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Synergism of antiproliferative effects of young green barley and chlorella water extracts against human breast cancer cells

Marta Kinga Lemieszek[1,A-F] ✉, **Wojciech Rzeski**[1,A,F]

[1] *Department of Medical Biology, Institute of Rural Health, Lublin, Poland*
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Abstract

Introduction and Objective. Breast cancer is the most common type of tumour in terms of incidence and mortality among women. In the light of recent data that has revealed the beneficial impact of increasing plant-based food consumption on the risk of breast cancer, the use of young green barley and chlorella, the chemopreventive properties of which have been previously reported, seems to be a reasonable therapeutic strategy in this type of cancer. Nevertheless, there are only a few scientific reports focused on the influence of the mentioned products on breast cancer development; thus, the aim of the study was to enrich knowledge resources in this area.

Materials and method. The chemopreventive effect of water extracts of chlorella (CH) and young green barley (YGB) and their mixture (MIX) was investigated in human breast adenocarcinoma T47D cells and human skin fibroblasts HSF by LDH, MTT and BrdU assays. Changes in cell morphology in response to tested extracts were examined in light microscopy.

Results. Tested extracts were not toxic against HSF and did not affect their proliferation and morphology. Simultaneously, extracts increased the permeability of T47D cell membranes and inhibited their proliferation. Microscopic observation confirmed the results of biochemical assays and additionally suggested necrosis induction in T47D cells in response to tested compounds. Obtained results demonstrated that MIX induced stronger beneficial changes than their components.

Conclusions. The study revealed the chemopreventive properties of the investigated green food products against breast cancer cells, without any side-effects in human skin fibroblasts. The beneficial properties discovered of the tested extracts on cancer cells enhanced by their concomitant administration, and in the case of antiproliferative effects YGB and CH, revealed synergism of action.

Key words

chemoprevention, Hordeum vulgare, functional food, Chlorella pyrenoidosa, breast cancer cells, super-food

INTRODUCTION

Breast cancer is the most commonly occurring neoplasia in women. At the end of 2020, there were 7.8 million women with diagnosed breast cancer in the past 5 years, making this type of tumour the world's most prevalent cancer. Furthermore, in 2020 alone, there were 2.26 million new cases of breast cancer, and 0.67 million women died because of this disease. Despite the fact that since the 1980s significant improvements in the survival rate in patients with breast cancer have been observed, mostly because of early detection programmes, women all over the world lose more disability-adjusted life years (DALYs) because of this cancer than any other type of tumour [1]. The high incidence and limitations of therapeutic strategies (significant cost of therapy, drug resistance, serious side-effects) indicated the importance of pursuing prevention strategies which are much safer, successful and cheaper than common oncological interventions. According to several scientific reports, next to the best-described risk factors for breast cancer development, such as gender, age, gene mutations, and family history of this disorder, indicate also poor eating habits with particular emphasis on a lower intake of raw fruits and vegetables [2, 3, 4, 5], chemoprevention seems to be a reasonable therapeutic option.

The presented strategy is in the line with the well-known recommendation to consume at least 5 servings of fruits and vegetables daily in order to decrease the risk of both cancer and heart disease. Considering the increase in consumption of plant-based food as a chemopreventive strategy for the reduction of the risk of breast cancer development, the Temple and Gladwin study is worth mentioning. They reviewed cohort of over 200 patients, as well as case-control studies that investigated the correlations between the risk of cancer and fruit and vegetable intake, indicating that cancer prevention is more effective when consumers obtained a wide variety of phytochemicals from their diet, and not from supplementation with pure anti-cancer compounds [6].

In light of the above-mentioned data, it was decided to verify the possibility of using in breast cancer prevention mixture of two popular super-foods, i.e. chlorella (*Chlorella pyrenoidosa*) and young green barley (*Hordeum vulgare*), which in recent years have celebrated triumphs on Internet forums dedicated to functional food as a panacea for various ailments, including cancer. Despite the media hype, there is still little scientific data on the chemopreventive properties of these green food products. So far, the anti-cancer effect of young barley products, as well as pure phytochemicals isolated from them, was proved in several human cancer cell

✉ Address for correspondence: Marta Kinga Lemieszek, Department of Medical Biology, Institute of Rural Health, Lublin, Jaczewskiego 2, 20-090 Lublin, Poland
E-mail: martalemieszek@gmail.com

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lines, including breast cancer (MCF7, MDA-MB-231) [7, 8], colon cancer (HT-29, LS180) [9, 10, 11, 12], leukemia (Jurkat) [13], lung cancer (A549) [9], lymphoma (BJAB, NALM6) [13], and prostate cancer (DU145) [8]. Simultaneously, the anti-proliferative properties of both chlorella extracts and compounds isolated from them, has been observed in human cancer cells derived from breast (MCF7) [14], colon (HCT116, HCT-8, HT-29, Caco-2) [11, 15, 16, 17], and glial cells (A172) [15]. There are also single reports from *in vivo* studies indicating the anti-cancer effects of both chlorella and young green barley products. Among them, the research by Kubotka et al. should be emphasized which proved in two independent studies that chlorella as well as young green barley prevented N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats [7, 14]. Li et al. discovered that extract from barley grass prevented colitis-associated colorectal cancer in murine model [18]. The beneficial effect of dried *Chlorella pyrenoidosa* powder on the development of diethylnitrosamine-induced hepatocarcinogenesis in rats was reported by Takekoshi et al. [19]. On the contrary, research conducted on mammary tumour-bearing mice strain Balb/c by Khalilnezhad et al. revealed that powder of *Chlorella vulgaris* administered to mice for 42 days at a dose of 200 mg/kg promoted tumour growth, while other investigated concentration (300 mg/kg) did not affect the analyzed process [20].

