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Effects of Harvesting Period on Phytochemicals of Wheatgrass (*Triticum aestivum*, WK 1204 Variety)

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Abstract

Wheatgrass is a plethora of essential phytochemicals considered to exhibit numerous benefits on human health. Therefore, the study of phytochemicals with varying stages of growth is essential. This study aims to determine the optimum harvesting period of wheatgrass based on its phytochemical content. During the growth of wheat seed (WK 1204 variety), the phytochemicals such as chlorophyll, total phenol content, flavonoids and tannins were extracted by 80% acetone and 80% methanol from wheatgrass harvested on days 6, 7, 8, 9 12 and 15, respectively. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, chlorophyll and flavonoid contents significantly ($p < 0.05$) increased and reached the maximum level on day 9. Total phenol content was found to be increasing significantly ($p < 0.05$), while the tannin content was decreasing considerably ($p < 0.05$) on day 15. The optimum harvesting period was found on day 9. The DPPH radical scavenging activity, chlorophyll and flavonoid content significantly ($p < 0.05$) increased and reached the maximum level, i.e., 92.27 ± 1.92%, 6.63 ± 0.053 mg g^{-1} and 183.64 ± 33.49 mg QE g^{-1}, respectively, on day 9 of cultivation. The total phenol content was found to be increasing significantly ($p < 0.05$) from 291.67 ± 5.69 mg GAE g^{-1} on day 6 to 446.67 ± 5.77 mg GAE g^{-1} on day 15, while the tannin content was declining significantly ($p < 0.05$) from 11.74 ± 0.29 mg GAE g^{-1} on days 6, 7 and so on to 3.36 ± 0.47 mg GAE g^{-1} on day 15. Therefore, the optimum harvesting period of wheatgrass was found to be day 9 in terms of phytochemical analysis.

Keywords: cereal grass; DPPH scavenging activity; harvesting period; phytochemicals

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INTRODUCTION

Cereal grasses are gaining popularity due to the presence of antioxidants, chlorophylls, bioactive compounds, enzymes and vitamins, and their use in the preparation of a healthy beverage (Mujoriya and Bodla, 2011). Wheatgrass is taken as a shoot of *Triticum aestivum* obtained from the cotyledons of the common wheat plant belonging to the Gramineae family. *Triticum* is a genus of annual and biennial grasses, yielding various types of wheat and is found in almost all parts of the world (Mogra and Rathi, 2013). *Triticum aestivum* is mentioned as the herbal system of medicine in Ayurveda and is known for its immunomodulatory, antioxidant, astringent, laxative, diuretic and antibacterial effects (Ashok, 2011). Wheatgrass can be freshly juiced or powdered after harvesting and consumed by both humans and animals to reap its extraordinary benefits. Because of its high chlorophyll content,

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it is even referred to as "green blood" (Gore et al., 2017).

Wheatgrass juice is an abundant source of essential vitamins, particularly vitamins A, C, E and B complex. It is rich in minerals such as calcium, phosphorus, magnesium, alkaline earth metals, potassium, zinc, boron and molybdenum (Aydos et al., 2011). Several enzymes, including protease, amylase, lipase, cytochrome oxidase, transhydrogenase and superoxide dismutase, are involved in their pharmacological actions (Padalia et al., 2010). The antioxidant activity of the wheatgrass could be attributed to the presence of bioflavonoids such as apigenin, quercetin and luteolin. Indole compounds such as choline and laetrile also play an important role in wheatgrass's therapeutic activity (Kulkarni et al., 2006). The variety WK 1204, obtained for this study from the Agriculture Botany Division, Nepal Agricultural Research Council (NARC), Khumaltar, Kathmandu, Nepal, is best suited for cultivation in the mid and high hills. It has a strong plant structure with elongated spikes and waxy semi-erect leaves. The grains are medium in size and oval in shape, and they can be stored for a long time (Joshi et al., 2017).

Chlorophyll is a green pigment found in most plants that gets its name from the Greek words *chloros* (green) and *phyllon* (leaf) (Inanc, 2011). Both compounds have a tetra-pyrrole ring structure, with the only difference being the nature of the central metal atom–magnesium (Mg) in chlorophyll and iron (Fe) in hemoglobin (Padalia et al., 2010), which is why wheatgrass is referred to as "green blood". Because the pH factor of human blood and wheatgrass juice is 7.4, wheatgrass juice is quickly absorbed into the blood (Kumar et al., 2016).

