



Phytochemical Properties of Processed Chickpea varieties of Haryana (India)

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ABSTRACT

In present study, we investigated the nutraceutical potential of seed coat, dehulled raw dal, cooked dal, whole seed of dark coloured *desi* chickpea and light coloured *kabuli* chickpea. Locally grown varieties of Haryana (India) named HC-1 and KC-1 were under investigation. Total phenolic content, o-dihydric phenols, hydrophilic and hydrophobic phenols, flavonoid contents were determined in methanolic extracts of both cultivars. Free radical scavenging efficiencies were estimated by adoption of DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay method. Similarly, antioxidant activities in terms of peroxide inhibition were measured by using ferric thiocyanate solution. Results concluded that phenolic contents varied widely in seed parts as well as in cooked cotyledons and correlated ($p < 0.05$) significantly with antioxidant properties in most of the extracts. *Desi* chickpea genotype was found to be better source as nutraceutical food than that of *kabuli* chickpea.

Keywords: Chickpea, antioxidant, flavonoids, phenolics, free radical scavenging activity.

INTRODUCTION

Various antioxidants such as tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), propyl gallate and butylated hydroxyanisole (BHA) are synthetically produced and found to have scavenging properties suitable enough for the treatment of various ailments. However, due to their wide range of toxicity their application in the medical science have not been appreciated so much. Therefore, the screening of medicinal and food plants

for antioxidant properties have been performed efficiently for the last few decades. Polyphenols found in fruits, grains, vegetable and legumes possess different bioactivities like antioxidation, anticancer, antiaging, cardiovascular protection, inhibition of unnecessary cell proliferation (Miller *et al.*, 2000; Anderson *et al.*, 1999). Chickpea (*Cicer arietinum* L.) is an important leguminous crop and has been extensively grown in parts of north and east Africa, southern Europe, middle eastern countries and India as their major pulse crop (Iqbal *et al.*, 2006).

In south Asian subcontinent, two morphologically different forms of chickpea are being grown which are different in size, shape and color. One produce small seeds having angular surface and yellowish-brown to dark brown in color and popularly known as the *desi* variety whereas the other produce relatively large seeds having round surface and yellowish-white to light-brown color and popularly known as the *kabuli* variety. Temperate environmental conditions are more favoured by the *Kabuli* chickpea whereas semi-arid climate is conducive for *desi* chickpea. The coats of colorful seeds varieties viz. pink, red and black are rich in anthocyanin pigments, phenolic compounds and antioxidant (Mazza and Miniata, 1993). Reviewing the existing literature confirms that few research has been done to measure phenolic activity and antioxidant potential of *desi* chickpea seeds (Zia-Ul-Haq *et al.*, 2008; Segev *et al.*, 2011). But in India, pulses are processed and cooked in a number of ways. These different treatments and cooking methods can alter the physical characteristics and chemical compositions of the food. Some common reports state that thermally cooked foods have less nutritional value as compared to fresh foods due to decline in specific physicochemical features (Barros, 2011; Zhang, 2004). While other studies conclude that cooking enhances antioxidant activities by dissolving antioxidant compounds from insoluble and bounded parts of food (Turkman *et al.*, 2005; Dewanto *et al.*, 2006). However, taken together, studies on effect of processing on chickpea local varieties developed in Haryana (India), no information is available in the literature regarding phytochemical constituents and their antioxidant properties. After thorough investigation and considering above facts and information this study was undertaken to measure the chemical composition of whole seeds, seed coats, decoated raw dal and cooked dal of *desi* and *kabuli* chickpea as well as to analyse the anti-oxidant and anti-radical activity of the methanolic extracts of the samples. Studying such local legume will furnish information to the consumers and manufacturers of value added foods for betterment of human health.

MATERIALS AND METHODS

Seed material

For experimental analysis healthy seeds of *desi* chickpea var. Haryana chana-1 (HC-1) and *kabuli* chickpea var. *kabuli* chana-1 (KC-1)

were provided by the Pulses section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University (India). These seeds were cleaned to remove foreign materials, damaged seeds and immature seeds. After sorting, major portion of seeds from each variety were dehusked to obtain seed coat and dal. Dehusked dal was divided into two sets. The first set was kept for extraction which constitutes intact dehusked seeds and second part was cooked using distilled water in 1:10 (w/v) ratio at 100°C, dried at 55°C. Thus, we obtained four samples of *desi* chickpea and four samples of *kabuli* chickpea, 100 g each for further analysis.

