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PHARMACOLOGICAL EVALUATION OF TUMOUR INCIDENCE, APOPTOSIS AND HISTOPATHOLOGICAL INTERPRETATION OF NEOPLASTIC LESIONS WITH DAIDZEIN A PHYTOESTROGEN IN DMBA INDUCED MAMMARY TUMOURS

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ABSTRACT

Phytoestrogens as potential sources of chemo prevention in mammary carcinogenesis were proved in our earlier studies. Daidzein, a phytoestrogen was used to explore its anti - cancer potential using Wistar rats models and to evaluate its apoptotic activity in mammary carcinogenesis induced by 7, 12-dimethylbenz (a) anthracene (DMBA). This chemical carcinogen was administered (25mg/rat/s.c) in divided doses to induce mammary cancers in female wistar rats. Daidzein 50mg/kg; 100mg/kg; Tamoxifen as reference standard (40mg/kg) was administered, from the first day of the study. The growth of tumors was monitored. The tumour incidence, tumour volume, histopathological analysis and macroscopic changes showed that neoplastic lesions in the drug treated groups was comparatively reduced and their mortality lesser than the DMBA treated groups, at the end of the study.

KEYWORDS: Chemoprevention, Daidzein, DMBA, histopathological analysis, macroscopic changes, mammary carcinogenesis, phytoestrogens, Tamoxifen, tumour incidence and tumour volume.

INTRODUCTION

Mammary carcinogenesis, globally and in the western world in particular is a vulnerable cause of death worldwide having a tendency to metastasize if not treated promptly.[1] Phytoestrogens are xenoestrogens of plant origin having estrogen like activity which may act as weak modulators of estrogen receptors in humans. Lignans, Coumestans and Isoflavones are listed as the three classes of Phytoestrogens.[2] Isoflavones are a subgroup of flavonoids. Among commonly consumed foods, isoflavones are found in dietary-relevant amounts only in the soybean. The two primary isoflavones in soybeans are daidzein and genistein and their respective glucosides genistin and daidzin. The isoflavone Daidzein[3] was abundantly present in soybeans and soy proteins of leguminous plants. Daidzein was documented to possess weak estrogenic effects in rats breast cancers in our earlier studies and as also quoted in some literatures.[4] There exists a major degree of ambiguity in the effect of these isoflavones in the prevention/treatment of breast cancers.

The process of carcinogenesis occurs due to the generation of reactive oxygen species/free radicals which alters the redox balance, causing oxidative stress resulting in damage to regular cells. 7, 12 (DMBA Dimethylbenz [a] anthracene was used to induce chemical carcinogenesis, with a cascade of events leading to Oxidative stress.[5] Using rat models of chemically induced tumourigenesis, the effect of the tumour incidence and increase in tumour latency in young adult females was compared with Tamoxifen as the conventional anti-tumour agent.

Since the result of several epidemiologic studies of this association are highly variable, experimental evidence suggestive of estrogenic activity of Soy isoflavones could also be potentially risk enhancing. However, the dose of the compounds the duration and route of administration and the time of exposure have posed numerous unanswered questions. Thus the present study was designed to evaluate the tumour incidence, tumour volume, histopathological analysis and macroscopic changes of Daidzein in DMBA induced mammary carcinogenesis in comparison with Tamoxifen[6], a SERM used as an adjuvant in most types of breast cancer.

MATERIALS AND METHODS

Wistar albino Female rats 7 - 8 weeks old, 150 – 180 gms were used for the study. The animals were obtained from Central Animal House, RMIHS, (Reg no. 160/1999/CPCSEA) Annamalai University, India & approved the experimental design. The rats were housed in

polypropylene cages at room temp, (27 ± 2°C) with relative humidity 55 ± 5%, in an experimental room. The LD cycle of about 12: 12 hr was maintained. Animals were maintained as per the principles and guidelines of the IAEC in accordance with the laws of animal care and use. Animals were also provided standard pellet feed and water *ad libitum*.

DMBA and biochemical's such as reduced glutathione were obtained from Sigma – Aldrich Chemicals, Pvt, Ltd, Bangalore, India. DMSO from Sun Pharma, India. Heparin, Thiobarbituric acid (TBA) and related chemicals were from Hi – Media Laboratories. Daidzein from Ascent Scientific Ltd UK, Tamoxifen from Mankind Pharma. All other chemicals used were of Analar grade.