Phenolic compounds are considered a major group of semi-water soluble compounds with one or more benzene rings normally found in fruits and vegetables. Such compounds are thought to be a diverse, bioactive and pervasive category of plant secondary metabolites with numerous health benefits. Phenolic compounds play a major role in decreasing the incidence of cardiovascular diseases, colon cancer, liver disorders, obesity and diabetes (Rasouli et al., 2017). Flavonoids are an important class of natural products belonging to a class of plant secondary metabolites with a polyphenol structure, widely found in plants, and thus playing a variety of biological activities in plants and animals. They have been classified into different subgroups i.e. chalcones, flavones, flavonols and isoflavones (Panche et al., 2016).

Tannins are galloyl esters or oligomeric and polymeric proanthocyanidins developed by the secondary metabolism of plants synthesized by biogenetic pathways. They are major poly-phenolic secondary metabolites distributed widely in the range of 5 to 10% of dry vascular plant materials and are found mainly in bark, stems, seeds, roots, buds and leaves. They are broadly classified into 2 major groups i.e. hydrolyzable tannins and condensed tannins (Das et al., 2020). According to research conducted by Li et al. (2014), free radicals have been the reasons behind the tremendous impact on human health. To scavenge superfluous free radicals and maintain the balance of homeostasis in the human body as well as to assure the prevention and treatment of diseases, the consumption of antioxidants has become essential. Antioxidants are compounds that are capable of neutralizing the excess of free radicals, protecting the cells against their toxic effects and contributing to disease prevention.

A lack of nutrient antioxidants is one of the causes of several chronic and degenerative diseases. Consumption of foods rich in such bioactive compounds, such as chlorophyll and polyphenols, including flavonoids and tannins, exhibits a great deal of antioxidant activity in the human body. The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical method is an antioxidant assay that uses electron transfer to produce a violet solution that is stable at room temperature. In the presence of an antioxidant molecule, DPPH is reduced, resulting in a colorless solution. The DPPH assay is a simple and quick way to evaluate antioxidants using spectrophotometry (Garcia et al., 2012).

Changes in the phytochemical content of wheatgrass were deemed necessary in this case to optimize the wheatgrass harvesting period. According to Ghimire et al. (2021), the significant correlation between phenolic compounds, mineral content and antioxidant activity could be found in different periods of harvest on *Atractylodes japonica.* Similarly, significant effects of wheatgrass lengths on antioxidant and phenolic content of wheatgrass have been found according to research carried out by Agrawal et al. (2015). Research conducted by Kaur et al. (2021) also suggested that

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nutritional composition and antioxidant activity are significantly affected by growing conditions.

Wheatgrass has been widely recognized as a miraculous food with potential health benefits. However, the potential health benefits of wheatgrass are still unknown for a large number of people in Nepal. Only a few pieces of research have been conducted in Nepal in terms of variety selection, soil conditions, growing conditions, harvesting periods, post-harvest processing and evaluation of phytochemicals present on wheatgrass by varying their harvesting period. Therefore, a detailed study of the wheatgrass WK 1204 variety was deemed relevant in the context of Nepal and has the potential to contribute to agricultural sustainability through crop quality improvement (Adhikari et al., 2018). This study covered cultivation practices, post-harvest handling and phytochemical evaluation in the context of Nepal, and it will pave the way for other types of research in the future.

MATERIALS AND METHOD

Materials

Wheat seeds (*Triticum aestivum*) of the WK 1204 variety were obtained from NARC, Khumaltar, Kathmandu, Nepal. This research was conducted at the Central Campus of Technology, Tribhuvan University, Dharan, Nepal in April 2018.

The DPPH (97%), gallic acid (98%) and quercetin (99.5%) were purchased from HiMedia, India; Folin-Ciocalteau reagent (2N) and acetic acid (99.5%) from Thermo Fischer Scientific, India; sodium carbonate (99.5%) and sodium nitrate (99%) from Qualigens Fine Chemicals, India; and methanol (99%), chloroform (99%) and acetone (99%) from Merck, India.

