**Revue Agrobiologia**
www.agrobiologia.net
ISSN (Print): 2170-1652
e-ISSN (Online): 2507-7627

# BIOACTIVE COMPOUNDS OF THYME (*THYMUS FONTANESII* BOISS. & REUT.) AND NATIVE SAVORY (*SACCOCALYX SATUREIOIDES* COSS. & DUR.) AGAINST CUTANEOUS LEISHMANIASIS

MOSTEFA SARI Fouzia[1*], ALLEM Rachida[2] and TOUMI Mohamed[3]

1.University of Djillali Bounaama, Faculty of Life and Nature and Ground Sciences, Department of biology, Laboratory of Water, Rocks and Plants, Khemis Milian, Ain Defla, Algeria
2.University of Hassiba Ben Bouali, Faculty of Sciences, Department of Biology, Laboratory of Natural Bioresources, Chlef, Algeria
3.Higher National School, Department of Natural Sciences, Laboratory of Ethnobotanic and Natural Substances, Kouba, Algeria.

*Reçu le 09/12/2019, Révisé le 10/06/2020, Accepté le 16/06/2020*

## Abstract

**Description of the subject:** Leishmaniasis occupies the second position in the list of the most prevalent diseases in the world, and the standard drugs employed in its treatment pose serious side effects for patients.
**Objective:** The aim of this work is to test the viability of *Leishmania major* cells treated by the essential oils and flavonoids extracts of two plants : *Thymus fontanesii* Boiss. & Reut. and *Saccocalyx satureioides* Coss. & Dur.
**Methods:** The essential oils were extracted by hydrodistillation, and their chemical composition were determined by the gas chromatography coupled with mass spectrometry (GC-MS); and flavonoids were dosed by the spectral method. The antiparasitic assay was carried out by *in vitro* culture method in RPMI 1640 medium added with different concentrations of plant extracts.
**Results:** The major compounds of these two oils are: carvacrol (52.138%) and borneol (13.678%). The thyme EO showed a more pronounced leishmanicidal activity on the promastigotes forms of *Leishmania major* $2.21\times10^6\pm0.03$ for the lowest dose of thyme EO (9 µg/ml ) and $1.04\times10^6\pm0.075$ for the highest dose of thyme EO (181 µg/ml ).
**Conclusion:** The results of the antiparasitic tests seem very promising because both plant extracts have acts on the parasitic species tested even in low concentration.
**Keywords:** *Thymus fontanesii* Boiss. & Reut. ; *Saccocalyx satureioides* Coss. & Dur. ; essential oils ; flavonoids ; antileishmania.

# MOLÉCULES BIOACTIVES DU THYM (*THYMUS FONTANESII* BOISS. & REUT.) ET DE LA SARIETTE INDIGÈNE (*SACCOCALYX SATUREIOIDES* COSS. & DUR.) CONTRE LA LEISHMANIOSE CUTANÉE

## Résumé

**Description du sujet:** La leishmaniose occupe la deuxième position dans la liste des maladies les plus répondues dans le monde, et les médicaments employés dans son traitement posent de sérieux effets secondaires.
**Objectifs:** Le présent travail a pour objectifs de tester la viabilité des cellules de *Leishmania major* en présence des huiles essentielles et des extraits flavonoïdiques de deux plantes : *Thymus fontanesii* Boiss. & Reut. et *Saccocalyx satureioides* Coss. & Dur.
**Méthodes:** Les huiles essentielles ont été extraites par hydrodistillation. Leur composition chimique a été déterminée par la chromatographie en phase gazeuse couplée à la spectrométrie de masse CG-SM ; et les flavonoïdes ont été dosés par méthode spectrale. L'essai antiparasitaire a été réalisé par culture *in vitro* en milieu RPMI 1640 additionné de différentes concentrations des extraits végétaux.
**Résultats:** Les feuilles des deux plantes sont riches en flavonoides et en huiles essentielles (HE). Ces dernières sont de chemotype carvacrol (52,138%) et borneol (13,678%). L'HE du thym a montré une activité leishmanicide plus prononcée sur les formes promastigotes de *Leishmania major* ($2,21\times10^6\pm0,03$ pour la dose la plus faible d'HE de thym (9 µg/ml ) et $1,04\times10^6\pm0,075$ pour la dose la plus élevée d'HE de thym (181 µg/ml).
**Conclusion:** Les résultats des tests antiparasitaires semblent très prometteurs car les deux extraits végétaux ont agit sur l'espèce parasitaire testée même à faible concentration.
**Mots clés:** *Thymus fontanesii* Boiss. & Reut. ; *Saccocalyx satureioides* Coss. & Dur. ; Huiles essentielles ; Flavonoïds ; Activité antileishmanienne.