INDUCTION OF MAMMARY CANCER

Mammary carcinogenesis was induced in female Wistar rats by *subcutaneous* injection of 25mg/rat of DMBA, in two divided doses in the 4^{th} week & 5^{th} week. The drug was formulated as emulsion in sunflower oil (0.75ml) and physiological saline (0.25ml) to each rat in groups II toV.[7]

Experimental design

36 female Albino Wistar rats were divided into 5 groups of six rats each. Group I – Normal controls with 2% DMSO as vehicle, *po;* Group II – DMBA (25mg/rat/S.C in 2 divided doses) at 4^{th} & 5^{th} week; Group III– Tamoxifen (40mg/kg, po) + DMBA; Group IV–Daidzein – (50mg/Kg/, po) + DMBA and; Group V – Daidzein – (100mg/kg, p.o) + DMBA. The drugs were dissolved in 2% DMSO and administered from the 1^{st} day till the 4^{th} week before the exposure to the carcinogen and continued throughout the experimental period with a wash out period of a fortnight. The experiment was terminated at the 16^{th} week and evaluated for chemo preventive effect. The rats were sacrificed by cervical dislocation at the completion of study.

TUMOUR INCIDENCE, MULTIPLICITY AND VOLUME

Palpable tumors or any abnormal masses were counted, removed, weighed and cut into smaller sections.[8] This was fixed in 10% formalin for histomorphology and analysis of Bcl-2 and Bax expression by Immunohistochemical studies. For each group, occurrence of mammary lesions was determined according to the different types of volume using the formula.

V= n(4/3)π(D1/2)(D2/2)(D3/2)

D1, D2, D3 are the three diameters (cm) of the tumour; The number in parentheses indicates total number of rats bearing tumours.

$$Oc(\%) = \frac{\text{No of Tumours}}{\text{No of total mammary tumour found in eachgroup at the end of the experiment}}$$

IMMUNO HISTOCHEMISTRY

Mammary glands were fixed overnight in 10% neutral-buffered formalin, dehydrated with a series of descending ethanol concentration and embedded in paraffin. Endogenous peroxides were blocked by incubation with 3% H_2O_2 and washed. Sections of the tissues were then incubated with rabbit serum for 10min at ambient temperature Subsequently, the sections were incubated overnight with a mouse/goat polyclonal anti-Bax antibody and anti-Bcl-2 anti-body at 4^0C, followed by the addition of biotynylated rabbit anti-mouse/goat Ig G secondary anti-body. To verify the binding specificity for Bax & Bcl-2, some sections, were also incubated with primary or the secondary anti –body. Immunohistochemical[9] staining was processed in accordance with the manufacturer's instructions and visualized by use of diaminobenzidine (DAB) staining. Samples were observed in four randomly selected optical fields under microscopy (x200).

HISTOPATHOLOGICAL STUDY

The breast tissue was sliced into 5mm pieces and fixed in neutral formalin (10%) solution for 3 days. Tissue was washed in running water followed by dehydration with alcohol of increasing strength for 12 hrs. Final dehydration was carried out using absolute alcohol with about three changes at 12 min intervals followed by cleaning using xylin with changes at 15-20 min intervals. The pieces were subjected to paraffin infiltration in automatic tissue processing unit. After removal of excess formalin by repeated washing, tissues were embedded in paraffin.[10] The blocks were cut using a microtone to thickness of 5 microns, fixed on glass slides using albumin and stained with Hematoxylin and Eosin.

STATISTICAL ANALYSIS

The values are expressed as Mean ± SD Statistical comparisons were performed by analysis of Variance (ANOVA), followed by Duncan's Multiple range test and Bonferroni's test

(using SPSS version12.0 for Window) P-Values ($P < 0.05$) was considered to be statistically significant.

RESULTS

Table 1: Tumour Incidence and Tumour Volume.

