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Original Article

Peiminine Alleviates Breast Carcinoma via Reprogramming the PI3K/Akt/mTOR Pathway *in vitro* and *in vivo*

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ABSTRACT
Background: The breast cancer is most frequently diagnosed cancer in the women worldwide. Our study investigated the anticancer effect of peiminine, an alkaloid obtained from Fritillaria thunbergii, against breast carcinoma. **Materials and Methods:** The toxicity study investigated LD_{50} and the subsequent doses of peiminine for carcinogenic study. The *in vitro* chemotherapeutic assessment was performed on MCF7 cells through MTT assay and flow cytometry. The breast cancer was developed in rats via induction of DMBA (5 mg/kg, i.v.) and sustained for 24 weeks. The induction of breast cancer and the chemotherapeutic effect of peiminine were assessed through histopathological analysis of rat mammary tissue, followed by immunohistochemical analysis, cell proliferation assay and apoptosis assay by TUNEL method. **Results:** The IC_{50} value of peiminine in MCF7 cell was found to be 5 µg/mL which demonstrate a significant induction of apoptosis and enhanced caspase-3 expression in MCF7 cells in a dose dependent manner. The complex also caused cell cycle arrest at S phase and G2/M phase dose dependently. Additionally, peiminine therapy decreased the hyperplastic lesions of mammary tissue and restored the normal histopathological characteristics of breast tissue. Furthermore, peiminine treatment downregulated the expression of carcinogenic markers such as PI3K and Akt increased the expression of apoptotic markers including p53 and Bax. Peiminine therapy also decreased the cellular proliferation and enhanced the apoptotic events. **Conclusion:** In conclusion, the breast cancer progression was significantly reduced via induction of apoptotic events and inhibition of cell propagation which allowed constructing of suitable mechanism for peiminine mediated chemotherapeutic approach.

Keywords: Peiminine, Apoptosis, Breast carcinoma, Chemotherapy, Cell proliferation.

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INTRODUCTION

Mammary carcinoma is the most recurrent carcinogenic disorder that significantly causes of death for women globally, as per the WHO. According to the GLOBOCAN 2020, mammary carcinoma is the most often diagnosed malignancy with an estimated 2,261,419 newly reported cases and 684,996 deaths in 2020.[1] The leading cancer type in women is breast cancer which contributes 18% of the global breast cancer incidences.[2] The modification of cellular physiology and signaling ways of normal cell led to the carcinogenic manifestation through the abnormal mutation of several regulatory proteins thus causes cellular propagation.

The deactivation of cancer suppressor proteins and carcinogenic modification of PI3K/Akt/mTOR transduction pathway caused by PIK3CA mutations is one of the main contributors to the emergence of Estrogen Receptor (ER) regulated breast cancer.[3] The upregulation of PI3K/Akt/mTOR signaling pathway is directly related with the cellular survival, growth and propagation of malignant cells through cellular metabolism and restructuring of cytoskeleton.[4] Consequently, causes the deactivation of apoptosis in the malignant cells[5] which triggers the cancer cell survival, escalate invasiveness, angiogenesis.[6] As well, the stimulation of this carcinogenic signaling pathway have also connected with the resistance to Human Epidermal growth factor Receptor 2 (HER2)-mediated treatment, endocrine therapy and anticancer therapy in mammary carcinoma.[7] Hence, the recent therapeutic strategies are focusing on the development of alternative chemotherapeutic agents with distinct cytotoxic activity which can modify resistance and sensitize the cancer cells to chemotherapies.

Recently, peiminine, an alkaloid which is obtained from Fritillaria thunbergii, which has been showed the advance interest among scientists due to its extensive pharmacological activities including anti-inflammatory,[8] antioxidant,[9] antitussive and bronchial smooth muscle relaxant activity.[10] Peiminine

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is also associated with inhibition of osteoarthritis,[11] atopic dermatitis,[12] acute lung injury,[13] and myocardial injury.[14] The potent anticancer activities have also been noted for peiminine. Recent studies demonstrated that, peiminine elicits apoptosis and autophagy in the HCT-116 colon cancer cell line.[15] Additionally, peiminine caused the alterations of metabolic pathway of amino acids, carbohydrates and lipids which eventually prevented the proliferation of colon cancer through the regulation of PI3K/Akt/mTOR pathway.[16] In accordance to this peiminine mediated reduction of LINC00659 expression and upregulation of miR-760 expression is directly associated with the inhibition of colony formation, cell viability and metastasis of the colon cancer cells.[17] Furthermore, peiminine caused apoptosis, autophagy and arrest of G0/G1-phase in osteosarcoma cells through the alteration of ROS/JNK signaling pathway.[18] Peiminine also induced apoptosis in HepG2 hepatocellular cancer cells through the downregulation of Bcl2, procaspase-3/8/9 and PARP expression with increased expression of Bax and caspase-3/8/9.[19] Downstream activity of cell proliferation and colony formation has also been noted in the glioblastoma multiforme cells due to peiminine therapy which involved the blocking of autophagic flux and initiation of cell cycle arrest through downregulation of Akt and GSK3β expression.[20]

Till now there is absence of documented confirmation that indicating anticancer activity of peiminine via the initiation of apoptosis on mammary carcinoma model. Thus, the current investigation is aiming on the peiminine directed chemotherapeutic assessment on mammary carcinoma via *in vivo* and *in vitro* experimentation.

MATERIALS AND METHODS

Chemicals and reagents

Peiminine (#18059-10-4), 3,3′-diaminobenzidine (DAB) (#D12384), proteinase K (#1.07393), biotinylated goat anti-rabbit IgG (#21537) and streptavidin peroxidase (#S5512) have been purchased through Sigma Aldrich Chemical Co. (St. Louis, MO). Anti-rabbit PI3K (#GTX100462), Akt (#GTX121937), Bax (#GTX109683), PCNA (#GTX100539) and Anti-mouse p53 (#GTX70214) were procured through GeneTex International Corporation (Global). The apoptotic assay kit (#MK500) been purchased via Takara Bio Inc. (Kusatsu, Japan). All the supplementary chemicals have been obtained through the local suppliers at its pure form.

