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Modulatory effect of *Brassica oleracea* L. var. *italica* extract in chemically induced mammary carcinomas in rats.

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Broccoli, super detoxifying agent, crude extract was evaluated for anti- breast cancer property *in-vitro* and *in-vivo* aspects. The *in-vitro* anti-cancer effect was applied on MCF7, human breast cancer cell line, obtained from Sweden. Breast cancer in rats was induced chemically by 7, 12-dimethylbenz-(a)-anthracene (DMBA) administration. Antioxidant activity, anti-inflammatory biomarkers, estrogen and progesterone hormones, rate growth cancer limiting enzymes activities (aromatase and α 1- Na^+/K^+ ATPase) and tumor biomarker (carcinoembryonic antigen, CEA) were determined. In *in-vitro* study, broccoli extract decreased the MCF-7cell growth (12.50%). In addition, the extract showed antioxidant activity and selective anti-inflammatory effect. Broccoli extract showed promising anti-breast cancer effect as it inhibited cancer growth rate limiting enzymes, aromatase (4.07 ± 0.70 and 3.90 ± 0.63 μg Eq/ml) and Na^+/K^+ ATPase (0.48 ± 0.06 and 0.89 ± 0.24 μg Eq/ml). In addition, it suppressed CEA (306.00 ± 0.87 and 149.00 ± 1.00 μg Eq/ml) for the protective and therapeutic groups, respectively, which were significantly amplified as a response for breast cancer induction (8.33 ± 1.34 , 5.70 ± 0.91 and 524.17 ± 1.02 μg Eq/ml, respectively). Broccoli extract regulated sexual hormones production as it induced progesterone production and depleted estrogen levels that significantly affected by breast cancer induction. It could be concluded from research results that broccoli has anti-breast cancer activity proved in both *in-vitro* and *in-vivo* evaluations which may be attributed to its orchestral activity approach includes inhibition aromatase, Na^+/K^+ ATPase and cyclooxygenases-2 activities, antioxidant activity and sex hormone regulation.

.Keywords: Breast cancer, Broccoli, Na^+/K^+ ATPase inhibitors, aromatase inhibitor, estrogen, progesterone, antioxidants, cyclooxygenases.

INTRODUCTION

Broccoli, *Brassica oleracea* L. var. *italica*, is a vegetable belongs to *Cruciferae* or alternatively *Brassicaceae*. It has been used in a wide range of biological activities including gastro-protective, antimicrobial, antioxidant, hepatoprotective,

cardioprotective, anti-obesity, antidiabetic, anti-inflammatory and immunomodulatory activities as well as prevention of renal damage. In addition, broccoli is considered as a good source of health improving components including glucosinolates, anthocyanins, flavonoids and hydroxycinnamic

acids. Glucosinolates are exclusively produced in the Brassica plants (Owis, 2015).

In 2012, cancer incidence was elevated to 14 million new cases per year; breast cancer comprised 1.7 million, representing 11.9% (WHO, 2015). Breast cancer is the most common cancer in women, including almost one-third of all malignancies with a predominance in the developing countries. It is the second main cause of the cancer-related death among females all over the world. Lifestyle, hormones and obesity after menopause are important risk factors in the development of breast cancer (WHO, 2015). The synthetic cancer therapy led to multifarious side effects, there is a tendency to turn back to the natural medications, which are therapeutically effective and more acceptable. The phytochemicals from medicinal plants have a remarkable effect against cancer, such as the phenolic compounds known with its anticancer ability on several cancer cell lines and promoter for apoptosis (Tariq et al. 2017). Most living organisms have a self-defense system to protect themselves against the oxidative stress. Amplification of oxidative stress is considered as one of the mechanisms for cancer presentation. Protection of DNA from damage presented by oxidative stress is presented by antioxidants components which also reduce the abnormal cell division (Menon et al. 2016). Prostaglandins, presented as cyclooxygenase-2 (COX-2) activity product, have promoting effect on the mitogenesis through influencing fibroblasts, osteoblasts in mammary cells. Meanwhile, COX-2 can induce the mutagenesis and angiogenesis as well as the raised cell migration and apoptosis (Mazhar et al. 2006). The most important way to face breast cancer is determinants development prevention (Chauhan et al. 2009) by different modulators like aromatase inhibitor and cyclooxygenase inhibitors. Aromatase is the enzyme that converts androgens into estrogens through the aromatization process. Aromatase inhibitors are drugs that play as blocker of estrogen production or receptors blocker for estrogen. Aromatase inhibitors are used to treat breast cancer in the postmenopausal women and the gynecomastia in men. Based on this idea, using aromatase inhibitors that decrease estrogen production at the site of the cancer has been demonstrated to be an impact therapy for the postmenopausal women with hormone-sensitive breast cancer (Howell et al. 2005). Hence, the combination between COX-2 inhibitors and aromatase inhibitor drugs lead to reduce aromatase activity and prostaglandin

synthesis, resulting tumors inhibiting. In addition, other investigations demonstrated potent anti-cancer activities of the Na^+/K^+ ATPase inhibitors that affect tumor cell metabolism and growth (Konstantinos et al. 2014). Carcinoembryonic antigen (CEA) is a glycoprotein normally found in the embryonic endodermal epithelium and is founded in a high level with patients in primary malignancies like breast, ovarian and prostate cancers (Locker et al. 2006). Through this point of view, this research aimed to evaluate the role of Broccoli inflorescence aqueous alcoholic extract in treating chemically induced-breast cancer in animals relevant to enzymes and hormones that may control the progression of cancer.

MATERIALS AND METHODS

Plant material and extraction

Broccoli were organically cultivated in the Experimental Farm of Sekem Company in Bilbes, Sharqia Governorate, Egypt (50 km North Cairo) during the seasons of 2013/2014. Broccoli inflorescences were collected in April 2014. The inflorescences were exposed to heat shock at $100^\circ\text{C}/1\text{min}$ to stop enzymes activity that may convert phytochemicals and then they were air dried while they completed dryness in oven at 40°C . The dried powder (1kg) was exhaustively extracted with 70% EtOH by shaken soaking at room temperature. The filtrate was collected and was evaporated under reduced pressure until dryness. The extract was then lyophilized to be free from any water or solvent residues. The remained powder was then incorporated into the bio-assay.

