Efficacy of *Vitex agnus* in lowering prolactin level in mammary tumor induced SD rats

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ABSTRACT

Introduction and Aim: Breast cancer accounts for about 30% of all cancers in women. The present study aims to see the role of prolactin and *Vitex agnus* fruit extract in breast cancer progression in mammary tumor induced Sprague Dawley (SD) rats.

Materials and Methods: Thirty-day old inbred SD female rats of body weight 70-80 grams were taken for this study. The rats were induced with N- Methyl-Nitroso-Urea for mammary tumor development. After the development of palpable and visible tumor the rats were treated with anti-prolactin drug (Cabergoline) and a prolactin lowering herb *Vitex agnus* Castus (VAC) for two months. After the treatment the rats were sacrificed for antioxidants estimation and histopathological section examination.

Results: The rats treated with anti-prolactin drug showed benign tumors with hyperplasia and lactational change proving the presence of prolactin in the tumor tissue, whereas the plant extract showed mammary tumor regression by the presence of foamy macrophages in the histopathological sections. Results also showed treatment with cabergoline increased the GSH level and decreased the MDA level compared to tumor induced group.

Conclusion: Prolactin may have a potential role in progression of breast cancer and *Vitex agnus* extract showed a prolactin lowering effect and facilitated in regression of the tumor.

Keywords: Breast cancer; prolactin; cabergoline; *Vitex agnus*; histopathology; GSH; MDA.

INTRODUCTION

Breast cancer is one of the leading health problems throughout the world. One in ten of all new cancers diagnosed worldwide each year is a cancer of the female breast. It is also the principal cause of death from cancer among women globally. Breast cancer accounts for about 30% of all cancers in women and is one of the leading causes of cancer-related mortality in females (1, 2). Metastatic breast cancer develops in 30-40% of patients with breast carcinoma and is essentially incurable with standard therapy (3).

Recent studies done on breast cancer shows, the emerging factor that has gained maximum focus in the association of prolactin with breast cancer. Several studies done on breast cancer from past few decades have led to the possibility that prolactin plays an important role in human breast cancer. Prolactin, a pituitary hormone, is mainly involved in the production of milk protein and plays a very important role in the mammary gland development (4). The literature reveals that prolactin belongs to the same gene family as Growth Hormone and placental lactogenes (PLs). The PRL receptor (5); (PRLR) and the GH receptor (GHR; 6, 7) are members of the cytokine receptor superfamily. Ligand binding induces homo-dimerization of two receptor molecules followed by activation of tyrosine kinases in the Janus family, phosphorylating members of the STAT family and proteins involved in the MAP kinase signaling pathway. Previous studies done on mammary tissue show the presence of receptors for both GH and PRL. It is evident from the studies that breast cancer and mammary tumor cell lines express the PRLR (8). A study showed that if the prolactin receptor was blocked it resulted in the inhibition of growth in cultured tumor cells (9). From the literature it is also seen that GHR is also expressed in mammary tumors and tumor cell lines (10). For a long time, plants have played an important role in human life, and the use of plants as treatments is still very important (11). From the study it has been estimated that more than 80% of the world's population utilizes plants as their primary source of medicinal agents (12), due to many reasons but primarily due to the high cost of Western pharmaceuticals and also because the traditional medicines are generally more acceptable from a cultural and spiritual perspective. Studies are showing that even in the Western world, use of the herbal medicines is steadily growing, with approximately 40% of the population reporting use of herbs to treat medicinal illness (13).

Vitex agnus Castus is an anti-prolactin herb which works by acting on the pituitary gland and the hypothalamus. These two structures are responsible for the release of hormones or for triggering hormone responses throughout the reproductive system (14). Many of the in-vitro studies done on *Vitex agnus* describe the dopaminergic effects of *Vitex* via a dose-dependent binding of dopamine-2 receptors, yielding a potent inhibition of prolactin in cultured pituitary cells (15).

Therefore, the present study aimed to evaluate the role of prolactin and assess the anti-prolactin effect of *Vitex agnus* Castusseed extract in breast cancer etiology, progression and treatment.