In vitro assessment

Cell culture

MCF7 (HTB-22) breast carcinoma cell line which have been procured through American Type Culture Collection (ATCC), United States, which has been incubated in DMEM growth media, 10% FBS (foetal bovine serum), 100 U/mL penicillin and 100 µg/mL streptomycin. The cells been cultured at 37°C in 5% CO_2 and 95% relative humidity.

Cell viability assessment

The cell viability assay was performed by MTT (3-(4,5dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The MCF7 cells were seeded in 96-well plate and incubated at 37°C in 5% CO2 for one day. The cells have been treated with peiminine and incubated for one day. Next day, MTT solution was treated on the cells and incubated for 3 hr. The absorbance was measured at 560 nm. The percentage of cell viability was calculated as:

$$\% \text{ Cell viability} = 100 - \% \text{ of cytotoxicity}$$

Flow cytometry

The cellular apoptosis has been investigated through flow cytometry with annexin V-FITC apoptosis detection kit. The MCF7 cells have been seeded in 6-well plate and treated with peiminine for 24 hr. The cells been labeled with annexin V-FITC and Propidium Iodide (PI) and examined by FACSCelesta flow cytometer (BD Biosciences, San Jose, CA, United States). The results obtained were analyzed by Modfit tools.

Determination of caspase-3 expression

The expression of caspase-3 in MCF7 cells have been investigated through flow cytometry. The cells been treated by anti-rabbit caspase-3 polyclonal antibody for half an hour at 37°C in unlighted state and analyzed by flow cytometry.

In vivo assessment

Animal husbandry and maintenance

Experimental animal has been conducted as per the Institutional Animal Ethical Committee (IAEC) of Baoding First Central Hospital (Approval no. 2022BCH420) and complied with the ARRIVE guidelines.

The female Wistar rats (70-120 g) of six-week-old have been obtained from Baoding First Central Hospital Animal Center for chemotherapeutic analysis. All the experimental animals have been reserved in the polypropylene cages with free access to food and tap water, at 22±3°C with relative humidity 50% to 58% and 12 hr light/dark sequences. The experimental animals been accustomed for 7 days prior to the starting of experiment.

Experimental protocol

After the period of acclimatization, the experimental animals have been dispersed randomly into 5 individual groups in which each group comprised with 6 animals. 7-8 weeks old Wistar rats (excluding group I) has been injected DMBA (0.5 mg/100 g body weight) in corn oil by tail vein single i.v injection. Subsequently, the rats have been treated with peiminine by oral route that was

sustained for twenty-four weeks. All investigational animals been divided as:

Group I- normal control animals, Group II- introduce chemical carcinogen DMBA and designated as carcinogen control, Group III-DMBA induced carcinoma+administered 0.25 mg/kg peiminine, Group IV- DMBA induced carcinoma+administered 0.5 mg/kg peiminine, Group V-DMBA induced carcinoma+administered 1 mg/kg peiminine.

Histological assessment of mammary tissue

The experimental animals have been ether anaesthetized and thoracic and abdominal inguinal breast tissues been isolated. Fixation of mammary tissues were completed in 10% formalin, after that the tissues are embedded with paraffin wax. The paraffinized tissues have been incise into 5 μm thickness and positioned onto the slides. The slides were stained with hematoxylin and eosin and visualized by light microscope.

Antioxidant assessment of mammary tissue

Mammary tissues have been homogenized (10% w/v) with 0.1 M of phosphate buffer (pH 7.0) for antioxidant experimentation. Then these homogenized tissues were centrifuged for 15 min, supernatant been collected and assessed for enzymatic study.[21] In accord with Sinha *et al*., (1972) homogenized breast tissue was utilized for the catalase assessment. Absorbance had been showed at 620 nm and the catalase assessment has been stated as μmol of H_2O_2 consumed/min/mg protein.[22] The SOD activity has been evaluated in accordance with the scheme elaborated through Awasthi *et al*., Superoxide Dismutase (SOD) assessment been revealed by units/min/mg protein.[23] The assessment of GPx had been evaluated as per the scheme mentioned thru Rotruck *et al*., 1973.[24] Glutathione Peroxidase (GPx) assessment been explained in μmol of GSH consumed/min/mg protein.

Immuno histochemical assessment

The isolated mammary tissue was embedded with paraffin, incises into 5 μm thickness and mounted onto the poly-L-lysine coated slides. The sections were incubated with anti-rabbit PI3K (1:500), Bax (1:500), Akt (1:500), anti-mouse p53 (1:1000) and anti-rabbit PCNA antibody (1:500) at 4°C temperature for 24 hr. Then the slides been introduced to HRP conjugated secondary antibody for half an hour. DAB was utilized as primary stain and counterstaining have been done with hematoxylin and eosin staining. Then, the tissue section was visualized by light microscope (OLYMPUSCX 21i TR) and quantification of immune responsive cells was obtained through ImageJ software (version1.8.0).

Apoptosis assesses through TUNEL scheme

The mammary tissues were mounted on poly-L-lysine coated slides which have been introduced in proteinase K (20 μg/mL in PBS) for 10 min at room temperature. Then the slides had been introduced in Terminal Deoxynucleotidyl Transferase (TDT) buffer solution and further the slides been preserved in TdT solution comprising of TdT and dUTP at 37°C for 1 hr 30 min. The slides have been incubated with anti-digoxigenin peroxidase at room temperature for half an hour. DAB was used for primary staining and counterstaining was done with H and E staining.

Assessment of apoptotic index as well as labeling index

The % of PCNA-positive cells/total number of cells was determined as Labeling Index (LI). The % of TUNEL-positive cells/total number of cells was expressed as Apoptotic Index (AI).

Statistical assessment

The results have been expressed as mean±Standard Deviation (SD). The one-way Analysis of Variance (ANOVA) followed by post-hoc test (Tukey's test) been performed to determine statistical magnitudes by using Graph pad prism software (Version 5). The variants had been hypothecated to be statistically significant, with p value less than 0.05 ($p<0.05$).

