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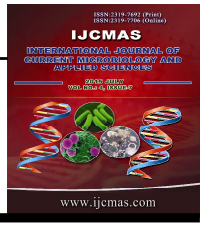

Original Research Article

The Anti-angiogenic Effect of *Chamomila recutita* Aqueous Extract Determined Using a Modified Chicken Chorioallantoic Membrane *ex ovo* assay

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Keywords

Chick chorioallantoic membrane assay, Chicken egg *ex ovo* cultive, Anti-angiogenic effect, *Chamomila recutita*, Caffeic acid

A B S T R A C T

Chamomile aqueous extracts have been shown to have antitumoral and anti-inflammatory activity. Various doses of either chamomile extract, Vascular Endothelial Growth Factor (VEGF) and Hydrocortisone (HC) were investigated for angiogenesis effects by an *in vivo* CAM assay in a shell-less culture method. We found that the number of viable embryos was higher when UV light was used than for eggs cultivated without UV light (22.3 vs. 7.6 %, $p<0.05$). After 10 days of cultivation, the mean survival rate of the embryos cultivated in petri dishes was lower than in weighting boats (26 vs. 47%, $p<0.05$). The effects of various doses of VEGF, HC and chamomile aqueous extracts were tested using a modified *ex ovo* CAM assay method. As expected, the VEGF (positive control) displayed an angiogenic activity (EC50: of 5.4 ng/mL), whereas HC (negative control) had an anti-angiogenic activity (IC50: 2.4 µg/mL). The chamomile extracts were able to inhibit vessel growth (anti-angiogenesis) (IC50: 28 µg/mL) due to its caffeic acid content (IC50: 1.8 µg/mL). Our findings indicate that *Chamomilla recutita* aqueous extract has an anti-angiogenic activity and our modified *ex ovo* CAM assay increased the mean survival rate of the embryos for evaluation of angiogenic effect.

Introduction

Angiogenesis is the complex biological process of forming new vessels from pre-existing ones. This physiological phenomenon is required for wound healing and tissue growth and is therefore essential for human health. Furthermore, angiogenesis is also related to pathological conditions such as cancer, contributing to tumor growth and metastasis, rheumatoid arthritis and psoriasis (Folkman, 2006, 1976; Hanahan and Folkman, 1996).

The use of animal models for the study of angiogenesis is an important tool for screening new agents that might inhibit or activate angiogenesis. The chick embryo is a

well-known animal model and the chorioallantoic membrane (CAM) assay is particularly widely used (Ribatti, 2008; Vargas *et al.*, 2007). This technique can be performed either *in ovo* or *ex ovo* (shell-less), with each approach having its own advantages and limitations (Vargas *et al.*, 2007). Microorganism contamination can be problematic for both the *in ovo* or *ex ovo* approaches. The *ex ovo* method may be advantageous when it is necessary to assay a large number of samples simultaneously. The survival rate of eggs cultured *ex ovo* is a major success-limiting factor of this technique (Ribatti, 2010, 2008).

Due to the presence of various classes of bioactive compounds, chamomile is a widely used medicinal plant. Polyphenolic compounds (including flavonoids, organic acids, essential oils and other minor compounds) are the principal constituents of chamomile flowers. *In vitro* and *in vivo* experiments show that these substances are correlated with its antitumoral, antioxidant, anti-inflammatory and anxyolitic effects. Because of chamomile's antitumoral and anti-inflammatory activity, the aim of this study was to evaluated the influence of chamomile extract on angiogenic activity using an *ex ovo* CAM assay approach (McKay and Blumberg, 2006)

Materials and Methods

Plant material

Chamomilla recutita (L.) Rauschert Asteraceae was cultivated locally in the Curitiba Metropolitan Region, Brazil. This was authenticated at the Curitiba Municipal Botanical Garden and a voucher specimen was deposited in the herbarium under number 386495. The flower buds were detached from the stem, dried at room temperature and macerated at 60°C for 6 h with distilled water using a 1:10 ratio. Aqueous extracts were filtered and evaporated at 60°C under vacuum and the residues freeze-dried and stored in vials at -20°C until use.

For the CAM assay, 500 mg/mL chamomile stock solutions were prepared in distilled water. Working solutions of *Chamomilla recutita* extract were prepared by further dilution of the stock with distilled water (to concentrations of 1000, 300, 100, 30, 10, 3 and 1 µg/mL).