* Auteur correspondant: MOSTEFA SARI Fouzia, E-mail: msarifouzia@yahoo.fr

## INTRODUCTION

Leishmaniasis is a parasitic disease which, according to the clinical manifestations, is defined by three aspects : Visceral leishmaniasis (Black-Sickness), cutaneous leishmaniasis and mucocutaneous leishmaniasis. The causal agent is a parasite belonging to the *Leishmania* genus, which is transmitted by the bite of female sandfly of the phlebotominae family [1]. A patient presenting clinical signs (skin or mucosal lesions) and biological signs (positive parasitological culture) and/or ; for mucocutaneous leishmaniasis only ; positive serology), is considered as a case of cutaneous leishmaniasis. Lesions caused by *Leishmania major* develop most rapidly and are often inflamed or exudative [2]. The World Health Organization (WHO) has considered the leishmaniasis as a tropical affliction which occupies the second position in the list of the six most prevalent diseases in the world [3], Indeed, 12 million people are affected by this disease, among whom 50000 die each year. Statistics showed that there is approximately 2 million new cases of cutaneous leishmaniasis in 70 countries of the world every year [4]. Algeria is classified the first in the Mediterranean region as regards the number of cutaneous leishmaniasis new cases per year (10,000 cases) during years from 2004 till 2008 [5]; it is mainly caused by *L. major* [6]. The standard drugs employed in the treatment pose serious side effects for patients : Atovaquone for example, may cause hepatotoxicity, blood dyscrasias, gastrointestinal and mild neurological side-effects; Dehydroemetin causes weakness, muscular pain, hypotention, precordial pain and cardiac arrhythmias [7]. Nephrotoxicity is the most serious side effect of Amphotericin which is used to treat leishmaniasis [8], but it may cause also cardiac toxicity according to Gil and Rivas [9]. Even though the Glucantime is the first-line treatment for cutaneous leishmaniasis [10], it still causes many side effects when administered in the organism : burning sensation, erythema, pruritus, secondary infection, nausea, vomiting, urticaria, necrosis, sporotrichoid lesions, dizziness, dyspnea and anaphylactic chock [11].

The toxicity exhibited by most of these compounds, and the cost of treatment by many of them has prompted the search for alternative drugs that meet the following conditions : oral administration, fewer side effects, lesser toxicity and lowerprice [12-14].Moreover, some reports have suggested that the *Leishmania* species exhibited different degrees of resistance to conventional drugs, which justifies the ongoing search for leishmanicidal molecules [15]. Natural compounds such as flavonoids have shown promise to reverse multidrug resistant phenotype in *Leishmania* species. It constitutes a well-known class of natural inhibitors of different proteins [16]. The antimicrobial properties of plant volatile oils and other constituents of a wide variety of plants have been evaluated [17-20]. The *Thymus fontanesii* Boiss. & Reut. is one of the most used aromatic plants in the kitchen and for the preservation of dishes, but also for therapeutic purposes against several types of affections or physiological disturbances of the human body systems such as the digestive tract, the respiratory system, the circulatory system, the skin and the nervous system [21-27]. The *Saccocalyx satureioides* Coss. & Dur. is another endemic plant of Algeria which is known for its various therapeutic effects [28,29]. The aim of this work is to extract and quantify the volatile (essential oils) and non-volatile (flavonoids) compounds of two medicinal plants : *Thymus fontanesii* and *Saccocalyx satureioides* as well as to determine the chemical composition of essential oils by gas chromatography-mass spectrometry (GC-MS) analysis. The extraction of essential oils was carried out by hydrodistillation using a Clevenger-type apparatus. The dried leaves of the studied plants have been immersed in distilled water. The hydrodistillation was realised under conditions of temperature (175°C)
and extraction time (2h 30 min) that allow the best yield and good quality of the essential oils. Finally, we tested the viability of *Leishmania* promastigote cells treated by the essential oils and flavonoid extracts.

## MATERIALS AND METHODS

### 1. Chemicals and reagents

Ethyl Acetate, absolute ethanol, sodium carbonate, hydrochloric acid, physiological water, n-butanol, Roswell Park Memorial Institute (RPMI) 1640 medium, heat-inactivated fetal bovine serum (FBS) and fetal calf serum (10%) were provided by Sigma (St. Louis, MO, USA). Nicolle Mac Neal Novy (NNN) medium, Formol, trypan blue, penicillin, aluminum chloride, sodium carbonate, streptomycin, quercetin, Tween 80 and Glucantime® were purchased from Sigma (St. Louis, MO, USA).

### 2. Microbial species (parasite)

The *Leishmania major* species were supplied to us by the Parasitic Eco-epidemiology and population genetics laboratory of the Pasteur Institute of Algeria (Algiers, Algeria). Reference strain of *Leishmania major* MHOM/DZ/2009/LIPA100 was maintained as promastigotes at 26°C in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 µg of streptomycin/mL and 100 U of penicillin/mL.

### 3. Plant material

The *Thymus fontanesii* Boiss. & Reut. was collected from the region of Chlef situated in the northwest of Algeria in May 2015, and *Saccocalyx satureioides* Coss. & Dur. was collected in the region of M'sila situated in the northeast of Algeria in April 2015. The plants were identified according to the identification key of Quezel and Santa [30]; this identification was confirmed by the digital herbarium of France: the Herbarium of the Montpellier University II (MPU). The samples were washed, then dried. The leaves were taken out of the aerial parts to serve for the preparation of extracts.

### 4. Preparation of the plant extracts

#### 4.1. Extraction of flavonoids

The flavonoids were obtained by liquid - liquid extraction according to the method of Lee et *al*. (1995) in Bouchelta et *al*. [31], The plants leaves were powdered, then the organic phase was extracted by using a mixture of distilled water / analytical ethanol (v:v) which is heated at 90°C under reflux during four hours. The extract was filtered through a filter paper (pore diameter of 0.45 µm). Two volumes of analytical n-butanol were added, then acidification of the solution by the HCl (10%) to the pH 3 was done. Then, the butanoic phase was evaporated under reduced pressure. The residue was again dissolved in 2 volumes of the mixture distilled water/ analytical ethyl acetate for one hour. The potential of Hydrogen (pH) of the organic phase was adjusted (pH=9) by addition of sodium carbonate. After 15 minutes of rest, the organic phase was evaporated under reduced pressure, weighed and dissolved by ethanol (1%) for the biological tests.

