

GC-MS Analysis, *In Vitro* Antioxidant and Cytotoxic Studies of Wheatgrass Extract

Garima Shakya, Sankar Pajaniradje, Muddasarul Hoda, Varalakshmi Durairaj and Rukkumani Rajagopalan*

Department of Biochemistry and Molecular Biology, School of Life Sciences, Pondicherry University, Puducherry, India

Address for Correspondence

Dr. R. Rukkumani,
Assistant Professor,
Department of
Biochemistry and
Molecular Biology,
School of Life
Sciences,
Pondicherry
University,
Puducherry-605014
India.

E-mail:
ruks2k2@yahoo.co.in

ABSTRACT

Cancer is the second leading cause of death worldwide and oxidative stress is one of the factors responsible for causing cancer. Antioxidants play a very important role in prevention of cancer. Therefore, efforts have been made to identify natural antioxidants having anti-cancer potential. Various herbs, fruits and vegetables have diverse phytochemicals that display antioxidant properties and help in reducing the risk of cancer. Wheatgrass has a potent antioxidant efficacy and has been used as a health drink to cure many diseases in folk medicine. In the present study, a number of solvent extracts of wheatgrass was tested for their antioxidant ability. The most effective solvent extract was further evaluated for cytotoxic effect in Hep2 cell lines and apoptotic induction was demonstrated by propidium iodide (PI) fluorescent staining. The bioactive constituents were analysed by GC-MS. Methanol extract (ME) showed the highest quantity of phenols and flavonoids. PI staining showed apoptotic features like nuclear fragmentation and chromatin condensation and GC-MS analysis showed the presence of nine bioactive phytoconstituents in methanol extract. Hence we conclude that wheatgrass has good antioxidant and cytotoxic property and being a natural product, could be a good candidate for cancer prevention and treatment.

Keywords: Wheatgrass, Antioxidant activity, Antiproliferative activity, Hep2 cells, GC-MS, Apoptosis.

INTRODUCTION

Despite intensive research for the prevention and treatment of cancer, the number of deaths due to cancer is continuously increasing and it remains a leading cause of morbidity and mortality in

the world¹. Although the efficacy of chemotherapy for majority of cancer types has increased, these drugs cause several toxic effects, ultimately leading to reduction in the quality of life².

Free radicals or reactive oxygen species, generated from normal metabolism within the body or from the exogenous sources, cause oxidative stress and are found to be the root cause for the development of many diseases, including cancer³. However, cells have highly specialised antioxidant defence system to protect themselves from free radical damage. Endogenous and exogenous antioxidants work interactively and synergistically in neutralizing the free radicals. Thus, antioxidants are recommended to be taken in the diet. Synthetic antioxidants are very commonly used in food industry. However synthetic antioxidants are found to have various toxic effects, and hence their use is restricted. Therefore much emphasis is given to identify natural antioxidants, without undesirable side effects⁴.

Since ages, more than 3000 plant species have been used for the chemotherapy and chemoprevention. According to World health organisation, 80% of population in some Asian and African countries still depend upon tradition herbal medicine for the prevention of many diseases, most of which involve plant extracts¹. The effectiveness of the plant extracts is mainly due to the presence of bioactive constituents like phenolics, flavonoids and others⁵. Studies have suggested that polyphenols due to the presence of double bond and hydroxyl group, display high antioxidant properties which help in reducing the risk of development of various degenerative diseases, including cancer¹. Therefore, in recent years many efforts have been made to identify natural, potent anticancer agents with low toxicity and high antioxidant activity⁶.

Wheat (*Triticum aestivum*) germinated over a period of 6-10 days is called wheatgrass. It is also known as “living food” and it is a rich source of

vitamins, antioxidants and minerals. Wheatgrass also contains Vitamin A, B₁, C and E, β -carotene, Ferulic acid, Vanilic acid, many minerals and trace elements including Calcium, Iodine, Selenium and Zinc. Wheatgrass is also known to contain antioxidant enzymes like Superoxide Dismutase and Cytochrome Oxidase. Wheatgrass extract is a rich source of chlorophyll that is found to be responsible for inhibiting the metabolic activation of carcinogens. In India, wheatgrass is consumed in the form of tablet or as a juice to maintain good health. Wheatgrass juice is seen to have healing properties in various degenerative diseases and is effective in the treatment of thalassemia, distal ulcerative colitis, and benefits other parts of the body^{7,8}.

Consumption of natural antioxidants from plant sources have been shown to decrease the risk of development of various oxidative stress related diseases. The objective of this study is to identify the most effective solvent extract of wheat grass, to determine the total phenolic and flavonoid and other phytochemical contents and to compare the antioxidant activity of various solvent extracts of wheatgrass. Further, we have evaluated the *In Vitro* cytotoxic effect of the most effective solvent extract.

MATERIALS AND METHODS

Chemicals

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Dulbecco's Modified Eagle Medium (DMEM), Trypan blue, antibiotics and fetal bovine serum (FBS) and the standards - BHA (2-tert-butyl-4-methoxyphenol), gallic acid, quercetin, ascorbic acid, BHT (butylated hydroxyl toluene), BSA (Bovine serum albumin), Propidium Iodide (PI), were purchased from Sigma–Aldrich, Bangalore, India. All other reagents and chemicals were of analytical grade. The

Hep2 cell lines were purchased from NCCS (National Centre for Cell Science) Pune, India. Wheatgrass powder was purchased from Eden Park Agro Products Pvt Ltd, under the brand name of "Green Heart", who is the grower, manufacturer & exporter of Wheatgrass.

