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Biochemical investigation on symbiotic products of soybean Okara in milk processing

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Abstract

There is growing interest in the potential role of bioactive ingredients from agro-industrial wastes, such as the soybean milk that also called Okara (O). In this study,we investigated the biological efficacy of milk supplemented with okara(M:O) at three different ratios (100: 0, 80: 20, and 70:30) to improve their nutritional and biological properties levels. The results indicated an enhancement in the phenolics and flavonoids contents in okara due to supplementation, the antioxidants capacity determined with ABTS and DPPH, and the anticancer activity against three cancerous cell lines including, Human breast MCF-7,colon HT-29, and prostate PC3 cancer cell lines as well as one non-cancerous normal breast MCF-12Fcell lines. The effect of milk supplemented okara showed an increase in antioxidant activity relative tothe supplementation ratio. Moreover, the Total phenolic TPC and flavonoid contentsTFC analysis showed an increase in the milk TPC and TFC contents after supplementation. In addition, okara enhances the anticancer activity against tested cancer cell lines. The results indicated a ratio-dependent effect in all tested cell lines. Surprisingly, milk supplemented with okara at a 20% ratio displayed the highest anti-proliferation activity of MCF-7compared to a 30% ratio. No significant cytotoxic effect was observed due to MCF-12F. Moreover, the oxidative stress enzymes including SOD, CAT, GSH, and GPxWere determined. The results indicated an increase in the enzyme activity of supplemented milk. The mode of action was also evaluated using gene expression of pro and anti-apoptosis key markers Caspase-3, P53, Bax, and BCl-2. The data indicated that all pro-apoptosis markers were upregulated when the anti-apoptosis marker BCL-2 was down regulated. It was concluded that fortification of dairy products with 30 % Okara enhances physical and biological activity and health-promoting products.

Keywords: Okara, dairy products, cytotoxicity, Human breast MCF-7, colon HT-29, and prostate PC3 cancer cell lines.

1. Introduction

There is no statistical data is provided for Egypt for okara production. However, the annual amounts of okara production provide a serious disposal issue; its accumulation may cause an environmental risk, while it occasionally can be used to feed animals directly [1-2]. Soybean derivatives are commonly consumed and offer consumers both quantity and quality of their valuable nutrient contents, they can be an alternative used to enhance healthy lifestyles and dietary limitations [3-5]. Okara's chemical composition showed high contents of proteins, as well as different secondary metabolites including, saponins, isoflavones, and other substances, in this context it can help prevent cancer and heart disease, lessen the effects of osteoporosis, and lower LDL levels, among other health benefits. A by-product of

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processing soybeans to make soymilk is okara. Its appearance seems to be neutral and yellowish-white color with a smooth taste.

Okara preparation is conducted on the insoluble fraction that is produced when crushed soybeans are hydrothermally treated. Thus, the remaining residue has a high moisture content of up to 80%, therefore, increasing the possibility of mold and bacterial infection, which poses a serious environmental risk. The fact that this by-product still has a lot of healthy ingredients has increased interest in functional foods [6-11]. Due to the high moisture content the dietary fiber content inokara can attract microorganisms and therefore microbial infection. The chemical composition of okara showed that it is enriched in total carbohydrates and total protein contents. In addition, Okarais a source of important free fatty acidsincluding linoleic, palmitic, stearic, oleic, and linoleic acids.