Chemicals

7, 12 di-methylbenzo-a-anthracene (DMBA) purchased from Sigma Aldrich, USA. Liver and kidney functions, lipid profile and antioxidant parameters kits were purchased from Bio diagnostic, Egypt. ELISA kits for cyclooxygenases activity (COX-1 and COX-2), aromatase, Na^+/K^+ ATPase, carcinoembryonic antigen (CEA), estrogen and progesterone were purchased from Sunlong Biotech Co., LTD, PingShui Street, Gong Shu District, Hangzhou, Zhejiang, China, Email: Sales@Sunlongbiotech.Com. Ethylene diamine tetra acetate (EDTA), Sodium dihydrogen phosphate, disodium monohydrogen phosphate were purchased from Fin Chem Ltd.

In-vitro anti-breast cancer assay

The *in-vitro* anti-cancer activity of the Broccoli crude extract was assayed using cell viability of Human Caucasian breast adenocarcinoma (MCF7) by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan (Mosmann, 1983). MCF-7 cells were generously provided by professor, doctor Stig Linder, Professor in Oncology and Pathology department in Karolinska Institute, Sweden. Under a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA), the procedure was carried out. MCF7 cells were suspended in RPMI 1640 medium, (El-Menshaw et al. 2010). Amicroplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595nm was used. The reference wavelength was 620nm. The percentage of change in viability was calculated according to the formula; $(\text{Reading of extract} / \text{Reading of negative control} - 1) \times 100$

In-vitro anti-cancer activity of Broccoli crude extract was conducted and determined by the Bioassay-Cell Culture Laboratory, National Research Centre, El-Tahrir St., Dokki, Cairo 12622, Egypt.

In-vivo anti-breast cancer

Acute toxicity study

According to the method described by Bruce, the acute toxicity of Broccoli crude extract was carried out. Mice (20-25 g) 8 mice each group was tested in a dose pattern started from 500 mg/kg body weight and increased up to 8000 mg/kg body weight with a rate of 500 mg/ kg body weight (Bruce, 1985). Control group received only the normal saline. All groups were observed for any gross effect or mortality during 48h and then animals were followed up for one week to observe any behavioral changes. The extract found to be safe up to 6000 mg/kg b. wt.. The recorded LD₅₀ was 600 mg/kg b. wt.

Chemically breast cancer induction

Mammary gland tumors were induced by a single dose of 65 mg of DMBA/ kg body weight diluted in soy oil given in an intragastric route. All rats in an average weight (100-130 g) received DMBA at the age of 60 days. The mammary cancer was obtained after 16 weeks (Yerma et al. 1988).

Maintenance of animals

The experimental animals were young virgin Sprague-Dawley female rats. They were obtained

from animal house of National Research Centre. This type of the rats live an average of 3 years, starting its reproductive function, which lasts for about 1 year, at 50 to 60 days of age.

One hundred fifty young virgin Sprague-Dawley female rats were bred in the animal house under ideal conditions of temperature (25 ± 5 °C), humidity ($60 \pm 5\%$), and light (12 dark: 12 light). they were fed on appropriate ration in pellets and filtered water.

Experimental design

Animals were acclimatized for 2 weeks on the laboratory conditions and then were randomly divided into 3 groups:

Group I (-ve control group) that included 30 rats and were force-fed saline at all the experimental period.

Group II (+ve control group) that was divided into two subgroups:

extract control subgroup (n=30) in which the animals were force fed saline up to the 16th week and then were administered the extract at a dose of 600 mg/ kg body weight (as 0.1 of the LD₅₀) orally for three months.

Cancer control subgroup. (n=30) that were chronically received DMBA at 65 mg/kg body weight as one intragastric dose and administered saline for 3 months, they maintained in lab condition until the end of the experiment.

Group III (treated group) that was divided into two equal subgroups;

protective subgroup (n=30) that was received extract at dose of 600 mg/ kg/day/ 3months and then was treated with DMBA. Animals were then remained to reach the end of the experiment.

Therapeutic subgroup (n=30) that firstly was received DMBA since the 16 weeks from administration and then the animals were treated with extract at dose of 600 mg/kg/day/ 3 months.

After 28 weeks, the end of the experiment, animals were fasted for 18 hours and then were anesthetized. Blood samples were collected from the retro orbital plexus. Serum was obtained by centrifugation at 4000 rpm for 10 min using Sigma labor zentrifugen. Organs were collected, washed in ice saline solution and were weighted freshly for the chronic toxicity evaluation.

Biochemical assessment

The biochemical assessment carried out for all sera samples to evaluate toxicity biomarkers, antioxidant parameters, anti-inflammatory biomarkers, rate growth cancer biomarkers and hormonal levels.

Toxicity biomarkers

liver function tests; total protein concentration, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were spectrophotometrically assessed according to (Henry, 1964 and Reitman and Frankel, 1957) respectively. The kidney function tests; urea, uric acid and creatinine, were estimated spectrophotometrically as described by Tabacco et al. 1979, Gochman and Schmitz, 1971 and Faulkner and King, 1976 respectively. Total lipids and total cholesterol were determined based on the methods of Estadella et al. 2004 and Allain et al. 1974 respectively.

Antioxidants parameters:

antioxidants, either non enzymatic like glutathione (GSH) concentration or enzymatic like glutathione reductase (GR), glutathione S-transferase (GST), Glutathione peroxidase (GPx) and catalase (CAT) activities were determined spectrophotometrically according to the methods of Griffith, 1980, Goldberg, and Spooner, 1983, Paglia and Valentine, 1967, Habig et al. 1974 and Beers, and Sizer 1952, respectively.

Anti-inflammatory biomarkers;

COX-1 and COX-2, rate growth cancer enzymes activities; aromatase, and α 1-Na, K ATPase and tumor biomarker; CEA and sexual hormones; estrogen and progesterone were determined using ELISA kits of Sunlong Biotech Co., LTD

RESULTS

In vitro anti-cancer effect of broccoli crude extract

Cytotoxic effect of Broccoli crude extract was evaluated using Human Caucasian breast adenocarcinoma (MCF7) using cell viability assay. The extract at 100 ppm showed kill percentage against MCF7 cells reached to 45.38% with IC_{50} , 98 μ g/ml.

