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The possible protective effect of pumpkin seed extract on mammary carcinoma in rats: An experimental study

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ABSTRACT

Objective: To evaluate the potential protective effect of pumpkins' seed extract on oxidative stress and cyclin D1 expression associated with mammary gland carcinoma in rats.

Design: Randomized controlled experimental study.

Animals: Forty female Sprague Dawley rats.

Procedures: Rats were allocated equally to four groups (10 rats each); group 1 (control group); group 2 received 7, 12 dimethylbenzanthracene (DMBA) subcutaneously in the mammary region to induce carcinoma. Group 3 received pumpkin seed extract at 300 mg/kg body weight orally, and group 4 was treated with both pumpkin seed extract and DMBA. Animals were euthanized after 8 weeks of treatment, and tissues from mammary gland were collected and divided into three portions. The first portion was used to determine antioxidant and oxidative stress markers; the second one was stored in RNA for later estimation of Cyclin D1 expression, and the last portion was stored in neutral buffered formalin (10%) for histopathological examination.

Results: Levels of Nitric oxide, Malondialdehyde and Reduced Glutathione, as well as activity of Glutathione-S-transferase and superoxide dismutase (SOD) showed a significant decline in rats supplemented with pumpkin seed extract and subjected to induced mammary carcinoma in comparison with diseased non-supplemented rats ($P < 0.05$). In addition, there was a down-expression in cyclin D1 expression in rats supplemented with pumpkin seed extract.

Conclusion and clinical relevance: Pumpkins' seed extract can alleviate the oxidative stress and cyclin D1 expression associated with experimentally induced mammary carcinoma in rats. Further studies are needed to get an evidence for the use of pumpkin seed extract in the clinical practice.

Keywords: Mammary carcinoma, Cyclin D1, Pumpkin seed.

1. INTRODUCTION

Mammary gland carcinoma is the most common malignancy among females and is the second most prevalent after lung cancer [1]. Medicinal plants are used as an alternative treatment for cancer [2]. There are more than 3000 plants worldwide have anticancer characteristics [3]. Some patients utilize herbal remedies without biomedical treatment, or in combination with anticancer treatments [4].

Some selected medicinal plants for cancer prevention and therapy; *Crocus Sativus* (Saffron) is a small perennial plant of oriental origin. There are studies that indicate the potentiality of saffron extracts on inhibiting tumorigenesis and the delay of tumor development in a several of experimental *invivo* and *invitro* systems [5]. *Vitex agnus-castus* (Chaste tree) is native of the Mediterranean region. *Vitex agnus-castus* fruits (VACF) has anti-tumor effects in human cancer cell lines [6]. *Withania Somnifera* (Ashwagandha) is a short hairy shrub and grows in semi-tropical and temperate regions. The major active consists are alkaloids and steroidal lactones (withaferin A). In mice, it has been shown that withaferin A may have anti-metastatic effect [7].

Pumpkin is used as a processed food worldwide [8]. Pumpkins are gourd squashes of the genus "*Cucurbita*" and the family "*Cucurbita ceeae*". Based on the texture and form of stem, the pumpkin species are categorized to *Cucurbita ceeaepepo*, *Cucurbita ceeamoschata*, and *Cucurbitaceae maxima* [9]. Pumpkin's seed contains a high level of phytoestrogens [10]. The mechanism of action of phytoestrogens is through attaching to estrogen receptors (ERs) because its structure is similar to estradiol, and they have an anti-proliferative effect on tumor cells [11]. The aqueous extract of pumpkin seed has a high concentration of phenolic ingredients, which are considered more effective scavengers of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals [12]. It has also been used as anti-inflammatory [13], antioxidant [14], anti-ulcer [15], urinary disinfectant [16], antidiabetic [17], anti-tumor [18], and antibacterial [19].

Cyclin D1 protein encoded CCND1 gene, is situated on chromosome 11q13 [20]. Many lines of evidence point to a critical function for cyclin D1 in breast cancer production. The cyclin D1 gene is enlarged in up to twenty percent of human breast cancers [21], while cyclin D1 protein is greatly expressed in over fifty percent of human carcinoma [20, 22]. The overexpressed cyclin D1 is found in all histological types of human breast cancer [20]. It can

be found at the early stages of breast cancer progression, as ductal carcinoma in situ, but not in pre-malignant lesions [23]. Many trials have indicated a relationship among 11q13 enlargement and biomarkers of phenotype as (estrogen receptors) status. A positive relation between cyclin D1 mRNA and ER mRNA in primary breast cancer has been shown [24].

The aim of the current study was to evaluate the potential protective effect of pumpkins' seed extract on oxidant/antioxidant status and cyclin D1 expression in rats' model with mammary gland carcinoma.

