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Simran Tandon et al. / Journal of Pharmacy Research 2011,4(12),

Research Article
ISSN: 0974-6943

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Antioxidant Profiling of *Triticum aestivum* (wheatgrass) and its Antiproliferative Activity In MCF-7 Breast Cancer Cell Line

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Received on:20-08-2011; Revised on: 15-09-2011; Accepted on:10-11-2011

ABSTRACT

The present study was undertaken to put forward the scientific evidences of wheatgrass as an alternative therapy to treat cancer. The aim was to investigate the antiproliferative effects of wheatgrass extract on the selected model of breast cancer cells i.e. MCF-7 cell line and effect of extraction medium and sample preparation on total antioxidant capacity (TAC) of wheatgrass plant. Antioxidant properties of different wheatgrass extracts were studied by estimating Total Phenol Content (TPC), DPPH ((1, 1-diphenyl-2-picrylhydrazyl) and FRAP (Ferric reducing antioxidant power) Assay. Significant antiproliferative effect and cell death was observed in a dose dependent manner of wheatgrass extract on MCF-7 cells. The results indicates that TAC of the samples processed with one step procedure is more substantial as compared to two step procedure and three step procedure. The best results were obtained when the one -step procedure for sample preparation and 70 % ethanol (ethanolic) as an extracting agent were applied. Thus, this study provides preliminary data about the anticancer activity of wheatgrass extract and its potential to be considered as an anticancer agent.

Key words: *Triticum aestivum*, TPC, FRAP, DPPH, MTT, MCF-7.

INTRODUCTION

Variables like solvent, extraction method and extraction time, all influence the amount of antioxidants which can be extracted. Recently, a new three step method was proposed for sample preparation based on discriminating water-soluble, water-insoluble and protein antioxidants within same plant; the same protocol is followed in our work for extraction of different extracts from wheatgrass [1].

Antioxidants are chemical compounds that can bind to free oxygen radicals thus preventing these radicals from damaging healthy cells which could lead to cancer. The young grass of the common wheat plant, *Triticum aestivum* is known as wheatgrass, family Poaceae. Wheatgrass is known to be a rich source of vitamins, antioxidants and minerals. It also contains Vitamin A, B1, C and E, many minerals and trace elements including calcium, iodine selenium and zinc. Wheatgrass is known to contain antioxidant enzymes Superoxide dismutase (SOD) and cytochrome oxidase that have the potential to convert Reactive oxygen species (ROS) to a hydrogen peroxide and an oxygen molecule[2]. Chlorophyll, one of the primary components in the wheatgrass extract, was found to augment blood formation and strengthen the immune system through inhibition of metabolic activation of carcinogens[3-4]. It also possesses the ability to inhibit oxidative DNA damage[5]. Few clinical trials have been accomplished that have shown on consumption of wheatgrass juice, the number of transfusions in patients with thalassemia major is decreased[6]. Reduction in the overall disease activity index and the severity of rectal bleeding in cases of distal ulcerative colitis on consumption of wheatgrass juice has also been observed[7].Some preliminary studies have suggested that the observed beneficial results could be because of the antioxidant activity, but a detailed study has not been done[8].

In the present study, we investigated the free radical scavenging activity and antiproliferative activity of various extracts of *Triticum aestivum* by estimating Total Phenolic Content, DPPH and FRAP , followed by cell proliferation assay using MTT assay on breast cancer cell line MCF-7.

MATERIALS AND METHODS

Materials

Plant material

Seeds of *Triticum aestivum* (strain PBW343) were obtained from HAU (Chaudhary Charan Singh University, Hisar) for the following study. The seeds were soaked overnight. Next day, the seeds were germinated in trays containing soil. The trays were monitored daily and watered as per the need. Wheatgrass plant of 6-7 inches was used as an earlier study revealed that maximum antioxidant activity was observed in a wheatgrass plant of the aforesaid length[9].

