Original Article

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Anti - Angiogenic Activity of 4-Chromanol (C9H10O2) in Ex Vivo and in Vivo Animal's Study

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Abstract

Background: Angiogenesis plays a crucial role in wound healing, involving the formation of new blood vessels from existing ones, through the invasion of the wound clot and the creation of a microvascular network in the granulation tissue. This process is primarily regulated by signals from the serum and the extracellular matrix (ECM) environment. Objective: This research aims to assess the potential antiangiogenic activity of 4-chromanol. Materials and Methods: Male albino rats were used in the study. 4-chromanol was prepared by dissolving it in DMSO. The antiangiogenic effect of 4-chromanol was evaluated using ex vivo rat aorta ring assays and in vivo Chorio Allantoic Membrane (CAM) assays. The inhibition of blood vessel growth by 4-chromanol was assessed, and the inhibition zone was measured as the Mean Inhibition Region (mm ± SD). Cell viability and proliferation were determined using the MTT assay, and the data were statistically analyzed. Additionally, 4-chromanol was tested for its effect on vascular endothelial growth factor gene expression. Results: The data showed significant inhibition of blood vessel growth by 4-chromanol compared to the negative control (received DMSO 1%). 4-chromanol exhibited potent inhibition of blood vessel growth compared to the negative control. Conclusion: The study results suggest that chloroform and methanol extracts of phoenix dactylifera seeds displayed significant antiangiogenic activity. Notably, 4-chromanol exhibited the most significant antiangiogenic activity in both rat and CAM assays.

Keywords:

Anti – angiogenic, ECM, chromanol, Chorio Allantoic.

Introduction

Angiogenesis, was process which include the new Blood Vessels formation from that the existing ones. The term of the angiogenesis means that the words of "angio" refer to that blood vessels and "genesis" refer to that creation [1].

During the physiological state, it was highly controlled process, that it was plays an important part in the embryonic development, also the Wound Healing process and in Menstrual Cycle. The Angiogenesis is also being showed in non-malignant diseases which include, 1 Diabetic Retinopathy, 2 periodontitis, 3 The ischaemic diseases and 4 The autoimmune disease which include as the Connective Tissue Diseases , and also Psoriasis [2, 3].

Also angiogenesis cause to provide THE nutrients and ALSO THE oxygen for tumor cell that cause waste product to be removed, and the process of formation of new vessel allow the cancer cell to be metastasize and also to be proliferate to remote areas by access to the recently make the blood and also the lymphatic system, insufficiency of that blood donated, also, it could stop the tumor development, and cause the tumor To be shrinkage and the death of cancer cells [4]. The old studies reveal of that in the vacation of the angiogenesis, the tumours was perhaps to get larger to about 1–2 mm^3 in the diameter prior to the ceased the growing and the death, other tumour cells perhaps can grow more than(2 mm^3) in the size in cell rich with angiogenesis [5]. Angiogenesis is a firmly regulated process. Strict control is necessary to ensure that a new vasculature is only make when and where it is required,

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and organisms have several "off" and "on" switches to facilitate this [6]. So, it is important to maintain stability between pro-angiogenic and anti-angiogenic signals, which is known as the "angiogenic switch". This steady stability is maintained through the action of cellular signaling pathways, specifically through the stimulation of growth factor receptors [7].

The primarily activated factors are HIFs (hypoxia inducible factors) which in turn they up–regulate multiple pro–angiogenic genes directly or indirectly. Among the up–regulated genes, VEGF-A (vascular endothelial growth factor–A) is the major one and also responsible for the proliferation and migration of cells during this process [8]. Angiogenesis is split into two main types: sprouting angiogenesis and intussusceptive angiogenesis. These occur both in adult organisms and *in utero*, taking place in nearly all organs and tissues[9].

The activity of angiogenesis could be expressed as different steps. First one, was the Angiogenic stimulation led to rise the endothelial cell (EC) Permeability and also the Cellular Multiplication, which THAT was continues as new capillary that sprout. And the. Second was the basement membrane components lead to protolysis is important process to cause invasion of that ECs to the Stroma of that the Neighboring tissue, in that the helper activity of that was plasminogen activator system and also matrix metalloproteinase (MMPs) is needed.

Third, the migration of ECs cause activation of the lumen formed Then, also the new capillary channel was formed. Finally, that the capillary was rigid through the erection of the basement membrane, and the adherent junctions, and also E Cs angiogenesis inhibitors, also known as anti-angiogenics, have been approved to treat cancer [10]. These prevent tumors from developed new blood vessels, cessation the supplying of nutrients and oxygen. In this way, angiogenesis inhibitors cause "starve" tumors which is the purpose of preventing them from growing and metastasizing, or to shrink them [11].

4-chromanol This compound that belongs to class of the organic compounds which known as 2 ,2 -dimethy l- 1-benzopyrans. which are the organic compounds that containing a 1- benzopyran moiety which have two (methyl groups) at the 2-position, also has been improved as anti-inflammatory and anti-carcinogen. The goal behind this study was to determine the possibility of antiangiogenic activity of 4-chromanol

Materials and Methods:

THE works were DONE in laboratory of tissue culture of THE Pharmacology Department / OF THE College of Medicine / OF Al-Nahrain University. THIS WORK was begun on august 2022 and was ended on NOVOMBER 2023. THIS Experiments had been done after the approvement of Ethics of Committee of Al-Nahrain University / of College of Pharmacy (the Letter No. SY/2/2/1012, that Dated at 24 November, 2020).

Animal:

The Animals used in the study are: 1) include Albino male rats (used for RAR anti - angiogenic assay) were have been obtained from the animal house of Pharmacology Department / College of Medicine / Al-Nahrain University. That They were permitted for get of the water and the food and spend at 28-30ºC in the cages in animal house.

