Academic Sciences
International Journal of Pharmacy and Pharmaceutical Sciences
ISSN- 0975-1491
Vol 6, Suppl 1, 2014

Research Article

THE ANTI ANGIOGENIC ACTIVITY OF *VITEX AGNUS CASTUS* LEAVES EXTRACTS

HAYDER B. SAHIB*1, ADEEB A AL-ZUBAIDY1, SHALLAL M HUSSAIN2, GHAITH ALI JASSIM3

1 Al-Nahrain University/ College of Medicine/ Pharmacology Department. 2 Al-Mustansiriya University- Iraqi Centre for cancer and medical, 3 Al-Mustansiriya University-College of Pharmacy, Baghdad-Iraq. Email: haider_bahaa@yahoo.com

Received: 04 Jan 2014, Revised and Accepted: 19 Feb 2014

ABSTRACT

Objective: This study aimed to investigate the possible anti angiogenic activity of *Vitex agnus castus* leaves extracts

Methods: The leaves were dried and grounded into fine powder, and extracted sequentially with Chloroform, methanol and water. *Ex-vivo* rat aorta anti angiogenic assay was used to identify the most anti angiogenic extract, active extract has been chosen for dose response testing, by preparing serial concentrations of that extract and applied on aorta rings of the rat, the active extract was tested on human umbilical ventricular endothelial cell line as *in vitro* assay. Free radical scavenging activity has been tested by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) to discover which extract has the highest free radical scavenging activity and as a mean to elucidate the possible mechanism of extract action.

Results: Methanol extract has the highest percentage of blood vessels growth inhibition in comparison to other extracts $P< 0.05$. However, there were no significant differences between chloroform and water extract $P> 0.05$. Methanol extract showed dose dependant inhibitory activity on rat aorta assay the IC_{50} was (21.73 μg/ml). Methanol extract showed dose response activity against human umbilical ventricular endothelial cells (HUVEC) and IC_{50} was (42.09μg/ml). Methanol extract of *Vitex agnus castus* leaves showed the most potent free radical scavenging activity in comparison to other extracts; IC_{50} was (126.79μg/ml).

Conclusion: The results showed that methanol extract of *Vitex agnus castus* has potential anti angiogenic activity and this activity may be due to the high activity in free radical scavenging capability.

Keywords: Anti angiogenesis, *Vitex agnus castus*, free radical scavenging activity, Human umbilical ventricular endothelial cells line (HUVEC).

INTRODUCTION

Angiogenesis, the formation of new blood vessels from existing ones, is an 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 receive the stimulatory signals and secretion of matrix metalloproteinase (MMPs) and heparanase, which cause the decomposition of the extra cellular matrix (ECM). The tight junction between the endothelial cells is then altered, and the cells project 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]. Angiogenesis processes can be described as developmental or disease-associated, although both types share many mechanistic features, the differences might only be related to their regulatory control [3]. Tissue repair is a self-limiting process that occurs due to hypoxia near the site of tissue injury, while progressive tumour growth creates ongoing hypoxia and acidosis that do not regress as normally occurs after injury [4]. *Vitex agnus castus* Known as chaste berry herb. It is widely known to use medicinally in homeopathic regulation of amenorrhea, infertility, menopause due to estrogenic effects of phytoesterogens present in *Vitex agnus castus* [5].

Strategies for anti-angiogenic therapies

Agents that inhibit or suppress angiogenesis usually do so by interfering with the critical steps. Possible targets for therapeutic intervention in angiogenesis include six broad categories of therapeutic strategies for anti-angiogenesis: [6]

1- Inhibition of extra cellular cells (ECs) activation.

2- Inhibition of ECs proliferation.

3- Inhibition of ECs migration.

4- Disruption of the organization of a three-dimensional (3D) structure (formation of capillary tubules and loops).

5- Interference with the biosynthesis and remodelling of basement membrane (BM) and extra cellular matrix (ECM) (for example, inhibition of proteases secreted by the extra cellular cells ECs).

6- Induction of ECs apoptosis and direct killing of ECs.

The goal of this study is to identify which extract has the highest anti-angiogenic activity and to find out the most likely mechanism by which the extract showed its effect.

MATERIALS AND METHODS

Extraction

The leaves were collected from the plant on March 2013. Leaf specimen was labeled and annotated with date of collection and locality. A voucher specimen number (2) was deposited at the Herbarium College of pharmacy, Karbala University. 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 sequentially by adding 33.3gm in each of the twelve flasks with 200ml of chloroform with continuous shaking by using the shaker water bath for eight hours, and then the mixture filtered, the filtrate kept in umber bottle and the residue dried and extracted with methanol and water sequentially. Extraction process repeated three times with each solvent. Each extract was concentrated using a rotary-evaporator under vacuum. The extract was then kept in desiccators at room temperature prior to the experiment [7].

Experimental animal

Male Sprague Dawly rats with 12-14 weeks of age were used in the experiments. All the animals were allowed to free access to food and tap water. The animals were obtained from the Animal House Facility, National Iraqi center for Cancer scientific research, kept at 28-30 º C. The experiments were approved by the Animal Ethical Committee Al-Nahrain University College of Medicine/Baghdad-Iraq.

