

Research Article**Chemoprevention of breast cancer by *Psidium guajava* Linn.**Prachi D. Karia^a, Laxmi A. Patil^b, Mitul S. Vakani^c, Gaurav M. Chauhan^c, Sarita S. Gupta^c, S. P. Rathod^d, Kirti V. Patel^{b*}^aITM School of Pharmacy, Dhanora Tank Road, Paldi Village, Halol Highway, Near Jarod, Vadodara, Gujarat, India^bFaculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India^cDepartment of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India^dParul Institute of Pharmacy and Research, Vadodara, Gujarat, India

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Abstract

Objective: The present study was performed to investigate the chemo-preventive potential of Pulp of *Psidium guajava* (POPG) in breast cancer and elucidating its mechanism of action by assessing its effect on key processes like apoptosis, angiogenesis and metastasis. **Materials and Methods:** The cytotoxicity assay of POPG was performed on MCF-7(ER+), MDA-MB-231(Triple negative) and MDA-MB-453 (HER2+) human breast cancer cell lines. Assessment of anticancer potential of POPG was done through measurement of growth rate, feed consumption efficiency, tumor parameters, estrogen and progesterone expressions & nucleic acid content in *in-vivo* study. The mechanism for anticancer potential was screened by *in-vitro* studies involving Migration assay (metastasis), Annexin V- FITC assay (apoptosis) and Chick Chorioallantoic Membrane assay (angiogenesis). Statistical analysis was done by ANOVA followed by bonferroni's post-hoc test. **Results:** The IC₅₀ value of POPG on MCF-7 cells was significantly less than other two cell lines, indicating POPG to be more potent inhibitor of ER+ cells *in-vitro*. Confirmatory results were obtained in MNU induced mammary carcinoma. POPG attenuated tumor parameters, expression of estrogen and progesterone receptor, nucleic acid content and increased latency period. POPG prevented MCF-7 cell migration (66.67%) suggesting inhibition of metastasis. POPG significantly increased apoptotic rate, especially late apoptotic population (6.1%). The mean zone of inhibition in CAM assay was found to be 1.13±0.33 implying inhibition of neovascularization. **Conclusion:** POPG thus depicts chemoprevention of breast cancer and this could be attributed to its ability to induce apoptosis, curtail angiogenesis, prevent metastasis and is mediated through inhibition of estrogen and progesterone expression.

Keywords: Breast cancer, MCF-7 cells, methylnitrosourea, *Psidium guajava***Introduction**

Breast cancer continues to be the most frequently occurring cancer in women around the world. For women, the three most commonly diagnosed cancers are breast, lung and bronchus, and colorectal. Representing one-half of all the cases; breast cancer alone is expected to account for 30% all new cancer diagnoses in women. An estimated 41,070 breast cancer deaths will occur in

2017 (Siegel et al., 2017). In India, it is the cancer of breast alone which is expected to cross the figure of 200000 by the year 2021 (Takiar et al., 2010).

Based on the stage of diagnosis, breast cancer is treated with a multidisciplinary approach involving surgery, radiation and systemic therapy including chemotherapy or hormonal therapy (Harfindal and Helms, 2006). Surgery alone may increase the chances of relapse so combinations of chemotherapeutic agents are given. The side effects of chemotherapy depend on the individual, the drug used, the schedule and dose used. These side effects can include fatigue, risk of infection, nausea, vomiting, mouth sores, hair loss, anorexia, diarrhea and bone marrow suppression. Hormonal modifiers like Tamoxifen are given in estrogen

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and progesterone positive breast cancer. The use of Tamoxifen is established but side effects like endometrial cancer and thromboembolic complexities are issue of concern. Moreover, chances of resistance development are higher. Radiation therapy causes swelling of the breast, redness and/or skin discoloration/hyperpigmentation and pain/burning in the skin.

Unfortunately, in spite of improved diagnostic skills and breakthrough in effective treatment, breast cancer continues to be the leading cause of cancer deaths among women worldwide. Cancer being a multifaceted disease should be targeted on multiple pathways. The complexity of this hyperproliferative disease requires the adoption of prevention as promising and rational way to control it. Several new studies have discovered that most patients on cancer therapy are concurrently self-medicating with one or several complementary and alternative medicines (Rockwell et al., 2005). Among Complementary and alternative medicines, natural herbal medicine is the most commonly used group of treatment. Herbal treatment is the oldest used system of medicine in the world with more than 2000 years history (Ma et al., 2011) The herbs prevent malignancy by promoting detoxification, modify the activity of precise hormones and enzymes, diminish lethal side effects and complications of chemotherapy and radiotherapy (Sakarkar and Deshmukh, 2011). Moreover, phytoconstituents resulting from the herbs such as *Vinca rosea*, *Taxus species*, *Allium sativum*, *Panax pseudoginseng*, *Taxus wallichiana*, *Tinospora cordifolia*, *Viscum album*, *Withania somnifera*, *Zingiber officinale* etc. have been used in numerous preparations to assist the body to battle cancer more efficiently and also decrease the harmful side effects of chemotherapy and radiotherapy. Vinca alkaloids, Docetaxel and Paclitaxel hold their names in FDA approved list for treatment of breast cancer. Thus, an attempt was made in the current study to identify an easily available common herb and evaluate its potential in the treatment or prevention of breast cancer. Hence, Pulp of *Psidium guajava* was selected as a plant for evaluating anticancer potency.

