

Efficacy of *Pleurotus ostreatus* (Jacq. Ex Fr.) P.kumm. on 7,12-dimethylbenz(a)anthracene induced mammary carcinogenesis in female Sprague-Dawley rats

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ABSTRACT

Neoplastic growth of the breast is the most common malignancy in women worldwide and its incidence has increased in most countries. In this study, we have evaluated the efficacy of *Pleurotus ostreatus* (Jacq. Ex Fr.) P.kumm. (*P. ostreatus*) an edible mushroom on modulating levels of xenobiotic metabolizing enzymes, hormonal status of estrogen and progesterone receptor (ER/PR), protein expressions and histopathological analysis in 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis using a rat model. DMBA was induced by single subcutaneous injection at a dosage of 25 mg in 1 mL vehicle. The ethanolic extract of *P. ostreatus* (POEet) was administered orally at a concentration of 600 mg/kg bwt as pre- and post-initiation stage of treatment throughout the experimental period which was also compared with standard tamoxifen (TAM) (10 mg/kg bwt). At the end of 16 weeks, our results showed the elevated phase I and depleted phase II metabolizing enzymes, over expression of (ER/PR) and the expression pattern of the proteins such as fas, fasL, caspase 3, caspase 8, caspase 9, Bax were found to be down regulated whereas p53, Bcl2, cox-2 and cyclin D1 were markedly upregulated in DMBA-induced Sprague-Dawley rats, which were significantly reversed on *P. ostreatus* administration. Moreover, pre-treatment with *P. ostreatus* showed improved response when compared to that of posttreatment. Based on scientific appraisal, we conclude that the dietary consumption of *P. ostreatus* might offer maximum protection against DMBA-induced mammary carcinogenesis and improving human health if used as a regular basis.

Focal points:

- Benchside
 - The potential of *Pleurotus ostreatus* on DMBA induced rat mammary carcinogenesis were determined by the analysis of xenobiotic metabolizing enzymes, hormonal status of ER/PR, as well as the expression of protein using western blotting techniques and histological examination of liver and mammary tissues.
- Bedside
 - The pharmaceutical potential of *P. ostreatus* are analysed for is markedly available, edible one with high protein as well as fiber with low fat content and cost effective for patient convenience.
- Industry
 - Mushrooms are considered as a nutraceutical functional food which can contain enriched prolific produced of novel “mycochemicals” responsible for the human health potential on various diseases and malignancies.
- Community
 - Standardization and refinement of mycochemical from mushrooms were help to develop the new novel active compounds which contribute for better health and also help to reduce disease burden.

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- Regulatory agencies
 - The *P. ostreatus* contain several bioactive compounds which might be the key factor for the anticancer effect. This will need to be taken for labeling and patterning the compounds and tested in clinical trials.
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1. Introduction

Breast cancer is the most frequent type of malignancy among women in global population, makes up about one-tenth of all new cancer diagnosis worldwide [1]. Although the etiology of breast cancer is multifactorial. Significantly breast cancer risk factor include age, early age at menarche, late age of menopause, and late age at first pregnancy, obesity, oral contraception, hormone replacement therapy, diet, family history, lactation and prior history of benign breast diseases [2]. Although, a vast number of cancer researches have been devoted in the development of anti-neoplastic drugs, the prognosis of the diseases is often challenging due to increased side effects. Thus the pursuit for anti-cancer drugs takes a compelling urgency for alternate preventive approaches through dietary means with more efficacy and low toxicity are considered to be the winning strategy in reducing the morbidity and mortality of breast cancer [3].

Edible mushrooms are often used in the traditional system of medicine for the treatment of human ailments. In worldwide, mushrooms have been attracted the attention of the public due to presence of chemical composition particularly for antitumor property. *Pleurotus ostreatus* (Jacq.ex.fr) P.kumm. is a traditional Chinese medicinal and edible fungus which is famous for its delicious taste and high quantities of proteins, carbohydrates, minerals and vitamins as well as low fat [4]. Chemical investigations from different region of the world confirmed that the lectins, polysaccharides, polysaccharide-peptides, polysaccharide-proteins have been identified in *Pleurotus* mushrooms and many of these compounds have been found to have promising biological effects which protect the body against free radicals that damage body cells to induce cancer. These compounds are regarded as biological response modifier (BRM) they cause no harm and place no additional stress on the body, but help the body to adopt to environmental and biological stress [5]. We earlier reported that supplementation of *P. ostreatus* exhibits the modulatory effect on oxidant/antioxidant status in DMBA induced mammary carcinoma in experimental rats [6].

The present study was designed to ascertain the inhibitory effect of *P. ostreatus* on 7,12-dimethylbenz(a)anthracene induced rat mammary carcinoma by assessing the changes in phase I and phase II enzymes levels, hormonal status with respect to histological grading as well as the expression pattern of proteins.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin, cytochrome C, 1-chloro-2,4-dinitrobenzene (CDNB), 2,6-dichlorophenolindophenol (DCPIP), methylene blue, reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide (NADPH), sodium dithionite, 7,12-dimethylbenz(a)anthracene (DMBA) and tamoxifen were purchased from Sigma Chemical Pvt. Ltd., Bangalore, India. Antibody's used for western blotting were purchased from Santa Cruz Biotechnology, CA, USA and Neo Markers USA. All other chemical and solvents used were of analytical grade.

2.2. Material

P. ostreatus mushrooms were collected in and around areas of Udthagamandalam, Nilagiri district, Tamil Nadu. The mushroom was taxonomically identified and a voucher specimen (No: 233) was deposited in the herbarium of Botany, Department of Botany, Annamalai University.

2.3. Preparation of mushroom ethanolic extract

The fresh fruiting bodies of *P. ostreatus* were dried in shade conditions and the dried materials were pulverized in a blender to get coarse powder. For *P. ostreatus* fruiting bodies ethanolic extraction, five grams of the powder was extracted with 100 mL of 95% ethanol using a Soxhlet apparatus. The solvent was evaporated on a rotary evaporator (Buchi Rotarvapour, Switzerland) under reduced pressure and controlled temperature (40–50 °C) [7]. A dark semisolid material (6% yield) thus obtained was stored at 4 °C until use. A known amount of the residual extracts were suspended in distilled water and was orally administered to the animals by gastric intubation.