Rat aorta ring anti -angiogenesis assay

The angiogenesis assay used in this method is according to that developed by Brown and coworkers. Freshly excised thoracic tissues were rinsed with Hanks Balanced Salt Solution containing 2.5μg/ml amphotericin B (Sigma, St. Louis. MO). The tissue specimens were then cleaned of per adventitial fibro adipose material and residual blood clots. This was then cut into

1mm thick aortic ring segments under a dissecting microscope (Motic, Taiwan). The assay was performed in a 48-well tissue culture plate (Coster Corning, USA). 500µl of 3mg/ml fibrinogen (Calbiochem, USA) in serum free M199 growth medium (Gibco, UK) was added to each well with 5mg/ml of aprotinin (Sigma-Aldrich, Germany) to prevent fibrinolysis of the vessel fragments. Each tissue section was placed in the center of the well and 15µl of thrombin (50NIH U/ml) (Sigma-Aldrich, Germany) in 0.15M NaCl added. Immediately after embedding the vessel fragment in the fibrin gels, 0.5ml of medium M199 supplemented with 20% HIFCS (Gibco, UK), 0.1% έ-aminocaproic acid (Sigma-Aldrich, Germany), 1% L-Glutamine (Sigma-Aldrich, Germany), 1% amphotericin (Sigma-Aldrich, Germany), 0.6% gentamicin (Sigma-Aldrich, Germany) were added to each well. 100µg/ml of the test substance was added to the complete growth medium, and each treatment was performed in six replicates. Control cultures received medium without the test substances. The sample extract was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany), and diluted in M199 growth medium to make the final DMSO concentration 1% vessels were cultured at 37°C in 5% CO_2 in a humidified incubator (Binder, Germany) for five days. Fresh medium was added on day four of the experiment. Suramin, a well recognized anti-angiogenic agent was purchased from (Sigma-Aldrich, Germany) was used as a positive control. The extent of blood vessel growth was quantified under 40X magnification using an inverted microscope (Olympus, Japan) on day five of the procedure with the aid of a camera (Lieca CCD, Japan) and (Lieca QWin) software packages. The magnitude of blood vessel growth inhibition was determined according to the technique developed by Nicosia and coworkers [8]. Briefly, the length of the tiny blood vessel outgrowths from the primary explant was measured. The data is represented as mean ± standard deviation (SDV). The experiment was repeated three times using six replicate per sample. The percentage of blood vessels inhibition was determined according to the formula:

Blood vessels inhibition = $1- (A_0/A) \times 100$

Where

A_0= distance of blood vessels growth in µm.

A=distance of blood vessels growth in the control in µm. [9]

Dose response study on the crude extract of *Vitex agnus castus* with rat aorta assay (anti-angiogenesis)

Serial dilution from the Vitex agnus castus methanol extract were prepared in the following concentrations. 200, 100, 75, 50, 25, 12.5 and 6.25µg/ml, of the methanol extract were dissolved in DMSO, and diluted in the growth medium (M199) to make the final DMSO concentration 1%. Wells with no samples treatment were received medium with 1% DMSO used as the negative control. The data was represented as mean ± SDV. 100µg/ml suramin was used as a positive control. The IC_{50}, which is the concentration that inhibit the blood vessels growth by 50%, was calculated by using the linear regression equation for the extract. Where Y= the percentage of inhibition and, X= Concentration.

Human Umbilical ventricular endothelial cell line proliferation inhibition

(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromid) (MTT) assay was used as a measure of cell line proliferation according to Mosmann Method [10]. The cells were between passages 4-7. The cells were treated with several concentrations of Vitex agnus castus extract for 48 hrs. MTT was prepared by adding 5mg/ml in PBS (phosphate buffer saline). 20µl of MTT was used per well and the plates were incubated at 37 º C, in 5% CO_2 for 5hrs. The plates were removed from the incubator and the supernatant was aspirated. DMSO (200µl) was added to each well. The plates were shaken vigorously for one minute at room temperature to dissolve the dark blue crystals. The absorbance reading was taken at 570nm and the reference at 650nm by using micro-plate reader. 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 that for the 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 ± SDV 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 logarithmic correlation equation.

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

The free radical scavenging activity of the *Vitex agnus castus* extracts were measured by DPPH method. One ml of 0.1 mM solution of DPPH in methanol was added to 2ml *Vitex agnus castus* extracts with the following concentrations (0.5, 0.25, 0.12, 0.062, 0.031, 0.015, and 0.007mg/ml). After 30min, absorbance was measured at 517nm. All concentrations of extracts were tested in triplicate. Percentage reduction of DPPH (Q) was calculated according to the formula below. [11]

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

Where

A_0= Absorbance of control

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

Experimental design and analysis of data

The experiment design used for this study was Rationalized Complete Block Design (RCBD). The results were reported as means ± standard deviation (SDV). One way analysis of variance (ANOVA) followed by Tukey test comparison t-test (2-tailed) was used to compare between treatments groups. The differences between the means are considered significant at the 5% confidence level. The concentration that inhibit 50% from the blood vessels growth, cells proliferation (IC_{50}) this value was analyzed by logarithmic equation. The statistic analysis was carried out by using SSPS 16.0, the level of significance was set at $P<0.05$.

