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Baicalein Inhibits Migration of Breast Cancer Via Inflammatory and Apoptosis Cell Signaling Pathway

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Abstract

Background: Breast carcinoma is one of the leading carcinomas in women in developed countries. Breast carcinoma is manifestation of different biological structures with distinct physiological features and clinical consequences. Baicalein, a form of flavonoid, isolated from *Radical Scutellariae* exerts specific pharmacological properties along with antioxidant nature. The current study evaluated the anti-mammary effect of baicalein on 7, 12-Dimethylbenz (α) anthracene (DMBA) induced breast cancer and explore the possible mechanism.

Methods: A single dose of DMBA (80 mg/kg) was injected in the mammary gland in the female rats to induce tumor. The female rats were divided into five groups- Group I- Normal control group; Group II- DMBA control (80mg/kg of BW); Group III- DMBA treated rats received baicalein 5 mg/kg of BW; Group IV- DMBA treated rats received baicalein 10 mg/kg of BW; Group V- DMBA-treated rats received baicalein 20 mg/kg of BW. At the end of experiment, tumor burden, incidence and volume were studied. Together, the markers of mammary tumors such as carcinoembryonic antigen (CEA), Lipid-bound Sialic Acid (LSA), Alpha-fetoprotein (AFP), Serum Total Sialic Acid (TSA), and Cancer Antigen 15–3 (CA 15–3) were estimated. Also, the pro-inflammatory cytokine, inflammatory mediators and apoptosis proteins were also studied.

Results: Compared to the DMBA control group, dose-dependent treatment with baicalein ($P<0.001$) significantly decreased the levels of AFP, CEA, LSA, TSA, and CA 15–3. Baicalein significantly ($P<0.001$) down-regulated the hepatic parameters such as alanine transaminase (ALT), aspartate transaminase (AST) , pro-inflammatory cytokines viz., interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6); inflammatory mediators such as nuclear factor kappa-B (NF-kB). Compared to DMBA control group rats, Baicalein significantly ($P<0.001$) reduced Bcl-2 expression and increased Bax and caspase-3 expression.**Conclusion:** Altogether, the above finding shows that the preventive effect of baicalin on DMBA induced mammary gland in Wistar rats.

Keywords: Breast cancer; DMBA; Baicalein; Inflammation; Apoptosis

Introduction

From the decades, Cancer is one of the biggest challenges globally. In 2015, 15.2 million cases of cancer reported and 8.9 millions cancer patients' deaths reported [1,2]. Among the women, breast, colorectal, bronchus, and lung cancers are commonly diagnosed. According to a previous study in 2016, 29% of new cancer cases diagnosed in women [3]. The cases of breast cancer were mostly reported in China, followed by the United States. Breast cancer is the most common cancer diagnosed worldwide and the leading cause of cancer death among women. [4]. In developed countries, breast cancer is commonly caused by cancer death compared to the less developed countries [3].

Mutations, age and exposure to oestrogen in tumor suppressor genes such as BRCA1 and 2), environmental toxins (polycyclic aromatic hydrocarbons) and obesity are common risk factors for breast cancer. [5]. The most frequently used drugs for treating breast cancer are tamoxifen (hormonal therapy) and chemotherapy drugs including paclitaxel (anti-microtubule) and doxorubicin (cytotoxic antibiotic), both the treatment widely used for metastatic breast cancer and estrogen dependent breast cancer [6,7]. Moreover, these treatment possess the limitation

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due to serious side effects such as endometrial cancer with thromboembolic, cardiotoxicity and many more serious side effects [8]. Due to the limitations of above discuss treatment, urgent need to develop the novel chemo-protective agent to treat breast cancer with more efficacy and safety to recover breast cancer as well as decrease the pain to the patient with low cost [9,10].

Carcinogenesis is a multiple process comprising initiation, expansion and progression with various epigenetic and genetic events [11]. Environmental factors (chemical or biological origin) may function as carcinogenesis initiators, promoters, or both. DMBA is a commonly used carcinogen to induce breast cancer [11,12]. DMBA is commonly absorbed via the respiratory, intestinal and skin tracts and through intraperitoneal and intravenous injection, inhalation and ingestion. DMBA commonly acts either by promoting or initiating mutations in the carcinogenesis responsible genes [11–13]. DMBA activates the aryl hydrocarbon receptor (AhR) of the cellular cytosolic receptor, which translocates AhR into the nucleus and interacts with the AhR proteins for nuclear translocation. AhR-dependent upregulation of cytochrome enzymes P450 (CYP1A1 and CYP1B1) that metabolize DMBA into a mutagenic intermediate epoxy that forms adducts of DNA is a result. These DNA adducts lead to carcinogenesis associated with polycyclic aromatic hydrocarbons (PAH) caused by mutation and malignant transformation. [11,14]. PAH is clearly implicated in the process of carcinogenesis, especially DMBA, the most potent breast and skin carcinogenesis.

