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ORIGINAL ARTICLE

Embryonic Vascular Toxicity of *Calotropis Procera*; Evaluation of Early Anti-Vasculogenic Property and Molecular Aspects Using A Chick's Extra-Embryonic Membrane Model

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ABSTRACT

Background: *Calotropis procera Aiton* (*C. procera*) is used in folk medicine to cure various diseases. However, the use of herbs in human medicine is sometimes associated with adverse effects. Chick embryo is a preclinical model relevant to assess adverse effects of drugs and herbs. Therefore, the current study aimed to assess the alteration of vascular branching patterns in the chick's extra-embryonic membrane following *C. procera* treatment. Besides, the alteration in molecular cues involved in early embryonic vasculogenesis, such as vascular endothelial growth factor A (VEGF-A) was also quantified.

Methods: In an experimental study, 30 fertile chicken eggs were divided into three equal treatment groups; sham control, and *C. procera*-treated groups whose cases were treated with *C. procera* extract at doses of 50 or 100 mg per kg of egg-weight.

Results: Quantification of extra-embryonic membrane vasculature showed that anti-vasculogenic effect of the herbal extract was revealed by a reduction in vessels area, total vessels length, vascular branch and increased lacunarity. The alterations were made in a dose-dependent manner. The relative expression levels of VEGF-A mRNA was also decreased in the herbal-exposed extra-embryonic membrane.

Conclusion: Concerns about the side effect of *C. procera* during pregnancy were confirmed by data presented in this study. We concluded that altered early vascular development and gene expression might eventually lead to developmental defects in embryo following *C. procera* consumption. Therefore, the use of this herb must be limited at the time of fetal growth especially at the dosage higher than 50 mg per kg.

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Introduction

During pregnancy, the normal development of the fetus has always been a major concern. Some factors that influence this biological phenomenon are the drug toxicity and compounds that affect the genesis and proliferation of the vascular network. Consumption of certain herbs during pregnancy is associated with an increased risk of fetal defects, congenital malformations, histopathological injuries, and growth retardation (1-3). Herbs may also possess the vasculo-toxic property and affect the embryonic peripheral vascular development (4, 5).

Vasculogenesis is the de novo formation of vessels from angioblasts and it is the critical steps involved in embryo survival and development. Several pathways, growth factors, and proteins are known to be crucial for the regulation, promotion and inhibition of this process. The most important protein is the vascular endothelial growth factor A (VEGF-A) which is generated by mesenchymal tissue (6, 7). Determination of the adverse effects of chemical elements and drugs requires the use of a preclinical model. The chick's extra-embryonic membrane (EEM) is promoted to study the effects of vasculogenic/anti-vasculogenic factors and compounds (8, 9). The vascular plexus of the chick's EEM is simple and contains undifferentiated vessels, which allow it to branch progressively during embryonic growth (10).

The *Calotropis procera* (*C. procera*) is used in folk medicine to cure various diseases. Medicinal properties associated with various parts of the plant include anti-inflammatory, anthelmintic, antioxidant, analgesia, antipyretic, antiproliferative, antinociceptive, antibacterial, larvicidal, antifungal, and molluscicidal activity (11, 12). Today, it is often dispensed in the treatment of digestive disorders including diarrhea, constipation, and stomach ulcers; for painful conditions including a toothache, cramps, and joint pain; and for parasitic infections including elephantiasis and worms. Some people use *C. procera* for syphilis, boils, inflammation, epilepsy, hysteria, fever, muscular spasm, warts, leprosy, gout, snakebites, and cancer (13-16).

It is well established that the use of *C. procera* in human medicine is sometimes associated with adverse effects such as diarrhea, vomiting allergy, and ocular toxicity (17, 18). Although increasing consumption and production of various compounds of *C. procera* are predicted in some regions of the globe, little has been published about the toxic and pathological effects of this compound on the embryonic vasculature. In addition, the exact mechanisms by which it affects vascular genesis and expansion are not yet fully understood.

