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Therapeutic effect of hydroethanolic extract of *Trianthema portulacastrum* L. against N-Nitroso-N-Methylurea-induced mammary tumors in Wistar rats

Narendra Kumar[1], Rajendra Singh[1,*,+], M Saminathan[1], KP Singh[2], K Dhama[1], A Arun Prince Milton[3] & NP Kurade[4]

[1]Division of Pathology; [2]Centre for Animal Disease Research and Diagnosis, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly 243 122, Uttar Pradesh, India

[3]Division of Animal Health, ICAR Regional Complex for NEH Region, Umiam 793 103, Meghalaya, India

[4]ICAR-National Institute of Abiotic Stress Management, Baramati, Pune 413 115, Maharashtra, India

E-mail: [+]rajendra_singh5747@rediffmail.com

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This study evaluated the therapeutic action of hydroethanolic extract of *Trianthema portulacastrum* L. (TPE) on N-nitroso-N-methylurea (NMU)-induced mammary tumors in Wistar rats. A hydroethanolic was prepared and subjected to qualitative and quantitative phytochemical screening. After acclimatization, Wistar rats were divided into 4 groups of 6 rats each: Group A (vehicle control), Group B (TPE control), Group C (TPE treatment) and group D (NMU control). NMU (50 mg/kg body weight) was injected intraperitoneally at 50, 80 and 110 days of age. After the induction of palpable tumors, the rats were administered 200 mg/kg bw of TPE by oral gavage for 2 months. The treatment with TPE significantly ($p<0.05$) decreased tumor incidence, frequency, size and malignancy in comparison to the tumor-bearing rats that were not administered TPE. Immunohistochemical analysis revealed that TPE treatment significantly reduced the expression of PCNA, VEGF, ER-α and ER-β, and caused non-significant reductions in matrix metallopeptidase-9 (MMP-9). Caspase-3 expression significantly increased in TPE-treated rats in comparison with NMU-treated controls. The qRT-PCR results showed PCNA and ER-β expression was down regulated and caspase-3 expression was up regulated in the TPE-treated group. The present study showed the *in vivo* therapeutic action of TPE extract on NMU-induced mammary tumors. TPE exhibited antitumor activity through its antiproliferative, antiangiogenic, pro-apoptotic, and estrogen receptor-modulatory properties.

Keywords: Cancer biomarker, Hydroethanolic extract, Mammary cancer, N-Nitroso-N-methylurea, *Trianthema portulacastrum*, Therapeutic effect

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Cancer is one of the most complex and aggressive diseases in both humans and animals. A total of 1,806,590 new cases of tumors will be detected, and 606,520 cancer death were projected to arise in the United States of America in 2020[1]. Breast cancer constitutes a major problem worldwide; it comprises one-tenth of all new cancer diagnoses and is a most important cause of mortality in women[2]. The causes for the initiation of mammary tumour are sex, obesity, sedentary lifestyle, alcohol, diet, hormone transition during menopause, ionizing radiation and aging[3]. The current treatments available for early-stage breast cancer, such as chemotherapy, radiation therapy, surgery, immunotherapy, hormone therapy, vaccines and targeted therapy, are costly, have many side effects and kill normal cells in addition to cancer cells[4]. Recently, several studies have shown that naturally occurring chemotherapeutic substances are capable of the inhibition and reversal of different stages of carcinogenesis[5,6]. Therefore, it is of interest to explore the possibility of using plant-based phytochemicals or other dietary chemicals, such as chemotherapeutic agents[6,7].

The weed *T. portulacastrum* L. (family *Aizoaceae*), which is widely present in tropical countries including India. The plant is a versatile component of the indigenous Ayurveda and Unani medicines[8]. The principal constituents of *T. portulacastrum* are trianthenol, ecdysterone, 3-acetylaleuritolic acid, leptorumol, 5,2-dihydroxy-7-methoxy-6,8-dimethylflavone, 3,4-dimethoxycinnamic acid, p-methoxybenzoic acid, 5-hydroxy-2-methoxy-benzaldehyde and betacyanin[9,10]. *T. portulacastrum*, known traditionally as Biskhapra, has been widely used for the treatment of urinary system disorders, ascites, blood pressure, anemia, inflammation, night blindness

*Corresponding author

and as an analgesic, stomachic and laxative[8-10]. Experimentally, TPE have been proven to exert nephroprotective, hepatoprotective[11], antioxidative[12], anti-inflammatory and hypolipidemic activities[13]. Recently, Bishayee and Mandal[14] reported that ethanolic TPE showed chemopreventive activity against 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors in rats. Mandal and Bishayee also reported that *T. portulacastrum* prevented DMBA-induced mammary neoplasia by anti-inflammatory mechanism controlled by NF-κB and Nrf2 signaling pathways[15].

DMBA and NMU-induced mammary tumors in rats are widely accepted as a model for the study of cancer pathogenesis and the development of chemotherapeutic and chemoprevention agents for breast cancer[7,14,16-18]. The dynamic process of tumor development can be evaluated through the expression of tumor markers, such as PCNA, caspase-3, VEGF, estrogen receptors and MMP-9, in cancer cells in comparison with their expression in normal cells; this has been effectively used in the diagnosis and prognosis of tumors[18]. Investigations on tumor cell proliferation, invasiveness, angiogenesis and apoptosis can help detect the tumor stage as well as the therapeutic response[7,14,19]. The present study aimed to assess the therapeutic action of TPE on the expression of cancer molecular markers in NMU-induced mammary tumors in Wistar rats. A total of 6 biomarkers (PCNA, VEGF, caspase-3, ER-α, ER-β, and MMP-9), which are responsible for the multistage progression of cancer, were studied in tumor tissues, and the mRNA expression of the PCNA, caspase-3 and ER-β genes was evaluated to understand mechanism of tumor regression.

Methodology

Plant materials

T. portulacastrum was collected from the ICAR-IVRI campus, Izatnagar, Barielly district, Uttar Pradesh, India. The plant was authenticated by the CSIR-National Botanical Research Institute (NBRI), Lucknow, India and a voucher specimen (LWJ-029) was submitted in its herbarium.