MATERIALS AND METHODS

Animals

After obtaining the permission from the institutional animal ethical committee dated 28/02/14, healthy, 30 days old inbred Sprague Dawley female rats of body weight 70-80 grams were taken for this study. Animals were maintained according to prescribed guidelines of a committee for the purpose of control and supervision of experts on animals (CPCSEA), Govt. of India, for the use of laboratory animals. All animals were maintained under normal day-night environment in temperature controlled institutional animal house. Animals were housed in polypropylene cages; paddy husk was used for bedding. Animals were given water *ad libitum* and standard rat feed pellet was used for feeding.

Induction of tumor

Preparation of the carcinogen

Mammary tumor was induced by injecting N-Methyl-N-Nitrosourea dissolved in normal saline with pH- 4 maintained by adding 3% glacial acetic acid. The SD rats were given a single intraperitoneal dose of 50 mg/kgbw of MNU and kept for observation for the mammary tumor development.

Collection of plant material

The plant *Vitex agnus* Castusalso known as *Vitex negundo* or *Vitex nirgundi* was collected from Vijaynagar; Mysore, Karnataka and the fruits and leaves of the plant were air dried and powered for the extraction.

Preparation of *Vitex agnus* extract

Hydro-alcoholic extract of the plant seeds was prepared using Soxhlet apparatus where 100gm of dry seed powder was suspended in 100 ml of 50% methanol (1:1 ratio) and refluxed at 50^{o} C for 72 hours. The crude extract was concentrated by rotary flash vacuum evaporator and the concentrate was collected in an air tight container which was stored at 4^{o}C for subsequent use. Stock and working standard solutions of the concentrated crude extract were prepared for the study.

The dosage was selected on the basis of previous literature as 200mg/kg bw administered orally once daily for 2 months (16). Cabergoline (anti prolactin drug) was given at a dose of .05mg/kg bw once daily through oral route for 2 months.

Histopathology

Histopathological section of the mammary tumor of the SD-rats treated with / without plant extract was done on the sixteenth day after finishing the treatment plan. The rats were anesthetized and the tumor was excised from the mammary region for the same. The tumor tissue was kept at 10% buffered formalin and then sectioned for Hematoxylin and Eosin staining.

Anti-oxidant estimation

***Reduced glutathione (GSH;* 17)**

The samples (0.5ml of serum) were treated with 1.5mL of precipitating solution and kept for 10 minutes for the precipitation to complete and then filtered through a Whatmann No.1 filter paper. 500µL of the filtrate is taken and to this 2mL of phosphate solution and 250µL of DTNB solution is added. Simultaneously a blank is maintained containing 200µL of distilled water, 300µL of precipitating solution, 2mL of phosphate solution and 250µL of DTNB. The intensity of the yellow color formed is spectrophotometrically read immediately (within ten minutes) at 412nm against the blank.

Lipid peroxidation (LPO; 18)

100µL serum is diluted to 500µL with distilled water. To the diluted sample 1mL of TCA-TBA-HCl reagent is added and vortex is done for 2 minutes. The samples are kept in boiling water bath for 15 minutes. The reaction mixture is cooled and centrifuged. The supernatant is taken and the optical density of the pink color formed is read at 535nm.

Estimation of prolactin

Quantitative estimation of serum prolactin levels was done by using a commercially available in vitro Enzyme Linked Immuno-Sorbent Assay (ELISA) kit procured from Ray Biotech.

Principle: The assay is based on biotinylated double antibody sandwich technology which employs a prolactin specific antibody.

Statistical analysis

The statistical analysis was done by ANOVA followed by Tukey's post hoc test.

RESULTS

Antioxidant estimation

The antioxidant status (Table 1) was found to be significantly lowered in tumor induced group and significantly increased in *Vitex* seed extract treated group. The GSH levels were significantly reduced in the tumor induced and cabergoline treated groups. Themalondialdehyde (MDA) levels were found to be increased in tumor induced groups as compared to control and treated group showing increased lipid peroxidation in tumor tissue. Cabergoline treated group also showed a decrease in MDA level and an increase in antioxidant level compared to tumor induced group. However, plant

extract group showed better response than Cabergoline treated group.