As a by-product of making tofu or soymilk, okara has a composition of roughly 50% dietary fiber, 25% protein, 10% fat, and other nutrients. Several reports showed that okara contains high fiber content, with low production cost, and can be considered an ideal raw material as a valuable source for fiber preparation. To avoid diabetes, obesity, and hyperlipidemia, it may also be used as a dietary supplement [12-17].Okara has high levels of proteins, lipids, fibers, bioactive ingredients, and polysaccharides. Okaraalso showed high contents of secondary metabolites such assaponins, phenolics,lignans, flavonoids, phytosterols, and isoflavones (genistein and daidzein) among other secondary metabolites, okara exhibited high contents ofisoflavones, glucosides, aglycones andacetyl genistin with a high percentage of 30, 28, 15, and 0.89% are existed in okara, respectively. These substances offer a wide range of physiological and medicinal qualities, including the ability to prevent cardiovascular disease, act as antioxidants, and work as chemo-preventive agents for cancer patients undergoing treatment [17-20]. An extensive investigation of okara has been conducted in the last two decades in pharmaceutical uses of okara derivatives as an anticancer against breast cancer [21]. In this study, we provide solid data about the use of okara alternatively as a supplementary component in milk processing, highlighting the chemical composition and biological activities.

2. Materials and Methods.

2.1 Okara preparation

Soybean seeds were obtained from the Food Technology Research Institute FTRI, Agriculture Research Center, Giza Egypt. Soybean seeds were cleaned and washed by floatation to remove all undesired materials. Subsequently, the seeds were blanched in 100°C hot water for 25 minutes and then soaked in a solution of 1 kg / 5 liters. Following this, the soybeans were filtered using a muslin cloth to extract the milk and retrieve the residue known as okara. After that okara was mixed with Buffalo Milk at two different ratios 80:20 and 70:30 Milk: Okara, respectively.

Total flavonoids and phenolics content

Total phenolics and total flavonoid contents TFCcontents were measured usingthe Folin–Ciocalteu and aluminum chloride method as previously described by[22 and 23].

Antioxidant's activity
2.3.1 DPPH assay

The potential of radical scavenging of the above-mentioned samples was evaluated by the DPPH method as previously described by [24]. 950 µl of 50 mM DPPH was mixed with 50 µl of containing different concentrations (0,5, 10, 25, 50, 100 µg/ml). The OD was measured after 15 min of incubation under dark at 517 nm using a UV-VIS spectrophotometer (Hitachi, Model 100-20). The potential radical scavenging %was determined from the next equation:

Antioxidants %= $(A_{control}-A_{sample})/A_{control}) \times 100$ where $A_{control}$is the absorbance of DPPH and A_{sample} is the absorbance of samples [25].

ABTS assay

Radical scavenging of generated ABTSradical of the above-mentioned samples was determined following the protocol proposed by [26].An immediately prepared mixture between ABTS 7 mM solution with potassium persulphate $K_2S_2O_8$2.45mM (1:1) was incubated under dark for 24 h before use. The absorbance was adjusted to A= 0.7±0.03 with waterat

734 nm. A mixture of 50 µl containing different concentrations (0,5, 10, 25, 50, 100 µg/ml) was added to 950 µl of $ABTS^+$. After incubation for 10 minutes in a dark environment, the absorbance at 734 nm was measured using a UV-VIS spectrophotometer (Hitachi, Model 100-20). The samples were measured in triplicate, and antioxidants % was calculated using the following equation:

Antioxidants % $=((A_{control}-A_{sample})/A_{control}) \times 100$ where $A_{control}$is the absorbance of ABTS,and A_{sample} is the absorbance of the test compound.

2.3 Cytotoxicity and cell viability

The cytotoxic effect of milk and milk supplemented with okara were tested at different concentrations (0, 5, 20, and 30µg/ml)for their anticancer activity using three cancerous cell lines, Human breast cancer MCF-7, human colon HT-29, and prostate cancer PC3 in comparison to non-cancerous normal breast MCF-12F cell line. In brief, cells were seeded at 3×10^4in RPMI-1640complete medium containing 10 % FBS, 100 µg/ml penicillin/streptomycin at 37°C,and 5% CO_2 in a tissue culture flask. The cells were sub-cultured at 2×10^3 cell/well in 150 µl of medium and were seeded into a 96-well plate (Gibco, USA) for 24 h before treatment with 5, 20, and 30 µg/ml of okara, milk, and milk supplemented okara stored for another 24 h [28].