Psidium guajava traditionally employed intensively as folklore remedy for a wide spectrum of gastrointestinal diseases in India. *Psidium guajava* possess antibacterial, antispasmodic, anti-inflammatory, analgesic, anti-diarrheal, hepatoprotective and anti-diabetic activity (Barbalho et al., 2012). The anticancer activity of guava is proven in prostate cancer (Chen et al., 2010). Aqueous extract of leaves inhibited LNCaP cell proliferation and down-regulate expressions of androgen receptor and prostate specific antigen. Treatment with leaves also significantly diminished tumor size in a xenograft mouse tumor model (Chen et al., 2010). There is also molecular evidence that cytotoxic activities of guava may act via repression of the NF-kB pathway mainly inhibiting NF-kB transactivation level (Kaileh et al., 2007; Ojewole, 2006). The ethanol extract from guava leaf

possess prostaglandin endoperoxide H synthase inhibitory activity, an enzyme responsible for synthesis of prostaglandins which play important role in inflammation and carcinogenesis (Kawakami et al., 2009). *Psidium guajava* contains myricetin (Miean and Mohamed, 2001), which is reported to be aromatase inhibitor (Paoletta et al., 2008) and anti-cancer (Lu et al., 2006) *in-vitro*. *Psidium guajava* fruits and leaves are enriched with anti-oxidative compounds that are able to suppress huge harming impacts of reactive oxygen species (ROS) which can be related to its anticancer activity (Feng et al., 2015; Thaipong et al., 2006).

In view of above mentioned facts, the present investigation was designed to evaluate the anticancer effects of *Psidium guajava* Linn. on mammary carcinoma.

Materials and Methods

Materials

MCF-7, MDA-MB-231 and MDA-MB-453 human breast cancer cell lines were procured from NCCS, Pune. Methylnitrosourea (MNU), Propidium iodide were procured from Sigma Aldrich. MTT, DMSO, Culture media, fetal bovine serum, penicillin G-streptomycin solution were procured from Himedia. Annexin V-FITC assay kit was procured from BD sciences.

Preparation of pulp of *Psidium guajava* (POPG)

Ripe fruits of *Psidium guajava* Linn. were collected from local market of Baroda and authenticated by senior scientist Dr. Geetha, Plant Breeding Department, National Research Centre for Medicinal and Aromatic Plants, Boriavi. All of the guavas were free from physical and pathological defects. The pulp was freshly prepared by mechanically crushing the *Psidium guajava* fruits and was standardized to maintain specific gravity in the range of 1.05 to 1.35. It was prepared in the doses of 100mg/kg, 200 mg/kg and 400 mg/kg.(Rai et al., 2007). A voucher specimen (voucher number MSUPC-016) of the same is deposited at Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara, India.

Cell cultures

Human breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-453 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) -high glucose supplemented with 10% fetal bovine serum (FBS), 1% penicillin G-streptomycin solution at 37°C in a 5% CO₂ incubator.

MTT Assay

Cell growth inhibitory assay was performed by MTT method on human breast cancer cell lines as described

(Parvathaneni et al., 2014) with various concentrations (10-1000 µg/ml) of POPG for 24, 48, and 72 h.

Scratch Motility Assay

Anti-metastatic potential of POPG (820 µg/ml) was studied on MCF-7 cells for 24 hours by Scratch Motility Assay (Tang et al., 2013).

Annexin V- FITC apoptosis assay

Apoptosis was studied on MCF-7 cells using Annexin V-FITC/propidium iodide (PI) double staining as described with POPG (820 µg/ml) for 24 hrs (Zhu et al., 2014).

Chick Chorioallantoic Membrane (CAM) Assay

Anti-angiogenic potential of POPG was studied using Chick Chorioallantoic Membrane assay as described (Karia et al., 2018).

In-vivo MNU induced mammary carcinogenesis

Nulliparous female Sprague Dawley rats were obtained from Zydus Research Centre, Ahmedabad. The animals were housed in a group of 6 rats per cage under well-controlled conditions of temperature ($22 \pm 2^\circ\text{C}$), humidity ($55 \pm 5\%$) and 12hrs/12hrs light-dark cycle. The animals had free access to conventional laboratory diet and distilled water *ad libitum*.

The experiment was carried out as per guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and The Prevention of Cruelty to Animals act (PCA), 1960. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department, The Maharaja Sayajirao University of Baroda (MSU/IAEC/2015-16/1601).

Mammary cancer was induced by a single dose of 50mg/kg body weight of MNU, dissolved in 0.9% saline adjusted with acetic acid (pH=4) and then administered intraperitoneally. Experimental protocol was of 100 days (Jagadeesan et al., 2013).

The rats were randomly divided into 7 groups. The normal control (group 1) animals received saline. All groups except group 1 received single dose of 50mg/kg b.w; i.p MNU diluted in 0.9% saline adjusted with acetic acid at pH 4.0. Group 2 served as model control. Group 3 (Vehicle control) animals received sesame oil as per the body weight, Group 4 served as standard control and received Tamoxifen (1mg/kg b.w; s.c) (Martin et al., 1996). Group 5 To 7 were test groups and received POPG viz 100mg/kg; 200 mg/kg and 400mg/kg respectively (Rai et al., 2007). The experimental duration was for 100 days.

During experimental period, the rats were palpated for tumors every two weeks. Growth rate using formula ($\text{Final body weight} / \text{Initial Body weight}^{(1/\text{Periods}-1)}$) and feed consumption efficiency using formula ($\text{Weekly body weight gain} / \text{Weekly food}$

consumption)*100 were calculated. At the end, animals were euthanized humanely for assessing different parameters. Tumor parameters (Parvathaneni et al., 2014) involved weight, number of tumors, volume, tumor incidence and latency period (Jagadeesan et al., 2013). Estrogen and Progesterone receptor expressions were quantified by immunohistochemistry (Parikh et al., 2005; Thordarson et al., 2001). The nucleic acids were extracted for measurement of DNA and RNA (Rengarajan et al., 2013).

Statistical analysis

All the data were expressed as mean \pm SEM. The results were compared using a computer based fitting program (Prism, GraphPad version 5, GraphPad Software, Inc). Statistical difference between the means of the various groups were analyzed using ANOVA followed by post hoc Bonferroni's test with P value < 0.05 .