Preparation of extracts

For the preparation of extracts the samples were ground using electric grinder and converted into fine powder. Powdered samples were then extracted separately by refluxing for six hours using methanol. Extracts produced after refluxing were filtered and the filtrates were concentrated under reduced pressure on rotating evaporator at 40°C. These concentrated filtrates were stored at 5°C for analyses of phytoconstituents.

Determination of total phenolic content

Determination of total phenolic content was done by Folin- Ciocalteu reagent using gallic acid as standard (Shahidi and Naczk, 2001). To a 50 ml volumetric flask 1.0 ml extract, 1.0 ml Folin–ciocalteu reagent (50% v/v) and 2.0 ml of Na₂CO₃ (20%w/v) were added and mixed and final volume was made to 50 ml. The mixture was allowed to settle for 30 minutes and then centrifuged at 6000 rpm for 5-7 minutes. After centrifugation, solution was measured colorimetrically at 730 nm using Shimadzu UV-Vis spectrophotometer (UV-2600). A blank was also prepared by following same aforementioned procedure without sample. After multiplication with the dilution factor the concentration of phenolic content was expressed as equivalent to milligrams of gallic acid per gram of extract (mg GAE/g) by using standard plot.

Determination of hydrophilic and hydrophobic phenolic contents

For the determination of hydrophilic and hydrophobic phenolic contents 50 ml of crude extract was fractionated to obtain constituting hydrophilic and hydrophobic components. It was done by mixing the

extract with deionised water and n-butanol (100ml each) in separating funnel as per Wettasinghe's method (2000). The mixture was then allowed to stand until separate layers visible. Separated layers were then concentrated using rotavapor at 40°C temperature. After measuring the weight of each fraction the phenolic content of each fraction was determined as per Shahidi and Naczki method.

Preparation of standard curve of o-dihydric phenols

Preparation of standard curve was done by estimating o-dihydric phenols in methanol extracts by Arnow's method (Kim *et al.*, 2003) using catechol as standard. 0.4 ml of extracted solutions were added to 1 ml 0.5N HCl, 1 ml Arnow's reagent and 2 ml 1N NaOH. Final volume was made to 10 ml using double distilled water. The intensity of resulting orange red colour was measured colorimetrically at 515 nm using spectrophotometer. Standard curve was made by plotting absorbance against amount of catechol used (mg). A blank was also prepared by following same aforementioned procedure without extract.

Determination of flavonoids

The aluminium chloride colorimetric assay, as described by Zhishen *et al.* (1999) with modification was used. Briefly, 1 ml of diluted (1:4) extracts was added. A blank solution using doubled distilled water was prepared. Then 0.3 ml 5% NaNO₂ was added to the testing samples, followed by 0.3 ml 10% AlCl₃, 2 ml of 1M NaOH and the total volume was made 10ml with dilution and mixed thoroughly. Then the absorbance was measured at 510 nm against blank. Total flavonoid contents were expressed as mg catechin equivalent per gram of the extract (mg CAE/g).

2, 2'-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

2, 2'-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was used to evaluate the antioxidant activity of the both the genotypes. The antioxidant response of extracts against DPPH free radical was estimated by method of Hatano *et al.*, 1988. Methanol treated extracts were dried completely and weighed. The dried mass of methanol extracts was redissolved in required volume of methanol to make the stock solution (5 mg/ml).

Different concentrations (for *desi* chickpea 0.5 to 3 mg/ml and for *kabuli* chickpea 0.5 to 4 mg/ml) were made by appropriate dilutions with 100% methanol from the stock solution. In extracts of different concentrations, 2.0 ml of DPPH solution (0.025 g L⁻¹ in 50% methanol) was added and the mixture was shaken and absorbance was measured at 515 nm at every 5 minutes interval until the reaction subsided. After 2 hours the percent absorbance was declined corresponding to the percentage of DPPH scavenged which was an expression of antioxidant activity. During this process butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) with conc. 0.1-1.0 mg/ml were used as standard solutions having same preparatory method. Similarly, a control sample was also prepared. By using Microsoft Excel Software, a quadratic regression equation ($y = ax^2+bx+c$) was obtained. By putting $y = 50\%$ in the equation $y = ax^2+bx+c$; it was converted to the form $ax^2+bx+c = 0$. IC₅₀ was calculated from the equation $ax^2+bx+c = 0$ by using the formula:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

where, $x = IC_{50}$ (mg/ml)

Calculation

The calculation for DPPH scavenged (% DPPH_{sc}) was done by following formulae:

$$\%DPPH^*_{sc} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ and A_{sample} is the absorbance of control and the sample. Based on the results obtained after calculation a graph was made by plotting percent DPPH free radical scavenging activity or inhibition percentage (y-axis) against extract concentration (x-axis).