In vivo anti-cancer effect of Broccoli crude extract

Effect of the Broccoli extract on the relative weight of organs

DMBA is a polycyclic aromatic hydrocarbon used as carcinogenesis in experimental models. In this experiment, it caused enlargements in liver, heart, spleen, lung, breast and total weight which were shown as weights increments of these organs (4.59 ± 0.86 , 0.52 ± 0.16 , 0.95 ± 0.30 , 0.66 ± 0.12 and 10.56 ± 0.56 g/100g, respectively) as

compared to the negative control (2.78 ± 0.20 , 0.44 ± 0.11 , 0.39 ± 0.05 , 0.80 ± 0.41 and 2.27 ± 0.20 g/100 g, respectively), whereas kidney was shrunk ($P < 0.05$). On the other hand, receiving Broccoli extract showed recovering effects on all organs towards the normal without significant changes, Table (1). No toxicity symptoms appeared in the organs of positive control animals, therefore, the relative weight of their organs did not change significantly.

Effect of the Broccoli crude extract on the toxicity biomarkers

Liver function:

Liver functions, including AST and ALT activities and total protein content, were determined as a part of the Broccoli extract chronic toxicity evaluation. Induction of breast cancer chemically showed hepatotoxicity represented as a significant augment on the liver enzymes levels (161.00 ± 2.11 and 78.75 ± 2.45 U/L for AST and ALT, respectively). Marked depletion in total protein, 3.37 ± 0.95 mg/dl, was recorded in comparison with negative control (8.89 ± 1.11 mg/dl), Figure (1). Administration of Broccoli extract, either as a protective or as a therapeutic agent significantly reduced AST activity to less than half of cancer control value. A significant reduction was recorded in ALT in animals treated with extract concurrence with a significant increase in total protein content. Liver function of extract positive control did not affect significantly in respect to negative control, $P < 0.05$.

Kidney functions:

Regarding kidney function, disturbances were observed in kidney performance as a response to DIMBA administration as data represented in Figure (2). Uric acid, urea and creatinine concentration were significantly elevated to 3.06 ± 0.77 , 12.43 ± 1.67 and 4.06 ± 0.53 mg/dl, respectively, as compared to those of negative control (2.43 ± 0.57 , 9.87 ± 1.25 and 3.23 ± 0.71 mg/dl, respectively). Broccoli extract as protective or as therapeutic application provoked remarkable amelioration in kidney performance explained a significant reduction in uric and creatinine concentration compared to cancer control.

Lipid profile:

Concerning lipid profile, induction of breast cancer presented remarkable increment in lipid and cholesterol concentration in sera as a result of liver disturbance (161.01 ± 2.07 and 184.45

± 8.00 mg/dl, respectively) in comparison with the negative control (86.59 ± 2.33 and 67.67 ± 3.95 mg/dl, respectively), Figure(3). Broccoli extract enhanced liver characters to inhibit the accumulation of lipid and cholesterol. There are no significant differences were recorded between extract protective and therapeutic groups on lipid or cholesterol concentration, Figure (3). Lipid and cholesterol concentration values of either extract positive control or treated group were lower than negative control group, $P < 0.05$.

Effect of Broccoli crude extract on antioxidant characters

Induction of breast cancer was associated with increment in oxidative stress status explained as remarkable depletion in glutathione concentration (GSH) and activities of antioxidant enzymes, including glutathione reductase (GR), glutathione S- transferase (GST), glutathione peroxidase (GPx) and catalase (CAT) by about 73.46, 75.94, 68.62, 63.62 and 78.53% as sequentially compared to those of the negative control (Table. 2), $P < 0.05$. Extract positive control, significantly amplified GSH and antioxidant enzymes activities in comparison with the negative control. The protective effect of Broccoli crude extract suppressed oxidative stress appeared as significant augment in the GSH concentration (9.11 ± 1.00 mmol/dl.), GR (11.23 ± 1.08 μ mol/ mg protein/ min), GST (5.19 ± 1.23 μ mol/ mg protein/ min), GPx (4.59 ± 1.00 μ mol/ mg protein/ min) and CAT (19.85 ± 2.30 μ mol/ mg protein/ min), compared to cancer control. In comparison with cancer control, receiving extract in a therapeutic route exhibited significant increments in GSH concentration and enzymes activities; GR, GST, GPx and CAT (4.42 ± 1.03 mmol/dl, 5.45 ± 0.91 , 4.67 ± 1.11 , 2.23 ± 1.12 and 13.74 ± 2.46 μ mol/ mg protein/ min, respectively). Nevertheless, Broccoli extract protective effect was more promising than its therapeutic effect.

Effect of Broccoli crude extract in the anti-inflammatory biomarkers

COX-1 and COX-2 were determined using ELISA kits. Induction of mammary cancer in rats amplified COX-2 to reach 326.00 ± 1.38 ngEq/ ml (Figure 4), although it highly decreased COX-1 (102.00 ± 2.14 ngEq/ ml) in respect to the values of the negative control (121.50 ± 0.79 and 470.00 ± 1.35 ngEq/ ml), $P < 0.05$. Broccoli extract represented selectivity as anti-inflammatory agent shown as a significant inhibition in COX-2 and a

significant elevation on COX-1. Broccoli extract, either as protective or therapeutic, reduced COX-2 (248.50 ± 0.97 and 191.75 ± 1.22 ngEq/ ml, respectively) comparing to cancer control. In addition, COX-1 was magnified by about five-fold higher than COX-1 of cancer control. Regarding positive control, COX-1 was significantly amplified to 647.50 ± 1.67 ngEq/ ml whereas COX-2 was significantly reduced to 90.00 ± 1.34 ng Eq/ ml as a response to extract administration in respect to negative control. COX-2/ COX-1 ratio of cancer group was significantly magnified by about twelve-fold higher than the ratio of the negative control. The extract showed anti-inflammatory effect represented as a significant reduction in COX-2/ COX-1 ratio to reach 0.42 and 0.36% for the protective and therapeutic groups, respectively, as compared to cancer control.

Influence of Broccoli crude extract in rate limiting cancer growth enzymes and tumor biomarker (CEA)

Aromatase:

Induction of breast cancer enhance production of aromatase which induced tumor growth signed as increasing in total breast weight. Data presented in Table (3) showed that aromatase activity was elevated around ten-fold higher in the breast cancer group than in negative control (8.33 ± 1.34 and 0.80 ± 0.24 μ g Eq/ ml, respectively). Broccoli extract reduced the amount of aromatase in cells. The protective and therapeutic efficacy of broccoli extract represented as a significant inhibition in aromatase activity (4.07 ± 0.70 and 3.90 ± 0.63 μ gEq/ml, respectively) in respect to the cancer control. Aromatase activity of positive control was remained close to that of the negative control (0.89 ± 0.23 and 0.80 ± 0.24 μ g Eq/ ml, respectively), $P < 0.05$. There is no significant difference was noticed between the protective and therapeutic effect of the Broccoli extract.