#### 4.2. Extraction of essential oils

The plant volatile fraction was extracted by hydrodistillation using the Clevenger type apparatus. We have added 25 g of dry leaves and 250 ml of distilled water in a 500 ml flask, then joined the condensing apparatus to the flask, then heating at 175°C for 2 h and 30 min. The choice of temperature and extraction time was made following several preliminary tests and by referring to the results obtained by Rouatbi et *al*. [32]; these conditions are better to improve the yield and chemical quality of essential oil. The recovery of the essential oil is easy and direct in the hydrodistillation method, because the oil accumulates in the bulb above the Clevenger tap, to be directly collected in bottles by opening the tap. The yield of essential oils was calculated using the following formula [33]: Yield (%)=(m/M)×100, Yield (%) : The yield of essential oils (expressed as a percentage).

$m$ : Weight of the extracted oil (g),

$M$ : Weight of plant material (g).

### 5. Determination of flavonoids content

The determination of total flavonoids content was realized by the spectral method described by Jain et *al*. [34] which is based on the evaluation of the colorimetric intensity of the complex resulting from the reaction of the aluminum ion with flavonoids compounds. The plant leaves were powdered then put in methanol (80%). After a centrifugation, 1.5 ml of methanolic extract was added to 1.5 ml of aluminum chloride (2%). After 10 minutes of incubation, we proceed to the reading of absorbance at 415 nm. The flavonoids concentration was determined by referring to a calibration curve using quercetin as standard. The control was prepared in the same way by replacing the extract with methanol.

### 6. GC/MS analysis of Essential oils

The identification of the essential oil constituents was realized by using a gas Chromatographic system (CG) of Shimadzu corporation autosystem type (TQ 8030 model, CAT 225-23,011-44, serial number : 020705106220 J1, Japan), With a capillary column fused-silica RTX-5MS (length: 30 m, diameter: 0.25 mm, thickness: 0.25 µm), equipped with a flame ionization detector (FID). The temperature of the injector and the detector was 220°C and 290°C respectively. The oven temperature was programmed at 40°C during 3 min then increased to 280°C at the rate of 3°C/min. The acquisition duration is 40 min and the vector gas flow (Helium) is 1 ml/min. The sample (essential oil) was diluted (the dilution factor is 1/1000) in the n-hexane, then injected manually in the splitless mode.

The GC/MS is of Shimadzu corporation autosystem type (TQ 8030 model, CAT 225-23,011-44, serial number : 020705106220 J1, Japan), with a capillary column fused-silica RTX-5MS (length: 30 m, diameter : 0.25 mm, thickness : 0.25 µm), equipped with a mass selective detector of (HP 5973) type in the following impact mode: ionization energy : 70 ev. The source and quadripol temperatures are 220°C and 290°C, respectively. We identified the components of essential oils by comparing their retention time to those of the standards of the informatic database, then we determined the contents (in percentage) from the chromatogram peaks surface.

### 7. Preparation of the parasitic inoculum

The inoculum was prepared from *Leishmania* culture on Nicolle Mac Neal (NNN) medium, in the RPMI 1640 medium added of fetal calf serum 10 % and L-glutamine (200 mm) 1 % and penicillin 1000 µg/ml and the streptomycin 100 µg/ml (final pH is equal to 7.2). We adjusted the inoculum to a concentration of $10^6$ parasite / ml.

### 8. Antileishmanial test

The evaluation of antipromastigote effect of essential oil and flavonoid extracts on promastigotes of *L. major* was carried out according to the modified method described by Rahman et *al*. [35] and Mazoir et *al*. [36] : The parasitic inoculum was distributed in the wells of the sterile 96-well cell cultures plates (100 µl per well).

Wells are beforehand filled by various concentrations of the plant extracts prepared in the RPMI 1640 medium supplemented by 10% of fetal bovine serum (essential oils and flavonoid extracts):

9 µg/ml, 36 µg/ml, 72 µg/ml and 181 µg/ml. The controls were realized by replacing the essential oil by tween 80 and the flavonoid extract by the ethanol. After 72 hours of incubation at 26°C, we realized a counting of the promastigotes forms of living *Leishmania* having previously fixed in the formalin and colored in the trypan blue for the parasite viability. Sterile fetal bovine serum (FBS), tween 80 (Solvent) and ethanol (solvent) were used as negative controls, while Glucantime® was used as positive control. The experiments were replicated three times. The concentration that inhibited culture growth by 50% ($IC_{50}$) was determined by dose–response regression analysis, plotted by the Excel stat version 2009 software [37].

### 9. Statistical analysis

Statistical differences were evaluated by the variance analysis (ANOVA) and the Newman and Keuls test at the 5% threshold, using the Statistica version 6.1 software (StatSoft, France).

## RESULTS

### 1. Extraction of flavonoids and essential oils

The leaves of *Thymus fontanesii* contain 84.23±2.26 mg EQ/g of dry sample of flavonoids and 30.86±0.638 mg/g of essential oil (EO) ; *Saccocalyx satureioides* contains lower quantities of flavonoids and essential oil than those reported in thyme, which are 45.28±0.7 mg EQ/g of dry sample and 25.26 ±0.745 mg / g respectively.

### 2. GC/MS analysis of essential oils

The results reported in the table 1 showed that the essential oil of *T. fontanesii* contains a high proportion of monoterpene compounds, mainly the monocyclic monoterpenes (carvacrol 52.138%, gamma-terpinene 4.606%, thymol 1.895%. It also contains bicyclic monoterpene compounds (alpha-pinene 2.634% and beta-pinene 0.755%), and few linear monoterpenes (beta-myrcene : 1.456 %) and bicyclic sesquiterpenes (beta-caryophyllene : 0.216 %, delta-cadinene : 0.085%). According to these results, terpene compounds represent 96.03% of the total identified compounds in the *T. fontanesii* essential oil; Based on the chromatogram profile, the thyme essential oil could be considered as a carvacrol chemotype.