Sl.no	Parameters	Control	DMBA	DMBA + Tamoxifen	DMBA + Daidzein 50mg/kg	DMBA + Daidzein 100mg/kg
1	Tumour incidence	0	100%	20%	6%	33%
2	Number of tumours	0	(6)/6	(1)/6	(4)/6	(2)/6
3	Tumour Volume	0	16.2±1.3	0.65±0.15	3.01±r0.25	0.84±0.25

Values are expressed as Mean ± SD for 6 animals in each group (n=6), the number in parentheses indicates total number of rats bearing tumors. *P<0.05 as compared to control, DMBA. Values that are not sharing a common superscript letter in the same row differ significantly at p<0.05 (DMRT).

Table 2: IMMUNOHISTOCHEMICAL MARKERS OF APOPTOSIS – Bax and Bcl-2

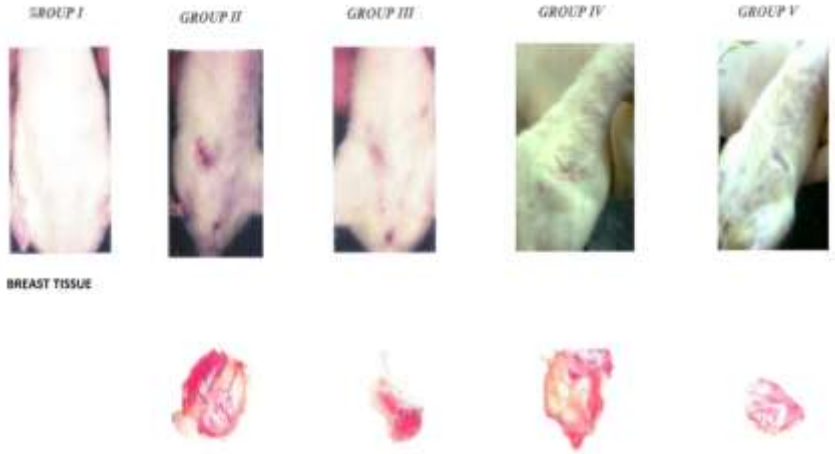
GROUPS	BAX	Bcl-2
GROUP- I	3+,4+,4+	0,0,1+
GROUP-II	0,1+,1+	4+,4+,4+
GROUP-III	3+,2+,3+	0,1+,2+
GROUP-IV	1+,2+,3+	2+,3+,4+
GROUP-V	2+,3+,3+	0,1+,2+

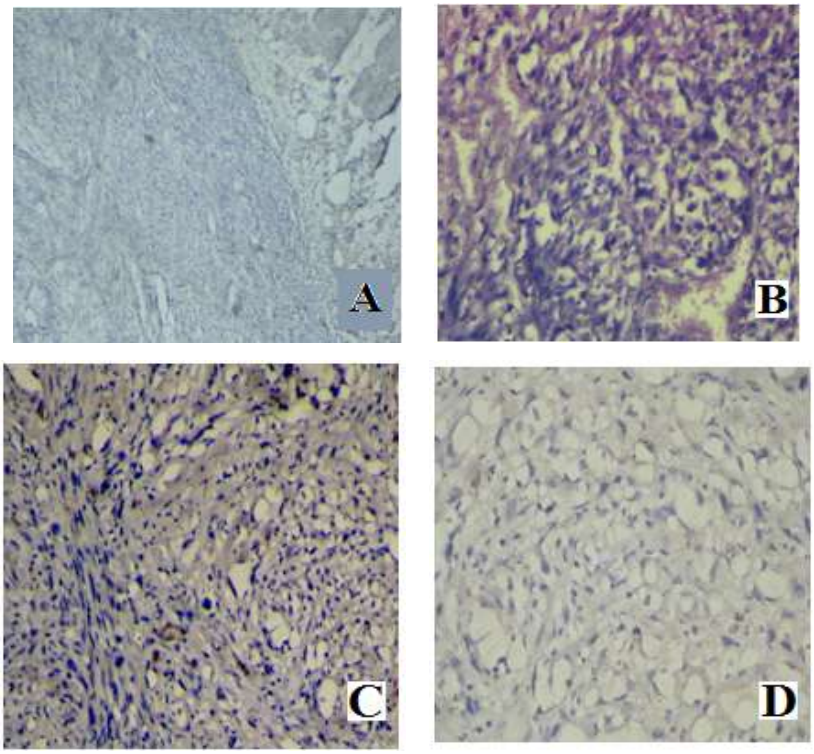
The slides were counterstained with hematoxylin for identification of tissue architecture. The level of expression of both proteins was evaluated by using a semi quantitative scale (0, lack of expression; 1+, weak expression; 2+, medium expression; 3+, strong expression; and 41+, very strong expression) as recommended by Krajewski *et al.*,1994.