RESULTS

Figure 1 and images 1 show the effect of the three extracts on blood vessels growth of rat aorta. 100 µg/ml of the chloroform extract (CE), methanol extract (ME) and water extract (WE) were administered on rat aorta embedded in complete growth medium of M199. The blood vessel growth was quantified according to the method mentioned above, and is presented as mean percentage ± SDV. CE, WE and ME, extracts significantly inhibit blood vessels growth at day five. From these three active extracts, ME showed high percentage of anti-angiogenic activity in comparison to other extracts. 100 µg/ml of ME inhibited blood vessels growth by 93.81± 0.95% while the two remaining extract CE and WE inhibited blood vessels growth by 59.9 ± 7.38%, and 67.09 ± 5.81%, respectively. There was significant difference in terms of blood vessels inhibitions between ME, CE, WE and negative control ($P<0.05$) and there were significant difference between ME and CE and WE, in term of blood vessels inhibition ($P<0.05$). ME and suramin the positive control showed significant difference in activity ($P<0.05$). 100 µg/ml from suramin used as positive control inhibited the blood vessels growth by 100%. However there was no significant difference between chloroform extract and water extract ($P>0.05$).

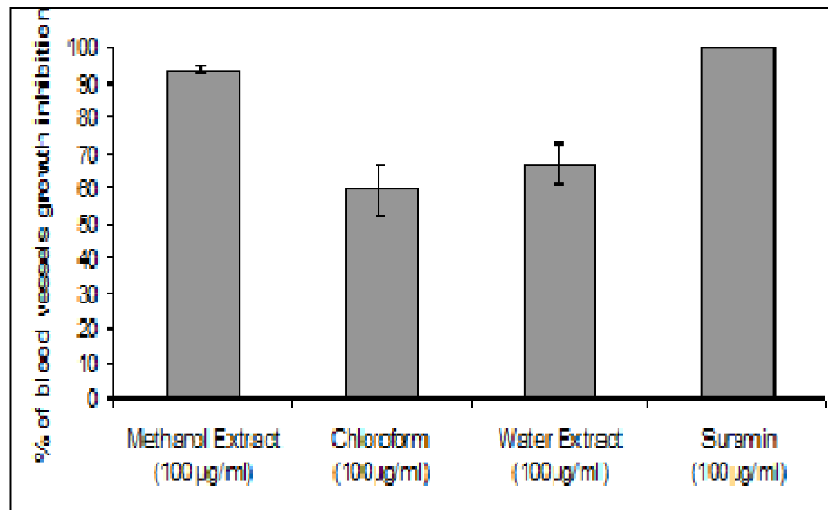

Fig. 1: The anti angiogenic activity of chloroform, methanol and water extracts on blood vessels growth of rat aorta. 100µg/ml of chloroform extract (CE), methanol extract (ME) and water extract (WE) of *Vitex agnus castus* leaves extract. Suramin was used as a positive control and the inhibition of blood vessels growth was quantified after 5 days, (n=18). Results are expressed as mean percentage changes in blood vessels growth on rat aorta ± SDV. The experiment was repeated three times, with six replicate per sample. There was significant difference as compared between the ME, CE and WE ($P < 0.05$). There was significant difference between ME and suramin ($P < 0.05$). However, ME gave the high percentage of response overall extracts. No significant differences between Chloroform extract (CE) and water extract WE ($P > 0.05$).