2) also Fertilized chicken eggs (used for CAM anti - angiogenic assay) have been obtained from the egg hatchers from altaji

The Rat aorta ring anti-angiogenic ex vivo assay (RAR):

That Assay had been done as same as the typical Protocol that done by the Brown and the co-workers, with the little minor's differences. 12 to 14 weeks (Albino_ Male_ Rats) have been anesthetized with chloroform then abandon through Cervical Dislocation. Then the Thoracic Aorta is extracted, then have been rinsed with the serum- free media, remove excessive fat surrounding the aorta with a sharp blade and section aorta into 20 rings of 1 mm width[12]. Also, this assay was used ((48 – well tissue culture plate)). The 300µl of the Fibrinogen and (5mg/ml) of the the Aprotinin to the serum free medium was added to that each Well and putted at the center of the well of the ring tissue were placed. Then there was added to the each well (10µl) of the Thrombin; that has been produced to ((50NIH))U/M in (0.15M) of NaCl and after that WHERE incubated the tissue culture for about 30 min in the humidified (5%) CO2 incubator at the 37°C in order to solidify and also for the formation of the fibrin gel[13].

After that also was added for the each well (300µl) of theM199 provided with the ((20%)), the Heat Inactivated of THE Fetal Bovine Serum, (0.1 %) of aminocaproic acid, (1%) of THEL-glutamine and (0.6%) of the Gentamycin. The tested substances (4-chromanol) were have been processed by the dissolving in DMSO and to dilute in ((M199)) for making LAST ((DMSO)) concentration of1%. Each concentration was replicated in 3 times after that then experiment was reprise 3 times. and then the cultured plates putted in (5 %) CO2 of moistened Incubator FOR five days at 37° C. Tt the Day (4), upper surface was changed with the fresh Medium.

1 % of DMSO with tissue rings only represented the negative control and the tissue rings with vitamin C represented the positive control. then the result represented by mean of the inhibition of the Vessel growth that assess as the Mean of the INHIBTION

percent of ((Negative Control ± SD)). EXTENT of the Blood Vessels growth forbidden had been known by Technique developed by THE Nicosia [14] and was been quantified in Manually under the (40x) Magnification by the using of the camera and software. Percentage of the Blood Vessels forbidden had been calculated under that formula:

Blood vessels inhibition % = = 1- (A0/ A) × 100

In which: A0=the Distance of the Blood Vessel Growth OF tested substance in mm; and A= the Distance of Blood vessel growth of negative control in mm.

The Dose-Response study on 4-chromanol with rat aorta assay (anti-angiogenesis)

The Serial dilution FOR Test substance HAD been produced by dissolving the substance in DMSO in order for making the stock solution of the 1% conc. After that have been diluted at (M199) Growth Medium with the different potencies :200, 100, 50, 25, 12.25 and µg/ ml. Negative Control considered as Wells without the tested substance which that achieved medium in addition to 1% DMSO. That Data had been meaned as the mean of ± SD. concentrations which inhibit of about 50% of the Blood Vessel growing (IC50) had been calculate through the use of ((logarithmic equation)). Where Y= = represent PERCENTAGE OF INHIBTION , the X= CONSTRATION [15].

-Chorioallantoic membrane (CAM) in *vivo assay*

The Fertilized chicken eggs were having been Cleaned of the waste by the use (of 70%) of the ethanol then have been incubated for about ((72 hours at 37°C)) also use of humidity for about 60%. The horizontal position was that the egg had been putted and rotated in different times.

That Then after about 72 hours, the two ml of albumin had been draw out using pin point tip that breakdown at side and then close up in order to give the best visual of CAM that had been formed, that CAM will break from pocket that was joined to Egg Shell," and AFTER THAT had incubat for about ((24 hours)). mini–Square Window with (3-4 cm) of shell then produced THEN tested Samples HAD been submersed earlier to the circular discs of that Filter papers that putted above CAM and then covered by a sterile sticky tape, the window was covered to keep the humidity, and after that the eggs incubated one more for about 48 hours.

The constrained zone has been captured and also calculated. tested sample that is 4-chromanol was prepared (10mg/ml) and 50µl, final dose as 0.5mg/disc, 4-chromanol that putted above Filter Paper discs- and then lifted for drying prior to move to CAM. The procedure had done under sterile state [16, 17].

Cell viability *in vitro* assay (MTT assay):

Cell viability and proliferation was been evaluated by the using of 4-chromanol, a 3- -(4, 5-dimethyl thiazol- 2 -yl) -2, 5- diphenyl tetrazolium bromide((MTT)) assay; the microtitration colorimetric method in according to(Mosmann method) [18].

Briefly, HUVEC cells suspensions were have been seed that have a concentration of (1×10^4 /ml) into the * 96-well * culture plates with - 200 μl - of complete growth medium.

Then cells where were been setted in the - CO_2 incubator- at (37°C for 24 hours) in order to permit them for the adherence. 4-chromanol were dissolving in (DMSO) with the different concentration in order to prepare the serial dilutions with the media, then after that the supernatant medium was changed with the fresh medium containing of 4-chromanol. Then in -co2 incubator- the cells were incubated again for (48 hours at 37°C).

-The preparation of MTT had been done by the addition of (5mg/ml) of - (phosphate buffer saline) , and by adding (20 μl of 5 mg/ml) , MTT to each of well and after that incubating for about - 4 hours- for the determination of the cell viability.- 200 μl- dimethyl sulfoxide was been added by the removal of medium and the plates that agitated vigorously for about * 1-2 minutes * for dissolving the Formazan Blue Crystals, The absorbance *A* That measured to (570 nm) and reference to the(650nm) utilize the - microplate reader enzyme-linked immunosorbent assay (ELISA).

