

ESSENTIAL OIL OF *THYMUS PECTINATUS* FISCH&MEY. VAR. *PECTINATUS*: CHEMICAL FORMATION, ANTIMICROBIAL, ANTIOXIDANT, ANTISPASMODIC AND ANGIOGENIC ACTIVITIES

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Abstract

Background: Thymus species are well-known as medicinal plants. It was aimed to investigate the chemical composition of *Thymus pectinatus* (TP) and its antioxidant, antimicrobial, antispasmodic, and angiogenic activities.

Materials and Methods: After essential oil of TP (EO-TP) was obtained with cleverger distillation system, it was analyzed for chemical composition with GC-MS. To study antispasmodic activity, eight male Wistar albino rats each weighing approximately 250-300 g were used.

Results: The results of the analysis revealed 19 components, which equals to 92.93 of the essential oil. The following are the main components; *thymol* (48.77%), *m-cymene* (9.15%), *isoborneol* (5.19%), *trans-caryophyllene* (4.43%), *carvacrol* (3.91%) and *γ-terpinene* (3.54%). It has been found that microorganisms subjected to whole all of microbiological tests are highly resistant to EO-TP. 2,2- diphenyl-1-picrylhydrazyl, hydroxyl radical, along with superoxide radical scavenging, and lipid peroxidation says were utilized to show the antioxidant activities of the EO-TP. In measuring its effect, 0.1 mg/mL and 0.5 mg/mL dosage of the EO-TP was applied to the rat ileum as a result of which the magnitude and the frequency of the contractions were observed to be significantly.

Conclusion: It was also understood that EO-TP had angiogenic effect upon the vein system of the embryos of chicks. There is a need for further studies to clarify the mechanisms of antimicrobial, antioxidant and angiogenic effects and to determine the constituents of EO-TP responsible from these effects, especially the angiogenic.

Keywords: *Thymus pectinatus*, essential oil, angiogenic, antioxidant, antimicrobial, antispasmodic activity, shell-less chick embryo culture

Introduction

People have always made use of plants for various purposes, including curing people with a minimum cost (Baytop, 1999; Çelik et al., 2010; Sezik et al., 1992; Yeşilada et al., 1993). Discovering the fact that essential oil of the plants can be medicinal, either the patient takes it by the mouth or applies to the skin, people have been forced to have better understanding of these plants such as their characteristics whether they have side effects or not. Essential oil of plants is invaluable in this sense. There are numerous components consist of such an essential oil all of which can be used in a great variety of purposes in our daily lives from foods to cosmetics or from aroma therapy to phytotherapy (Baytop, 1999; Çelik et al., 2010; Sezik et al., 1992; Sökmen et al., 1999; Yeşilada et al., 1993).

So far, Thymus has caught great attention in the researchers' world and various studies have been conducted on different kinds of it both in laboratory environments and on living organisms. The area of usage for the *Thymus* is wide in Turkey especially in alternative medicine. It can appear as a flower as well as a leave or can be consumed as oil. *Thymus* is good for the bronchitis and relieving intestinal gas when used orally. *Thymus* also serves as a diuretic and it works up on appetite. On the other hand, it can cure arthritis rheumatism if it is applied to the skin.

Essential oil of *Thyme* is also used as a germ-killer in mouthwashes. In some situations of baldness of scalp, essential oil of *Thyme* is used to fight bacterial and fungal infections (Dob et al., 2006; Göze et al., 2009; Hadian et al., 2008; Sökmen et al., 2004a; Sökmen et al., 2004b; Tepe et al., 2004; Tümen et al., 1999). This is why more information is necessary regarding the composition of this essential oil or whether they have side effects and so on.

As a physiological process, angiogenesis means the formation of blood vessels through already existing ones and is possible with endothelial proliferation as well as migration of the endothelial cells and the formation of tubes. This process might be desirable particularly when tissues' need of (intense) oxygen increase or it might end with some unwelcome situations such as cell expansion in neoplasia (Elluru et al., 2009; Lokman et al., 2012; Liu et al., 2008; Mojzis et al., 2008; Ribatti, 2008; Tufan and Satiroglu-Tufan, 2003).

