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Vitex Berries Attenuates Chemically-Induced Mammary Carcinomas in Rats through modulation of the cancer growth rate-limiting enzymes activities: aromatase and Na+/K+ ATPase.

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Abstract

Vitex (*Vitex agnus-castus*) berries have shown potent antioxidant, selective anti-inflammatory against cyclo-oxygenase-2 (COX-2), and cytotoxicity effect against human breast cancer cells (MCF-7), as we recorded in our previous study". Additionally, we found that Ethanolic extract (70%) and its ethyl acetate fraction have exhibited anti-tumor ability on Ehrlich's Ascites Carcinoma in mice model in our previous study. The present study was planned to evaluate the protective and therapeutic effects of berries ethanolic extract and its ethyl acetate fraction on breast cancer using a 7, 12-dimethylbenz(a)anthracene-induced breast cancer model. Results showed that Vitex materials exhibited protective and therapeutic effects on chemically-induced breast cancer Sprague-Dawley female rats, where breast cancer biomarkers including the number of tumors devolved, weight and volume of tumor mass, and carcinoembryonic antigen level were significantly reduced compared to the cancer control group. Interestingly, the therapeutic effects of vitex materials were better than the protective effects, and ethyl acetate fraction was more effective than ethanolic extract. Finally, the obtained data showed that Vitex ethanolic extract (70%) and its ethyl acetate fraction can protect and treat chemically-induced breast cancer with a good safety margin. Vitex struggled with chemically-induced breast cancer through inhibition of the overexpression of cancer growth rate-limiting enzymes; Na^+/K^+ ATPase and aromatase as well as estrogen overproduction. Also, Vitex prevented overexpression of COX-2 and oxidative stress.

Keywords: *Vitex agnus-castus* hydroethanolic extract and ethyl acetate fraction, 7,12-dimethylbenz(a)anthracene-induced breast cancer in rats, aromatase and Na+/K ATPase activities, estrogen and progesterone, COX-2 overproduction.

Introduction

Breast cancer is the most common malignant tumor among females all over the world. It is the second most common type of cancer. Breast cancer has two types; hormone-dependent (HR-positive) and hormone-independent (ER-negative). ER-negative breast cancer proliferates and grows without estrogen, whereas ER-positive proliferates and grows with estrogen. ER-positive ER-positive is the most common among females worldwide **[1].** Hormone therapy drugs can inhibit or avoid the development of hormone-dependent cancers via decreasing estrogen and progesterone products or blocking their functions on breast cancer cells. The most important hormone therapy drugs for ER-positive management and treatment are aromatase inhibitors (AIs) or selective estrogen receptor modulators (SERMs) drugs **[2]**. AIs are aromatase blockers like anastrozole (Arimidex®) and letrozole (Femara®) that prevent the convertion of testosterone to estrogen. SERMs, such as tamoxifen, block the role of estrogen by competing for estrogen receptors **[3].** Tamoxifen and Raloxifene are approved by The FDA to reduce the risk of developing breast cancer in women. But these drugs have many side effects, including hot flashes, night sweats, vaginal dryness, and disruption in premenopausal women's menstrual cycle. In accordance, aromatase inhibitor drugs increase the risk of bone loss, mood swings, depression, loss of libido, heart attack, angina, heart failure, and hypercholesterolemia **[4-5]**. Therefore, searching for another alternative approach for breast cancer of natural origin is important. Several natural products affect the hormones in experimental models of breast

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cancer and women, predisposing them to serve as prodrugs for hormone therapy. Many studies demonstrated the ability of Broccoli to inhibit aromatase activity and reduce estrogen production in chemically-induced breast cancer in rats **[6]** and in breast cancer women **[7-8]**. Also, there are polysaccharides produced from Bacillus sp. showed a promising role in reducing estrogen production and inhibiting aromatase activity in the experimental model **[9]**. From this point on we planned this study to discover new substances that could act as hormonal therapy.

Vitex, *Vitex agnus-castus* (*Verbenaceae)*, is commonly known as a chaste tree, chaste berry, Abraham's balm, lilac chaste tree, and monk's pepper. It is natively grown in warm temperate and subtropical regions in European, Mediterranean, and Central Asia countries. It is a deciduous shrub that grows to 1-5 m. It has slender, finger-like leaves and purple-black berries. Peppery-taste berries are the medicinally used part **[10]**. Many secondary metabolites were found in the fruits as terpenoids, flavonoids, iridoids, and phenol carboxylic acids. Flavonoids are casticin and luteolin-7-glycoside, apigenin, artemetin, vitexin, orientin, isovitexin, isoorientin, kaempferol, penduletin, and eupatorin. Iridoid glycosides are agnoside and aucubin. Many phenolic acids were identified in the fruits like 3,4-dihydroxybenzoic acid, 5-hydroxy2-methoxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, p-hydroxybenzoic acid, p-hydroxyphenylethanol-p-coumarate, and chlorogenic acid. In non-polar fraction, several terpenoids are found as vitexilactones, vitetrifolines, spatulenol, 8-epimanoyl oxide, aromadendren-4α, 10α-diol, 3-epi-corosolic acid, 3-epi-maslinic acid, and ilelatifol D. Essential oil of the fruits contains α-pinene, sabinene, β-pinene, β-myrcene, p-cymene, limonene, 1,8-cineole, cis-sabinene hydrate, trans-sabinene hydrate, cis-p-ment-2-en-1-ol, trans-p-ment-2- en-1-ol, trans-verbenol, δ-terpineol, terpinene-4-ol, krypton, α-terpineol, trans-carveol, β-citronelol, and α-terpinyl acetate **[11]**. Various pharmacological effects of Vitex have been demonstrated. Also, many Vitex formulas on the market have been used. Vitex preparation (Mastodynon®) treats women's fertility disorders such as pregnancy and spontaneous menstruation in women with amenorrhea and/or subsequent pregnancy, or concentrations of luteal hormones. Vitex showed an ameliorative effect on premenstrual aggravations like mouth ulcers, acne, and premenstrual edema. Additionally, it decreased headaches, breast tenderness, bloating, fatigue, sweet cravings, nervousness, lack of concentration, depression, mood swings, and aggressiveness in patients with PMS **[11].** Recently, Vitex berries hydroethanolic extract has had a therapeutic and protective effect on 7,12-dimethylbenz(a)anthracene-induced prostate cancer in rats **[12].**

Our previous study **[13] is** evident that vitex berries' alcoholic hydroethanolic extract and its fractions; petroleum ether, ethyl acetate, chloroform, and remaining aqueous) have antioxidant capacity. They recorded free radicals, ROS, and RNS scavengers, and recorded reducing power, metal chelation, and lipid peroxidation inhibition activities. All vitex materials exhibited selective anti-inflammatory against COX-2, not COX-1. In addition, vitex materials occurred anti-prostate cancer against PC3 with low IC_{50} value. The best antioxidant capacity, anti-inflammatory activity, and anticancer activity were represented by hydroethanolic extract and ethyl acetate fraction. Therefore, the hydroethanolic extract and ethyl acetate fraction were incorporated in an anti-tumor experiment using Ehrlich Ascetic Carcinoma (EAC) in mice. Both materials showed an anti-tumor efficacy that ameliorated hematological parameters, decreased tumor volume and weight, and increased animal life span.

The present study was planned to evaluate the protective and therapeutic effects of berries ethanolic extract and its ethyl acetate fraction on chemically-induced breast cancer in rats model based on its ability to influence hormonal homeostasis.

Materials and methods

Chemicals

7, 12 di-methylbenzeα-anthracene (DMBA) was purchased from Sigma Aldrich, USA. Spectrophotometric kits were obtained from Bio Diagnostics, Egypt. ELISA kits for cyclooxygenases activity (COX-1 (**Cat NO.:**SL1228Ra) and COX-2 (**Cat NO.:**SL0218Ra)), aromatase (**Cat NO.:**SL1145Ra), Na^+/K^+ ATPase, carcinoembryonic antigen (CEA, **Cat NO.:**SL1244Ra), estrogen (**Cat NO.:**SL0271Ra) and progesterone (**Cat NO.:**SL0596Ra) were obtained from Sunlong Biotech Co., LTD, PingShui Street, Gong Shu District, Hangzhou, Zhejiang, China, Email: Sales@Sunlongbiotech.Com.

Plant material and extraction

Berries of *Vitex agnus-castus* were obtained from the organic farm of the SEKEM company group, in Egypt in July 2021. The accession number of the voucher specimen is EGY-MAZHAR-03250501 and the herbarium specimen was deposited in Shehab Mazhar botanical garden, Egypt Each kilogram of berries was exhaustively extracted by hydroethanolic 70% (3 L) by shake soaking several times (3 times) at room temperature. The filtrates were combined and

then evaporated by a rotary evaporator at 40°C. The remaining residue was lyophilized (15 g) and was kept under -20°C. Hydroethanolic extract (15 g) was fractioned by petroleum ether (1 L), chloroform (1 L), ethyl acetate (1 L), and butanol (1 L). The yield of fractions was 5 g petroleum ether, 4 g chloroform, 3 g ethyl acetate, and 2 g remained aqueous. The fractions were concentrated under reduced pressure and lyophilized for bioassays.

Evaluation of anti-breast cancer effect of Vitex materials

Ethical protocol

The experiment was performed in The Central Animal House of the National Research Centre, Dokki, Egypt in July 2021. The study design and methods were accepted by ethical guidelines (Ethical Committee for Animal Care and Use, Egypt). The study design was accepted by the Medical Research Ethics Committee of the National Research Centre in Egypt (**No 19/295 in 2019)**.

Animals

Young virgin Sprague-Dawley female rats were utilized to discover the ability of the two vitex berries materials, hydroethanolic extract and ethyl acetate fraction, in the prevention and treatment of mammary carcinomas in females. Two hundred thirty female rats were purchased from the animal house of the National Research Centre. Rats were maintained in the laboratory conditions (25 ± 2°C, moisture 60 to 65% with a 12: 12 h light to dark), food and water were *ad labium*.

Chemical induction of mammary carcinomas in female rats

Female rats with age 60 days and weighing 100-130 g were used in this study. Based on the model of **Yerma et al. [14]**, mammary carcinomas were made. Rats have orally administered a single dose of DMBA; 65 mg/ kg body weight in corn oil. After 5 months, the breast cancer was checked using pathological examinations.

Experimental Scheme

The anti-breast cancer effect of Vitex hydroethanolic extract and its ethyl acetate fraction was evaluated at doses 180 and 165 mg/kg/day, respectively which equals 1/10 of the $LD_{50.}$ Their LD_{50} was 1800 and 1650 mg/kg previously estimated in our previous study **[12-13].** We dissolved tested materials in a saline solution. Animals received tested materials orally by stomach tube. 230 female rats were divided into four main groups: 1) negative group, 2) cancer group, 3) Vitex hydroethanolic extract, and 4) ethyl acetate fraction group.

The negative group was 20-rat that received saline solution for 8-months.

The cancer group was 30-rats that received a single dose of DMBA (65 mg/kg body weight) orally and kept in laboratory conditions for five months. These rats were administered saline solution for the following 3-months, the time that equals Vitex treatment.

The Vitex hydroethanolic extract group, ninety rats, was classified into 3-subgroups as follows.