Table 1: Chemical composition of the *T. fontanesii* essential oil

| Compound | % of area | Retention time Rt (min) |
|---|---|---|
| Alpha-thujene | 0.415 | 7.603 |
| Alpha-pinene | 2.634 | 7.994 |
| Camphene | 0.168 | 8.693 |
| Verbenene | 0.050 | 9.006 |
| Beta-pinene | 0.755 | 10.264 |
| Beta-Myrcene | 1.456 | 11.329 |
| Alpha-phellandrene | 0.134 | 12.028 |
| Alpha-terpipene | 1.227 | 12.885 |
| Gamma-Terpinene | 4.606 | 15.917 |
| Terpineol | 0.100 | 16.404 |
| Para cymenyl | 0.159 | 17.845 |
| Linalool | 2.376 | 19.069 |
| Borneol | 0.120 | 23.262 |
| 4-Terpinenol | 0.183 | 24.018 |
| Carvacrol methyl ether | 0.451 | 28.693 |
| Thymol | 1.895 | 33.127 |
| Carvacrol | 52.138 | 34.939 |
| P-Cymen-3-ol | 0.572 | 35.151 |
| Beta-Caryophyllene | 0.216 | 39.952 |
| 6.alpha-Cadina-4,9-diene | 0.052 | 43.542 |
| Ledene | 0.289 | 44.617 |
| Alpha-Amorphene | 0.071 | 45.778 |
| Delta-Cadinene | 0.085 | 46.357 |
| Spathulenol | 0.708 | 49.619 |
| Alpha-Cadinol | 0.101 | 53.186 |
| Adamantane | 0.083 | 67.905 |

***3.* The chemotype of *S. satureiodes***

essential oil is also determined by the same chemical class (terpene compounds) as that of the thyme essential oil except that the majority molecules are not the same ; It is about two constituents: the borneol (13.678%) and the thymol (8.726%) ; These latter are followed by : camphene 3.697%, alpha-pinene 2.430%, gamma-terpinene 1.453% and carvacrol 1.404% (Table 2). This chemical group represents 88.70% of the total identified compounds, and the great majority of this latter are represented by bicyclic monoterpenes (49.348%); followed by monocyclic monoterpenes (32.731%), then the monocyclic sesquiterpenes, bicyclic and tricyclic (6.621%). The essential oil of *S. satureioides* also contains other compounds belonging to various chemical classes but in very small proportion (Table 2).

***4. Antileishmanial test***

A significant difference (Anova, $p < 0.0001$) of the number of viable *Leishmania major* was recorded with different treatments (Fig. 1 and Table 4). The minimum of viable cells was recorded in the presence of the Glucantime® (Control 4), In comparison with the other controls ($p<0.0001$).

This standard antiparasitic was less effective against the studied parasite species with regard to both plants extracts, because the difference is clearly significant ($p<0.0001$) ; Except the group treated with 9 µg/ml of *S. satureiodes* flavonoid extract (SF1), where we noted no significant difference by comparing it with this control ($p=0.813$). However, the lowest dose of the *T. fontanesii* flavonoid extracts showed less efficiency with regard to the control 4 ($p<0.0001$) (Tab. 3). The most remarkable inhibitive effect of the parasitic growth is obtained with the highest *T. fontanesii* essential oil dose (181 µg/ml), followed by the TE3 (72 µg/ml of thyme essential oil) treatment.

According to our results, the essential oils of both plants are endowed with higher antileishmanial effect compared to flavonoid extracts at the same concentration ($p<0.001$). We also noticed that the *S. satureiodes* essential oil is less effective ($IC_{50}$=437.632 µg/ml) against the promastigote forms of tested *Leishmania major*, than the *T. fontanesii* essential oil ($IC_{50}$=268.5 µg/ml) ($p<0.001$) : *p* (TE1 vs SE1)=0.000175, *P* (TE2 vs SE2)=0.00335, *p* (TE3 vs SE3)=0.00503, *p* (TE4 vs SE4)=0.000719.

The same observations were made with flavonoid extracts; indeed, those of *T. fontanesii* gave the best effect ($IC_{50}$=674.367 µg/ml) with regard to those of *S. satureiodes* at the same concentration, with a statistically significant difference ($p$<0.001), the concentration of the latter extract required for 50% inhibition is 781.73 µg/ml. The Newman and Keuls test revealed 8 homogeneous groups, where the group «A» represent the controls CRL1, CRL3 and CRL2 in which the viable *Leishmania major* number is highest. Unlike these groups, the number of *Leishmania major* in control CRL4 represented by the group «C» is lower than that of SE1 (group «B») and similar to that of SF1 (group «C»). The minimum of viable individuals was observed in the groups treated by dose 3 and 4 of thyme essential oil.

