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Combinatorial Usage of Sumac Unriped Fruit Extract *(Rhus Coriaria)* and Tannic Acid Enhanced Synergistic Anti-Angiogenic Effect on Chick Chorioallantoic Membrane Assay

Ayça ÜVEZ[1], Şima KILIÇ[2], O. B. Burak ESENER[1], Duygu ÜNDER[3], Fatma Zerrin SALTAN[4], Konstantinos DIMAS[5], Elif İlkay ARMUTAK[1]

[1]Department of Histology and Embryology, İstanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, İstanbul, Turkey
[2]İstanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, İstanbul, Turkey
[3]Department of Clinical Pharmacy, Marmara University, Faculty of Pharmacy, İstanbul, Turkey
[4]Department of Pharmacognosy, Anadolu University, Faculty of Pharmacy, Eskişehir, Turkey
[5]Universityof Thessaly, Faculty of Medicine, Department of Pharmacology, Larissa, Greece

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ORCID IDs of the authors: A.Ü. 0000-0002-3875-2465; Ş.K. 0000-0002-3768-1589; O.B.B.E. 0000-0001-9444-3598; D.Ü. 0000-0001-9563-8469; F.Z.S. 0000-0001-8739-0256; K.D. 0000-0001-7481-9148; E.İ.A. 0000-0002-7359-6568

Abstract

Rhus coriaria (sumac) naturally grows worldwide and contains many phytochemicals. Tannic acid is frequently used for treating medical conditions. The aim of this study is to determine the synergistic effects of methanolic and acetonic extracts of *Rhus coriaria's* from unriped fruits in combination with tannic acid on angiogenesis by using the Chick Chorioallantoic Membrane (CAM) assay. The effects of methanol and acetone extracts of Sumac (MES and AES, respectively, at a final concentration of 5 mg/mL for both) and tannic acid (Tan 1.25 and 5 mg/mL) alone or in combination were examined for their effect on angiogenesis by applying the CAM assay. CAM assay results showed that AES is more effective than MES on suppressing angiogenesis. Tannic acid started to show antiangiogenic effects at the concentration of 5 mg/mL. When AES and tannic acid were used in combination at a dose of 5 mg/mL each, a very strong antiangiogenic effect was observed (score; 1.1±0.131, $p<0.001$). The combinatorial usage of AES and tannic acid may result in strong suppression of angiogenesis, an effect that is shown in this study for the first time. Based on this observation, combinatorial usage of sumac extracts and tannic acid could be promising as a new antiangiogenic therapy.

Keywords: Angiogenesis, chick chorioallantoic membrane assay, sumac, tannic acid

Introduction

Angiogenesis is a complex mechanism in which new capillaries are formed from existing blood vessels and where epithelial cells play an active role. Although angiogenesis is necessary in most physiological events, it is also a serious problem in many pathological conditions. In this context it is well known to play an important role in the development and spread of a tumor and it is known to be a key factor that may determine the prognosis of cancer cases (Auerbach et al., 2003; Folkman, 1971; Ribatti et al., 2007). Therefore, the researchers have reported the need to focus on natural and synthetic drugs that target angiogenesis as a tumor under siege strategy in cancer treatment. In this context drugs such as bevacizumab and ramucirumab have been developed to target angiogenesis in solid tumors (Al-Abd et al., 2017). Recenlty, the use of herbal derived substances (phytochemicals) that could result in the suppression of angiogenesis have gathered the interest of many research groups worldwide and some studies already demonstrate that such substances may have promising effects on several cell lines (Kumar et al., 2016; Kunnumakkara et al., 2008; Singh et al., 2015; Su et al., 2005).

Sumac grows naturally in countries of eastern Asia as Afghanistan and Iran and of Europe as Turkey, Greece, Bulgaria, Italy, and up to France (Köroğlu, 1989). Its natural habitat is known to be rocks and coasts. *Rhus coriaria* is called by several different names such as "titre, somalık, tutum, tatari, tetri, somak" in 4 different regions of Turkey (Güner, 2012). *Rhus coriaria* (used in the manufacturing of leather) and *Rhus cotinus* (dyer's sumac) are

