

In vitro effects of different essential oils and their major components on *Fasciola hepatica*

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Abstract

This study evaluated *in vitro* *Cinnamomum verum*, *Syzygium aromaticum*, *Origanum vulgare*, *Thymus vulgaris* essential oils activity and its major components eugenol, carvacrol and thymol on the motility and *Fasciola hepatica* integument surface. The trematodes were incubated in solutions containing these compounds in concentrations of 0.025%, 0.05% and 0.1% and had their essential oils motility recorded at 3, 12, 15 and 24 hours after incubation. The chromatographic analysis revealed eugenol as the major compound of *C. verum* and *S. aromaticum*, while *O. vulgare* and *T. vulgaris* presented carvacrol as the main component. The images obtained by scanning electronic microscopy of the specimens after 24 hours of incubation revealed the tegumentary alterations presence such as blebbing, spine loss and extensive sloughing with basal lamina exposure. The results obtained in the *in vitro* test demonstrated that essential oils and major components had a dose-dependent effect on the motility and integument surface of *F. hepatica*, having the essential oils of *O. vulgare* and *T. vulgaris* and the components carvacrol and thymol presenting the best results at lower concentration and time.

INTRODUCTION

Fasciolosis is a worldwide distribution disease that generates great economic losses for bovine animals due to a systemic presentment that causes weight loss, milk production loss, lower carcass yield and livers condemnation during slaughter, besides the risk of human infection (Jacobs et al. 2015). In Brazil, in addition to the several localities considered endemic for the disease, direct losses of US \$ 210 million / year (Molento et al. 2018) are estimated as a result of bovine fasciolosis, representing a challenge for rural producers, veterinarians and public health.

The current attempts to control fasciolosis aim to decrease the intermediate host population and the herds treatment with antiparasitic drugs, however, the impossibility of using molluscicides and the increasing parasitic resistance found among the *Fasciola* spp. populations (Monteiro 2017; Kelley et al. 2016; Bowman 2014) has led researchers from all over the world to seek new control alternatives for this helminthiasis.

Since the essential oils have a varied composition of bioactive substances, several researchers have investigated the effects of these compounds on microorganisms and multicellular organisms, finding promising results in the use of these substances as antimicrobials, insect repellents and antiparasitics (Buckle 2003).

The objective of this work was to test the *Cinnamomum verum*, *Syzygium aromaticum*, *Origanum vulgare* essential oils *in vitro* activity, *Thymus vulgaris* and the major components carvacrol, eugenol and thymol on the motility and tegumentary surface of *Fasciola hepatica* adult especimens.

MATERIALS AND METHODS

Essential oils and their major compounds

For this experiment the essential oils and their major compounds were purchased from commercial sources and the main information can be found in Table 1.

Table 1
Commercial sources where essential oils and their major compounds used on this experiments was obtained.

ESSENTIAL OIL	COMPANY
<i>Cinnamomum verum</i> (leaves)	Destilaria Bauru LTDA
<i>Syzygium aromaticum</i> (buds)	Quinarí
<i>Origanum vulgare</i> (leaves)	Quinarí
<i>Thymus vulgaris</i> (leaves)	Quinarí
MAJOR COMPOUND	COMPANY
Carvacrol	Sigma-Aldrich
Eugenol	Vetec Química Fina LTDA
Thymol	Neon Comercial

In order to confirm the purity of the essential oils and identify their main compounds, the gas chromatography coupled to mass spectrometry (GC-MS) method was used. A capillary column of fused silica Rtx-5MS (30 m long, 0.25 mm internal diameter) was used with helium as the carrier gas. The temperatures were 220°C for the injector and 300°C for the detector. The initial column temperature was 60°C, programmed for an increase of 3°C per minute until reaching the maximum temperature of 240°C (Pinheiro et al., 2015). The components identification was performed by comparing their mass spectra with the reference data from the NIST / EPA / NIH 08 Mass Spectral Library and by the Kovats Index (Adams, 2007).

For component quantification, the essential oils were analyzed on a gas chromatograph equipped with a flame ionization detector (GC-FID). The stationary phase was the capillary column Rtx-5MS (30 m long and 0.25 mm internal diameter). Nitrogen was used as carrier gas. Temperature programming was the same as previously reported for GC-MS analysis. The injector and the detector temperatures were 240°C and 250°C. 10 mg of essential oil sample was diluted in 1 mL dichloromethane and 1 µL of the mixture was injected (Pinheiro et al. 2015).