RESULTS

Cell viability assay

The *in vitro* study has been demonstrated in Figure 1. TCell viability assay of peiminine demonstrated a significant inhibition of cell viability dose dependently (Figure 1A). The cell viability of MCF7 cells were found to be 89.54%, 72.86%, 51.29%, 38.43% and 12.59% at the concentration of 1, 2, 5, 10 and 15 μg/mL respectively. The IC_{50} value of the complex was then calculated to be 5 μg/mL. The subsequent doses for chemotherapeutic study were further determined as 2.5 μg/mL (IC_{25}), 5 μg/mL (IC_{50}) and 7.5 μg/mL (IC_{75}).

Flow cytometry

The treatment with peiminine displayed an increase in the percentage of apoptotic cells in MCF7 cell line dose dependently (Figure 1B, C). The % apoptotic cells were determined as 15.64%, 38.24% and 52.81% at IC_{25}, IC_{50} and IC_{75} of the complex. The cell population was also increased dose dependently in the early and late apoptotic stage due to peiminine therapy (Figure 1D).

The flow cytometric data for cell cycle phase distribution has been depicted in Figure 1E, F. A significant increase in the cell population at G0/G1 phase, S phase and G2/M phase was observed in complex treated group. At G0/G1 phase the cell population was found to be 65.28%, 61.45%, 50.29% and 41.51% in untreated, IC_{25}, IC_{50} and IC_{75} group respectively. Likewise, at S phase and G2/M phase the cell population was found to be 4.21%, 7.02%, 13.87%, 16.26% and 21.47%, 23.85%, 29.24%, 36.47% in untreated, IC_{25}, IC_{50} and IC_{75} group respectively.

Table 1: Effect of peiminine on the expression of Bax, PI3K, *p*53 and Akt in breast tissues.

Groups	Bax§	PI3K§	p53§	Akt§
Control	7.25±0.07	8.01±0.09	8.08±0.08	8.72±0.06
DMBA	4.08±0.08*	15.47±0.07*	4.01±0.08*	19.4±0.06*
peiminine 0.25 mg/kg	5.65±0.07*#	12.4±0.06*#	6.6±0.1*#	15.05±0.08*#
peiminine 0.5 mg/kg	9.05±0.09*#$	9.58± 0.06*#$	9.61±0.08*#$	12.1±0.1*#$
peiminine 1 mg/kg	11.7±0.06*#$α	7.28±0.06*#$α	14.4±0.06*#$α	10.1±0.07*#$α

§Each score represents the results of 6 slides per rat and 6 rats per group, mean±SD (*n*=6). Each field was selected randomly for evaluation of percentage of immune-positive cells. The results were compared using ANOVA, followed by a Tukey's multiple comparison post-hoc analysis

*Significant difference as compared to the control (*p*<0.05).#Significant difference as compared to the DMBA control (*p*<0.05).

$Significance difference as compared to the peiminine 0.25 mg/kg (*p*<0.05).αSignificance difference as compared to the peiminine 0.5 mg/kg (*p*<0.05).

Table 2: Cell proliferation and apoptosis in breast.

Groups	PCNA-LI§	AI (%)§	*R*= PCNA-LI /AI
Normal control	22.06±0.01	0.17±0.01	129.35±4.7
DMBA	36.07±0.08*	0.06±0.01*	651.62±66.36*
peiminine 0.25 mg/kg	27.78±0.08*#	0.09±0.01*#	337.73±34.65*#
peiminine 0.5 mg/kg	21.9 ±0.1#$	0.1 ±0.01*#	216.46±14.19#
peiminine 1 mg/kg	18± 0.1*#$α	0.12±0.01*#$	150±8.4#$

LI= Labelling index, PCNA-LI =percentage of PCNA labelled cells/total number of cells counted, AI=Apoptotic index. R= PCNA-LI/AI. AI was calculated as the percentage of TUNEL positive cells/total number of cells counted. Values represent mean±SD. The results were compared using ANOVA, followed by a Tukey's multiple comparison post-hoc analysis

§Total number of six slides were evaluated per rat. Each field consisted of approximately 500 cells.

*Significant difference as compared to the control (p<0.05).

#Significant difference as compared to the DMBA control (*p*<0.05).

$Significance difference as compared to the peiminine 0.25 mg/kg (*p*<0.05).

αSignificance difference as compared to the peiminine 0.5 mg/kg (*p*<0.05).

Peiminine increases caspase-3 expression

The treatment with peiminine showed a dose dependent increase in the caspase-3 expression in MCF7 cell line (Figure 1G). The untreated group showed caspase-3 labeled cells in M1 quadrant. Whereas, a substantial increase in the population of caspase-3 marked cells in M2 quadrant have been observed in peiminine treated group.

Histopathological assessment of breast tissue

The histopathology and *in vivo* antioxidant assay has been depicted in Figure 2. Histological assessment of breast tissue was showed in the (Figure 2(i)). The normal control group (group I) was presented the normal morphology of breast tissue that displayed Alveoli (*a*), Acinus (*ac*), Terminal Duct lobular units (*td*), Serous Gland (*sg*) and alveolar septa (sg) of breast tissue (Figure 2(i)A). The carcinogen control group (group II) demonstrated the atrophy of glands with Periductal Stromal Fibrosis and fatty tissue (*psf*), Atrophy of Serous Glands (*asg*) enclosed by stromal fibrosis, atrophy of glands (*ag*) bounded by fatty tissue and atypical hyperplasia (*ah*) (Figure 2(i)B). The 0.25 mg/kg administered group exhibited the atrophy of glands (*ag*), atrophy of serous glands (*asg*) and periductal stromal fibrosis and fatty tissue (*psf*) (Figure 2(i)C). The 0.5 mg/kg group showed the mucinous and serous glands hyperplasia (*h*) (Figure 2(i)D). Moreover, 1 mg/kg group represented a significant degeneration of hyperplasia or cellular dissemination of mammary tissue (Figure 2(i)E) and depicted the normal morphological profile of the breast tissue.