1- **Subgroup 1:** 30-rats received saline solution for 5-months, the time that equals cancer induction, and then orally administered the extract (180 mg/ kg/day) **[12]** for the following 3-months. **This subgroup represented the positive control of Vitex hydroethanolic extract.**
2- **Subgroup 2:** 30-rats were administered the hydroethanolic extract firstly 180 mg/kg/day for 3-months. Then they orally administered one dose of DMBA (65 mg/kg) and were maintained in laboratory conditions for another 5-months. **This subgroup is considered the protective group of hydroethanolic extract**.
3- **Subgroup 3:** 30-rats were first administered a single dose of DMBA (65 mg/ kg body weight) and kept under laboratory conditions for 5-months. Then they force-fed 180 mg extract /kg/day **[12]** for 3-months. **This subgroup is considered the treated group of hydroethanolic extract**.

The ethyl acetate fraction of the vitex hydroethanolic **extract group** constituted ninety-rat that were classified into 3-subgroups.

1- **Subgroup 1:** 30-rats received saline solution for five months and then orally administrated the extract (165 mg /kg) for another 3-months. **This subgroup is considered the positive control of the vitex ethyl acetate fraction.**
2- **Subgroup 2:** 30- rats received the ethyl acetate fraction (165 mg/kg **[13]**) for 3-months. Then they force-fed 65 mg/ kg of DMBA and maintained it in laboratory conditions for the following 5-months. **This subgroup is considered the protective group of the ethyl acetate fraction.**
3- **Subgroup 3:** 30-rats were administered a single dose of DMBA (65 mg/ kg) first and kept under laboratory conditions for 5-months. Then they force-fed an ethyl acetate fraction (165 mg/kg **[13]**) for 3-months. **This subgroup is considered the treated group of the ethyl acetate fraction.**

The experiment was accomplished after eight months, and the animals were fasted overnight. Each rat was intraperitoneally injected with 0.2 ml/ 100 g body weight of solution of 87 mg ketamine/ kg and 13 mg/ xylazine, for anesthesia **[15]**. Blood samples were obtained from the retro-orbital plexus

and were centrifugated (4000 *g* for 10 min at 4°C) by Sigma Laborzentrifugen (Osterode am Harz, Germany) to isolate serum. Organs were isolated and weighted for the safety profile assessments.
Gross assessment of tumor mass by using a scale to measure its weight and determine volume by measuring 3 dimensions length, width, and height, and multiplying them.

Biochemical assays

Anti-inflammatory and cancer biomarkers

COX-1, COX-2, aromatase, α1-Na^+/K^+ ATPase, CEA, and sexual hormones; estrogen and progesterone were determined using ELISA kits.

Antioxidants parameters

Glutathione (GSH) concentration **[16]** as well as antioxidant enzymes activities; glutathione reductase (GR) **[17],** glutathione *S*-transferase (GST) **[18],** Glutathione peroxidase (GPx) **[19]**, catalase (CAT) **[20]** and superoxide dismutase (SOD) **[21],** were spectrophotometrically determined.

Safety profile assessments

Liver performance was evaluated by determination of total protein (TP) **[22]** and albumin levels **[23]** and aspartate aminotransferase (AST), and alanine aminotransferase (ALT) **[24]** activities. The difference between the total protein and albumin is globulin **[25].** The kidney function assessments: urea **[26]**, uric acid **[27],** and creatinine **[28]** were estimated spectrophotometrically. Total cholesterol (TC) **[29]**, high-density lipoprotein cholesterol (HDL-C) **[30],** and triglycerides (TG**) [31]** were determined. Low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), and the risk ratio were calculated according to **[32, 30, 33].**

Statistical analysis

The results showed as the mean±SE (n=20). Results were analyzed by ANOVA one-way using the IBM-SPSS statistics program (version 25) followed by *post hoc* for multiple comparisons. $P \leq 0.001$ was considered a significant difference.

Results

We planned this study to evaluate the anti-breast cancer effect of vitex hydroethanolic extract and its ethyl acetate fraction as well as their mechanism. To evaluate the anti-breast cancer effect, we estimated

three breast cancer biomarkers; i) relative weight of the breast, ii) carcinoembryonic antigen (CEA a cancer biomarker), and iii) breast tumor characterization (number of tumors developed, weight and volume of tumour). Also, we study the anti-breast cancer mechanism of vitex materials by studying the effect of vitex materials on i) cancer growth rate-limiting enzymes; aromatase and Na/K ATPase activities, ii) COX-2 overexpression, iii) oxidative stress, and iv) estrogen overproduction. Finally, we estimated the effect of Vitex materials on the safety profile of rats to know their toxicity or safety. We mentioned the relationship between each biomarker and breast cancer in the discussion section.

The influence of Vitex in breast cancer biomarkers

In the current study, three cancer biomarkers including relative weight of the breast, carcinoembryonic antigen (CEA), and breast tumor characterization were investigated.

The relative weight of the breast

Vitex hydroethanolic extract or its fraction insignificantly affected the relative weight of the breast in the positive groups compared to the negative control.

As shown in **Fig. 1,** the relative weight of the breast was elevated in cancer-control rats by about 4.65 times more than the negative control ($P \leq 0.001$). Administration of vitex extract or its fraction in the protective route resulted in a significant reduction in the breast relative weight as they recorded 8.11±0.31 and 7.60±0.74% compared to the cancer control (10.56±0.56%) ($P \leq 0.001$). Similarly, hydroethanolic extract or ethyl acetate fraction decreased breast relative weight significantly (7.60±0.61 and 7.23±0.98%, respectively) in the therapeutic groups compared to the cancer control ($P \leq 0.001$). Interestingly, the effect of two vitex materials on the relative weight of breasts in the same group was nearly equal.

Carcinoembryonic antigen (CEA)

The level of CEA of the positive control groups remained stable after administration of vitex materials either hydroethanolic extract or ethyl acetate fraction for three months, compared to the negative control; 281.36±2.01, 277.24±1.92, and 279.97±1.04 pg/ml, sequentially **Fig. 1**.

Conversely, breast induction provoked CEA production in cancer controls (524.17±1.02 pg/ ml) greater than the negative control (279.97±1.04 pg/ ml). Contrariwise, vitex hydroethanolic extract and ethyl acetate fraction treatments significantly depleted CEA production in the protective (349.11±2.32 and 338.19±1.51 pg/ ml, respectively) and therapeutic groups (300.16±1.62 and 291.27±2.05 pg/ ml, respectively).

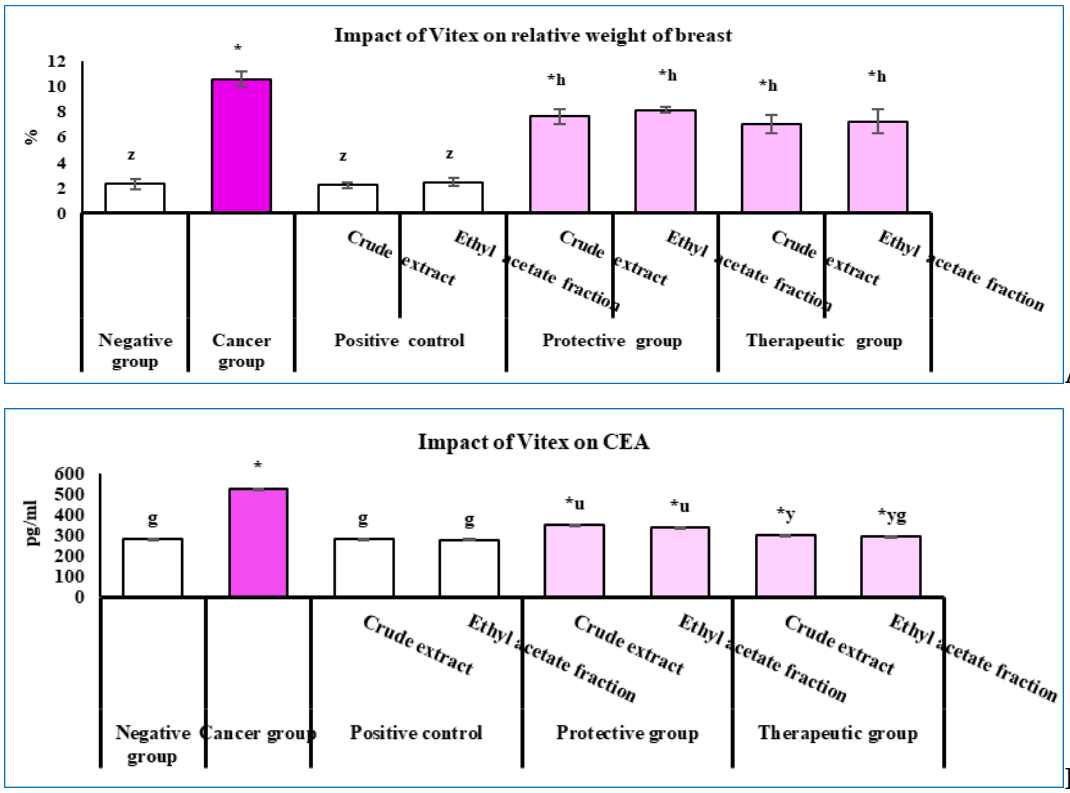


Fig. 1 Effect of Vitex hydroethanolic extract and ethyl acetate fraction in cancer biomarkers: the relative weight of the breast (A) and CEA (B).

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters. Carcinoembryonic antigen (CEA).

Breast tumor characterization

The tumor of breast cancer was characterized by the determined number of cancers developed, weight, and volume of tumor mass as represented in **Table. 1**. Breast cancer induction using DMBA developed the cancer number; 13.50±0.83, with a weight; of 1.15±0.12 g, and volume; of 1.33±0.11 cm^3.

Table 1. The effect of vitex materials on breast tumor characterization.

Parameter	Cancer group	Protective Group		Therapeutic group	
		Hydroethanolic extract	Ethyl acetate fraction	Hydroethanolic extract	Ethyl acetate fraction
Average % of rat body weight gain	390±1.38	280.22±1.54*	264.13±1.62*	257.61±1.89*	231.33±1.17*
Weight difference % concerning negative control (223.15g)	74.77±1.28	25.57±1.01*	18.36±1.13*	15.44±0.97*	8.18±1.01*
No. of cancers developed	13.50±0.83	8.74±0.25*	7.61±0.18*	6.30±0.12*	5.04±0.15*
Weight of tumor mass (g)	1.15±0.12	0.74±0.04*	0.64±0.03*	0.54±0.03*[b]	0.51±0.02*[b]
The volume of tumor mass (cm^3)	1.33±0.11	0.86±0.04*	0.74±0.04*	0.62±0.03*[a]	0.59±0.03*[a]

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.

In contrast, in protective groups, animals that received hydroethanolic extract or ethyl acetate fraction appeared smaller cancers developed number (8.74±0.25 and 7.61±0.18) than the cancer control (13.50±0.83) ($P\leq$ 0.001). Accordingly, they significantly decreased breast cancer weight (0.74±0.04 and 0.64±0.03 g for hydroethanolic extract and ethyl acetate fraction, respectively) and volume of tumor mass (0.86±0.04 and 0.74±0.04 cm^3, respectively) comparing to the cancer control; 1.15±0.12 g and 1.33±0.11 cm^3 ($P\leq$ 0.001). The efficacy of ethyl acetate fraction as a cancer protective agent was more promising than hydroethanolic extract.

