ORIGINAL ARTICLE

# *Hordeum vulgare* (Barley grass) Scavenge Free Radical and Inhibits Formation of Advanced Glycation End Products Formation.

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## ABSTRACT

**Introduction:** Advanced glycation end products (AGEs) formation is due to glycation of lipids, proteins, and nucleic acids. AGEs play a vital role in Diabetes Mellitus (DM) and cardiovascular complications and is enhanced by oxidative stress. *Hordeum vulgare* (Barley Grass) is a cereal plant of grass family poaceae and has been reported to exhibit antidiabetic and antioxidant characteristics and prevention of cardiovascular disease. Current experiment focused on the evaluation of antioxidant and anti-advanced glycation end products formation effect of *Hordeum vulgare* water extract. **Methods:** Free radical scavenging ability of *Hordeum vulgare* water extract, was evaluated using DPPH, nitric oxide, hydrogen peroxide, lipid peroxidation, FRAP and metal ion chelating. Total phenolic content of *Hordeum vulgare* was also analysed. In-vitro antiglycation activity was done using BSA-MGO, BSA-Glu. Phytochemical screening was performed to detect the presence of flavonoids, saponins, tannins, steroids and terpenoids. **Results:** The results showed *Hordeum vulgare* water extract ameliorated the formation of AGE and scavenge free radical from DPPH, Nitric oxide, hydrogen peroxide, Lipid peroxidation, FRAP and Metal ion chelating. Phytochemicals such as saponins, tannins, flavonoids, steroids and terpenoids were detected in *Hordeum vulgare* water extract. **Conclusion:** *Hordeum vulgare* water extract reveals antiglycation and free scavenging properties.
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**Keywords:** *Hordeum vulgare*, AGE, Antioxidant, Diabetes mellitus, Oxidative stress

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## INTRODUCTION

Glycation is a condensation reaction of sugar and protein by linking the reducing end carbonyl groups of sugar to the amino groups under high temperature (1). Advanced glycation end products (AGEs) are formed from Mailard reactions. They are produced through three (3) stages, initial step is the formation of glycosylamines/Schiff bases, as well as their amadori rearrangement product, the intermediate stage involves break down of amadori products into several fission components of the highly reactive sugar - amino complex compound. The final stage involves precipitation of amino compounds and sugar particles into polymerized protein and brown pigments (2).

Maillard reaction can either happen endogenously through non-enzymatic reaction or exogenous through intake of foods processed under high temperature. Endogenous AGEs formation can happen inside the body because of high dietary sugar intake and hyperglycemia condition (3). Factors affecting exogenous AGEs formation are processed food that are high in protein and sugar content. Lifestyle like diet and smoking plays vital role in the rate of formation of AGE (4). Increase in the formation and accumulation of AGE increases the risk of DM and associated complications. Oxidative stress and protein glycation are linked under hyperglycemic conditions in diabetic patients (5). Disparity between free radicals and antioxidant system will lead to oxidative stress (AOS). Accumulation of reactive oxygen species without effective action on the exogenous and endogenous antioxidant systems, causes generation of highly reactive electrophilic compounds. This reactive component reacts with amino group of proteins to generate reactive dicarbonyl which is a precursor of

AGE. These reactive dicarbonyl compounds will glycate with proteins to form AGEs (6).

Antioxidants delays damage of cells through quenching or inhibition of free radical generation (7). They stabilize or prevent free radicals damage by donating electrons to damage cells. They also transform free radicals into products in the body, thereby eliminating them out of the system (8). Intake of antioxidant rich foods such as vegetables and fruits are effective in the protection against chronic diseases resulting from free radicals (9). Antioxidants shows health benefits because of the presence of vitamins and polyphenols. Interaction of free radicals and macromolecules such as protein, lipids and nucleic acid stimulates apoptosis which causes various physiological, cardiovascular, and neurological disorders (10). The current study evaluated the ability *Hordeum vulgare* (barley grass), water extract to scavenge free radical generation and inhibition of accumulation of AGE. *Hordeum vulgare* (barley grass) is the world's fourth most important cereal crop. It is mostly famous for its high amount of fibers such as β-glucan which is a soluble fibre that has some impressive health-related benefits such as lowering blood cholesterol, reducing coronary heart disease risk and slows glucose absorption as well as maintain glycemic control (11). Previous studies shows that young leaves of *Hordeum vulgare* contain high flavonoid which exhibit a high antioxidant activity (12). Antioxidant property is useful in the treatment of the disease caused by oxidative stress damage (13). Current experiment reports *Hordeum vulgare* (barley grass) water extract possess in vitro antioxidant and anti-AGE activities.

