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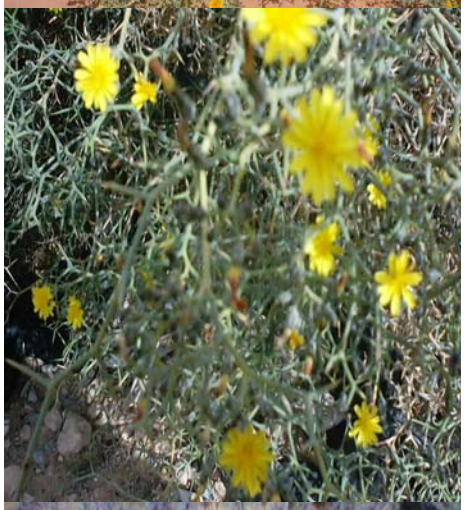

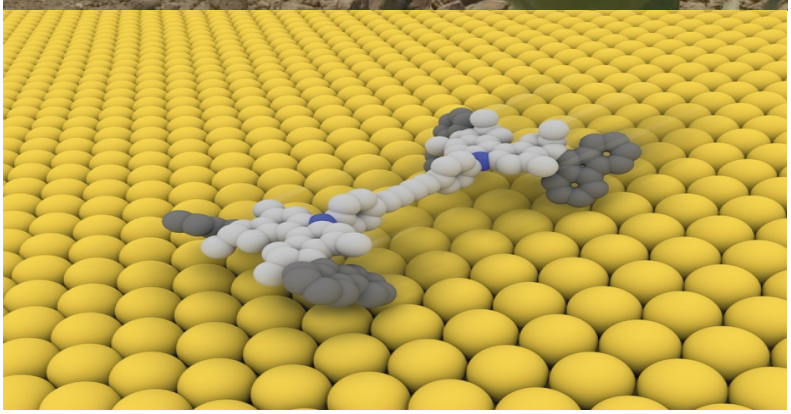

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Chemical composition, acute toxicity, antimicrobial and anti-inflammatory activities of *Thymus fontanesii* essential oil from Algeria

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Abstract *The aim of the present study was to determine the chemical composition and to evaluate the acute toxicity, antimicrobial and anti-inflammatory activities of Thymus fontanesii essential oils (TFEO). The oils were obtained by hydrodistillation from the aerial parts of T. fontanesii at yield of 2.4±0.2%. Using GC and GC/MS techniques, 24 compounds were identified representing more than 98% of the oil composition. The main constituents were carvacrol (54.7±1.2%), p-cymene (17.5±0.3%) and ɣ-terpinene (8.8±0.6%). Using the disc diffusion and broth microdilution methods against six microbial strains, the antimicrobial evaluation showed that TFEO exhibited good antibacterial activity against all the strains tested except Pseudomonas aeruginosa. The acute toxicity test of TFEO was conducted in mice by gavage in single doses of 100-3000 mg/kg. However, the mortality rate as well as the acute toxicity of the oral administered oil increased progressively with increasing dose (LD_{50}=875mg/kg). Anti-inflammatory activity of TFEO was evaluated using carrageenan-induced paw edema in mice. The paw edema was reduced by the TFEO at doses of 50 mg/kg (22.8%) and 100 mg/kg (62.2%). The TFEO was found to possess potent anti-inflammatory activity.Results of the present study indicate that TFEO has a noteworthy potential for the use in pharmaceutical formulations.*

Key Words: *Thymus fontanesii, hydrodistillation, chemical composition, acute toxicity, antimicrobial activity, anti-inflammatory activity*

