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Milk Supplemented with *Okara* Soymilk Improves the Physical and Biological Properties of Processed Milk

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Abstract

Agro-waste industries are focused on the transformation and processing of raw materials derived from agricultural sources, including plant or animal, and contributed to the generation of large amounts of organic residues. *Okara* is a major by-product of soymilk and tofu production, a large quantity of *okara* produced annually pose a significant environmental problem. Herein, we demonstrated yogurt supplemented with okara at 20 and 30% to enhance milk properties biological benefits as an alternative source. The chemical composition indicated that *okara* contains high contents of crude fiber composed of cellulose, hemicellulose, and lignin, about 25% protein, 10-15% oil, but little starch or simple carbohydrates. As well as 20% of Okara yogurt supplementation increases the potential antioxidants activity determined by DPPH and ABTS assays. In addition, the fermented yogurt/okara displayed anticancer activity against HCT-116 and HepG-2 cell lines and no significant cytotoxic effect was detected against BJ-1 fibroblast normal cells. Overall obtained data indicated that *okara* can act as a suitable replacement for digestible milk to reduce calorie intake. Dietary fiber in okara can be added in milk as functional composition to promote digestion of protein, lipid, and other nutrients.

Keywords: Okara, soybean, soymilk, yogurt, antioxidants, anticancer

1. Introduction

The conversion and processing of agricultural raw materialsincludingplants or animalsis the main focus of the agro-industries, which also produce a significant number of organic leftovers. These components, which can be either liquid or solid, are not utilized in the production process. They pose a significant issue since they are reportedly thrown into the environment without any practical purpose, which can contaminate soil, surface water, and ground water if not handled appropriately [1].One important byproduct of the manufacture of tofu and soymilk is okara [2]. The annual production of large amounts of okara presents a major disposal challenge. It is largely made up of cellulose, hemicellulose, and lignin, with just a small amount of starch or simple carbohydrates, roughly 25% protein, and 10% to 15% oil [3]. Okara, also known as draff, is the residue that remains from ground soybeans after the water extractable fraction of water needed to make soy milk and tofu is extracted. [4], *tofukasu*[5], or *soy pulp*, *douzha* (Chinese), *bejee* (Korean), and *tempegembus* [6]. About 1.1 kg of fresh okara is produced from every kilogram of soybeans processed for soy milk [7]. The chemical composition of okara indicated that contains 11% oil, 20% bean protein, and 30% of bean solids [8]. Large amounts of okara are produced; in 1986, the tofu producing sector in Japan produced over 700 000 tons of okara. [9]. It would be ideal if okara could be used as a human food. Accordingly, okara has higher-quality protein than other soy products; for example, okara's protein efficiency ratio

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was 2.71, but soymilks were only 2.11 [10]. Nevertheless, the proportion of total amino acids to essential amino acids was comparable to that of soy milk and tofu [11]. It seems that fermented okara has a very high antioxidant content [12], phenolic compounds, flavonoids, and radical scavenging activities. There could be clear benefits to using fermented okara in a diet. It can serve as a good substitute for food that is easily digested in prepared meals in order to cut down on caloric consumption [13]. Because it is a meal that has antioxidant action, much like vitamin E, it can lower blood cholesterol levels and lower the body's number of free radicals. It has been demonstrated that okara can be added to dairy products as an enrichment to prevent lipid oxidation, cut expenses, and create useful goods [10]
The highest concentration of phenolic compounds with antioxidant activity was found in milk that had okara added. These bioactive substances found in milk may have an anti-free radical effect on people. Thus, although okara is a residue, it may be regarded as an antioxidant ingredient and provides a low-cost and effective source of antioxidants for food items. Okara, which is thrown out as a residue, may one day be utilized as an antioxidant component to enhance food. [12]. As a nutritional supplement, okara can be added to biscuits and snacks to help prevent obesity, hyperlipidemia, and diabetes [6]. It raises dietary fiber and lowers calorie intake [14].
As a functional component, dietary fiber from okara can be added to food to aid in the digestion of protein, fat, and other nutrients. In addition, fermentation can be utilized to enhance the nutritional makeup of okara for celling and culturing [15].