The present study was aimed to answer the questions: (i) Does *C. procera* alter the early development of the EEM-vasculature? And (ii) Does *C. procera* alter the expression of VEGF-A in the extra-embryonic vascular plexus? To answer these questions, a chick embryo model was used. Computerized programs were also applied to quantify the branching pattern and morphometric evaluation of vessel density in the chick's EEM in order to demonstrate the *C. procera*-induced anti-vasculogenic activity. Finally, the results are combined with real-time PCR data to assess the effect of the drug on molecular expression, which is associated with vascular genesis.

Materials and Methods

In an experimental study, the effect of the *C. procera* on early embryonic vasculogenesis was determined by analysis of vascular branching pattern and morphometric analysis of capillary density from the chick's EEM. Briefly, 30 fertile chicken eggs (Ross 308) with the average egg-weight of 53.6±0.7 g were purchased from the Mahan Breeder Company, Kerman, Iran. In this company, the breeder birds were reared under the standard condition to attain optimum bird performance.

For extraction of the essential oil from *C. procera*, the fresh leaves and stems of the milk weed of *C. procera*, from *Asclepiadaceae* family were collected at the beginning of November in Larestan's Arad area, south of Fars province, Iran and dried in a dark room for 20 days at ambient temperature. Then, the dried materials (stems and leaves) were grinded to small parts and 70 g of the resulting mixture was added to 1.5 L of methanol followed by vigorous shaking on a shaker for 48 hours at room temperature. Afterward, the filtration of solution and evaporation of its solvent at 45°C under vacuum condition provided the green viscous oil that was used without further purification. A milky sap outflow from the milkweed was obtained by wounding its growing branches' tips, buds, and leaves (19).

Regarding the embryo treatment and image acquisition, fertilized eggs were incubated in an electrical incubator (Belderchin Damavand Co. PLC-DQSH, Tehran, Iran) at 37.5°C and 60% relative humidity. A pinpoint hole of approximately 0.5 mm was made in the egg shell and the outer shell membrane at the wider end after 24 h of incubation. The eggs were treated with 50 µl of either *C. procera* extract or sterile phosphate buffered saline, as sham control. The eggs were re-treated at 2 different time points: 24 and 48 h following the first treatment (e.g., 48 and 72 h total developmental stage, respectively).

The herbal extract was applied in a single drop on the inner shell membrane, using a 50 µl Hamilton syringe. This method of treatment has described by others (20, 21). After each treatment, the exposed hole was sealed with warmed paraffin (Merck, Darmstadt, Germany) and the eggs were placed back into the incubator under the mentioned condition. On day 4 of incubation, live embryos were selected and those that died during the incubation period (e.g., due to the effect of the herbal extract on the blastodisc and vascular plexus or manipulation) were excluded.

Finally, treated embryos were assigned to three groups as follow: group 1 (n=10): phosphate buffered saline treated group (control group), groups 2 (n=10) and 3 (n=10): herbal extract-treated groups, in which, embryonated eggs were treated with herbal extract at doses of 50 or 100 mg per kg egg-weight, respectively. A window of 25 mm by 25 mm was made in the eggshell, to allow microscopic imaging of the EEM-vasculature. High-resolution images (4000×3000 pixels) were captured using a stereomicroscope (Luxeo 4D Stereozoom Microscope, Labomed, CA, USA) attached to Canon SX200 camera supported by Luxeo software (Figure 1, parts a, e, and i). The captured images were saved as *.tif files using a 14.5 inch PC (Intel Core i3-390M, 2.66 GHz). The experiment was performed according to the suggested European Ethical Guidelines for the care of animals in experimental investigations.

The computerized vascular branching pattern analysis was performed using the image analyzer software such as MATLAB® (Mathworks Matlab R2015a), ImageJ® 1.48 (National Institutes of Health, Bethesda, Maryland, USA) and Digimizer®4.3.0 (MedCalc Software, Mariakerke, Belgium). Firstly, a certain area was extracted from the captured images. The extracted area, approximately 212 mm^2 containing 1870×3035 pixels, was identified at the right-lateral vitelline vascular plexus (Figure 1, parts a, d, and g).