Reagents and Chemicals

Hydrocortisone, Vascular Endothelial Growth Factor 165(VEGF), caffeic acid and chlorogenic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Animals

Fertilized eggs of *Gallus gallus* were obtained from a commercial poultry farm located in the Curitiba metropolitan region and maintained at ambient temperature until use (above 20°C). This project was approved by the Ethical Committee for Animal Experimentation of the Federal University of Paraná and registered under number 23075.047773/2010-61.Eggs weighing<60 or >70 g were excluded. Eggs were incubated in a commercial egg incubator (Chocmaster CHM 240) at 37°C and 60% relative humidity (RH).

Egg microbiologic quality control

Microbiologic analyses were performed accordingly to (Downes and Keith, 2001)were entire eggshells were washed with 100 mL of sterile tryptic soy broth (TSB) and a serial dilution was prepared in 0.9% saline solution. The various dilutions were pour plated, covered with tryptic soy agar (TSA), and cultivated for 48 h. Results were expressed as number of total colony

forming units (CFUs) per mL. Eggs were incubated for 3 days with use of UV light for 1h every 12 h. The eggs were then opened (Dohle *et al.,* 2009)under a laminar flow and disposed on to sterile weighing boats (KASVI K30-8080) that were enclosed in culture petri dishes (TPP 93100). At this point, egg embryos were classified as stage HH 20(Hamburger and Hamilton, 1993).

CAM assay

The egg embryos remained in an unaltered conditions until the 10^{th} day of incubation, at which point the embryos were removed from the incubator and 6 mm cellulose discs (LABORCLIN) impregnated with either phosphate buffer solution, VEGF (as positive control), hydrocortisone (as negative control) or chamomile aqueous extract at concentrations of 1, 3, 10, 30, 100, 300, 1000 and 3000 ng/µL. All analysis were made in triplicate and returned to the incubation chamber for a further 2 days.

Photomicrography technique

The egg embryos were removed from incubation and a photomicrography was taken of each disc to count the total number of blood vessels surrounding the disc. This process was accomplished using a 30% milk powder solution injected inside the CAM sack and submitted to image analysis using the Image J program. The total blood vessels for each disc were plotted graphically (Graph Pad Prism, version 5.0) to obtain either 50% inhibition (IC50) or excitatory (EC50) concentrations.

HPLC analyses

The HPLC system consisted of a Varian SYS-LC-240 E, 410/50492 autosampler, a 335/EL06019048 photodiode array detector, a 230/01513 solvent delivery module and the Varian Star workstation software. Separation was achieved using a microsorb-mv C18 reverse phase column (250 × 4.6 mm I.D., 5 µm) using amobile phase consisting of (A) 0.1% phosphoric acid in water (pH 2.0) and (B) acetonitrile. A gradient system was used; 0–5 min, 30% B, 5–10 min, 40% B, 10–15 min, 50% B, 15–20 min, 70% B; ata flow rate of 0.7 mL/min and UV absorbance set at 321 nm with sample injection of 10 µL. The mobile phase and samples were filtered through a 0.22 µm (HPTE) filter before use. Aqueous and hydroalcoholic (50 mg/mL) extraction and standard curve production were performed according to Wang *et al.* (2008).

Statistical analysis

Statistical analyses were performed using Graph Pad Prism software version 5.0 and results expressed as mean ± standard deviation (SD) from 3 experiments using 60 eggs each. A suitable statistical analysis was performed for each experiment, including one way ANOVA, the student *t*-test and nonlinear regression analysis.

Results and Discussion

Egg decontamination using ultraviolet germicidal irradiation

Prior to the egg decontamination test, the number of contaminating viable microorganisms was determined (initial egg contamination). The initial egg contamination value was $3.5 \times 10^4 \pm 0.4$ CFU/eggshell. After initial counting, UVGI treated eggs were analyzed for eggshell contamination, which was found to be 25.7 ± 4 CFU/eggs hell at soon after the first treatment (D0) but reduced to zero after 24 h. These values remained constant until 72 h incubation, with was the day of egg opening ($p<0.05$; $F = 54.67$; $df = 4$) (Figure 1).

To evaluate the significance of UVGI treatment on egg contamination, eggs were cultured in both the presence and absence of UVGI light. As verified in figure 2, the number of viable embryos when UV light was used was statistically higher (22.3%) than for eggs cultivated without the use of UV light (7.6%) ($p<0.05$; $t= 7.138$; $df = 4$).

Egg quality can be described as a function of their eggshell and cuticle (Board and Halls, 1973) with the bacterial colony forming unit (CFU) raging from 10^2 in clean eggs to up 10^6 CFU/eggshell in contaminated eggs. Despite the high contamination rate of the external eggshell, the interior of the egg is practically sterile (Coufal *et al.,* 2003) and natural egg hatching can occur with high survival rates.

