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Breast Cancer Migration Is Inhibited By *Ficus Glomerata Roxb* Leaf Column Elute Via An Inflammatory And Apoptotic Cell Signalling Pathway

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Abstract

Among developed countries, breast carcinoma is one of the most common cancers in women. Breast carcinoma is the result of the fusion of several biological structures, each with its own physiological characteristics and clinical repercussions. The *Ficus* genus has been reported to have important pharmacological properties in a variety of plants. The current study examined at the anti-mammary action of *Ficus glomerata* leaf column elute (FGLCE) in 12-Dimethylbenz (α) anthracene (DMBA) driven breast cancer and the mechanism behind it. To produce tumors in female rats, a single dosage of DMBA (25 mg/kg) was injected into the mammary gland. The female rats were divided into five groups- Group I. Control group; Group II- control + FGLCE (100mg/kg of BW); Group III- DMBA treated rats; Group IV- DMBA + FGLCE treated; Group V- DMBA + DOX (5 mg/kg) treated rats. Tumor burden, incidence, and volume were measured at the end of the experiment.The lipid profile (TC, FFA, PL, and TG) in plasma, liver tissue, and mammary tissue were all calculated.At a dose of 25 mg/kg body weight, DMBA inhibits the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione peroxidase (GPX).DMBA at a dose concentration 25mg/ kg body weight xenobiotic markers increased in group III rats When compared to the control group and glycoprotein p53, and TNF-α, the dose-dependent treatment with FGLCE ($P<0.05$) significantly reduced .Hepatic markers such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), tumor necrosis factor- (TNF-α), and inflammatory mediators such as nuclear factor kappa-B(NF-kB) were considerably ($P<0.05$) down regulated by FGLCE. FGLCE significantly ($P<0.05$) reduced apoptotic regulator (Bax), B-cell lymphoma 2 (Bcl-2), and Breast Cancer gene 1 (BRCA-1) expression when compared to DMBA control group rats.Therefore, the findings suggest that FGLCE has a protective effect against DMBA-induced mammary gland in rats.

Keywords: Breast cancer; DMBA; Ficus Glomerata leaf column elute (FGLCE); Inflammation; Apoptosis

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women around the world, and it is also the leading cause of cancer death in women[1, 2].Breast cancer cases were predominantly reported in Europe, then the United States[1, 3].Breast cancer is more usually caused by cancer death in developed countries than in developing countries[1, 4].In 2020, 16.3 million cancer cases will be recorded, with 9.8 million cancer patients expected to death[5, 6].Breast cancer is caused by mutations, age, and oestrogen exposure in tumor suppressor like BRCA1 and 2, environmental toxins including polycyclic aromatic hydrocarbons, and obesity[6, 7].

Tamoxifen (hormonal therapy) and chemotherapy medications such as paclitaxel (anti-microtubule) and doxorubicin (cytotoxic antibiotic), both widely utilised for metastatic breast cancer and estrogen-dependent breast cancer, are the most commonly used drugs for treating breast cancer[8, 9].Furthermore, these treatments are limited by major side effects such as endometrial cancer with thromboembolic complications, cardiotoxicity, and a slew of other serious complications[10, 11]. Due to the limits of the above-mentioned treatments, there is an urgent need to create a novel natural protective agent for breast cancer that is more efficacious and safer in reversing breast cancer and reducing patient suffering at a cheap cost.

In animal research, DMBA is often used to cause breast cancer[12].The respiratory, intestinal, and cutaneous tracts, as well as intraperitoneal and intravenous injection, inhalation, and ingestion, are all common routes of DMBA absorption[13]. DMBA usually works by inducing or beginning mutations in the genes involved in carcinogenesis[14, 15].The aryl hydrocarbon receptor (AhR) of the cellular cytosolic receptor is activated by DMBA, which translocates AhR into the nucleus and interacts with AhR proteins to facilitate nuclear translocation[16, 17]. As a result, AhR-dependent over expression of cytochrome P450 (CYP1A1 and CYP1B1) enzymes, which metabolise DMBA into a carcinogenic intermediate epoxy that produces DNA adducts, occurs[18, 19].Carcinogenesis linked to polycyclic aromatic hydrocarbons (PAH) is induced by mutation and malignant transformation triggered by these DNA adducts.Previous research suggests that DMBA is lipophilic in nature, and that breast tissue contains a significant amount of adipose tissue, in which the DMBA can concentrate on the epithelium contact before metabolic activation, and that this property certainly correlates with DMBA activity at the mammary level.

According to World Health Organization (WHO) reports, traditional medicine is used for primary health care in 82 % of developing countries[20].Various phyto-constituents derived from traditional medicine have been shown to exhibit a variety of pharmacological actions in previous research[21].Phytoconstituents obtained from plants are not only essential for proper body function, but also have a positive and protective effect on health or play a key part in the treatment of a variety of disorders[22, 23].Anti-inflammatory, anti-cancer, antibacterial, antihypertensive, and anti-diabetic phytoconstituents derived from traditional plants have several health benefits[24, 25].*Ficus glomerata,* also known as the cluster of fig Gular, is a moraceaceous plant that is native to Australia, Southeast Asia, and the Indian subcontinent. In traditional medical systems, the genus *Ficus glomerata* has been shown to offer tremendous medicinal value in several parts of the world (leaves, stem, root, fruit, seeds, latex and even whole plant).Gular has been used to treat diarrhoea, diabetes, hypertension, gastric ulcers, wound healing, and other ailments.We attempted to investigate the anticancer effect of FGLE on DMBA-induced breast cancer and the possible routes implicated due to its potential anti-inflammatory action.