Table 2: Chemical composition of the *Saccocalyx satureioides* essential oil

| Compound | % of area | Retention time Rt (min) |
|---|---|---|
| Alpha-thujene | 0.415 | 7.604 |
| Alpha-pinene | 2.430 | 7.980 |
| Camphene | 3.697 | 8.784 |
| Sabinene | 0.154 | 10.110 |
| Beta-pinene | 0.270 | 10.254 |
| Beta-Myrcene | 0.315 | 11.242 |
| Alpha-terpipene | 0.481 | 12.813 |
| Beta-Limonene | 0.811 | 13.777 |
| Gamma-Terpinene | 1.453 | 15.715 |
| Terpineol | 0.302 | 16.356 |
| Alpha-Terpinolene | 0.172 | 17.633 |
| 4-Thujanol | 0.291 | 18.529 |
| Camphor | 0.292 | 21.460 |
| Borneol | 13.678 | 23.999 |
| Bornyl formate | 0.246 | 27.677 |
| Alpha-terpinenyl acetate | 0.333 | 32.723 |
| Thymol | 8.726 | 33.412 |
| Carvacrol | 1.404 | 33.831 |
| Acetyl thymol | 0.342 | 36.193 |
| Copaene | 0.111 | 37.195 |
| alpha-Gurjunene | 0.418 | 39.282 |
| trans-Caryophyllene | 0.570 | 39.908 |
| Alpha-humulene | 0.152 | 41.991 |
| Germacrene B | 0.599 | 44.685 |
| alpha.-Muurolene | 0.170 | 44.993 |
| alpha.-Elemene | 0.098 | 45.971 |
| delta-Cadinene | 0.856 | 46.410 |
| Spathulenol | 0.868 | 49.620 |
| Globulol | 0.387 | 49.912 |
| Veridiflorol | 0.436 | 50.367 |
| Ledol | 0.549 | 50.964 |
| alpha.-Cadinol | 0.550 | 53.215 |
| t-Muurolol | 0.232 | 53.952 |

Figure 1 : Viable *Leishmania major* MHOM/DZ/2009/LIPA100 population number treated by flavonoids and essential oils of *T. fontanesii* and *S. satureioides*

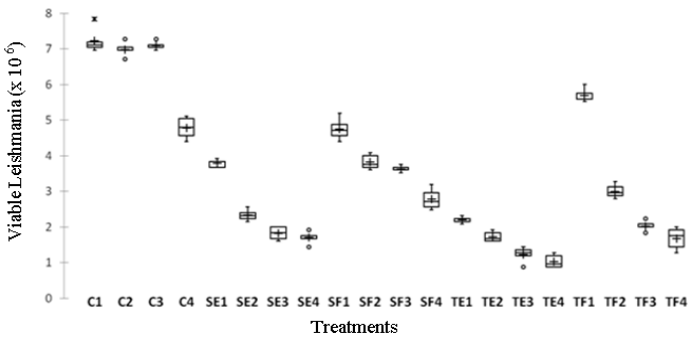

Table 3 : Statistical differences between groups

| Treatments | Probability | Significant |
|---|---|---|
| C1 vs TE | < 0.0001 | Yes |
| C1 vs TF | < 0.0001 | Yes |
| C1 vs SE | < 0.0001 | Yes |
| C1 vs SF | < 0.0001 | Yes |
| C1 vs C4 | 0.002 | yes |
| C1 vs C3 | 0.989 | No |
| C1 vs C2 | 1.000 | No |
| C2 vs TE | < 0.0001 | Yes |
| C2 vs TF | < 0.0001 | Yes |
| C2 vs SE | < 0.0001 | Yes |
| C2 vs SF | < 0.0001 | Yes |
| C2 vs C4 | 0.008 | Yes |
| C2 vs C3 | 1.000 | No |
| C3 vs TE | < 0.0001 | Yes |
| C3 vs TF | < 0.0001 | Yes |
| C3 vs SE | < 0.0001 | Yes |
| C3 vs SF | < 0.0001 | Yes |
| C3 vs C4 | 0.032 | Yes |
| C4 vs TE | < 0.0001 | Yes |
| C4vs TF | < 0.0001 | Yes |
| C4 vs SE | 0.012 | Yes |
| C4 vs SF | 0.355 | No |
| SF vs TE | < 0.0001 | Yes |
| SF vs TF | 0.001 | Yes |
| SF vs SE | 0.378 | No |
| SE vs TE | < 0.0001 | Yes |
| SE vs TF | 0.294 | No |
| TEF vs TE | 0.081 | No |

Table 4: Number of viable *Leishmania major* treated by *T. fontanesii* and *S. satureioides* extracts (expressed as means ± SEM)

| Treatments | Controls | | | | Plant extracts (µg/ml) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | | 9 | 36 | 72 | 181 |
| Number of promastigotes in millions (Mean ± SEM) | 7.23± 0.14 | 6.99± 0.08** | 7.10± 0.047** | 4.78± 0.12* | TE | 2.21± 0.03* | 1.73± 0.058* | 1.23± 0.084* | 1.04± 0.075* |
| | | | | | TF | 5.70± 0.076* | 3.01± 0.07* | 2.03± 0.058 * | 1.68± 0.123* |
| | | | | | SE | 3.79± 0.042* | 2.34± 0.061* | 1.82± 0.072 * | 1.71± 0.070* |
| | | | | | SF | 4.75± 0.123*, ## | 3.82± 0.082* | 3.63± 0.036* | 2.78± 0.118* |

C1 : Inoculum of *Leishmania* added of RPMI and FBS, C2 : Inoculum of *Leishmania* added of RPMI and FBS and Tween 80, C3 : Inoculum of *Leishmania* added of RPMI and FBS and Ethanol, C4 : Inoculum of *Leishmania* added of RPMI and FBS and Glucantime® , TE : *T. fontanesii* essential oils , TF : *T. fontanesii* flavonoid extract, SE : *S. satureioides* essential oil, SF : *S. satureiodes* flavonoid extract, SEM : Standard error of mean. * : $p < 0.001$ significant from control 1, ** : $p > 0.05$ Non significant from control 1, ## : $p > 0.05$ Non significant from control 4.