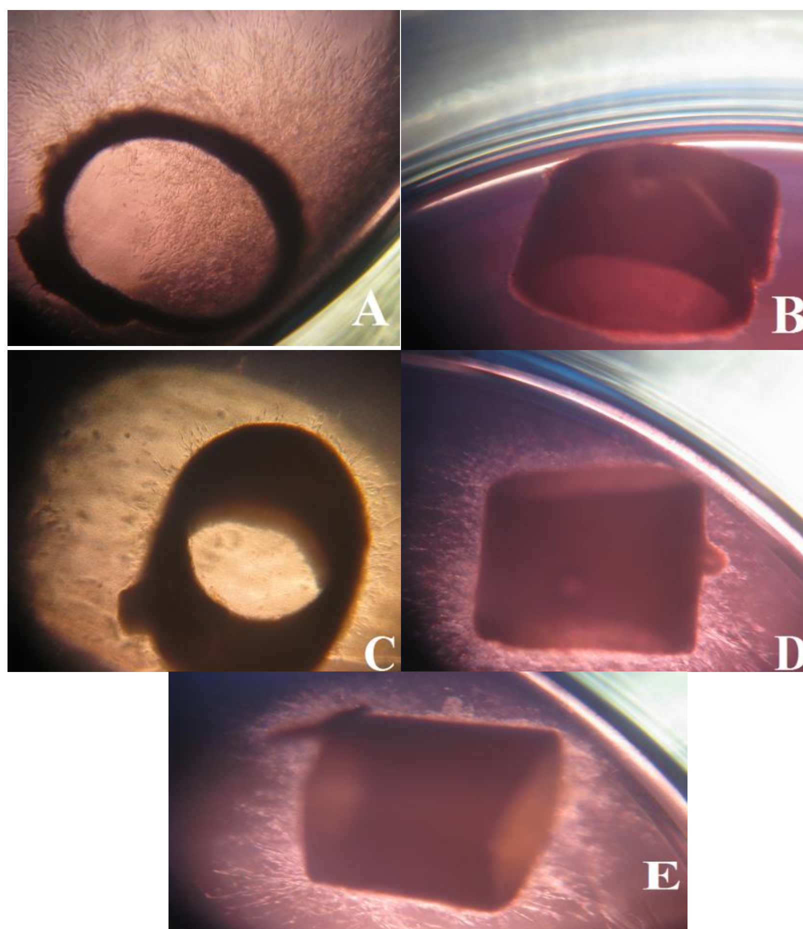

Image 1: The Images of aorta rings that have been treated with different solvent extracts, and the two controls. A, B, C, D, and E represented the activity of the negative control the one received the vehicle used to dissolve the sample with, positive control (suramin), methanol extract, chloroform and water extract respectively. The concentration used in this assay was 100µg/ml. The images been captured at day five of the experiment. Methanol extract (ME) gave highest anti-angiogenic activity in comparison to the other extracts. However chloroform extract (CE) and water extract (WE) gave potent inhibition in comparison to the negative control, but lower level than the methanol extract and positive control.

Dose response relationship for methanol extract of *Vitex agnus castus* on blood vessels inhibition on rat aorta ring

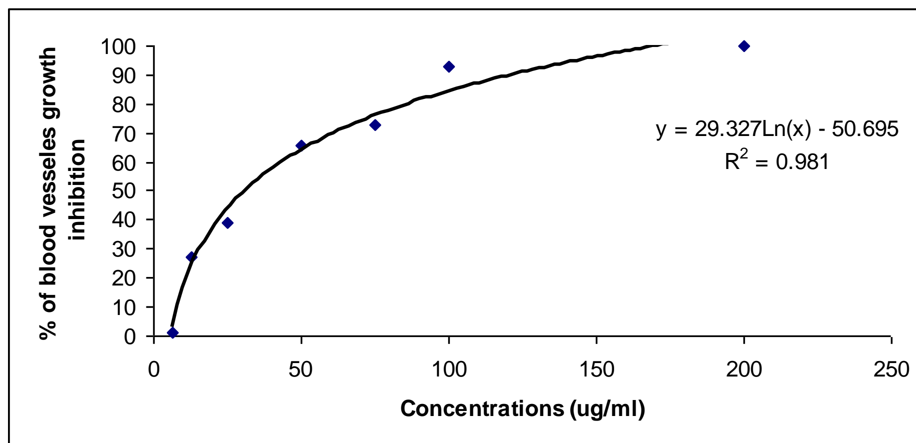

Fig. 2: Dose response curve of *Vitex agnus castus* methanol extract on rat aorta ring assay. The data indicates significant dose dependant inhibitions (n=18). The experiments were repeated three times with six replicate per sample. The data is represented as mean ±SD, of blood vessels growth inhibition. IC_{50} was calculated by using linear regression equation.

Figure 2 and images 2 show the dose response curve of the serially diluted methanol extract (ME) that was added to the rat aorta. Six concentrations used ranging from 200, 100, 75, 50, 25, 12.5, 6.25 and 3.125µg/ml. These concentrations showed significant dose dependent inhibition activity (P<0.05) percentage of inhibitions were represented as mean ± SDV as follows 100%, 93.15±3.12%, 72.75±3.6%, 65.64±3.9%, 39.3±1.27%, 27.07±1.21%, and 1.24±0.12% for the above concentration respectively. Blood vessels were quantified after five days of extract addition, where (n=18). The IC_{50} value was calculated from the linear regression equation Y= 29.327ln(x) – 50.695 equating to 21.73µg/ml. Where Y= the percentage of inhibition and X=concentration.

