Blackcurrant Incorporated Biofunctional Cookie Digesta Shows Chemical and Cellular Biological Activity Against Human Liver Cancer Cells, HepG2

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Abstract

The improved understanding of the underlying mechanisms of oxidative damage and/or chronic diseases is of high priority in dietary research. Although the chemical extraction of biofunctional molecules from different fruits and cereals have been studied extensively, the impact of food processing and digestion on bioactivity has not been studied systematically. The aim of this study was to investigate the biofunctional potential of blackcurrant powder incorporated into wholemeal wheat and barley cookies in after simulated in vitro digestion. The incorporation of blackcurrant significantly ($p<0.05$) increased the total phenolic content (about 60 %), significantly improved oxygen radical absorbance capacity (about 25 %) of the cookie digesta. Additionally cellular antioxidant and anti-proliferative activity (lowest EC_{50} value 1.02 mg/mL) on human liver cancer cell model, HepG2 was significantly enhanced. Bioactive metabolites of blackcurrant incorporated cookies were significantly supressed the inflammatory cytokine genes IL-1β (about 3-fold), IL-6 (about 0.5-fold) and NF-kB (about 2-fold) regulation and upregulated satiety gene NUCB-2/Nesfatin-1 (about 5-fold) compare with wholemeal wheat and barley control cookies. The exerted synergistic effects of this study suggest that there may be a new and effective option to prevent and control chronic diseases in human.

Keywords: Oxidative stress; blackcurrant; biofunctional cookie; simulated digestion; bioactive metabolites; inflammatory cytokines

Introduction

Oxidative stress induced by free radicals is a physiological mechanism that has long been recognised as common denominator of numerous life-threatening diseases such as cancer. Reactive oxygen species (ROS) levels regulate the oxidative stress, which causes the degradation of cellular integrity and tissue functions[1-3]. Dysregulation of normal cellular respiration promotes the release of the highly diffusible ROS free radicals such as super oxide anion (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2), mediating oxidative injury of aging[4], carcinogenesis[5], atherosclerosis[6],

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metabolic syndrome[3, 7], diabetes[8] and chronic neuronal disorder such as stroke, Parkinson's (PD) and Alzheimer's disease (AD)[9, 10]. The uncontrolled formation of ROS and their deleterious effects (redox reactions) can be neutralized by substances named "antioxidants", which act as enzymatic cofactors and inhibit autoxidation[11, 12].

Berries are rich in bioactive compounds including phenolics, flavonoids, anthocyanins and vitamins[13]. Meta-analysis studies have shown that the consumption of berries exerts a wide range of biological activities in lowering the risk of chronic diseases like cancer, diabetes, aging and neurodegeneration[14-16]. Blackcurrants are one of the richest sources of bioactive compounds compared with other berries, that contain a number of polyphenols, including phenolic acids, flavonoids, flavonols, anthocyanin, proanthocyanidins, vitamins and minerals[17]. Recently, much research has been focussed on blackcurrants due to their richness in bioactive compounds, such that they are considered as a functional food due to their diverse health benefits. Very limited studies have demonstrated the blackcurrant exerts a neuroprotective effects against oxidative stress induced neural inflammation in human cell lines.

Cereal grains including wheat and barley are an excellent source of basic nutrients mainly carbohydrate and protein. Cereal grains contain high amounts of bioactive compounds such as fibre, vitamins, minerals and phenolic compounds; however, they are depleted during the refining[18]. There is a suggestion from epidemiological studies that the consumption of whole grains might reduce the risk of mortality including developing the type 2 diabetes, cardiovascular diseases, and total and specific cancers[19-21], but no particular studies have been conducted to identify the biological activity of whole grains on cell model after digestion.

Therefore, the objective of this study was to investigate the synergistic effects of bioactive compound rich cookies on the proliferation of human cancer cells and their cellular antioxidant activity.

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Materials and methods

Chemicals and reagents

Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-2carboxylic acid (Trolox), 2,2´-azobis (2-amidinopropane) dihydrochloride (ABAP), 2´7´-dichlorofluorescin diacetate (DCFH-DA) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA). Chlorogenic acid, ρ-coumaric acid, ferulic acid, vanillic acid, caffeic acid, 3,4-hydroxybenzoic acid and quercetin were purchased from Aladdin Industrial Corporation (Shanghai, China). Chromatographic grade of acetonitrile and methanol were purchased from ANPEL Scientific Co. Ltd. (Shanghai, China). William's medium E (WME), fetal bovine serum (FBS), insulin and other cell culture reagents were purchased from Gibco Biotechnology Co. (Grand Island, NY, USA).

Sample preparation

Previously developed cookies[22] (wholemeal wheat cookie; wholemeal wheat + 15 % blackcurrant powder cookie; wholemeal barley cookie; wholemeal barley + 15 % blackcurrant powder cookie) were grinded before *in vitro* digestion.

