

The Anti-proliferative Activity of *Vitex agnus-castus* Leaves Methanol Extract against Breast and Prostate Cancer Cell Line

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ABSTRACT

Objectives: The aim of this study was to investigate the capability of *Vitex agnus-castus* methanol extract which showed anti-angiogenic activity by Hayder and co-worker in previous study, to inhibit cancer cell line proliferation, and to identify the possible mechanism of action.

Methods: The anti-proliferative activity of the methanol extract tested by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on breast cancer cell line (MCF7) and Prostate cancer cell line (PC3) which were purchased from American type culture cell (ATCC). 1, 1-diphenyl -2-picrylhydrazyl (DPPH assay) has been used to identify the free radical scavenging activity.

Results: Dose response relationship was shown. No significant cytotoxic activity of methanol extract has identified on MCF7 and PC3, the IC₅₀ were 46.52µg/ml and 90.01µg/ml respectively. Methanol extract presented free radical scavenging capability the IC₅₀ was (126.79mg/ml).

Conclusion: These finding showed that methanol extract had a significant dose-dependent efficiency against the growth of the cells MCF7 and PC3. At the same time, the extract did not show any cytotoxic action at the applied dose, so no toxic effect against the MCF7 and PC3 cell line can be expected *in vitro*. The capability of the *Vitex agnus-castus* methanol extract in scavenging the free radicals may elucidate the mechanism of action on cell line.

Keywords: *Vitex agnus-castus*, Methanol extract, Cell line, Anti-proliferation.

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INTRODUCTION

Cancer well-known pathologically as malignant neoplasia, is an extensive group of diseases involving unregulated cell growth. In cancer, cells divide and develop violently, forming malignant tumors, which may attack nearby cells¹. The cancer may also spread to more distant parts of the body over the lymphatic system or bloodstream. Not all tumors are cancerous; benign tumors do not attack neighboring tissues and do not spread throughout the body. There are over 200 different known cancers that affect humans¹. Cancer is one of the main causes of death globally often with poor clinical prediction. The causes of cancer are various, complex, and only partially understood². Many things are known to increase the risk of cancer, including tobacco use, dietary factors, certain infections, exposure to radiation, lack of physical activity, obesity, and environmental pollutants³. These factors can cause a conventional damage gene or association with existing genetic faults within cells to cause cancerous mutations⁴. Approximately 5-10% of cancers can be found directly to inherited genetic faults⁵. Many cancers could be prohibited by avoid smoking, eating more vegetables, fruits and whole grains, eating less meat and refined carbohydrates, keeping a good physical shape and weight, exercising, minimizing sunlight exposure, and being vaccinated against some infectious diseases⁶. *Vitex agnus-castus*, also called *Vitex*, *Chaste Tree*, *Chasteberry*, *Abraham's Balm*⁷, is a native of the Mediterranean region. It is one of the few temperate-zone species of *Vitex*, which is on the whole a genus of tropical and subtropical flowering plants⁸. *Vitex agnus-castus*, is a shrub belonging to the genus *Vitex* of the Verbenaceae family. It is common on riverbanks and on shores in the Mediterranean region and in many places in Asia. *Vitex agnus-castus* is a deciduous

shrub which extends heights of 5m⁹. Study done on 2014 approved that methanol extract of *Vitex agnus-castus* had a potent anti-angiogenesis activity and, as angiogenesis consider one of the cancer treatment approach, methanol extract nominated to be tested against two cancer cell line to find whether this extract has anti-cancer activity or not⁹.

MATERIALS AND METHODS

Assessment of proliferation inhibition of cancer cell line¹⁰

The (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT assay was used to identify the cell line proliferation capability in according to Mosmann method. All of the cells were between passages 4-7. The cells were treated with numerous concentrations of *Vitex agnus-castus* extract for 48 hrs. MTT was prepared by adding 5mg/ml in PBS (phosphate buffer saline). 20 μ l of MTT was used per well and the plates were incubated at 37°C, in 5% CO₂ for 5hrs. The plates were removed from the incubator and the supernatant was removed. (200 μ l) of DMSO was added to all wells. The plates were shaken vigorously for one minute at room temperature to dissolve the dark blue crystals. The absorbance was taken at 570nm and the reference at 650nm by using enzyme-linked immunosorbent assay (ELISA). The absorbance of cells cultured in control media was taken to represent 100% viability. The viability of treated cells was determined as a percentage of untreated control. Each concentration was tested in quadruplicate, and the experiment was repeated twice. The concentration of the cells in each well was 1x10⁴, the percentage of cell line inhibition was determined as the mean \pm SD, using the following equation.

$$1-(A_0-A_1)/(A_2-A_1)$$

A_0 = Absorbance of sample

A_1 = Absorbance of blank

A_2 = Absorbance of control

IC_{50} values were calculated by the linear and logarithmic correlation equation.