Results

Effect of POPG on cell inhibition (MTT assay) on human breast cancer cell lines

In the present study, POPG (10, 100, 200, 300, 500, 1000 µg/ml) showed decreased cell proliferation in MCF-7 human breast cancer cell line in concentration- and time-dependent manner (regression) when compared as seen by descending IC_{50} values (820 ± 1.22 µg/ml, 680 ± 1.34 µg/ml and 600 ± 1.03 µg/ml for 24 h, 48h and 72 h respectively) (Figure 1(a)). The pattern observed for % cell inhibition in MDA-MB-453 cells was in accordance with those observed for MCF-7 cells but the % cell inhibition was comparatively weaker in case of MDA-MB-453 as depicted by IC_{50} (1000 µg/ml for 72 h) (Figure 1 (b)). The pattern obtained for % cell inhibition on MDA-MB-231 was completely different than that observed for MCF-7 and MDA-MB-453 cells. In case of MDA-MB-231 cells, the % cell inhibition was weak after 24 h as compared to 72 h. The IC_{50} value was higher than 1000 µg/ml (Figure 1 (c)). These results clearly demonstrated POPG potential to inhibit cell proliferation in MCF-7 human breast cancer cell line than in other two cell lines.

Effect of POPG on Scratch Motility Assay in MCF-7 human breast cancer cell line

Using scratch motility assay, a continuous and rapid movement was observed for all cells. The movement of cells was clearly evident in control wells at 24 hours, in which a highly confluent monolayer region gradually migrated to cell free 'scratch region'. In POPG treated well, the migration of cells was reduced and the reduction in migratory effect was found to be 66.67% (Figure 2).

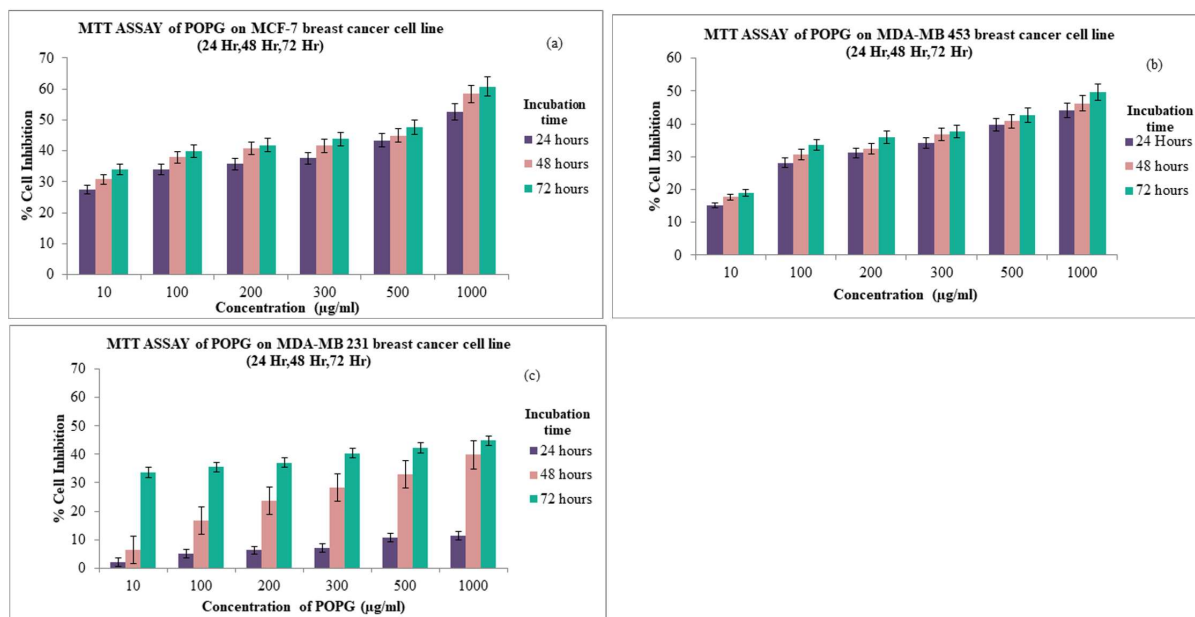


Figure 1. Effect of POPG on the cell inhibition on (a). ER+ MCF-7 human breast cancer cell line (b). HER2+ MDA-MB-453 human breast cancer cell line (c). Triple negative MDA-MB-231 human breast cancer cell line. Values are expressed as mean \pm SEM. (N=3)