Cell Culture

Human Breast cancer cell line- MCF-7 was used as an *in vitro* model for our study and obtained from NCCS, Pune, India. The cells were cultured in DMEM medium supplemented with 1% (v/v) antibiotic combination of Penicillin-Streptomycin and 10 % (v/v) FBS, maintained at 37°C in a 5 % CO_2 incubator. Cells at exponential stage were used for experimentation.

Preparation of different extracts

Fresh wheatgrass was dried, cut into small pieces and 10 % (w/v) wheatgrass extracts were prepared by maceration using pestle and mortar, with different solvents such as water, 70% ethanol, 0.1 % TCA, sodium acetate buffer pH 5 and potassium phosphate buffer pH 7.4. The homogenates were centrifuged at 15000 r.p.m for 30 minutes at 4°C and the resulting supernatants (one- step, crude fraction) were analyzed for future experiment. In the two-step procedure residues left behind, except that from the ethanol slurry, were re-extracted with acetone under agitation for 30 min at room temperature, centrifuged at 15 000 r.p.m for 15 min at 4ºC and the supernatants were collected for further analysis (acetone fraction). For the three-step procedure, initial supernatants from phosphate and acetate buffers were further processed by adding 60% TCA to a final concentration of 1%. After centrifugation at 12 000 r.p.m for 15 min at 4ºC, obtained supernatants (low-molecular fraction) were evaluated further. The protein residues were resuspended in a detergent solution (6 M Urea, 0.1 M acetate buffer, pH 5) and used in the analyses (protein fraction)[1].

Methods

Phytochemical Screening

The aqueous extract of wheatgrass was subjected to various chemical tests in order to determine the presence of secondary plant constituents.

Test for Reducing Sugars:

To 2 ml of the extract, 5 ml of a mixture (1:1) of Fehling's solution A and Fehling's solution B was added and boiled in a water bath for five minutes. A brick-red precipitate indicated the presence of free reducing sugars[10].

Test for the presence of Anthraquinones:

0.5 g of the extract was shaken with 10 ml of benzene, filtered and 5 ml of 10% ammonia solution added to the filtrate. The mixture was shaken; the presence of a pink, red or violet colour in the ammoniacal (lower) phase indicated the presence of anthraquinones[11].

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Test for Saponins:
Aqueous extract was obtained by boiling on the water bath. The extract was transferred into a test tube and shaken vigorously then was left to stand for 10 minutes and the result noted. A thick persistent froth indicates saponins[10].

Test for Flavonoids:
Extract of the sample was reduced to dryness on the boiling water bath. The residue was treated with dil. NaOH, followed by addition of dilute HCl, solubility and colour was noted. A yellow solution with NaOH, which turns Colourless with dil. HCl, confirms flavonoids[10].

Test for Tannins:
0.5 g of the extract was dissolved in 5 ml of water followed by a few drops of 10 % ferric chloride. A blue-black, green, or blue-green precipitate would indicate the presence of tannins[12].

Test for Alkaloids:
0.5 g of extract was stirred with 5ml of 1 % aqueous hydrochloric acid on a water bath, filtrate was second was treated with Dragendorff's reagent. Turbidity or precipitation with either of these reagents would indicate the presence of alkaloids in the extracts[13].

Test for terpenoids (Salkowski test):
5 ml of extract was mixed in 2 ml of chloroform, and concentrated sulfuric acid (3 ml) was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoids[14].

Determination of Total Phenolic Content
The phenolic compounds in the various extracts were determined using the Folin-Ciocalteu reagent in a manner proposed by Singleton and Rossi [15], based on the reduction of mixture of phosphate tungsten and molybdenum oxides complex by phenolics to give blue coloured product. To 50 µl of a sample/standard, 3.5 ml water, followed by 250 µl FC (2 N Folin-Ciocalteu) reagent was added. The mixture was mixed and incubated it for 8 minutes at room temperature. To the above mixture 750 µl of 20% sodium carbonate solution was added and incubated for 2 hrs at room temperature. After incubation, the absorbance was taken at 765 nm. The total phenolic content (TPC) was expressed as mg Gallic Acid Equivalents/gram of dry extract.

