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COMPARISON OF POLYPHENOLS, FLAVANOIDS, ANTIOXIDANT AND FREE RADICAL SCAVENGING CONTENT OF FREEZE DRIED WHEATGRASS EXTRACT FROM THREE DIFFERENT WHEAT SPECIES

Amandeep Saini, Shweta Sinha and *Jagtar Singh

*Department of Biotechnology, Panjab University, Chandigarh, India

ABSTRACT: Oxidative stress is the leading cause of numerous age-related and chronic disorders that can be controlled by using antioxidants. Thus, there is growing concern to screen natural antioxidants from plant source that will enhance the nutritional quality of human foods to prevent disease and mitigate age-related symptoms. Wheatgrass (WG) has a potent antioxidant efficacy therefore, the present study is aimed to evaluate and compare the phytoconstituents from *T. dicoccum*, *T. durum* and *T. aestivum*. The WG were freeze dried and methanolic extract were prepared. To test antioxidant activity, total phenolic, flavanoid and antioxidant content along with free radical scavenging assay were performed in order to provide natural antioxidants in food and pharmaceutical supplements. The extracts were prepared from day 6, 9, 12, 15 WG from all three wheat species. Our results found that *T. dicoccum* have highest polyphenol, flavanoid and antioxidant contents with 192 GAE/g, 109 QE/g and 116 AAE/g dry weight of the dry extract in day 15 sample. The IC_{50} value in DPPH assay was 6.32 µg/mL for *T. dicoccum* followed by 9.2 µg/mL for *T. durum* and 11 µg/mL for *T. aestivum* respectively. In conclusion, comparing three wheat species of WG our result showed maximum antioxidant and free radical scavenging activity in *T. dicoccum* followed by *T. durum* and *T. aestivum* in day 15 sample that can be explored for therapeutic applications.

Key words: Wheatgrass, total antioxidant, total flavanoid, total phenolics, 2,2-diphenyl-1-picrylhydrazyl.

*Corresponding author: Jagtar Singh, Department of Biotechnology, Panjab University, Chandigarh, India jagtar72@gmail.com, Copyright: ©2017 Jagtar Singh. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Abbreviations

FR: Free Radicals, HbF: fetal hemoglobin, ROS: Reactive Oxygen Species, FCR: Folin-Ciocalteau Reagent, WG: wheatgrass, TAC: Total Antioxidant Capacity, TPC: Total Phenolic Content, TFC: Total Flavonoid Content, DPPH: 2,2-diphenyl-1-picryhydrazyl, GAE: Gallic Acid Equivalent, QE: Quercetin Equivalent, AAE: Ascorbic Acid Equivalent, IC_{50}: Inhibitory Concentration for 50% inhibition.

INTRODUCTION

Oxidative stress due to free radicals (FR) and reactive oxygen species (ROS) produced in human body as byproduct of frequent physiological and biochemical processes are the major risk factor for numerous disorders (Halliwell and Gutteridge, 1990; Young and Woodside, 2001). ROS are able to react with lipids, proteins and nucleic acids that damage the biomolecules of the body leading to age-related and chronic diseases including atherosclerosis, diabetes mellitus, cancer, heart and neurodegenerative diseases *etc.* (Hailiwell, 1991; Hemnani and Parihar, 1998; Israhad and Chaudhuri, 2002). Oxidative or cellular damage can be blocked by using antioxidants that are capable of donating electrons to a free radical to neutralize it (Lobo *et al.*, 2010).

Natural plant products exhibit various bioactive components such as antioxidant, anticarcinogenic, antimutagenic and anti-aging activities. Hence, there is growing concern to enhance and supplement the nutritional quality of human foods by exploring the components present in foods we consume (Tester and Langridge, 2010; Kumar *et al.*, 2011).

Wheat grass (WG) is the shoot obtained from wheat plant (family Poaceae) has various therapeutic qualities as it is rich source of nutrients, chlorophyll, minerals, vitamin E and amino acids (Walters, 1992). The major WG source of India is *Triticum aestivum* that has been reported to contain high levels of antioxidants and provide defence against oxidative DNA damage as well as ROS (Kulkarni *et al.*, 2006; Rana, 2011; Rimple *et al.*, 2016). The antioxidant property of WG can be increased by applying pulsed electric field (PEF) to the wheat seeds (Leong *et al.*, 2016).