Table 1: Antioxidant parameters of the experimental groups

Parameters	Control	Tumor induced	Cabergoline treated	Vitex treated	p-value
GSH (µmol/ml)	7.33± 0.09	3.15±0.07	5.27±0.08	7.94±0..06	$< 0.001^{*\$}$
MDA (µmol/ml)	0.91±0.11	1.55±0.02	0.95±0.02	0.72±0.03	$< 0.05^{*\#\$}$

Results are expressed as Mean ± SD
Symbols *, #, $ indicate comparison between Tumor induced vs Control
Tumor induced vs Cabergoline and Tumor induced vs Vitex treatment groups respectively.

ELISA of prolactin levels

The prolactin levels of the groups were estimated in the groups in order to re-evaluate the role of f prolactin in the mammary tumor progression and if the reduction of prolactin levels reduces the tumor progression. A significant increase in Prolactin levels were observed in the tumor induced and cabergoline treated groups. A significant decrease in prolactin levels was observed in the Vitex treated group when compared to the tumor induced and cabergoline treated group.

Table 2: Levels of prolactin in experimental groups

Parameters	Control	Tumor induced	Cabergoline treated	Vitex treated	p-value
Prolactin (ng/ml)	0.55±0.19	$1.17\pm0.40^{*\#}$	$1.01\pm0.42^{*}$	$0.89\pm0.32^{*\#\$}$	P<0.05

Results are expressed as mean ± SD
Symbols *, #, $ indicate comparison between Tumor induced vs Control,
Tumor induced vs cabergoline and Tumor induced vs Vitex treatment groups respectively

Histopathology

The histopathological section of normal control rats showed the mammary ducts, breast parenchyma and sebaceous ducts (Fig.1). The tumor induced SD rats showed infiltrating ductal carcinoma (IDC) with sheets of malignant ductal cells and stromal infiltration, tubular adenosis and malignant ductal cells infiltrating the squamous epithelial layer of the skin (Fig.2). The histopathological section of Cabergoline (anti prolactin drug) treated group showed intraductal papilloma, lactational change, and adeno-myoepithelial hyperplasia (AMH; Fig.3). The histopathological section of *Vitex agnus* seed extract treated group showed atrophic ducts and absence of malignant tumor cells and fibroblastic proliferation (Fig. 4). The findings are tabulated in Table 3.

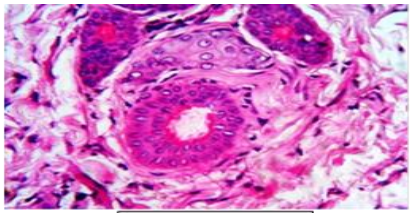

Fig 1. Control

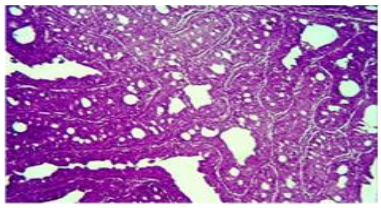

Fig 2. Tumor Induced

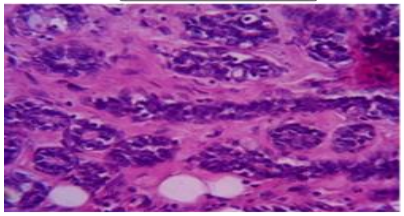

Fig 3. Cabergoline treated

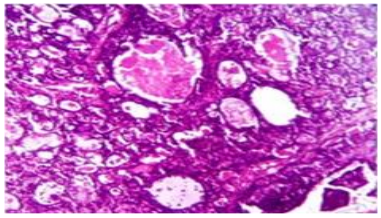

Fig 4. *Vitex agnus* seed extract treated

Fig.1: Shows mammary gland, sweat glands and the breast tissue of rats. **Fig.2:** shows ductal carcinoma, necrosis & stromal infiltration, tubular adenosis & cribriform Ductal Carcinoma *in Situ* (DCIS) with single intraperitoneal dose of MNU at 50 mg/kg. **Fig.3:** Intraductal papilloma, benign and unremarkable breast tissue, lactational change Adenomyoepithelial hyperplasia (AMH) **Fig.4**: Shows atrophic ducts and absence of malignant tumor cells and fibroblastic proliferation in the stroma. Presence of foamy macrophages or histocytes was observed which indicates the tumor regression.

