Inhibition of NF-κB, Bcl-2 and COX-2 Gene Expression by an Extract of *Eruca sativa* Seeds during Rat Mammary Gland Carcinogenesis

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Abstract

The effect of *Eruca sativa* seed extract (SE) on nuclear factor kappa B (NF-κB), cyclooxygenase-2 (COX-2) and B-cell lymphoma-2 (Bcl-2) gene expression levels was investigated in rat mammary gland carcinogenesis induced by 7,12 dimethylbenz(a)anthracene (DMBA). DMBA increased NF-κB, COX-2 and Bcl-2 gene expression levels and lipid peroxidation (LP), while, decreased glutathione-S-transferase (GST) and superoxide dismutase (SOD) activities and total antioxidant concentration (TAC) compared to the control group. After DMBA administration, SE treatment reduced NF-κB, COX-2 and Bcl-2 gene expression levels and LP. Hence, SE treatment reduced inflammation and cell proliferation, while increasing apoptosis, GST and SOD activities and TAC. Analysis revealed that SE has high concentrations of total flavonoids, triterpenoids, alkaloids and polyphenolic compounds such as gallic, chlorogenic, caffeic, 3,4-dicaffeoyl quinic, 3,5-dicaffeoyl quinic, tannic, cinnamic acids, catechin and phloridzin. These findings indicate that SE may be considered a promising natural product from cruciferous vegetables against breast cancer, especially given its high antioxidant properties.

Keywords: DMBA - breast cancer - *Eruca sativa* - NF-κB - bcl-2 - COX-2 - qRT-PCR

Introduction

Breast cancer is the second most common cancer in the world. The most common types of breast cancer are ductal and lobular carcinoma (Zeeneldin et al., 2013). Environmental toxic agents especially polycyclic aromatic hydrocarbons (PAHs) are considered risk factors for breast cancer. PAHs result from incomplete combustion of coal, oil, petrol, wood, tobacco, charbroiled meats, garbage and other organic materials. Some of them are used in the industries of dyes, plastics and pesticides (Reynaud and Deschaux, 2006). They have carcinogenic and mutagenic effects since they alter the function of cell membranes and the associated enzyme systems. The most extensively studied PAHs are 7, 12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene. DMBA is an immunosuppressor and a powerful organ-specific laboratory carcinogen, it serves as a tumor initiator (Saha and Hait, 2012).

Several studies have suggested that phytochemical products could be a useful strategy to prevent the deleterious effects of xenobiotics including carcinogens and mutagens through the modulation of genes which control cell proliferation, cell cycle, apoptosis, signal transduction, oncogenesis and transcription regulation (Shaban et al., 2013). Rocket plant (*Eruca sativa*) is a member of the Brassica plant family. Eruca sativa has a wide spread medicinal use. It is used in Unani and Arab traditional medicine as a stomachic, diuretic, stimulant and for the treatment of scurvy. Epidemiological and experimental data provide strong evidence that Brassicaceae species and cruciferous vegetables inhibit tumorigenesis. Like other cruciferous vegetables, the preliminary phytochemical studies of rocket seeds revealed the presence of carotenoids, flavonoids, phenolics, vitamins and glucosinolates which metabolized into isothiocyanates (Melchini and Traka, 2010).

Nuclear factor kappa B (NF-κB) is a nuclear transcription factor that regulates the expression of various genes, such as B-cell lymphoma-2 (Bcl-2) and cyclooxygenase-2 (COX-2), that involved in cell proliferation, adhesion, angiogenesis, apoptosis, cytoprotection, carcinoge-nesis, and inflammation. The activation and regulation of the expression of these gene products leading to cell cycle arrest, suppression of proliferation and induction of apoptosis (Escarcega et al., 2007). Apoptosis represents a physiological process for removing damaged or infected cells and maintaining tissue...
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During tumorigenesis, cancer cells develop the ability to avoid apoptosis through the upregulation of anti-apoptotic proteins and/or downregulation of proapoptotic signalling pathways. Bel-2 family of both pro- and antiapoptotic proteins are central regulators of apoptosis (Yip and Reed, 2008). COX-2 is an inducible enzyme, stimulated by cytokines, growth factors, oncogenes or tumour promoters during inflammation or malignancy. Overexpression of COX-2 has been detected in a number of tumors, such as breast, lung, pancreatic and colorectal cancers (Sobolewski et al., 2010).