2.4. Animals

The whole experiment was carried out according to the guideline of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), New Delhi, India and approved by the animal ethical committee of Annamalai University (Reg. No:160/1999, Proposal number: 947 CPCSEA). The study was conducted on six week old adult female Sprague-Dawley rats, weighing approximately 130–150 g were obtained from National Institute of Nutrition, Hyderabad and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The rats were housed in polypropylene cages at room temperature (27 ± 2 °C) with relative humidity 55 ± 5%, in an experimental room. In Annamalainagar, the LD (light:dark) cycle is almost 12:12 h. The rats were maintained as per the principle and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian National Law on animal care and use. The rats had free access to standard pellet diet (Amrut Laboratory Animal Feed, Mysore Feed Limited, Bangalore, India) and water *ad libitum* were available to the animals throughout the experimental period and replenished daily. The standard pellet diet comprised of 21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorous, 3.4% glucose, 2% vitamin and 55% nitrogen free extract (carbohydrate) and it provides metabolizable energy of 3600 kcal/kg.

2.5. Experimental design

Animals were assorted into six groups of six animals each according to the following experimental regimen. Animals in Groups 1 were induced with DMBA 25 mg in 1 mL of vehicle (0.5 mL of sunflower oil in 0.5 mL of saline) [8]. In Group 2 and 3 rats was received (600 mg/kg bwt) POEet extract as pre-initiation and post-initiation phase alone with DMBA. Group 4 rats were treated with tamoxifen (TAM) (10 mg/kg bwt) along with DMBA. Group 5 rats received (600 mg/kg bwt) POEet alone and group 6 rats was treated as control. The experiment was first terminated at the end

of 12 weeks (pre-initiation phase) and subsequently terminated at the end of 16 weeks (post-initiation phase). During sacrifice, blood sample were collected for plasma separation and the mammary, liver tissues were excised immediately and stored in a liquid nitrogen container to avoid protein degradation after that the tissues were minced and homogenized (10% W/V) in 0.1 M phosphate buffer (pH 7.0) and centrifuge at 4 °C 12,000g for 30 min and supernatant was collected and used for further biochemical and molecular studies. The liver and mammary tissues were used for histological studies.

2.6. Tumor volume

Tumor volume was measured as described by Escrich et al. [9]. $V = 4/3\pi(d1/2) \times (d2/2)^2$, where d1 and d2 are the two diameter of the tumor (d1 > d2). At sacrificing, the volume of each tumor calculated using its three diameters was

$$V = 4/3\pi (d1/2) \times (d2/2) \times (d3/3); (d1 > d2 > d3)$$

2.7. Histological assay

For histological assays, three rats from each group were perfused with physiological saline followed by formalin (10% formaldehyde). The mammary and liver tissues were excised immediately and fixed in 10% formalin. The mammary and liver tissues were sliced and embedded in paraffin wax, 3–5 μm thick sections were cut in a rotary microtome and were stained with hematoxylin and eosin. The specimens were evaluated with a light microscope. All histopathological changes were examined by the pathologist.

2.8. Immunohistochemical study

Paraffin embedded tissue section were dewaxed and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H₂O₂ in methanol for 10 min. The antigen retrieval was achieved by microwave in citrate buffer solution (2.1 g citric acid/LD. H₂O; 0.37 g EDTA/LD. H₂O; 0.2 g Trypsin) (pH 6.0) for 10 min, followed by washing with tris-buffered saline (TBS) (8 g NaCl; 0.605 g Tris) (pH 7.6). The tissues section was then incubated with power Block TM reagent (Biogenex, San Ramon, CA, USA). Universal proteinaceous blocking reagents were incubated for 15 min at room temperature to block nonspecific binding sites. The tissues sections were then incubated with the respective primary antibody (rabbit monoclonal antibodies) (Dako, Netherland) overnight at 4 °C for ER. The bounded primary antibody was detected by incubated with (mouse polyclonal antibody) (BioGenex, San Ramon, CA, USA) the secondary antibody conjugated with horseradish peroxidase (BioGenex, San Ramon, CA, USA) for 30 min each at room temperature. After rising with TBS, the antigen-antibody complex was detected using 3,3'-diaminobenzidine (Sigma, USA). When accepted color intensity was reached, the slides were washed, counter stained with hematoxylin, and covered with a mounting medium.

For negative control, the primary antibody was replaced with TBS. Positive control for each antibody was also processed simultaneously. The percentage positive tumor expressing these proteins was graded as follows: 3+ = strong staining, more than 50% of cells were stained; 1+ = weak staining, between 5% and 25% of cells were stained; 0 = negative, less than 5% of cell staining.

2.9. Biochemical assessments

Cytochrome P450 and cytochrome b5 content were assayed by the method of Omura and Sato [10]. Cytochrome P450 was

determined by using the carbon monoxide difference spectra. Reduced cytochrome P450 combines with carbon monoxide to yield a pigment with an absorbance maximum at 450 nm. Cytochrome b5 was measured from the difference spectrum between reduced and oxidized cytochrome b5. The activity of glutathione-S-transferase (GST) was determined as described by Habig et al. by following the increase in absorbance at 340 nm using CDNB as the substrate [11]. The activity of DT-diaphorase was assayed as described by Ernster [12]. This method involves measurement of reduction at 550 nm using NADPH as the electron donor and 2,6-dichlorophenolindophenol as the electron acceptor. Glutathione reductase activity was determined as described by Carlberg and Mannervik [13]. This method involves measurement of reduced glutathione formation at 340 nm due to the oxidized glutathione (GSSG) is reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH). The protein content was estimated by the method of Lowery et al. [14].

2.10. Western blotting analysis

Western blotting was performed to analyze the expression pattern of cox-2, p53, cyclin D1 Bax, Bcl-2, Fas, FasL, Caspase-3, Caspase-8 and Caspase-9 by using the method of Laemmli [15]. The mammary tissues sample were homogenized in a buffer [5 mM sodium azide, 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaHCO₃ (pH 7.0)]. The homogenate was centrifuged at 12,000 g for 30 min at 4 °C to remove debris. Sample containing 50 μg of total cellular proteins were loaded and separated using 10% SDS polyacrylamide gel electrophoresis. The resolved proteins were blot transferred onto a PVDF membrane (Millipore). The membranes were incubated with the blocking buffer containing 5% w/v non-fat dry milk and then incubated with the primary antibody in 10 mL of antibody-diluted buffer (Tris-buffered saline and 0.05% Tween-20 with 5% milk) with gentle shaking at 4 °C for 8–12 h. After this, membranes were incubated with their corresponding secondary antibodies (anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase) for 2 h at room temperature. Membranes were washed thrice with TBST for 30 min. After extensive wash in TBST, the bands were visualized by treating the membranes with 3,3'-diaminobenzidine tetrahydrochloride (Western blot detection reagent). Bands were scanned using a scanner and quantified by Image J, a public Java image processing software, Wayne Rasband, NIH, Bethesda, MD, USA. Percentage of expression was calculated by keeping expression of protein in control animals as 100%.

