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Main Compounds, Antioxidant Activity and Lethal Effect of *Thymus pallescens* Essential Oil on Protoscoleces of Hydatid Cysts

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Abstract

THIS work aimed to investigate the chemical constituents, in-vitro scolicidal, and antioxidant activities of *Thymus pallescens*. Hydrodistillation and gas chromatography with flame ionization detection and gas chromatography-mass spectrometry were used for the extract and determination of compounds in essential oil, respectively. The antioxidant activity of essential oil was assessed by DPPH assay and FRAP assay. Collected aseptically from the sheep livers infected with hydatid cysts slaughtered at the Tiaret abattoir, the protoscoleces used in the present study were washed previously with sterile normal saline. Essential oil scolicidal activity was carried out at the dose (4.55 and 9.1 mg/ml) during 5, 10, and 15 min. An average essential oil yield of 1.87 ± 0.30 % (w/w) was obtained in the present study. Carvacrol (57.31%) was found to be the major compound followed by γ-terpinene (14.09 %) and p-cymene (10.64 %). Moderate antioxidant activity was shown for this essential oil with an IC50 value of 1090.03 ± 153.59 µg/ml and an EC50 value of 1043.51 ± 130.86 µg/ml. All protoscoleces were killed only after 5 min of exposure to the dose of 9.1 mg/ml. Good scolicidal and moderate antioxidant activities of *Thymus pallescens* essential oil was shown in the present study.

Keywords: Antioxidant activity, Carvacrol, Chemical constituents, Essential oil, Scolicidal activity, *Thymus pallescens*.

Introduction

Considered a neglected and potentially fatal parasitic zoonosis [1], cystic echinococcosis (CE) is an extremely endemic cosmopolitan zoonosis, It is especially prevalent in parts of South America, North Africa, China, and the Middle East [2]. It is caused by the larval stages of the taeniid helminth *Echinococcus granulosus* [3]. Dogs and other canids are definitive hosts for the parasite's adult stage, colonizing their intestines [4]. Infection can occur in intermediate hosts (humans, cattle, sheep, pigs, and horses) through ingestion of infectious eggs shed in dog feces through close contact or environmental contamination, resulting in the development of CE-containing protoscoleces (larvae stage) in various parts of the body (liver, lungs…etc.) [5, 6]. In humans, the disease poses a health and economic threat (leading to increased medical costs and risk of death) [1, 7]. In animals, this leads to direct and indirect economic losses such as a decrease in milk production, meat quality, wool, skin, and reproduction [7, 8].

According to the American WHO-IWGE classification [9], treatment of cystic echinococcosis depends on several factors; such as the type of cyst, its size, its location, and the presence/absence of

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complications. The treatment consists of the complete exeresis of the cyst, regardless of its location. If the cyst with all its layers cannot be completely removed, it is necessary to associate protoscolicidal agents to avoid the intraoperative dissemination of fluid rich in protoscolex during surgery and thus avoid the recurrence of cystic echinococcosis [10].

However, many conventional scolicidal agents cause undesirable side effects such as sclerosing cholangitis, hepatic necrosis, and methemoglobinemia [11, 12]. The World Health Organization has reported that discovering a new scolicidal agent that is more effective and produces fewer adverse effects is crucial [13]. Currently, herbs are notable sources of very useful therapeutic agents against many pathological infections [14]. Over the last few decades, many plant species belonging to different families have been used to neutralize the contents of hydatid cysts in vitro, ex-vivo, and in-vivo models [5, 15-18].

One of the most widespread genera in terms of number in the family Lamiaceae is *Thymus* (with more than 350 species growing mainly in the Mediterranean basin and Asia) [19-20]. Among these species, *Thymus pallescens de Noé* (synonym *Thymus fontanesii* Boiss. et Reut.) is a native plant to northern Algeria and is commonly used as a food preservative and in Algerian popular medicine [21]. However, *Thymus* essential oil is a readily accessible natural source of antibiotics, antioxidants, and other medicinal applications, not toxic, and rich in phenolic compounds [22].