## MATERIALS AND METHODS

### In-vitro antioxidant activity

#### *DPPH radical scavenging activity*

DPPH radical scavenging activity was evaluated according to method described by Okechukwu et al 2019 (14), One milliliter (1 ml) of sample at concentration of (10-100 µg/ml) was evaluated, BHT and DPPH without extract was used as negative control. The absorbance was measured at 517 nm against blank using a 96 well plate reader. The results were expressed in terms of $IC_{50}$value.

The percentage of DPPH free radical inhibition was calculated using the equation (1) below

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control-Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Where Abs control = Absorbance of the control; Abs sample = Absorbance of the sample

#### *Nitric oxide radical scavenging activity*

The experiment was performed according to the method described by Mridula et al 2021 (15) with minor modification. A mixture of 0.5 ml of sample (10-100 µg /ml), 1 ml of sodium nitroprusside (10 mM) and 0.5 ml of phosphate buffer saline were vortexed for 5 seconds. After incubation for 150 mins in dark at room temperature, 1 ml of sulfanilic acid reagent (0.33 % in 20 % glacial acetic acid) were added to each test tubes of the mixture. After vortex and incubation for 5 minutes, 1 ml of 0.1 % N-1-naphthyl ethylene diamine dihydrochloride was added to each test tubes and incubated at 25°C for 30 minutes. The absorbance of pink colored chromophore formed was measured at 540 nm against blank using 96 well plate reader. BHT was used as positive control. The percentage of NO free radical inhibition was calculated using the equation 3.1 above. The results were expressed in terms of $IC_{50}$value.

#### *$H_2O_2$ radical scavenging activity*

$H_2O_2$ radical scavenging activity was measured following method described by Mridula et al 2021 (15) with minor modifications. A solution of $H_2O_2$ (43mM) containing 100 ml of sodium phosphate buffer (0.1 M; pH 7.4) was prepared. Sample of extract concentration range of 10-100 µg/ml and at volume of 200 µl was added to 1.2 ml of $H_2O_2$ and 600 µl of phosphate buffer and vortexed. The absorbance value of the reaction was measured at 230 nm against blank using a 96 well plate reader. 800 µl of phosphate buffer and 1.2 ml of $H_2O_2$ against blank was used as negative control. The percentage of $H_2O_2$ free radical inhibition was calculated using the equation in section (1). The results were expressed in terms of $IC_{50}$value. The positive control used was BHT.

#### *Lipid peroxidation radical scavenging activity (TBARS)*

Lipid peroxidation was measured by thiobarbituric acid reactive species (TBRAS). Egg homogenate is known to be rich in lipid medium and substrate, therefore it was used for this experiment. The experiment was done according to established method by (15). Egg homogenate was used as a rich lipid medium and substrate. Lipid peroxidation activity was measured by thiobarbituric acid reactive species (TBRAS). One milliliter (1mL) of sample at concentrations (10-100 µg /ml), was mixed with 0.5 mL of 10 % v/v of egg homogenate. Distilled water and FeSO4 (0.07 M) were added to the mixture to induce lipid peroxidation and incubated for 30 minutes. After incubation acetic, TBA, SDS and TCA were added, vortexed and heated at 95°C for 60 minutes. Similar replicate was prepared using same method but incubated without TBA and used as negative control and BHT was used as positive control. After incubation butanol was added, centrifuged at 5000 rpm for 10 minutes, absorbance of organic layer was measured at 532 nm and % inhibition was calculated using above equation (1).

#### *Chelating Capacity*

Chelating capacity was measured using the method as reported by Mridula et al 2021 (15) with minor

modification. One milliliter (1mL) of sample ranger 10-100 µg/ml, was mixed with $FeSO_4$ (0.1mM) and ferrozine (0.25 mM) incubated at room temperature for 10 minutes. Absorbance was read at 562 nm against a blank solution using 96 well plate reader and EDTA was used as a positive control. The percentage of ferrous ion chelating capacity was calculated using the equation (1). The results were expressed in terms of $IC_{50}$ value.