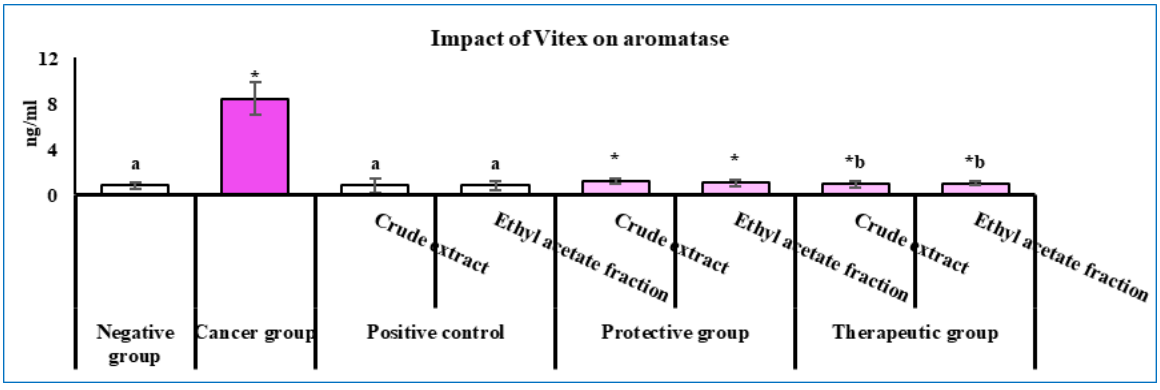
Administration of hydroethanolic extract and ethyl acetate fraction in therapeutic route suppressed cancers developed number (6.30±0.12 and 5.04±0.15, respectively) less than the cancer control (13.50±0.83) ($P\leq$ 0.001). Accordingly, hydroethanolic extract and ethyl acetate fraction significantly reduced tumor mass weight (0.54±0.03 and 0.51±0.02 g, respectively) and volume (0.62±0.03 and 0.59±0.03 cm^3, respectively) compared to the cancer controls ($P\leq$ 0.001). It is evident from the obtained results that tumor characterization of therapeutic group rats was better than the prophylactic group values.

The influence of Vitex in cancer growth rate-limiting enzymes:

Aromatase activity

Vitex materials: hydroethanolic extract and ethyl acetate fraction, exhibited an insignificant change in the activity of aromatase in the positive groups, compared to the negative control; 0.81±0.62, 0.80±0.37 and 0.82±0.31 ng/ ml, respectively **Fig. 2**.

Aromatase in cancer control was highly activated by about 10.26 folds than the negative control ($P\leq$ 0.001). Conversely, vitex hydroethanolic extract significantly inhibited aromatase activity when it was administered both as a protective (1.10±0.26 ng/ ml) and therapeutic (0.91±0.25 ng/ ml), in comparison to the cancer control (8.41±1.42 ng/ ml). Similarly, vitex ethyl acetate fraction statistically suppressed aromatase activity as a protective (1.04±0.31 ng/ml) or therapeutic (1.00±0.17 ng/ml), concerning the cancer control ($P\leq$ 0.001). Vitex materials adjusted aromatase activity toward normal ranges, compared to the negative control.



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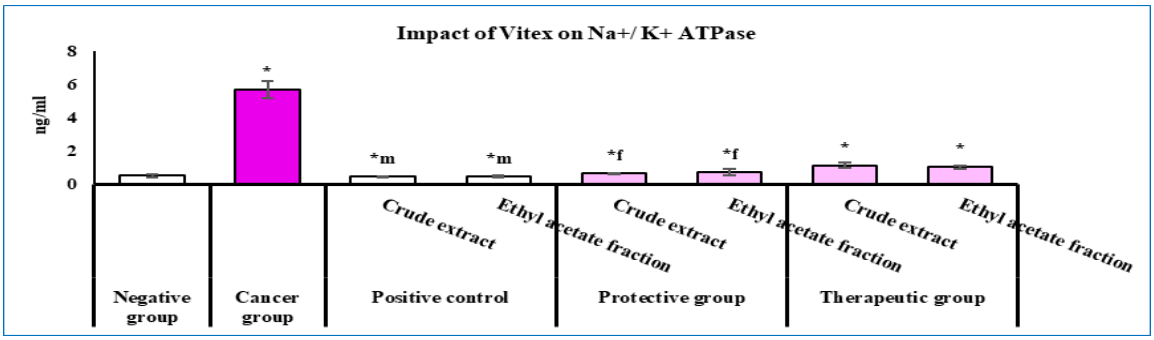

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Fig. 2 Cancer rate-limiting enzymes; aromatase (A) and Na/K ATPase (B) in DIMBA-induced breast cancer female rats treated with Vitex berries hydroethanolic extract and its ethyl acetate fraction.

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.

Na^+/K^+ ATPase activity

Administration of vitex hydroethanolic extract and ethyl acetate fraction for three months significantly prohibited Na^+/K^+ ATPase activity in the positive groups, compared to the negative control; 0.44±0.22, 0.45±0.64, and 0.50±0.11 ng/ ml, respectively ($P \leq 0.001$) **Fig. 2**.

DMBA stimulated Na^+/K^+ ATPase activity in the cancer control (5.70±0.91 ng/ ml) by about 11.40 folds than the negative control ($P \leq 0.001$). Meanwhile, vitex hydroethanolic extract and ethyl acetate fraction remarkably frustrated Na^+/K^+ ATPase activity in the protective group (0.66±0.41 and 0.70±0.19 ng/ ml, respectively) in comparison to the cancer control ($P \leq 0.001$). Additionally, Na^+/K^+ ATPase activity in the therapeutic group was considerably inhibited by the administration of vitex hydroethanolic extract or ethyl acetate fraction to 1.12±0.57 and 1.01±0.21 ng/ml, respectively, compared to the cancer group ($P \leq 0.001$). Worth mentioning that Vitex materials' therapeutic action was more pronounced than the protective effect. In addition, insignificant differences were recorded between the effect of hydroethanolic extract or ethyl acetate fraction on Na^+/K^+ ATPase activity of the same group.

The influence of Vitex in inflammation rate-limiting enzymes

An insignificant difference was observed between COX-1 activity in positive rats that received hydroethanolic extract or ethyl acetate fraction and the negative control. Meanwhile, both vitex materials exhibited selective anti-inflammatory action towards COX-2. Therefore, COX-2 of positive rats was significantly reduced by administration of hydroethanolic extract or ethyl acetate fraction (16.82 and 10.06%, respectively) in comparison to the negative control ($P \leq 0.001$) **Fig. 3**.

Conversely, DMBA showed inflammatory action features where DMBA significantly elevated COX-2 up to 2.68 folds higher than the negative control and dramatically dropped COX-1 to the quarter, compared to the negative control. In the protective group, vitex hydroethanolic extract or ethyl acetate fraction significantly increased COX-1 activity up to 281.50 and 288.97%, respectively, compared to the cancer control. On the other hand, COX-2 of the protective group was statistically depleted by about 53.77 and 54.11% by administration of hydroethanolic extract and ethyl acetate fraction, respectively, compared to the cancer control ($P \leq 0.001$). The protective effect of vitex two materials on COX-1 and COX-2 is equal. In the therapeutic group, vitex hydroethanolic extract and ethyl acetate fraction restored COX-1 activity towards normalization, hence it considerably raised by 302.22 and 317.30%, sequentially, relative to the cancer control. Conversely, Vitex hydroethanolic extract and ethyl acetate fraction significantly declined COX-2 in the therapeutic-treated rats to 57.58 and 63.16%, respectively, in comparison to the cancer control ($P \leq 0.001$). Interestingly, ethyl acetate fraction decreased COX-2 to reach values close to the negative control; 120.11±1.83 and 121.50±0.79 pg/ ml, sequentially. The therapeutic effect of Vitex materials was better than the protective effect.

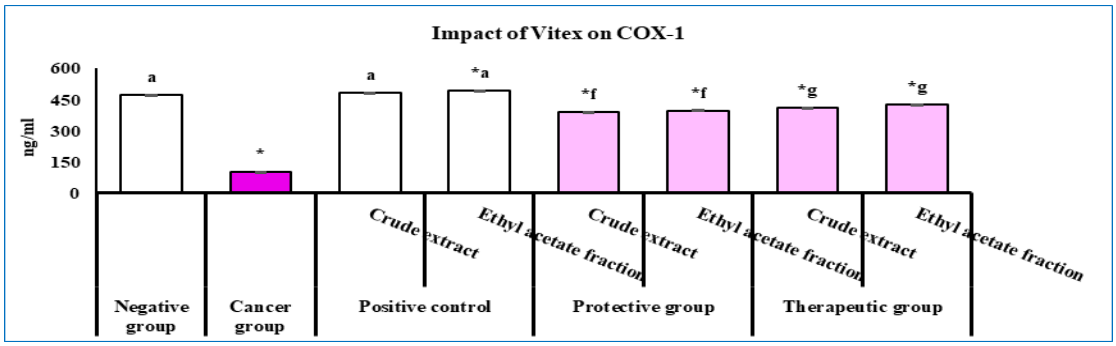
The influence of Vitex on oxidative stress

Vitex materials remarkably improve oxidative stress presented as modulation in either reduced antioxidant biomarkers or magnified lipid peroxidation (represented as MDA). Ethyl acetate fraction significantly decreased lipid peroxidation in the positive groups, meanwhile, hydroethanolic extract reduced it insignificantly, compared to the negative control **Fig. 4**.

DMBA administration evoked lipid peroxidation exhibited a significant increment in MDA level by about 6.67 folds to negative control ($P \leq 0.001$). Vitex hydroethanolic extract and ethyl acetate fraction statistically blockaded lipid peroxidation in the protective group (2.27±0.22 and 2.07±0.20 mmol/ l as MDA), and significantly restored MDA of the therapeutic group towards the normal level (2.22±0.18 and 2.00±0.09 mmol/ l, sequentially), in comparison to the cancer control; 15.00±0.55 mmol/ l ($P \leq 0.001$). Generally, vitex materials reduced MDA close to the negative control.

Antioxidant biomarkers were statistically affected by vitex materials **Fig. 5**. In the positive groups, vitex hydroethanolic extract and ethyl acetate fraction significantly augmented GSH concentration and activated GR, GST, GPx, CAT, and SOD, in comparison to the negative control ($P \leq 0.001$).

In contrast, DMBA administration was associated with oxidative stress conditions occurring in a significant depletion in GSH (0.85±0.21 mg/ dl), and significant suppression in GR (0.94±0.13 µmol/ mg protein/ min), GST (1.11±0.25 µmol/ mg protein/ min), GPx (0.41±0.02 µmol/ mg protein/ min), CAT (4.04±0.15 U/ mg protein), and SOD (6.95±0.07 U/ mg protein) activities in comparison with the negative group ($P \leq 0.001$). In protective group, both hydroethanolic extract and ethyl acetate fraction exhibited significant antioxidant properties as they significantly provoked GSH (4.46±0.38 and 5.10±0.41 mg/ dl) and activated GR (5.02±0.34 and 5.33±0.20 µmol/ mg protein/ min), GST (5.56±0.47 and 6.48±0.44 µmol/ mg protein/ min), GPx (2.54±0.33 and 2.98±0.28 µmol/ mg protein/ min), CAT (21.22±1.31 and 22.32±1.05 U/mg protein) and SOD (12.54±0.26 and 11.80±0.57 U/ mg protein) respectively, in comparison to the cancer control ($P \leq 0.001$).