Antioxidant status

Carcinogen control group have been exhibited a substantial decrease of GST, CAT as well as SOD levels been notified as comparison with normal control group ($p<0.05$). Peiminine treated group exerted the superior intensities of GST, CAT and SOD, suggestively in mammary tissue wherein 1 mg/kg group displayed significantly over activation of antioxidants within mammary tissue as comparison with normal control group and other peiminine treated groups ($p<0.05$) (Figure 2(ii)).

Immunohistochemical assessment

Immunohistochemical assessment of breast tissue depicted in Figure 3 was performed for evaluation of protein expression which comprises proapoptotic protein Bax, *p*53 (tumor-suppressor protein), Akt (growth regulatory proteins) and PI3K. Normal control group (group I) was showed a higher mutation of *p*53 in normal epithelial tissues together with alveolar ducts and terminal end bud (Figure 3(i)A). While carcinogen control

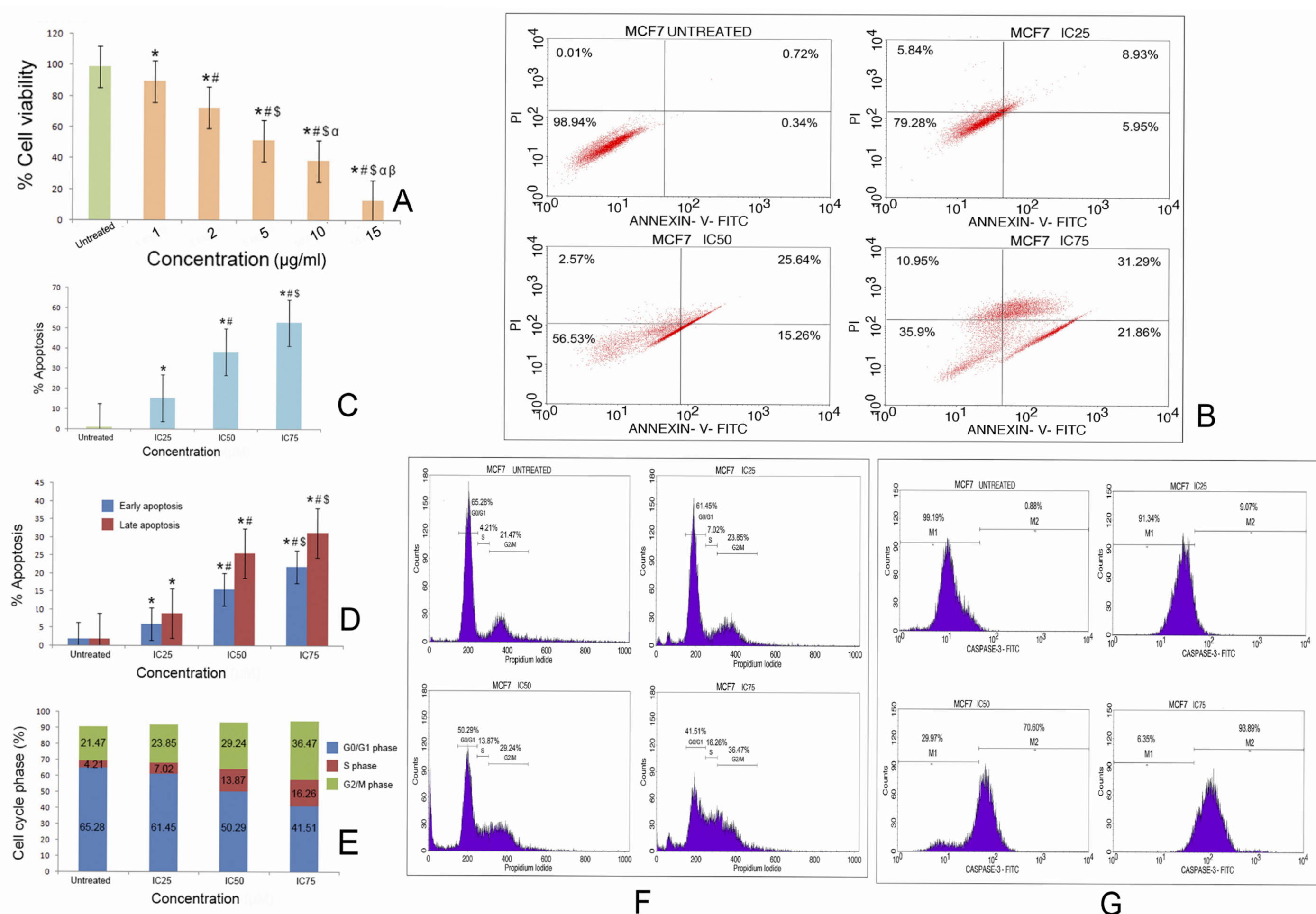

Figure 1: The effect of peiminine on MCF7 cell line at 24 hr. (A) Effects of peiminine on cell viability where * represented $p<0.05$ as compared to untreated group. Similarly, # represented $p<0.05$ as compared to 1 μg/mL concentration, $ $p<0.05$ as compared to 2 μg/mL concentration, α $p<0.05$ as compared to 5 μg/mL concentration and β $p<0.05$ as compared to 10 μg/mL concentration (B) detection of apoptosis in MCF7 cells by Flow cytometry. Percentage of (C) total apoptosis (D) early and late apoptosis where * represented $p<0.05$ as compared to untreated group. Similarly, # represented $p<0.05$ as compared to IC_{25}, $ $p<0.05$ as compared to IC_{50} (E) quantitative of distribution of MCF7 cells in different phases of cell cycle (F) analysis of cell cycle phase distribution of MCF7 cells (G) Determination of caspase-3 expression on MCF7 cells.