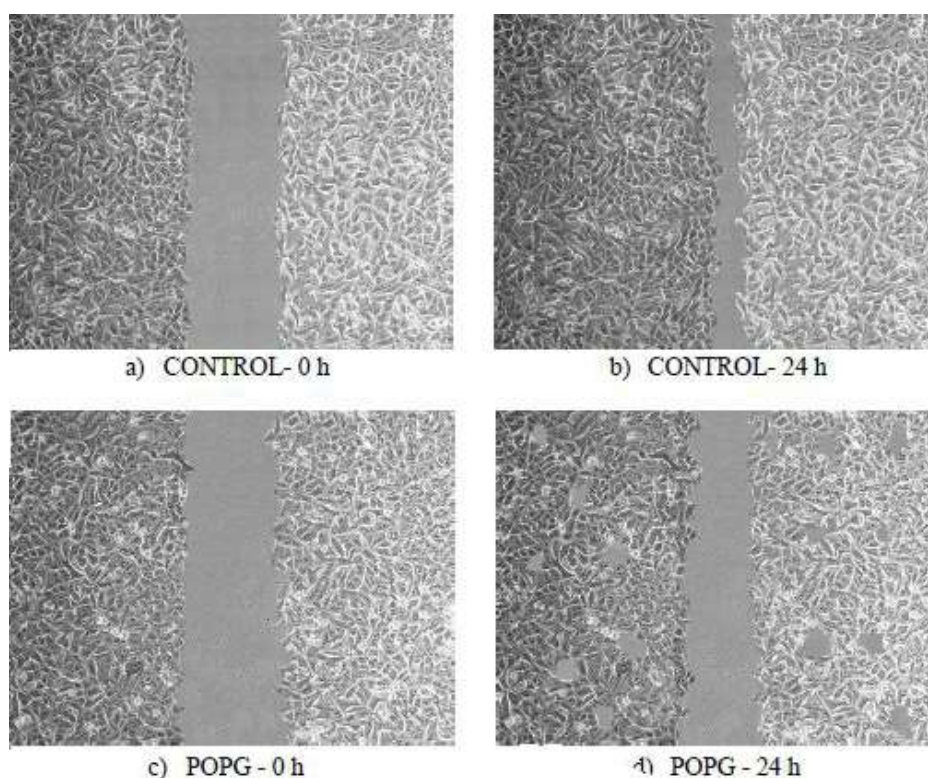


Figure 2. Effect of POPG on the cell migration (metastasis) on MCF-7 human breast cancer cell line. (a) Control- 0 h- MCF-7 cells with scratch. (b) Control 24 h- Migration of cells and restoration of monolayer in scratched area. (c) POPG- 0 h- MCF-7 cells with scratch (d) POPG 24 h- Inhibition of cell migration and less restoration of monolayer in scratched area as compared to control cells. The photographs are taken in Nikon Eclipse TS100. Magnification: 10X.

Effect of POPG on Apoptosis on MCF-7 human breast cancer cell line (Annexin V- FITC binding assay)

The results of Annexin V/PI double-staining assay demonstrated that the apoptosis of MCF-7 cells was observed after treatment with POPG for 24 h. As shown in Figure 3, after treatment with

POPG, viable cell percentage was reduced to 54% which was 72.1% in control cells. The early apoptotic populations were found to be 2.5% whereas 6.1% cells were found to be in late apoptotic phase after treatment with POPG. The necrotic phase constituted 5.3% cell population. The

Table 1. Effect of POPG on Tumor parameters in MNU induced mammary carcinogenesis

Groups	Tumor Incidence	Tumor burden	Tumor multiplicity	Tumor weight (g)	Tumor volume (mm ³)	Tumor volume	Tumor latency period (days)
Normal control	0	0	0	0	0		0
Model control	5	6	1	6.4± 1.46 ^{###}	99.29± 1.18 ^{###}		45.17±9.21 ^{###}
Vehicle control	6	6	1	6.2± 1.55	89.74± 1.18		53.23±19.41
Standard control	5	5	0.84	0.88±0.29 ^{***}	2.02±6.33 ^{***}		82.17±16.43 ^{**}
POPG 100 mg/kg	6	6	1	3.27±0.64 [*]	23.57± 3.45 ^{***}		68.67±13.43
POPG 200 mg/kg	6	6	1	2.6±0.66 ^{**}	12.04± 1.79 ^{***}		71.5±15.90
POPG 400 mg/kg	5	5	0.84	1.4±0.33 ^{***}	5.16± 2.54 ^{***}		75±1.63 [*]

Values are expressed as Mean ± SEM of 6 animals. Values are statistically evaluated using ANOVA analysis followed by Dunnett's Post hoc test. Significant values were compared with ^{###}P<0.001 normal control vs. model control; ^{*}P<0.05 model control vs. all other groups; ^{**}P<0.01 model control vs. all other groups ^{***}P<0.001 model control vs. all other groups

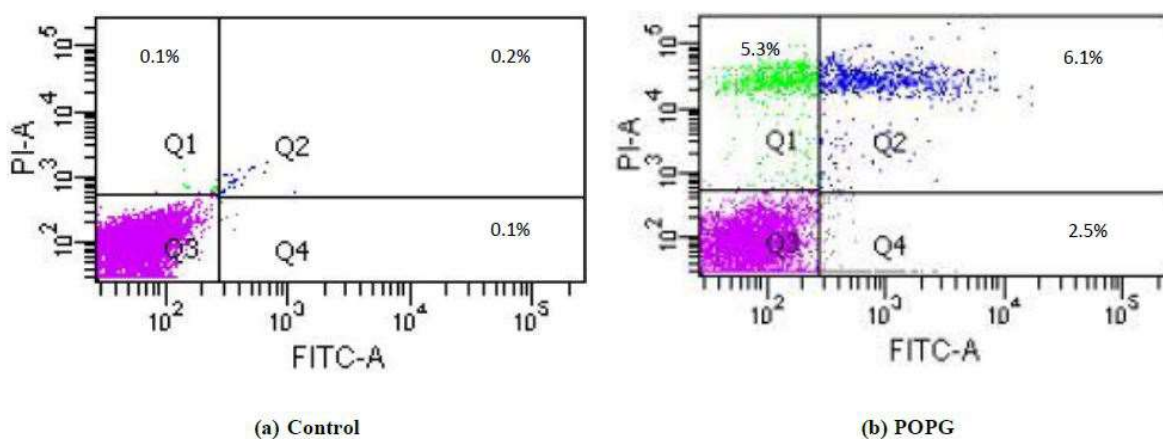


Figure 3. Effect of POPG on the apoptosis on MCF-7 human breast cancer cell line: Q1: Necrosis; Q2: Late Apoptosis; Q3: Viable cells; Q4: Early Apoptosis. (a) Control MCF-7 cells. (b) MCF-7 cells treated with POPG: Percentage of late apoptotic and necrotic population is increased. The analysis was done by FACSDiva Version 6.1.3.

untreated cells did not show any significant apoptosis.

Effect of POPG on Chick Chorioallantoic Membrane Assay in fertilized eggs

The antiangiogenic activity of POPG was investigated using a CAM assay. The increase in the blood vessel total diameter was observed in the PBS control group over the 48 h treatment and monitoring period. Starting from the first day of POPG treatment, a decrease in the vasoproliferation was observed. POPG produced a significant decrease in the development of angiogenesis in a chick embryo without any sign of thrombosis and hemorrhage over 48 h treatment and monitoring period (Figure 4). The zone of inhibition was found with 1.13±0.33 mm.