1. Introduction:

The Lamiaceae family is one of the most employed as world-wide source of spies and a consolidated source of functional ingredients [1], within this family, the genus *Thymus* has received particular attention due to their food related biological properties [2].The genus *Thymus* is one of the most taxonomically complex genera and includes 250-350 species and varieties of wild growing evergreen species of herbaceous perennials and subshrubs, native to Southern Europe, North Africa and Asia [3-5].Among the various biological properties

reported for *Thyme*, some are very well established, such as antioxidant, insecticidal, antibacterial, antifungal, antiviral and anti-inflammatory activities [6-9]. All these activities are related to the high content of monoterpens, phenolic compounds, especially thymol and carvacrol, and of other compounds more or less biologically active including eugenol, p-cymene, γ-terpinene, linalool, germiniol and broneol [10-13]. *Thymusfontanesii* is one of the eleven species presented in the flora of Algeria [14]. This species is a spontaneous aromatic plant endemic to Algeria and Tunisia [15], the aerial parts of *Thymusfontanesii* species have been highly recommended, they were commonly used as herbal teas, condiment and spices, so as for various medicinal purposes [10]. In addition, its oils are used for their antimicrobial [16] and antidermatophytic activities [17]. A lot of papers have been published on the chemical composition and antimicrobial activity of *T. fontanesii* essential oil from Algeria [14, 16, 17], but the toxicity and the anti-inflammatory activity was not been reported before. Therefore, the present paper is aimed to characterize and evaluate the acute toxicity, the antimicrobial and the anti-inflammatory activities of *T. fontanesii* essential oil from Algeria.

2. Materials and methods:

2.1. Plant materials and isolation of essential oil:

The areal parts of *T. fontanesii* were collected during the flowering period (Jun 2014) from Tarik Ibn Ziad (Northwest of Algeria – latitude: 35.993611035°59’36’’N; longitude: 2.1433330 2°8’35’’E; altitude: 630 m; mean annual temperature: 16.4°C; mean annual rainfall: 545 mm). The identification is done by Mr. Benissad botanist at Hamma Botanical Garden (Algiers). A voucher specimen was deposited in the Herbarium of the Agronomic Department of Khemis Miliana University. The dried leaves (50 g) were subjected to hydrodistillation for 2 hours using a Clevenger-type apparatus. The oils were collected and stored at 4°C in the dark. Tests were carried out in triplicate.

2.2. Essential oil analysis:

10 mg of essential oil was dissolved in 5 ml of diethyl ether. The essential oils were analyzed by gas chromatography coupled to a flame ionization detector (GC-FID) and by gas chromatography coupled to a mass spectrometer (GC-MS).

GC-FID analysis:

The analysis of the oil was carried out by means of an Agilent technology HP GC 6890 system with a flame ionization detector (FID), using a capillary column coated with 5 % phenyl-methylsiloxane (30 m x 0.25 mm x 0.25 µm film thickness Agilent Technologies, Hewlett-Packard, CA, USA). Temperature program was as follows: 40°C during1 min, then raised in a first ramp to 200°C at 6°C/min, followed by a second ramp to 280°C at 30°C/min, and finally stayed at 280°C during 2 minutes. Injection was realized in splitless mode at 280°C; the volume injected was 1µL of diluted oil (10 mg of oil/5 mL diethyl ether). Detector temperature was fixed at 300°C; Carrier gas was helium at 1 mL/min.

GC-MS analysis:

GC/MS was performed with an Agilent HP 6890 GC system coupled with an Agilent HP 5973 Network Mass Selective Detector operated by HP Enhanced ChemStation software. Analytical conditions have been fixed as follows: Agilent HP-5MS capillary column (30m × 0.25 mm, df = 0.25 μm), a split-splitless injector at 250°C (splitless mode), temperature program: from 40°-250°C at 6°C/min, mobile phase: carrier gas was helium at 1 mL/min. The mass spectra have been recorded in EI mode (70 eV), scanned mass range: from 35 to 500

amu. Source and quadrupole temperatures were fixed at 230°C and 150°C, respectively. The identification of the components was performed on the basis of chromatographic retention indices and by comparison of the recorded spectra with computed spectral library (Wiley 275.L, Adams 2001). For sesquiterpene hydrocarbons, further confirmations were obtained by comparing the mass spectra with data from the literature [18, 19]. Retention indices (RI) were calculated by means of a mixture of homologue *n*-alkanes (C_7-C_{30}) analyzed under the same chromatographic conditions as for the analysis of essential oils [18].

