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Antiangiogeneic Activity of *Annona Reticulata* Seeds Extract

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Abstract:

Background: Annona reticulata used in traditional medicines for epilepsy, dysentery, constipation, cardiac problems and as insecticide. Annona reticulata seeds extracts contain active acetogenins compounds.
Aim of study : The study aimed to investigate the possible antiangiogenic activity of Annona reticulata extracts.
Material and method: The sequential extraction process with two solvents used to extracts grinded seeds of A. reticulata in the following order according to increment in polarity: n-hexane and ethanol.CAM assay and Ex-vivo rat aorta assay was used to identify the antiangiogeneic activity of both extracts and determine the most biological active extract.
Results: According to CAM assay, there was a statistically higher percent of inhibition zone during exposure to bevacizumab and the two extracts of A. reticulata at both concentrations when compared with DMSO, as a negative control group (p-value ≤ 0.05). Meanwhile, n-hexane extract at only higher concentration (500 µg/5µl) showed a significantly greater zone of inhibition compared with bevacizumab group (p-value ≤ 0.05).Data of rat aorta rings assay that represented as mean of blood vessels growth inhibition revealed a statistically higher percentage of inhibition in bevacizumab and both extracts of A. reticulata treated group compared with negative control group (p-value ≤ 0.05). In this study, the percent of growth inhibition after exposure to n-hexane was approach to that of bevacizumab, where no significant difference was observed between both groups (p-value > 0.05), while group that treated with ethanol extract showed statistically lower percent of growth inhibition compared with n-hexane and bevacizumab treated groups (p-value ≤ 0.05). The results of this assay for the five concentrations of ethanol and n-hexane extract showed a significant reduction in blood vessels growth in a concentration- dependent manner (P ≤ 0.05) compared with negative control. The IC50, n-hexane was 74.95µg/ml while the value of IC50 for ethanol 95.89.
Conclusion : According to the results of these assays the n-hexane was considered as more potent antiangiogenic extract compared to ethanol.

Keywords: *Antiangiogenesis, Annona reticulata, CAM, rat aorta ring assay.*

Introduction:

Angiogenesis is the multiple steps process through which the formation of new blood vessels from pre-existing one take place, is present in two types either physiological or pathological, the pathological angiogenesis play an important role in cancer growth and metastasis (1,2). For this reason the antiangiogeneic therapy consider one of the important ways to fight cancer (3,4).

One of the proangiogenic protein that stimulate the new blood vessels formation is vascular endothelial growth factor (VEGF), so the first angiogeneic inhibitors that developed was Bevacizumab, which is act by binding to VEGF lead to inhibit its binding to receptors . The treatment protocol with angiogeneic inhibitors may associated with various adverse effects or resistance mechanisms so new goals was usage of the herb medicine as alternative or adjuvant therapy to it (5,6).

Annona reticulata is belong to Annonaceae family (7), that located in tropical and subtropical regions (8). This plant studied for their many biological activities like Antioxidant , Anti-inflammatory, Analgesic, CNS depressant activity, Antipyretic and anticancer activity against various cancerous cell lines (7,9,10) The major active constituents that respon-

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sible for most of these activity are acetogenins that extracted from most parts of this plant. This study aimed to evaluate the antiangioeneic effect of A.reticulata seed n-hexane and ethanol extracts through CAM and rat aorta rings assay and determine which one is more biological active.

Materials and Methods:

Plant material and extraction

Seeds A.reticulata were collected from the local market in Baghdad. Authentication was done in Pharmacognosy and Medicinal Plants Department- College of Pharmacy / Mustansiriyah University. Soxhlet apparatus was used to extract the plant seeds using successive solvent extraction method with two solvents, using less polar solvent (n-hexane) then more polar one (ethanol). The concentrated extract transferred in beakers (pre-weighed), dried under a stream of cold air to determine the percent of yield according to following formula (11,12):

$$\text{The yield of extract} = \frac{\text{weight of material obtained}}{\text{weight of start material}} \times 100$$

The extracts were then stored in glass amber containers until use. Anti-angiogeneic activity of the extracts was examined by CAM assay, rat aorta ring assay.