2. Materials and Methods

2.1 Okara preparation and supplementation

Soybeans were obtained from soybean factory, Food Technology Research institute, Agriculture Research center, Ministry of Agriculture, Giza Egypt. The soybean seeds were cleaned and washed by floatation to remove all undesired materials, after cleaned beans were blanched in hot water at 100°C for 25 min and soaked in water 1 kg/5L. Soybeans were washed with hot water at 100°C, the obtained solution was filtered through a muslin cloth to remove the milk and recover the residue called okara.

Yogurt supplementation

During yogurt preparation milk was mixed with Okara obtained from the previous step and followed by yogurt preparation and the final product was used directly into further analysis.

2.2 Sensory evaluation

Random volunteers were selected for different parameters panel test including, appearance, flavor, texture, and overall acceptability.

2.3 Chemical composition of okara

2.3.1 Moisture content

An appropriate amount of okara were accurately weighted and dried in an oven at 105°C until a constant weight was reached according to A.O.A.C [16].

2.3.2 Crude protein

The total nitrogen was determined by using kjeldahal method A.O.A.C [16]. The crude protein was then calculated by multiplying the total nitrogen by factor of 6.25.

2.3.3 Crude fibers

A known weight of the dried samples (ca. 0.2g) was digested with sulfuric acid (200 ml, 1.25%), then with sodium hydroxide solution (200 ml, 1.25%), then shed several times with diethyl ether.
The residue was dried at 110 °C then ignited at 550°C in muffle furnace until a constant weight was reached as described by A.O.A.C. [16].

2.3.4 Ash contents

Ash process was carried by ignition in muffle furnace at 550c until a constant weight according to A.O.A.C [16].

2.3.5 Lipid content

Lipid content was determined by extraction with hexane using a soxhelt apparatus A.O.A.C [16].

2.3.6 Total carbohydrates

Total carbohydrate was calculated by difference as follow:
Total carbohydrate = (dry weight 100g) - (protein + lipid + Fibers + ash) according to t according to A.O.A.C [16].

2.3.7 Determination of minerals content

Minerals contents for okara were determined according to A.O.A.C [16] USING Atomic Absorption spectrophotometer (perkin-Model 3300, USA).

2.3.8 Determination of water-soluble vitamin

Vitamin C content of okara was determined using HPLC according to described by Romeu-Nadal et al., (2006). Vitamin B complex (B1, B2, B3, B6, and B12) content of okara was determined using HPLC

according to the method described by Batifoulieret al., (2005)

2.3.9 Determination of fat-soluble vitamins

Vitamins A, D, E and K were determination in purslane leaves using HPLC according to the methods described by Noll (1996), plozzaet al., (2012), pyka and sliwiok (2001) and preez –ruiz et al., (2007), respectively.

2.3.10 Determination of fatty acid

Fatty acid composition was analyzed by gas chromatography GC according to the method described by Cossignani et al., (2005) Fatty acid methyl esters were fractionated using Agilent 6890 series GC apparatus provide with a DB -23 column (60 m×0.32 mm×0.25Mm) was used. Oven temperatures were 150 C ramped to 195 C at 5C/min, ramped to 220 C/min and flow rate was 1.5 ml/min.

2.3.11 Determination of amino acids

Amino acid composition of okara was analyzed using automatic amino acid analyzer (AAA 400 INGOS Ltd, Czeeh Republic). The samples (100 mg) were hydrolyzed with 10 ml of 6 N HCl in a sealed tube at 110c in an oven for 24 h. After hydrolysis, the acid was evaporated in vacuum evaporator under reduced pressure at 80 °C. The HCl free residue was dissolved in 2 ml of loading buffer (0.2M, pH 2.2) to inject into the apparatus [17].

