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Full Length Research Paper

Investigation of effects of essential oils of *Origanum minutiflorum* O Schwarz PH Davis and *Cyclotrichium niveum* (Labiatae) plants on angiogenesis in shell-less chick embryo culture

Ismihan Goze[1]*, Ali Cetin[2] and Aysegul Goze[3]

[1]Vocational School of Health Services, Cumhuriyet University, TR-58140 Sivas, Turkey.
[2]Department of Obstetrics and Gynecology, School of Medicine, Cumhuriyet University, TR-58140 Sivas, Turkey.
[3]Faculty of Dentistry, Cumhuriyet University, TR-58140 Sivas, Turkey.

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The aim of this study was to investigate the effect of essential oils extracted from *Origanum minutiflorum* and *Cyclotrichium niveum* (Labiatae) plants to the vascularization systems of the chick embryos in a chorioallantoic membrane model. The aerial parts of *O. minutiflorum* and *C. niveum* were subjected to hydrodistillation. Essential oils were applied to the center of the blastodisc of 50 µl using a precision micropipette prior to the transfer of shell-less cultures to the incubator. Antiangiogenic effect of essential oil of the study plants was determined with the use of chorioallantoic membrane model. Numbers of main, branching and capillary blood vessels were counted. In the statistical analysis, when the groups were compared with regard to the main blood vessel (MBV) and branch blood vessel (BBV) development, no significant difference ($p > 0.05$) was observed. However, when the groups were compared with regard to capillary vessel (CV) development, the difference between the data of the groups was found significant ($p < 0.05$). One of the tested compounds, *C. niveum* essential oils, showed antiangiogenic effect; while the other, *O. minutiflorum* showed no antiangiogenic effect. This study examined the effect of exposure to *O. minutiflorum* and *C. niveum* essential oils on extraembryonic vascular development in the chick embryo area vasculosa (AV) in shell-less culture for the first time. Essential oils extracted from *C. niveum* has antiangiogenic effect; while *O. minutiflorum* essential oils had no antiangiogenic effect.

Key words: Angiogenesis, essential oil, *Cyclotrichium niveum, Origanum minutiflorum*, shell-less chick embryo culture.

INTRODUCTION

It is a well known fact that people benefit from natural plants as medicine. With use of medicinal plants, investigations have been performed all over the world in order to find more productive and economical medicines. Medications used to cure disorders require continuous changing to improve their effectiveness. With this purpose, many studies have been made comprising antioxidant and antimicrobial activities and determining other effective agents of plants (Candan et al., 2003; Sokmen et al., 2004; Tepe et al., 2005a, 2005b).

Origanum species are among the plants recognized as "*Oregano*" (Kekik) in Turkey. In the genus *Origanum* Lamiaceae family, 24 species are represented in Turkey with 16 of them endemic (Baser, 2002; Davis et al., 1998; Demirci et al., 2004). *Origanum minutiflorum* (O. Schwarz and P.H. Davis) that is widespread in the eastern Mediterranean region and southwestern Anatolia in Turkey is known locally as “toga kekik”, “dağ kekik” (mountain kekik) (Baser, 2002; Unlu et al., 2007). *Origanum* oil, mainly rich in carvacrol, is used as a painkiller in rheumatism by rubbing externally on painful

*Corresponding author. E-mail: igoze58@mynet.com. Tel: +3462219333.

limbs (Baser, 2002; Unlu et al., 2007; Baser, 2008). The aromatic oregano water, rich in carvacrol, is consumed to check gastrointestinal disorders, reduce blood cholesterol and glucose level and also for tumor suppressive activities (Baser, 2002; Baser, 2008). In recent years, a lot of reports have been published about the chemical composition and biological activity of the essential oils from *O. minutiflorum* (Baydar, 2005; Dadalıoglu and Evrendilek, 2004; Unlu et al., 2002).

