

Vasomodulatory effects of semi-purified fractions of garlic aqueous extract on chick chorioallantoic membrane

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Abstract: *Allium sativum* (As), commonly known as garlic, has been used for a long time, for its therapeutic effects. Recent studies showed the ability of As to modulate vascular activity. The present study aimed to investigate the vasomodulatory effects of aqueous extract of As and to analyse the molecular nature of the active components. Experiments were performed on chick chorioallantoic membrane. Fractions of garlic were directly injected using micropipette on a high vessel density area. Our results clearly indicated that garlic increased permeability and induced vasodilatation of blood vessels and capillaries. These effects were dose-dependent and had been observed just few minutes after the onset of treatment. The active component responsible of these effects, which had a low molecular weight seems to be of peptide nature and appeared different from Diallyl Sulfide (DAS) and Diallyl Disulfide (DADS).

Keywords: *Allium sativum*, chorioallantoic membrane, vasodilatory effect.

INTRODUCTION

Allium sativum (As), commonly known as garlic has been widely studied for its medicinal properties. Indeed, more than a thousand scientific papers over the past decade have gradually confirmed the traditional recognized health benefits of garlic intake.

It has been widely recognized for its preventive and curative properties of cardiovascular disorders by lowering serum cholesterol and triglycerides, inhibiting blood coagulation and platelet aggregation and lowering blood pressure (Warshafsky *et al.*, 1993; Silagy and Neil, 1994; Reuter, 1995; Sendl, 1995). Many other studies suggested possible cancer preventive effects of garlic preparations (Caragay, 1992). Indeed, experimentally garlic and its associated components are reported to suppress cancer risk and to alter the biological behaviour of tumours in breast, colon, skin, uterine, oesophagus and lung cancer (Hussain *et al.*, 1990; Ipetal., 1992; Shukla *et al.*, 1999; Sumiyoshi and Wargovich, 1990; Wargovich *et al.*, 1988). Garlic had been described for several other medicinal properties including antimicrobial, antiarthritic, hypoglycaemic and antitumor effects (Rivlin, 2006; Wu *et al.*, 2005; Matsuura *et al.*, 2006; Block, 1955).

As is characterised by its large variety of components including sulfurones like allicin and non-sulphur compounds like saponins and flavonoids. All these compounds had been described for providing various health benefits (Amagase, 2001).

The aim of the present study is to determine vasomodulatory effects of as. Thus, we have chosen to use the chick chorioallantoic membrane (CAM) as a model. The CAM is a highly vascularised extra-embryonic membrane that mediates exchange of gas and nutrients during embryonic chick development. It is formed between embryonic days of development 3 and 10 of gestation period by fusion of the allantois mesodermal layer (extending out of the embryo) with the mesodermal layer of the chorion (Kleibeuker *et al.*, 2014).

Due to its extensive vascularisation, the CAM has been used to study the morpho-functional aspects of angiogenesis *in vivo* and to investigate the activity of pro-angiogenic and anti-angiogenic molecules (Ribatti, 2010). CAM contains a well-developed vascular network and the vasodilatation that follows manipulation may be easily distinguished with direct macroscopic observation.

MATERIALS AND METHODS

Plant material and extractions

Garlic (*Allium sativum* L.) was purchased from local market. Bulbs of garlic were divided into separate cloves. These later were manually peeled, weighed and ground with an electric mincer. In order to obtain an aqueous suspension, the blended raw garlic was then dissolved in double-distilled water at a concentration of 4g/ml on the basis of the weight of starting fresh material. Aqueous suspension was then, centrifuged at 10.000g for 15min, 4°C. (Beckman J20). Supernatant was aliquoted and stored at -80°C until use.

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Aqueous extract was subjected to the extraction with ethanol as described previously (Qureshi *et al.*, 1983), with slight modifications as follows: Briefly, 1 volume of aqueous extract was precipitated twice with 7 volumes of ethanol and centrifuged at 10,000 for 15min at 4°C.

Supernatant was dried using a rota vapor, dissolved in double-distilled water and referred as “ethanol-soluble fraction” (AS). Pellet was dissolved in double distilled water and referred as “ethanol-insoluble extract” (AP).