Cultivation of wheat seeds

The cultivation of wheat seeds (*Triticum aestivum*) of WK 1204 variety was carried out by soil cultivation method according to Wigmore (1985). Wheat seeds were washed, soaked for 12 hours and sprouted for another 12 hours by wrapping them in a moist muslin cloth. Finally, the sprouted wheat seeds were sown in soil-filled trays and then covered. The trays were uncovered after 2 days, held in indirect light and watered daily at every 8 hours. The grasses were harvested on days 6, 7, 8, 9, 12 and day 15 after sowing.

Experimental design

The experimental design was based on variations in wheatgrass harvesting days. The grasses were harvested on days 6, 7, 8, 9, 12 and day 15 after sowing. These were then collected for further analysis. The analysis was carried out in triplicate for each sample.

Preparation of wheatgrass extract

The extracts were prepared by using the maceration technique, as described by Ahmad et al. (2014) with slight modifications. In a nutshell, 10 g of wheatgrass shoots were properly crushed with a mortar and pestle, steeped in 100 ml of 80% methanol for 12 hours at room temperature near 20 to 25°C, and filtered through Whatman No. 41 filter paper. The filtrate was transferred to 250 ml brown (amber) glass bottles, sealed and stored at 4 ± 2°C.

Phytochemical screening of the extracts

The phytochemical contents of the extract were qualitatively analyzed according to the standard procedure as described by Jaradat et al. (2015).

Phenols and tannins

Two milliliters of 2% solution of $FeCl_3$ were mixed with the crude extract. Black or blue-green color indicated the presence of tannins and phenols.

Flavonoids

Shinoda test: after pieces of magnesium ribbon and concentrated HCl were mixed with crude plant extract for a few minutes, a pink-colored scarlet appeared, indicating the presence of flavonoids.

Alkaline reagent test: when 2 ml of 2% NaOH solution was mixed with plant crude extract, an intense yellow color was formed, which turned colorless when 2 drops of diluted acid were added to the solution, indicating the presence of flavonoids.

Saponins

Five milliliters of distilled water were added to the crude plant extract in a test tube and shaken vigorously. The lack of foam formation indicated the absence of saponins.

Glycosides

Liebermann's test: 2 ml of acetic acid and 2 ml of chloroform were mixed with entire plant crude extract. The mixture was then cooled and added with concentrated H_2SO_4. The green color

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indicated the entity of the aglycone steroidal part of glycosides.

Salkowski's test: a concentrated H_2SO_4 (approximately 2 ml) was added to the entire plant crude extract. A reddish-brown color indicated the entity of steroidal aglycone part of the glycoside.

Keller-kilani test: a mixture of acetic acid glacial (2 ml) with 2 drops of 2% $FeCl_3$ solution was added to the plant extract and concentrated H_2SO_4. A brown ring was produced between the layers, which indicated the entity of cardiac steroidal glycosides.

Steroids

Two milliliters of chloroform and concentrated H_2SO_4 were mixed with the entire plant crude extract. In the lower chloroform layer, red color was produced, indicating the presence of steroids. Another test was performed by mixing 2 ml of each acetic acid with concentrated H_2SO_4 and crude extract with 2 ml of chloroform. The green color indicated the entity of steroids.

Terpenoids

Two milliliters of chloroform were mixed with the plant extract and evaporated on the water path, and then boiled with 2 ml of concentrated H_2SO_4. A grey color indicated the entity of terpenoids.

Quantitative analysis of photochemical

Determination of total phenol content

Determination of total phenol content in wheatgrass extract was carried out with Folin-Ciocalteau reagent as described by Waterhouse (2002) with slight modifications. Briefly, 2.5 ml of 10% Folin-Ciocalteau reagent and 2.5 ml of 7.5% sodium carbonate were added to 0.5 ml of methanolic extract. It was then incubated at 45°C for 45 minutes and finally, absorbance was measured in triplicates at 765 nm using UV-Vis single-beam spectrophotometer (LT-291). Total phenol values were calculated using the standard curve equation and expressed in terms of gallic acid equivalent i.e., mg GAE g^{-1} of dry mass.

Determination of flavonoid content

Total flavonoid content was determined using the aluminum chloride assay method as described by Barek et al. (2015) with minor modifications. In brief, 0.2 ml of 5% sodium nitrate was added to 2 ml of the extract solution and stood for 5 minutes. 0.2 ml of 5% aluminum chloride was added and stood for 5 minutes. Two milliliters of 1N sodium hydroxide was then added and the final volume was made up to 5 ml with distilled water. It was later incubated for 15 minutes at room temperature (20 to 25°C) and finally, absorbance was measured at 510 nm in UV-Vis single-beam spectrophotometer (LT-291). Flavonoid content was expressed in terms of quercetin equivalent i.e., mg QE g^{-1} of dry mass.