### *Ferric Reducing Antioxidant Power (FRAP)*

Ferric Reducing Antioxidant Power (FRAP) was measured by adding 0.2 mL of sample at 10-100 µg/ml to a mixture of FRAP reagent which is prepared by mixing sodium acetate buffer (0.3 M; pH 3.6), 2, 4, 6-tri-(2-pyridyl)-s-triazine (TPTZ) solution (20 mM) and $FeCl_3.6H_2O$ (20 mM) at the ratio of 10:1:1. Ten minutes (10 Mins) was allowed for the mixture to incubate at room temperature. The experiment applied method described by Gayathri et al 2014 (16) with minor modifications. Ascorbic acid was used as standard reference control and the percentage of ferric ions inhibition was calculated using the equation (1). The increase in absorbance was measured using a 96 well plate reader at 593 nm. Ascorbic acid was used as positive control and the results were expressed in terms of $IC_{50}$value.

### Total Phenolic Content

Total Phenolic Content of Vulgare water extract were performed by using the Folin-Ciocalteu's reagent (Merck). Folin-Ciocalteu's reagent were mixed with extract and sodium carbonate. The mixture was centrifugated, left in dark for 30 minutes and absorbance was measured at 765 nm using 96 well plate reader. Total phenolic content of sample extract was expressed as gallic acid equivalent (GAE) in mg per 1g of sample extract. Experiment was done following established method described by Mahboubi et al 2015 (17).

### In-vitro anti-glycation assays

### *Bovine-Serum Albumin (BSA-Glucose) assay*

Reagent for BSA-Glucose assay were prepared by mixing 10 mg/ mL of Bovine Serum Albumin (BSA), 500 mM of glucose and extract at concentration of 10µg/mL-50µg/mL in 0.2M sodium phosphate buffer (pH 7.4). Sodium azide (0.5mM) was added to the mixture after 5 minutes incubation. The tubes were incubated for 7 days at 37°C in dark and aminoguanidine was used as a positive control. Glycation was measured as fluorescence intensity (excitation wavelength of 370 nm and emission wavelength of 440 nm) using Omega micro plate reader. The experiment was performed following method proposed by Mridula et al 2021 (15). The percent inhibition was calculated by using Equation (1).

### *BSA-Methylglyoxal (BSA-MGO) assay*

Bovine serum albumin (BSA)-Methylglyoxal assay was performed according to the Mridula et al 2021 (15) Bovine serum albumin (BSA) (20mg/mL) and methylglyoxal (60mM) were dissolved in 0.1M sodium phosphate buffer (pH 7.4). 3mL of reaction mixture containing 1mL of Bovine serum albumin (BSA), 1mL of methylglyoxal and 1mL of extract at different concentrations (10µg/mL-50µg/mL) were prepared. The reaction mixture was mixed thoroughly and incubated for 5 minutes. After incubation, 0.5mL of sodium azide (0.2g/L) was added to each tube. The tubes were tightly capped and incubated at 37°C for 7days in dark conditions. Aminoguanidine was used as a positive control inhibitor for glycation of proteins. After 7 days of incubation, the samples were measured at fluorescence intensity (excitation wavelength of 370nm and emission wavelength of 420 nm) using Omega micro plate reader. The experiment was carried out in triplicates. The percentage of inhibition was calculated by using the Equation (1).

### Phytochemical screening

Colorimetric tests were performed for phytochemical analysis of *Hordeum vulgare* water extract. Test for Saponins and flavanoids were done separately following the method developed by Ali et al 2018 (18). Distilled water of volume 5 mL water was mixed with 0.1 g of *Hordeum vulgare* water extract,the mixture was filtered and shaken for 30s. Formation of froth confirms the presence of saponins, and the presences of a yellowish orange colour confirms the presence of flavonoids.

Test for tannin was conducted according to the method proposed by Ali et al 2018 (18). *Hordeum vulgare* water extract at 1 g was mixed with 10 ml of distilled water, 2 mL of diluted colorless filtrate was mixed with 2 drops of $FeCl_3$ reagent. The presence of blue or blackish green colour confirms the presence of tannin.