Na⁺/K⁺ ATPase:

The vast increment of the cell pump power is considered one of the earliest events in the cell proliferation. Hence, inhibition of the Na⁺/K⁺ pumps lead to a block in the cell proliferation. The other parameter that was used for monitoring controlling tumor growth is Na⁺/K⁺ ATPase (Table 3). Breast cancer induction amplified Na⁺/K⁺ ATPase by about eleven-fold higher than that of the negative control (5.70 ± 0.91 and 0.50 ± 0.11 μ g Eq/ ml, respectively). The anti-breast cancer efficacy of Broccoli crude extract in this research

Table 1. Effect of Broccoli crude extract on vital organs of normal and cancer rats, chronic toxicity effect through 90 days.

Parameter Group		Total weight (Mean)	Organs weight (g/ 100 g)						
			Liver	Kidney	Spleen	Lung	Heart	Brain	Total Breast
-ve control		156.80 ±1.25	2.78 ±0.20 ^a	1.01 ±0.35 ^a	0.39 ±0.05 ^a	0.80 ±0.41 ^a	0.44 ±0.11 ^a	0.99 ±0.31	2.27 ±0.37 ^a
+ve control groups	Cancer group	150.5 ±2.13	4.59 ±0.86	0.71 ±0.09	0.95 ±0.30	0.66 ±0.12	0.52 ±0.16	1.06 ±0.24 ^a	10.56 ±0.56
	Broccoli	121.20 ±2.01	2.72 ±0.55 ^a	1.00 ±0.44 ^a	0.41 ±0.04 ^a	0.76 ±0.31 ^a	0.43 ±0.14 ^a	1.04 ±0.19 ^a	2.31 ±0.44 ^a
Treated group	Protective	90 ±0.95	3.86 ±0.72 ^b	0.86 ±0.22	0.50 ±0.06 ^b	0.70 ±0.17	0.43 ±0.18 ^a	1.07 ±0.40 ^a	7.03 ±0.87 ^b
	Therapeutic	111.50 ±2.41	4.03 ±0.58 ^b	1.09 ±0.35	0.45 ±0.03 ^b	0.74 ±0.22 ^a	0.46 ±0.17 ^a	1.06 ±0.41 ^a	6.51 ±1.11 ^b

The presented data are mean of 20 replicates ± SD. Data were analyzed using ANOVA one-way followed with post hoc for multiple comparisons. Appearance of letters means insignificant difference between groups that have the same letter as compared to –ve controls.

Table 2. Antioxidant status of induced breast cancer animals and animals treated with Broccoli crude extract determined in sera samples.

Groups	Subgroups	Glutathione concentration (mmol / dl)	Glutathione reductase ($\mu\text{mol}/\text{mg protein}/\text{min}$)	Glutathione-s-transferase ($\mu\text{mol}/\text{mg protein}/\text{min}$)	Glutathione Peroxidase ($\mu\text{mol}/\text{mg protein}/\text{min}$)	CAT activity ($\mu\text{mol}/\text{mg protein}/\text{min}$)
Negative group	-ve control	3.24 \pm 1.12	3.99 \pm 0.89	3.57 \pm 1.00	1.63 \pm 0.51	19.10 \pm 3.12 ^a
Positive control	Cancer group	0.86 \pm 0.51* (-73.46%)	0.96 \pm 0.63* (-75.94%)	1.12 \pm 0.35* (-68.62%)	0.43 \pm 0.22* (-63.62%)	4.10 \pm 2.15* (-78.53)
	+ve extract	8.06 \pm 0.67 ^{*a}	9.94 \pm 2.13*	8.51 \pm 0.94*	4.06 \pm 0.68 ^{*a}	19.56 \pm 1.58 ^a
Treated groups	Protective	9.11 \pm 1.00 ^{*a}	11.23 \pm 1.08*	5.19 \pm 1.23 ^{*a}	4.59 \pm 1.00 ^{*a}	13.85 \pm 2.30
	Therapeutic	4.42 \pm 1.03*	5.45 \pm 0.91*	4.67 \pm 1.11 ^{*a}	2.23 \pm 1.12*	19.74 \pm 2.46 ^{*a}

The presented data are mean of 20 replicates \pm SD. Decreasing percentage in cancer control corresponding to -ve control. Data were analyzed using ANOVA one-way followed with post hoc for multiple comparisons. Appearance of * means significant difference between groups and -ve controls while appearance of letters means insignificant difference between groups that have the same letter as compared to cancer controls.

Table 3. Cancer rate limiting enzymes in DIMBA- induced breast cancer female rats as treated with Broccoli crude extract.

Groups	Subgroups	Aromatase ($\mu\text{g Eq/ ml}$)	$\text{Na}^+ \text{K}^+ \text{ATPase}$ ($\mu\text{g Eq/ ml}$)	CEA ($\mu\text{g Eq/ ml}$)
Negative control	-ve control	0.80 ± 0.24^a	0.50 ± 0.11^a	279.97 ± 1.04^a
positive groups	Cancer control	$8.33 \pm 1.34^*$	$5.70 \pm 0.91^*$	$524.17 \pm 1.02^*$
	+ve extract	0.89 ± 0.23^a	0.57 ± 0.12^a	281.09 ± 0.94^a
Treated groups	Protective	$4.07 \pm 0.70^{*b}$	0.48 ± 0.06^a	$306.00 \pm 0.87^*$
	Therapeutic	$3.90 \pm 0.63^{*b}$	$0.89 \pm 0.24^*$	$149.00 \pm 1.00^*$

The presented data are mean of 20 replicates \pm SD. Data were analyzed using ANOVA one-way followed with post hoc for multiple comparisons. Appearance of * means significant difference between groups and -ve controls while appearance of letters means insignificant difference between groups that have the same letter as compared to cancer controls. Carcinoembryonic antigen (CEA)