2.11. Statistical analysis

Statistical analysis was performed using SPSS software package, version 16.0. The values were analysed by One Way Analysis Of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). All these results were expressed as mean ± SD for six rats in each group *P*-values < 0.05 were considered as significant.

3. Results

3.1. Effect of POEet on body weight changes

Table 1 represents the body weight changes during initial and final stage of experimental animals. There was a significant descent in the body weight of DMBA induced tumor bearing animals (*P* < 0.05) compared to control. On the contrary, POEet treatment as both pre- and post-initiation stage on animals showed a gradual ascent in body weight next to TAM (standard drug for breast cancer treatment) treated animals compared with DMBA

Table 1
Effect of POEet on the changes of bodyweight of control and experimental animals.

Groups	Initial body weight (grams)	Final body weight (grams)
DMBA	152.57 ± 13.16	110.8 ± 10.34 ^a
DMBA+Pre-POEet (600 mg/kg bw)	154.76 ± 13.63	145.64 ± 12.61 ^b
DMBA+Post-POEet (600 mg/kg bw)	151.27 ± 13.12	128.56 ± 10.43 ^c
DMBA+Post-TAM (10 mg/kg bw)	169.81 ± 14.86	163.53 ± 13.66 ^d
POEet alone (600 mg/kg bw)	156.47 ± 14.24	180.33 ± 14.88 ^e
Control	153.85 ± 13.75	185.73 ± 17.10 ^e

Values are expressed as mean ± SD for six animals in each group. Values not sharing a common superscript differ significantly at a *p*-value of < 0.05 Duncan's multiples range test (DMRT). Comparison – a – (*p* < 0.05) DMBA untreated group (Group 1) compared with control group (Group 6); b, c, d – (*p* < 0.05) POEet treated group (Group 2, 3, 4) compared with DMBA untreated group (Group 1); e – POEet alone and control (Group 5, 6) non significant.

Table 2
The incidence of mammary tumor in control and experimental animals.

Groups	Tumor incidence	Tumor number	Tumor volume (mm ³)
DMBA	100%	6/6	4326.30 ± 396.12 ^a
DMBA+Pre-POEet	50%	3/6	2700.28 ± 236.08 ^b
DMBA+Post-POEet	66%	4/6	3950.04 ± 324.06 ^c
DMBA+Post-TAM	33%	2/6	1580.04 ± 134.23 ^d
POEet alone	–	0/6	–
Control	–	0/6	–

Tumor incidence was measured using the formula: Tumor incidence (%) = Total no. of tumor bearing rats/Total no. of rats × 100. Tumor volume was measured using the formula: $V = (4/3)\pi(D1/2)(D2/2)(D3/2)$ Where, D1, D2, D3 are the diameters (in mm³) of the tumor. Values are expressed as mean ± SD for six animals in each group. Values not sharing a common superscript differ significantly at a *p*-value of < 0.05 Duncan's multiples range test (DMRT). Comparison- a – (*p* < 0.05) DMBA untreated group (Group 1) compared with POEet treated group (Group 2, 3, 4).

untreated animals (*P* < 0.05). Importantly, the pre-initiation treated animals were more pronounced in improving the body weight than the post-initiation treatment. However, there was no significant differences were noticed in body weight of animals between control and POEet alone treated groups.

3.2. Effect of POEet on tumor volume and tumor incidence

Table 2 shows the carcinogenic parameter like tumor incidence and tumor volume. The tumor was recorded from the period of time carcinogen administration to the palpable detection. There was a considerable tumor progression in untreated animals when compared with treated animals. The study demonstrated that 100% tumor incidence with a mean tumor volume of 4326.30 mm³ in DMBA induced animals. The tumor did not disappear totally in POEet treated animals but a significant decreases in prevalence was found when compared with DMBA animals (*P* < 0.05). Comparatively the animals treated with POEet as pre- and post-initiation stage, the maximum reduction was observed at a pre-POEet treated animals next to TAM treated rats showed 33% of tumor incidence with mean tumor volume of 1580.04 mm³. However, there was no significant changes were observed in POEet alone and control treated animals. The morphological appearance in DMBA, DMBA+Pre-POEet (600 mg/kg bw) and DMBA+Post-POEet (600 mg/kg bw) treated animals were showed in Fig. 1.

3.3. Effect of POEet on biotransformation enzymes activities in control and experimental animals

The level of phase I and Phase II biotransformation enzymes in the mammary and liver tissues of control and experimental animals is shown in Tables 3 and 4. The activities of Phase I enzyme (Cytochrome P450 and Cytochrome b5) was significantly elevated and Phase II enzyme (GST, GR and DT-Diaphorase) was depleted in DMBA induced tumor bearing animals when compared with control animals. Whereas, oral supplementation of POEet as pre- and postinitiation stage to DMBA induced tumor bearing animals showed significant decreases in prevalence in enzyme levels. Notable, with pre-POEet treated animals exhibited more prominent activities as significantly reduced the levels of phase I enzymes