The genus *Cyclotrichium* is presented in Turkish flora by five species of which two are endemic and this endemic species grow in eastern Anatolia (Davis et al., 1998). One of the members of this genus *Cyclotrichium niveum* - know as “dağ nanesi” in Turkish - has been widely used as herbal tea in addition to its medicinal uses since ancient times. *C. niveum* is an annual herb used as a traditional medicine of Sivas (Turkey) for treating influenza, nausea and muscle pain disorders (Gulcin et al., 2008; Tepe et al., 2005a). There are limited numbers of reports about the genus *Cyclotrichium* spp (Baser et al., 1994; Cetinus et al., 2007; Tepe et al., 2005a). The chemical composition and antioxidant activity of *C. niveum* has previously been reported (Başer et al., 1994; Cetinus et al., 2007).

Although in recent years, immense progress has been made in our understanding of molecular mechanisms and cellular regulation of angiogenesis in important diseases like cancer, clinical development of antiangiogenic agents for the therapy of cancer remains challenging. Since solid tumors account for more than 85% of cancer mortality in humans, tumor growth and metastasis are dependent on blood vessels. Therefore, nowadays, targeting tumor angiogenesis is one of the most widely studied areas to find new therapeutic strategies. In screening potential drug candidates against angiogenesis, a broad range of plant products were screened for antiangiogenic effects (Elluru et al., 2009; Mojzis et al., 2008; Liu et al., 2008; Oner et al., 2007).

Angiogenesis is a complex biological process that occurs normally in development, turnover and remodeling of mature vascular networks (Bryan and D’Amore, 2007; Guran et al., 2004). Angiogenesis is the formation of new vessels by endothelial sprouting, that is, endothelial cell migration, proliferation and tube formation. Angiogenesis is useful in some cases such as tissue infarcts when oxygen necessity increases; conversely in some cases it may be harmful (Staton et al., 2006; Tufan and Satıroglu-Tufan, 2003). By the surrounding neoplastic cells increasing excessively and without control, it may cause the tumor to be nourished and oxygenated and thus encourage the growth of the tumor.

Essential oils are natural, complex and multi-component systems composed mainly of terpenes as well as some other non-terpene components. They and their constituents can hopefully be considered in the future for more clinical evaluations and possible applications and as adjuvants to current medications (Edris, 2007). The aim of this study was to examine the effect of *O. minutiflorum* and *C. niveum* essential oils exposure on extraembryonic vascular development in area vasculosa (AV) of chick embryo in shell-less chick embryo culture.

MATERIALS AND METHODS

C. niveum was collected from Divrigi (1200 m above sea level), Sivas in Turkey and *O. minutiflorum* was collected from Isparta in Turkey (1035 m above sea level) in July 2006 during its flowering season. These were botanically identified by Erol Donmez, PhD, Department of Biology, Cumhuriyet University, Sivas in Turkey. The voucher specimens have been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas, Turkey (CUFH-Voucher No: ED 8732/ *O. minutiflorum* /CUFH-Voucher No: ED 9912/*C. niveum*).

Isolation of the essential oil

The air-dried and finely ground aerial parts of two plants submitted for 3 h water distillation using a clevenger-type apparatus (*C. niveum* yields 1.6% v/w, *O. minutiflorum*) yielded 2.9% v/w. The oils were dried over anhydrous sodium sulphate and, after filtration, stored at +4°C. Pulegone was the major component in *C. niveum* essential oil. The pulegone content was found as 76.84 mL essential oil (Cetinus et al., 2007). The major compounds of *O. minutiflorum* were carvacrol (79.34 mL), *p*-cymene (32.6 mL) and γ-terpinene (21.4 mL) (Unlu et al., 2007).

Chicken embryos

Fertilized eggs were used and incubated at 37.5°C in a humidified egg incubator for the desired period of time. Fertilized eggs belonged to 33 week old Ross 308 species hens. 60 eggs were used in the study. Development of chick embryos was illustrated in the beginning of the experiments.