In vitro digestion

The *in vitro* simulated digestion method developed previously[23], was followed with some modification for four sequential phases enzymatic digestion. The total digestion process was carried out at 37 °C in a water bath. The samples (2.0 g) were completely dispersed in 20 mL RO water. 0.5 mL α-amylase solution (6.5 mg enzyme in 5 mL of 1 mM $CaCl_2$; pH 7.0) was added to each sample similar oral digestion and incubated for 10 min. The pH was then adjusted to 3 with 6 M HCL for the gastric phase digestion. 0.1 g pepsin was added to the oral digesta and incubated for 2 h. Then, pH of the digesta was adjusted to 7.5 with 0.9 M $NaHCO_3$ for the intestinal process. The enzyme solution (5 mL of 0.1 g pancreatin, 0.6 g lipase and 0.625 g bile extract in 0.1 M $NaHCO_3$; pH 7.5) was added to represent small intestinal digestion and incubated for 4 h. For the large intestinal digestion, the pH

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was adjusted to 4.0 and then 60 µl viscozyme L was added to the digesta, incubated for 6 h to simulate the colon digestion. 2 mL of each physiological phases digested aliquots were collected and stored at – 40 °C for further analysis.

Determination of total phenolic content

The total phenolic contents (TPC) of the digested samples were measured by using Folin-Ciocalteu colorimetric reagents as described previously[22]. Gallic acid was used as a standard to determine TPC and unit of measurement was expressed as milligram gallic acid equivalent per gram sample, dry weight ((mg GAE/g sample, DW).

Determination of phenolic acids composition by RP-HPLC

The phenolic acid composition of digested samples were identified and quantified by chromatographic analysis as described before[24] with some modification. The HPLC (Waters Co., Milford, MA, USA) system was equipped with a Sunfire C18 reverse phase column (250 x 4.6 mm, 5 µm particle size, Waters, USA) at 280 nm wavelength. The samples were eluted with a gradient system consisting of binary elution phase A (0.1 % trifluroacetic acid in Milli-Q water) and phase B (acetonitrile) with a flow rate 1.0 mL/min. The gradient conditions were 0-5 min 10 % B, 5-20 min 25 % B, 20-25 min 35 % B, 25-40 min 90 % B, 40-50 min 10 % B, 50-60 min 10 % B. The chromatographic peaks were analysed by their retention times and compare with the pure standard compounds in specific wavelength. The contents of phenolic acids were determined and results were expressed as mg/100 g DW.

Measurement of total antioxidant capacity

Oxygen radical absorbance capacity (ORAC) assay was performed according to the literature[25] to determine the total antioxidant capacity of the tested samples. Four trolox concentrations (between 6.25 µM to 50 µM) and optimized concentration of samples were used for the analysis. ABAP solution was prepared fresh with phosphate buffer and added immediately before reading the samples. Multi-Mode Microplate Reader (FilterMax F5, Molecular Devices, USA) with fluorescence intensity

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(excitation of 485 nm and emission of 535 nm) was used and run for 35 cycle every 5 min[26]. Data were processed by extrapolation on a calibration curve and unit of measurement was expressed as micromol Trolox equivalents per gram sample, dry weight (µmol TE/g sample, DW).

Cell culture

Human liver cancer cells HepG2 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Conditioned medium (CM) was Williams medium E (WME) supplemented with 5 % FBS, 10 mM N-2-hydroxyethlypiperazine-*N´*-2-ethanesulfonic acid (HEPES), 2 mM L-glutmine, 5 µg/mL insulin, 0.05 µg/mL hydrocortisone, 50 units/mL penicillin, 50 µg/mL streptomycin and 100 µg/mL gentamycin, were maintained at 37 °C and 5 % CO_2 in an incubator humidified condition[27].

Cellular antioxidant activity (CAA) of digesta

The CAA assay was conducted with HepG2 cells with 12 to 35 passages as following the method developed by Wolfe and Liu[28]. The CAA values were calculated by dividing the the EC_{50} of quercetin standard by the EC_{50} of the sample and the results were expressed as µmol of quercetin equivalent (QE) per 100 g of DW.

Determination of cytotoxicity and anti-proliferative activity

The cytotoxicity of digested samples were investigated by modified methylene blue assay[29]. Cells were treated with the different concentrations of digested samples and the viability was compared to the control after 24 h incubation. The reduction of cell viability by >10 % was considered to be cytotoxic.

The antiproliferative efficacy of digested samples was evaluated on HepG2 cancer cell lines following the modified methylene blue assay[29]. Precultured cells were treated with different concentrations of digesta and incubated for 72 h in humidified atmosphere (37 °C and 5 % CO_2). The anti-proliferative effects were assessed by the EC_{50}.

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Quantitative real-time PCR and regulation of gene expression