*Negative control represented The cells that have been received only 1% DMSO was and the viability of cells treated with 4-chromanol was been measured as mean percent of that negative control ± SD, Cell growth viability and inhibition were have been calculated using:

Cell viability (%) = =((A_0/A)) x 100

Cell inhibition (%) = = (1- cell viability) (%)

Where:

A_0 = = Absorbance of the samples

A = = Absorbance of the negative control

That Inhibitory concentration ((IC_{50})) values were calculated by using - linear and logarithmic equations.

Gene expression analysis:

Huvec cells were been exposed to * 200 µg/ml * of 4-chromanol for about 12 h, * TRI Reagent * ® solution kit has been used for - total RNA extraction- was done for (($5x10^6$ treated cells)) -in the (RNA Extraction Procedure) all the solutions HAD BEEN prepared BY the Autoclaved 0.1% DEPC (diethyl pyrocarbonate) treated water., all (plastic and the glassware) were treated with *DEPC* THEN autoclaved.

-The protocol included that then after centrifugation of sample suspension to separate the cells and then discarded the supernatant, (1 ml) of tri - reagent was

added by vigorous pipetting in order to lyse cells. Also, another centrifugation was done and supernatant was then transferred to new tube.

Then The separation phase done by the adding of -0.2 ml- chloroform with the additional centrifugation for about 15 minutes in order to acquire a- two phase's solution- ,then aqueous (RNA - containing phase) represented is the upper phase, 9 red organic DNA and protein – containing phase) represented is the lower phase. Then In the RNA precipitation step,(the aqueous phase) was moved to another tube and(0.5 ml) isopropyl alcohol was added then after that , centrifuged in order to get RNA pellet which that washed with 75% ethanol then after that air dried for about 10 minutes.1then , DEPC water was added to re-suspend then the RNA to obtain the pure solution [19].

The quantity and quality of RNA were had been qualified used THROUGH measure that the optical density (OD) at (260 nm and 280 nm) by a nanodrop -2000 -Ultraviolet Spectrophotometer-. Prior to cDNA synthesis, the RNA must be treated with -dsDNase I- and then have been incubated at -37°C - for about 2 minutes in order to get rid of the traces amounts of DNA impurities.

cDNA synthesis was done by using a called Revert Aid™ first strand (cDNA synthesis kit (, in which that the main reaction component used for reverse transcription that was 1RNA template, 2Moloney murine leukemia virus reverse transcriptase,3 buffer,4 Oligo(dT)$_{18}$ primer, 5dNTPs ((Deoxynucleoside triphosphate)), 6Dithiothreitol (DTT),7 RNase inhibitor, and8 RNase - free water. Then after that the reaction tube have been Mixed and centrifuged then incubated for 60 about min for about at 42°C. Then the resulted product used in PCR [20-22].

RESULTS

Rat aorta ring anti – angiogenesis ex vivo assay

The differentiation between 4-chromanol and the negative control:

The differences between the aortic rings which treated with (200µg/ml) of 4-chromanol and then implanted in the Complete Growth Medium of (M199), WHICH revealed that a notable differentiation shown of- blood vessels inhibition- for about (91% ±0.7µg/ml0 at* ($p<0.05$) of theday5 of a trial as then make comparisons with rings of the negative control that get only the? (1% DMSO) as shown in Table 1.

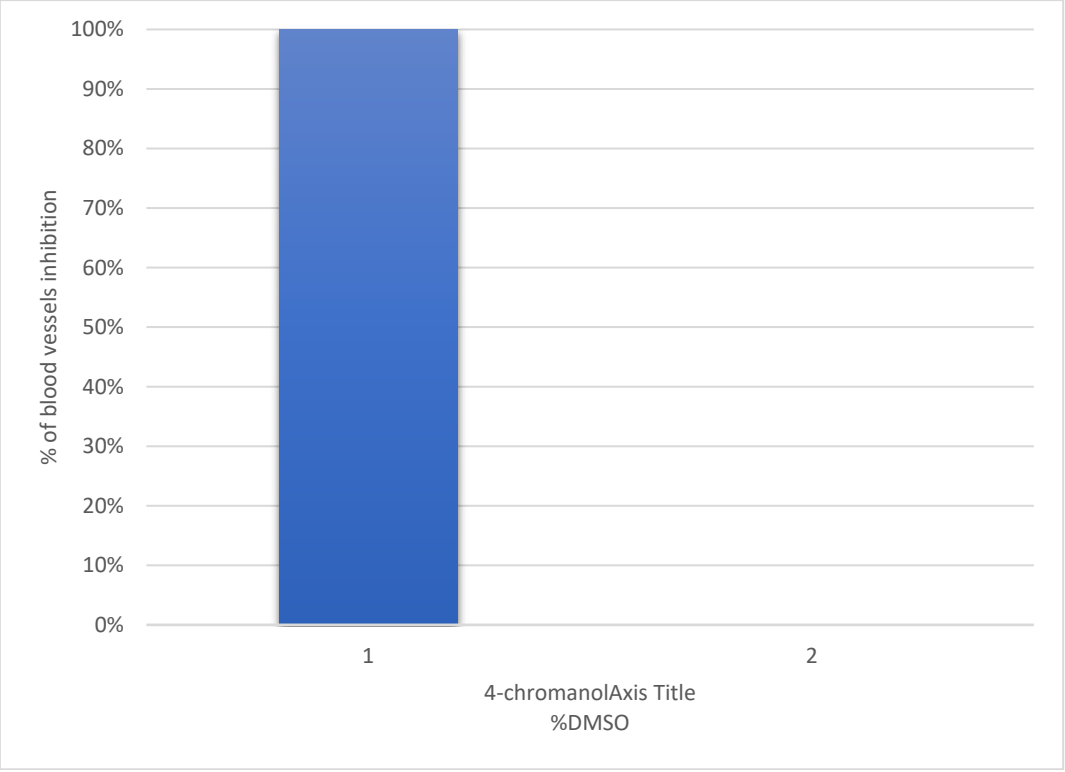
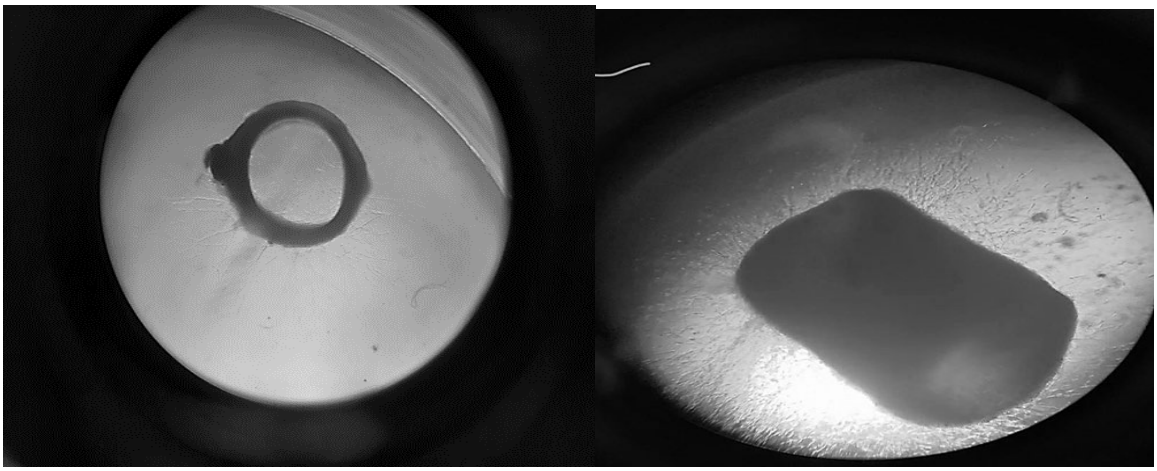