Determination of tannin content

Tannin was determined using the method as proposed by Mythili et al. (2014) with slight modifications. 7.5 ml of distilled water, 0.5 ml of 10% Folin-Ciocalteau reagent and 1 ml of 35% sodium carbonate were added to 0.1 ml of extract. The final volume was made up to 10 ml with distilled water and mixed well. It was kept for 30 minutes at room temperature (20 to 25°C). The absorbance was measured at 725 nm using UV-Vis single-beam spectrophotometer (LT-291). Tannin content was expressed in terms of (+)-catechin equivalent i.e., mg (+)-catechin g^{-1} of dry mass.

Determination of DPPH free radical scavenging activity

Free radical scavenging activity was determined using the method as defined by Vignoli et al. (2011) with minor modifications. Concisely, 2 ml of 0.1 ml methanolic DPPH solution was added to 1 ml of the extract. It was then incubated in the dark for 30 minutes. The absorbance was measured at 517 nm against control (1 ml of 80% methanol + 2 ml of 0.1 mM methanolic DPPH solution) in UV-Vis single-beam spectrophotometer (LT-291). Percentage scavenging activity was calculated with the following formula Equation (1).

$$\% \text{ DPPH radical scavenging activity} = \left(\frac{A_c - A_s}{A_c}\right) \times 100 \quad (1)$$

Where, A_c is absorbance of control and A_s is absorbance of sample

Determination of chlorophyll

Chlorophyll content was determined as per the standard procedure given by Ranganna (1986). The extract was prepared using 80% acetone. The wheatgrass sample was cut into small pieces. A total of 5.0 g of sample was taken and ground

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to a fine pulp in mortar and pestle with about 10 ml of 80% acetone. The pulp was centrifuged at 5000 rpm for 5 minutes. The green supernatant was transferred to a 50 ml volumetric flask. The sediment was scraped in the centrifuge tube and ground again in the same mortar and pestle with a small amount of 80% acetone to extract the residual chlorophyll. The mixture was again centrifuged as done earlier and the extract was pooled in the 50 ml volumetric flask (containing the previous supernatant). The extraction process was repeated until no perceptible green color in the residue was observed. The volume was made up to 50 ml by adding 80% acetone. The extract was held in the refrigerator for 10 minutes to lower its temperature. The absorbances of the extracts were measured in a spectrophotometer at 663 and 645 nm using 80% acetone as the blank. The calculations were carried out using the empirical formula Equations (2 and 3).

$$\text{Chl b}, \frac{\text{mg}}{\text{g}}\text{ tissue} = \big(22.9(\text{A645})\text{-}4.68(\text{A663})\big) \times \frac{\text{V}}{1000 \times \text{W}} \quad (2)$$

$$\text{Total chlorophyll}, \frac{\text{mg}}{\text{g}}\text{ tissue} = \text{chl a+chl b (Calculated above)} \quad (3)$$

Where, A is absorbance at specific wavelengths; V is final volume of chlorophyll extract; W is fresh weight of tissue extracted

Statistical analysis

The data were analyzed by using IBM SPSS Statistics (Version 26) predictive analytics software, for Analysis of Variance (ANOVA) at a 5% level of significance. The obtained data were subjected to one-way ANOVA and the significance of the means was tested by using Tukey's honest significant difference (HSD) method.

RESULTS AND DISCUSSION

The harvesting period of wheatgrass was optimized by measuring phytochemicals (total phenol content, tannin, flavonoid and chlorophyll) and DPPH radical scavenging activity, qualitatively and quantitatively.

Qualitative analysis for phytochemicals

The analysis of a methanolic extract of wheatgrass revealed the presence of phenols, flavonoids, tannins, glycosides, steroids and terpenoids whereas saponins were found to be absent. Similar results for preliminary phytochemical analysis were found in the research carried out by Suryavanthana et al. (2016), where extraction was carried out in different solvents like aqueous, methanol, ethyl acetate, chloroform and relevant phytochemicals were characterized in wheatgrass. The presence and absence of different phytochemicals during preliminary analysis depends on the plant biochemistry as well as the nature of the extraction solvent. Hence, from the preliminary phytochemical analysis, it was discovered that wheatgrass is a natural food consisting of a wide range of essential phytonutrients and has an extensive benefit on human health.