2.4 Cell viability by MTT

The cell viability was assessed using MTT assayas previously reported by[29]. After incubation for 24 h, 100 µl/well of MTT (0.5 mg/ml) was added and incubated at 37°C for another 3 h. The purple crystals were dissolved in 100 µlSDS 10%/well with shaking for 1 h at 37°C. The absorbance was measured at 549 nm using a microplate reader (ELX 800; Bio-Tek Instruments, Winooski, VT, USA). The concentrations that induce 50% inhibition (IC_{50}) were calculated.

2.5 Effects of milk-supplementedokara (OM) on the oxidative stress enzyme.

The oxidative stress-involved enzymes were determined inMCF-12F normal breast cell lines. In brief, $1x10^6$ cells were seeded in an RPMI-1640 complete media into 12 well plates incubated at 37°C and 5% CO_2 for 24 h. The cell medium in each well was replaced with serum-free medium (SFM) containing 20 µl/ml treated with okara, milk, and milk-supplemented with okara stored for (20, 30%).Before being trypsinized (Trypsin 0.05%/0.53 mM EDTA), rinsed twice in PBS, and resuspended in 1 milliliter of PBS containing 0.1% Triton X-100, the cells were cultured for 48 hours. Next, the cells were subjected to 2 × 10 s of sonication at 100 Hz on ice (Vibra-cell, Sonics & Material). The collected cells were centrifuged for 10 minutes at 4 °C at maximum rpm. In the supernatant, the enzyme activity was determined [30].

2.6 RT-qPCR of inflammatory gene

Gene expression of inflammatory-related geneswas conducted with MCF-12F normal breastcells exposed to 50 µg/mL doses of milk-supplementedokara, M:O 80:20 and 70:30 ratios for 24 h [16]. Briefly, RNA was isolated using of Total RNA Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. Synthesized of cDNA was performed using the QuantiTect Reverse Transcriptase Kit (Qiagen, Germany) [31]. The primers used in this analysis are shown in Table 1.

Table1: Primer sequence of inflammatory Key markers

Target gene	Primer sequence
TNF-α	For: 5′ATGAGCACAGAAAGCATGA 3′ Rev: 5′GAATGAGAAGAGGCTGAGA 3′
IFN-γ	For: 5'-GCGCAAAGCCATAAATGAAC-3' Rev: 5'-CTCAGAAAGCGGAAGAGAAG-3'
Cox2	For: 5'-CCGGGTACAATCGCACTTAT-3' Rev: 5'-GGCGCTCAGCCATACAG-3
IL-6	For: 5′CTCTGGGAAATCGTGGAAAT 3′ Rev: 5′CCAGTTTGGTAGCATCCATC 3′
IL-10	For: 5′GGGAAGACAATAACTGCACC 3′ Rev: 5′GCTGGTCCTTTGTTTGAAAGA 3′

The gene expression was expressed as a fold-change value for each gene compared to the expression levels of *GAPDH* as a HK gene, which was calculated using the equation$2^{-\Delta\Delta Ct}$.

2.6 Statistical Analysis

The statistical analyses were done using CoStat 6.45 for Windows. Duplicate data were included in each of the four different treatments, and the total data was displayed as mean ± standard deviation (SD). The treatments were considered statistically significant at $p < 0.05$.

3. Results and Discussion

Total phenolics and flavonoidcontents

The total phenolic contents of milk and milk supplemented with okaraare shown in Table 2. The total phenolic contentsTPC of in milk supplemented with okara80:20 and 70:30 was significantly higher than in untreated milk, where the total phenolic contents were 5.34, 6.98, and 1.23 mg of gallic acid/g dry sample, respectively. A similar pattern of total

flavonoid content was detected when milk supplemented with okara80:20 and 70:30 exhibited2.94 and 3.65 mg/g, respectively compared to untreated milk of 0.93 mg/g dry weight (Quercetin equivalents). The results indicated that okara increases the levels of TPC and TFC in milk Table 2.