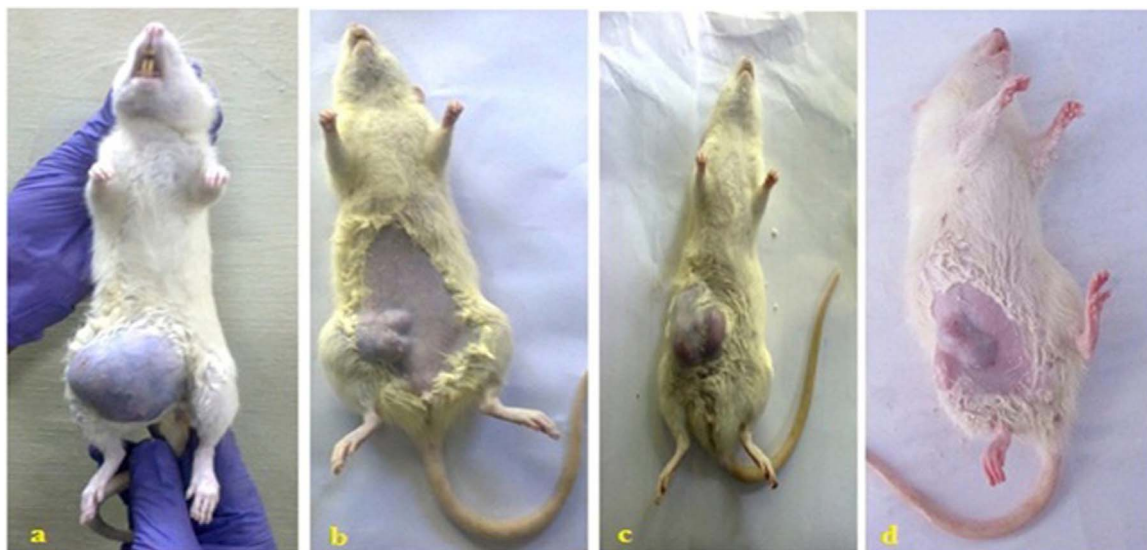


Fig. 1. The gross appearance of mammary carcinoma in DMBA, DMBA+(Pre- & post-POEet) and DMBA+TAM treated female Sprague-Dawley rats. (a) Shows the mammary carcinoma in DMBA induced female Sprague-Dawley rat, (b) and (c) the gross appearance of the mammary carcinoma in DMBA+pre- & post-POEet (600 mg/kg bw) treated female Sprague-Dawley rats and (d) shows the gross appearance of mammary carcinoma in DMBA+post-TAM (10 mg/kg bw) treated female Sprague-Dawley rats.

and increased significantly the level of phase II enzymes compared with post-POEet treated animals. However, TAM showed greater modulatory effects on xenobiotic metabolizing enzymes compared to other groups. There was no significant changes were observed in POEet alone treated rats when compared to control animals.

3.4. Histopathological changes in breast and liver tissues of control and experimental group of animals

Histopathological evaluation was carried out to characterize the biological response factors. (Fig. 2 A–F) depicts the photomicrograph of H & E staining of mammary tissue of control and experimental animals. DMBA induced animals sections (Fig. 2A), showed altered ductal epithelial lining indicating ductal adenocarcinoma with abnormal cellular proliferation. Administration of POEet resulted in attenuation of abnormalities in the mammary histopathology. Dramatically decreases in prevalence in tumor with ductal gland were evident in pre-POEet treated animals (Fig. 2B), showed florid ductal hyperplasia when compared with post-POEet treatment animals (Fig. 2C). In TAM treated animals sections showed further more increased in ductal epithelial architecture as ductal dysplasia respectively. Control and POEet alone treated animals displayed no signs of pathological changes however exhibiting normal ductal architecture.

Fig. 2 G–L represents histopathological assessments of liver tissues sections of control and experimental animals. In DMBA induced tumor bearing animals shows loss of architecture with nuclear pleomorphism and sinusoidal dilatation with feathery

degeneration. On oral administration of pre-POEet treated animal sections showed improved hepatocyte with glandular cytoplasm with uniform nuclei. Conversely, in Post-POEet treated animal showed inflammatory cell infiltrate around portal triad. Notably, the pre-POEet treatment was more pronounced in improved hepatocyte than the post-POEet treatment animals. In TAM treated animals showed near normal hepatocyte. Whereas, POEet alone and control treated animals displayed normal liver architecture.

3.5. Immunohistopathological changes in breast tissues of control and experimental animals

Fig. 3 A–F represents the immunohistochemical (IHC) analysis of hormones status of estrogen receptor (ER) and progesterone receptor (PR) respectively (Fig. 3 G–L) in control and experimental animals. DMBA induced tumor bearing animals showed over expression of the hormonal receptor when compared to control animals. On oral supplementation of POEet treated animals significantly down regulated expression of the hormone receptors. Consequently, the pre-POEet treated animals were showed improved down regulated receptor expression when compared to that of post-POEet treated animals. Whereas, TAM treated animals showed a near normal hormone receptor expression. In control and POEet alone treated group showed mild expression of ER and PR.

3.6. Western blotting analysis in breast tissues of control and experimental animals

Western blot analysis illustrates the molecular changes occurred during tumorigenesis. We sought to determine the apoptotic proteins, inflammatory protein, cell cycle regulatory protein markers by investigating some important regulators involved in the above signaling pathways (Figs. 4 and 5). The expression pattern of the proteins such as fas, fasL, caspase 3, caspase 8, caspase 9 and Bax were found to be down regulated. Whereas, COX-2, p53, Bcl2, cox-2 and cyclin D1 was markedly upregulated in DMBA induced positive control group. However, supplementation of POEet (Pre- & Post-initiation stage) altered the above mentioned protein expressions to near normal. Moreover, it is noteworthy that, compared to post- initiation POEet animal group the pre-treated POEet animals were showed remarkably altered the expression of markers to near normal which also compared with standard TAM treated animals. Moreover, no significant changes were observed in control and control treated groups.

Table 3

Effects of POEet on Phase I enzymes in Liver and mammary tissues of control and experimental animals.

Groups	Cyto P450 (U ^a /mg protein)		Cyto b5 (U ^b /mg protein)	
	Liver	Mammary	Liver	Mammary
DMBA	1.72 ± 0.15 ^a	1.54 ± 0.12 ^a	1.78 ± 0.16 ^a	1.62 ± 0.13 ^a
DMBA + Pre-POEet	1.23 ± 0.10 ^b	1.15 ± 0.08 ^b	1.18 ± 0.07 ^b	1.12 ± 0.08 ^b
DMBA + Post-POEet	1.59 ± 0.13 ^c	1.40 ± 0.12 ^c	1.54 ± 0.10 ^c	1.43 ± 0.10 ^c
DMBA + Post-TAM	0.98 ± 0.07 ^d	0.78 ± 0.06 ^d	0.92 ± 0.08 ^d	0.78 ± 0.06 ^d
POEet alone	0.80 ± 0.06 ^e	0.51 ± 0.04 ^e	0.71 ± 0.06 ^e	0.65 ± 0.04 ^e
Control	0.79 ± 0.06 ^e	0.46 ± 0.03 ^e	0.70 ± 0.05 ^e	0.63 ± 0.05 ^e

U^a – micromoles of cytochrome P450; U^b–micromoles of cytochrome b5. Values are expressed as mean ± SD for six rats in each group. Values that are not sharing a common superscript letter in the same rows differ significantly at a *p*-value of < 0.05 Duncan's multiples range test (DMRT). Comparison- a – (*p* < 0.05) DMBA untreated group (Group 1) compared with control group (Group 6); b, c, d – (*p* < 0.05) POEet treated group (Group 2, 3, 4) compared with DMBA untreated group (Group 1); e – POEet alone and control (Group 5, 6) non significant.

Table 4

Effects of POEet on Phase II enzymes in liver and mammary tissues of control and experimental animals.

