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Original Article

Anti Angiogenic Activity of *Vitex Agnus Castus* Methanol Extract *In Vivo* Study

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Abstract

This study aimed to investigate the anti-angiogenic activity of *Vitex agnus castus* methanol extract *in vivo*. Eggs were incubated for three days, small whole made on the fine pinpoint. The next day, the egg's sac was penetrated and a small frame was made in the shell. The window was resealed and the eggs were returned to the incubator until day 10 of chick embryo development, 20 µl of 500mg/ml of the methanol extract was transferred to the Chick Embryo Chorioallantoic Membrane (CAM), and eggs incubated for 72 hours (n = 6); The zone of inhibition was calculated as mean of inhibition area in millimetre (mm) ±standard deviation (SD). Functional groups of the chemicals component inside the extract has been identified by Fourier transform infrared spectroscopy FT-IR and High performance liquid chromatography HPLC was used to identify the most likely causative agent. The results showed that the zones of inhibition area more than 10 mm. FT-IR showed that some of the identified functional group may relate to flavonoids. Casticin has been identified in methanol extract. Because of the above results the mechanism of anti-angiogenic activity for the methanol extract of the *Vitex agnus castus* may be related to the Casticin which has the ability to block the VEGF-receptor, thereby inhibiting the angiogenesis process.

Key word: Angiogenesis, Anti-angiogenic Activity, CAM assay, Casticin, *in vivo* study, *Vitex agnus castus*

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1. Introduction

The formation of new blood vessels from an existing one is the definition of Angiogenesis process; Angiogenesis is very important physiological process in wound healing, placenta formation, embryonic growth, and tumour development [1]. To form a new blood

vessel, the endothelial cells need to have the stimulatory signals and secretion of matrix metalloproteinase (MMPs) and heparanase, which cause the interruption of the extra cellular matrix (ECM). The tight junction between the endothelial cells is then altered, and the cells are projected throughout the newly created space. Here, the newly formed endothelial cells organize into fresh capillary tubes, allowing the sprouting vessels to progress towards the source of a fresh blood supply [2, 3]. Angiogenesis can occur either through sprouting or non-sprouting systems. The sprouting mechanism occurs by ramification of new capillaries from pre-existing vessels. The non-sprouting reaction results from the expansion, breaking, and fusion of pre-existing vessels produced by the proliferation of the endothelial cells within the wall of a blood vessel [4]. Trans luminal bridges can also occur in enlarged vessels produced by non-sprouting angiogenesis [5, 6]. This type of angiogenesis can be manifested at the same time with sprouting angiogenesis during the vascularisation of organs or tissues, such as the lungs and heart. The mechanisms for the metastasis of the non-sprouting angiogenesis are not clearly understood, but VEGF, which has a vital role in developmental, physiologic, and pathologic neo-vascularisation, is a candidate effectors molecule. VEGF stimulates the proliferation and migration of endothelial cells and induces metalloproteinase expression and plasminogen activity; [7]. *Vitex agnus castus*, the chaste tree is a shrub belonging to the genus Vitex of the Verbenaceae family. It is widespread on riverbanks and on shores in the Mediterranean region and in Asia. *Vitex agnus castus* is a deciduous shrub which reaches heights of up to 5m The leaves are opposite, hand-shaped, composed of five to seven radiating leaflets which are borne on a main stalk. The leaflets are linear, lance-shaped, toothed, dark green above and grey beneath with a very close felt. In August to October flower panicles with numerous flowers are formed [8]. Finding a new antiangiogenic extract may be of great value in targeting diseases related to this process, to be administering either together with the conventional drugs or alone. The aim of this study was to evaluate the antiangiogenic activity of methanol extract that Hayder and co-workersidentified in study done on 2014 but *in vivo* this time. Moreover, this study aimed to identify the bioactive constituent as a mean to elucidate the mechanism of action.

2. Materials and Methods

2.1. Plant Materials

Vitex agnus castus leaves were collected in February 2011. The species

was identified by Dr Ibraheem Saleh Al-Gobori from Al-Mustansirya University, Pharmacognosy department and the Voucher specimen (No. 1) has been deposited at college of medicine, Al-Nahrian University, Baghdad, Iraq.

