Total Phenolic and Flavonoid Content and Antioxidant Activity of Amaranth Based Gluten Free Cookies

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Abstract: Flavonoids and phenolic compounds are essential in our diets for their role in combating lifestyle disorders. Antioxidant activity of foods may protect us from diseases such as cancer, diabetes and cardiovascular diseases. This study was undertaken with an aim to develop bakery products using a pseudocereal and legume based flour instead of refined wheat flour which may have a higher phenolic and flavonoid content. Cookies were developed from composite flour made of raw and popped amaranth flour, pearl millet flour and chickpea flour. 0.01g/ml extracts of composite flour and cookies were prepared using distilled water for analyzing total phenolic content (TPC) and 80% ethanol for analyzing total flavonoid content (TFC) and antioxidant activity (AOA). AOA was measured using DPPH free radical scavenging assay and FRAP assay. The composite flour and cookies had a TPC of 277.22 mg GAE/ 100g and 315.56 mg GAE/100g respectively. The TFC was 312.50 mg QE/100g and 250 mg QE/100g respectively. DPPH free radical scavenging assay showed 26.97% and 40.82% AOA by 10 mg/ml extract of composite flour and cookies respectively. FRAP assay showed a reducing power of 151.6 mg GAE/100g and 204 mg GAE/100g for the flour and cookies respectively. The results of the study show that amaranth based composite flour and cookies may offer health benefits due to their high phenolic and flavonoid content and good antioxidant activity.

Key words **– Raw amaranth, popped amaranth, pearl millet, chickpea, phenolic content, flavonoids, antioxidant activity**

I. INTRODUCTION

Amaranth is a multi-purpose crop. Its grains and leaves can be used as food and feed and it can also be grown as an ornamental plant (Mlakar, Turinek, & Jakop, 2009). The amaranth grain is an excellent source of good quality protein and lipids (Salcedo-Chávez, Osuna-Castro, Guevara-Lara, Dom\'\inguez-Dom\'\inguez, & Paredes-López, 2002) with higher content of minerals like calcium, potassium, phosphorus, as well as dietary fiber (Eggum, Pedersen, Knudsen, & others, 1990). Amaranth is a pseudocereal and lacks gluten. Gluten is a protein present mainly in wheat, but also in barley and rye. It triggers an immune-response in some people. Such people are said to be suffering from celiac disease. Celiacs have to depend on gluten free foods throughout their life. Since most of the gluten free foods are predominantly starch based, they usually lack minerals, vitamins, fibre and good quality protein (de la Barca, Rojas-Martínez, Islas-Rubio, & Cabrera-Chávez, 2010). Thus, pseudocereals like amaranth, buckwheat and quinoa have become very popular these days. Not only among the celiacs, they are also getting popular among healthy individuals. This is because of their nutritional content. Amaranth has a higher protein, fat, mineral and fibre content than many cereals (Amare, Mouquet-Rivier, Rochette, Adish, & Haki, 2016). It has been found that amaranth has high content of lysine, so the biological value of amaranth protein is high (Hozová, Buchtová, Dodok, & Zemanovič, 1997).

Researchers have found antioxidant activity in amaranth grain which is because of the phenolics, flavonoids, tocopherols and anthocyanins present in it (Klimczak, Małecka, & Pachołek, 2002), (Escudero, Albarracin, Lucero Lopez, & GIMÉNEZ, 2011). This antioxidant activity may have some beneficial effect on health. Amaranth has shown protective effects on serum and liver intoxication and amaranth oil has shown a reduction in serum triglycerides and low-density lipoproteins in animal studies (Muyonga, Andabati, & Ssepuuya, 2013).

Many researchers have tried to develop bakery products using amaranth flour alone or in combination with other grains like oats, buckwheat, chickpea etc. Mixing of raw and popped amaranth grain flours has also been tried for making bread and cookies (de la Barca et al., 2010). Popping improves rheological properties of the dough (Zapotoczny, Markowski, Majewska, Ratajski, & Konopko, 2006). An increase in total flavonoid content and antioxidant activity in popped grains has also been shown in studies.

In this study, raw amaranth flour was mixed with popped amaranth flour, pearl millet flour and chickpea flour and composite flour was developed. Pearl millet flour and chickpea flour were chosen on the basis of previous literature and preliminary studies. Organoleptically acceptable cookies were made and subjected to analysis of total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity by DPHH and FRAP assays.