Figure (1): Anti – angiogenesis activity of 4-chromanol and negative controls in *ex vivo* aortic ring model as a percentage of blood vessels growth inhibition.

Table (1): percentage of inhibition of growth of blood vessels done by 4-chromanol, negative control

The COMPOUND	The Percentage of inhibition ± SD
4-chromanol	91% ± 0.7
DMSO 1%	0



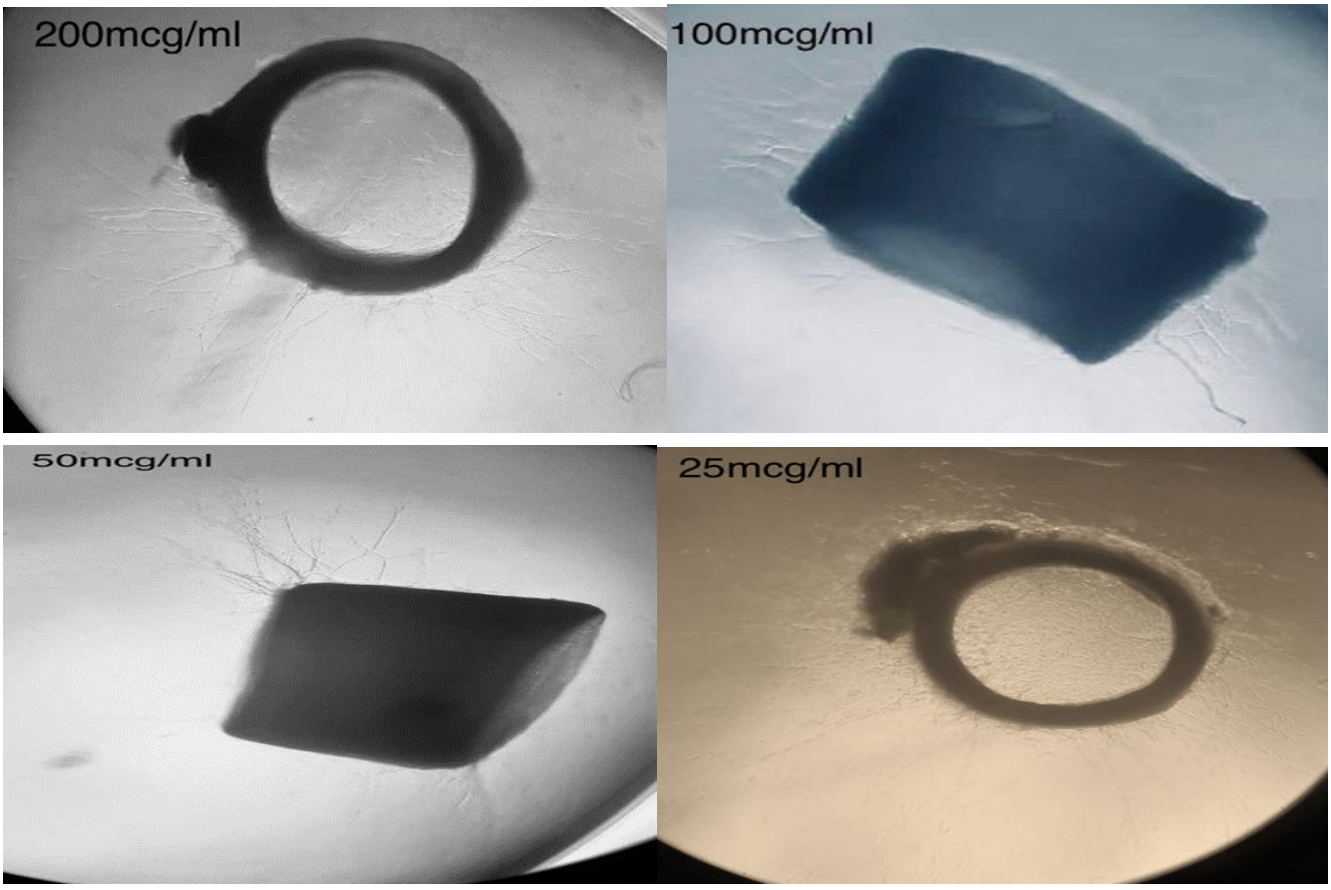
(A) (B)

