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Effect of Soaking, Cooking and Germination on Chemical Constituents and Bioactive Compounds as well as their Cytotoxic Activities of Black Bean Extracts

Eshraq BK[1]*, Mona AM[1], Sayed AF[2] and Emam AA[2]

[1]Crops Technology Department, Food Technology Research Institute, Agricultural Research Center, 12613 Giza, Egypt
[2]Biochemistry Department, Faculty of Agriculture, Cairo University, 12613 Giza, Egypt

Abstract

Legumes are the basic diet in many populations, especially in Africa. They hold high nutritional value and they promote the human health. The aim of this investigation was to study the influence of different processing such as soaking, cooking and germination on chemical composition, phenols, tannins and flavonoids contents, as well as effects on their antioxidant and anticancer activity of black beans. Phenolic compounds of raw and processed black beans was evaluated by HPLC. The antioxidant activity was evaluated by DPPH. Anticancer activities were evaluated on five different cell lines (colon (HCT), breast (MCF7), lung (A549), prostate (PC3) and Hela (HELA). The obtained results indicated that black beans showed high protein, ash and fiber content 26.54, 5.22 and 5.58%, respectively. Total phenols, tannins and isoflavonoids decreased as prolonging soaking time. The reduction percent reached high level in cooked beans. High DPPH antioxidant activity for raw black beans was observed. After 24 h of germination, e-vanellic acid showed the high value 56.4 mg/100 g followed by ferulic acid 45.38 mg/100 g. In soaking plus cooking treatments the level of kaempferol and naringin increased. There were differences in total phenols, flavonoids and tannins content have been observed between raw and processed black beans samples that influence the antioxidant activity. Although antioxidant activity was decreased in the processed samples. Ethanol extracts of different processing exhibited cytotoxicity activities on cancer cell lines, raw sample proved to be the most active in anti-tumoral followed by germinated sample (48 h). This study demonstrated that phenolic compounds of black beans is related with soaking, cooking process and germination, and also with their anticancer activity. Strong anticancer activity toward (MCF7) cell line was observed. Anticancer activity realized a noticeable reduction of tumor inhibition after 48 h of germination.

Keywords: Legumes; Soaking; Germination; Cooking; Antioxidant; Cytotoxic; Cell line

Introduction

Legumes commonly used in various food preparations and formulations for both adults and children as weaning/complimentary foods in Egyptian diet. They considered as good sources of nutritionally valuable minerals and natural dietary antioxidants and considered as potent scavengers/mopping agents of free radicals thereby which can be utilize to alleviating symptoms associated with chronic diseases.

The consumption of meat caused increases the risk of cardiovascular diseases and some types of cancer. Legumes contain a number of bioactive substances including enzyme inhibitors, phytates, lectins, phenolic compounds and oligosaccharides, which play metabolic roles in humans that frequently, consume these foods. These compounds may have protective effects against cancer [1,2].

Polyphenolic compounds of legumes consist of tannins, phenolic acids and flavonoids. The legumes with the highest polyphenolic compounds content are the dark, highly pigmented varieties, such as red kidney beans and black beans. Legumes vary based on their total phenolic contents and antioxidant activity. Legumes with the highest total phenolic content (lentil, red kidney and black beans) extract had the highest antioxidant capacity. However, beans contain compounds that can negatively affect their nutritional value, such as polyphenols (especially tannins in beans) also oligosaccharides and phytase. Some of these compounds are thermolabile, disappearing after cooking and the others thermostable, but their concentrations are reduced by dissolution in water [3].

However, Ramirez-Ca'rdenas et al. [4] pointed out some studies that state that low concentrations of phenolic compounds have protective effect against cancer and. Cardiovascular diseases The positive or negative effects of these compounds seem to be more closely associated with their concentration in the beans, which varies according to type of bean, as well as their interaction with other diet's components [4,5].

The over production of free radicals may lead to oxidative stress, an imbalance between the antioxidant mechanisms and the production of free radicals is observed. Oxidative stress has been showed to be associated with a variety of diseases and pathological condition, such as cancer, diabetes, obesity and autoimmune diseases [6,7].

This work aimed to study the effect of cooking and germination processing methods, on total phenols, tannins, flavonoids content as well as antioxidant and anticancer activity.

Materials and Methods

Materials

Bean: The present research work was carried out to study black bean. N (*Phaseolus vulgaris*) which purchased from the local market.

