

In vitro antioxidant and antihaemolytic potential of *triticum aestivum* grass

Abstract

Triticum aestivum belongs to family Poaceae. Its grass is rich in nutrient. In present study, four different extracts (petroleum ether, ethyl acetate, n-butanol, aqueous) of *Triticum aestivum* grass were used to test the antioxidant and antihaemolytic activity. In phytochemical screening n-butanol and aqueous extract were found to possess high concentration of flavonoids when content compared with other phytochemicals were present in moderate and low concentration. Different parameters i.e. reducing power, NO radical scavenging activity, DPPH radical scavenging activity, Superoxide dismutase assay were used to test antioxidant power of *Triticum aestivum* grass. n-butanol and aqueous extracts scavenge the free radicals maximally. Maximum antihaemolytic activity was found in n-butanol extracts. Thus, it is deduced that *Triticum aestivum* shows maximum antioxidant and antihaemolytic activity in n-butanol and aqueous extract in comparison to other two extracts.

Keywords: *triticum aestivum*, antioxidant, antihaemolytic, sod, phytomedicine

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Sonia Johri, Neha Khan

Assistant Professor, ITM University, India

Correspondence: Sonia Johri, Assistant Professor, ITM University, Gwalior, Diploma in clinical pathology and diagnostic technology, and Bachelor's in Pharmacy, Jiwaji, University, Gwalior, India, Email johrisonia@gmail.com

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Introduction

Plants are the good source of phenolic and polyphenolic compound having potent antioxidant activities which can be exploited in preparation of food and pharmaceutical product.¹ Being a rich source of secondary metabolites, medicinal plants are used by native people from ancient times and play a pivotal role as therapeutic and lifesaving drugs.² Herbal medicines are adventitious over synthetic medicines as they have varied properties and 70% of the world population is dependent on such population.³ Use of phytomedicine is gaining importance as they have the capability to treat many diseases without any side effects and used in preparation of creams, decoctions, syrups and infusions.

T. aestivum (wheat) belongs to Poaceae family is maximum edible cereal rich in vitamins, minerals and proteins as compared to mature cereal plant.⁴ It is rich in chlorophyll, minerals (Ca, K, Na, Mg, S) and contain 17 amino acids, vitamins and active enzymes.⁵ It is usually used as a herbal medicine in many diseases like thalassemia and myrloidysplastic syndrome.^{6,7} In addition is also been supposed to strengthen the immune system and increase the life span of cancer patients by preventing the spread of cancer cells.⁸

Oxidative balance is defined to be a state when rate of production and removal of free radicals is balanced. Any change in the decrease rate of removal or increase production of free radicals leads to the generation of ROS which finally results in oxidative stress. An antioxidant can scavenge the free radicals due to their redox hydrogen donors and singlet oxygen quencher.⁹ Due to insufficient number of antioxidants, free radicals cannot be neutralized and results in oxidative stress are generated. Oxidative stress is lead to several diseases like cardiovascular diseases, cancer and aging.¹⁰

Haemolytic anaemia is a condition in which red blood cells are destroyed and removed from the bloodstream before their normal lifespan is over resulting in reducing number of cells. It may be caused due to various causative agents such as environmental toxicants, malaria, and dengue etc.¹¹ Many researchers supported the study of mechanism of haemolytic actions of several agents on RBCs and it has been reported that haemolytic injury is related with oxidative stress. Haemolytic agents have been reported to cause membrane lipid per oxidation and denaturation of cytoskeletal protein.¹²

Wheatgrass fresh juice is used as a nutritional supplement since decades.¹³ The aim of this study was to evaluate the antioxidant and antihaemolytic properties of *T. aestivum* grass in four different extracts and its use to cure haemolytic anaemia.

Materials and Methods

Extraction of plant material

The grass of *T. aestivum* was cultivated, chopped, dried in shade and powdered with a mechanical grinder. 50g of powder was weighed and extracted with soxhlet apparatus using various solvent according to their polarity i.e. petroleum ether, ethyl acetate, n-butanol and water. After solvent extraction, it was evaporated to obtain a powdered extract for various biochemical analyses.

Qualitative phytochemical screening of *T. aestivum* grass in four extracts

Preliminary phytochemical screening of the extracts was performed for the presence of alkaloids, flavonoids, steroids, tannins, saponin and phenol using the standard procedures.