Experimental animals

A healthy apparent experimental rats used in this study were chosen and supplied by the animal house of Iraqi Center for Cancer and Medical Genetics Research (ICCMGR), University of Mustansiriyah / Baghdad –Iraq. All the animals were allowed to access food and tap water freely, and preserved in plastic cages under controlled circumstances of temperature (22±2 C0) and light (12-12 hrs of light/dark cycle), The experiments were accepted by the ethics committee for animal's experimentation in the College of Pharmacy/ Mustansiriya University.

Chick chorioallantoic membrane assay (CAM assay)

36 fertilized chicken eggs were used and obtained from the Agricultural Research Office / Ministry of Agriculture, Baghdad- Iraq. The eggs were put in an incubator for 3 days under constant humidity at 37 C0. Then (2-3) ml of albumin was withdrawn from eggs after spraying with 70% alcohol. The holes of eggs were closed by sterile adhesive tape and incubated again until reach the day 9 of experiment. On day 9, the window was made and reclosed with tape and any remaining materials inside eggs were removed from the area quickly (13,14). The n-hexane and ethanol seeds extract were prepared by weighing 0.05 and 0.1 g, dissolved in 10 µl of Dimethylsufoxide (DMSO) and complete volume to 1000 µl by adding 990 µl of distilled water. Then using 5 µl of the prepared solution and put on filter paper disc to insert into the fertilized eggs on day 10, then reclosed the eggs and return to incubator for 72 hr. DMSO was used as a negative control at concentration of 1% v/v, while bevacizumab was used as a positive control at concentration 238µg/ 9.5 µl; 6 CAM were used for each control and test sample. The scoring of CAM was described as (+) for 3-6 mm, (++) for 7-9 mm, and (+++) for >10 mm zone of inhibition (15) .

Rat aorta ring assay (ex vivo study)

Rat aorta ring assay is an ex-vivo assay used to evaluate angiogeneic and anti- angiogeneic substance activity. The method used in this assay was according to Brown and his co-workers, with some modification (16). Two healthy albino rats were adopted to laboratory conditions for 7 days before experiment, anesthetized by ketamine (dose of 50 mg /kg, intraperitoneally) then excision and identification of thoracic aorta, which isolated by cutting both ends of it (17,18). Freshly excised thoracic tissues were rinsed with cold phosphate buffer saline (BPS) previously prepared and washing the dissected tissue several times with it. Then the tissue specimen cleaned by removing any adipose tissue and blood clots. After that, the tissue sample was putted with Minimum Essential Medium Eagle (MEM) media supplied with high concentration of penicillin and streptomycin until cutting into 1-2 mm thickness under dissecting microscope (19). The rings were cultured in 96 tissue culture plate, 100 µl (MEM) media was used for culturing {prepared of each 500 ml of media by adding 50 ml of fetal bovine serum (FBS) + 0.5ml of penicillin + 0.25 ml streptomycin +10 ng/ml epidermal growth factor (EGF)} (20,21). N –hexane and ethanol extracts were added to the complete growth Medium and prepared in five serial concentrations (6.25, 12.5, 25, 50, and 100 µg /ml), each treatment was performed in six replicates. Negative control culture received just 1% of DMSO with medium only, positive control culture received bevacizumab at concentration of 1µM /ml (0.59mg) (22,23). Vessels were cultured at 37˚C in 5% CO2 in a humidified incubator for five days. The length of branches was measured under inverted microscope (at 40x and 100x magnification) with the aid of camera and software program (micro-capture, 6.9.12). Data were represented as mean ± standard deviation (M±SD). The blood vessels growth inhibition percent was determined according to the following formula (24,25) :-

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

A0 = distance of blood vessels growth in µm.

A = distance of blood vessels growth in the control in µm

Rat aorta assay Dose - response plot and IC50 of n-hexane and ethanol seeds extracts of A. reticulate

Dose- response plot was drawn as x and y-axis on Excel program to estimate IC50, which represent the concentration that inhibit 50% of blood vessels growth and calculated using the linear regression equation for each extract (26), where: Y= percentage of blood vessels growth inhibition , X= concentration of Annona reticulata extract.

