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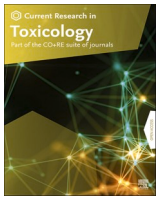

Modulation of wheatgrass (*Triticum aestivum Linn*) toxicity against breast cancer cell lines by simulated microgravity

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ABSTRACT

This study scrutinizes the effects of simulated microgravity on the antioxidant and cytotoxic potential, along with the phytochemical content of wheatgrass (*Triticum aestivum Linn*). To imitate microgravity, wheatgrass seeds were germinated in a 3D-clinostat at different rotations per minute (5, 10, 15, and 20 rpm), together with terrestrial gravity control, over 10 days. After germination, the methanolic extracts were analyzed using UPLC-Triple Quad LCMS for their phytochemical composition and tested for their hydrogen peroxide, nitric oxide, and DPPH scavenging activities. The cytotoxic effects of these extracts were evaluated against normal skin fibroblasts, normal breast cells (MCF-10), and breast cancer cells (MCF-7 and MDA-231). The findings showed an extended root growth in wheatgrass germinated under microgravity (WGM) compared to under gravity (WGG). Additionally, WGM extracts demonstrated increased H2O2–, NO–, and DPPH-scavenging activities and a higher content of polyphenols and flavonoids than WGG extracts. These effects were amplified with an increase in clinostat rotations. Moreover, WGM extracts were found to contain a unique set of bioactive compounds (compounds that were detected in the microgravity-germinated wheatgrass but were either absent or present in lower concentrations in wheatgrass germinated under standard gravity conditions.), including pyridoxine, apigenin, and tocopherol, among others, which were absent in WGG. The UPLC-Triple Quad LCMS analysis revealed these unique bioactive compounds in WGM. Notably, WGM extracts showed enhanced cytotoxic effects against normal skin fibroblasts, normal MCF-10, MCF-7, and breast cancer MDA-231 cell lines, with increased cytotoxicity correlating with the number of clinostat rotations. Particularly, WGM extract (at 20 rpm) demonstrated significantly stronger cytotoxicity against MCF-7 breast cancer cells. Further in-depth gene expression analysis of MCF-7 cells exposed to WGM revealed a significant downregulation of genes integral to breast cancer pathways, tyrosine kinase signaling, and DNA repair, complemented by upregulation of certain cell survival and cytotoxic genes. These alterations in genetic pathways associated with cell survival, hormone responses, and cancer progression may elucidate the enhanced cytotoxicity observed in WGM extracts. Our findings underscore the potential of microgravity as a tool to enhance the cytotoxic capabilities of wheatgrass against cancer cell lines, presenting a promising direction for future research in the field of space biology and its implications for terrestrial health.

Introduction

Triticum aestivum Linn, commonly known as wheat, is a vital grain with wide-reaching applications and nutritional benefits (Langridge and Reynolds, 2021). This global staple is not only a rich source of dietary fiber, vitamins, minerals, and numerous bioactive compounds but also harbors antioxidant properties that contribute to disease protection (Popoola, 2022). Such bioactive compounds, predominantly located in the grain's outer shell or bran, include flavonoids and phenolic compounds. Germination of wheat grains enhances their nutritional value, with studies suggesting that germinated grains offer superior nutritive value compared to their non-germinated counterparts (Benincasa et al., 2015). An interesting aspect of this germination process is the increase in antioxidant compounds such as Vitamins C, Vitamin E, and β-carotene. Initially barely detectable in dry grains, these antioxidants increase during germination, peaking after seven days in darkness (Flores and

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Shaa, 2016). The germination of the common wheat plant results in young grass called wheatgrass, which belongs to the Poaceae (Gramineae) family. This germinated form of *Triticum aestivum Linn* is highly valued in traditional medicine, owing to its nutrition.

The nutrient composition of sprouted grains is significantly influenced by various growth conditions, including elements like humidity, temperature, length of germination, and the medium and circumstances for cultivation (Benincasa et al., 2019). Interestingly, the gravitational context, especially conditions of microgravity as experienced in space missions, is proven to affect plant growth and composition. As an illustration, wheat demonstrated higher concentrations of minor secondary metabolites when grown under microgravity compared to normal gravity (Kordyum and Chapman, 2017). Increasing rates of clinostat rotations per minute could potentially incite an escalated stress response in the sprouting *T. aestivum* (Ran et al., 2016). In turn, this may cause the antioxidant systems within the wheatgrass to enhance their operations to neutralize this stress. Contributing to these antioxidant activities are phenolic and flavonoid compounds, known for their redox abilities, which serve as hydrogen donors, reductive agents, and singlet oxygen quenchers. Studies have found that the roots of *T. aestivum* tend to extend more in microgravity conditions compared to normal circumstances, exhibiting increased antioxidant levels and activity. Additionally, an enhanced antidiabetic impact has been observed in extracts of *T. aestivum* cultivated in the absence of gravity (Al-Awaida et al., 2020).

Plant-derived antioxidants are recognized for their capacity to mitigate oxidative harm by neutralizing free radicals, thereby providing enhanced protection against diseases like cancer and heart coronary (Yıldırım and Kaya, 2017, Nunes et al., 2020). Various studies have shown that wheatgrass extract can induce cell death and reduce the count of leukemia cells, inhibit metastasis in oral cancer cells, decrease the risk of diminished bone marrow function in chemotherapy patients, and improve enzymes that help lower blood glucose levels, as well as alter cholesterol profile (Mohan et al., 2013, Shakya et al., 2016, Gore et al., 2017, Bar-Sela et al., 2007, Cho et al., 2016).

Despite these extensive studies, no research to date has investigated the effect of microgravity on wheatgrass composition, its antioxidant, and cytotoxic capacities. This represents a significant gap in the literature, the filling of which holds immense potential for understanding the therapeutic capabilities of this plant species.

This study aims to address this gap by examining the impact of microgravity conditions on the germination of T. aestivum, its compositional changes, and its potential therapeutic applications. Specifically, we will focus on the changes in antioxidant content under microgravity and how these changes might influence the cytotoxic potential of *T. aestivum* extracts on specific cancer cell lines – MCF-7, MDA-MB-231, and leukemia cells.

Materials and methods

The 3D clinostat

In our experiment, we employed a 3D clinostat manufactured by Rumrock for Synthetic Engineering, model 12021, designed to simulate microgravity conditions for plant or animal cells. Earth's gravitational field, with an average magnitude of 9.8 m/s2 at any given point on its surface, impacts the sedimentation of cellular organelles in living organisms, informing their sense of gravitational direction. This clinostat, effectively a random positioning machine, disrupts the unidirectional pull of gravity, creating conditions akin to weightlessness in space(Yotov et al., 2022).

The 3D clinostat uses two motors to induce three-dimensional rotations. The primary motor operates at a constant angular velocity, while the secondary motor has a variable angular velocity. This combination allows us to adjust the rotational speed to 5, 10, 15, or 20 rotations per minute. It's worth noting that throughout our experiment, each motor consistently rotated in the same direction(Yotov et al., 2022).

Experimental conditions such as temperature and relative humidity were rigorously controlled. The temperature was held steady at 21 °C, while relative humidity was kept within the range of 50–60% - conditions recognized as ideal for plant growth in a laboratory environment.

This specific 3D-clinostat has demonstrated its effectiveness as a tool for investigating plant responses under simulated microgravity. The insights derived from our work contribute to the broader understanding of plant behavior in conditions resembling those of space(Hu et al., 2023).

Preparation of wheatgrass extracts

To mitigate microbial growth and safeguard sterility, an autoclave was employed to sterilize all apparatus and containers designated for the germination process. We selected uniformly sized seeds from the Ammon cultivar of *Triticum aestivum Linn* grains to diminish variability in the experiment. These seeds were subjected to a thirty-minute treatment with a 1.25% sodium hypochlorite solution at room temperature, an approach designed to neutralize potential contaminants(Al-Awaida et al., 2020).