Effect of POPG on Tumor Parameters in MNU induced mammary carcinogenesis

Oral administration of SD rats bearing MNU induced mammary cancer with POPG significantly decreased the mammary cancer. The incidence rate in untreated MNU group was found to be 83.33%. The weight and volume of tumor in positive control animals were found to be 6.4±1.47 g and 99.29 ±3.19 mm³. The

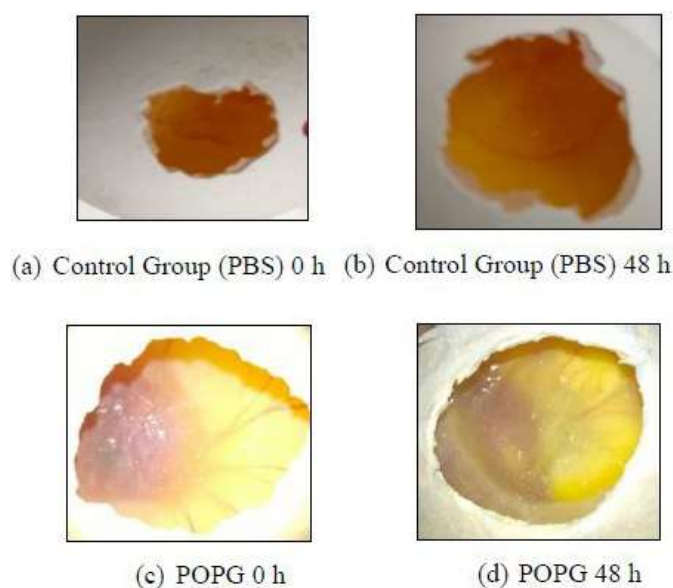


Figure 4. Effect of POPG (30µg/ml) on angiogenesis in the Chick Chorioallantoic Membrane of fertilized eggs: (a) Control group 0 h (b) Control group after 48 h- no hindrance in growth and neovascularization of CAM was observed. (c) POPG 0 h (d) POPG 48 h- Zone of inhibition with loss of blood vessels

weights and volume were significantly curtailed in all treatment groups. The latency period of cancer bearing group II was found to be 45 days. Tamoxifen (82.17 ± 16.43 ; $P < 0.001$) and all doses of POPG (100mg/kg: 68.67 ± 13.43 ; 200mg/kg: 71.5 ± 15.90 and 400mg/kg: 75 ± 15.90) prolonged latency period. No significant changes were observed in group III vehicle control animals (Table 1).

Effect of POPG on Growth Rate in MNU induced mammary carcinogenesis

When body weight was evaluated as % growth rate, significant difference was found from 63 day. From then, the growth rate of model control animals decreased significantly ($P < 0.05$) till the end. In vehicle control, the growth rate curve runs parallel to model control suggesting no significant difference. On treatment with POPG (100mg/kg, 200mg/kg and 400 mg/kg) and tamoxifen, the growth rate curve resembles to normal control and

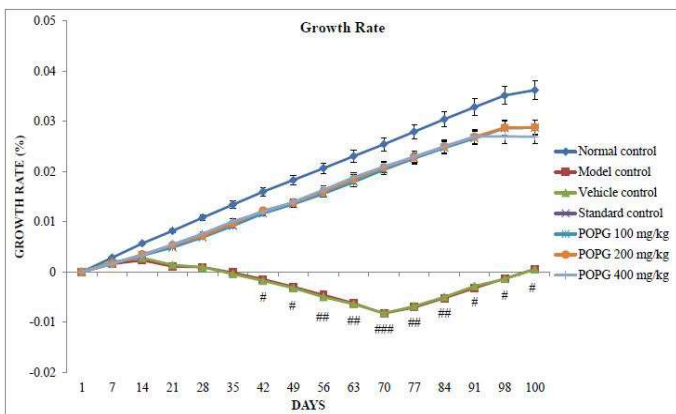


Figure 5. Effect of POPG on Growth Rate in MNU induced mammary carcinogenesis. Values are expressed as mean \pm SEM of 6 animals. Values are statistically evaluated using Two way ANOVA analysis followed by Bonferroni's post hoc test. Significant values were compared with normal control vs. model control (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$)

is significantly different from model control (Figure 5).

Effect of POPG on Feed Consumption Efficiency in MNU induced mammary carcinogenesis

Food intake was calculated as feed consumption efficiency. The model control animals showed decreased in feed consumption efficiency. There was no significant difference found between treated groups (Figure 6).

Effect of POPG on Estrogen and Progesterone receptor expressions in MNU induced mammary carcinogenesis

Immunohistochemical analysis revealed that the breast tumor tissue of cancer bearing group II animals expressed significantly higher number of positive stained estrogen and progesterone nuclei. The % positive stained ER and PR cells in model control were found to be $60\% \pm 1.03$ and $73.34\% \pm 1.29$ respectively when compared to normal breast tissue (ER- $30.56\% \pm 0.98$; PR- $43.66\% \pm 1.38$). Treatment with highest dose of POPG and Tamoxifen significantly decreased expression of estrogen and progesterone as compared to model control. The % positive stained ER and PR cells for POPG were found to be $42\% \pm 1.13$ and $56.66\% \pm 2.69$ respectively. The % positive stained ER and PR cells for tamoxifen were found to be $31.66\% \pm 0.89$ and $46.66\% \pm 1.78$ respectively (Figure 7 and 8).

Effect of POPG on Nucleic Acid contents in MNU induced mammary carcinogenesis

Within the tumor tissues of group II cancer bearing animals, the significant increased ($P < 0.05$) levels of nucleic acids. (DNA: 5.72 ± 1.02 ; RNA: 4.69 ± 1.07) were observed when compared to group I control animals (DNA: 3.66 ± 1.03 ; RNA: 2.47 ± 1.42). Ironically, these rises were attenuated by treatment groups ($P < 0.05$) dose dependently. The