The WG extract from common wheat plant *T. aestivum* is also called as "green blood" as it gets immediately absorbed into the bloodstream and gives energy in about 20 min that lasts throughout the day (Singhal *et al.*, 2012; Chauhan, 2014). It is observed to be advantageous for blood cells, bones, glands, kidneys and other parts of the body (Aydos *et al.*, 2011; Sri Jaya and Gayathri, 2009) and consumed most often as fresh juice or as tablets, capsules and liquid concentrates. Advanced DNA based technology revealed that WG is able to increase 3-5 fold fetal hemoglobin (HbF) production (Reynolds, 2005). The antioxidant and high chlorophyll content of WG found to be effective against various degenerative disorders including thalassemia and hemolytic anemia (Padalia *et al.*, 2010), reduce chemotherapy myelotoxicity (Dey *et al.*, 2006; Bar-Sela *et al.*, 2007), rheumatoid arthritis (Kumar *et al.*, 2011; Bar-Sela *et al.*, 2015), diuretic (Popovic *et al.*, 2014), diabetes mellitus (Saravanan and Ponmurugan, 2011). *In vivo* studies on rat also found that it can treat Alzheimer's disease (Bitra *et al.*, 2014) and act as a potent anti-hyperglycaemic agent in type II diabetes (Shakya *et al.*, 2016). Glycogen level has been increased by 7-fold in *T. aestivum* treated diabetic rats which actually get lowered in diabetes (Mohan *et al.*, 2013). This reveals that the extract has the potential to regenerate the pancreatic *β*-cells (Chika and Bello, 2010).

Keeping in view the therapeutic qualities of WG, the present study was designed to individuate the presence of antioxidant in the WG obtained from three different wheat species namely *T. dicoccum* (Emmer wheat), *T. durum* (Durum wheat), *T. aestivum* (Bread wheat) using total phenolic, total flavonoid, total antioxidant capacity, free radical scavenging activity assay and compare the harvest time in order to be used as herbal medicine.

METHODS AND MATERIALS

Plant samples

The wheat seeds of all three different species – *T. dicoccum* (HW1095), *T. durum* (variety HI8663) and *T. aestivum* (variety HD2967) were obtained from Indian Institute of Wheat and Barley Research, Karnal, Haryana, India. All the samples were provided by Dr. Arun Gupta (Principal Scientist, Economic Botany, DWR, Karnal) (Figure 1).

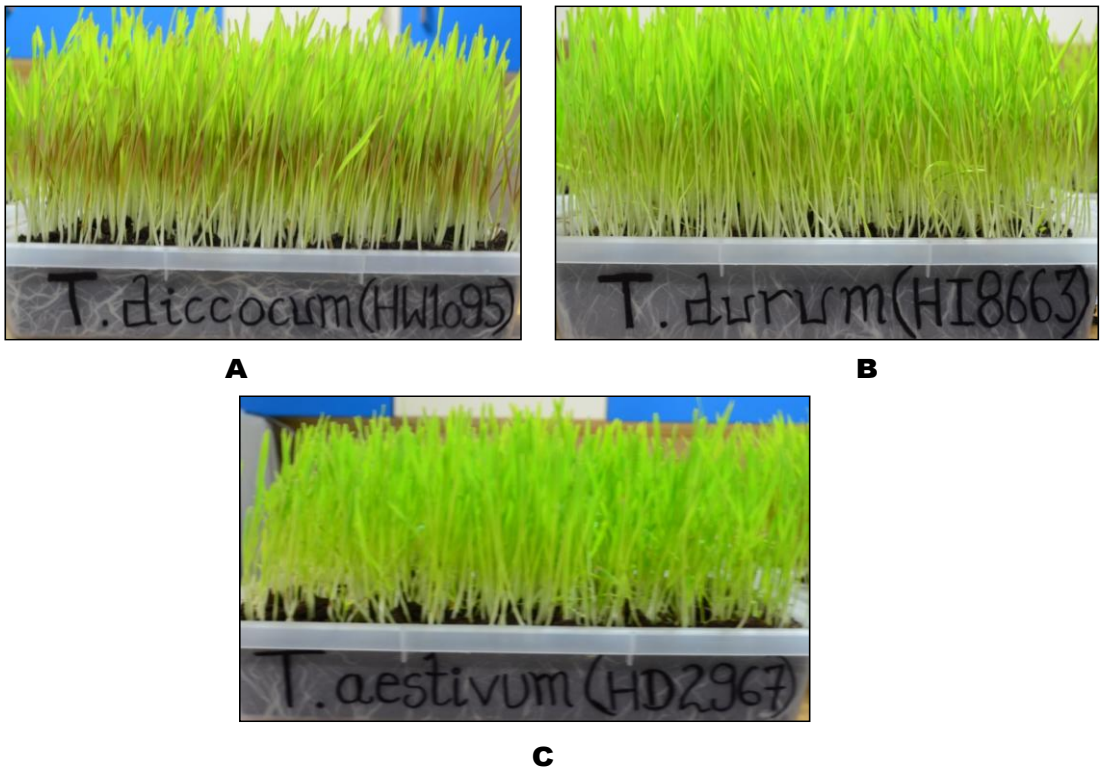

Figure 1. Wheatgrass of all three different wheat species- (A) *T. dicoccum* (HW1095), (B) *T. durum* (variety HI8663), (C) *T. aestivum* (variety HD2967).