Sample preparation

Wheatgrass powder was subjected to soxhlet extraction by using various solvents like double distilled water, methanol, acetone and chloroform for about 24h. Each solvent extract was evaporated to dryness. Water extract was immediately lyophilized and used for further studies. As wheatgrass is consumed in a form of juice, freshly prepared crude wheatgrass sample was also used [wheatgrass powder (mg/ml) dissolved in double distilled water for 1h and supernatant was used for various experiments].

Cell Lines and culture conditions

Hep2 cells were cultured in T-25 flask maintained in 1x DMEM supplemented with 10% FBS at 37°C in CO₂ incubator in an atmosphere of humidified 5% CO₂ and 95% air. Methanol extract (ME) powder of wheatgrass was dissolved in 0.2% DMSO. Different concentrations of ME (100- 1000 µg/ml) were used for the determination of IC₅₀.

Phytochemical analysis

Total phenolics estimation

The total phenols of all extracts were estimated by colorimetric assay by Folin Ciocalteu reagent as described by Mc Donald *et al*⁹. Total phenol results were expressed as µmole of gallic acid equivalents/g of extract (GAEs), which is the standard reference compound.

Total flavonoids estimation

Flavonoid contents in the extracts were determined by a colorimetric method developed by Zhishen *et al*¹⁰. The results were expressed as µmole of quercetin equivalents/g of extract.

Estimation of protein content

Protein content was estimated by Lowry *et al*¹¹. Bovine albumin serum (BSA) was used as the standard.

Qualitative analysis of phytochemicals

The screening of phytochemical constituents was carried out with all the extracts of wheatgrass to analyze the presence of different bioactive component according to standard methods¹².

In Vitro antioxidant activities

Ferric reducing power assay

The reducing power of the sample was determined by the method of Barros *et al*¹³. BHA was used as the standard.

Total antioxidant assay

The antioxidant activity of the wheatgrass extracts was evaluated by the phosphomolybdenum method according to the procedure previously reported by Prieto *et al*¹⁴. Ascorbic acid was used as the positive control.

Metal chelating activity

The metal chelating assay was performed according to the method previously described by Chan *et al*¹⁵ with BHT as the positive control. The metal chelating ability (%) of wheatgrass extract was calculated by using following equation:

$$\text{Metal chelating ability (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of sample of wheatgrass extract and standard.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the method described by Klein *et al*¹⁶. Percentage hydroxyl radical scavenging was calculated by the formula as mentioned in metal chelating activity. BHT was used as the standard.

Hydrogen peroxide scavenging activity

The ability of wheatgrass extracts to scavenge hydrogen peroxide was determined by the method of Ruch *et al*¹⁷. The percentage of hydrogen peroxide scavenging by the extract was calculated by a formula mentioned in metal chelating ability. BHT was used as the standard compound.

Nitric oxide radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and its quantity was determined using griess reagent, using the modified method of Marcocci *et al*¹⁸. The percentage of Nitric oxide scavenging by the wheatgrass extracts was calculated by a formula as mentioned in metal chelating ability. BHA was used as the standard.

Antiproliferative activity

Cell viability was determined by using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay according to a previously described protocol¹⁹. Hep2 cells were harvested by trypsinization and resuspended at a final concentration of 2×10^4 cells/ml in fresh DMEM with 10% FBS. Aliquots of 100 μ l cell suspension were plated in 96-well tissue culture plates. In order to detect the cytotoxicity of the cells, cells were treated with different concentrations of ME and incubated for 24h. After 24h, 20 μ l of a

5 mg/ml MTT solution was added to each well, and the plate was incubated for 4h, allowing viable cells to reduce the yellow MTT to dark-blue formazan crystals, which were dissolved in 100 μ l of DMSO. The absorbance in individual well was determined at 570 nm using microplate reader [Molecular Devices]. The cell viability was calculated as percentage of viable cells and then plotted on a graph.

Growth inhibition (%) = $(A_{570 \text{ nm of treated cells}} / A_{570 \text{ nm of controlled cells}}) \times 100$

Propidium iodide (PI) staining

PI staining was done to detect the DNA integrity²⁰. Briefly, Hep2 (3×10^6 cells/mL) cells were grown in 6 well plates and treated with ME of wheatgrass for 24h. Cells were washed with ice cold PBS and fixed in 70% ethanol. 0.5 mL of PI buffer containing 0.1% Triton X-100, 0.1% sodium citrate, 5 μ L of RNase A (1mg/mL) and 5 μ L of PI (50 μ g/mL in PBS) was added and incubated at 37°C for one hour and the fluorescence was observed under fluorescent microscope (Olympus).

Gas chromatography – Mass spectroscopy analysis (GC-MS)

A GC-MS study was performed at Indian Institute of Crop Processing Technology (IICPT) Thanjavur, to study the phytochemical components present in the methanol extract of wheatgrass. GC-MS analysis was carried out on a GC CLARUS 500 PerkinElmer system comprising a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 \times 0.25 mm ID \times 1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a

constant flow of 1 ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) with injector temperature of 250°C; ion-source temperature of 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da²¹.