The present study is to investigate the therapeutic effect of Eruca Sativa seed ethanolic extract against DMBA-induced mammary gland carcinogenesis through the study of gene expression of NF-κB, COX-2 and Bel-2, as well as lipid peroxidation and antioxidant parameters. In addition, histopathological examination of breast and profile tests of liver and kidney were determined. Moreover, the characterization of Eruca Sativa seed ethanolic extract was studied.

Materials and Methods

Eruca sativa seeds extract (SE) preparation

Three kilograms of Eruca sativa seeds obtained from a market (Alexandria, Egypt) were grained. Grained seeds were soaked in 95% ethanol in an incubator shaker (150 rpm) at 30°C for two days (Harborne, 1988). The extract was concentrated to dryness using rotary evaporator (Heidolph) to yield dried ethanolic extract, which was 12.89% (~386.7 g) of the starting material. Then the ethanolic extract was converted to powder using lypholizer (TELSTAR, Cryodost), which was 11% (~330 g) of the starting material.

Characterization of SE

Qualitative phytochemical analysis. Phenols and tannins, flavonoids, saponins, glycosides, steroid, terpenoids and alkaloids were detected in SE which dissolved in water (1:1 w/v) (Harborne, 1973; Trease and Evans, 1989; Sofowora, 1993).

Quantitative phytochemical analysis. The ethanolic extract was analyzed for total phenolic contents (Taga et al., 1984), HPLC (phenolic compounds), total flavonoid contents (Zhishen et al., 1999), total triterpenoids (Bai et al., 2007), total alkaloids (Fazel et al., 2008) and total antioxidant capacities (Tyagi et al., 2010). Moreover, anti- lipid peroxidation assay (Oikawa et al., 1979), reducing power (Oyaizu, 1986) and diphenyle–α-picrylhydrazyl (DPPH) radical scavenging assay (Amarowicz et al., 2000) were evaluated.

Experimental animal model

Twenty-eight female Sprague–Dawley rats (weighing about 80-100 g at age 40 days) were obtained from Misr University for Science and Technology, Cairo, Egypt. Rats were examined for health status, housed and handled under ethical conditions according to the international rules of animal care. Rats were divided into four groups (seven rats/group). Experimental animal design was as follows: Control (C) group (administered orally with corn oil), DMBA (D) group (administered with DMBA dissolved in corn oil, 20 mg/100 g body weight) (Carroll et al., 2010), DMBA and SE (D-SE) group (administered with DMBA as group D and then the rats were treated orally with SE (500 mg/kg body weight/day for four weeks)) (Behrens and Karber, 1970) and SE (SE) group (administered orally with SE only (500 mg/kg body weight/day for four weeks)). Rats were palpated every day for mammary tumors. The cumulative number of tumor masses was calculated by each day’s palpation of rats. Tumor volume (V) was determined by the following equation: \[ V = (L \times W^2)/2 \] where L is the length and W is the width of the tumor. During treatment, animals were observed daily for signs of morbidity and mortality. Body masses were recorded initially, once weekly and at the end of the experimental period. At the end of the experimental period, feeding was stopped 12 h before dissection, then the rats were sacrificed and the mammary tissues of each animal were dissected and immediately put in RNA later and kept at -80°C for the molecular study analysis.

Quantitative real time polymerase chain reaction (qRT-PCR)

RNAs extraction. From mammary gland rat’s tissue, total RNA was isolated using Gene JET RNA Purification Kit (Thermo Scientific, USA). The extracted RNAs from different samples were quantified and qualified (purity) using NanoDrop Spectrophotometer. Finally, total RNAs samples were normalized (same concentration) to avoid any false increase in gene expression levels.

