

Antiproliferative, apoptotic and antioxidant activities of wheatgrass (*Triticum aestivum* L.) extract on CML (K562) cell line

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Aim: Plant-based diet supplements help the prevention and therapy of several kinds of cancer because they contain micronutrients, a class of substances that have been shown to exhibit chemopreventive and chemotherapeutic activities. In the present study the effects and oxidant/antioxidant status of aqueous and ethanol extracts of wheatgrass were tested in human chronic myeloid leukemia CML (K562) cell line.

Materials and methods: K562 cell lines were treated with 10% (w/v) concentration of aqueous and ethanol wheatgrass extracts. Cytotoxicity and apoptosis were determined morphologically and by MTT and DNA laddering. MDA level and CAT, SOD, and ADA activities of the cell lines were measured.

Results: Both preparations inhibited the growth of leukemia cells in a time-dependent manner. The most apoptotic and antiproliferative effect was seen in the cell line treated with aqueous extract at 48 h ($P < 0.001$). Increases in MDA level and CAT and SOD activities were observed.

Conclusion: Wheatgrass extract has an antioxidant activity, inhibits proliferation of leukemia cells, and induces apoptosis; thus, this finding may represent a novel therapeutic approach for the treatment of CML.

Key words: Wheatgrass extract, chronic myeloid leukemia cell line, antiproliferative effects, apoptosis, antioxidant enzyme activity

Buğday çimi (*Triticum aestivum* L.) ekstraktının KML (K562) hücre serisinde antiproliferatif, apoptotik ve antioksidan aktiviteleri

Amaç: Bitki kaynaklı diyet takviyeleri çeşitli kanserlerin önlenmesi ve tedavisinde kullanılırlar. Çünkü içerdikleri mikromineralerin bağlı olduğu sınıftaki maddeler kemopreventif ve kemoterapötik aktiviteye sahiptirler. Bu çalışmada kronik myeloid lösemi KML (K562) hücre serisinde, buğday çiminin su ve etanol ekstraktlarının etkisi ve oxidant/antioksidan durumu incelendi.

Yöntem ve gereç: K562 hücre serileri buğday çiminin % 10 (w/v) konsantrasyondaki su ve etanol ekstraktları ile muamele edildi. Sitotoksisite ve apoptozis morfolojik olarak, MTT ve "DNA laddering" ile saptandı. Hücre serilerinin MDA seviyeleri ve CAT, SOD ve ADA aktiviteleri ölçüldü.

Bulgular: Her iki ekstrakt da zamana bağlı olarak lösemi hücre serilerinin büyümesini inhibe etti. En fazla gözlenen apoptotik ve antiproliferatif etki 48. saatte buğday çiminin sudaki ekstraktı ile muamale edilmiş hücre serilerinde görüldü ($P < 0,001$). MDA seviyesi, CAT ve SOD aktivitelerinin yükseldiği saptandı.

Sonuç: Buğday çimi ekstraktı antioksidan aktiviteye sahip olup, lösemi hücrelerinin proliferasyonunu inhibe etti ve apoptozisi indükledi. Bu yönleriyle, elde edilen sonuç KML tedavisinde uygun yeni terapötik yaklaşımların olabileceğini düşündürmektedir.

Anahtar sözcükler: Buğday çimi ekstraktı, kronik miyeloid lösemi hücre serisi, antiproliferatif etki, apoptozis, antioksidan enzim aktivitesi

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Introduction

Wheat (*Triticum aestivum* L.) is an important component of the human diet, particularly in developing countries. Epidemiological studies have shown that the consumption of whole grain and whole-grain products are protective against chronic diseases such as cardiovascular disease, diabetes, and cancer (1-4). Wheat germinated over a period of 6-10 days is generally called wheatgrass (5). During germination, vitamins, minerals, and phenolic compounds including flavonoids are synthesized in wheat sprouts, and wheat sprouts reach the maximum antioxidant potential (5).

Wheatgrass contains vitamin C and E, β -carotene, ferulic acid, and vanilic acid the concentrations of which increase with the germination period (6), and wheatgrass also contains chlorophyll, which was found to be responsible for inhibiting the metabolic activation of carcinogens (7,8). There are reports on the antimutagenic effect of oxidative DNA damage towards benzo(a)pyrene induced mutagenicity (9). Falcioni et al. demonstrated the inhibition effect of wheatgrass on oxidative DNA damage (10). It has been shown that wheatgrass extracts contain significant amounts of phenolic compounds including flavonoids (11). Phenolic compounds of plant products are mainly responsible for the antioxidant activity to reverse the effect of ROS mechanism by various pathways, and they have a potent effect to reduce incidence of cancer (12). Reactive oxygen species (ROS) are produced as a by-product of various metabolic processes, mainly during respiration, in living organisms. Normal physiological concentrations of ROS usually have a role of regulation of cell activities, whereas higher concentrations cause oxidative damage. Oxidative stress is considered to be implicated in the pathophysiology of many diseases including cancers. The generation of ROS is prevented by ROS-interacting enzymes (catalase, superoxide dismutase) and additional antioxidants like medicinal plants (green tea, wheat). Recently, investigators have focused on the antioxidant potential of plant tissue.