Statistical Analysis

The results are presented as means \pm SD of triplicate observations. All the data were analyzed using the SPSS 13-Windows Students version software. Statistical analysis was done by analysis of variance (ANOVA) followed by Tukey's test. $p \leq 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Currently, there is a growing trend in the use of natural antioxidants from plant sources, because of their efficacy and minimal toxicity. Thus antioxidant and radical scavenging activities of medicinal plants have been extensively studied. Plant phenolics have shown to possess antioxidant, hypocholesterolemic, hypolipidemic, antihypertensive, antidiabetic and anticancerous properties^{1,5}.

Phytochemical analysis

Phenolics are powerful chain breaking antioxidants; contributing directly to the antioxidant action. The high antioxidant potential of phenolics may be due to the presence of hydroxyl group. Flavonoids are also important for human health and act through scavenging or chelating the metals ions⁴. So, it was our interest to analyze how the total phenolic and flavonoid contents influence the antioxidant and cytotoxic activity of the plant extracts. We observed that the

methanolic extract (ME) showed the highest phenolic and flavonoid contents followed by aqueous extract (AqE), acetone extract (AE), crude extract (CrE) and chloroform extract (CE) (Fig. 1A. and 1B.).

For any plant, it is important to determine its nutritional quality, which is the nutritional value of that particular plant. Protein content is an important parameter to determine the nutritional value²². Thus in this study we evaluated the total protein content of different extracts. ME showed highest protein content than AqE > AE > CrE > CE as is represented in Fig. 1C. This result shows that ME extract has good nutritional value compared to all other extracts. Some proteins can also act as antioxidants and Okamoto *et al*²³ have indicated that glycated protein has a higher scavenging ability for the hydroxyl radicals.

Qualitative analysis of phytochemicals

Phytochemical analysis with all the extracts showed the presence of different types of active compounds such as alkaloids, saponins, amino acids and proteins, carbohydrates, cardioglycosides, coumarin, terpenoids, tannins, flavonoids and phenolics (Table 1). Tannin was absent in chloroform extract. All the extracts (especially ME) showed significant antioxidant activity that may be due to the presence of these potent compounds such as alkaloids, flavonoids, phenolics, saponins, tannins, coumarin etc.

Antioxidant activity

Antioxidants are the substances that inhibit the oxidation of oxidizable substrate in the chain reaction and play a significant role in the prevention of many degenerative diseases. It may function as a free radical scavenger, metal chelator, reducing agent and quencher of singlet oxygen⁴. To determine the antioxidant capacity of various solvent extracts of wheatgrass, we

assayed the reducing power, total antioxidant activity, metal chelating activity, hydroxyl, hydrogen peroxide and nitric oxide radical scavenging activity.

Ferric reducing power assay

Reducing power assay measures the hydrogen donating ability of the phenols and/or hydroxyl containing groups. It converts Fe^{3+} /ferricyanide complex to the ferrous form. Generally the reducing properties of any compound are due to the presence of reductones, which are known to possess good antioxidant property. It acts by breaking the free radical chain reaction by donating the hydrogen atom. The reducing activity of any compound may serve as a potential indicator of its antioxidant property. So reducing power of the sample was analyzed to determine the relationship between the antioxidant effect and the reducing power⁴. The reducing activity is depicted in Fig. 2A. and activity was found in the order, ME >AqE >AE >CrE >CE, which was in correlation with the presence of total phenolics and flavonoids content in the respective extracts. The reducing power of the extract was observed to rise as the concentration of the extract was gradually increased. These results suggest that all the extract possess phenols or some other compounds with hydrogen donating ability.

Total antioxidant assay

Total antioxidant assay was done to evaluate the ability of plant extracts to reduce the Mo (VI) to Mo (V) followed by the formation of a green phosphate/Mo (V) complex at acidic pH²⁴. ME showed the highest total antioxidant activity followed by AE> AqE> CrE> CE as shown in the Fig. 2B. This was also in correlation with the total phenolic, flavonoid and other phytochemical constituents and the activity was found to increase with the increase in the sample concentration.

Metal chelating activity

Metal chelation is one of the important antioxidant mechanisms that retard metal-catalysed oxidation. Ferrous ions are one of the most effective pro-oxidants present in food systems²⁴. As Fe^{2+} causes the production of oxy-radicals and lipid peroxidation, minimizing its concentration can give a protection against oxidative damage. In this assay, Ferrozine can quantitatively form complexes with Fe^{2+} . The formation of complex is decreased if the samples possess chelating activity. Therefore, measurement of the rate of colour reduction helps to estimate the chelating activity of the samples²⁵. Fig. 2C. shows that ME and AqE has good metal chelating activity, which is found to increase in a dose dependent manner, whereas CrE shows its activity only at 1 mg/ml concentration. The iron binding ability of these extracts suggests that their protection against peroxidation may be related to their iron binding capacity. All other extracts like AE and CE did not show any activity. This finding suggests that the compounds which have metal chelating activity especially for ferrous ion is not present in these (AE and CE) extracts²⁴.

Hydroxyl radicals scavenging activity

Hydroxyl radicals are the most active reactive oxygen species that can easily pass through the cell membrane and react with most of the biomolecules like proteins, polypeptides, nucleic acids and lipids causing tissue damage and cell death. Scavenging hydroxyl radical is an important antioxidant mechanism for protecting the living cell^{24, 26}. Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals using ascorbic acid–iron–EDTA. The Hydroxyl radicals are formed by the oxidation reaction with dimethyl sulphoxide (DMSO), which is detected by treatment with nash reagent.