Cell lines

Breast cancer cell line (MCF-7), and Prostate cancer cell line (PC3) were used in this study. The entire cell lines were maintained in its specific medium; RPMI-1640, (Gibco.UK) were used to maintain MCF-7 and PC3. Heat inactivated foetal calf serum (HIFCS) was used by 10% the serum purchased from (Gibco, UK), and 1% pen/strep (Penicillin/streptomycin) (Sigma-Aldrich, Germany) was added to those mediums to make complete growth medium as mentioned in the growth medium sheet provided with cell line. Each medium that was made have complete growth medium prior to the experiments. Sequential dilutions from *Vitex agnus-castus* were prepared by dissolving the samples in DMSO and diluting it with the medium used for each cell line. The final DMSO concentration in the medium was 1%; the control wells received 200 μ l from the medium with the final DMSO concentration, while the samples were added to the well in quadruplicate, incubated in the incubator at 37°C, with 5% CO₂ for 48hrs. MTT added on the cell and incubated for 4 hours prior to the absorbance measurements at 570nm¹⁰.

1, 1-diphenyl -2-picrylhydrazyl (DPPH) scavenging activity

The free radical scavenging activity of the *Vitex agnus-castus* methanol extract was measured by DPPH. One ml of 0.1 mM solution of DPPH was added to 2ml *Vitex agnus-castus* methanol extract, with the following concentrations (0.5, 0.25, 0.12, 0.062, 0.031, 0.015, and 0.007mg/ml); The absorbance was measured after 30min, at 517nm. All concentrations of methanol

extract was tested in triplicate. Percentage reduction of DPPH (Q) was calculated according to the formula below¹¹.

$$Q=100 \times (A_0-A_C)/ A_0$$

Where,

A_0 = Absorbance of control

A_C =Absorbance of the two samples after 30 min incubation.

RESULTS AND DISCUSSION

Activity of *Vitex agnus-castus* methanol leaves extracts on MCF7 hormonal dependent breast cancer cell line

Figure 1 shows the dose response curve for the *In vitro* screening of *Vitex agnus-castus* methanol extract on hormonal dependant breast cancer cell line MCF7, which was in passage 7 the results showed a dose-dependent inhibition on the cell growth after 48hr. The extract concentrations used were 200, 100, 50, 25, 12.5 and 6.25 μ g/ml, with each concentration in quadruplicate and the experiments were repeated twice. The data is represented as the mean \pm standard deviation (SD). The percentages of the MCF7 cell proliferation inhibition were 79.6 \pm 0.03%, 75.93 \pm 0.05%, 45.35 \pm 0.09%, 26.36 \pm 0.03%, 20.89 \pm 0.05% and 14.44 \pm 0.05% for methanol extract at each concentration mentioned above respectively. The IC_{50} value was deduced from the graph for the methanolic extract of *Vitex agnus-castus*, was calculated by using the following linear regression equation below: $Y=19.48\ln (X)-24.83$, where Y=the percentage of inhibition and X= concentration. The IC_{50} value for ME was 46.52 μ g/ml.

Activity of *Vitex agnus-castus* methanol leaves extracts on PC3 Prostate cancer cell line

Figure 2 shows the dose response curve of *In vitro* screening of *Vitex agnus-castus* methanol extract on PC3 prostate cancer cell line, which were in passage 6.