2. Materials and methods

2.1 Chemicals and reagents

DMBA were purchased from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India. TNF-, NF-B, Bcl-2, Bax, BRCA-1, p53, PCNA, and -actin monoclonal antibodies, as well as polyclonal anti-goat and anti-rabbit antibodies, were obtained from Santa Cruz Biotechnology in the United States. S.D. Fine Chemicals in Mumbai and Fisher Inorganic and Aromatic Limited in Chennai, India provided all other analytical chemicals.

2.2 Plant collection and Extract Preparation

2.2.1 Collection of plant materials

The fresh leaves of *Ficus glomerata* were collected in the month of August (2022) from pulugandiur (Vill), Krishnagiri (Dt), Tamilnadu, India.

2.2.2 Processing of plant Extract

The leaves of *Ficus glomerata* was collected from natural habitat and washed thoroughly with saline water and shade dried. The dried leaves are uniformly using mechanical grinder to make fine powder. Then the coarse powder 9g was extracted with 150ml ethanol in a hot soxhlet apparatus for 48 hr. The ethanolic extracts were then distilled evaporated and dried.The crude extracts thus obtained is subjected to column chromatography.

2.2.3 Column chromatography for purification of crude sample

Column chromatography is commonly used purification technique isolation of pure, pharmacologically active constituents from plants remain a long and tedious process. It is necessary to have methods available for efficient separation from plant extracts, to isolate the purify compound. The column chromatography (length: 450mm; Bore: 30mm) was performed using 60 – 120 mesh silica gel to elute out individual components from the crude plant extract. The column was rinsed with hexane and completely dried before use. The column was filled 3/4th with mixture of solvents ratio (1:2:0.5) (Distilled water: Acetic acid: Chloroform) and 20g of silica gel was packed 2/3rd of the column length with simultaneous draining of the solvent to aid proper packing. The packing was performed after activating the silica gel gently poured on the top of the column with constant tapping to avoid air bubbles and cracks after mixing with solvents. Add 2mm layer of sea sand into the top of the column after settled the gel. The column was run with solvent of (Hexane & Ethanol 1:5) after loading with the crude plant extract (2-3g) mixed with activated silica gel. The fractions collected were dried for further analysis.

2.3 Experimental animal

Six to seven weeks old female Sprague-Dawley rats (weighed 120–130 g), were purchased from Biogen, Bangalore, India. Rats were kept at the Rajah Muthiah Medical College and Hospital, Annamalai University, Chidambaram, Tamilnadu, India's Central Animal House. Guidelines must be followed after receiving proper approval from the Institutional Animal Ethics Committee for the Control and Supervision of Experimental Animals (CPCSEA approval number: 971). The rats were kept in a controlled environment with a temperature of 24°C, humidity of 50%, and a 12-hr light/dark cycle. Amounts of food and drink are offered ad libitum.

2.4 Breast cancer induction

A single intraperitoneal DMBA injection (80mg/kg) was used for breast cancer induction. Briefly, DMBA (80 mg/kg) soluble in phosphate buffered saline (PBS). All rats were received DMBA treatment except normal rats [26].

2.5 Experimental protocol

All rats were divided into five groups after effective breast cancer induction: normal control (group I); control + FGLE (100 mg/kg BW): group II; DMBA (25 mg/kg BW): group III; DMBA + FGLE (100 mg/kg BW): group IV; and DMBA + DOX (5 mg/kg BW): group V. The rats in both groups were given treatment for up to 140 days. Food intake, water intake, and body weight of all group rats

were measured at daily intervals. To obtain the blood sample, all of the group rats were anaesthetized at the end of the process. Blood samples were centrifuged at 10,000 rpm for 15 minutes at 4°C for future research, and the serum was kept at -80°C.

2.6 Biochemical analysis

The liver and mammary tissues that were removed were washed in ice cold saline. A known amount of tissue was homogenised in 0.1M Tris-HCl buffer (pH 7.4) at 4°C in a Potter-Elvehjem homogenizer with a Teflon pestle at 600 rpm for 3 min in a Potter-Elvehjem homogenizer with a Teflon pestle the homogenate was centrifuged for 10 min at 3000g at 4°C. The supernatant was collected as tissue homogenate and utilised to test a variety of biochemical parameters. The procedure [27]was used to isolate microsomes from liver and mammary tissue. The method [28]was used to calculate the microsomal protein content. The method [29]was used to calculate the plasma TBARS concentration. The concentration of TBARS in breast tissue was calculated using the method [30].The method [31]was used to calculate the concentrations of LOOH in plasma and mammary tissue. The method [32]was used to measure SOD activity in plasma and breast tissue. The method [33]was used to measure CAT activity in plasma and mammary tissue. The method [34]was used to determine GPX activity in plasma and mammary tissue. The method [35]was used to determine GSH levels in plasma and mammary tissues. The method [36]was used to calculate the amounts of Cyt p450, Cyt-b5andcyt-C in liver and mammary tissue microsomes. The approach [37]was used to measure GST activity in liver and mammary tissues. The method [38]was used to measure GR activity in liver and mammary tissues.