group (group II) has been displayed a lower expression of *p*53 (Figure 3(i)B). Peiminine treated group at the different doses such as 0.25, 0.5 and 1 mg/kg body weight a suggestively (*p<0.05*) increases the mutation of p53 have been noticed at terminal ducts as well as alveolar region (Figure 3(i) C-E) as comparison with carcinogen control group (Table 1). Bax protein mutation has been identified in the cap cells of normal control group and entire terminal end bud (Figure 3(ii) A). In carcinogen control group, the apoptotic protein Bax been dramatically decreased (Figure 3(ii)B). However, the peiminine treatment groups was demonstrated a considerably (*p<0.05*) higher proliferation of Bax in alveolar duct area and terminal end buds (Figure 3(ii) C-E) (0.25, 0.5 and 1 mg/kg) in associated with carcinogen control group (Table). In normal control group, significantly depicted the mutation of PI3K in mammary tissue (Figure 3(iii) A) at terminal end buds but lower mutation been designated at the cap of cell layer. PI3K mutation has been considerably higher in the carcinogen control group (Figure 3(iii)B) in hyperplastic breast tissue as comparison with normal control group. On the other hand, a substantial (*p<0.05*) decrease of PI3K mutation have been detected for peiminine treatment group (Figure 3(iii) C-E) (0.25, 0.5 and 1 mg/kg) (Table). The proliferation of Akt has been noted in the entire terminal end bud and cap cells for the experimental animals of normal control group (Figure 3(iv)A). Akt expression has been drastically amplified in carcinogen control group (Figure 3(iv)B). While the peiminine treatment group was designated a significant (*p<0.05*) lower mutation of Akt in alveolar duct zone and terminal end buds (Figure 3(iv) C-E) (0.25, 0.5 and 1 mg/kg) in associated with carcinogen control group (Table).

Cellular proliferation assessment

The expression of PCNA and TUNEL positive cells has been demonstrated in Figure 4. The results of peiminine treated group in the cell proliferation of mammary tissue were depicted in

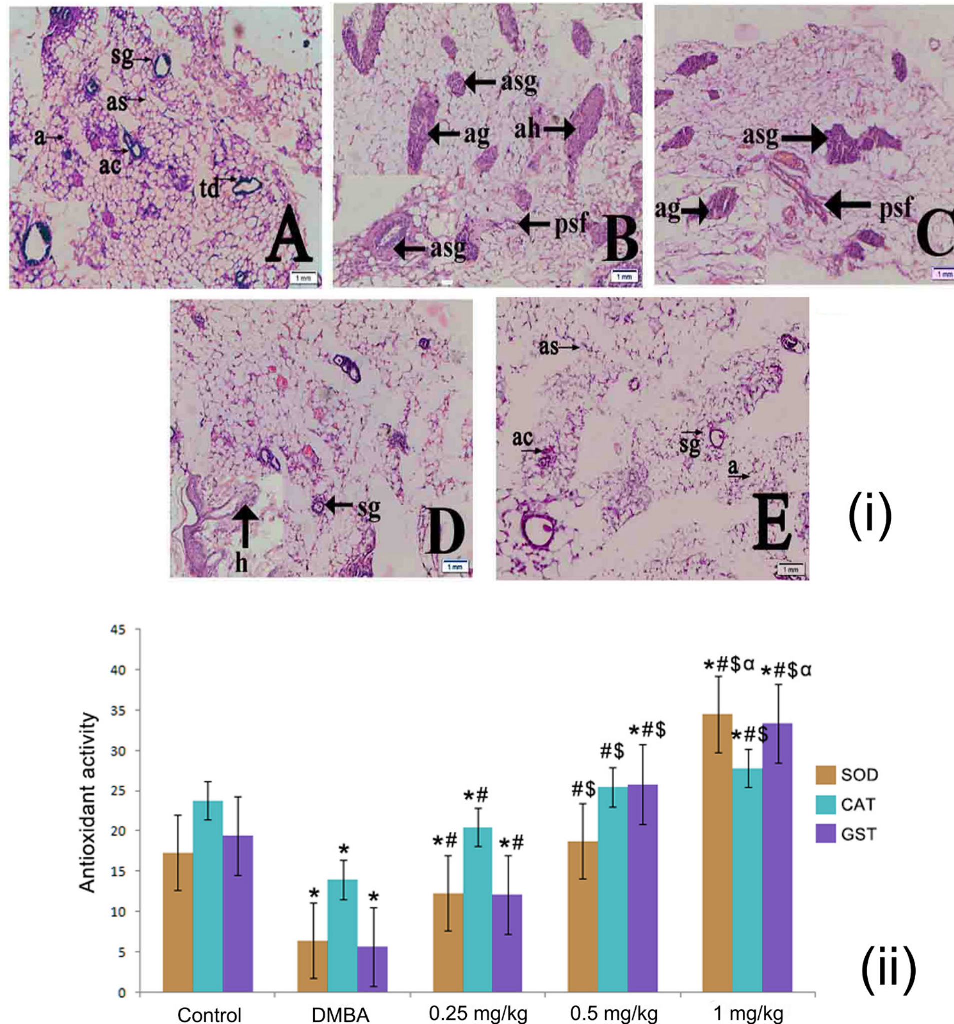

Figure 2: (i) Histological appearance of mammary tissue of (A) normal control showing Terminal duct lobular units (*td*), alveoli (*a*), alveolar septa (sg), Acinus (*ac*), serous gland (*sg*) (B) DMBA control shows atrophy of glands with periductal stromal fibrosis and fatty tissue (*psf*), atrophy of glands (*ag*) with surrounding fatty tissue, atrophy of serous glands (*asg*) with surrounding stromal fibrosis, atypical hyperplasia (ah). (C) Mammary tissue of DMBA induced group treated with 0.25 mg/kg peiminine showing atrophy of serous glands (*asg*), atrophy of glands (*ag*) and periductal stromal fibrosis and fatty tissue (*psf*) (D) Mammary tissue of DMBA induced group treated with 0.5 mg/kg peiminine showing hyperplasia of serous and mucinous glands (*h*) (E) Mammary tissue of DMBA induced group treated with 1 mg/kg peiminine having almost normal architecture. (ii) Effect of peiminine on *in vivo* antioxidant enzymes SOD (superoxide dismutase) and CAT (catalase), (glutathione) GST. * Significant difference as compared to normal control group (p<0.05). $^{\#}$ Significant difference as compared to carcinogen control group (p<0.05). $^{\$}$ Significant difference as compared to 0.25 mg/kg group (p<0.05). $^{\alpha}$Significant difference as compared to 0.5 mg/kg group (p<0.05).