Evaluation of *in vitro* Antioxidant activity

DPPH radical-scavenging activity
The free radical scavenging activity of wheatgrass extracts based on principle, when DPPH ((1, 1-diphenyl-2-picrylhydrazyl) reacts with phenolic compounds, it is reduced to 1, 1–diphenyl-2-picryhdrazine due its ability to donate H atom. The change in colour **(DPPH) + (H-A)** $_{\text{purple}}$ **→ DPPH-H + (A)** $_{\text{yellow}}$ was measured. The DPPH method is described as a simple, rapid and the most convenient method. It is independent of sample polarity for screening of many samples for free radical scavenging activity[17]. A solution of 0.002 % (w/v) DPPH in methanol was prepared and 1.5 ml of this solution was mixed with of different concentrations of the extracts in 1:1 dilution in methanol. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517 nm[16]. The IC_{50} value was defined as the concentration (µg/ml) of extracts that scavenges the DPPH radicals by 50%.
% scavenging activity was calculated by the

following formula = $\frac{A_{cont} - A_{test}}{A_{cont}}$ X 100

Where A_{cont} = Absorbance of DPPH radical + methanol;
A_{test} = Absorbance of DPPH radical + standard/ sample extract.

Ferric Reducing Antioxidant Power Assay
FRAP assay was followed according to modified method of Benzie and Strain. Fresh FRAP reagent was prepared by mixing the following reagents: 25 ml Sodium acetate.trihydrate buffer (300 mM) pH 3.6, 2.5 ml TPTZ (10mM, in 40 mM HCl), 2.5 ml of $FeCl_3.6H_2O$ (20 mM) and 3 ml H_2O. The temperature of the solution was raised to 37°C before using for further work. Wheatgrass extracts (150 µl) were allowed to react with 2850 µl of FRAP solution for 30 minutes in the dark condition. Readings of the coloured product (ferrous tripyridyltriazine complex) was taken at 593 nm[16].

Evaluation of *in vitro* anti-proliferative activity

MTT Assay:
The 3-(4,5-dimethylthiazol-2-yl) 2,5diphenyltetrazolium bromide (MTT) assay that differentiates dead from living cells was adapted from the literature received [18-21]. Cells were seeded into a 96-well microplate (10^5cells/well) and incubated for 48 hrs at 37°C to achieve 70-80 % confluency; they were treated with varying concentrations (40-400 µg/ml) of wheatgrass extracts for a further period of 48 hours at 37°C. Following this, 30 µl of MTT reagent was added to each well and was incubated for 4 hrs at 37°C. Isopropanol-HCl (50 µL) was added to each well after the incubation was over to solubilize the formazan product and kept at room temperature for 15-20 min. The absorbance (A) was then read at a wavelength of 570 nm[22]. The decrease in absorbance in the assay measures the extent of decrease in the number of viable cells following exposure to the test substances calculated by using the following formula:

% Inhibition of cells = $\frac{A_{cont} - A_{test}}{A_{cont}}$ X 100.

Statistical analysis
The experimental results were expressed as mean ± standard error of mean (SEM) of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Duncan's Multiple Range test using GraphPad Instat. *P* values < 0.05 were regarded as significant and *P* values < 0.01 as very significant.