2.7 Western Blot Analysis

For 30 min, breast tissue was homogenised in an ice-cold lysis RIPA buffer with a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). The lysate was centrifuged for 10 min at 4°C at 13,000 rpm, and the supernatant was used to quantify protein concentration using the Nanodrop 2000. (Thermo Scientific, USA). 50 g of protein from cell extracts were electrophoresed on a 12% SDS-PAGE gel and transferred to a PVDF membrane using a transblot semi-dry device (Biorad, USA). Before putting together the transfer system, immerse the PVDF membrane for 10 min in methanol and the blotting sheets in cold transfer buffer.The sandwich, blotting paper, membrane, gel, and blotting paper were placed in the transfer equipment, along with a few drops of transfer buffer, and exposed to a 20 V electric current under cold conditions for 1 hr. The sandwich was withdrawn from the transfer system after the transfer. To ensure equal loading, the membrane was dyed with 0.5 % ponceau in 1% acetic acid. PVDF membranes[38]were blocked with non-fat milk (5% (w/v) for 6 hours before being incubated overnight 37° C in blocking solution with primary antibodies (Sigma-Aldrich, USA).The PVDF membranes were then washed three times with TBST at a 10min interval, and the generated bands were identified with a chemiluminescence substrate. Image Studio software was used to capture the photographs (LI-COR, USA).

2.8 Immunohistochemistry Analysis

Tissue sections were cut to a thickness of around 4 µm from paraffin-embedded tissue. After mounting and deparaffinizing the paraffin-embedded slices in xylene, they were rehydrated with an ethanol/H_2O gradient. The slides were allowed to cool to room temperature for additional 20 min after a 10min heat-mediated antigen retrieval process. After that, a 15min peroxidase block (to inhibit endogenous peroxidase enzyme activity) and a 15min power block were applied (to prevent non-specific antibody binding to strongly charged locations).After incubation with the diluted primary antibodies (p53 and PCNA) solution, the sections were treated with the super enhancer solution (for 30 min) and super sensitive Poly-HRP solution (for 2 hr, 1:200). (for 30 min) After colour development using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and counterstaining with haematoxylin, the sections were inspected under a light microscope and images were obtained.

2.9 Histopathology

After 30 min in a 60 °C oven, the slides were immersed in xylene for 5 min, 4 min, and then absolute ethanol for 3 min and 2 min, respectively. The slides were placed in a water bath for 2-3 min after 7 min in hematoxylin. After 1 min of dehydration with 70% ethanol, the slides were stained with the working eosin solution for 11 min. The slides were dehydrated in 95% ethanol for 30s before being washed three times in 100% ethanol for 30s each time before being mounted in a neutral deparaffinated xylene (DPX).

2.10 Statistical analysis

All data is presented as a mean standard error of the number of experiments. One-way analysis of variance (ANOVA) was used to determine statistical significance in SPSS version 17.0 (SPSS, Cary, NC, USA), and Duncan's Multiple Range Test was used to determine individual comparisons (DMRT). A significance difference between groups was defined as a value of $p<0.05$. At $p<0.05$, values with the same superscript do not differ significantly from one another.

3. Results and Discussion

3.1 Tumor weight

Table 1 shows the effect of FGLCE on DMBA-induced breast cancer in Sprague-Dawley rats. In the normal control group of rats, there was no sign of a tumor. The tumor incidence in rats treated with DMBA was 100%. When FGLCE (100 mg/kg) was given to DMBA-treated rats, two animals developed breast tumors with a 25% tumor incidence. The average tumor size in the DMBA-treated rats was 24.852.94 mm^3. At 100 mg/kg, DMBA+FGLCE therapy resulted in a 20% tumor incidence and a tumor volume of 9.341.98. Treatment with DMBA+DOX resulted in a 25% reduction in tumor size, with an average tumor size of 9.185.08 mm3 and a dose of 5 mg/kg, respectively. In rodents, the compound 7,12 dimethylbenz (a) anthracene (DMBA) has been frequently used to produce breast cancer[39]. DMBA is a polycyclic aromatic hydrocarbon that is also a common workplace carcinogen linked to breast cancer[40]. The current experimental approach used a single intraperitoneal injection of DMBA (80 mg/kg) to induce breast cancer. After 20 weeks of testing, huge tumors were discovered in experimental animals given a high dose of DMBA, while normal rats were given saline only and exhibited no evidence of breast cancer.

Table 1. showed the effect of FGLCE on DMBA induced breast cancer in rats

Groups	Totalnumber of tumor (n)	Tumor incidence (%)	Tumor volume (mm3)/rat
Control	(0)/8	0	0
Control + FGLE (100 mg/kg BW)	(0)/8	0	0
DMBA (25 mg/kg BW)	(8)/8	100	24.85 ± 2.94
DMBA + FGLE (100 mg/kg BW)	(2)/8	25	9.34 ± 1.98
DMBA + DOX (5 mg/kg BW)	(2)/8	25	9.18 ± 5.08

Tumor volume was measured using the formula $V = 4/3\pi$ (D1/2) (D2/2) (D3/2),where D1,D2 and D3 are the three diameters (in mm) of the tumor; () indicatestotal number of rats bearing tumors. Values are expressed as means ± S.D for eight rats in each group. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

3.2 Body Weight

Table 2 shows the effect of FGLE on normal and DMBA-induced community rats. Rats in the conventional control group showed an increase in body weight from the start of the experiment to the end. In DMBA-treated rats, there was an increase in body weight compared to the beginning body weight. When compared to the normal and other treated rats in the group, the reduced body weight was seen. The group of rats given FGLE had a rise in body weight that was dosage dependent. FGLE (100 mg/kg) raised body weight and brought it close to normal values.

Table 2. Effect of FGLCE plant elute on body weight changes in control and experimental animals.

Groups	Initial Weight (g)	Final Weight (g)
Control	136.80 ± 11.63[a]	168.61 ± 12.83[a]
Control + FGLE (100 mg/kg BW)	138.44 ± 12.63[a]	169.54 ± 12.97[a]
DMBA (25 mg/kg BW)	141.19 ± 9.43[a]	108.50 ± 8.26[b]
DMBA + FGLE (100 mg/kg BW)	140.38 ± 10.68[a]	164.97 ± 12.62[a]
DMBA + DOX (5 mg/kg BW)	141.94 ± 9.17[a]	166.30 ± 12.66[a]

Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT.