2. MATERIALS AND METHODS

2.1. Chemicals

7, 12 Dimethyl Benzantracene, DMBA (Sigma Aldrich, UK) was used to induce mammary gland carcinoma. It was dissolved in an emulsion of sunflower oil (0.75 ml) and physiological saline (0.25 ml) [25]. To get pumpkin seed extract, seeds were air-dried and milled to a powder. The powder (50 g) was dissolved in 500 ml of distilled water. The mixture was then boiled for 45 minutes, filtered and the resultant extract was evaporated to dryness. The solid extract yield 6.5% (w/w) obtained was stored at 4 °C [26].

2.2. Animals and samples collection

This experiment was conducted on 40 female Albino Sprague Dawley rats (130±10 g). The rats were obtained from a local animal house in Giza governorate. They were housed in standard laboratory conditions and they had an access to water and commercial diet. Animals were allocated equally into 4 groups (10 rats each). Group 1 (control group) was fed on normal ration and received an injection of one ml of saline solution for four weeks. Group 2 (Cancer group), the rats of this group were injected subcutaneously with 7, 12 DMBA in the mammary region at a dose rate of 7.5mg/kg body weight, once a week for four consecutive weeks [25]. Group 3 (pumpkin-supplemented group), in which rats received an aqueous solution of pumpkin seed extract orally by gavage (300 mg/kg. bw) [27]. Group 4 (treatment group), the rats of this group was given an aqueous solution of pumpkin seed extract (300 mg/kg.bw) orally for four weeks, then were injected with 1 ml DMBA (7.5mg/kg.bw) subcutaneously in mammary region once a week for four consecutive weeks. At the end of the 8th week, the rats were euthanized under diethyl ether anesthesia. Mammary tissues were obtained and divided to three parts, the first part was homogenized in ice-cold PBS (pH 7.4), then the homogenate was centrifuged and stored at -20 °C for the measurement of the oxidative stress and antioxidant parameters [Nitric oxide (NO) [28], Malondialdehyde (MDA) [29], superoxide dismutase (SOD) activity [30], Reduced Glutathione (GSH) concentration [31], Glutathione-s-transferase (GST) activity [32]. The second part was stored in RNA for later estimation of Cyclin D1. However, the third part was kept in neutral buffered formalin 10 % for histopathological examination [33].

2.3. Gene expression of Cyclin D1

Total RNAs were extracted using RNA Mini Kit (RNeasy, 74104). Equivalents of 2 (µg) of RNA were transferred into cDNA, using instructional manual. cDNA was synthesized using high capacity cDNA Reverse transcription kit (Cat. No. EP0441) to act as a template for quantitative PCR by using Quantitect SYBR green PCR kit (Cat. No. 204141). The PCR reaction was performed using Pikoreal, ThermoScientific, USA and started with an initial denaturation step of 94°C for 5 minutes. A forty cycles of PCR reaction was performed that initiated with denaturation step at 94°C for 15 seconds, annealing step at 55° C for 30 seconds using the following primer pair of both cyclin D1 and B actin (55° C for 30 seconds) as a house keeping gene which was listed in (Table 1). Finally, an extension step was performed at 72° C for 30 seconds for completion of the amplification step. The determination of relative expression of cyclin D1 was calculated as fold expression in comparison with control, where GAPDH was house-keeping gene [34].

2.4. Statistical analysis

Data was analyzed using SPSS v.17, statistical software program. One- way ANOVA with post hoc LSD comparison test was used to detect significant differences among different groups [35]. Data was presented as mean and standard error of mean.

Table 1. List of primer pairs used in the experiment.

Gene	Primer sequence (5' -3')	Reference
Rat B-actin	TCCTCCTGAGCGCAAGTACTCT GCTCAGTAACAGTCCGCCTAGAA	Banni et al. [55]
Cyclin D1	CCATGCTTAAGACTGAGGAGAC TGAGCTTGTTCCACAGAAGC	Wang et al. [56]

3. RESULTS

3.2. Oxidative stress and antioxidant parameters

Levels of Nitric oxide, Malondialdehyde and Reduced Glutathione, as well as activity of Glutathione-S-transferase showed a significant decline after pumpkin seed extract administration in the treatment group (G4) and pumpkin supplemented group (G3) in comparison to cancer group (G2), but the activity of superoxide dismutase (SOD) were reduced (Table 2).

3.2. Cyclin D1 gene expression

Cyclin D1 level was significantly decreased in G2 than G1 (control group). A precipitous decrease of cyclin D1 was also noticed in G3 and G4 than in G2 (Figure 1).