RESULTS AND DISCUSSION
The following work was carried out to explore the antioxidant and antiproliferative activities of wheatgrass plants by deducing the effect of different extraction methods and sample preparations. Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. Free radicals are molecules with incomplete electron shells which make them more chemically stable than those with complete electron shells. On exposure to various exogenous factors like tobacco, smoke, alcohol and UV radiation, leads to free radical formation[23]. Significant work has been done showing positive linear correlation between the total content of Phenolic compounds and the antioxidant activities. The methanolic and aqueous extracts of Chinese medicinal plants show a positive correlation between the Total Phenolic Content and antioxidants [24]. The extraction of polyphenols entirely depends upon the extraction method and solvent system used and in support of this it was reported that use of methanol as an extraction solvent resulted in higher antioxidant activities [25]. Aqueous ethanol and methanol extracts of red and black currant contained more polyphenols as compared to its crude aqueous extract [26]. Also it was found methanol was a better solvent in extraction of low molecular weight antioxidants, while aqueous acetone extracts yielded more of high molecular weight antioxidants [27]. Most commonly used solvent for polyphenols extraction was reported to be ethanol[28]. Till date, one step procedure and two step procedure were followed by researchers, but a new approach was developed, which used three step procedure based on extraction of water soluble, water insoluble and protein antioxidants from the same sample was done[1]. In our study we used three step procedures to check the total antioxidant capacity and antiproliferative activity based on different extraction medium and solvent system. The results of phytochemical screening of the crude aqueous extract revealed that wheatgrass is a rich in secondary metabolites as evidenced by the presence of reducing sugars, saponins, flavonoids, alkaloids, anthraquinones, tannins and terpenoids(Table 1).Work done by other workers also indicated the presence of saponins, flavonoids, tannins, anthraqiunones, reducing sugar and tripernenoids[29].

Table 1: Qualitative phytochemical screening of aqueous wheatgrass extract

Compound	Observation	Results
Reducing sugar	Blue colour of Fehling turns to brick red	+ve
Saponins	Appearance of frothing	+ ve
Flavonoids	Appearance of yellow colour	+ ve
Alkaloids	Appearance of turbidity	+ ve
Terpenoids	Reddish green colour	+ ve
Anthraquinones	Pink colour	+ ve
Tannins	Bluish green	+ ve

Total Phenolic Content
Previous studies revealed that most commonly used synthetic antioxidant Propylgallate(PG) butylatedhydoxyanisole (BHA), and butylatedhydoxytoluene (BHT) were toxic to liver and also carcinogenic[30-31], so research has been directed towards more of naturals than that of synthetic antioxidants. Plants are rich in natural antioxidants [32-33]. Among these, the Phenolic compounds have ability to scavenge free radicals, super oxide and hydroxyl radicals through oxidation reactions. The estimation of TPC of wheatgrass extracts was carried using straight line equation ($y = 0.0132x - 0.0093$, $r^2 = 0.999$). The total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram of dry weight of extract. The results obtained in Table.2 indicated that ethanolic extract has highest TPC content in comparison to other wheatgrass extracts. Similar work was done by which showed ethanolic extract showed highest TPC content as compared to aqueous extract [9].

Table 2: Total Phenolic Content of different extracts of wheat grass

Sample (crude extracts)	TPC (mg GAE/gram of dry extract)
Ethanolic	06.48 ± 0.23
Potassium phosphate	02.44 ± 0.06***
Sodium acetate	02.75 ± 0.19***
0.1% TCA	02.56 ± 0.10***
Aqueous	03.08 ± 0.30***

*Values are Mean ± SEM; n=3), Significant at ***p < 0.001 One way ANOVA was used to compare different extracts of wheatgrass with extract showing highest activity (70 % Ethanolic extract).) [*p – least significant,**p – significant, ***p – extremly significant].*

FRAP

According to Benzie and Strain [34], at low pH- the reduction of ferric tripyridyl triazine (Fe^{+3}) TPTZ complex to ferrous form (Fe^{+2}) gives intense blue color product, which can be monitored by measuring the change in absorption at 593 nm. The reaction is non-specific, any half reaction that has lower redox potential, under reaction conditions than that of ferric ferrous half reaction will drive the ferrous Fe III to Fe II ion formation. Therefore change in absorbance is directly related to reducing power of the electron donating antioxidants present in the reaction mixture. Table.3 represents the FRAP value of different extracts of wheatgrass. The FRAP value were calculated using the standard curve equation y = 0.6178x - 0.0053 and r^2 = 0.997. The FRAP value of known antioxidant Ascorbic acid obtained was 12.13 ± 0.98 mM Fe II/gram.