In vitro test of motility inhibition

Adult *F. hepatica* copies recovered from bile ducts of slaughtered cattle in southern state slaughterhouses of the Espírito Santo were referred to the Pharmaceutical Production Laboratory of Exact, Natural and Health Sciences Center of the Federal University of Espírito Santo, where the parasites with higher motility and macroscopically preserved structures were washed in physiological solution and transferred to Petri dishes containing RPMI 1640 culture means until the *in vitro* test, time as described by Jeyathilakan et al. (2010).

The compounds were tested at concentrations of 0.025% (m v⁻¹), 0.05% (m v⁻¹) and 0.1% (m v⁻¹). As a positive control, 0.5% albendazole sulfoxide (v v⁻¹) was used as the negative control and the combination of solvents Dimethyl sulfoxide (DMSO) 0.25% (v v⁻¹) and 0.25% Tween 80 (v v⁻¹). The culture means chosen for solution preparation and parasite maintenance was RPMI 1640 means, as proposed by Jeyathilakan et al. (2010).

The *in vitro* test began within 60 minutes after transfer of the specimens to RPMI. The specimens were individually distributed in Petri dishes containing the test solutions and the groups divided into three parasites per experimental unit in triplicate, totaling nine parasites per treatment. Motility was evaluated after 3h, 12h, 15h and 24h of immersion in the respective treatments, and the presence of motility was recorded with value 1 and absence with value 0.

At the end of the incubation the specimens were fixed in 10% formaldehyde solution (v v⁻¹) for analysis by scanning electron microscope (SEM).

Scanning Electron Microscopy

Samples fixed in 10% formaldehyde (v/v) were sectioned in their anterior third, washed in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.2–7.4) and immersed in solution containing 1% osmium tetroxide, 0.1 mol L^{-1} cacodylate and 1.25% potassium ferrocyanide. After 60 minutes the specimens were washed repeatedly in distilled water, dehydrated in an ascending ethanol series and dried with carbon dioxide at the critical point. The samples were fixed in aluminum stubs, metallized with gold and observed in a scanning electron microscope (SEM) JEOL® model JSM-6610LV operating at 20kV. The regions selected for visualization were: ventral face of the cephalic cone in the magnification of 70X, oral suction cup in the magnification of 200X and integument of the ventral anterior region in the magnifications of 100X and 600X.

Statistical analysis

In order to determine the tested products efficacy, the following formula, defined by Wood et al. (1995), was used:

$$Efficacy (\%) = \frac{Controlgroupaverage - Treatedgroupaverage}{Controlgroupaverage} \times 100$$

The *in vitro* motile inhibition test results were submitted to variance analysis and the averages compared by the Tukey test, which were considered significant if $p < 0,05$.

RESULTS AND DISCUSSION

Chromatographic analysis

GC-MS analysis identified 37 compounds in the analyzed essential oils and confirmed carvacrol as the major compound in the *O. vulgare* and *T. vulgaris* essential oil (Table 2). Similar studies have also identified carvacrol was the major compound in *O. vulgare* essential oil (Tomanić et al. 2022; Gong and Ren 2020; Szczepanik et al. 2017). However, in studies by Ali (2021), Gedikoğlu et al. (2019) and Nemati et al. (2017) thymol was the main compound in the *T. vulgaris* essential oil. In our study, the essential oil of *T. vulgaris* also identified the presence of o-cymene and thymol with percentage values similar to carvacrol (Table 2), suggesting that these three compounds may vary their concentration in the oil.

Table 2
Chemical constituents Percentage, Kovat indexes calculated ($IK_{calc.}$) and tabulated ($IK_{tab.}$) of the *C. verum* (CV), *S. aromaticum* (SA), *O. vulgare* (OV) and *T. vulgaris* (TV) essential oils.