Although there are some reports on inhibition of in vitro metabolic activation of carcinogens by wheat sprout extracts (7,8) and the antimutagenic effect of oxidative DNA damage towards benzo(a)pyrene

induced mutagenicity (9), there are no reports on whether wheatgrass extract has an effect on human cancer/leukemia cell lines.

Considering the rich antioxidant and vitamin contents of wheatgrass, this study investigated possible effects and oxidant/antioxidant status of aqueous and ethanol extracts of wheatgrass on the human chronic myeloid leukemia (CML) (K562) cell line characterized by the BCR/ABL fusion gene.

Materials and methods

Preparation of wheatgrass extracts

The package of organic hard red wheat seed that we used was a commercial product (Işık organic agriculture products) certified organic by the "BCS GmbH" (TR-OT-001) (certificate number: 8467). The seeds were harvested from a commercial planting located in Manisa, Turkey, in 2007.

The seeds of wheat (*Triticum aestivum* L.) were procured and washed with tap water, followed by distilled water. The seeds were soaked in distilled water for 8 h and transferred to the containers and the wheat plants were grown in soil. The wheatgrass was collected on day 10. Only wheatgrass of uniform size and shape, without injuries was selected. The plants were washed, wiped and cut into small pieces. They were homogenized with a clean pestle and mortar using distilled water and ethanol (10% w/v). The extracts were centrifuged at 15,000 rpm for 20 min at 4 °C and the supernatants were stored at -20 °C until further use (5).

Cell culture and extract preparation

The K562 (CML) human myeloid cell line was obtained from Oregon Health and Science University, Cancer Institute, Portland, USA. These cells were maintained in RPMI-1640 medium, supplemented with 20% heat inactive fetal Calf Serum (FCS) and 2 mM L-Glutamine, antibiotics (1,000,000 U/mL penicillin and 1 g/mL streptomycin), at 37 °C in a humidified atmosphere of 5% CO₂ in air. The K562 cell line was treated with (5×10^6 cells/mL in a 6-well plate) 10% (w/v) concentration of aqueous and ethanol wheat grass extracts for 0, 24, and 48 h. Untreated cells were used as a negative control for each condition. All experiments were performed in triplicate.

MTT assay for cell viability

The extent of the cell proliferation and cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cell proliferation and viability were determined by MTT (Cell proliferation Kit I, Roche, Germany) assay after wheatgrass extract treatments according to the manufacturer's protocol. A Thoma slide was used to count the cells in the culture flask. In brief, 1×10^5 cells were incubated in 96-well plates. Cells were treated with 10 μL /well of MTT reagent for 4 h at 37 °C and then treated with 100 μL /well of solubilization solution at 37 °C. After this incubation period, a water-insoluble formazan dye was formed. Following the solubilization, the formazan dye was quantitated by measuring absorbances of OD_{550} - OD_{690} with a SOFT max Pro 3.12 program. The blank control contained cell culture medium only. At least 3 independent experiments were performed.

Apoptosis determination

Giemsa staining of nuclei was performed to detect morphological changes. Briefly, cells were washed with phosphate-buffered saline (PBS) (pH 7.3), fixed with methanol:acetic acid (3:1) and then stained with 5%. Cells were examined with a light microscope for the determination of fragmented/condensed of nuclei and condensation of chromatin with intact cytoplasm. Finally, the cells fixed on the cover slips were analyzed, and photographed under a fluorescence microscope (BH-2, Olympus, Japan). At least 1000 cells were counted in each experiment and the percentage of apoptotic cells was calculated.

DNA fragmentation assay: Apoptosis was also confirmed by DNA laddering by using an Apoptotic DNA Ladder Kit (Roche, Germany). DNA samples were electrophoresed on 1.5% agarose gel containing ethidium bromide, visualized under UV light, and photographed.