A wide range of plant products were studied in terms of their antiangiogenic effects in the search for drugs which have the potential to be used against angiogenesis (Demirci et al., 2003; Demirci et al., 2004; Demirci et al., 2005; Elluru et al., 2009; Göze et al., 2009; Göze et al., 2010; Göze et al., 2015; Liu et al., 2008; Lokman et al., 2012; Mojzis et al., 2008; Öner et al., 2007; Ribatti, 2008; Tufan and Satiroglu-Tufan, 2003; West et al., 2001; Yıldız et al., 2013).

Angiogenesis and anti-angiogenesis are mostly studied in-vivo and via the chorioallantoic membrane (CAM) of the chick embryos (Demirci et al., 2003; Demirci et al., 2004; Demirci et al., 2005; Elluru et al., 2009; Göze et al., 2010; Göze et al., 2015; Liu et al., 2008; Mojzis et al., 2008; Öner et al., 2007; West et al., 2001; Yıldız et al., 2013).

The present study focuses on the essential oil of *Thymus pectinatus* (EO-TP) in terms of its composition, antimicrobial, antispasmodic, antioxidant activities as well as its angiogenic effects. EO-TP composition, antimicrobial (Vardar-Unlu et al., 2003) and antioxidant activities have already been studied and presented; yet, its angiogenesis effect and antispasmodic activities have not been questioned before (Başer et al., 1992; Başer et al., 1999; Vardar-Unlu et al., 2003).

Materials and Methods

Essential oil of plant as the subject of this study was obtained from *TP* which is common in the Sivas province. The *TP* were collected from Yoncabayiri, Cengelli Mountain (1750 m) in İmranlı, and a district of Sivas. Another copy of the voucher was produced and sent to the Herbarium of the Department of Biology, Cumhuriyet University in Sivas in Turkey to be kept (CUFH-Voucher No: ED 11010).

Separation of the essential oil

Water distillation of the Clevenger distillation system was applied for three hours to the parts of the *TP* which have been air dried (yield, 4.1% v/w). Then the essential oil became dried over anhydrous sodium sulphate. When the filtration was finished, the dried oil was kept at +4°C and then it was analyzed using GC-MS described by Adams, 2001; 2007. EO-TP was examined by a Shimadzu (Kyoto, Japan) QP5000 GC-MS apparatus, which has the features of a GL Sciences (Tokyo, Japan) capillary column (TC-5; 30m x 0.25mm id.; film thickness 0.25 µm) and a 70 eV electron ionization quadrupole detector. In analyzing the components, a comparison was made between the duration and retention time (NBS75K library data of the GC-MS system).

Using DPPH assay in determining the antioxidant activity

Here, the alcoholic DPPH solutions were diminished with the help of the antioxidant donating hydrogen. A sound absorption band was found at 517 nm in the DPPH solutions, which turns into a dark violet in color (Blois, 1958; Burits and Bucar, 2000; Cuendet et al., 1997). The study sets an example for the present study in using the synthetic antioxidant reagent Butylated hydroxytoluene (BHT) as the positive control. All tests were performed three times.

β-Carotene/linoleic acid assay

This method was employed to detect the antioxidant capacity by means of analyzing the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides which are brought about by the linoleic acid oxidation. Here, when there is a reaction emerges with the radicals who are formed by linoleic acid oxidation, the yellow color of β-carotene fades. In investigating the antioxidative capacity of the essential oil, the study conducted by Dapkevicius et al., (1998) was benefitted from by making a comparison with that of BHT within the same concentration and of 350 µL of EtOH / C₂H₅OH.

Microbial strains

Antimicrobial activities of the essential oil were tested by being subjected to three gram positive and five gram negative bacteria whilst for its antifungal activities a fungus was used, employing the disc diffusion method. The following are the microorganisms that were used and of which cultures were provided by the culture collection of the Contagious Disease Research. Refik Saydam National Type Culture Collection Unit, Department of Microbiology

Reference Laboratory, Public Health Institution located in Ankara, Turkey. *Staphylococcus aureus* ATCC-25923, *Bacillus subtilis* ATCC-6633, *Pseudomonas aeruginosa* ATCC-27853, *Klebsiella pneumonia* NCTC-5046, *Escherichia coli* ATCC-35218, *Salmonella typhi* WCTC- 9394, *Candida albicans* ATCC-10231, *Corynebacterium diphtheria* RSHM-633 and *Proteus vulgaris* RSHM-96G22. All these cultures were provided by the culture collections of the Contagious Diseases Research. Bacterial strains were cultured throughout a night at 37°C in Mueller-Hinton agar (CM 337, Oxoid, Basingstoke, UK) and the ferment was cultured at 30°C throughout a night in Sabourad dextrose agar (CM41, Oxoid). The tests were performed three times. Average and standart deviation (SD) calculations were used to obtain inhibition zone diameters.