Reagents

Aluminum Chloride ($AlCl_3$), 2,2-diphenyl-1-picryhydrazyl (DPPH), Trolox (Siga-Aldrich, St. Louis, USA), Methanol, Potassium Ferricyanide, Ferric chloride, Ascorbic acid, Sodium sulphate, Ammonium molybdate, Potassium acetate, Quercetin, Folin–Ciocalteu Reagent (FCR), Sodium carbonate (Na_2CO_3) (Himedia, Mumbai, India), Sulphuric acid (Rankem), Distilled water, Liquid Nitrogen.

Sample preparation

About 100g of wheat seeds from three different species were soaked in tap water for 48 hours and were allowed to sprout in dark for next 48 hours at room temperature. These sprouted wheat seeds of different species were evenly placed on soil bed in different plastic trays and kept in dark. After 3 days, the sprouts begin to develop into young leaf blades, called as WG. Trays were regularly checked for growth of white mould.

The WG from all 3 different wheat species were collected on day 6, 9, 12 and 15 (after the completion of sprouting period). The samples of various days were freeze dried using liquid nitrogen and grinded into fine powder using pestle and mortar. The freeze dried powered samples were stored in -20°C until further analysis.

Extract preparation

For the phytoconstituent analysis, the extracts from different wheat species of all days were prepared through Ultrasonic-Assisted Extraction (UAE) method. The UAE was performed at room temperature with 85% methanol as the extracting solvent. 20g of freeze dried WG powder of each species of various days were dissolved in 200mL of 85% methanol for a time period of 30 minutes (30 sec ON and 30 sec OFF cycles, amplitude 35%) and centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was collected and filtered through Whatman filter paper no.1 into clean flask and then concentrated to dryness using rotary evaporator at 40°C. The dried extracts were dissolved in 85% methanol as stock solution and stored at 4°C from which working solutions of different concentrations were made.

Screening of *in vitro* antioxidant activity of phytochemicals

The quantification of the antioxidants was performed with the aid of various spectrophotometric assays.

1) Total Phenolic Content (TPC)

The TPC of the extracts was determined using FCR method (Singleton and Rossi, 1965). Briefly, 200µl of both standard and samples of varying concentrations (10-100 µg/mL) were mixed with 1mL FCR (1:10) and 800µl of sodium carbonate (7.5% w/v). The mixture was vortexed thoroughly and incubated in the dark for 30 min at room temperature. After incubation, the absorbance was measured at 765nm against blank. Gallic acid (GA) was used as standard. The results were expressed as Gallic Acid Equivalent (GAE)/g dry weight using the formula: T = CV/M; where, T is TPC mg/g of plant extract as GA, C is concentration of the positive control established from calibration curve in mg/mL, V is the volume of the extracts in mL and M is the weight of the extract in g.

2) Total Flavonoid Content (TFC)

The TFC of the extracts was determined using aluminum chloride method given by Chang *et al.* (2002). Briefly, 200µl of both standard and samples of varying concentrations (50-500 µg/mL) were added to 600µl of methanol. Thereafter, 40µl of 10% aluminum chloride and 40µl of 1M potassium acetate was added, volume was made up to 2mL with distilled water. Then the mixture was vortexed thoroughly and after incubation for 30 min at room temperature, absorbance was measured at 415 nm against a blank. Quercetin was used as the standard. The results were expressed as Quercetin Equivalent (QE)/g extract.

3) Phosphomolybdenum Assay/ Total Antioxidants Capacity (TAC)

The TAC of the extracts was estimated by molybdate assay Prieto *et al.* (1999). Briefly, 200µl of both standard and samples of varying concentrations (30-300 µg/mL) were mixed with 2mL of TAC reagent (3.24mL sulphuric acid + 0.397g sodium sulphate + 0.494g ammonium molybdate, volume made up to 100mL with distilled water). Thereafter, the mixture was vortexed thoroughly and after incubation for 90 min at 95°C, the absorbance was measured at 695 nm against blank. Ascorbic acid was used as the standard. The results were expressed as Ascorbic Acid Equivalent (AAE)/g dry extract.

4) Free Radical Scavenging Activity/ DPPH assay

The DPPH assay of extract was measured by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay by the method given by Brand-William, (1995) with slight modifications (Bursal and Gulcin, 2011). Methanolic solution of DPPH was prepared by dissolving 100µM of DPPH in 100mL of methanol. Briefly, 50µl of both standard and samples of varying concentrations (20-300µg/mL) were mixed in 1950µl of methanolic solution of DPPH. The mixture was vortexed thoroughly and incubated in the dark for 30 min at room temperature.