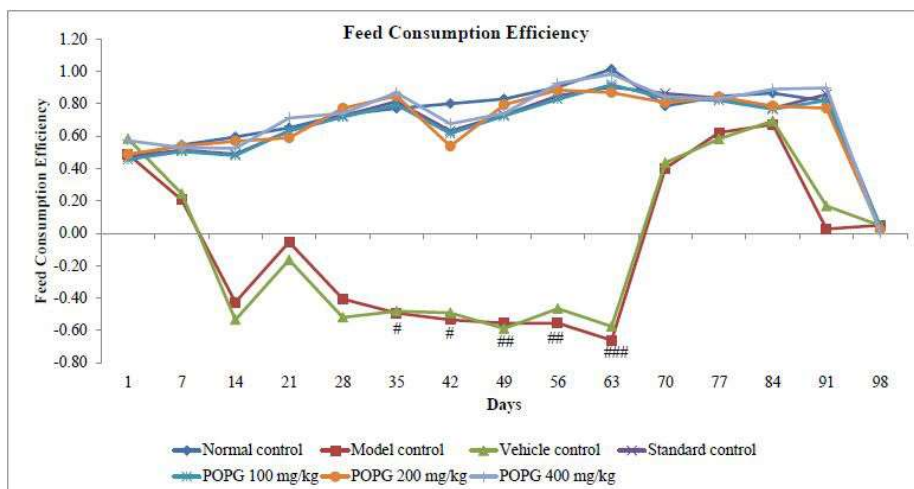


Figure 6. Effect of POPG on Feed Consumption Efficiency in MNU induced mammary carcinogenesis. Values are expressed as mean \pm SEM of 6 animals. Values are statistically evaluated using Two way ANOVA analysis followed by Bonferroni's post hoc test. Significant values were compared with normal control vs. model control (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$)

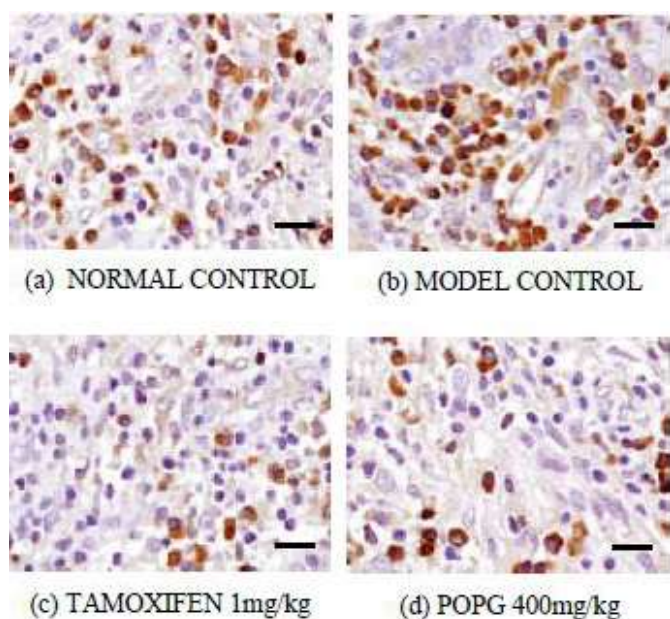


Figure 7. Effect of POPG on the expression of Estrogen in MNU induced mammary carcinogenesis. (a) Normal control (b) Model control- More positively stained brown nuclei representing higher expression levels of estrogen receptor (c) Tamoxifen- Purple hematoxylin estrogen negative stained nuclei are more in number (d) POPG- Positively stained brown nuclei are reduced. The photographs are taken in Nikon Eclipse TS100. Magnification: 10X; Scale bar: 100 μ m

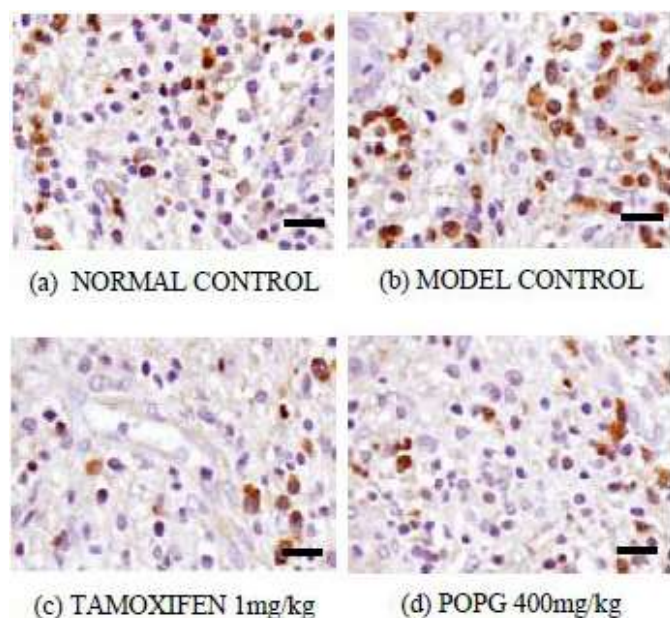


Figure 8. Effect of POPG on the expression of Progesterone in MNU induced mammary carcinogenesis. (a) Normal control (b) Model control- More positively stained brown nuclei representing higher expression levels of progesterone receptor (c) Tamoxifen- Purple hematoxylin progesterone negative stained nuclei are more in number (d) POPG- Positively stained brown nuclei are reduced. The photographs are taken in Nikon Eclipse TS100. Magnification: 10X; Scale bar: 40 μ m

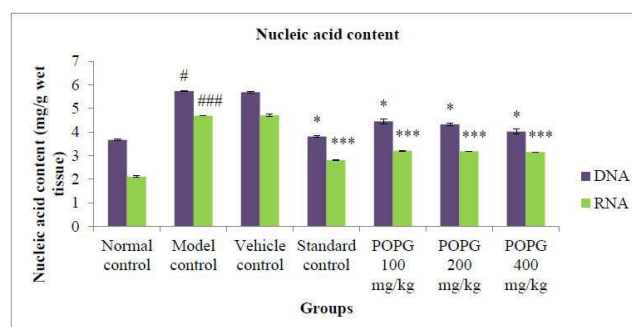


Figure 9. Effect of POPG on nucleic acid content in MNU induced mammary carcinogenesis. Each bar represents Mean \pm SEM of 6 animals. Values are statistically evaluated using One Way ANOVA analysis followed by Bonferroni's post hoc test. Significant values were compared with normal control vs. model control ($\#P < 0.05$; $\###P < 0.001$); model control vs. all other groups ($*P < 0.05$ $***P < 0.001$).

percentage reduction in DNA content in Tamoxifen and POPG (100, 200, 400mg/kg) treated group were 33.50%, 22.37%, 24.69% and 29.90% respectively. The percentage reduction in RNA content in Tamoxifen and POPG (100, 200, 400mg/kg) treated group were 40.21%, 31.81%, 32.37% and 33.01% respectively (Figure 9).