Once sterilized, the grains were thoroughly rinsed using autoclaved distilled water, maintained at approximately 10 °C, for 15 min. Subsequently, the sterilized seeds underwent a twenty-four-hour soaking process in autoclaved tap water at a steady 21 °C, facilitating the onset of germination. The grains were then sown in a container equipped with drain holes, leveraging the superior aeration and drainage attributes of perlite as the substrate(Islam et al., 2022).

Throughout the ten-day germination phase, the seeds received nourishment from autoclaved Hoagland's solution, favored for its beneficial mineral content, and were consistently kept at a temperature of 21 °C. Post-harvest, the plants were gently rinsed with autoclaved distilled water for around three minutes, a measure designed to remove any lingering perlite or nutrients(Al-Awaida et al., 2020). During the entire germination process, both earth gravity and simulated microgravity conditions, achieved at 5, 10, 15, and 20 rotations per minute, were utilized to cultivate wheatgrass for ensuing experiments. Grains that failed to germinate were systematically discarded.

Following the harvest, the wheatgrass was shade-dried for seven days under conditions of 25 °C temperature and 70–90% relative humidity (Napagoda et al., 2020, Al-Awaida et al., 2020, Al-Awaida et al., 2018). After drying, the plants were ground into a fine powder. This powdered plant material was subjected to an extraction process using anhydrous, 99.8% methanol, and a Maceration extraction apparatus. We took precise measures to ensure the complete removal of methanol post-extraction with a rotary vacuum evaporator. Thereafter, the extracts from both gravity and microgravity conditions were dissolved in 0.1% DMSO (Al-Awaida et al., 2020). Considering the thorough extraction procedure, we anticipate that the residual influence of methanol on our results is practically non-existent.

The concentrated extracts obtained from the procedure were then stored frozen at −20 °C for subsequent use(Al-Awaida et al., 2018, Al-Awaida et al., 2020). In our experimental design, Wheatgrass germinated under gravity (WGG) served as a control to Wheatgrass germinated under microgravity (WGM) at varying rotation speeds.

Hydrogen peroxide-, nitric oxide-, and DPPH-scavenging activity assays

We assessed the ability of WGG and WGM extracts to neutralize hydrogen peroxide, nitric oxide, and DPPH radicals, using a range of concentrations: 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95, 0.98 μg/ml. Ascorbic acid, prepared at the same concentrations, served as a reference substance for comparison.

For the computation of IC_{50} values, we employed GraphPad Prism Software Version 8.0. This enabled us to accurately determine IC_{50}

through a Dose-Response curve, ensuring the accuracy of our results.

Hydrogen peroxide-scavenging assay: The method by Ruch et al. (Ruch et al., 1989) was adopted to evaluate the hydrogen peroxide-scavenging capacity of WGG and WGM extracts at varying rotations (5, 10, 15, and 20 rotations/minutes). A hydrogen peroxide solution (43 mM, 0.6 ml, pH 7.4) was added to 3.4 ml of varied extract concentrations (pH 7.4). After 10 min, the absorbance of the reaction mixture at 230 nm was determined. As a control, the reaction mixture without the extract was utilized. As a reference substance, ascorbic acid was used.

Nitric oxide-scavenging assay: Sodium nitroprusside compound was dissolved in an aqueous solution at physiological pH to spontaneously generate nitric oxide, which then interacts with oxygen to produce nitrite ions that are quantified by Griess reagent (Rao, 1997). The NO scavenger would be expected to compete with oxygen over NO and hence reduce the formation of nitrite ions. In our experiments, various concentrations of WGG and WGM extracts (5, 10, 15, and 20 rpm) were dissolved in dimethyl sulfoxide (DMSO), then mixed with sodium nitroprusside (5 mM) in 10 mM phosphate-buffered distilled water and incubated at 25 °C for 150 min. A control experiment was conducted with the same amount without any of the test extracts. At intervals, 0.5 ml of each of the incubated solutions was diluted with 0.5 ml of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2% H_3PO_4). The reaction of nitrite with Griess reagent generates a chromophore which is measured at an absorbance of 546 nm. Potassium nitrite was used as a standard to calculate the concentration of nitrite ions in the test solutions. Lower nitrite concentration represents higher NO-scavenging activity of the tested extracts.

DPPH-scavenging assay: According to Chen et al, (Chen et al., 1999), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate the free radical scavenging capabilities of wheatgrass extracts. Five mL of 0.04% DPPH radical solution were added to solutions containing the different extracts. The mixtures were vortexed and kept in dark conditions for 30 min. The optical density (OD) was measured at 517 nm wavelength. Anhydrous, 99.8% methanol was used as a negative control. Ascorbic acid was used as a positive control.

Determination of total polyphenol and flavonoid contents in wheatgrass extracts

In our assessment of total polyphenol and flavonoid contents, a standardized weight of 1000 μg/ml of dried wheatgrass powder was used.

The total polyphenolic content of WGG and WGM (5, 10, 15, and 20 rotations /minutes) was estimated using the Folin–Ciocalteu assay (Kaur and Kapoor, 2002). 50 μL wheatgrass extract and 475 μL 5% sodium carbonate solution were mixed. After 3–5 min, 475 μL of 50% Folin–Ciocalteu reagent was added and incubated at room temperature for 1 h. The TP concentration was determined by measuring the absorbance of the mixture at 724 nm with an ultraviolet–visible spectrophotometer and then estimating the results using a reference curve based on gallic acid. The results are presented as mmol of equivalents of gallic acid (GAEs) per 100 mg of dry plant weight (mmol GAE 100 mg − 1 DW).

The total flavonoid (TF) content of WGG and WGM (5, 10, 15, and 20 rotations/minute) was determined according to Nurcholis et al, (Nurcholis et al., 2021). 50 μL of plant extract and 40 μL of 5% potassium nitrite in 600 μL of ddH2O were incubated for 6 min at room temperature. Then 70 μL of 4.26% aluminum chloride solution was added and incubated for 5 min at 25 °C, followed by 240 μL of 1 M sodium hydroxide. The absorbance of the reaction result was measured at 510 nm, and the TF concentration was determined using a rutin-based standard curve. The results are reported in terms of mmol of rutin equivalents (REs) per 100 mg of plant dry weight (mmol RE 100 mg − 1 DW).

Determining the percentage of bioactive compounds in wheatgrass extracts

Bioactive compounds percentage was performed using a UPLC -Triple Quad LCMS (Model:

Shimadzu LC 30 + 8030 MS/MS), with SIL-30C autosampler with cooler, CTO-30 column oven, LC-30A pump, and CBM-20A system controller. The PrimeSIL (Wesley Technologies, Inc., USA) C18 column (4.6 cm × 25 cm × 5 μm) with a guard column was used with an isocratic mobile phase of 90% acetic acid–water (A) and 10% MeOH (B). The elution gradient was isocratic 10% B for 5 min, 10–100% B over 20 min, 100% B for 15 min, and re-equilibration of the column, using a flow rate of 200 μL/min. The autosampler temperature was maintained at 4 °C, and the injection volume was 10.0 μL. For mass spectrometry, an electrospray ionization (ESI) interface was used with positive and negative screening modes at 3 kV capillary voltage, 120 °C source block temperature, and 45 °C desolation gas temperature (Abraham et al., 2020).

In our study, the specific bioactive compounds we targeted and identified include flavonoids (apigenin, rutin, Quercetin, Kaempferol, Isoorientin, Isoscoparin), phenols, terpenes, quinones, phytosterols, carbohydrates (β-Glucan, Cellulose), fatty acids (Linolenic Acid), vitamins (pyridoxine, tocopherol, Vitamin A, Folate, γ-Tocopherol), coumarins (ellagic acid, Salicylic Acid, O-Coumaric Acid), and others like γ-Cryptoxanthin. The content of the extract from these bioactive compounds was determined based on the retention time of their respective standards.