Oxidant/antioxidant parameters

Oxidant (malondialdehyde [MDA]) level) and antioxidant (catalase [CAT] and superoxide dismutase [SOD] activities) parameters and adenosine deaminase (ADA) activity were measured in the cell line at the beginning, and after 24 h and 48 h.

MDA concentration, given in nmol/million cells, was determined by using the thiobarbituric acid reaction. The activity of CAT was given in international unit (IU)/mL cell and SOD activity in U/mL cell. One unit for SOD activity was defined as the amount of protein causing 50% inhibition of the nitroblue tetrazolium salt (NBT) reduction rate. The activity of CAT was determined by measuring the absorbance decrease of hydrogen peroxide (H_2O_2) at OD_{240} .

MDA level and CAT, SOD, ADA activity analyses were performed as described in the references respectively (13-17).

Statistical analyses

Data were summarized as median (minimum-maximum). Differences among groups were evaluated by Kruskal-Wallis and Friedman tests. Multiple comparison tests were used to find out which groups differ from which others. P values less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS 11.5 (Statistical Package for the Social Sciences) for Windows.

Results

Apoptotic effect of wheatgrass ethanol extract on K562 cells was 1.6 and 2.4 times higher than that of the controls at 24 h and 48 h, respectively (Figure 1) and apoptotic effect of wheatgrass aqueous extract on K562 cells was 4.3 and 4.6 times higher than that of the controls at 24 h and 48 h (Figure 2), respectively. Although there were no significant differences between apoptotic effects of aqueous and ethanol extracts of wheatgrass, the highest apoptotic effect was observed on K562 cells treated with aqueous extract of wheatgrass at 48 h (Figure 3).

MTT results

All MTT values of aqueous and ethanol wheatgrass extract-treated K562 cells were found to be significantly different ($P < 0.05$) compared to the values of their controls. The vitality rate of the cells decreased by 14% in ethanol wheatgrass extract-treated cells and increased by 24% in their controls (Figure 4), and the vitality rate of the cells decreased by 39% in aqueous wheatgrass extract-treated cells and increased by 50% in their controls at 24 h (Figure 5).

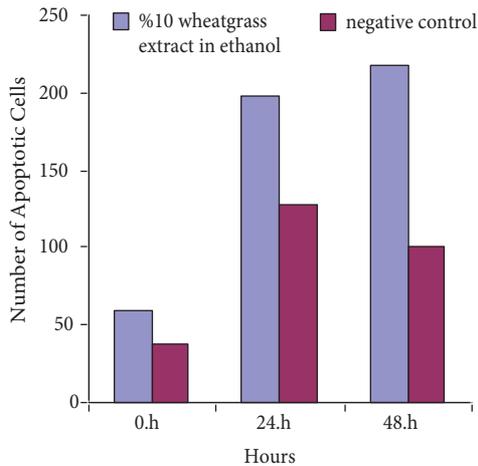


Figure 1. Apoptotic effect of wheatgrass ethanol extract on K562 cells.

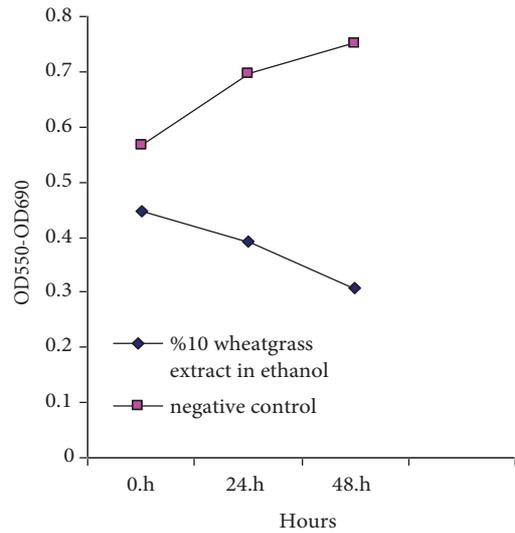


Figure 4. Antiproliferative effect of wheatgrass extract in ethanol on K562 cells.

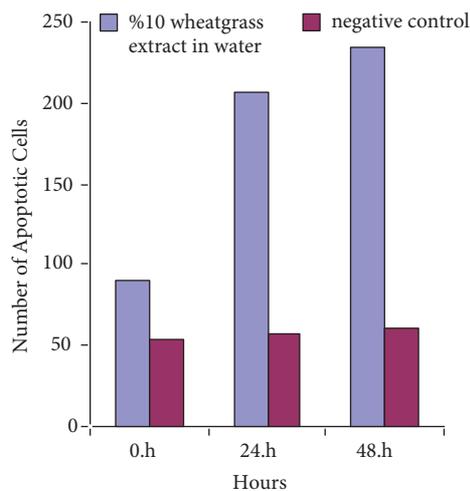


Figure 2. Apoptotic effect of wheatgrass aqueous extract on K562 cells.