*Hordeum vulgare* water extract at 5 mg was dissolved in 1 mL of chloroform, mixed with 1 mL acetic acid and few drops of concentrated $H_2SO_2$ for the test of steroids and terpenoids according to the method by Ali et al 2018 (18). The appearance of pink or violet colour ring at bottom indicates the presence of steroids, the appearance of reddish brown at bottom layer indicates the presence of terpenoids.

## RESULTS

### In-vitro antioxidants capacity

### *DPPH radical scavenging capacity*

The antioxidant capacity of *Hordeum vulgare* was evaluated using DPPH scavenging assay. Based on the $IC_{50}$ (µg/mL), *Hordeum vulgare* extract showed significantly higher inhibition of DPPH radical with $IC_{50}$ = 43.80 ±0.22 µg/mL followed by the BHT at $IC_{50}$ = 73.13 ±0.16 µg/mL as shown in Figure 1(a).

### *Hydrogen Peroxide Radical Scavenging capacity*

Based on the $IC_{50}$ (µg/mL), *Hordeum vulgare* indicate significant ($p<0.01$) higher hydrogen peroxide radical scavenging effect with $IC_{50}$ value of 11.13 ± 2.17 µg/ml compared to our standard, BHT, 18.60 ± 0.25 µg/ml as shown in Figure 1(b).

### *Nitric Oxide Scavenging capacity*

The results shows that there is no statistically significant difference detected in the activity between *Hordeum vulgare* and BHT ($p>0.05$) with respect to their IC_50 values of 5.14 ± 1.33 µg/ml and 3.27 ± 0.93 µg/ml respectively, shown in Figure 1(c). Therefore, the inhibition of NO radicals by *Hordeum vulgare* is as effective as BHT.

### *Chelating Capacity*

The effects of Hordeum and EDTA on the metal chelating ion was evaluated using chelating capacity activity as shown in Figure 1(c). Comparative $IC_{50}$ indicates that EDTA had significant ($p<0.001$) stronger iron cheating capacity with $IC_{50}$ 5.29 ± 0.28µg/ml compared to *Hordeum vulgare* with $IC_{50}$ value of 7.42 ± 0.17 µg/ml as shown in Figure 1(d).

### *Lipoperoxidation radical scavenging capacity (TBRAS)*

The results show that BHT demonstrates significant ($p<0.01$) higher effect on the inhibition of MDA formation compared to *Hordeum vulgare* with the $IC_{50}$ values of 9.48 ± 1.75 µg/ml and 15.87 ± 1.88 µg/ml respectively as shown in Figure 1(e).

### *Ferric Reducing Antioxidant Power (FRAP)*

The reducing ability of *Hordeum vulgare* and ascorbic acid was evaluated using Ferric reducing capacity. According to results based on $IC_{50}$, ascorbic acid had significantly ($p<0.01$) higher reduced capacity with $IC_{50}$ = 0.32 ± 0.35 µg/ml than *Hordeum vulgare* with $IC_{50}$ value of 33.11 ± 0.3 µg/ml as shown in Figure 1(f).

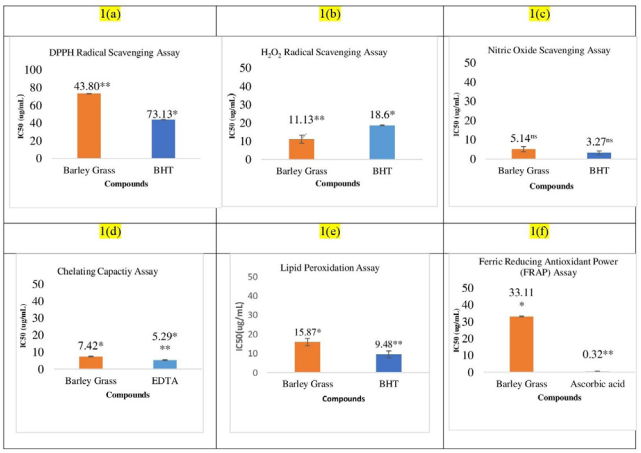


**Figure 1: Antioxidant profile of *Hordeum vulgare* water extract and standard drug (BHT and EDTA).** (a) DPPH radical scavenging capacity; (b) H2O2 radical scavenging capacity; (c) NO radical scavenging capacity ; (d) Chelating capacity; (e) Lipoperoxidation radical scavenging capacity; (f) Reducing power capacity. Result is showed as $IC_{50}$ value. The data were statistically analyzed by One-way ANOVA followed by Dunnet's' post hoc test. Significant at (***p < 0.001, **p < 0.01, *p < 0.05).