Image (1): (A) Anti – angiogenesis effect of 100µg/ml of 4-chromanol in *ex vivo* aortic ring model and negative control (B). NOTE: white arrows represent the growth of micro-blood vessels

Dose response the effect in the rat aortic ring anti-angiogenesis assay

The Dose response curve of 4-chromanol on rat aortic ring model:

-Dose response curve showed five serial dilution concentrations ((200,100, 50, 25, 12.5µg/ml)). In the Table 2 the data showed that the inhibition percentage ± SD and the significant dose dependent inhibition of blood vessels at) ($p<0.05$)). logarithmic equation used to calculate the IC50 where Y= represent inhibition percentage and X= the concentration m ic50was =4.28 µg/ml. The experiment involved exposing blood vessels to different concentrations, and the dose-response curve and Table 2 indicate a significant dose-dependent inhibition. The IC50 value of 4.28 µg/ml provides a quantitative measure of the concentration at which the inhibition is at 50%.



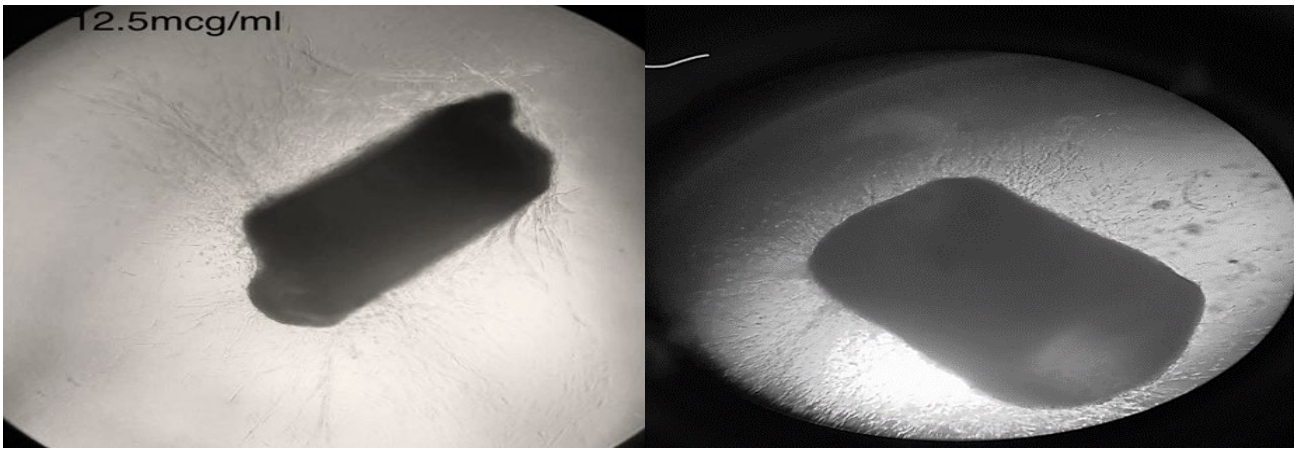


Image (2): The dose response effect to the serial concentrations of 4-chromanol in rat aortic rings model. Note: white arrows represent the growth of micro-blood vessels

Table (2): The Serial concentrations with percentage of inhibition for 4-chromanol

The Concentration (µg/ml)	% Of blood vessels inhibition ±SD
200	91%± 0.7
100	88%± 7
50	80%± 3.5
25	74%± 7
12.5	64%± 3.5

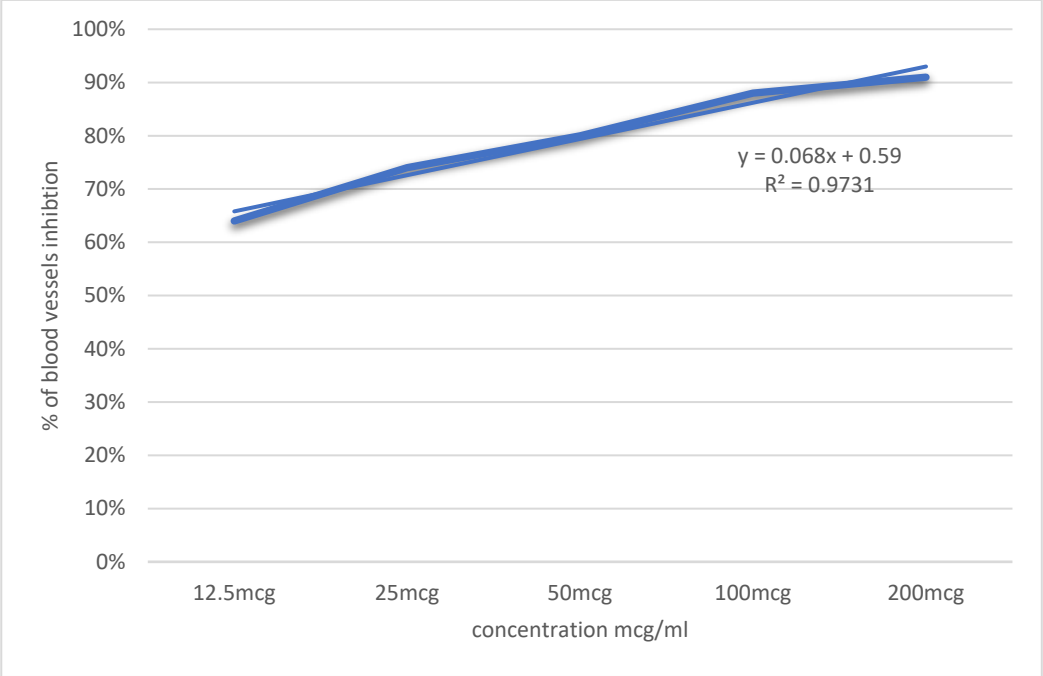


Figure (2): The dose response curve of serial dilutions of 4- chromanol in rat aortic rings assay