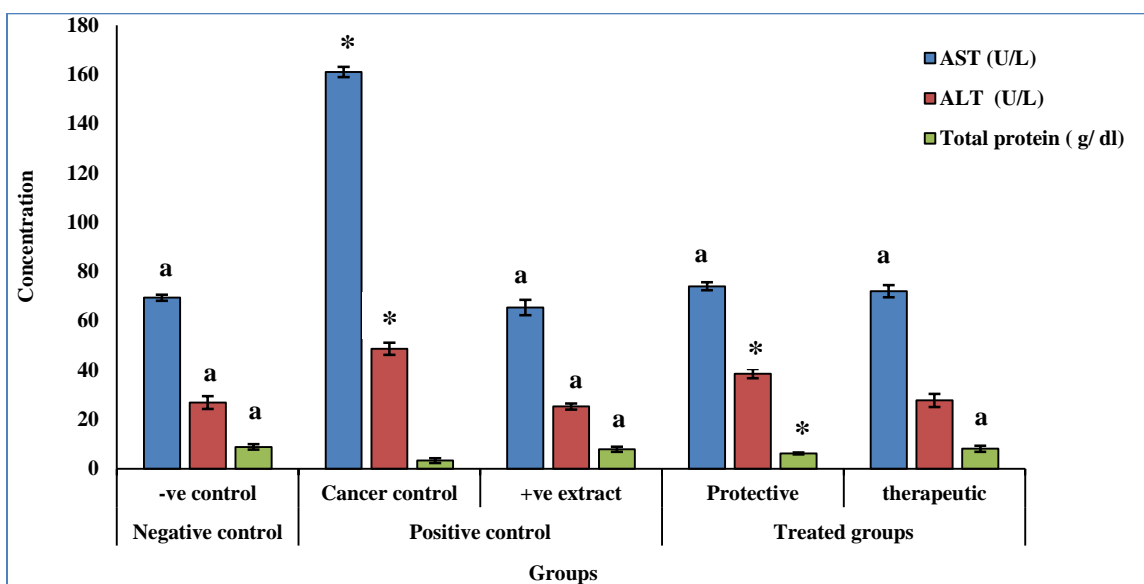


Fig.1 Effect of Broccoli crude extract on liver functions in DMBA induced breast cancer in female rats. The presented data are mean of 20 replicates \pm SD. Data were analyzed using ANOVA one-way followed with post hoc for multiple comparisons. Appearance of * means significant difference between groups and –ve controls. Appearance of letters means insignificant difference between groups that have the same letter as compared to –ve controls.

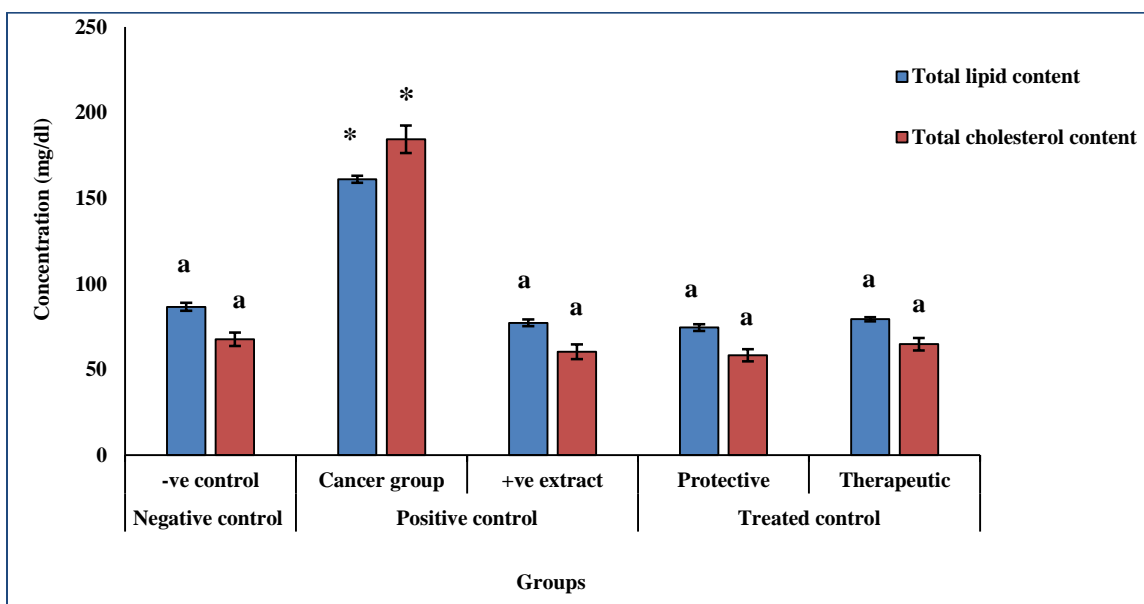


Fig. 2 Impact of Broccoli crude extract on total lipid and cholesterol in DMBA induced breast cancer in female rat. The presented data are mean of 20 replicates \pm SD. Data were analyzed using ANOVA one-way followed with post hoc for multiple comparisons. Appearance of * means significant difference between groups and –ve controls while appearance of letters means insignificant difference between groups that have the same letter as compared to –ve controls.

