Effect of microwave roasting on antioxidant and anticancerous activities of barley flour

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Abstract
The antioxidant and anticancerous activities of native and microwave roasted barley flour were compared in three different solvents (methanol, ethanol and water) using various antioxidant assays. The extracting solvents significantly affected the antioxidant potential and inhibition of DNA damage capabilities of barley flour extracts. Among the different solvents methanol was found to be the most suitable for extraction, as the extracts showed highest antioxidant as well as anticancerous activities and also prevented DNA damage to the maximum extent. Microwave roasting resulted in a mixed response toward the antioxidant potential of barley, as % inhibition of DPPH and reducing power increased while all other parameters such as OH radical scavenging activity, H2O2 radical scavenging activity and total phenolic content showed a decrease. An increase of 13.24% in methanol, 8.14% in ethanol, and 6% in water was reported in case of DPPH & an increase of 21.37% in methanol, 21.43% in ethanol, and 15.23% in water was seen in case of reducing power as a result of roasting. A decrease of 34.39%, 121.47% and 50% in TPC values was reported as a result of microwave roasting for Methanolic, Ethanolic and Aqueous extracts, respectively. However, all the tests showed an overall increase in a dose dependent manner as the concentration of extracts increased. Roasting also resulted in a decrease in the anticancerous potential and the inhibition of DNA damage by the barley extracts irrespective of the solvents used. The anti-proliferative activities of the native and microwave roasted barley flour extracts were tested on Colo-205, T47D and MCF7 cell lines. Barley flour extracts inhibited cancer cell growth which was more for native barley flour (39.81%) than roasted barley flour (22.91%).

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1. Introduction

Barley (Hordeum vulgare) is an important cereal crop ranking fifth in the world production and it plays an important role in human nutrition (Madhujith et al., 2006; Sharma and Gujral, 2010). Barley is a widely consumed cereal among the most ancient cereal crops. Almost 80–90% of barley production is used as animal feeds and malt. However barley is now gaining renewed interest as an ingredient for the production of...
functional foods due to high concentrations of bioactive compounds such as β-glucans and polyphenols (Jadhav et al., 1998). Barley is also reported to contain B-complex vitamins, tocotrienols, and tocopherols (Madhujith et al., 2006; Sharma and Gujral, 2011) which are known to inhibit nonenzymatic lipid peroxidation and are widely recognized for antioxidant and antiradical properties. The abundant contents of phenolic compounds in barley reveal that barley may serve as an excellent dietary source of natural antioxidants for disease prevention and health promotion.

Loss of nutritional components, generation of health deteriorating compounds, non-ecofriendly and economic considerations are major setbacks for the processing industry. Due to these considerations minimally processed foods are gaining importance in day to day life. It is well documented that the minimally processed foods have more health benefits (Shahidi, 2009; Sharma and Gujral, 2011). Roasted foods are one of the minimally processed foods that have been used all over the world from the ancient times. In India, barley is widely consumed in the roasted form called sattu. Microwave cooking is becoming a common heating method because it is time saving and the short-comings of sand roasting e.g., lack of temperature control and contamination with sand can be eliminated by switching over to microwave cooking.

2. Materials and methods

2.1. Materials

The indigenous hull-less variety of barley (H. vulgare) was procured from Regional Research Station, Ladakh. All chemicals used were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and calf thymus DNA were procured from sigma Aldrich, whereas, Folin–Ciocalteu reagent, gallic acid, sodium phosphate buffer, potassium ferricyanide, trichloroacetic acid, ferric chloride, ascorbic acid, H₂O₂, KH₂PO₄, thiobarbituric acid, phosphate buffer, hydrogen peroxide, BHT, FeSO₄, phosphate buffer, 1.5% agarose gel Ethidium bromide and Tris. Tris/boric/EDTA gel buffer were procured from Hi-media laboratories.

2.2. Microwave cooking of barley

Hulled barley (400 g) was conditioned to a moisture content of 10% so as to eliminate the effect of differences in moisture content on microwave roasting behavior. Barley was kept in microwave oven (LG, Intellocoook, 2450 MHz, 900 W) and roasted for 120 s at 900 W. Microwave roasting was carefully optimized in such a way that it resulted in grain with maximum expansion and no burning.