Chromatography on Cep-Pak C18 cartridge

Ethanol-soluble fraction was further subjected to chromatography on Cep-Pak C18 reverse phase cartridge. Cartridge was first, submitted to extensive washing, first with ethanol and then, with double distilled-water. Ethanol soluble fraction was then, loaded into the cartridge conducting to two yielded fractions: a polar fraction (phile) and a non-polar one (phobe). Phile fraction is none absorbed and was eluted with double distilled-water. Whereas, Phobe fraction was eluted with 10% ethanol.

The non-polar fraction was dried using rotavapor, dissolved in double distilled water and subjected to thin layer chromatography.

Thin Layer Chromatography (TLC) Analysis

Phobe extract was subjected to TLC on silica-gel plates 60 F₂₅₄ (Merck, Germany). A mixture of butanol/acetic acid/water (12/3/5) was used as a mobile phase. After migration, the plates were air-dried at room temperature

for 20 min. The spots were visualized after exposing the plates to UV light and removed as previously described (Skipski *et al.*, 1964). Briefly, the area of each spots was scraped using a razor blade and re-suspended in water. The mixture was then agitated, centrifuged, and the supernatant fluid stored at -80°C until use.

Chick chorioallantoic membrane assay

Fertilized white chicken eggs were purchased from Poulina (Tunis, Tunisia). Eggs were placed horizontally in the incubator as soon as embryogenesis starts and are kept under constant 70% relative humidity at 37°C. On the 7th day of development, eggs were gently cracked. Care must be taken to avoid causing damage to the embryos.

The chick embryos were individually placed into Petri-dishes and placed under a binocular stereo microscope.

Application of semi-purified fractions of garlic

Fractions of garlic were directly injected using micropipette on a high vessel density area. In order to follow the immediately effects, macro photos were made every 100 seconds and videos sequences were recorded. Experiences were repeated 6 times.

RESULTS

Effects of as on vascular pattern in CAM assay: Time course study

The chick embryos were exposed to a single application of raw garlic extract 80µg. A clear dilatation of blood vessels and capillaries was observed in the 100 first