Hydroethanolic extraction

The entire plant (excluding the roots) was air-dried in the shade and ground. The ground plant powder was extracted in hydroethanolic solvent (distilled water and ethanol; 1:1) in the ratio of 1:6 (plant powder: solvent) by using Soxhlet apparatus for 8 h at 80°C. The extract was passed through Whatman No. 1 filter paper and evaporated in a water bath until it dried to thick, sticky paste. For further use, the sticky paste was suspended in normal saline to a final concentration of 50 mg/mL.

Qualitative phytochemical analysis

The qualitative phytochemical analysis of hydroethanolic extract of *T. portulacastrum* was conducted to detect the alkaloids, steroids, terpenoids, flavonoids, glycosides, saponins, carbohydrates and phenolic compounds, by using the procedures described by Kokate[20], Harborne[21] and Thimmaiah[22].

Quantitative analysis of phytochemical constituents

Total phenolic content estimation

The total phenolic content of the hydroethanolic extract of *T. portulacastrum* was measured by using the Folin–Ciocalteu (FC) reagent[23]. Linearity was noticed in the range of 0.25–1 mg/mL. The total phenolic content in the hydroethanolic extract was calculated through comparison with the standard curve and expressed as mg gallic acid equivalent (GAE) per g of extract.

Estimation of total flavonoid content (Aluminum chloride colorimetric method)

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino[24]. Catechin was used to construct the calibration curve. The total flavonoid content was calculated as mg catechin equivalent (CE) per g of extract.

Chemicals and antibodies

NMU, 3-Aminopropyl) triethoxysilane (APES) and Extr Avidin peroxidase was obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies PCNA (PC10, sc-56), VEGF (C-1, sc-7269), caspase-3 (H-277, sc-7148), ER-α (H-184, sc-7207), ER-β (H-150, sc-8974) and MMP-9 (c-20, sc-6840), secondary antibodies [goat anti-mouse IgG-B (sc-2039) and goat anti-rabbit IgG-B (sc-2040)] and normal goat serum (sc-2043) were procured from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Various chemicals like TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), DNase I (MBI Fermentas, USA), RNAlater™ (Qiagen, Hilden, Germany), 3,3'-Diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA, USA), Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, USA), and real time One Step Prime Script™ RT-PCR kit (Takara Bio Inc, Japan) were procured from standard company.

Experimental animals

Female Wistar rats, approximately 30 days old, were provided by Laboratory Animals Resource (LAR) Section of ICAR-IVRI, Izatnagar, and maintained in the experimental animal shed, Division of Pathology. The rats were housed in polypropylene cages under suitable environmental conditions (30–55% relative humidity; 27±2°C temperature) in a 12-h light/dark cycle. The rats were acclimatized for 1 week and provided standard diet and water *ad libitum*. The experimental design was approved by the Institute Animal Ethics Committee (IAEC), vide letter no F.1-53/2012-13/JD(R), dated 16/08/2014.

Experimental design

After acclimatization, the rats were divided into 4 groups. In group A (n=6), the rats received an intraperitoneal injection of acidified saline only and served as the vehicle control. In group B (n=6), the rats received 200 mg/kg bw TPE entire experiment and acted as TPE control. In group C (n=6), the rats were administered with 200 mg/kg bw TPE for 2 months after the appearance of palpable tumors. In group D (n=6), the rats with tumors received only normal saline and served as the NMU control.

Chemical induction of mammary tumors

Immediately prior to its use, NMU was dissolved in 0.9% acidified saline (pH 4.0) to a final concentration of 5 mg/mL and was intraperitoneally administered at a concentration of 5 mg/100 g bw. The injection was administered in ventral midline, half way between the two sites of 3rd and 4th pair of mammary glands[18].

Clinical observation

The rats were palpated daily to detect any tumor growth, and their bw were measured at weekly intervals. The size (diameter) and gross appearance of developed tumors were recorded on alternate days. Tumor frequency was calculated from the average number of tumors per tumor-bearing rat. Inhibition of tumor multiplicity=(Total no. of tumors in the carcinogen control)–(total no. of tumors in TPE treated)×100/Total no. of tumors in the carcinogen control.

Histopathological studies

The rats were killed and subjected to necropsy at the end of the experiment. At necropsy, the tumors were observed for position, size, color and consistency. The tissues from normal mammary glands and the mammary tumors were collected in 10% neutral buffered formalin for the preparation of 5-µm-thick paraffin-embedded sections and stained with hematoxylin and eosin (H&E). The stained tumor sections were classified and graded in accordance with the method of Russo and Russo[16]. Carcinomas and normal mammary gland samples were also collected in RNAlater^{TM} (RNA Stabilization Reagent) for mRNA extraction and stored at -80^{o}C.

Immunohistochemical staining

The duplicate paraffin sections collected on clean APES-coated glass slides were dew axed and rehydrated through a graded ethanol series and distilled water. Antigen retrieval was achieved by microwaving the samples in 10 mM tri-sodium citrate buffer for 15 min and then washed with PBS. Endogenous peroxidase was blocked by incubation with 3% H_2O_2, and then samples were incubated with 5% serum for block non-specific antigen binding. The sections were incubated overnight in humidified chamber at 4°C with primary antibodies targeted against PCNA, VEGF, caspase-3, ER-α, ER-β, and MMP-9 (Table 1), incubated with biotinylated secondary antibody for 30 min at RT, and then incubated with ExtrAvidin peroxidase for 30 min at RT. DAB was used to detect the Ag-Ab complex. When acceptable color intensity was obtained, the slides were washed and counter-stained with Mayer's hematoxylin. The slides were rehydrated using an ascending gradient series of alcohol and mounted in DPX mountant.