Address for Correspondence: Elif İLKAY ARMUTAK • **E-mail:** elif@istanbul.edu.tr
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economically important *Rhus* species that grow in our country (Köroğlu, 1989). Sumac is widely used as a spice and as a medicinal plant in folk medicine in Turkey (Köroğlu, 1989). *Rhus coriaria* contains different phytochemicals in its leaves, fruits, and seeds such as tannins, polyphenols, flavonoids, flavones, organic acids, and essential oils (Ardalani et al., 2016; Elagbar et al. 2020). Phenolic compounds such as gallic acid, quercetin, malic acid, rosmarinic acid, volatile oil, cyanidin, peonidin, pelargonidin, and delphnidin glucosides, besides vitamins and minerals, have been detected in different parts of sumac (Abu-Reidah et al., 2015; Koşar et al., 2007). Sumac has also been extensively studied for several kinds of bioactivity owing to its phytochemically enriched content. Thus, sumac has been reported to show important properties such as astringent, antimicrobial, diuretic, anti-inflammatory, antipyretic, antioxidant (Ünder and Saltan, 2019). Moreover, fruits of sumac have been reported to exhibit antidiabetic, scolidal, DNA-protective, analgesic, anticancer, and antiviral activities in the literature (Candan and Sökmen, 2004; Chakraborty et al. 2009; Giancarlo et al. 2006; Moazeni and Mohseni, 2012; Mohammadi et al., 2016; Parsania et al. 2017; Rajabi and Mousa, 2017). Sumac consumption, owing to its well-known therapeutic potential, is increasing day by day (Köroğlu, 1989). Also recently, combinations with various other natural substances and herbal extracts have been used to increase the therapeutic efficacy of *Rhus coriaria* (Abedi Gaballu et al., 2015; Adwan et al., 2008; Özcan, 2003).

One of these compounds is tannic acid, a type of hydrolyzed tannin that is found in the shell, root, leaf, fruit, and seed parts of plants such as chestnut, oak, tea, and sumac (Türkan et al., 2019; Ünder and Saltan, 2019). Tannic acid is frequently used in medical conditions, such as wound healing, body burns, bleeding and chronic diarrhea. In addition, various studies reported that tannic acid is a powerful antioxidant; has anti-angiogenic, anti-inflammatory, and antiproliferative effects; increases apoptosis; and suppresses tumor growth in various types of cancer (Chen et al. 2003; Türkan et al., 2019). Importantly, various studies demonstrate that combinatorial usage of tannic acid can show synergistic effects (Ünder and Saltan, 2019).

Chick Chorioallantoic Membrane (CAM) assay is an *in vivo* method commonly used to detect the anti-angiogenic properties of various compounds. CAM assay, which is shown as an alternative model for animal studies, has attracted the attention of researchers in recent years owing to its easy application, rapid results, and low cost (Cimpean et al. 2008; Özgürtaş, 2009).

Although there is a limited number of studies investigating the anti-angiogenic effects of *Rhus coriaria* extract (sumac) and tannic acid alone, which are well-known to exhibit, as discussed above, multifaceted therapeutic activities, no studies were found in which both were used in combination. Thus, this study was aimed to investigate the anti-angiogenic effects of methanolic and acetonic extracts of *Rhus coriaria*'s unriped fruits, tannic acid, and their combinations in the CAM model.

Material and Methods

Chemicals

Tannic acid was purchased from Sigma Aldrich. (403040 Sigma-Aldrich, Steinheim, Germany). Acetone (HPLC grade) was obtained from Merck (Germany). (±)-Thalidomide was purchased from Sigma-Aldrich, Germany. UltraPure Agarose was purchased from Invitrogen (Carlsbad, CA, USA).

Plant material

The unriped fruits of *Rhus coriaria* were collected from Diyarbakır-Kahta province in Turkey in October 2018. They were deposited in the Herbarium of Anadolu University, Faculty of Pharmacy (ESSE 15483).

Extraction method

The unriped fruits of *Rhus coriaria* (10 g) was dried. Plant material was extracted with 70% acetone in a shaker bath set at 20°C for 3 days. The extract was filtered and evaporated at 40°C using a rotavapor until dryness. The yield of extract was calculated as 18.1% (w/w). Also, methanolic (80% methanol) extract of the same plant material was performed by Accelerated Solvent Extraction (ASE) method. In this extraction method, 40°C heat and 1,500 psi pressure and 10 minutes were performed with ASE 300 Dionex equipment (Dionex Corp., USA). The yield of extract was determined as 6.8% (w/w) (Kossah et al. 2010; Pandey and Tripathi, 2014).