Ferric thiocyanate (FTC) method

The FTC method was given by Kikuzaki and Nakatani (1993). This method was used for the evaluation of antioxidant activity of the extracts under study. After making required dilutions with methanol, 0.5 to 3 mg/ml of *desi* chickpea and 0.5 to 4 mg/ml *kabuli* chickpea samples were made. After that, samples were mixed with 5 ml of linoleic

acid emulsion and final volume was made to 10 ml using 0.2 M phosphate buffer (pH 7.0) and incubation was done at 37°C for 96 hours (4 days). After incubation aliquots of 0.1 ml were drawn from the incubated mixture after 24 hours interval and mixed with 30% ammonium thiocyanate, 20 mM ferrous chloride in 3.5% HCl and final volume was made to 10 ml with 75% ethanol and allowed to stand for 10 minutes. The color developed was measured colorimetrically at 500 nm using spectrophotometer. For this experiment butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as standard solutions and by using quadratic regression equation ($y = ax^2 + bx + c$) calculations were done as described earlier. A control mixture was prepared simultaneously following same procedure without the test sample.

Calculation: Observed antioxidant activity was expressed as:

Antioxidant activity (%) = $\{1 - (\text{increase in abs. of sample} / \text{increase in abs. of control})\} \times 100$

Statistical analysis

Four replications of each sample were used for statistical analysis for minimising random experimental error. Values obtained were expressed as mean \pm S.E. Both one way and two way analysis of variance (ANOVA) and F-test were carried out to assess significant differences in between means ($p < 0.05$). Correlation analyses of polyphenolic composition and their antioxidant activities were

carried out using Pearson correlation programme in Online Statistical Analysis (OPSTAT www.hau.ernet.in).

RESULT AND DISCUSSION

Extract yield of all the four treatments of each pulse crop varied widely. In case of *desi* chickpea, seed coat extract had highest yield (7.01) followed by whole seed extract (6.87), raw dal extract (5.89) and cooked dal extract (4.60) in g/100g seed. Similarly *kabuli* chickpea had also highest extract yield in case of seed coat extract (8.34) but least in case of raw dal extract (3.10). As per Nithiyantham *et al.*, 2012, the percentage of extract yield of chickpea seeds when processed differentially was in range from 1.33 to 28.06%. The variation in yields of different extracts of Haryana chana-1 and *Kabuli* chana-1 is attributed to polarities of various compounds present in various seed parts and analogous differences have been explained in literature (Jayaprakasha *et al.*, 2001 and Hasmida *et al.* 2014).

As per results analysed for estimation of total phenolic content in *desi* chickpea extracts, seed coat contained highest amount of total phenolics i.e. 25.24 mg GAEg⁻¹. *Desi* chickpea whole seed extract contained 18.72 mg GAE g⁻¹ which were similar to those found in the literature (Xu and Chang, 2008) i.e. 14.4 mg GAE/g and Nithiyantham *et al.*, 2012 19.42 mg GAEg⁻¹, but lower than those noted in other legumes faba bean (major and minor) 30.93 mg