2.3. Extraction of barley flour for antioxidant assays

0.3 g each of native as well as roasted barley was dissolved separately in 10 ml of methanol, ethanol and water, and then stirred for 2 h on a magnetic stirrer followed by the centrifugation for 10 min at 3500 rpm. The supernatants obtained from native and roasted barley were labeled as N⁰, N¹, N² & R⁰, R¹, R² respectively and superscripts ‘m’, ‘e’ and ‘a’ indicate methanol ethanol and aqueous solvents, respectively. 300 µl each of N⁰, N¹, N², R⁰, R¹, R² & R³ was kept for TPC determination in triplicates. Rest of the quantity was then evaporated at 40 °C. The dried samples were redissolved in their respective solvents to make the stock sample of varied concentrations (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml & 50 µg/ml).

2.4. Antioxidant activity of microwave roasted barley flour

2.4.1. DPPH radical scavenging activity

DPPH radical scavenging activity of the extract solutions (viz; methanolic, ethanolic and aqueous) was determined according to the method of Gaulejac et al. (1998) with some minor changes. 100 µl each of native as well as roasted barley sample extracts in different solvents of varied concentrations (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml & 50 µg/ml) was added to 2.9 ml of 6 × 10⁻⁵ mol/l methanolic solution of DPPH. The absorbance at 517 nm was measured with a spectrophotometer (Hitachi U-2900) after the solution was allowed to stand in the dark for 60 min. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

Percentage inhibition was calculated by using the formula:

\[
\text{% inhibition} = \frac{A_{\text{control}517} - A_{\text{sample}517}}{A_{\text{control}517}} \times 100
\]

where \( A_{\text{control}517} \) is the absorbance of the control and \( A_{\text{sample}517} \) is the absorbance of the extract.

2.4.2. Determination of total phenolic content

The TPC of the barley extract was determined according to the Folin–Ciocalteu spectrophotometric method (Singleton and Rossi, 1965) with some modifications. 100 µl each of N⁰, N¹, N², R⁰, R¹, R² & R³ was mixed with 2.5 ml of 10-fold diluted Folin–Ciocalteu’s phenol reagent and allowed to react for 5 min. Then, 2 ml of 7.5% Na₂CO₃ solution was added to each of the samples, and the final volume was made up to 10 ml with deionized water. After 1 h of reaction at room temperature, the absorbance at 760 nm was determined. The measurement was compared to a standard curve of gallic acid (GA) solution, and the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry barley (mg GAE/g db).

2.4.3. Reducing power

The reducing power was determined by the method of Oyaizu (1986) with minor changes. 100 µl of each extract with varying concentrations (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml) was mixed with sodium phosphate buffer and potassium ferricyanide. The mixture was incubated at 50 °C for 20 min followed by the addition of trichloroacetic acid and then centrifuged at 3000 rpm for 10 min. The upper layer was mixed with deionized water and FeCl₃, and then the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.4.4. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of extracts was assayed by the method of Wu et al. (2007) with certain modification. The reaction mixture contained 25 mM calf thymus DNA (1 ml), 10 mM ferric chloride (200 µl), 100 mM ascorbic acid (200 µl), 2.8 mM H₂O₂ (200 µl) in 10 mM KH₂PO₄ (pH 7.4) and various concentrations (10 µg/ml, 20 µg/ml, 30 µg/ml,
40 μg/ml, 50 μg/ml) of native as well as roasted barley extracts in different solvents (methanol, ethanol & water). The reaction mixture was incubated at 37 °C for 1 h. Then 1 ml of 1% thiorbarbituric acid and 1 ml of 3% trichloroacetic acid were added and heated at 100 °C for 20 min. The TBARS was measured spectrophotometrically at 532 nm. The results were expressed as percentage inhibition of DNA oxidation.

Percentage inhibition was calculated by using the formula:

\[
\% \text{ inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]

where \(A_{\text{control}}\) was the malondialdehyde produced by Fenton reaction treated alone, and \(A_{\text{sample}}\) was the malondialdehyde produced in the presence of extract.

2.4.5. \(H_2O_2\) scavenging activity

The ability to scavenge hydrogen peroxide was evaluated according to the method of Ruch et al. (1989). A solution of \(H_2O_2\) (2 mM) was prepared in a phosphate buffer (pH 7.5). The native and roasted barley extracts at different concentrations (10 μg/ml, 20 μg/ml, 30 μg/ml, 40 μg/ml, 50 μg/ml) were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 15 min against a blank solution containing phosphate buffer without hydrogen peroxide. BHT was taken as standard. The scavenging activity of extract on \(H_2O_2\) was expressed as:

\[
\% \text{ inhibition} = \frac{A_0 - A_1}{A_0} \times 100
\]

where \(A_0\) is the absorbance of the control and \(A_1\) is absorbance in the presence of extract and known standards.

