreased the specific activity of the peel. Furthermore, since apple contains 13.51 g of total soluble sugar per 100 g of fruit and it is also known that sugar has a positive correlation with cancer proliferation (Warburg effect). This finding suggested that dose dependent increase in CFU assay might be the effect of increased sugar concentration in the extract.

Therefore, the anticancerous-based assay warrants further investigation using other anticancerous-based bioassays.

## Chapter-6

### SUMMARY AND CONCLUSION

Fruits and vegetables, containing abundant dietary fiber, vitamins and minerals, in particular large amounts of phytochemicals (Baliga *et al.*, 2011;Deng *et al.*, 2013;Fu *et al.*, 2010;Xia *et al.*, 2010;Fu *et al.*, 2011;Deng *et al.*, 2012 and Li *et al.*, 2014), are recommended by nutritionists because of their health benefits (Giampieri *et al.* 2014 ; Hoffman *et al.* 2016). Phytochemicals in these natural products are considered to be responsible for positive health outcomes. Particularly, it is widely noted that plants produce plenty of antioxidants to combat the oxidative stress induced by oxygen and light in the natural environment (Lobo *et al.*, 2010). Oxidative stress performs an essential role in multiple chronic diseases (Forbes-Hernandez *et al.*, 2014 ; Huang *et al.*, 2016 and Halliwell *et al.*, 1999). Therefore, antioxidants in fruit and vegetables have been extensively explored for their effects on several diseases. Epidemiological and nutritional studies suggested that one's fruit and vegetable consumption is inversely proportional to the prevalence of chronic diseases like coronary heart problems, cancer, and Alzheimer's disease (Lock *et al.*, 2005 and Gescher *et al.*, 1998).

Apple (*Malus domestica* Borkh.; Rosaceae) is one of the most economically and culturally significant, nutrient-rich fruit grown in all temperate zones (Spengler, 2019). The whole fruit is eatable except seeds; apart from that, many other products are produced from them: Ciders and juices, jams, compotes, tea, wine, or dry apples. They are irreplaceable in human nutrition since they increase immunity, have a positive effect on stress resistance, and contain many bioactive substances that are beneficial for humans. There is no doubt that apples are healthy and have many health benefits, but it was modern medicine, based on evidence, not experience, which had to prove their usefulness for human health ( Boyer and Liu, 2004).

The biochemical and genetic variation among the various underutilized apple genotypes is imperative for their efficient utilization as nutritious food as well as for their effective conservation. Therefore, the present investigation entitled " A Study on Biochemical and Nutraceutical Potential of Some Underutilized Apple Varieties of Kashmir " was undertaken in the Biochemistry and Molecular Biotechnology Laboratory, Division of Basic Sciences and Humanities, Faculty of Horticulture, Sher-e-Kashmir, University of Agricultural Sciences and Technology, Kashmir during the year 2018-19 and 2019-20 with the following objectives:

1. To study the nutritive composition of some underutilized apple genotypes of Kashmir.
2. To chemoprofile and evaluate the antioxidant/anticancerous potential of selected underutilized apple genotypes of Kashmir

In the study, the fruits of the four selected apple genotypes were collected from KVK Shopian and the local market were subjected to biochemical investigation.

In the current study, the Ambri genotype was found to have the highest moisture content (83%) highest total soluble sugars (13.51 g/100g), crude fiber (1.61 g/100g), total phenolic content (137.83mg/100g) and total flavonoid content (113.31mg/100g). Carotenoid content (0.051 mg/100g), vitamin C content(4.37mg/100g), potassium content (8604 ppm) and magnesium content were maximum in Yellow Crab Apple genotype. The antioxidant activity was found to be highest in red-coloured crabapple genotype (1319.08  $\mu$ M FRAP/100g) along with anthocyanin content(13.29mg/100g) and calcium content (74.2 ppm). The study revealed that all the genotypes are the best source of one or the other parameter but in general Ambri and crabapples can be comparatively regarded as the best genotypes due to more than one beneficial selected trait present in them.

Therefore, from the current study, it is concluded that the fruits of selected

apple genotypes have a great level of divergence among them at both nutritional and biochemical levels. The wide differences among the selected genotypes led to the identification of promising genotypes especially with respect to their nutritional quality and biochemical agents. Further, they have a great potential to be used as health-promoting foods as they possess high nutraceutical content and antioxidant activity. In nutshell, the study suggests that the selected apple genotypes have promising potential as a means of food biofortification.

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## **CERTIFICATE**

Certified that all the corrections/amendments as suggested by External Examiner **Dr. Sanjay Guleria**, SKUAST Jammu during Viva-Voce examination held on 19-07-2021 have been incorporated in the manuscript entitled “**A Study on Biochemical and Nutraceutical Potential of Some Underutilized Apple Varieties of Kashmir**” submitted by **Mr. Ajaz Bashir (Regd. No. MSBS/2018/08)**.

**(Dr. Imtiyaz Murtaza)**  
Chairman  
Advisory Committee