CAM (chick embryo chorioallantoic membrane) assay

Fertilized eggs were supplied from Mudurnu Piliç- Pak Tavuk Company (İstanbul). Experiment groups were designated as Tannic acid 1.25 and 5 mg/mL (Tan 1.25 and 5 mg/mL), Acetone Extract of Sumac 5.0 mg/mL (AES), Methanol Extract of Sumac 5.0 mg/mL (MES) alone, and their combinations (MES 5.0 mg/mL + Tan 1.25 mg/mL, AES + Tan 1.25 mL/mg, AES + Tan 5.0 mg/mL). (±)Thalidomide (Sigma-Aldrich, Germany) 5 mg/mL were used as positive control. All extracts and tannic acid were dissolved in a 2.5% (w/v) agarose (UltraPure Agarose, Invitrogen) solution at the requested concentrations immediately before use. Pellets (10 µL) were prepared by dropping on circular stainless steel with a 5 mm diameter and applied on to the chick chorioallantoic membrane (CAM).

The in vivo CAM assay was performed as described previously (Bürgermeister et al., 2002; D'Arcy and Howard, 1967; Gurel-Gurevin et al., 2018). All studies with fertilized chicken eggs were performed in an incubator at 36.5°C and relative humidity of 80%. Before the addition of the agents/combinations to be tested fertilized eggs were pre-incubated at 36.5°C for 72 hours. Subsequently 8-10 mL of albumin were aspirated by syringe with a 21G needle from the bottom of the egg. A hole was opened on the top of the egg shell using forceps, then holes were covered with parafilm® and eggs were further incubated again at the same conditions for another 72 hours. Then, pellets (1 pellet/egg) were placed on CAM and were closed again well with parafilm®. After 24 hours of incubation with pellets,

Table 1. Semi-quantitative scoring system for evaluating antiangiogenic effects on CAM (Ikitimur-Armutak et al. 2017; Krenn and Paper, 2009)

Scale	Anti-angiogenic effect	Effects observed on CAM after treatment
<0.5	Inactive	No change (normal embryo growth).
0.5-0.75	Weak	No capillary free area.
		Area with reduced density of capillaries around the pellet not larger than its own area.
>0.75-1	Strong	Small capillary free area or area with significantly reduced density of capillaries.
		Effects not larger than double the size of the pellet.
>1	Very strong	Capillary free area around the pellet at least doubles the size of the pellet.

Average score = [Number of eggs (score >1) × 2 + *number of eggs (score* >0.75 - 1*)* × 1] / *(total number of eggs scored)*

Table 2. Anti-angiogenic effect results of positive controls, sumac extracts, and tannic acid alone and their combinations on CAM assay

Treatment Groups (n)	Concentration (µg/pellet)	Average score	Anti-angiogenic effect	Tox (%)
MES 5 mg/mL (n=45)	50	0.27±0.043	No effect	-
AES 5 mg/mL (n=45)	50	0.75±0.051	Strong effect	-
Tan 1.25 mg/mL (n=45)	12.5	0.2±0.023	No effect	-
Tan 5 mg/mL (n=45)	50	0.55±0.029	Weak effect	-
MES 5 mg/mL +Tan 1.25 mg/mL (n=45)	50+12.5	0.33±0.040	No effect	-
AES 5 mg/mL +Tan 1.25 mg/mL (n=45)	50+12,5	0.8±0.047	Strong effect	-
AES 5 mg/mL +Tan 5 mg/mL (n=45)	50+50	1.1±0.131***	Very strong effect	-
Agarose (blank) (n=30)	%2.5, w/v	0.2±0.2	No effect	-
Thalidomide (Positive control) (n=30)	50	0.86±0.021	Strong effect	-

Significantly different ***p<0.001 compared to thalidomide.
MES: Methanolic extract of sumac; AES: Acetonic extract of sumac; Tan: Tannic acid; Tox: Toxicity; n: Total egg number for each group

the anti-angiogenic effect was evaluated by measuring the CAM area under the stereo-microscope (Leica LED2500- Leica MC170, Wetzlar, Germany) with Leica Application Suite, Las V4.7 (Leica). We used 10 eggs for positive and negative control and 15 eggs for treatment groups. All samples are tested in triplicate independent experiments. We used 375 fertilized eggs for experiments.

