

# Wheat Seedlings as Food Supplement to Combat Free Radicals: An *In Vitro* Approach

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Ravikumar, *et al.*: Antioxidant Activity of Wheat Seedlings

The present study was designed to evaluate the antioxidant activity of 5 organic solvent extracts (petroleum ether, *n*-hexane, chloroform, ethyl acetate and methanol) of wheat grains, 3, 5 and 7 days old wheat seedlings. To determine the antioxidant activity of five extracts of four different samples, 1,1-diphenyl-2-picrylhydrazyl and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity, total phenolic content and ferrous reducing power ability were carried out. 1,1-Diphenyl-2-picrylhydrazyl radical scavenging effect of chloroform and ethyl acetate extracts of 3 days old wheat seedlings was higher than wheat grains. Chloroform, ethyl acetate and methanol extracts of 3 days old wheat seedlings exhibited higher 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging effect than extracts of other samples. The phenolic content was high in chloroform, ethyl acetate and methanol extract of 5 days old wheat seedlings. When compared with wheat grain, reducing power ability was high in chloroform, ethyl acetate and methanol extract of wheat seedlings, especially in 3 and 5 days old wheat seedlings. From the above results, it was concluded that chloroform, ethyl acetate and methanol extract of 3, 5 and 7 days old wheat seedlings showed better antioxidant activity than the wheat grain extracts. Hence, the results of the present study suggest the intake of wheat seedlings as a food supplement to combat the diseases caused by free radicals.

**Key words:** Wheat grains, 1,1-diphenyl-2-picrylhydrazyl, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), phenolic content, reducing power ability

In the present era, people are affected by various diseases due to changes in culture and lifestyle. The changes in food habits and working pattern causes stress which leads to biochemical changes. Research findings relate oxidative stress to a number of diseases like cancer, diabetes, Alzheimer's disease, coronary heart diseases, and aging<sup>[1,2]</sup>. Scientific reports emphasize the importance of dietary antioxidants as they may decrease the risk of these diseases and also improve general health<sup>[3]</sup>. Various parts of plants such as fruits, leaves, seeds, and oils contain many natural antioxidants like flavonoids, tannins, coumarins, curcuminoids, xanthenes, phenols, and terpenoids<sup>[4]</sup>. Phenols which are good sources of natural antioxidants are present in cereals and legumes<sup>[5]</sup>. Slavin *et al.*<sup>[6]</sup> reported the whole grains to be an excellent source of carbohydrates, dietary fiber, protein, E and B-group vitamins, and minerals. Several research studies revealed that the whole grain diet reduces the body mass index. Moreover reduced risk of cardiovascular disease<sup>[7]</sup>, colorectal cancer<sup>[8]</sup>

and hypertension in women<sup>[9]</sup> is also related to the whole grain diet.

Wheat is one of the major cereals in India and constitutes the staple food in North India<sup>[10]</sup>. Wheat bran, germ, and endosperm are together known as wheat kernel. The antioxidants reported from wheat are carotenoids, tocopherols, flavonoids, and phenolic acids<sup>[11]</sup>. Ferulic acid is one of the phenolic compounds available in wheat which has strong antioxidant activity<sup>[12]</sup>. The reports of phytochemicals isolated from the wheat are only limited. Wheat germ extract contains 2,6-dimethoxy-1,4-benzoquinone (DMBQ) and 1,4-benzoquinone (BQ)<sup>[13]</sup> and the

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presence of hydroxybenzoic acids (p-hydroxybenzoic acid, vanillic acid and syringic acid), hydroxycinnamic acids (p-coumaric acid, ferulic acid and ferulic acid derivatives) and a flavonoid (apigenin) was reported by Hernández *et al*<sup>[14]</sup>.

The antioxidant activities of whole wheat and milling fractions have been widely elucidated<sup>[15]</sup> against oxidation of DNA, proteins, and membrane lipids which are biologically important molecules<sup>[16]</sup>. The different parts of wheat are also tested for their antioxidant activity. Wheat bran extracts are proved to scavenge free radicals of 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS<sup>+</sup>), and peroxide anion (O<sub>2</sub><sup>-</sup>)<sup>[11]</sup>. They also have been reported to inhibit the peroxidation of human LDL cholesterol<sup>[16]</sup>, phospholipid liposomes, and hydrogen peroxide<sup>[17]</sup>. The roasted defatted wheat germ<sup>[5,18]</sup>, the oil from wheat germ<sup>[19]</sup> and wheat germ protein hydrolysates<sup>[20]</sup> have also been reported to exhibit antioxidant activity and the antioxidant activity of the coloured wheat seeds may be attributed to the natural pigments of wheat seeds<sup>[21-27]</sup>. Onyenecho and Hettiarachchy<sup>[28]</sup> demonstrated that oxidation of bulk oils was inhibited by phenols from ethanol extracts of wheat bran. Species, genotypes, and growing locations are also having pronounced effect on the difference in the antioxidant properties of wheat<sup>[29-31]</sup>.