2 MATERIALS AND METHODS

Whole grains such as amaranth, pearl millet, chick pea and popped amaranth grains and other ingredients like curd, sugar, shortening, coco powder, flavours, baking powder and chocolate chips were purchased from local market. Grains were washed and dried at 60° C in a tray drier for 3-4 hours. After cooling they were ground in a domestic stone mill to obtain flour which was sieved through 60 mesh sieve. Flours were stored in air tight containers and stored at less than 25°C with all other ingredients.

2.1 Preparation of composite flour

Using mixture experiment design approach, the composite flour (CF) was standardized by mixing raw amaranth flour, popped amaranth flour, pearl millet flour and chickpea flour in different proportions. Cookies were made with them and best combinations were selected on the basis of spread factor, hardness and sensory scores. Finally, cookies were prepared from this flour for further analysis. The results and details of this methodology will be discussed in a separate paper.

2.2 Preparation of cookies

Cookies were prepared by the method of Dubey (2007) with minor modifications. Shortening and sugar were creamed till white and fluffy. Curd, liquid glucose, ammonium bicarbonate, sodium bicarbonate, salt and flavours were mixed in water and the added to the mixture. It was creamed for five more minutes. Flour, baking powder and coco powder were mixed and added to the cream mixture and kneaded lightly to make soft dough. Dough was divided and rolled into small cookie balls and put in a baking tray. Chocolate chips were sprinkled on the balls and cookies baked at 170°C for 20 minutes. Cookies were cooled on a wire rack and packed in LDPE pouches and stored.

2.3 Total Phenolic content

For estimation of total phenol content (TPC), sample was ground to a fine powder from which 0.5g was taken and dissolved in 50 ml deionized water. After 1 hour, it was filtered through Whatmann 4 and centrifuged at 2000 g. The supernatant was used as sample extract (10mg/ml) for both cookies and composite flour.

TPC was estimated by Folin-Ciocalteau method using methods described by Georgé, Brat, Alter, & Amiot (2005) with modifications. 1 ml of sample and standard Gallic acid (0, 20, 40, 60, 80, 100, 120 µg/ml) were taken in the test tubes and the volume was made up to 5 ml in each test tube with distilled water. 0.5 ml of Folin-Ciocalteau reagent was mixed and shaken. After 5 minutes, 1.5 ml of 20% sodium carbonate was added and volume made up to 10 ml with distilled water. It was allowed to incubate for 2 hrs at room temperature. After 2 hours intense blue colour was developed. Then the absorbance was measured at 750 nm using spectrophotometer. The assays were performed in duplicates. The calibration curve was plotted using standard Gallic acid. The data for TPC of flour and cookies was expressed as mg of Gallic acid equivalent (GAE).

2.4 Total Flavonoid content

For estimation of flavonoids, sample was ground to a fine powder from which 0.5 g was taken and dissolved in 50 ml of 80% ethanol (v/v in distilled water), filtered through Whatmann 4 and centrifuged at 2000g. The supernatant was used as sample extract (10mg/ml) for both cookies and composite flour.

Total flavonoid content (TFC) was measured by the aluminum chloride colorimetric assay (Eghdami & Sadeghi, 2010) with modifications. 1 ml each of aliquots of both the samples and standard quercetin solution (100, 200, 300, 400, 600, 800, 1000 µg)

were taken in test tubes and 4 ml of distilled water was added to each test tube. 300 µl of 5% $NaNO_2$ was then added to each test tube. After 5 minutes, 300 µl of 10% $AICI_3$ was added. After 1 minute, 2 ml of 1M NaOH was added and volume was made up to 10 ml with distilled water. It was allowed to incubate for 1 hour at room temperature. After incubation absorbance was measured at 510 nm. Standard curve of quercetin was plotted and flavonoid concentration was calculated. TFC was expressed as mg of quercetin equivalent (QE).