This is not true for eggs used either in *ex ovo* or *in ovo* angiogenesis assays, since an eggshell rupture is needed in order to access the CAM vessels. Although widely used (Vargas *et al.,* 2007), the *in ovo* assay is technically difficult, time consuming and is limited to one sample per egg. Although, a large number of samples can be tested in each egg when using the *ex ovo* approach, this method is hampered by high contamination rates. To overcome this problem, the eggshell, which is the major contamination site, must be disinfected without use of chemical agents.

UVGI has been long used in medical facilities to decontaminate highly infected areas, especially when sporulated microorganisms(e.g., *Mycobacterium tuberculosis*) are involved (Memarzadeh *et al.,* 2010).

In our analysis of eggshell contamination, we encountered a high bacterial count, some of which were related to Gram-positive sporulated microorganisms. Therefore, we implemented UVGI inside the incubation chamber, significantly reducing the egg contamination rate. The presence of microbial development after the first use of UVGI is a clear indication of bacterial spore germination, since this is resistant to UV light. A second UV application reduced egg shell contamination to zero, suggesting its effectiveness against bacterial contamination due to spore germination.

Dew effect over egg contamination

We found that while 32.3 ± 1.4% of the embryos were alive after 12 days of incubation when no dew was formed soon after introducing the eggs into the incubator. Whereas only 13.0 ± 1.5% of the embryos survived when dew was formed soon after the eggs were placed into the incubator ($P<0.05$; $t =1.105$; $df = 4$) (Figure 3).

These data show that the presence of water over the eggshell, in the form of dew, was strongly correlated with embryo death after eggshell opening and transfer of the egg content to Petri dishes.

Visual analysis of the dead embryos showed bacterial growth over the entire Petri dish. The bacterial sample was partially identified as fastidious Gram-positive *cocci*.

Although an adult chicken is considered a homoeothermic animal, chicken egg embryos need an external font of heat and therefore require artificial incubator conditions. The RH is also important because eggshells are porous to gases and water, and small changes in these characteristics can have profound effects on embryos survival(De Reu *et al.,* 2006).

Of these two factors, RH has a greater impact on embryo survival, since dehydration can easily occur inside the incubator. At 37°C, the temperature normally used for egg incubation, the HR of

the incubator can easily drop below 30%, leading to embryo dehydration. This process is even more dramatic on *ex ovo* cultured eggs.

To avoid dehydration, a water vapor generator was used to continuously force water mist inside the incubator, thus maintaining a RH of ~60%. However, the formation of dew soon after the eggs are placed inside the chamber is a problem observed with this method, as previously reported by Sparks and Board (1985), eggs washed with water facilitate the migration of microorganisms by augmenting eggshell pores.

Green and Dyer (2009) stated that the formation of dew occurs rapidly when ambient HR is high and material surface temperature is low. This process was observed in our experiment when eggs maintained at room temperature (20°C) were placed inside the incubator (60% RHand 37°C). To avoid this natural process, at the moment of transferring the eggs, the incubator should have low humidity (e.g., 30%).Only after the egg temperature has reached the critical temperature of 29°C (using Dyer formula) should the chamber humidity be increased to 60%.

Egg opening and *ex ovo* culture

Eggs were cultured in two different systems: 1) using only a cell culture Petri dish; and 2) weighting boat adapted to fit inside the Petri dish (Figure 4).

After 10 days of cultivation, the number of living embryos was determined. The mean survival rates of embryos cultivated with and without the weighting boat were 47 and 26% respectively, and were statistically different ($p<0.05$; $t = 7.236$; $df = 4$) (Figure 5).

The eggs lost during *ex ovo* culture were observed to have a characteristic oval shape. Inside the normal egg, three structures can be easily seen: the *chalazae*, albumen and yolk. The vitelline, blastoderm and perivitelline layer are most important for embryo formation (Brake *et al.*, 1997) not easily seen. The vitelline is responsible for egg nutrition, especially for its vitamin and lipid content. The blastodermis related to embryo formation and the perivitelline layer protects the yolk content from extravasation into the albumen.

The *chalazae* and albumen respond to ensure the centralization of the egg yolk and absorb shock to protect the fragile vitelline layer (Stadelman and Cotterill, 1995). The albumen also has a role in antimicrobial protection, having a low pH and antimicrobial enzyme activity (e.g., lysozyme activity) (Greenfield and Bigland, 1971).

During egg opening for *ex ovo* culture, depending on the surface area of the plate, albumen slowly flows off the egg yolk. This process permits direct air contact with the blastoderm and easily dehydrates the egg embryo, ultimately leading to death. A second problem of using plates with a high surface area is the absence of the antimicrobial effect of the albumen over the egg yolk, leading to egg contamination if any bacteria survive shell treatment.