Statistical analysis

The statistical analysis was performed using SSPS 16.0 for Windows Software Program. All data were expressed as mean ± standard deviation (SD). To compare among tested groups, the one way analysis of variance (ANOVA) was used. P-value ≤ 0.05 was considered to indicate a statistically significant difference. The IC50 for both extracts within rat's

aorta ring assay test was determined and calculated according to linear regression equation on Excel program as follow: y = m x + b, where y is the percentage of inhibition and it set to be 50%, m is the slope of the standard curve, x is the concentration of compound tested, and b is the y intercept of the line of standard curve.

Results:

Yield percent of A.reticulate seeds extracts

The yield of extract obtained by extraction process gave the lowest percentage of 36.44 gm (11.2 %) for n-hexane extract and the highest percentage of 44.4 gm (13.6 %) for ethanol extract. These results were obtained from 325 gm of Annona reticulata dried seeds.

Effect of A.reticulate seeds extracts on blood vessels growth inhibition depending on chick chorioallantoic membrane (CAM) assay:

The inhibition zone of blood vessels which calculated from images in figure (1) was more than 10 mm (+++) for bevacizumab and each extract, these results also showed in table (1). According to the mean level for inhibition zone, there was a statistically higher percent of inhibition during exposure to bevacizumab (18.64%) when compared with DMSO, as a negative control group (6.393%) (P-value= 0.004). The two extracts of A. reticulata at both concentrations also showed a significantly higher percent of growth inhibition when compared with negative control (p-value ≤ 0.05). There was no significant difference in the extent of inhibition zone between bevacizumab treated group and groups treated with ethanol extract (17.105% & 22.123% is mean of inhibition zone for ethanol extract at 250 & 500 µg/5µl respectively) p value > 0.05. Meanwhile, the n- hexane extract at only higher concentration (500µg/5µl) showed a significantly greater zone of inhibition (27.77 %) compared with bevacizumab group (p-value=0.026).

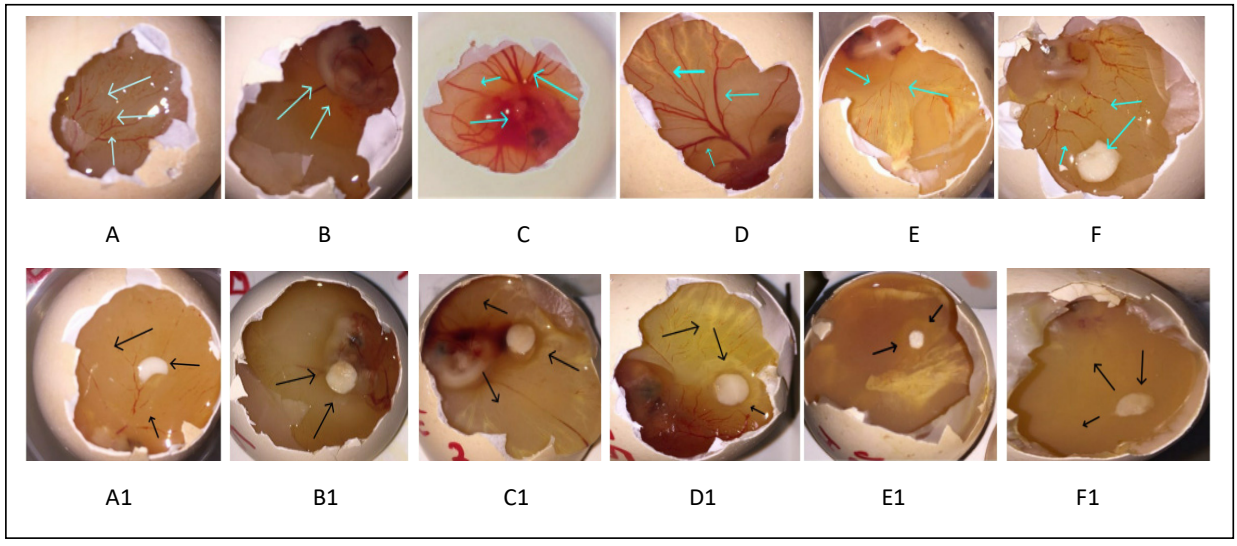


Figure (1): *Effect of Annona reticulata seed extracts on blood vessels during chick chorioallantoic membrane (CAM) assay. Treated groups were: (A) DMSO, (B) bevacizumab, (C, D, E & F) ethanol and n- hexane extract of Annona reticulata seeds at 250 & 500 µg/5µl, respectively before and after (represented by no.1) treatment.*