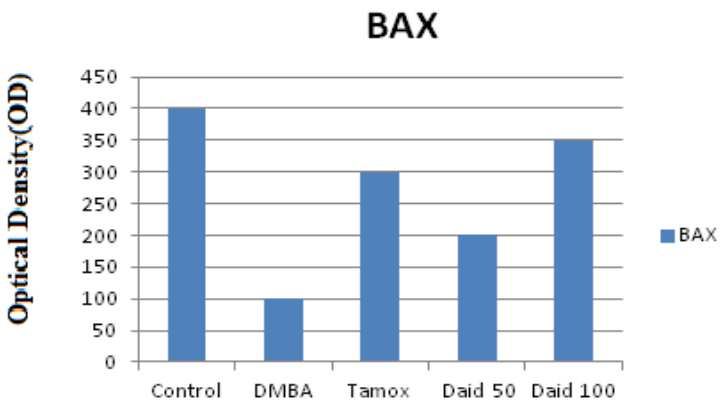
Normal ductal and alveolar cells surrounding various mammary lesions and tumors served as a positive control for Bcl-2 and Bax expression. The top sections of each slide, which were not treated with the corresponding antibody, were used as negative controls. Relevance values of Bcl-2 and Bax expression were those of epithelial cells in ducts and alveoli in the same tissue section where hyperplasic lesions and tumors were identified.

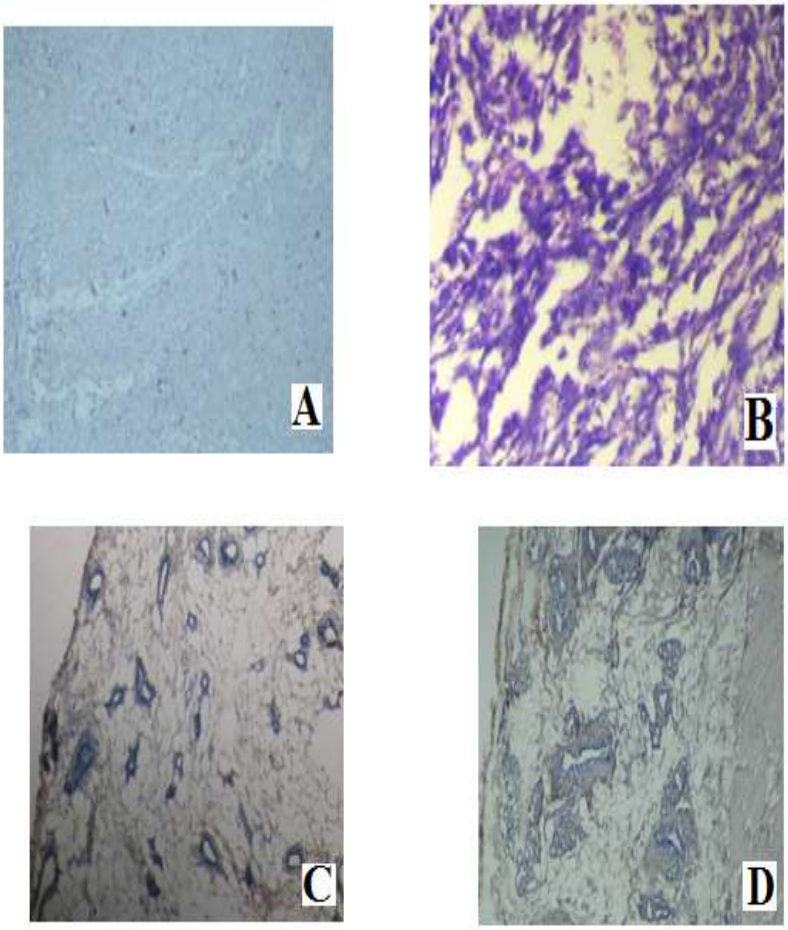
Semi-quantitative analysis of optical density of BAX and Bcl-2 Values are shown as means ± SD (n= 6). *P < 0.05 vs control.