Table 2. Total phenolic and flavonoid contents.
Fresh Okara (O), Milk (M), Milk-supplemented 20% Okara (MO20), Milk-supplemented with 30 % Okara (MO30).

Sample	TPC mg (QE)/g	TFC mg (QE)/g
Milk	114.41	35.82
Okara	1.11	0.33
OM20	2.1	0.63
OM30	74.12	22.48

Antioxidants activity

The antioxidant properties of the samples were evaluated using DPPH and ABTS radical scavenging assays Figure 1. Similar antioxidant activity was observed using both methods with slight differences. However, DPPH and ABTS assays showed milk-supplemented okara dose dependent activity. This result also indicated a synergistic effect for individuals. In this context, milk supplemented with okara (70:30) displayed higher antioxidant activity of 98.54% at 100 µg/ml. The antioxidant activity of milk supplemented with okara (MO)was enhanced as has been reported [32]. supplementation compared to okara and milk controls

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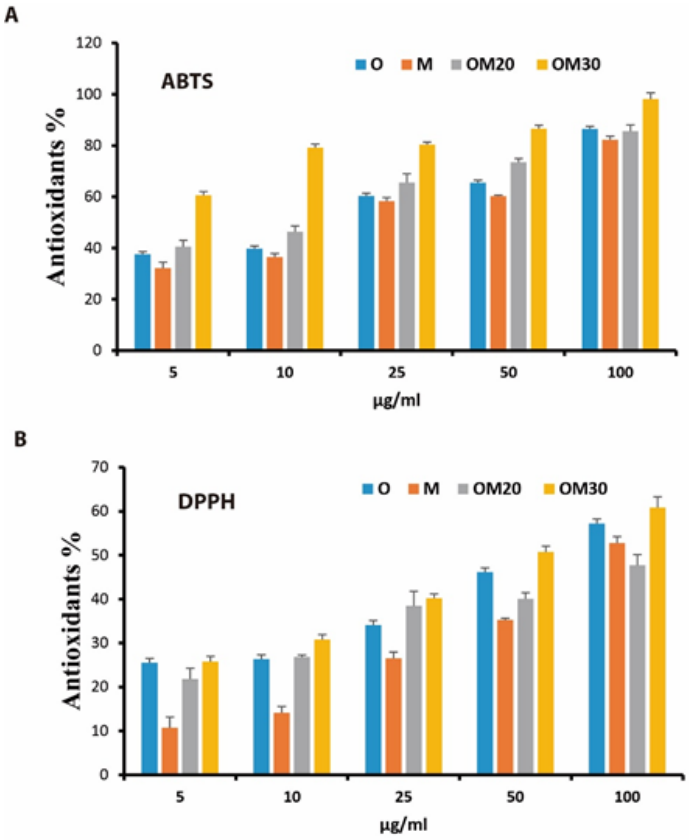

Figure 1. Antioxidant activity of milk supplemented with okara using ABTS (A) and DPPH (B). Fresh Okara (O), Milk (M), Milk-supplemented20% Okara (MO20), Milk-supplementedwith30 % Okara(MO30).

Cytotoxicity and Anticancer activity

The effect of (MO) on cancer cell growth was evaluated using MTT assay at 5, 20, and 30 µg/mL for 48 h Figure 2. The results of the growth inhibitory assay showed that a Milk-supplemented with 30 % Okara(MO30) dose of 30 µg/mL displayed the highest antiproliferation of two cancerous cell linesHT-29 and PC3 cell lines compared to respective control Figure 2 B and C, respectively. However, no significant cytotoxic effects were observed against the MCF-12 F normal breast cell line Figure 2 D.