- Alkaloids:** To 1ml of extract, 2–3 drops of Wagner's reagent were added. The appearance of pale or white precipitate indicated the presence of alkaloids.¹⁴
- Steroids:** To 2ml of extract, 2ml of chloroform and 2ml of concentration sulphuric acid was added. Tubes were shaken and allowed to stand. Formation of red colour chloroform layer indicates the presence of steroids.¹⁵
- Tannins:** 3 ml of extracts was treated with 1% lead acetate solution. A red or yellow colour precipitate was formed, indicating the presence of tannins.¹⁴
- Saponins:** To 3ml of extracts, few drops of sodium bicarbonate was added and shaken vigorously for 3 min. Honey comb froth was formed, showing the presence of saponins.¹⁵
- Phenolic:** To 1ml of extracts, 2ml of distilled water and few drops of 10% ferric chloride solution were added. Formation of blue or green colour indicates the presence of phenols.¹⁴

f. Flavonoids: To 2 ml of each extract was added few drops of 20% sodium hydroxide, formation of intense yellow colour is observed, and by adding 70% hydrochloric acid yellow colour disappeared. Disappearance of yellow colour indicates the presence of flavonoids in the extract.¹⁶

Reducing power assay

2.5ml phosphate buffer (0.2M, pH 6.6) and 2.5ml 1% potassium ferrocyanide was mixed in 1ml of different fraction of plant extract at various concentration (20, 80, 120, 240µg/ml) diluted in distilled water. The test tubes were incubated at 50°C in water bath for 10 min. followed by addition of 2.5ml 10% TCA and centrifuge at 3000 rpm for 10 min. 2.5ml of upper layer was collected, and 2.5ml distilled water was added followed by 0.5 ml 0.1% FeCl₃ (freshly prepared). Increase in absorbance was measured at 700 nm against a suitable blank.¹⁷

NO radical scavenging activity

NO radical scavenging activities of plant extract in different fraction were examined by Royer et al.¹⁸ To 200 µl sodium nitroprusside (5Mm), 800µl extracts (0.1–1 mg/ml) was added dissolved in PBS (25mM, pH 7.4). The mixture was incubated for 2.5 hr at 37 °C under normal light and followed incubation in dark for 20 min., 600µl Griess reagent (1% sulphanilamide, 0.1 % naphthyl ethylene diamine hydrochloride in 2% phosphoric acid) was added and incubated for 40 min. at room temperature and absorbance was measured at 540nm against a suitable blank (2ml H₂O and 0.6 ml Griess reagent). Control (1.6 ml H₂ O, 400µl SNP and 600µl Griess reagent) was prepared and percent of inhibition was calculated by using this equation.

Percentage inhibition = $\frac{\text{OD of control} - (\text{OD of extract} / \text{OD of control}) \times 100}{1}$ (1)

DPPH free radical scavenging activity

DPPH free radical scavenging activity of plant extracts in different fraction was examined by Blois method.¹⁹ with minor modification. To 2ml of plant extract taken at various Concentrations (20–100µg/ml), 1 ml of DPPH solution (0.1 Mm in methanols) was added, shaken well and incubated at 37 °C for 30 min in dark. Decrease in absorbance was measured at 517nm spectrophotometrically against a suitable blank and control tube which contains methanol and DPPH without extract. The percentage of inhibition was calculated by using equation 1.

Superoxide dismutase (sod) assay

This assay was done by the method of Kakkar et al.²⁰ Fresh wheatgrass (0.5g), were ground with 5.0ml of sodium phosphate buffer (50mM, pH 6.4) centrifuged at 2000g for 10g and supernatant were collected used for the assay.

Assay mixture contained 1.2ml of Sodium pyrophosphate buffer (0.025M, pH 8.3), 0.1ml of phenazine methosulphate (186µM), 0.3ml of nitro blue tetrazolium (300µM), 0.2ml of plant extract at different concentration (20–100µg/ml) and distilled water in a total volume of 2.8ml. The reaction was initiated by addition of 0.2ml NADH (780µM). The mixture was incubated at 30 °C for 90 secs. 1ml glacial acetic acid to stop the reaction. Reaction mixture was shaken with 4 ml n-butanol, and allowed to stand for 10 min. It was centrifuged at 2000g for 10min. and butanol layer was collected. The intensity of chromogen n-butanol layer was measured at 560nm. The percent of inhibition was calculated by using equation (2).

Percentage inhibition = $\frac{\text{OD of control} - \text{OD of extract}}{\text{OD of control} \times \text{mg of protein}} \times 100$ (2)

Antihaemolytic activity/ membrane stabilizing activity

The following two methods were used for conducting *in vitro* membrane stabilizing assay:

i. Hypotonic solution–induced haemolysis: This method was done by method of Shinde et al.²¹ 5ml of whole blood of a healthy person in heparinized tube was collected. The blood was centrifuged at 3000g for 10 min. supernatant was removed and RBCs were washed three times with sodium chloride isotonic solution (154mM NaCl) in 10mM Sodium phosphate buffer (pH 7.4) through centrifugation using the same volume as supernatant. Finally, RBCs were resuspended in the same volume of isotonic buffer solution. 0.5 ml of RBCs suspension was mixed with 5ml of hypotonic solution (50mM NaCl in 10mM sodium phosphate buffer pH 7.4) containing 0. 5ml plant extracts (10mg/ml). The control sample was prepared by 0.5 ml suspension mixed with hypotonic buffered saline. The mixture was incubated for 10 min. at room temperature, centrifuged at 3000g for 10min. and the optical density of supernatant was measured at 540nm.