Assessment of wheatgrass methanol extract cytotoxicity on breast Cancer, Non-Tumorigenic breast Epithelial, and normal skin fibroblast cell lines

MCF-7 and MDA-231 (cancerous cell lines), along with MCF-10 and skin fibroblast HDFa (non-cancerous cell lines), were seeded at a density of $1x10^4$ cells per well in 96-well Clear Flat Bottom Polystyrene TC-Treated plates using a 1 × 8 Stripwell™ format. The cells were cultured in their respective media to a final volume of 100 μL/well (Gołąbek-Grenda et al., 2023). The cancerous cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with fetal bovine serum (FBS) or bovine calf serum (BCS), antibiotics (penicillin–streptomycin), and glutamine. For the non-cancerous cell lines, MCF-10 cells were cultured in Mammary Epithelial Cell Growth Medium (MEGM) while HDFa cells were cultured in DMEM, supplemented with FBS or BCS, antibiotics, and glutamine. Plates were then incubated in a humidified atmosphere (37 °C, 5% CO2) for 24 h to allow cell adherence (Al-Awaida et al., 2018).

Subsequently, cells were exposed to the different concentrations of wheatgrass methanol extracts (WGG and WGM), and dissolved in 0.1% DMSO immediately before use. Equal volumes of 0.1% DMSO were used as vehicle control. All treatments, including untreated cells as viability controls, were conducted in triplicate for 72 h(Al-Awaida et al., 2018).

Cell viability was determined using the MTT assay with the CellTiter Non-Radioactive Cell Proliferation Assay Kit® (Promega; Madison, USA). The optical densities were recorded at 570 nm using a plate reader (Multiscan FC; Thermo Scientific, Waltham, MA, USA) (Al-Awaida et al., 2018).

The cytotoxic potential of the extracts was compared against a chemotherapeutic agent, Doxorubicin (Sigma, USA), used as a positive control. For the computation of IC_{50} values, we employed GraphPad Prism Software Version 8.0. This enabled us to accurately determine IC_{50} through a Dose-Response curve, ensuring the accuracy of our results.

Gene expression analysis

The 5X IC_{50} represents a concentration five times that of the IC_{50}. These precise concentrations were selected to evaluate the effect of the

extracts on MCF7 cells at both a dose that induces a half-maximal response (IC_{50}) and at a significantly higher level of toxicity (5X IC_{50}). The use of 5X IC_{50} was intended to explore the maximum changes in gene expression within a 24-hour window without resulting in cell death. In our study, we concentrated our efforts on analyzing the gene expression of the most affected cells, specifically MCF7 cells, after being treated with the WGM extract derived from plants grown at a rotation speed of 20 rpm.

RNA extraction and cDNA synthesis: RNeasy® Mini kit (Qiagen GmbH) was used(Özgür et al., 2023). Cells were collected and lysed in guanidine–thiocyanate-containing buffer (Rneasy lysis buffer) and then homogenized by vortexing. Next, 1 ml of 70% ethanol was added to the lysate. Samples were then applied to the Rneasy Mini spin columns where RNA was allowed to bind to the membrane of the column whilst contaminants were washed away. High-quality RNA was eluted in Rnase-free water. Binding, washing, and elution were performed by spinning the samples at 12,000 rpm in a microcentrifuge (Qiagen GmbH). The optical density ratio (OD260/OD280), which ranged between 1.9 and 2.2 for all measured samples, was used to evaluate the purity of extracted RNA. All RNA samples were stored at –80℃ until used for cDNA synthesis. A total of 0.5 μg of RNA was used to synthesize cDNA for each sample using the RT2 First Strand Kit® (Qiagen GmbH), whereby genomic DNA was eliminated and then reverse-transcribed to cDNA.

Real time-qPCR: The Breast cancer profiler PCR array (cat. No. PAHS-131ZR, Qiagen GmbH) and cell death profiler PCR array (cat. No. PAHS-212ZR, Qiagen GmbH) kits were used(Subayyil et al., 2023). The kits included primers for 84 genes that are relevant to Breast cancer and cell death, in addition to 12 genes for quality control purposes.

A diluted cDNA aliquot was mixed with the RT2 SYBR® green Master Mix (glucose metabolism RT2 profiler PCR array; cat. No. PAHS–006Z; Qiagen GmbH) and loaded into 96–well array plates. The qPCR reactions were performed using a CFX 96 thermocycler (Bio–Rad Laboratories, Inc.) with the following thermocycling conditions: Initial denaturation of 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. Data analysis was performed using the 2–ΔΔCq method available from the Biosciences company (Qiagen GmbH) web portal. Data were normalized across all plates to the expression of β–actin housekeeping gene. The threshold cycle values of the control wells were all within the ranges recommended by the PCR array user manual. Fold change represents normalized gene expression in the test sample divided by normalized gene expression in the control sample. Fold regulation represents fold change values. Fold change values > 1 indicate upregulation.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. Values are expressed as the mean ± SEM, and data were analyzed by using two-way ANOVA, followed by Tukey's multiple comparison test, with the level of significance set at $P < 0.05$.

Results

Germination of Triticum aestivum Linn. Under microgravity results in longer roots but not shoots compared to its germination under gravity conditions

The effect of microgravity on *Triticum aestivum Linn.* (wheatgrass) germination resulted in significantly longer roots compared to those grown under gravity conditions, which exhibited an average root length of 4.6 ± 0.35 cm. Microgravity conditions, simulated through various rotations per minute (rpm) on a 3D-clinostat, resulted in extended root lengths: 8.07 ± 0.24 cm at 5 rpm, 8.80 ± 0.4 cm at 10 rpm, 11.1 ± 0.31 cm at 15 rpm, and 14.80 ± 0.61 cm at 20 rpm. Statistical analysis confirmed these differences as significant when comparing gravity vs. all microgravity conditions, as well as when comparing the microgravity conditions of 5 rpm vs. 15 rpm and 20 rpm, 10 rpm vs. 20 rpm, and 15 rpm vs. 20 rpm. However, there were no significant differences in root length between 5 rpm and 10 rpm or 10 rpm and 15 rpm (Fig. 1). These results underscore a potential correlation between increased clinostat rotations and wheatgrass root elongation under simulated microgravity. On the other hand, no significant difference between gravity and microgravity conditions (5, 10, 15, and 20) was observed in maximum shoot length (Fig. 1).

Germination under microgravity enhances the antioxidant activity of *Triticum aestivum Linn.* Extract.

The methanolic extract of *T. aestivum* germinated under microgravity conditions (5, 10, 15, and 20 rpm) showed a significant increase in hydrogen peroxide scavenging activity with a lower half-maximal inhibitory concentration (IC_{50}) value (30.35 ± 4.391, 23.62 ± 0.955, 15.11 ± 2.886 and 6.24 ± 1.47, respectively) compared to gravity (45 ± 8.543) (Fig. 2A). In addition, no significant difference between ascorbic acid (11.027 ± 1.92) and WGM (15 and 20 rpm) was noted ($P > 0.05$). On the other hand, a significant difference between ascorbic acid (11.027 ± 1.92) and microgravity conditions (5 and 10 rpm) was observed. The methanolic extracts of T. aestivum germinated under microgravity conditions (20 rpm) exhibited significantly higher hydrogen peroxide scavenging activity compared to extracts of T. aestivum germinated undergravity and microgravity conditions (5, 10 rpm) (Fig. 2A).

The methanolic extract of WGM (10, 15, and 20 rpm) exhibited greater nitric oxide-scavenging activity with a lower IC_{50} (206.6 ± 19.354, 139.49 ± 22.457 and 98.782 ± 13.974, respectively) compared to WGG (492 ± 27.34). Moreover, the methanolic extracts of WGM (5, 10, 15, and 20 rpm) exhibited significantly lower nitric oxide scavenging activity with higher IC_{50} compared to ascorbic acid (Fig. 2B).

The methanolic extract of WGM (10, 15, and 20 rpm) showed significantly greater DPPH-scavenging activity with lower IC_{50} (9.763 ± 1.45, 7.493 ± 0.994 and 5.176 ± 0.381, respectively) compared to WGG (18.3 ± 1.92). In addition, no significant difference was observed between ascorbic acid and microgravity conditions (15 and 20 rpm) (7.493 ± 0.99 and 5.176 ± 0.38) (Fig. 2C).