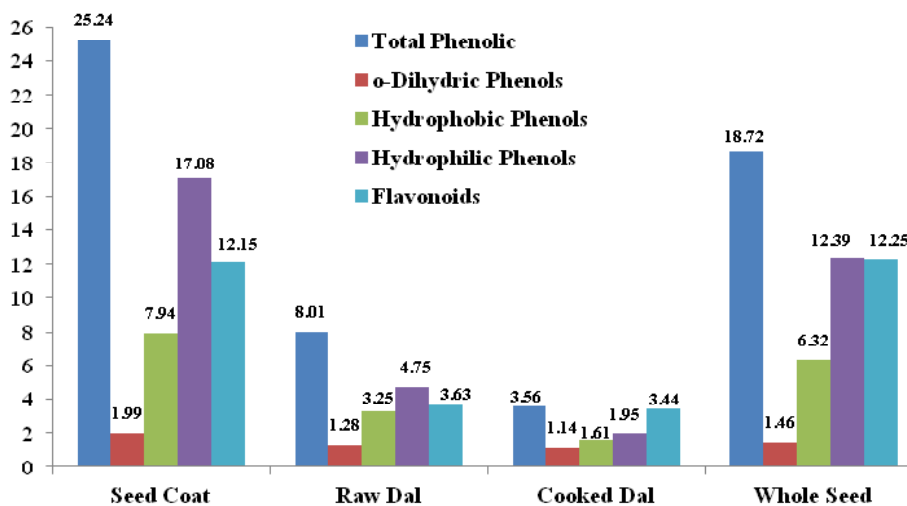


Fig. 1 : Phytoconstituents of *desi* chickpea extracts

GAEG⁻¹, 42.44 mg GAEG⁻¹ respectively. Rest of the extracts contained significantly reduced amount. Raw dal extract contained 8.07 mg GAEG⁻¹, cooked dal extract contained 7.60 mg GAEG⁻¹. Similarly, in *kabuli* chickpea genotype, phenolic content was highest in seed coat (6.82 mg GAEG⁻¹). Corresponding value for total phenolics in raw dal, cooked dal, whole seed extract was 1.85, 1.72, 5.45 mg GAEG⁻¹. Raw dal and cooked dal phenolic contents were statistically at par with each other. According to studies done so far it has been found that the function of seed coat is to protect the cotyledon by acting as a protective barrier which is composed of phenolic compounds in high concentrations (Shahidi *et al.*, 2002; Troczynska *et al.*, 1997).

In *desi* chickpea variety, for 100 g sample, the o-dihydric phenols were observed as 0.013 % in seed coat extract, 0.007% in raw dal extract, 0.005% in cooked dal extract and 0.012 % in whole seed extract while in *Kabuli* genotype order follows as: seed coat (0.56%) > whole seed (0.45%) > raw dal (0.057%) ~ cooked dal (0.056%). Lopez-Amoros *et al.* in 2006 stated that chickpea possess varying concentrations of hydroxybenzoic phenolic compounds, vanillic acid, p-hydroxybenzoic, protocatechuic, trans-ferulic acid, cis and trans p-coumaric acid.

Segev *et al.*, (2011) stated that the *kabuli* chickpea cotyledon has very less phenolic content and consists mainly hydroxybenzoic acids, free and

combined hydroxycinnamic acids and (+)-catechin. Graphical representation for various phytochemical contents in *desi* and *kabuli* chickpea extracts is given in Fig. 1 and Fig. 2 respectively.

In *desi* chickpea extracts, it was observed that total phenolics reported in seed coat extract was 25.24 mg GAEG⁻¹ and it consisted of 32% (w/w) and 68% (w/w) of hydrophobic and hydrophilic phenols (ratio of approx. 1:2, w/w) respectively (Fig. 1). In case of, raw dal extract, it contained 3.25 mg GAEG⁻¹ hydrophobic and 4.75 mg GAEG⁻¹ hydrophilic phenols which constituted 41% (w/w) and 59% (w/w) of total phenolics (8.01 mg GAEG⁻¹). Similarly, cooked dal extract possessed 1.61 mg GAEG⁻¹ hydrophobic (73%, w/w) and 1.95 mg GAEG⁻¹ hydrophilic phenols (27%, w/w). Whole seed methanolic extract had total phenol with the value of 18.72 mg GAEG⁻¹ and it exhibited 66% and 34% (w/w) for hydrophilic (12.39 mg GAEG⁻¹) and hydrophobic (6.32 mg GAEG⁻¹) phenol respectively

Hydrophobic and hydrophilic phenols of all the four treatments of *kabuli* chickpea varied widely (Fig.2). In seed coat extracts, hydrophobic and hydrophilic phenols were 2.48 mg GAEG⁻¹ and 4.34 mg GAEG⁻¹ which exhibited 36% and 64% of total phenolics respectively. In case of raw dal and cooked dal extract hydrophobic phenols were (46% of total phenol) 0.85 mg GAEG⁻¹ and (46% of total phenol) 0.79 mg GAEG⁻¹ while hydrophilic phenols were (54% of total phenol) 0.96 mg GAEG⁻¹ and