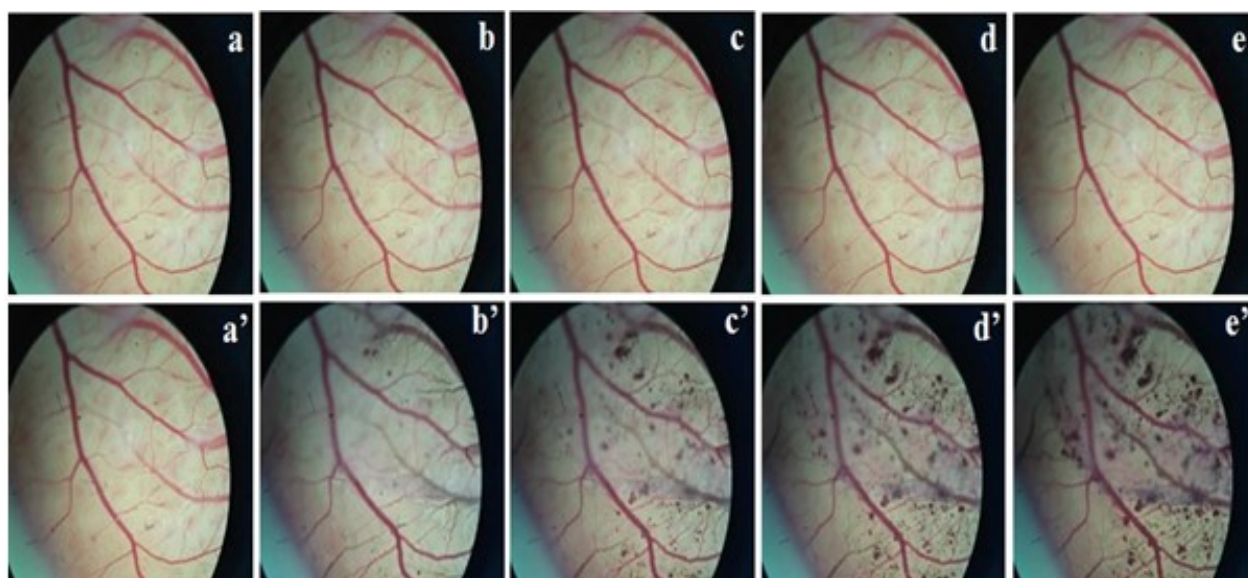


Fig. 1: Kinetic study of the effect at different times after application of 80 µg of raw garlic extract. At t=0 sec (fig. 1a'), t=100 sec (fig. 1b'), t=200 sec (fig. 1c'), t=300 sec (fig. 1d') and t=400 sec (fig. 1e'). Results showed hyperemia response with time dependent increasing when compared to control (application of saline solution at 9‰), at t=0 sec (fig. 1a), t=100 sec (fig. 1b), t=200 sec (fig. 1c), t=300 sec (fig. 1d) and t=400 sec (fig. 1e). Experiences were repeated 6 times.

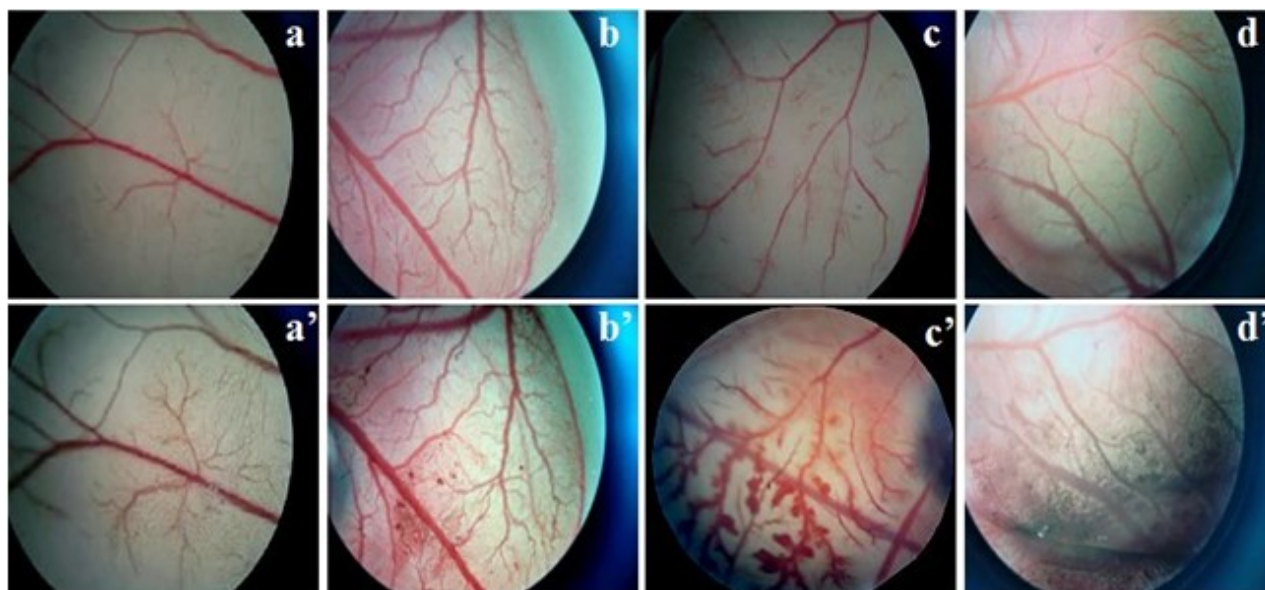


Fig.2: Dose-dependent effect: Focal application of 40 μ g [fig. 2a (0 sec) and fig. 2a' (400 sec)], 60 μ g [fig. 2b (0 sec) and fig. 2b' (400 sec)], 80 μ g [fig. 2c (0 sec) and fig. 2c' (400 sec)] and 160 μ g [fig. 2d (0 sec) and fig. 2d' (400 sec)] of raw garlic extract. Focal application induced a characteristic dose-dependent hyperemia within 400 sec after application. Results showed a prominent reaction for high concentrations and the weaker reactions for lower concentrations. Experiences were repeated 6 times.