Anti-angiogenic effects of samples were determined by using a scoring system (Table 1) (Ikitimur-Armutak et al. 2017; Krenn and Paper, 2009). The anti-angiogenic scores were calculated using the following formula (Krenn and Paper, 2009; Gurel-Gurevin et al., 2018).

Average score = [Number of eggs (score >1)×2 + *number of eggs (score* >0.75-1)×1]/(*total number of eggs scored)*

The CAM assay does not require an ethics committee approval for animal experimentation as the eggs are not considered to carry live animals until day 17 of development in most countries. Institutional Animal Care and Use Committee of New England (IACUC) reports that researchers do not need ethics committee approval for CAM assay experimentation, by reason of chick embryos are not able to experience pain until day 15 of incubation (IACUC, 2001; NIH, 1991; Ribatti, 2017).

Statistical analysis

Statistical analysis was performed using One-way ANOVA followed by Dunnett's multiple comparisons post hoc test using Graphpad Prism 6.0 statistical software for Windows (San Diego, CA, USA). p-values<0.05 were considered statistically significant. Results are expressed as the mean ± standard deviation.

Results

The effects of methanolic and acetonic extracts of sumac (MES and AES) and tannic acid (Tan 1.25 and 5 mg/mL) alone and their combinations on angiogenesis were examined by CAM assay. Thalidomide as a positive control and agarose as a negative control were used. The results are summarized in Table 2.

The CAM assay results, revealed that 5 mg/mL concentration of MES (score; 0.27±0.043) had no antiangiogenic effects as compared to positive control thalidomide (score; 0.86±0.021) (Figure 1 and Figure 2). However, AES at the same concentration (score; 0.75±0.051) showed strong antiangiogenic effects as positive control thalidomide. Tannic acid alone showed no antiangiogenic effect when compared to thalidomide at the concentration of 1.25 mg/mL (score; 0.2±0.023), but demonstrated a weak antiangiogenic effect compared to the positive control at the concentration of 5 mg/mL (score; 0.55±0.029) (Figure 2).

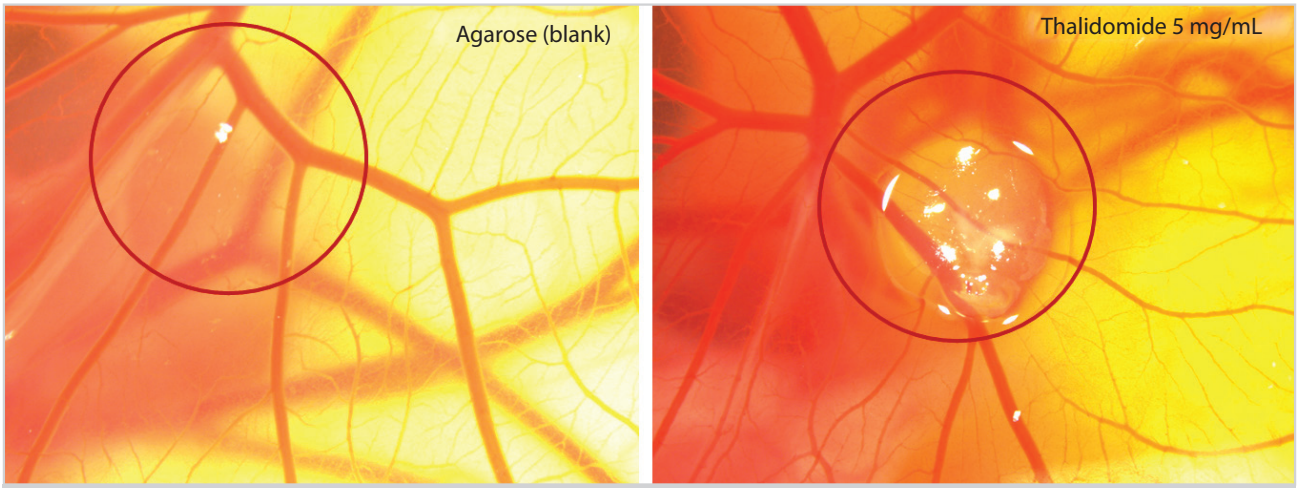

Figure 1. Effects of agarose as a negative control and thalidomide (5 mg/mL) as a positive control on CAM