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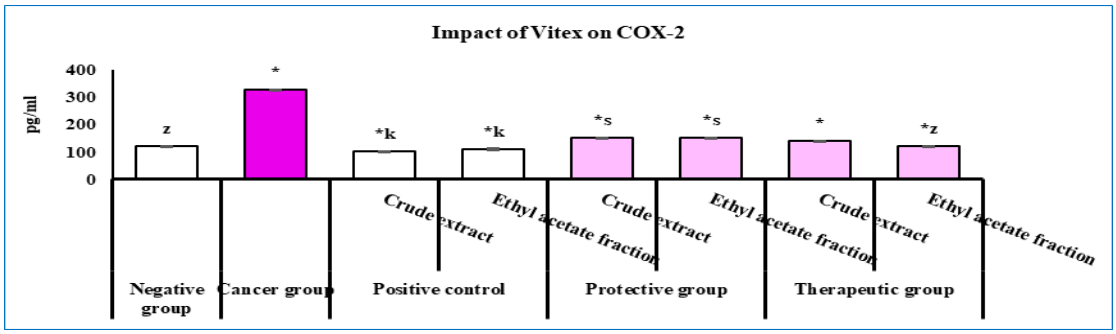

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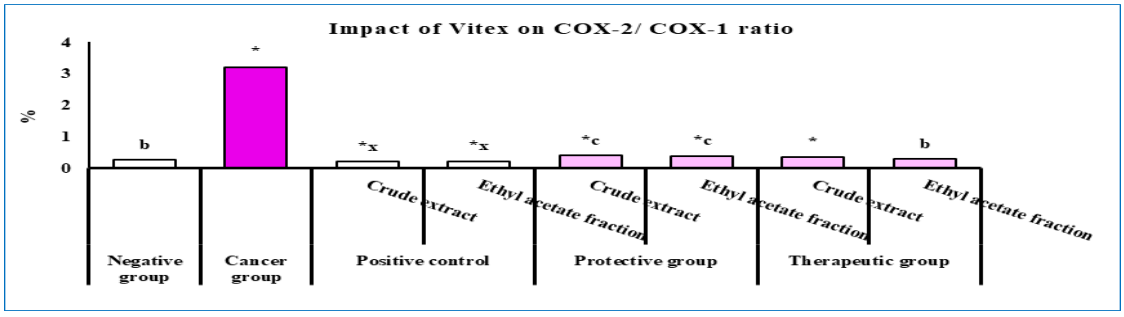


C

Fig. 3 Rat inflammatory rate-limiting enzymes; COX-1 (A), COX-2 (B), and COX-2/COX-1 ratio (C) of breast cancer rats and treated with vitex berries hydroethanolic extract and its ethyl acetate fraction.
The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.

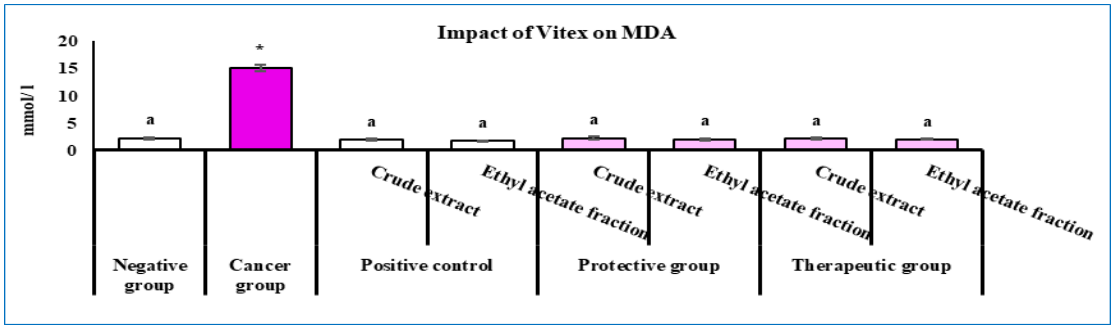
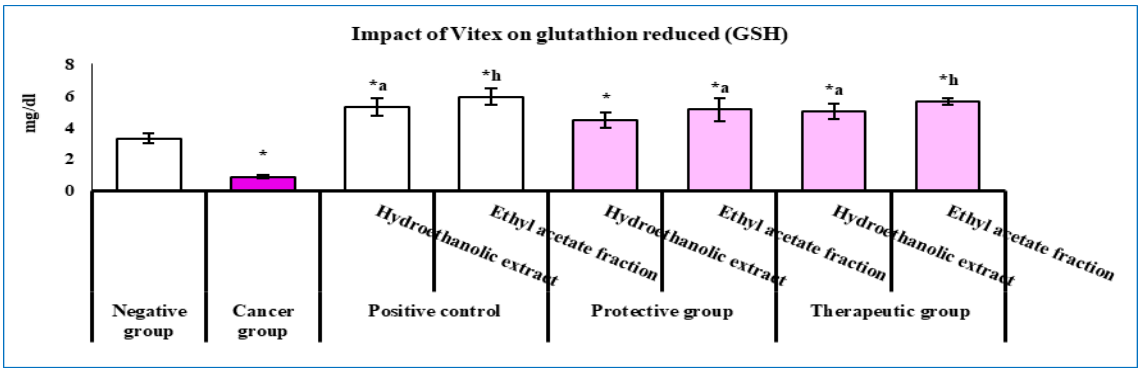


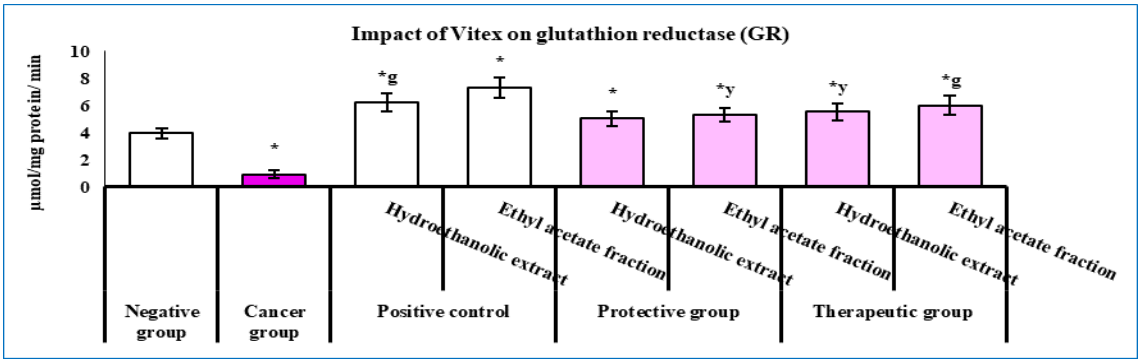
Fig. 4 MDA level of breast cancer rats treated with Vitex berries hydroethanolic extract and its ethyl acetate fraction.
The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.

Similarly in the therapeutic group, vitex hydroethanolic extract and ethyl acetate fraction regained GSH (4.99±0.98 and 5.62±1.23 mg/ dl) and activated GR (5.52±0.19 and 6.01±0.58 µmol/ mg protein/ min), GST (5.18±0.44 and 5.54±0.45 µmol/ mg protein/ min), GPx (2.40±0.19 and 2.78±0.37 µmol/ mg protein/ min), CAT (18.20±1.11 and 20.16±1.03 U/mg protein), and SOD (11.41±0.41 and 11.80±0.33 U/ mg protein) respectively, in comparison to the cancer control ($P \leq 0.001$).

Finally, vitex ethyl acetate fraction was more promising than hydroethanolic extract and the therapeutic effect of vitex was more pronounced than the protective effect.



A



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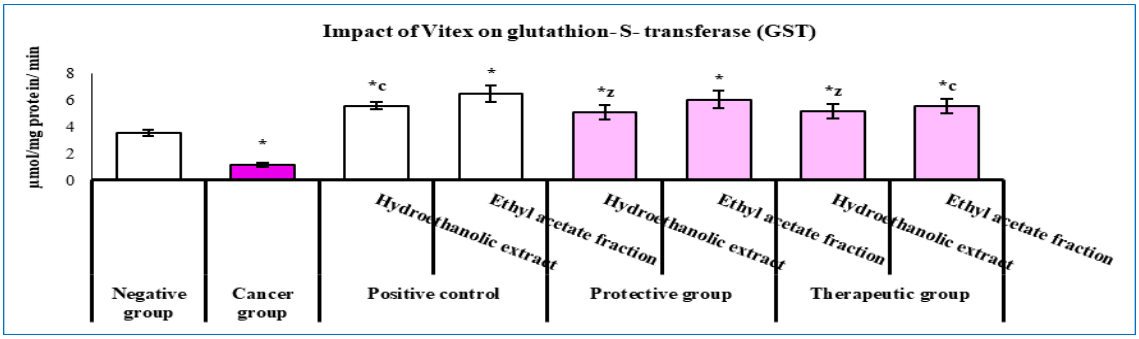

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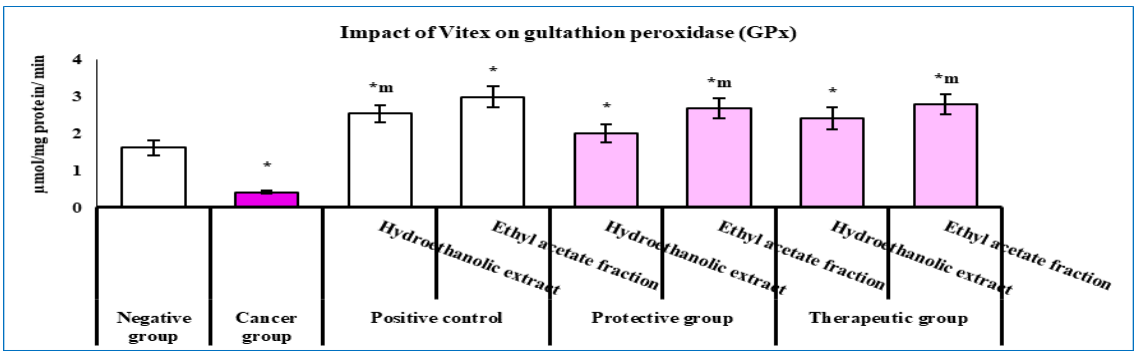


D

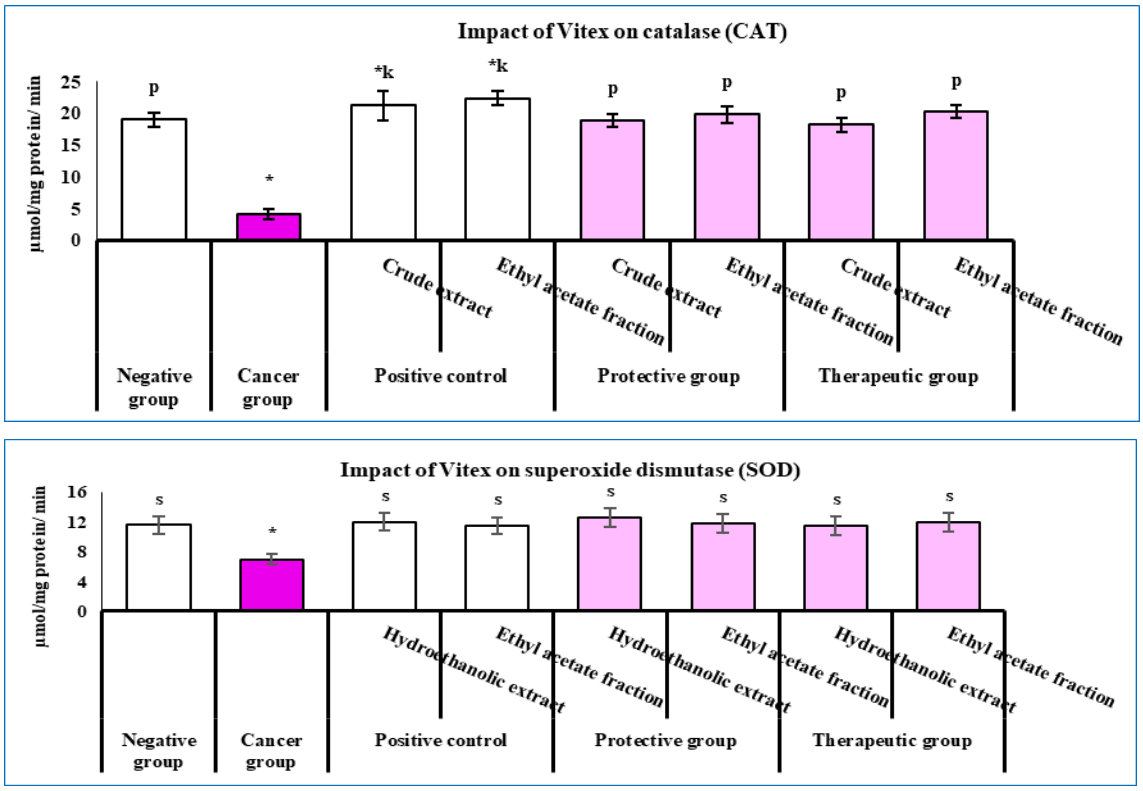


Fig 5 Antioxidant profile of rats with breast cancer treated with vitex hydroethanolic extract and its Ethyl acetate fraction determined in sera samples. GSH (A), GR (B), GST (C), GPx (D), CAT (E), SOD (F).