Shell-less chick embryo culture

Shell-less cultures were prepared using the technique as used in the study of Tufan and Satiroglu-Tufan (2003). Briefly, fertilized chicken eggs were preincubated for 48 h at 37.5°C. Prior to explanation, eggs were placed horizontally, sprayed with 70% ethanol and permitted to air-fly for 10 min to reduce contamination from the egg surface and also to ensure that the embryo was properly positioned. This also allowed the eggs to cool down. The contents of the eggs were then transferred under aseptic conditions into culture system consisting of a clear, semipermeable polyethylene film secured with elastic rubber bands in the mouth of a cylindrical, transparent plastic cup (diameter: 7 cm, height: 7 cm) and presterilized under ultraviolet light for 1 h, by cracking the underside edge. Only cultures with the blastodisc positioned to the uppermost side of the yolk were used in the experiments. Following the appropriate experimental procedure, each shell-less culture was covered with a sterile plastic petri dish lid and incubated at 37.5°C with saturated humidity (Nüve A. S., Ankara, Turkey) for 48 h (Tufan and Satiroglu-Tufan, 2003; West, 2001). The solutions of *O. minutiflorum* and *C. niveum* was prepared by the mixtures of essential oils obtained by hydrodistillation with 10% ethyl alcohol (EA) (9 particles out of 10 are 10% EA and the remaining part is essential oils).

For this process, embryos were divided into 4 groups, each group consisting of 15 subjects: group 1, was left as the control

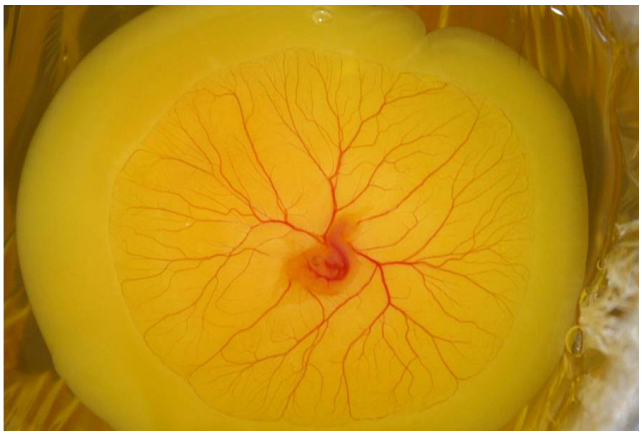

Figure 1. Representative picture of chorioallantoic membrane of an egg exposed to mixtures of 10% E.A. and *C. niveum* (9 parts out of 10 are 10% E.A. and the remaining part is *C. niveum*).

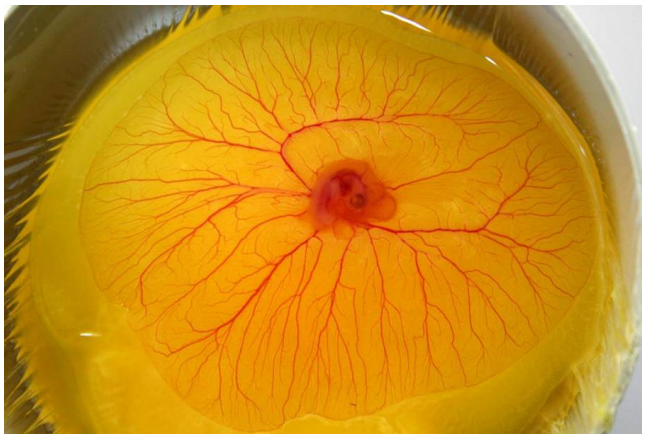

Figure 2. Representative picture of chorioallantoic membrane of an egg exposed to mixtures of 10% EA and *O. minutiflorum* (9 parts out of 10 are 10% EA and the remaining part is *O. minutiflorum).*

group; group 2 was applied with 10% EA; group 3 was administered the mixtures of 10% EA and *O. minutiflorum* and group 4 received the mixtures of 10% EA and *C. niveum.*

Material application was made to the embryos incubated in the incubator during the 72 h of incubation. From the liquids prepared for each embryo, 50 µl liquid was taken with a micropipette and was applied in the center of the blastodisc. This process was applied with one dose only.

Aiming to check incubation conditions, control group was placed in each incubator. By checking heart beats, embryos were controlled. On the 8th day of incubation, the vascular development of the subjects was photographed. 40 eggs were photographed and numerical measurements were made on these photographs (Figures 1 - 3).

Photographs were evaluated using an acetate paper called “point counter” resembling a graphic paper divided into 0.3 mm x 0.3 mm squares (Weibel, 1979). The procedure studied is named as “square grade”. The acetate divided into squares with 0.3 mm dimensions was placed on the photographs and ensured to stay stable.