Chemicals: 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and Folin-Ciocalteu reagent were obtained from LOBA chemie, India and bile extract from Win Lab Laboratory chemicals reagents, Mumbai, India. All other chemicals used were of analytical reagent grade.

***Corresponding author:** Eshraq BK, Crops Technology Department, Food Technology Research Institute, Agricultural Research Center, Cairo University, 12613 Giza, Egypt, Tel: +9647722232228; E-mail: eshraqbadawi@yahoo.com

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Methods

Preparation of raw materials

Raw black bean: Raw bean seeds were milled in a laboratory mill (IKA-Laboratechnic, Janke and Kunkel Type: MFC, Germany) to obtain a whole meal flour and kept at -20°C until analysis.

Soaking of black bean: Bean seeds were soaked in distilled water for 2 h, 6 h and 12 h with a ratio 1:5 w/v and the soaked water changed twice. At the end of soaking time, the soaked water was discarded. The seeds were rinsed twice in distilled water then dried at 45 ± 5 °C overnight in drying oven. The dried soaked seeds were milled in a laboratory mill to obtain whole meal flour and kept at

-20 °C until analysis. Different treatments were carried out on the soaked seeds.

Cooking of soaked black bean: Soaked seeds were cooked by boiling in sufficient amounts of distilled water for 30 min, then submerged in distilled water, and finally dried, milled and kept at -20 °C until analysis.

***Germination of soaked black bean*:** Soaked seeds were germinated, placed in plastic boxes, covered with cotton cloth and left at room temperature (25-27 °C) for 24 h, 48 h and 72 h. After that, germinated grains were sprayed with distilled water three times intervals then dried. The shoot and root portions were manually removed. The seeds were milled and kept at -20 °C until analysis.

Chemical and biochemical analyses

Proximate analyses: Moisture, protein, fats, crude fiber and ash contents of the raw black bean and processed were determined according to the methods of A.O.A.C. [8]. Total carbohydrate was calculated by difference. The estimated parameters were related to the untreated beans (control).

Determination of antinutritional factors

Determination of phenols

Determination of total phenol: Total phenol was determined as described by Singleton and Rossi [9]. One gram sample was mixed with 10 mL 80% methanol in a dark bottle and shaking for 2 h. Then the mixture was filtrated. The color was developed by Folin-Ciocalteu reagent and sodium carbonate. 0.250 mL was mixed with 0.250 mL Folin–Ciocalteau reagent, 0.50 mL of 10% sodium carbonate (Na_2CO_3) and the volume was completed to 5 mL with distilled water. After incubation in dark at room temperature for 30 min, the absorbance of the reaction mixture was measured at 725 nm against blank on a spectrophotometer (UV-Vis spectrophotometer, Labomed Inc., USA). Gallic acid was chosen as a standard to prepare the standard curve. Phenols were expressed as mg/100 g sample on dry weight basis.

Determination of total flavonoid: Total flavonoid was determined according to the method of Zhishen et al. [10]. Sample (1 g) was mixed with 10 mL 80% methanol in a dark bottle and shaking for 2h. Total flavonoids extract (0.4 mL) were added to 4 mL H_2O. Then 0.3 mL 5% $NaNO_2$ was added. After 5 min 0.3 mL 10% $AlCl_3$ was added. After 6 min 2 mL of 1M NaOH were added and the total volume was made up to 10 mL with distilled water. The pink color was measured at 510 nm against a blank reagent on a spectrophotometer (UV-Vis spectrophotometer, Labomed Inc., USA). Catechin served as standard compound was used for preparing the calibration curve. Total flavonoid was calculated as mg/100 g on dry weight basis.

Determination of tannins: Tannins were determined as described by Price et al. [11]. One gram sample was mixed with 10 mL 1% methanol/HCl solution in a in a dark bottle and shaking for 20 min at room temperature. Then the mixture was filtrated. The tannins in the supernatant were estimated by using 1 mL of supernatant and 5 mL vanillin/HCl mixture (mixing equal volumes of 2%vanillin in methanol and 8% methanol/HCl) in a test tube and kept for 20 min at room temperature. The formed color was determined at 500 nm by using spectrophotometer (Uv-Vis spectrophotometer, Labomed Inc., USA). Catechin was used to prepare the standard curve. Tannins were calculated as mg/100 g on dry weight basis.