Table 2. Effect of pumpkin seed extract on selected antioxidant and oxidative stress markers in rats with experimental mammary gland.

Groups	MDA (nmol/g)	NO (µmol /L)	GSH (mg/g)	GST (U/g)	SOD (U/g)
G1	4.16±1.5 ^b	4.37± 0.4 ^b	68.06± 12.8 ^b	0.9±0.1 ^b	1325.5±100 ^b
G2	29.67±2.02 ^a	31.57± 3.8 ^a	144.8± 8.1 ^a	4.6± 0.7 ^a	2234.4±37 ^a
G3	9.36±1.4 ^c	3.47± 1.5 ^b	21.16± 1.7 ^c	0.62± 0.1 ^b	1746.7±251 ^{ab}
G4	18.66± 2.6 ^b	8.06± 0.8 ^b	67.53± 10.9 ^b	1.33± 0.1 ^b	1969.4± 136 ^{ab}

Values represent means ± standard errors. Values in the same column with different superscript letters are differing significantly (P <0.05).

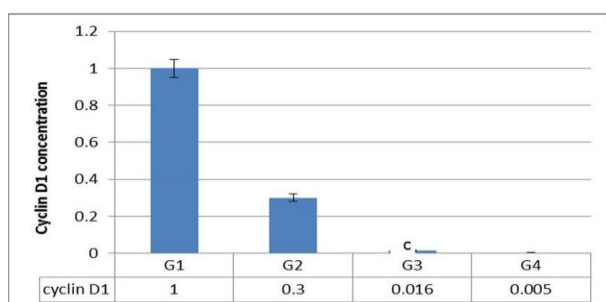
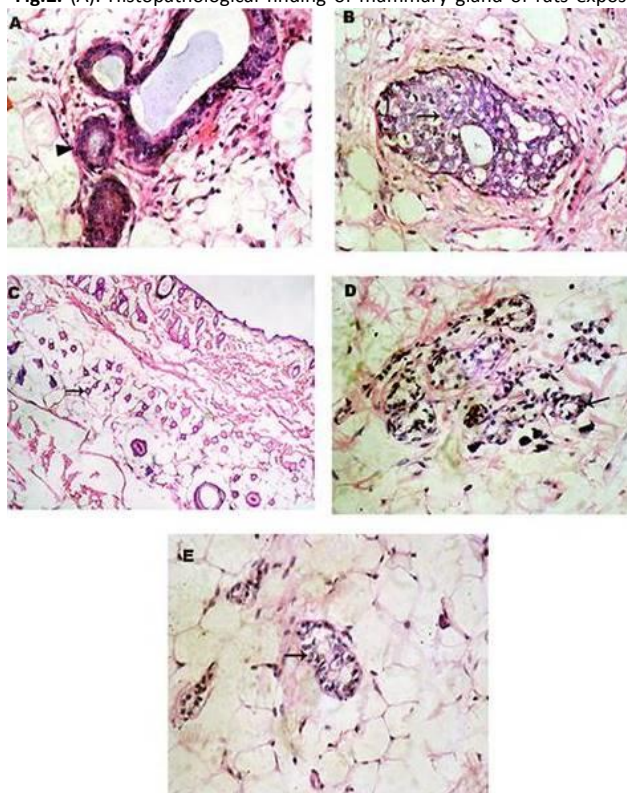


Figure 1. Cyclin D1 relative expression in rats' model of breast carcinoma. Results were expressed as means with its standard errors, where columns contained different letter is considered a significant result.

3.3. Histopathological examination

As compared to normal tissue (Figure 2A), mammary gland of carcinoma- induced group displayed neoplastic cells with pleomorphic hyperchromatic, prominent nucleoli and scanty basophilic cytoplasm in the lumen of secretory lobule (arrow) forming solid adenocarcinoma (Figure 2B). In pumpkin-supplemented rats (G3), the mammary tissues displayed normal features (Figure 2C). However, in G4, necrosis of neoplastic cells (arrow) and secretory acini became lined with only one layer and normal lining epithelium lining lobule (arrow) and normal fibrofatty stroma (Figure 2D-E).

Fig.2. (A). Histopathological finding of mammary gland of rats exposed to



7,12 dimethylbenzanthracene (DMBA) and treated with pumpkin seeds' extract. Mammary gland of G1 (A), G2 (B), G3 (C), and G4 (D, E).