Semi-quantitative analysis of immunoreactive cells

PCNA immunostaining was evaluated through counting positive and negative nuclei from a minimum of 1000 neoplastic cells in 8–10

Table 1 — Antibodies used for immunohistochemistry in this experiment

Antibodies	Clone/Origin	Blocking serum (5%)	Primary antibody dilution	Secondary antibody dilution
PCNA	Mouse monoclonalantibody	Normal goat	1:500	1:100
VEGF	Mouse monoclonalantibody	Normal goat	1:200	1:100
Caspase-3	Rabbit polyclonal antibody	Normal goat	1:200	1:100
ER-α	Rabbit polyclonalantibody	Normal goat	1:200	1:100
ER-β	Rabbit polyclonalantibody	Normal goat	1:200	1:100
MMP-9	Goat polyclonalantibody	Normal donkey	1:150	1:100

representative high-power fields. Other markers were analyzed by using the method proposed by Franchi et al. 0, no stained cells; 1, ≤25% stained cells; 2, >25% and ≤50% stained cells; 3, >50% and ≤75% stained cells; and 4, >75% stained cells[25].

RNA extraction and reverse transcription

Total RNA was extracted from mammary tumors and normal mammary glands by using TRIzol® reagent and phenol-chloroform extraction. Reverse transcription was performed with RevertAid First Strand cDNA synthesis kit by using random primers. An aliquot of 500 ng RNA was used to synthesize cDNA, and 1 µL reverse transcriptase was used for the subsequent experiments.

Quantitative single step real-time polymerase chain reaction (qRT-PCR)

Real-time PCR was conducted in an Applied Biosystems 7500 thermocycler using SYBR Green I. Each PCR mixture contained 10 µL 2×One-step SYBR® RT-PCR Buffer 4, 100 ng total RNA, and 10 µM of each primer (Table 2). Gene expression was measured through quantification of the mRNA with respect to a calibrator sample (mRNA from a normal mammary gland). All quantifications were normalized with endogenous control GAPDH. The relative value obtained for quantification was expressed as $2^{-\Delta\Delta Ct}$, where ΔCt represents difference between the Ct value of sample and that of GAPDH in the same sample, and $2^{-\Delta\Delta Ct}$ is difference between the ΔCt value of a sample and that of the calibrator sample (normal mammary gland).

Statistical analysis

SPSS software (version 17) was used for the statistical analyses. All data are expressed as mean±SE. ANOVA was used to detect difference between various groups. Post hoc analysis was performed by using Tukey's test. Probability (p) values of less than 0.05 indicated statistical significance.

Results

Phytochemical analysis

The preliminary phytochemical screening of the hydroethanolic extract of *T. portulacastrum* revealed the presence of steroids, alkaloids, terpenoids, glycosides, flavonoids, phenolic compounds, saponins, anthraquinones and carbohydrates. The total phenolic content in the extract was found to be 93±3.9 mg/g GAE. The total flavonoid content in the extracts was found to be 65±2.1 mg/g CE.

General observations

There was no evidence of acute toxicity after the administration of NMU or TPE. No differences in food consumption, water intake, and behavioral changes were observed among groups throughout the experimental period (24 weeks). However, the NMU control rats were more depressed, weaker, had lower body weight, and experienced hair loss.

TPE Reduces size and number of NMU-induced mammary tumors

Most of the tumors in the NMU-treated rats were large, solid in consistency (Fig. 1a), and locally invasive, with multiple growths that subsequently coalesced to become a large tumor mass overlain by necroses and ulcers. The tumors had a more prominent blood supply in the NMU control (Fig. 1a), whereas the TPE-treated group had no prominent blood supply (Fig. 1b). The treatment of rats with TPE also reduced the number and size of tumor (Fig. 1c & Fig. 1d). The size of the tumors was 5–6.5 cm

Table 2 — List of primers used for RT-PCR and qRT-PCR

Target gene	Primer sequence (5′-3′)	Annealing temperature	Product size (bp)	Reference
GAPDH	F: GTTACCAGGGCTGCCTTCTC R: GGGTTTCCCGTTGATGACC	56°C	168	[26]
PCNA	F: GAGTGGGGAGCTTGGCAAT R: ACAACAAGGGGTACATCTGC	55°C	198	This study
VEGF	F: CAGCTATTGCCGTCCAATTGA R: CCAGGGCTTCATCATTGCA	56°C	131	[27]
Caspase-3	F: GCTGGACTGCGGTATTGAGA R: CGTACAGTTTCAGCATGGCG	55°C	179	This study
Estrogen-α	F: TAAGAACCGGAGGAAGAGTTG R: TCATGCGGAATCGACTTG	45°C	623	[28]
Estrogen-β	F: AAGTAGCCGGAAGCTGACAC R: CCGGGACCACATTTTTGCAC	57°C	197	This study
MMP-9	F: CCACCGAGCTATCCACTCAT R: GTCCGGTTTCAGCATGTTTT	59°C	159	[29]

in diameter in the NMU control group (Fig. 1e) and 3–4 cm in the TPE treatment group (Fig. 1d) and led to multifocal necrotic areas (Fig. 1f). The tumor frequency was 3.5 times higher in the NMU control, whereas it reduced to 1.5 times in TPE treatment group. The inhibition of tumor multiplicity was 57.14%, which indicated that TPE reduced tumor multiplicity in NMU-induced mammary carcinogenesis.

TPE Induces intratumoral necrosis

In the NMU control group that comprised 6 rats, a total of 21 tumors developed: 20 were malignant (17 invasive carcinoma and 3 non-invasive *in situ* carcinoma) and 1 was benign. In the TPE-treatment group, 9 tumors were observed in 6 rats; 2 were benign and 7 were malignant (3 invasive carcinoma and 4 non-invasive *in situ* carcinoma). Histopathologically, the tumors in the NMU-control group showed epithelial proliferation both in the lumen of the mammary duct and the alveoli, which resulted in a fused glandular pattern (Fig. 1g). The tumors also showed various combinations, such as papillary and cribriform carcinomas of both *in situ* and invasive types. The tumor sections exhibited fewer areas of necrosis, prominent blood vessels and multi-layered epithelium around the alveoli. The neoplastic cells showed pleomorphism, nuclear enlargement and many mitotic structures (Fig. 1g). In the TPE-treatment group, the alveoli were lined by a multilayered epithelium surrounded by extensive areas of necrotic debris, fewer mitotic structures and fewer blood vessels (Fig. 1h). Vehicle control and TPE control groups showed normal histopathological changes in the mammary gland characterized by fully differentiated alveoli lined by a single layer of low cuboidal epithelium (Fig. 2a & Fig. 2b).