2.5. Inhibition of oxidative DNA damage

The hydroxyl radical-mediated DNA strand breaks were measured by the procedure described by Yeung et al. (2002) with some minor modifications. Briefly, 0.5 μg of DNA was incubated with 1 μl of 1 mM FeSO4, 1 μl of 10% \(H_2O_2\), 3 μl each of native as well as roasted extract (50 μg/ml) in different solvents (viz; methanol, ethanol & aqueous) and the final volume was made up to 15 μl with 50 mM phosphate buffer (pH 7.0). The mixture was incubated in a water bath at 37 °C for 30 min. After incubation, the sample was immediately loaded in a 1.5% agarose gel along with 3 μl ethidium bromide, containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, and electrophoresed in a horizontal slab apparatus in Tris/boric/EDTA gel buffer. The gel was then photographed under UV light.

2.6. Antiproliferative effects of barley extract on different human cancer cell lines

Cell proliferation inhibitions were investigated according to Mosmann, 1983 with minor modifications. Colo-205 (human colon cancer cell line), T47D (human ductal breast epithelial tumor cell line) and MCF7 (human breast adenocarcinoma cell line) were used for the MTT human cancer assay. The cells were plated in 96 well plates at 5000–7000 cell density per well. Cells were grown overnight in 100 μl of 10% FBS. After 24 h cells were replenished with fresh media and the extracts of the sample solution were added to the cells. The aqueous extracts of native and microwave roasted barley (50 μg/ml each) were added to wells in triplicates. Cells were incubated with the extract for 24 h. After 24 h 20 μl of MTT dye (5 mg/ml) was added to each well and further incubated for 3 h. Before read-out, precipitates formed were dissolved in 150 μl’s of DMSO using vortex for 15 min. All the steps after MTT addition were performed in the dark. Absorbance was measured at 590 nm.

2.7. Statistical design

Results were expressed as mean of triplicate analyses. A one-way analysis of variance and Duncan’s test were used to establish the significance of differences among the mean values at 0.05 significance level. The statistical analyses were performed using SPSS software.

3. Results and discussions

3.1. Assay for antioxidant activity

3.1.1. DPPH

The DPPH radical scavenging activity of the samples is ranked in the following order: methanol > ethanol > water, in both native as well as roasted barley (Fig. 1). There was no significant difference between the yields of methanolic and ethanolic extracts (Moure et al., 2000). The observed differential scavenging activities of the extracts against the DPPH system could be due to the presence of different compounds in the fractions (Sahreen et al., 2010). An increase in the values of % inhibition with an increase in concentration of the extract in a dose dependent manner was seen in all the three solvents indicated by their correlation coefficients (Data not shown). An average increase of 13.24%, 8.14% & 6% was seen as a result of microwave roasting in case of methanolic, ethanolic and aqueous extracts, respectively. A significantly higher increase in percentage inhibition of DPPH free radicals by the methanolic extract suggests methanol as a more stabilizing solvent for antioxidants. Correlation coefficients for methanolic extracts before and after microwave roasting suggest a more facilitated release of antioxidants. IC50 values were calculated and it was lowest for methanolic solvents. Microwave roasting resulted in a decrease of IC 50 values from 39.853 μg/ml to 36.182 μg/ml in case of methanol & 40.597 μg/ml to 38.712 μg/ml in case of ethanol & 41.866 μg/ml to 40.691 μg/ml in case of aqueous solvents. Thus among all the three solvents methanol was most suitable in terms of % inhibition of DPPH generated free radicals and microwave roasting resulted in an increase in the antioxidant potential, which may be due to greater release of antioxidants. Microwave roasting results in the production of various compounds like melanoids, which may also impart antioxidant activity to the extract. Holtekjolen et al. (2008) also reported similar results upon baking of bread prepared by incorporating barley flour. Vitali et al. (2009) observed that the baking of fiber incorporated biscuits leads to a significant (p< 0.05) increase in antioxidant activity.