Identification of phenolic acid and flavonoid compounds

HPLC analysis of phenolic compounds: Phenolic compounds were detected by HPLC according to the method of Goupy et al. [12] as follows: the aqueous extracts were centrifuged at 10000 rpm (in ICE Micro-MB Centrifuge/ NARP 64606 instrument) for 10 min and the supernatant was filtrated through a 0.2 µm Millipore membrane filter, then 1-3 mL were collected in a vial for injection into HPLC Agilent (Series 1200) equipped with auto sampler injector, solvent degasser, ultraviolet (UV) detector set at 280 nm and quaternary HP pump (Series 1100). The column [Agilent 5HC-C18 (2) 250 × 4.6 mm] temperature was maintained at 35 °C. Gradient separation was carried out with methanol and acetonitrile as a mobile phase at flow rate of 1 mL/min. Phenolic acid standards from sigma Co. were dissolved in a mobile phase and injected into HPLC. Retention time and peak area of the tested samples were calibrated against standard solutions of different phenolic compounds concentration by the data analysis of HEWLLET Packed (HP) software.

HPLC analysis of flavonoid compounds: Flavonoid fractions were also identified by HPLC according to the method of Mattila et al. [13] as follows: the aqueous extracts were centrifuged at 10000 rpm (in ICE Micro-MB Centrifuge/ NARP 64606 instrument) for 10 min and the supernatant was filtrated through a 0.2 µm Millipore membrane filter, then 1-3 mL were collected in a vial for injection into the previous HPLC Agilent (Series 1200) and HP software were used. The ultraviolet (UV) detector was set at 330 nm and the other conditions were set as that previously used in the fractionation of phenolic compounds.

Determination of antioxidant activity

Radical scavenging ability using DPPH method: The antioxidant activity of plant methanol extracts was determined based on the radical scavenging ability in reacting with a stable DPPH free radical according to Williams et al. [14]. One gram sample was extracted with 10 mL 80% methanol for 2h as described above. Briefly, 2.4 mg of DPPH in 100 mL methanol were prepared and 3.9 mL of this solution were added to 0.1 mL of methanolic extract. The mixture was shaken vigorously and allowed to stand in the dark for 30 min at room temperature. Then the absorbance was measured at 515 nm by using spectrophotometer (Uv-Vis spectrophotometer, Labomed Inc., USA). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Methanol was used as blank. The corresponding blank readings were taken and the capability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH^{\cdot} \text{ scavenging activity } (\%)=[(A_0-A_1/A_0)] \times 100$$

Where: A_0=the absorbance of the control reaction (containing all reagents except the test compounds).

A_1=the absorbance in the presence of the tested extracts after 30 min.

Measurement of potential cytotoxicity by Sulfo-Rhodamin Blue (SRB) assay: Potential cytotoxicity of the compound(s) was tested using the method of Skehan et al. [15]. Cells were plated in 96-multiwell plate

(10^4 cells/well) for 24 hrs before treatment with the compound(s) to allow attachment of cell to the wall of the plate. Different concentrations of the compound under test (0, 1, 2.5, 5 and 10 µg/mL) were added to the cell monolayer triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) for 48 hrs at 37 °C and in atmosphere of 5% CO_2. After 48 hrs, cells were fixed, washed and stained with Sulfo-Rhodamine-B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris / EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified compound [15]. New colorimetric cytotoxicity assay for anti-cancer drug screening.

Statistical analysis: The collected data from three repetitions of any experiment were statistically analyzed in triplicate. Data were presented as mean values ± SD. Data were analyzed to one-way analysis of variance (ANOVA) and least significant difference (LSD) at $p<0.05$ followed by Duncan's new multiple range tests to assess differences between group's means [16].

Results and Discussion

Table 1 shows the major chemical constituents of black beans at different treatments. Protein content of raw beans was high 26.54%. This result is in agreement with Vargas-Torres et al. [17], Carmona-Garcı´a et al. [18]; and Osorio-Dı´az et al. [19] they reported that protein content of beans ranged from 21.2 to 23.4%. Content of protein in bean was reported by Berrios et al. [20] which ranged from 25.9% to 23.3%, as slightly lower 22.4%. Fernández- Quintela et al. [21] found that protein content in beans ranged from 18.9 to 24.2%. The crude protein was significantly decreased after different treatments compared with raw bean except for soaked beans for two hours and germinated beans for 24h. Nutrients loss may be attributed to the leaching of soluble nitrogen, minerals and other nutrients in desired solution as reported by Shaker et al. [22].