qRT-PCR. Using SYBER Green 1-step qRT-PCR Kit (Thermo Scientific, USA), gene expression of BCI-2, NF-κB and COX-2 (target genes) and β-actin (reference gene) were quantified by Real-Time PCR System (Thermo Scientific PikoReal) with the use of specific primers sequences (Forward/Reverse) 5’-GGTGGCACCCTGTGGCCACCTG-3’/5’-CTTCACTTTGGGCCCAGATAAGG-3’ for BCI-2 gene (Saleha, 2010), 5’-CTGGCACTTCTTCTCAAAAGC-3’/5’-CCAGGTGACAGAGGGCTCAAA-3’ for NF-κB gene (Yar et al., 2011), 5’-CTGATCCCGCCCGGCTCTGGTG-3’/5’-CTTCACTTGTGGCCCAGATAGG-3’ for COX-2 gene (Rani and Kansal, 2011) and 5’-GTGGGCCTGCCTAGGCAACCAA-3’/5’-CTCTTGTAGTGCAGCAGCAATTC-3’ for β-actin gene (Todorova et al., 2006; Saleha, 2010). qRT-PCR was performed in a reaction mixture of 10 μl using 0.1 μl versus enzyme mix, 5 μl 1-step QPCR SYBER mix (1X), 0.5 μl RT-enhancer, 0.5 μl forward and reverse primers (10 pm), 0.2-9.9 μl water (PCR grade) and 0.5-3.4 μl RNA template (50 ng). qRT-PCR program was applied as one cycle of cDNA synthesis at 50°C for 15 min, one cycle of Thermo-start enzyme activation at 95°C for 15 min and followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 1 min and extension at 72°C for 30 sec.

Antioxidant and oxidative stress profiles

One gram of the mammary gland tissue was
homogenized in 5 volumes of cold 0.1 M sodium phosphate buffer saline (PBS), pH 7.4 using Teflon glass homogenizer at 4°C. The total homogenate was centrifuged using cooling centrifuge at about 7000 rpm for 20 min at 4°C. The supernatant (MH) was stored at -20°C until used for the determination of malondialdehyde (MDA) level, superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities (Nishikimi et al., 1972; Habig et al., 1974; Ohkawa et al., 1979). Moreover, total antioxidant concentration (TAC) was determined colorimetrically in serum (Nishikimi et al., 1972).

Liver and kidney profile tests

Alanine aminotransferase (ALT) activity, albumin, protein, urea and creatinine levels were determined in serum (Gornall et al., 1949; Reitman and Frankel, 1957; Doumas et al., 1971).

Histopathological studies

Mammary tissues were fixed in 10% formalin for 24 hour, washed with running water, dehydrated in ascending grades of alcohol (70%, 80%, 95% and absolute alcohol) and cleaned by immersion in xylene followed by impregnation in melted paraffin wax in oven at 60°C for 1 hr. The specimens were embedded in paraffin and were left to solidify at room temperature. Using a rotary microtome, sections of 5µm thick were cut and were mounted on clean glass slides. Finally, the samples were stained with conventional hematoxylin and eosin (H&E) stain for examination under light microscope for any histopathological changes.

Statistical analysis

Statistical analyses were performed using the statistical software, SPSS (Version 13). Results were presented as means (X) ± standard deviation (SD).

Figure 1. SE Characterization (A). HPLC analysis of some phenolic compounds in SE (B). Anti-lipid peroxidation activity of SE (C). Reducing power of SE (D). DPPH radical scavenging activity of SE

Figure 2. Tumor Incidence, Volume and Body Masses of Studied Groups (A). Tumor incidence of rats during the experimental period (B). Tumor volume of rats during the experimental period (C). Body masses of rats during the experimental period. Where, a: significant with the C group, b: significant with the D group, at p < 0.05. Group C: Control group; group D: rats injected with DMBA; group D-SE: rats injected with DMBA and then treated with SE; group SE: rats treated with SE only
Results

SE characterization

The qualitative analysis showed that SE contains phenols, tannins, flavonoids, saponins, glycosides, steroid, terpenoids and alkaloids. On the other hand, the quantitative analysis showed that SE contains total phenolic, flavonoid, alkaloid, triterpenoid compounds and total antioxidant concentration (TAC) with concentrations 0.15 mg/g SE as gallic acid equivalents, 0.61 mg/g SE as catechin equivalents, 0.04 mg/g SE as berberine equivalents, 0.73 mg/g SE as ursolic acid equivalents and 0.14 mg/g SE as ascorbic acid equivalent, respectively. HPLC analysis of some phenolic compounds in SE revealed the presence of gallic acid, chlorogenic acid, caffeic acid, 3,4-dicaffeoyl quinic acid, 3,5-dicaffeoyl quinic acid, catechin, phloridzin, tannic acid and cinnamic acid with concentration 4.254, 4.39, 19.59, 2.12, 11.96, 28.37, 0.0011, 0.527 and 0.00268, respectively as mg/g SE (Figure 1A). The results showed that SE has anti-lipid peroxidation, reducing power and DPPH radical scavenging activities (Figure 1B, C and D).