Disk diffusion assay

Being used in order to observe the antimicrobial activities of the EO-TP, this method took its place in the related issue of the National Committee for Clinical Standards (NCCLS, 1997; NCCLS, 1999).

Antispasmodic activity

To study antispasmodic activity, eight male Wistar albino rats each weighing approximately 250-300 g were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and the experiments were approved by Cumhuriyet University-Medical Faculty, Animal Ethics Committee. Rats were acclimatized to a 12 h light/dark cycle at 23 °C with food and water available ad libitum.

Preparing to test the TP effect upon the rat ileum contractions

Male rats were dilled and got subjected to cervical dislocation in order to be inactivated. Then 2 cm of their ileums were extracted and vertically placed into the Tyrode solution composing of (mM); NaCl 136.0, KCl 5.0, MgCl₂ 0.98, CaCl₂ 2.0, NaH₂PO₄ 0.36, NaHCO₃ 11.9, glucose 5.5, bubbled with air (37°C, pH 7.4). First, no intervention was made and the natural contractions of the ileum were examined. After the steady state lasting for 1 hour, numerous drugs were included with different dosages as; 0.1 mg/mL, 0.5 mg/mL and 1 mg/mL. The study was conducted in line with the relevant study previously carried out by Yildirim et al., 2005.

Chicken embryos

Tufan and Satiroglu-Tufan's studies serve as an example to prepare shell-less cultures; for instance fertilized chicken eggs were previously incubated at 37.5°C for 48 hours. (Nüve A. S., Ankara, Turkey) The above mentioned fertilized eggs (n=40) were taken from the chickens which are members to the Ross 303 species and which are 33 week old.

TP solution was composed by mixing the essential oils which are separated through hydrodistillation with 10% of ethyl alcohol (EtOH / C₂H₅OH) (9 of 10 equal to 10% EtOH / C₂H₅OH and the rest is the essential oil).

This study required the embryos to be separated into three groups of 10. While group 1 was left untouched as the control group, 10% EtOH / C₂H₅OH was given to the group 2 and a combination of 10% EtOH / C₂H₅OH and TP was given to the group 3. First an egg was subjected to the combination of 10% EtOH / C₂H₅OH and TP (9 out of 10 equals to %10 EtOH / C₂H₅OH and the rest is the essential oil) then its embryos were exposed to material application which were previously incubated for 72 hours. 50 µL was sucked through a micropipette from the solution made for the embryos and it was poured into the middle of the blastodisc. This was conducted with just one dose. Incubation conditions were checked by placing control groups together with study groups into each incubator. The viability state of embryos was controlled by checking heart beats. During the angiogenesis stage embryos were photographed at 8th day to evaluate the integrity of vascular development. On these photographs taken from 30 eggs at 8th day, numerical measurements were performed. In order to analyze the photographs "point counters" were used. Point counters were in fact acetate paper looking like a graphic paper with sizes of 0.3 mm X 0.3 mm squares. This process is named as "square grade" (Weibel, 1979). The acetate paper was put upon the photographs in order to make it stay still. "Point counting" method was made use of to count the photographs. In order to understand how often the vessels are formed, the above mentioned methods were applied and the results were shown in a Table 4. Kruskal-Wallis ANOVA test and post hoc Tukey test was used to analyze the data (Statistical Methods, 2001).

Results

Chemical composition of the essential oil

The outcomes of the GC-MS analysis of the chemical formation of the EO-TP were presented in Table1. 19 components were found to form the chemical composition of the essential oil which equals to 92.93% (by GC-MS). The following are the major components; *m-thymol* (48.77%), *m-cymene* (9.15%), *isoborneol* (5.19%), *trans-caryophyllene* (4.43%), *carvacrol* (3.91%) and *γ-terpinene* (3.54%) which account for 74.99% of the essential oil.