In the present study AqE showed the highest activity whereas CrE showed the least activity, all other extracts activity was found to be in the sequence AE> CE> ME as represented by Fig. 2D. ME had less hydroxyl radical scavenging ability compared to other extracts but its activity was still significant. Hydroxyl radicals are the quick initiators of the lipid peroxidation, thus the quenching of hydroxyl radical activity by these extracts could be directly related to the prevention of lipid peroxidation.

Hydrogen peroxide scavenging activity

Fig. 2E. represents the hydrogen peroxide scavenging activity of various extracts. Highest scavenging of hydrogen peroxide radical was shown by ME, rest all the extracts showed a significant activity except AE, which showed no activity. Scavenging of H₂O₂ by the extracts may be attributed to the phenolics, flavonoids, hydroxyl containing compounds or compounds containing double bonds, which can donate electrons to H₂O₂, thus neutralize it to water²⁷. The differences in the H₂O₂ scavenging capacities between the extracts may be attributed to the structural features of their active components, which determine their electron donating ability²⁷. AE showed no activity, which may be because of the presence of some hindering or interfering substance in this extract²⁸.

Nitric oxide radical scavenging activity

Nitric oxide radical is also implicated in various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis. NO reacts with superoxide radicals and forms highly reactive peroxynitrite anion (ONOO⁻), which is highly toxic²⁹. The plant extracts may have the ability to counteract the generation of the nitric oxide radicals and in turn may be able

to protect the individual from its ill effects. The extract directly competes with the oxygen in reaction with nitric oxide and prevents nitrite formation. The Fig. 2F. gives the radical scavenging activity and it was in the order AqE>CE>ME, where as the CrE and AE showed no activity.

There are various factors that affect the extraction of the active components from the plants like their chemical nature, the extraction method used, particle size, storage time and conditions, as well as the presence of interfering substances. Moreover, other substances, which are soluble in the same solvent, will also be present in the extract. Therefore the plant extracts are the mixture of different classes of active components like phenolics, flavonoids and/or other substances which could synergistically activate or hinder or interfere in their antioxidant function²⁸. Thus the antioxidant component and activity are dependent upon the extraction solvent and the nature of the sample.

In our results, AE has not shown any activity in the various assays which may be because of the presence of hindering or interfering substance in this extract. Whereas we found that ME had highest phenolic, flavonoid and other phytochemical contents and accordingly it had shown the highest antioxidant activity in all the assays except for hydroxyl radical scavenging and nitric oxide radical scavenging assays, which though low, was still significant. The high antioxidant activity of ME could also be because of the presence of some other active compounds (as shown in GC-MS report, Fig. 6.) which could have been activated or promoted the antioxidant effect of the ME. AqE had the second highest phenolics and flavonoids and accordingly it showed significant antioxidant activity in various assays next to ME. Thus considering the good antioxidant ability of ME, we

selected ME for further analysis of the antiproliferative activity.

Antiproliferative activity

MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a yellow water-soluble tetrazolium salt. The succinate dehydrogenase systems present in the active mitochondria of metabolically active cells are able to convert the dye to a water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. Thus, the amount of formazan formed can serve as an estimate of the number of mitochondria and hence the number of living cells in the sample³⁰. In Fig. 3, ME showed a significant reduction in the proliferation of the Hep2 cell line and IC₅₀ was found to be 600 µg/ml after 24h treatment.

Morphological Changes

The cytotoxicity effect of the drug leads to the morphological changes in the cell structure. Fig. 4. clearly demonstrates that, the increase in the drug concentration causes the cell morphology to change (cell shrinkage, disorganized structure) as compared to the control.

Fluorescent staining with Propidium Iodide

The morphology of Hep2 cell nucleus was examined using PI, a fluorescent DNA-binding agent. PI was taken up by apoptotic cells and the fragmented apoptotic bodies were seen. The results of our study (Fig. 5.) showed fragmented apoptotic bodies upon treatment with ME of wheatgrass. It induced nuclear damage like chromatin condensation and nuclear fragmentation, which are the indicators of apoptotic death, in cancer cells.

GC-MS analysis

Fig. 6, GC-MS analysis of methanol extract of wheatgrass powder, shows the

presence of nine bioactive constituents. Almost all the compounds found in the GC-MS analysis are found to possess hydroxyl group and/or double bonds that could have possibly contributed for free radical scavenging activity of the ME of wheatgrass. It is very well known fact that the antioxidant properties of the various compounds are due to the presence of numerous double bonds and hydroxyl groups that can donate electrons through resonance to stabilize the free radicals³¹. The presence of electron donating groups like free hydroxyl and alternate double bonds might have helped in terminating the free radical chain reaction.

Squalene is one of the active compounds with peak area of 8%, found in our GC-MS reports. Squalene is found to have good antioxidant and antitumor activity. *In Vitro* experiments have suggested that squalene is a highly effective singlet oxygen scavenging agent. Moreover, squalene is also found to inhibit aberrant hyperproliferation, an event that precedes mammary tumorigenesis *In Vivo*³². Rao *et al*³³ have reported that squalene inhibits the azoxymethane (AOM)-induced colonic aberrant crypt foci thus showing chemopreventive activity against colon carcinogenesis. Budiyo *et al*³⁴ showed that the application of olive oil (a rich source of squalene) effectively prevented UVB induced murine skin tumour, possibly because of its antioxidant activity. From these reports it can be suggested that squalene might have played a significant role in promoting the antioxidant and antiproliferative activity of ME of wheatgrass.