After incubation, the absorbance was measured at 517 nm against blank. Ascorbic acid was used as standard. The free radical scavenging activity was calculated according to the equation: % inhibition= $[(A_o-A_e)/A_o]$ x100 % where A_o is the absorbance reading of the control, A_e is absorbance reading in the presence of sample extract.

Statistical analysis

All the experiments were performed in duplicates and averaged. Statistical analysis for all the assays was obtained by interpolation from linear regression analysis using Microsoft Excel 2007.

RESULTS

The present study shed some light on the use of WG as healthy food in terms of antioxidant activity and therapeutic applications. WG obtained from all the species of wheat showed remarkable antioxidant and free radical scavenging activity at all days of their growth and reached at their maximum on day 15.

1) Analysis of polyphenols

The concentration of TPC was expressed as GAEs by reference to the GA standard calibration curve. The calibration equation for Gallic acid was y= 0.010x - 0.079, (R^2=0.997), where x is the GA concentration in mg/l and y is the absorbance reading at 765nm (Figure 2). The TPC for *T. dicoccum* for day 6, 9, 12 and 15 were 114, 136, 172 and 192 GAE/g of dry weight respectively. The TPC for *T. durum* for day 6, 9, 12, 15 was 89, 97, 123 and 142 GAE/g of dry weight respectively. The TPC for *T. aestivum* for day 6, 9, 12, 15 was 64, 73, 110 and 132 GAE/g of dry weight of extract respectively (Figure 3).

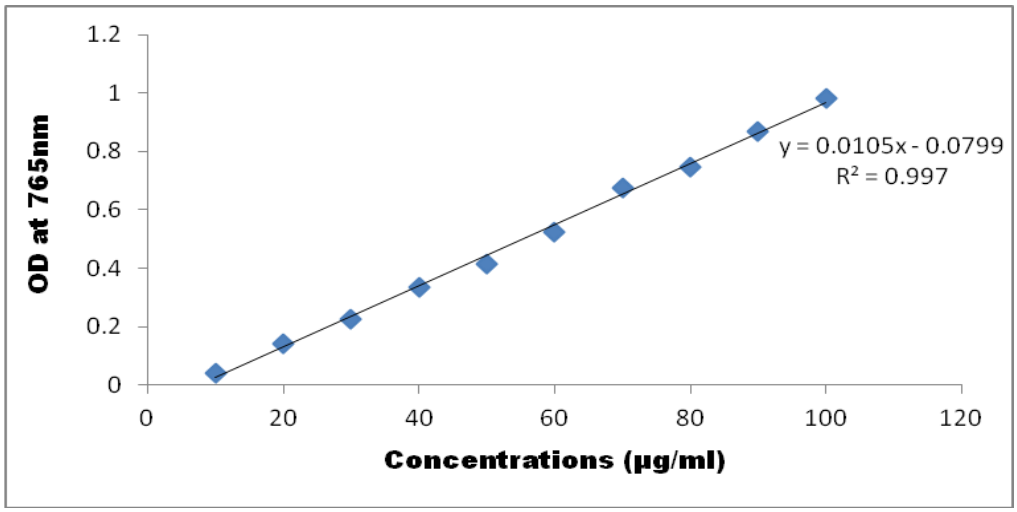

Figure 2. Standard curve for Gallic acid.