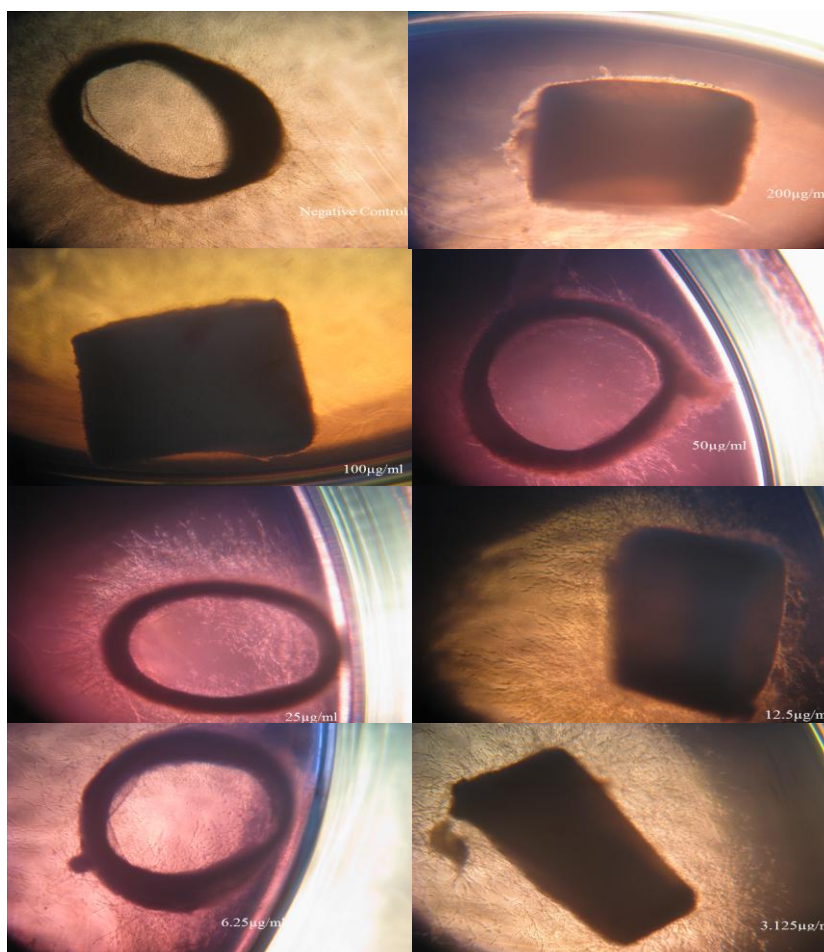

Image 2: Dose response curve of *Vitex agnus castus* methanol leaves extract, (n=18). The result shows significant dose dependant inhibition for the blood vessels growth. The blood vessels quantification was carried on day 5. Six replicate for each concentration. The experiment repeated three times. The data were represented by mean ± SDV.

Cytotoxicity against HUVEC cell line

Figures 3 shows the *in vitro* screening of methanol extract (ME) on HUVEC cells, which were in passage 4. The results showed a dose-dependant 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 ± SDV. The percentages of the HUVEC cell proliferation inhibition were 79.37 ±0.003%, 76.83±0.004%, 64.85±0.0009%, 40.56±0.03%, 8.08±0.006% and 3.8±0.0009% 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=25.07ln(X) - 43.82, where Y= the percentage of inhibition and X= concentration. The IC_{50} value for ME was 42.09µg/ml. Vincristine was used as a positive control, and 0.015 µg/ml of the Vincristine inhibited the HUVEC cell growth by 55.35±5.34%.

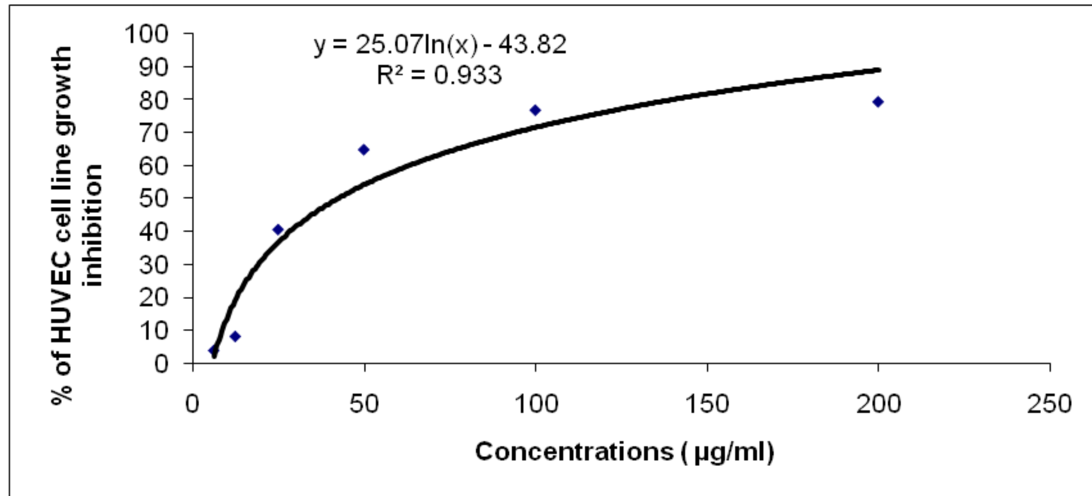

Fig. 3: The *in vitro* screening of methanol extract on Human umbilical vein endothelial cells (HUVEC).

DPPH Assay

Figure 4, 5, and 6 showed the dose response curve of methanol (ME), chloroform (CE) and water extract (WE), on DPPH scavenging activity. The data is represented as mean ± SDV. Serial dilution of the concentration range 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, IC_{50} of the DPPH scavenging activity, of ME, CE and WE, was calculated by the linear regression equation. The equations were as follows, Y=0.103X+36.94, for the ME extract, while for the CE Y=0.087X+34.47. WE free radical scavenging activity calculated through Y= 7.72ln(x) +8.125 where Y= Percentage of DPPH scavenging activity and X=concentration. Y is the percentage of scavenging and it is set to be 50%. The IC_{50} of DPPH scavenging activity for ME 126.79µg/ml. CE showed 178.5µg/ml. WE scavenging activity was 223.6µg/ml. quarecetine used as positive control and it showed scavenging activity through the equation 6.47ln(x) +51.77 the IC_{50} was 1.30µg/ml.