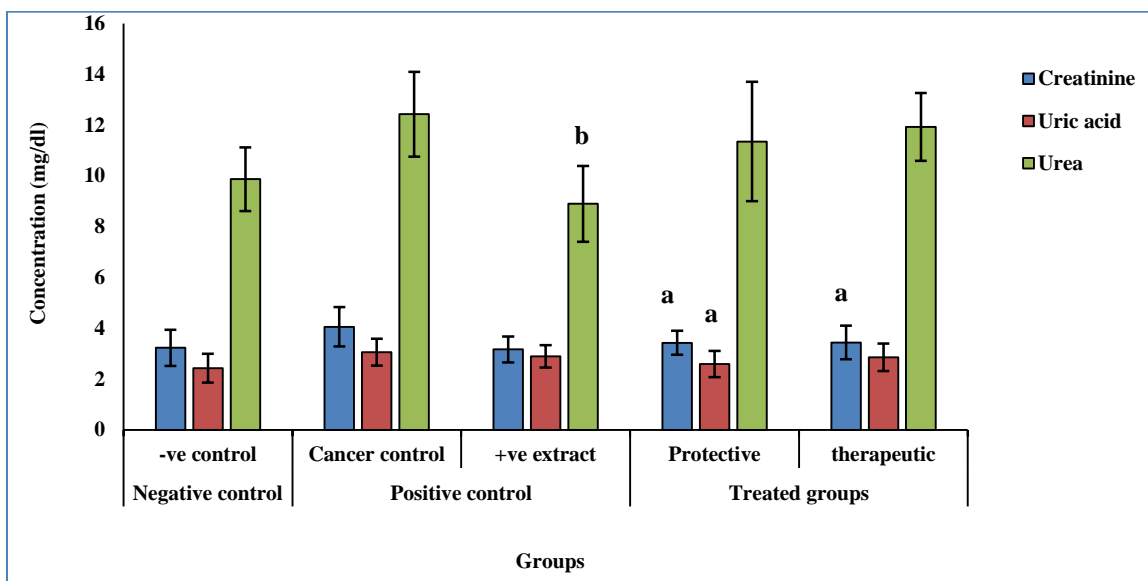


Fig.3 Effect of Broccoli crude extract on kidney functions in DMBA induced breast cancer in female rats. The presented data are mean of 20 replicates \pm SD. Data were analyzed using ANOVA one-way followed with post hoc for multiple comparisons. Appearance of * means significant difference between groups and –ve controls while appearance of letters means insignificant difference between groups that have the same letter as compared to –ve controls.

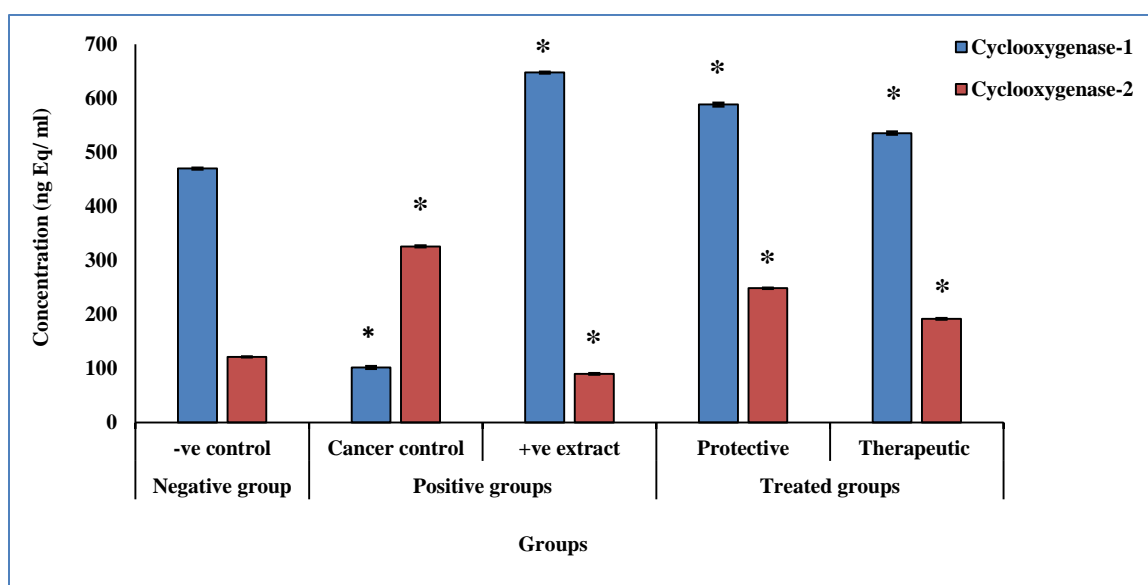


Fig. 4 Rat inflammatory rate limiting enzymes as affected by breast cancer induction and treated with Broccoli crude extract. The presented data are mean of 20 replicates \pm SD. Data were analyzed using ANOVA one-way followed with post hoc for multiple comparisons. Appearance of * means significant difference between groups and –ve controls while appearance of letters means insignificant difference between groups that have the same letter as compared to cancer controls.

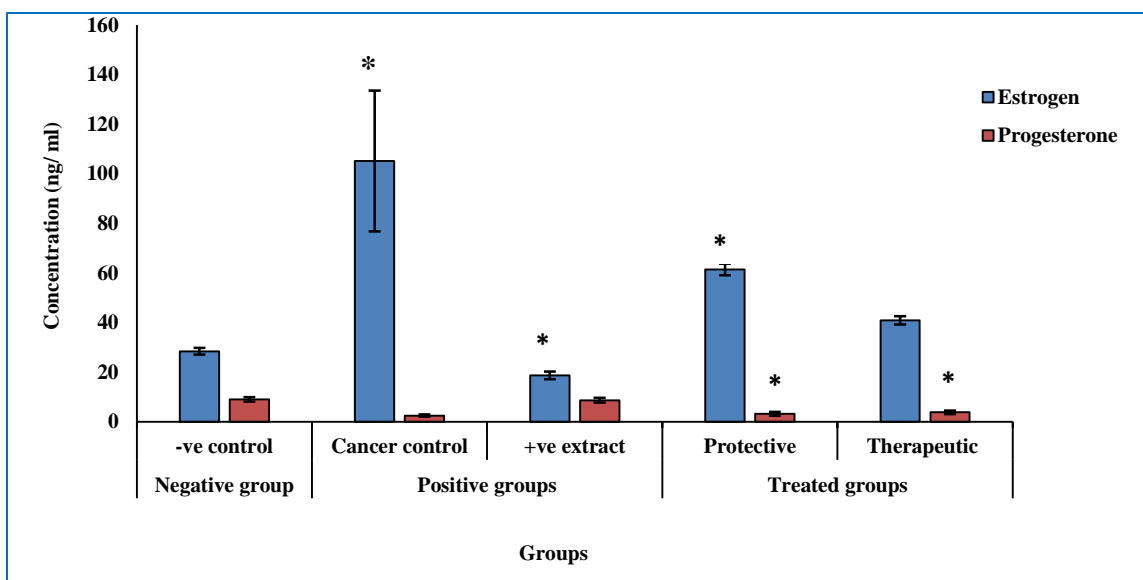


Fig.5 Rat sex hormones as affected by breast cancer induction and treated with Broccoli crude extract. The presented data are mean of 20 replicates \pm SD. Data were analyzed using ANOVA one-way followed with post hoc for multiple comparisons. Appearance of * means significant difference between groups and –ve controls while appearance of letters means insignificant difference between groups that have the same letter as compared to cancer controls.

explained by the suppression of Na^+/K^+ ATPase activity, which significantly decreased to 0.48 ± 0.06 and 0.89 ± 0.24 μg Eq/ ml for the protective and the therapeutic groups, respectively, as compared to the cancer group (5.70 ± 0.91 μg Eq/ ml). The extract keep Na^+/K^+ ATPase activity around those recorded in positive and negative control, $P < 0.05$.