Several studies have investigated the scolicidal activity of various *Thymus* essential oils [23-26]. A previous study carried out by Selles et al. [26] determined the chemical composition, scolicidal, and antioxidant activities of the essential oil of *Thymus fontanesii*. The objectives of the present study were to determine the chemical composition and to confirm the scolicidal and antioxidant activities of *Thymus pallescens* collected from another region, namely the Sebaïne locality (Tiaret region, Algeria).

Material and Methods

Extraction of essential oil

Leaves of *Thymus pallescens* were collected in May 2022 from the Sebaïne locality, Tiaret, Algeria (35◦ 27′ 22″ N and 1◦ 36′ 13″ E). The plant was identified by AIT HAMOU (botanist at the Faculty of Life and Natural Sciences, Ibn Khaldoun University, Tiaret, Algeria), and dried in obscurity at room temperature. A mixture of twenty grams of *Thymus pallescens* dry leaves with 500 ml of distilled water was subjected to hydrodistillation for 3 h at the end of extraction the oily phase was recovered and dried by anhydrous sodium sulfate and stored in a refrigerator at 4 C° in a sealed dark glass bottle until its use. The extracted essential oil (1.87 ± 0.30% (w/w)) was calculated by dividing the weight of essential oil obtained during each hydrodistillation by the weight of dry matter used initially..

Analysis of essential oil

The determination of the chemical composition of the essential oil was carried out by Sarl Pyrenessences Analysis (France), according to the ISO 11024 standard using gas chromatography equipped with a flame ionization detector (GC/FID) and by gas chromatography coupled with mass spectrometry (GC-MS).

Collection of protoscoleces

Sheep livers naturally infected by hydatid cysts were collected from the Tiaret municipal slaughterhouse and transferred to the Parasitology Laboratory (Veterinary Sciences Institute, University Ibn Khaldoun of Tiaret). The cyst fluid was aseptically aspirated by a syringe then transferred into the glass cylinders and allowed to settle for 30 min to facilitate the precipitation of protoscoleces. After removing the supernatant, the protoscoleces were washed three times with sterile normal saline (0.9% NaCl) [27]. Finally, a 0.1% eosin staining test was used to assess the viability of protoscoleces (observation under an optical microscope of muscular movements and the impermeability of the protoscolex). Live protoscoleces were stored at 4°C in a dark container containing normal saline and for until use.

Scalicidal activity

Scalicidal effect of Thymus pallescens

To evaluate the scolicidal activity of *Thymus pallescens* EO, concentrations of 4.55 and 9.1 mg/ml were previously prepared separately and dissolved in tween 20/ physiological normal saline solution (1/10 v/v) and then mixed well using a vortex mixer (Dragon Lab MX-S) to ensure good dispersion of the essential oils. In each test tube, half ml of protoscoleces solution and half ml of different concentrations of *Thymus pallescens* EO tested were added. The tubes were mixed gently and incubated for 5, 10, and 15 minutes at 37°C, then removing the supernatant [17]. The mortality rate of protoscoleces was studied by adding 0.5 ml of 0.1% eosin and then incubating for 15 min at room temperature. At the end of this coloring, a drop of the mixture was placed on a slide and the percentage mortality of protoscoleces was examined by counting at least 1300 protoscoleces under a light microscope [28]. In addition, physiological normal saline solution and a mixture tween 20/ physiological normal saline solution were used as control groups. Each tests were performed in triplicate.

Viability

The assessment of the muscle movements of protoscoleces under an optical microscope was carried out according to the method described by

Miman et al. [29] which consists of exposing for 15 min a mixture of a drop rich in protoscoleces with a drop of 0.1% eosin solution. Afterward, a drop of the mixture is placed on a slide covered with a coverslip. Protoscoleces stained red are considered dead, while colorless ones are alive (figure 1).