Table 3: Histopathological findings of the different experimental groups

Groups	FA	ADH	DPA, IDP	AME	PDC	DCIS	Macrophages histiocytes
Control	-	-	-	-	-	-	-
Tumor MNU induced	-	-	-	-	+	+++	-
cabergoline	++	-	-	-	-	-	-
Vitex treated	+	-	-	++	-	-	+

FA: Fibroadenosis, ADH: Adenoductal hyperplasia, DPA: Ductal papillary adenoma, IDP: Intra ductal papilloma, AME: Adenomyoepitheilum, PDC: Papillary ductal carcinoma and DCIS: Ductal carcinoma *in situ*

DISCUSSION

Breast cancer is a major public health problem throughout the world. Metastatic breast cancer develops in 30–40% of patients with breast carcinoma and is essentially incurable with standard therapy (1). The cause for breast cancer still remains under search but the diagnostic test to evaluate the early detection of breast cancer has always been under the prime focus. Hormones have played a major role in the development of breast cancer (19). Post-menopausal women have always been under the risk of developing cancer due to the fluctuations in the estrogen levels. In the recent research on the diagnosis of breast cancer has led to the information that prolactin is secreted in the breast tissue and increase in the levels of prolactin from the mammary tissue can be the major reason for the development of the breast cancer. Studies carried out on mammary cancer cell lines have given a brighter insight about the role of prolactin but there is a lack of sufficient literature about *In-vivo* studies to find out the role of prolactin in mammary cancer development (20). The present study is an *In-vivo* study to find out the role of prolactin in progression of breast cancer.

The antioxidant estimation showed a decrease in GSH levels and higher MDA levels in tumor induced group. This is suggestive of the oxidative stress present in the tumor tissue. Plants have always been a great source of antioxidants providing a properly oxidized state of the tissue. *Vitex agnus* treatment showed significant improvement in both MDA and GSH suggestive of its antioxidant potential (21, 22). Treatment with cabergoline showed improvement in lipid peroxidation as indicated by the MDA levels compared to other groups. Therefore, the antioxidant estimation of the groups indicated the oxidative stress in the tumor induced group and improvement in the groups where the tumor progression was reduced due to the decrease in prolactin levels. The prolactin levels estimated through rat ELISA kits showed increased levels of prolactin the tumor induced and cabergoline treated groups. This can be attributed to the production of local prolactin in the vicinity of mammary tissue. The higher levels of prolactin in the cabergoline treated animals confirms the mammary tissue release of prolactin as cabergoline is known to inhibit the pituitary release of prolactin. The presence of prolactin in spite of pituitary inhibition confirms the local release of prolactin. The Vitex agnus treated group showed significant decrease in the prolactin levels indicating the prolactin lowering activity of this herb extract.

The histopathological section of MNU induced rats showed the presence of stromal infiltration and ductal carcinoma *in situ* (DCIS) marking the presence of malignant tumor when compared to normal rats showing normal sweat glands and

sebaceous glands. Rats treated with cabergoline at a dose of 0.5mg/kgbw for 2 months showed Intraductal papilloma, benign and unremarkable breast tissue, adenomyoepithelial hyperplasia (AMH) along with lactational change indicating the presence of prolactin in the breast tissue. In Vitex treated group the histopathological section shows the presence of atrophic ducts and absence of malignant tumor cells along with fibroblastic proliferation in the stroma. Also presence of foamy macrophages or histocytes was observed which indicated the tumor regression. Therefore, from the histopathological study it was evident that prolactin was present in the mammary tissue inspite of its pituitary inhibition by cabergoline and the prolactin lowering herb administered showed the regression of mammary tumor.

CONCULSION

The presence of prolactin in mammary tumor in spite of its pituitary inhibition with cabergoline was evident and *Vitex agnus* treatment helped in reduction of prolactin levels and hence regression of mammary tumor which can be used in the management of breast cancer.

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