The images were converted into the 8-bit format and processed to extract the schematic pattern of the vascular plexus (Figure 1, parts b, e, and h). Finally, their color level was reduced to a dichotomic binarized (black and white) format and transformed into skeletonized pictures (Figure 1, parts c, f, and i). The skeletonized picture presents the structural shape of the object. The vascular branching pattern

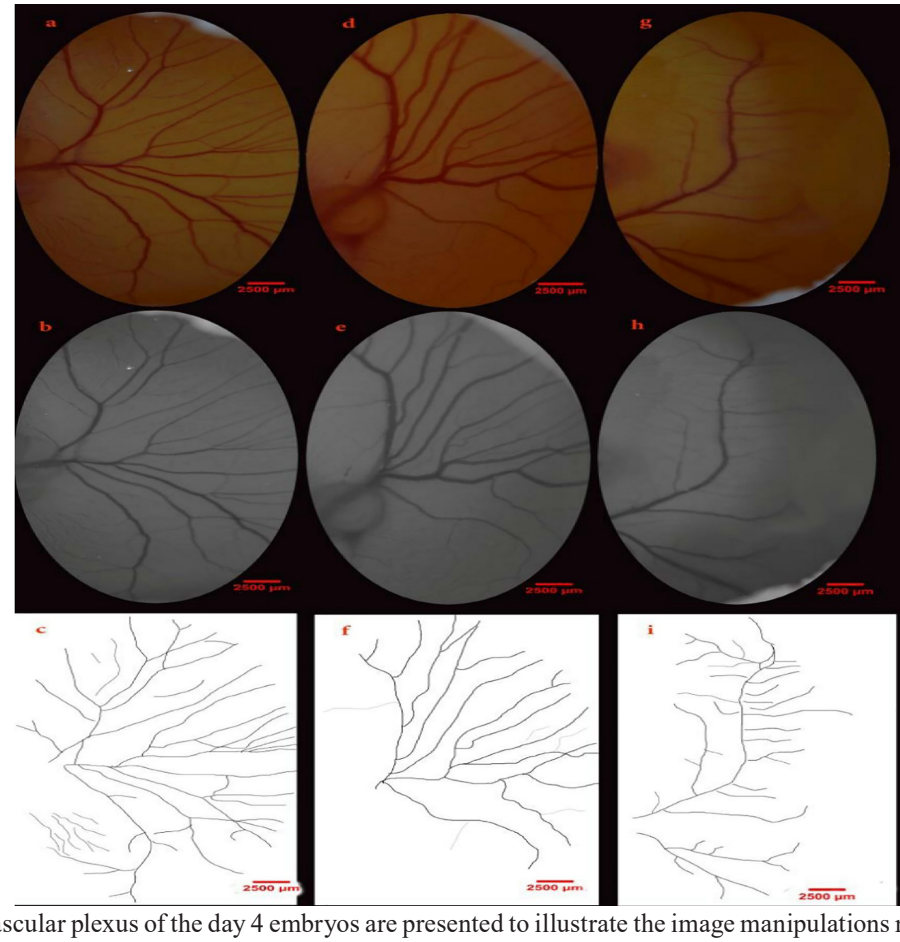

Figure 1: The vascular plexus of the day 4 embryos are presented to illustrate the image manipulations required to vascular branching pattern analysis. The images are captured from the embryo of the control (a-c) and *Calotropis procera* at a doses of 50 (d-f) or 100 (g-i) mg per kg egg-weight. (a, d, and g) A certain area, 212 mm^2 containing 1870×3035 pixels, was identified at the right-lateral vitelline vascular plexus. (b, e, and h) The extracted areas were converted into the 8-bit format. (c, f, and i) The vascular branching pattern was ascertained from the skeletonized pictures.

was specifically estimated for changes in parameters including vessels area, total vessels length, vascular branch and lacunarity (22, 23). The lacunarity indicates the areas without any vessel branch.

For morphometric analysis of capillary density, a defined area inside the right-lateral vitelline plexus was chosen and the contrast enhanced (Figure 2a). The effort has been made to choose the constant areas in each case in order to avoid subjectivity in the analysis. The selections were converted to a binarized format (Figure 2b). From those, areas without any branch vessels were selected for analysis. Five such areas per case were identified and the percentage of the areas containing black pixels was calculated (Figure 2c). The black pixels of the images indicate the red color, or blood, in the original images. The mean of all areas quantified in each image is expressed as the mean capillary area (MCA).