Table 1: Chemical composition of *Thymus pectinatus* Fisch & meyer var. *pectinatus*

	RT	LRI	Components	<i>Thymus pectinatus</i> Fisch&mey.var. <i>pectinatus</i>
1	12,770	939	α -pinene	1.95
2	13,555	953	camphene	0.58
3	16,418	991	β -myrcene	1.92
4	18,000	1015	α -terpinene	1.86
5	18,185	1082	m-cymene	9.15
6	18,531	1032	1,8-cineol	2.75
7	20,667	1060	γ -terpinene	3.54
8	23,900	1153	3-thujanol	1.49
9	25,980	1155	isoborneol	5.19
10	27,238	1169	endo-borneol	0.25
11	28,500	1177	terpinene-4-ol	2.79
12	37,258	1290	m-thymol	48.77
13	38,625	1299	carvacrol	3.91
14	40,845	1351	α -terpinyl acetate	0.54
15	45,983	1406	trans-caryophyllene	4.43
16	46,327	1408	junipene	0.50
17	46,625	1485	β -selinene	0.58
18	47,500	1534	cis-nerolidol	1.52
19	48,275	1619	(-)-spathulenol	1.21
			Total	92.93%

^aRT: Retention Time ^bLRI: Linear Retention Indices (OV-5 column)

Antimicrobial activity

For this study, it would not be wrong to say that EO-TP prevented 8 of 9 microorganisms which were tested from growing; *C. albicans*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *S.typhi*, *B. subtilis*, *C. diphtheriae*, *P. vulgaris* and *E. coli* (Table 2).

Table 2: Antimicrobial activity of the EO-TP using agar disc diffusion method

Microorganisms	<i>Thymus pectinatus</i> Fisch.&mey.var. <i>pectinatus</i>		Gentamycin ^d	Nystatin ^e
	Disc Diffusion Method ^b	MIC ^c		
<i>Staphylococcus aureus</i>	90±1.52	10.10	23±0.54	-
<i>Escherichia coli</i>	44±0.88	31.50	16±0.20	-
<i>Pseudomonas aeruginosa</i>	15±1.01	62.50	20±0.28	-
<i>Salmonella typhi</i>	54±1.16	21.00	10±0.18	-
<i>Klebsiella pneumoniae</i>	80±1.18	35.50	20±0.40	-
<i>Proteus vulgaris</i>	67±1.68	49.25	22±1.45	-
<i>Bacillus subtilis</i>	90±1.35	13.25	29±0.80	-
<i>Corynebacterium diphtheriae</i>	60±1.22	15.50	23±0.15	-
<i>Candida albicans</i>	52±1.43	31.50	-	25±0.16

^aResults are means of three different measurements. ^bAgar disc diffusion method, diameter of inhibition zone (mm) including disk diameter of 6 mm; ^cMinimum inhibitory concentrations (MIC), ^dAntibacterial, ^eAntifungal

Antioxidant activity

It was detected that The IC₅₀ value of EO-TP was 160 mg/mL and the IC₅₀ value for BHT was 10.5 mg/mL. The β-carotene-linoleic acid assay is also responsible from 72% of the prevention along with the EO-TP as presented in Table 3.

Table 3: *Thymus pectinatus* Fisch.&mey.var.pectinatus essential oil the in vitro free radical DPPH and β-carotene-linoleic acid system

	Inhibition IC ₅₀ (μg/mL) (DPPH)	Inhibition % (β-carotene-Linoleic acid)
<i>Thymus pectinatus</i> <i>Fisch.&mey.var.pectinatus</i>	0.160	72
BHT	0.0105	100

Antispasmodic activity

The effect of EO-TP was measured by observing the natural contractions on the ileum of the rat. It was detected that the magnitude of the contractions were reduced significantly while the time span between the contractions got significantly longer at 1mg/mL dose. When the dose was increased to 1 mg/mL the contractions disappeared. Figure 1 presents the related results.