Quantitative analysis of phytochemicals

Quantitative analysis of phytochemicals was carried out for chlorophyll, total phenol, flavonoids, tannins content and DPPH radical scavenging activity.

The chlorophyll contents of the extracts from wheatgrass harvested on days 6, 7, 8, 9, 12 and 15 were found to be increasing in order, reaching a maximum level (16.26 ± 0.076 mg g^{-1}) on day 9, then decreasing to 13.36 ± 0.355 mg g^{-1} on day 15 (Figure 1). Statistically, there was a significant difference ($p < 0.05$) in chlorophyll content of the extracts of wheatgrass harvested on days 6, 7, 9 and 15, whereas it was not significantly different ($p > 0.05$) on days 8 and 12. A similar result was observed by Murali et al. (2016), where the optimum chlorophyll was obtained on day 9. Kohler (1944) has suggested that chlorophyll reaches a peak concentration at or near the jointing stage when the leaf blades and sheaths entirely make up the aerial part of the plant. As the seedling grows and the leaves unfold, more and more surface areas get exposed to sunlight, allowing it to engage in photosynthetic activity. Similarly, the research carried out by Anwar et al. (2015) and Özköse et al. (2016) confirmed the reduced value of chlorophyll content of wheatgrass in the second cut as compared to the first cut when grown under laboratory conditions.

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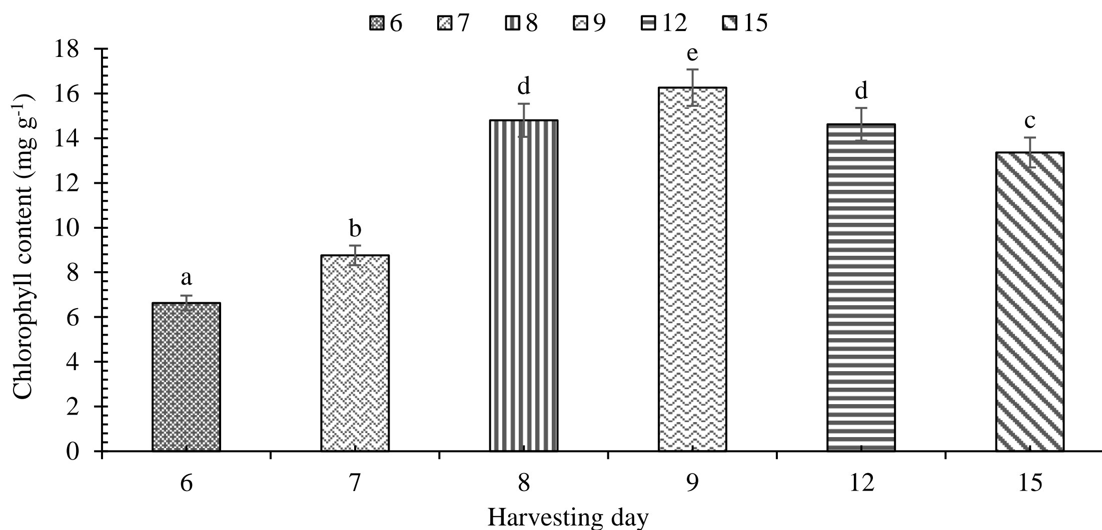

Figure 1. The effects of growing days on the chlorophyll content

Note: The values in the figure are the mean values for the parameters in terms of their growth days. Values on top of the bar with a similar alphabet are not significantly different at a 5% level of significance. Vertical error bars represent the standard deviation of the values.