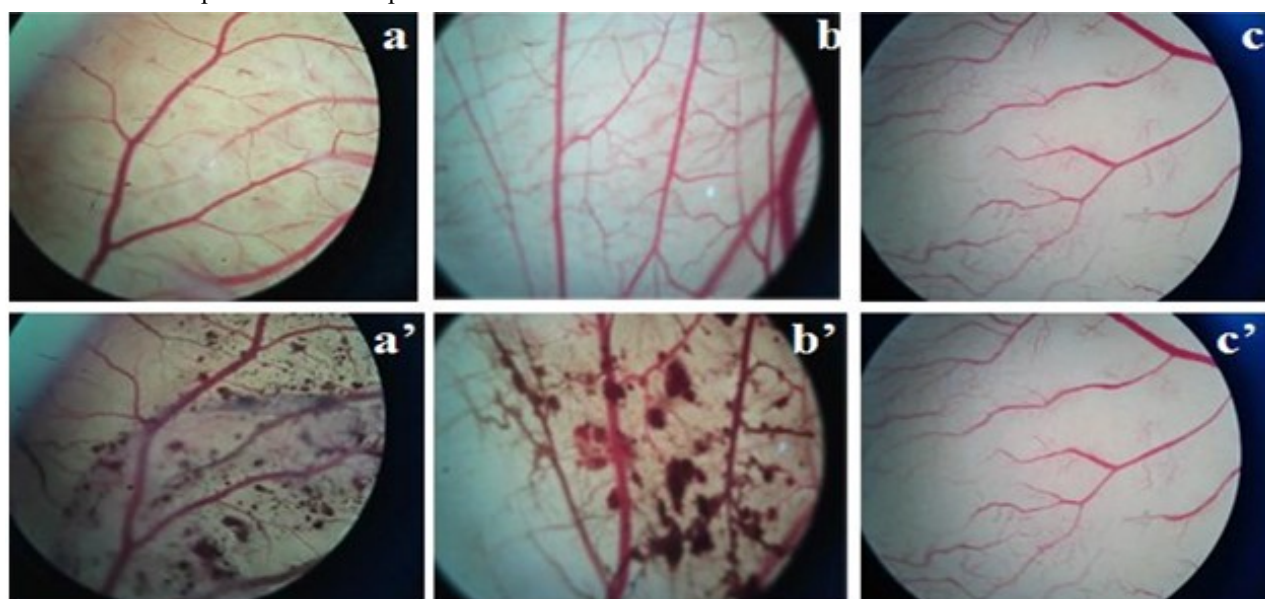


Fig. 3: Focal application of AS, phobe and AP fractions. No visible effects were observed with application of CA [fig. 3c (0 sec) and fig. 3c' (400 sec)] while vasorelaxant effects were observed with SA [fig. 3a (0 sec) and fig. 3a' (400 sec)] and phobe [fig. 3b (0 sec) and fig. 3b' (400 sec)] fractions. Experiences were repeated 6 times.

seconds after application (fig. 1b'), followed by a progressively important hemorrhage respectively after 200 sec (fig. 1c'), 300 sec (fig. 1d'), and 400 sec (fig. 1e'). No visible effects or changes of morphology were seen with normal saline solution (control) [fig. 1a (20 sec), 1b (100 sec), 1c (200 sec), 1d (300 sec) and 1e (400 sec)]. The photos were captured under dissecting microscope.

Effects of as on vascular pattern in CAM assay: Dose course study

Different concentrations of As: 40 μ g (fig. 2a'), 60 μ g (fig. 2b'), 80 μ g (fig. 2c') and 160 μ g (fig. 2d') were applied on the chick embryos for 400 sec. The data indicated a progressive marked increase in vessels diameter recorded for all the fractions doses used. Among four

concentrations, 160µg As showed distinct prominent speaker reaction (hemorrhage).