Phytol, another important compound with peak area of 14%, found in GC-MS has also shown anticancerous activity by induction of apoptosis. The antitumor activity of phytol has been detected in various cell lines like HT29 (Human colon

cancer), P388 (mouse lymphocytic leukemia cells), MG63 (osteosarcoma cells) and AZ521 (gastric cancer cells). On the basis of these reports it can be suggested that phytol could also have contributed for the cytotoxic effect of ME of wheatgrass in Hep2 cells³⁵.

In a recent report 11,14,17-Eicosatrienoic acid, an omega -3 polyunsaturated fatty acid have been reported to possess protective effect against UV- induced skin damage by suppressing the expression of IL-1 β , COX-2 and MMP-13 at mRNA and protein levels and by regulating in NF- κ B signalling pathways³⁶. Various reports have suggested that omega-3 polyunsaturated fatty acid has found to augment the cancer therapy, helps in prevention and treatment of colon rectal cancer³⁷. In our GC-MS result we have also found the methyl ester of 11,14,17-Eicosatrienoic acid with highest peak area of 36%, as a constituent of ME, which could also have contributed for the antiproliferative effects of ME of wheatgrass.

Thus from our GC-MS result, we assume that the antioxidant and antiproliferative activity of ME could be due to the presence of various bioactive constituents especially squalene, phytol, methyl ester of 11,14,17-Eicosatrienoic acid, along with the presence of potent polyphenolics, flavonoids, alkaloids, saponin, tannin, coumarin etc (found in the phytochemical analysis).

CONCLUSIONS

In our study, methanol extract was found to be a good solvent extract, which showed a positive correlation between total phenolics, flavonoids and other phytochemical constituents and the antioxidant properties. It has also been found to inhibit the proliferation of Hep2 cells which could be by induction of apoptotic like nuclear fragmentation and chromatin

condensation. GC-MS report of wheatgrass powder revealed the presence of nine bioactive constituents, containing numerous double bonds and free hydroxyl groups. So we suggest that the antioxidant and antiproliferative activity of the methanol extract of wheatgrass could be due to the presence of phenolics and flavonoids along with the nine different bioactive components. Thus, the present study ascertained that the wheatgrass is an important source of natural antioxidant. Therefore its consumption may play an important role in reducing the oxidative stress and preventing various degenerative diseases that include cancer. However further studies are required to identify the mechanism of action of wheatgrass as anticancerous agent.

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Table 1. Phytochemical estimation

Particulars	Water Extract	Crude Extract	Methanol Extract	Acetone Extract	Chloroform Extract
Alkaloids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Tannins	+	+	+	+	-
Amino acid and Protein	+	+	+	+	+
Carbohydrate	+	+	+	+	+
Cardioglycoside	+	+	+	+	+
Saponins	+	+	+	+	+
Coumarin	+	+	+	+	+
Terpenoids	+	+	+	+	+
Sterol and Steroids	-	-	-	-	-
Quinone	-	-	-	-	-

“+” indicate the presence of compound in the extract and “—” indicate the absence of compound in the extract.