2.3. Antimicrobial activity:

2.3.1. Microorganisms:

The antimicrobial activity was evaluated using Gram+ bacteria: *Staphylococcus aureus* (ATCC 25923), *Sercina Lutea* (ATCC 13562), *Bacillus Subtilis* (ATCC 6633), Gram- bacteria: *Escherichia coli* (ATCC 25922), *pseudomonas aeruginosa* (ATCC 27853), and the yeast *Candida albicans* (ATCC 2231). All microorganisms were provided from the microbiology laboratory of SAIDAL Antibiotical, Algeria.

2.3.2. Antimicrobial screening:

The antimicrobial activity of TFEO was determined by the disk diffusion method, which is based on the spread of antimicrobial compounds in solid medium [20-22].A suspension of the tested microorganism in log phase (0.1 mL) was spread on Mueller Hinton Agar (MHA) for bacteria and Sabouraud Dextrose Agar (SDA) for yeasts in sterile petri dishes (90 mm diameter). Filter paper discs (9 mm diameter) were individually impregnated with 15 µL of pure EO and placed on the inoculated agar surface. Petri dishes were allowed to stand for 30 minutes at room temperature before incubation at 37°C during 24hours for bacteria and at 25°C during 48hours for yeast. The effect of essential oil was reflected by the appearance around disc with a transparent circular zone corresponding to the absence of growth. The diameter of inhibition zone was measured in mm, whose the larger diameter of the area indicate the more susceptible strain [23].

2.3.3. Determination of the minimum inhibitory concentration (MIC):

The broth microdilutionmethod was used to determine the minimum inhibitory concentration (MIC) described by Moussaoui et al.[21] and Kasrati et al. [24]. The investigated oil was dissolved in 4% dimethylsulphoxide (DMSO). The tests were performed in Muller Hinton Broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for yeast. A fresh overnight culture, in log phase of the tested microorganism was used to prepare the cell suspension adjusted to 1-2 x 10^3 cells/mL for fungal strains and approximately to 10^6 UFC/mL for bacteria, and 100 µL were added to each test tube. Subsequently, 100 µL of each dilution of samples (EO and antibiotic) were added. Negative controls were prepared with the (DMSO) and microorganisms. The test tubes were incubated at 37°C during 24hours for bacteria and 25°C during 48hours for yeast. The MIC was definedasthe lowest concentration of the EO at which the microorganism did not show visible growth. Microbial growth was analyzed by the turbidity of the culture medium.

The same methods were applied for the antibiotic (Amoxicillin) as positive control.

2.4. Acute toxicity:

Acute toxicity studies were carried out using the method described by Tahraoui et al. [25], with slightmodifications. Healthy strain mice of either sex (obtained from SAIDAL Antibiotical, Algeria), weighing between 20 and 25 g were divided in 4 groups of 5 mice.Animals, housed five per plastic cage, were maintained in a room at a temperature of 25

± 0.3°C for 12hours. Mice had free access to tap water and regular rodent food, except for a short fasting period before the treatment with single doses of the oil. The TFEO (dissolved in 50% of Tween 80 + 50% of physiological saline (0.09%) and adjusted to 10 mL/kg per dose) was administered by gavage at doses of 0, 100, 500, 1000 and 3000 mg/kgbody weight. The animals were observed for general behavioral changes, signs of toxicity and mortality continuously for 1hour after treatment, then intermittently for 4hours, and thereafter over a period of 24hours [26]. The mice were further observed for up to 14 days following treatment [27]for behavioral changes and signs of toxicity and/or death, and the latencyof death. The DL_{50} values were determined according to the method of Litchfield and Wilcoxon [28].