(Figure 4(i)) which represented the chemotherapeutic activity of peiminine in the DMBA induced breast carcinoma. A distinctive nuclear localization with brown pigment due to the chromogen treatment was represented in cells with PCNA labelling that been required for cellular quantification. The normal control group designated a reduced level of PCNA marked cells (Figure 4(i)A). The percentage of PCNA marked cells been determined through the labeling index (LI) and carcinogen control group (Figure 4(i) B) represented a significant level of LI (Table 2). Conversely, the peiminine treatment group was showed a substantial ($p<0.05$) decreases the L1 level (Figure 4(i) C-E) (0.25, 0.5 and 1 mg/kg) (Table).

Apoptosis assay through TUNEL method

The TUNEL analysis has been carried out for demonstrating the manifestation of apoptosis in the cells of hyperplastic breast tissue. The apoptotic cells been recognized through the brown coloration caused by chromogen treatment (Figure 4(ii)). DMBA control group (Figure 4(ii)B) denoted the decrease amount of TUNEL positive cells, on an average of 3 to 5 apoptotic cells, in an around 700 cells as compared with normal control group (Figure 4(ii)A). A significantly ($p<0.05$) higher mutation of TUNEL positive cells have been showed for peiminine treatment group (Figure 4(ii) C-E) (0.25, 0.5 and 1 mg/kg) in associated with carcinogen control group. Usually 8 to 9 apoptotic cells, in an around 700 cells been noticed for peiminine treatment

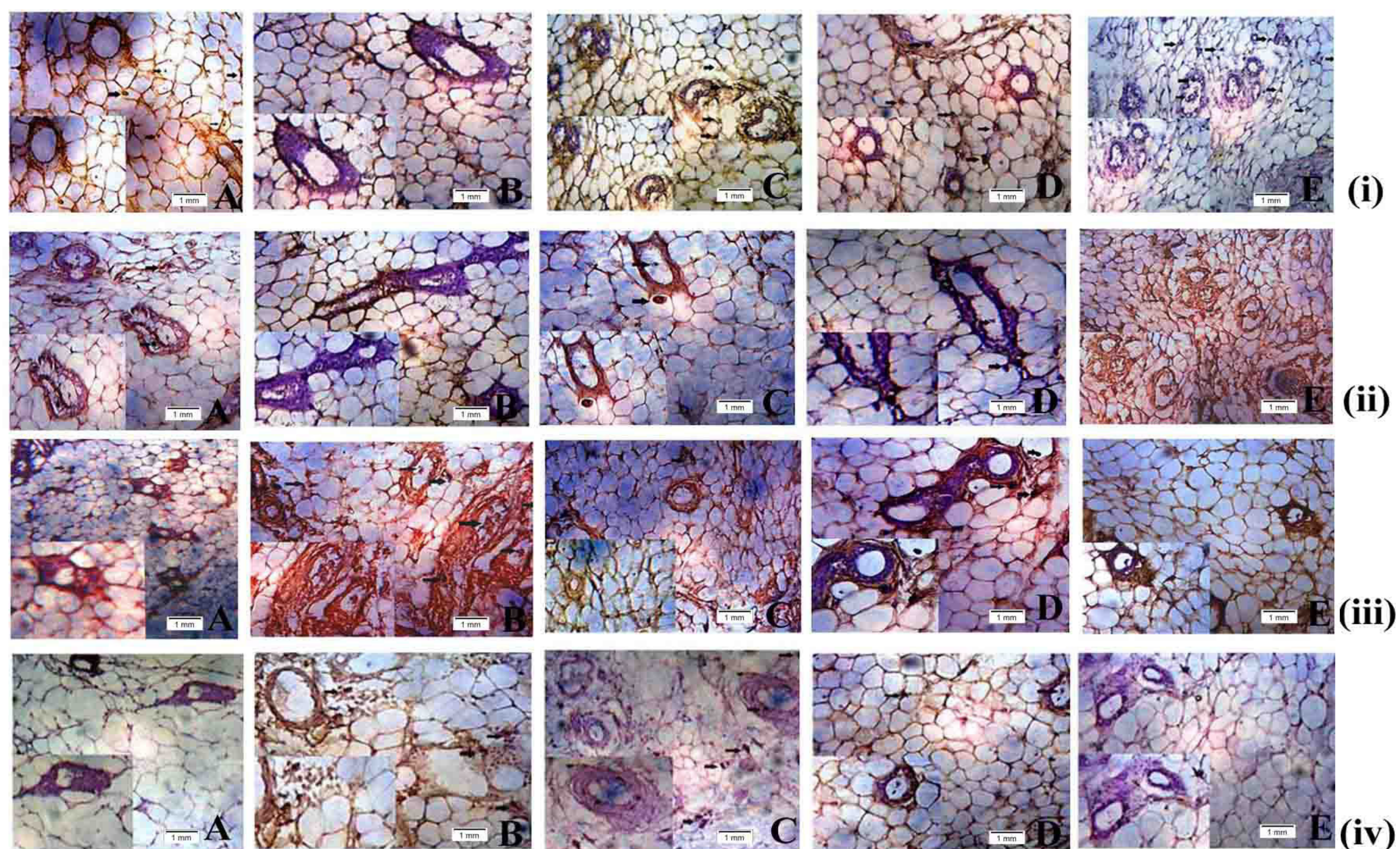

Figure 3: The immuno histochemical analysis of the (i) p53, (ii) Bax, (iii) PI3K and (vi) Akt expressions in the breast tissues of different groups of rats (A) the normal control (B) carcinogen control (C) 0.25 mg/kg of peiminine treated (D) and (E) 0.5 and 1 mg/kg peiminine treated. All images at 40X.

group. Herein, the overlapping happens between the condensed chromatin of apoptotic body and the brown stain that associated with the TUNEL assessment through the initiation of apoptosis.