Increase in antioxidant content of 3 to 5 days wheat sprouts was due to germination<sup>[32]</sup> and the content of fatty acids like palmitic acid, linoleic acid, and oleic acid were also found to be high in wheat sprouts<sup>[33]</sup>. Previous findings reported that aqueous and ethanol extracts of wheat grass have potent antioxidant activity and inhibit proliferation of leukemia cells<sup>[34]</sup> and the antioxidant properties were changed by lighting effects<sup>[35]</sup>.

The objective of the present study was to evaluate the antioxidant activities of five different extracts (petroleum ether, *n*-hexane, chloroform, ethyl acetate, and methanol) of wheat grains (WG), 3 days, 5 days and 7 days old wheat seedlings (WS) using DPPH, ABTS, Phenolic content and Ferrous reducing power ability.

## MATERIALS AND METHODS

1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, sodium carbonate, ascorbic acid, potassium

dihydrogen phosphate, potassium hydrogen phosphate, potassium ferricyanide, trichloro acetic acid, ferric chloride, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, mercuric chloride, potassium iodide, sulfuric acid, hydrochloric acid,  $\alpha$ -naphthol, magnesium ribbons, acetic anhydride, sodium hydroxide, and ninhydrin were purchased from HiMedia Laboratories Pvt. Ltd., India. Folin-Ciocalteu's reagent was obtained from S d fine-Chem. Ltd., India. All the solvents were of analytical grade and purchased from Sisco Research Laboratories Pvt. Ltd., India.

### Collection and preparation of plant material:

Wheat (*Triticum aestivum*) seeds (Variety: HD2833-Pusa Tripti) were obtained from the Indian Agricultural Research Institute (IARI), Wellington, The Nilgiris, India. One thousand five hundred grams of wheat seeds were taken, washed and soaked in distilled water for 12 h and kept for germination up to 3, 5 and 7 days as each 500 g and WG (without germination, 500 g) were taken after thoroughly washed with distilled water. The WG and germinated WS were shade dried and homogenized to fine powder which was used for further processing.

### Preparation of extracts:

Wheat grains and different days (3, 5 and 7 days) of wheat seedlings powder were taken and extracted separately with solvents of increasing polarity (petroleum ether, *n*-hexane, chloroform, ethyl acetate, and methanol) using a Soxhlet apparatus for 48 h, filtered and the extracts were concentrated to 2% of the original volume using Rotavapor, (Buchi-R210, Switzerland). The final yields of the extracts were used for studies.

### DPPH radical scavenging activity:

The antioxidant activities were determined using DPPH as a free radical<sup>[36]</sup>. Sixty microliters each of all the five extracts of WG, 3, 5 and 7 d old wheat seedlings (30 mg/ml concentration) were made up to 0.1 ml with methanol to which 3.9 ml (0.025 g/l) of the DPPH solution was added. A control solution was prepared without the addition of the extract and methanol was used as blank. The absorbance was measured at 515 nm. The analysis was performed in triplicate and the scavenging activities of the extracts were calculated using the following equation: DPPH radical scavenging activity (%) =  $[(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$ . Where

$Abs_{control}$  = absorbance of DPPH+methanol;  
 $Abs_{sample}$  = absorbance of DPPH+WG/WS extracts.

#### ABTS radical scavenging assay:

The total antioxidant activity of all the five extracts was measured by ABTS radical cation decolorization assay according to the method of Re *et al.*<sup>[37]</sup> described by Siddhuraju and Manian<sup>[38]</sup>. ABTS radical was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12-16 h at room temperature. Prior to assay, this solution was diluted in methanol and equilibrated at 30° to give an absorbance at 734 nm of 0.700±0.02 and in the present study, the stock solution was obtained in the ratio of 1:53 v/v. One ml of the ABTS stock solution was added to 4 µl of test sample (10 mg/ml concentration). The control was prepared without test sample solution. After the initial mixing, all the tubes were kept at 30° exactly for 30 min and absorbance was measured at 734 nm and here, methanol was used as blank. The analysis was done in triplicate and the percentage scavenging activities of the extracts were calculated using the following equation: ABTS radical scavenging activity (%) =  $[(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$ . Where  $Abs_{control}$  = absorbance of ABTS solution+methanol;  $Abs_{sample}$  = absorbance of ABTS solution+WG/WS extracts.