2.5. Anti-inflammatory activity:

In this part of the experiment, the anti-inflammatory activity of the TFEO was investigated on carrageenan-induced inflammatory paw edema [29], with some modifications. The EO was dissolvedin 50 % of physiological saline (0.09%) + 50% of Tween 80, and administered by gavage for pretreated group of five mice at doses of 50 and 100 mg/kg. The mixture of physiological saline (0.09%) + Tween 80 was given to the negative group at the same volume. One hour after administration, 0.1 mL of 0.5% carrageenan solution was injected into the right paw of each mouse in all groups. Prior to carrageenan injection, the mice paw volume was measured with a plethysmometer. Increasing of carrageenan induced inflammatory paw volume was measured at 1, 2, 3 and 4 hours over the injection. The anti-inflammatory activity of TFEO was compared with that of 50 mg/kg Diclofenac sodium.
The anti-inflammatory activity was expressed as paw edema rate and inhibition rate of edema in treated mice with regard to control mice was calculated. The paw edema rate and inhibition rate of each group were calculated as follows:

$$\text{Paw edema rate (\%)} = (V_t - V_0)/V_0 \quad (1)$$
$$\text{Inhibition rate (\%)} = (E_c - E_t)/E_c \quad (2)$$

Where Vt is the right paw volume (treated paw) and V_0 is the volume of the left paw (untreated paw); E_c is the paw edema rate of the control group and E_t is the paw edema rate of the treated group [30].

2.6. Statistical analysis:

All experiments were repeated three times and the data were expressed as mean ± standard deviation (SD).

3. Results and discussion:

3.1. Essential oil yield and chemical composition:

The hydrodistillation of the aerial parts of *T.fontanesii*gave yellow oil with a yield of 2.4 ± 0.8 % (w/w), was compared to what has been reported by Ghannadi et al.and Farah et al. [15, 31]Who found the yield of the essential oil from aerial parts of *T.fontanesii* by hydrodistillation to be respectively (1.9% and 2%).
Chemical composition of the volatiles constituents of the essential oil are presented in Table 1. Twenty-four compounds, representing 98.6% of the total essential oil, were identified.The major constituents were carvacrol (54.7 ± 1.2%), p-cymene (17.5 ± 0.3%) and γ-terpinene (8.8 ± 0.6%).The oil consisted mainly in oxygenated monoterpenes (61.6%) and hydrocarbon

monoterpenes (35.8%). Sesquiterpenoids represented only 1.2% of the total composition.Our results are in agreement with on other study [16].
Other previous study has shown different quantitative volatile constituents on Algerian *T. fontanesii* reported the major compounds to be thymol (67.8%), p-cymene (13%) and γ-terpinene (15.9%) with a low concentration of carvacrol (1.7%) [31]. This large variability in the chemical composition of the *T. fontanesii* essential oil could be due that several factors, including local climate and environment (temperature, sun, rain, etc.) of the season, location and nutriments [32], and the intraspecific chemotypes [33].The same constituents were reported in oil composition of many Thymus species samples in different climatic context, such as *T.vulgaris* [34], *T.piperella* [35] and *T.capitatus* [36].

3.2. Antimicrobial activity:

According to disc diffusion results, the inhibition zones of bacteria were 11 ± 0.02 to 36 ± 0.3 mm (Table 2). Maximum activity was observed against *Candida* species (42 ± 0.2 mm) followed by Gram+ bacteria (25 ± 1.07 mm to 36 ± 0.3 mm) and Gram- bacteria (11 ± 0.02 mm to 24 ± 0.5 mm) compared with the antibiotic (Amoxicillin) (14 ± 0.8 to 45 ± 0.03 mm) except *Pseudomonas aeruginosa*, it is notorious for its involvement in nosocomial infections, and seem to be the least sensitive to the action of essential oil with smallest inhibition zone (11 ± 0.02 mm) and to the antibiotic tested (14 ± 0.8 mm). This resistance appears to be the results of the impermeability of the bacterial membranes, and the presence of efflux mechanisms, protecting the bacteria against the action of essential oils [24, 37].
For the broth microdilution method, the essential oil of *T.fontanesii* showed differences in microbial susceptibility. Oil was the most active with MIC values of (0.48 mg/mL to 7.81 mg/mL) (Table 2) with exception of *P. aeruginosa* (15.62 mg/mL), similar susceptibility of this bacterium to the other thyme [22, 24]. The antimicrobial activity of *T. fontanesii* essential oil is in agreement with that reported by Bekhechi et al. [16].