Discussion

Carcinogenesis is an extremely dynamic, nonlinear process following unpredictable pathways. After immense research into its etiology, pathways and mechanisms, it still remains to be a vulnerable disease. Despite the advancements in diagnosis and treatment modalities, the mortality and morbidity associated with cancer is colossal.

According to report of American Cancer Society- Cancer Facts and Figures in 2017, in female breast cancer tops the list followed by lung and colorectal. An estimated 2,52,710 new cases of breast carcinoma are expected to be diagnosed in 2017 accounting 30% of all cancer (Siegel et al., 2017).

Since antiquated times, plants and plant-derived compounds have furnished tremendous backing in conventional medication framework, and have been used as source of new potential drugs in modern pharmaceutical industries. Several new studies have discovered that most patients on cancer therapy are concurrently self-medicating with one or several complementary and alternative medicines (Rockwell et al., 2005). Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products and the plant kingdom has been the most significant source.

The cancer cells acquire certain unusual capabilities like uncontrolled cell proliferation, metastasis, invasiveness due to oxidative stress, sustained angiogenesis, resistance to

apoptosis and genetic mutation (Hanahan and Weinberg, 2000). The sustained proliferation is the most defining feature. The ability of anticancer drug to inhibit cell proliferation can be explored using cancer cell lines. One such widely used cell line based assay is MTT assay. MTT Cell Proliferation and Viability Assay is a safe, sensitive, in vitro assay for the measurement of cell proliferation or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. To estimate this property, different human breast cancer cell lines viz. MCF-7, MDA-MB 453 and MDA-MB-231 were used. MCF-7 is ER+ cell line, MDA-MB-453 is HER+ cell line whereas MDA-MB-231 was used as a model of triple-negative breast cancer (Holliday and Speirs, 2011). Based on present findings, the value of IC₅₀ of POPG in MCF-7 cells was found to be significantly less than that of MDA-MB-231 and MDA-MB-453 cells, which indicated the higher inhibitory potency of POPG in MCF-7 cells than in other two cell lines. Furthermore, regression analysis showed that the effect of POPG was dose- and time- dependent on MCF-7 cells at highest concentration.

In course of metastasis, cancer spread beyond the place of origin into the other parts of body. Recurrence and metastasis of breast cancer after initial diagnosis and treatment are one of the major challenges for current therapeutic methods. Prevention of metastasis is very crucial for success of breast cancer therapy and survival of patients after diagnosis and treatment (Weigelt et al., 2005). Hence investigating the migration inhibition potential of POPG was undertaken and results suggested that it has potentially inhibited the migration of breast cancer cell line MCF-7. The effect might be contributed to its property to inhibit cell to cell contact.

Cell apoptosis is a normal physiological process of orderly controlled cell death for maintaining stable internal environment of the whole organism. Compounds that can induce cell cycle arrest and apoptotic cell death are generally considered to be potential anticancer drugs (Pistritto et al., 2016). The experimental data provided evidence for POPG induced apoptosis in MCF-7 human breast cancer cell line, implying a strong correlation between inhibition of cell-proliferation and apoptosis. The pathways for POPG-induced apoptosis need to be further elucidated.

Severe pathological processes such as solid tumour growth and metastasis are angiogenesis- dependent and a novel approach in cancer therapy and prevention is the use of agents with antiangiogenic activity (Samant and Shevde, 2011). Anti-angiogenesis effect was studied using Chick Chorioallantoic Membrane. In the chick embryo, the chorioallantois is formed between days 4 and 5 of development, when the outer mesodermal layer of the allantois fuses with the mesodermal lining of the chorion, and a network of blood vessels is gradually formed between the two layers. The central portion of the CAM

is fully developed by day 8 to 10 at which time it becomes capable of sustaining tissue grafts, while the outskirts of the CAM are still developing and expanding until the CAM fully envelopes the embryo at day 12 of incubation (Deryugina and Quigley, 2008). In our study, we introduced filter paper disk containing POPG on Day 8 and observed it for next 48 hours. The results indicates that POPG has antiangiogenic effect, which might be phyto-constituents like lycopene which possess anti-angiogenic property (Chen et al., 2012).

To validate *in-vitro* results, mammary carcinogenesis was induced in rodents by intra peritoneal administration of N-methyl N- nitrosourea (MNU) (Macejova and Brtko, 2001). MNU is a water soluble direct alkylating carcinogen, and highly specific carcinogen for mammary gland. MNU induced mammary carcinomas are aggressive, more estrogen dependent and locally invasive. Mammary carcinomas arising from MNU- induced hyperplastic alveolar nodule contain transformed c-Ki-ras proto-oncogene with the present of specific G-35 A-35 point mutation in codon 12, which results in the substitution of normal glycine with the aspartic acid (Macejova and Brtko, 2001). With this, amplification of cyclin D1 gene, IGF2, loss of expression of the mitogenic growth factor gene, heparin binding growth factor midkine gene and mutation in the tumor suppressor p53 gene are seen in mammary tumors. Literature survey reveals that injecting 50mg/kg b.w (i.p.) MNU to nulliparous Sprague Dawley (SD) female rats induces mammary carcinoma (Jagadeesan et al., 2013; Parikh et al., 2005; Parvathaneni et al., 2014).