2.5 Free radical scavenging activity

The decrease of absorption of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution after addition of sample was measured. 2 ml aliquots of ethanol with different concentrations (2, 4, 6, 8 and 10 mg/ml) of sample extract were positioned into test tubes with a control test tube containing 2 ml ethanol only. 4 ml DPPH solution was added to all the test tubes. The mixture was vortexed and then incubated for 30 minutes. Absorbance of sample and control was measured at 517 nm (Brand-Williams, Cuvelier, & Berset, 1995). The free radical scavenging activity was calculated by the formula:

$$\%\ Scavenging\ activity = \frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control} \times 10$$

2.6 In vitro antioxidant assay (FRAP)

For measuring anti oxidant activity of samples by FRAP (Ferric Reducing Ability of Plasma), 10 ml solutions of distilled water with different concentrations (50, 100, 150, 200, 250, 300, 350 µg) of gallic acid and sample extracts were prepared in test tubes. To these, 1 ml of 0.2 M phosphate buffer and 1 ml of 1% potassium ferricyanide was added. The mixture was incubated at 50°C for 30 minutes. Reaction mixture was rapidly cooled and then 2.5 ml of 10% trichloroacetic acid was added to stop the reaction. The solutions were centrifuged at 2000g for 10 minutes. From the supernatants, 2.5 ml aliquots were pipetted out of this and positioned into test tubes. 2.5 ml water and 0.5 ml of 1% ferric chloride solution was added. The colour changed to green. The mixture was allowed to stand for 10 minutes and then absorbance was measured at 593nm. The results were recorded in terms of mg gallic acid equivalent per 100g of sample (Chlopicka, Pasko, Gorinstein, Jedryas, & Zagrodzki, 2012)

2.7 Statistical analysis

Correlation coefficients were calculated and regression equations were predicted for all the parameters. Mean ± s.d. values were calculated.

III RESULTS AND DISCUSSION

3.1 TOTAL PHENOLIC CONTENT

Standard curve was plotted between the concentration of standard Gallic acid (200µg/ml) and slope was calculated Fig. 1. The concentrations and absorbance showed a positive correlation (r^2=0.997). TPC results of CF and cookies are given in Table 3.1.

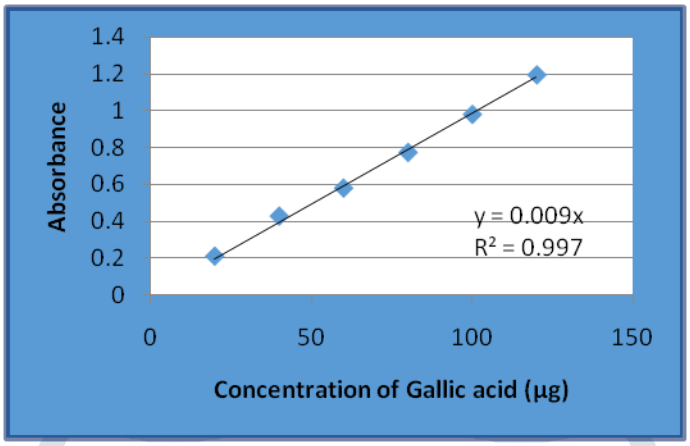

Fig. 1 Standard curve between concentration of gallic acid (µg) and absorbance

Table3.1 Total polyphenol Content (mg GAE/100g) of composite flour and cookies

Sample	TPC (mg of GAE)/100g
Composite flour	277.22±30.64
Cookies	315.56±87.9

Folin-Ciocalteau (FC) method is based on the chemical reduction of the reagent, which is a mixture of molybdenum and tungsten. The reduction gives a product exhibiting intense blue colour with a λ_{max} at 765nm. The intensity of the colour is directly proportional to the presence of phenols in the mixture (Waterhouse AL, 2002).

TPC of composite flour and cookies is found to be 277.22mgGAE/100g and 315.56mgGAE/100g respectively. TPC of amaranth flour has been reported as GAE 271mg/100g (Chlopicka et al., 2012), 43 mg/100g (Gorinstein et al., 2007) and 363 mg/100g (Muyonga et al., 2013). It can be seen that there is a huge variation in the reported values of TPC. This could be due to the solvent mixture used for phenol extraction. Researchers have used a wide variety of solvents like distilled water, ethanol, methanol and methanol-HCl; there are also variations in the technique like the time and speed of centrifugation, temperature of solvents etc. TPC of pearl millet has been reported to be 278 mg/100g (Elyas, El Tinay, Yousif, & Elsheikh, 2002) and that of chickpea has been found to be 18.3.mg/100g in one study (Fratianni et al., 2014). Thus, TPC of CF is close to the values of amaranth and pearl millet reported by others.