2.2. Extraction

The leaves were collected from white flowered plant. The plant was oven dried at 40^{o}C. The dried leaves were separated and then ground into powder. The dried powder leaves (400gm) were extracted by maceration method sequentially by adding 33gm to each of the twelve flasks with 200ml of chloroform with continuous shaking by using the water bath for eight hours at 40°C, then filtration done by using filter paper watman 20cm, the filtrate kept for concentration with rotary evaporator while the residue dried and extracted with methanol and water sequentially. Each extract was concentrated using a rotary-evaporator under vacuum and then dried. The lyophilized extract was then kept in desiccators at room temperature prior to the experiment [9].

2.3. Chic Chorioallantoic Membran Assay (CAM Assay)

The Fertilized chicken eggs were obtained from Ebaa Research centre, Baghdad, Iraq. The Eggs were incubated at 37 °C with 60% humidity. At day three of incubation a small whole made on the fine pinpoint and the egg left to be incubated at 37 °C for one more day. On day four the egg's sac punctured and a small window was made in the shell. The window was resealed with adhesive tape and eggs were returned to the incubator until day 10 of chick embryo growth. On day 10, sample prepared as 500mg/ml. 20 µl placed on round disc of filter paper left to dry and then transferred to the cam and eggs were resealed and returned to the incubator for 72 hours until day 14 (n = 6 chicken embryos per sample); the zone of inhibition was photographed and calculated [10].

2.3.1. Quantification and Immaging of CAM

Six CAMs were used in each control and experimental group. The responses may be graded (+ 3-up to 6 mm; ++ 6 up to 9mm; +++ > 10 mm3. the quantification of zone of inhibition was done by using image analyzer (BIOCOM Visiolab TM 2000) [11].

2.4. Fourier Transforms Infrared Spectroscopy

2.4.1. Sample Preparation

This assay is important for functional group identification of methanol extract of *Vitex agnus castus*. Potassium bromide

(KBr) was transported out of the oven into a mortar. 2 % of the extract was added on the KBr, then mixed and grinded to a fine powder. The sample made was very finely ground. The two stainless steel disks have been taken out of the desiccators; and placed a piece of the pre-cut cardboard (in the tin can next to the oven) on top of one disk and filled the cut out hole with the finely grounded mixture. After that, the second stainless steel disk was put on top and transferred the sandwich onto the pistil in the hydraulic press. With a pumping movement, the hydraulic pump handle moved downward. The pistil started to rising until it reached the top of the pump cavity. Left for a few seconds and with the small lever on the left side, release the pressure. The disks removed and pulled away from each other. The film removed, which should be homogenous and transparent in appearance. Then Inserted into the Infra-Red (IR) sample holder and attached with foil tape. After that the spectrum Run. [12].

2.5. High-performance Liquid Chromatography (HPLC)

2.5.1. Preparation of Sample

Vitex agnus castus leaves methanol extract was taken in a volumetric flask using methanol: water (1:1) solvent and sonicated for 10-15 min make it the final volume 10 ml and were filtered through 0.45µm membrane filters (Whatman) finally used for HPLC analysis.

2.5.2. Analysis of Flavonoids

The analysis of flavonoids components was achieved using an Agilent 1100 Series HPLC system with a quaternary solvent delivery system, an online degasser, an auto-sampler, a diode array detector (DAD) used for the analysis. The column was Phenomenex Luna C18 (5µm, 250 mm X 4.6 mm). The analysis was performed by isocratic elution with a flow rate of 1mL/min. Column temperature was set to 30°C. The mobile phase was a mixture of trifluoroacetic acid 0.1% in water (A), trifluoroacetic acid 0.1% in methanol (B), trifluoroacetic acid 0.1% in acetonitrile (C) with a ratio of 60:20:20 (A:B:C). All the solvents were filtered through a Millipore filter (0.45µm) before use and degassed in an ultrasonic bath. 10µL volumes of the standard solution and sample were injected into the system. Standard solutions were analysed. Qualitative analysis was performed by measuring at 335 nm for casticin. The chromatographic run time was 25 min and the column void was 2 min. The system was controlled and data analysis was performed by Agilent Chemstation software.