DMBA is commonly used to induce breast cancer in animal experiments [15]. After metabolic activation in breast tissue, the carcinogen metabolites interact with the proliferative cells in the terminal of end buds, subsequent mutations and forming DNA adducts, which further turn and contribute to their transformation to malignant cells [16]. Previous published research suggests that the DMBA is lipophilic in nature and breast tissue contains the considerable quantity of adipose tissue, in which the DMBA can concentrate on the epithelium contact before the activation of metabolic and this property certainly correlates with the DMBA activity at the mammary level [15].

Reports from the World Health Organization (WHO) indicate that 80% of developing countries use traditional medicine for their primary health care. [17]. Previous published studies have suggested that various phyto-constituents obtained from traditional medicine have several pharmacological activities [17,17]. Phyto-constituents obtained from the plants are not only required for normal functioning of the body, but also having the beneficial and protective effect on health or also contribute an important role in ameliorating the various diseases [18–20]. Phyto-constituents obtained from traditional plants having the numerous health benefits such as anti-inflammatory, anti-cancer, antimicrobial, anti-hypertensive, and anti-diabeticetc.. Baicalein is the phyto-constituent of flavonoids family and derived from various plants used as traditional Chinese Medicine [21]. Due to the excellent free radical scavenging activity, its attenuate the oxidative stress in neuronal cells and cardiomyocytes. Baicalein showed anti-inflammatory activity and use against various diseases such as diabetes, inflammation, caridiopathy, atherosclerosis and many more [22,23]. Due to the potential anti-inflammatory effect of Baicalein, we are tried to explore the anticancer effect of baicalein on the DMBA induced breast cancer and probable pathways involved.

Material and methods

Chemical

Baicalein (98%) and DMBA were purchased from Sigma Aldrich (St. Louis, MO, USA). Pro-inflammatory cytokines kits were procured from Invitrogen (Invitrogen Corporation, Frederick, USA). Rest of the chemicals and solvents used in the current experimental study were analytical grade and purchased from commercial sources.

Experimental animal

For the existing experimental protocol, Wistar rats (130–160 g) were used. The rats received and maintained the normal laboratory environment from the animal house (temperature 21 ± 2°C; 70% relative humidity and final 12 h per day and night light cycle). Both rats were acclimatized after being procured from the animal house for 10 days. With the regular pellet diet and water ad libitum, the rats were fed.

Breast cancer induction

A single intraperitoneal DMBA injection (80 mg/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 [24].

Experimental protocol

Following successful breast cancer induction, all rats were divided into the following groups: normal control: group I; DMBA control received DMBA (80

mg/kg): group II; DMBA control received baicalein 5 mg/kg: group III; DMBA control received baicalein 10 mg/kg: group IV and DMBA control received baicalein 20 mg/kg: group V. Both group rats were treated for up to 140 days.

All group rats were estimated at daily time intervals for food intake, water intake and body weight. All the group rats were anaesthetized at the end of the experimental procedure to collect the blood sample. For further study, blood samples collected were centrifuged at 10,000 rpm for 15 min at 4°C and the serum collected was stored at −80°C.

Tumorgenicity marker

Breast tumor markers such as carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP) and Cancer Antigen 15–3 (CA 15–3) were estimated using the enzyme immunoassay kit UBI Magiwell (USA) according to the manufacture instructions. Lipid bound Sialic Acid (LSA) and total Sialic Acid (TSA) were calculated with minor modifications using the previous published method. [25].

Lipid parameters

Lipid parameters such as total cholesterol (TC), high-density lipoprotein (HDL), total cholesterol (TC) and triglyceride (TG) were estimated using the standard Fortress Diagnostics Restricted manufacturing instruction kits. (Muckamore, United Kingdom).