Table 1: Chemical composition of the essential oil of *Thymus fontanesii* from Algeria (mean of triplicates).

Compounds	**RI[a]**	**RI[b]**	**Area (%)**
α-Thujene	930	924	1.2 ± 0.02
α-Pinene	939	930	3.2 ± 1.2
Camphene	954	944	0.2 ± 0.01
β-Pinene	979	973	0.1 ± 0.01
β-Myrcene	991	989	1.5 ± 0.09
α-Phellandrene	1003	1002	0.2 ± 0.01
δ-3 Carene	1011	1008	tr
α-Terpinene	1017	1014	1.7 ± 0.6
p-Cymene	**1025**	**1023**	**17.5 ± 0.3**
Limonene	1029	1027	1.1 ± 0.01
γ-Terpinene	**1060**	**1059**	**8.8 ± 0.6**
cis-Sabinene hydrate	1070	1067	0.3 ± 0.01
Linalool	1097	1100	3.7 ± 0.8
Borneol	1166	1167	0.3 ± 0.02
Terpinen-4-ol	1177	1178	2.2 ± 0.03
Carvacrol methyl ether	1245	1244	0.4 ± 0.02

Thymol	1290	1295	0.3 ± 0.01
Carvacrol	**1299**	**1311**	**54.7 ± 1.2**
α-Gurjunene	1411	1412	0.2 ± 0.09
β-Caryophyllene	1419	1422	0.4 ± 0.02
Aromadendrene	1447	1442	0.2 ± 0.01
allo-Aromadendrene	1460	1464	tr
Spathulenol	1587	1583	0.3 ± 0.04
Caryphylleneoxide	1583	1589	0.1 ± 0.01

tr : traces (< 0.1%)

RI[a]: Retention indices (Adams)

Monoterpenes :	**35.8**
Oxygenated monoterpenes :	**61.6**
Sesquiterpenes :	**0.8**
Oxygenated sesquiterpenes :	**0.4**
Identified compounds :	**98.6**

The high antibacterial activities of TFEO can be attributed to the presence of phenolic compounds, particularly the carvacrol (54.7 ± 1.2%) which is well known for its broad-spectrum antimicrobial activity, effective against bacteria, yeasts and fungi [38-40].
Furthermore, other Thymus oils rich in carvacrol were previously demonstrated to have potent antimicrobial activities in vitro [24, 11]. A synergetic action between carvacrol and other compounds, such as its precursor p-cymene has been previously suggested. Ultee et al. [41] reported that p-cymene is a very weak antibacterial compounds but swells bacterial cell membranes to a greater extent than carvacrol doses.

Table. 2: Microbial inhibitions zones and Minimum Inhibitory Concentrations (MIC) of *Thymus fontanesii* essential oil and antibiotic (Amoxicillin).

	EO		Amoxicillin	
	D (mm)	MIC (mg/mL)	D (mm)	MIC (mg/mL)
Gram-bacteria				
E.coli	24 ± 0.5	1.95	28 ± 0.4	0.48
P.aeruginosa	11 ± 0.02	15.62	14 ± 0.8	3.90
Gram+ bacteria				
S. Lutea	35 ± 0.8	0.48	42 ± 1.3	0.12
B.subtilis	25 ± 1.07	0.97	38 ± 0.9	1.95
S.aureus	36 ± 0.3	7.81	22 ± 1.2	0.12
Yeasts				
C.albicans	42 ± 0.2	0.97	45 ± 0.03	0.06