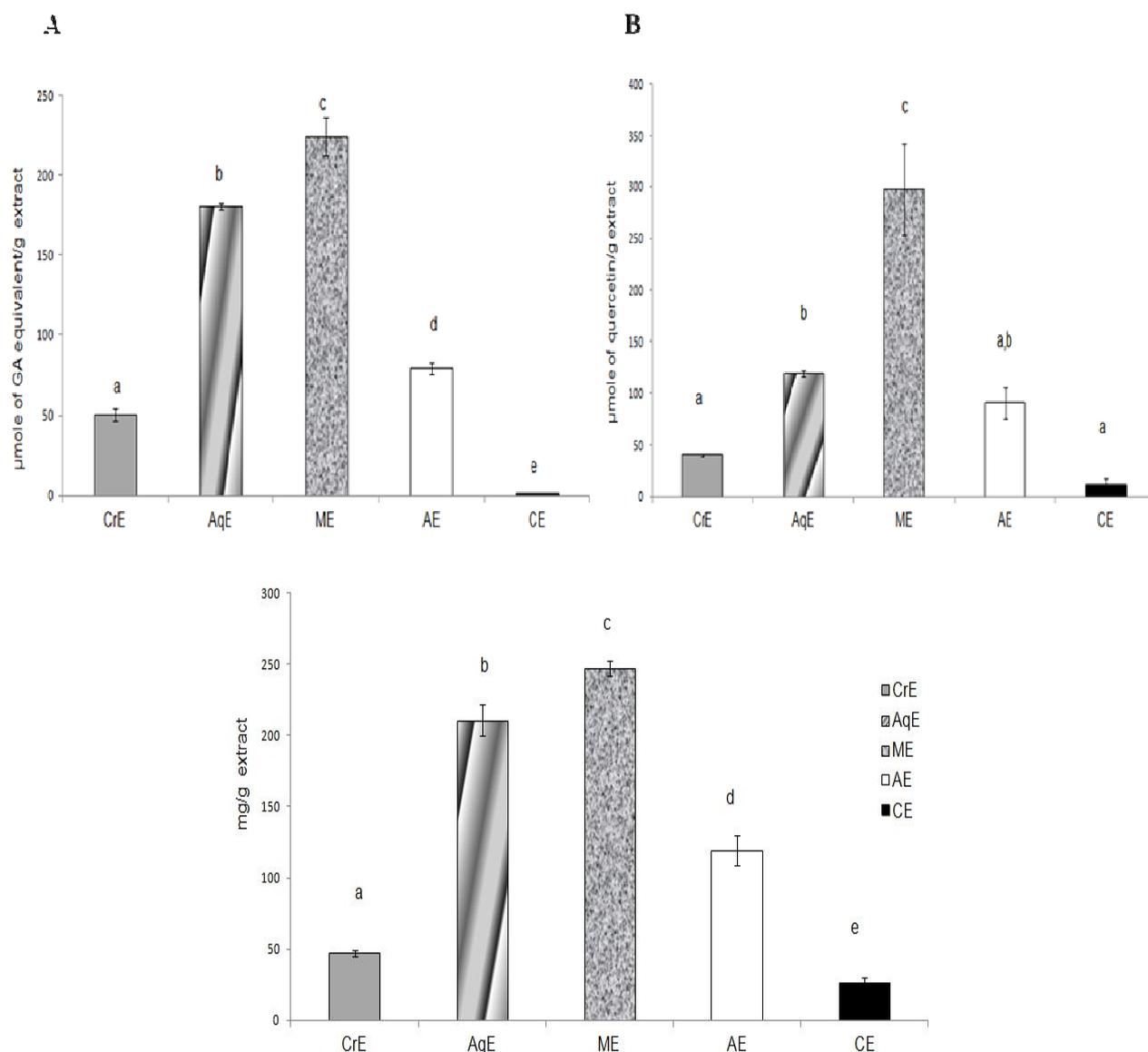


Figure 1. Phytochemical analysis

Values are mean \pm SD of triplicate observation in each group. ANOVA followed by Tukey's test. Bars sharing a common superscript do not differ significantly at $P \leq 0.05$