OBJECTIVE

As mentioned above, there are only a few scientific reports (mostly *in vitro* studies) focused on the influence of the mentioned green food products on breast cancer development. Thus, the aim of the study was to enrich the modest resources of knowledge in this area. The study was conducted in human breast cancer T47D cells as well as human skin fibroblasts. Water extracts of chlorella and young green barley were examined in terms of their cytotoxicity, anti-proliferative properties, influence on cell morphology and cell death induction. Furthermore, because of the fact that most people usually consume simultaneously two or more pro-healthy products, the chemopreventive effects of the mixture of young green barley and chlorella were also examined.

MATERIALS AND METHOD

Reagents. The chemicals were purchased from Sigma-Aldrich Co. LLC, unless otherwise indicated. Both dried chlorella (Chlorella pyrenoidosa) and powder of young green barley juice (Hordeum vulgare) were obtained from Green Ways (Prague, Czech Republic). The detailed procedure of young green barley (YGB) and chlorella (CH) extracts preparation have been presented previously [11].

Cell lines. The human breast adenocarcinoma cell line T47D was obtained from the European Collection of Cell Cultures (ECACC, Centre for Applied Microbiology and Research, Salisbury, UK). The human skin fibroblasts (HSF) were a laboratory strain established by the outgrowth technique, from skin explants of young persons, in the Department of Medical Biology, Institute of Rural Health, Lublin, Poland. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and a solution of penicillin and streptomycin Solution. Cultures were kept at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Cytotoxicity assessment – LDH assay. Cells were seeded on 96-well plates at $5x10^4$ cells/ml. The next day, the growth medium was exchanged for fresh medium with the extracts used separately or together as the mixture (YGB and CH in a 1:1 ratio). Working solutions of the extracts were prepared in a medium with reduced 2% amount of FBS. Cytotoxicity of the compounds was examined after 48 hours of cell treatment using *in vitro* Toxicology Assay Kit Lactate Dehydrogenase, according to the manufacturer's instructions. Absorbance was quantified spectrophotometrically at 450nm using a microplate reader (BioTek ELx800, Highland Park, Winooski, [VT], USA).

Evaluation of cell metabolic activity – MTT assay. Cells were seeded on 96-well plates at $3x10^4$ cells/ml. The next day, the growth medium was exchanged for fresh medium with the extracts used separately or together as the mixture (YGB and CH in a 1:1 ratio). Working solutions of the extracts were prepared in a regular cell culture medium. Assessment of cell proliferation was performed after 96 hours of cell treatment. In order to do this, cells were incubated for 4 hours with MTT solution (5 mg/ml), the crystals of formazan were then dissolved for 16 hours in 10% SDS in 0.01N HCl. The colour product was recorded on a microplate reader at 570 nm wavelength.

Evaluation of cell proliferation – BrdU assay. Cells were seeded on 96-well plates at $5x10^4$ cells/ml. The next day, the growth medium was exchanged for fresh medium with the extracts used separately or together as the mixture (YGB and CH in a 1:1 ratio). Working solutions of the extracts were prepared in a regular cell culture medium. After 48 hours of cell treatment, their proliferation was examined by Cell Proliferation ELISA BrdU (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. Absorbance was recorded on a microplate reader at 450 nm wavelength.

Cell morphology examination – MGG staining. Cells were seeded at a density of $5x10^4$ cells/ml on Lab-Tek Chambers Slide (Nunc). On the following day, the growth medium was exchanged for fresh medium supplemented with YGB and/or CH at the concentration of 500 µg/ml. Investigated extracts were prepared in a regular cell culture medium. After 48 hours of treatment, cells were stained according to the May-Grünwald-Giemsa method. Changes in cell morphology were observed in light microscope MW 50 (OPTA-TECH, Warsaw, Poland), while micrographs were prepared in Capture V2.0 software (OPTA-TECH, Warsaw, Poland).

Statistical analysis. The collected data were presented as the mean and standard error of the mean. Statistical analyses were performed using a one-way ANOVA test with Dunnett's or Tukey's *post-hoc* tests, and the column statistics for comparisons. Statistical significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

The influence of water extracts of young green barley and chlorella on viability and proliferation of human skin fibroblasts and human breast cancer cells. The most important feature of chemopreventive agents is their selectivity, understood as the effective elimination of cancer cells with lack or at least low toxic effect against normal cells. In order to examine green food products' chemopreventive selectivity, in the first step, their impact on the viability and proliferation of human skin fibroblast was conducted using LDH, MTT and BrdU assays.