Very low-density lipoprotein (VLDL) and Low-density lipoprotein (LDL) were estimated by the Friedewald et al. formula [26].

Hepatic parameters

Hepatic parameters such as alanine transaminase (ALT) and aspartate transaminase (AST) were estimated using the reagent kits following the manufacture instruction Fortress Diagnostics Limited (Muckamore, United Kingdom).

Reverse transcription polymerase chain reaction (RT-PCR)

A quick RNA mini Prep kit was used for extracting total RNA from the tumor tissue sample according to manufacture instruction. RT-PCR was used for estimating the apoptotic and anti-apoptotic gene estimation using the cDNA verso kit with a temperature scale of 42°C for 30 min, 32 cycles of 94°C for the next 30 sec, 56°C for the next 30 sec and finally 72°C for next 30 sec. Table 1 shows the primers used for estimating genes.

Pro-inflammatory cytokines and inflammatory mediators

Pro-inflammatory cytokines include IL-1β, TNF-α, IL-6 and inflammatory mediators such as NF-kB estimated using standard kits (Invitrogen Corporation, USA) according to manufacture instruction.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 (San Diego, CA, USA) software. All the findings have been presented as mean±S.D. The comparisons between groups were performed using one-way variance analysis (ANOVA) followed by multiple comparison tests from the Tukey. The $p<0.05$ value is considered important statistically.

Results

Tumor weight

The effect of baicalein on DMBA-induced breast cancer in Wistar rats is shown in Table 1. There was no evidence of a tumor in the normal control group of rats. Rats treated with DMBA showed an average tumor burden of 90 g. Baicalein (20 mg/kg) given in DMBA treated rats showed that the 2 rats having breast tumor with 1.2 g tumor burden. 91.6% tumor incidence was observed in the DMBA treated rats show average tumor 24.5±4.91 g. Baicalein treatment at a dose of 5, 10 and 20 mg/kg showed 80, 55.45 and 20% tumor incidence, respectively. Baicalein treatment showed 28, 72, and 99% inhibition with decrease the average tumor size 12.8±3.56 g, 4.1±1.34 g and 0.27±0.04 g at doses of 5, 10 and 20 mg/kg, respectively.

Body weight

The effect of baicalein on normal and DMBA induced community rats is demonstrated in Table 2. Up-regulation in the body weight of rats up to the end of the experimental study was demonstrated by standard control group rats. The increased body weight relative to the initial body weight was seen in DMBA treated rats. The reduced body weight was seen compared with the normal and other treated rats in the group. The group of rats treated with Baicalein displayed increased body weight at dose-dependent levels. Baicalein (20 mg/kg) increased body weight and nearly achieved normal levels of body weight.

Tumorigenicity marker

Tumorgenicity markers such as AFP, CEA and CA 15–3 showed in figure 1. DMBA-treated rats showed a reduced level of tumorigenicity markers such as AFP, CEA and CA 15–3 as the comparison to the normal group. Baicalein treatment significantly

(P<0.001) reduced the level of AFP (figure 1a), CEA (figure 1b) and CA 15–3 (figure 1c) at dose-dependently. Baicalein (20 mg/kg) treatment showed the potential effect of all dose in reducing the level of tumorigenicity marker.

Hepatic parameters

Compared to normal group rats, DMBA treated rats showed a higher degree of hepatic parameters. Baicalein treatment substantially (P<0.001) decreased the dose-dependent levels of AST (Figure 2a) and ALT (Figure 2b).

Lipid parameters

Lipid parameters such as TC, TG, LDL, VLDL and HDL presented in figure 3. DMBA-treated rats displayed increased levels of TC, TG, LDL, VLDL and reduced the level of HDL levels compared to normal rats. Baicalein treatment significantly decreased the level of total cholesterol, triglyceride, low-density protein, low-density protein and increased the level of high-density lipoprotein (figure 3).

LSA and TSA

In the DMBA treated rats, the level of LSA and TSA increased in breast cancer and similar findings were observed. In comparison with DMBA-induced breast cancer rats, dose-dependent treatment with baicalein substantially (P<0.001) reduced the amount of LSA and TSA (Figure 4).

Apoptosis markers

Apoptosis markers such as Bax, caspase-3 and Bcl-2 altered during breast cancer. DMBA induced group rats showed decreased expression of Bax, Caspase-3 and increased expression of Bcl-2 and dose-dependently treatment of baicalein significantly (P<0.001) up-regulated the level of caspase-3, Bax and down-regulated the level of Bcl-2 (figure 5).