Fat content was 2.15% in raw beans, which significantly decreased after all processing treatments compared with raw beans. Granito et al. [23] discovered the similar amount of lipids in the investigated cultivars of bean, but Candela et al. [24] reported value of 3.50% in bean. The reduction may be due to the physiological changes during germination, which require energy to proceed, thus little part of seed oil was utilized to produce such energy. Regarding to ash and fiber content, data in the same table showed that raw black beans contained high ash and fiber 5.22% and 5.58%, respectively.

After different processing treatments, ash and fiber content were significantly decreased compared with raw beans. Mubarak [25] reported that germination and cooking processes caused significant decreases in ash content. Similar values were reported in bean by Granito et al. [23]. Slightly higher value (4.65%) was given by Berrios [20] and (4.87%) by Candela et al. [24]. However total carbohydrate content was significantly increased after different treatments relative to raw beans (66.10%).

The effect of soaking on total phenol, flavonoid and tannin content of black beans is given in Table 2. Total phenols showed a decrement level over soaking treatment. For instance, inhibition percent reached (1.88%, 29.69% and 32.10%) after 2, 6 and 12 h of soaking.

It cleared that total phenols was decreased as prolonging the soaking time. Same trend was observed in flavonoid content, since it reached 65.80 mg/100 g after 12 h of soaking compared with raw beans 89.82 mg/100 g. Also tannins content showed decrement by soaking treatments, it reached 174.20, 147.35 and 86.99 mg/100 g after 2, 6, 12 h of soaking.

On the basis of the above mentioned discussion, the decline of total phenols, flavonoid and tannins content of black beans were increased by increasing the time of soaking and this may indicate that these components had lost in soaking water. These results are in agreement with Olivera et al. [26] and [27] and Ramirez- Cardeves et al. [4] who found a greater reduction in the content of tannins in soaked beans.

With respect to the effect of heat treatment (Table 2) it is clearly noticed that the reduction percent reached highest level in cooked beans after soaking 12 hours. The reduction level of total phenols, flavonoids and tannins content was 148.77, 65.86 and 56.94 mg/100 g respectively.

Regarding the germination effect on the same components, Table 2 indicates that total phenols, flavonoids and tannins showed a descending pattern of reduction. For instance total phenols reduction after 24, 48 and 72 h of germination recorded 37.16, 43.41 and 50.40% respectively. Flavonoids reduction was 29.9, 38.11 and 49.38%, while tannin recorded 60.17, 64.30 and 65.73%. These results are in agreement with López et al. [28]. Who reported that after boiling and germination, was decrease in phenol concentrations of dark beans was observed.

DPPH antioxidant activity

In the past, the antioxidant characteristics of food have been studies since reactive oxygen species are widely believed to be included in many diseases such as cancer, diabetes, autoimmune con, various respiratory diseases, eye diseases, and schizophrenia Cai et al. [29]. DPPH antioxidant activity was highest in raw black beans compared to processed beans (Figure 1). The effects on DPPH were contributed by the high level of total phenols in raw beans. while the lowest value was obtained after 72 h of germination treatment. These results are agree with Aguilera et al. [30] and Xu and Chang [31] and Amarowicz and Pegg [32]. In the case of germination, the value of antioxidant activity depends on the days of germination, due to the variances in the enzymatic activity along the germination period Randhir et al. [33].

HPLC analysis of phenolic compounds:

Beans are good source of phenolic components. In this study sample phenolic compounds identified results at different treatments are shown in Table 3. The levels of phenolic compounds in raw black beans are 0.148 mg/100 g for gallic acid, 1.809 mg/100 g for benzoic acid, 6.627 mg/100 g for colorogenic acid, 20.153 mg/100 g for catechein, 1.717 mg/100 g for epi-catechin, 0.738 mg/100 g for p-coumaric and 1.278 mg/100 g for ferulic acid. From the present

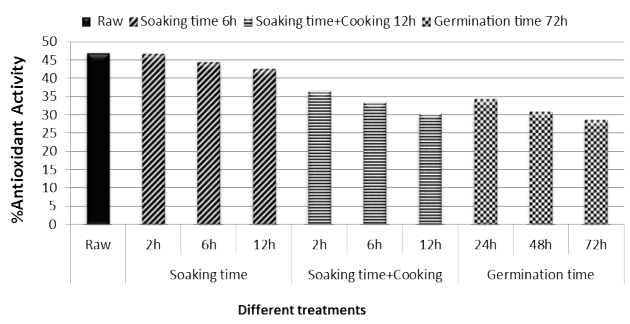

Figure 1: DPPH Activity scavenging of black bean at different processing treatments.