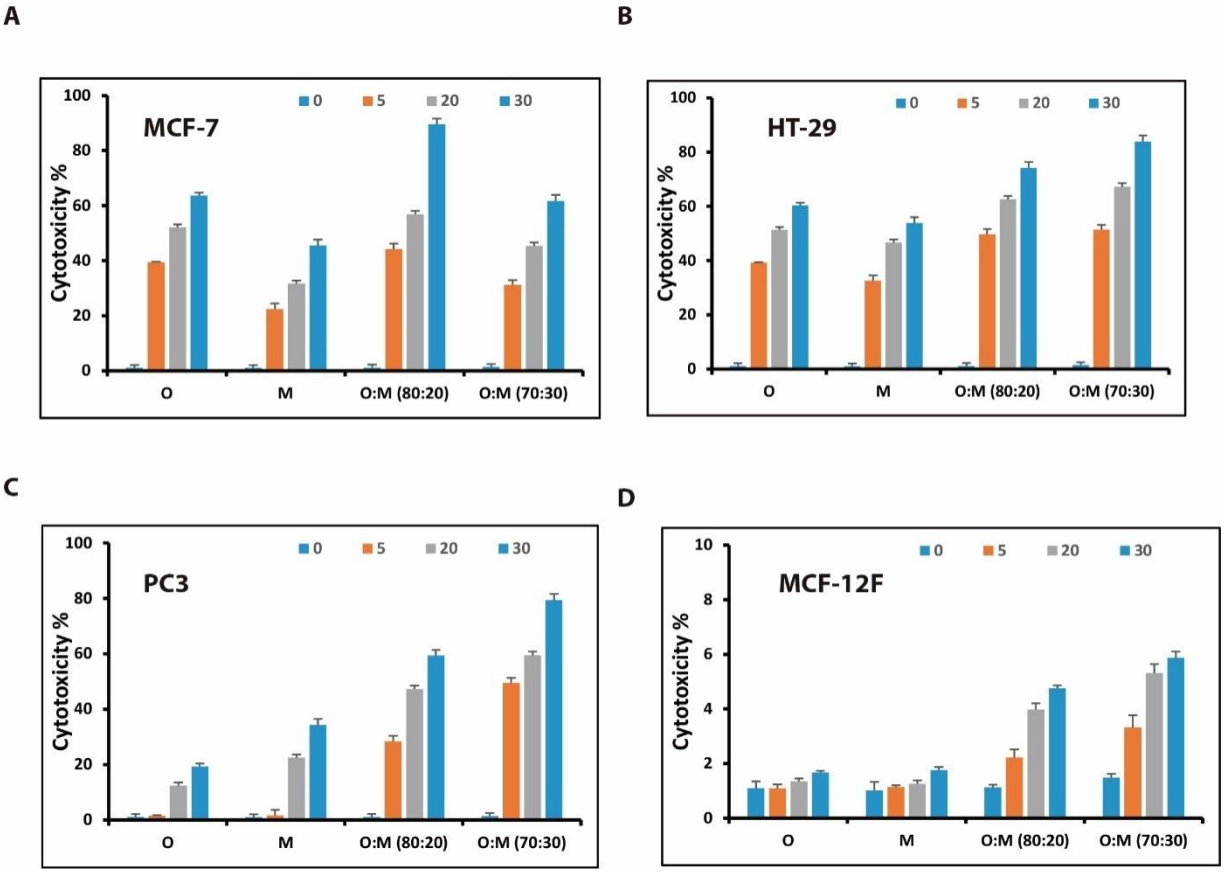

Figure 2. Cytotoxic activity Human breast MCF-7 (A), colon HT-29 (B) and prostate PC3 cancer cell lines (C) as well as one non-cancerous normal breast MCF-12F cell lines (D). Cells were incubated for 48 h at different concentration 5,20,30 µg/ml.

Effects of okara-supplemented milk on the oxidative stress enzymes.