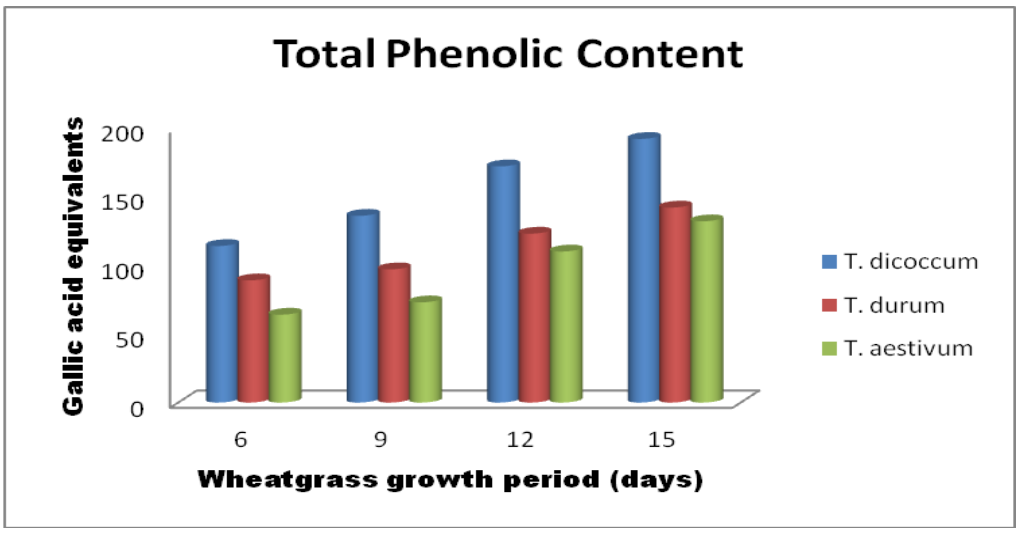

Figure 3. Comparison of the total phenolic content of *T. dicoccum*, *T. durum* and *T. aestivum* with growth period.

2) Evaluation of flavonoid content

The concentration of TFC was expressed as QEs by reference to the quercetin standard calibration curve. The calibration equation for Quercetin was y= 0.001x + 0.017, (R^2=0.999), where x is the Quercetin concentration in mg/l and y is the absorbance reading at 415nm (Figure 4). The TFC for *T. dicoccum* day 6, 9, 12, 15 were 36, 52, 87 and 109 QE/g dry weight respectively. The TFC for *T. durum* for day 6, 9, 12, 15 were 29, 34, 69 and 83 QE/g dry weight respectively. The TFC for *T. aestivum* for day 6, 9, 12, 15 were 23, 29, 43 and 64 QE/g dry weight of extract respectively (Figure 5).

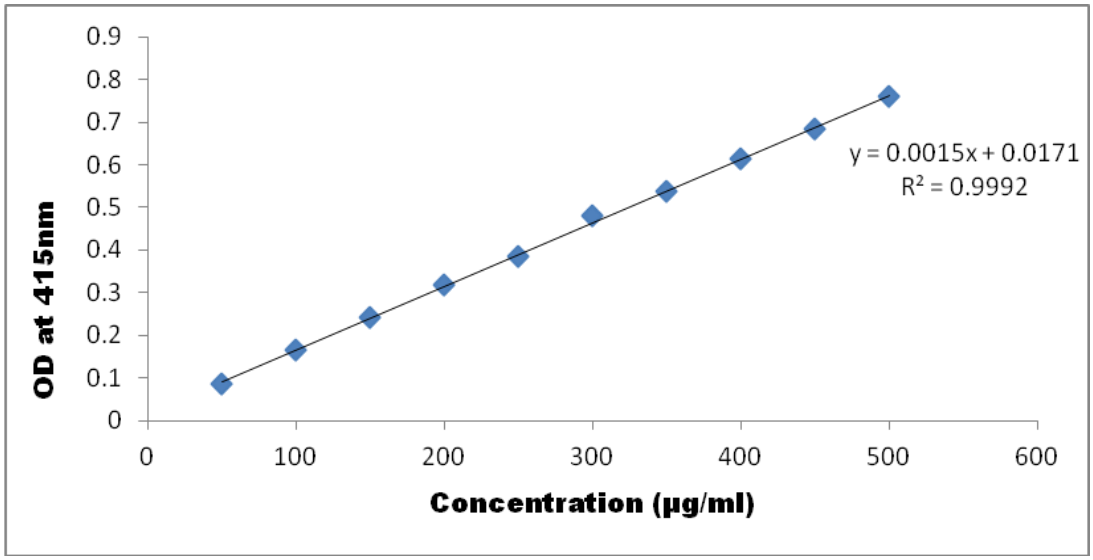

Figure 4. Standard curve of Quercetin.