Cytokines and inflammatory mediators

In breast cancer, cytokines and inflammatory mediators significantly increased and similar findings were observed in rats treated with DMBA. The DMBA-induced breast cancer group of rats reported a substantial decrease in dose-dependent levels of TNF-alpha, IL-1β, IL-6 and inflammatory mediator NF-kB after treatment with baicalein (P<0.001) (Figure 6).

Discussion

7,12 dimethylbenz (a) anthracene (DMBA) has been widely used to cause breast cancer in rodents. [16]. DMBA is a polycyclic aromatic hydrocarbon that is also a widely used occupational carcinogen to cause breast cancer. [1]. A single intraperitoneal injection of DMBA (80 mg/kg) was used for breast cancer induction in the current experimental procedure. Large tumors were found in experimental animals treated with a large dose of DMBA after 20 weeks of the experimental trial, while normal rats received saline only and showed no signs of breast cancer. [3].

Biochemical parameters such as AFP, CEA and CA 15–3 play an essential role to identify the cancerous cells [27]. In addition to treatment, these criteria were regularly tested to test the cancerous condition for detection, prognosis and evaluation of development [27]. Tumor markers such as AFP are the major plasma proteins produced via liver and yolk sac during fetal expansion. It is assumed to be the fetal form of albumin serum [28,29]. AFP is often used as a biomarker for identifying tumor subgroups [27]. CA 15–3 isolated from the Mucin-1 gene is used as a breast cancer tumor marker. The reaction to breast cancer care and disease recurrence is used as a monitor [30]. CEA are glycoproteins of Glycosylphosphatidyl Inositol (GPI) that act as functional carcinoma ligands of E-selectin and L-selectin that are involved in metastasis and adhesion of cancer cells [30]. During fetal expansion, CEA production starts in the gastrointestinal tissue, but before birth, the production of CEA ceases [31]. Furthermore, boosted CEA level commonly occurs in the serum in the expansion of various types of cancers, which shows that it's a prominent tumor marker [31,32]. In breast cancer, the level of all tumor markers increased and similar findings were observed in rats treated with DMBA and the group of rats treated with baicalein demonstrated a decrease in the level of tumour markers indicating a chemo protective effect against breast cancer.

The end moiety of Sialic acid is the carbohydrate chain and plays a significant role in the functioning of glycoconjugates and mostly present in the gangliosides and glycoproteins and the level boosted during cancer [33]. During the cancer, these glycoconjugates start secretion into the circulation via enhanced turnover or defend the malignant cells. Sialic acid is observed either as lipid bound sialic acid (LSA) or total sialic acid (TSA) in glycolipids and glycoproteins. The level of sialic acid quickly boosted during the breast cancer [34]. A similar momentum was observed in the treated rats and baicalein treatment down-regulated the level of TSA or LSA and suggesting the anticancer effect on the breast cancer.

Inflammatory pathways play a key role in tumor

activation, promotion, angiogenesis and metastasis [28,35]. Pro-inflammatory cytokines include IL-1β, TNF-α, IL-6 and inflammatory mediators such as NF-kB is the major marker associated with cancer axis inflammation [36,37]. An essential feature in treating chemical-induced breast cancer is the reduction of cytokines and inflammatory mediators. [38]. Baicalein treatment significantly (P<0.001) reduced the level of TNF-α, which was observed in high amount during the DMBA induced breast cancer. Higher IL-1β levels are correlated with breast cancer aggressiveness and invasiveness, and higher tumor grades. It is well proved that NF-kB is complex pro-inflammatory proteins molecules that manage the gene involved in the invasion, apoptotic process, cell proliferation and inflammation [39]. NF-kB is a nuclear transcription factors that activated via cytokines such as TNF-α, IL-1β and IL6 [5]. Previous published research showed that the cytokines are the intracellular short acting mediators involve in cancer pathogenesis [5,11]. IL-6 is the putative NF-kB attachment [39]. Other pro-inflammatory cytokines, such as IL-1β, play a key role in breast cancer development by controlling the function of immune cells and tissue in the micro-environment of the tumour. During DMBA-induced breast cancer, up-regulated levels of pro-inflammatory cytokines, including TNF-a, IL-1β and IL-6, play an important role in the differentiation and proliferation of tumor cells, apoptosis, malignant development, invasion, migration of cells and attachment to metastases.