3.3 Antioxidant activity

Tables 3, 4 and 5 illustrate the enzyme and non-enzymic antioxidant status in Erythrocytes, liver tissue, and mammary tissue fractions. The levels of non-enzymic antioxidants like GSH fractions in Erythrocytes, liver tissue, and mammary tissue of control and experimental rats, as well as the activities of enzymic antioxidants like SOD, CAT, and GPX. When breast carcinoma-bearing rats were compared to normal control rats, antioxidant status (both enzymic and non-enzymic) was found to be considerably decreased ($p < 0.05$). When Groups IV animals were given FGLCE extract, all of these changes were reversed to near-control levels.When rats were given FGLE extract, antioxidant levels were higher than when they were given conventional DOX. Reactive oxygen species (ROS) produced during the metabolism of DMBA can disperse to different targets within the cell or even spread the injury to healthy cells outside the cell. Despite having a plentiful supply of antioxidants, the liver is particularly sensitive to ROS-induced damage. As a result, ROS-induced lipid and protein oxidation occurs in the presence of weakened antioxidant defences [40].Low levels of GSH and reduced activities of GPx, SOD, and CAT in the mammary tumours and liver of DMBA-treated animals in this study can lead to superoxide anion and H_2O_2 accumulation, which can lead to oxidative stress, oxidation of critical –SH groups, and conformational changes in functional proteins[41].

Table 3. Effect of FGLC plant extracton SOD, CAT, GPx in erythrocytes and GSH in plasmaof control and experimental animals.

Groups	SOD (U*/mg of Hb)	CAT (U**/mg of Hb)	GPx ($U^{@}$/mg of Hb)	GSH (mg/dL)
Control	4.58 ± 0.34[a]	157.88 ± 12.02[a]	12.47 ± 0.94[a]	36.84 ± 2.80[a]
Control + FGLE (100 mg/kg BW)	4.56 ± 0.34[a]	158.50 ± 12.13[a]	12.54 ± 0.96[a]	36.87 ± 2.82[a]
DMBA (25 mg/kg BW)	2.65 ± 0.20[b]	109.77 ± 8.35[b]	7.25 ± 0.55[b]	20.35 ± 1.54[b]
DMBA + FGLE (100 mg/kg BW)	4.02 ± 0.30[c]	154.86 ± 17.22[a]	11.03 ± 0.84[c]	32.59 ± 2.28[c]
DMBA + DOX (5 mg/kgBW)	4.49 ± 0.31[a]	160.49 ± 12.22[a]	11.88 ± 0.90[c]	35.62 ± 2.70[a]

Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).
U*= Enzyme concentration required to inhibit the chromogen produced by 50% in one min under standard condition.
U**= µmole of hydrogen peroxide decomposed/min.
U@ =µmole of GSH utilized/min.

Table 4. Effect of FGLCE plant extracton SOD, CAT, GPx and GSH inliver tissue of control and experimental animals.

Groups	SOD (U*/ mg protein)	CAT (U**/mg protein)	GPx (U@/mg protein)	GSH (µg /mg protein)
Control	7.82 ± 0.61[a]	62.15 ± 4.73[a]	6.14 ± 0.45[a]	6.65 ± 0.50[a]
Control + FGLE (100 mg/kg BW)	7.83 ± 0.59[a]	61.70 ± 4.72[a]	6.09 ± 0.41[a]	6.68 ± 0.51[a]
DMBA (25 mg/kg BW)	5.42 ± 0.38[b]	32.48 ± 2.45[b]	3.52 ± 0.23[b]	4.02 ± 0.30[b]
DMBA + FGLE (100 mg/kg BW)	7.08 ± 0.61[c]	52.78 ± 4.04[c]	5.34 ± 0.43[c]	6.35 ± 0.41[a]
DMBA + DOX (5 mg/kg BW)	7.65 ± 0.48[ca]	58.47 ± 4.45[a]	5.90 ± 0.41[a]	6.51 ± 0.51[a]

Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).
U*= Enzyme concentration required to inhibit the chromogen produced by 50% in one min under standard condition.
U**= µmole of hydrogen peroxide decomposed/min.
U@ =µmole of GSH utilized/min.

Table 5. Effect of FGLCE plant extraction SOD, CAT, GPx and GSH inmammary tissue of control and experimental animals.

Groups	SOD(U*/ mg protein)	CAT (U**/mg protein)	GPx (U@/mg protein)	GSH (µg /mg protein)
Control	14.61 ± 1.11[a]	52.24 ± 3.05[a]	13.32 ± 1.01[a]	15.20 ± 1.35[a]
Control + FGLE (100 mg/kg BW)	14.58 ± 1.11[a]	52.22 ± 3.19[a]	13.36 ± 1.08[a]	15.15 ± 1.16[a]
DMBA (25 mg/kg BW)	9.20 ± 0.70[b]	28.46 ± 2.16[b]	7.34 ± 0.42[b]	7.52 ± 0.52[b]
DMBA + FGLE (100 mg/kg	13.89 ± 1.06[a]	50.20 ± 3.84[a]	12.48 ± 0.95[a]	14.25 ± 1.09[c]
DMBA + DOX (5 mg/kg BW)	14.02 ± 1.10[a]	51.76 ± 3.94[a]	12.98 ± 0.84[a]	14.86± 1.13[ac]

Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).
U*= Enzyme concentration required to inhibit the chromogen produced by 50% in one min under standard condition.
U**= µmole of hydrogen peroxide decomposed/min.
U@ =µmole of GSH utilized/min.