CEA:

Our findings appeared the role of Broccoli extract in suppression chemically-induced breast cancer, where a large pool of CEA is expressed in the plasma membrane (Table 3). The CEA was significantly magnified by about two-fold higher than that of the negative control to reach 524.17 ± 1.02 $\mu\text{gEq/ml}$ as a response to breast cancer induction in respect to the negative control (279.97 ± 1.04 $\mu\text{gEq/ml}$), ($P < 0.05$). Normal level of CEA was recorded in Broccoli extract treated animals, indicating the efficacy of the extract in preventing tumor development. Upon the extract

administration either on protective group or on therapeutic group, they significantly reduced CEA level to 306.00 ± 0.87 and 149.00 ± 1.00 $\mu\text{gEq/ ml}$, respectively, compared to cancer group; 524.17 ± 1.02 $\mu\text{gEq/ ml}$. In the positive control, CEA level was around that found in the negative control.

Impact of Broccoli crude extract on the Sex-Hormone status of induced breast cancer female rats

Breast cancer group animals are characterized by a high estrogen level, which was elevated by about four-fold higher than that of the negative (105.14 ± 3.21 and 28.35 ± 1.35 ng/ ml, respectively). In addition, progesterone level of cancer group was declined by about quarter-one of the negative control progesterone (2.45 ± 0.40 and 8.95 ± 0.88 ng/ ml, respectively), Figure (5). Broccoli extract exhibited significant reduction in estrogen level of protective and therapeutic groups (61.35 ± 2.31 and 40.87 ± 1.67 ng/ ml, respectively) in comparison with estrogen level of

cancer control (105.14 ± 3.21 ng/ ml). On the other hands, administration of broccoli extract, either as a protective or a therapeutic agent, showed a significant augment on the progesterone level (3.17 ± 0.74 and 3.76 ± 0.67 ng/ml, respectively) in respect to cancer control (2.45 ± 0.40 ng/ ml). The depletive effect of the extract on estrogen appeared on the positive control animals also. Whereas, progesterone level did not change significantly, in comparison with negative control.

DISCUSSION

This study was conducted to evaluate the possibility of using *Brassica oleracea* L. var. *italica* inflorescence crude extract to treat chemically induced-breast cancer in rats. Three strategies were followed in this work to illustrate the anti-breast cancer activity of Broccoli and expecting its mechanism which include reduction oxidative stress, regulation of inflammatory enzymes (COX-1 and COX-2), inhibition of rate cancer growth limiting enzymes (aromatase and Na^+ , K^+ ATPase). Oxidants are able to produce mutagenesis through stimulating the cell division. When a cell with a damaged DNA divides, cell metabolism and duplication become disrupted, resulting increment in mutation, which is the important factor in carcinogenesis. Antioxidants are responsible for the defense system of cell against reactive species. Antioxidants are divided into three types included endogenous antioxidants, natural antioxidants and synthetic antioxidants. The endogenous antioxidants involve non-enzymatic antioxidants as glutathione, α -lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, L-carnitine, melatonin and enzymatic antioxidants as glutathione peroxidases (GPxs), glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT). These antioxidants can be decreased oxidative damage to DNA and inhibiting any abnormal cell division (Menon et al. 2016). Concerning our study, administration of broccoli extract, either as protective agent or as therapeutic has anti-oxidative stress evident in a remarkable elevation in the glutathione concentration and enzymatic activities (GR, GST, GPx, CAT and SOD) in respect to breast cancer control. Therefore, anti-breast cancer activity of Broccoli crude extract may be attributed to its antioxidant activity. Tumorigenesis process associate with over production of cyclooxygenase (COX)-2 enzyme that lead to over production of prostaglandins

(PGE₂). PGE₂ can directly promote cancer cells by inhibiting apoptosis and enhance the cell migration. Also, PGE₂ increase cyclic AMP (cAMP) production and growth of the cell, as well as it induces cAMP-dependent protein kinase A (PKA) which in turn regulates the differentiated cell functions. The PKA-mediated phosphorylation of cAMP-responsive element binding protein (CREB) and regulation of the transcription through interaction cAMP-response elements and CREB are considered the main pathways that change gene expression in the cancer cells (Crusz and Balkwill 2015, & Liu et al. 2005). Activating transcription factor 4 (ATF-4) organizes the genes expression contributory in oxidative stress, amino acid synthesis, differentiation, metastasis, and angiogenesis. The expression of ATF-4 is enhanced by different external stimuli in the cancer microenvironment and controls in several processes that would control in the cancer progression (Ameri and Harris 2008). This work demonstrated that, the Broccoli extract blocked COX-2 enzyme when it used as a protective or as a therapeutic agent. The Broccoli anti-breast cancer ability may be due its COX-2 inhibitory activity.

The estrogen hormone has promoting effect on the proliferation concurrent with suppressive effect on the apoptosis. Apoptosis is the highly coordinated process of cell death. A wide variety of stimuli can be initiated the apoptosis process like the developmental signals, the cellular stress and disruption of the cell cycle. Breast cancer differs than most cancer types in being under the hormonal control. Estrogen has a promoting effect on the progression of breast cancer ERs1 through two pathways: affecting the cell cycle and inducing specific growth factors and their receptors. In addition, estrogen prevents the induction of apoptosis through changing the expression of the Bcl-2 family of proteins. When MCF-7 breast cancer cells treated with estrogen, pro-apoptosis Bax was decrease and anti-apoptosis Bcl-2 mRNA and protein were increased (Wang et al. 2001). Whereas, estrogen inhibitors or anti-estrogen treatment promote apoptotic cell death in MCF-7 tumors. In MCF-7 cell line, the depletion of estrogen resulting induction of apoptosis and inhibition of proliferation concurrent with over expression of estrogen receptors, p27 and p21 protein levels, and a reduction in Bcl-2, cyclin D1, and Rb protein expression (Cimpean et al. 2016).

Tamoxifen, the anti-estrogen reference drug, has been outperformed chemotherapy in the

estrogen receptor-positive breast cancer postmenopausal patients. Unfortunately, the Tamoxifen resistance is developed through its partial agonist properties. Aromatase inhibitors are a new class of drugs that prevent estrogen synthesis through blocking the aromatase enzyme and do not have estrogenic effect. Letrozole, the aromatase inhibitor reference drug is to be more effective than Tamoxifen, either in an animal's breast cancer model or in patients. Letrozole drug causes retreating in the MCF-7 human breast cancer cells associated with aromatase gene (MCF-7Ca) (Chumsri et al. 2011).