ii. H₂O₂ induced haemolysis: This method was done by Ebrahimzadeh et al.²² 5ml whole blood was collected in heparinized tube, centrifuged (10 min. at 1500g) RBCs were separate out from plasma and buffy coat was obtained, after three washes in 10 volumes of 10mM/L PBS, washed RBCs were diluted in PBS to obtained 4% suspension. To 2ml RBCs suspension, 1ml of plant extracts (10mg/ml) was added and incubated for 5 min. at room temperature, 0.5ml H₂O₂ was added shaken well and incubated at 37 °C for 30 min. Supernatant was collected and absorbance was read at 540 nm. Percentage inhibition of was calculated by using equation 1.

iii. Statistical analysis

All data were expressed as mean+standard deviation from three repeats (n=3) experiments. The free radical scavenging activity was calculated by using Graph Pad Prism 7 ink. Software.

Results and discussion

The qualitative test for alkaloids, flavonoids, tannins, phenols, saponins, and steroids from four different extract. *T. aestivum* grass was investigated. The Table 1 explains the phytochemical screening indicates the presence of alkaloids, flavonoids, tannins, phenols, saponins, steroids. Flavonoids were present in very high concentration which indicates rich antioxidant property. Studies have shown that many of the phytochemicals possess anti-inflammatory, anti-diabetic and antimicrobial activities.²³ In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents.²⁴ Wheatgrass proved to be an effective radical scavenger in all antioxidant assay.²⁵

Figure 1 shows the reducing power of four extracts of wheatgrass at 700 nm. At physiological pH (7.4), ferrous ions (Fe²⁺), in the presence of oxygen and phosphate ions (PO₄²⁻), exist only transiently before being auto-oxidized to ferric ion (Fe³⁺). During this process, an electron is transferred from iron to oxygen to form a superoxide radical anion and hydroperoxyl radical (HO₂·) by Fenton reaction. Highest reducing abilities 1.008 were observed in aqueous extract of wheatgrass followed by n-butanol extracts (0.760) at 240µg/ml concentration. The high reducing power which was concentration-dependent of the aqueous extract of wheat grass may be attributed

to the fact that the extract possessed the ability to be effective, under physiological conditions, in reducing the transition state of iron and consequently, the rate at which superoxide and hydroperoxyl radicals are generated from the metal. A strong relationship between the total phenolic content and reducing activity in fruits and vegetables has been reported.²⁶ Therefore, the reducing power of the extract may be attributed to its phenolic content.

Table 1 Phytochemical screening of *T. aestivum* in four extracts.

Extract	Alkaloids	Flavonoids	Tannins	Phenols	Saponins	Steroids
Petroleum ether +	++	+	+	+	+	+
Ethyl acetate	+	++	+	+	+	+
n-butanol	++	+++	+	+	+	+
Aqueous	++	+++	+	+	+	+

+: low concentration; ++: Moderate concentration; +++: high concentration

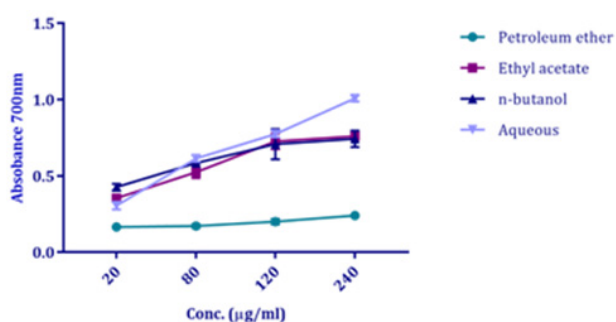


Figure 1 Reducing power of *T. aestivum* grass in four extracts.

Figure 2 represents the NO scavenging activity of wheatgrass in four extracts. The maximum scavenging activity was found in aqueous extract 60.7 at 1mg/ml as compared to others extracts.

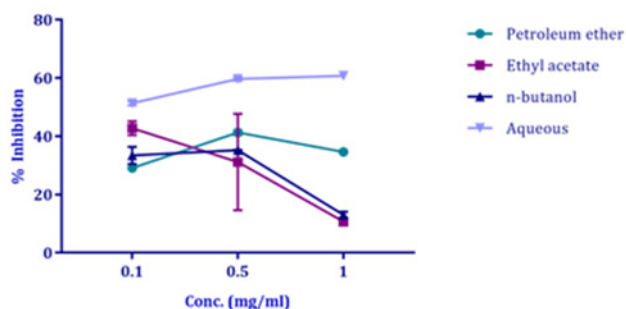


Figure 2 NO radical scavenging activity of *T. aestivum* grass in four extracts.