## DISCUSSION

In the light of the obtained results, we can say that both plants essential oil and flavonoid extracts have an important leishmanicidal effect at low concentration (9 µg/ml). Our results are more promising than those observed by Mikus et *al*. [38], which evaluated the effect of different essential oils as well as of isolated mono- and sesquiterpenes on the viability of *Leishmania major* promastigotes. As demonstrated by this study, the inhibition of promastigote *L. major* proliferation is dose-dependent. Similar observation was made by Bosquiroli et *al*. [39] who studied the inhibitive effect of *Piper angustifolium* essential oil on the intracellular proliferation of the *L. Infantum* amastigote forms in peritoneal macrophages from BALB/C mice. Various studies (*in vivo* and *in vitro* tests) have contributed to the identification of some very active natural compounds against leishmaniasis disease [40, 41]. According to Bauri et *al*. [42], The phenolic compounds, such as the thymol and the carvacrol and the borneol, are very effective against a multitude of parasites; they act on the oxidative phosphorylation mechanism thus preventing the energy generation, and also affect the glycoproteins of the parasites cell surface, and cause consequently their death. Based on the results of the chromatographic analysis of both studied plants essential oil (*T. fontanesii* and *S. satureioides*), we can say that this high antiparasitic activity is explained by their high carvacrol, thymol, borneol and d-terpinene contents [43]. Youssefi et *al*. [44], evaluated the toxicity of carvacrol, thymol and linalool on amastigotes and promastigotes of *L. infantum* by *in vitro* and *in vivo* Methods; their assay showed promising antileishmanial activity of thymol and carvacrol, with IC50 values of 7.2 (48 µM) and 9.8 µg/mL (65 µM), respectively; while linalool at all concentrations did not affect *L. infantum* promastigote viability [44]. The synergistic effect of carvacrol is supported by previous studies where this monoterpene increased the leishmanicidal activity of ascaridole [45]. Moreover, components present in low concentrations can act as synergists to improve the efficiency of the main constituents of the essential oils through a variety of mechanisms[46]. To test compound interactions, the activity of mixtures of the major EO components in binary combinations were evaluated. Mixtures were assayed at final concentrations of 100 and 10 µg/mL containing 50% of each compound. For comparison purposes, the expected activity was calculated as the sum of the activities obtained separately by the compounds of the mixture at the concentration present in the mixture. Nine of the binary combinations tested at 100 µg/mL resulted antagonistic, while at a concentration of 10 µg/mL seven combinations were synergistic. Among the synergistic, four included carvacrol and four terpinene [43]. Other studies were carried out to estimate the extracts leishmanicide activity of plants belonging to various families [47-49]. Fournet et *al.* [20], collected the results of their studies and those of the other researchers who tested different phytochemical extracts of more than 50 Bolivian medicinal plants species, on three *Leishmania* species agent of cutaneous leishmaniasis in Bolivia: *Leishmania amazonensis*, *L. braziliensis* and *L. donovani* . Based on their finding, the majority of the tested extracts did not show antileishmania activity at the concentration of 100 µg/ml. According to García et *al*. [50], the organic extracts (Ethanol, hexan, chloroform, butanol and ethyl acetate) of three species belonging to *Pluchea* genus (*P. carolinensis*, *P. odorata* and *P. rosea*) of the Asteraceae family, were efficient against *L. amazonensis* at 20 mg/ml. This dose appears clearly higher than those tested in our experiments. Therefore, both plants extracts tested in our study have most important Leishmanicidal effect compared to *Pluchea*; our findings reinforce the immense value of the Lamiaceae family and the *Thymus* and *Saccocalyx* genus.

## CONCLUSION

Our study demonstrated the effectiveness of the plant species extracts tested. Thus, the bioactive molecules contained in these extracts could be used as an active ingredient in the formulation of a new semi-synthetic drug for the treatment of cutaneous leishmaniasis. Research remains to be developed, mainly on the type of pharmaceutical preparations, methods of administration, stability of the final product ; and *in vivo* tests must be performed to determine some parameters related to the product such as the half-life of the active ingredients, effective and toxic doses, and the treatment duration.

## FUNDINGS

This work was funded by Djillali Bounaama University of Khemis Miliana (Ain Defla), and Institut Pasteur d'Algérie (Algeria).

## REFERENCES

**[1]. Gupte S. (2016).** *The short textbook of paediatrics, The Health science*, 12th ed., Jaypee Brothers medical LTD, page 386, New delhi, London, Philadelphia.

**[2]. Berger S. (2016).** *Infectious deseases of Germany*, Gideon, Los Angeles, California, USA.

**[3]. Kumar A. (2013).** *Leishmania* and Leishmaniasis, Springer sciences and business media, pages 1-4, New york.

**[4]. Kobets T., Grekov I. & Lipoldová M. (2012).** Leishmaniasis: Prevention, Parasite Detection and Treatment. *Curr Med Chem*. 1 : 1443-1474.

**[5]. Bounoua L., Kahime K., Houti L., Blakey T., Ebi K.L., Zhang P., Imhoff M. L., Thome K. J., Dudek C. , Sahabi S. A., Messouli M., Makhlouf B., ElLaamrani A. and Boumezzough A. (2013).** Linking Climate to Incidence of Zoonotic Cutaneous Leishmaniasis (*L. major*) in Pre-Saharan North Africa. *Int. J. Env. Res. Public He*. 10 : 3172-3191.

**[6]. Harrat Z., Pratlong F., Dereure J., Daniau M., Rioux J.A., Belkaid M. and Dedet J.P. (1996).** *Leishmania infantum* and *L. major* in Algeria. *T Roy Soc Trop Med Hyg*. 90 : 625-629.

**[7]. Bennett P.N. and Brown M.J. (2007).** *Clinical pharmacology e-book: with student consult access*, 10th ed, Elsevier health sciences, page 246, Spain.

**[8]. Walker R. and Whittlesea C. (2011).** *Clinical Pharmacy and Therapeutics*, 5th ed, Elsevier Health Sciences, Page 662, London.