Treatments	Protein%	Fiber%	Oil%	Ash%	TC%
Raw	26.54 ± 0.15^{a}	5.97 ± 0.34^{a}	2.15 ± 0.04^{a}	5.22 ± 0.04^{a}	66.10 ± 0.37^{f}
Soaking					
2 h	25.98 ± 0.29^{a}	5.26 ± 0.04^{b}	1.98 ± 0.02^{b}	4.55 ± 0.02^{b}	67.50 ± 0.36^{e}
6 h	24.71 ± 0.28^{b}	4.96 ± 0.05^{c}	1.78 ± 0.03^{d}	4.39 ± 0.01^{c}	69.12 ± 0.28^{d}
12 h	23.67 ± 0.15^{c}	4.56 ± 0.10^{d}	1.62 ± 0.06^{f}	4.22 ± 0.04^{d}	70.49 ± 0.36^{c}
Soaking+cooking					
Soaking 2 h	25.11 ± 0.19^{a}	5.12 ± 0.03^{bc}	1.79 ± 0.04^{d}	4.40 ± 0.05^{c}	68.70 ± 0.22^{d}
Soaking 6 h	23.04 ± 0.16^{c}	4.71 ± 0.03^{d}	1.62 ± 0.02^{f}	3.99 ± 0.12^{e}	71.35 ± 0.20^{b}
Soaking 12 h	22.28 ± 0.34^{d}	4.54 ± 0.04^{d}	1.56 ± 0.01^{g}	3.49 ± 0.18^{f}	72.68 ± 0.28^{a}
Germination					
24 h	26.28 ± 0.28^{a}	4.33 ± 0.01^{e}	2.02 ± 0.02^{b}	4.12 ± 0.02^{d}	67.59 ± 0.30^{e}
48 h	25.07 ± 0.90^{b}	4.02 ± 0.08^{f}	1.85 ± 0.01^{c}	3.36 ± 0.02^{g}	69.72 ± 0.51^{d}
72 h	23.98 ± 0.35^{c}	3.78 ± 0.01^{g}	1.69 ± 0.01^{e}	3.04 ± 0.06^{h}	71.30 ± 0.09^{c}

Table 1: Proximate analysis of black beans at different treatments (dry weight basis).

Treatments	Total phenols	Reduction%	Flavonoids	Reduction%	Tannins	Reduction%
Raw	293.28 ± 2.48^{a}	0.00	89.82 ± 0.09^{a}	0.00	214.34 ± 6.49^{a}	0.00
Soaking						
Soaking 2 h	287.77 ± 7.24^{a}	1.88	84.9 ± 0.27^{b}	5.45	174.20 ± 7.44^{b}	19.48
Soaking 6 h	206.21 ± 6.36^{b}	29.69	72.94 ± 0.30^{c}	18.80	147.35 ± 3.18^{c}	32.51
Soaking 12 h	199.15 ± 0.93^{c}	32.10	65.80 ± 0.24^{e}	26.74	86.99 ± 4.13^{f}	60.34
Soaking+cooking						
Soaking 2 h	189.91 ± 0.55^{d}	35.25	71.91 ± 1.72^{c}	19.94	87.64 ± 1.11^{ef}	60.41
Soaking 6 h	157.07 ± 0.46^{g}	46.44	69.61 ± 1.09^{d}	22.50	69.61 ± 3.52^{d}	68.83
Soaking 12 h	148.77 ± 0.90^{h}	49.27	65.86 ± 1.11^{e}	26.68	56.94 ± 1.25^{h}	74.73
Germination						
24 h	184.30 ± 1.58^{e}	37.16	62.96 ± 0.23^{f}	29.90	90.56 ± 3.32^{e}	60.17
48 h	165.98 ± 0.19^{f}	43.41	55.59 ± 0.45^{g}	38.11	81.87 ± 2.73^{fg}	64.30
72 h	145.47 ± 0.74^{h}	50.40	45.47 ± 0.28^{h}	49.38	79.27 ± 4.49^{g}	65.73

Table 2: Relations between (soaking, cooking and germination) and Total phenol, flavonoid and tannin content (mg/100 g).