HepG2 cancer cells were seeded into 6-well plates at a density 1 x 10^5 cells/mL and incubated at culture condition (37° C and 5 % CO_2). After 24 h incubation, the growth medium was removed from the well and it was washed with PBS. Treatment cells were incubated with medium containing digesta for 12 h and the corresponding control cells were treated with medium only. All the RNA was extracted from the cells by using the RNA Extraction Kit (Dongsheng Biotech, Guangzhou, China), according to the manufacturer's protocol. Isolated total RNA (2 µg) was then reverse-transcribed into cDNA for PCR using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Biotechnology, Dalian, China), according to the manufacturer's protocol. The qPCR on cDNA was performed using SYBR® Premix Ex Taq™ Kit (Takara Biotechnology, Dalian, China) in the Bio-Rad MiniOption™ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using GAPDH as internal control. Primers were obtained from Sigma-Aldrich and nucleotide sequences were: 5′-GTCAGTGGTGGACCTGACCT-3′ (forward) and 5′-AGGGGTCTACATGGCAACTG-3′ (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH); 5′-GTGTGAAAGCAGCAAAGAGGC-3′ (forward) and 5′-CTGGAGGTACTCTAGGTATAC-3′ (reverse) for interleukin-6 (IL-6); 5′-AAAAGCTTGGTGATGTCTGG-3′ (forward) and 5′-TTTCAACACGCAGGACAGG-3′ (reverse) for interleukin-1β (IL-1β); 5′-CAAAGTAGACCTGCCCAGAC-3′ (forward) and 5′-GACCTCTCTCTAATCAGCCC-3′ (reverse) for nuclear factor-kappa B (NF-kB); 5′-AGTGGGAGGCTAAGCAAAGA-3′ (forward) and 5′-CAGATCACTTGTTGCCGCTT-3′ (reverse) for nucleobindin-2 (NUCB2)/nesfatin-1. Relative mRNA expression level for each gene was calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Results were expressed as mean ± standard deviation (SD) of multiple experiments. One-way analysis of variance (ANOVA) was Tukey's multiple comparison test were employed using statistical software Minitab (version 17, Minitab Inc). Dose-effect analysis was employed using Calcusyn software (Version 2.0, Biosoft, Cambridge, UK). Significance differences were determined at $P<0.05$.

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Result and discussion

Gallic acid equivalent total Phenolic content, TPC

This study investigated the effect of digestion on the TPC release from the experimental cookies using a simulated gastrointestinal digestion. The results from the different digestion phases (oral, gastric, intestinal and colon) were compared and are presented in **Table 1**. The TPC value of bioactive cookies was significantly increased ($P<0.05$) at the end of gastrointestinal digestion. The highest TPC release from food matrix was shown after completion of intestinal phase in WCC and BCC. This could be due to polyphenols that are bound to proteins in the wholemeal flour being released at the time of protein digestion[30]. Proteins in wholemeal flour are digested at different rates from upper to lower gastrointestinal tract and the pH environment of gastrointestinal tract could manipulate the phenolics release[31]. In contrast to the previous study, this simulated gastrointestinal digestion study showed a three-fold higher TPC than the organic solvent extraction (methanolic extraction, 70 % v/v)[22]. This implies the slow-release of the bound phenolics that are present in the cell wall materials of the wholemeal flour, under different physiological conditions.

The mean TPC value (free and bound phenolics) of in the combination of blackcurrant powder with wholemeal flour containing W15B and B15B samples were about two-folds higher than the wholemeal flour control cookies. The TPC of cookies incorporating blackcurrant were significantly increased in gastric phase and then declined during intestinal and colon digestion. This could be due to the higher flavonoid content of blackcurrant powder, which was released during upper gastrointestinal digestion and may exert a potential health benefit, such as cytoprotection from the oxidative stress in the GIT[32]. The lower pH condition of the gastric phase may also enhance the rapid cleavage and release of anthocyanins content of blackcurrant. A significant decrease in TPC concentration was observed after the pancreatic bile salt digestion (mimic of intestinal digestion) in mild alkaline environment. It is noteworthy that the total phenolics were significantly affected (about 15 %) by the change in pH, suggesting the phenolics stability at the pH change, which is in agreement with previous studies[32,

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33]. Isomerization and interaction with other food components may also attenuate the stability of TPC concentration during in digestion[34]. Additionally, bioactive polyphenols act as effective inhibitors of digestive enzymes, carbohydrate and protein binding affinity, for example, complex interaction of α-amylase and proanthocyanidins, free amino acids of protein-phenolics and starch-phenolics interaction[22]. Some of the phenolics may degrade and could be oxidised into other chemicals of the assay[33]. Light and O_2 also are the two most important factors attenuating the TPC concentration during *in vitro* digestion[34]. However, the higher TPC concentration of the bioactive cookies release at different phase of digestion may exert potential in local and systemic health benefits.

Table 1. Total phenolic content of cookie digesta

Phase	WCC	W15B	BCC	B15B
Oral	44.37 ± 5.37^{d}	205.23 ± 9.00^{d}	41.04 ± 0.30^{d}	254.25 ± 3.08^{d}
Gastric	141.01 ± 4.79^{c}	375.81 ± 2.92^{a}	112.02 ± 3.32^{c}	376.55 ± 4.24^{c}
Intestine	211.28 ± 1.96^{a}	354.51 ± 3.89^{b}	183.50 ± 2.48^{a}	360.86 ± 4.16^{a}
Colon	193.7 ± 25.00^{b}	310.61 ± 6.97^{c}	162.32 ± 2.23^{b}	311.63 ± 3.64^{b}

Data expressed as MEAN mg GAE/100 g sample, *n* = 3; values with different letter in each column are significantly different ($p < 0.05$)

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

Trolox equivalent antioxidant activity

The antioxidant activity of bioactive compounds of cookie digesta was determined by oxygen radical absorbance capacity (ORAC) assay **(Table 2)**. Blackcurrant with wholemeal wheat and barley flour contained higher phenolics and showed significantly ($P<0.05$) higher ORAC value in the assay. It is noteworthy that the presence of blackcurrant in the cookie resulted in higher total phenolics in the