Moreover, adhesion of the perivitelline layer to the plate during embryo development can lead to rupture, disrupting the blood vessel network and therefore killing the embryo.

The use of a weighting boat with a narrow base can circumvent the above problems and thereby increase egg embryo survival rate. A further increase in embryo survival rate could be achieved, but was not used in our work (Crum *et al.*, 1985).

Angiogenic assay on chorioallantoic membrane (CAM)

The live embryos obtained after 10 days incubation using dry eggs, treated with UV light and disposed on weighting boats were tested for angiogenic activity in order to verify if the proposed methodology is suitable for this assay. Our initial attempt to investigate the blood vessels failed because of the color similarity between the blood vessel and the surrounding media. To overcome this problem, milk powder solution (30% [w/v]) was injected inside the CAM sack (between the two layers) as a contrast agent (Figure 6).

The process of milk injection should be performed carefully, since the two CAM membranes can often stick together. An erroneous injection can be easily noticed as the milk flows outside the CAM sack. When done correctly, the milk will be trapped inside the CAM and only the upper vessels of the CAM will be observed (Figure 6).

Blood vessels are important physiological structures that realize tissue gas exchange, provide nutrients for cell development and remove metabolic wastes. This tissue is formed by endothelial cells, smooth muscle cells surrounded by collagen, elastin and glycoproteins, which form the extracellular matrix (Hanahan and Folkman, 1996). The formation of blood vessels can occur in two ways: vaculogenesis and angiogenesis. Vasculogenesis only occurs during fetal development, with all other physiological and pathological blood vessels developing by angiogenesis (Moreira *et al.,* 2007). Since angiogenesis is involved in a series of pathological conditions (cicatrization and tumorigenesis), several studies have aimed at discovering new drugs to treat these conditions. A central difficulty associated with this kind of study is the use of a suitable animal model in which the molecular mechanisms of various substances can be tested (Ribatti *et al.,* 2001).

The most common animal model is the chorioallantoic membrane (CAM) assay, in with different materials impregnated with the test substance are dropped over the membrane and, after some time (usually 48 h), angiogenesis or anti-angiogenesis can be evaluated (Auerbach *et al.,* 2003).

This evaluation can be accomplished with embryos either inside (*in ovo*) or outside the eggshell (*ex ovo*); both approaches have advantages and disadvantages. Here we used the *ex ovo* assay because it is more straightforward to perform and a greater number of tests can be performed per egg (Vargas *et al.,* 2007).

Photomicrography analysis using image J

A high level of contrast was obtained when photomicrography was submitted to image analysis. As shown in figure 7, even CAM vessels photographed with milk powder solution as a contrast have a low resolution and are sometimes difficult to identify. However, we found that a combination of image analysis and contrast application allowed individual blood vessels to be fully visualized.

To count blood vessels, the use of a white contrast agent was helpful, allowing us to differentiate the red vessels from the yellow yolk below. The counting sensitivity was further improved by image treatment.

Angiogenic/anti-angiogenic CAM assay using *ex ovo* methodology

To verify the methodology, the responses to various doses of VEGF, hydrocortisone and chamomile aqueous extract were tested each in a CAM assay.

Figure.1 Comparative data between non-treated (ST) and treated eggs at 0 (D0), 24 (D1), 48 (D2) and 72 h (D3). *** are statistically different from ST ($p < 0.05$) and # are statistically equal to each other ($p > 0.05$)

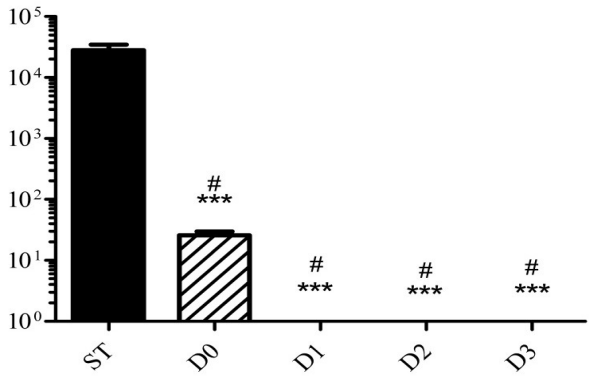


Figure.2 Comparative data of viable embryos for UV-treated and non-treated eggs. The percentage of viable embryos was statistically different between treatments ($p<0.05$)