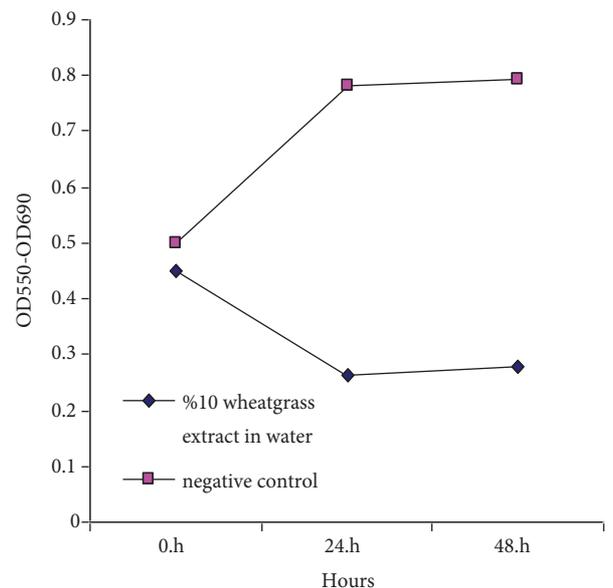


Figure 5. Antiproliferative effect of wheatgrass aqueous extract on K562 cells.

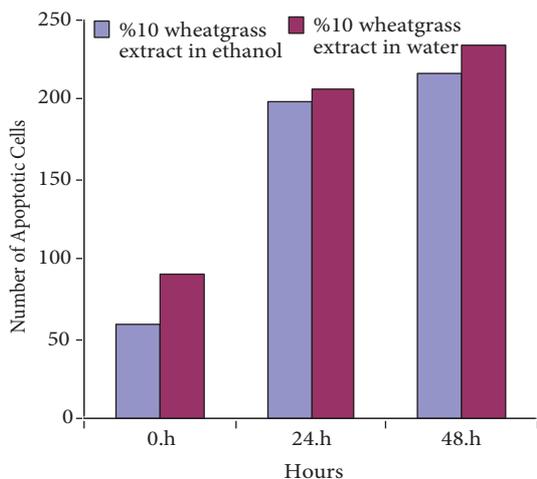


Figure 3. Comparison of apoptotic effects of both wheatgrass extracts.

While there was significant difference for antiproliferative effect on K562 cells between ethanol and aqueous extracts of wheatgrass at 24 h ($P < 0.05$), there was no significant difference at the 48 h. The vitality rate decreased by 29% and 35% in ethanol extract of wheatgrass-treated cells and in aqueous extract of wheatgrass-treated cells and increased by 34% and 57% in their controls, respectively at 48 h.

Oxidant/antioxidant results

In the present study, increases in MDA level and CAT and SOD activities were observed in the extract added groups at 0, 24, and 48 h in the cell line.

The data presented in Tables 1-3 show a significant increase of CAT activity in the aqueous extract added group. In both aqueous and ethanol extract added groups, SOD activity was significantly increased. Although MDA levels increased, there were no statistically significant differences in MDA levels between 0, 24, and 48 h in all the groups.

Discussion

CML is defined as a clonal myeloproliferative disorder, which is derived from oncogenic conversion of hematopoietic stem cells (18). The Philadelphia (Ph) chromosome, which functions because of a reciprocal translocation fusing the 5' sequence of the *bcr* gene with the upstream exon 2 sequence of the *c-abl* proto-oncogene on chromosome 22, is detected in fundamentally all cases of CML. The

fusion production, named BCR-ABL, is a protein (210 kDa) that it is larger than the normal Abl protein (160 kDa), and in which the tyrosine kinase is constitutively active (19). The chimeric BCR-ABL oncoprotein, which has tyrosine kinase activity and involves nuclear import and export signals, is the main molecular signal of CML.

In recent years, it has increasingly been recognized that malignancy may not exclusively result from enhanced cell proliferation but also from decreased physiological cell death, i.e. apoptosis (20). Apoptotic induction has been a new target for innovative mechanism-based drug discovery (21,22). Chemoprevention, a relatively new strategy to prevent cancer, depends on the use of nontoxic chemical substances, to block, reverse, or retard the process of carcinogenesis. Plant-based diet is regarded one of the potential chemopreventive agents (23,24). If a plant-derived extract induces apoptosis and has antiproliferative and antioxidant effects, it might protect normal cells from the damage caused by ROS while inducing apoptosis and inhibiting proliferation in tumor cells.