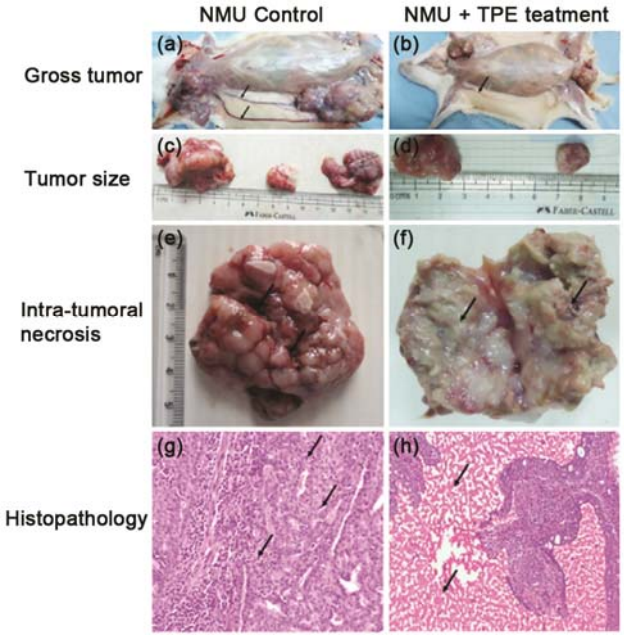

Fig. 1 — Histopathological changes in the mammary gland of Vehicle control (a) and TPE control (b) groups. Normal mammary gland revealed fully differentiated alveoli lined by single layer of low cuboidal epithelium. H & E ×100.

Antiproliferative effect of TPE

The expression patterns revealed by immunohistochemical (IHC) analysis revealed was used to assess the effect of TPE on cellular proliferation in NMU-induced mammary tumor. The level of PCNA expression in all groups showed significant variation. Tumor sections from the NMU-control rats had more than 75% PCNA-positive cells, which indicated an increase in cell proliferation (Fig. 3a). A significant ($p<0.05$) reduction in PCNA expression in the TPE-treated group was indicative of the antiproliferative effect of TPE (Fig. 3b & Fig. 4A). The mRNA expression of PCNA in normal mammary gland, NMU control and TPE-treated tumors was measured by qRT-PCR. In the TPE-treated group, PCNA expression was down regulated in comparison with the NMU control, but up regulated in the NMU control in comparison with normal mammary glands (Fig. 4B).

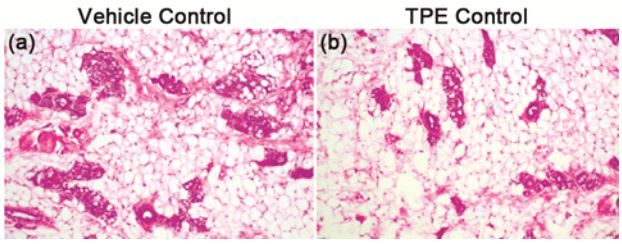

Fig. 2 —TPE-mediated gross and histopathological changes in NMU-induced mammary tumors in rats. a) Mammary tumors are multiple, large, and grayish lobulated mass. The L1 mammary tumor is connected to a coalesced mass of mammary tumors (L4, L5, and L6) by two prominent blood vessels (arrow). b) TPE treatment caused a reduction in mammary tumor size, number, and blood supply (arrow). c) Dissected tumor mass measuring 5.5 cm in diameter in the NMU control. d) Dissected tumor mass in the TPE-treated group measuring 2.8 cm in diameter. e) Dissected tumor mass from the NMU control showing multi-lobuled solid mass without necrosis (arrow). f) A cut section of the tumor mass from the TPE-treated group showing extensive areas of necrosis (arrow). g) Invasive papillary carcinoma: Pleomorphic cancer cells are arranged in solid sheets with marked desmoplasia and mitotic figures (arrow). H&E ×200. h) Invasive papillary carcinoma with comedo pattern: Inward papillary growths within lumen filled with necrotic eosinophilic mass (arrow). H & E ×200.