#### Total phenolic content:

The total phenolic content was determined according to the method described by Siddhuraju and Becker<sup>[39]</sup>. In the test tubes, 40 µl each of all the five extracts of WG, 3, 5 and 7 d old wheat seedlings (30 mg/ml concentration) were taken and made up to volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm. The analysis was performed in triplicate and the results were expressed as gallic acid equivalents (GAE) using the following linear equation based on the calibration curve:  $y = mx + c$  ( $y$  = absorbance,  $x$  = phenolic content, and  $c$  = intercept). The values of the equation was found to be  $Y = 0.045x - 0.033$  with  $R^2 = 0.997$ .

#### Reducing power ability:

The reducing power of the extracts was determined according to the method described by Oyaizu<sup>[40]</sup>. In the five test tubes, 40 µl each of all the five

extracts of WG, 3, 5 and 7 days old wheat seedlings (30 mg/ml concentrations) were taken and made up to 1 ml with methanol, further mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [ $K_3Fe(CN)_6$ ] (2.5 ml, 1%). After incubating the mixture at 50° for 20 min, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and  $FeCl_3$  (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as standard. A control solution was prepared without the addition of the extract. The analysis was performed in triplicate and the results were expressed as mean±standard deviation.

#### Statistical analysis:

All the values were presented as mean±standard deviation and the statistical analyses were done using the SPSS (version20) package for windows. Using this software, one way ANOVA followed by Tukey's method was performed to find the significant differences among the samples.

## RESULTS

#### DPPH radical scavenging activities:

Lower the absorbance of the reaction mixture, higher the free radical scavenging activity and is expressed in percentage scavenging. When the DPPH free radical scavenging activities of petroleum ether and hexane extracts of wheat grains (WG) and 3, 5 and 7 days old wheat seedlings (WS) were analysed, no significant difference was observed (fig. 1). The chloroform (65.60, 55.83 and 45.28%) extract of 3, 5 and 7 days WS and methanol extract (72.76, 48.49 and 47.8%) of 5, 3 and 7 days WS, respectively, exhibited better activity than WG (38.96 and 33.69%) whereas ethyl acetate extract of 3 days WS (64.83%) only had higher activity than WG (50.53%). Of the seedlings, chloroform, ethyl acetate of 3 day WS and methanol extract of 5 day WS had higher DPPH radical scavenging activity and there was a decreased activity in 7 day WS. The observed results showed significant ( $P < 0.05$ ) differences at 5% level.

#### ABTS radical scavenging activities:

The decolourization of ABTS radical cation assay is used to determine the antioxidant activity. ABTS

with potassium persulfate in water at overnight results in the generation of ABTS radical cations<sup>[37]</sup>. Reduction in color is directly proportional to the reduction of ABTS radical<sup>[41]</sup>. In this study, ABTS radical scavenging activities of five extracts of four (WG, 3, 5 and 7 day WS) samples were evaluated to assess the antioxidant activity. From the results (fig. 2), it was inferred that chloroform (22%), ethyl acetate (27.4%) and methanol extracts (42.47%) of 3 day old wheat seedlings exhibited higher ABTS radical scavenging activity than the WG (18.08, 19.49 and 14.06%), 5 day (18.31, 29.6 and 27.08%) and 7 day (18.59, 6.07 and 29.34%) old wheat seedlings, respectively and petroleum ether (4.58, 1.54, 4.91 and 6.71%) and *n*-hexane extracts (2.57, 4.61, 6.46 and 3.66%) of WG, 3, 5 and 7 day old wheat seedlings exhibited trivial ABTS radical scavenging activity. The observed results showed significant ( $P<0.05$ ) differences at 5% level.