Antioxidant Activity

DPPH Free Radical Scavenging Activity

The method previously described by Blois [30] was used to evaluate the free radical scavenging activity of various concentrations *Thymus pallescens* EO tested using 1,1-diphenyl-2-picryl hydrazyl (DPPH). One ml of various concentrations of *Thymus pallescens* EO was added to a 200 µl solution of DPPH free radical in ethanol (0.5 mM). A control group (containing all reagents except the tested essential oil) was also prepared. After that, the mixture was shaken and kept in complete darkness for 30 min. The absorbance was read at 517 nm. The scavenging activity was estimated, by using the equation.

$$\text{DPPH I\%} = \left(\frac{\left(A_{control} - A_{sample}\right)}{A_{control}}\right) \times 100$$

Where: $A_{control}$: Control reaction absorbance; A_{sample}: Testing specimen absorbance.

The results are expressed as IC_{50}. All tests were performed in triplicate, and results were expressed as mean values ± standard deviation (SD). VitC and Gallic acid were used as the positive controls.

*Ferric reducing power assay (FRAP***)**

The determination of the ability of EO to reduce Fe^{3+} to Fe^{2+} was performed according to the method previously described by Vijayalakshmi and Ruckmani [31] with some adjustments. This assay consists of incubation at 50°C for 20 min of a mixture containing an equal volume of various concentrations of *Thymus pallescens* EO**,** phosphate buffer (0.2 M, pH 6.6), and $C_6N_6FeK_3$ (1%). At the end of this incubation, 0.5 ml of trichloroacetic acid (10%) was added to the mixture and then centrifuged at 3000 rpm for 10 min. After centrifugation, 1 ml of supernatant was taken and mixed with the same volume of distilled water and 0.5 ml of $FeCl_3$ (0.1 The mixture was allowed at room temperature in the dark for 10 min before measuring the absorbance at 700 nm. The results are expressed as EC_{50}. All tests were performed in triplicate, and results were expressed as mean values ± standard deviation (SD). Ascorbic acid was used as the positive control.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's highly significant difference (HSD) post hoc test was performed by R software (version 4.3.1/2023-06-16) to investigate the differences between the test and control groups.

Results

Chemical composition

GC/MS and GC/FID analysis allowed the identification of one hundred and seven compounds, representing 100% of the total components of *Thymus pallescens* EO. Carvacrol was the major compound of this EO (57.31%). Table 1 summarizes the main compounds of *Thymus pallescens* EO.

Antioxidant activity

Regarding the antioxidant activity, table 2 summarizes the IC_{50} and EC_{50} for *Thymus pallescens* EO and standards tested by DPPH free radical scavenging activity and ferric reducing power.

Scolicidal activity

In the present study, *Thymus pallescens* EO exhibited high scolicidal activity. A 100% mortality rate at the doses of 9.1 mg/ml after only 5 min of exposure was observed (figure 2). However, at a concentration of 4.55 mg/ml, this EO killed the protoscoleces with rates of 20.03 %, 31.48 %, and 67.42 % after 5 min, 10 min, and 15 min of exposure, respectively. Additionally, very highly significant ($P<0.001$) and significant ($P < 0.05$) differences were found between this EO at doses of 9.1 mg/ml and 4.55 mg/ml and controls, respectively.

Discussion

Yield

In the current study, the yield of EO extracted from *Thymus pallescens* dried leaves and produced by simple hydro-distillation ranged from 1.87 ± 0.30% (w:w). A similar result with a rate of 1.7 to 1.9 % was reported by Benchabane et al. [20] for *Thymus pallescens* harvested from the center of northern Algeria and extracted by Clevenger apparatus at different durations of distillations. Likewise, the rates of *Thymus pallescens* EO collected from Ain Defla (Algeria) by steam distillation, and steam diffusion were 1.54±0.07% and 1.50±0.04% respectively [32]. However, the same authors obtained a lower yield (1±0.05%) using the hydro distillation method. The higher yield was mentioned by Hazzit et al. [19], Hazzit et al. [33], and Selles et al. [26], from *Thymus pallescens* collected from Lakhdaria, Oued Rhiou, and Maalab (Algeria) with a yield ranging from 2.06% to 6.2%.