Tumor incidence and its volume

Tumor incidence was significantly increased by 100% at day 120 after DMBA injection A (Figure 2A). Also, the tumor volume in DMBA (D) group was significantly increased by 100% compared to the control group (C). The results showed that the treatment with SE after DMBA (D-SE group) decreased the tumor volume significantly by 99.9% compared to the D group (Figure 2B).

Body masses of rats

Body masses of the rats at the day 120 (100% tumor appearance) were decreased significantly by about 18.4% and 16.2% in D and D-SE groups, respectively (Figure 2C). While in SE group, body masses had a non-significant change compared to the C group. At the day

Figure 3. Genes Expression of Nf-κb, Bcl-2 and COX-2 of Studied Groups. (A). Gene Expression level of Nf-κb during the experimental period (B). Gene Expression level of Bcl-2 during the experimental period (C). Gene Expression level of COX-2 during the experimental period. Where, a: significant with C group, b: significant with D group, at p < 0.05. Group C: Control group; group D: rats injected with DMBA; group D-SE: rats injected with DMBA and then treated with SE; group SE: rats treated with SE only

Figure 4. Alterations in the Levels of Lipid Peroxidation and Antioxidants of Studied Groups. (A) SOD activity (B) GST activity (C) Total antioxidant concentration (D) MDA levels. Where, a: significant with C group, b: significant with D group, at p < 0.05. Group C: Control group; group D: rats injected with DMBA; group D-SE: rats injected with DMBA and then treated with SE; group SE: rats treated with SE only
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160 (after treatment by SE), body masses were decreased significantly by about 19% in D group compared to the C group. On the other hand, body masses were increased significantly by about 7% in D-SE group compared to the D group. While in SE group, body masses had a significant increase by about 19% compared to the C group.

Gene expression of NF-κB, Bcl-2 and COX-2

The expression levels of NF-κB, Bcl-2 and COX-2 genes in D group were significantly increased by about 341%, 261% and 603%, respectively compared to the C group. While, the expression level in D-SE group was significantly decreased by about 99%, 75% and 100% respectively compared to the D group. The expression level of NF-κB, Bcl-2 and COX-2 genes in SE group was significantly decreased by about 97%, 82% and 57%, respectively compared to the C group (Figure 3A, B and C).

Antioxidant and oxidative stress profiles

SOD activity was decreased significantly in D group by about 66% compared to the C group. Whereas its activity

Table 1. Liver and Kidney Profile Functions

| Test            | C          | D          | D-SE       | SE
|-----------------|------------|------------|------------|------------|
| Albumin (g/dl)  | 3.87 ± 0.51| 2.40 ± 0.36a| 4.04 ± 0.14b| 4.90 ± 0.69a
| % change from C | 0          | -38        | 5          | 27         |
| % change from D | --         | 0          | 69         | --         |
| Total protein (g/dl) | 6.95 ± 1.31| 5.45 ± 0.27a| 6.81 ± 0.48b| 6.96 ± 0.26|
| % change from C | 0          | -21        | -2         | 0.1        |
| % change from D | --         | 0          | 25         | --         |
| ALT (U/ml)      | 30 ± 1.91  | 34 ± 5.45  | 31± 3.43   | 30 ± 0.00  |
| % change from C | 0          | 13         | 3          | 0          |
| % change from D | --         | 0          | -9         | --         |
| Creatinine (mg/dl) | 0.69 ± 0.39| 0.91 ± 0.02a| 0.56 ± 0.37b| 0.58 ± 0.16a|
| % change from C | 0          | 32         | -19        | -17        |
| % change from D | --         | 0          | -38        | --         |
| Urea (mg/dl)    | 26 ± 5.5   | 48 ± 3.3a  | 21 ± 5.6b  | 20 ± 2.0a  |
| % change from C | 0          | 87         | -19        | -22        |
| % change from D | --         | 0          | -57        | --         |