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.

The influence of Vitex on inducible biomarkers of breast cancer

Lipid profiles

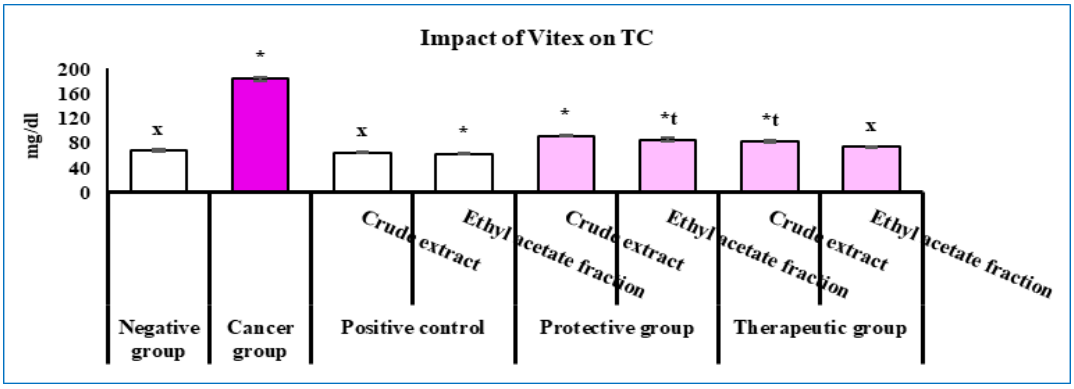
A remarked hypolipidemic effect appeared after oral administration of Vitex materials for three months. In the positive groups, all TC, TG VLDL-C, and LDL-C were significantly reduced than the negative control, concurrent with insignificant change in HDL-C (**Fig. 6**).

Oppositely, breast cancer induction amplified TC (+170.07%), TG, and VLDL-C (+93.82%) and significantly declined HDL-C (-28.55%) in comparison to the negative control ($P \leq 0.001$). Additionally, LDL-C was amplified by about 11.40 folds relative to the negative control. In the protective group, lipid profiles were restored away from the cancer group. Vitex hydroethanolic extract and ethyl acetate fraction statistically minimized TC (-50.10 and -53.48%), TG and VLDL-C (-38.28 and -3.67%), and LDL-C (-75.17 and -79.75%), sequentially, and statistically maximized HDL-C (+37.49 and +41.22%), ($P \leq 0.001$). In the therapeutic group, Vitex recovered the lipid profile disruption that occurred by DMBA oral administration. Vitex hydroethanolic extract and ethyl acetate fraction, significantly reduced TC (-55.52 and -60.07%), TG and VLDL-C (-43.11 and -47.77%), and LDL-C (-81.93 and -87.66%) and statistically maximized HDL-C (+44.12 and +45.15%), respectively, in comparison with cancer group ($P \leq 0.001$). The effect of ethyl acetate fraction in the lipid profile was more effective than the hydroethanolic extract.

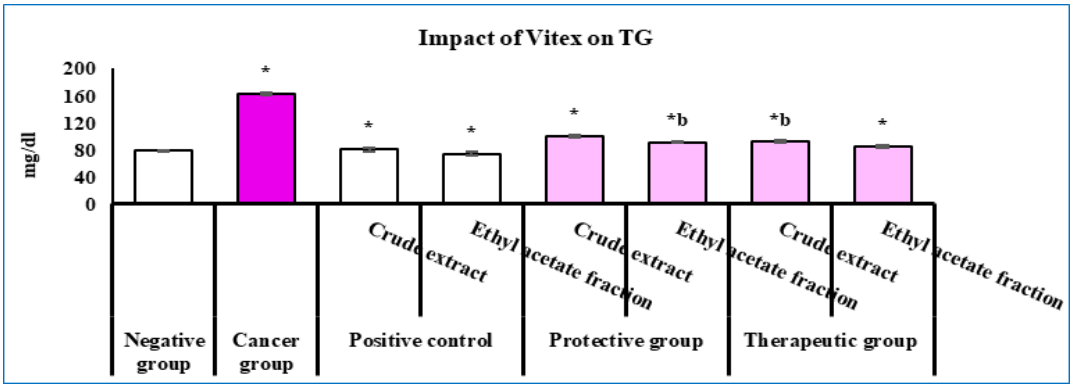
Hormonal status

Orally administration of vitex hydroethanolic extract or ethyl acetate fraction insignificantly affects estrogen or progesterone levels in the positive groups, compared to the negative control (**Fig. 7**).

Concomitantly, a significant hormonal imbalance occurred by DMBA administration. Where estrogen level was increased by about 3.36 folds and progesterone level was decreased by about 30%, relative to the negative control ($P \leq 0.001$). In the protective group, rats orally administrated either hydroethanolic extract or ethyl acetate fraction produced estrogen less than the cancer control by about 36.51 and 44.70%, respectively, and they produced progesterone higher than the cancer control by about 79.14 and 99.34%, sequentially ($P \leq 0.001$).


Impact of Vitex on TC
mg/dl
200
160
120
80
40
0
x
*
x
*
*
*t
*t
x
Crude extract
Ethyl acetate fraction
Crude extract
Ethyl acetate fraction
Crude extract
Ethyl acetate fraction
Negative group
Cancer group
Positive control
Protective group
Therapeutic group

A


Impact of Vitex on TG
mg/dl
200
160
120
80
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*
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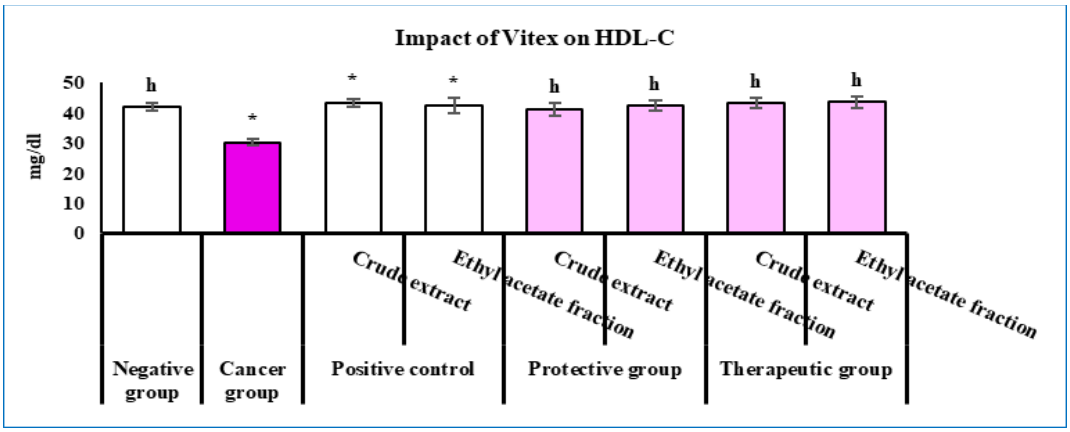
Crude extract
Ethyl acetate fraction
Crude extract
Ethyl acetate fraction
Crude extract
Ethyl acetate fraction
Negative group
Cancer group
Positive control
Protective group
Therapeutic group

B


Impact of Vitex on HDL-C
mg/dl
50
40
30
20
10
0
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*
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Crude extract
Ethyl acetate fraction
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Ethyl acetate fraction
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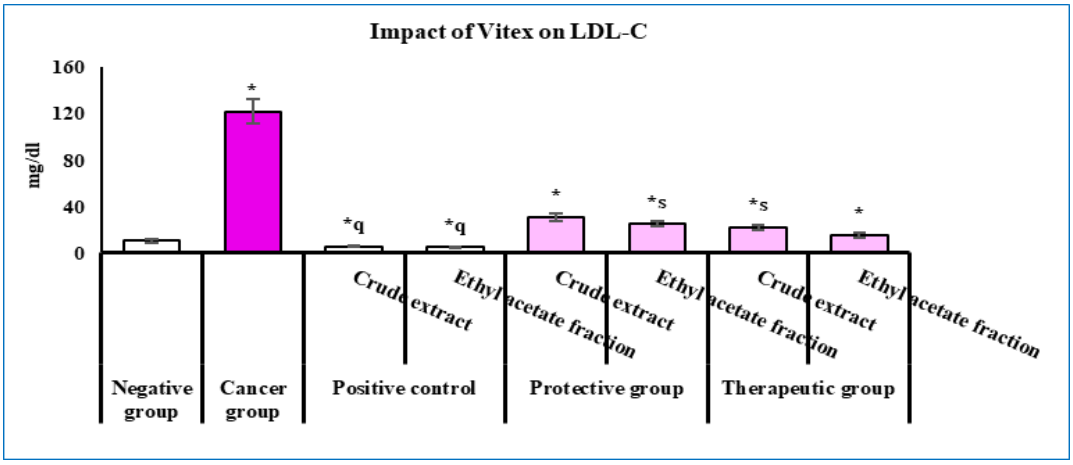
Negative group
Cancer group
Positive control
Protective group
Therapeutic group

C


Impact of Vitex on LDL-C
mg/dl
160
120
80
40
0
*
*q
*q
*
*s
*s
*
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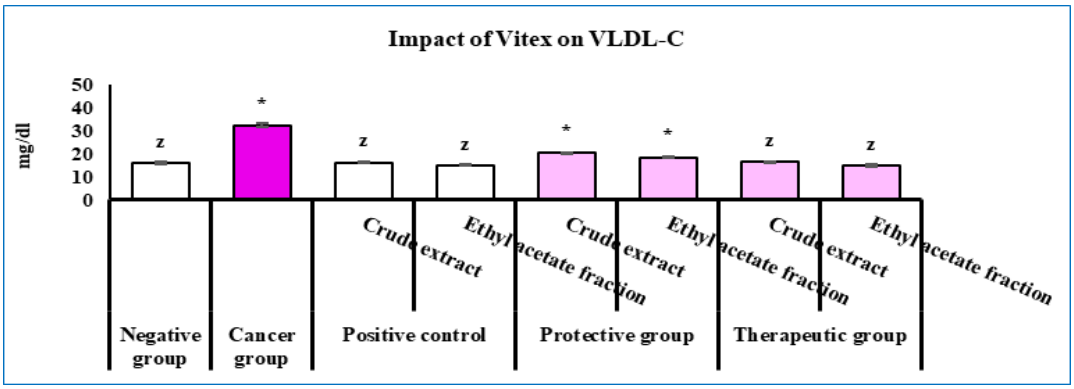
Negative group
Cancer group
Positive control
Protective group
Therapeutic group