Effects of different semi-purified fraction of as on vascular pattern in CAM assay

In order to characterize the active component, which is responsible of the observed effects, different semi-purified fractions were tested. Results showed that ethanol soluble fraction (AS)(fig. 3a') and non-polar fraction (Phobe)(fig. 3b')induced the same effects that raw garlic extract. Whereas, any visible effect was observed after treatment with the insoluble ethanol fraction (AP) (fig. 3c').



Fig. 4: Phobe extract (1mg) was subjected to reverse-phase C18 silica gel was subjected to TLC on silicagel plates 60 F₂₅₄. A mixture of butanol/acetic acid/water (12/3/5) was used as a mobile phase. UV light revealed 5 different spots of Phobe extract.

Thin layer chromatography (TLC) analysis of phobe extract

Phobe extract was further subject to TLC on silica-gel plates. Data from fig. 4 showed the presence of 5 spots in Phobe extract.

Effects of the five spots scraped from TLC in CAM assay

The five spots scraped from TLC were tested. Results showed that only one spot (fig. 5e') induced the same effects that raw garlic extract when compared with all others spots (fig. 5a', b', c' and d').

Effects of diallyl sulfide and diallyl disulfide in CAM assay

Any visible effect was observed after treatment with the diallyl sulfide (fig. 6a') and diallyl disulfide (fig. 6b').

DISCUSSION

The chorioallantoic membrane, a highly vascularized tissue, serves as a model to determine the direct effect of compounds on vessels. The CAM is a useful tool because it is a relatively rapid assay and it is amenable to local administration of tested agents.

The present study showed that local application of As and its different fractions on CAM vascular pattern induces a clear dilatation of blood vessels. This dilatation could be explained by a relaxing effect on the smooth muscular cells of the blood vessels (Aqel *et al.*, 1991). The vasorelaxant effect of As would be dependent on the presence of endothelium. In fact, As may be responsible of the stimulation of the endothelial relaxant factors synthesis and of a strong inhibition of the endothelial contracting ones (Ashraf *et al.*, 1999).

To our knowledge, there has been no study dealing with the effect of As on the permeability of the capillaries. The appearance of hemorrhagic spots on CAM, leads us to assume that a strong blood extravasations arises following an increase of the capillaries' permeability. Many factors would be involved in this extra vasation phenomenon, among which prostaglandins, histamine and nitric oxide (Trustov, 1987).

Furthermore, the modulation of the vascular permeability would result from the VEGF synthesis (Vascular Endothelial Growth Factor) by endothelial cells (Keck *et al.*, 1989). The synthesis of the VEGF is partly regulated by NO. The increase of the VEGF expression stimulates the NO synthase at the level of the endothelial cells (Krolf and Waltenberger, 1998). Thus synthesized, the NO is responsible of an increase of the vascular permeability. Moreover, it has been shown that AGE acted in increasing the levels of NO (Nagae *et al.*, 1994) by stimulating the activity of the NO synthase *in vivo* and *in vitro* (Das *et al.*, 1995). Our previous data have already, shown that pre-treatment with L-NAME as well as DPI totally inhibits the production of NO induced by As. This study suggests that As promotes the production of NO²⁻/NO³⁻ via the stimulation of constitutive NOS (Mokni *et al.*, 2006). Furthermore, a link have been shown between the synthesis of NO and the effect on vasomotri city and vascular permeability. Thus, the NO is considered as a diffusible substance, which ensures the endothelium mediation that depends on the vascular relaxation (Vanhoutte, 1999). It is also involved in the modification of the vascular permeability (Krolf and Waltenberger, 1998).