The results showed a dose-dependent inhibition on the cell growth after 48hr. The extract concentrations used were 200, 100, 50, 25, 12.5 and 6.25µg/ml, with each concentration in quadruplicate and the experiments were repeated twice. The data is represented as the mean ± SD. The percentages of the PC3 cell proliferation inhibition were $60.3 \pm 0.05\%$, $54.76 \pm 0.04\%$, $44.04 \pm 0.01\%$, $19.84 \pm 0.03\%$, $3.9 \pm 0.03\%$ and $3.8 \pm 0.04\%$ for methanol extract at each concentration mentioned above respectively. The IC₅₀ value was deduced from the graph for the methanolic extract of *Vitex agnus-castus*, was calculated by using the following linear regression equation below: $Y=18.93\ln(X)-36.39$, where Y=the percentage of inhibition and X=concentration. The IC₅₀ value for ME was 90.01µg/ml.

Free radical scavenging activity

Table 1 shows the percentage of DPPH scavenging activity of the ME, of *Vitex agnus-castus*. The data is represented as mean ± SD. Concentrations ranged of 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156 and 0.0078mg/ml was used throughout. Methanol was used as a solvent, each concentration was triplicate, IC₅₀ of the DPPH scavenging activity, of ME, was calculated by the linear regression equation. The equations were as follows.

$Y=0.103X + 36.94$, for the ME extract, where Y=Percentage of DPPH scavenging activity and X=concentration. Y is the percentage of scavenging and it is set to be 50%.The IC₅₀ of DPPH scavenging activity for ME 126.79mg/ml. The positive control used was quercetin and it showed scavenging activity through the equation $6.47\ln(x) + 51.77$, the IC₅₀ was 1.30mg/ml. Figure 3, and 4 showed the dose response curve of free radical scavenging activity of the ME and respectively.

Methanol extract of *Vitex agnus-castus* showed strong anti-angiogenic activity in *ex-vivo* study on rat aorta; and the anti-angiogenic agents may have anti-tumour activity. Most of the clinically used anti-tumour agents possess significant cytotoxic activity in cell culture systems¹². In the present study, the *in vitro* effect of methanol extract of *Vitex agnus-castus* was evaluated with different cell lines to investigate if these compounds have any cytotoxicity against breast and prostate cancer cell lines. Selective cytotoxicity is a desired feature of a new candidate anticancer agent. The cytotoxicity for ME was tested by the MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay, The MTT assay is a colorimetric assay for assessing cell viability. NAD (P) H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present¹³. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. MTT assays are usually done in the dark since the MTT reagent is sensitive to light whereby the reduction of MTT provides information about mitochondrial function through the activity of succinate dehydrogenase¹⁰. ME decrease the viability of MCF7 and PC3 which are hormonal dependant breast cancer cell line and prostate cancer cell line respectively. To consider any agent as cytotoxic against cell lines, its IC₅₀ should be less than 20µg/ml¹⁴. These finding showed that ME had a significant dose-dependent efficacy against the growth of the cells MCF7 and PC3. At the same time, these agents did not have any cytotoxic activity at the applied dose. They had toxicity at high concentration, so no toxic effect against the above named cell line can be expected *in vitro* from these agents, as

this extract had high percentage of anti-angiogenesis activity, with no cytotoxicity on endothelial cells and other cell lines⁸. Their anti-angiogenic activity is not related to the cytotoxicity, but may relate to other mechanisms such as anti-oxidant activity. Feng and co-workers attribute the mechanism of action which identified by Western blotting analysis to the inhibition of the expression of heme oxygenase-1 and the active forms of caspases-3, -8 and -9. It is concluded that the cytotoxic activity of *Vitex* extract may be attributed to the effect on cell growth, the cell death occurs through apoptosis. Other study done by Hirobe *et al.* (2000) reported previously that the methanol extract of *Vitex agnus-castus* contained eight flavonoids and that seven of them had cytotoxic activity against mouse lymphocytic leukemia P388 cells¹⁵. Free radical scavenging activity for the methanol extract was important to be test to understand the mechanism of action. The presence of flavonoids and terpenes in *Vitex agnus-castus* may elucidate the anti-angiogenesis mechanisms, and the cell line proliferation inhibition. Anti-oxidants are well known for having potent anti-angiogenic activity, amongst those that have been identified include vitamin C, vitamin D, vitamin E, vitamin A, rosmarinic acid, 3-hydroxyflavone, 3', 4'-dihydroxyflavone and 2', 3'-dihydroxyflavone¹⁶. Free radicals are atoms or molecules with an unpaired electron¹⁷.