Groups	GST (U ^a /mg protein)		GR (U ^b /mg protein)		DT-D (U ^c /mg protein)	
	Liver	Mammary	Liver	Mammary	Liver	Mammary
DMBA	0.90 ± 0.07 ^a	1.38 ± 0.10 ^a	15.65 ± 1.39 ^a	2.69 ± 0.22 ^a	0.35 ± 0.02 ^a	0.18 ± 0.01 ^a
DMBA + Pre-POEet	1.23 ± 0.10 ^c	1.90 ± 0.14 ^b	21.48 ± 1.94 ^c	3.57 ± 0.32 ^b	0.50 ± 0.04 ^c	0.29 ± 0.02 ^c
DMBA + Post-POEet	1.08 ± 0.08 ^b	1.67 ± 0.14 ^c	18.86 ± 1.52 ^b	3.12 ± 0.27 ^c	0.42 ± 0.02 ^b	0.23 ± 0.02 ^b
DMBA + Post-TAM	1.36 ± 0.10 ^d	2.23 ± 0.19 ^d	23.75 ± 2.09 ^d	3.98 ± 0.35 ^d	0.59 ± 0.04 ^d	0.34 ± 0.02 ^d
POEet alone	1.45 ± 0.12 ^d	2.50 ± 0.22 ^e	24.48 ± 2.02 ^d	4.45 ± 0.43 ^e	0.61 ± 0.05 ^d	0.40 ± 0.03 ^e
Control	1.47 ± 0.09 ^d	2.59 ± 0.23 ^e	25.85 ± 2.02 ^d	4.47 ± 0.42 ^e	0.64 ± 0.04 ^d	0.43 ± 0.04 ^e

Units: a – micromoles of CDNB conjugated with GSH per minute; b – micromoles of NADPH oxidized per hour; c – micromoles of 2,6-dichlorophenol indophenols reduced min⁻¹. Values are expressed as mean ± SD for six rats in each group. Values that are not sharing a common superscript letter in the same rows differ significantly at a *p*-value of < 0.05 Duncan's multiples range test (DMRT). Comparison- a – (*p* < 0.05) DMBA untreated group (Group 1) compared with control group (Group 6); b, c, d – (*p* < 0.05) POEet treated group (Group 2, 3, 4) compared with DMBA untreated group (Group 1); e – POEet alone and control (Group 5, 6) non significant.

4. Discussion

The present study revealed the evidence to indicate the anticancer effect of POEet on DMBA induced mammary carcinogenesis

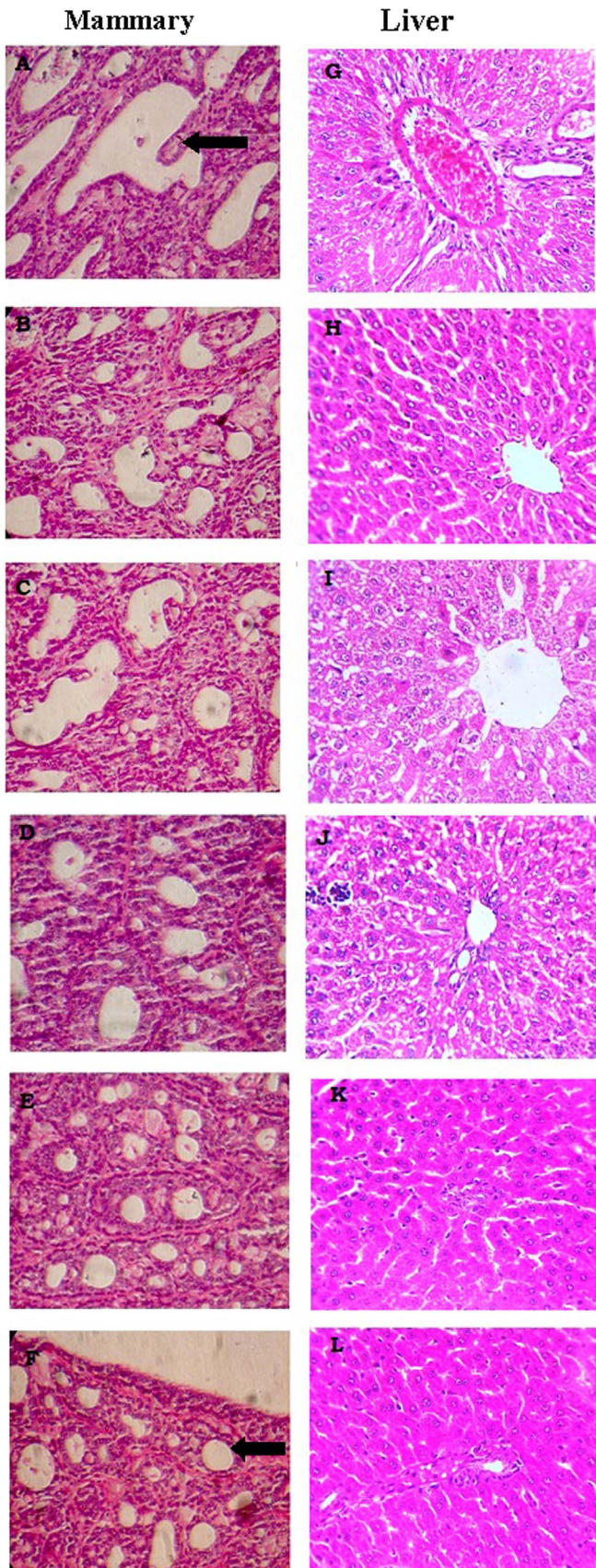


Fig. 2. Photomicrographs of histopathological changes in the mammary and liver tissues of control and experimental rats (hematoxylin and eosin staining showed at 40 ×). (A) and (G) DMBA, (B) and (H) DMBA+Pre-POEet (600 mg/kg bw), (C) and (I) DMBA+Post-POEet (600 mg/kg bw) (D) and (J) DMBA+Post-TAM (10 mg/kg bw), (E) and (K) POEet alone (600 mg/kg bw), (F) and (L) control.