Table (1): *Effect of A. reticulata seed extracts on growth inhibition zone of blood vessels within CAM assay.*

		Zone of inhibition area (mm)/scroing						
Group	concentartion	Egg1	Egg2	Egg3	Egg4	Egg5	Egg6	Percent of inhibition (mean ± SD)
DMSO (negative control)	1% v/v	5.37/+	6.17/+	8.4/++	7.15/++	4.22/+	7.05/++	6.393± 1.34[a]
Bevacizumb (postive control)	238µg/9.5µl	13.9 / +++	20.9 / +++	25.5 / +++	19 / +++	20.65 / +++	11.9 / +++	18.64 ± 4.55[b]

Ethanol extract (low concentration)	250µg/5µl	11.4 / +++	11.67 / +++	26.32 / +++	13.75 / +++	24.52 / +++	14.97 / +++	17.105±6.02[b]
Ethanol extract (high concentration)	500µg/5µl	23.1 / +++	16.35 / +++	35.92 / +++	25.17 / +++	13.85 / +++	18.85 / +++	22.123 ±7.32[b]
n-hexane extract (low concentration)	250 µg/5µl	17.77 / +++	40 / +++	22.1 / +++	18.55 / +++	28.9 / +++	13.7 / +++	23.5 ± 8.72[b]
n- hexane extract (high concentration)	500 µg/5µl	32.02 / +++	34.97 / +++	25.15 / +++	23.92 / +++	33.37 / +++	17.2 / +++	27.77 ± 6.25[c]

Data of growth inhibition were expressed as mean ± SD. Different lowercase letters (a,b,c) indicate significant difference among groups, where P-value ≤ 0.05 consider significant difference. Score of inhibition zone were as follow: (+) mean (3-6mm), (++) mean (7-9 mm), while (+++) mean (> 10 mm) inhibition. Note: n= 6 for each group.

Effect of A. reticulata seed extracts on blood vessels growth inhibition depending on rat aorta ring assay (ex vivo assay):

Data of rat aorta rings assay that represented as mean of blood vessels growth inhibition revealed a statistically higher percentage of inhibition in bevacizumab treated group (63%) compared with DMSO group (0.0%) which considered as negative control (p-value =0.00). Also Also, n-hexane and ethanol extracts of A. reticulata showed significantly higher percentage of growth inhibition compared to DMSO group (p-value = 0.00). In this study, the percent of growth inhibition after exposure to n-hexane at concentration (100 µg /ml) (59%) was approach to that of bevacizumab (63%), where no significant difference was observed between both groups (p-value = 0.313) while ethanol treated group showed statistically lower percent of growth inhibition (51.3%) compared with n-hexane and bevacizumab treated groups (p-value = 0.05 & 0.01, respectively).Figure (2) shows the percentage of growth inhibition after exposure to bevacizumab and A.reticulata seed extracts (100 µg / ml).