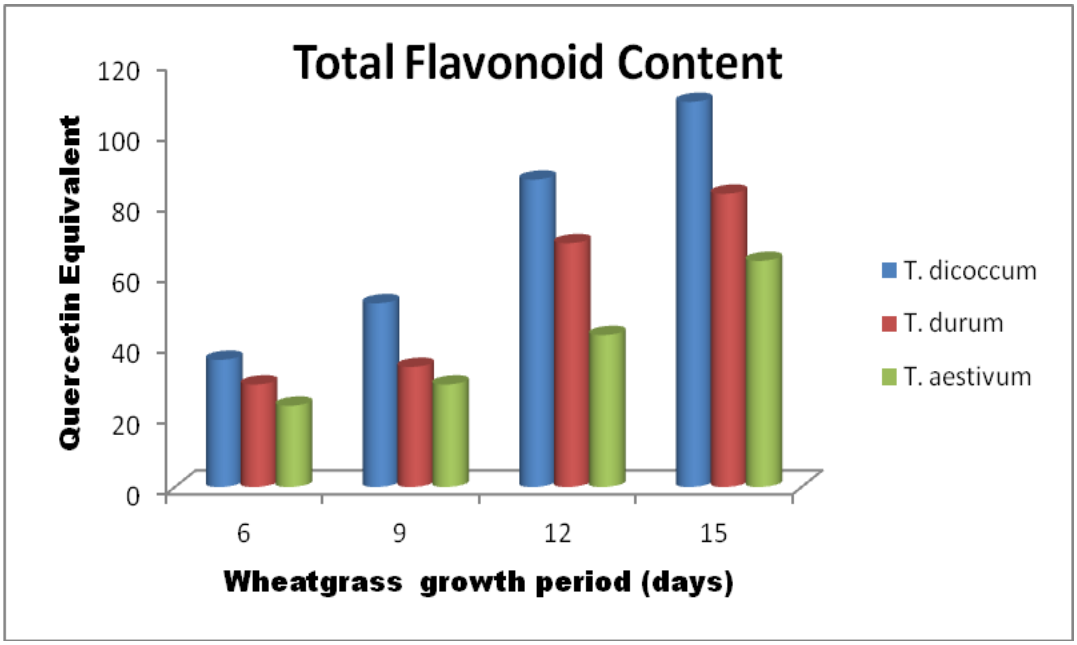

Figure 5. Comparison of the total flavanoid content of *T. dicoccum*, *T. durum* and *T. aestivum* with growth period.

3) Evaluation of antioxidant capacity

The concentration of TAC was expressed as AAEs by reference to the ascorbic acid standard calibration curve. The calibration equation for Ascorbic acid was y= 0.003x - 0.019, (R^2=0.998), where x is the ascorbic acid concentration in mg/l and y is the absorbance reading at 695nm (Figure 6). The TAC for *T. dicoccum*, *T. durum* and *T. aestivum* were 116, 72 and 49 AAE/g of dry weight of the extract respectively (Figure 7).

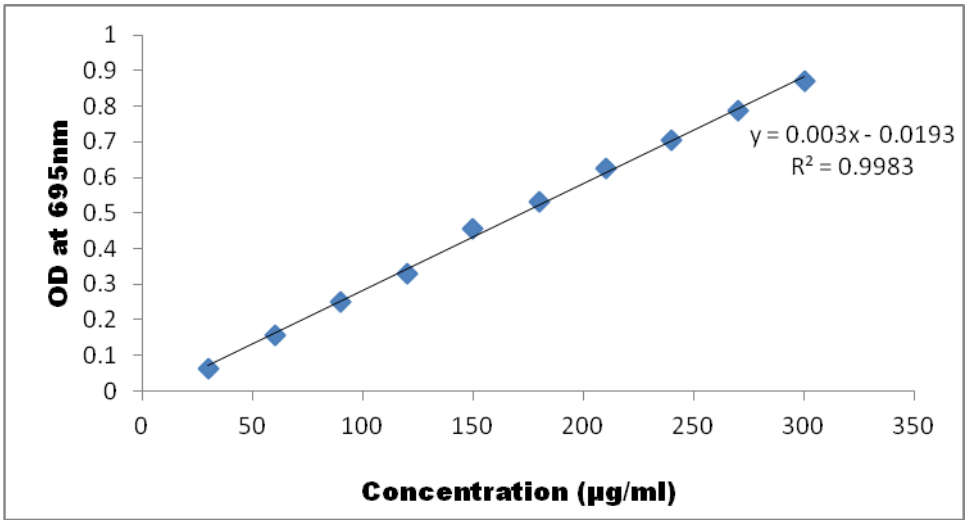

Figure 6. Standard curve of Ascorbic acid.