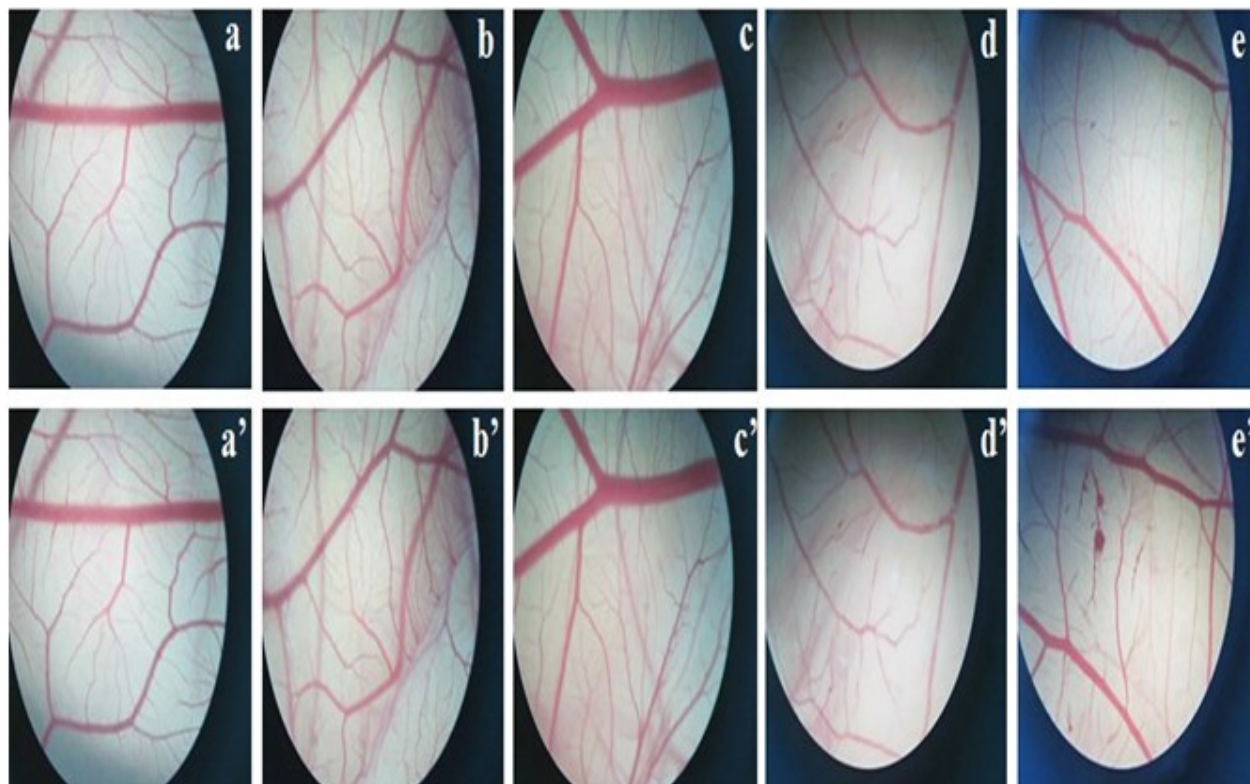


Fig. 5: Focal application of different purified fractions obtained with TLC. Focal application of spots 1,2,3 and 4 respectively [fig. 5a, 5b, 5c, 5d (0 sec) and fig. 5a', 5b', 5c', 5d' (400 sec)] and Focal application of spot 5 (fig. 5e (0 sec) and 5e' (400 sec)]. Vasomodulatory effects were observed with the spot 5 only. Experiences were repeated 6 times.