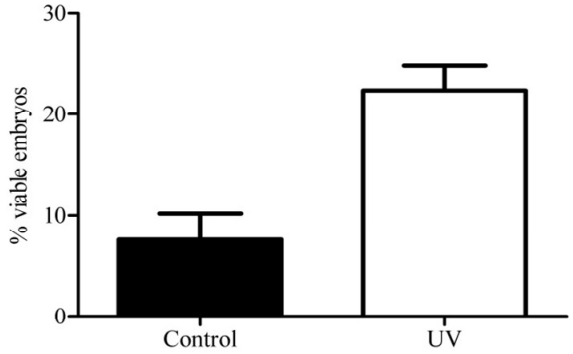


Figure.3 Percentage of viable embryos for eggs incubated without (dry) or with (humid) dew present over the eggshell

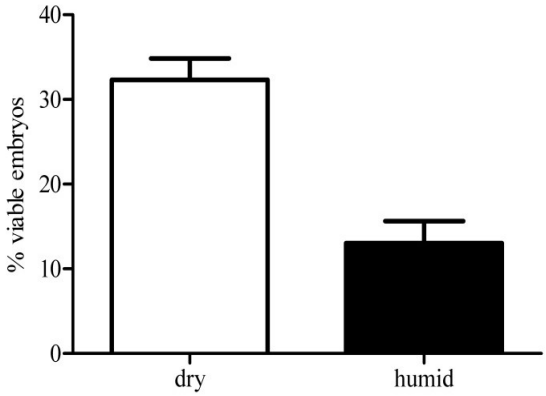


Figure.4 Comparative images of a cultivated egg without a weighting boat (panel A) and an egg with a weighting boat (panel B). In panel A the embryo has been cultured for 120 h and in panel B for 72 h

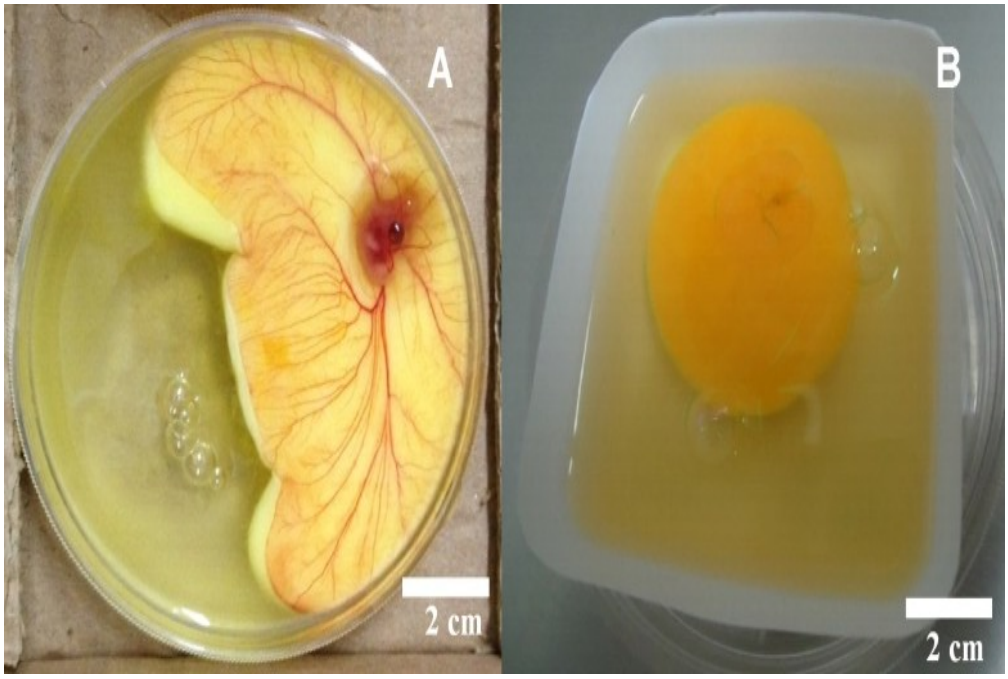


Figure.5 Percentage of viable embryos for eggs incubated either with or without a weighting boat. Error bars show the standard error of the mean (SEM)