Higher total phenolic and total flavonoid contents in *Triticum aestivum Linn extracts* germinated under microgravity (10, 15, and 20 rpm) compared to gravity conditions

The methanolic extract of WGM (10, 15, and 20 rpm) showed a significant increase in the total phenolic content (173.6 ± 8.89, 194.3 ± 18.64, and 297.8 ± 25.97, respectively) compared to WGG (65.9 ± 10.95) (Fig. 3A). However, no significant difference was observed between the methanolic extract of WGM (5 rpm) (78.7 ± 13.54) compared to extract of WGG. On the other hand, we noted a significant increase in the total phenolic content of the methanolic extracts of WGM (10, 15, and 20) compared to WGM (5 rpm) (78.7 ± 13.54) (Fig. 3A).

Moreover, the flavonoid content in the methanolic extracts of WGM (10, 15, and 20 rpm) was significantly higher (5.36 ± 0.28, 6.1 ± 0.52, and 7.16 ± 0.98, respectively) than in the extract of WGG (3.68 ± 0.54). A significant increase in the total flavonoid content was noted in the methanolic extracts of WGM (10, 15, and 20 rpm) compared to WGM (5 rpm) (1.6 ± 0.4). (Fig. 3B).

Furthermore, analysis of wheatgrass extracts through UPLC -Triple Quad LCMS (Model: shimadzu LC 30 + 8030 MS/MS) revealed significant differences between WGG and WGM in the contents of multiple bioactive compounds. The phytochemical composition of the plant extracts in this study indicated a wide range of potentially beneficial chemicals such as flavonoids, phenols, terpenes, quinones, phytosterols, carbohydrates, fatty acids, vitamins, and coumarins. Some of the phytochemical compounds were only detected in WGM extract such as

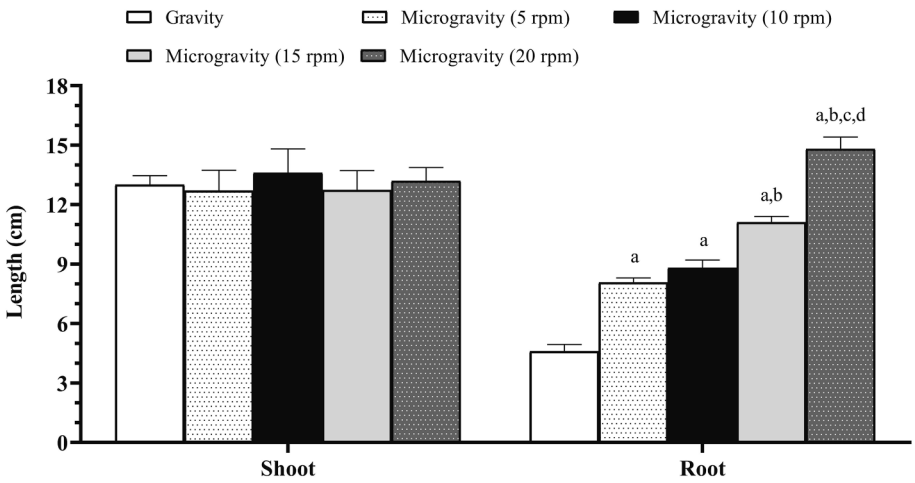


Fig. 1. Average shoot and root length of *Triticum aestivum* seeds germinated under gravity and microgravity conditions (5, 10, 15, and 20 rpm). Values are expressed as mean ± SEM. Data in a–e were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests with the significance level set at $P < 0.05$. **a** refers to gravity versus different conditions of microgravity (5, 10, 15, and 20 rpm); **b** refers to microgravity (5 rpm) versus microgravity (10, 15, and 20 rpm); **c** refers to microgravity (10 rpm) versus microgravity (15 and 20 rpm); **d** refers to microgravity (15 rpm) versus microgravity (20 rpm).

pyridoxine, apigenin, rutin, tocopherol, ellagic acid, Quercetin, Kaempferol, β-Glucan, Vitamin A, Folate, and Linolenic Acid (Table 1). On the other hand, many compounds were only detectable in the WGG extracts including Cellulose, γ-Tocopherol, Isoorientin, Isoscoparin, Salicylic Acid, O-Coumaric Acid, and γ-Cryptoxanthin (Table 1).

In vitro cytotoxicity effect of methanol extract of *t. Aestivum* against breast cancer, fibroblast, and non-tumorigenic epithelial breast cell lines

Compared to WGG, the methanolic extracts of WGM (5, 10, 15, and 20 rpm) exhibited greater cytotoxic effects against normal skin fibroblasts, normal MCF-10, and breast cancer MDA-231 cell lines with $IC_{50} > 125$ μg/mL. Additionally, the methanolic extract of WGM (20 rpm) showed significant strong cytotoxicity against MCF-7 cells with a lower IC_{50} (18.72 ± 1.23 μg/mL) compared to WGG IC_{50} (549.49 ± 13.00 μg/mL), and compared to WGM (5, 10 and 15 rpm) with IC_{50} of (313.02 ± 11.37, 297.45 ± 10.16, and 72.65 ± 16.40 μg/mL, respectively) (Fig. 4). These findings indicate that the cytotoxic effects of WGM extract is enhanced with increased number of clinostat rotations.

A differential expression in breast cancer and cell death genes in MCF7 cells exposed to extracts of wheatgrass germinated under microgravity versus gravity conditions

The extracts of wheatgrass germinated under microgravity (20 rpm) and gravity were tested at concentrations of IC_{50} and 5X-IC_{50} on MCF-7 cells for 24 h. Cells were then collected to study gene expression. In the breast cancer pathway, estrogen receptor 1 (ESR1) gene expression was significantly downregulated in response to WGM but not in WGG, with a more dramatic downregulation in the 5X-IC_{50} versus IC_{50} extracts (-16.02 vs. −3.64, respectively), indicating a dose–response effect. This paralleled the expression pattern in the Cyclin D1 gene (CCND1), which is a downstream signal to ER pathway. Moreover, progesterone receptor (PGR) gene expression was significantly downregulated with WGM (5X-IC_{50}) but not with other conditions, while Androgen receptor (AR) gene expression was also significantly downregulated with WGM on both concentrations but not with WGG (Table 2).

Our experiments suggest a downregulation in the tyrosine kinase signaling pathways. The EGFR and TGFB1 expression levels were significantly downregulated with WGM 5X-IC_{50} but not with the other conditions. Also, IGF1 gene expression was downregulated under all conditions but WGM 5X-IC_{50}. Importantly, the gene expression of AKT - the downstream signal to these tyrosine kinase pathways - was significantly downregulated with both WGM concentrations but not with WGG conditions (Table 2).

As for DNA repair, tumor suppressor, and/or pro-apoptotic genes, a significant downregulation in BRCA1, RB1, APC, BAD, DFFA, CASP2, CASP6, and CASP7 genes was noted with WGM extract 5X-IC_{50} but not with other conditions. Moreover, exposure of MCF-7 cells to WGM at 5X-IC_{50} has also significantly upregulated genes involved in cell survival including CCNA1, JUN, MAPK8, and BCL2A1. However, this was also accompanied with an upregulation in the growth arrest and DNA-damage inducible protein (GADD45α) which exhibits antiproliferative and pro-apoptotic effects. Conversely, other oncogenes and cell survival genes including BCL2 and ERBB2 were downregulated with WGM extracts at both concentrations, while CCNE1, BIRC3, BIRC5, and CTSS were downregulated only with WGM IC_{50}. The pro-metastatic MMP9 was also downregulated with WGM IC_{50} Furthermore, with WGM 5X-IC_{50}, the gene expression of the proinflammatory TNF and its receptor TNFRSF11B were significantly downregulated, while a significant induction in IL6 was observed. Additionally, the antioxidant gene GSTP1 was significantly downregulated with both concentrations of WGG and WGM compared to control (Table 2).