#### Total phenolic content:

Total phenolic content of all the five extracts of WG, 3, 5 and 7 day old WS were evaluated and the results were tabulated (Table 1). Phenolic content was high in chloroform, ethyl acetate and methanol extract of 5 d WS. When compared with WG, the total phenolic content started to increase in 3 and 5 d WS then decreased in 7 d WS, whereas petroleum ether and *n*-hexane extracts of WS had very low level of

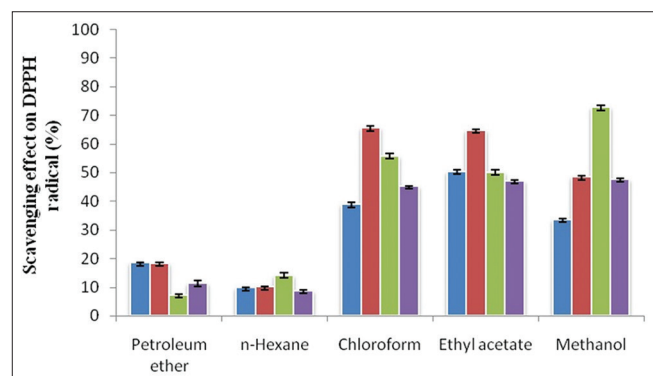


Fig. 1: 1,1-diphenyl-2-picrylhydrazyl radical scavenging activities of extracts.

■ Wheat grains; ■ 3 days old wheat seedlings; ■ 5 days old wheat seedlings; ■ 7 days old wheat seedlings.

phenols. While comparing all the samples based on the polarity, significantly the chloroform extract had high phenolic content and it decreased when go for methanol extract. Further, the significant differences were not observed between chloroform and ethyl acetate extract of all the four samples. The observed results showed significant ( $P<0.05$ ) differences at 5% level.

#### Reducing power ability:

The reducing power of all five extracts of WG and different days of wheat seedlings were investigated and results were given in Table 2. From the observed results, among the 4 samples, reducing power ability was higher in chloroform, ethyl acetate and methanol extracts of WS than in WG. There was a gradual increase in the reducing power ability of WS from 3 to 5 day and thereafter decline in 7 day WS. Further, the higher reducing power ability was observed in hexane and chloroform extracts of 3 d WS than 5 and 7 day WS. The petroleum ether and *n*-hexane extracts of WG and 7 day WS did not show significant activity but petroleum ether extract of 5 day WS and *n*-hexane extract of 3 day WS showed reducing power ability. There is no significant ( $P>0.05$ ) difference between the samples at 5% level.

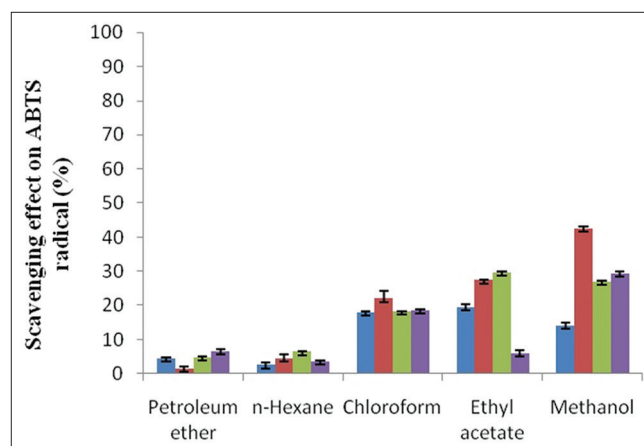


Fig. 2: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activities of extracts.

■ Wheat grains; ■ 3 days old wheat seedlings; ■ 5 days old wheat seedlings; ■ 7 days old wheat seedlings.

TABLE 1: TOTAL PHENOLIC CONTENTS OF EXTRACTS

Samples	Petroleum ether	Hexane	Chloroform	Ethyl acetate	Methanol
Wheat grains	3.82±0.28	5.55±0.08	8.41±0.06	7.4±0.09	3.54±0.05
3 days old wheat seedlings	3.48±0.04	2.16±0.08	10.29±0.06	10±0.09	8.46±0.07
5 days old wheat seedlings	1.86±0.07	2.12±0.03	12.05±0.06	12.19±0.08	10.62±0.09
7 days old wheat seedlings	2.32±0.04	1.91±0.05	10.77±0.04	10.44±0.05	6.19±0.05

Results are expressed as mean±SD in mg GAE/g of extract ( $n=4$ ,  $P<0.05$ ), significant differences at 5% level. GAE: Gallic acid equivalent, SD: standard deviation

**TABLE 2: REDUCING POWER ABILITY OF EXTRACTS**

Samples	Petroleum ether	Hexane	Chloroform	Ethyl acetate	Methanol
Wheat grains	1.6±1.26	6.6±0.67	11.81±0.43	7.22±1.05	0.9±0.64
3 days old wheat seedlings	5.27±0.43	33.19±1.05	35.97±0.64	13.4±0.84	19.31±0.43
5 days old wheat seedlings	12.01±0.84	9.24±0.64	20.49±1.05	14.24±0.84	26.6±1.26
7 days old wheat seedlings	2.32±0.04	1.91±0.05	10.77±0.04	10.44±0.05	6.19±0.05