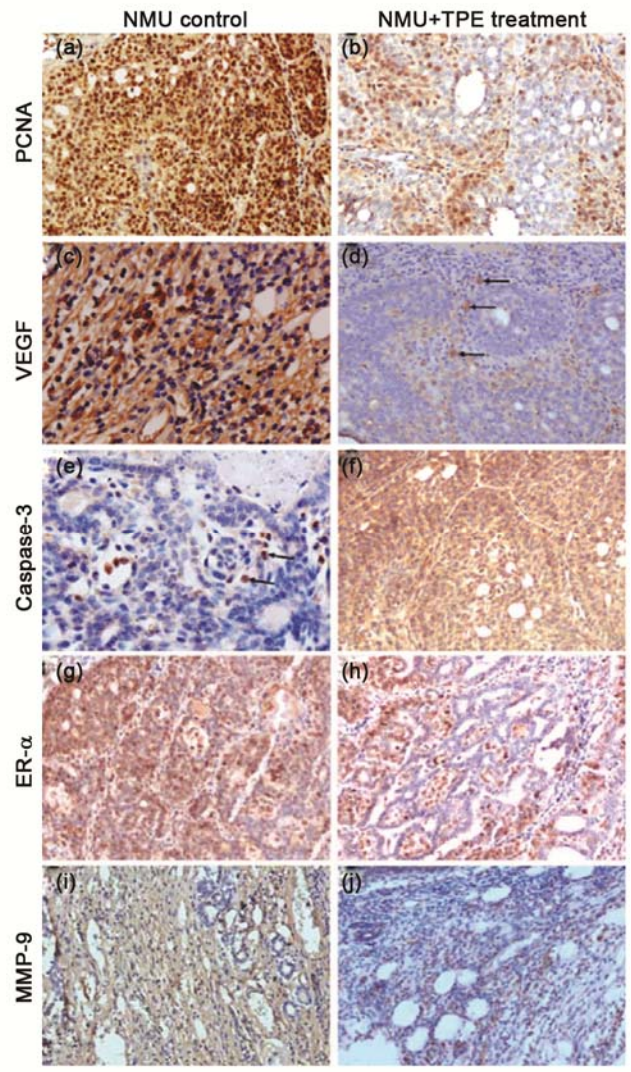

Fig. 3 — Representative images of the immunohistochemical staining of samples from the NMU control and the TPE-treated groups. a) Invasive cribriform carcinoma: More than 85% cells exhibiting strong PCNA-positive brown nuclear immunostaining. IP-DAB-MH ×200. b) Invasive solid cribriform carcinoma: Cancerous cells showing moderate nuclear reaction to PCNA. IP-DAB-MH ×200. c) Invasive tubular adenocarcinoma: Strong diffuse cytoplasmic expression of VEGF. IP-DAB-MH ×200. d) *In situ* solid cribriform carcinoma: Neoplastic cells showing mild cytoplasmic expression of VEGF (arrow). IP-DAB-MH ×200. e) Invasive solid tubular carcinoma: Few neoplastic cells showing cytoplasmic reaction to caspase-3 (arrow). IP-DAB-MH ×400. f) *In situ* solid cribriform carcinoma: More than 85% cancerous cells showing strong cytoplasmic expression of caspase-3. IP-DAB-MH ×200. g) Papillary carcinoma: Neoplastic cells showing strong nuclear reaction to ER-α. IP-DAB-MH ×200. h) Papillary adenoma: Distinct ER-α-positive nuclear immunostaining of tumor cells. IP-DAB-MH ×200. i) Invasive tubular adenocarcinoma: Strong positive MMP-9 immuno-expression in stroma of the tumor. IP-DAB-MH ×200. j) Tubular adenoma: mild MMP-9 expression in the cytoplasm of tumor cells infiltrating the connective tissue. IP-DAB-MH ×200.

TPE Inhibits intratumor angiogenesis

To assess whether TPE inhibited angiogenesis in NMU-induced mammary tumors, the expression of VEGF was assessed in tumor sections by IHC analysis. Elevation in the expression of VEGF was noticed in NMU control rats, which was indicative of an increase in angiogenic activity (Fig. 3c). However, significantly ($p<0.05$) decreased VEGF expression in TPE-treated group indicated antiangiogenic activity of TPE (Fig. 3d).

TPE Induces apoptosis in mammary tumors

To determine the extent of apoptosis in NMU-induced tumors, we assessed caspase-3 expression by IHC analysis and qRT-PCR. The amount of caspase-3 immunopositive cells was much lower in the NMU control (Fig. 3e) in comparison with the TPE-treated group; the frequency of immunopositive staining was more in treated group (Fig. 3f). The mRNA expression of caspase-3 was upregulated in the TPE-treated group, but downregulated in the NMU control (Fig. 4B).

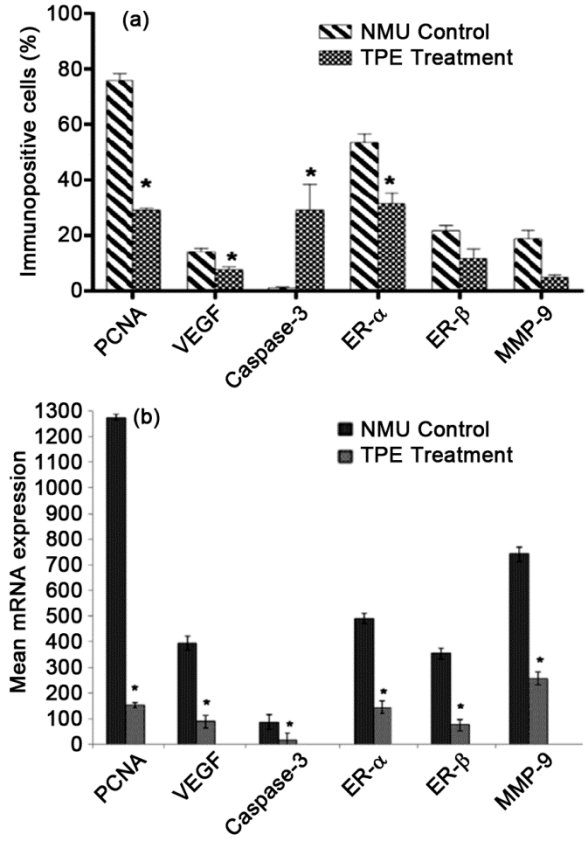

Fig. 4 — Immunohistochemical (A) and mRNA (B) expression index for different markers in the NMU control and TPE-treated groups. Means with an asterisk (*) indicate statistically significant differences ($p<0.05$).

TPE Modulates expression of ER-α and ER-β

The expression of ER-α and ER-β was assessed by IHC staining to evaluate the involvement of ER-α and ER-β signaling in NMU-induced mammary tumors (Fig. 3g & Fig. 4A) and the possible changes after TPE treatment. A significant decrease ($p<0.05$) in ER-α (Fig. 3h) and ER-β expression was observed in the TPE-treated group in comparison with the NMU control (Fig. 3g). The immunohistochemical profile clearly indicated an elevation in the expression of ER-α- and ER-β-positive cells in the NMU-induced mammary tumors (Fig. 4A). The mRNA expression of ER-β was decreased in the TPE-treated group, but was upregulated in the NMU-control group (Fig. 4B).

TPE Inhibits metastasis in mammary tumors

To assess whether TPE inhibited metastasis in NMU-induced mammary tumors, the expression of MMP-9 was assessed in tumor sections by IHC analysis. Tumor sections from the NMU control rats showed a higher cytoplasmic expression of MMP-9 (Fig. 3i). A non-significant reduction in MMP-9 expression in the TPE-treated group indicated that TPE may exert metastasis-inhibiting activity (Fig. 3j).