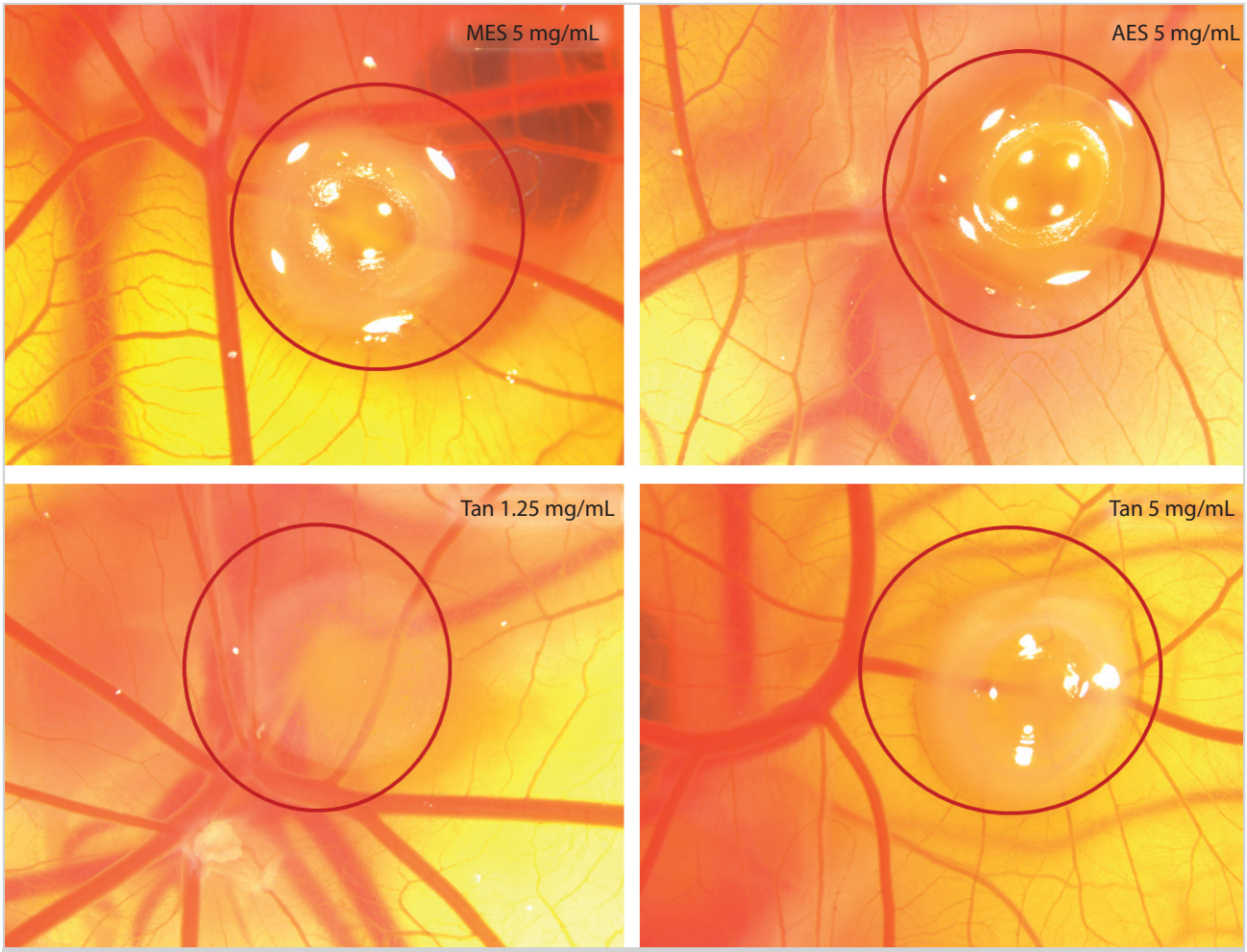

Figure 2. Anti-angiogenic effects of methanolic extract of sumac (MES; 5 mg/mL), acetonic extract of sumac (AES; 5 mg/mL), and tannic acid (Tan; 1.25 mg/mL and 5 mg/mL) alone on CAM