**[9]. Gil C. and Rivas L. (2018).** Drug Discovery for Leishmaniasis, *Royal Society of Chemistry*, 60: 17-18.

**[10]. Katsambas A.D., Lotti T.M., Dessinioti C. and D'Erme A.M. (2015).** *European Handbook of Dermatological Treatments*, 3rd ed, Springer-Verlag Berlin Heidelberg, page 498, London.

**[11]. Acton Q.A. (2013).** *Cutaneous Leishmaniasis*: New Insights for the Healthcare Professional, Scholarly, page : 15, USA.

**[12]. Murray H.W. (2001).** Clinical and Experimental Advances in Treatment of Visceral Leishmaniasis Antimicrob. *Agents Chemother*. 45: 2185–2197.

**[13]. Melby P. (2002).** Recent developments in leishmaniasis. *Curr Opin Infect Dis*. 15 : 485-490.

**[14]. Palumbo E. (2009).** Current treatment for cutaneous leishmaniasis: A review. *Am J Ther*. 16:178-182.

**[15]. Rai M.K. and Kon K.V. (2013).** *Fighting Multidrug Resistance with Herbal Extracts, Essentiel Oils and Their Components*, First ed., Elsevier, pages 95-107, London.

**[16]. Boumendjel A., Boutonnat J. and Robert J. (2009).** ABC Transporters and Multidrug Resistance, Drug discovery and developpement, Volume 11, John Wiley & Sons, page 206, New jersey.

**[17]. Dorman H.J.D. and Deans S.G. (2000).** Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl Microbiol*. 88: 308–316.

**[18]. Schnitzler P., Koch C. and Reichling J. (2007).** Susceptibility of Drug-Resistant Clinical Herpes Simplex Virus Type 1Strains to Essential Oils of Ginger, Thyme, Hyssop, and Sandalwood. American Society for Microbiology, *Antimicrob Agents Ch*. 51: 1859–1862.

**[19]. Molina-Garza Z.J., Bazaldua-Rodriguez A.F., Quintanilla-Licea R. and Galaviz-Silva L. (2014).** Anti-Trypanosoma cruzi activity of 10 medicinal plants used in northeast Mexico. *Acta Trop*. 136: 14–18.

**[20]. Fournet A., Angelo Barrios A. and Munoz V. (1994).** Leishmanicidal and trypanocidal activities of Bolivian medicinal plants. *J. Ethnopharmacol*. 41 : 19-37.

**[21]. Wang L., Waltenberger B., Pferschy-Wenzig E-M., Blunder M., Liu X., Malainer C., Blazevic T., Schwaiger S., Rollinger J.M., Heiss E.H., Schuster D., Kopp B., Bauer R., Stuppner H., Dirsch V.M. and Atanasov A.G. (2014).** Natural product agonists of peroxisome proliferator-activated receptor gamma (PPARg): a review. *Biochem Pharmacol*. 92: 73–89.

**[22] . Bounatirou S., Smiti S., Miguel M.G., Faleiro L., Rejeb M.N., Neffati M., Figueiredo A.C., Barroso J.G. and Pedro L.G. (2007).** Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus Hoff.* et *Link. J. Food Chem*. 105: 146–155.

**[23]. Juhas S., Bujnakova D., Rehak P., Cikos S., Czikkova S., Vesela J., Ilkova G. and Koppel J. (2008).** Anti-Inflammatory Effects of Thyme Essential Oil in Mice. *Acta vet. Brno*. 77 : 327–334.

**[24]. Juliano C., Mattana A. and Usai M., (2000).** Composition and *in vitro* antimicrobial activity of the essential oil of Thymus berba-barona Loisel growing wild in Sardinia. *J. Essent Oil Res*. 12 : 516–522.

**[25]. Duke J. A., Bogenschutz-Godwin M. J., Cellier J. and Duke P.A. (2002).** *Handbook of Medicinal Herbs*, Second ed., CRC Press LLC, pages 730-731, London New York Washington.

**[26]. Aouati K.S., Mebarki N., Ayadi A., Chader H., Nabiev M. and Mansouri M. (2011).** Evaluation of the antidermatophytic activity of a Pasty formulation with the *Thymus fontanesii* Boiss and Reut. essential oil. *Ann Dermatol Vener*. 138, Supplement, A193.

**[27]. Bekhechi C., Atik Bekkara F., Abdelouahid D.E., Tomi F. and Casanova J. (2007).** Composition and Antibacterial Activity of the Essential Oil of *Thymus fontanesii* Boiss. and Reut. from Algeria. *J. Essent oil Res*. 19: 594-596.

**[28]. Zerroug M.M., Laouer H., Strange R.N. and Nicklin J. (2011).** The Effect of Essential Oil of *Saccocalyx Satureioides* Coss. et Dur. On the Growth of and the Production of Solanapyrone a by *Ascochyta rabiei* (Pass.) Labr. *Adv in Environ Biol*. 5: 501-506.

**[29]. Bendahou M., Benyoucef M., Muselli A., Desjobert J.M., Paolini J., Bernardini A.F. and Costa J. (2008).** Antimicrobial Activity and Chemical Composition of *Saccocalyx satureioides* Coss. and Dur. Essential Oil and Extract Obtained by Microwave Extraction. Comparison with Hydrodistillation. *J. Essent oil Res*. 20: 174-187.

**[30] . Quezel P., Santa S. (1963).** *Nouvelle flore d'Algérie et des regions désertiques méridionales*, Tome II, Centre National de la Recherche Scientifique(CNRC), pages 804- 807, Paris 7 France.

**[31] . Bouchelta A., Boughdad A. and Blenzar A. (2005).** Effet biocide des alcaloides, des saponines et des flavonoïdes des extraits de *Capsicum frutescens* L. (Solanaceae) sur *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae). *Biotechnol. Agron. Soc. Environ*. 9: 259-269.