The study revealed that the investigated extracts in the whole range of tested concentrations (2.5–1,000 μg/ml) were not cytotoxic against human skin fibroblasts HSF, and did not affect their proliferation determined by evaluation of both cell metabolic activity and DNA synthesis (Fig. 1). Obtained results correspond with earlier observations by the authors of the current study which showed that juice, as well as young green barley water extract used in a similar range of concentrations, did not impact on the integrity of HSF cell membranes [9]. However, it needs to be highlighted that the mentioned water barley extract was prepared in a different way and than from different raw material than the extract investigated in the presented study. Furthermore, data collected by Czerwonka et al. were obtained after a shorter time of HSF exposure to barley products (24 h), and the cell toxicity was determined using a different method (neutral red assay). Due to the similar time of cells treatment (48 h) as well as used assays (MTT and BrdU test), it is worth mentioning the reaserch by Gromkowska-Kępka et al., who also showed that the water extract of young barley juice at concentrations ranging from 5 – 100 μg/ml did not alter the proliferation of normal human skin fibroblasts (NHSF) [21]. Robles-Escajeda et al., who investigated the water extract obtained from the powder of barley young leaves of *Hordeum vulgare* in human dermal neonatal foreskin Hs27 fibroblasts, revealed that the tested extract at concentration of 1500 μg/ml was not cytotoxic (assessment after 24 h of cell treatment; flow cytometry of cells stained with propidium iodide), and did not alter fibroblast proliferation (investigation after 96 h of cell treatment; cell counting) [13].

In the case of chlorella, the results of research by Jaafar et al. has to be mentioned. They demonstrated that water extract of *Chlorella vulgaris* in a concentration ranging from 100 – 800 μg/ml did not cause any unwanted changes in young human diploid fibroblasts (HDFs). They also observed a significant increase in cells viability in response to the extract at concentrations 400, 500 and 600 μg/ml [22]. However, Jaafar et al. investigated extract obtained from a different strain of Chlorella in a different way, and what seems to be the most important cell viability was conducted after a 24 hours shorter time of treatment than the presented studies [22].

As previously mentioned, a good chemopreventive agent next to low toxicity against normal cells also has to be characterized by a great ability to eliminate cancer cells. In order to evaluate these properties, the influence of young green barley and chlorella extract on the viability and proliferation of human breast cancer cell line T47D was conducted using LDH, MTT and BrdU assays.

Water extract of chlorella in the wide range of tested concentrations (5–1,000 μg/ml) effectively eliminated breast cancer cells (Fig. 2). The extract of young green barley also revealed beneficial chemopreventive properties, in particular, a cytotoxic effect was observed in concentrations from 100 – 1,000 μg/ml, while antiproliferative properties were noted in the concentration ranging from 25–1,000 μg/ml (MTT test), or from 10–1,000 μg/ml (BrdU test). Both green food extracts revealed a dose-dependent cytotoxic effect against T47D cells, wherein the level of LDH released through damaged cell membranes of cancer cells in response to YGB and CH at the highest tested concentration increased by 28.8% and 43.5%, respectively. Investigated extracts also effectively inhibited the proliferation of breast cancer cells. YGB as well as CH at concentrations of 1,000 μg/ml decreased the metabolic activity of T47D cells by 36.5% and 56.0%, respectively. A more specific and selective BrdU assay also confirm the antiproliferative effect of the tested extracts, which at the highest tested concentration decreased DNA synthesis in breast cancer cells by 23.5% (YGB) or 47.6% (CH). MTT and BrdU results obtained from CH investigation correspond with data provided by Kubatka et al., who showed a significant decrease in both metabolic activities, as well as DNA synthesis in human breast adenocarcinoma cell line MCF-7 in response to *Chlorella pyrenoidosa* in the concentration ranging from 19.5–1250 μg/ml (MTT test), or from 4.9–1,250 μg/ml (BrdU test). Viability of MCF-7 after 72 h of treatment with chlorella at the concentration 1,250 μg/ml lowered over 20% of the control, while the proliferation of breast cancer cells after 48 h of exposure to the tested compound at the same concentration reached around 15% of control. It has to be noted that studies conducted by the Kubatka team with the same raw material as used in the presented studies, revealed that the investigated compound more strongly inhibited the proliferation of MCF-7 cells than influenced their metabolism. Furthermore, the above-mentioned studies also demonstrated the anti-cancer effect of *Chlorella pyrenoidosa* in a well-known model of N-methyl-N-nitrosourea (NMU) induced mammary carcinogenesis in female rats. Chronic chlorella administration with diet in rats with induced mammary carcinogenesis revealed that the tested compound in the amount of 30 g/kg suppressed tumour frequency by 61% and lengthened tumour latency by 12.5 days, compared with the controls [14].