2.5.3. Calibration

Five different concentrations of casticin were prepared in methanol ranging between 1-100 µg/mL. Triplicate 10µL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak areas obtained from injections were plotted against the concentrations to establish the calibration graph [13].

3. Results and Discussion

3.1. Chicken Embryo Chorioallantoic Membrane Assay (CAM ASSAY)

A study conducted by Hayder and co-workers in 2015 showed that methanol extract of *Vitex agnus castus* had potent anti-angiogenic activity in *ex vivo* rat aorta antiangiogenesis assay and significantly inhibited HUVEC cell line as *in vitro* assay [14]. From this point the current research has launched CAM Assay as one of the several classical *in vivo* models for studying angiogenesis [15]. Table 1 showed that the zones of inhibition area is more than 10 mm, so the score is three plus. Figure 1 showed the blood vessels growth inhibition; (A) representing the control and (B) representing the treated blood vessels of CAM. The results found that CAM treated with methanol extract of *Vitex agnus castus* leaves showed prominent anti-angiogenesis. A large number of vessels stopped radiated from underneath the disc which carried the 10 mg of extract. Moreover, the vessels were sparse, disorganized, with a light yellow appearance.

3.2. Functional Groups Identification of *Vitex Agnus castus Methanol Extract*

The peak absorbance of *Vitex agnus castus* leaves methanol extract identified the existence of eleven functional groups with different absorbance. As shown in figure 2 each of which has been mentioned in table 2. The functional groups of the chemicals in the leaves of methanol extract tested by FT-IR; FTIR data showed that phenols and hydroxyl as a functional

Table 1. The zone of blood vessels inhibition Chick Chorioallantoic Membrane Assay (CAM Assay) assay.

Eggs	zone of inhibition Area (mm) ±SD	Scoring
1	12	+++
2	14	+++
3	8	++
4	12	+++
5	11	+++
6	7	++
Mean ± SD	10.66±2.6	+++

Table 2. The peaks absorbance of Vitex agnus castus methanol extract functional group (Prati *et al*., 2010).

No. of Peaks	Peaks Absorbance	Functional group
1	773.48	Aromatic C=C
2	1074.39	Aromatic C-H
3	1165.04	Amide C=O Stretch
4	1274.99	Aromatic C=C
5	1375.29	C-H
6	1446.66	Carboxylic Acid O-H Stretch
7	1516.1	Carboxylic Acid O-H Stretch
8	1606.76	Aromatic C=C
9	1695.49	Aromatic C= O
10	2926.11	Alkyl C-H Stretch
11	3352.39	Alcohol/Phenol O-H Stretch

groups exist, and this agree with previous studies done on *Vitex agnus castus* which showed that flavonoids may exist in the extract [16].

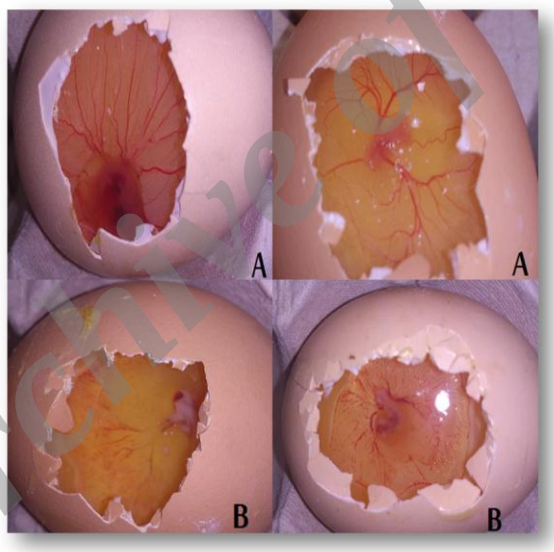

Figure 1. The blood vessels growth inhibition, were image (A) represent the control and (B) represent the treated blood vessels of (CAM).