The percentage of TUNEL positive cells have been determined via the Apoptotic Index (AI) (Table). R value was designated as the ratio of cellular proliferation to apoptosis. The elevated R value in carcinogen control group was represented that the higher proliferative action of cancerous cells but the peiminine treated group exerts significantly reduced the R value caused by the initiation of apoptosis.

DISCUSSION

Recently, the antiquated chemotherapeutic drugs have become outdated owing to their various adverse activities as well as the development of drug resistance that assigning the advancement of new drugs to contract malignant prevalence even though evading the allied side events. Furthermore, the natural products may be the utmost sustainable possibility as of their prospective antineoplastic effects and less adverse events.[25] Therefore, the chemotherapeutic activity of peiminine on DMBA-induced breast cancer in Wistar rat model has been examined and probable molecular mechanistic pathway of peiminine as an antineoplastic agent has been further hypothesized.

The chemotherapeutic activity of peiminine was investigated against MCF7 breast carcinoma cell line. The cell viability assessment demonstrated the compound significantly prevented the viability of MCF7 cells dose dependently. Furthermore, the IC_{50} value was also calculated from cell viability assay which was found to be 5 μg/mL. The flow cytometric analysis demonstrated the induction of apoptosis in MCF7 cell line upon the treatment with peiminine. A significant number of cells at apoptotic stage were identified in the peiminine treated group, where the treatment with 7.5 μg/mL complex showed maximum apoptosis (52.81%) in MCF7 cells. The loss of cell cycle regulation followed by uncontrolled cellular growth is one of the important aspects of cancer cells.[26] Thus, the regulation of cell cycle through the induction of cell cycle arrest is a major chemotherapeutic strategy which allows the accumulation of DNA damage and promotes apoptosis in cancerous cells. The peiminine activity on cell cycle phase distribution has also been investigated in MCF7 cells through flow cytometry. The result from the cell cycle analysis designated that, the treatment with peiminine caused cellular arrest in S phase and G2/M phase of cell cycle and ultimately stimulated the apoptotic events in breast cancer cells.

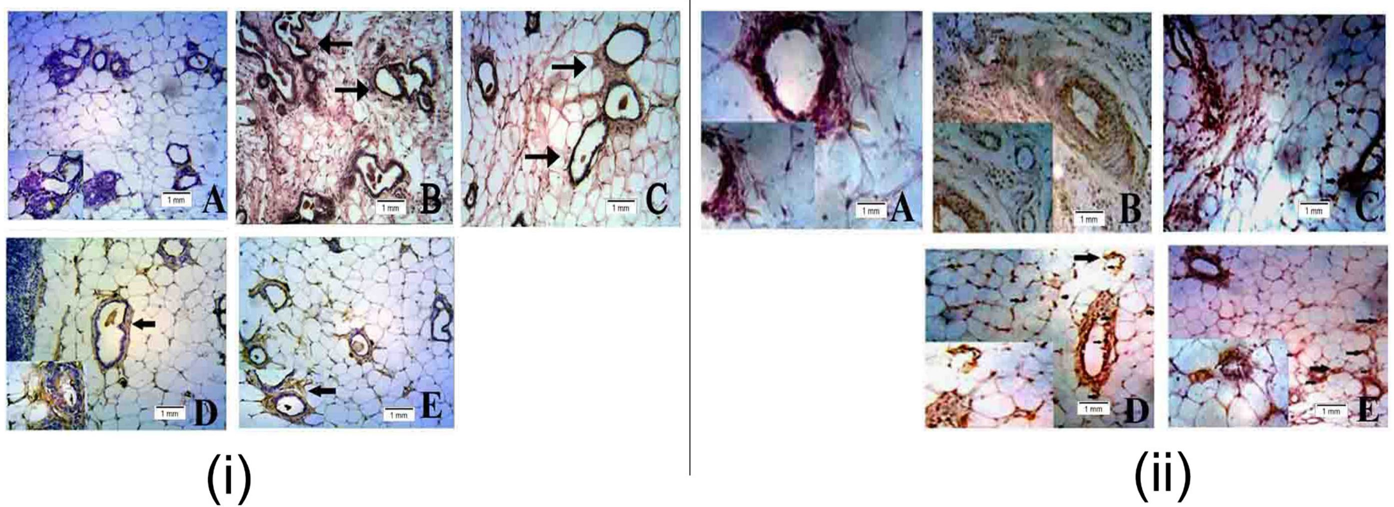

Figure 4: [i] The immuno histochemical analysis of expression of PCNA of different group of rats (A) the normal control (B) carcinogen control (C) 0.25 mg/kg of peiminine treated (D) and (E) 0.5 and 1 mg/kg peiminine treated. All images at 40X. [ii] TUNEL assay of apoptotic (A) the normal control (B) carcinogen control (C) 0.25 mg/kg of peiminine treated (D) and (E) 0.5 and 1 mg/kg peiminine treated. All images at 40X.

Peiminine
Induction
Inhibition
PI3K
PCNA
p53
Bax
Akt
Bcl2
Cytochrome c
Cyt c + Apaf 1 + Procaspase 9
mTOR
Proliferation of cancer cells
Cas-9
Procaspase 3
Cas-3
Cell growth
Cellular proliferation
Cell survival
Apoptosis

Figure 5: The molecular mechanistic pathway of peiminine in mammary cancer.

The initiation of carcinoma via chemical carcinogens is multiphase method that concerned with activating of normal cells to cancer cell and then allowing those cells to invade into surrounding tissue.[27] In this investigation the breast carcinoma has been manifested in Wistar rat model by the management of DMBA that was resembled with human mammary carcinoma. Together with, the histology, hyperplastic evolution in the pre-carcinoma and carcinoma lesions is moderately similar with the human mammary carcinoma prevalence.[28] DMBA administered in experimental rats causing the formation of hyperplastic lesions and change the normal architectural association of breast tissue due to the higher amplification of cellular propagation, which have been further evaluated by histological assessment. Peiminine treatment fruitfully reversed the mammary tissue's cellular architectural modifies to normalcy by preventing the initiation of hyperplastic lesions and reduced the cellular propagation that clearly demonstrates the chemotherapeutic effect of drug on mammary carcinoma.