Constituents	Kovats Index		Essential Oils (%)			
	$IK_{calc.}$	$IK_{tab.}$	CV	SA	OV	TV
1,8-cineole	1028	1035	–	–	–	2,07
α -copaene	1371	1376	0,58	–	–	–
α -phellandrene	999	1005	0,79	–	–	1,37
α -humulene	1448	1454	0,53	1,89	–	–
α -pinene	940	939	0,74	–	1,52	4,53
α -terpineol	1186	1189	0,26	–	–	0,77
α -terpinolene	1085	1088	–	–	–	1,15
α -thujene	926	931	–	–	0,62	–
β -carene	1012	1011	–	–	0,91	–
β -phellandrene	1026	1031	0,58	–	–	–
β -mircene	990	991	–	–	1,45	1,81
β -pinene	977	980	0,21	–	0,35	0,60
γ -terpinene	1058	1062	–	–	7,45	0,71
δ -2-carene	1005	1001	–	–	–	0,36
δ -3-carene	1012	1011	–	–	–	0,42
Benzyl benzoate	1760	1762	3,48	–	–	–
Borneol	1163	1165	–	–	–	2,88
Boryl Acetate	1283	1285	–	–	–	1,73
Camphene	953	953	0,23	–	0,35	3,16
Camphor	1141	1143	–	–	–	0,61
Carvacrol	1300	1298	–	–	67,67	23,93
Caryophyllene oxide	1577	1581	0,58	0,46	0,25	–
Cinnamaldehyde	1267	1266	1,05	–	–	–
Cinnamyl acetate	1442	1440	1,94	–	–	–
Eugenol	1361	1356	77,95	87,66	–	–
Eugenyl acetate	1525	1524	3,59	–	–	–
Isoborneol	1153	1156		–	–	1,58
Limonene	1026	1031	–	–	–	3,01
Linalool	1097	1098	2,30	–	2,42	4,68
Longifolene	1397	1402	–	–	–	0,33

Constituents	Kovats Index		Essential Oils (%)			
	IK _{calc}	IK _{tab}	CV	SA	OV	TV
o-cymene	1022	1022	0,74	–	11,60	22,57
Safole	1283	1285	1,02	–	–	–
Terpinen-4-ol	1173	1177	–	–	–	1,28
Thymol	1291	1290	–	–	3,91	20,23
Thymol methyl ether	1241	1235	–	–	0,21	–
Trans-caryophyllene	1413	1418	3,16	9,99	1,30	–
Not identified	2732	–	0,27	–	–	0,23
Total (%)	–	–	100	100	100	100

Eugenol was the main compound in the essential oils of *C. verum* and *S. aromaticum*. For *C. verum* essential oil, eugenol also was the compound with the highest percentage in the studies by Gogoi et al. (2021), Lokuge et al. (2018) and Li et al. (2015) studies. Qin et al. (2023), Ghallab et al. (2020) and Boughendjioua (2018) identified eugenol as the main compound in *S. aromaticum* essential oil.

Essential oils and main compounds on the *F. hepatica* motility in vitro activity

The results obtained with the efficacy calculation at each concentration over time (Table 3) demonstrate that the motility inhibition had a dose-dependent pattern in all treatments, being the 0.1% dose (m v^{-1}) the best treatment. Fixing the time of 24h as a reference, it was observed that there was inhibition of motility in 100% of the specimens at a concentration of 0.1% (m v^{-1}) in all treatments, however is worthy mentioning that treatments ORV, THV, CAR and THY showed maximum inhibition percentage earlying at concentrations of 0.25% and 0.5% (m v^{-1}).

Table 3

Treatments efficacy (%) with essential oils and their majorities, on *F. hepatica*, at different concentrations and exposure time.

	3h			12h			15h			24h		
Treatment	0,025*	0,05*	0,1*	0,025*	0,05*	0,1*	0,025*	0,05*	0,1*	0,025*	0,05*	0,1*
CV	0	0	0	0	0	100	0	0	100	11,11	0	100
AS	0	0	100	0	0	100	0	11,11	100	100	11,11	100
EU	0	0	100	0	0	100	0	0	100	22,22	0	100
OV	100	100	100	44,44	100	100	88,89	100	100	100	100	100
TV	66,67	100	100	66,67	100	100	88,89	100	100	100	100	100
CA	66,67	100	100	66,67	100	100	100	100	100	100	100	100
TI	88,89	88,89	100	100	100	100	100	100	100	100	100	100
PC	17,78	17,78	17,78	15,56	15,56	15,56	26,67	26,67	26,67	48,89	48,89	48,89

(*) = concentration expressed as % (m v^{-1}); CV – *C. verum*; SA – *S. aromaticum*; EU – eugenol; OV – *O. vulgare*; TV – *T. vulgaris*; CA – carvacrol; TH – thymol; PC – positive control.

Statistical analysis (Table 4) showed that there was a difference between the treatments and the positive and negative controls ($p < 0.05$), with a 10.9% coefficient of variation, which according to Ferreira (1991) means excellent precision in the results.