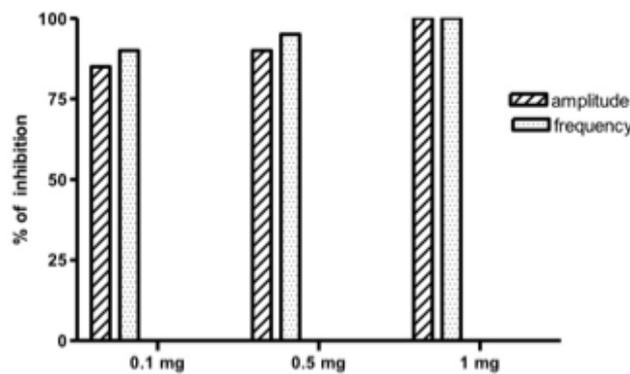


Figure 1. Effect of EO-TP on rat ileum spontaneous contractions.

Angiogenic activity

In order to decide the angiogenic activity, 10 photographs that were taken for each group was analyzed the main blood vessels (MBV), branching blood vessels (BBV) and capillary vessels (CV) in the intersections (Figure 2) were counted. First, the MBV, BBV and CV values of the groups were compared with those of the control group and then with each other regarding the parameters that were set in the first step. In terms of MBV and BBV growth, significant distinction was not found between the groups ($p > 0.05$). On the other hand, when it comes to the CV growth, the group showed significant distinction from each other ($p < 0.05$) (Table 4).

Table 4: Main, branching and capillary blood vessel numbers of the study groups

Groups	Main blood vessel (MBV)	Branching blood vessel (BBV)	Capillary vessel (CV)
Control	0.023+0.002	0.039+0.003	0.29+0.01
% 10 EtOH / C ₂ H ₅ OH	0.026+0.003	0.004+0.006	0.36+0.03
% 10 EtOH / C ₂ H ₅ OH +TP	0.025+0.002	0.036+0.030	0.42+0.03
	Kw =3.77 $p > 0.05$	Kw=2.02 $p > 0.05$	Kw=31.14 $p < 0.05$

MBV:main blood vessel; BBV:branching blood vessel; CV:capillary vessel; EtOH / C₂H₅OH: ethyl alcohol

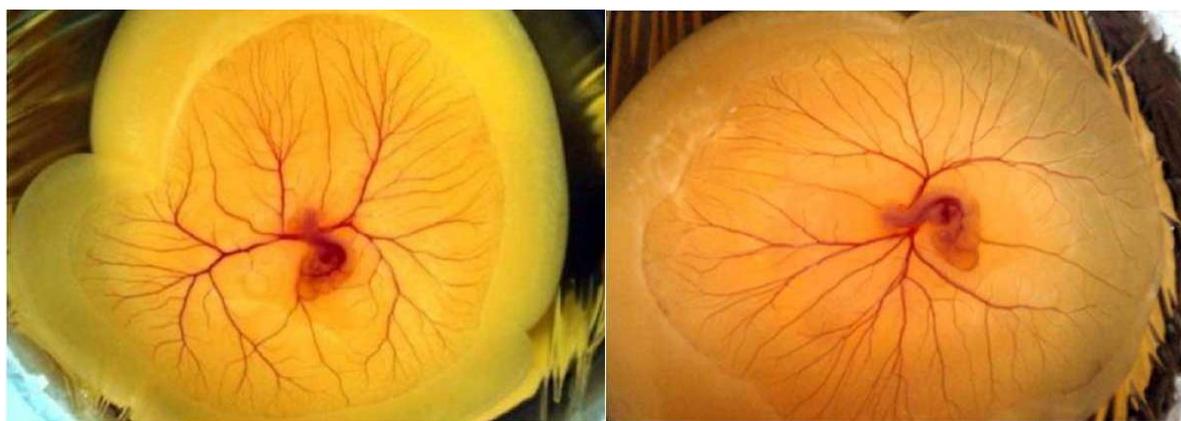


Figure 2: Angiogenic effect of EO-TP and control group

Discussion and Conclusion

Antimicrobial and antioxidant activities of the EO-TP in laboratory environment have been discussed (Başer et al., 1992; Başer et al., 1999; Vardar-Unlu et al., 2003).