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gastric phase, while higher total antioxidant activity of the same samples was exhibited in the intestinal and colon phases. This could be due to the compositional changes of phenolic acid released during the individual digestion phase. Additionally, not all phenolic compounds have greater antioxidant and/or biological properties. For example, the anthocyanins of the blackcurrant might have higher antioxidant activity ability and this lead to the significant differences observed[13]. The potential radical scavenging ability was strongly increased (about 10 %) by the simulated digestion of wholemeal flour cookie compared with chemical extraction of the previous study[22]. This could be due to the fraction of free/bound phenolics ratio of the samples and their release during digestion[35]. However, the mean ORAC value was significantly increased when blackcurrants were incorporated to the wholemeal flour cookie after simulated digestion, which have about 20 % higher peroxyl radical neutralizing capacity than the control cookies. Furthermore, the enzymatic hydrolysis of cookie components such as carbohydrate and protein digestion may enhance the release of phenolics that are attributed to potential radical scavenging activity. Thus, the blackcurrant has greater antioxidant capacity and its incorporation into food could be appropriate protection against diseases associated oxidative stress.

Table 2. Extracellular antioxidant activities (ORAC values) of cookie digesta

Digestion phase	WCC	W15B	BCC	B15B
Oral	15.34 ± 3.93^{d}	29.92 ± 4.24^{d}	9.63 ±2.9^{d}	36.29 ± 10.02^{d}
Gastric	53.76 ± 8.71^{c}	64.74 ± 9.38^{c}	40.12 ± 7.86^{c}	58.65 ± 8.16^{c}
Intestine	89.75 ± 2.56^{a}	114.78 ± 10.60^{a}	89.33 ± 3.54^{a}	110.41 ± 11.23^{a}
Colon	86.51 ± 6.57^{b}	112.4 ± 5.49^{b}	81.28 ± 10.84^{b}	109.10 ± 9.28^{b}

Data expressed as MEAN NetAUC Trolox equivalents (µmol TE/g sample, DW); *n = 3,* values with different letter in each column are significantly different ($p < 0.05$)

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WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder **Quantification of individual phenolic compounds**

The HPLC analysis was performed to evaluate the individual phenolic acids of simulated gastrointestinal digesta of cookies. The relative distribution of identifiable phenolic acids (PAs) for different sample/digestion phase is listed in **Table 3**. The different chromatographic profiles were observed at the end of different digestion phase of incorporation of blackcurrant in wholemeal flour cookies. Nine common peaks, which had acceptable heights and were clearly separated, were calculated with relative areas of the standard. The number of peaks was generally consistent in same group of samples, but the areas of the common peaks were varied greatly at different phases of the digesta, which indicated the concentration variation of PAs. The mean value of highest total PAs content were observed in B15B (263 mg/100 g DW) and W15B (258 mg/ 100 g DW) digested samples, which were contained 15 % blackcurrant powder. Among all identifiable PAs fraction of the wholemeal flour cookie digesta, ferulic acid was the most dominant fraction, ranging from 16.49 to 48.16 mg/100 DW, followed by p-coumaric, p-hydroxybenzoic, vanillic and syringic acid respectively. Sinapic acids were detected only in WCC digesta, while chlorogenic acids were detected in BCC digesta. Quercetin-3-rutinoside was the major phenolic acids in W15B and B15B and ranged from 35.77 to 89.66 mg/100 g DW, followed by quercetin derivatives and kaemferol-3-glucoside, confirming that the incorporation of blackcurrant powder strongly contributed to the total PA in the cookie. The individual PAs quantified in the present study were similar to other previous studies have been reported[18, 36, 37]. Oral phase digesta were lower PAs content, this is due to the very short residence time[38]. Most of the PAs were exhibited in concentrated form in the simulated lower gastrointestinal digestion phase, was indicating the PAs slow release as carbohydrate and protein digestion progressed. A slight loss of PAs was identified mostly in colon phase, which could be due to transformation of pH to an alkaline environment and unstable properties of PAs, as discussed above[33]. However, the abundance of PAs were markedly increased with the addition of blackcurrant powder to the cookies, which may have

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significant antioxidant properties in local and systemic health effects and protect against all forms of cancer including digestive cancer.