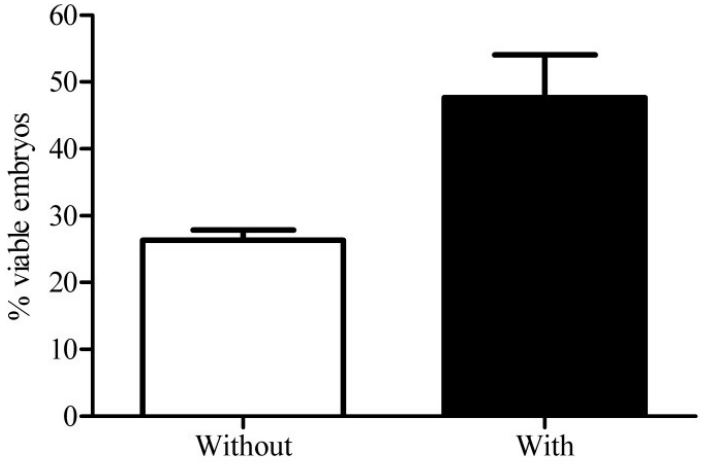


Figure.6 Schematic sequence of our *ex ovo* methodology for angiogenesis evaluation. A) Transfer plate for cell culture of the embryo with 3 days of incubation; B) Embryo at 13 days of incubation, here it is possible to observe the vascular networks forming the chorioallantoic membrane (CAM); C) Three disks containing the test sample are placed onto the CAM; D) Embryo after 15 days of incubation and 2 days post disc placement; E) Contrast injection into the CAM; F) The contrast spreads into the allantois and discs are photomicrographed. It can be seen that the embryo remains alive, allowing the count of vessels around the disk

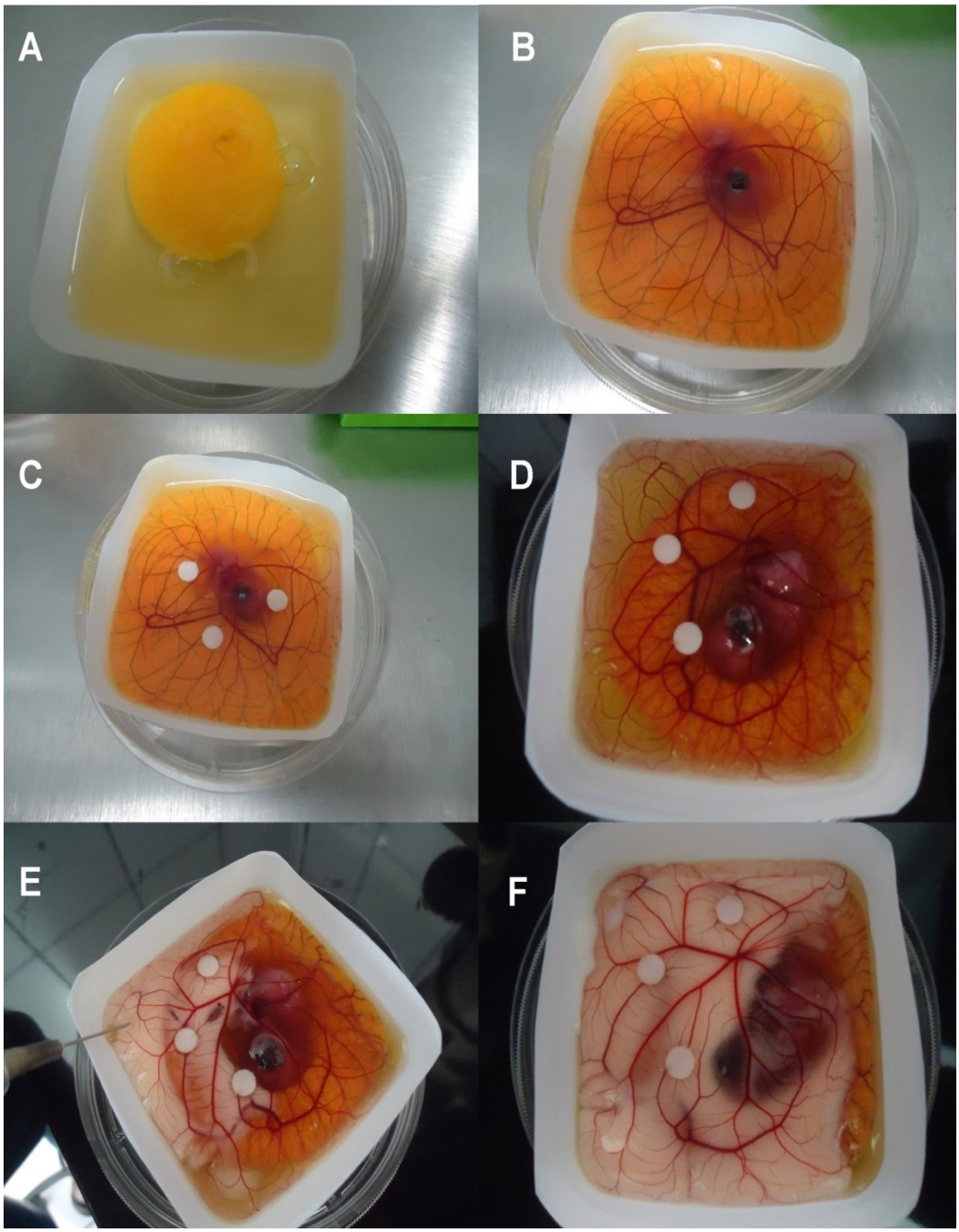


Figure.7 Egg embryo treated with saline to demonstrate image analysis. A) Cellulose disc impregnated with PBS in its original image form; B) after background subtraction resulting in visual enhancement of the blood vessel borders; C) after applying the invert function of the image J software