Table 4
F. hepatica motility absence average at different concentrations and exposure time, obtained from Tukey test for the unfolding of each product and concentration within each time level.

	3 h			12 h			15 h			24 h		
Treatment	0,025*	0,05*	0,1*	0,025*	0,05*	0,1*	0,025*	0,05*	0,1*	0,025*	0,05*	0,1*
NC	1,0 c	1,0 c	1,0 c	1,0 c	1,0 c	1,0 c	1,0 b	1,0 b	1,0 b	1,0 cd	1,0 cd	1,0 cd
PC	1,5 bc	1,5 bc	1,5 bc	1,2 c	1,2 c	1,2 c	1,2 b	1,2 b	1,2 b	1,3 bc	1,3 bc	1,3 bc
CV	1,0 c	1,0 c	1,0 c	1,0 c	1,0 c	2,0 a	1,0 b	1,0 b	2,0 a	1,1 cd	1,0 d	2,0 a
AS	1,0 c	1,0 c	2,0 a	1,0 c	1,0 c	2,0 a	1,0 b	1,1 b	2,0 a	2,0 a	1,7 ab	2,0 a
EU	1,0 c	1,0 c	2,0 a	1,0 c	1,0 c	2,0 a	1,0 b	1,0 b	2,0 a	1,2 bcd	2,0 a	2,0 a
OV	2,0 a	2,0 a	2,0 a	1,5 bc	2,0 a	2,0 a	1,9 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a
TV	1,7 ab	2,0 a	2,0 a	1,7 ab	2,0 a	2,0 a	1,9 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a
CA	1,7 ab	2,0 a	2,0 a	1,7 ab	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a
TH	1,9 a	1,9 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a	2,0 a
Averages followed by the same letter in the column do not differ by Tukey's test ($p \leq 0.05$). (*) concentration expressed in % ($m v^{-1}$); NC – negative control; PC – positive control; CV – <i>C. verum</i> essential oil; SA – <i>S. aromaticum</i> essential oil; EU – eugenol; OV – <i>O. vulgare</i> essential oil; TV – <i>T. vulgaris</i> essential oil; CA – carvacrol; TH – thymol.												

The World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of anthelmintics in ruminants affirm an antiparasitic drug is considered effective when it is more than 90% effective, being considered highly effective when reaches value greater than 98% (Wood et al. 1995). Thus, it can be stated that ORV and THV were out with the highest activity among tested essential oils, while among the major components THY and CAR were in the best efficacy values, being THY the treatment with the best efficacy in the shortest time (3h) and concentration (0.025%).

In the CIV, SYA and EUG treatments, the best inhibition values were observed only at the highest concentration tested, initiating at 3 hours on SYA and EUG treatments and at 12 hours in CIV treatment.

When compared to the positive control, ORV, THV, CAR and THY treatments have always been shown to be more effective at *F. hepatica* motility inhibiting. Moreover, the similarity between the results obtained with the essential oils and their respective major components suggests that these were the main responsible for the action of the tested oils.

The available literature about the use of *O. vulgare* and *T. vulgaris* essential oils and the main components Thymol and Carvacrol as anthelmintics are still scarce, and although studies suggest that the substances own anthelmintic potential (Štrbac et al. 2022; Ferreira et al. 2016; Pensel et al. 2014), no records of their actions were found in *F. hepatica*, opening new perspectives for control of this trematode.

Scanning Electron Microscopy observations

After 24 h of treatment, both the specimen exposed to treatment with albendazole sulfoxide (positive control) and the specimen exposed to the treatment containing only the solvents (negative control) maintained preserved spines and tegument (Fig. 1). On the other hand, different integumentary lesions levels were verified in the treatments with essential oils and major components (Figs. 2 and 3).

SEM images revealed that all treatments presented integument lesion, which showed a dose-dependent pattern directly proportional to the dose used (Table 5).

Table 5

Changes in *F. hepatica* specimens analyzed after 24 hours of exposure to the treatments with essential oils and major components at concentrations of 0.025% (m v⁻¹), 0.05% (m v⁻¹) and 0.1% (m v⁻¹).