In vivo chick chorioallantoic membrane (CAM) assay for 4-chromanol:

Inhibition zone HAD BEEN steadied DURING day7of experiment., Blood vessels in CAM had been subjected for deterioration, also disorder and the presence of pale yellowish appearance at $p<0.05$ That The inhibition showed by presence of vascular zone around the disc which have (0.5mg/ml) of 4-chromanol by showing in Table 3

Table 3. The Zone of Inhibition of Blood Vessels Growth of 4-chromanol Using the Chick Chorioallantoic Membrane (CAM) assay

Eggs number	Zone of inhibition
1	21
2	20
3	22
4	16
5	10
Mean ± STD	17.8 ± 4.91

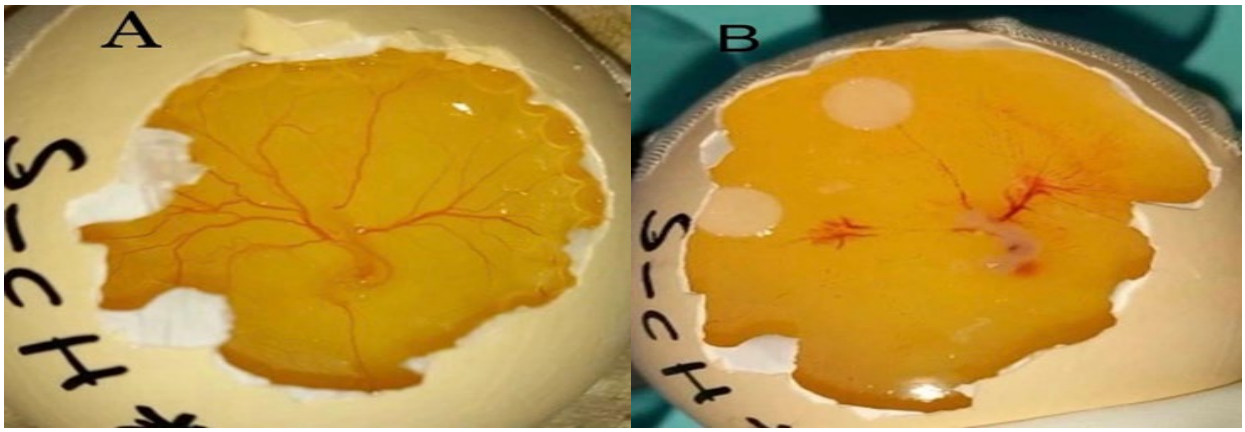


Image (3): The *in vivo* Chick Chorioallantoic Membrane (CAM) assay; where (A)=treated group with DMSO 1% as negative control, (B)=treated group with 4-chromanol

Assessment of proliferation inhibition of human umbilical vein endothelial cells (HUVEC) cell line

Cell viability was evaluated used by MTT assay on the human umbilical vein endothelial cells obtained from (ATCC), passage 7, that authenticated and tested. The data was represented as (mean ±SD) and (IC50) values were had been calculated from the algorithmic equation and was 6.7 mcg/ml as seen in figure 3.

The data obtained from the MTT assay was represented as (mean ± SD), where SD stands for standard deviation. This representation provides information about the central tendency (mean) and the variability (standard deviation) in the measured values.

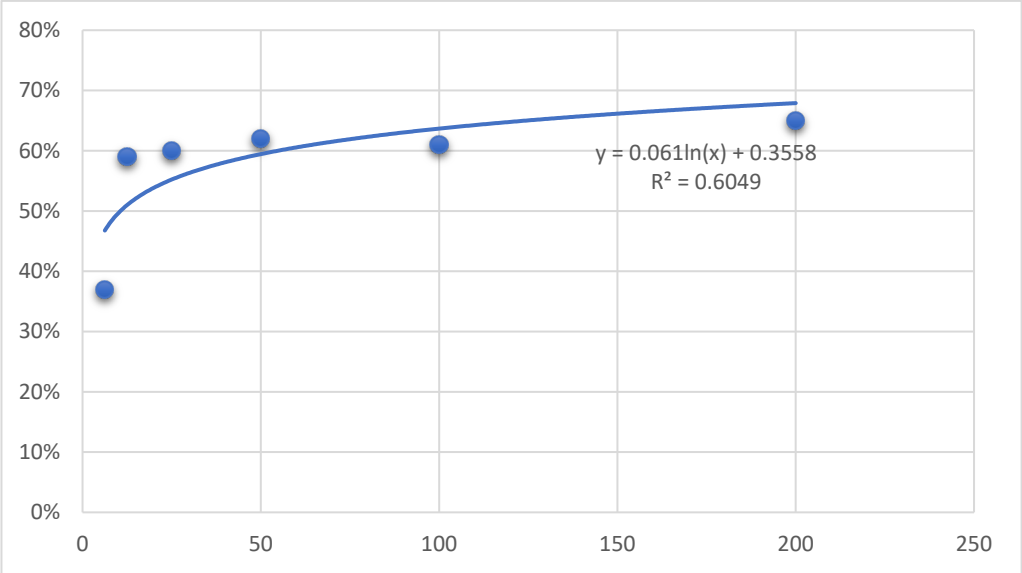


Figure (3): Dose response curve of 4-chromanol on HUVEC cell

Effect of 4-chromanol on cell viability

The following Table 4 and Figure 3 had been showed that there was the significant dose dependent decrease in the cell viability on HUVEC cells at (P<0.05) after (48 h) of treatment. The findings suggest that 4-chromanol has a notable impact on the viability of HUVEC cells, and this effect is likely to be related to the dosage administered.

Table 4: Serial concentrations and their percentage of cell viability of 4-CHROMANOL on HUVEC cell lines.