**[32]. Rouatbi. M., Duquenoy. A., Giampaoli. P. (2007).** Extraction of the essential oil of thyme and black pepper by superheated steam, *J. Food Eng*. 78 : 708–714.

**[33]. Elyemni M., Louaste B., Nechad I., Elkamli T., Bouia A., Taleb M., Chaouch M. and Eloutassi N. (2019).** Extraction of Essential Oils of *Rosmarinus officinalis* L. by Two Different Methods: Hydrodistillation and Microwave Assisted Hydrodistillation. *Sci. World J*. 6 pages.

**[34]. Jain S., Jain A., Sharma P., Singh Solanki S. and Paliwal P. (2012).** Phytochemical Investigation and Antioxidant Properties of leaves of Daemia extensa Containing Phenolic and Flavanoid Compounds. *J. Nat. Prod. Plant Resour*. 2: 281-287.

**[35]. Rahman.A-U., Choudhary. M.I. and Thomson.W.J. (2005).** *Bioassay Techniques for Drug Development*, Taylor & Francis , pages : 55, Amsterdam.

**[36]. Mazoir N., Benharref A., Bailen M., Rein M., Gonzalez-Coloma A., Rafael A. and Martinez-Diaz R.A. (2011).** Antileishmanial and Antitrypanosomal Activity of Triterpene Derivatives from Latex of Two Euphorbia Species. *Verlag. Z. Naturforsch*. 66 : 360-366.

**[37]. Machado M., Santoro G., Sousa M. C., Salgueiro L. and Cavaleiro C. (2010).** Activity of essential oils on the growth of *Leishmania infantum* promastigotes. *Flavour and Fragrance Journal*, 25(3), 156-160.

**[38]. Mikus J., Steverding D., Harkenthal M. and Reichling J. (2000).** *In vitro* Effect of Essential Oils and Isolated Mono- and Sesquiterpenes on *Leishmania major* and *Trypanosoma brucei*. *Planta Medica*. 66:366-373.

**[39]. Bosquiroli L.S.S., Demarque D.P., Rizk Y.S., Cunha M.C., Marques M.C.S., Matos M.F.C., Kadri M.C.T., Carollo C.A. and Arruda C.C.P. (2015).** *In vitro* anti-*Leishmania infantum* activity of essential oil from *Piper angustifolium*. *Rev Bras de Farmacogn*. 25 : 124-128.

**[40]. Torres-Santos E.C., Moreira D.L., Kaplan M.A.C., Meirelles M.N. and Rossi-Bergmann B. (1999).** Selective effect of 2',6'-dihydroxy-4'methoxychalcone isolated from Piper aduncum on *Leishmania amazonensis*. *Antimicrob Agents Ch*. 43 : 1234–1241.

**[41]. Muzitano M.F., Tinoco L.W., Guette C., Kaiser C.R., Rossi-Bergmann B. and Costa S.S. (2006).** The antileishmanial activity assessment of unusual flavonoids from Kalanchoe pinnata. *Phytochemistry*. 67: 2071–2077.

**[42]. Bauri R.k., Tigga M.N. and Saleeb Kullu S. (2015).** A review on use of medicinal plants to control parasites. *Indian J. Nat Prod and Resour*. 6: 268-277.

**[43]. Guardo NI, Sainz P, González-Coloma A, Burillo J. and Martinez-Díaz RA. (2017).** Trypanocidal effects of Essential Oils from Selected Medicinal Plants. Synergy among the Main Components. *Nat. Prod. Commun*. 12 :709-712.

**[44]. Youssefi M.R., Moghaddas E., Tabari M.A., Moghadamnia A.A., Hosseini S.M., Farash B.R.H., Ebrahimi M.A., Mousavi N.N., Fata A., Maggi F., Petrelli R. , Dall'Acqua S., Benelli G. and Sut S. (2019)**. *In Vitro* and *In Vivo* effectiveness of Carvacrol, Thymol and Linalool against *Leishmania infantum. Molecules*. 24: 2072-2082.

**[45]. Pastor J, García M, Steinbauer S, Setzer WN, Scull R, Gille L. and Monzote L. (2015)**. Combinartions of ascaridole, carvacrol and caryophyllene oxide against *Leishmania*. *Acta Tropica*, 145, 31-38.

**[46]. Neiro LS, Olivero- Verbel HJ. and Stashenko E. (2010)**. Repellent activity of essential oils: A review. *Bioresource Technology*, 101: 372-378.

**[47]. Maldonado E.M., Salamanca E., Giménez A. and Sterner O. (2016).** Antileishmanial metabolites from Lantana balansae. *Rev Bras Farmacogn*. 26 : 180-183.

**[48]. Grecco S.D.S., Reimão J.Q., Tempone A.G., Sartorelli P., Cunha R. L.O.R., Romoff P., Ferreira M.J.P., Fávero O.A. and Lago J.H.G. (2012).** *In vitro* antileishmanial and antitrypanosomal activities of flavanones from *Baccharis retusa* DC. (Asteraceae). *Exp Parasitol*. 130 : 141–145.

**[49]. Bahmani M., Saki K., Ezatpour B., Shahsavari S., Eftekhari Z., Jelodari M., Rafieian-Kopaei M. and Sepahvand R. (2015).** Leishmaniosis phytotherapy: Review of plants used in Iranian traditional medicine on leishmaniasis. *Asian Pac J. Trop Biomed.* 5 : 695–701.

**[50]. Garcia M., Perera W.H., Scull R. and Monzote L. (2011).** Antileishmanial assessment of leaf extracts from *Pluchea carolinensis*, *Pluchea odorata* and *Pluchea rosea*. *Asian Pac J. Trop Med*. 7: 836-840.