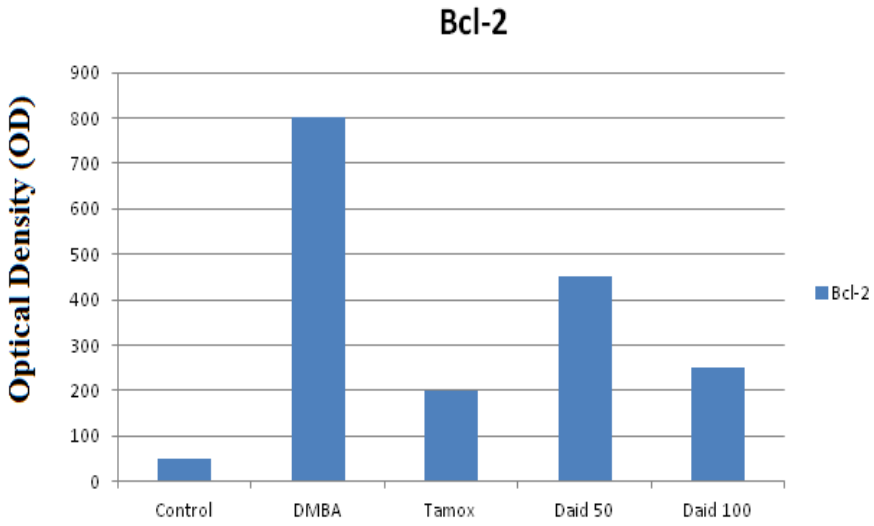
MAMMARY CANCERS



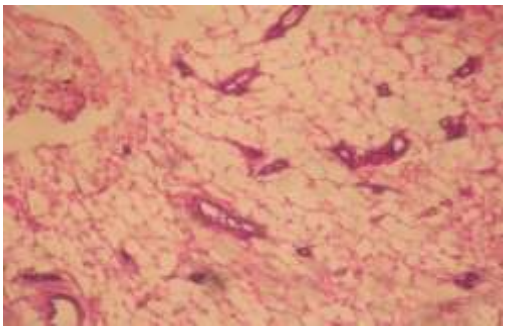




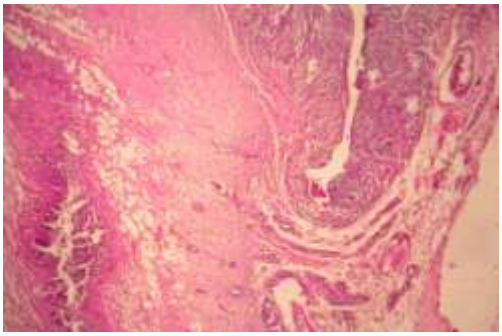

A
B
C
D


Bcl-2
Optical Density (OD)
900
800
700
600
500
400
300
200
100
0
Control
DMBA
Tamox
Daid 50
Daid 100
Bcl-2

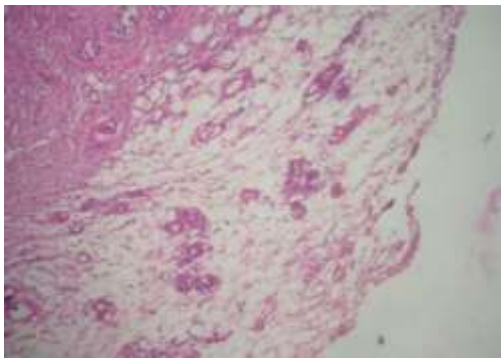
HISTOPATHOLOGICAL ANALYSIS



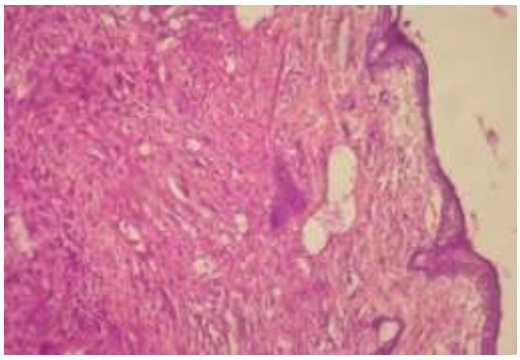
Group I – Normal controls with 2% DMSO as vehicle, po.