Changes										
Treatment	Concentration	b	d	f	nr	s	sr	ss	sw	w
CV	0,025*						+			+
	0,05*			+			+			+
	0,1*			+		+		+		+
SA	0,025*	+		+		+		+		+
	0,05*						+			
	0,1*			+		+		+		+
EU	0,025*					+				
	0,05*						+			+
	0,1*			+		+		+	+	
OV	0,025*			+	+		+			
	0,05*			+		+		+		+
	0,1*		+	+		+		+		+
TV	0,025*		+	+			+			
	0,05*	+		+		+		+		
	0,1*		+	+		+		+		+
CA	0,025*		+	+		+		+		+
	0,05*			+		+		+		+
	0,1*		+	+		+		+		+
TH	0,025*		+	+		+		+		+
	0,05*	+	+	+		+		+		
	0,1*	+		+	+	+	+	+		

(*) concentrations expressed in % (m v⁻¹). (+) present change; CV – *C. verum* essential oil; SA – *S. aromaticum* essential oil; EU – eugenol; OV – *O. vulgare* essential oil; TV – *T. vulgaris* essential oil; CA – carvacrol; TH – thymol; sw – swelling; b – blebbing; ss – empty spine socks; s – sloughing; w – wrinkle; f – furrowing; nr – spine number reduction; sr – spine size reduction; d – disruption.

Among the changes observed in SEM, the blebbing presence, empty spine socks and sloughing were distinguished by the severity they represent, whose presence may be considered indicative of tegumentary lesion.

Barros et al. (2003) reported that in damaged cells the blebbing presence correlates with imminent cell death, a finding supported by Charras (2008), Fackler and Grosse (2008), and Wickman et al. (2013), which state that apoptosis and necrosis processes are closely linked to the formation of bubbles on the cell surface. Authors such as Saowakon et al. (2009) and Stitt and Fairweather (1993) observed the bubbles formation in the *F. hepatica* specimens integuments submitted to *in vitro* incubation with triclabendazole. Similarly, Meaney et al. (2003) observed this phenomenon occurrence on the *F. hepatica* specimens integument surface recovered from rats treated with an oral dose of clorsulon, whereas McKinstry et al. (2003) obtained the same results, however after treatment with nitroxinil. According to Buchanan et al. (2002) the bubble formation phenomenon has been observed in studies with other helminths and is believed to be an attempt by the parasite to replace the surface membrane damaged by the drug action.

Several authors have verified the spines loss in *F. hepatica* treated with anthelmintic fasciolicides, such as triclabendazole (Saowakon et al. 2009), nitroxinil (McKinstry et al. 2003) and clorsulon (Meaney et al. 2003), reinforcing the premise that the compounds tested had a similar action to that of the mentioned drugs. According to McKinstry et al. (2003) and Toner et al. (2010), the empty spine socks presence indicates the tegumental syncytium destruction or detachment, resulting in the spines joint removal and sometimes basal lamina exposure. This detachment or sloughing, reported by several authors when observing the *in vitro* and *in vivo* anthelmintics, also characterizes an important alteration, whose presence could cause death to the parasite. In this study, the alterations classified as wrinkle, together with the tegumentary absence (sloughing), correspond to what the authors characterize as basal membrane exposure and indicate the complete integument destruction in the injured region.

Thus, the literature findings suggest that treatments with a higher presence of blebbing (b), empty spine socks (ss), sloughing (s) and wrinkle (w) lesions have provoked more severe tegumentary lesions among the others. When analyzed in Table 4 it is observed that the highest number of lesions, being these also more severe, occurred in the concentration of 0.1%. However, when the treatments were evaluated separately, it was possible to conclude that the treatments ORV, THV, CAR and THY resulted in severe alterations also in lower concentrations, allowing to affirm that these compounds had greater action on the parasites tegument in comparison with the controls and other treatments and also to suggest that the major compounds were the main responsible for causing the observed lesions. In addition, similarities between the SEM findings obtained in this study and the findings obtained by other authors when submitting *Fasciola* spp. to the treatment with anthelmintic fasciolicides allows to consider a possible anthelmintic activity of the compounds tested on *F. hepatica*.

Despite the promising results obtained with the *in vitro* test, it is worth noting that further studies are needed to prove the anthelmintic potential of these products, as well as their action and possible use in the fasciolosis control.

CONCLUSIONS

The essential oils and major components had a dose-dependent effect on the *F. hepatica* motility, having the *O. vulgare* and *T. vulgaris* essential oils and the carvacrol and thymol components presenting the best effects in the shortest time in concentration. MEV showed that the highest tegumentary lesions number occurred at a concentration of 0.1% (m v⁻¹) in all treatments, with *O. vulgare* and *T. vulgaris* essential oils, and the carvacrol and thymol components produced severe damage at lower concentrations. The joint analysis of the findings allows to affirm that the major components were the main responsible for the tested essential oils action.