## In-vitro anti glycation assays

### *In-vitro AGEs-Glucose-BSA and MGO-BSA assays*

The inhibitory activity of *Hordeum vulgare* and aminoguandine on the advanced glycation end products was evaluated using Glucose - BSA and MGO-BSA glycation assays. Based on Illustration of data from Glucose BSA protein model, barley grass shows a significantly ($p<0.001$) higher inhibition potency of AGEs with $IC_{50}$ =31.02±1.703 ug/ml compared to Aminoguanidine with $IC_{50}$ =10.65±0.957 ug/ml as shown in Figure 2(a). However, Aminoguanidine showed significantly ($p<0.0001$) higher inhibition of AGEs with $IC_{50}$ =2.02± 0.889, compare to *Hordeum vulgare* with $IC_{50}$ =0.51± 0.66 in MGO-BSA protein model as shown in Figure 2(b).

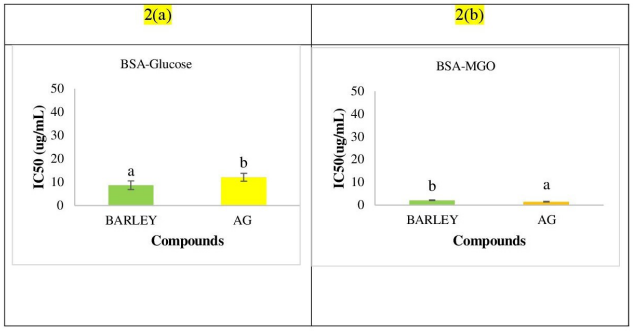


**Figure 2: In-vitro anti-glycation activity of *Hordeum vulgare* water extract and standard drug (aminoguandine).** (a) GLUCOSE-BSA protein glycation inhibition activity; (b) MGO-BSA protein glycation inhibition activity Result are showed asIC50 value. The data were statistically analyzed by One-way ANOVA followed by Dunnet's' post hoc test. significant at (***p < 0.001, **p < 0.01, *p < 0.05).

## Total Phenolic Content Assay

The total phenolic content (TPC) of Barley grass extract is determined by Folin-Ciocalteu reagent method expressed as gallic acid equivalent (GAE) in mg per gram of sample extract. The concentration of TPC of *Hordeum vulgare* is determined from gallic acid standard curve. According to the experiment, TPC of *Hordeum vulgare* increased with increasing concentration with lowest TPC of 1.007 mg GAE/g extract at 10 µg/ml and highest TPC of 3.005 mg GAE/g extract at 100 µg/ml. shown in Figure 3.

## Phytochemical screening assay

Phytochemical compounds including saponins, flavonoids, tannin, steroids and terpenoids are present in the water extracts of *Hordeum vulgare*.

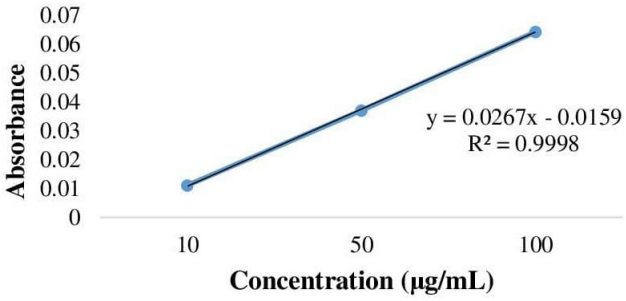


**Figure 3: Gallic acid standard curve**

**Table I: Phytochemicals present in *Hordeum vulgare* water extracts**

| Phytochemical | Present (✓) Absent (X) |
|---|---|
| Saponins | ✓ |
| Flavonoids | ✓ |
| Tannin | ✓ |
| Steroids | ✓ |
| Terpenoids | ✓ |