Data from the current study on young barley examination also correspond with the results of the research by Kubatka et al., who performed their study on on the same raw material of *Hordeum vulgare* (dried grass juice) [7]. Results of MTT and BrdU assays conducted after 72 h of MCF-7 cells treatment with young barley, revealed a significant decrease of breast cancer cells' metabolic activity in response to the tested product in a whole range of investigated concentrations (31.2 μg/ml – 1,000 μg/ml; the range of changes 21.7% – 62.1%), while inhibition in DNA synthesis was observed from the concentration 62.5 μg/ml and intensified in a dose-dependent manner (range of changes 15.4% – 23.9%). Further studies conducted by Kubatka et al. in an above-mentioned animal model of NMU-induced mammary carcinogenesis have shown that young barley in the concentration of 3 g/kg inhibited cancer frequency by 37%, while in a 10-times higher concentration, suppressed tumour development only by 13%. Nevertheless, young barley at the higher tested concentration (30 g/kg) shorten tumourlatency by 10 days in comparison with untreated rats [7].

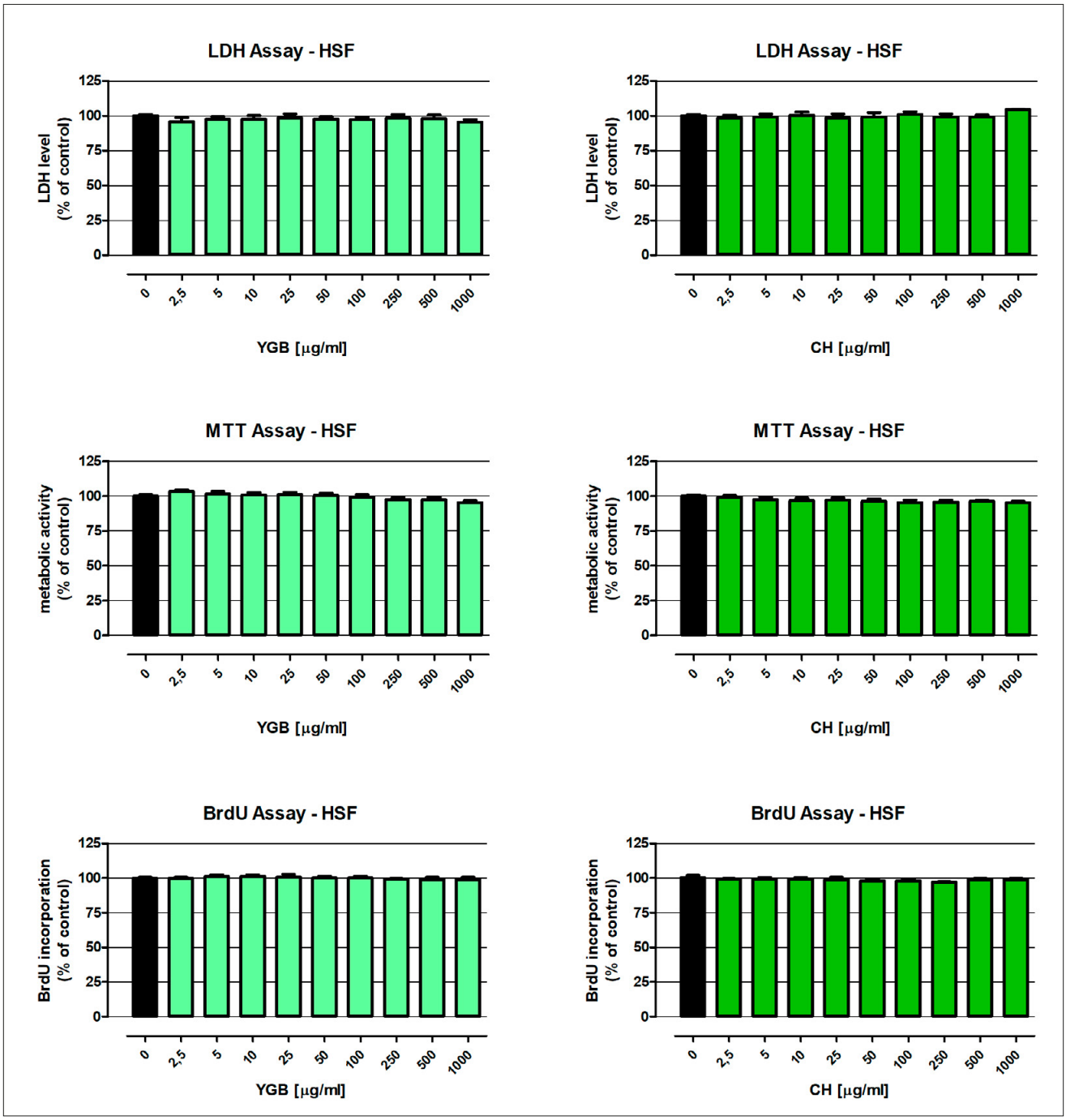

Figure 1. Influence of water extracts of young green barley and chlorella on viability and proliferation of human skin fibroblasts HSF cells. Cells were incubated with culture medium alone (control) or extracts of young green barley (YGB) and chlorella (CH) at concentrations ranging from 2.5 – 1,000 µg/ml. Cell viability was determined after 48 h of treatment by examination of cell membrane integrity using LDH assay. Cell proliferation was evaluated after 96 h and 48 h of treatment by assessment of both metabolic activity (MTT assay) and DNA synthesis (BrdU assay), respectively.
Results presented as mean ± SEM of at least 4 measurements. One-way ANOVA test; post-test: Dunnett.