Compounds of the essential oil

This study presents the analysis of the formation of the EO-TP and suggests that the main components are the following; *thymol* (48.77%), *m-cymene* (9.15%), *isoborneol* (5.19%), *trans-caryophyllene* (4.43%), *carvacrol* (3.91%) and *γ-terpinene* (3.54%). The study conducted by Başer et al., 1992, puts forward that the main components of the EO-TP are the following; *m-thymol* (35%), *borneol* (17.70%) and *p-cymene* (12%). On the other hand, another study made by Başer et al., 1999 the components of the EO-TP were detected as the following; *p-cymene* (20.74%, 8.88% and 14.62%), *γ-terpinene* (11.11%, 11.12% and 12.13%) and *thymol* (47.81%, 61.67% and 52.48%). Vardar-Unlu et al., (2003) stated in their study that EO-TP included *thymol* 49.8%, *γ-terpinene* 16.11%, *p-cymene* 14.3%, *carvacrol* 3.7% and *borneol* 2.7%. These variations might be explained by the shifts in the weather conditions, different seasons or geographical regions or geological features.

Antioxidant activity

The results of the relevant study Vardar-Unlu et al., (2003) and this study has similar results although this study revealed that *TP* showed intense antioxidant activity. It was observed that *TP* had strong antioxidant activity. DPPH assay was used for determining the free radical scavenging capacities of the EO-TP. Free radical scavenging capacities of the essential oil was identified in DPPH assay. It was detected that The IC_{50} value of EO-TP was 0.160 $\mu\text{g/mL}$ and the IC_{50} value for BHT was 0.0105 $\mu\text{g/mL}$. That is why it can be said that EO-TP had relatively more intense antioxidant activity when compared to that of BHT.

Antimicrobial activity

The present study found out that microorganisms except for the *P. aeruginosa* was resistant to the EO-TP. Vardar-Unlu et al., (2003) stated that there was a wide range of microorganisms resistant to EO-TP, which shows the compliance of the present study with the other related studies.

Antispasmodic activity

The effect of EO-TP was directly observed on the natural contractions on the ileum of the rat. It was seen that the magnitude of the contractions was significantly reduced and the time span between the contractions got significantly longer when it was applied at 0.1 mg/mL dose and that the contractions were completely gone under 1 mg/mL dose. It was found that both amplitude and frequency at 0.1 mg/mL and 0.5 mg/mL were significantly decreased.

Angiogenic activity

EO-TP was detected to have angiogenic effect. This study revealed that *T.P.* expands blood vessels and leads to the formation of new vessels.

Demirci et al. (2004, 2005) studied on the chorioallantoic membrane (CAM) of the fertilized chicken egg and analyzed the total biological activity of the plant essential oil (100 g/pellet) including anti-angiogenic and anti-inflammatory

activity (Demirci et al., 2003; Demirci et al., 2004; Demirci et al., 2005). That study proved that the angiogenic effect of the essential oil of *Phlomis linearis* is not strong at all. Another study carried out by the same researchers studied the angiogenic effect of the essential oil of the *Origanum onites* L and *Salvia* species by means of CAM assay and reached the conclusion that the essential oil has no significant antiangiogenic characteristics (Demirci et al., 2004; Demirci et al., 2005).

Angiogenic effects of the *Origanum minutiflorum* (OM) and *Cyclotrichium niveum* (CN) (*Labiatae*) essential oil were investigated by Göze et al., (2010; 2015) employing the CAM assay. This study showed that the essential oil of OM exhibited angiogenic characteristic while contrarily CN exhibited antiangiogenic effect (Göze et al., 2010). Another study carried out by the same researchers put forward that the essential oil of *Juniperus excelca* did not have a strong angiogenic effect (Göze et al., 2015). EO-TP was found to have angiogenic effect in general; however, further research is necessary to better understand which components are to be held responsible for this situation.

Due to the doses found are not toxic, can be used as an antimicrobial, antioxidant for the treatment of various diseases such as influenza and colds. However, angiogenic capillary activity has been found to increase activity in capillary vessels, so it has been found that it must be used with caution especially in cancerous cells.

As a conclusion, it can be said that EO-TP can be regarded as an untouched reservoir in terms of its antimicrobial and antioxidant effects. TP was also proven to have angiogenic characteristics. Further investigations should be conducted to understand what components account for the growing number of vein formation in the egg CAM in the ovarium and also to discover the pathological relatedness of the outcomes of the present study.

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Conflict of interest: The authors declare that there is no conflict of interest with respect to this study.

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