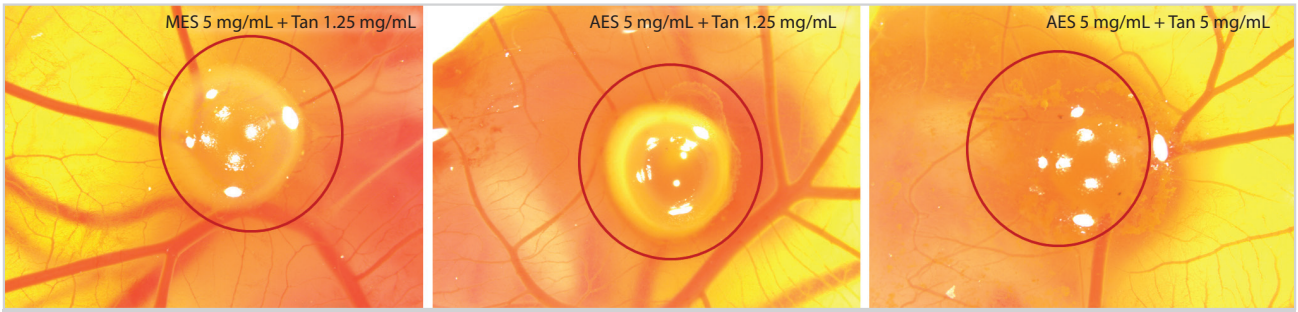

Figure 3. Anti-angiogenic effects of combinatorial groups (MES 5 mg/mL +Tan 1.25 mg/mL; AES 5 mg/mL +Tan 1.25 mg/mL and AES 5 mg/mL +Tan 5 mg/mL) on CAM

To further test any synergistic effect, methanolic and acetonic sumac extracts were initially combined with low doses of tannic acid and the effect on CAM was examined. When tannic acid was combined at a concentration of 1.25 mg/mL with a 5 mg/mL concentration of methanolic and acetonic sumac extracts, scores were similar to that of sumac extracts alone (score of MES 5 mg/mL + Tan 1.25 mg/mL: 0.33±0.040; score of AES 5 mg/mL + Tan 1.25 mg/mL: 0.8±0.047) (Figure 3); therefore, we concluded that no significant synergistic effect occured in these combinations of low concentration tannic acid and sumac extracts. Therefore we further aimed to study a potential synergistic effect at high concentrations of tannic acid. In these experiments the combination of AES at 5 mg/mL with 5 mg/mL of tannic acid showed very strong antiangiogenic effect as compared to the positive control i.e., thalidomide (score; 1.1±0.131; $p<0.001$) (Figure 2). To conclude, our data demonstrated that combination of sumac acetone extract and tannic acid (which alone show only weak anti-angiogenic effects) shows a synergistic effect, which was superior from all single treatments.

Discussion

Bioactive substances (phytochemicals) derived from plants have nowadays gained too much appreciation as alternative treatments of many diseases (Benkeblia, 2004; Catalgol et al., 2012; Noureen et al., 2019; Penumathsa and Maulik, 2009). Phytochemcilas such as curcumin obtained from ginger, resveratrol and quercetin obtained from predominantly grapes, rosmarinic acid from rosemary are frequently used in pharmacological studies due to their multifaceted therapeutic potential (Anand David et al. 2016; Koushki et al., 2018; Nadeem et al., 2019). In this context, extracts and bioactive phytochemicals are also frequently investigated in angiogenesis suppression studies, which have gained increased attention recently (Beladjila et al., 2019; Kuo et al.; 2016; Wang et al., 2010).