Chemopreventive properties of barley grass extract have also been reported by Woo et al., who demonstrated in the MTT test a significant decrease in proliferation of human breast adenocarcinoma cell line MDA-MB-231 after 72 h of treatment with the tested extract in concentration from 10 – 500 µg/ml. Furthermore, next to the antiproliferative properties of young barley extract they also indicated its proapoptotic effect in investigated cell lines [8].

Enhancement of antiproliferative and cytotoxic effects of the mixture of green food products against human breast cancer cells. There is strong scientific evidence of a significantly stronger anti-cancer effect of the extracts or mixtures of a few phytochemicals than any influence by the pure substances present in these compositions [6, 23, 24]. In pharmacology, the above-mentioned phenomenon of the enhancement of beneficial properties of the combination of two or more compounds, which are used together induced biological effects greater than the sum of the effects of the individual chemicals, is called synergism. The synergism explained why the diet rich in fruits and vegetables revealed stronger healthy properties, including chemopreventive

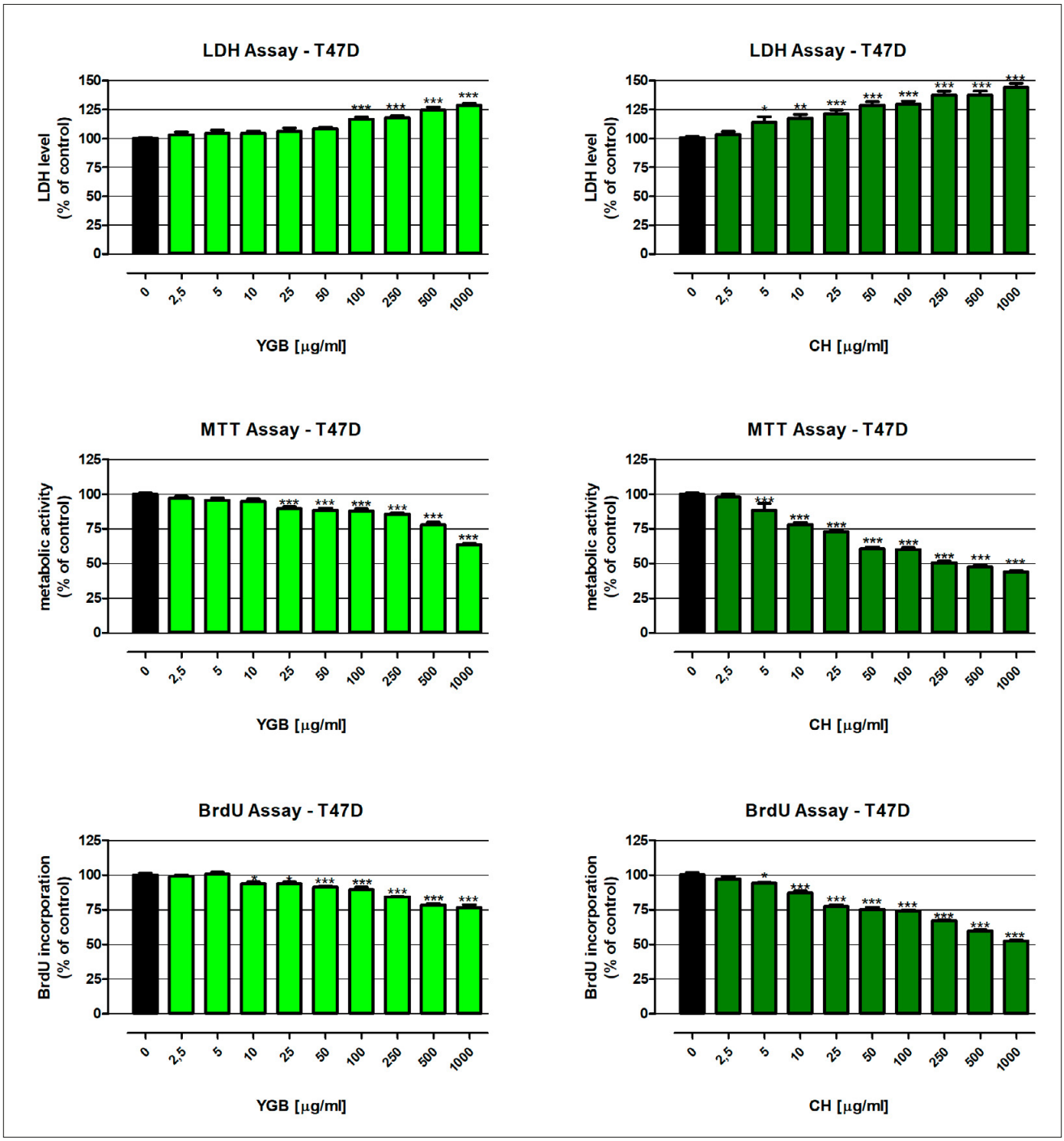

Figure 2. Alterations in viability and proliferation of human breast adenocarcinoma cell line T47D in response to water extracts of young green barley and chlorella. Cancer cells were incubated with culture medium alone (control) or young green barley (YGB) and chlorella (CH) extracts at concentrations ranging from 2.5 – 1,000 µg/ml. Cell viability was determined after 48 h of treatment by examination of cell membrane integrity using LDH assay. Cell proliferation was evaluated after 96 h and 48 h of treatment by assessment of both metabolic activity (MTT assay) and DNA synthesis (BrdU assay), respectively. Results are presented as mean ± SEM of at least 4 measurements.
Significantly different,:* p< 0.05; ** p< 0.01; *** p< 0.001 vs. control (untreated cells). Data were analysed using one-way ANOVA test followed by the post-test: Dunnett.

effects, than pure anti-cancer substances isolated from these products. In the presented studies it was decided to check the influence of the administration together of young barley and chlorella extract on their chemopreventive properties; nevertheless, it needs to be highlighted that the investigated extracts themselves are a combination of many bio-active compounds.

Enhancement of chemopreventive effects of investigated extracts used together as a MIX were evaluated in human breast adenocarcinoma cell line T47D by LDH, MTT and BrdU tests. The cytotoxic effect of MIX was significantly stronger than changes induced by young barley in corresponding concentration, while LDH release in response to MIX and chlorella in corresponding concentration was on similar levels (Fig. 3). On the contrary, MTT and BrdU assays demonstrated that the mixture of the extracts more strongly inhibited the proliferation of breast cancer cells than its components used separately in corresponding concentrations. Furthermore, it