D

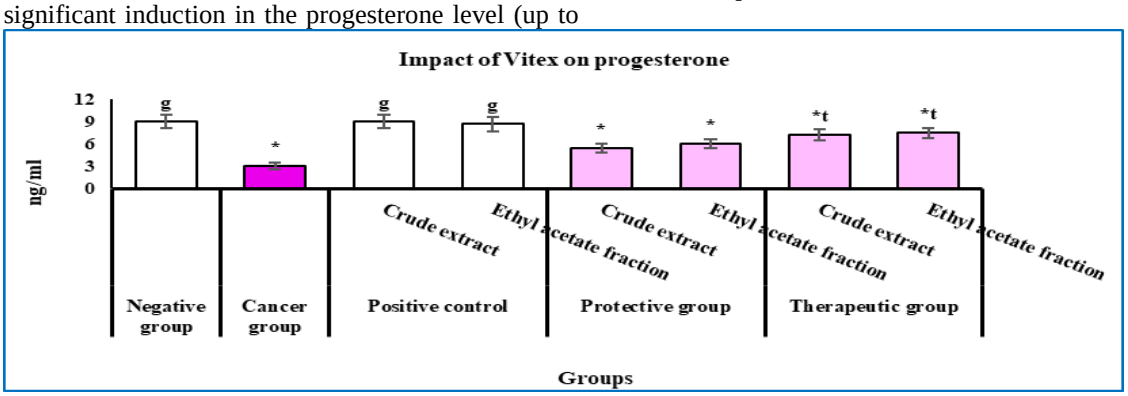


E

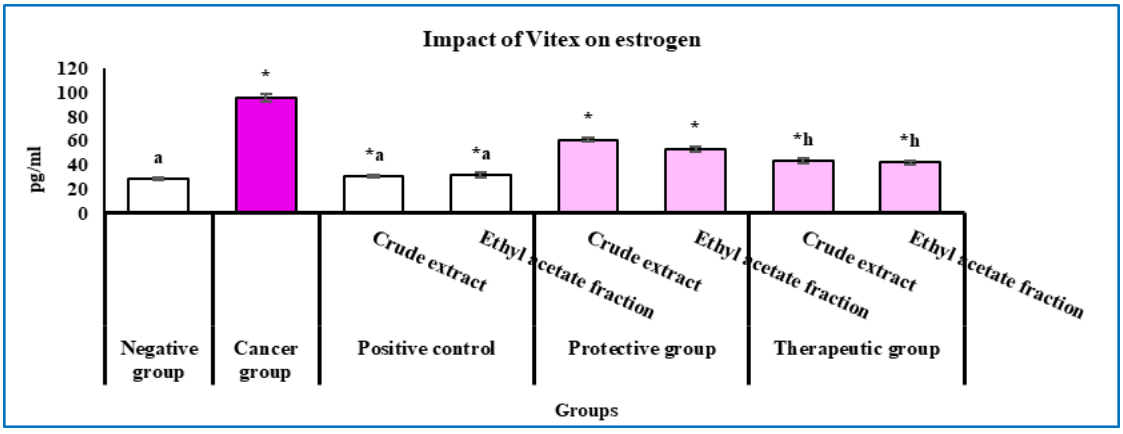
Fig. 6 Impact of Vitex berries hydroethanolic extract and its ethyl acetate fraction on lipid profile in DMBA-induced breast cancer in the female rat. Total cholesterol (A), triglycerides (B), HDL-C (C), LDL-C (D), and VLDL-C (E).

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.

Rats in the therapeutic group restored their hormonal balance by orally administering hydroethanolic extract or ethyl acetate fraction represented a significant reduction in estrogen level (up to 54.29 and 56.32%) concurrent with a significant induction in the progesterone level (up to 137.09 and 146.03%) relative to the cancer control ($P \leq 0.001$). The effect of ethyl acetate fraction on hormonal balance in protective or therapeutic rats was better than hydroethanolic extract. In addition, the therapeutic effect of Vitex materials was better than the protective effect.



A



B

Fig. 7 The impact of vitex berries hydroethanolic **extract and its ethyl acetate fraction on rat sex hormones; progesterone (A) and estrogen (B) of DMBA-induced breast in female rats.**

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.

The influence of Vitex on the safety profile of breast cancer female rats

In the current study, the safety of vitex hydroethanolic extract and ethyl acetate fraction was estimated by determining the relative weight of vital organs (**Table 2**), liver function (**Fig. 8**), and renal function (**Fig. 9**). DMBA administration caused harmful changes in female rats that considered as hepatotoxicity and nephrotoxicity whereas vitex extracts administration appeared safe characters.

The relative weight of the vital organs

Data presented in **Table 1** show that administration of vitex hydroethanolic extract or its ethyl acetate fraction for three months kept the relative weight of organs. Therefore, there are insignificant differences were recorded among the relative weight of organs of hydroethanolic extract-control, ethyl acetate fraction-control groups, and the negative control.

DMBA oral administration produced a significant increase in the relative weight of the liver, spleen, heart, and breast of the cancer control group, compared to that of the negative control ($P \leq 0.001$). In addition, the relative weight of the brain of cancer control did not alter significantly. Conversely, the relative weight of kidneys and lungs in cancer control was significantly decreased in comparison with the negative control.

Vitex hydroethanolic extract or its fraction returned the relative weight of organs of the therapeutic and protective groups towards normal ranges, whereas enlarged organs including the liver, spleen, heart, and breast were reduced significantly compared to the cancer control. Concurrently, shrank organs such as kidneys and lungs were significantly increased compared to cancer control.

Generally, the therapeutic effect of vitex materials was more pronounced than its protective effect, and ethyl acetate fraction was more effective than hydroethanolic extract.

Liver performance

DMBA administration that enlarged liver cancer control significantly harmed liver functions, compared to the negative control ($P \leq 0.001$). DMBA statistically raised AST and ALT activity to 2.32 and 1.81 folds higher than the negative control. On the contrary, TP, albumin, globulin, and GSH concentrations were significantly reduced than the negative control by about 2.64, 2.07, 3.65, and 2.93 folds, respectively **(Fig. 8)**.

Data in **Fig. 8**. indicate that vitex materials did not show any harmful effect on liver performance. Therefore, liver function, including TP, albumin, globulin, and GSH concentrations as well as AST & ALT activities of hydroethanolic extract-control and ethyl acetate fraction-control groups were close to that of the negative control.

Vitex materials administration for three months restored liver function in the protective group near normal ranges. Vitex: hydroethanolic extract and ethyl acetate fraction significantly decreased AST (-53.94 and -56.21%), and ALT activities (-40.92 and -39.92%), respectively, lower than the cancer control, concurrently, with a significant rise in TP (+ 124.63 and + 95.55%), albumin (+ 88.07 and + 103.67%), globulin (+ 188.45 and + 78.72%), and GSH (+ 149.80 and + 117.41%) concentrations relevant to the negative control ($P \leq 0.001$).

In the therapeutic group, vitex materials decreased AST (74.46 ± 1.11 and 71.26 ± 1.17 U/l) and ALT activities (25.50 ± 1.16 and 27.15 ± 0.99 U/l) less than 50% relative to the cancer control. On the contrary, TP (7.93 ± 1.13 and 7.21 ± 1.02 mg/dl), albumin (3.83 ± 0.37 and 4.91± 0.43 mg/dl), globulin (4.10 ± 0.46 and 2.30 ± 0.23 mg/dl), and GSH (6.47 ± 1.03 and 6.87 ± 1.01 mg/g tissue) levels of the therapeutic group were considerably increased more than two-fold relative to the cancer control. The effect of hydroethanolic extract was near to the ethyl acetate fraction in liver performance. In addition, the protective effect of the tested materials was close to its therapeutic effect.

Kidney performance

Administration of vitex for three months insignificantly changed creatinine levels of the positive group, compared to the negative control whereas urea concentration was significantly reduced by two vitex materials (-48.23 and -33.24%), meanwhile, ethyl acetate fraction only significantly induced uric acid by about 10.70% in comparison with the negative control (**Fig. 9**).

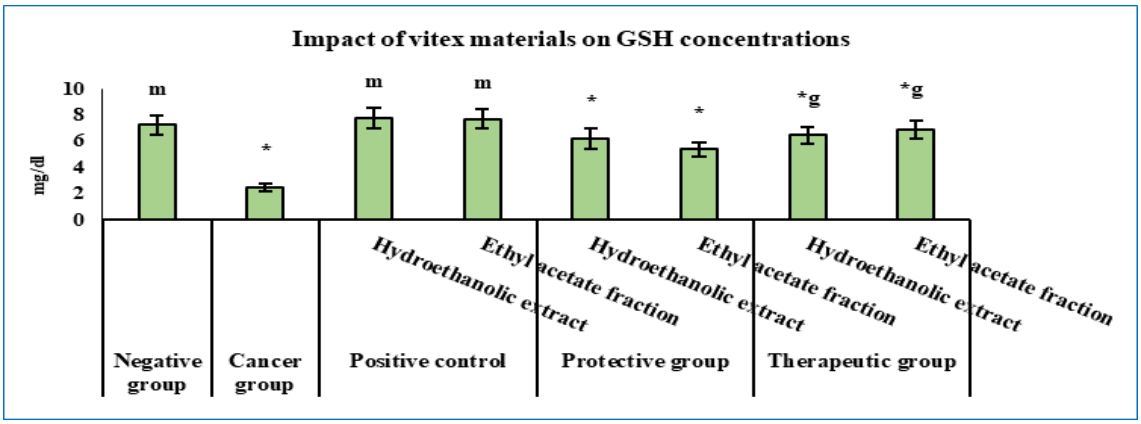
DMBA is considered a disruption in kidney function. DMBA augmented creatinine (4.06 ± 0.77 mg/dl), uric acid (3.06 ± 0.53 mg/dl), and urea (12.43 ± 1.67 mg/dl), compared to the negative control ($P \leq 0.001$).