2.4 Determination of total phenolic and total flavonoid contents

The concentrations of total polyphenols content (TPC) and total flavonoids content (TFC) of the investigated extracts were determined using Folin-Ciocalteu and aluminum trichloride ($AlCl_3$) methods of [18, 19], respectively. Measurements were carried out using UV-Vis spectrophotometer (Jenway 6800, UK) at 750 nm for TPC and 510 nm for TFC. Gallic acid (GA) and quercetin (QE) were used as a standard for TPC and TFC measurements, respectively. Quantification was calculated according to standard curve of gallic acid (10-100 mg/L) and a standard curve of quercetin (10-100 mg/L), respectively.

2.5 HPLC analysis of phenolic and flavonoid compounds

The polyphenol and flavonoid components of the extracts were analyzed using Agilent 1260 infinity HPLC Series (Agilent, USA), equipped with Quaternary pump, the column used was: a KineteX®5µm EVO C_{18} 100 mm × 4.6 mm, (Phenomenex, USA), operated at 30 ^{o}C. The separation was achieved using a ternary linear elution gradient with HPLC grade water 0.2% H_3PO_4 (v/v) (A), methanol (B) and acetonitrile (C). VWD detector was set at 284 nm. The samples volume injected was 20 µL and the flow rate used was 0.7 mL/min. Identification of the phenolic compounds was carried out by comparing the retention times (RT) and UV spectra with those of standards stored in a database. Quantification was performed using an external standard method with reference samples of polyphenols.

2.6 Antioxidants activity

2.6.1 DPPH assay

The radical scavenging potential was evaluated using DPPH assay as previously described by [20] with some slight modification. In this assay, 192 µl of 50 mM DPPH was vortexed with 8 µl of diluted sample in 96 well plate. The mixture was incubated under dark condition at room temperature for 15 min. Butylated hydroxy toluene BHT was used as slanderedantioxidants against 8 µl distilled water as negative control. The absorbance was measured at 517 nm using a spectrophotometer. The potential radical scavenging activity was calculated according to the following equation inhibition (%) = $((A_{control}-A_{sample})/A_{control}) \times 100$ where $A_{control}$is the absorbance of control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound.

2.6.2 ABTS assay

ABTS assay was used according to previously described by [21]. In brief, equal volume of freshly prepared ABTS 7 mM and potassium persulphate 2.45 mM were mixedunder dark at 4^{o}C for 24 h before use. The absorbance of ABTS was adjusted with distilled water to the appropriate absorbance (0.7±0.03) at 734 nm. The ABTS reagent 950 µl was mixed with 50 µl of each sample and incubated for 15 min in dark at room temperature. The absorbance was measured at 734 nm. Each sample was measured in triplicate, and the scavenging% was calculated using the following: inhibition (%) =$((A_{control}-A_{sample})/A_{control}) \times 100$ where $A_{control}$is the absorbance of control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound.

2.7 Cell lines and culturing conditions

The combinations and controls were tested for their anticancer activity against two cancerous cell lines, including colorectal adenocarcinoma (HCT-116, ATCC® CCL-247™) and hepatocellular carcinoma (HepG2, ATCC® HB-8065™), and one noncancerous noncancerous skin fibroblast BJ-1 (ATCC® CRL-2522™) was tested for cytotoxicity. Cell lines were

subcultured in DMEM/high glucose supplemented with 2 mM L-glutamine, 10% FBS and 1% penicillin/streptomycin kept in Corning® 75cm² U-Shaped canted neck cell culture flask with vent cap (Corning, New York, USA). Then, sub-confluent cultures (70–80%) were trypsinized (Trypsin 0.05%/0.53 mM EDTA) and spilt depending on the seeding ratio [22-24]. The cell viability was determined using MTT assayin cells treated with yogurt and yogurt supplemented with okara according to our previous reports [25-26]. Cells were seeded into 96-well microtiter plates with 3×10^4 cells per well in a complete media and incubated at 37°C under a humidified atmosphere of 5% CO_2. After 24 h the medium was replaced by serum free medium (SFM) containing 5, 10, 50 and 100µg/ml of each sample. After incubation at 37°C for another 24 h, 100 µL/well of MTT (0.5 mg/mL PBS) were added and incubated at 37°C for 3 h. The formed purple formazan crystals were dissolved in 100 µLSDS 10%/well with shaking for 1 h at ambient temperature. The absorbance at 549 nm was read on a microplate reader (ELX 800; Bio-Tek Instruments, Winooski, VT, USA). The concentrations causing 50% cell growth inhibition (IC_{50}) were calculated.