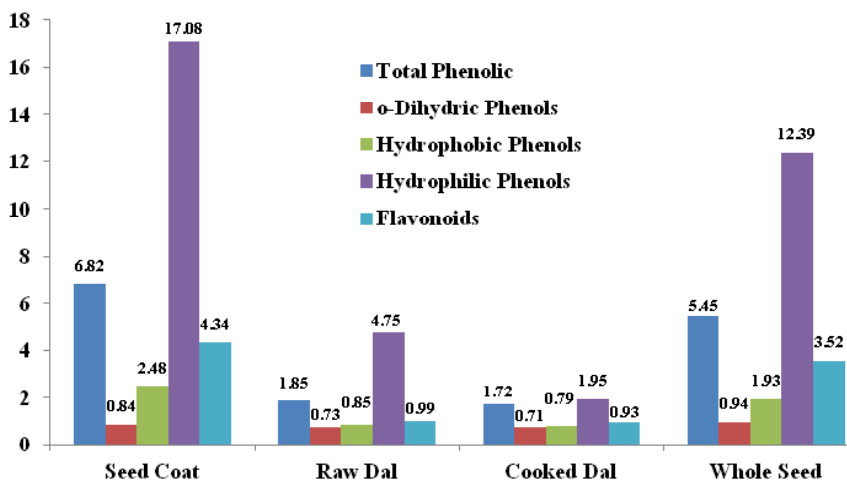


Fig. 2 : Phytoconstituents of *kabuli* chickpea extracts

(54% of total phenol) 0.94 mg GAEg⁻¹ respectively. Similarly, in whole seed extract 37% and 63% of total phenols was constituted by hydrophobic and hydrophilic phenols.

In present studies, total phenolics including o-dihydric phenols, hydrophobic and hydrophilic phenols were observed to be decreased on cooking. Segev *et al.*, 2011, reported 85% decrease in total phenolics by cooking in *kabuli* chickpea. Similarly, Hwang *et al.*, 2012, found that boiling and steaming significantly decreased the ascorbic acid content, total phenolics, and anti-oxidant potential as compared with the other cooking methods. Reduced total phenolics in boiled or steamed foods have been attributed to the solvation of phenolic constituents into the cooking water (Zhuang *et al.*, 2012). Therefore, fractions of hydrophilic phenols were high in some extracts while few extracts (mainly cooked dal) were having high hydrophobic phenolic content. Also, extraction ability of phenolic compounds is a function of polymerization of phenolic compounds, interaction of phenolics with various food constituents, type of solvent, solvent polarity, temperature and time of reaction (Marathe *et al.*, 2011; Oomah *et al.*, 2011).

In *desi* chickpea extracts, it was observed that flavonoid content was highest in seed coat (12.15 mg CAEg⁻¹) analogous with result obtained for total phenolics. Corresponding values for flavonoid content in raw dal, cooked dal, whole seed extracts were 3.63, 3.44, 12.25 mg CAEg⁻¹. Flavonoids in raw dal and cooked dal were statistically at par with each other. Similarly, in *kabuli* genotype, the methanolic extract of seed coat showed maximum flavonoid content (3.13 mg CAEg⁻¹) followed by whole seed extract (1.90 mg CAEg⁻¹), raw dal extract (1.18 mg CAEg⁻¹) and minimum in cooked dal extract (1.08 mg CAEg⁻¹). As reported by Sharma *et al.*, 2013, the total flavonoid content in selected *desi* and *kabuli* chickpea cultivars ranged from 0.15 mg QE g⁻¹ of flour to 0.36 mg QE g⁻¹ of flour. Total flavonoid content of the *desi* cultivar was found significantly higher than *kabuli* cultivars of chickpea which is in conformity to current studies. Ladjal and Chinabe (2015) reported that total flavonoid content of the chickpea (12.7 mg QEg⁻¹) and pea (12.5 mg QEg⁻¹) extracts was found significantly ($p < 0.05$) less as compared to the lentil extract (25.9 mg QEg⁻¹). Whereas, concentrations of glycosides of quercetin, luteolin and apigenin was

found significantly ($p < 0.05$) higher in the seed coat of both peas as reported by Duenas *et al.* (2006).