Treatments	Gallic	Benzoic acid	e-Vanillic	colorogenic	Caffeine	Catechein	Epi-Catechin	P-Coumaric	Ferulic
Raw	0.148	1.809	23.625	6.627	0.620	20.153	1.717	0.738	1.278
Soaking+Cooking									
Soaking 2 h	0.131	21.916	27.055	1.903	1.630	13.198	3.319	1.006	1.853
Soaking 6 h	0.334	25.546	25.393	0.983	0.811	3.743	2.645	1.537	2.183
Soaking 12 h	0.147	14.590	12.940	0.430	0.450	2.515	0.982	0.865	0.769
Germination									
24 h	1.501	29.928	56.942	2.104	2.463	21.360	3.157	1.730	4.539
48 h	0.466	10.853	48.150	1.996	1.392	16.253	2.377	1.463	2.075
72 h	0.467	8.947	32.410	0.786	2.107	11.911	2.221	0.918	0.918

Table 3: Composition of the phenolic compounds (mg/100 g).

results there are differences in the level of the phenolic compounds as affected by different processing treatments. In soaking plus cooking treatment the level of the phenolic compounds decreased compared with raw beans. Although soaking for 6 h plus cooing showed increase in some phenolic acids compared with the other two soaked plus cooked samples. Regarding to germination process, germinated beans at 24 h had much higher phenolic compounds compared with the other samples e-vanellic acid showed the highest value 56.942 mg/100 g followed by ferulic 4.539 mg/100 g. Data showed decreasing in phenolic compounds levels as prolonging the germination period [34,35].

In a study of quantitation of phenols in seeds of pink beans Joseph et al. [36] found that levels of ferulic were 36.0 mg/kg while epi-catechin was 2.27 mg/100 g. In germination treatment 24 h e-vanillic acid had the highest concentration of 56.94 mg/kg followed by ferulic acid 45.38 mg/kg while epi-catechin had the lowest concentration 3.15 mg/100 g. The low value of epi-catechin could be attributed to an epimerization reaction which conuers epi-catechin to its epimer catechin during the extraction process Khandelwal et al. [37]. The same authors reported that total phenolics and tannin content was decreased significantly in germinated green gram compared to Bengal gram, red gram and lentil. Loss of total phenolics and tannins content could be as high as 96% in germinated kidney bean as shown by Shimelis and Rakshit [38]. However, Duenas et al. [39] found that germination increased total phenolics content in lupin seeds after 9 days; the same results were reported by Chai [40] using germinated peanut. The observed reduction in tannin content after germination was a result formation of hydrophobic association of tannins with seed proteins and enzymes. In addition, loss of tannins during germination attribute to the leaching of tannins into the water Shimelis and Rakshit [38] and binding of polyphenols with other organic components such as protein or carbohydrate Saharan [41]. In addition, during the period of soaking and germination, the enzyme polyphenol oxidase may be activated, resulting in degradation and consequent losses of polyphenols Saxena

et al. [42], Khandewal et al. [37]. Luthria and Pastor- Corrales [43] found that caffeic acid was quantified at 1.1 mg/100 g in black beans, p-coumaric acid was 12.4 mg/ 100 g, ferulic acid was 26.6 mg/100 g and sinapic acid 9.4 mg/100 g. The data are greater than our finding results. This difference may be attributed to various factors such as storage conditions, variety, assay procedure, growing and agronomic practices (irrigation, fertilization, past management), maturity and weather changes.

HPLC analysis of flavonoid compounds:

Flavonoids compositions at different processing treatments are presented in Table 4. In raw black beans, some flavonoids have been identified, 0.134 mg / 100 g for kaempferol, 0.559 mg/100 g for hespirtin, 1.313 mg/100 g for quercatrin, 1.125 mg/100 g for rosmarinic, 8.098 mg/100 g for hespiridin, 1.548 mg/100 g for rutin, 2.346 mg/100 g for naringin and 2.407 mg/100 g for luteolin. In soaking plus cooking treatments, the level of kaempferol and naringin were increased while other identified flavonoids decreased. Quercetin was higher in raw (1.31 mg/100 g) and in soaked beans for 6 h plus cooking. The lowest concentration was observed at soaking 12 h plus cooking treatment for that component (0.047 mg/100 g). Naringin showed high concentration at 2 and 6 h soaking plus cooking (7.373 and 11.312) mg/100 g compared with raw beans. From the same Table it is noticed that the level of kaempferol was increased at 24 h and 48 h of germination which were 2.496 and 2.138 mg/100 g respectively compared with raw beans 0.134 mg/100 g, main while that compound decreased at 72 h of germination. Result in the same Table showed increase in quercetin in germination at 24 and 48 h (2.530 and 2.154 mg/100 g, respectively) compared with raw beans (0.133 mg/100 g). The highest concentration was observed for naringin (10.74 mg/100 g) at 24 h of germination followed by luteolin (9.486 mg/100 g). Kaempferol was increased by about 19 fold compared with raw beans at 24 h of germination, while naringin concentration was increased by about 4 fold relative to raw beans. In the case of germination, the concentration of flavonoids depends on the days of germination, due to the variances in the enzymatic activity along the germination period Randhir et al. [33] and Kao et al. [34] reported that during soaking of soy bean, 12-57% of isoflavonoids were lost in the water. The same authors explained that leaching of isoflavonoids to the soaking water depend on time and temperature and rises by increasing temperature and time.