Table3: FRAP value of different extracts of wheatgrass

Sample (crude extracts)	FRAP Value(mMFe (II)/gram of dry extract)
Ethanolic	0.61 ± 0.010
Potassium phosphate	0.10 ± 0.000 ***
Sodium acetate	0.16 ± 0.005 ***
0.1% TCA	0.14 ± 0.000 ***
Aqueous	0.10 ± 0.002 ***

*Values are Mean ± SEM; n=3), Significant at ***p < 0.001 One way ANOVA was used to compare different extracts of wheatgrass with extract showing highest activity (70 % Ethanolic extract) [*p – least significant,**p – significant, ***p – extremly significant]*

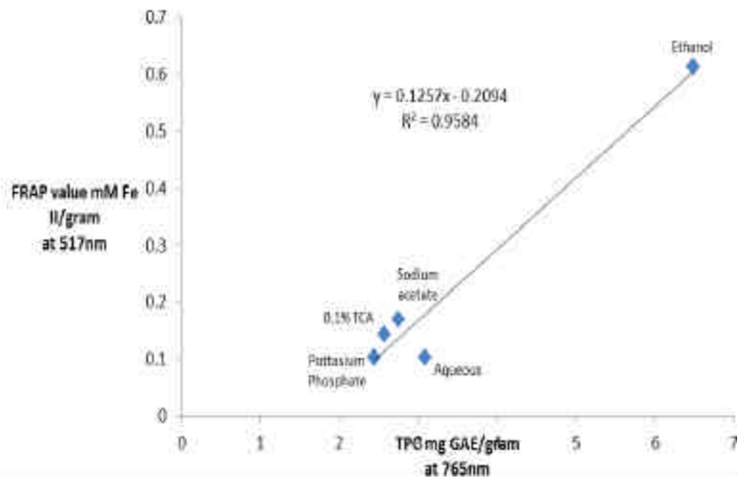

Figure1: Correlation between the TPC and FRAP value of different wheatgrass extracts.

The figure indicates the positive correlation between the total phenolic content and the FRAP value of different extracts of wheatgrass, signifying the relation between the polyphenols and antioxidant activity.

DPPH

The DPPH method is considered as a simple, rapid and the most convenient method. It is independent of sample polarity for screening of many samples for radical scavenging activity [17]. The use of methanol as extract solvent restrict the cellular compounds responsible for scavenge the DPPH radical, only the substances soluble in methanol (e.g. carotenoids, fatty acids) are involved in this scavenging process. High reduction of DPPH is related to the high scavenging activity performed by particular sample [35]. IC_{50} was calculated as amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by 50%. The lower the IC_{50} value, the higher is the free radical scavenging activity of the sample. Table.4 shows that ethanolic extract has highest IC_{50} of 140.32 µg/ml as compared to other extracts of wheatgrass, while IC_{50} of known antioxidant Ascorbic acid was 6.33 µg/ml; similar work was done using two extracts of wheatgrass (Aqueous and ethanol) according to which ethanolic extract showed significantly higher IC_{50} value as compared to aqueous extract [9].

Table 4: IC_{50} value of free radical scavenging activity of different extracts of wheat grass.

Sample(crude extracts)	IC_{50} (µg/ml)
Ethanolic	177.701
Potassium phosphate	1076.68***
Sodium acetate liquid	764.316*
0.1% TCA liquid	993.411**
Aqueous	646.056 *

*Values are Mean ± SEM; n=3), Significant at *p < 0.05 One way ANOVA was used to compare different extracts of wheatgrass with extract showing highest activity (70 % Ethanolic extract). [*p – least significant,**p – significant, ***p – extremly significant]*

Antiradical activity assay is based on the reduction of 1, 1-diphenyl-2-picrylhydrazyl (DPPH). Due to the presence of an odd electron it gives a strong absorption maximum at 517 nm. As this electron becomes paired off in the presence of a hydrogen donor, i.e. a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured. The anti-radical activity of known antioxidant Ascorbic acid was found to be 0.168±0.003 Table.5, shows that ethanolic extract has highest anti-radical activity as compared to other extracts.

Table 5: Anti- Radical activity of different wheatgrass extracts.