Declarations

ETHICS IN ANIMAL EXPERIMENTATION

This project was developed under the approval of the Committee on Ethics in Animal Experimentation of the Federal University of Espirito Santo under the protocol 13/2016, on July 1, 2016.

CONFLICT OF INTEREST STATEMENT

There were no conflicting interests that could have influenced this study conduction and reporting.

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Figures

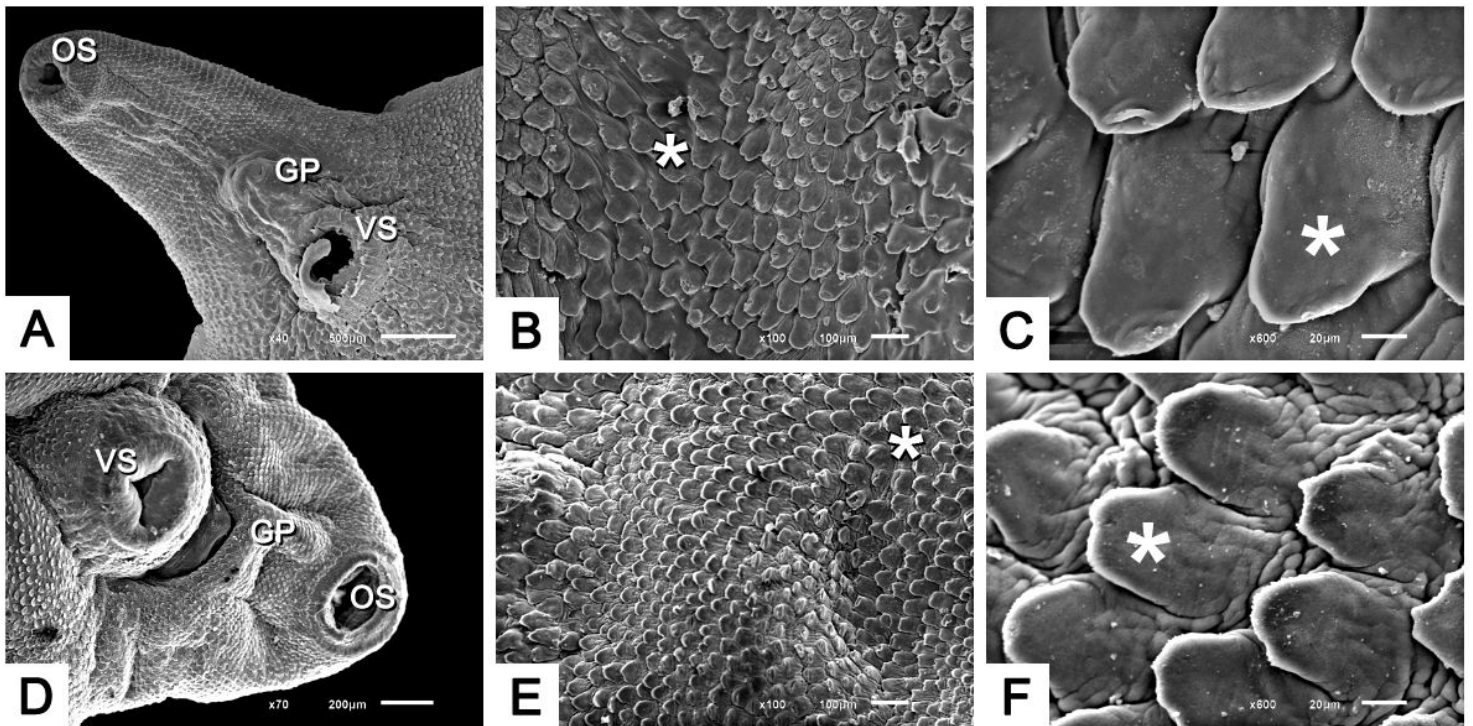


Figure 1

Scanning electron micrographs of the tegumental surface of adult *F. hepatica* specimens after 24 h of *in vitro* exposure. (A-B) Specimen fixed in 10% formalin and untreated; (C-D) Negative control using a mixture of RPMI-1640 medium, dimethyl sulfoxide and tween 80. (E-F) Positive control using 0.5% albendazole sulfoxide. The structures marked correspond to: OS, oral sucker; VS, ventral sucker; GP, genital pore; *, spine. In the images above, it is observed that spines and integument remained intact after the time of exposure.