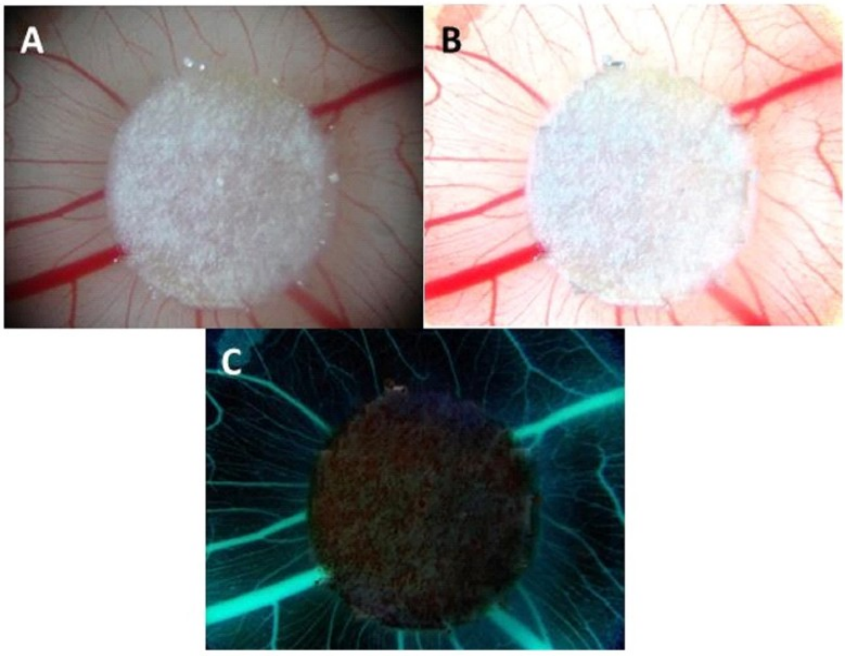


Figure.8 Excitatory dose response curve (EC50) of VEGF and the inhibitory (IC50) dose response curve of Hydrocortisone chamomile aqueous (AE) and Hydroalcoholic extract (HAE). Curve obtained from non-linear regression of relative blood vessel (%) in each dose

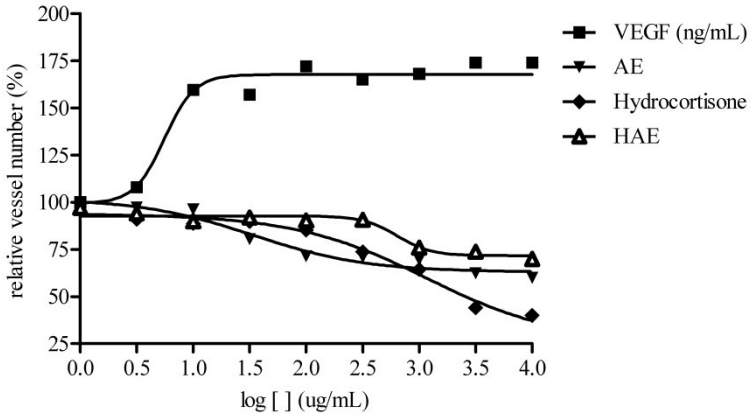


Figure.9 Inhibitory dose response curve (IC50) of caffeic acid. Curve obtained from non-linear regression of relative blood vessel (%) in each dose