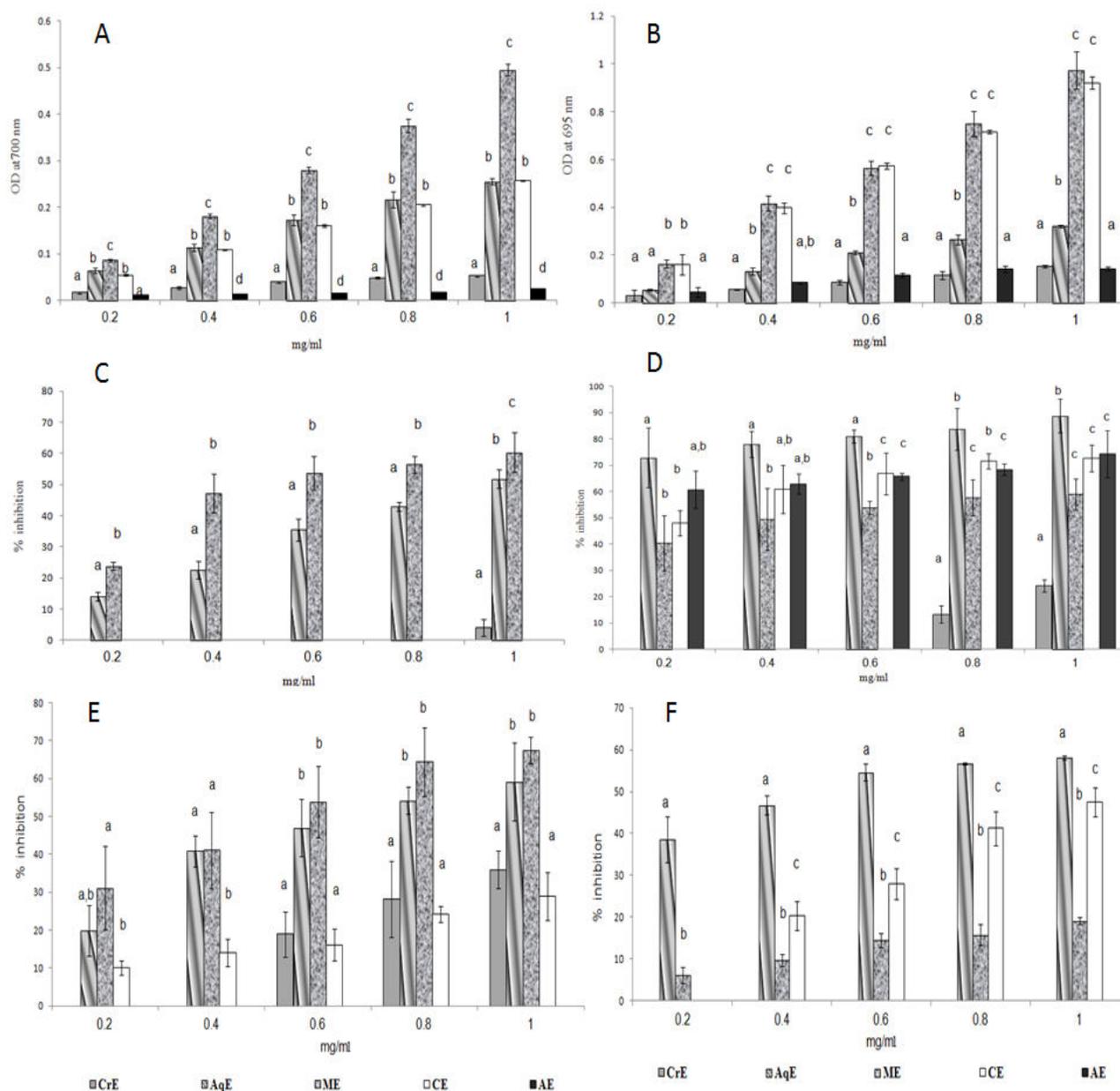


Figure 2. Antioxidant activity

(A) Ferric reducing activity, (B) total antioxidant activity, (C) metal chelating activity, (D) hydroxyl radical, (E) hydrogen peroxide radical, (F) nitric oxide radical scavenging activity of different extracts of wheatgrass

Values are mean \pm SD of triplicate observation in each group. ANOVA followed by Tukey's test. Bars sharing a common superscript do not differ significantly at $P \leq 0.05$

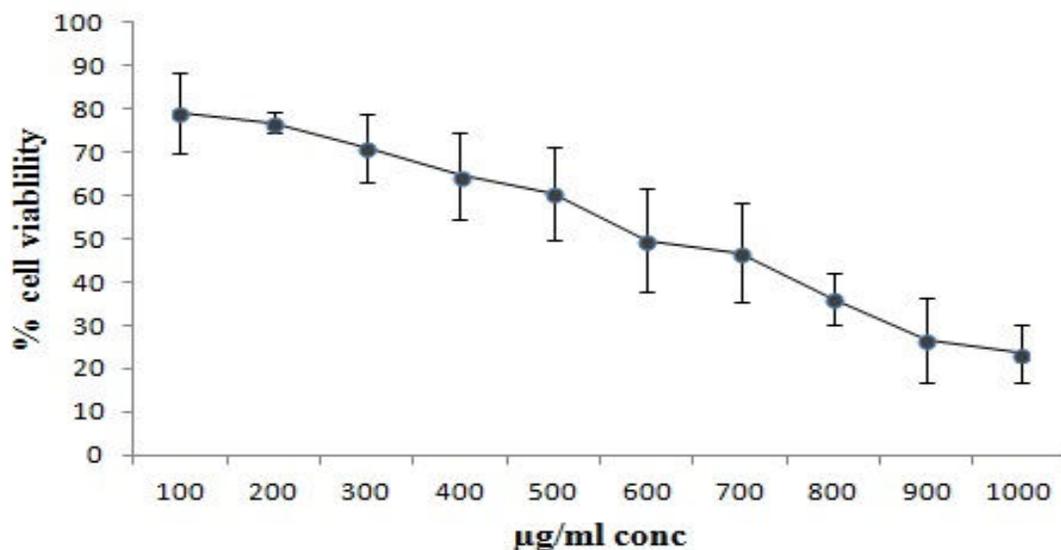


Figure 3. Antiproliferative activities of methanol extract (ME) of wheatgrass (MTT assay)

Antiproliferative effects of different concentrations of methanol extract of wheatgrass on Hep2 cells after 24h treatment. (IC₅₀- 600 µg/ml)

Values are means ± SD of triplicate observations from one representative of at least three experiments with similar results