Relative expression level of VEGF-A gene was determined by quantitative real-time PCR (qPCR) assay. Briefly, total RNA was extracted from the chick's EEM using the RNeasy® mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol (n=4 per treated group). RNA concentration (ng) and purity (260 nm: 280 nm) were determined spectrophotometrically using the NanoDrop ND-1000 (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE, USA). The cDNA was synthesized by the Primescript™ RT reagent kits (Takara Bio, Inc., Shiga, Japan) and reverse transcription was carried out on 500 ng of the total RNA at 37 °C for 15 min.

The PCR reaction was performed in duplicate with the Rotorgene cycler system (Rotorgene 3000 cycler system, Corbett Research, Sydney, Australia) using a SYBR Green assay (SYBR Premix Ex Taq™ II, Takara Bio, Inc., Shiga, Japan) according to the recommended protocol. The specific primers and reference gene sequences are listed in Table 1 (21). The primers amplified 86 bp fragments in the VEGF-A mRNA genes. At first, a holding treatment at 95ºC for 1 min was performed and then amplification was done for 40 cycles (10 s at 95ºC for denaturation of DNA, 15 s at 60ºC for primer annealing and 20 s at 72ºC for extension). Expression levels were calculated relative to the expression levels of the selected reference gene.

Statistical analysis was performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance followed by Tukey's test was applied to assess the significance of differences in the vascular parameters and gene expression. A p-value of <0.05 was considered as statistically significant.

Results

Regarding vascular branching patterns, at the time of image acquisition (day 4 of incubation), the embryos were at Hamburger–Hamilton

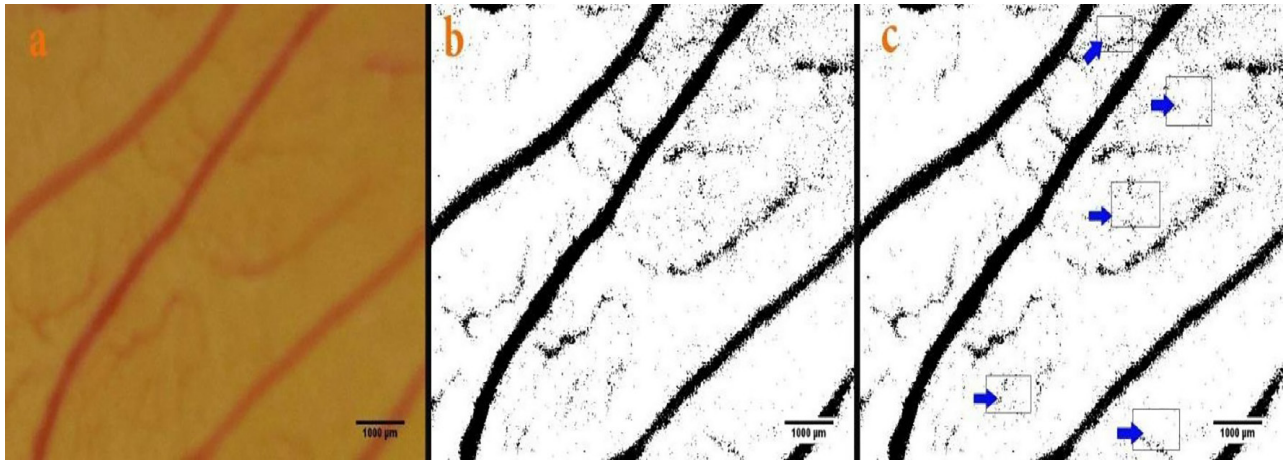

Figure 2: The mean capillary area (MCA) quantified from the chick's extra-embryonic membrane at day 4 of the incubation period. (a) The defined area inside the right-lateral vitelline vascular plexus. (b) The selection has been converted to a binarized image. (c) Five areas (arrows) without any branch vessels are selected and the percentages of the areas containing black pixels were calculated for quantification of the MCA. The black pixels of the image indicate the red color, or blood, in the original image.