Some authors studied the effects of thermal processes on model solutions of phenolic compounds; these studies were led especially on flavonoids. The data indicated that flavonoids in aqueous solutions showed different sensitivity to heat processing refer to their structures. However, their structure showed significant degradation under temperature above 100 °C. Rutin had a higher stability compared to it's a glycon form (quercetin) was observed Buchner et al. [35]; Friedman [44]; Makris and Rossiter [45]. These findings are attributed to the prevention of carbanion formation due to the glycosylation of the 3-hydroxyl group in the C-ring Buchner et al. [35]; Friedman [44]. Luteolin had more stability to heat than luteolin-7-glucoside and rutin when heated at 180 °C for 180 min Murakami et al. [46]. The degradation of flavonoids was not only a function of temperature and magnitude of heating; it refers also to other parameters such as pH, phytochemicals, and structure and even the presence or absence of oxygen. Indeed, original flavonol concentration had no effect on the degradation of rutin and quercetin. Moreover, under weak basic Buchner et al. [35]; Friedman [44] and neutral Friedman [44] reaction conditions, more degradation of rutin and quercetin was observed Buchner et al. [35]. The absence of oxygen highly decreased quercetin degradation and prevents rutin breaking up during heating. The presence of oxygen was shown to accelerate quercetin and rutin degradation due to the presence of the reactive oxygen species Buchner et al. [35] and Makris and Rossiter [45]. Colorogenic acid was observed to protect rutin against degradation when a mixture of the two substances was heated at 180°C Murakami et al. [46].

Anti-cancer activity

The black beans extract was found to express HCT, MCF7, A549, PC3 and HELA cancer inhibitory activity when tested at concentrations of 5-50 µg/mL toward MCF-7 cell line followed by A549, HELA, HCT and PC3 cell line, 20.8, 21.8, 23.6 and 32.3 µg/mL, respectively.

Tables 5 and 6 (Figure 2) shows the cytotoxic effects of raw, soaked with cooked, and germinated black beans extracts on breast cancer cell line (MCF7). Strong anticancer activity toward MCF7 cell line was observed for raw bean extract, which IC_{50} recorded 17.3 µg/mL, which had the lowest the IC_{50} value, the highest the anticancer activity of the extract.

Regarding to heat treatment, results revealed that the anticancer activity of bean extract was dependent on the soaking time before cooking (Figure 3). More specifically, bean extract (soaking 12h + cooking) exhibited higher anticancer activity than the other two cooked bean extracts (soaking 2h and 6h + cooking).

Cytotoxic activity of bean extracts during different germination periods (24, 48 and 72 h) are given in the same Figure 3. From which

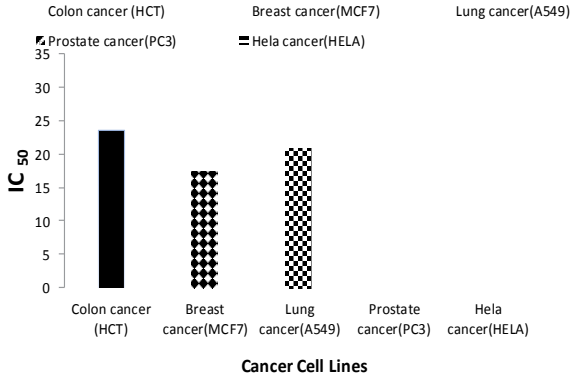

Figure 2: The inhibition of cell growth by different cell lines of black bean.