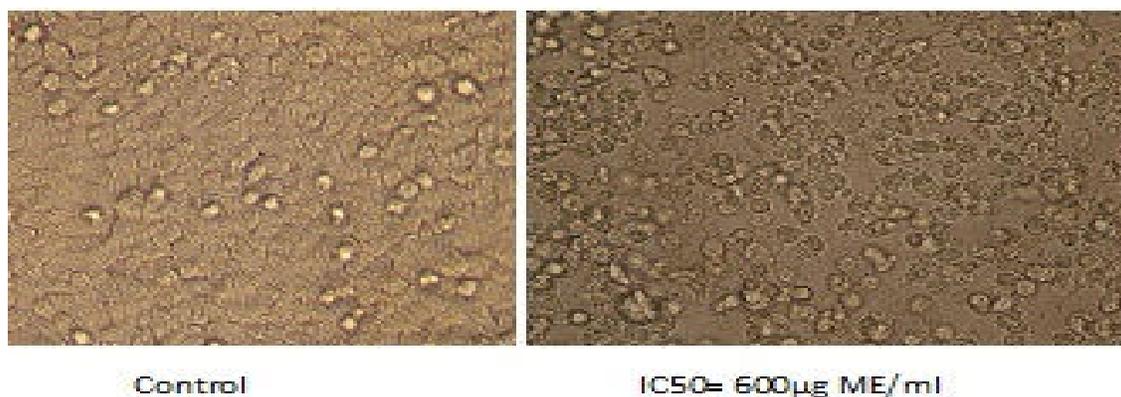
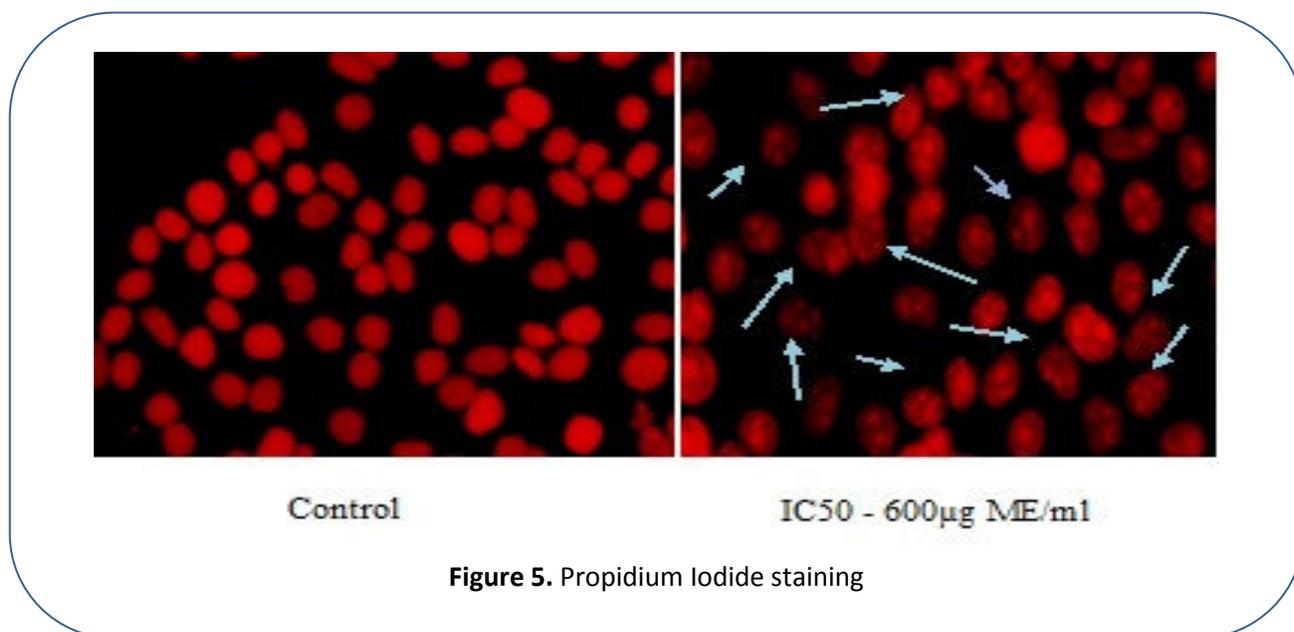


Figure 4. Morphological changes of Hep2 cells

Changes in the morphology of Hep2 cells after 24h treatment with IC₅₀ concentration of methanol extract of wheatgrass.



Changes in the morphology of nuclear chromatin of Hep2 cell after 24h treatment with IC50 concentration of methanol extract of wheatgrass

No.	RT	Name of the compound	Molecular formula	MW	Peak area %	Structure
1.	11.40	1,14-Tetradecane diol	C ₁₄ H ₃₀ O ₂	230	18.0	
2.	12.42	Hexanoic acid, 2-methyl-	C ₇ H ₁₄ O ₂	130	2.0	
3.	12.88	1,2-Benzenedicarboxylic acid dipheptyl ester	C ₂₂ H ₃₄ O ₄	362	8.0	
4.	14.46	Cyclohexane, ethenyl-	C ₈ H ₁₄	110	4.0	
5.	14.55	11,14,17-Eicosatrienoic acid, methyl ester	C ₂₁ H ₃₆ O ₂	320	36.0	
6.	14.68	Phytol	C ₂₀ H ₄₀ O	296	14.0	
7.	19.50	Furan-2-carboxamide, 5-benzoyl-N-(2-dimethylaminoethyl)	C ₁₆ H ₁₈ N ₂ O ₃	286	6.0	
8.	20.51	Didodecyl phthalate	C ₃₂ H ₅₄ O ₄	502	4.0	
9.	24.32	squalene	C ₃₀ H ₅₀	410	8.0	

GC-MS Chromatogram

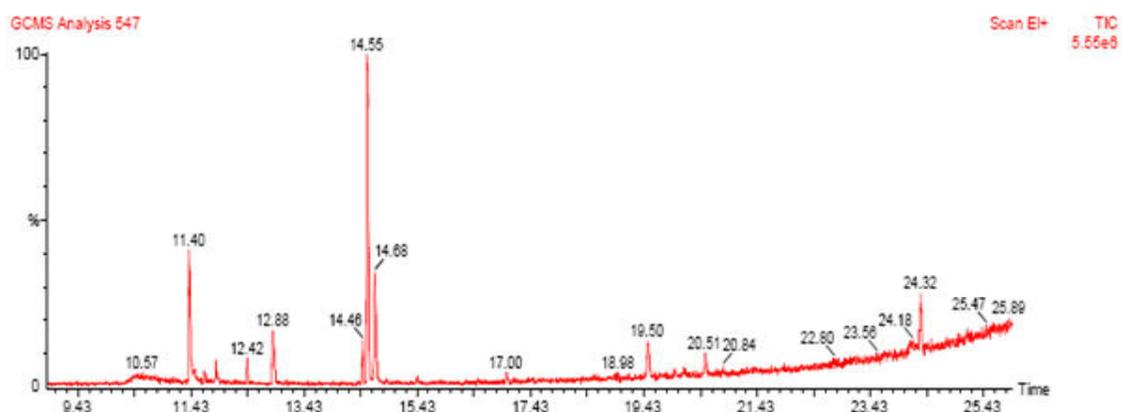


Figure 6. GC-MS analysis for different components in methanol extract of wheatgrass