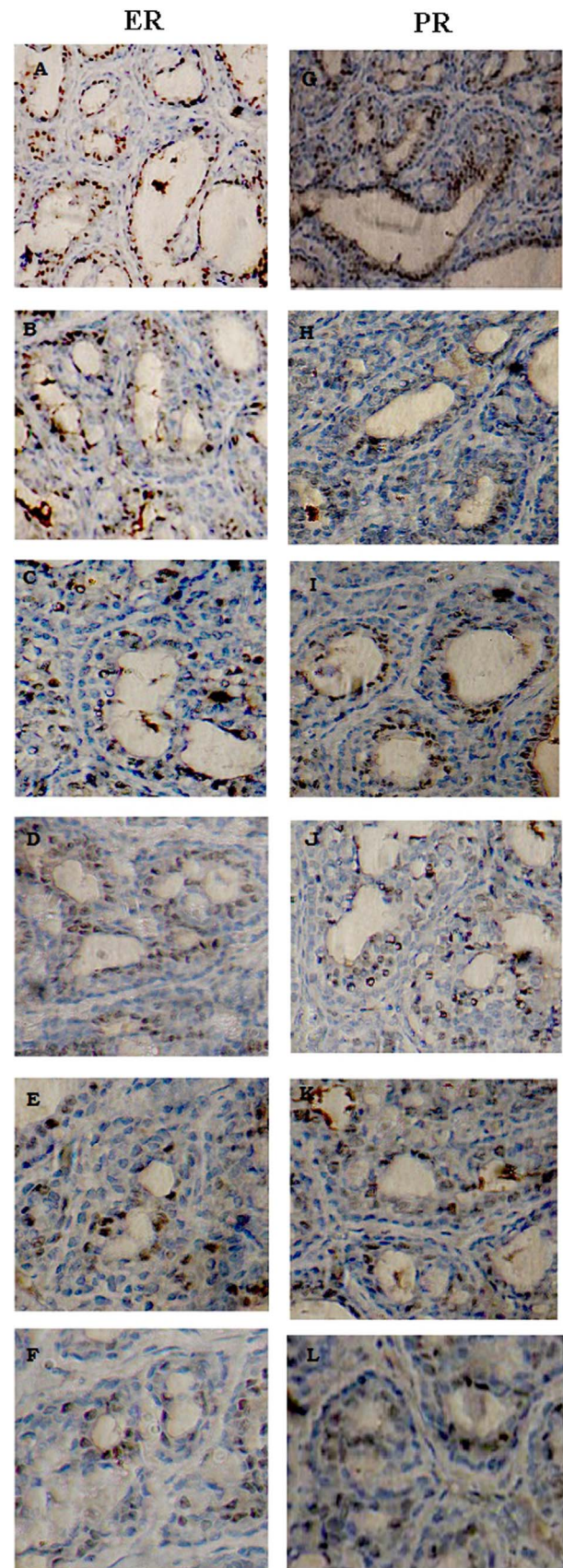


Fig. 3. Photomicrographs of estrogen receptor (ER) and progesterone receptor (PR) immunopathological changes in the mammary tissues of control and experimental animals. (40 ×) (A) and (G) DMBA, (B) and (H) DMBA+Pre-POEet (600 mg/kg bw), (C) and (I) DMBA+Post-POEet (600 mg/kg bw) (D) and (J) DMBA+Post-TAM (10 mg/kg bw), (E) and (K) POEet alone (600 mg/kg bw), (F) and (L) control.

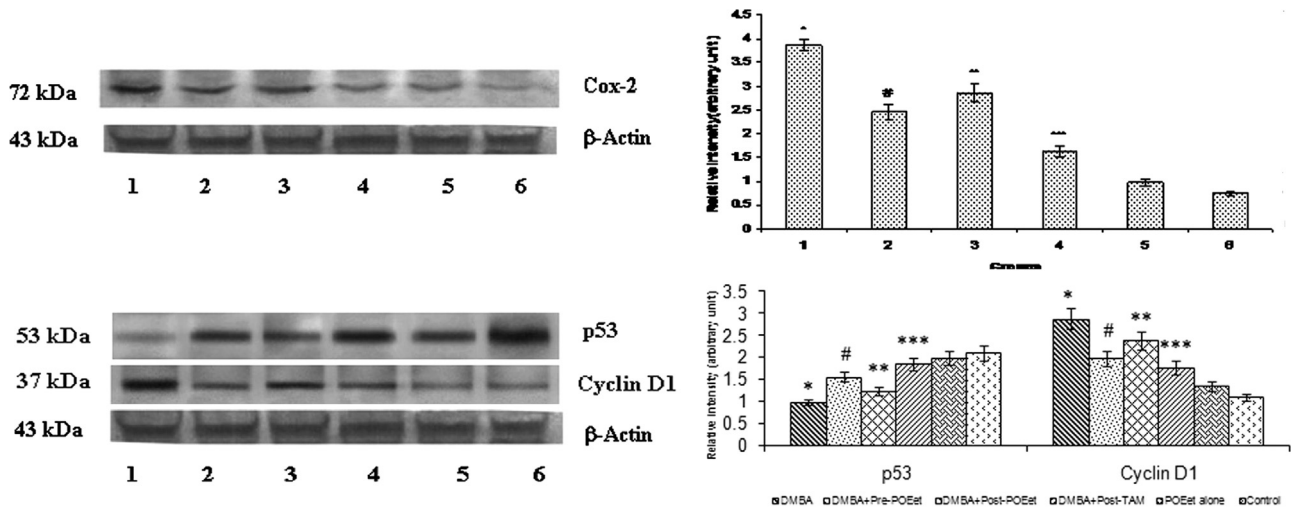


Fig. 4. Western blot analysis of inflammatory protein and the cell cycle regulatory proteins and expression Cox-2 in mammary tissue. (1) DMBA, (2) DMBA+Pre-POEet (600 mg/kg bw), (3) DMBA+Post-POEet (600 mg/kg bw) (4) DMBA+Post-TAM (10 mg/kg bw), (5) POEet alone (600 mg/kg bw), (6) control. Band intensities were scanned by densitometer. Histograms of densitometric analysis represent the ratio of Cox-2, p53 and Cyclin D1 expression. Values that do not share a common superscript in the same column differ significantly at $p < 0.05$ (DMRT). * ($p < 0.05$) DMBA untreated group (Group 1) compared with control group (Group 6); #, **, *** ($p < 0.05$) POEet treated group (Group 2, 3, 4) compared with DMBA untreated group (Group 1).