Table 1: The specific primers and reference gene sequences for quantitative real-time PCR

Gene (*Gallus gallus*)		Primer Sequence (5′–3′)	Product size (bp)
VEGF-A	Forward	CAATTGAGACCCTGGTGGAC	86
	Reverse	TCTCATCAGAGGCACACAGG	
GAPDH	Forward	CCTCTCTGGCAAAGTCCAAG	176
	Reverse	GGTCACGCTCCTGGAAGATA	

developmental stages 22-24. In the control group, a rich plexus of vitelline circulation was around the embryo (Figure 3a). The blood, after circulating in the rich plexus of vessels, finally made its way either directly into vitelline veins, or to the sinus terminalis which acts as a collecting vessel, and then from the sinus terminalis to the vitelline veins. In the *C. procera* group of 100 mg per kg egg-weight, a disturbing pattern of the EEM-vasculature was seen in embryos. Vascular disruption was demonstrated by decreased branching (Figure 3b).

The analysis of vascular branching pattern following *C. procera* treatment was presented in Table 2. The herbal extract altered the vascular branching pattern and it was associated with embryos, which received the highest dosage of the herb. In the embryos of group 3 (treated with 100 mg *C. procera* extract/kg egg-weight), the parameters of the vessel plexus decreased when compared with controls ($P<0.05$). In the mentioned group, the lacunarity increased. The vascular pattern of the embryos in group 2, which were treated with a *C. procera* extract at a dose of 50 mg/kg egg-weight, was normal as embryos in the control group.

Considering capillary density, there was a significant loss of MCA from the plexus of the treated embryos in the group that received *C. procera* at doses of 100 mg/kg egg-weight (control: 15.43±1.72; 50 mg/kg egg-weight: 14.82±1.45; 100 mg/kg egg-weight: 6.83±2.11; $P<0.05$). Statistical analysis revealed that embryos that received larger volumes of herbal extract, exhibited a decrease in MCA in a dose-dependent manner. The relative expression of the VEGF-A gene was determined using qPCR assay at day 4 of the incubation period. The relative mRNA expression levels of VEGF-A decreased in the *C. procera*-treated group at doses of 100 mg/kg egg-weight compared to the control (Figure 4).

Discussion

Today, in developing societies, there are trends towards the use of new medical systems that involve the use of herbal remedies. In these societies, herbal plants are applied in the treatment of various ailments. They are also considered in primary health care programs (24, 25). Herbal plants are an integral part of ethnoveterinary medicine, too. In spite of extensive applications and numerous properties of herbs, considering their adverse effects on animal and human health still, needs to be

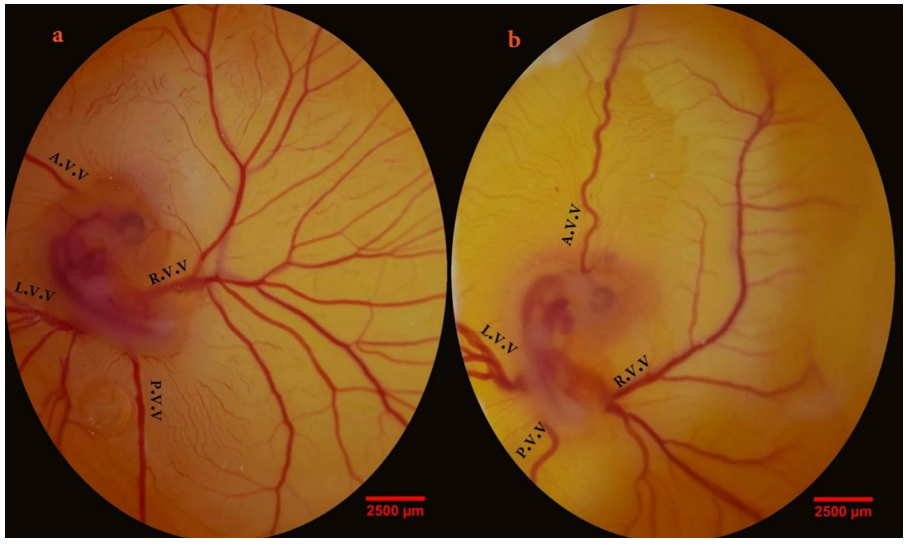

Figure 3: Embryonated eggs were treated three times at 24, 48 and 72 h of the incubation period. (a) Control embryo with normal extra-embryonic membrane vasculature is seen. (b) Embryonated egg received *Calotropis procera* extract at the dosage of 100 mg per kg egg-weight. Vascular disruption is demonstrated by decreased branching. A.V.V., anterior vitelline vein; L.V.V., left lateral vitelline vessel; P.V.V., posterior vitelline vein; R.V.V., right lateral vitelline vessel.