Yield can be influenced by harvest time and stage of development [19]. These authors found that a maximum yield of 4.6% was obtained at the full flowering stage while a minimum yield of around 0.9% was displayed at the start of the vegetative cycle. Additionally, the region and harvest period, type, and duration of distillation can influence yield.

Chemical composition

In the present study, carvacrol is the major component of *Thymus pallescens* EO (57.31 %), followed by γ-terpinene (14.09 %), p-cymene (10.64 %), β-myrcene (2.65 %), limonene (2.09 %), α-terpinene (1.92 %), α-pinene (1.40 %), linalool (1.34 %), and α-thujene (1.28%). While this EO contains a small amount of thymol (0.60 %) (Table 1). A similar composition was reported by Selles et al. [26] who recorded that carvacrol is the main compound of this essential oil followed by γ-terpinene, p-cymene, α-terpinene, linalool, and α-pinene with slight percentage differences. Similarly, these authors recorded little thymol in this EO (0.49 %). However, low rates of β-myrcene (1.56 %), limonene (0.69 %), and α-thujene (0.36 %) were noted by the same authors for this EO. In the current research, the results are in agreement with those carried out by Moutassem et al. [34] which showed that carvacrol was the major component (56.64%) of *Thymus pallescens* EO harvested from various regions of Mascara (Algeria). Similarly, Sadjia et al. [32] and Benchabane et al. [20]showed that carvacol was the main chemical constituent with rates respectively of 79.4 - 86.3 % depending on the extraction method used and from 63.3-68.2 % impacted by the distillation time. Likewise, Hazzit et al. [19] noted that *Thymus pallescens* EO collected from four regions of Algeria (Sidi Aissa, Boussaada, Kadiria, and El-Asnam) were a carvacrol chemotype (44.4–57.7%) followed by p-cymene (10.3–17.3 %) and γ-terpinene (10.8–14.2 %). Contrariwise, the chemical composition of *Thymus pallescens* EO collected from Oued Rhiou (Algeria) was dominated by thymol (49.3 %), while carvacrol only represents a small amount (9.0%) [19].

Figueiredo et al. [35] noted that the geographical location of *Thymus pallescens* is Algeria and Tunisia and that the chemical composition of its essential oil is as follows: Thymol >1-68 %, Carvacrol 9-65 %, γ-terpinene 6-17 %, p-cymene 5-17%, Linalool >1-7 %, α pinene >1-6%.

The difference in *Thymus pallescens* EO composition can be explained by hydrodistillation time [20], method of extraction [36], and part of the plant [19]. In addition, geographic location, season, and moment of harvesting plant affect the composition of essential oil [37-38].

Antioxidant activity

Reducing power and DPPH assay were used to determine the ability of EO to reduce ferric ions (Fe3+) to ferrous ions (Fe2+) and the capacity to scavenge the stable DPPH•. IC_{50} and EC_{50} values of reducing power and DPPH scavenging activity of *Thymus pallescens* EO and standards were summarized in Table 2.

This study showed an IC_{50} of *Thymus pallescens* EO of 1090.03 ± 153.59µg/ml. Higher results were cited by Ziani et al. [39], Benchabane et al. [20], and Hazzit et al. [40] with an IC_{50} of 103g/ml, 149.8 mg/ml, and 410.2 mg/ml, respectively. Several studies have reported low IC_{50} of the essential oil of specimens of *Thymys fontanesii* harvested from various regions of Algeria ranging from 15.58 µg/ml to 346.08 µg/ml [26, 41-42].

In the current investigation, less IC_{50} was recorded by ascorbic acid (4.45 µg/ml) and gallic acid (3.19 µg/ml). A very highly significant difference (P=0.00000752) between standard molecules and *Thymus pallescens* EO was demonstrated.

However, the comparison of the result of the present research with other species of *Thymus* shows a powerful activity of *Thymus vulgaris* with an IC_{50} of 0.19 µg/ml and 0.5 µg/ml, respectively [43-44]. This potent DPPH free radical scavenging activity can be explained by its richness in thymol [45]. Likewise, a strong IC_{50} (0.08 mg/ml) was shown by Zaïri et al. [22] for the *Thymus capitatus* EO carvacrol chemotype. Additionally, He et al. [38] reported a low IC_{50} of *Thymus quinquecostatus celak* collected from different regions of China with a value ranging from 0.512 mg/ml to 0.931 mg/ml.