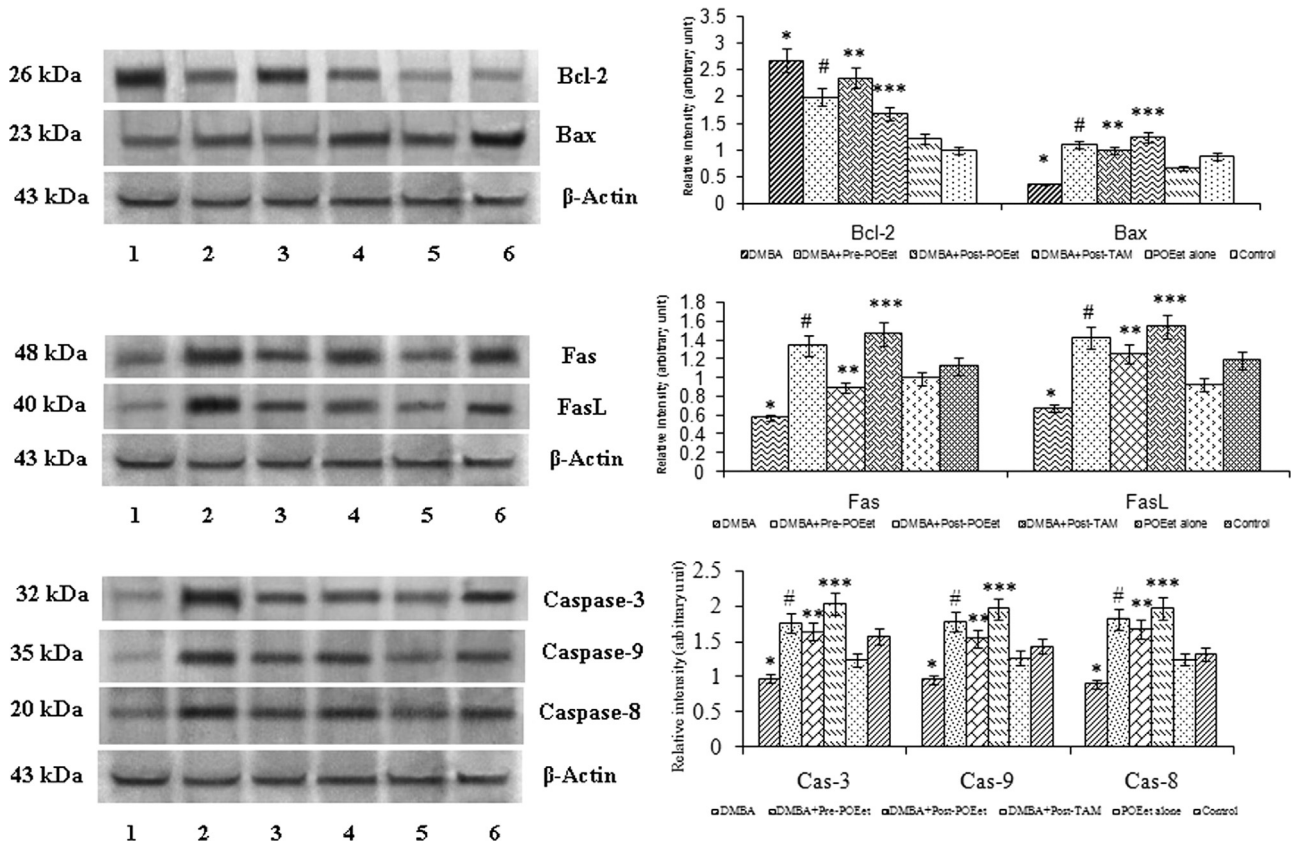


Fig. 5. Western blot analysis of the apoptotic protein expression in mammary tissues. (1) DMBA, (2) DMBA+Pre-POEet (600 mg/kg bw), (3) DMBA+Post-POEet (600 mg/kg bw), (4) DMBA+Post-TAM (10 mg/kg bw), (5) POEet alone (600 mg/kg bw), (6) control. Band intensities were scanned by densitometer. Values that do not share a common superscript in the same column differ significantly at $p < 0.05$ (DMRT). * ($p < 0.05$) DMBA untreated group (Group 1) compared with control group (Group 6); #, **, *** ($p < 0.05$) POEet treated group (Group 2, 3, 4) compared with DMBA untreated group (Group 1).

in Sprague-Dawley rats in both pre- and post-initiation phase. It is well known that during cancer condition excessive energy expenditure, malabsorption, metabolic alterations of the host leads to loss of weight. However, when compared to DMBA induced tumor bearing animal oral administration of POEet showed a gradual improvement in body weight. Earlier reports also

suggested that the *P. ostreatus* had a rich content of protein and the superior quality of this mushroom may be because of this genus contain complete proteins with the well distribution of essential amino acids, as well as non-essential amino acids, this might be the key factor for the improved body weight of the POEet treated rats [6,16].

In addition POEet effectively decreased tumor volume which might be due to the inhibitory action of the drug on tumor growth in animals. The potential preventive response of POEet was also reflected in the reduced total tumor incidence in DMBA-exposed animals. The observed tumor development inhibitory impact of POEet might represent to a selective toxic environment to multiplying cells which would eventually hold back the advancement of breast cancer [17]. However, the inhibitory effect of POEet against mammary carcinogenesis which may due to the presence of insoluble β -glucans, steroids and proteins. Additionally, since POEet treatment did not affect food intake, water intake, behavioral characteristics and growth rate of experimental animals it may be conclude that the observed mammary tumor-inhibitory effect of POEet is devoid of any toxic manifestation. Present results were inline with our recent toxicological study of *P. ostreatus* have been described the high margin of safety up to the dose of 5000 mg/kg bwt on experimental animals [18].

Liver plays pivotal role in the activation and elimination of toxic xenobiotics. In addition, the metabolic activation and detoxification of DMBA *in vivo* are known to occur primarily in the liver and also in other organs like mammary gland [19]. Both proximate and ultimate metabolites of DMBA-induced carcinogenesis are formed in liver cells that can be transported to mammary gland resulting in DMBA-DNA adducts [20]. Conversely two main groups of biotransformation enzymes participate in metabolism of carcinogens, namely phase I (Cyto P450 and Cyto b5) which converts hydrophobic compounds to more water-soluble moieties and phase II (GST, GR, DT-diaphorase) which catalyse conjugation reactions and further facilitates the excretion of the product [21]. In our present study, we have observed elevated activities of the phase I enzymes and depleted phase II enzymes in the liver and mammary tissues of DMBA induced animals which could be due to the presence of DMBA eliciting substrate induced activation of these enzymes and this is well in accordance with the previous finding of Mathivadhani et al. [22].

However, oral administration of POEet significantly reverse back to normal level this may due to the presence of the bioactive compound like β -glucan, ergosterol and triterpenoids content which possible interact with AhR-ARNT pathway leading to decreased induction of the CYP₁ protein. This may also contribute to the reduction in tumor measured in DMBA induced tumor bearing groups and not affected in control and drug alone treated groups. In line with the above results are well in accordance with previous findings have also suggested that high level of phase II enzyme may leads to an enhanced DMBA detoxification and elimination [23]. In addition, previously in our laboratory studies of *Ganoderma lucidum* had been showed to exhibit both the capacity to induce phase II detoxification enzymes and ability to inhibit phase I enzyme activity had produced effect against DMBA-induced mammary gland carcinogenesis in female Sprague-Dawley rats [24]. Taken together from these findings balance between Phase II and Phase I enzymes that favor an increased detoxification rate of carcinogens is more important for cancer.