3.1.2. Total phenols

A decrease in total phenolics was seen in barley flour extract as a result of microwave roasting (Fig. 2). It has been reported that microwave cooking leads to reduction in the total phenolic content of barley extract (Sharma and Gujral, 2011). A decrease in TPC was also reported by Zhang et al. (2010) for
roasted and microwave heated buckwheat flour. Thermal degradation of heat susceptible phenolic compounds leads to the decrease in the total phenolic content (Randhir et al., 2008). The decrease in total phenolic content could be due to the alteration in molecular structure of phenolic compounds, which may lead to the decrease in extractability due to the degree of polymerization (Altan et al., 2009). Longer heat treatment in the microwave oven leads to a greater decrease in the TPC. It was also seen by Zhu et al. (2010) for γ-irradiated rice grain. The total phenolic content of methanolic, ethanolic and aqueous solutions was found to be 16.92 mg GAE/g db, 33 mg GAE/g db & 14.1 mg GAE/g db, respectively, while after microwave roasting of barley TPC of methanolic, ethanolic & aqueous solvents decreased to 12.59 mg GAE/g db, 14.9 mg GAE/g db & 9.4 mg GAE/g db, respectively. The results revealed that the extraction solvents affected the total phenolic content in the following decreasing order: ethanol > methanol > water. Similar results were also observed by Inglett et al. (2011). Variation in the total phenolic content of three extracts is attributed to the polarities of different solvents. As a result of microwave roasting, a significant decrease of 34.39%, 121.477% & 50% was seen in case of methanolic, ethanolic and aqueous extracts, respectively. A lesser percentage decrease was seen in case of methanolic solvent suggesting that methanol had a more protective effect on the antioxidant compounds extracted during the process (Inglett et al., 2011). It has been seen that the phenols present in a sample are responsible for the antioxidant activity of barley extract and in our study higher TPC was found in ethanolic solvent, which is not in consent with the results of DPPH and reducing power discussed above. The lower antioxidant potential of ethanolic solvent despite of higher TPC may be caused either by the extraction of phenolic compounds similar to vitexin and iso vitexin, which do not have any antioxidant activity (Inglett et al., 2011) or the higher antioxidant potential of methanolic solvent (as indicated by higher % inhibition of DPPH free radicals and higher reducing power) than other solvents which may be due to the production of melanoids and stabilization of phenolic compounds.

3.1.3. Reducing power

The antioxidant activity is indicated by the reducing power (Lee et al., 2007). The electron donor compounds act as the reducing agent and help in reducing the oxidized intermediates of lipid peroxidation reactions and hence there might be the primary and secondary antioxidants (Sharma and Gujral, 2011). The reducing power of the antioxidant compound is related to the presence of reductones. Furthermore, the antioxidant activity of these reductones is due to the breakage of free radical chain reaction by donating a hydrogen atom and thus preventing lipid peroxidation (Gordon, 1990). There was an
increase in the antioxidant activity of the barley flour due to microwave roasting (Fig. 3). Similar results were obtained by Sharma and Gujral (2011). The products produced by the miltiand reaction, which are generated during microwave roasting may have enhanced the reducing power (Woffenden et al., 2002). The average values of reducing power varied significantly among different solvents both in native as well as roasted barley. The reducing power of the sample can be ranked as methanolic > ethanolic > water, in native as well as roasted barley. While studying the effect of roasting with respect to a particular solvent, the average values also varied significantly. As a result of microwave roasting, an increase of 21.37%, 21.43%, and 15.23% was seen in methanolic, ethanolic and aqueous extracts, respectively. Methanolic extract has the higher antioxidant activity, which might be due to its greater hydrogen donating capability (Shimada et al., 1992).

3.1.4. Hydroxyl radical scavenging activity

In this study it was found that the microwave treated barley showed lower hydroxyl radical scavenging activity than the native one, which may be due to structural changes in phenolic compounds induced by microwave treatment (Fig. 4). Structural changes to phenolic compounds lead to change in free radical scavenging activity, e.g., at ortho position, the methoxylation of hydroxyl groups of phenolic compounds, as in ferulic acid results in lowering the rate constant of reduction of phenolic antioxidants with hydroxyl radicals (Bors et al., 1984). Similar results were reported in proso and little millets, which showed significantly lower hydroxyl radical scavenging activity than the uncooked sample (Chandrasekara et al., 2012). Methanolic solvents showed better Hydroxyl radical scavenging activity in comparison to other solvents. This decrease was in agreement to the results obtained in case of total phenolics where a minimum decrease was seen in case of methanolic extracts. Among all the solvents used, highest scavenging activity was seen in methanolic extracts for both native as well as roasted barley following the trend methanol > ethanol > aqueous. Similar results were seen by Sumathi et al. (2011) in rhizomes and leaves of Curcuma amad-a. This also confirms the superiority of methanolic solvent. Roasting resulted in a significant decrease of 28.11%, 29.88% and 52.11% in hydroxyl radical scavenging activity in methanol, water and ethanolic solvents, respectively. Better activity in methanolic extracts might be due to greater extraction of antioxidants from the sample or better stabilizing action on the extracted antioxidant compounds.