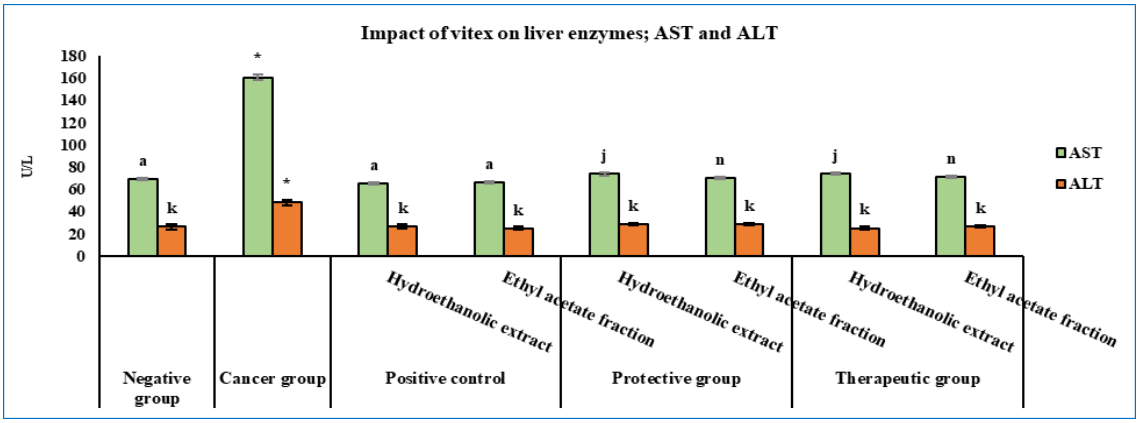
Conversely, in the protective group, vitex; hydroethanolic extract or its ethyl acetate fraction significantly decreased creatinine (3.68 ± 0.36 and 3.79 ± 0.22 mg/dl), uric acid (2.62 ± 0.27 and 2.64 ± 0.33 mg/dl), and urea (7.15 ± 0.52 and 9.02 ± 0.98 mg/dl), respectively, concerning the cancer control ($P \leq 0.001$). In addition, in the therapeutic group, vitex materials; hydroethanolic extract, and ethyl acetate fraction, restored kidney functions toward normalization or near to normal. Vitex hydroethanolic extract and ethyl acetate fraction statistically reduced creatinine (– 13.80 and – 13.05%), uric acid (- 22.88 and – 11.77%), and urea (- 56.56 and – 49.96%), sequentially, relative to the cancer control ($P \leq 0.001$).

Table 2. Effect of Vitex hydroethanolic extract and ethyl acetate fraction on vital organs of chemically-induced mammary cancer in female rats and sub-chronic toxicity margin through 90 days.

Parameter / Group		Organ's weight (g/ 100 g)						
		Liver	Kidney	Spleen	Lung	Heart	Brain	Total Breast
-ve control		2.78±0.20^{a}	1.01±0.35	0.39±0.05^{q}	0.80±0.14^{s}	0.44±0.11	0.98±0.31^{y}	2.27±0.37^{z}
Cancer group		4.69±0.86*	0.71±0.09	0.95±0.30*	0.66±0.12*	0.52±0.16	1.06±0.24*	10.56±0.56*
Positive group	Hydroethanolic extract	2.81±1.01^{a}	0.98±0.31	0.41±0.11^{q}	0.81±0.13^{s}	0.43±0.14	1 ±0.13^{y}	2.21±0.23^{z}
	Ethyl acetate fraction	2.80±1.00^{a}	0.97±0.19	0.41±0.18^{q}	0.80±0.21^{s}	0.44±0.11	0.98±0.11^{y}	2.42±1.04^{z}
Protective Group	Hydroethanolic extract	3.29±0.97*^{g}	1.02±0.23	0.83±0.24*	0.80±0.22^{s}	0.51±0.14	1.01±0.14^{y}	8.11±0.31*^{h}
	Ethyl acetate fraction	3.25±0.65*^{g}	1.00±0.21	0.85± 0.20*	0.79±0.17^{s}	0.46±0.92	1.02±0.22*	7.00±1.14*^{h}
Therapeutic group	Hydroethanolic extract	3.27±1.02*^{g}	1.01±0.15	0.63±0.16*	0.80±0.15^{s}	0.49±0.21	1.02±0.12*	7.60±1.01*^{h}
	Ethyl acetate fraction	3.27± 0.81*^{g}	1.00±0.31	0.91±0.15*	0.78±0.15^{s}	0.45±0.13	1.00±0.16^{y}	7.23±0.98*^{h}

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.





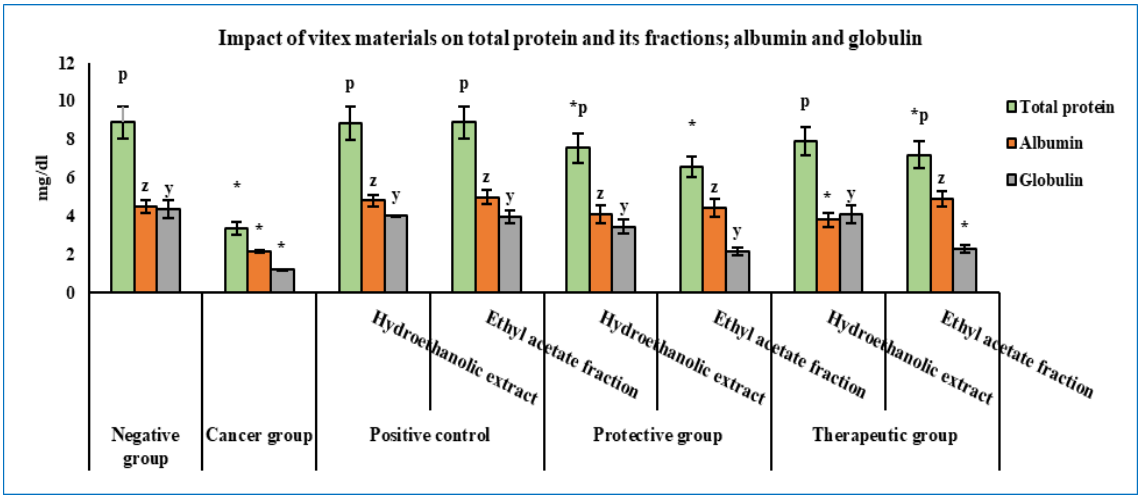


Fig 8. Impact of vitex hydroethanolic extract and ethyl acetate fraction on liver function protein and glutathione in DMBA induced breast cancer in the female rat.

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letters.

Generally, the effect of hydroethanolic extract and ethyl acetate fraction in creatinine and uric acid was close in the protective and therapeutic groups. Meanwhile, their effect on urea differed, where hydroethanolic extract was more effective than ethyl acetate fraction. In conclusion, the therapeutic effect of Vitex on kidney function was more promising than its protective effect.

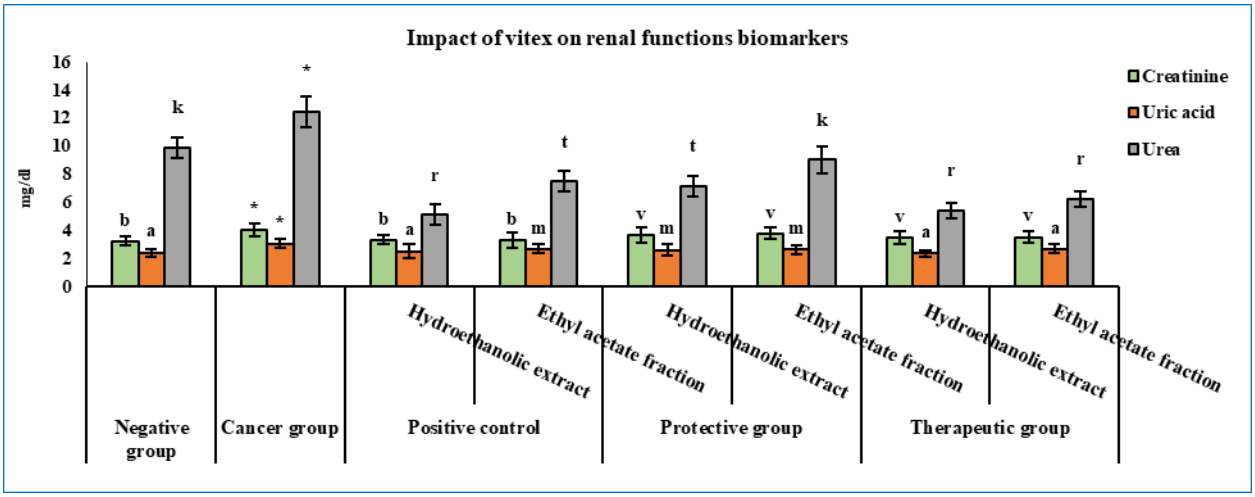


Fig. 9. Effect of Vitex berries hydroethanolic extract and its ethyl acetate fraction on kidney function in DMBA-induced breast cancer in female rats.

The results presented as a mean± SE (n=20). Results were analyzed by ANOVA one-way followed by post hoc for multiple comparisons. * indicates significant results compared to ve- -controls, while the letters indicate a non-significant difference between groups with similar letter.

Discussion

The current study was performed to evaluate the ability of two vitex materials: hydroethanolic extract and its ethyl acetate fraction, to treat and/or prevent breast cancer. 7,12 dimethylebenz-(a)-anthracene (DMBA)-mediated mammary carcinomas in Sprague Dawley female rats were used as an experimental model. DMBA administration stimulated inducible breast cancer biomarkers, including hyperlipidemia, estrogen, cancer growth rate-limiting enzymes (aromatase and Na^+/K^+ATPase), pro-inflammatory (COX-2), and oxidative stress. Meanwhile, DMBA inhibited preventive biomarkers of breast cancer, including antioxidant enzymes, anti-inflammatory COX-1, and progestogen. Taken together, DMBA produced mammary carcinomas in rats representing a significant increment in the relative weight of the breast, CEA concentration, No. of cancer developed, the weight of tumor mass, and the volume of the tumor mass.

Conversely, the administration of Vitex materials for three months recorded promising results. Vitex, as a protection or treatment caused considerable improvement in DMBA-mediated breast cancer in rats. Vitex significantly ameliorated inducible biomarkers of breast cancer that support i) balance in lipid metabolism, ii) modulation in estrogen production, iii) suppression of aromatase and Na^+/ K^+ATPase activities, iv), inhibiting COX-2 activity, and v) reduction of MDA. Concurrently, vitex materials promoted preventive biomarkers of breast cancer as significant i) activation in antioxidant enzymes activity, ii) stimulation of cell maintenance by induction of COX-1 activity, and iii) increasing progestogen production. Compared to the cancer control, vitex statistically reduced the relative weight of the breast, CEA level, No. of cancer developed, the cancer's weight, and the volume of the tumor mass. The obtained results agreed with **Youness et al. [6]** on Broccoli, **Ibrahim et al. [9]** on acidic exopolysaccharide produced from marine *Bacillus amyloliquefaciens* 3MS 2017.

In the following, the explanation of the Vitex materials efficacy approach in the treatment and prevention of mammary carcinoma induced by DMBA in rats. Vitex is struggling with DMBA-induced mammary carcinoma in rats through two paths: reducing inducible biomarkers concurrently with inducing prevention biomarkers of breast cancer.

Firstly, vitex materials considerably reduced inducible breast cancer biomarkers, including lipid metabolism disruption, increased estrogen hormone, and activated cancer growth rate-limiting enzymes activity such as aromatase, Na^+/K^+ ATPase, inducible COX-2 expression, and the lipid peroxidation biomarker.