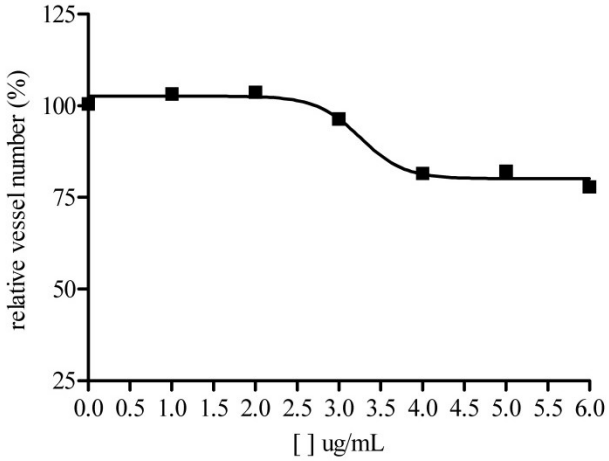
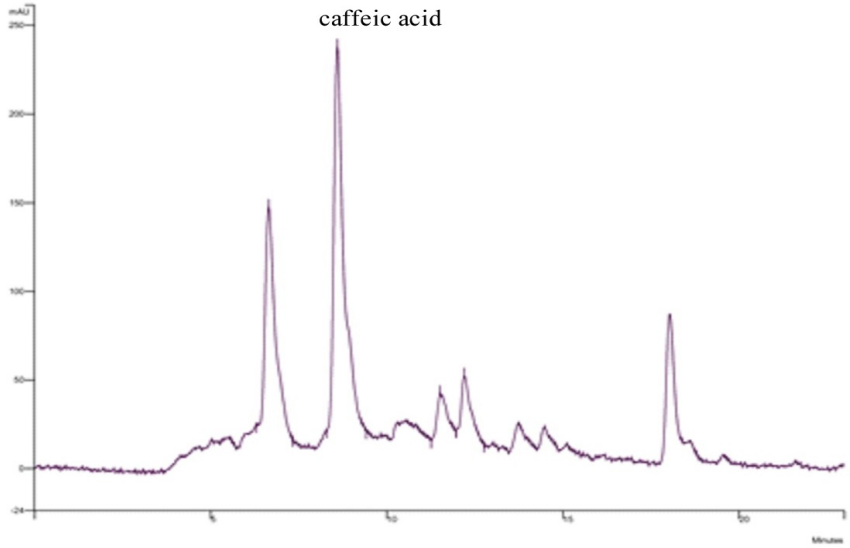


Figure.10 Chromatogram of HPLC analysis of aqueous chamomile extract (panel A) and chlorogenic, caffeic and ferulic acid standards (panel B)



It was possible to observe an active angiogenic activity of the VEGF (EC50: 5.5 ng/mL) and an anti-angiogenic activity for the hydrocortisone and aqueous extract, with IC50s of 1,175, 34.12 and 638 µg/mL, respectively (Figure 8). These data demonstrate the potency of VEGF's angiogenic inducing capacity versus hydrocortisone's anti-angiogenic capacity. Therefore, we can speculate that the VEGF levels in the organism are much lower than the corticoid levels.

In our HPLC analysis, we identified caffeic acid as the principal constituent of the chamomile aqueous extract, therefore, a range of caffeic acid concentrations were also tested for angiogenic activity. Caffeic

acid was found to have an anti-angiogenic activity, with an IC50 of 1.8µg/mL (Figure 9).

These data show that chamomile has an anti-angiogenic activity due to the presence of caffeic acid. Therefore, the ethonopharmacological use of chamomile is in line with our results, since the popular use of chamomile includes its anti-inflammatory activity. According to Srivastava *et al*.(2009), traditional uses of chamomile includes the treatment of inflammation of the skin and mucous membranes, in the form of aqueous extract or alcoholic tincture.

HPLC analysis

We found that the aqueous chamomile extract was more effective on angiogenesis than the hydroalcoholic extract. In order to determine the possible active compound, liquid chromatography was performed and compared with chlorogenic, caffeic and ferulic acid standards (Figure 10).

We observed that caffeic acid was the major constituent of the aqueous extract with a 99.84% correlation between the spectral overlay of the standard and the second peak of chamomile aqueous extract chromatogram. Using a caffeic acid standard curve, the chamomile aqueous extract (50 mg/mL) solution was found to contain55.05µg/mL caffeic acid.

According to Kim *et al*.(2009), caffeic acid effectively inhibits VEGF-induced proliferation of retinal endothelial cells in a concentration-dependent manner. In his work, Kim states that caffeic acid's anti-angiogenic activity is related to its antioxidant activity, which in turn inhibits the expression of VEGF from endothelial cells.

Here we show that an *ex ovo* methodology for angiogenesis evaluation can be accomplished by reducing microbial contamination using physical decontamination methods such as UVGI. Moreover, the use of a weighting boat increases the embryo survival rate by reducing the probability of perivitelline membrane rupture (and subsequent embryo death).An advantage of this modified *ex ovo* methodology is that a reduced number of eggs are required, since each egg can be used to evaluate more than one test disc. VEGF and hydrocortisone were used to validate this methodology, with their angiogenic and anti-angiogenic effects observed here being concordant with the literature. It was also possible to observe that *Chamomilla recutitta* extract has an anti-angiogenic activity and that this is correlated to its caffeic acid content.

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