Aromatase inhibitors and anti-estrogens induce disruption in the cell cycle progression and activation of apoptosis. They have anti-proliferative effects in the cell cycle profile of MCF-7Ca cells. They increased the percentage of cells in the G0-G1 phase of the cell cycle and reduce the fraction of cells in the S and G2-M phases. After three days of Letrozole treatment, cells in the G0-G1 phase were increased by about 83.42%, especially sub-G1 phase corresponding to 49.70% in the control, while the cell number in S phase and G2-M phase was significantly reduced (Mourindsen et al. 2001).

According the above mentioned theory, aromatase inhibitors are considered as chemo preventive agents that significantly contribute in monitoring, prevention and treating mammary cancer. In our study, Broccoli extract showed an aromatase inhibitory effect that may be attributed to its anti-breast cancer ability.

It is evident that reported role of ion channels and pumps in cell proliferation, migration, apoptosis and differentiation. Recently, it is known that the cancer progression is associated with both ion channels and ion pumps. The P-type ATPase pumps: SERCA and the Na^+/K^+ ATPase pumps are the main pumps that associate with the cancer. The Na^+/K^+ ATPase is consisted of a catalytic α -subunit with ten trans-membrane segments and a heavily glycosylated β -subunit. The pump is responsible for preservation of physiological electrochemical gradient which is important for cell survival and for several cell functions. In glioblastoma cells, Na^+/K^+ ATPase is intensively expressed (Litan and Langhans 2015). Specific Na^+/K^+ ATPase inhibitors, Digoxin and Ouabain, reduced proliferation in glioblastoma cell lines concurrence with increment in the apoptotic (Lange and Yee 2008). Broccoli crude extract recorded a significant reduction in Na^+/K^+ ATPase pump when used as protective or as therapeutic agent, indicating that broccoli extract may exert its

anti-breast cancer activity as one of Na^+/K^+ ATPase inhibitor.

Progesterone is an ovarian steroid hormone found in several tissues as the brain, breast and reproductive organs. Estrogen induces expression of the progesterone receptors in breast tissue. Hence, progesterone is dependent on the estrogen to mediate lobulo-alveolar development. Postmenopausal hormone replacement therapy; Progestins combined with estrogen as a means to block estrogen-induced endometrial growth. There is no doubt the role of estrogen as a potent breast mitogen, hence estrogen receptor inhibitors and estrogen-producing enzymes (aromatases) are the first-line cancer therapies. However, progesterone action in breast cancer; it acts as a promoter or an inhibitor is grossly understudied and remains controversial (Higdon et al. 2007). Although, the combination natural progesterone with synthetic progestins (like medroxyprogesterone acetate) showed a reduction in the breast cancer risk, they significantly elevated when the same drug combination with estrogen in postmenopausal women was done.

17β -estradiol the endogenous estrogen metabolizes into 16α -hydroxyestrone (16α -OHE1) or 2-hydroxyestrone (2OHE1). Unlike 2OHE1, 16α -OHE1 has a high estrogenic effect, it promotes the proliferation of estrogen-sensitive breast cancer cells in culture. Therefore, when metabolism 17β -estradiol shifted to 2OHE1 and not metabolize to 16α -OHE1 could be declined the risk of estrogen-sensitive cancers, such as breast cancer. When postmenopausal women increased cruciferous vegetable intake for one month elevated urinary ratio of 2OHE1: 16α -OHE1, indicating that cruciferous vegetables can modify estrogen metabolism towards 2OHE1. Recently, many studies found that the increasing cruciferous vegetable intake was significantly lower breast cancer in women (Higdon et al. 2007).

In the recent study, anti-breast cancer effect was associated with the Broccoli treatment was evident by a remarkable decrease on the estrogen level and a significant increase on the progesterone of DIMBA- induced breast cancer in animals. The anti-estrogenic effect of Broccoli may be the exact cause of its anti-breast cancer ability.

Generally, from mentioned results could be deduced the potential role of the Broccoli crude extract as an inhibitory or recovery agent for breast cancer progression. Broccoli extract anti-

breast cancer efficacy may be attributed to its ameliorative effect on the body oxidant status, selective inhibitory effect on COX-2, inhibition of Na^+/K^+ ATPase and aromatase concurrently with the withdrawal of carcinoembryonic antigen level estimated in animal sera. The ability of Broccoli extract to exert these important effects may be attributed to its highly content from glucosinolates and sulfur compounds. Broccoli, a Cruciferous vegetable, is rich with glucosinolates and sulfur-containing compounds. Broccoli is also rich in glucoraphanin and glucobrassicin which were the glucosinolates precursor of sulforaphane and indole-3-carbinol (Higdon et al. 2007). Indoles and isothiocyanates, the compound found after broccoli preparation (chewing, drying, extraction and digestion), have been proved as inhibitor for development of many types of cancer involved the breast cancer. Indoles and isothiocyanates appeared several potential ways might be to prevent cancer progression, including inhibit of DNA damage, an anti-inflammatory effect, induce apoptosis, and inhibit tumor blood vessel formation and tumor cell migration. Epidemiological studies of the Egyptian National Cancer Institute have shown that people who eat a diet rich in broccoli have a lower risk of some cancers. In addition, sulforaphane compounds found in broccoli with high concentration has been shown to reduce the number of acute lymphoblastic leukemia cells in the lab setting and have both preventive and therapeutic properties in solid tumors (Owis Al. 2015)

CONCLUSION

In conclusion, our *in vitro* and *in vivo* studies clearly elucidated that *Brassica oleracea* L. var. *italica* crude extract could prevent and treat breast cancer with induction of apoptosis through antioxidant and anti-inflammatory activities as well as aromatase and Na^+/K^+ ATPase inhibitory properties. Broccoli has a good margin of safety represented as no toxicity symptoms were appeared in this investigation

CONFLICT OF INTEREST

The authors declare that all researchers familiar with this work contributed in all this work items without conflicts

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AUTHOR CONTRIBUTIONS

ER and AY designed and performed the experiments and also wrote the manuscript. AY, SA performed animal treatments, AM collect the tissue and data analysis. WA prepared the extract. All authors read and approved the final version.

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