TPC of cookies is higher than CF which could be due to several reasons. There could have been a release of bound phenolics due to heat (Jannat et al., 2010). However, reduction or no change in TPC is also reported by others (Muyonga et al., 2013). Thus there are conflicting reports on this. Another difference between CF and cookies is the presence of coco powder in the latter. Coco powder is rich in polyphenols like theaflavin, epigallocatechin gallate and resveratrol. It has been found to have GAE of 611mg/100g; more than tea and red wine (Lee, Kim, Lee, & Lee, 2003). Thus it must have contributed to the TPC in cookies. Lastly, it can be speculated that sugars present in cookies might have interfered with the TPC test as Folin's method is sensitive to sugars (Waterhouse AL, 2002), this could have shown a little higher value of TPC in cookies as compared to CF.

However, the results show that both CF and cookies developed in this study have got good TPC which would result in some antioxidant activity in the product.

3.2 Total Flavonoid Content

Standard curve was plotted between the concentration of standard quercetin solution (200µg/ml) and slope was calculated (Fig. *2*). Quercetin concentration showed positive correlation with the absorbance (r^2=0.891). The results are presented in Table 3.2..

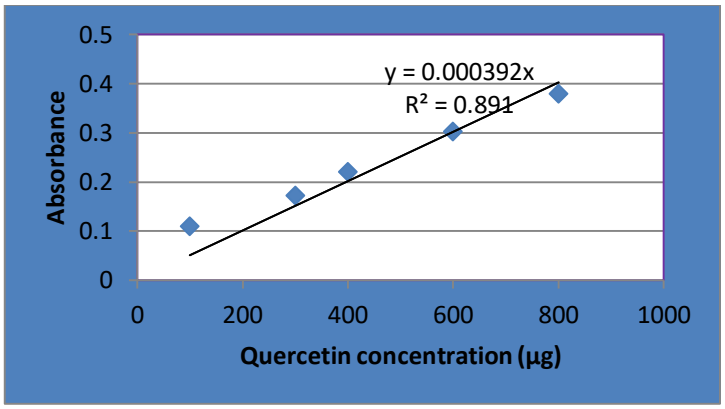

Fig. 2 Standard curve between quercetin concentration(µg) and absorbance

Table 3.2. Total flavonoid content (mg QE/100g) of composite flour and cookies

Sample	TFC (mg of QE)/100g
Composite flour	312.50±17.67
Cookies	250±35.5

$AlCl_3$ forms acid stable complexes with keto and hydroxyl groups of flavones and flavonols. Also, it makes acid labile complexes with the orthodihydroxyl groups of flavonoids (Chang, Yang, Wen, & Chern, 2002). The absorbance is measured which is directly proportional to the amount of flavonoids in the solution.

The flavonoid content of CF is found to be 312.5mg QE/100g. The flavonoid content in cookies is 250mg/100g. Flavonoid content of amaranth flour has been reported as 6.5 mg/100g (Chlopicka et al., 2012), 75mg/100g (Gorinstein et al., 2007), 67.6 mg/100g (Paśko, Sajewicz, Gorinstein, & Zachwieja, 2008). Again, there are variations in the flavonoid content analysed in different studies. This depends on the cultivar also. In some studies, researchers were not even able to detect flavonoids, however, this cannot be interpreted as absence of flavonoids.

Flavonoid content of pearl millet has been reported as 172.1-248.3 mg catechin equivalents/100g (Siroha, Sandhu, & Kaur, 2016). Flavonoid content of Chickpea was found to be 250 mg CE/100g to 450 mg CE/100g (Segev et al., 2011)
Although, studies have not shown very high content of flavonoids in amaranth, the composite flour contains pearl millet as well as chickpea flour which could have contributed to its high flavonoid content. In CF, TFC was found to be higher than TPC. This is not possible theoretically. However, sometimes, Folin's method does not completely reflect the TPC of a sample; this can lead to lower TPC values. Such results have been reported by (Gorinstein et al., 2007).
TFC of cookies is quite high (250mg QE/100G). It has been seen that heating process increases flavonoid content. As explained by (Muyonga et al., 2013), 'This was attributed to enhanced extractability of bound flavonoid compounds resulting from heat-induced disruption of the plant cell wall'. Also, in cookies, coco powder must have contributed to the TFC. Flavonoid content of coco powder was reported as 564 mg epicatechin equivalents/100g (Lee et al., 2003). These flavonoids could be contributing to antioxidant activity of cookies.