Our finding recorded higher EC_{50} (1043.51 µg/ml) for *Thymus pallescens* EO comparatively to ascorbic acid (EC_{50} = 23.39 µg/ml). A lower result was noted by Selles et al. [26] with a value of 869.82 µg/ml *Thymus fontanesii* EO. Likewise, low EC_{50} values (63.8 µg/ml to 112.6 µg/ml) of various samples of *Thymus fontanessi* EO were reported by Sid Ali et al. [41]. A higher concentration was found in a study realized by Ziani et al. [39] with an EC_{50} of 63 mg/ml. A highly significant difference (P=0.00187) between standard molecules and *Thymus pallescens* EO was demonstrated.

The high content of this essential oil in phenolic components (carvacrol) explains this good antioxidant activity. Moreover, hydro distillation time [20], method and the solvents used [46], geographic location, radiation exposure [21], the chromatographic profile of the putative antioxidant, and its polarity also have consequences on the antioxidant properties [38, 43].

Scolicidal activity

In our investigation, *Thymus pallescens* EO exhibited strong scolicidal activity at very low concentrations and in a short time. A lethality of 100% at 5 min of exposure was recorded by this essential oil (figure 2). While the dose of 4.55 mg/ml killed 20.03%, 31.48%, and 67.42% after 5 min, 10 min and 15 min of exposure, respectively.

Selles et al. [26] noted that the essential oil of *Thymus fontanesii* possesses a powerful scolicidal activity. Since this EO at the concentration of 9.25

mg/ml kills 100% of protoscoleces after only 5min of exposure and showed a significant difference with the controls ($P < 0.05$). The same authors noticed no significant difference in scolicidal activity at the concentration of 4.625 mg/ml and the controls. Likewise, Hizem et al. [25] recorded that *thymus capitatus* EO exerts a protoscolicidal effect of 100% at doses of 2 and 3 mg/ml after 5 min and 1 min, respectively. Several previous studies report the potent scolicidal activity of various essential oils with carvacrol major compound depending on dose and time. Mahmoudvand et al. [47] mentioned that 10 mg/ml *Nigella sativa* EO kills 100% of protoscoleces after 10 min of exposure. Similarly, at a concentration of 0.05% *Origanum minutiflorum* EO causes mortality of all protoscoleces after 5 min of exposure [48]. Whereas, Soleimani et al. [49] report that *Origanum vulgar* EO at a concentration of 1% requires 15 min to kill all protoscoleces. Likewise, Moazeini et al. [50] found that 1mg/ml of *Satureja hortensisn* EO kills 100% of protoscoleces after 20 min. However, 2 mg/ml of these EOs need 10 min to kill all protoscoleces. Nevertheless, at a dose of more than 17µg/ml, *Zataria multiflora* EO kills protoscoleces after 10 min of exposure [51].

Additionally, previous investigations have shown a powerful scolicidal activity of various EOs but with a longer action time and/or a higher concentration than that observed in the present study [5, 15-16,18, 52-54].

Our study was characterized by low concentration and short time of acting. These criteria are in agreement with the main criteria for an ideal scolicidal agent reported by Moazeni and Larki [55]. This strong scolicidal activity of *Thymus pallescens* EO is due probably to the richness of this essential oil in carvacrol. Previous studies reported that carvacrol has a strong effect against protoscoleces this activity may be linked to the hydroxyl group, which may act as proton translocators [56].

Furthermore, this experiment allowed us to observe morphological changes in dead protoscoleces such as loss of hooks, contraction of soma, and modification in the shape (square shape) (figure 3). Similar morphological changes were shown by Selles et al. [26]. These authors assume that carvacrol (the major compound of this essential oil) is responsible for these modifications.