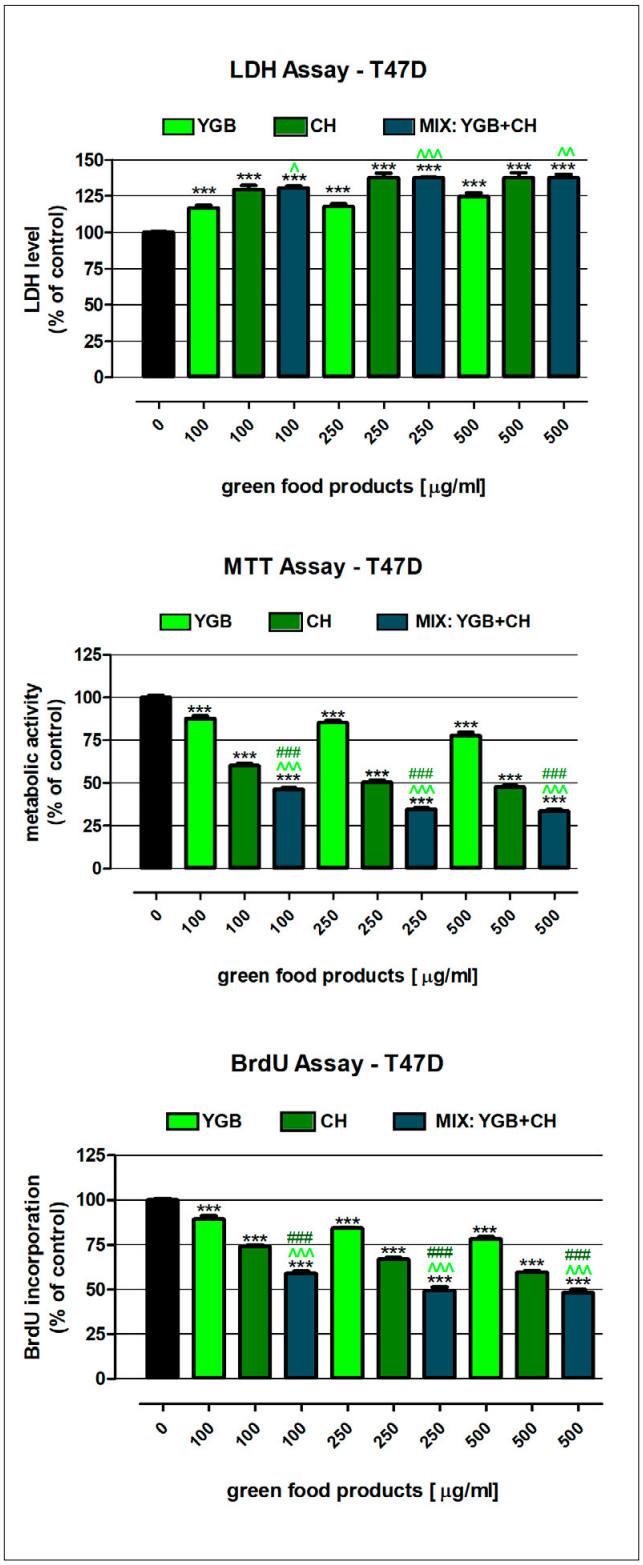

Figure 3. Enhancement of the antiproliferative and cytotoxic effects of the mixture of green food products against human breast adenocarcinoma cell line T47D. Cancer cells were treated with young green barley (YGB) and chlorella (CH) extracts at concentrations 100, 250 and 500 μg/ml used separately or together as a MIX, rastion – 1:1. The control was untreated cells. Cell proliferation was assessed after 48 and 96 h of incubation using BrdU and MTT assay, respectively. The cytotoxicity of green food products was measured after 48 h of treatment using LDH assay. Results are presented as mean ± SEM of at least 4 measurements.
Significantly different: *** p< 0.001 vs. control; ^ p< 0.05 MIX vs. YGB (comparison with corresponding concentrations), ^^ p< 0.01; ^^^ p< 0.001 MIX vs. YGB (comparison with corresponding concentrations of YGB); # p< 0.05; ## p< 0.01; ### p< 0.001 MIX vs. CH (comparison with corresponding concentrations). Data were analysed using a one-way ANOVA test followed by the post-test: Tuckey.

needs to be highlighted that both the metabolic activity and DNA synthesis in T47D cells treated with MIX 100 μg/ml and MIX 250 μg/ml, were significantly lower than the sum of the effects of the individual ingredients administered in the same concentrations.

According to the best knowledge of the authors of the current study, the presented data is the first to demonstrate the synergism of chemopreventive actions of young green barley and chlorella extracts' against breast cancer cells. The favorable effects discovered of the mixture of green food products may be explained by the accumulation of anti-cancer phytochemicals in the examined extracts. Previous studies by the authors indicated that the carbohydrate-protein complex is responsible for the chemopreventive and immunomodulatory effects of the young green barley extract. The extract contained 26.9% of proteins and 64.2% of sugars, in particular glucose (54.7%), fructose (42.7%), mannose (2.6%) and galactose (less than 0.5%) [12]. The carbohydrate-protein complexes are also present in the extract of chlorella; the amount of proteins – 22.3%, sugar amount – 12.0% (a detailed evaluation of sugar composition has not yet been completed).