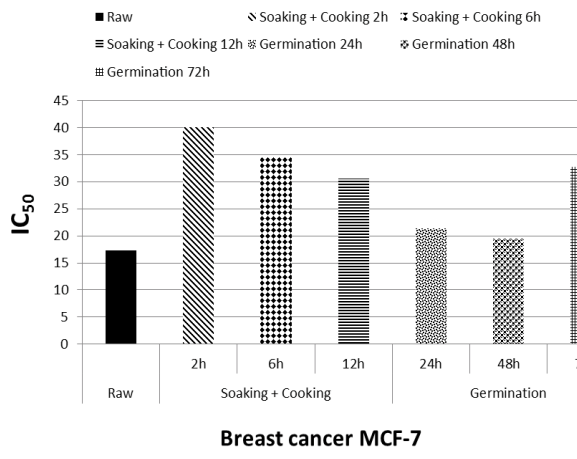

Figure 3: The inhibition of cell growth by different treatments of black bean.

Treatments	Kaempferol	Hespirtin	Quercetin	Quercetrin	Rosmarinic	Hespiridin	Rutin	Naringin	Luteolin
Raw	0.134	0.559	1.313	1.069	1.125	8.098	1.548	2.346	2.407
Soaking +Cooking									
Soaking 2 h	1.418	3.179	0.444	0.294	0.865	2.140	2.939	7.373	1.295
Soaking 6 h	1.330	0.948	1.130	0.283	1.311	1.933	1.061	11.312	4.085
Soaking 12 h	0.261	0.579	0.047	0.060	0.107	2.198	0.454	0.974	0.391
Germination									
24 h	2.496	0.698	2.530	1.108	2.126	2.687	3.539	10.747	9.486
48 h	2.138	0.322	2.154	0.501	1.202	15.201	2.715	9.536	2.888
72 h	1.457	0.737	0.219	0.153	0.969	6.860	1.300	3.748	0.601

Table 4: Composition of flavonoid compounds (mg/100 g).

Cell lines Conc. µg/mL	Colon cancer (HCT)	Breast cancer (MCF7)	Lung cancer (A549)	Prostate cancer (PC3)	Hela cancer (HELA)
0.00	1.00	1.00	1.00	1.00	1.00
5.00	0.846	0.867	0.958	0.876	0.825
12.50	0.793	0.659	0.773	0.738	0..720
25.00	0.461	0.260	0.371	0.608	0.425
50.00	0.61	0.194	0.176	0.241	0.386
IC50	23.6	17.30	20.80	32.3	21.8

Table 5: The inhibition of cell growth by different cell lines of black bean.

Treatments Conc. µg/mL	Raw	Soaking 2 h+Cooking	Soaking 6 h+Cooking	Soaking 12 h+Cooking	Germination 24 h	Germination 48 h	Germination 72 h
0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
5.00	0.867	0.925	0.926	0.960	0.853	0.785	0.858
12.50	0.659	0.837	0.852	0.825	0.680	0.727	0.697
25.00	0.260	0.630	0.585	0.535	0.428	0.320	0.539
50.00	0.194	0.419	0.370	0.359	0.346	0.308	0.396
IC50	17	40	34.6	30.6	21.3	19.5	32.7

Table 6: The inhibition of cell growth by different treatments of black bean

it is clear that the anticancer activity ascending with subsequent improvement of MCF7 cell line inhibition, IC_{50} was 19.5 µg/mL as a result of 48h of germination. On contrary, anticancer activity realized a noticeable reduction of tumor inhibition after 72 h of germination, IC_{50} was 32.7 µg/mL. The previous results support the view that prolonging the period of germination could enhanced the enzymes activity which affecting on the total phenolic content, the time affecting on the anticancer activity. Although IC_{50} of black bean extract increased after 72 h of germination, It conceder had cytotoxic activity.

Our results are in agreement with previous studies of Chan et al. [47] that demonstrated the antiproliferative activity of some components of beans, different from phenolic compounds, on human breast cancer (MCF7), human hepatoma (Hep G2) and nasopharyngeal carcinoma (CNE1 and CNE2) cell line.

Conclusion

The current study showed that ethanolic extracts of black beans have antioxidant and anticancer activities. The results observed that black beans had high total phenols, tannins and flavonoids. There were differences in these bioactive components as a result of soaking, cooking and germination processes. Anticancer activity realized a noticeable reduction of tumor inhibition. Soaking 12 h followed by cooking for 30 min and germination for 48 h exhibited higher anticancer activity relative to the other processing. It is clear that the anticancer activity ascending with subsequent improvement of MCF7 cell line inhibition.

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