CONCLUSION

Methanol extract of *Vitex agnus-castus* showed no significant cytotoxic activity against cancer cell lines (PC3 and MCF7). However, the data showed a dose related activity; which may be attributed to the existence of antioxidant agents.

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Table 1. The percentage of DPPH scavenging activity of the methanol extract (ME) of *Vitex agnus-castus* extracts and Quercetin

Concentrations µg/ml	ME	Quercetin
500	88.95±0.005	89.51±0.03
250	59.22±0.28	87.9±0.02
125	54.03±0.006	83.7±0.02
62.5	45.96±0.011	79.83±0.01
31.25	41.93±0.011	76.61±0.03
15.62	37.9±0.023	70.96±0.007
7.81	33.06±0.007	61.29±0.02

Caption:

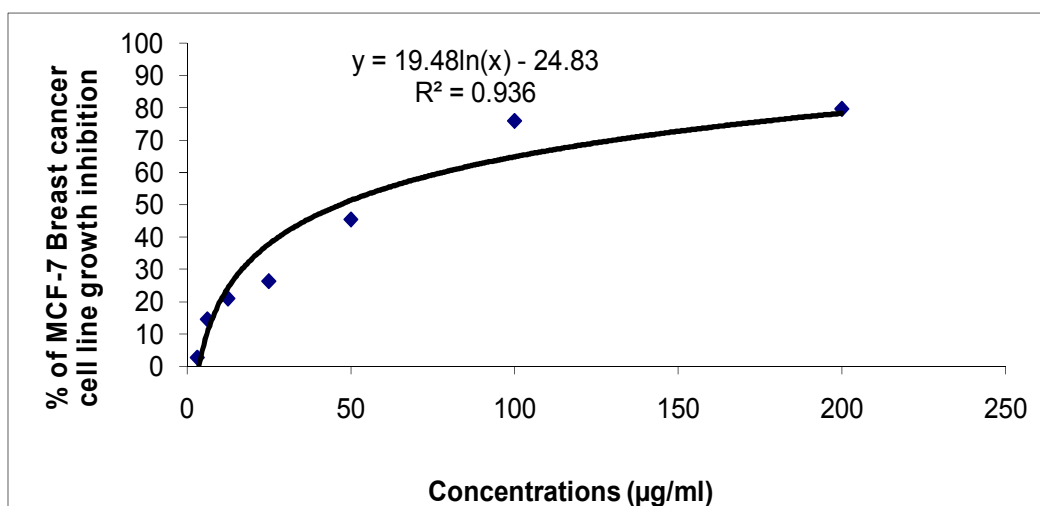


Figure 1. Cell proliferation inhibition activity of methanolic extract of *Vitex agnus-castus* on MCF7

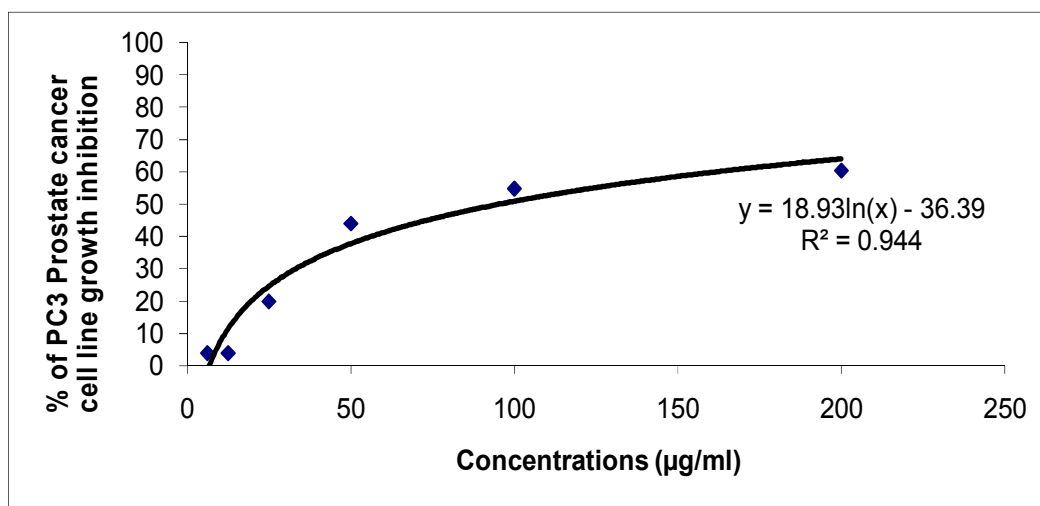


Figure 2. Cell proliferation inhibition activity of methanolic extract of *Vitex agnus-castus* on PC3 cell line