*Values are expressed as mean ± S.D. for six rats. Where, a: significant with C group, b: significant with D group, at p < 0.05. The positive values in the % change mean there was an increase and the negative values mean there was a decrease. Group C: Control group; group D: rats injected with DMBA; group D-SE: rats injected with DMBA and then treated with SE; group SE: rats treated with SE only. ALT: Alanine aminotransferase

Figure 5. Histological Examination of Mammary Gland Tissue of Studied Groups. (A) C group (H&E, x400) Examination of this group showed a dense collagenous tissue, blood vessels and islands of glandular tissue surrounded by dense fibrous and adipose tissue. The breast acini and tubules (arrows) are lined by inner epithelial layer (with dense cytoplasm) and outer myoepithelial layer (with clearer cytoplasm) (B) D group (H&E, x200): Administration of rats with DMBA revealed a neoplastic growth made up of trabeculae, ducts, and nests of malignant ductal cells (arrows) having rounded to ovoid vesicular nuclei with prominent nucleoli and indistinct cell borders. The cytoplasm is abundant and eosinophilic. Mitotic figures are discerned. The stroma is desmoplastic and infiltrated by lymphocytic infiltrate mainly lymphocytes. Areas of necrosis are identified as well as foci of carcinoma in situ, whether the comedo form or the solid form. Foci of dystrophic calcification are noted in some sections (C) D-SE group (H&E, x100): In addition examination of mammary gland tissues of rats which treated with SE after DMBA administration revealed occasional nests of viable tumor cells left (arrows) (D) SE group (H&E, x100): Administration of SE alone showed normal branched breast ducts (arrows) with normal distribution of fat tissue as control.
in D-SE group was increased significantly by about 235% compared to the D group. After SE administration SOD activity was increased significantly by about 21% compared to the C group (Figure 4A). GST activity was decreased significantly in D group by about 42% compared to the C group. While its activity in D-SE group was increased significantly by about 112% compared to the D group. SE administration significantly increased GST activity by about 54% compared to the C group (Figure 4B). TAC was decreased significantly in D group by about 37% compared to the C group. While its activity in D-SE group was increased significantly by about 109% compared to the D group. SE administration caused a non significant increase by about 17% compared to the C group (Figure 4C). The level of MDA of D group was increased significantly by about 275% compared to the C group. While its level in D-SE group was decreased significantly by about 37% compared to the D group. SE administration caused a non significant decrease by about 1.25% compared to the C group (Figure 4D).

Liver and kidney profile functions

Albumin and protein levels were significantly decreased in D group compared to the C group, while, their levels in D-SE group were significantly increased compared to D group. SE administration increased albumin level (significantly) and protein level (non significantly) compared to C group. ALT activity was nonsignificantly increased in D group as compared to C group, while, its level was decreased as compared to D group. SE administration had no affect on ALT activity as compared to C group. Creatinine and urea levels in D group were significantly increased compared to C group, while, their levels in D-SE group were significantly decreased compared to D group. SE administration significantly decreased creatinine level but nonsignificantly increased urea level compared to C group (Table 1).

Histological examination of mammary gland tissues

Examination of the C group showed a dense collagenous tissue, blood vessels and islands of glandular tissue surrounded by dense fibrous and adipose tissue. The breast acini and tubules are lined by inner epithelial layer with dense cytoplasm and outer myoepithelial layer with clear cytoplasm (Figure 5A). Administration of rats with DMBA (D group) revealed a neoplastic growth made up of trabeculae, ducts, and nests of malignant ductal cells having rounded to ovoid vesicular nuclei with prominent nucleoli and indistinct cell borders. The cytoplasm was abundant and eosinophilic. Mitotic figures were discerned. The stroma was desmoplastic and infiltrated by lymphocytic infiltrate mainly lymphocytes. Areas of necrosis were identified as well as foci of carcinoma in situ, whether the comedo form or the solid form. Foci of dystrophic calcification were noted in some sections (Figure 5B). In addition, examination of mammary gland tissues of rats which treated with SE after DMBA administration revealed proliferated dilated breast ducts embedded into the surrounding fat tissue with no residual tumor tissue could be identified (Figure 5C). Administration of SE alone showed normal branched breast ducts with normal distribution of fat tissue as control (Figure 5D).