Tumor progression, growth, metastasis and maintenance are assumed to be typically arbitrated by shifts in apoptosis-related proteins [40]. Dysregulation of apoptosis is well established as the primary reason for breast cancer development, and 2 main pathways lead to the apoptosis cycle such as the death receptor adjudicated extrinsic apoptosis pathway and the intrinsic adjudicated mitochondrial pathway [41]. Apoptosis family protein like Bcl-2, also known as antiapoptotic proteins, functions by neutralizing the pro-apoptotic proteins like Bax protein. Bax play a significant role in defending the tumor cells to undergo apoptosis[41,42]. Bax protein is present in the cytosol and is transferred to the mitochondria to induce apoptosis, however Bax, like Bcl-2 and Bcl-xl, also functions as anti-apoptotic proteins. The mitochondrial system involving the protein pro-anti-apoptotic portion, was regulated by Bcl-2. Bax and Poor translocate to the outer mitochondrial membrane during the apoptosis process, improving the secretion of Cytochrome C [43]. Various cells removed during the apoptosis through boosted the Bcl-2 and Bcl-xl expression. DMBA induced breast cancer rats exhibited reduced expression of Bax, caspase-3 and increased the expression of Bcl-2 [43]. Baicalein significantly (P<0.001) down-regulated Bcl-2 expression and up-regulated Bax and caspase-3 expression, implying a protective effect against breast cancer.

Conclusion

Baicalein significantly reduced the tumor weight, tumor incidence and tumor burden. Baicalein significantly (P<0.001) increased the body weight and reduced Tumorgenicity marker, sailic acid, hepatic parameters and altered the lipid parameters. Baicalein significantly (P<0.001) increased the level of caspase-3, Bax and reduced the level of Bcl-2. Baicalein significantly (P<0.001) decreased thecytokines and inflammatory mediators. Based on the result, we may infer that baicalein is an effective chemotherapeutic agent for breast cancer, and therefore needs further molecular research, especially clinical study, to further assess this effect.

Abbreviation

DMBA=7, 12-Dimethylbenz (α) anthracene
CEA=Carcinoembryonic Antigen
LSA=Lipid-bound Sialic Acid
AFP=Alpha-fetoprotein
TSA=Total Sialic Acid
CA 15-3=Cancer Antigen 15-3
TNF-α=Tumor necrosis factor-α
IL-1β=Interleukin-1β
IL-6=Interleukin-6
NF-kB=Nuclear factor kappa-B
PBS=Phosphate buffer saline
AhR=Aryl hydrocarbon receptor
CYP1A1=Cytochrome enzymes P450
PAH=Polycyclic aromatic hydrocarbons
WHO=World Health Organization
TC=Total cholesterol
HDL=High density lipoprotein
TG=Triglyceride
LDL=Low density lipoprotein
VLDL=Very low density lipoprotein
AST=Aspartate transaminase
ALT=Alanine transaminase
RT-PCR=Reverse transcription polymerase chain reaction