In our study, we tested two extracts of sumac for angiogenesis suppresion activity. Methanol extract of sumac at the 5 mg/mL concentration showed no anti-angiogenic effects. On the contrary the acetone extract of sumac demonstrated a strong anti-angiogenic effect at the same concentration. This difference most probably occurs due to the the difference in the composition of extracts obtained using different organic solvents, as in the studies of Pinelo et al. (2004) and Do et al. (2014). *Rhus coriaria* is known to contain many antioxidants such as phenolics, flavonoids, proanthocyanidins, and saponin (AL-Jubory et al., 2010; Batiha et al., 2020; Sakhr and El Khatib, 2020). Also, in their study in which *Salacia chinensis* was investigated using different solvents to produce extracts, Ngo et al. (2017) reported that acetone is more suitable for extraction of phenolics, flavonoids, proanthocyanidins, saponin and antioxidant capacity compared to other solvents. Based on these studies, acetone extract of sumac may have a richer composition in phytochemicals than methanol extract, which could explain its stronger anti-angiogenic effect. Thus, it would be of great interest to analyze the consistency of these two extracts and compare them in an effort to identify phytochemcials that could be responsible for the superior antiangiogenic efficacy of the AES. We have to point out here that this is the first study demonstrating that AES shows such a strong antiangiogenic effect on a CAM model. Mirian et al. (2015) studied the cytotoxicity and anti-angiogenic effects of methanol extract of *Rhus coriaria*'s oleo gum resin on human umbilical vein endothelial cell (HUVEC) and retinoblastoma cell line and reported a dose-dependent anti-angiogenic effect. In this study Mirian et al. (2015) also reported that *Rhus coriaria* can be a better choice to treat types of metastatic cancer due to its low toxicity and high anti-angiogenic effect. However, in our study when methanol and acetone extracts of unriped fruits from *Rhus coriaria*'s was investigated on angiogenesis at the same concentration, methanol extract of sumac did not show any anti-angiogenic effect but acetone extract of sumac demonstrated strong anti-angiogenic effect. An explanation to this could actually be the use of different parts of the plant for the preparation of extracts.

In another study, the effect of ethanol extract of *Rhus coriaria* fruit was investigated on the malignant behavior mechanism of triple negative breast cancer cells (TNBC) by using many methods such as matrigel invasion analysis, wound healing migration analysis, vascular tube formation analysis, tumor growth, and metastasis analysis on chick embryos. In this *in vitro* studies

an increased dose-dependent anti-angiogenic effect of *Rhus coriaria* fruit was reported (El Hasasna et al., 2016). In our study, a single dose of sumac extract was used and the data obtained from the acetone extract of sumac were similar to the *in vitro* results of El Hasasna et al. (2016).

Recently, several studies report synergistic effects of natural compounds combined with tannic acid (Carvalho et al., 2018; Dusane et al., 2015; Rivero et al., 2010). Geng et al. (2019) reported that the use of tannic acid with cisplatin increased mitochondrial pathway-mediated apoptosis in liver cancer cells. In another study, the effects of tannic acid on human cholangiocarcinoma cells were examined together with various chemotherapeutics and tannic acid with mitomycin C and 5-fluorouracil reported to suppress tumor growth in a synergistic manner (Naus et al., 2007). Chang and Wang (2013) reported that tannic acid and quercetin might be protective against cancer when administered together. Also, one study reported that tannic acid may show synergistic effects with some antiviral agents (Vilhelmova-Ilieva et al., 2019). Furthermore, some food quality studies reported that tannic acid combinations could be used to improve food tastes and shelf life (Al-Hijazeen et al., 2018; Moawad et al., 2017). Rekha et al. (2015), examined the effect of tannic acid obtained from Memecylon Malabaricum on vascularization at the concentration of 10 mg/mL by using the CAM assay and they reported a strong antiangiogenic effect. As the aim of our study was to show a synergistic effect between a weak in terms of anti-angiogenic efficacy concentration of tannic acid and the acetone extract of sumac, we did not test concentrations of tannic acid higher than 5 mg/mL. Our study though suggests that testing higher concentrations of tannic acid may not be necessary as the result of the combination was found to be extremely strong in terms of angiogenesis suppression resulting in superior activity even compared to thalidomide, the positive control (Table 2).

In conclusion, our data clearly show that the acetone extract prepared from the immature fruits of sumac is more effective than the methanol extract in suppressing angiogenesis and that tannic acid demonstrated weak but concentration dependent antiangiogenic effects. More significantly when acetone sumac extract and tannic acid were combined, they strongly suppressed angiogenesis underlining a synergistic activity, an effect that is demonstrated for the first time. Thus, we could suggest that combinatorial use of sumac acetone extracts and tannic acid can be a potential new antiangiogenic therapy for diseases, such as cancer. Obviously, toward this aim more studies on angiogenesis *in vitro* and *in vivo* are needed, which now are ongoing in our laboratories.

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Peer-review: Externally peer-reviewed.

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