Taken together, a differential gene expression profile was observed between MCF-7 cells exposed to extracts of WGG versus WGM (20 rpm). The induction of genes that regulate cell survival and death was more pronounced with WGM versus WGG extract. This was also accompanied by a suppression in major pathways that promote cancer survival including sex hormone receptors and tyrosine kinase signaling. This might explain the superior growth inhibitory effect of the extract from wheatgrass germinated under microgravity (20 rpm) compared to wheatgrass germinated under gravity conditions.

Discussion

This study aims to investigate the effect of germinating wheatgrass under simulated microgravity versus earth gravity conditions on plant growth, phytochemical content, antioxidant activity, and cytotoxic effects against human normal and cancer cell lines. Our study is the first to demonstrate that *T. aestivum* germinated under microgravity exhibited increased cytotoxic effects, root length, total phenolic content, total flavonoid content, hydrogen peroxide-, nitric oxide-, and DPPH-scavenging activities compared to the *T. aestivum* germinated under gravity conditions. These effects of microgravity were enhanced by increasing the number of clinostat rotations to approach zero gravity.

Our results reveal that the wheatgrass roots, when exposed to microgravity conditions, grow significantly longer compared to those germinated under gravity conditions. In contrast, shoot growth did not display any significant difference between the microgravity and gravity conditions.

One plausible explanation for this could be the effect of the 3D-

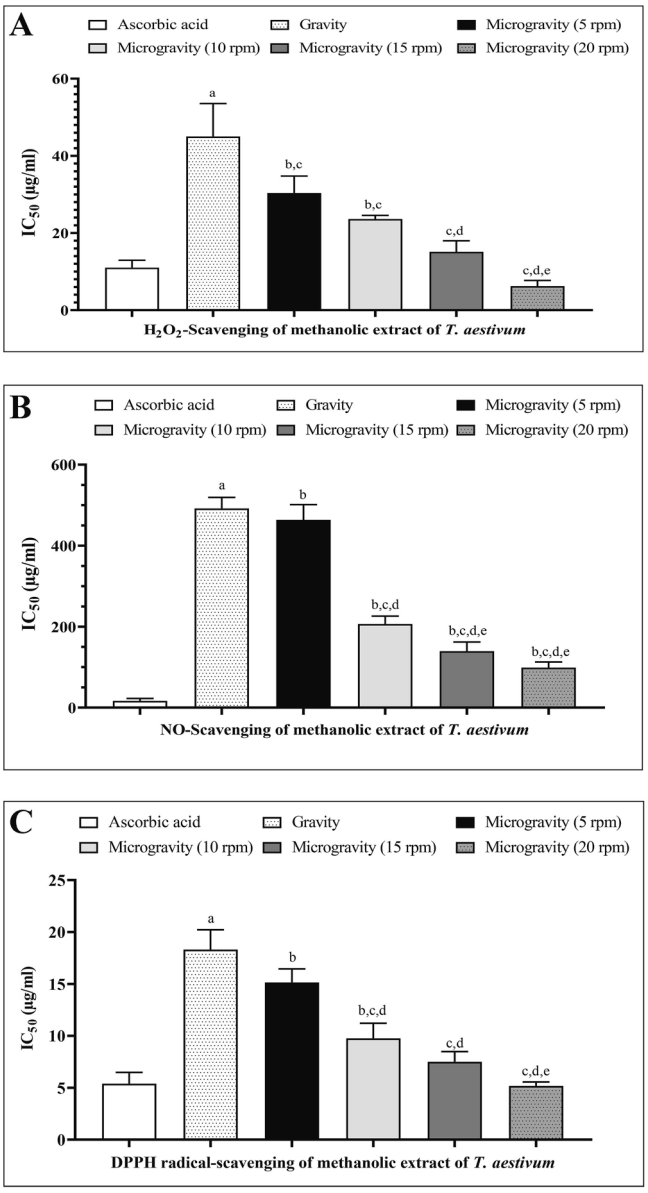


Fig. 2. A) Hydrogen peroxide; B) Nitric oxide; and C) DPPH-scavenging activities of methanolic extracts of *T. aestivum* germinated under gravity and microgravity conditions. Values are expressed as the mean ± SEM. Data in a–f were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests. **a** refers to gravity condition versus ascorbic acid; **b** refers to different conditions of microgravity (5, 10, 15, and 20 rpm) versus ascorbic acid; **c** refers to gravity condition versus different conditions of microgravity(5, 10,15, and 20 rpm); **d** refers to microgravity condition (5 rpm) versus microgravity (10, 15 and 20 rpm) conditions; **e** refers to microgravity condition (10 rpm) versus microgravity (15 and 20 rpm) conditions; **f** refers to microgravity (15 rpm) condition versus microgravity (20 rpm) condition.

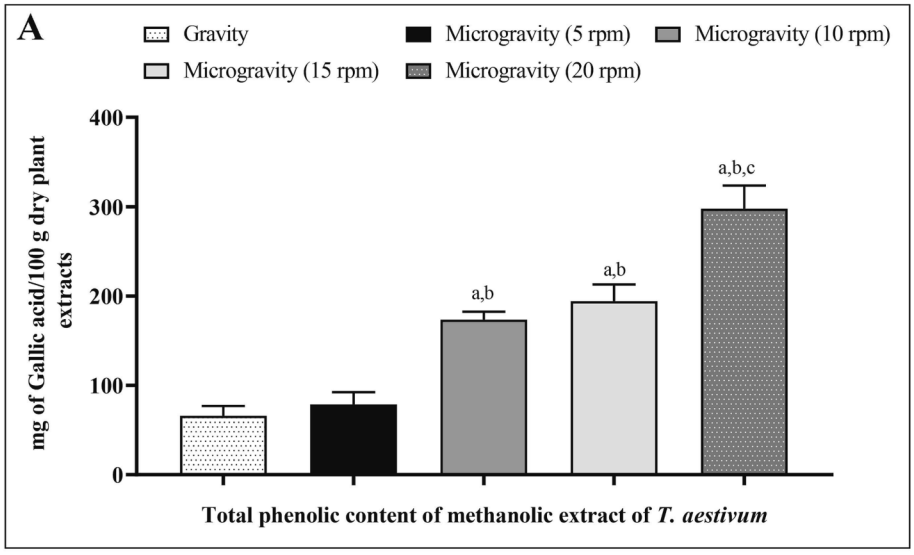
clinostat's rotation on root orientation and growth behavior. It's possible that the roots, in trying to find a stable orientation in the absence of a clear gravitational vector, extended longer to anchor the seedling. As for the shoots, the expected elongation might have been counteracted by other environmental conditions, such as the distribution of light or possible changes in hormonal signaling in the absence of gravity. Another perspective is the involvement of the "gravitropic set-point angle (GSA)", a genetically defined set point where the roots and shoots orient themselves at specific angles relative to the gravity vector. Variations in GSA are known to affect plant responses to gravity, and it's conceivable that Triticum aestivum Linn. may possess a unique GSA that makes its roots more responsive to alterations in gravity than its shoots.

Wheatgrass methanolic extract was reported to contain a wide range of bioactive compounds having cytotoxic effects against cancer cells (Qamar et al., 2018, Zendehbad et al., 2014). The phytochemical composition of the wheatgrass extracts in our study included a wide range of potentially beneficial chemicals such as flavonoids, phenols, terpenes, quinones, phytosterols, carbohydrates, fatty acids, vitamins, and coumarins. These compounds were detected in previous investigations on wheatgrass methanolic extracts (Qamar et al., 2018). Some phytochemical compounds including pyridoxine (Pugachev et al., 2021) (Qamar et al., 2018), apigenin (Noori et al., 2022), rutin (Saleh et al., 2019), tocopherol(Tam et al., 2018), ellagic acid(Baradaran Rahimi et al., 2020), Quercetin(Mohammed et al., 2021, Qamar et al., 2018), Kaempferol (Wang et al., 2019), β-Glucan (Wani et al., 2021) and Linolenic Acid (Dhar Dubey et al., 2019) were only detected in WGM but not in WGG extracts in our investigation.