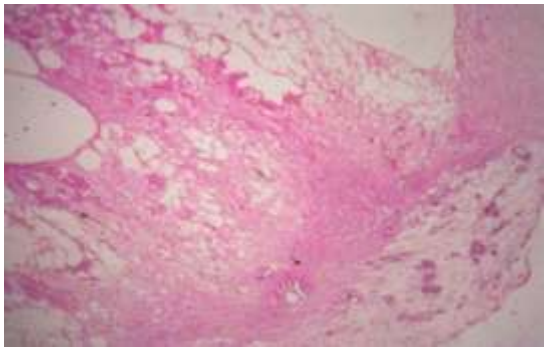
Group II – DMBA (25mg/rat/S.C in 2 divided doses) at 4th & 5th week.



Group III– Tamoxifen (40mg/kg, po) + DMBA.



Group IV–Daidzein – (50mg/Kg/, po) + DMBA.



Group V – Daidzein – (100mg/kg, p.o) + DMBA.

Group 1- Section shows normal mammary gland.

GROUP II -Section study shows breast tissue with features suggestive of infiltrating ductile carcinoma with extensive stromal involvement.

GROUP III - Section study shows reactive changes & minimal infiltration with few malignant epithelial cells.

GROUP IV - Section study shows glandular & stromal components of breast tissue with features of infiltrating ductile carcinoma stroma shows moderate distribution of moderate epithelial cells.

GROUP V - Section study shows vacuolative changes in glandular and stromal components of breast tissue, minimal distribution of malignant epithelial cells seen.

DISCUSSION

Cancer cells are frequently under persistent oxidative stress because of oncogenic stimulation, increased metabolic activity and mitochondrial malfunction.[11] The present study was designed to evaluate the anticancer effect of Daidzein, in comparison with Tamoxifen an anti-estrogen used in ER+ breast cancers. The incidence of neoplastic lesions in the experimental model with DMBA as chemical carcinogen was described. The number of rats that exhibited neoplasms, their multiplicity as well as the volume of the tumours was assessed. For each group, occurrence of mammary tumours was determined by using the standard formula for tumour incidence followed by statistical analysis using DMRT.

The incidence and volume being observed in the 7, 12 DMBA treated group (**Vide table1**) was found to be increased both in number and volume. Oral administration of Daidzein (50mg/kg&100mg/kg) to groups IV & V showed a reduction in the tumour incidence (30%, 60% respectively) as comparable with 80% reduction in tumour mass with Tamoxifen. The

type of lesion observed in our study was malignant, poorly differentiated Adenocarcinoma exhibiting both tubular and papillary patterns.

Morphological analysis of the DMBA induced tumours in mammary glands based on its general characteristics revealed infiltrating adenocarcinoma.[12] Tumour growth was associated with discharge/ulceration of the nipple area, in addition to the presence of large, soft, bulky nodules ulcerating through the skin. (vide fig) Some tumours revealed central haemorrhage or cyst formation. However the tumour incidence and multiplicity was markedly reduced in the Daidzein treated group V, at doses of 100mg/kg. Rats administered DMBA subcutaneously or intraperitoneally[13] manifested cancers of lobular in situ, comedocarcinoma and carcinoma simplex type. DMBA treatment is known to change the normal differentiation process of the mammary gland into an invasive carcinoma. There fore the study of in situ carcinoma in this setting has revealed the importance of this model in predicting the aggressiveness of breast cancer.[14] 7, 12 DMBA induced mammary carcinogenesis is a suitable model to study the in vivo tumourigenesis in rats. The oxidation of DMBA by Cyt P450 enzyme produces metabolites, that form covalent adducts with DNA and result in formation of depurinated sites within DNA. Excess production of reactive Oxygen species as a result of metabolic activation, causes oxidative damage to the structure and function of DNA, protein and lipids, contributing to neoplastic transformation.[15] Daidzein significantly decreased the proliferation of MCF-7 cells in a concentration and time-dependent manner. One study indicated that, Daidzein by activation of Caspase 9, presumably triggered the mitochondrial –mediated cell death pathway. The subsequent disruption of the mitochondrial membrane potential releases apoptosis promoting factors and cell death.