3. Results and Discussion

3.1 Sensory evaluation

Sensory evaluation, including appearance, flavor, texture, and acceptability of yoghurt and yogurt supplemented with Okara showed in Table 1.The yogurt samples' sensory scores ranged from 1 (poor) to 9 (excellent). The significantly higher ($p<0.05$) compared to sensory scores for appearance, flavor, texture, and acceptability of control.

Table 1: The average scores for sensory evaluation of plain and fortified Y, O, and Y: O

Sample	A	F	T	AC
Y	8.1 ± 0.3^{a}	7.4 ± 0.1^{a}	7.1±0.2^{a}	6.6±0.1^{a}
O	3.2± 0.2^{b}	3.43± 0.3^{b}	4.5±0.4^{c}	5.2±0.4^{b}
Y:O (80:20)	6.5± 0.1^{c}	6.9± 0.3^{b}	6.4±0.3^{ab}	6.4±0.4^{b}
Y:O (70:30)	6.3±0.2^{d}	5.6±0.2^{d}	5.76±0.1^{ab}	5.1±0.2^{d}

A: Appearance, F: Flavor, T: Texture and AC: Acceptability

3.2 Chemical composition

3.2.1 Proximate analysis of okara and yogurt supplemented with okara

After yogurt supplementation with okara a slight reduction in the protein contents, ash and carbohydrates were observed as shown in Table 2. However, the fiber contents were increased from 0.0% to 0.7 and 0.98% due to supplementation with 20 and 30% of okara, respectively. As well as a slight increase in the moisture contents were observed Table 2. These results are nearly similar to those of [28] who found moisture was 6.51% in okara. The protein content was found to be 33.64%. This result is slightly lower than that of [29] who found okara had 40.0% protein and was nearly similar to that have been found by [30-34] who found protein was 33.4%.

Table 2: Proximate analysis of yogurt supplemented with okara

Parameters	Okara (O)	Yogurt (Y)	Y: O (80:20)	Y: O (70:30)
Moisture	81.00 ± 2.31	82.52 ± 4.00	82.97 ± 2.00	82.56 ± 1.99
Protein	6.50 ± 0.21	4.00 ± 0.98	4.50 ± 1.76	4.70 ± 1.79
Fat	3.89 ± 0.11	6.98 ± 2.00	6.10 ± 1.90	6.00 ± 1.89
Ash	0.88 ± 0.02	0.90 ± 0.017	0.87 ± 0.026	0.90 ± 0.027
Fiber	3.12 ± 0.07	0.00	0.70 ± 0.011	0.98 ± 0.017
Carbohydrates	4.61 ± 0.13	5.20 ±0.105	4.87 ± 0.111	4.96± 0.201

Y: O: Yogurt: Okara

3.2.2 Mineral contents

Due to yogurt supplementation, there is a slight reduction in the element contents of Na and K from 623 to 460 and 411 ppm in case of Na and from 1801 to 1589 and 1680 ppm in case of K after 20 and 30% of okara, respectively. Interestingly, P, Mg, Fe and Zn were increased. In this context, no significant differences were observed in the Ca contents Table 3.