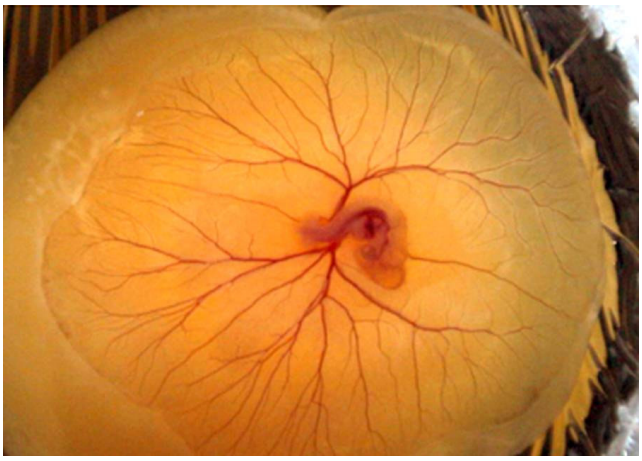

Figure 3. Representative picture of chorioallantoic membrane of an egg from the control group.

Intersection points in the area where all embryos and vessels cover were counted first (total area, T), then the number of intersection points of the number of main vessels directly coming out of the embryo were counted (main blood vessel, MBV) and divided by the number of points in the total area MBV/T.

Intersection points in the area that the first branches sprouting from main blood vessels covered were counted (branch blood vessel, BBV) and were divided by number of points in the total area: BBV/T. The same counting process was also made for the capillary vessel (CV) network separating from branching blood vessels (CV: CV/T).

Photographs taken for each group were counted one by one using “point counting” method and the frequency of the vascularization was calculated as explained above and the values obtained were placed in the table. Data were analyzed with Kruskal-Wallis ANOVA test and post hoc Tukey test.

RESULTS

We evaluated 10 photographs for each group and counted intersection points. The MBV, BBV and CV values of the control group were compared with the same values of each group, respectively (Figure 4). Each group was compared with the control values first, then compared with each other (Table 1).

In the statistical analysis, when the groups were compared with regard to MBV and BBV development, there was no significant difference ($p > 0.05$). However, when the groups were compared with regard to CV development, the difference between the data of the groups was found significant ($p < 0.05$).

DISCUSSION

In this study, we investigated the antiangiogenic effect of essential oils of *O. minutiflorum* and *C. niveum* as members of the Labiatae family. These grow naturally in our country (Turkey). We found that *C. niveum* essential

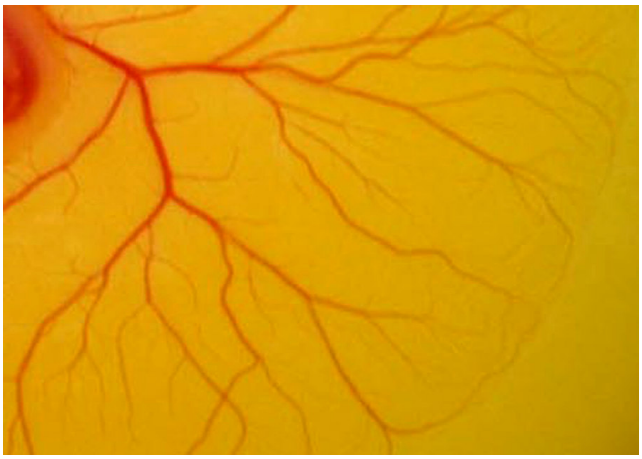

Figure 4A. Representative picture of chorioallantoic membrane of an egg exposed to essential oil of *C. niveum*.

Figure 4B. Representative picture of chorioallantoic membrane of an egg exposed to essential oil of *O minutiflorum*.