Discussion

Cancer is a complex and multistage disease, with fatal consequences, which can be induced by radiation, chemicals, or genetic factors[3]. Millions of new cases of mammary cancer in bitches and queens and breast cancer in women are diagnosed, which result in a high number of fatalities[1]. The various available treatment options for cancer involve huge expenses, many side effects, and kill normal cells in addition to cancer cells; therefore, phytochemicals may be a preferably option with respect to economics, reduced side effects and to increase the rate of survival of cancer patients[7,19]. Plants contain various bioactive principles, including phenolic compounds, flavonoids, alkaloids, triterpenes and tannins, which are responsible for the biological effects that include antioxidant, antitumor, antibacterial, and antiviral activities[6,19,30]. In the present study, phytochemical analysis revealed that TPE contained alkaloids, steroids, terpenoids, flavonoids, glycosides, saponins, carbohydrates, and phenolic compounds, which were concordant with the observations of previous studies[9,31,32].

In the present study, we hypothesized that TPE extract could prevent or reduce the incidence and progression of mammary tumors. To prove this hypothesis, an NMU model in Wistar rats was used. NMU, an alkylating carcinogen, is known to induce mammary tumors through a G-35 point mutation in the c-Ki-*ras* proto-oncogene in the 12th codon[33]. Furthermore, in rodent models, the mammary gland is a source of hormone-dependent neoplasms, which are, in many ways, similar to many common malignant tumors diagnosed in women and dogs [16,17]. To explore the mechanism by which TPE exerted its therapeutic effect in NMU-induced mammary tumors, the rats were administered 200 mg/kg bw TPE for 2 months after the induction of tumors. During the experiment, no side effects of NMU (other than cancer) and TPE were observed in the rats. In the treatment group, the number and size of various malignant tumor types were reduced in comparison with the NMU control. This might result from the reduced proliferation of cancer cells, decrease in angiogenesis and extensive apoptosis found in tumor tissues of the TPE-treated group, as evidenced by a corresponding decrease in PCNA, VEGF, ER-α, and ER-β, and an increase in caspase-3 biomarkers, as shown by IHC and qRT-PCR. These findings were also supported by a significant decrease in the mitotic index in the TPE-treated group in the H&E-stained sections.

As the PCNA expression level varies with the cell cycle stage of the mammalian cell[34]. In the present study, a substantial elevation of the expression of PCNA was seen in the NMU control, which is a known carcinogen[18]. Conversely, a significant reduction was seen in treatment group, which suggested an anti-proliferative mechanism of TPE. The same observations were also reported in the use of a DMBA-induced rat model to evaluate the chemopreventive effect of TPE[14]. Further, the mRNA expression of PCNA was down regulated in the TPE group in comparison with the NMU control, which supported the antiproliferative effect of TPE. Angiogenesis is essential both for normal and abnormal tissue growth. Cancer arises owing to an imbalance between positive and negative angiogenic factors. VEGF, a multifunctional cytokine, is mitogenic to vascular endothelial cells and known to play a crucial role in angiogenesis during tumor growth[35]. Nowadays, the inhibition of angiogenesis is a novel strategy in antitumor therapy and its clinical potential has attracted significant attention[36]. Our results showed that TPE treatment resulted in a decrease in vascularization observed by gross examination, histopathology analysis and VEGF expression in comparison with the NMU control,

which was an indication of its anti-angiogenic role. This observation was further supported by an increase in intra-tumoral necrosis and decrease in the size and number of tumors because of inadequate blood supply to tumor cells. Our findings supported the possibility of anti-angiogenic properties as the basic mechanism of cancer therapeutic effects of naturally occurring dietary compounds[37].

Apoptosis is characterized by morphological changes, chromatin condensation, DNA fragmentation and formation of apoptotic bodies. Defects in apoptosis regulatory mechanisms can result in malignant transformation and tumor progression[38]. In the present study, a substantial increase in the induction of apoptosis by TPE was observed in comparison with the NMU control. TPE significantly increased both the protein and mRNA expression of the executioner caspase, caspase-3, which might be the reason for the reduction in the size and number of tumors that occurred in our study. These results clearly showed the apoptosis-inducing property of TPE. More apoptotic cell death may remove neoplastic clone cells and suppress the neoplastic process[39]. The results corroborate an earlier report of Bishayee and Mandal[14], who reported a significant increase in DNA fragmentation and the proapoptotic protein Bax and less expression of anti-apoptotic protein Bcl-2 in mammary tumors triggered by DMBA; thus, this indicated the apoptosis-inducing activity of TPE in mammary tumours. A substantially decreased expression of caspase-3 in NMU control rats indicated the dysregulation of apoptosis in NMU-induced mammary tumors.

Estrogen is a potent mitogen and required for development of normal mammary gland. Estrogen also plays a role in the induction and progression of mammary carcinoma by mediating through estrogen receptors, ER-α and ER-β[40]. ER-α is the major ER subtype present in the mammary epithelium. For this reason, targeting the estrogen receptors or blocking their action by using anti-estrogen compounds from dietary sources has recently emerged as an increasingly important treatment for breast cancer. In the present study, a substantially elevated expression of ER-α and ER-β in the mammary tumors of NMU control rats indicated an accelerated proliferation of tumor cells that resulted from the increased expression of estrogen receptors. Our findings were consistent with the finding of a previous study[41]. Interestingly, we found that both protein expression and mRNA expression of ER-α and ER-β significantly reduced in TPE-treated group, which suggested that ERs were a novel target of TPE in mammary cancer. These results are supported by a recent evidence, showing that the inhibition of estrogen receptors is an essential component of the mechanism of cancer preventive effects of naturally occurring dietary compounds[42].