Table 3: HPLC analysis of phenolic acids composition in cookie digesta

Sample	Phase	p-HA	VA	SA	CA	ρ-CA	FA	Q-3-R	Q	K-3-G	Total PAs
WCC	Oral	5.39 ± 0.1	4.67 ± 0.5	9.27 ± 0.1	nd	1.7 ± 0.0	21.77 ± 1.3	nd	nd	nd	42.8 ± 2.0
WCC	Gastric	8.46 ±0.8	17.12 ±1.1	14.61 ± 0.6	6.75 ± 0.1	7.83 ± 0.8	46.92 ± 2.3	nd	nd	nd	101.69 ± 5.7
WCC	Intestine	16.89 ± 0.8	19.13 ± 2.7	17.42 ± 1.1	5.60 ± 0.1	19.70 ± 0.7	57.51 ± 2.8	nd	nd	nd	136.25 ± 8.2
WCC	Colon	19.82 ± 0.4	19.54 ± 1.3	17.80 ± 0.7	4.72 ± 0.2	16.05 ± 1.3	48.16 ± 1.6	nd	nd	nd	126.09 ± 5.5
W15B	Oral	3.82 ± 0.2	9.14 ± 0.3	3.01 ± 0.1	nd	12.89 ± 0.6	16.17 ± 0.5	66.50 ± 2.1	9.28 ± 0.5	6.21 ± 0.0	127.02 ± 4.3
W15B	Gastric	15.57 ± 1.7	13.96 ± 0.2	14.75 ± 1.1	4.13 ± 0.0	29.00 ± 1.1	40.56 ± 1.4	89.66 ± 2.7	36.22 ± 0.7	25.46 ± 0.8	269.31 ± 9.7
W15B	Intestine	18.45 ± 1.6	13.01 ± 0.1	16.58 ± 0.8	5.14 ± 0.0	31.58 ± 1.6	44.69 ± 1.8	83.00 ± 1.8	27.15 ± 1.6	24.84 ± 0.8	264.44 ± 10.1
W15B	Colon	12.95 ± 0.7	19.73 ± 0.3	17.50 ± 0.1	9.64 ± 0.1	27.40 ± 0.9	43.07 ± 1.5	73.10 ± 1.9	28.79 ± 1.3	26.02 ± 1.8	258.2 ± 8.6
BCC	Oral	8.35 ± 0.8	6.96 ± 0.9	nd	15.87 ± 0.6	2.96 ± 0.0	16.49 ± 0.6	nd	nd	nd	50.63 ± 2.9
BCC	Gastric	12.66 ± 0.8	16.06 ± 0.8	10.71 ± 0.0	19.36 ± 0.8	11.09 ± 0.9	29.32 ± 1.5	nd	nd	nd	99.2 ± 4.8
BCC	Intestine	12.78 ± 0.1	15.69 ± 0.1	6.21 ± 0.0	23.49 ± 0.2	19.23 ± 1.5	27.55 ± 0.9	nd	nd	nd	104.95 ± 2.8
BCC	Colon	10.56 ± 0.2	20.70 ± 0.8	4.59 ± 0.1	16.19 ± 0.4	12.94 ± 0.9	28.12 ± 0.7	nd	nd	nd	147.1 ± 3.1
B15B	Oral	17.16 ± 0.2	8.29 ± 0.0	nd	nd	nd	17.16 ± 0.7	35.77 ± 0.6	9.29 ± 0.1	6.05 ± 0.0	93.72 ± 1.6
B15B	Gastric	27.65 ± 1.3	18.80 ± 0.6	10.71 ± 0.0	2.66 ± 0.1	5.59 ± 0.3	54.49 ± 1.6	85.47 ± 2.2	48.81 ± 1.3	10.89 0.2	265.07 ± 7.6
B15B	Intestine	28.74 ± 1.2	12.95 ± 0.2	4.84 ± 0.1	11.27 ± 0.3	18.37 ± 0.1	42.89 ± 1.9	79.50 ± 1.8	37.17 ± 1.5	24.20 ± 0.2	259.93 ± 7.3
B15B	Colon	29.10 ± 1.7	11.84 ± 0.1	3.21 ± 0.0	17.79 ± 0.1	19.03 ± 1.1	45.47 ± 1.6	75.53 ± 2.6	33.68 ± 0.6	27.51 ± 0.6	263.16 ± 8.4

Data expressed as mg phenolic acid (PA) /100 g sample, DW; *n = 3,* values with different letter in each column are significantly different ($p < 0.05$)

Compounds are presented as: p-HA: p-hydroxybenzoic acid; VA: Vanilic acid; SA: syringic acid; CA: caffeic acid, *p*-CA: *p*-coumaric acid, FA: ferulic acid; Q-3-R: quercetin-3-rutinoside; Q: quercetin; K-3-G: kaemferol-3-glucoside

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

Cytotoxic and anti-proliferative effect against HepG2 cell model

The *in vitro* anti-proliferative activity and cytotoxicity of cookie digesta were evaluated in a dose dependent manner on the human liver cancer cells, HepG2. The cytotoxic effects were tested after completion of each digestion phase individually of all samples, by human liver cancer cells, HepG2.

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The CC_{50} value represents the cytotoxicity of the samples, which was observed in a dose-dependent manner. The CC_{50} value was high up to 200 mg/mL of oral and gastric phase and up to 120 mg/mL of intestinal and colon phase **(Table 4)**. The minimal stimulatory effects of lower gastrointestinal digesta could be due to the enzymes (pancreatic bile salt digestion) used in the simulated digestion and/or the biofunctional compounds released in the system, have been reported in previous studies[27, 39].

The concentration with greater activity and no cytotoxicity are of interest to potential cellular investigation. Anti-proliferation activities of the tested samples were investigated with the concentration having no cytotoxic effects on HepG2 cells. Results of anti-proliferative effects are presented as median effective dose (EC_{50}), where lower EC_{50} value indicates higher inhibition of proliferation, presented in **Figure 1 and Table 4**. The blackcurrant incorporated samples, W15B and B15B, digested metabolites effectively inhibited the cancer cells, HepG2, proliferation at the last phase of the digestion. The EC_{50} values were significantly different after each digestion phase and the lowest EC_{50} value was observed at the colon phase, followed by intestinal, gastric and oral phase respectively of all samples. The significantly lowest EC_{50} value of each sample was W15B (1.02 mg/mL), B15B (1.97 mg/mL), WCC (58.92 mg/mL) and BCC (61.92 mg/mL) respectively, this reflects (negative correlation) the cellular antioxidant activity (CAA value) in each digestion phase. Complex cellular antioxidant capacity and individual bioactive compounds present in the digesta could have the potential anti-proliferative effects in human cancer cells[40]. In addition, the anti-proliferative effects of the cookie increased might be due the improved cellular uptake by digestion[41].