3.3. High-performance Liquid Chromatography (HPLC) of *Vitex Agnus Castus Leaves Extract*

HPLC chromatogram of *Vitex agnus castus* leaves methanol extract (Figure 3) shows the existing of casticin **(1)** as a major component at retention time R_t 10.7 min. The presence of casticin was proved by comparison with the HPLC chromatogram of standard markers casticin as shown in Figure 4. The retention time of the peak obtained from the methanol extract chromatogram was similar to the standard markers in Figure 4. Therefore, the peak with retention time of 10.7 min, in

methanol extract were identified as casticin (1).

significant amounts of casticin existing in the methanol leaves extract. These findings

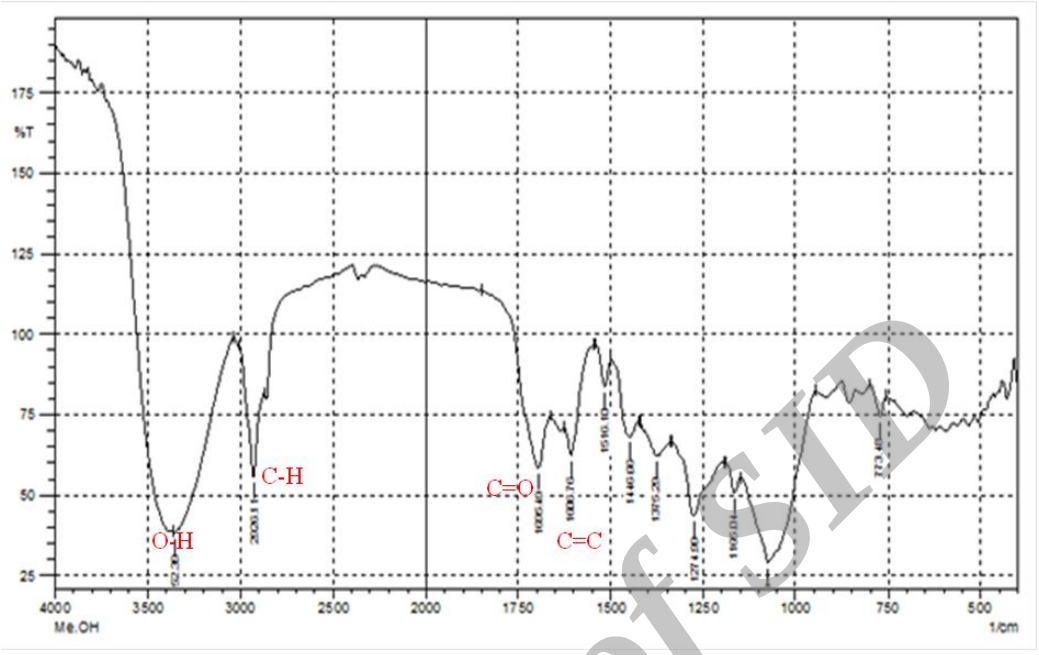

Figure 2. The peaks absorbance of Vitex agnus castus leaves methanol extract functional group.

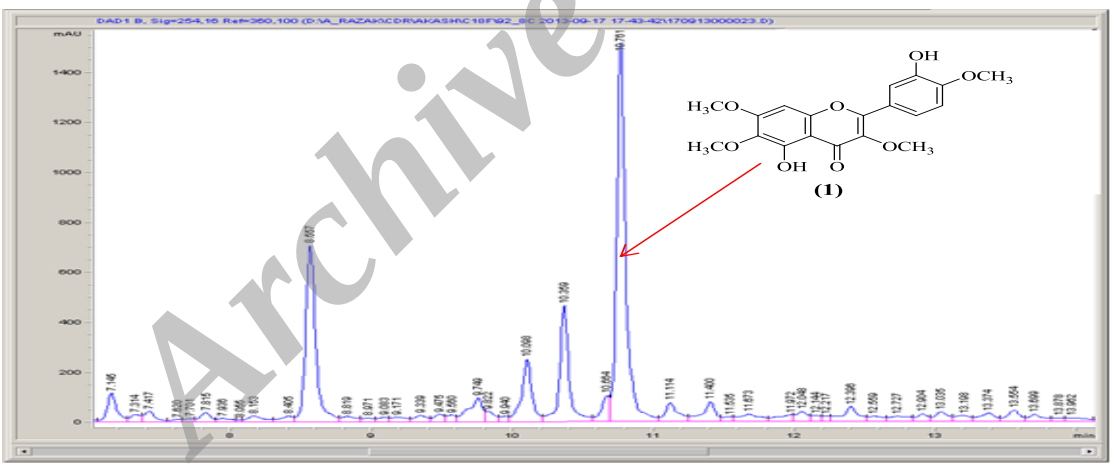

Figure 3. HPLC chromatogram of Vitex agnus castus methanol leaves extract.