The total phenol content was found to be increasing along with the progress in the growth of wheatgrass. The total phenol contents of extracts from wheatgrass harvested on days 12 and 15 were 421.33 ± 4.16, 446.67 ± 5.77 mg GAE g^{-1} respectively (Figure 2). The total phenol contents among all the six harvested days were found to be significantly different ($p < 0.05$). Similarly, the research conducted by Durairaj et al. (2014) confirmed the presence of total phenolic and flavonoid components in aqueous extract of wheatgrass by carrying out GC-MS analysis. A similar result was obtained by Kulkarni et al. (2006) in a study of an aqueous extract of wheatgrass. Chalorcharoenying et al. (2017) also reported that total phenolic content which was low at the sprout stage in corn was found exceptionally high and significant at the seedling stage. Research carried out by Saini et al. (2017) strengthened the significant increment in total phenol content along with its growth period. Correspondingly, Kim et al. (2018) signposted that a longer germination period resulted in the generation of higher levels of phenolic acids (gallic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid and p-coumaric acid).

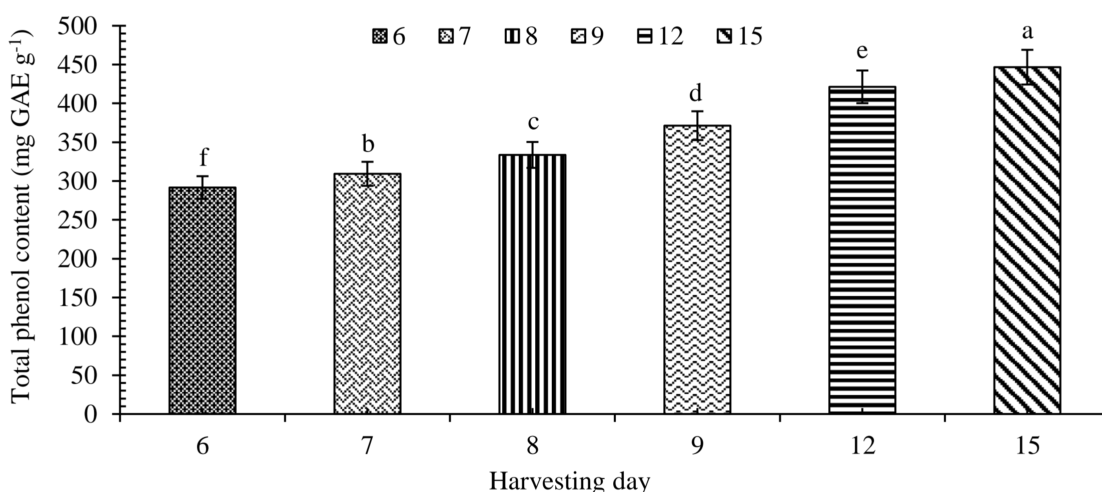

Figure 2. The effects of growing days on the total phenol content

Note: The values in the figure are the mean values for the parameters in terms of their growth days. Values on top of the bar with a similar alphabet are not significantly different at a 5% level of significance. Vertical error bars represent the standard deviation of the values.

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Total flavonoid contents of the extract from wheatgrass harvested on days 6 (86.98 ± 0.0038 mg QE g^{-1}), 7, 8 and 9 were found to be in increasing order and reached a maximum (183.64 ± 33.49 mg QE g^{-1}) on day 9 (Figure 3). However, the flavonoid content decreased from 173.97 ± 0.002 to 119.60 ± 37.67 mg QE g^{-1} within day 12 to day 15. Statistical analysis showed that the flavonoid content on day 6 was not significantly different ($p > 0.05$) from those on days 7, 8 and 15, but was significantly different ($p < 0.05$) from that on day 9. A substantial difference ($p < 0.05$) was also seen in between the flavonoid content of extract from wheatgrass harvested on days 12 and 15 (Figure 3). The decrease in bioactive compounds after maxima were significant in both wheat and barley by the research carried out by Niroula et al. (2019). According to Kaushal (2017), the flavonoid content of wheatgrass increased until day 9 and started decreasing from day 10, whereas Skoczylas et al. (2017) reported that the flavonoid content on day 15 was found to be decreasing, compared to the samples harvested on day 10.