Sample(crude extracts)	Anti-radical activity ($1/IC_{50}$)
Ethanolic extract	0.0055 ± 0.00
Aqueous extract	0.0013 ± 8.81***
0.1%TCA	0.00098 ± 1.15***
Potassium phosphate	0.0009 ± 0.00***
Sodium acetate	0.0012 ± 3.33***

*Sodium acetate 0.0012 ± 3.33***,Values are Mean ± SEM; n=3), Significant at ***p < 0.001 One way ANOVA was used to compare different extracts of wheatgrass with extract showing highest activity (70 % Ethanolic extract). [*p – least significant,**p – significant, ***p – extremly significant].*

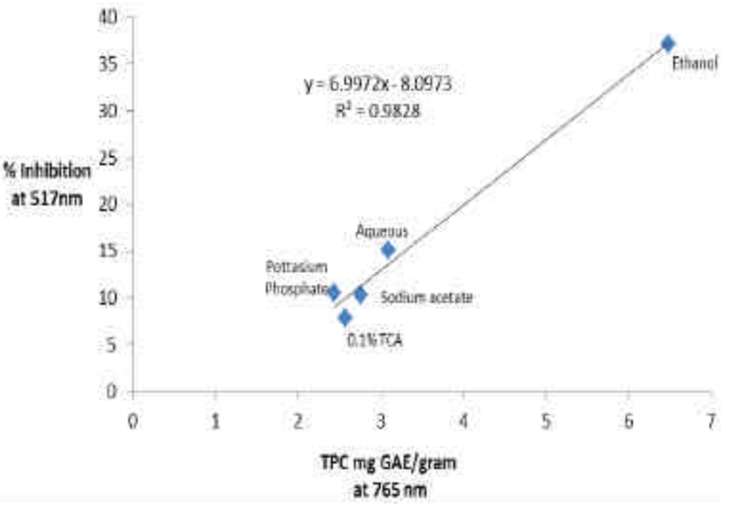

Figure 2: Correaltion between TPC and % (percent) inhibition of DPPH by different extracts of wheatgrass.

The figure indicates the significant relation between the free radical scavenging activity and total phenolic content between the different extracts of wheatgrass, indicating the positive correlation be tween the polyphenols and antioxidants.

MTT assay

The MTT assay was performed to evaluated the antiproliferative effect of the wheatgrass extraction on breast cancer cell line i.e. MCF-7. From the above experiment IC_{50} of the ethanolic extract was found to be 140.32 µg/ml i.e. 50% of cell death was observed at this particular concentration of ethanolic wheatgrass extract which proved to be highest as compared to other extracts, whereas

the IC_{50} of Cisplatin which was used as a positive control was found to be 12 µg/ml. Table.6 represents the IC_{50} of different extracts of wheatgrass.

Table 6: IC_{50} of different extracts of wheatgrass.

Sample(crude extracts)	IC_{50}(µg/ml)
Ethanolic	140.32
0.1 % TCA	196.05***
Aqueous	168.46***
Potassium phosphate	456.87***
Sodium acetate	408.27***

*Values are Mean ± SEM; n=3), Significant at ***p < 0.001 One way ANOVA was used to compare different extracts of wheatgrass with extract showing highest activity (70 % Ethanolic extract).). [*p – least significant, **p – significant, ***p – extremly significant].*

The results obtained from the various assays for total antioxidant activities and cell cytotoxicity studies have indicated that wheatgrass is rich in water soluble antioxidants as compared to water-insoluble and protein soluble antioxidants ,which showed no detectable activities.

CONCLUSION

The work done so far indicates the importance of wheatgrass in terms of antioxidant and antiproliferative properties. From the results obtained in this study, crude ethanolic extract proved to show highest free radical scavenging activity as well as the highest cell killing, which correlates well with the role of antioxidants in reduction of viability of cancer cells. However, further investigations are required to identify the mechanism behind the killing of cancer cells.

ACKNOWLEDGEMENT

We are thankful to Professor and Head, Department of Biotechnology and Bioinformatics, Jaypee University of Information and Technology, Waknaghat Campus for providing facilities for doing this work.

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Source of support: Nil, Conflict of interest: None Declared