3.3 Free radical scavenging activity

Free radical scavenging activity (FRSA) was estimated using DPPH (radical scavenging assay. 1,1-diphenyl-2-picryl picrylhydrazyl (α,α-diphenyl-β-picrylhydrazyl (DPPH) is a stable free radical with a delocalization of an electron over the whole molecule. It does not form dimers as other free radicals form. In this form it has a deep violet colour. When it is mixed with a substrate which can donate a hydrogen atom, it gets reduced and turns colourless in reduced form. The substrates which can donate a hydrogen atom are said to have an antioxidant activity (Molyneux, 2004). Substances such as phenols and flavonoids have a strong antioxidant or free radical scavenging activity. The activity is measured by measuring the absorbance of a control DPPH solution and solutions of DPPH with different concentrations of substrate at 517 nm. EC_{50} (Efficient Concentration) value is calculated which is defined as the amount of sample required to decrease the absorbance of DPPH by 50%. In this study, a standard curve was plotted between sample concentrations and % reduction in DPPH absorbance. The concentrations and absorbance values correlated well.

The correlation between concentration and absorbance was negative, because absorbance decreases as substrate concentration increases. % scavenging activity was calculated and it was also plotted against concentration. Using regression equations, the data was extrapolated and EC_{50} value was calculated as 22.78 mg of composite flour extract and 12.99 mg/ml of cookie extract.

The results are presented in Table and Table and graphs are shown in Fig. 3 and Fig. 4

Table 3.3 Free radical scavenging activity of composite flour extract at different concentrations

Concentration of CF extract (mg/ml)	**% scavenging activity**
2	13.11
4	16.10
6	18.73
8	24.34
10	26.97
22.78	EC_{50}

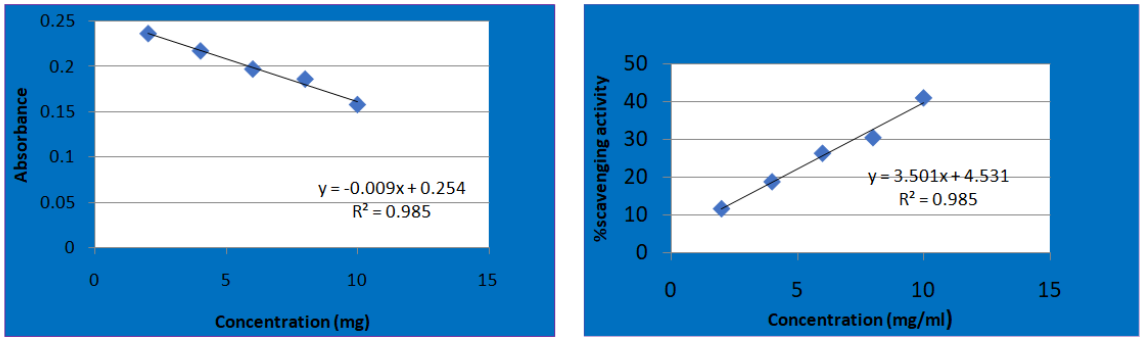

Fig. 3 Graphical representation showing negative correlation between concentration of CF extract and absorbance of DPPH (left) and positive correlation between concentration and % scavenging activity

Table 3.4 Free radical scavenging activity of cookie extract at different concentrations

Concentration of cookie extract (mg/ml)	% scavenging activity
2	11.61
4	18.73
6	26.22
8	30.34
10	40.82
12.99	EC_{50}

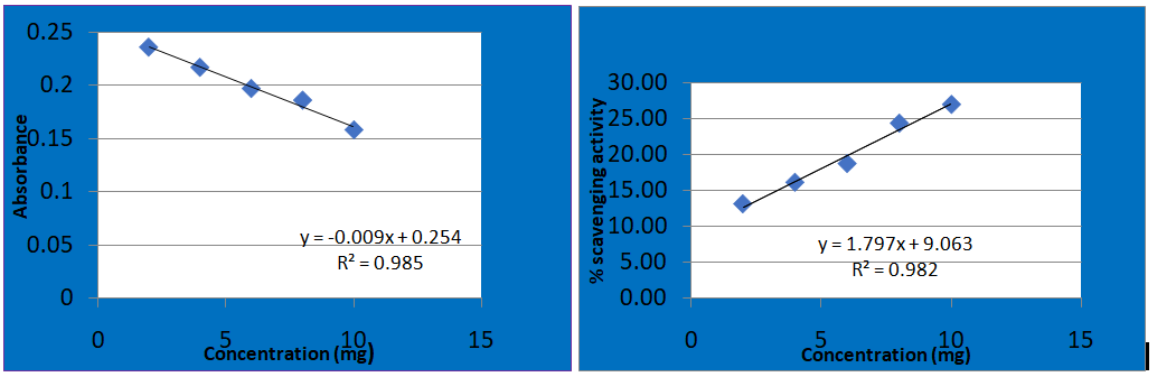

Fig. 4 Graphical representation showing negative correlation between concentration of cookie extract and absorbance of DPPH (left) and positive correlation between concentration and % scavenging activity