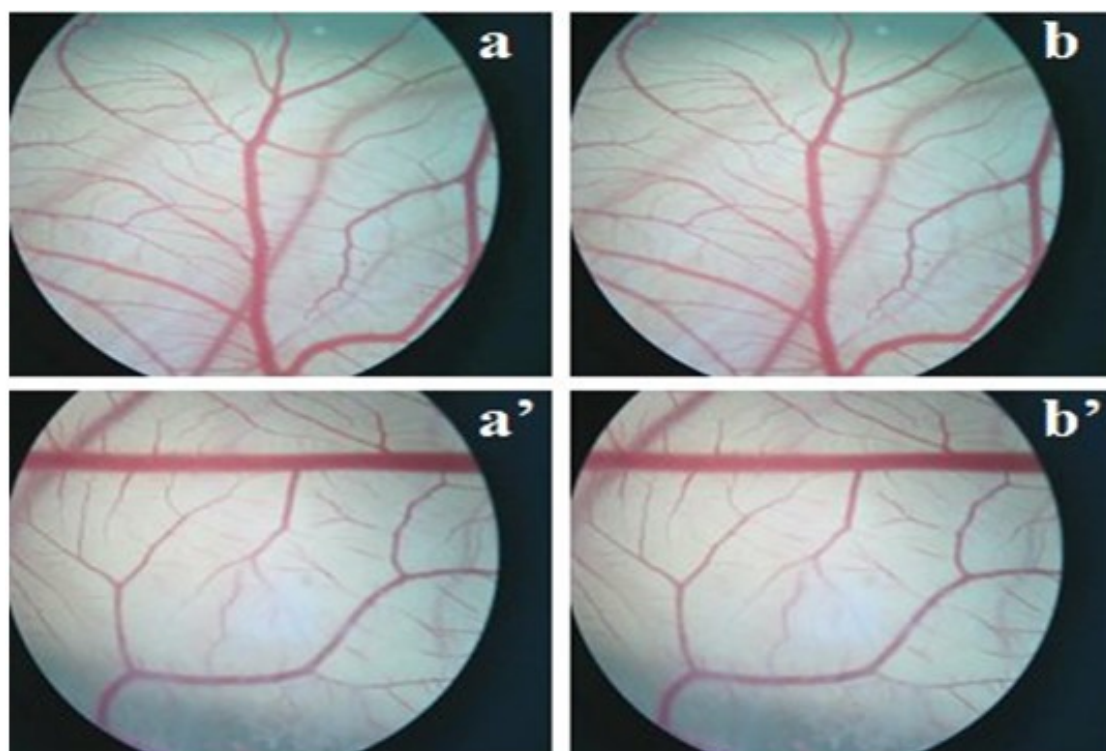


Fig. 6: Focal application of diallyl disulfide [fig. 6a (0 sec) and fig. 6a' (400 sec)] and diallyl disulfide [fig. 6b (0 sec) and fig. 6b' (400 sec)]. Note the unchanged vascular pattern in the two cases. Experiences were repeated 6 times.

In order to determine the nature of the active ingredient that is responsible of the physiological effects of as, different fractions have been prepared and tested. In fact, the application of the supernatant alcohol, a partially purified fraction, on CAM leads to the same effects observed during the application of as. In comparison to the supernatant alcohol, the hydrophobic fraction, tested in the same conditions as induced a clear vasodilatation and leads to the appearance of hemorrhagic spots.

Furthermore, we have studied the sensitivity of the semi purified fractions to heat. Our results clearly showed that the heat treatment abolishes totally the vasodilatory effect previously observed (data not shown). Furthermore, our results had clearly shown that the application of diallyl sulfide and diallyl disulfide, two purified molecules of as, have no effect on CAM vascular pattern.

Finally, the migration of the hydrophobic fraction on a silica sheet shows the presence of five spots, this indicates that the fraction contains five compounds of different hydrophobicity. The five spots had been tested on the CAM. The results clearly indicated that the last spot, meaning that the spot with the least molecular weight reproduces the same physiological effects of As. Thus, it could be concluded that the vasodilatory effects of As could be induced by a compound of nature peptide, of low molecular weight and different of diallyl sulfide and diallyl disulfide, as it was previously found by kaye *et al.* (2000). Whereas, studies by ku *et al.* (2002) had shown that allicin, the principally active metabolite of Garlic, induces a NO-dependant relaxation in rat pulmonary arteries (Ku *et al.*, 2002). On the other hand, it was been reported that Garlic-derived organic polysulfides, which are converted by erythrocytes into hydrogen sulfide (H₂S), are responsible of relaxing vascular smooth muscle and thus, inducing vasodilatation of blood vessels (Ginter and Simko, 2010).

CONCLUSION

As aqueous extract induced an increase of permeability and vasodilatation of blood vessels and capillaries in CAM of chick embryo. Furthermore, this research work has demonstrated that the active component responsible of these effects seems to be of peptide nature and appeared different from Diallyl Sulfide (DAS) and Diallyl Disulfide (DADS). Further experiments are needed to determine the exact molecular nature of this active principle, which could be very interesting to inhibit angiogenesis in the case of cancer.

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