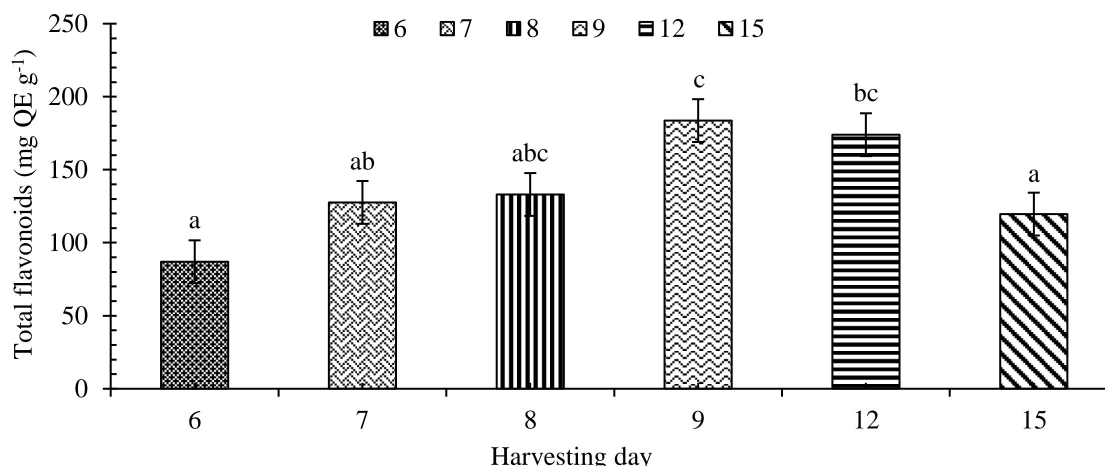

Figure 3. The effects of growing days on total flavonoid content

Note: The values in the figure are the mean values for the parameters in terms of their growing days. Values on top of the bar with a similar alphabet are not significantly different at a 5% level of significance. Vertical error bars represent the standard deviation of the values.

The tannin content of wheatgrass extracts continued to decrease along with the increase in growing days. Tannin content was found to be maximum (13.39 ± 0.71 mg (+)-catechin g^{-1} on day 6, which kept decreasing to 11.74 ± 0.29; 10.66 ± 0.30; 9.65 ± 0.35; 5.53 ± 0.36 and 3.36 ± 0.47 mg (+)-catechin g^{-1} on day 7, 8, 9, 12 and 15 respectively (Figure 4). Statistical analysis revealed that the tannin contents of extracts from wheatgrass harvested on these days were found to be significantly different ($p < 0.05$). Ayet et al. (1997) also reported the degradation of tannins in lentils during the period of germination procedure. Khandelwal et al. (2010) and Pal et al. (2016) also concluded the significant reduction of tannin content in germinated green gram and horse seed grams respectively. Megat-Rusydi and Azrina (2012) confirmed the reduction in the tannin content of soybean and peanut during germination. The hydrophobic association of tannins with seed proteins and enzymes may lead to a reduction in tannin content after germination. Besides, the leaching of tannins into the water may also be the reason for the loss of tannins during germination (Kassegn et al., 2018).

DPPH radical scavenging activity of the extracts from wheatgrass harvested on day 6 (78.88 ± 1.92%) increased to 83.38 ± 1.92% on day 7 and 91.11 ± 1.92% on day 8, and attained the maximum value of 92.27 ± 1.92% on day 9. The value decreased to 87.77 ± 1.92% on day 12 and finally reached 84.46 ± 1.92% on day 15 (Figure 5). Statistically, there was a significant difference ($p < 0.05$) between the DPPH radical scavenging activity of the extracts from wheatgrass harvested on days 6, 7 and 12. On the other hand, DPPH radical scavenging activity between day 8 and day 9, as well as between day 7 and day 15 was not significantly different ($p > 0.05$). Kaushal (2017) reported the maximum DPPH radical

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scavenging activity on wheatgrass harvested on day 9 after which started decreasing from day 10. Similar results were obtained by Skoczylas et al. (2017), where the DPPH activity of the harvested wheatgrass on day 15 was lower than that harvested on day 10.