Free radicals arbitrarily react with lipids, proteins and nucleic acids causing oxidative stress and damage in these macromolecules leading to age-related and chronic diseases.^{27,28} Flavonoids or bioflavonoids are ubiquitous group of polyphenolic substances which can be correlated to the maintenance of antioxidant status.²⁹ Numerous studies have shown that flavonoids possess potent antioxidant activities capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals.³⁰ The antioxidant potential of wheatgrass extracts was investigated in the search for new bioactive compounds from natural resources. The obtained results for DPPH agree with the phenolic contents which was determined for each sample. Plant polyphenols act as reducing agents and antioxidants by the hydrogen-donating property of their hydroxyl groups.³¹ The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability.³²

Hence, we can conclude that polyphenols are responsible for the observed antioxidant activity in this study. Figure 3 shows the DPPH radical scavenging activity. The highest scavenging activity was observed in n-butanol extract (60.3 at 100µg/ml) followed by ethyl acetate extract (52.7 at 80µg/ml). Though the DPPH radical scavenging abilities of the extracts were less than those of ascorbic acid the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

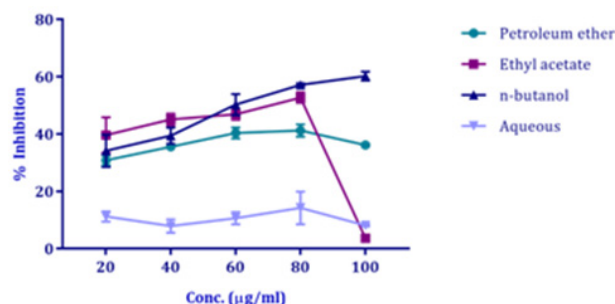


Figure 3 DPPH free radical scavenging activity of *T. aestivum* grass in four extracts.

Superoxide dismutase activity from the fresh sample was examined at various concentration of extract. Table 2 represent the SOD activity of wheat grass. Percentage of inhibition was found to be maximum at 60–80µg/ml. SOD is thought to play a very important role in protecting living cells against toxic damage to free radicals. The enzyme catalyses the dismutation of two superoxide radicals (O₂⁻) into O₂ and H₂O₂.³³ Table 2 shows the activity of pure superoxide dismutase assay at different concentration. Result indicates maximum inhibition at 60–80µg/ml concentration. This concentration is effective to reduce O₂⁻ radical. The SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen, thereby reducing the likelihood of superoxide anion reacting with nitric oxide to form reactive peroxynitrite.³⁴

Table 2 Superoxide dismutase activity of *T. aestivum* grass.

S.No.	Concentration (µg/ml)	% Inhibition
1	20	21.9+0.6
2	40	37.1+3.7
3	60	40.9+0.63
4	80	39.3+2.2
5	100	36.5+2.5

Data represent as mean + SD (n=3).

Table 3 explains the antihaemolytic activity. In case of hypotonic solution induced haemolysis, the maximum level of membrane stabilizing activity was observed by n-butanol fraction of wheatgrass which was 75% by H₂O₂ induced haemolysis and 82% by hypotonic solution induced haemolysis. The minimum antihaemolytic activity was exhibited by petroleum ether extract of wheatgrass. The ranges of antihaemolytic of all extracts were between 65–82%. The extract was quite effective in membrane stabilization studies and stabilization of erythrocyte which could be correlated to the stabilization of the lysosomal membrane.³⁵ Therefore, the high membrane stabilizing activity of the extract may be due to the presence of flavonoids or saponins since tannins were minimally present in the extract. The findings on the aqueous extract of wheat grass leaves in this study are like those reported by Sadique *et al.*³⁶ & Olugbenga *et al.*³⁷

Table 3 Induced haemolytic activity of *T. aestivum*.

Fractions	H ₂ O ₂ Induced Antihemolysis Activity (10mg/ml)	Hypotonic Solution Induced Antihemolysis (10mg/ml)
Control	100%	100%
Petroleum ether	65%	62%
Ethyl acetate	68%	68%
n-butanol	82%	75%

Conclusion

In this study, *T. aestivum* grass shows maximum antioxidant and antihemolytic activities in aqueous and n-butanol extracts in comparison to petroleum ether and ethyl acetate extracts. It may be due to the presence of high flavonoid content in these two extracts. These extracts have potent antioxidant and antihemolytic activities. Further study needs to show the antioxidant and antihemolytic activities more effectively. Further investigation both *in vivo* and *in vitro* needs to be explored for antianemic and antioxidant efficacy of the *T. aestivum* grass extracts.

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Conflicts of Interest

Authors declare no conflict of interest.

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