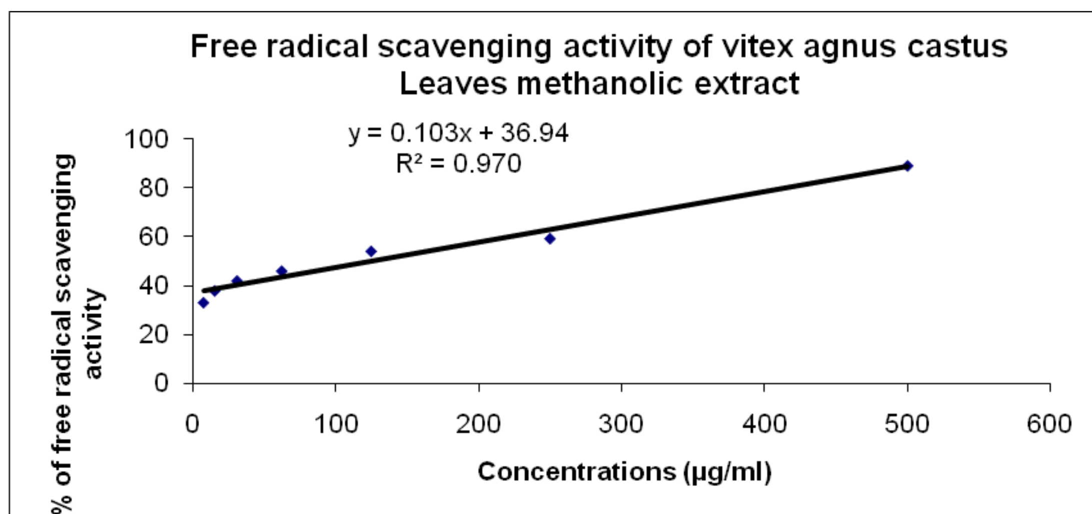

Fig. 4: The free radical scavenging activity of *Vitex agnus castus* methanol extract

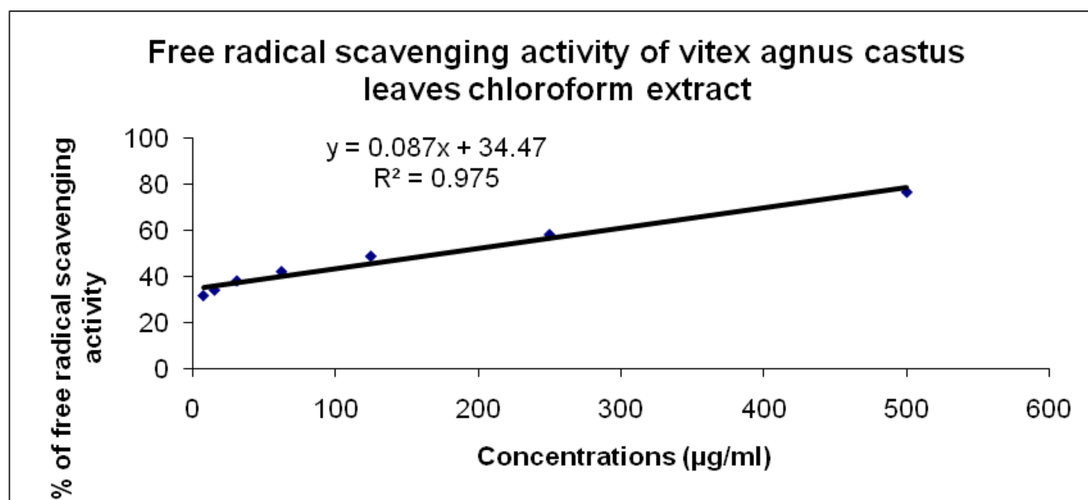

Fig. 5: The free radical scavenging activity of *Vitex agnus castus* chloroform extract

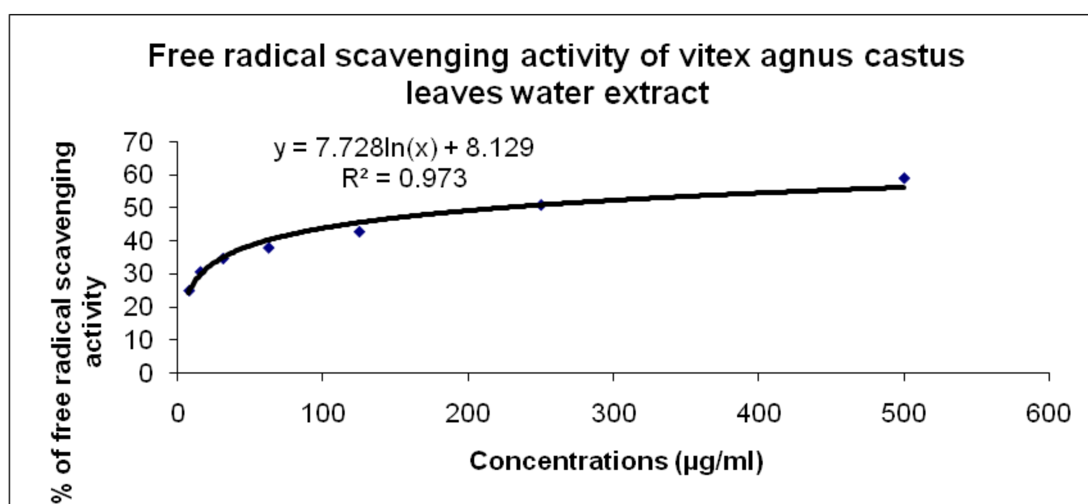

Fig. 6: The free radical scavenging activity of *Vitex agnus castus* water extract

DISCUSSION

In this study the extraction process done sequentially to ensure that the majority of the active constituents have been extracted and isolated in accordance to their polarity to be ready for use in the experiments, to test the pharmacological activity as anti-angiogenic activity [7]. Extraction method used in this study was (cold method) the cold extraction method was used in order to avoid any loss or destruction to the compounds inside the leaves from exposure to high temperature [12]. Many factors affecting the extraction yield; such as the shaking, time of macerations, types and concentrations of the solvents used, heat degree of the water used to warm the container that contain the powder of the leaves of *Vitex agnus castus*, these factors explain the variations in the percentage of yield of the extract, all these factors been taken in consideration during extraction process to get the enough amount of extracts [13].