Matrix metalloproteinases (MMPs) are involved in the breakage of extracellular matrix that play a crucial role in several stages of tumor progression, including invasion, angiogenesis and metastasis. Tumor cells produce increased levels of MMP enzymes that destroy the basement membranes and allow metastasis[43]. In the present study elevated levels of MMP-9 were observed in the NMU control, whereas reduced levels were observed in the TPE treatment group, which indicated the metastatic preventive potential of the extract. Elevated levels of MMP-9 in the NMU control correlated with increased number of tumors, greater malignancy and non-mammary tumors in the kidneys, lungs and ovaries (data not shown). Similar observations were made in breast cancer brain metastasis in rats by Mendes *et al.*[44]. The reduced level of MMP-9 in the TPE treatment group was consistent with other dietary compounds, such as curcumin, which prevent metastasis through the down regulation of MMP-2 and MMP-9 and the up regulation of expression of TIMP1 and TIMP4 in breast cancer cells[45,46].

Conclusion

In conclusion, the results of our present study showed the therapeutic effect of a hydroalcoholic extract of TPE on NMU-induced mammary tumors in rats. TPE treatment slowed down the tumor growth rate, reduced the tumor frequency and size and showed more intratumoral necrosis and less malignancy in comparison with the NMU control. Our results showed that the therapeutic effect of TPE could be attributed to its antiproliferative, antiangiogenic, apoptosis-inducing and metastasis-inhibiting properties. These findings suggest that TPE is a promising cancer therapeutic agent, owing to its non-toxicity in the host. However, further investigations are necessary to identify the detailed molecular mechanisms responsible for its anticancer action and a comprehensive phytochemical analysis is required.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

References

1 Siegel RL, Miller KD & Jemal A, Cancer statistics, 2017, *CA: A Cancer Journal for Clinicians*, 67 (2017) 7–30.

2 Bray F, McCarron P & Parkin DM, The changing global pattern of female breast cancer incidences and mortality, *Breast cancer Research*, 6 (2004) 229–239.

3 Kerr J, Anderson C & Lippman SM, Physical activity, sedentary behaviour, diet, and cancer: an update and emerging new evidence, *Lancet Oncology*, 18 (2017) e457–e471.

4 Khosravi-Shahi P, Cabezón-Gutiérrez L & Custodio-Cabello S, Metastatic triple negative breast cancer: Optimizing treatment options, new and emerging targeted therapies, *Asia-Pacific Journal of Clinical Oncology*, 14 (2018) 32-39.

5 Surh Y, Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances, *Mutation Research*, 428 (1999) 305–327.

6 Saminathan M, Rai RB, Dhama K, Tiwari R, Chakraborty S, *et al.*, Systematic review on anticancer potential and other health beneficial pharmacological activities of novel medicinal plant *Morinda citrifolia* (Noni), *International Journal of Pharmacology*, 9 (2013) 462–492.

7 Narendra K, Singh R, Kurade NP, Saminathan M, Ranjan R, *et al.*, Immunohistochemical evaluation of chemopreventive effect of hydro-alcoholic extract of garlic (*Allium sativum*) in N-methyl-N-nitrosourea (NMU) induced rat mammary tumours, *Indian Journal of Veterinary Pathology*, 39 (2015) 210–216.

8 Gaddeyya G & Kumar PKR, A comprehensive review on ethnobotany and photochemistry of an herbal weed *Trianthema portulacastrum* L, *Journal of Pharmacognosy and Phytochemistry*, 4 (2015) 25–31.

9 Shivhare MK, Singour PK, Chaurasiya PK & Pawar RS, *Trianthema portulacastrum* L. (bishkhapra), *Pharmacognosy Reviews*, 6 (2012)132–140.

10 Yamaki J, Venkata KCN, Mandal A, Bhattacharyya P & Bishayee A, Health-promoting and disease-preventive potential of *Trianthema portulacastrum* Linn. (Gadabani) -An Indian medicinal and dietary plant, *Journal of Integrative Medicine*, 14 (2016) 84–99.

11 Banu GS, Kumar G & Murugesan AG, Effect of ethanolic leaf extract of *Trianthema portulacastrum* L. on aflatoxin induced hepatic damage in rats, *Indian Journal of Clinical Biochemistry*,24 (2009)414–418.

12 Kumar G, Banu GS & Pandian MR, Evaluation of the antioxidant activity of *Trianthema portulacastrum*L, *Indian Journal of Pharmacology*, 37 (2005)331–333.

13 Anreddy RN, Porika M, Yellu NR & Devsrakonda RK, Hypoglycemic and hypolipidemic activities of *Trianthema portulacastrum* Linn. plant in normal and alloxan induced diabetic rats, *International Journal of Pharmacology*,6 (2010)129–133.

14 Bishayee A & Mandal A, *Trianthema portulacastrum* Linn. exerts chemoprevention of 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis in rats, *Mutation Research*, 768 (2014)107–118.

15 Mandal A & Bishayee A, *Trianthema portulacastrum* Linn. displays anti-inflammatory responses during chemically induced rat mammary tumorigenesis through simultaneous and differential regulation of NF-κB and Nrf2 signaling pathways, *International Journal of Molecular Sciences*, 16 (2015) 2426–2445.

16 Russo J & Russo IH, Atlas and histologic classification of tumors of the rat mammary gland, *Journal of Mammary Gland Biology and Neoplasia*, 5 (2000) 187–200.

17 Chan MM, Lu X, Merchant FM, Iglehart JD & Miron PL, Gene expression profiling of NMU-induced rat mammary tumors: Cross species comparison with human breast cancer, *Carcinogenesis*, 26 (2005)1343–1353.

18 Saminathan M, Rai RB, Dhama K, Ranganath GJ, Murugesan V, *et al.*, Histopathology and immunohistochemical expression of N-Methyl-N-Nitrosourea (NMU) induced mammary tumours in Sprague- Dawley rats, *Asian Journal of Animal and Veterinary Advances*,9 (2014)621–640.

19 Saminathan M, Rai RB, Dhama K, Jangir BL, Suresh S, *et al.*, Effect of *Morinda citrifolia* (Noni) fruit juice on antioxidant, hematological and biochemical parameters in N-Methyl-N-Nitrosourea (NMU) induced mammary carcinogenesis in Sprague-Dawley rats, *International Journal of Pharmacology*, 10 (2014) 109–119.