Our reports are in harmony to above studies. Injecting MNU (50mg/kg b.w; i.p.) to nulliparous SD female resulted in mammary tumors. Tumor was induced after 45 days post MNU injection. Five out of six rats developed mammary carcinoma suggesting 83.33% tumor incidence. Tumor burden i.e. total number of tumors in MNU injected groups was found to be 6. One rat developed two mammary tumors. Tumor multiplicity i.e. number of tumors per rat was found to be 1. Tumor weight and volume were found to be 6.4±1.47 g and 99.29 ±3.19 mm³ respectively. The data proposed successful induction of mammary carcinomas in MNU injected rats.

Pulp of *Psidium guajava* (POPG) was used as treatment for MNU induced mammary carcinogenesis. Based on literature survey, 100mg/kg, 200 mg/kg and 400 mg/kg were selected for preventing mammary carcinogenesis (Chen et al., 2010; Gakunga et al., 2013; Ojewole, 2006; Rai et al., 2007). The results of the study have indisputably demonstrated that suppression of carcinogen-induced tumor incidence and multiplicity is caused by

administration of POPG. The latency period of tumor appearance was lengthened in POPG 400 mg/kg as compared to model control ($P < 0.05$). However, tumor incidence, multiplicity and latency period of POPG (100 mg/kg and 200 mg/kg) was not significantly different from model control. It is worthy of mention that tumor weight and tumor volume was significantly lower than model control in all treated groups. This confirms that POPG inhibit cancer cell proliferation which might be due to oncosuppressive potential of POPG.

It has been documented that there is a significant loss in the body weight and in contrast a considerable increase in the organ and as well as tumor weight in cancer conditions. It is also seen in humans possessing breast cancer. The growth rate, parameter of body weight, of the MNU injected animals was declined when compared to control groups. This may be due to the changes in energy metabolism during tumor formation and in addition an increased level of MDA also plays an important role in the initiation of tumor development and in the decrease of body weight (Rengarajan et al., 2013). Also, the feed consumption efficiency was decreased in model control but not in different experimental test groups during study period was found to be unaltered. This feature is of paramount importance because nutritional depreciation causing body weight loss may parallel a decrease in tumor volume.

Further confirmation was obtained by expression studies of Estrogen (ER) and progesterone (PR) expressions by immunohistochemistry. ER activation in breast and uterus enhances cell proliferation which is necessary for growth and maintenance of tissues (Thordarson et al., 2001). When the response to estrogens by the endocrine system is deregulated, ER activation might eventually result in tumor formation. Studies suggests that reduced levels of ER- α in the mammary gland predict low breast cancer risk. Literature revealed that MNU induces estrogen dependent tumors (Thordarson et al., 2001). Overexpression of ER can be due to binding of growth factors also like IGF-I, IGF II, EGF and TGF. PR-B is required for normal mammary gland development; PR+ cells in the mammary gland may interact primarily with stromal components to mediate proliferative signaling of nearby or neighboring PR-null cells via the action of locally acting growth factors. Both the rapid actions and the transcriptional activity of PR-B contribute to breast cancer cell proliferation in response to progesterone. Our findings are in line with previous studies demonstrating that MNU injected rats showed increased expression levels of ER and PR (Thordarson et al., 2001). These levels were significantly reduced in both standard and treatment group (POPG 400 mg/kg). The effect might be contributed to decreasing levels of endogenous hormones and cytokine levels. It also possesses NF-Kb (Choi et al., 2008) and aromatase inhibitory activity which decrease gene mutation and

transcription (Duke and Beckstrom-Sternberg, 1994).

Nucleic acids damage is a sensitive indicator and a prospective biological target for many initiators of carcinogenesis. Elevation of DNA adducts formation and oxidative base lesions have been reported in the normal adjacent and tumor tissues of breast cancer patients. These findings suggest that an accumulation of DNA damage may contribute to breast carcinogenesis. Hence, the determination of DNA content plays an important role in tumorigenesis. The increased DNA content in cancer-bearing breast may be due to the increased expression of enzymes which are necessary for DNA synthesis in tumor cells with repression of many enzymes related to differentiated cell function. RNA levels were also found to be increased in the cancerous condition; the uncharacteristic increased content of DNA may lead to an increased transcription that leads to the increased RNA content of tumor cells. Jagadeesan et al. (2013) proved that diosgenin treatment to MNU induced mammary carcinoma female rats showed decrease in DNA and RNA levels. In our study, the model control animals, 56% and 90% hike in DNA and RNA levels were observed as compared to normal control.

The percentage reduction in DNA content in POPG (100, 200, 400mg/kg) treated group were 22.37%, 24.69% and 29.90% respectively. The percentage reduction in RNA content in POPG (100, 200, 400mg/kg) treated group were 31.81%, 32.37% and 33.01% respectively. The levels were brought back to be nearly the usual level, which intimate the anti-tumor property of the drugs that slow down the progression of tumor growth, since the size and weight of the tumor is well linked with the tumors DNA content in malignant conditions. The effect might be due to the intervention strategies of the POPG in nucleic acid biosynthesis which ultimately results in the inhibition on the rate of development of tumors through controlled nucleic acid biosynthesis and exhibits the tumor inhibitory effect during POPG treatment. Tamoxifen treated animals showed 33.5% and 40.21% inhibition in DNA and RNA levels as compared to model control.

In conclusion, POPG was found to be more potent cytotoxic in MCF-7 estrogen positive human breast cancer cell line than in MDA-MB-453 and MDA-MB-231. The POPG is found to be apoptotic as they increased the early and late apoptotic cell population. POPG is found to be anti-angiogenic and anti-metastatic. The results of methyl nitrosourea (MNU) induced mammary carcinogenesis is in accordance to *in-vitro* cell lines studies. The cancer manifesting from MNU is Estrogen and Progesterone positive; which is more prevalent in humans also. The mammary gland differentiation, prevention of

mammary tumor induction, augmentary changes in nucleic acid & receptor status suggests chemo-preventive potential of POPG in MNU induced mammary carcinogenesis. The chemo-preventive activities are likely mediated through a number of mechanisms involving inhibition of cell proliferation, metastasis, angiogenesis, overexpression of hormones and nucleic acid content.

Conflicting interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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