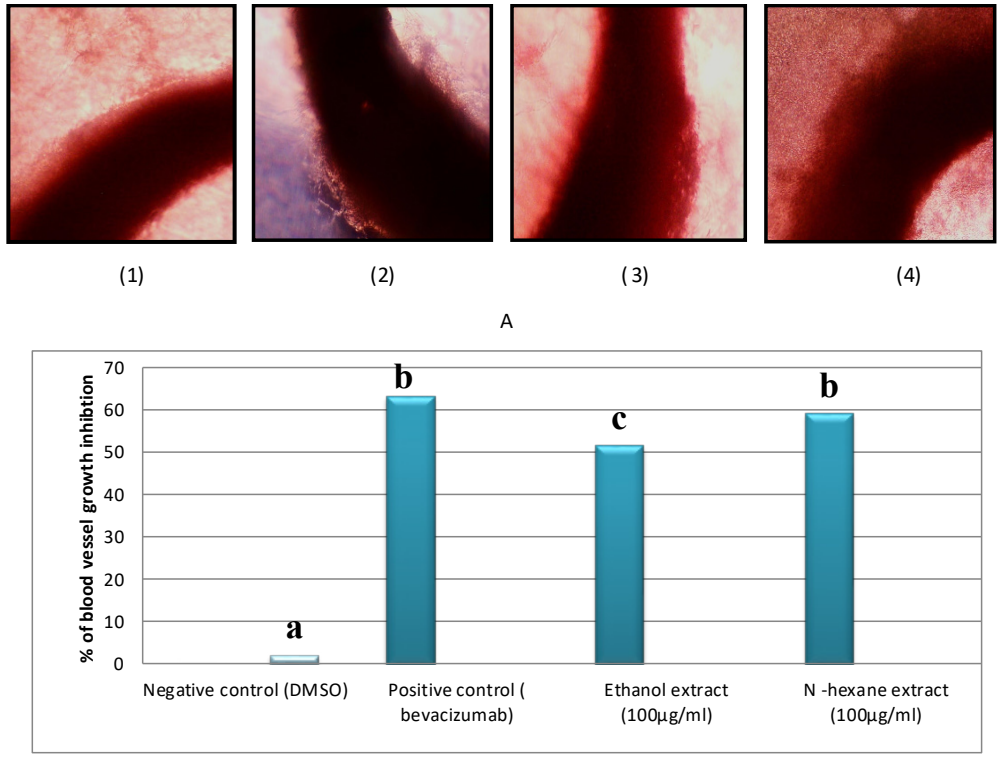
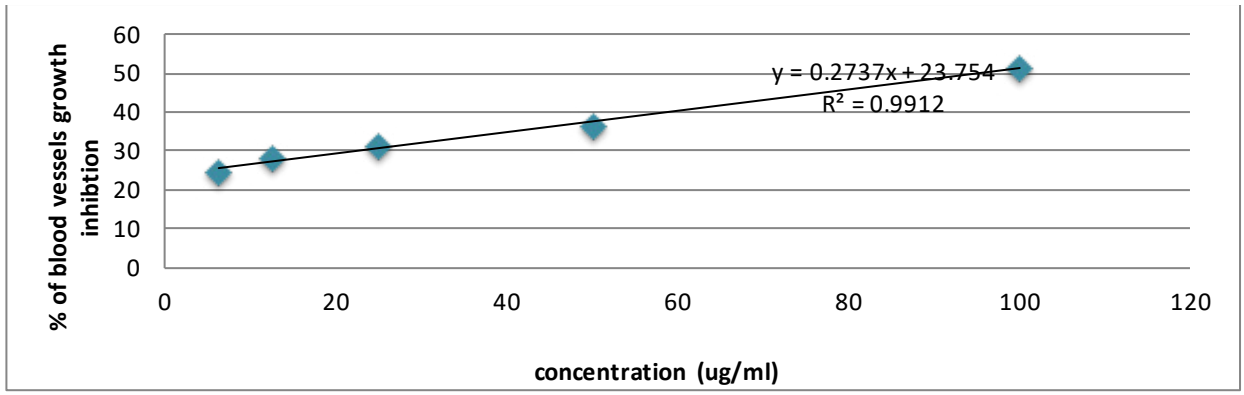


Figure (2): *Effect of Annona reticulata seed extracts (100µg/ml) on blood vessels growth inhibition depending on rat aorta ring assay (ex vivo assay).(A) shows the images of aorta rings treated with(1) DMSO, (2) bevacizumab, (3) ethanol extract (100µg/ml), and (4)) n-hexane extract (100µg/ml). (B) Data were expressed as mean ± SD. Different lowercase letters (a,b,c) indicate significant difference among groups, where P- value ≤ 0.05 considered statistically significant difference..*