Conc. Mcg/ml	HUVEC Viability ± SD	
200mcg	65%±	0.29
100mcg	61%±	0.34
50mcg	62%±	0.15
25mcg	60%±	0.25
12.5mcg	59%±	0.17
6.25mcg	37%±	0.1

Gene expression analysis using real time PCR:

The resulting outcomes showed significant Downregulation of the VEGF expression in the 4-chromanol Treated group by (73%) as shown in figure 4 below.

The target gene analyzed in this study is VEGF, which is a crucial signaling protein involved in the formation of new blood vessels (angiogenesis). A 73% downregulation means that the expression of VEGF in the 4-chromanol treated group is reduced by 73% compared to a control group or baseline.

Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method and the data is presented in Table (5).

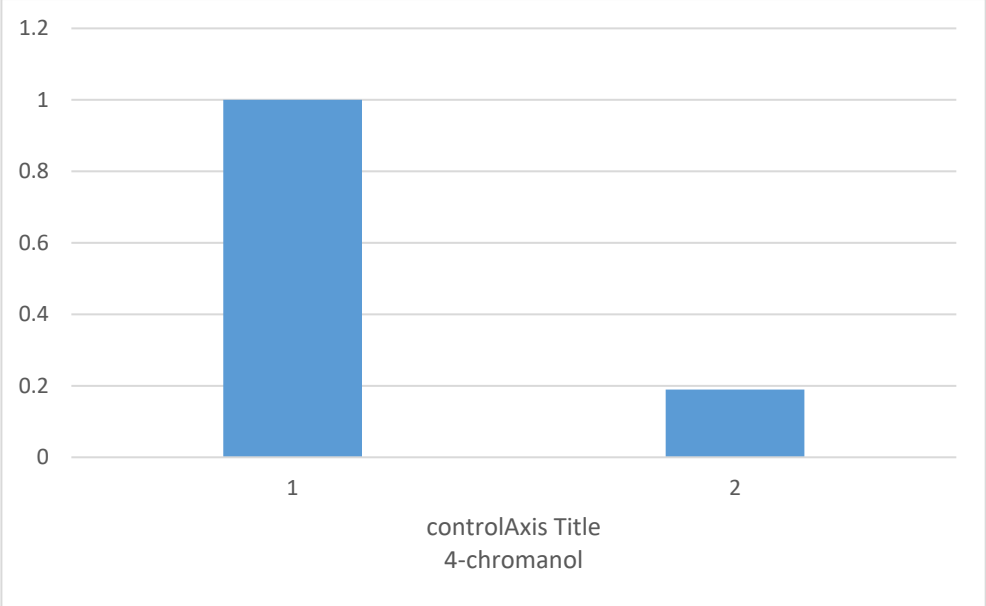


Figure (4): Fold of gene expression of VEGF gene after exposure to 200 µg/ml 4-chromanol depending on ΔΔCt method.

Table (5): Results of relative quantification of PCR by livac method.

Sample	CT VEGF	CT HK	ΔCTE	ΔCTC	ΔΔCt	Expression Fold Change2^-ΔΔCt	
C	17.39	18.04	0	0	0	1	control
1	23.08	21.87	1.21	-0.65	1.86	0.275476279	4-chromanol

Discussion

Cancer is a generic term for huge group of diseases that could affect any part of the body. Most important the feature of the cancer is that the rapid creation of an abnormal cells that was grown after usual boundaries.[23] Cancer could be develop anywhere in body and was named for that the part of the body where it was started , While A tumor was a lump or a growth. Some of the lumps are cancer other are not, of tow type benign and malignant, in which cancer could be spread to other parts of the body while benign tumors did not [24].

Angiogenesis which means by forming of new blood vessels for growing of tissues, means that the same as neovascularization. It was important in enlargement of baby ("good") for situation of tissue repair, But bad in That situation of cancer., the new blood vessels were required to make sure of an sufficient provide of an oxygen and then utrients to cells Before that tumor could grow to size larger than a few millimeters in size .also It's believed that an absence of oxygen ((hypoxia)) in the tumor would provoke angiogenesis, In the response to hypoxia, cancer cells could either emitted signals by themselves or by the influence other cells to emit signals. the example of messengers is VEGF or vascular endothelial Growth factor[25]. That cancers required the evolution of the new blood vessels in order to grow and to metastasize so angiogenesis is the important for cancer. so, for the cancers to increase in size in order to be larger than of one millimeter (1 mm), angiogenesis require to be occurred. growth of cancer done by secreting the substances that cause stimulation of angiogenesis which is known as switched off." during the new blood vessels were used for the repering of wound or after the menstruation, then process of ("switched on") again, but had been usually for short period of the time. also, number of the substances that had been play an inhibitory role for stopping of angiogenesis. like: Angiostatin , Endostatin [26].

The anti – angiogenic activity in ex vivo rat aorta ring assay:

-Aortic ring model is that the most usually used an assay for the angiogenesis as was depend on the rat

aortic for explanation of the capacity of form of new vessels in the collagen gels, also Fibrin or the basement membrane. That model had been an advantage of both in the vivo and in the vitro of angiogenesis. The aortic ring assay was one of the most commonly utilized tests of angiogenesis. Different of the reasons which include :1 popularity comprising , 2cost effectiveness, 3reproducibility, 4 simplicity of the use and5 worthy correlation with in the vivo studies [27].

From that the current work, the anti-angiogenic effect of the 4-chromanol was been first investigated using rat aorticring model, the results assessed during about five days of the ring implants culturing, done by the determining that of the blood vessel length extensions. because that growth of the blood vessel achieved their maximum on the day 5, this interval was represented as the greatest appropriated for Quantification.