Table 3: Mineral contents in yogurt supplemented with okara

Mineral	Okara (O)	Yogurt (Y)	Y: O (80:20)	Y: O (70:30)
Na	230±11	623±30	411±21	460±20
K	240±13	1801± 51	1680±39	1589±40
Ca	1400±36	1681± 43	1600±41	1700±42
P	8501±48	1003± 31	3085±35	2397±44
Mg	450±7.11	162± 3.74	263±2.37	220±2.88
Fe	22.61±0.17	2.04± 0.051	8.08±0.118	6.11±0.085
Zn	22.11±1.10	2.02± 0.087	7.89±0.19	5.81±0.15
CU	0.09±0.002	0.194± 0.004	0.097± 0.002	0.087± 0.002

Y: O: Yogurt: Okara

3.2.3 Fatty acid profiling

The fatty acid contents of okara are shown in Table 4. The results showed that linoleic acid represents the major content (52.00%), followed by oleic acid (21.51%) and linolenic acid (6. 01%). The results indicate that okara has high concentration of essential fatty acids.Okara contain palmitic acid (10.99%),stearic acid (5.00%),oleic acid (22.38%), vaccenic acid 1.15%, Arachidic acid 0.60%, Gondolic acid 0.48%, behenic acid 0.40%

Table 4: Fatty acid content at Okara, yogurt and supplementation

Fatty acids	Okara (O)	Yogurt (Y)	Y: O (80:20)	Y: O (70:30)
Palmitic acid C16:0	10.99%	31.85%	31.08%	30.41%
Stearic acid C 18:0	5.00%	14.55%	12.43%	12.33 %
Oleic acid C 18:1	21.51%	28.96%	23.84%	23.44%
Vaccenic acid C 18:1	1.15%	3.00%	2.78%	2.74 %
Linoleic acid C 18:2	52.00%	5.44%	6.14%	9.21%
Linolenic acid C 18:3	6.01%	0.73%	0.74%	1.02 %
Arachidic acid C 20:0	0.60%	0.19%	0.27%	0.28%
Gadolic acid C 20:1	0.50%	0.20%	0.15%	0.18%
Behenic acid C 22 :0	0.40%	0.00%	0.11%	0.15%

3.2.4 Vitamins of okara

The vitamins contents showed that okara have low vitamin contents Table 5. However, the supplementation of yogurt with okara showed a slight reduction in the vitamin contents. In general, vitamin data represented in Table 5 showed that, nicotinic (0.101), folic acid (0.029), B6 (55.001), riboflavin (0.020), thiamin (36.600), and B12 (0.677).

Table 5: Vitamins content of okara, yogurt and supplementation

Fatty acids	Okara (O)	Yogurt (Y)	Y: O (80:20)	Y: O (70:30)
Vit C	0.080±0.002	30.00±1.101	21.11±0.888	21.00±1.212
Vit A	3.00±0.099	201±10.00	150±3.111	170±2.989
Vit D	0.15±0.002	161±3.111	99±2.00	120±2.303
Vit E	0.068±0.002	0.079±0.001	0.050±0.001	0.048±0.002
Vit K	0.108±0.004	0.090±0.003	0.091±0.002	0.071±0.001
Folic acid	0.029±0.002	0.060±0.002	0.058±0.002	0.050±0.002
Vit B_1	36.600±2.00	0.609±0.033	12.010±0.500	15.99±0.766
Vit B_2	0.020±0.001	1.851±0.069	2.799±0.119	2.800±0.118
vit B_6	55.001±2.001	60.00±1.897	50.111±1.80	49.89±1.599
Nicotine acid	0.101±0.003	1.287±0.029	1.000±0.025	1.140±0.029
Vit B_{12}	0.677±0.014	0.398±0.015	0.463±0.029	0.439±0.031

Vitamins content (ppm) mean ±SD (triplicate)

3.3 Phenolics and flavonoids profile

Phenolic and flavonoids contents were fractionated using HPLC analysis Table 6. The most abundant phenolic and flavonoids compounds present in okara were E-vanilic and Hesperidin with a content value of

760.00 and 3.81 µg/g. The phenolic and flavonoid contents in yogurt were extremely low. However, yogurt supplementation with okara showed acute reduction in the PC and FC.