## DISCUSSION

Several reports have linked accumulation of advanced glycation end products (AGEs) as influencer of reactive oxygen species generation and implicated to hyperglycemia (19). Current experiment shows free radical scavenging activity (antioxidant) of *Hordeum vulgare* water extract. Antioxidants prevents damage of cells by reactive oxygen species (ROS) and its derivatives (20). Natural antioxidant potentials of extracts is recommended to be determined through a combination of inhibition of lipid peroxidation and electron/radical scavenging activity (21); (22). The present result reflects the free radical scavenging ability of *Hordeum vulgare* water extract, which is attributed to the presence of reductones (23). Reductones plays active role in reducing properties of $Fe^{(3+)}$- ferricyanide complex to the ferrous form. Reductones has been reported to possess antioxidant properties through its donation of hydrogen atom that breaks free radical chain and it prevents formation of perioxide, when it reacts with certain peroxide precursors (24). Presence of phenolics in *Hordeum vulgare* water extract may have been the reason it is able to scavenge of $H_2O_2$ radicals. This is because phenolics donates electrons to $H_2O_2$ and neutralises it to water (25). Transition metals enhances the generation of free radicals which damages cellular systems, thus agents that stabilizes these transition metals will minimize radical-induced damage (26). Chelating activity of *Hordeum vulgare* water extract maybe through the conversion of ferrous sulphate to ferrozine (27). Free radicals influence the deterioration of polyunsaturated lipids into Malondialdehyde (MDA). Increase cellular content of MDA indicates lipid peroxidation (LPO), and MDA is the major oxidative product of peroxidized polyunsaturated fatty acids. Present study shows *Hordeum vulgare* water extract, showed a significant decrease in MDA content indicating the prevention of lipid peroxides, because stimulation of antioxidant enzymes play an important role in scavenging reactive oxygen species (28).

Accumulation and formation process of AGE involves the release of many free radicals, carbonyl species, and reactive dicarbonyl species, that are detrimental to cellular systems. One of such compounds is methylglyoxal (MG), which is very reactive and can cause dicarbonyl stress, influencing normal physiological functions (5). Free radicals and its derivatives are critical in the formation of AGE, antioxidants activity closely linked to anti-AGE property, this is because the antioxidants can scavenge free radicals and its derivatives. Antioxidants are also able to trap dicarbonyl species or break down glycation end products which will lead to inhibition of AGEs (29,30). Free radical scavenging property of *Hordeum vulgare* water extract may have contributed to the observed inhibition of AGE formation. This is because free radical scavenging capability is critical in non-enzymatic protein glycation and autoxidation of glucose which leads to inhibition of AGE formation. The inhibition of BSA-glucose and BSA-MGO interaction demonstrates the fact that *Hordeum vulgare* water extract was able to react with carbonyl groups and dicarbonyl intermediates to prevent formation of AGE.

Phenolics are secondary metabolites found in plants that possess therapeutic benefits such as radical scavenging, carbonyl trapping, metal iron chelation, anti-AGEs, antioxidants, and anti-diabetic activity (31). Hydroxyl groups and their derivatives present on the phenol rings of phenolic compounds plays a major role in stabilization property and may be responsible for the antioxidant activity of phenolic compounds (32). Previous report by Grzegorczyk-Karolak et al 2016 (33), shows that polyphenols from Scutellaria alpina L. and S. altissima L. possesses antioxidant and anti – AGE activity via multiple mechanisms such as radical scavenging, reducing power, inhibition of lipid peroxides. Several researchers have reporter a very strong correlation between antiglycation activity, antioxidant, and total phenolic content in herbs, spices, and plant extracts (34,35). Anti -AGE activity of *Hordeum vulgare* water extract may be connected to its antioxidant property and presences of phenolic compounds (36,4,37).

## CONCLUSION

*Hordeum vulgare* water extract inhibits the formation and accumulation of AGE, which may be because of its free radical scavenging property, decrease of reactive carbonyl and presence of phenolic component. Further research is ongoing to determine the effect of *Hordeum vulgare* water extract on glycation pathway and interaction with RAGE receptor.

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