The concept of Vitex ameliorated the lipid metabolism disruption: In postmenopausal women, obesity and metabolic syndrome are the main modifiable risk factors for breast cancer progression. Using over 1 million Korean patients, **Kitahara et al. [34]** found that high cholesterol concentration positively correlates with prostate and colon cancers in men and breast cancer in women. In addition, a cohort study in 1996–2003 reported that lipophilic statins like simvastatin were linked to decreased cancer recurrence **[35]**. Furthermore, cholesterol involves estrogen receptor-positive breast cancer in mice via stimulation of aromatase over-expression. On the other hand, cholesterol encourages the estrogen receptor-positive breast cancer progression in mice through the metabolite 27-hydroxycholesterol (27HC) which accelerates the growth of cancer cells. CYP27A1 is the enzyme that is responsible for 27HC production. In human breast cancer, CYP27A1 levels were readily expressed in higher-grade cancers **[36]**. Concomitantly, aromatase which is a key step in the synthesis of estrogens is expressed in undifferentiated adipose fibroblasts and breast tumors. Therefore, increasing breast adipose tissue increases estrogen production via aromatase-over-expressing fibroblasts. Cancer cells require cholesterol to proliferate **[37]**. Therefore, vitex materials could prevent cell cancer proliferation regarding this concept.

Vitex suppressed the expression of inducible COX-2 enzyme concept: COX-2 protein is predominantly confined to the tumor epithelium, with negligible expression in normal epithelium. The relation between COX-2 over-expression and breast cancer was confirmed as i) elevation in COX-2 protein levels in 40% of invasive breast carcinomas, ii) the inverse relationship between COX-2 protein levels and disease-free survival, iii) COX inhibitors can reduce the growth rate of implanted tumors, iv) complete ablation of COX-2 reduced the mean tumor multiplicity, v) COX-2 contributes to tumor growth as was observed in COX-2 null animals, vi) transgenic over-expression of COX-2 is sufficient to induce mammary neoplasia in multiparous animals **[38]**. Additionally, COX-2 overexpression is involved in breast cancer via i) stimulation of angiogenesis, ii) activation of aromatase, iii) reduction of apoptosis, iv) stimulation of cell proliferation, v) depression of immune system, and vi) promotion of cell invasiveness. The relation between COX-2 over-expression and angiogenesis is demonstrated by several phenomena as follows; i) decrease in mammal blood vessels COX-2 null models, ii) angiogenesis-linked genes were considerably downregulated as VEGF (vascular endothelial growth factor), *Ang1* and *Ang2* (Tie-2 ligands), and *Flk-1* & *Flt-1* (vascular endothelial growth factor receptors), iii) COX-2 over-production in mouse mammary gland promoted tumorigenesis. Accordingly, we suggested that the breast cancer inhibitory effect of vitex materials in part attributed to its anti-inflammatory action exhibited in this study as suppression of COX-2.

Vitex reduced the elevated estrogen levels concept: Approximately 70% of all tumors express the estrogen receptor (ER), a transcription factor that is induced by estrogen binding and that adjusts the production of several genes implicated in tumorigenesis **[39]**. It was confirmed in rodents that estrogens or their catechol metabolites are carcinogens in mammary glands. The metabolite, estrogen 3,4-quinone can create unstable adducts with adenine and guanine in DNA, resulting in depurination and mutation either *in vitro* or *in vivo*

[40]. Additionally, elevated endogenous estrogen concentrations, a magnitude breast cancer risk. 16-hydroxylated metabolites of estrogen, specifically 16-α-hydroxy estrone, induce cancer cell growth and proliferation of breast cells in animal models **[41]**. Therefore, an estrogen deprivation strategy including, antiestrogens (Tamoxifen) or estrogen blockers (Fulvestrant) and aromatase inhibitors (Anastrozole and Ovariectomy) is used for the treatment of breast cancer. In this study, estrogen level was significantly minimized by vitex materials. So, the anti-breast cancer of vitex materials could be attributed to estrogen regression.

Vitex inhibited cancer growth rate-limiting enzymes activity; aromatase, and Na^+/K^+ ATPase concept:

1) Aromatase activity*:* Based on the two facts; i) the role of estrogen in breast cancer, which has already been introduced, and ii) the role of aromatase enzyme in estrogen synthesis, aromatase is involved in breast cancer. In hormone receptor-positive (HR+) breast cancer, aromatase is extremely overproduced in breast endothelial cells and the surrounding stroma increases local estrogen in the tumor microenvironment, which induces cancer progression via estrogen receptor stimulation. The third generation of aromatase inhibitors (AIs) exhibited a considered role in the endocrine protection and treatment of HR+ breast cancer. The International Breast Cancer Intervention Study II (IBIS-II) found that AIs reduced breast cancer incidence in postmenopausal women with high risk for breast cancer **[42-43]**. Therefore, the anti-breast cancer efficacy of Vitex material could be due to being an aromatase inhibitor.

2) Na^+/K^+ ATPase activity: Changed expression of the Na^+/K^+ ATPase has been demonstrated in mammary carcinoma. Microarray analysis reported a remarked (1.5-fold) elevation in the production of the *ATP1A1*gene (coding the α1-subunit of Na^+/K^+ ATPase) in breast cancer patient groups **[44]**. **Khajah et al. [45]** reported that Ouabain, a specific high-affinity sodium pump inhibitor, significantly inhibited cell proliferation, motility, and invasion. The antiproliferative effect of ouabain was in part transmitted through modulation of the cell cycle and apoptotic machinery. Therefore, it could be suggested that the anti-breast cancer property of vitex materials is due to its inhibitory effect on Na^+/K^+ ATPase.

Vitex reduced oxidative stress concept: Oxidative stress is linked to three stages of cancer: initiation, progression, and invasion. It causes DNA mutations leading to DNA injury, genomic instability, and the promotion of cell proliferation. Cancer cells release ROS amount is higher than normal cells, which increases the cell signaling pathways needed for cellular alternation and carcinogenesis. ROS promotes several growth factors, like VEGF and HIF-1α transcription factors leading to induction of cancer progression and metastasis **[46]**. Accordingly, malondialdehyde is significantly elevated in patients with cancer **[47]**. In the current study, vitex materials considerably restrained MDA production.

Secondly, vitex materials significantly stimulated breast cancer inhibitor biomarkers, including antioxidant biomarkers and progesterone.

Vitex activated antioxidant biomarkers*:* Tumor cells produce large amounts of ROS evident by mitochondrial defects and a decreased expression of antioxidant enzymes. **Elyasinia et al. [48]** found that SOD activity is lower in patients with breast malignancy. Catalase can modulate the cancer growth rate by degrading H_2O_2 and protecting certain proteins involved in the proliferation and migration from oxidative damage **[49].** GSH works as the organizer of cellular redox conditions preventing cells' damage to lipid peroxidation, ROS and NOS, and xenobiotics. On the other hand, GSH is a key signal transduction reaction as a regulator of; cell differentiation, proliferation, apoptosis, ferroptosis, and immune function. Molecular changes in the GSH system and disorders in GSH homeostasis involved in tumor initiation, progression, and treatment response **[50]**. In parallel, GSTs, the phase-II detoxification enzymes have a cytoprotective function and have an essential role in the detoxification of various carcinogens, and reactive metabolite intermediates, that are potentially involved in breast cancer **[46]**. In accordance, GPxs types are implicated in the protection from cancer as GPx1 can prevent oxidative DNA mutations and prohibit tumor progression. Additionally, the overproduction of GPx1 can inhibit tumor growth, indicating its suppressive action in tumorigenesis **[51].** According to the above mentioning, vitex materials could participate in cancer prevention and treatment as they significantly increased GSH with remarkable activation in antioxidant enzymes; GR, GST, GPx, CAT, and SOD activities.

Vitex stimulated progesterone production: The concept of progesterone (P4) as an inducer or reducer of breast cancer risk, has not been fully elucidated. A combination of estrogen with most synthetic progestogens or progestins elevates the breast cancer risk in postmenopausal women. Meanwhile, the combination of estrogen with natural progesterone has been linked to less breast cancer

risk **[52]**. Furthermore, low progesterone receptors (PR) are linked with overexpression of growth factor signaling and aggressive tumors **[53]** as progesterone modulates RNA Polymerase iii-mediated transcription and downstream translation which regulates overall tumor growth **[54]**. In accordance, Vitex materials significantly increased progesterone as a protective or a curative agent in comparison to cancer control.

Finally, we could report that vitex extract fought DMBA-induced mammary carcinomas in rats by seven mechanisms, i) suppression of COX-2, ii) inhibition of aromatase activity, iii) suppression of Na^+/K^+ ATPase activity, iv) reduction of oxidative stress, v) activation of antioxidant enzymes, vi) reducing estrogen, and vii) elevation progesterone. Vitex extract showed these properties due to its anti-androgenic effect that was documented by many researchers. Where, vitex contains dopamine, opioid, and estrogen receptor ligands, which modulate hormone levels and lower prolactin levels **[55]**. It regulates sex hormone levels, through regulation of the pituitary gland and LH (luteinizing hormone) production **[56]**. The positive effects of vitex on the increased levels of prolactin could also account for its anti-androgenic effects **[57]**. It was reported by **Mahady [58]** that vitex significantly inhibited basal and thyroid-stimulating hormone-stimulated prolactin secretion from rat primary pituitary cell cultures, *in vitro*. In addition, Vitex contains estrogen-like compounds, phytoestrogen, which are bound to estrogen receptors and produce the same effect as estrogen **[59]**. Increasing estrogen levels led to reduction in LH production, which decreases testosterone production **[60]** that decreases circulating estrogen, and ovulation, and it also elevates progesterone levels; it balances the estrogen/ progesterone ratio.

Additionally, many active components identified in the Vitex berries extract and its ethyl acetate fraction have anticancer action. For example, but not limited to, Casticin is a major bioactive flavonoid isolated from the Vitex species, recorded a potent anti-tumor ability via its multitarget properties and selectivity in cancerous tissues, which are investigated both at the cellular level and the molecular level on different signaling pathways **[61]**. Casticin suppresses breast cancer cell migration and invasion via down-regulation of the PI3K/Akt signaling pathway. **[62]**. Vitexin, the major component of vitex, has shown anticancer effects in the cancer cell line by inducing apoptosis by suppressing PI3K/Akt/mTOR signaling in human nonsmall cell lung cancer A549 cells (NSCLC). Vitexin inhibited NSCLC tumor growth, up-regulated Bax expression and cleaved caspase-3, and down-regulated Bcl-2 expression. Similarly, vitexin promoted ROS production through the activation of JNK and induced the expression of autophagy marker proteins Beclin-1, Atg5, and microtubule-associated protein light chain 3-II (LC3-II), which encourage autophagy induction in colorectal carcinoma cells. In addition, vitexin reduced tumor growth via decreasing p-p65 and Cyclin D1 expression in the NPC xenograft mouse model **[63]**. Agnuside is an iridoid glycoside isolated from an ethyl acetate fraction of crude extract of *Vitex agnus castus* berries. Agnuside showed a cytotoxic effect in COLO 320 cancer cells with an IC_{50} value of 15.99 µg/ml **[64]**. Aucubin is an iridoid glycoside, that recorded cytotoxic effects in breast cancer (model with mouse 4T1 cell line and BALB/c mice). Aucubin suppresses tumor growth by promoting tumor cell apoptosis **[65]**.

Conclusion

The current study confirmed our hypothesis that higher COX-2 expression results in higher aromatase expression, which, in turn, increases breast cancer progression via increases in estrogen production. Vitex materials, as a selective anti-inflammatory material, suppressed COX-2 overexpression, which protected and treated the carcinogenic features induced using DMBA-induced breast cancer in rats and reduced estrogen production. Furthermore, vitex exhibited significant inhibition of Na^+/K^+ATPase activity concurrent with significant activation in antioxidant enzymes. This study can be generalized to a broader study population and can be incorporated into application and formulation in clinical trials as the extract, depending on this study data, is completely safe.

Conflict of interest: The authors declare no conflict of interest.

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