3.1.5. Hydrogen peroxide scavenging activity

Hydrogen peroxide, which is a weak oxidizing molecule, can directly inactivate some enzymes by oxidizing essential thiol groups. Hydrogen peroxide may rapidly cross the cell membrane. As it enters the cell, \( \text{H}_2\text{O}_2 \) might react with the \( \text{Fe}^{2+} \) and \( \text{Cu}^{2+} \), which results in the formation of hydroxyl radicals, where there may be the occurrence of its toxic effects (Halliwell and Gutteridge, 1993). The hydrogen peroxide scavenging activity of native barley was seen greater than the roasted barley (Fig. 5). Same results were reported in proso little and pearl millets, which indicated the negative effect of processing on its antioxidant activity (Chandrasekara et al., 2012). An increase in the scavenging activity was seen with an increase in concentration (data not shown) but the hydrogen peroxide scavenging activity of barley samples was seen significantly higher for methanolic extracts than the rest of the solvents used for extraction (methanol > ethanol > water). Same results were reported in leaves and rhizomes, which showed the higher ability to scavenge \( \text{H}_2\text{O}_2 \) followed by the aqueous extract (Sumathi et al., 2011). These results are probably observed because of the absence of attached sugar moieties to the antioxidant constituents (Bradshaw et al., 2001). Microwave roasting leads to a significant decrease of 8.36%, 31.89% & 39.60% in hydrogen peroxide scavenging activity in case of methanolic, ethanolic and aqueous extracts, respectively.

![Figure 3](image-url)

*Figure 3* W, water; E, ethanol; M, methanol; N, native barley; R, roasted barley.
3.2. Protection against DNA damage

Hydroxyl radical is a very reactive radical formed from the reaction of many hydroperoxides with transition metal ions which can attack DNA, proteins and polyunsaturated fatty acids in membranes (Aruoma, 1999). Phenolic compounds consume these free radicals and thereby prevent damage to vital molecules present in cells and hence assist in countering cell death. The protective effect of barley extracts using different solvents was visible at a concentration of approximately 50 µg/ml of extract. The protective effect of native and roasted barley extracts in three different solvents is shown in Fig. 6. As indicated in Fig. 6, roasting leads to a significant decrease in the protective effect of barley extracts against DNA damage. However protective effect of barley extracts was highest in case of methanolic extract for both native as well as roasted barley.
3.3. Antiproliferative activity

Native and microwave roasted barley were selected for antiproliferative activity against three human cancer cell lines (Fig. 7). The extracts exhibited antiproliferative activities against Colo-205, T47D and MCF7 and significantly reduced the growth rate of cancer cells at an initial concentration of 50 μl/ml after 24 h treatment. However the maximum effect was seen in Colo-205. Interestingly, native barley extract showed highest antiproliferative effect against Colo-205, with significant inhibition at 50 μg/ml. Thermal processing caused reduction in the antiproliferative activity of barley which has also been reported by Xu and Chang, 2011. This may be due to the degradation of polyphenols as well as a reduction of saponins and phytic acids.

4. Conclusions

The result of this work has shown that barley has excellent anti-oxidant and anti-cancerous properties and can be compared to fruits and vegetables in this aspect. The study also revealed the Protective effect of barley against DNA damage and antiproliferative activity against Colo-205, T47D and MCF7, which became evident at a concentration of 50 μg/ml. All the parameters studied showed an increase in a dose dependent manner irrespective of the solvent used for extraction. Microwave roasting significantly affected the antioxidant and anticancerous activities of barley. It also reduced the protective effect of barley extracts against DNA damage. Among the different solvents used for extraction, methanol showed more promising results. Methanolic extracts showed highest values for all the antioxidant assays particularly in DPPH & reducing power. Microwave roasting results in generation of new compounds such as melanoidins which may strongly affect the parameters of study and need further chemical characterization, so that the mechanism of antioxidant and antiproliferative activities can be better understood and can be used in the field of medicine, cosmetology and pharmacy.

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