Table 1. Oxidant/antioxidant status in K562 cell line (at 0 h).

	GROUPS				P value
	Control with aqueous	Control with ethanol	Aqueous extract	Ethanol extract	
MDA	3.15 (2.58-3.31)	3.06 (2.90-3.15)	3.47 (3.31-4.19)	3.79 (3.23-7.82)	0.066
CAT	4.39 (3.66-5.12)	3.66 (2.93-5.86)	24.16(24.16-24.89)	5.86(4.39-6.59)	0.050
SOD	5.59 (3.80-6.70)	9.29 (6.60-9.61)	13.46 (11.68-13.65)	13.04 (12.84-13.07)	0.029
ADA	0 (0-1.45)	2.55 (0-6.72)	0.6 (0-1.02)	1.45 (0-2.21)	0.510

Values are expressed as median (minimum-maximum).

Table 2. Oxidant/antioxidant status in K562 cell line (at 24 h).

	GROUPS				P value
	Control with aqueous	Control with ethanol	Aqueous extract	Ethanol extract	
MDA	2.50 (2.26-2.74)	2.82 (2.42-3.55)	3.06 (2.82-3.55)	2.98 (2.98-3.06)	0.147
CAT	2.93 (2.20-2.93)	2.93 (2.20-2.93)	21.23 (19.76-21.23)	5.12 (5.12-5.86)	0.020
SOD	6 (5.79-6.27)	6.22 (5.80-6.41)	13.83 (13.76-14.04)	13.86 (13.52-13.98)	0.038
ADA	0.024 (0.01-0.03)	0.033 (0-0.04)	0.052 (0-0.24)	0.037 (0.03-0.18)	0.547

Table 3. Oxidant/antioxidant status in K562 cell line (at 48 h).

	GROUPS				P value
	Control with aqueous	Control with ethanol	Aqueous extract	Ethanol extract	
MDA	3.63 (3.47-4.03)	4.11 (3.23-4.68)	4.76 (4.36-5.08)	5.73 (4.60-19.03)	0.066
CAT	5.12 (4.39-5.86)	2.93 (2.93-3.66)	21.23 (20.50-21.96)	6.59 (5.12-9.52)	0.020
SOD	6.19 (4.96-6.57)	5.99 (5.08-8.73)	13.74 (12.52-13.79)	13.68 (12.31-13.83)	0.040
ADA	0.029 (0-0.06)	0.052 (0-0.09)	0 (0-0.14)	0 (0-0.02)	0.770

In this study, we investigated *in vitro* effects of wheatgrass extract on the human CML (K562) cell line and compared the effects of aqueous and ethanol extracts on K562 cells. Our results showed that both of the wheatgrass extracts inhibited the growth of leukemia cells in a time-dependent manner, compared to the controls. Although no significant differences were found between aqueous and ethanol extracts of wheatgrass, the highest apoptotic effect was observed on the K562 cell line treated with aqueous extract of wheatgrass at 48 h ($P < 0.001$). It was determined that at that time the death risk of K562 cells treated with aqueous extract of wheatgrass was 4.6 times higher than that of the untreated K562 cells. The results of this study also showed that there were increases in antioxidant enzyme activities, namely SOD and CAT activities, and these increases prevented oxidative reactions in part. Additionally, our results showed that the antioxidant activity of the aqueous extract of wheatgrass was better than that of ethanol extract.

With their antioxidant potential, wheatgrass extracts can be used as a dietary supplement in some diseases as well as in CML. In our previous study, it was found that wheatgrass extract induced apoptosis in 32Dp210 cells 6.2 times higher than in 32D cells (25). Although CAT and SOD activities

were increased in the extract added groups, it seemed that this compensatory change could not prevent cell death. Thus, the mechanism of the apoptosis might be based on some reasons other than oxidative stress. Our previous data show that wheatgrass extracts induce apoptosis in CML cells rather than in a normal cell line (25), and recent data have also shown their apoptotic and antiproliferative effects on the human CML cell line in addition to their antioxidant properties, which have possible protective effects from ROS damage in normal cells.

In conclusion, to the best of our knowledge, this is the first study to demonstrate that wheatgrass extracts exert significant anti-proliferative and apoptotic effects against K562 cells in a concentration- and time-dependent manner. Thus, it can be concluded that wheatgrass extract might have therapeutic value against CML. However, further investigations on a cellular or molecular level are necessary to describe possible mechanism(s) that cause these effects of wheatgrass.

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