3.4 Enzyme activities

The levels of phase I (cyt p450, cyt b5) and phase II (cyt -C) detoxifying agents in the liver and mammary tissues of control and experimental animals are shown in Tables 6 and 7. When DMBA treated animals were compared to control animals, the levels of cyt p450 and cyt b5 were considerably (p0.05) higher, and the activities of cyt-C were significantly higher. In comparison to group III animals, oral administration of FGLCE at dosage (100mg, /kg bw) significantly ($p<0.05$) lowered the level of phase I agents and raised the activity of phase II enzymes in the liver and mammary tissues of the (group III and IV) animals.However, when FGLE alone treated rats were compared to control animals, there was no significant change. Due to the activation of procarcinogens to ultimate carcinogens, the stimulation of phase I detoxifying enzymes such as cyt p450, cyt b5, and Phase II cyt-C systems is a potential cancer risk factor. As a result, nerolidol's reduction of phase I enzyme activity is critical for the chemo-preventive mechanism of DMBA-induced carcinogenesis.Phase II enzymes carry out conjugation processes, assisting in the conversion of phase I bio-transformed DMBA intermediates into less hazardous, water-soluble compounds that are easily eliminated from the body [42]. Increased activity of phase II detoxification enzymes in rats treated with nerolidol to metabolise and detoxify the carcinogenic DMBA drives the detoxification cascade. The findings show that nerolidol's bioactive molecules affect both phase I and phase II xenobiotic metabolism, suggesting that it could be an ideal

chemo-preventive medication for breast cancer. In this study, DMBA alone treated rats was found to be higher levels of Cyt P450, Cyt-b5, and cyt-C. Rats with tumor tissues had higher levels of phase I enzymes and phase II enzymes. These enzyme levels were considerably altered in FGLE treated mice.

Table 6. Effect of FGLCE plant extraction detoxification enzyme activities in liver tissue of control and experimental animals.

Groups	NADPH-cytochrome P450 reductase	NADH-cytochrome b5 reductase	NADPH-cytochrome C reductase
Control	6.92 ± 0.50^{a}	5.62 ± 0.41^{a}	17.35 ± 1.32^{a}
Control + FGLE (100 mg/kg BW)	6.90 ± 0.53^{a}	5.63 ± 0.48^{a}	17.30 ± 1.18^{a}
DMBA (25 mg/kg BW)	13.98 ± 1.06^{b}	11.85 ± 0.90^{b}	27.54 ± 2.09^{b}
DMBA + FGLE (100 mg/kg BW)	7.98 ± 0.61^{c}	6.03 ± 0.45^{a}	18.05 ± 1.37^{a}
DMBA + DOX (5 mg/kg BW)	7.60 ± 0.51^{ac}	5.92 ± 0.44^{a}	17.69 ± 1.47^{a}

NADPH-cytochrome P450 reductase - nmoles of cytochrome C oxidized/min/mg of protein
NADH-cytochrome b5 reductase - nmoles of ferricyanide reduced/min/mg of protein
NADPH-cytochrome C reductase - nmol of cytochrome C reduced/min/mg of protein
Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

Table 7. Effect of FGLCE plant extractondetoxification enzyme activities in mammary tissue of control and experimental animals.

Groups	NADPH-cytochrome P450 reductase	NADH-cytochrome b5 reductase	NADPH-cytochrome C reductase
Control	0.93 ± 0.06^{a}	0.56 ± 0.03^{ac}	1.20 ± 0.09^{a}
Control + FG (100 mg/kg BW)	0.96 ± 0.05^{ac}	0.53 ± 0.03^{a}	1.18 ± 0.09^{a}
DMBA (25 mg/kg BW)	1.48 ± 0.11^{b}	0.96 ± 0.05^{b}	4.63 ± 0.35^{b}
DMBA + FG (100 mg/kg BW)	1.05 ± 0.07^{c}	0.64 ± 0.03^{c}	2.15 ± 0.16^{c}
DMBA + DOX (5 mg/kg BW)	0.98 ± 0.05^{ac}	0.58 ± 0.04^{ca}	1.98 ± 0.16^{c}

NADPH-cytochrome P450 reductase - nmoles of cytochrome C oxidized/min/mg of protein NADH-cytochrome b5 reductase - nmoles of ferricyanide reduced/min/mg of protein NADPH-cytochrome C reductase - nmol of cytochrome C reduced/min/mg of protein Values are expressed as means ± S.D for eight rats in each group. Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

3.5lipid profile

The levels of lipid profile (TC, TG, PL, and FFA) and lipoprotein (HDL-C, LDL-C, VLDL-C) in plasma of control and experimental animals are shown in Tables 8, 9, and 10. TC, TG, PL, FFA, LDL-C, and VLDL-C levels in plasma were considerably ($p<0.05$) raised, while HDL-C levels were significantly ($p<0.05$) decreased. When DMBA alone treated mice are compared to control animals, the levels of PL and FFA in mammary tissue are lower.FGLE therapy at different dosage(100mg/kg bw) reduced TC, TG, PL, FFA, LDL, and VLDL levels significantly ($p<0.05$). When compared to group III animals, FGLCE treated rats HDL levels in plasma were considerably ($p<0.05$) higher, while PL and FFA levels in mammary tissue were lower. When FGLCE alone treated animals were compared to control animals, no significant differences were found. During the metabolic activation of DMBA into its active metabolite dihydrodiol epoxide, a large amount of reactive oxygen species (ROS) is produced[43].By-products of lipid peroxidation (LPO) are produced as a sign of oxidative stress. Increased amounts of LPO by-products in plasma in tumor-bearing mice confirmed oxidative stress. Reduced plasma and mammary enzymatic antioxidant activity are likely owing to fatigue of these enzymes as a result of scavenging overly generated free radicals in the system, and total body weight also dropped. Because in DMBA-treated rats, LPO plays a crucial role in the onset of tumor