The histological observation imply that pathological changes in the DMBA induced rats showing altered ductal epithelial lining indicating ductal carcinoma with abnormal cellular proliferation in mammary gland and show loss of architecture with nuclear pleomorphism and sinusoidal dilatation in the liver. Whereas, supplementation with POEet to DMBA induced tumor bearing rats showed attenuation of abnormalities as improved ductal architecture and hepatocyte with glandular cytoplasm with uniform nuclei. On other hand, POEet alone and control rats shows normal architecture. Thus POEet contain active constituents like fiber, non-starch polysaccharides (β -glucan), ergosterol and triterpenoids which may act as a free radical quencher and its role in conserving the pro-oxidant and antioxidant balance suggested that *P. ostreatus* as promising agent for hepatoprotection as well as

potent preventive agent for mammary cancer.

Appraisal of hormonal receptor expression is a focal part of the pathological evaluation of breast cancer. Since 1970, it has been hypothesized that ER and PR expression have played an indispensable role for diagnostic, prognostic and therapeutic implications [25]. Therefore, IHC analysis of DMBA-induced tumor bearing rats showed elevated level of ER and PR expression, which indicates development of hormone-dependent mammary carcinoma. However, oral supplementation of POEet in tumor bearing rats showed down regulated expression of ER and PR status. Thus POEet contain phytoestrogen constituents like ergosterol which may act as an anti-estrogen blocks in receptor mediated pathways [26]. In additional, the POEet supplementation to mammary cancer rats modulates the expression pattern of steroidal hormones levels which is consistent with our previous reports [24]. Moreover, our result were also collaborates with previous studies of Lakshmi et al., who also postulated that the chemopreventive effect of targetin in DMBA induced mammary carcinoma rats by exemplify the histological changes and immunohistological ER and PR status of mammary tissues [27].

Cyclooxygenases (COXs) are the rate limiting enzymes that catalyse the conversion of arachidonic acid to prostaglandins (PGs), which believed to play a key role in the development of inflammation and progression of various cancers [28]. Predominantly, cyclooxygenase inhibition of COX-2 isozyme blocking the prostaglandin (PG) cascade may have an impact on neoplastic development and improvement by inhibiting propagation of angiogenesis and metastasis. Based on the previous report of Pandi et al., we found that COX-2 may be a crucial factor in the development of mammary gland carcinogenesis and is usually associated with poor prognosis and short survival [29]. In our study we observed an increased expression of COX-2 in mammary tissue of DMBA group of experimental rats. Notably, a significant decrease in COX-2 expression in POEet treated animals compared to DMBA animals which indicate suppression of tumor development. In this connection, Kudryavtsev et al., also reported that overexpressed COX-2 cause increased PG production significantly contributed to tumor growth through the modulation of apoptosis and cellular proliferation [30].

A standout amongst the most widely recognized incidents required for human malignancy advancement is dysregulation of the cell cycle mechanism. In our study, we observed a decreased expression of p53 and over expression of cyclin D1 was observed in mammary tissue of DMBA induced experimental rats. However, increased p53 and diminished cyclin D1 expression in POEet treated animals compared to DMBA animals which indicate suppression of tumor development. Notably, Pre-POEet treated groups showed significant increased protein expression next to that of TAM when compared to (Post-POEet) treated group. In line with our findings, previously Rengarajan et al., 2013 also reported that expression of p53 was found to decrease in the mammary tissues of tumor bearing animals suggests that downregulation of p53 protein during DMBA induced mammary carcinogenesis [31].

Furthermore, we have focused on the apoptotic proteins involved in both intrinsic and extrinsic signaling cascades. Our results demonstrate the augmentation of anti-apoptotic protein Bcl-2 which is followed by a diminished expression of the pro-apoptotic protein bax in DMBA inducer group which resulted in increased bax and bcl-2 ratio. Consequently both intrinsic and extrinsic proteins such as fas, fasL, caspases 3, caspase 8, Caspase 9 and Bax showed decreased expression in DMBA induced mammary tumorigenesis which was in agreement with earlier reports [31]. Hence, supplementation of *P. ostreatus* to tumor bearing animals significantly upregulated the expression of Bax and down regulated the expression of Bcl-2 thereby decreases the Bax/Bcl-2 ratio which might be either due to the interference of

phytochemicals present in the POEet on the progressive phase of carcinogenesis. In addition, they effectively altered the apoptotic protein to near normal level thereby suggesting the involvement of both mitochondrial dependent and death receptor mediated apoptotic signaling pathways. Noteworthy, increased apoptotic protein expressions were observed in POEet (Pre-treated) tumor bearing group next to standard drug TAM when compared to Post-treated POEet. We therefore confirmed that POEet induced apoptosis *via* both intrinsic and extrinsic pathways which will serve as an effective chemotherapeutic agent for the treatment of mammary cancer.

5. Conclusion

In conclusion we demonstrated that the POEet significantly ameliorates the modulation of hormones and bio-transformation enzymes and molecular markers in DMBA induced cancer bearing animals. However, the antioxidant property of *P. ostreatus* through its bioactive compound like carbohydrates component mostly β -glucan content, terpenoids, proteins and steroids (ergosterol) which are derived from whole fruiting bodies of mushroom. As well as, the structural morphology of breast and histological analysis of mammary and liver tissues inevitably supports the biochemical alterations which prove the antineoplastic property of the *P. ostreatus*. On the basis of our data signify that the pre-treatment of *P. ostreatus* was more potent in inhibition of mammary cancer when compared to that of posttreatment. It is worth emphasizing that *P. ostreatus* can have considerable anticancer potential and conveniently incorporated in the diet as a nutritional supplement to improving human health if used on a regular basis.

Executive summary

- The *P. ostreatus* ethanolic extract contain several bioactive compounds like polysaccharides, alkaloids, steroids (ergosterol) and terpenoids, which might be the key factor for the anticancer effect of DMBA induced rat mammary carcinogenesis.
- The potentials of *P. ostreatus* to treat cancer is examined by investigating xenobiotic metabolizing enzymes, hormonal status of ER/PR, histological changes as well as the expression of proapoptotic and antiapoptotic proteins using western blotting techniques.
- However, it is worth emphasizing that the *P. ostreatus* can have considerable anticancer potential and conveniently incorporated in the diet as a nutritional supplement to improve human health if used on regular basis.

Conflicts of interest

None declared.

Ethical approval

None.

Funding source

None.

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