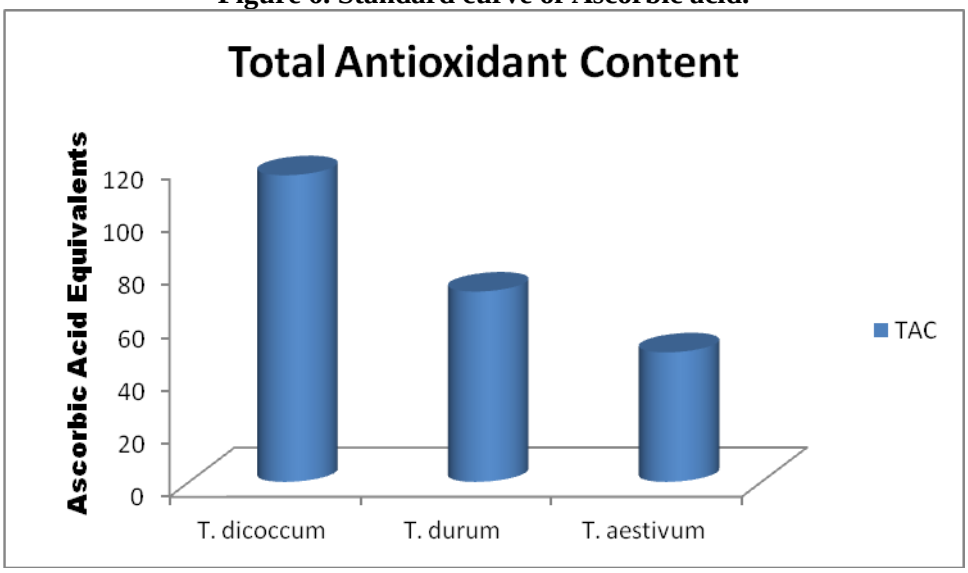

Figure 7. Comparison of the total antioxidant content of *T. dicoccum*, *T. durum* and *T. aestivum* with growth period.

4) Analysis of IC_{50} value

The free radical scavenging activity of the WG extract was investigated as % of inhibition (IC_{50} value of DPPH scavenging) with all the three wheat species. IC_{50} values of *T. dicoccum*, *T. durum* and *T. aestivum* were observed to be 6.32, 9.2 and 11µg/mL respectively (Figure 8 and 9).

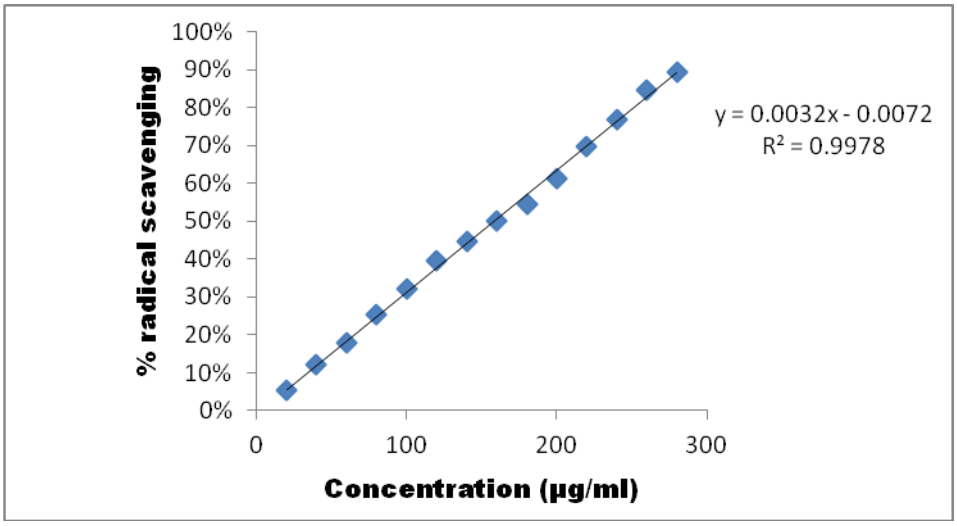

Figure 8. Standard curve of DPPH.

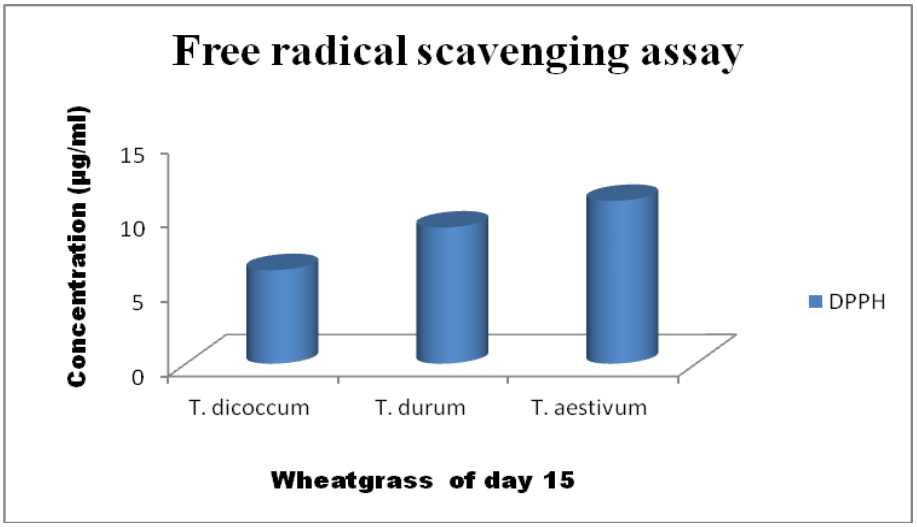

Figure 9. Comparison of the of IC_{50} values of *T. dicoccum*, *T. durum* and *T. aestivum* with growth period.