Rat aorta anti angiogenesis assay

The rat aortic ring anti-angiogenesis assay has been widely used in recent years to assess angiogenesis in whole or partial organ culture as an *ex vivo* assay [14]. In this study the main objective was to identify whether these extracts have anti-angiogenic activity and which one was the most active; it was important to screen the extracts against rat aorta *ex vivo* assay to pick up the most biological active extract for further experiment as a mean to confirm the action and elucidate the mechanism. Other assay employed is assessing the anti-proliferative activity against HUVEC as *in vitro* assay [15]. Quantification of blood vessels is achieved by measuring the length of the micro vessel outgrowths from the primary ex-plant. The anti-angiogenic activity of the plant crude extracts was quantified after five days of blood vessels culture, by measuring the length of the blood vessel outgrowths. As the blood vessel growth reached their maximum on day five, this period is considered as the most suitable period for blood vessels outgrowth quantification [16]. Suramin was used as a positive control in this study as it has been shown to act as anti-angiogenic agent [17]. The study reveals that CE, ME and WE have potent anti-angiogenic activity. ME of *Vitex agnus castus* was found to have the highest percentage of anti-angiogenic activity in comparison to other extracts. The other extracts, such as CE and WE, showed lower percent of anti-angiogenic activity. However, the anti-angiogenic activity exhibited by CE and WE remained significant, which was perhaps due to the presence of other active compounds having less concentrations or other compounds which may antagonised the active compounds [18]. Owing to its potent anti-angiogenic effect, ME was selected for further investigation. To determine their anti-angiogenic mechanisms and the chemical constituents those are important in its pharmacological activity. Dose response curve was tested for the ME against rat aorta anti-angiogenic assay to have an idea about the safety of the extract; as the IC_{50} level decreases the safety decrease and vice versa [19]. IC_{50} on blood vessels outgrowth was within the safe range [19]. Methanol extract tested against HUVEC cell line to see the effect of

extract on this cell line as angiogenesis process start from this type of cell, the IC_{50} of the methanol extract showed that this extract has no cytotoxic effect against this cell. According to sahib and coworkers if the IC_{50} of an extract on cell line is less than 20µg/ml, the extract consider toxic other wise it is safe, the results showed safe extract and it was important to test other mechanism of angiogenesis process [7]. Antioxidant concept is considered as one of the mechanisms which elucidate the antiangiogenesis process [20]; this fact was the trigger for the research team to investigate the antioxidant activity between the extracts

Free radical scavenging activity

Free radical scavenging activity for the three extract was important to be test to get an Idea about the mechanism of action and about the variation in the capability of blood vessels outgrowth inhibition. Free radicals are atoms or molecules with an unpaired electron [21]. Considerable evidence that free radicals induce oxidative damage to bio-molecules and play an important role in cardiovascular diseases, aging, cancer, inflammatory disease and a variety of other disorders. Anti-oxidants are well known for having potent anti-angiogenic activity [22]. This study has found that methanol extract of *Vitex agnus castus leaf* extract gave the most potent anti oxidant activity in comparisons to other extract and it showed the highest percentage of anti-angiogenic activity, as shown by the rat aortic ring assay. Its potency in inhibiting new blood vessel development could be contributed to its significant antioxidant behaviour, as shown in the DPPH scavenging assay. This may result in a decrease in the free radicals present, which are known to activate the hypoxia responsive element gene. During the process of angiogenesis the latter can acts as a trigger for vascular endothelial growth factor (VEGF), a key cytokine in angiogenesis activation [23]. The presence of significantly high phenols content in *Vitex agnus castus* may possibly play an important role in contributing to its anti-angiogenic potential by down regulating the activity of nitrous oxide (NO) production. This mechanism may be partly responsible for the pharmacological efficacy of several folkloric medicines. One of the potent angiogenic agents is transforming growth factor alpha TGFα; antioxidant agents can inhibit TGFα expression as one aspect of their ability to inhibit angiogenesis [24].

CONCLUSION

Angiogenesis important process in many diseases, such as in growing tumour, cataract, psoriasis and others; as *Vitex agnus castus* extracts showed potential inhibition activity against this process, this herbs may has promising activity against tumour as adjuvant with chemotherapy or in targeting angiogenesis related diseases.

ACKNOWLEDGEMENT

Authors would like to acknowledge Professor Dr Alaa Ghani Mubarak and Professor Dr Farqad the Dean and Deputy Dean of the college of medicine Al Nahrain University for offering the logic support to finish this work. Special thanks to Iraqi centre for cancer and medical genetic research, Al Mustansiriya University for offering the Animals and laboratory. The team would like to a knowledge Mrs Nada Ibrahim and Mr Ali Mussa for their help in the laboratory were the work had done as both of them working their as laboratory staff member.