20 Kokate CK, Practical pharmacognosy, India: New Delhi, Vallabh Prakashan Press, 1994, 107–113.

21 Harborne JB, Phytochemical methods, Chapman and Hall, London, 1998, 60-66.

22 Thimmaiah SR, Standard methods of biochemical analysis, 2nd ed. New Delhi: Kalyani Press, 2004.

23 Slinkard K & Singleton VL, Total phenol analysis: automation and comparison with manual methods, *American Journal of Enology and Viticulture*, 28 (1977) 49–55.

24 Woisky R & Salatino A, Analysis of propolis: some parameters and procedures for chemical quality control, *Journal of Apicultural Research*, 37 (1998) 99–105.

25 Franchi A, Santucci M, Masini E, Sardi I, Paglierani M, *et al.*, Expression of matrix metalloproteinase 1, matrix metalloproteinase 2, and matrix metalloproteinase 9 in carcinoma of the head and neck, *Cancer*, 95 (2002) 1902–1910.

26 Shibata M, Hariya T, Hatao M, Ashikaga T & Ichikawa H, Quantitative polymerase chain reaction using an external control mRNA for determination of gene expression in a heterogeneous *cell population*, *Toxicological Sciences*, 49 (1999) 290–296.

27 Gómez R, Simón C, Remohí J & Pellicer A, Vascular endothelial growth factor receptor-2 activation induces vascular permeability in hyperstimulated rats, and this effect is prevented by receptor blockade, *Endocrinology*, 143 (2002) 4339–4348.

28 Al-Bader MD, Estrogen receptors alpha and beta in rat placenta: detection by RT-PCR, real time PCR and Western blotting, *Reproductive Biology and Endocrinology*, 4 (2006) 13.

29 Deschner J, Rath-Deschner B & Agarwal S, Regulation of matrix metalloproteinase expression by dynamic tensile strain in rat fibrochondrocytes, *Osteoarthritis Cartilage*, 14 (2006) 264–272.

30 Saminathan M, Rai RB, Murugesan V, Kumar SS, Baqir M, *et al.*, Induction of cataract by N-methyl-N-nitrosourea (NMU) and anti-cataract activity of *Morindacitrifolia* fruit juice and ethanolic leaf extract in *rats*, *Indian Veterinary Journal*, 91 (2014) 18–20.

31 Sunder AS, Reddy ARN, Kiran G & Thirumurugu S, Anti-hyperlipidemic and antioxidant activity of methanolic extract of *Trianthema portulacastrum* in rats fed a high-fat diet, *Journal of Herbs, Spices & Medicinal Plants*, 16 (2010) 1–10.

32 Kavitha D, Parvatham R & Padma P, Assessment of *Trianthema portulacastrum* for its antimicrobial potential and investigation of their phytochemicals using HPTLC, GC-MS, and IR, *International Journal of Pharmacy and Pharmaceutical Sciences*, 6 (2014) 675–686.

33 Miyamoto S, Sukumar S, Guzman RC, Osborn RC & Nandi S, Transforming c-Kiras mutation is a preneoplastic event in mouse mammary carcinogenesis induced *in vitro* by N-methyl-N-nitrosourea, *Molecular and Cellular Biology*, 10 (1990) 1593–1599.

34 Paunesku T, Mittal S, Protić M, Oryhon J, Korolev SV, *et al.*, Proliferating cell nuclear antigen (PCNA): ringmaster of the genome.*International Journal of Radiation Biology*,77 (2001)1007–1021.

35 Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, *et al.*, Vascular endothelial growth factor and angiogenesis, *Pharmacological Reviews*, 56 (2004)549–580.

36 Liang Y, Wu J, Stancel GM & Hyder SM, p53-dependent inhibition of progestin induced VEGF expression in human breast cancer cells, *The Journal of Steroid Biochemistry and Molecular Biology*,93 (2005) 173–182.

37 Seo EJ, Kuete V, Kadioglu O, Krusche B, Schroder S, *et al.*,Antiangiogenic activity and pharmacogenomics of medicinal plants from traditional Korean medicine, *Evidence-Based Complementary and Alternative Medicine*, 2013 (2013) 131306.

38 Thompson CB, Apoptosis in the pathogenesis and treatment of disease, *Science*, 267 (1995)1456–1462.

39 Admas JM & Cory S, The Bcl-2 apoptotic switch in cancer development and therapy, *Oncogen*e, 26 (2007)1324–1337.

40 Thomas C & Gustafsson JA, The different roles of ER subtypes in cancer biology and therapy, *Nature Reviews Cancer*, 11 (2011)597–608.

41 Thordarson G, Lee AV, McCarty M, Van Horn K, Chu O, *et al.*, Growth and characterization of N methyl-Nnitrosourea- induced mammary tumors in intact and ovariectomized rats, *Carcinogenesis*,12 (2001)2039–2047.

42 Hahm ER & Singh SV, Diallyltrisulfide inhibits estrogen receptor-α activity in human breast cancer cells, *Breast Cancer Research and Treatment*, 144 (2014) 47–57.

43 Kohrmann A, Kammerer U, Kapp M, Dietl J & Anacker J, Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature, *BMC Cancer*, 9 (2009) 188.

44 Mendes O, Kim HT & Stoica G, Expression of MMP2, MMP9 and MMP3 in breast cancer brain metastasis in rat model, *Clinical & Experimental Metastasis*, 22 (2005)237–246.

45 Hassan ZK & Daghestani MH, Curcumin effect on MMPs and TIMPs genes in a breast cancer cell line, *Asian Pacific Journal of Cancer Prevention*, 13 (2012)3259–3264.

46 Phienwej H, Swasdichira IS, Amnuoypol S, Pavasant P & Sumrejkanchanakij P, *Tinosporacrispa* extract inhibits MMP-13 and migration of head and neck squamous cell carcinoma cell lines, *Asian Pacific Journal of Tropical Biomedicine*, 5 (2015) 738–743.