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Figures

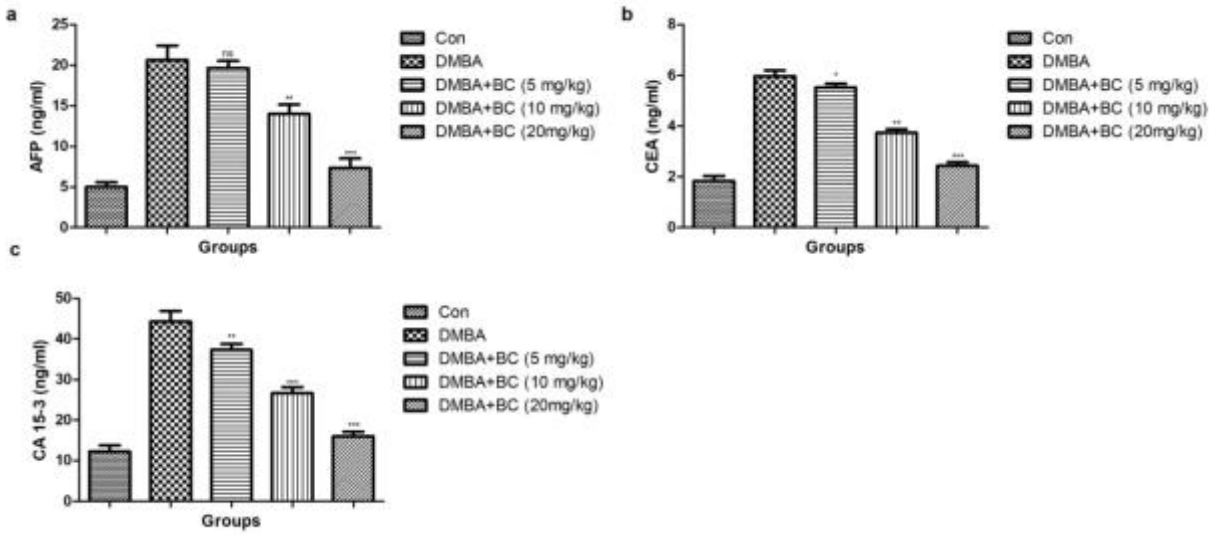

Figure 1. **exhibited the effect of baicalein on the Tumorgenicity marker against the DMBA induced breast cancer. . *p < 0.05, **p < 0.01, ***p < 0.001. #p < 0.05 shows the difference between the DMBA control and DMBA induced breast cancer treated group.**

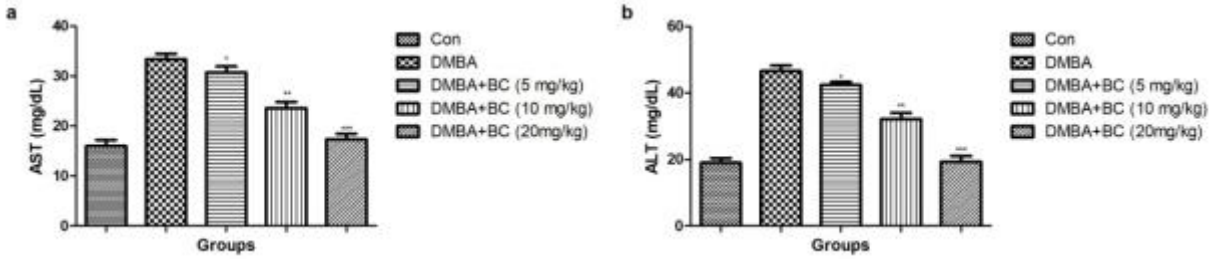

Figure 2. **exhibited the effect of baicalein on the hepatic parameter against the DMBA induced breast cancer. . *p < 0.05, **p < 0.01, ***p < 0.001. #p < 0.05 shows the difference between the DMBA control and DMBA induced breast cancer treated group.**

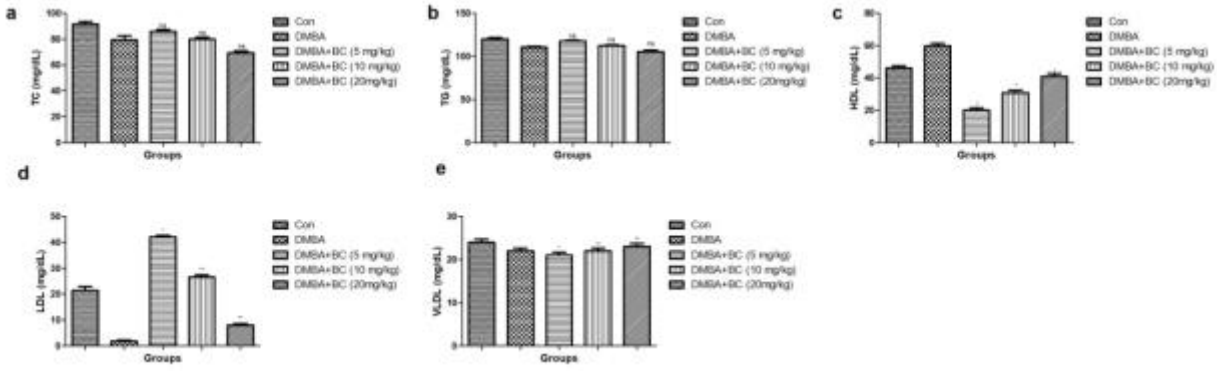

Figure 3. **exhibited the effect of baicalein on the lipid parameters against the DMBA induced breast cancer. . *p < 0.05, **p < 0.01, ***p < 0.001. #p < 0.05 shows the difference between the DMBA control and DMBA induced breast cancer treated group.**