REFERENCES

1. Lee, W L., Cheng MH., Chao, HT. & Wang PH. The role of selective estrogen receptor modulators on breast cancer: From tamoxifen or raloxifene. *Taiwan J. Obstet. Gynecol.,* (2008) 47**,** 24-31.
2. Folkman, J. Fundamentals concepts of the angiogenic process. *Curr. Mol. Med.,* (2003), 3, 643-651
3. Nieves BJ., D'Amore PA., Brayan BA.. The function of vascular endothelial growth factor. *Biofactors.*; (2009), 35(4):332-7
4. Hinsbergh, V., VM.W, PK.W.,. Role of fibrin and plasminogen activators in repair associated angiogenesis: *In vitro* study with human endothelial cells. *Exs Begul* (1997)*,* 79**,** 391-411.
5. Brazier NC, Levine MA. Drug-herb interaction among commonly used conventional medicines: a compendium for health care professionals. *Am J Ther*. (2003), 10 3:163-9.
6. Clifford, M.J. Angiogenesis Protocols*, Humana Press Inc*. New Jersey. (2001)185-205.
7. Sahib, H.B., Aisha, A.F., Yam, M.F., Asmawi, M.Z., Ismail, Z., Salhimi, S.M., Othman, N.H., & Abdul Majid, A.M.S. Anti-angiogenic and antioxidant properties of *Orthosiphon stamineus* Benth. Methanolic leaves extract. *International Journal of Pharmacology,* (2009), 5(2) 162-167.
8. Nicosia RF, Lin YJ, Hazelton D, Qian X. Endogenous regulation of angiogenesis in the rat aorta model. Role of vascular endothelial growth factor *A m.J.Pathol.*1997; 151: 1379–1386.
9. Brown, L.F., Beres, B., Jackman, R.W., Tognazzi, K., Guidi, A.J. & Dvork, H.F. Expression of vascular permeability factor (vascular endothelial growth factor and its receptors in breast cancer). *Hum. Pathol.,* (1995).26**,** 86-91.
10. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol Methods,* (1983)16**,** 55-63.
11. Oktay, M., Gulcin, I. & Kufrevioglu, OI. Determination of *in vitro* anti -oxidant activity of funnel (*Foeniculum vulgare*) seed extracts. *Lebensm-Wiss.U.-Technoli,* (2003) 36**,** 263-271.
12. Widsten, P., Laine, J.E., Qvintus-Leino, P. and Tuominen, S.: "Effect of High-Temperature Defibration on the Chemical Structure of Hardwood Holzforschung (2002) 56, 1, 51-59.
13. Keutgen, A., & Pawelzik, E. Modifications of taste-relevant compounds in 426 strawberry fruit under NaCl salinity. *Food Chemistry*, (2007). *105*(4), 1487-1494
14. Carolyn, A.S., Stephen, M.S., Simon, T., Russell, H., Nicola, J.B. & Claire, E.L. Current methods for assaying angiogenesis *in vitro* and *in vivo. Int. J. Exp. Path.,* (2004) 85**,** 233-248.
15. Carolyn A, Malcolm WR Reed, and Nicola J. A critical analysis of current *in vitro* and *in vivo* angiogenesis assays Int J Exp Pathol.; (2009). 90(3): 195–22.
16. Kathryn, J.B., Susan, F.M., Anna, B., Deborah, J.M., Mariam, D.F. & Christopher, R.P. Anovel *in vitro* assay for human angiogenesis *Laboratory Investigation,* (1996) 75**,** 539.
17. Vijay R, Michael T, Jessica L. Haug, T, Kimlinger K., Timothy H, Linda E. Wellik, Thomas E. Witzig, S. Vincent R, Alex A. Adjei, and Shaji Kumar S, a multikinase inhibitor, is effective in vitro against non-Hodgkin lymphoma and synergizes with the mTOR inhibitor rapamycin. *Am J Hematol*. (2012), 87(3): 277–283.
18. Zaharah, A. & Salbiah, H. Population density study of Misai kucing grown on In: Seminar on medicinal and aromatic plants current trend and perspectives. *Forest Research Institute,* Malaysia (2004) 98.
19. Wei Fang, Fan Peng, Tao Yi, Cong Zhang, Chunxi Wan, Huibi Xu Christopher Waikei Lam and Xiangliang Yang, Biological Activity and Safety of *Tripterygium* Extract Prepared by Sodium Carbonate Extraction *molecules* (2012).*17*, 11113-11123.
20. Ahn MR, Kunimasa K, Kumazawa S, Nakayama T, Kaji K, Uto Y, Hori H, Nagasawa H, Ohta TCorrelation between antiangiogenic activity and antioxidant activity of various components from propolis. *Mol Nutr Food Res*.. (2009); 53(5):643-51.
21. Fazilatun, N., Zahari, I., Nornisah, M. & Mas, R.H. M.H. Free-radical scavenging activity of organic extracts and of pure flavinoids of *Blumea balsamifera* DC leaves. *Food Chemistry,* (2004) 88**,** 243-252
22. Lobo V., A. Patil, A. Phatak, and N. Chandra Free radicals, antioxidants and functional foods: Impact on human health *Pharmacogn Rev*. (2010). 4(8): 118–126.
23. Goodwin, AM. *In vitro* assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvascular Research,* (2007)74**,** 172-183.
24. Kay Cheong Teo and Shu-Leong Ho. Monoamine oxidase-B (MAO-B) inhibitors: implications for disease-modification in Parkinson's disease Translational Neurodegeneration 2013, 2:19.