Thus, microgravity may upregulate the enzymes related to the phenylpropanoid pathway, which is involved in the biosynthesis of flavonoids and phenolic compounds. Some key enzymes in this pathway include phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and flavonoid 3′,5′-hydroxylase (F3′5′H). The actual enzyme would depend on the specific compound whose synthesis is being upregulated. Importantly, some of the compounds that were not detected in WGG extracts in our study were detectable in previous studies (Qamar et al., 2018), likely due to differences in analytical methods and plant growth environment (such as water and soil content).

Our study also evaluated the effect of simulated microgravity on the phytochemical profile, antioxidant, and cytotoxic activities of wheatgrass (*Triticum aestivum*). We found that microgravity enhanced the polyphenolic and flavonoid contents potentiated the antioxidant activity of wheatgrass extract with higher H2O2–, NO–, and DPPH-scavenging activities exerted by WGM than WGG extracts. This paralleled the higher polyphenolic and flavonoid content observed in WGM versus WGG extracts. Interestingly, these effects of microgravity were potentiated by increasing clinostat rotation numbers, indicating that the antioxidant content and activity of wheatgrass extract were inversely related to the force of gravity in which the plant was germinated. Our findings are novel and future studies must focus on revealing the mechanisms through which microgravity changes the phytochemical and antioxidant potential of wheatgrass. The escalating number of clinostat rotations per minute may induce a heightened stress response in the germinating T. aestivum. Consequently, the wheatgrass's antioxidant systems may be amplified to counteract this stress. The phenolic and flavonoid compounds, endowed with redox properties, act in the capacity of hydrogen donors, reducing agents, and quenchers of singlet oxygen to perform their antioxidant functions (Gülçin et al., 2007). Additionally, the hydroxyl-group-containing polyphenolics are essential components of plants that can protect their cells against oxidative stress (Jing et al., 2010).

We also investigated whether WGM and WGG extracts exhibit different effects on MCF-7 cell viability. The MTT cell viability assay we used in our study measures the metabolic activity of cells, which indirectly indicates the number of cells in a culture reservoir. Thus, reduced metabolic activity reflects reduced cell number due to cell cycle block and/or cell death(Al-Awaida et al., 2018, Basu and Haldar, 1998). We observed a reduction in cell viability in WGM-treated MCF-7 cells and hence, we decided to study the effect of the extract on the cell cycle progression. This is because deregulation of the cell cycle represents an important mechanism in cancer initiation and tumor development(Basu and Haldar, 1998, Zhao et al., 2020). Our results demonstrate that WGM extract blocked the cell cycle in the G0/G1 phase in a concentration-dependent manner (Table 2). This was accompanied by the down-regulation of positive regulators of G1/S transition, including cyclin D1, also in a dose-dependent manner. A previous study demonstrated that treating T47D cells with *Pistacia atlantica* extract was associated with the



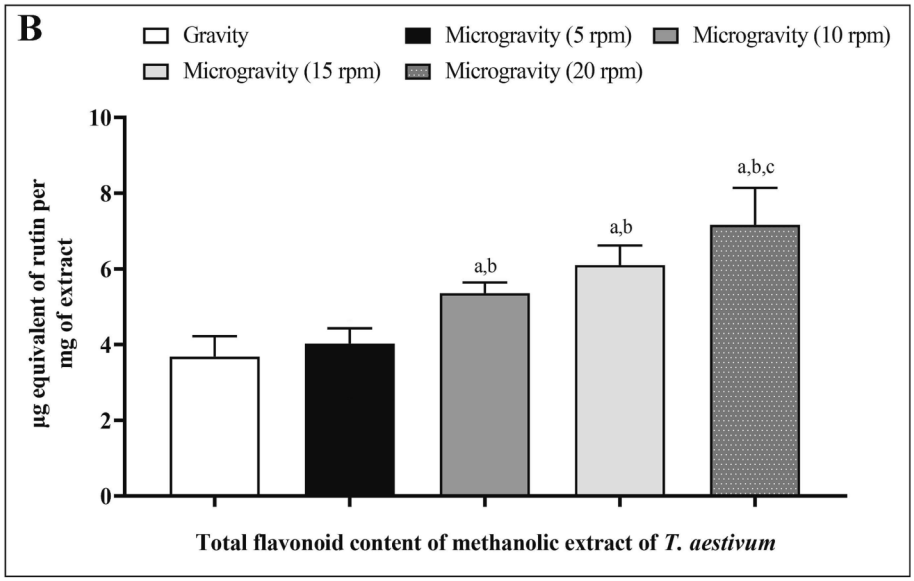


Fig. 3. Total phenolic and flavonoid content of methanolic extracts of *T. aestivum* germinated under gravity and microgravity conditions. The values are expressed as the mean ± SEM. Data in a–d were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests with the significance level set at $P < 0.05$. **a** refers to gravity condition versus different conditions of microgravity; **b** refers to microgravity condition (5 rpm) versus different conditions of microgravity (10,15 and 20 rpm); **c** refer to microgravity condition (10 rpm) versus microgravity (15 and 20 rpm) conditions; **d** refers to microgravity (15 rpm) condition versus microgravity (20 rpm) condition.

downregulation of cyclin D1 and a block at the G0/G1 phase (Rezaei et al., 2012). Furthermore, in our study, the anti-apoptotic BCL2 expression was downregulated with WGM extracts at both concentrations and this could be due to the presence of anti-cancer compounds such as apigenin. This is compatible with a previous study that used silver nanoparticles of apigenin on MCF-7 cells, which resulted in BCL-2 suppression, discharge of cytochrome *C* from mitochondria into the cytosol, accompanied by the initiation of cell death, leading to the killing of MCF-7 cells (Al-Otaibi et al., 2022).

Moreover, we detected the flavonol rutin, quercetin, ellagic acid, and Kaempherol in WGM extract, all of which exhibit anti-cancer effects. Rutin exhibits cytotoxic effects against the MCF-7 cell line by inducing apoptosis(Saleh et al., 2019), while quercetin triggers MCF-7 cell cycle arrest at the G2 phase, upregulates INXS, and downregulates UCA1(Rezaie et al., 2021). Ellagic acid triggers MCF-7 cell cycle arrest through TGF-/SMAD-dependent mechanisms(Chen et al., 2015). In the same cell line, Kaempferol upregulates pro-apoptotic enzymes and proteins, including caspase-3, caspase-7, caspase-9, p21, Bax, p53, PARP, and p-ATM (Diantini et al., 2012, Kang et al., 2022), and suppresses the anti-apoptotic proteins Bcl2, PLK-1, pAKT, pIRS-1, pMEK1/2, CDK1, cyclins A, B, D1, and E, and cathepsin D(Diantini et al., 2012, Kang et al., 2022, Choi and Ahn, 2008).

The genes BRCA1, RB1, APC, BAD, DFFA, CASP2, CASP6, and CASP7 mediate DNA repair, tumor suppression, and apoptosis(Rezaie et al., 2021, Chen et al., 2015, Green, 2022, Mohammed et al., 2021, Rezaei et al., 2012) (Green, 2022). These genes were significantly downregulated in MCF-7 cells exposed to WGM extracts. Moreover, exposure of MCF-7 cells to WGM at 5X-IC_{50} has also significantly upregulated genes involved in cell survival, including CCNA1, JUN, MAPK8, and BCL2A1. However, this was encountered by an upregulation in the growth arrest and DNA-damage inducible protein (GADD45α), which exhibits cytotoxic and pro-apoptotic effects. Additionally, the oncogenes and cell survival genes BCL2 and ERBB2 were downregulated with WGM extracts at both concentrations (IC_{50} and 5X-IC_{50}), while CCNE1, BIRC3, BIRC5, and CTSS were downregulated only with WGM IC_{50}. In addition, the pro-metastatic MMP9 was also downregulated with WGM IC_{50}. The downregulation of oncogenes favors cancer cell death and tumor suppression (Zhao et al., 2020). Overall, both cell death and survival genes were downregulated in response to WGM extracts, in a dose-dependent manner, which is consistent with reduced cell viability. On the other hand, the expression of most of these genes was not modulated by WGG extracts, suggesting stronger cytotoxic effect effects of WGM extracts.