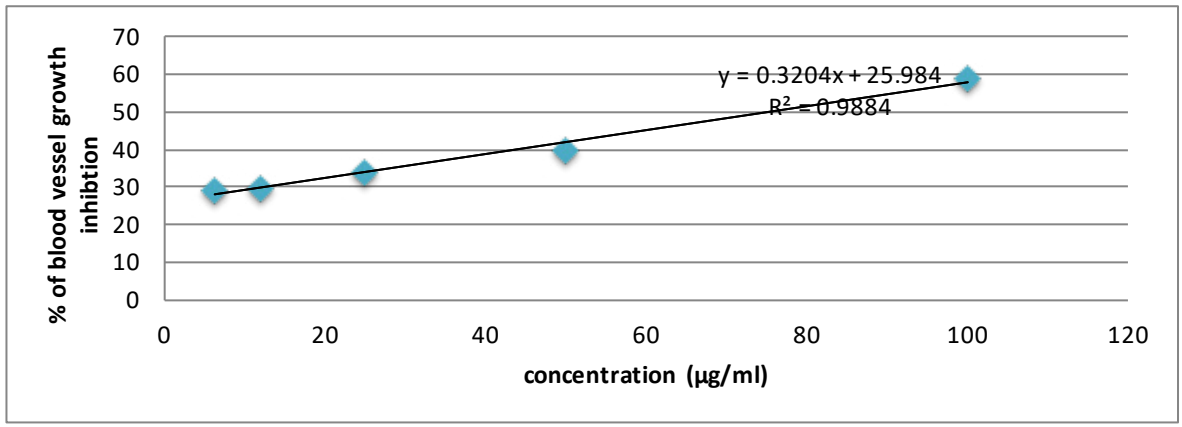
Dose-response curve of A. reticulata extracts n-hexane extract during rat aorta ring assay:

The results of this assay for the five concentrations of ethanol and n-hexane extracts showed a significant reduction in blood vessels growth in a concentration- dependent manner ($P \leq 0.05$) compared with negative control. Meanwhile, at 100 µg/ml concentration, n-hexane extract gave no significant difference in the percent of blood vessels growth inhibition ($P = 0.191$), compared with positive control.

The linear regression equations were used for calculation of IC50 for ethanol and n-hexane extract as shown in figures (3-A) and (3-B) respectively, where y = percentage of blood vessel growth inhibition and x= concentration of extract. The concentration that required to inhibit 50% of blood vessels by ethanol extract was calculated from the following equation: y= 0.2737 x + 23.754, so IC50 obtained = 95.89 µg/ml, and for n-hexane extract was calculated from the following equation: y= 0.3204 x + 25.984, so IC50 obtained = 74.95 µg/ml. According to the results that obtained from the percent of blood vessels growth inhibition and IC50, n-hexane extract was considered as more potent antiangiogenic agent than ethanol extract.



A



B

Figure (3-7): *Dose-response curve for inhibition growth of blood vessels after exposure to (A)ethanol extract (B) n-hexane extract of Annona reticulata, according to rat aorta ring assay.*

Discussion:

In the present study, the soxhlet apparatus and successive solvent extraction process were used to extract Annona reticulata seeds using two solvents (n-hexane and ethanol) that differ in their polarity to extract different components and study the biological effects of them (27, 28). The yield of extract in this study was 13.6% for ethanol extract (from 44.4 gm) and 11.2% for n-hexane extract (from 36.44 gm). These variations in the yield of extract can influence by many factors as solvent optimization, effect of extraction time, extraction temperature, and particle size of the sample (29).These results were better than that with a previous study which revealed the yield of extract from 100 gm seeds of Annona squamosa (another Annona species) was 3.6% for ethanol extract and 14.85% for n-hexane extract (30).

To study tumor angiogenesis and the probability of future

metastasis, CAM assay is one of the favorite used methods because it has low cost, rapid and simple technique, and allow to examine large number of samples to study their angiogenic and antiangiogenic activity (31,32). The percentages of CAM assay inhibition zone that obtained was (23.5 %, 27.77 %) for n-hexane extra and (17.105 %, 22.123 %) for ethanol extract at concentrations of (250 µg/5 µl and 500 µg/5 µl) respectively, so it follow a concentration-dependent manner (table 1). Both extracts had potent antiangiogenic activity and had a good inhibition for blood vessels growth compared with bevacizumab (18.64%). The highest and more significant percent of inhibition occurred with n- hexane extract at concentration of (500 µg/5 µl).