The anticancer activity of peiminine was evaluated by the establishment of experimental animal model counter to breast cancer is greatly valued for the investigation of human mammary carcinoma as rat mammary has extraordinary propensity for developing cancer that similar with human breast carcinoma.[29] The recent research depicts antineoplastic activity of peiminine on DMBA persuaded mammary carcinoma via the interruption of cancer cell dissemination, that triggers the apoptosis and decreases the mammary carcinoma occurrence through downstream effect of Akt and PI3K expression and the activation of apoptotic indicators including Bax and *p*53 in rat mammary carcinoma model. Additionally, this study delivers a summary of antineoplastic mechanism of this new chemotherapeutic drug.

The immunohistochemical assessment of mammary tissue have been conducted to examine the alteration of *p*53, Bax and survival and cellular proliferation proteins like Akt, PI3K to inaugurate the signaling pathway by which peiminine demonstrates its anticancer efficacy to counteract the breast cancer. Numerous revisions stated the alteration of tumor suppressor protein (*p*53), which has directly connected with cancer cell owing to the modification of cancer suppressive ways.[30] All through the stimulation of the intrinsic apoptotic pathway, which leads to start the caspase-dependent downregulation of cancer proliferation and ultimately results in cell death, increased *p*53 expression reduced the number of cells.[31,32] The results of this study prompted the overexpression of *p*53 and Bax that supports to initiation of apoptosis in mammary cancer cells. Additionally, the PI3K transduction scheme promotes cellular growth and inhibits malignant cell death via a number of cellular survival pathways that are facilitated with PI3K. In current research, the carcinogen control group displayed Akt and PI3Koverexpression whereas peiminine treated group displayed a substantial decreased in Akt and PI3K proteins expression in mammary carcinoma cells. As a result of peiminine treated group, carcinoma cells involve significant decreases of cell propagation and become more susceptible to apoptosis.

The Reactive Oxygen Species (ROS) shows an essential part in progression of cellular oxygen metabolism which interprets several cellular effects via stimulations of several signaling ways that accountable for cell progression and explosion at physiological concentrations. Nevertheless, ROS amplification causes the distraction of redox homeostasis that leads to ROS mediated damage of some essential macromolecules such as proteins, DNA and lipids.[33] Thus, eventually promotes the carcinogenesis.[34] Normal cells are enabling to defend themselves from the undesired activity of oxidants through the effects of various antioxidant enzymes like CAT, SOD and GST.[35] In this study, it has been established that the carcinogen control group displayed the declined activity of enzymes like CAT, SOD and GST whereas peiminine treated group displayed an upregulation of antioxidant levels in breast tissue that concern with the anticipation of ROS generation along with hinders the cell expression and dissemination.

Uncontrolled cellular proliferation is a crucial indicator of the occurrence of cancer. The modulation of various mechanistic ways that operates the cell cycle machinery is necessary for the control of cellular propagation. The cellular propagation has regulated by the several checkpoint pathways. In this research the peiminine treated group shows a substantial reduce in cellular proliferation which represented its anticancer effect on mammary carcinoma model. The cellular proliferative action of malignant cells in mammary tissue has been evaluated through Proliferating Cell Nuclear Antigen (PCNA) marker. The entire % of PCNA marked cells been considered through the labeling index where the distinctive nuclear localizations have been observed in cells marked with PCNA. For DMBA induced carcinogen control group, a superior labeling index been noticed which been lowered followed by peiminine treatment as observed in peiminine treated group.

In the early stage of mammary carcinoma, the apoptosis plays a crucial part in the prevention of tumorigenesis. In this investigation, TUNEL analysis been implemented for the evaluation of apoptosis initiation in carcinoma cells caused by the peiminine treatment. The management of cancer with peiminine (0.25, 0.5 and 1 mg/kg) exhibited a higher % of cells entering into apoptosis where a substantial lower apoptotic index been seen in carcinogen control group.

Since, all the results from this investigation, the chemotherapeutic action of peiminine on DMBA persuaded rat breast cancer with the prevention of cancer cell expression and propagation along with the initiation of apoptosis through the upstream expression of *p*53, Bax and downstream expression of PI3K, Akt remains intensely recognized (Figure 5). Additionally, this research

extremely affords new insight of prospective chemotherapy at a drug therapy regimen with a noticeably low dose in order to inhibit the expansion of cancer via reorganization of possible biomarkers that could be linked to apoptosis in breast carcinoma through activating intrinsic apoptotic signaling path.

CONCLUSION

In conclusion, the newer chemotherapeutic strategies in contest with mammary cancer are instantly directive as in the last years a little improvement was attained in this arena, despite the accessibility of novel and superior chemotherapeutic interventions. Our present research exhibited the chemotherapeutic activity of peiminine on DMBA persuaded the mammary cancer due to the modulation of PI3K/*p*53/Akt signaling way in rats. Subsequently, a satisfactory evaluation of the limits of this recent research, anticancer activity of peiminine should be confirmed in preclinical study and further it might be assessed as a potential drugs alternative in clinical research in nearby future prospective.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

J. Zhang, W. Zhao and J. Sun has prepared the manuscript. B. Zhou and H. Qiao has involved in work being competition, scientific analysis and manuscript writing. J. Zhang, J. Li and S. Ma were involved in scientific checking and manuscript writing. W. Zhao and J. Sun were involved in editing.

ETHICS APPROVAL

Experimental animal has been conducted as per the Institutional Animal Ethical Committee (IAEC) of Baoding First Central Hospital (Approval no. 2022BCH420) and complied with the ARRIVE guidelines.

SUMMARY

The article has enlightened upon the significance of peiminine on cancer cells which provide definitive evidence that it could stop, abrogate or postpone the progression of breast carcinoma by interacting with various biomarkers corresponding to the alteration of apoptotic events along with the interruption of the phase of angiogenesis, therefore performing the function of potential contender in the future of cancer chemotherapy. It is the first time where such as approach is being considered in the field of chemotherapy. In addition, we believe that the current research content should be highly valuable for your esteemed journal as it depicted the chemotherapeutic aspect of peiminine as a potent biomedicine.

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