Table 1
LC-MS-MS Phytochemical composition of *Triticum aestivum Linn* germinated under gravity and microgravity conditions.

Compound			Gravity		Microgravity (15 rpm)		*P*-value
Name	Molecular Formula	MW (g/mol)	RT (min)	PA (%)	RT (min)	PA (%)	
α-Pinene	$C_{10}H_{16}$	136.23	0.4	0	0.4	0.4 ± 0.02	0.9547
Vanillic Acid	$C_8H_8O_4$	168.15	0.7	**8.5 ± 0.5**	0.7	0.2 ± 0.02	<0.0001
Pyridoxine	$C_8H_{11}NO_3$	169.18	1	0	1	**14.3 ± 0.3**	<0.0001
Gallic Acid	$C_7H_6O_5$	170.12	1.2	**9.2 ± 0.2**	1.2	3.5 ± 0.5	<0.0001
Vitamin C	$C_6H_8O_6$	176.12	1.7	**7.2 ± 0.2**	1.7	0.5 ± 0.02	<0.0001
Caffeic Acid	$C_9H_8O_4$	180.16	2.2	**9.1 ± 0.1**	2.2	6.9 ± 0.9	<0.0001
Ferulic Acids	$C_{10}H_{10}O_4$	194.18	2.9	**1.1 ± 0.1**	2.9	0.9 ± 0.02	>0.9999
Syringic Acid	$C_9H_{10}O_5$	198.17	3.4	**6.2 ± 0.2**	3.4	0.4 ± 0.02	<0.0001
Sinapic Acids	$C_{11}H_{12}O_5$	224.21	4	**6.1 ± 0.1**	4	0.3 ± 0.14	<0.0001
Thiamin	$C_{12}H_{17}N_4OS^+$	265.36	5.2	0	5.2	0.1 ± 0.02	>0.9999
Linolenic Acid	$C_{18}H_{30}O_2$	278.4	6.6	0	6.6	**6.2 ± 0.2**	<0.0001
Oleic Acid	$C_{18}H_{34}O_2$	282.5	8	0	8	**7 ± 0.2**	<0.0001
Kaempferol	$C_{15}H_{10}O_6$	286.24	8.4	0	8.4	**10.2 ± 0.2**	<0.0001
Vitamin A	$C_{20}H_{30}O$	286.5	9.2	0	9.2	**1 ± 0.1**	0.0009
Ellagic Acid	$C_{14}H_6O_8$	302.19	10	0.9 ± 0.0.02	10	0.9 ± 0.02	>0.9999
Quercetin	$C_{15}H_{10}O_7$	302.23	10.8	0	10.8	**9.1 ± 0.1**	<0.0001
Isorhamnetin	$C_{16}H_{12}O_7$	316.26	11.4	0	11.4	**5.2 ± 0.2**	<0.0001
O-Coumaric	$C_{15}H_{18}O_8$	326.3	11.9	0.5 ± 0.02	11.9	**2.2 ± 0.2**	<0.0001
Riboflavin	$C_{17}H_{20}N_4O_6$	376.4	12.1	0	12.1	**1.5 ± 0.5**	<0.0001
β-Sitosterol	$C_{29}H_{50}O$	414.7	12.9	0	12.9	**4.5 ± 0.5**	<0.0001
Tocopherol	$C_{29}H_{50}O_2$	430.7	13.4	0	13.4	**4.4 ± 0.4**	<0.0001
Folate	$C_{19}H_{19}N_7O_6$	441.4	13.7	0	13.7	**2.2 ± 0.2**	<0.0001
β-Glucan	$C_{18}H_{32}O_{16}$	504.4	15	0	15	**3.2 ± 0.2**	<0.0001
Lignin	$C_{18}H_{13}N_3Na_2O_8S_2$	509.4	15.9	**6.9 ± 0.9**	15.9	1.6 ± 0.6	<0.0001
β-Carotene	$C_{40}H_{56}$	536.9	16.2	0.4 ± 0.02	16.2	0.9 ± 0.02	0.6751
Lutein	$C_{40}H_{56}O_2$	568.9	17.8	**10.8 ± 0.8**	17	1.1 ± 0.1	<0.0001
Rutin	$C_{27}H_{30}O_{16}$	610.5	18	0	18	**10.3 ± 0.3**	<0.0001
Salicylic Acid	$C_7H_6O_3$	138.12	0.5	**4.4 ± 0.4**	0.5	0	<0.0001
O-Coumaric Acid	$C_9H_8O_3$	164.16	0.6	**2.5 ± 0.5**	0.6	0	<0.0001
Apigenin	$C_{15}H_{10}O_5$	270.24	7	**0**	7	**11.3 ± 0.3**	<0.0001
Cellulose	$C_{12}H_{22}O_{11}$	342.3	12	**3.3 ± 0.3**	12	0	<0.0001
γ-Tocopherol	$C_{28}H_{48}O_2$	416.7	12.4	**4.6 ± 0.6**	12.4	0	<0.0001
Isoorientin	$C_{21}H_{20}O_{11}$	448.4	13	**2 ± 0.12**	13	0	<0.0001
Isoscoparin	$C_{22}H_{22}O_{11}$	462.4	14.6	**5.4 ± 0.4**	14.6	0	<0.0001
γ-Cryptoxanthin	$C_{40}H_{56}O$	552.9	17	**0.2 ± 0.02**	17	0	>0.9999

RT: retention time; **PA%**: Peak area percentage of standard or all components; P-value < 0.05 indicates statistical significance in the PA% between the extracts of plants germinated under microgravity (15 rpm) versus gravity conditions.

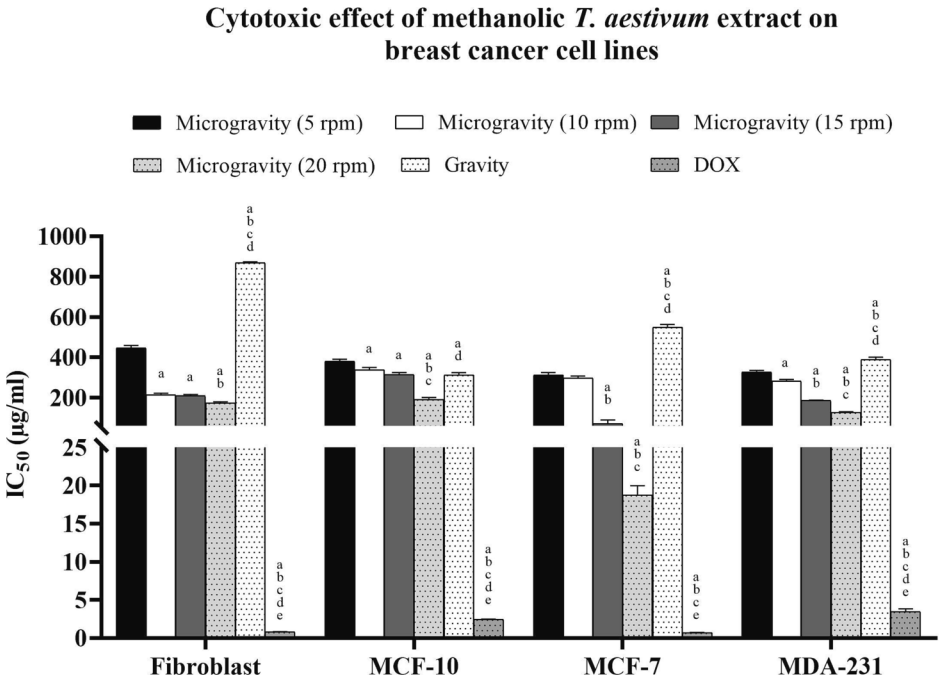


Fig. 4. In vitro cytotoxicity of methanolic extracts of *T. aestivum* germinated under gravity and different microgravity conditions (5, 10, 15, and 20 rpm) against breast cancer cell lines, the normal skin fibroblast and non-tumorigenic epithelial breast cell lines. **a** refers to microgravity condition (5 rpm) versus different conditions of microgravity (10, 15 and 20 rpm), gravity condition, and doxorubicin chemotherapy drug; **b** refers to microgravity condition (10 rpm) versus a different condition of microgravity (15 and 20 rpm), gravity condition and doxorubicin chemotherapy drug; **c** refers to microgravity condition (15 rpm) versus microgravity condition (20 rpm), gravity condition and doxorubicin chemotherapy drug; **d** refers to microgravity condition (20 rpm) versus gravity condition and doxorubicin chemotherapy drug; **e** refers to gravity condition versus doxorubicin chemotherapy drug.