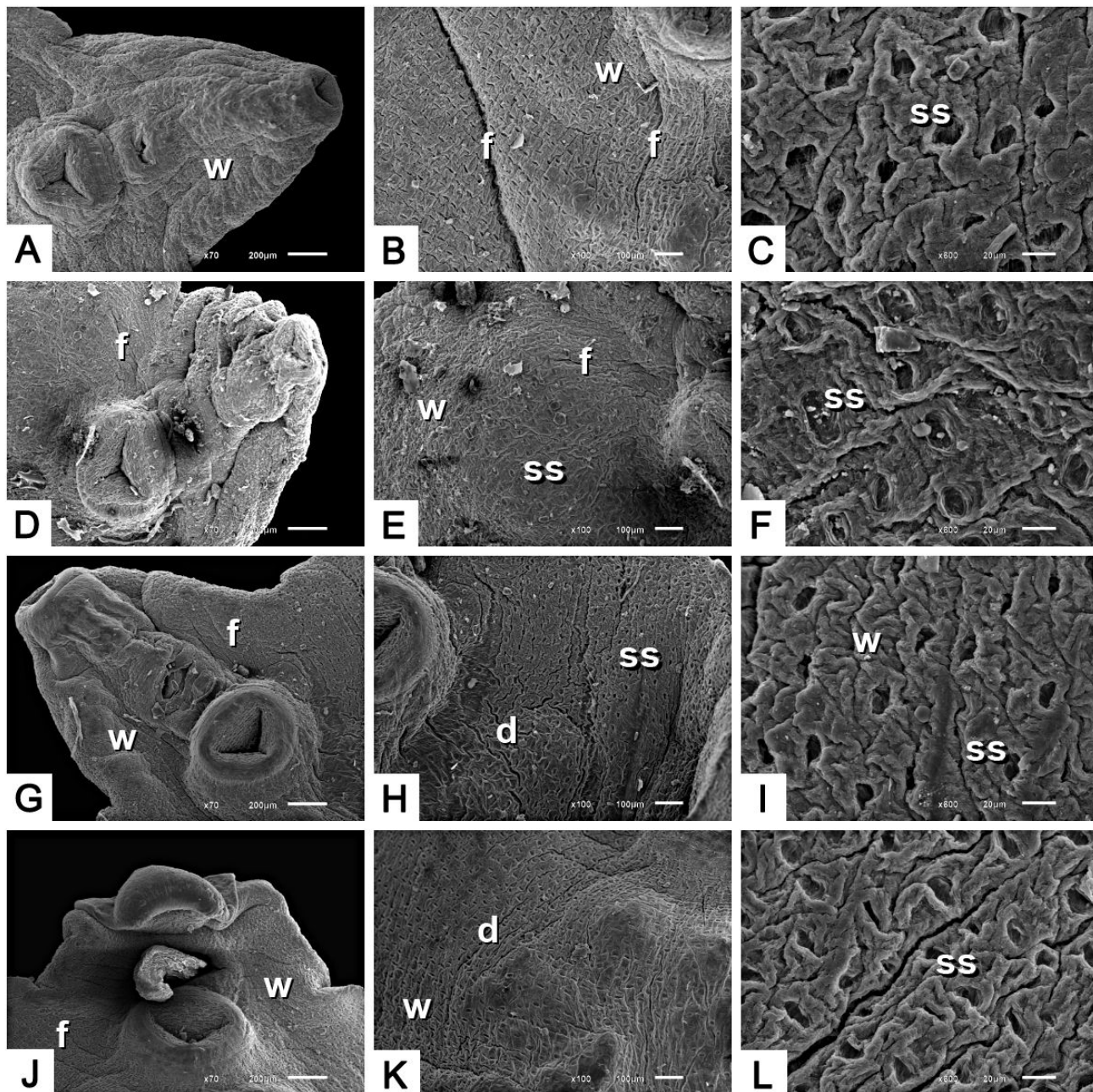


Figure 2

Scanning electron micrographs of the tegumental surface of adult *F. hepatica* specimens after 24 h of *in vitro* exposure. (A-B) *C. verum* (A, B, C); *S. aromaticum* (D, E, F); *O. vulgare* (G, H, I) and *T. vulgaris* (J, K, L) essential oils, all at 0.1% concentration (m v^{-1}). In the images above it is possible to observe significant tegumentary disruption (d), empty spine socks (ss), furrowing (f), sloughing (s) e wrinkle (w).