formation and changes in energy metabolism.The status of plasma LPO by products, body weight, and antioxidants were all restored to near-normal levels after oral administration of FGLCE, indicating that FGLCE possesses effective free radical scavenging properties during DMBA-induced mammary carcinogenesis. A previous study found that increased levels of TBARS in cancer cells are linked to free radical overproduction and can be used to determine the extent of tissue damage[44]. In this work, we found that DMBA-induced rats had higher amounts of TBARS and LOOH. Mammary tumor-induced rats had higher levels of TBARS, according to reported mammary tumor-induced rats had higher levels of TBARS, according to [45]. FGLCE, on the other hand, had a substantial effect on lipid peroxidation, showing that it had anti-lipid peroxidative properties.

Table 8. Effect of FGLCE plant extract on lipid profile in plasma of control and experimental animals

Groups	TC (mg/dL)	TG (mg/dL)	PL (mg/dL)	FFA (mg/dL)
Control	84.72 ± 6.45^{a}	74.17 ± 5.64^{a}	115.49 ± 8.79^{a}	11.58 ± 0.95^{a}
Control + FGLE (100 mg/kg BW)	86.53 ± 6.31^{a}	75.52 ± 5.77^{a}	113.77 ± 8.70^{a}	11.56 ± 0.78^{a}
DMBA (25 mg/kg BW)	143.83 ± 10.94^{b}	130.59 ± 9.94^{b}	162.47 ± 12.37^{b}	20.52 ± 1.56^{b}
DMBA + FGLE (100 mg/kg BW)	94.80 ± 7.25^{c}	82.50 ± 6.31^{a}	120.48 ± 9.22^{a}	13.28 ± 1.01^{c}
DMBA + DOX (5 mg/kg BW)	89.49 ± 6.81^{ac}	77.66 ± 5.25^{a}	119.32 ± 9.08^{a}	12.15 ± 0.81^{ac}

Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

Table 9. Effect of FGLCE plant extraction lipid profile in liver tissue of control and experimental animals

Groups	TC (mg/g wet tissue)	TG (mg/g wet tissue)	PL (mg/g wet tissue)	FFA (mg/g wet tissue)
Control	3.79 ± 0.28^{a}	8.82 ± 0.61^{a}	6.60 ± 0.50^{a}	5.62 ± 0.40^{a}
Control + FGLE (100 mg/kg BW)	3.72 ± 0.22^{a}	8.79 ± 0.63^{a}	6.88 ± 0.54^{a}	5.61 ± 0.43^{a}
DMBA (25 mg/kg BW)	9.62 ± 0.74^{b}	15.52 ± 1.18^{b}	11.62 ± 0.63^{b}	9.67 ± 0.73^{b}
DMBA + FGLE (100 mg/kg BW)	4.05 ± 0.30^{a}	9.42 ± 0.72^{a}	7.45 ± 0.57^{c}	6.14 ± 0.47^{a}
DMBA + DOX (5 mg/kg BW)	3.92 ± 0.29^{a}	9.05 ± 0.58^{a}	7.08 ± 0.41^{a}	5.86 ± 0.44^{a}

Values are expressed as means ± S.D foreight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

Table 10. Effect of FGLCE plant extraction lipid profile in mammary tissue of control and experimental animals.

Groups	TC (mg/g wet tissue)	TG (mg/g wet tissue)	PL (mg/g wet tissue)	FFA (mg/g wet tissue)
Control	6.23 ± 0.47^{a}	5.48 ± 0.42^{a}	16.34 ± 1.24^{a}	12.10 ± 0.72^{a}
Control + FGLE (100 mg/kg BW)	6.18 ± 0.32^{a}	5.46 ± 0.41^{a}	16.31 ± 1.17^{a}	12.12 ± 0.92^{a}
DMBA (25 mg/kg BW)	14.15 ± 1.08^{b}	12.05 ± 0.91^{b}	6.88 ± 0.42^{b}	6.75 ± 0.43^{b}
DMBA + FGLE (100 mg/kg BW)	6.48 ± 0.49^{a}	5.88 ± 0.45^{a}	15.38 ± 1.17^{a}	11.88 ± 0.81^{a}
DMBA + DOX (5 mg/kg BW)	6.30 ± 0.42^{a}	5.62 ± 0.43^{a}	15.92 ± 1.21^{a}	11.92 ± 0.92^{a}

Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

Table 11. Effect of FGLCE plant extraction lipoprotein in plasma of control and experimental animals

Groups	HDL-C (mg/dL)	LDL-C (mg/dL)	VLDL-C (mg/dL)
Control	36.61 ± 2.93^{a}	29.16 ± 2.21^{a}	16.94 ± 1.29^{ac}
Control + FGLE (100 mg/kg BW)	37.85 ± 2.89^{a}	29.56 ± 2.26^{a}	15.10 ± 1.15^{a}
DMBA (25 mg/kg BW)	20.49 ± 1.55^{b}	97.22 ± 7.40^{b}	26.11 ± 1.98^{b}
DMBA + FGLE (100 mg/kg BW)	32.07 ± 2.45^{c}	46.23 ± 3.54^{c}	16.50 ± 1.26^{ac}
DMBA + DOX (5 mg/kg BW)	36.17 ± 2.75^{a}	37.77 ± 3.07^{d}	15.53 ± 1.05^{ac}

Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

3.6 Hepatic parameters

Compared to normal group rats, DMBA treated rats showed a higher degree of hepatic parameters. FGLCE treatment substantially (P<0.05) decreased the dose-dependent levels of AST, ALT and ALPTable 12.