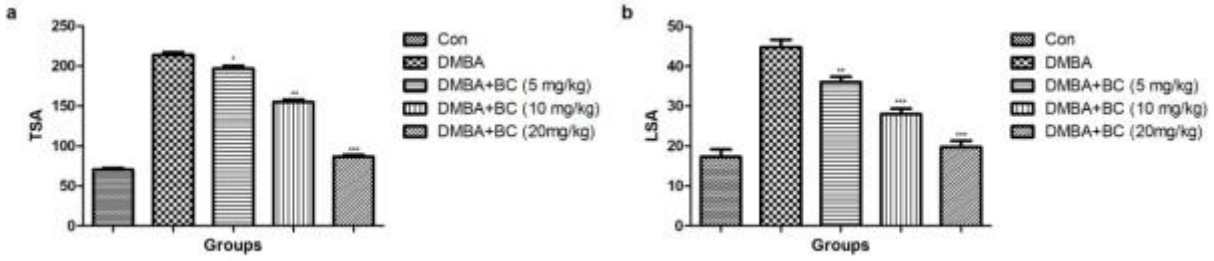

Figure 4. **exhibited the effect of baicalein on the sailic acid against the DMBA induced breast cancer. . *p < 0.05, **p < 0.01, ***p < 0.001. #p < 0.05 shows the difference between the DMBA control and DMBA induced breast cancer treated group.**

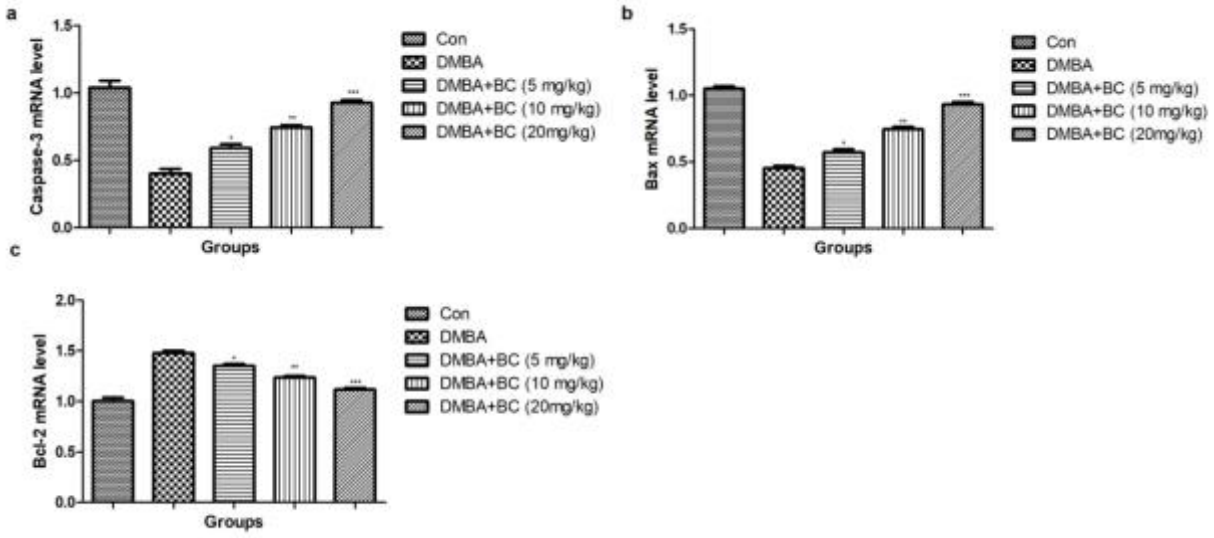

Figure 5. **exhibited the effect of baicalein on the apoptosis marker against the DMBA induced breast cancer. . *p < 0.05, **p < 0.01, ***p < 0.001. #p < 0.05 shows the difference between the DMBA control and DMBA induced breast cancer treated group.**