Alterations in morphology of human skin fibroblasts and human breast cancer cells in response to the mixture of green food products. Alterations in the morphology of human skin fibroblasts HSF and human breast adenocarcinoma cell line T47D were examined after 48 h of cell incubation with tested extracts, used separately or together as a MIX. In order to visualize morphological changes induced by the tested compounds, cells were stained with MGG, and representative images obtained by light microscopy (Fig. 4).

The current study confirmed earlier observations that the tested compounds, even at a high concentration (500 μg/ml), used together or separately, did not cause any changes in the morphology of HSF cells (Fig. 4A). Collected data correspond with the results of LDH, MTT and BrdU assays, as described above. Unfortunately, according to the best knowledge of the authours, there is a lack of other scientific reports examining the influence of young barley or chlorella on fibroblast morphology. Nevertheless, it is worth mentioning that the authors' previous studies conducted on human normal colon epithelial cells with the use of the same extracts, as well as their mixture, also showed no negative effect on the morphology of the tested cells [11]. On the contrary, the investigated green food products induced changes in breast cancer cells (Fig. 4B). First of all, the tested compounds significantly decreased the number of cells, and the observed effect was associated with both inhibition of cell proliferation and damage to intercellular connections, which correspond with the data obtained from MTT and BrdU assays. Furthermore, the tested compounds, especially extract of chlorella as well as the MIX, induced changes characteristic of necrosis like cytoplasmic oedema, loss of cell membrane integrity, and consequently, release of cell contents into the intercellular space. The indicated changes correspond with the results of LDH test.

It needs to be highlighted that the strongest antiproliferative and cytotoxic effects were observed in T47D cells treated with a mixture of green food products. Despite the fact that microscopic observation suggests the pro-necrotic ability of the investigated compounds against breast cancer cells, this observation still has to be proven in other specific