The outcomes of this WORK represent that 4-chromanol shows a insignificant dose dependent inhibition of the micro vessels sprouting as comparison to the untreated rings. 200µg/ml of 4-chromanol had been noticed to be the most anti-angiogenic effected on the rataortarings in compared to that another Concentrations, the quantification of the that angiogenesis on that system suggested that the determine of the number and the length of branching micro vessels. Heterocyclic compound is defined as the class of the cyclic organic compounds those have been at least one hetero atom that: nitrogen (N), oxygen (O) and, Sulphur (S) in cyclic ring system.

Heterocyclic compounds were abundant in plants and the animal products; and they are one of the important constituent of one half of natural organic compounds like: an Alkaloids, a natural dyes, a proteins, a drugs, enzymes etc. had a wide used in the pharmaceuticals, also agrochemicals and veterinary products.[28] Heterocyclic compounds are the primarily classified as one of saturated and unsaturated in which that the benzopyran one of classes of it. The benzopyran structural had been an important role in the different pharmaceutical molecules with the various range of the biological properties, which include that an anti-cancer [29],an anti-inflammatory ,an antibacterial [30] etc. In which the4-chromanol belongs to the class of organic compounds known as benzopyran.

4-chromanol it is was action include to bind or to interfere with the several molecular targets and different pathways, including the5-lipoxygenase, nuclear receptors, and also the nuclear-factor, the available data suggest that the chromanols are promiscuitively acting molecules that cause the inhibition of the enzyme activities, and bind to the cellular receptors, and cause modulation of mitochondrial function as well as gene expression [31].

From the mentioned studies that this support the results of study, it shows the inhibition of micro vessels outgrowth was done by 4-chromanol due to its nature as a derivative of benzopyran , chromanol structures regulate the expression of key pro-inflammatory enzymes like that interleukin (IL)-1b, , IL-6, and IL-8 as well as the tumor necrosis factor-a (TNF-a) and the respective formation of the signaling molecules [32], these the pro inflammatory which are considered to be the strong angiogenic factors perhaps due to its has been improved as anti-inflammatory, anti-carcinogen, , and also antioxidant as its vitamin E related compound [33],, anti-angiogenesis activity in Chick chorioallantoic Membrane in vivo Assay.

Chick Chorioallantoic Membrane (CAM) Assay:

In vivo the angiogenesis assays have permitted the important advance in the studying of the efficacy and in the determination of the mechanism of action of the several agents, in that condition (angiogenic enhancers or inhibitors). -The main reasons for the choice of that method are their 1cost, 2ease of use,3 reproducibility, and 4reliability [34], also it is also represented to be one of the most ethical in vivo angiogenesis assays. Had one big disadvantage which include is hard to be used for test of weakly pro-angiogenic drugs due to of its dense web of blood vessels.

In following study of CAM assay that used to verify the activity of anti-angiogenic of 4-chromanol in vivo. 4-chromanol at conc. of 0.5 mg/ml cause inhibition the blood vessels greatly via presence of the pale yellowish discoloration. Therefore, that the results in that assay supported the preceding exvivo results.

Cell viability *in vitro* assay (MTT assay):

-The most commonly of used colorimetric assay which is that utilizes the biomolecule which is3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, determined by((MTT)), that is breaked down by the mitochondria at living cells; this knows that the greater cell of the population cause decrease the MTT faster, the MTT is greatly used in the colorimetric assays that as it was yellow color prior to it breakdown into the purple formazan, also The formazandye is breakdown at solvent system where it was tested for the absorbance with the spectrophotometer , MTT assays is afacile to be evaluate the cell viability depend on the absorbance of the resulting Formazan Solution.

4-CHROMANOL test against THE HUVEC cellline for seeing effect 4-chromanol on this cellline as Angiogenesis process, the IC50 of the 4-chromanol showed that this substance has no cytotoxic effect against this cell. 4-chromanol has promising effect as strong anti-proliferative effect may be related as its vitamin E related compound that having antioxidant activity, Antioxidants like vitamin E, which protect our cells from the harmful effects of ROS that done by donating their electrons in order to neutralize the free radicals. That, cancer cells themselves maybe develop mutations that could be upregulate that their

development of antioxidants, cause providing of evidence of an evolutionary counterbalance system that mallows tumor cells to survive in the states of high oxidative stress. The lipid-soluble antioxidant (vitamin E) which protects the lipid membrane oxidation by the reacting with the lipid radicals that were produced in the lipid peroxidation chain reaction. This reaction cause removal of the free radicals, that prevent the peroxidation reaction from the damaging of cell membranes.

Gene expression analysis using Reverse Transcriptase-PCR:

the Over-expression of VEGF has been seen in the most human cancers compared with normal tissues. The VEGF is released by tumor cells and it was interacting with its receptor that (VEGF-R), on the nearby endothelial cells of normal blood. then the VEGF binds to the extracellular of domain of VEGF-R, that cause stimulating of the tyrosine kinase activity in the intracellular domain of VEGF-R, and cause triggers the downstream network of the cell signaling pathways that may lead to promote the endothelial cells proliferation and the new blood vessels formation. VEGF and VEGF-R had already become the targets for clinical angiogenesis inhibitors. So, angiogenesis inhibitors that caused regression of microvascular tissues and also reduction of the metastatic disease progression.

The Real-time PCR has been one of the most used methods of the gene quantitation as it had a big dynamic range, tremendous Sensitivity, and also could be THE highly sequence-specific, that have small to no post-amplification processing, and it is controllable for increasing sample [35], the Real-time PCR is the technique to that collecting data through the PCR process as it done, and the combining the amplification and of detection into the single step. that was done by the using of variety of different fluorescent chemistries that cause correlate PCR product concentration to fluorescence intensity [36].

In the present study, a -quantitative RT- PCR assay-demonstrated that 4-chromanolcause downregulated the expression of VEGF in HUVEC CELL by analyzing the mRNA expression, suggesting it's the potential involvement in the anti-angiogenic molecular mechanism. The resulting out comes had revealed a significant downregulation of the VEGF expression.

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