Free radical scavenging activity of cookies has been found to be more than flour. This could be because of the effect of heating on antioxidant activity (Devi, Vijayabharathi, Sathyabama, Malleshi, & Priyadarisini, 2014), (Muyonga et al., 2013), also due to higher TPC found in cookies. Also, flavonoid content of cookies is also good, though lower than in CF. Thus; DPPH assay shows that the CF and cookies both show some antioxidant activity which can be further studied using more concentrations and different sample extraction methods.

3.4 In vitro antioxidant assay (FRAP)

Another method to determine antioxidant activity was by FRAP and was expressed as mg of gallic acid per 100g dry weight. A standard curve was plotted between gallic acid concentrations and absorbance at 593 nm. There was a high positive correlation between concentrations and absorbance (r^2= 0.994). Concentration of sample was interpreted using the graph and ferric reducing power was calculated and expressed as mg gallic acid per 100 g. The FRAP results are presented in Table 3.5. Standard curve is shown in Fig. 5.

Table 3.5 Antioxidant activity by FRAP (mg GAE/100g)

Sample	FRAP (mg gallic acid equivalent/100g
Composite flour	151±16.0
Cookies	204±0.00

FRAP of amaranth flour and bread have been reported to be 38.6 mg Trolox equivalents/100g and 114.1 Trolox equivalents mg/100g respectively (Chlopicka et al., 2012). Composite flour in the present study has been found to have a FRAP of 151 mg GAE/ 100g which is quite high. Cookies have slightly higher reducing power of 204mg GAE/100g. Here again we find higher reducing power or antioxidant capacity of cookies than flour. 'FRAP reaction involves a single electron or hydrogen transfer mechanisms and functional groups in phenolic compounds play a crucial role in reducing the oxidized intermediates of peroxidation' (Sreerama, Sashikala, & Pratape, 2012).

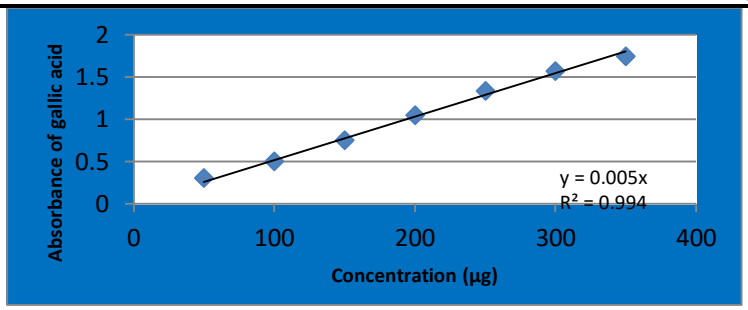

Fig. 5 Standard curve plotted between concentration of gallic acid (µg) and absorbance

Since the cookies have been found to have higher TPC, this could be the reason for higher reducing power in cookies. Cookies have also shown higher DPPH activity, thus the results are showing good correlation. Composite flour has also got good antioxidant capacity. In case of cookies, there are small chances of sugar having shown reducing power, since some amounts of reducing sugars are present in them.

IV CONCLUSION

Thus, we find that composite flour and cookies had very good TPC and TFC. Both have shown antioxidant capacity as shown by DPPH and FRAP assay. It is well known that flavonoids, polyphenolic substances and substrates showing antioxidant capacity are good for our health as they protect our body from free radical reactions which may lead to problems like diabetes, hypercholesterolemia, cancer etc. Normally, in products made of refined wheat flour, we do not get enough nutrients, in this case, polyphenols. But the flour and product in the present study have shown good antioxidant ability. The flour can be used in different preparations to increase phenolic content and flavonoid content of the diet. Cookies can be eaten as such. This will add to the nutritive value of our diet. Also, the study shows that amaranth based multi grain flours can provide us with adequate amount of polyphenols and flavonoids which have a protective effect on our body. In the current scenario of increasing lifestyle disorders, it is relevant to replace refined wheat flour with these grains. Also, in gluten free diets for celiacs, amaranth can be very effective in adding to the overall nutrient content.

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