Table 2
Changes in the expression of breast cancer and cell death genes in MCF7 cells treated with wheatgrass extract under Gravity and Microgravity (20 rpm) conditions.

Pathway	Gene Symbol	Gravity		Microgravity	
		5X-IC_{50}	IC_{50}	5X-IC_{50}	IC_{50}
Breast Cancer	CCNA1	–	–	16.07	–
	JUN	–	–	7.83	–
	IL6	−2.22	–	6.26	–
	MAPK8	–	–	2.53	–
	GSTP1	−2.5	−2.95	−2.21	−2.03
	ABCG2	−2.47	–	–	–
	CST6	−4.57	–	–	–
	RARB	–	−2.35	–	–
	RASSF1	–	−2.1	–	−3.01
	HIC1	–	−2.66	–	–
	ESR1	–	–	−16.02	−3.64
	CCND1	–	–	−7.45	−2.34
	BCL2	–	–	−5.14	−2.63
	AR	–	–	−3.85	−2.53
	BAD	–	–	−3.34	−2.27
	ERBB2	–	–	−2.49	−4.42
	AKT1	–	–	−2.4	−3.36
	PGR	–	–	−3.71	–
	APC	–	–	−3.01	–
	BRCA1	–	–	−2.49	–
	CCNE1	–	–	−2.35	–
	RB1	–	–	−2.26	–
	BIRC5	–	–	−2.07	–
	MMP9	–	–	−6.7	–
	EGFR	–	–	–	−3.12
	CDH1	–	–	–	−2.98
	TP53	–	–	–	−2.3
	TGFB1	–	–	–	−2.05
Cell Death	MAPK8	–	–	2.7	–
	BCL2A1	–	–	35.77	–
	GADD45α	–	–	12.61	–
	IGF1	−3.88	−5.63	–	−3.43
	TNFRSF11B	–	–	−21.41	−2.3
	BCL2	–	–	−12.05	–
	TNF	–	–	−3.98	–
	CTSS	–	–	−3.82	–
	BIRC3	–	–	−3.65	–
	CASP6	–	–	−3.44	–
	AKT1	–	–	−2.46	–
	CASP2	–	–	−2.22	–
	DFFA	–	–	−2.04	–
	CASP7	–	–	−2.02	–
	CD40LG	–	–	–	−2.34
	CCDC103	–	–	–	−2
	KCNIP1	–	–	–	−4.2

Fold change in gene expression of breast cancer and cell death genes are only reported if the change was statistically significant. Positive values represent upregulation while negative values represent downregulation in gene expression. **Breast cancer genes: GSTP1**: Glutathione S-transferase pi 1; **ABCG2**: ATP-binding cassette sub-family G (WHITE) member 2; **IL6**: Interleukin 6 (interferon, beta 2); **CST6**: Cystatin E/M; **CCNA1**: Cyclin A1; **JUN**: Jun proto-oncogene; **MAPK8**: Mitogen-activated protein kinase 8; **ESR1**:Estrogen receptor 1; **CCND1**: Cyclin D1; **BCL2**: B-cell CLL/lymphoma 2; **AR**: Androgen receptor; **BAD**: BCL2-associated agonist of cell death; **ERBB2**: V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian); **AKT1**: V-akt murine thymoma viral oncogene homolog 1; **GSTP1**: Glutathione S-transferase pi 1; **PGR**: Progesterone receptor; **APC**: Adenomatous polyposis coli; **BRCA1**: Breast cancer 1; early onset, **CCNE1**: Cyclin E1; **RB1**:Retinoblastoma 1; **BIRC5**: Baculoviral IAP repeat containing 5; and **MMP9**: Matrix metallopeptidase 9. **Cell death genes**: **IGF1**: Insulin-like growth factor 1 (somatomedin C); **MAPK8**: Mitogen-activated protein kinase 8; **BCL2A1**: BCL2-related protein A1; **GADD45α**: Growth arrest and DNA-damage-inducible alpha; **TNFRSF11B**: Tumor necrosis factor receptor superfamily member 11b; **BCL2**: B-cell CLL/lymphoma 2; **TNF**: Tumor necrosis factor; **CTSS**: Cathepsin S; **BIRC3**: Baculoviral IAP repeat containing 3; **CASP6**: Caspase 6; apoptosis-related cysteine peptidase; **AKT1**: V-akt murine thymoma viral oncogene homolog 1; **CASP2**: Caspase 2; apoptosis-related cysteine peptidase; **DFFA**: DNA fragmentation factor, 45 kDa, alpha polypeptide; and **CASP7**: Caspase 7 apoptosis-related cysteine peptidase; **HIC1**: Hypermethylated in cancer protein 1.

This conclusion is compatible with the dose-dependent downregulation in the sex hormone receptor genes ESR, PGR, and AR in response to the extract of WGM but not WGG. These receptors and their downstream signals have long been recognized as key promoters of hormone-dependent breast cancer (Green, 2022). These findings suggest that WGM extract exhibits anti-proliferative effects by regulating key pathways in breast cancer. Importantly, the higher phenolic and flavonoid contents in the WGM extract may at least in part explain its higher cytotoxic effects compared to the WGG extract. Many previous studies reported the cytotoxic effects of phenolic and flavonoid compounds against cancer cells(Imran et al., 2010).

The study's findings concluded that the microgravity environment generated by a 3D clinostat obtained germinated *T. aestivum* with a greater percentage of natural antioxidants and high level of antioxidant properties; the created wheatgrass has the ability as a cytotoxic treatment without any adverse effects and a low cost. This approach can be utilized to discover the therapeutic effects of WGM conditions for many other diseases.

Conclusion

This study underscores the potential utility of microgravity as a bioengineering tool for enhancing the antioxidant, cytotoxic capabilities and altering the phytochemical profile of wheatgrass (*Triticum aestivum Linn.*). The microgravity-germinated wheatgrass (WGM) not only displayed superior antioxidant activity and higher levels of bioactive compounds but also demonstrated an enhanced cytotoxic effect against breast cancer cell lines. The heightened cytotoxicity was found to correlate with increased clinostat rotations, with notable efficacy observed at 20 rpm. Furthermore, the observed gene expression changes in MCF-7 cells upon exposure to WGM extracts indicated a significant alteration in key pathways related to cell survival, hormone responses, and cancer progression. These findings contribute to the growing body of evidence that supports the potential applications of space biology in advancing our understanding of terrestrial health. Further research in this intriguing field could pave the way for the development of novel therapeutic approaches for cancer treatment.

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Author contributions

Experiments were designed by W.A. The experiments were performed by W.A, A.S., and H.A. Data analysis was accomplished by W.A.-A., A.S., H.A., and R.T.A. The manuscript was written by W.A., R.T.A., A. S., and H.J. The manuscript was edited by R.T.A.

CRediT authorship contribution statement

Wajdy Al-Awaida: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Hamzeh J. Al-Ameer:** Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. **Ahmad Sharab:** Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Rand T. Akasheh:** Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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