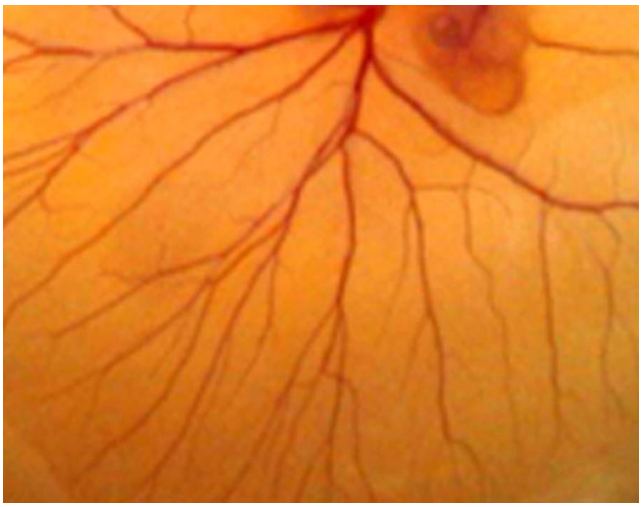

Figure 4C. Representative picture of chorioallantoic membrane of an egg with no exposure to essential oils of study plants.

oils has antiangiogenic effect; however, essential oils from *O. minutiflorum* has no antiangiogenic effect. In *O. minutiflorum* species, the active material was determined as carvacrol (Baser et al., 2002; Baydar, 2005; Dadalioglu and Evrendilek, 2004; Demirci et al., 2004., Sokmen et al., 2004; Unlu et al., 2007). The ratio of carvacrol differs according to its habitat or the sub species of the plant. In this study, we demonstrated that *O. minutiflorum* provides dilatation in the blood vessels and increase in the number of new vessels. *C. niveum* species have pulegone as the main component as determined by Baser et al. (1994) and Cetinus et al. (2007) and there is no information about the role of pulegone in vascularization.

The oils of the plants which contain essential oils are externally or orally consumed in traditional medicine. Thus, the toxic effects, metabolism and elimination of these oils need to be well known. There are a few studies investigating the antiangiogenic properties of essential oils of plants. Demirci et al. (2003) investigated anti-angiogenic effect of essential oils of *Phlomis linearis* Boiss. and Bal. of the Lamiaceae family growing in central, east and southeast Anatolia. These are endemic species for Turkey. They investigated the overall biological activity of the essential oil (100 µg/pellet) on the chorioallantoic membrane (CAM) of the fertilized hen's egg in order to examine the anti-angiogenic and anti-inflammatory activity. They demonstrated that the essential oils of *P. linearis* have very weak angiogenic effect. Demirci et al. (2004) investigated the antiangiogenic effect of the *Origanum onites* L. essential oils using the CAM assay. They found that the essential oil showed no meaningful antiangiogenic property. Demirci et al. (2005) studied the antiangiogenic effect of essential oils of *Salvia* species in the CAM assay and they concluded that the essential oils have no pronounced antiangiogenic effect.

Conclusion

Since essential oils from *C. niveum* has antiangiogenic effects, it is required that further analysis of its content and evaluation of its antiantiogenic effects in angiogenesis assays be carried out. We suggest that the volatile oils of *O. minutiflorum* may have a great importance in healing injuries. The impression acquired from this preliminary research is that volatile oils may accelerate treatment of wounds on all epithelial and mucosal surfaces, as long as the doses of the volatile oils are carefully determined and the pathological angiogenesis not activated. Thus, the essential oil of *O. minutiflorum* may also be utilized to treat bedsores and as aroma therapy.

Table 1. Main, branching and capillary blood vessel numbers of the study groups.

Groups	MBV	BBV	CV
Control	0.023 ± 0.022	0.039 ± 0.003	0.29 ± 0.01
10% EA	0.025 ± 0.003	0.040 ± 0.006	0.36 ± 0.02[a]
10% EA + % *Origanum minutiflorum*	0.023 ± 0.002	0.037 ± 0.001	0.43 ± 0.03[b]
10% EA + % *Cyclotrichium niveum*	0.022 ± 0.003	0.035 ± 0.04	0.33 ± 0.02[c]
	KW = 2.73	KW = 3.64	KW = 27.43
	p > 0.05	p > 0.05	p < 0.05

MBV = Main blood vessel; BBV = branching blood vessel; CV = capillary vessel; EA = ethyl alcohol.
[a, b, c]P < 0.05 vs Control. [b]P < 0.05 vs all other groups.

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