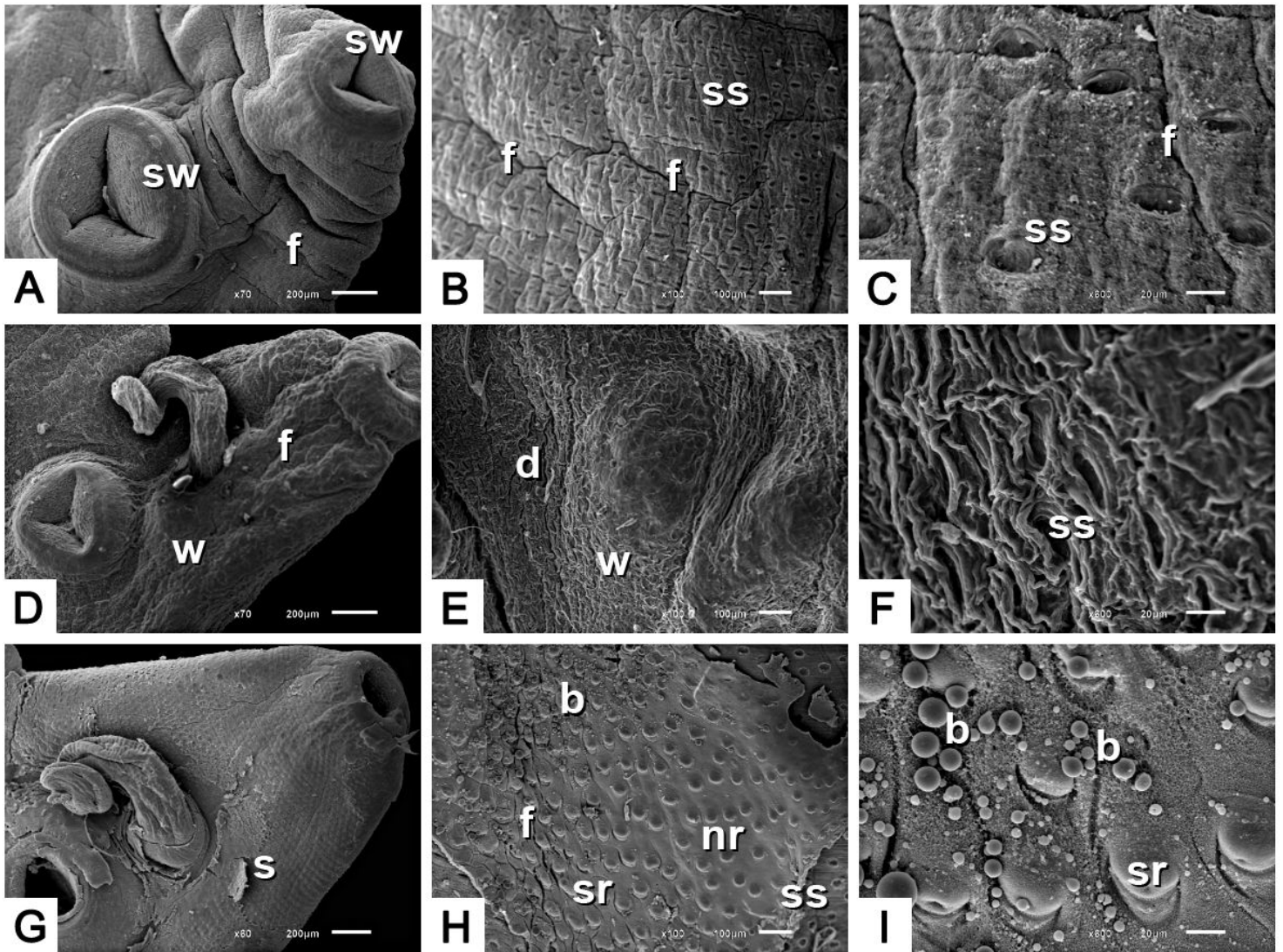


Figure 3

MEV of adult *F. hepatica* specimens after 24 h of *in vitro* exposure to major components eugenol (A, B, C); carvacrol (D, E, F) and thymol (G, H, I), all at the concentration of 0.1% (m v^{-1}). In the images above it is possible to observe significant tegumentary changes such as blebbing (b), disruption (d), empty spine socks (ss), furrowing (f), sloughing spine number reduction (nr), spine size reduction (sr), swelling (sw) and wrinkle (w).

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