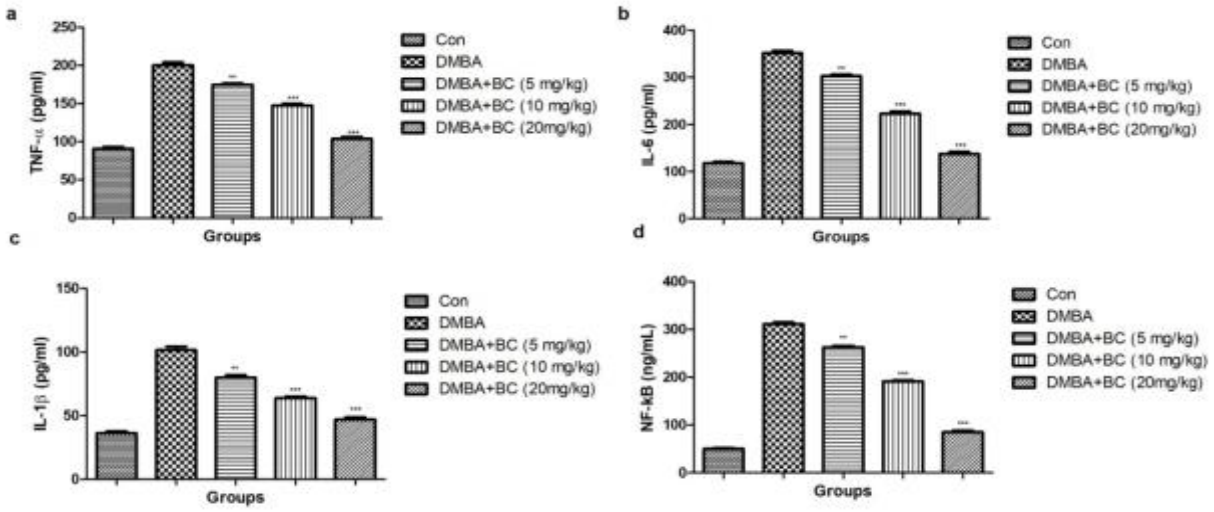

Figure 6. **exhibited the effect of baicalein on the cytokines and inflammatory against the DMBA induced breast cancer. *p < 0.05, **p < 0.01, ***p < 0.001. #p < 0.05 shows the difference between the DMBA control and DMBA induced breast cancer treated group.**

Tables

Table 1. **List of primers**

S. No	Gene	Primers	
		Forwarded	Reverse
1	Bcl-2	CCCCAGAAGAAACTGAACC	GCATCTCC TTGTCTACGC
2	BAX	GTTGCCCTCTTCTACTTTGC	ATGGTCACTGTCTGCCATG
3	Caspase-3	CTGGACTGCGG TATTGAGAC	CCGGGTGCGGTAGAGTAAGC
4	GAPDH	TCAAGAAGGTGGTGAAGCAG	AGGTGGAAGAATGGGAGTTG

Table 2. **showed the effect of baicalein on DMBA induced breast cancer in rats.**

S. NO	Group	Total rats/No of rats with tumors	Tumor burden (g)	Tumor incidence (%)	Inhibition	Average Tumor (g)	Inhibition (%)
1	**DMBA**	12/11	90.4	91.66	-	24.5±4.91	-
2	**DMBA+BC (5 mg/kg)**	10/8	65.5	80	28	12.8±3.56	48
3	**DMBA+BC (10 mg/kg)**	11/6	25.2	55.45	72	4.1±1.34	83
4	**DMBA+BC (20 mg/kg)**	10/2	1.2	20	99	0.27±0.04	99

Table 3. **illustrated the effect of baicalein on the body weight (g) of DMBA induced mammary gland in rats**

S. NO	Group	Days					
		1	**28**	**56**	**84**	**112**	**140**
1	**Control**	150.34±6.54	178.6±4.43	200.4±3.48	228.4±4.39	264±3.36	289±3.94
2	**DMBA**	155.1±5.83	163±5.87	171±6.98	182±6.53	198±6.98	208±5.38
3	**DMBA+BC (5 mg/kg)**	153±6.34^{ns}	169±5.13*	181±4.32*	197±4.34**	214±5.39***	237±3.84***
4	**DMBA+BC (10 mg/kg)**	158.3±5.43^{ns}	171±5.04**	188±4.12**	207±4.11***	222±4.45***	251±4.35***
5	**DMBA+BC (20 mg/kg)**	150.2±4.35^{ns}	165±4.78^{ns}	189±3.98***	216±3.56***	248±3.95***	276.6±3.84***

. *p < 0.05, **p < 0.01, ***p < 0.001. #p < 0.05 shows the difference between the DMBA control and DMBA induced breast cancer treated group.