Table 2: Vascular branching pattern analysis following *Calotropis procera* treatment

Variable	Group		
	Control	*Calotropis procera* (mg/kg egg-weight)	
		50	100
Vessels area (%)	44.4±0.31[a]	42.52±0.20[a]	20.90±0.38[b]
Total vessels length (Pixel)	6571.92±2.81[a]	6433.93±2.33[a]	3288.08±1.98[b]
Vascular branch	153±3.13[a]	149±2.22[a]	44±3.19[b]
Lacunarity	0.34±0.09[a]	0.33±0.12[a]	0.83±0.17[b]

Values are mean±standard error of mean. Values in a row followed by different superscripts differ significantly (a,b: $P<0.05$)

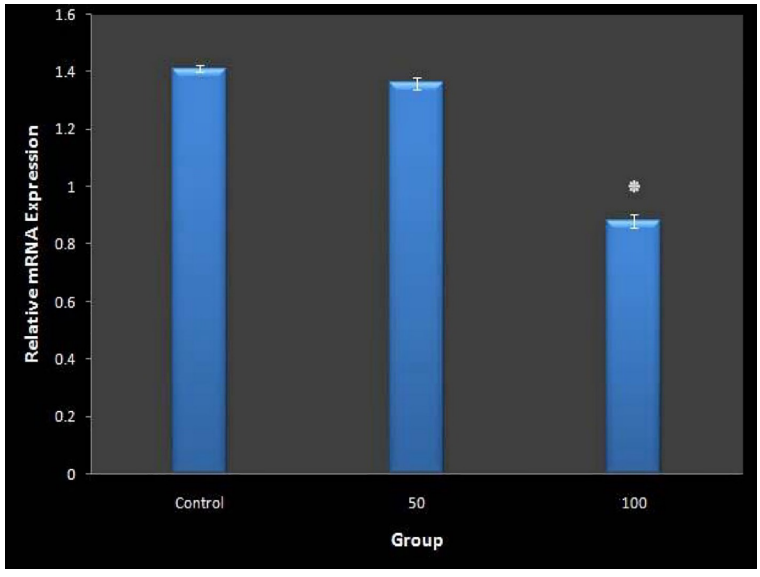

Figure 4: Relative mRNA expression levels of VEGF-A gene following *Calotropis procera* extract treatment. The expression of VEGF-A in the chick extra-embryonic membranes (n=4 per experimental group) was decreased in the *Calotropis procera*-treated group of 100 mg per kg egg-weight. The *Calotropis procera* extract was administered at doses of 50 or 100 mg per kg egg-weight (error bars show standard error of mean; *P<0.05, One-Way ANOVA).

justified. Furthermore, the drug toxicity is of great concern during pregnancy and in folk medicine, it is suggested that pregnant women should not use the *C. procera* (26, 27).

In this regard, some details of the embryonic vascular toxicity of the *C. procera* were evaluated in the present study using the chick's EEM model. Our study lends evidence towards the fact that the vascular disorders with alteration in the normal expression of the specific gene, which is associated with vascular genesis, can be generated by the *C. prccera* exposure during the growing period of the embryo. These disorders are characterized in two indications as follows.

The first one is the anti-vasculogenic property and alteration in the vascular branching pattern of the EEM, including a reduction in vessels area, total vessels length, vascular branch as well as an increase in lacunarity (28, 29).

The severity of the disorders was highest in the embryos that were treated with the highest dose of the herb (100 mg/kg egg-weigh). There are some studies in the literature, which focus on the vascular lesions following herbal treatment (28, 29). The anti-vasculogenic property of *C. procera* and the dosage, which the herb is able to cause vascular defects in embryo, is not clearly defined. Based on our results, it is concluded that *C. procera* can make the vascular injury at the dosage equal or greater than 100 mg per kg.