Table 12. Effect of FGLCE plant extraction liver marker enzymes in liver tissue of control and experimental animals.

Groups	AST	ALT	ALP
Control	8.35 ± 0.63^{a}	10.32 ± 0.78^{a}	1.62 ± 0.12^{a}
Control + FGLE (100 mg/kg BW)	8.32 ±0.50^{a}	10.32 ± 0.29^{a}	1.60 ± 0.10^{a}
DMBA (25 mg/kg BW)	18.02 ± 1.37^{b}	19.43 ± 1.47^{b}	2.80 ± 0.21^{b}
DMBA + FGLE (100 mg/kg BW)	10.29 ± 0.78^{c}	12.68 ± 0.96^{c}	1.98 ± 0.15^{c}
DMBA + DOX (5 mg/kg BW)	8.52 ± 0.64^{a}	10.98 ± 0.83^{a}	1.71 ± 0.13^{a}

AST and ALT - µmol of pyruvate liberated/mg of microsomal protein/min
ALP - µmol of phenol liberated/mg of microsomal protein/min
Values are expressed as means ± S.D for eight rats in each group.
Values not sharing a common superscript differ significantly at $p \leq 0.05$ (DMRT).

3.7 Western blot analysis

TNF-α and NF-κB p65 protein expression in mammary tissues of control and experimental rats after treatment with FGLCE plant extract. The LI-COR chemiluminescence substrate was used to acquire a Figure 1A shows western blot analysis of TNF-α and NF-κB protein expression. Figure 1B shows the measurement of protein using Image-studio software and densitometric analysis (LI-COR, USA.). The densitometry results are presented as relative density of protein bands adjusted to -actin and represent means SD from three immunoblots. At P 0.05, values without a common superscript (a,b,c) differ considerably (DMRT).

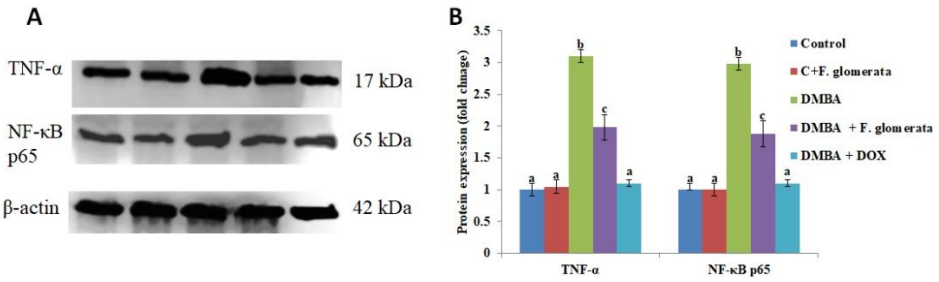

Figure 1. TNF-α and NF-κB p65 protein expression in mammary tissues of control and experimental rats after treatment with FGLCE plant extract

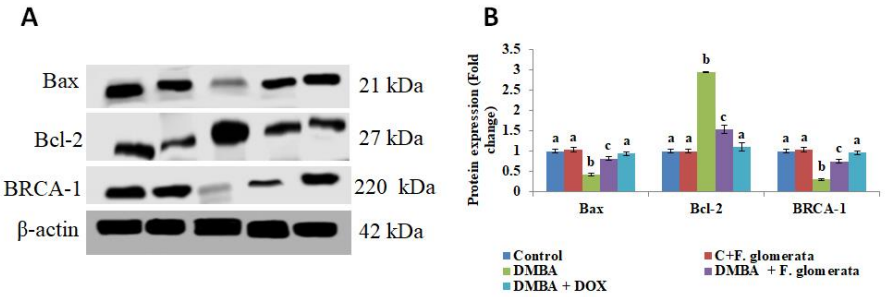

Figure 2. Effect of FGLCE plant extract on Bax, Bcl-2 and BRCA-1 protein expression of mammary tissues of control and experimental animals

Effect of FG plant extract on protein expression of Bax, Bcl-2, and BRCA-1 in mammary tissues of control and experimental rats. The LI-COR chemiluminescence substrate was used to acquire a Western blot analysis of Bax, Bcl-2, and BRCA-1 protein expression. Densitometric analysis with Image-studio software was used to quantify protein in Figure 2B. (LI-COR, USA.). The densitometry results are presented as relative density of protein bands adjusted to -actin and represent means ± SD from three immunoblots. At ($p < 0.05$), values without a common superscript (a, b, c) differ considerably (DMRT).

3.8 Immunohistochemical analysis

The immunohistochemistry examination of p53 and PCNA in mammary tissues of control and experimental rats is shown in Figures 3 and 4 (A-E). The protein expression of p53 and PCNA was significantly ($p < 0.05$) enhanced in Group I (A) control and Group II (B) FGLCE plant extract alone treated animals, however p53 expression was negative in Group II (C). DMBA-induced cancer-bearing rats in Group III (C) had positive p53 expression. The expression of p53 was moderately reduced in Group IV (D) DMBA + FGLCE plant extract treated rats. DMBA + Doxorubicin-treated animals in Group V (E) had p53 expression that was negative.While the protein expression of p53 and PCNA was considerably ($p<0.05$) reduced in group I (A) control and group II (B) FGLCE plant extract alone treated animals, PCNA expression was negative in group II (B) FGLCE plant extract alone treated animals. PCNA expression was high in DMBA-induced cancer-bearing rats in Group III (C). PCNA expression was considerably reduced in Group IV (D) DMBA + FGLCE plant extract treated rats. PCNA expression was negative in Group V (E) DMBA + Doxorubicin treated rats.