3.3. Acute toxicity:

After the acute toxicity test, we found that the mortality rate as well as the acute toxicity of the oral administered of TFEO increased progressively with increasing dose compared with control group (Table 3).The mice displayed indications of piloerection and asthenia with significant anorexia and their death became evident at 500 mg/kg BW treatment similar to that found in the mice treated with higher doses (1000 and 3000 mg/kg) while others (such as diarrhea and convulsions) were more pronounced. By contrast, treatment with 100 mg/kg BW did not record any clinical signs and mortality. Therefore, the results indicate that oral administration of TFEO has high acute toxicity in mice, since mortality or clinical signs were observed. The calculated acute toxicity of oral administration of TFEO was DL_{50} = 875 mg/kg. According to the Organisation for Economic Cooperation and Development (OECD), the oil tested falls in class 4 (a substance with oral lethal dose (DL_{50}) is between 300 and 2000 mg/kg), hence considered as toxic oil [42]. The observed toxicity of TFEO could be due to high bioavailability of the toxic compounds [25], more particularly the phenolic compounds, with special emphasis in thymol and carvacrol where these two constituents possess high degree of toxicity[9].

Table 3: Study of acute toxicity of *T.fontanesii* essential oil administered by gavage to mice

.

Doses (mg/Kg)	D/T	Mortality	Toxic symptoms
0	0/5	-	None
100	0/5	-	None
500	1/5	>48h	Anorexia, piloerection, asthenia
1000	3/5	>24h	Anorexia, piloerection, asthenia, diarrhoea
3000	5/5	>12h	Anorexia, syncope, piloerection, asthenia, diarrhoea, convulsions

3.4. Anti-inflammatory activity:

The carrageenan-induced paw edema is a widely used method to evaluate monsteroidal ant-inflammatory activity of diverse bioactive compounds;it is also used in the development of new drugs to assess the effect of pro-inflammatory agents on the acute phase of inflammation [43]. One hour after the carrageenan-induced inflammation, the control group continued to show edema, whereas the groups treated with TFEO at doses of 50 and 100 mg/Kg showed a significant decrease in edema compared to the control group (Table 4).

Table 4: Effect of *T.fontanesii* essential oil and Diclofenac sodium on carrageenan induced paw volume.

	Negative group	Diclofenac sodium	EO	
Time (h)	(Tween 80 + physiological saline)	50 (mg/kg)	50 (mg/kg)	100 (mg/kg)
1	20.8 ± 3.19	13.7 ± 1.01	16.6 ± 1.57	17.2 ± 1.91
2	22.2 ± 2.9	6.8 ± 1.67	17.1 ± 0.75	12.9 ± 1.86
3	20.8 ± 1.1	2.7 ± 0.48	18.7 ± 1.02	9.3 ± 1.62
4	15.2 ± 0.43	0.6 ± 0.02	13.2 ± 0.8	5.7 ± 0.54

Figure 1 showed that oral administration of TFEO at doses of 50 and 100 mg/kg inhibited the edema by (20.2 ± 1.8 and 17.3 ± 2.7 %) respectively during the first hour compared to 33.8 ± 2.1% of the positive control (Diclofenac sodium 50mg/kg) ($P < 0.0001$). In the second, third and fourth hours, the percentage of edema inhibition by 100 mg/kg of TFEO was (41.6 ±

2.4%, 55.2 ± 0.34% and 62.2 ± 2.7%) respectively, in unison the positive control also inhibited the inflammation by (69.9 ± 3.2%, 86.8 ± 1.9% and 96.4 ± 0.3%) ($P < 0.0001$) respectively. Furthermore, the inhibition of paw edema resulting from a 50 mg/kg dose of TFEO was not significantly compared with Diclofenac sodium. This evidence allows us to suggest that the anti-inflammatory actions of TFEO are related to the inhibition of some or more intracellular signaling path ways involved in the effects of several inflammatory mediators [44].