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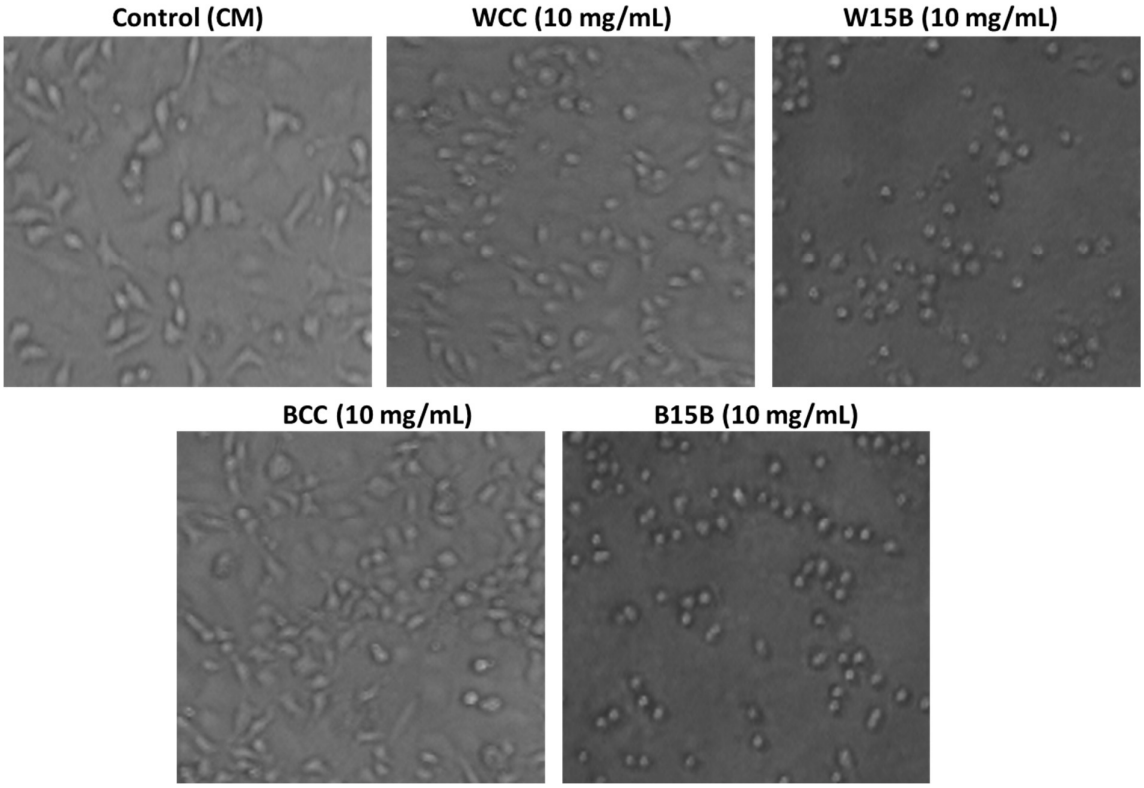

Figure 1. Anti-proliferative activity on HepG2 cell line (under microscope observation). Cells treated with antioxidant-DF for 72 h

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

Table 4: Antiproliferative activity and cytotoxicity of cookie digesta on HepG2 cell line

Antiproliferative activity (EC_{50}); Cytotoxicity (CC_{50})

	EC_{50}				CC_{50}
Digestion Phase	WCC	W15B	BCC	B15B	All sample (mg/mL)
Oral	252.7 ± 11.23[a]	38.38 ± 2.39[a]	257.8 ± 9.88[a]	46.53 ± 2.19[a]	>200
Gastric	89.38 ± 9.17[b]	30.69 ± 2.38[b]	84.87 ± 7.59[b]	33.47 ±1.52[b]	>200

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Intestine	61.59 ± 4.82[c]	7.648 ± 0.63[c]	68.13 ± 2.33[c]	6.88 ± 0.71[c]	>120
Colon	58.92 ± 4.10[d]	1.02 ± 0.11[d]	61.92 ± 01.89[d]	1.97 ± 0.08[d]	>120

Data expressed as mg /mL sample, DW; *n = 3,* values with different letter in each column are significantly different ($p < 0.05$)

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

Cellular antioxidant capacity, CAA on HepG2 cell model

Oxidative and/or pathological stresses are thought to be the leading cause of chronic diseases whereas antioxidants play a key role in cellular homeostasis[42]. Chemical assay of antioxidant capacity of bioactive compounds might be dissimilar in complex biological systems, as they might not be working as individual entities to neutralize the radical. Biologically, CAA assay is more relevant to physiological conditions, intracellular uptake, distribution and metabolism. To evaluate the antioxidant capacity of bioactive food digesta at the cell level, CAA assay was carried out with human liver cancer cell, HepG2, where DCFH-DA were used as probe with samples, and then ABAP were added to induced peroxyl radical oxidization. Two protocol "PBS wash" and "no PBS wash" after treated by samples were followed to examine the cellular uptake of the compounds. Data were presented in **Table 4**, as micromole quercetin equivalent per 100 gram, DW.