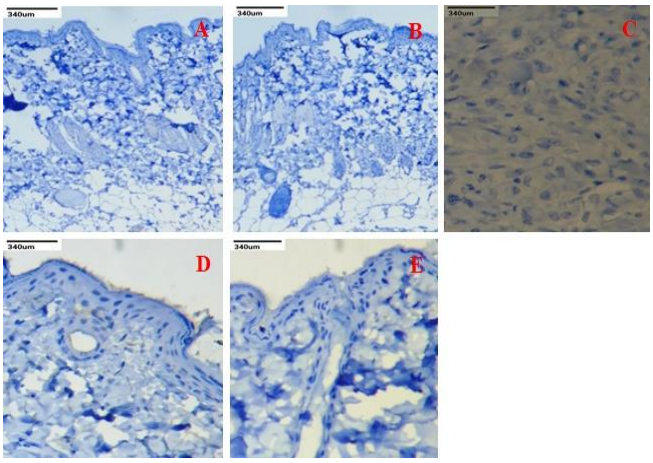

Figure 3. Effect of FGLCE plant extract on p53 expression of mammary tissues of control and experimental animals (Immunohistochemistry).

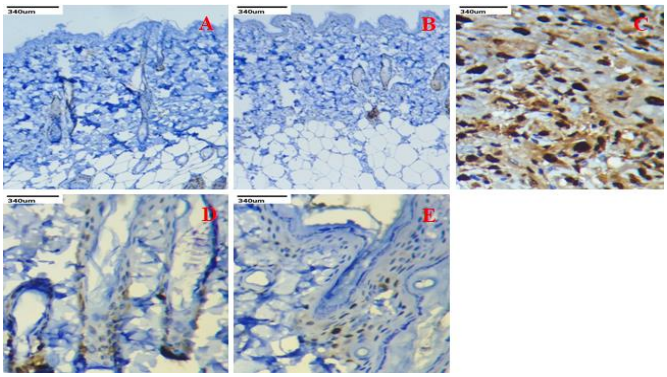

Figure 4. Effect of FGLCE plant extract on PCNA expression of mammary tissues of control and experimental animals (Immunohistochemistry).

3.9 Histological Analysis

The histological examination of AgNORs staining in mammary tissues of control and experimental animals is shown in Figure 5 a–f. The mammary tissues of the Group I (A) control and Group II (B) FGLCE plant extract alone treated mice had normal architecture. Infiltrating carcinoma in mammary tissue was seen in Group III (C) DMBA-induced cancer bearing mice, together with surrounding necrosis. Animals treated with DMBA + FGLCE plant extract in Group IV (D) had minimal tumour invasion. The mammary tissue architecture of Group V (E) DMBA + Doxorubicin treated rats was practically normal. The histological analysis of AgNORs staining in liver tissues of control and experimental rats is shown in Figure 6 a–f.The liver tissues of the Group I (A) control and Group II (B) FGLCE plant extract alone treated mice had normal architecture. Central venous congestion, sinusoidal congestion, and hepatocytes were seen in Group III (C) DMBA-induced cancer-bearing mice. Animals treated with DMBA + FGLCE plant extract in Group IV (D) had central venous congestion and moderate hepatocyte damage. The architecture of liver tissue in Group V (E) DMBA + Doxorubicin treated rats was nearly normal.

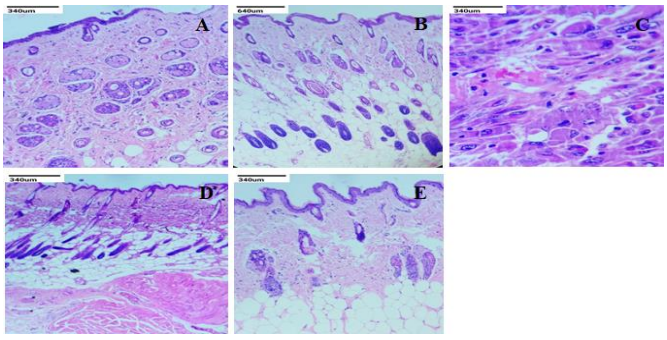

Figure 5. Effect of FGLCE plant extract on histopathological analysis of mammary tissues of control and experimental animals (hematoxylin and eosin staining).

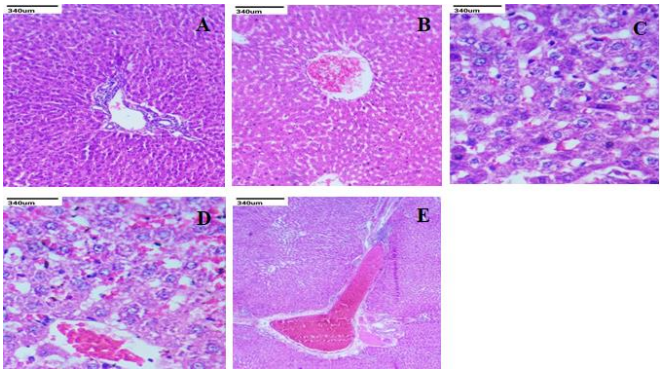

Figure 6. Effect of FGLCE plant extract on histopathological analysis of liver tissues of control and experimental animals (hematoxylin and eosin staining).

4. Conclusion

On the basis of these findings, it is possible to conclude that FGLCE treatment efficiently suppresses DMBA-induced breast cancer. To eliminate carcinogenic compounds, FGLCE relies on its anti-lipid peroxidative and antioxidant functions, as well as modulatory effects on phase I and II detoxification enzymes. Histopathological examinations of liver and mammary tissues back up the biochemical findings. To explain the probable mechanism of FGLCE in DMBA-induced mammary carcinogenesis, more research is needed at the molecular level.

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