Results are expressed as mean±SD in mg AAE/g of extract (n=4, P<0.05), significant differences at 5% level. AAE: Ascorbic acid equivalent, SD: standard deviation

## DISCUSSION

Natural antioxidants are present in many plants which play a major role in the reduction of free radicals and lead to the prevention of mutagenesis, carcinogenesis and aging<sup>[42]</sup>. Further, they reduce the risk of many diseases including cancer, diabetes, Alzheimer's disease, coronary heart diseases<sup>[1-3]</sup>. In the present study, radical scavenging activity of wheat grain, 3, 5 and 7 day old wheat seedlings were evaluated using DPPH and ABTS radical scavenging assay, the results of which showed that the chloroform, ethyl acetate and methanol extracts of WG, 3, 5 and 7 day old wheat seedlings had significant free radical scavenging activity. Further, petroleum ether and hexane extract of four samples showed very low free radical scavenging activity. From the results, chloroform, ethyl acetate and methanol extract of wheat seedlings showed better activity than the WG and of all the four samples, mostly 3 day old wheat seedlings showed higher radical scavenging activity.

In the present study, compared to WG, 3, 5 and 7 day WS had higher radical scavenging activity. It is interesting to note an earlier report which stated that thermal processing of 2 day wheat sprouts and 10 day seedlings increased the DPPH radical scavenging activity<sup>[43]</sup>. Total phenolic content is directly proportional to the antioxidant activity because phenolics are the main antioxidant components present in the plant extracts<sup>[44,45]</sup>. Previous report states that the single group of plant phenolics and the flavonoids were used in many applications like antibiotics, antiulcer, and antiinflammatory agents<sup>[46]</sup>. The present investigation demonstrated that chloroform extracts of all the four samples have more phenolics. Phenolic content of 2 day wheat sprouts (9%) and 10 day wheat seedlings (8%) were increased by thermal processing<sup>[43]</sup>. In the current study, when comparing with WG, phenolics are high in WS. Further, the results of this study showed that phenolics are high in 3 and 5 day seedlings and it started to decrease in

7 day wheat seedlings. From the earlier reports it was observed that 10 day old wheat seedlings have the lower content of phenolics than 2 day wheat sprouts which is in consistent with the results of present study.

Qingming *et al.*<sup>[47]</sup> reported that reducing power of the extracts significantly play a major role in exhibiting the antioxidant activity and it leads to the reduction of Fe<sup>3+</sup>/Ferricyanide complex to Fe<sup>2+</sup> form. The present study about reducing power ability of all the five extracts of 4 samples revealed that chloroform extracts of all the 4 samples have higher reducing power ability than the other extracts. Among these 4 samples, chloroform extract of 3 day old wheat seedlings had high reducing power ability. Liyana-Pathirana and Shahidi<sup>[15]</sup> stated that wheat grain has better reducing power capacity than wheat flours but in the present experiment, WG have lower reducing power ability than the wheat seedlings.

ABTS radical scavenging was not observed in the flour samples of 3 hard winter wheat varieties grown at five different locations of Eastern Colorado<sup>[31]</sup> but Lv *et al.*<sup>[48]</sup> reported that wheat flour of ten wheat varieties grown at maryland showed good ABTS radical scavenging activity. In the present study, WG and WS grown in India have potential ABTS radical scavenging capacity. The results of the present study is in confirmation with the report of Lv *et al.*<sup>[48]</sup> but contradict the report of Yu *et al.*<sup>[31]</sup>. Hence, it was concluded that the growing locations and varieties of wheat may have an effect on the radical scavenging activity.

From the results of all the assays, chloroform extract of all the four samples showed better antioxidant activity than the other extracts of 4 samples. When compared with WG, 3 and 5 day old wheat seedlings showed better antioxidant activity. The results also revealed that the antioxidant activity increased gradually when the wheat grains germinate up to 5 day and started to decrease in 7 day. Hence, it was

concluded that 3 day old wheat seedlings may help to prevent/combat diseases caused by oxidative stress.

The results of the present study recommend the 3 day old wheat seedlings as food supplement in the treatment of diseases caused by the free radicals. As wheat is already a staple diet, 3 day WS can be advised to the patients and can be tested for synergism with regular medications in combating free radicals.

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### Conflicts of interest:

There are no conflicts of interest.

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