Discussion

In the present study, the results showed that the SE contains many antioxidant compounds. These compounds are phenolics, flavonoids, alkaloids and triterpenoid. HPLC analysis of phenolic compounds demonstrated the presence of gallic, chlorogenic, caffeic, 3,4-dicaffeoyl quinic, 3,5-dicaffeoyl quinic, catechin, phloridzin, tannic and cinnamic acids. Previous studies reported that SE also contains glucosinolates, carotenoids, flavonoids (quercetin, kaempferol and isohamnetin), vitamin C, thiocyanates, isothiocyanates, glucorucin, erysolin, sulforaphane, phenylethyl isothiocyanates and 4-methylthiobutyliso-thiocyanate (Melchini and Traka, 2010). All these compounds have an antioxidant activity.

The results of the present study also revealed that DMBA administration increased tumor incidence and tumor volume. Histologically, there was a neoplastic growth consisting of atypical pleomorphic epithelial cells with large hyperchromatic overlapped nuclei and dilated ducts containing inspissated secretions, a picture of adenocarcinoma. There was high invasion of tumor cells to the surrounding and adjacent stroma beside destruction of the cells. The biochemical results showed that MDA level was increased but GST, SOD activities and TAC were decreased in mammary gland tissues as compared to the C group. This indicates that DMBA increased lipid peroxidation (LP), inhibited antioxidant enzymes and induced oxidative stress (OS) as it generated some free radicals (FRs) such as epoxides and quinones which are metabolites of DMBA. These FRs could bind covalently to nucleophilic sites on cellular macromolecules leading to peroxidation of native membrane lipids (Farmer and Mueller, 2013). DMBA increased the gene expression levels of NF-κB, COX-2 and Bcl-2 compared to the C group, so, it induced proliferation and inflammation but suppressed apoptosis in the breast tissues. The elevation in NF-κB expression level may be due to either mutation in genes encoding the NF-κB transcription factors themselves or in genes that control NF-κB activity such as IκB genes. Also, some tumor cells secrete some factors (cytokines, FRs and oxidized LDL) which activate NF-κB. FRs, epoxides and quinones may activate IκB kinase which phosphorylates IκB. The phosphorylated IκB is ubiquitinated and degraded by the proteasome system leading to translocation of NF-κB into the nucleus inducing the expression of more than 200 genes (Wu and Kral, 2005). Blocking of NF-κB causes tumor cell death, prevents their proliferation and increase their response to treatment (Escarcega et al., 2007). The elevation in COX-2 expression level may be due to the enhancement of transcription of the COX-2 gene by NF-κB. This concur with previous studies which showed that COX-2 over-expression is observed in 40% of breast cancers (Wu and Kral, 2005). Also, PGH2 (a product of COX-2) is converted by prostaglandin E2 synthase to PGE2, that modulates cell proliferation, cell death, and tumor invasion in many types of cancer such as lung, breast and colon (Sobolewski et al., 2010). Moreover, increasing
Moreover, glucoerucin and isothiocyanates (components of SE) inhibit the negative effects of OS, erucin and erysolin, glucoerucin and isothiocyanates. Previous studies showed that gallic, caffeic, tannic acids, Fenton reaction, inhibit the N-nitrosation reaction and stabilize unpaired electrons and scavenge ROS, terminate toxicity. Indicating the therapeutic role of SE against DMBA level with a significant decline in ALT activity in serum caused a significant elevation in total protein and albumin. DMBA improved the body masses at the day 160 and inhibition of angiogenesis and metastasis (Melchini and Traka, 2010). These results are in agreement with previous studies concerning about treatment with SE after carbon tetrachloride (Alam et al., 2007). However, SE considers a promising natural product from cruciferous vegetables against breast cancer; especially our results confirmed that SE has high antioxidant and anti-carcinogenic properties.

References


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