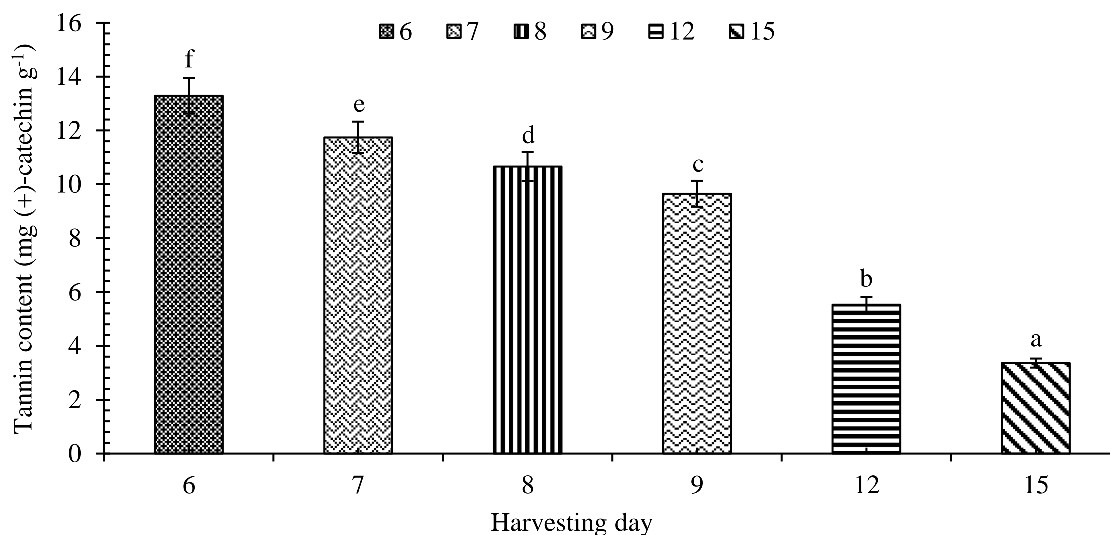

Figure 4. The effects of growing days on the tannin content

Note: The values in the figure are the mean values for the parameters in terms of their growth days. Values on top of the bar with a similar alphabet are not significantly different at a 5% level of significance. Vertical error bars represent the standard deviation of the values.

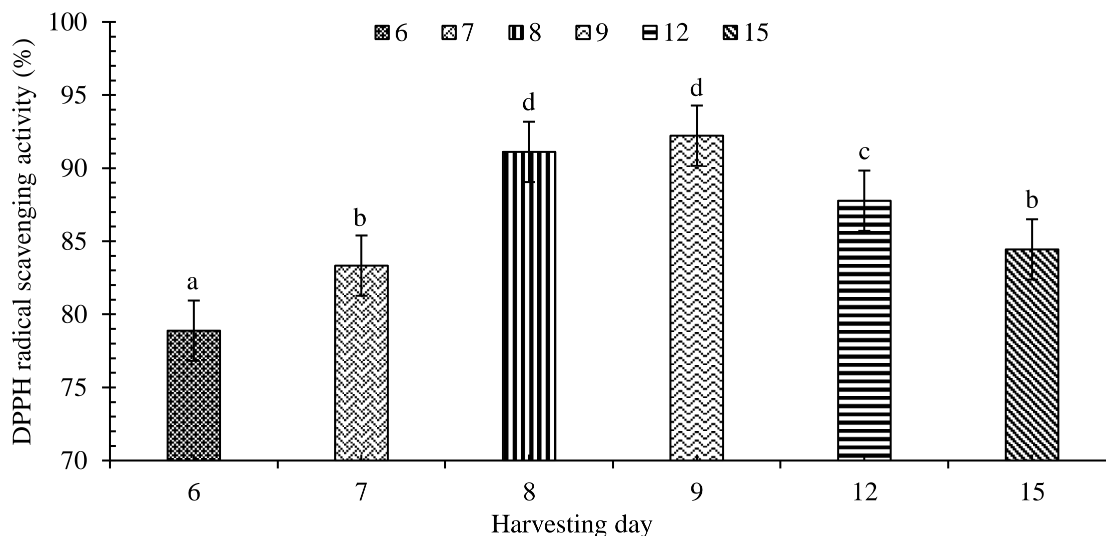

Figure 5. The effects of growing days on the DPPH radical scavenging activity (%)

Note: The values in the figure are the mean values for the parameters in terms of their growth days. Values on top of the bar with a similar alphabet are not significantly different at a 5% level of significance. Vertical error bars represent the standard deviation of the values.

CONCLUSIONS

The chlorophyll, flavonoid content and DPPH radical scavenging activity increased gradually, and reached the maximum results on day 9 and started decreasing onwards. Total phenol content was gradually increasing and reached the maximum value on day 15, whereas tannin content decreased gradually and reached the minimum value on day 15. Hence, wheatgrass harvested on day 9 had higher phytochemical content. Further analysis of phytochemical content during germination of several other types of cereal grass using

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several germination methods, germination period and blend of several types of cereal grass is highly recommended.

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