Conclusion

In conclusion, carvacol is the major compound of *Thymus pallescens* essential oil that has shown good potential as an antioxidant and scolicide. Based on our experimental results, we can suppose that this oil could be used as an alternative scolicide to routinely used chemicals during surgical excision of hydatid cysts. However, further ex vivo and in vivo studies are needed to confirm the use of this essential oil as a scolicidal agent during hydatid cyst surgery.

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Declaration of competing interest

The authors have no conflicts of interest in this study.

Ethical of approval

The samples were collected according to the protocol approved by the scientific committee of the Institute of Veterinary Sciences, Ibn Khaldoun University, Tiaret, Algeria (591/ Vice Rectorate of Third Cycle Higher Education and University Accreditation, Scientific Research and Postgraduate Higher Education / 2021).

TABLE 1. Main compounds of *Thymus pallescens* EO

Compounds	%
Carvacrol	**57.31**
γ-terpinene	14.09
p-cymene	10.64
β-myrcene	2.65
Limonene	2.09
α-terpinene	1.92
α-pinene	1.40
Linalool	1.34
α-thujene	1.28
Thymol	0.60

TABLE 2. Reducing power and DPPH assays of EO

	Reducing power (EC_{50}) µg/ml	DPPH (IC_{50}) µg/ml
Gallic acid	-	3.19 ± 0.41^{a}
Ascorbic acid	23.39 ± 0.81^{a}	4.45 ± 1.05^{a}
***Thymus pallescens* EO**	1043.51 ± 130.86^{b}	1090.03 ± 153.59^{b}

Values are expressed as mean ± standard deviation (n = 3). Values with different letters are statistically different at P <0.05 as measured by one-way analysis of variance (ANOVA)

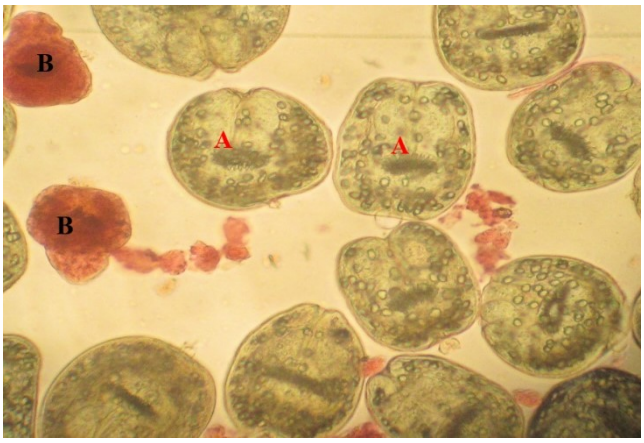

Fig. 1. A: Live protoscoleces; B: Dead protoscoleces of hydatid cysts after staining with 0.1% eosin GX40