Increased CAA values with no PBS wash treatment were observed at the end of digestion for samples B15B (122.70 µmol QE/100 g, DW) and W15B (113.40 µmol QE/100 g, DW), which is at least two to three-fold higher than the control cookie. The PBS wash treatment of the same samples also exhibited higher CAA values at the same ratio. The results indicate the potential of blackcurrant to enhance the antioxidant ability of the cookies. For example, bioactive compounds quercetin and kaempferol available in blackcurrant act as a powerful antioxidant have been reported[36, 43]. In comparison with

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the TPC value of the different digestion phases and the trolox equivalent antioxidant assay, unlike, the colon phase digesta were significantly ($P < 0.05$) higher than the CAA value in all samples. This indicates that some bioactive compounds could be available in the digesta which have powerful antioxidant ability in HepG2 cell model. The CAA values reflect the complex biological activity including absorption, metabolism and distribution, regardless of the scavenging free radicals of chemical assays. The PBS wash treatment CAA values were lower in the four digestion phases of all samples which compares with no PBS wash and is in agreement with other previous studies[28, 41]. This could be due to the physic-chemical properties of compounds present in the digesta, including molecular size, solubility, polarity and their bioavailability at the cellular level[1, 27, 44].

Table 4. Cellular antioxidant activity (CAA) of cookie digesta

	WCC		W15B	
Digestion phase	No PBS wash	PBS wash	No PBS wash	PBS wash
Oral	10.53 ± 0.82^{d}	6.31 ± 0.18^{d}	17.79 ± 1.03^{d}	10.67 ± 0.14^{d}
Gastric	22.81 ± 1.77^{c}	14.83 ± 0.91^{c}	37.04 ± 2.61^{c}	21.48 ± 1.83^{c}
Intestine	33.76 ± 1.16^{b}	22.50 ± 1.10^{b}	80.81 ± 2.67^{b}	52.52 ± 2.02^{b}
Colon	41.57 ± 2.49^{a}	24.94 ± 3.97^{a}	113.40 ± 8.32^{a}	67.48 ± 4.90^{a}
	BCC		B15B	
Phase	No PBS wash	PBS wash	No PBS wash	PBS wash
Oral	11.09 ± 0.95^{d}	5.95 ± 0.22^{d}	17.59 ± 1.13^{d}	10.99 ± 0.74^{d}
Gastric	21.62 ± 1.02^{c}	15.13 ± 1.11^{c}	33.40 ± 2.06^{c}	20.46 ± 1.79^{c}
Intestine	28.24 ± 2.11^{b}	17.41 ± 1.38^{b}	87.64 ± 3.31^{b}	55.50 ± 5.02^{b}
Colon	42.34 ± 3.60^{a}	29.64 ± 2.35^{a}	122.70 ± 5.81^{a}	73.99 ± 6.63^{a}

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Data expressed as µmol QE/ 100 g sample, DW); *n = 3,* values with different letter in each column are significantly different ($p < 0.05$)

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

IL-1B, IL-6, NF-kB and NUCB/Nesfatin 1 genes expression in HepG2 cell model

Proinflammatory cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6) and nuclear transcription factor-kB (NF-kB) are thought to be central regulators of inflammation and development of chronic diseases. Upregulation of NF-kB target genes including IL-1β and IL-6 genes expression are associated with chronic inflammation and mediate cell proliferation[45]. Controlling the expression of these inflammatory genes activates the immune response and promotes immunity. We compared the effects of cookie digesta on the relative expression of the three genes related inflammation, assessed by RT-qPCR to determine the biological effects of bioactive cookies in HepG2 cells. Cells were treated with 10 mg/mL of the digesta for 12 hours. All samples exhibited downregulation of IL-1β and IL-6 and NF-kB in digested samples (control vs tested samples) as shown in **Figure 2**. The downregulation pattern of these inflammatory genes was associated with an increased antioxidant value as reported in CAA analysis. The blackcurrant incorporation cookie digesta inhibited the expression of IL-6 and NF-kB genes slightly high in comparison with the control cookies. It is noteworthy that the inflammatory factor IL-1β was highly downregulated when treated with samples W15B (-0.36 ± 0.06) and B15B (0.21 ± 0.01). The reason for W15B and B15B treated samples showing regulation of gene expression may be due to the composition of the bioactive compounds, and their greater biological activity in a cell model, leading to potential anti-inflammatory/anticancer effects and/or corresponding diseases[40, 42].

Cumulative evidence suggests that upregulation of appetite regulator “nucleobindin-2/nesfatin-1 (NUCB/Nesfatin-1)” plays an important role in increasing satiety, glycaemic management and reducing

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body weight[46]. To determine the potential effects of bioactive model, the expression of NUCB/Nesfatin-1 was examined in HepG2 cells treated with digested samples. In the cell model, administration of the tested samples greatly influenced the expression of NUCB/Nesfatin-1 genes in all treatment **(Figure 1)**. The relative expressions of NUCB/Nesfatin 1 in both blackcurrants containing cookie digesta were upregulated by about three-fold compared with the control cookies digesta, and ten to fifteen-fold compared to the control. These results indicate that the bioactive compounds present in the cookies are a prominent regulator of anorexigenic NUCB/Nesfatin 1 mRNA expression.