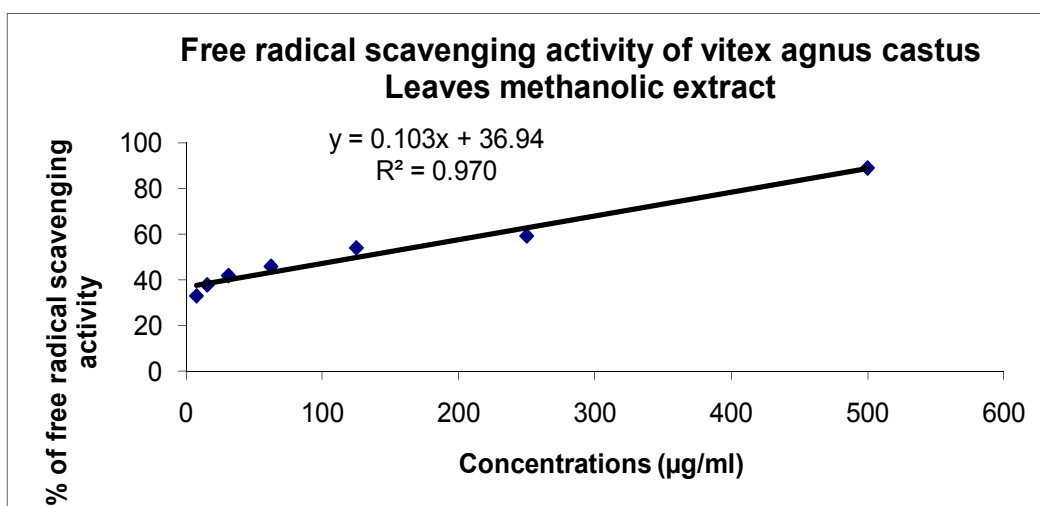


Figure 3. The dose response curve of serial dilutions of *Vitex agnus-castus* methanol leaves extract on DPPH free radical scavenging activity

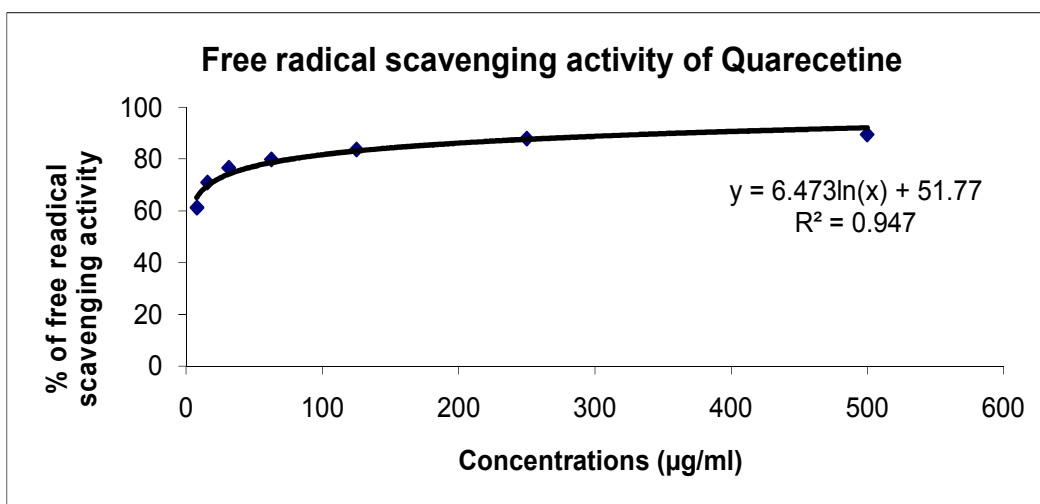


Figure 4. The dose response curve of serial dilutions of quercetin on DPPH free radical scavenging activity