Table 6: Phenolic and flavonoid compounds of okara, yogurt, yogurt supplementation

Phenolics					Flavonoids				
Compounds	**O**	**Y**	**Y + 20% O**	**Y+ 30% O**	**Compounds**	**Okara**	**Yogurt**	**Y + 20% O**	**Y+ 30% O**
Pyrogallol	120	0.26	28.21	34.89	Naringin	0.03	0.01	0.004	0.010
Protocatechuic	75.03	0.10	15.02	22.51	Hespirdin	3.81	0.02	0.75	1.13
Catechin	45.00	0.72	8.74	13.00	Rutin	0.35	0.02	0.06	0.12
Oleuropen	320	0.16	63.39	95.50	Rosmarinic	0.05	0.17	0.02	0.019
Vanillic	14.00	0.07	2.91	4.30	Quercetrin	0.30	0.04	0.05	0.088
p-coumaric	48.00	0.03	9.40	14.02	Quercetine	0.27	0.06	0.05	0.081
Iso – Ferulic	23.00	0.01	4.42	6.81	Hespirtin	1.31	0.03	0.25	0.40
E –vanilic	760.00	0.26	151.22	228.00	Kaempferol	0.45	0.02	0.009	0.15
Ellagic	129.00	0.14	27.00	41.12	Apigenin	0.17	0.04	0.028	0.04
Coumarin	116.00	0.01	23.32	34.71					
Coumaric acid	120	0.02	0.60	0.92					

3.4 Amino Acids

Amino acids fractionation indicated that Glutamic acid was the most abundant in okara with 1.609 mg/100mg amino acids. In general, the amino acids contents in yogurt supplemented with okara were severely increased in response to higher concentrations Table 7.

Table 7: Amino acids of okara and, yogurt and yogurt supplemented with 20 and 30%

Amino acid	Okara	Yogurt	Y + 20% O	Y + 30% O
Asp	1.00	0.365	0.958	0.988
Thr	0.413	0.246	0.544	0.600
Ser	0.440	0.228	0.547	0.580
Glu	1.609	0.983	2.01	2.99
Gly	0.330	0.082	0.274	0.290
Ala	0.271	0.137	0.386	0.406
Val	0.480	0.298	0.612	0.660
Ile	0.370	0.246	0.553	0.589
Leu	0.700	0.449	0.931	0.980
Tyr	0.529	0.257	0.465	0.480
Phe	0.571	0.252	0.508	0.580
His	0.241	0.123	0.264	0.270
Lys	0.502	0.368	0.893	0.901
Arg	0.550	0.133	0.415	0.450
Pro	0.478	0.466	1.03	1.50

*Amino acid represented as mg/100mg total amino acids

Antioxidants activity

The antioxidants activity was determined using DPPH and ABTS assays. In DPPH assay no significant differences were observed Figure 1A. However, the results of ABTS radical scavenger shower that Okara displayed high antioxidants activity Figure 1B. In addition, the antioxidants scavenging activity were increased after supplementation compared to okara and yogurt controls.