In our investigation, the *C. procera* extract was provided from the leaves and stems of the plant. Different parts of herb contain many active chemical groups with various biological activities. We did not evaluate extracts from other parts of the plant; therefore, the demonstration of the vascular lesions with the described extract does not exclude the possibility that other parts of the plant might cause similar lesions. Hence, it would be useful to identify further details involved in the vascular toxicity of the *C. procera* in the future.

The vasculo-toxic effect of the *C. procera* is supported by some experimental data on the drug biochemicals. According to most studies, the majority of the medical property of the *C. procera* is due to the various biochemicals present in the plant. The *C. procera* contain many biological and active groups including cardenolides, steroids, tannins, glycosides, phenols, terpenoids, sugars, flavonoids, alkaloids and saponins. Therefore, alteration in vascular development may be due to the effects of those mentioned biochemical. This may provide an explanation for the decrease in proliferation of the vascular network in the vascular beds of *C. procera*-treated EEM.

The next explanation to account for the vascular injury, due to *C. procera* administration would be to the cytotoxic activity of *C. procera*. It was showed by Juncker et al. (30) that the UNBS1450, a cardiotonic steroid which derived from *C. procera*, is a regulator of signaling pathways involved in cell death. Cytotoxic property of *C. procera* was also approved by other researchers (31, 32). Furthermore, certain inherent properties of *C. procera* could be associated with its adverse effects. For example, some findings indicate that the herb has a genotoxic activity that causes damage to DNA (33).

The second indication of the *C. procera* disorders

was a reduction in the expression of VEGF-A. Alteration in gene expression as well as vascular development may provide a link between *C. procera* exposure and developmental defects of the fetus. The *C. procera* was used at doses of 50 or 100 mg per kg egg-weight. The later dosage seems to have the tendency towards an adverse effect of *C. procera* on gene expression. We have shown that *C. procera* exposure affects the expression of VEGF (34).

To explain the reduced expression of that gene, we suggest the following mechanisms. The decrease of vascular branch and vasculogenesis, which is induced by *C. procera*, may cause the limited flow of bloodstream through the vessels. Alterations in blood flow may cause a reduction in shear stress, which is sensed by the endothelial cells (34). Generally, when shear stress increases, VEGF-A is up-regulated (35). Therefore, a suggested reduction in shear stress after *C. procera* exposure may decrease VEGF-A expression.

In the present study, methods that were used to quantify the anti-vasculogenic effect of *C. procera* extract were the computerized analysis of vascular branching pattern and calculation of the mean capillary area of the digitally acquired images from the EEM-vasculature. The chick EEM-vasculature is fractal because it lies within an almost two-dimensional plane and there is little crossing over of vessels (36). The time of treatment was also chosen to match with the previously documented sensitive time to teratogenesis, at which time vascular abnormalities had been noted (20, 21).

As far as the authors are aware, this is the first study to target the early anti-vasculogenic property of *C. procera* extract with the help of a chick's EEM model. The study provides elaborate data on the vascular toxicity of *C. procera* for the fetus. Our findings coincide well with previously reported data focused on the side effect and toxicity of herbs to the vascular system. Additionally, our data show that *C. procera* not only causes an adverse effect on the early vascular development but also alters the gene expression. We suggest that these alterations may result in devastating consequences in fetus and this phenomenon require further investigation.

Conclusion

Chick embryo is a preclinical model relevant to assess adverse effects of drugs and biochemicals, in which the interventions cannot be made in the human fetus due to ethical reasons, but will inspire researchers and clinicians for specific changes in the design of subsequent drug prescription. The results reported in this paper allow us to suggest that use of the *C. procera* during pregnancy should be considered as potentially vasculo-toxic, at least until further data are provided on safety for human fetus. Therefore, the herb consumption should be limited in pregnancy, particularly in industrialized societies, because there is an increasing reliance on the use of herbs in these societies. Furthermore, *C. procera* applied to the chick's EEM was vasculo-toxic at the dosage equal to or higher than 100 mg/kg. A lower dosage given in the various stage of pregnancy caused far less pronounced vascular lesions. Therefore, the use of safe alternatives herb should be the high priority at the time of fetus growth.

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Conflict of Interest

None declared.

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