In this experiment,the cell lysatecellular antioxidant and detoxification enzymeswere determined. The results exhibited an elevation in the enzymeactivity of SOD, CATGPxand GST in addition toGSH levels were significantly dose-dependent in milk supplementation with okara[Table 3]. The highest enzymes activity was detected in milk supplemented with 30 % of Okara compared to controls. In agreement with the antioxidants and anticancer obtained results the oxidative stress enzyme was enhanced in MCF-12F normal cell line in response to higher supplementation ratio at MO30 (30%). It wasconcluded that milk supplementation with okara has a potential enhancement of anti-inflammatory, anti-tumor, and antioxidant properties of milk.

Table 3: Effect of okara supplementation of milk on cellular antioxidants.

Sample	SOD U/10^6 cells	CAT U/10^6 cells	GSH U/10^6 cells	GPX U/10^6 cells	GST U/10^6 cells
M	1.78 ± 0.31^{c}	34.58 ± 2.12^{e}	45.34 ± 2.00^{c}	30.24 ± 2.90^{bc}	15.03 ± 1.35^{e}
O	2.62 ± 0.28^{c}	21.12 ± 3.84^{c}	25.21 ± 2.01^{c}	12.53 ± 2.17^{b}	8.51 ± 2.44^{e}
MO20	3.22 ± 0.21^{a}	63.04 ± 3.89^{ab}	45.22 ± 2.14^{c}	33.12 ± 2.21^{a}	24.31 ± 1.34^{c}
OM30	4.39 ± 0.29^{a}	84.06 ± 4.54^{a}	57.13 ± 2.65^{a}	47.21 ± 2.11^{a}	32.13 ± 2.14^{a}

Mean values highlighted with letters are significantly different ($P < 0.05$, values are mean ±SD).

Anti-inflammatory

The gene expression of various inflammatory markers that are involved in pro- and/or anti-inflammatory proteins was detected at the transcriptional and expression levels. Results obtained with RTq-PCR-based profiling of expressions of mRNAs of pro- and anti-inflammatory related genes showed that Milk supplemented with

okara induces down-regulation of the pro-inflammatory genes,TNFα, IFNγ, and Cox2, while the anti-inflammatory genesIL-6 andIL-10were upregulated (Figure 3). In agreement with our previously obtained data, okara supplementation to milk enhances the effects of anti-inflammatory.

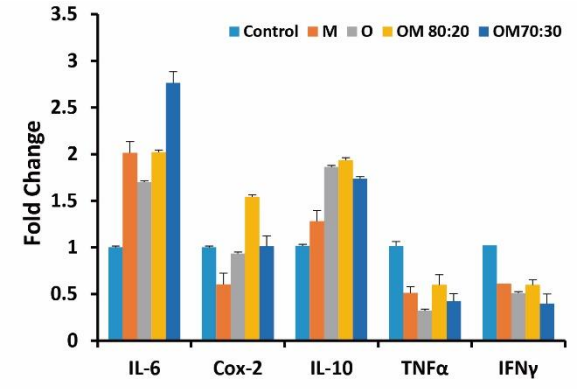

Figure 3. Gene expression profiling of inflammation markers of MCF-12F treated with 50 µg/ml of Milk (M), Okara (O), M:O 80:20 and O:M 70:30.

4 Conclusions

It was concluded that fortification of the dairy products by 30% Okara caused highly functional and health-promoting products as anti-inflammatory, anti-tumor, and antioxidant activities. It was concluded that okara can be used as a functional food ingredient, with high-quality nutritional properties, phytochemicals supplementation, and prebiotics potential. The effect of Okara-supplemention to dairy products (MO) by 20% (MO20) and 30%(MO30) on cytotoxicity and cell viability against four human cell lines were selected Human breast MCF-7, colon HT-29 and prostate PC3 cancer cell linesas well as one non-cancerous normal breast MCF-12F cell lines were studied. It was concluded that fortification of the dairy products by 30 % Okara caused highly functional and health-promoting products to have anti-inflammatory, anti-tumor,and antioxidant activities.

5. Conflicts of interest

The authors declare that there is no conflict of interests.

6. Formatting of funding sources

The authors declare that there is no funding support.

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