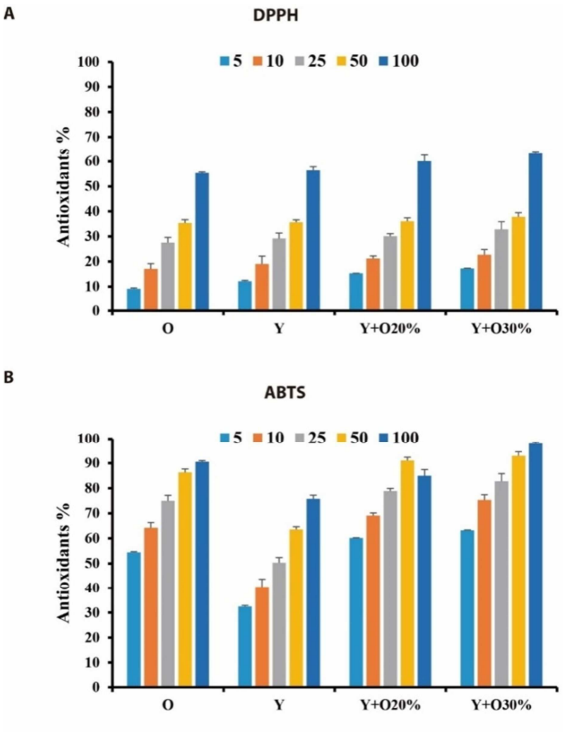

Figure 1: Antioxidants activity using DPPH (A) and ABTS (B) assays

Cytotoxicity and Anticancer

Yogurt supplemented with okara enhances the anticancer activity against two cell lines HepG-2 and HCT-116 Figure 2A and B . The anticancer activity displayed high anti-proliferation against HepG-2 followed by HCT-116. Yogurt supplementation with 30% was higher 20% of okara indicating that okara contains specific chemical constituents that increase the anticancer activity. However no cytotoxic activity against Bj-1 fibroblast normal cell line was observed Figure 2C.

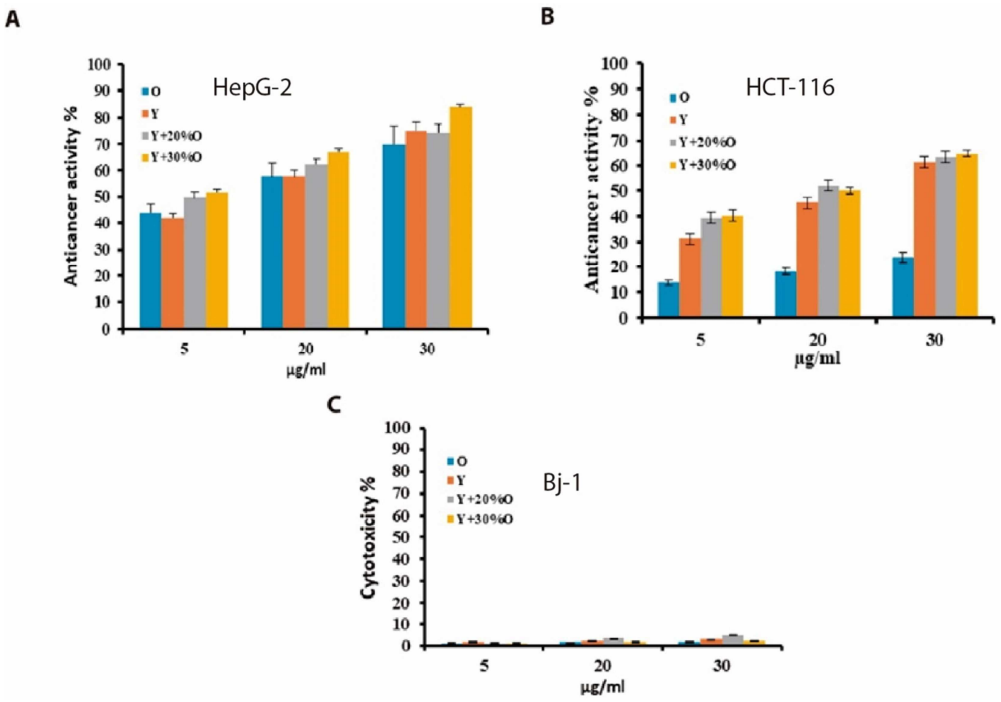

Figure 2: Cytotoxicity and anticancer activity using against HepG-2 (A), HCT-116 (B) and Bj-1 (C)

4. Conclusions

Agro-waste has been involved in multi fields including biofuel, pharmaceutics, and food processing. In this work, we investigate yogurt supplementation with okara milk extracted from soybean seeds. The overall obtained data showed an enhancement in the physical and biological properties of yogurt supplemented with okara. The results indicated that okara enhances the biological properties of yogurt in the antioxidants and anticancer activity. The antioxidants activity was elevated due to increases in the total phenolics and total flavonoids contents. The proximate analysis indicated a high level of free amino acids and fatty acids, in addition to several phenolic compounds detected by HPLC. However, the yogurt supplemented with Okara induces anticancer activity against HepG-2 and HCT-116 cell lines.

5. Conflicts of interest

The authors declare that there is no conflict of interests.

6. Formatting of funding sources

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