DISCUSSION

In the present investigation, in rats with mammary carcinoma, NO values were increased by about 7 folds than that in the control

group. Moreover, NO level showed a statistically significant decrease in G3 (pumpkin-supplemented group) and G4 (treatment group) in comparison to G2 (cancer group). In human gynecological cancer, it has been found that there was an association between NO synthase activity and tumor grade [36]. In pumpkin-supplemented groups, the NO decreased, suggesting the role of pumpkin to alleviate the oxidative stress with subsequent decrease of NO. Our finding is supported by the results of Kim et al., [37] who postulated that methanolic extract of pumpkin and other 47 species of Japanese plant contains secondary metabolites have a cancer preventive activity via reduction of excess NO.

MDA level was significantly increased in G2 compared to the control group. Similar findings have been previously reported by Gonenc et al. [38]. However, MDA levels showed a statistically significant decrease in G3 and G4 compared to G2. This result is in accordance with that observed in another study by Xu, [39] who suggested that pumpkin polysaccharide could reduce MDA activity in tumor- containing mice serum.

In the current study, the results of SOD activity are in accordance with that observed by Tas et al., [40]. SOD activity was significantly reduced in G3 and G4 compared to G2. In contrast, Xu, [39] stated that pumpkin's polysaccharide contents could increase superoxide dismutase activity in tumor- containing mice serum.

GSH was significantly increased in rats of G2 compared to those in G1. Likewise, in patients with breast cancer, there was an increase in the GSH [41]. On the contrary, GSH level in G3 was lower than that of G1. In an experiment in mice, GSH in the liver of pumpkin seed oil-treated rats was lower than that of control rats [42]. Correspondingly, GSH level in G3 and G4 was lower than in G2. In contrast, Xu, [39] stated that pumpkin polysaccharide could increase glutathione peroxidase activity (GPx) in tumor containing mice serum. It has been also noted that GPx was positively correlated with GSH in tumor as well as in normal tissue [43].

In rats with mammary gland carcinoma, GST activity was significantly higher than that in control rats. However, GST activity was significantly decreased in G3 and G4 compared to G2. On the other hand, Sakoda et al., [44] stated that the relationships between GST genotype and risk of breast cancer or fibrocystic breast conditions did not vary across intake levels of pumpkin and other vegetables .

Interestingly, cyclin D1 expression was significantly decreased in G2 when compared to G1. In a study carried out by Pusch et al. [45], the Cyclin D1 showed an importance in the cell cycle, where it reached a peak in mid G1 phase, decreased within s phase and remained low throughout the rest of cycle. Gillett et al., [46] stated that there was a significant inverse correlation between cyclin D1 staining and histological grade, as grade III infiltrating ductal carcinomas were more likely to be cyclin D1-negative, but grades 1 and 11 tumor lacked the protein. In G3 and G4 compared to G1, cyclin D1 level showed also a statistically significant decrease. The decrease in cyclin D1 expression has been explained by Kong et al., [47] and Dong et al. [48] who found that cucurbitacin E decreased expression of cyclin D1. Pumpkin has been found to contain 23, 24-dihydrocucurbitacin F, 23, 24-dihydrocucurbitacin D, cucurbitacin B and cucurbitacin [49]. In the same context, cyclin D1 level showed a statistically significant decrease in G3 and G4 in comparison to G2. This result coincides with that observed in previous studies [50].

In the present study, the results of histopathology in G1 are in accordance with those of Srivastava et al., [51]. However, for G2, a solid lobular adenocarcinoma, neoplastic cells with pleomorphic hyperchromatic, prominent nucleoli, and scanty basophilic cytoplasm in the lumen of secretory lobule were evident. Likewise, Barros et al. [52], observed adenocarcinoma with several

morphological types in the histopathological appearance of cancer group induced by DMBA. In another study by Russo and Russo, [53] prominent nucleoli, invasion of stroma and inflammatory infiltration were recorded in the histopathological appearance of malignant tumor of mammary gland of rat. On the other hand, mammary gland of G3 showed normal features of healthy mammary gland. Moreover, in G4 there was a regression of the pathological features of neoplasm, suggesting the beneficial role of pumpkin. Our finding is supported by the results of Medjakovic et al., [54] who stated that pumpkin seed extract inhibited cell growth in breast cancer and other types of cancer.

Conclusion

In rats with induced mammary gland carcinoma, supplementation of pumpkin seed extract could decrease Cyclin D1 gene expression and could improve oxidant/and oxidant status. The result of this study indicated that pumpkin seed extract may have a beneficial therapeutic effect on mammary gland cancer via improvement in the function of mammary gland and potentiation of the antioxidant status.

Conflict of interest: The authors declare that there is no conflict of interest.

Research ethics committee permission: The current study has been permitted by Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University.

Author contributions: W. A. conducted experimental protocol and drafted the manuscript; M. E. conducted the statistical analysis and revised the manuscript; M. H. performed histopathological examination; El-Said El-Sherbini revised the manuscript.

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