Evidence has been reported that bioactive polyphenols available in berries potentially inhibit the expression of inflammatory markers in animal and cell models[47, 48]. Previous studies have shown that the high phenolic content in berries potentially reduce pro-inflammatory cytokine IL-6, TNF-α[47, 49]. The biological activities of different berry extracts have been investigated widely, however that their enhanced potential activity after simulated digestion in the cellular level may be mediated synergistically by other compounds such as those contained in whole grains has not been shown before.

Taken together, the results indicate that the biofunctional ingredients especially polyphenols, flavonoids and anthocyanins present in the blackcurrant incorporated cookie played an important role in regulation of certain genes expression, which may lead to a therapeutic effect on certain diseases. The results suggest a synergy between blackcurrant and whole grain bioactive compounds.

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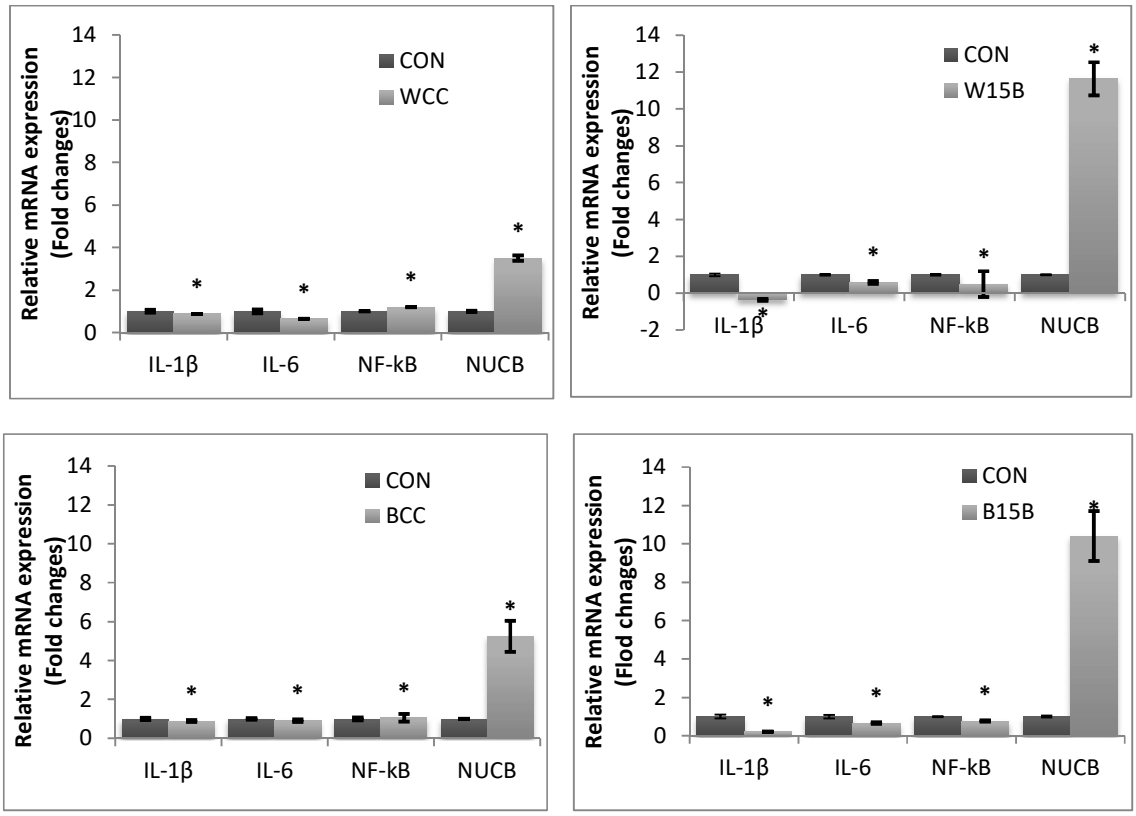

Figure 2: Relative mRNA expression (Fold change) of IL-1β, IL-6, NF-kB and NUCB after treated with WCC, W15B, BCC and B15B cookie digesta (10 mg/mL) for 12 hours; Asterisk (*) are significantly different ($p < 0.05$)

WCC- wholemeal wheat cookie control, W15B- wholemeal wheat + 15 % blackcurrant powder, BCC- wholemeal barley cookie control, B15B- wholemeal barley + 15 % blackcurrant powder

Conclusion

Biofunctional molecules from blackcurrant and whole grains synergistically contributed to the potential health benefits including improved cellular antioxidant activity, anti-proliferation and modulation of gene expression. Although the i*n vivo* digestion and cellular environment are much more complex, the findings clearly show that the bioactive compounds rich blackcurrant cookie potentially improved satiety and supressed inflammatory cytokine regulation, which are relevant to

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the pathogenesis. The underlying mechanisms behind those potential effects should be further investigated on human and/or animal studies.

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Abbreviations:

CAA: Cellular antioxidant activity

WCC- wholemeal wheat cookie control

W15B- wholemeal wheat + 15 % blackcurrant powder

BCC- wholemeal barley cookie control

B15B- wholemeal barley + 15 % blackcurrant powder

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