Bevacizumab is a humanized monoclonal antibody (anti-VEGF) that used as a positive control in this assay via inhibiting vascularization. It act via binding to circulating VEGF that consider as one of the most important pro-angiogenic factors, since VEGF over expressed in most tumor types and it's level increase in tumor progression or recurrence (33,34) . From data of the current study, it's shown that the extracts of A.reticulata have antiangiogenic effect and this may agree with previous literatures regarding anticancer effect with various parts of Annona species at molecular level. One mechanism of the A.muricata constituents is the inhibition of HIF-1α, which is one of the three isoforms of HIF family (HIF1-3) that when heterodimerize with aryl hydrocarbon receptor nuclear translocator (HIFβ/ ARNT) formed an active complex that began to express hundreds of genes, one of them is VEGF, to provide the hypoxic tissue and cells with oxygen and nutrients through formation a new blood vessels (35-37). So the components of the Annona family can act as inhibitors of HIF-1α and VEGF, this may account for their antiangiogenic activity in CAM assay.

The antiangiogenic activity for both extracts of A. reticulate seeds was quantified using a microcapture soft program through measuring a tiny blood vessels from cultured aorta rings and using an equation to calculate the percentage of blood vessels growth inhibition that was 51.3%, 59% and 63% for ethanol, n-hexane extract and bevacizumab (positive control) respectively. The results obtained by this assay support the results of CAM assay, where extract had antiangiogenic activity and significantly inhibit the blood vessels growth when compared with DMSO group which represent the negative control, as showed in figure (2). The antiangiogenic property for bevacizumab is well established and explained by its ability to inhibit the formation and progression of new blood vessels (38).One of the proposed mechanisms of Annona species antiangiogenic activity may related to the scavenging and antioxidant effect of their components. When the oxygen and nutrients demand of tissue increase, as what happen in tumor growth, the angiogensis process begin and a hypoxia/ re-oxygenation cycle occurred and reactive oxygen species (ROS) formed as a result for this cycle. The ROS allowed the angiogenesis either directly or indirectly by formation of active oxidation products, including peroxidized lipids. The family of Annona plants had antioxidant activity and this may attribute to the suppression of angiogenesis process (39,40).

The five concentrations of both extracts of A.reticulata gave a significant inhibition of blood vessels growth in a concentration-dependent manner, compared with DMSO as a negative control group. IC50 were determined by linear regression equation from dose-response curve figure (3) were equal to 95.89 µg/ml for ethanol extract and 74.95 µg/ml for n-hexane extract, whenever IC50 value decrease, the safety of extract will decrease too and vice versa. According to U.S. National Cancer Institute (NCI) Plant Screening Program, the crude extract considered not cytotoxic (in vitro) if IC50 > 20 µg/ml (41). In the present study, both extracts considered non cytotoxic because their IC50 values were greater than 20 µg/ml and the ethanol extract was less biologically active if compared with n-hexane extract, since its IC50 was greater. In the current study, n-hexane extract showed a higher percent of blood vessels growth inhibition and lower IC50 value than ethanol extract, These findings agree with pervious study of A.muricata seed extract on rats, where its n-hexane extract showed high increment in the catalase antioxidant activity, so protected the cells from oxidative stress and provided antiangiogenic activity (42).

According to this study both extracts had antiangiogeneic activity and the n-hexane extract is more biological active. However, to our knowledge, the antiangiogenic effect and other anti-tumor activates of A. reticultata is not verified till now. Therefore, using the plant as a crude extract without fractionation and over-extraction of specific compound have advantage in tumor therapy, since this may increase the ability of herbs to overcome the proposed multi-drug resistance (that may occurs with different chemotherapies and VEGF inhibitors) due to the synergistic effect of the compounds.

CONCLUSION:

Angiogenesis play important role in cancer growth and metastasis , A.reticulata extracts showed potential

Antiangiogeneic activity with highest effect observed by the n-hexane extract, this herb may give hope to act against tumor as adjuvant with chemotherapy.

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Conflict of interest: none

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