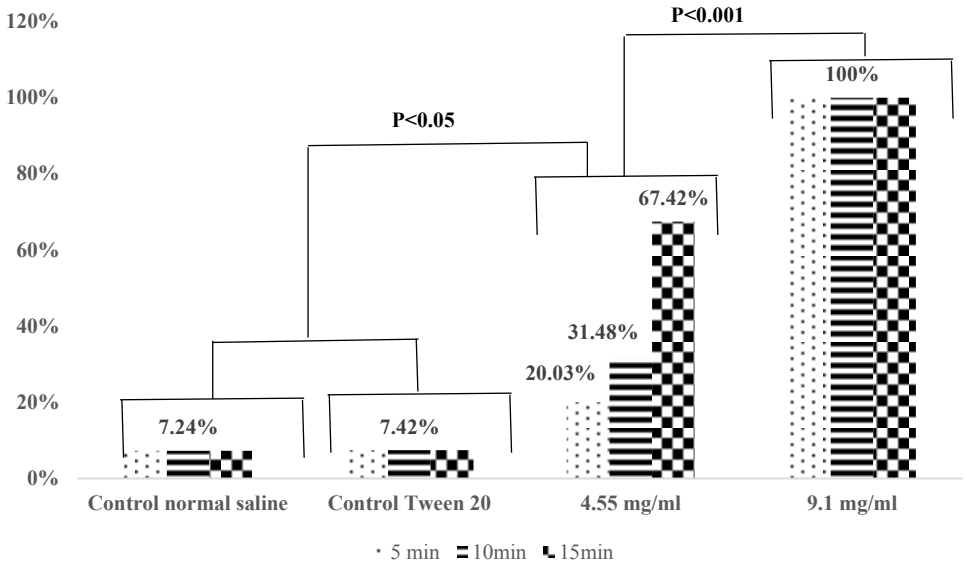

Fig. 2. Scolicidal activity of *Thymus pallescens* EO against protoscoleces.

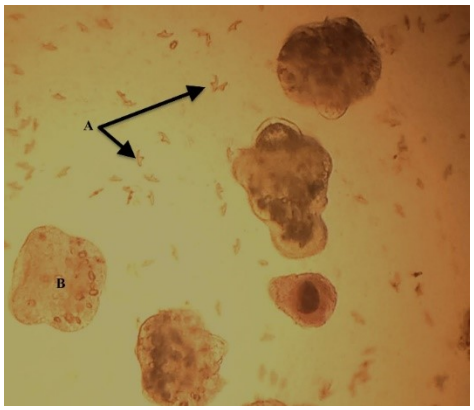

Fig. 3. Some modifications of the protoscolece. A: Hook release. B: Change in the shape of the protoscolece (Squar shape) GX40.

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المركبات الرئيسية ونشاط مضادات الأكسدة والتأثير القاتل لزيت الأساسي الزعتر على الرؤيسات الأولية للكيسات المائية

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الملخص

كان الهدف من هذا البحث هو دراسة المكونات الكيميائي، التأثير القاتل لزيت الأساسي للزعتر على حيوية الرؤيسات الأولية للمشوكة الحبيبية للكيس العذري (دراسة في الزجاج) وكذا أنشطة مضادات الأكسدة. تم استخراج الزيت الأساسي عن طريق التقطير المائي وتحليله بواسطة كروماتوغرافيا الغازالمدمج مع المطيافية الكتلية و كروماتوغرافيا الغازالمدمج مع كاشف التأين باللهب . تم استخدام طريقة DPPH و القوة الاختزالية لتقيم نشاط مضادات للأكسدة بواسطة. تم جمع الرؤيسات الأولية للمشوكة الحبيبية للكيس العذري المستخدمة في الدراسة الحالية بطريقة معقمة من أكباد الأغنام المصابة بأكياس الكيس المائي والتي تم ذبحها في مسلخ تيارت، وتم غسلها مسبقًا بمحلول ملحي عادي معقم. تم تقييم النشاط القاتل لزيت الأساسي للزعتر على حيوية الرؤيسات الأولية من خلال تعريض رواسب البروتوسكولكس لتركيزين من الزيت العطري (4.55، 9.1 ملجم / مل) وضمن جدول زمني لمدة 5، 10 و15 دقيقة. بلغ مردود استخلاص الزيت الأساسي 1.87 ± 0.30% (وزن/وزن). كان الكارفاكرول (57.31%) هو المركب الرئيسي للزيت الأساسي الذي تمت دراسته. أظهر نشاط مضادات الأكسدة بواسطة مقايسة DPPH قيمة IC50 تبلغ 1090.03 ± 153.59 ميكروغرام/مل، بينما أعطى اختبار قدرة الاختزال الحديدي قيمة EC50 تبلغ 1043.51 ± 130.86 ميكروغرام/مل. تم قتل جميع الخلايا الأولية بعد 5 دقائق فقط من التعرض لجرعة 9.1 ملغ/مل. وقد أظهرت الدراسة الحالية أن زيت الزعتر العطري له نشاط جيد في قتل الجراثيم ومضادات الأكسدة المعتدلة.

الكلمات الدالة: المكونات الكيميائي، النشاط القاتل الرؤيسات الأولية، النشاط المضاد للأكسدة، الزيت الاساسي، الزعتر، الكارفاكرول.