DISCUSSION

In the present study, WG obtained from three wheat species (*T. dicoccum, T. durum and T. aestivum*) were compared for antioxidant content and our results showed remarkable antioxidant and free radical scavenging activity in all the three WG that reached at their maximum on day 15, which is in concordance with the study done by Kulkarni *et al.* 2006.

A gas chromatography- mass spectrometry analysis has found that the young leaves of WG have ample load of bioactive compounds such as polyphenols, flavonoids, squalene, amyrins in varying percentage that can be used in pharmacological field (Durairaj *et al.*, 2014). The *T. aestivum* is the major source of WG in India that has been reported to have high quantity of antioxidant and effective against cancer (Bar-Sela *et al.*, 2007), rheumatoid arthritis (Kumar *et al.*, 2011), diuretic (Popovic *et al.*, 2014), diabetes mellitus (Saravanan and Ponmurugan, 2011), Alzheimer's disease (Bitra *et al.*, 2014) and anti-hyperglycaemic agent (Shakya *et al.*, 2016). This suggests that WG can be used as herbal medicine however, small *in vivo* trials and varied methodological attempts must be validated before any clinical recommendations to the patients (Bar-Sela *et al.*, 2015).

In our study, the highest content for both TPC and TFC was observed in *T. dicoccum* followed by *T. durum* and *T. aestivum* which significantly increased along its growth period and reached maximum on day 15. A study conducted on 18 ancient wheat species (12 *T. dicoccum* Schrank, 6 *T. monococcum* L.) and 2 bread wheat varieties grown in different regions of Turkey for their total phenolic and flavonoid contents. The results revealed 6.33±0.98 µmol GAE/g of TPC and 1.61±0.34 µmol catechin E/g of TFC in *T. dicoccum* which was much lower as compared to our results (Serpen *et al.*, 2008). In contrast, Benincasa *et al.* (2014) reported approximately 800 GAE/g of dry weight of TPC in a 12-day old WG cultivar (*T. durum* cv. 'Creso') which is the highest among *T. spelta, T. monococcum, T. dicoccum* and *T. aestivum*. Jangle and Padmanabhan (2016) reported 301.74 µg GAE/g total phenol and 10.29mg rutin E/g total flavonoid in a 10-day old WG juice from *T. aestivum* L. which is extremely high as compared to our study. In another study although, dry powder WG extract showed 210.15 ±2.14µmol of GAE/g of TPC and 160.25±2.17µmol of QE/g of TFC (Durairaj *et al.*, 2014) which was higher than our results, the species was not mentioned and the methodology varied.

In the present study, the TAC and IC_{50} value were also found to be highest in *T. dicoccum* followed by *T. durum* and *T. aestivum* which increased significantly along with the growth period of plants and reached maximum on day 15 for all species. In a study carried out in Turkey on 12 *T. dicoccum* Schrank, 6 *T. monococcum* L. varieties, TAC was found to be 18.31±0.31µmol Trolox E/g in *T. dicoccum* which was extremely lower from our findings (Serpen *et al.*, 2008). In contrast, a study found highest levels of total phenol (443 GAE/g) and DPPH radical scavenging activity (92.6%) in *T. durum* WG juice while comparing *T. durum, T. aestivum, Lolium perenne* L. and *Festuca arundinacea* Schreb (Ozkose *et al.*, 2016). Another study also reported 89-96% radical scavenging activity of WG from common wheat grains at 7-8" and 9-10" height (Agrawal *et al.*, 2015) which is incomparable. The DPPH radical scavenging activity for *T. aestivum* in the present study is 11 µg/mL which was observed to be highest as compared to 12.87% inhibition at 100 µg/mL in *T. aestivum* (Jangle and Padmanabhan, 2016).

Overall, WG from several wheat species was compared that gave a repulsive rather than conclusive result for therapeutic application of any wheat species. This might be due to the difference in environmental factors as well as the laboratory parameters thereby the extraction methodology. Our study clearly states that the WG from *T. dicoccum* (Emmer wheat) which is one of the oldest wheat in the world has higher antioxidant and free radical scavenging activity than both *T. durum* (Durum wheat) and *T. aestivum* (Bread wheat).

CONCLUSIONS

Our study concludes that WG from all three different wheat species showed maximum antioxidant and free radical scavenging activity on day 15. Comparing difference between the species, the WG showed maximum antioxidant and free radical scavenging activity in *T. dicoccum* followed by *T. durum* and *T. aestivum.* Several studies found the WG from *T. aestivum* as very promising herbal medicine that can cure various degenerative diseases. However, some studies including our results suggest that the WG from *T. dicoccum* species might potentially be valuable and extensive research work is needed in order to explore its therapeutic applications.

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