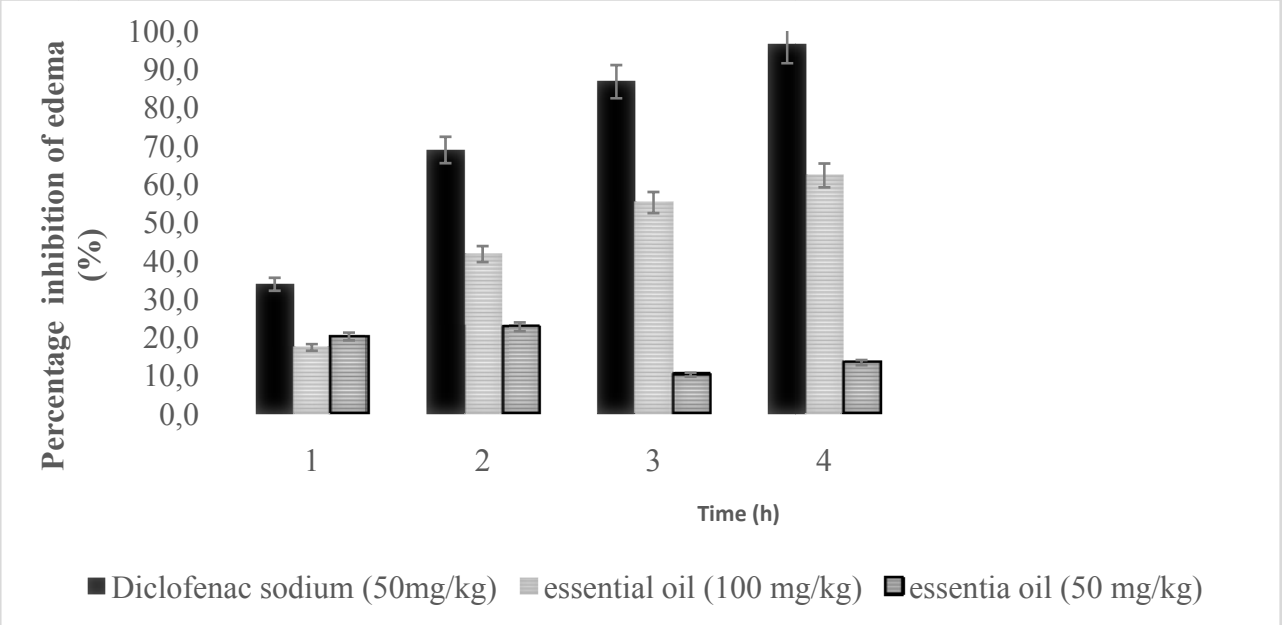

Figure 1: Percentage inhibition of edema by essential oil of *T. fontanesii* at different time

This study showed that oral administration of TFEO produces significant anti-inflammatory effects. Some essential oil compounds, particularly oxygenated monoterpenes (as carvacrol) have been reported to be useful in the management of inflammatory effects [45, 46]. In fact, the anti-inflammatory effect of carvacrol has been demonstrated bay in vitro and in vivo assays[47, 48, 49] Our results are in agreements with those reported in the literature for other EO of Thymus genus rich in oxygenated monoterpenes and showing a very strong anti-inflammatory effect [9, 30, 50]. The present investigation support the use of *T. fontanesii* essential oil for the treatment of various inflammatory disease such as bronchitis and rheumatism.

4. Conclusion:

T. fontanesii essential oil has been evaluated for their chemical composition, toxicity, antimicrobial and anti-inflammatory activities. Carvacrol (54.7 ± 1.2%) was found as a major compound, followed by p-cymene (17.5 ± 0.3%) and γ-terpinene (8.8 ± 0.6%). The oil showed a high antibacterial activity and is able to substantially decrease the MIC values against the most bacteria tested (0.48 mg/mL to 15.62 mg/mL); this activity is due to their phytochemical compounds. In addition, the phytochemical compounds may be involved for the anti-inflammatory activity and protective effect of the TFEO against inflammatory process particularly during the first and fourth hours of inflammation with an inhibition rate varied

from 17.3 ± 2.7 % to 62.2 ± 2.7% for the higher dose (100 mg/Kg), so it could be used for clinical practices. The acute toxicity of the EO administered increased progressively with increasing dose, with a LD_{50} of 875 mg/Kg, hence considered as toxic oil.Therefore, the application of essential oils in the treatment of human infections, which is suggested by traditional medicine, may be an interesting alternative to synthetic drugs.

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