This finding agreed with previous study managed to isolate casticin from the *Vitex agnus castus* by HPLC; Casticin as a standard was used to identify the existence of casticin in the *Vitex agnus castus* methanol extract [17]. The results showed may elucidate the pharmacological activity of the methanol extract as it may be related to the existence of casticin. Flavonoids have anti angiogenic activity through the polyphenols that they have. Polyphenols inhibit angiogenesis and cancer metastasis

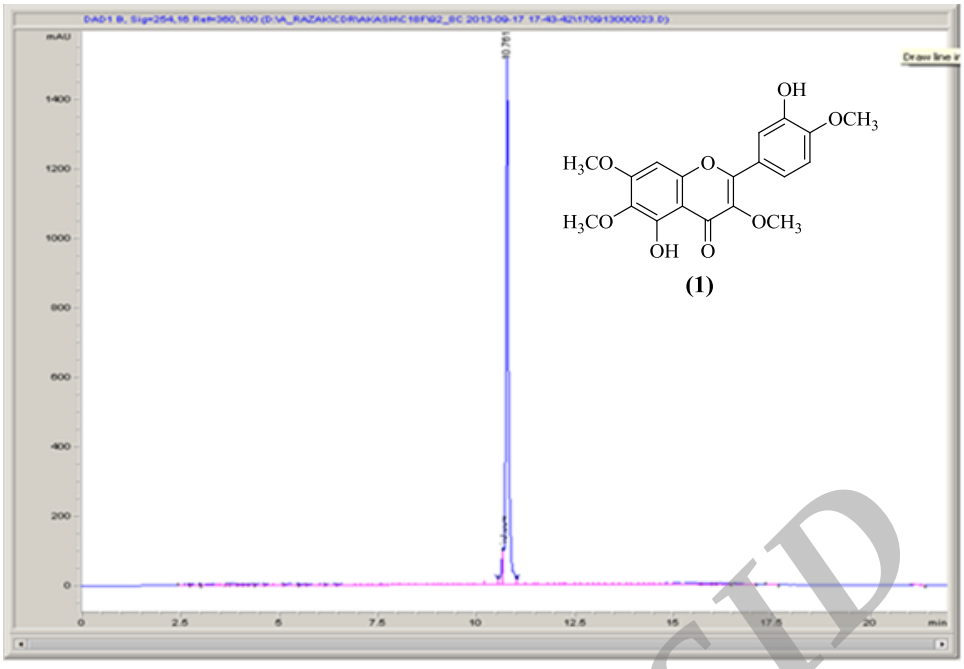

Figure 4. HPLC chromatogram of standard marker casticin (1).

through regulation of multiple signalling pathways. Specifically, flavonoids regulate expression of VEGF, matrix metalloproteinases (MMPs), EGFR, and inhibit NFκB, PI3-K/Akt, ERK1/2 signalling pathways; thereby causing strong antiangiogenic effects [18]. The data of this study may elucidate the significant anti-angiogenic activity possibly via its direct perturbation of the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), cyclooxygenase receptor (COX-2) and epidermal growth factor receptor (EGFR). The flavonoids identified in the extract has the ability to inhibit all these receptors [19]. Moreover, flavonoids plays another role in cancer cell proliferation inhibition by acting as an apoptotic agent via direct effects on mitochondria, leading to cytochrome-c release, which in turn directs the "downstream" caspase activation [20].

4. Conclusion

Methanol extract significantly inhibited the blood vessels growth in CAM. The mechanism may relate to the existence of flavonoids and polyphenols which have been identified by HPLC and FT-IR. These chemical groups proven to inhibit angiogenesis, proliferation of tumour cells, and endothelial cells.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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