Basically two methods were used for evaluation of antioxidant activities which are known as 2, 2'-diphenyl-1-picrylhydrazyl radical (DPPH) method and ferric thiocyanate (FTC) method. In both the experimental technique (DPPH as well as FTC method) for measuring antioxidant activities, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as standards. IC₅₀ value obtained for BHT and BHA solutions by DPPH method was 0.41 and 0.60 respectively (Fig.10). Corresponding (%) antioxidant activity or scavenging efficiency was 86.00% and 75.00%. Similarly by FTC method, IC₅₀ value observed for BHT and BHA solutions was 0.63 and 0.70 respectively. Inhibition percentage in terms of antioxidant activity by FTC method was 76.00% in BHT and 66.60% in BHA. IC₅₀ values obtained by DPPH method in *desi* chickpea extracts were 0.95, 1.32, 1.34, 1.14 mg/ml for seed coat, raw dal, cooked dal, whole seed respectively. The corresponding maximum antioxidant activity (%) at 3 mg/ml was 72.50, 64.20, 63.00, 67.60. But the % of inhibition or antioxidant activity of methanolic extracts by ferric thiocyanate method in cooked dal, raw dal, whole seed, seed coat was 58.00, 60.00, 63.20 and 67.00 respectively. Corresponding IC₅₀ values for all extracts were 2.07, 2.08, 1.77, 1.58 mg/ml. Analogous to results obtained for total phenolics, antioxidant activities were highest in seed coat extracts and lowest in cooked dal extracts.

As per the results obtained by DPPH method for *kabuli* chickpea, the % of inhibition of methanolic extracts in ascending order is: cooked dal (53.80) < raw dal (56.00) < whole seed (60.30) < seedcoat (67.00) while order for free radical scavenging efficiency of different extracts of *kabuli* chickpea follows: seed coat (62.50%) > whole seed (59.00%) > raw dal (52.00%) ~ cooked dal (51.00) by FTC method. Xu and Chang, (2008) reported that free radical scavenging capacity and antioxidant activity had been found significantly ($p < 0.05$) reduced after boiling in cool season edible legumes. The data for antioxidant activities is in accordance with the study of other research workers. Greenish brown seed coat of lentil had shown strong antioxidant activity and possessed high phenolic content whereas *kabuli* chickpea being made up of light colored seed

coat shown relatively weak antioxidant activity and comparatively less phenolic content. This result was also concluded by Marathe *et al.* (2011) for various legumes. Similar relationship between chemical structure of flavonoids and their antioxidant activities had been analysed by Arora *et al.*, 1998. They investigated the anti-oxidant properties of genistein and daidzein which help inhibit peroxidation of lipid in a liposomal system. According to them, presence of hydroxyl substituents on flavonoid skeleton boosts activity. However, different phenolics show different antioxidant activity ranging from strong antioxidant to weak antioxidant and due to this varying antioxidant activity strength they act as antagonists or synergists with themselves or with the other constituents of the extracts. (Moran *et al.*, 1997; Lein *et al.*, 1999). Therefore, different phenolic compounds respond differently toward antioxidant assays.

Correlation studies are used to study the interaction between polyphenolic compounds such as total phenolics, o-dihydric phenols, hydrophobic phenols, hydrophilic phenols, flavonoids and their corresponding antioxidant activities. Correlation studies had shown positive and prominent correlation between total phenolics, antioxidant activities and flavonoids. According to Shahidi (2002) it was stated that positive and prominent correlation existed between catechin content and antioxidant activity in beach pea seed coats. As per finding of Sancho *et al.*, 2013, some hydrophilic phenols and lipophilic phenols showed contribution towards total antioxidant activity in papaya fruit while others showed antagonistic effect. In conformity to the above results Yao *et al.*, 2010 explained the existence of positive correlation in between total phenolics and antioxidant activity in seven different colored grains.

Free-radical scavenging activity was observed in combined and free hydroxycinnamic compounds to a greater extent as compared to analogous hydroxybenzoics. It has also been found that few o-dihydric phenols, hydrophobic phenols and hydrophilic phenols were found to be non-significant and negatively correlated with each other. Extraction of different components of pulses can be done by adopting various processing methods such as cooking and soaking (Mubarak, 2005). Furthermore, it has been found that various components of phenolic substances were responsible for their contribution to antioxidant activity of varying degrees. Moreover, it was found that presence of various phenolic substances complex in the extract was responsible for synergistic effect whereas negative correlation existed when phenolic compounds are correlated in different modes in various assay systems. Hence, the results showed that in spite of cooking effect, the phytochemical constituents and antioxidant activity in seedcoat as well as cotyledon of chickpeas were strongly linked. The present study has highlighted the impact of solvent used for extraction, method of extraction, cultivar and its processing. Therefore, it can be concluded that raw and processed *desi* chickpeas are an important source of antioxidant compounds than that of *kabuli* genotype.

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