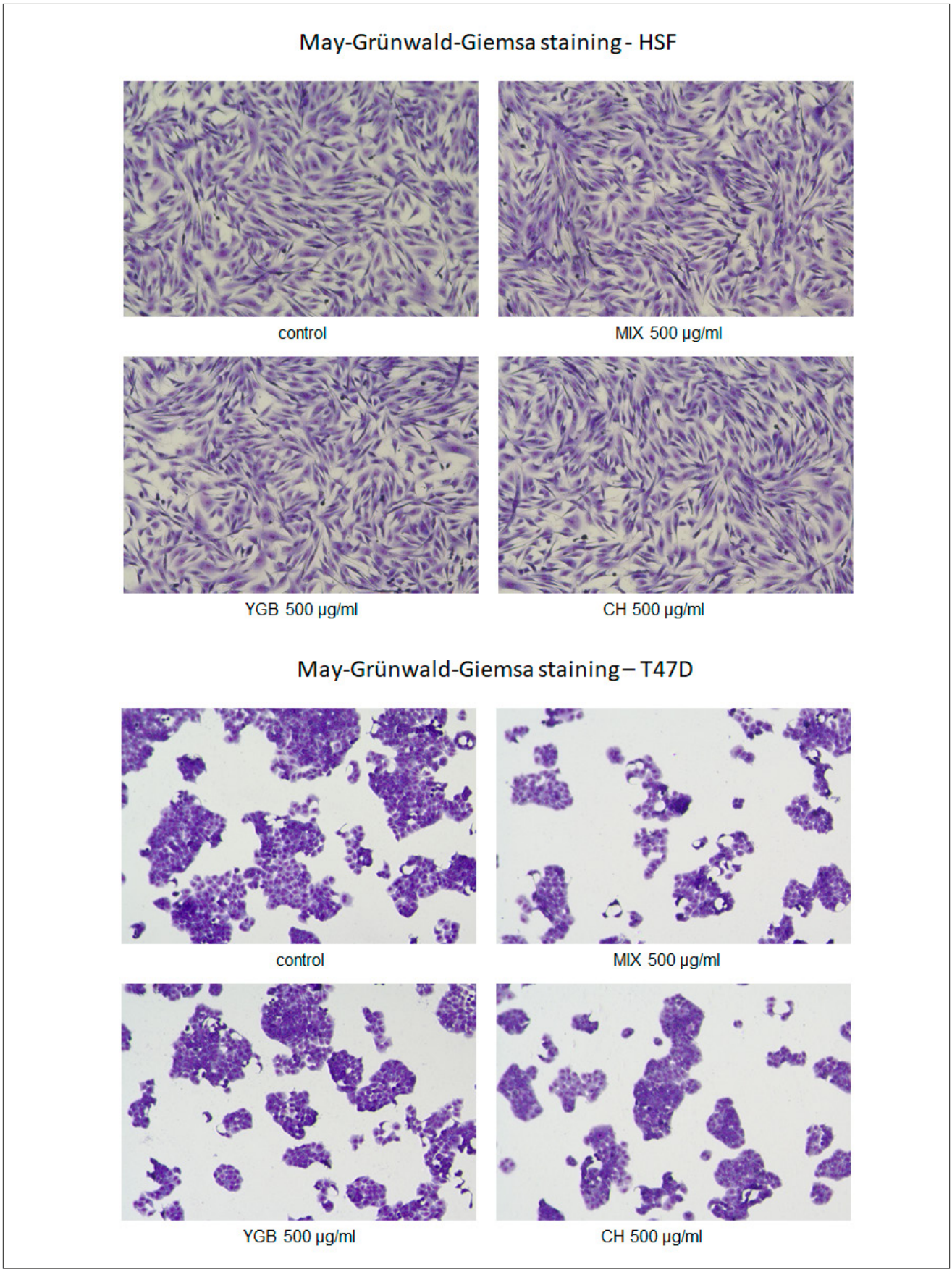

Figure 4. Impact of young green barley and chlorella extracts used together and separately on the morphology of human skin fibroblasts HSF and human breast adenocarcinoma cell line T47D. Cells were exposed for 48 hours to culture medium alone (control) or young green barley (YGB) and chlorella (CH) extracts at a concentration of 500 µg/ml, used separately or together in a 1:1 ratio as a MIX. Changes in cell morphology were visualized by the May-Grünwald-Giemsa staining method and examined under light microscopy. Representative pictures were obtained from two independent experiments. Magnification x100.

investigations, especially in the light of data collected by Kubatka et al. [7, 14]. Research conducted by Kubatka rt al. team on MCF-7 cells, revealed proapoptotic properties of young green barley [7], while chlorella treatment increased the amount of both early apoptotic and late apoptotic/necrotic cells [14]. Similarly, Woo et al. also proved programmed cell death induction in breast cancer MDA-MB-231 cells in response to barley grass extract [8]. Consequently, an unambiguous definition of the molecular mechanism of cell death induced in breast cancer cells by the investigated extracts, used together or separately, requires more detailed analysis.

CONCLUSIONS

The results obtained reveal that the chemopreventive effects of young green barley and chlorella extract in *in vitro* model of breast cancer and the observed favourable action of the investigated green food products, was associated with inhibition of both metabolic activity and DNA synthesis in cancer cells, as well as disruption of the cell membranes integrity of cancer cells (cytotoxic effect). Simultaneously, the tested compounds used together or separately had no negative effects on human skin fibroblasts. The described chemopreventive properties of the tested extracts intensified in a dose-dependent manner and were significantly enhanced by concomitant administration. Furthermore, the study revealed for the first time the synergism of antiproliferative effects of chlorella and young green barley in the culture of breast cancer cells. Obtained results encourage future *in vivo* and clinical research, as well as detailed chemical investigation, in order to specify the phytochemical composition of the tested extracts.

Conflicts of interest. There are no conflicts of interest relevant to the contents of this study.

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