Apoptosis, the programmed cell death is a critical event in tumourigenesis and tumour progression. Growth factors and other survival signals stimulate production of anti-apoptotic protein like Bcl-2, Bcl-X and Mcl-1.[16] These protein reside within the cytoplasm and in mitochondrial membrane where they control mitochondrial permeability and prevent leakage of mitochondrial protein that have the ability to trigger cell death. Cells are deprived of survival signals or their DNA is damaged or misfolded protein induce ER stress, sensors of damage or stress are activated.[17] The BH_3 only protein which include BAX and BAK form oligomers that insert into mitochondrial membrane and create channels that allow protein from inner mitochondrial membrane to leak out into the cytoplasm. The BH_3 only protein

may also bind and block the function of Bcl_{-2}, synthesis, which also tends to decline.[18] This effect of upregulation of Bax and down regulation of Bcl_2 as observed with Tamoxifen and Daidzein at higher doses was well corroborated in our study.

Daidzein supplementation resulted in the desquamation of neoplastic cells , as evidenced by a decrease in the of tumour incidence ,multiplicity and tumour volume. In vitro studies in in MCF-7 cells[19] has confirmed the involvement of the caspase dependant pathway in Diadzein induced apoptosis. Daidzein significantly decreased the proliferation of MCF-7 cells in a concentration and time-dependent manner. Other caspase independent mechanism of apoptosis have also been highlighted. The oxidative stress induced by DMBA, as observed in a previous study, alters cellular function, perturbs mitochondrial activity leading to caspase activation. One study indicated that, Daidzein by activation of Caspase 9, presumably triggered the mitochondrial –mediated cell death pathway. The subsequent disruption of the mitochondrial membrane potential releases[20] apoptosis promoting factors and cell cycle arrest.

The pleiotrophic response of the mammary stroma to tamoxifen treatment suggests that tamoxifen influences the expression of a master regulator of stromal function.[21] Research by other groups indicates that tumour growth factor (TGF) β, which acts on fibroblasts, immune and endothelial cells, in addition to epithelial cells, is a candidate regulator. In tamoxifen-sensitive breast tumour cells, growth inhibition has been shown to be mediated by activation of TGFβ.[22] Further, the ability of tumour cells to upregulate TGFβ and its receptor (tw-1).

The histopathological study showed the cells in DMBA group to be long interconnected nodular aggregates. The cells were moderate in size, deeply stained and invading the stroma.[12] (Russo J et al., 1989). When cellular hyperplasia was present, vacuolization was seen. These vacuolative changes observed with minimal infiltrative changes in the Daidzein treated groups is suggestive of a chemopreventive potential.

The main aim of this study was to investigate the possible protective effect of the isoflavone Daidzein, in inhibiting oxidative stress responses associated with DMBA-induced breast carcinogenesis in rat and its chemo preventive and apoptotic potential. Our current study demonstrated that Daidzein at dose levels of 50mg/kg did not show any significant regression in tumour growth, Reports of several studies indicate lesser protective association of Daidzein when administered in lower doses or after the induction of carcinogenesis. Results

of our study proved that 4weeks prior to tumour induction and continued till the end of the study period, reveal that there was a mediocre response at 50mg.kg dose but a statistically significant response at doses of 100mg/kg.

CONCLUSION

Phytoestrogens possibly may inhibit binding of the more potent endogenous estrogens and decrease their potential effects on breast cancer risk by competing for estrogen receptor. Isoflavon Daidzein and its metabolite equol, exhibits multiple biological effects and in some systems, operate through estrogen receptors, upon which they may act as agonists, antagonists or mixed agonist-antagonists and can thus be used for modulation of ERs functioning.[23] This strong evidence of apoptosis induced by Daidzein or equol contributes to its anti-tumour potential. Having the capability to modulate ER activity, isoflavone Daidzein and its derivatives thereof may be useful to treat or prevent a variety of diseases and conditions related to estrogen receptor functioning in mammals, preferably humans. Designing newer drugs with Daidzein or equol as its core structure could help in the prevention/treatment of breast cancer.

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