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Research Article

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Powerful anti-inflammatory, anti-Herpes and anticancer capacities of *Thymelaea microphylla* L. and TLC phenolic identification

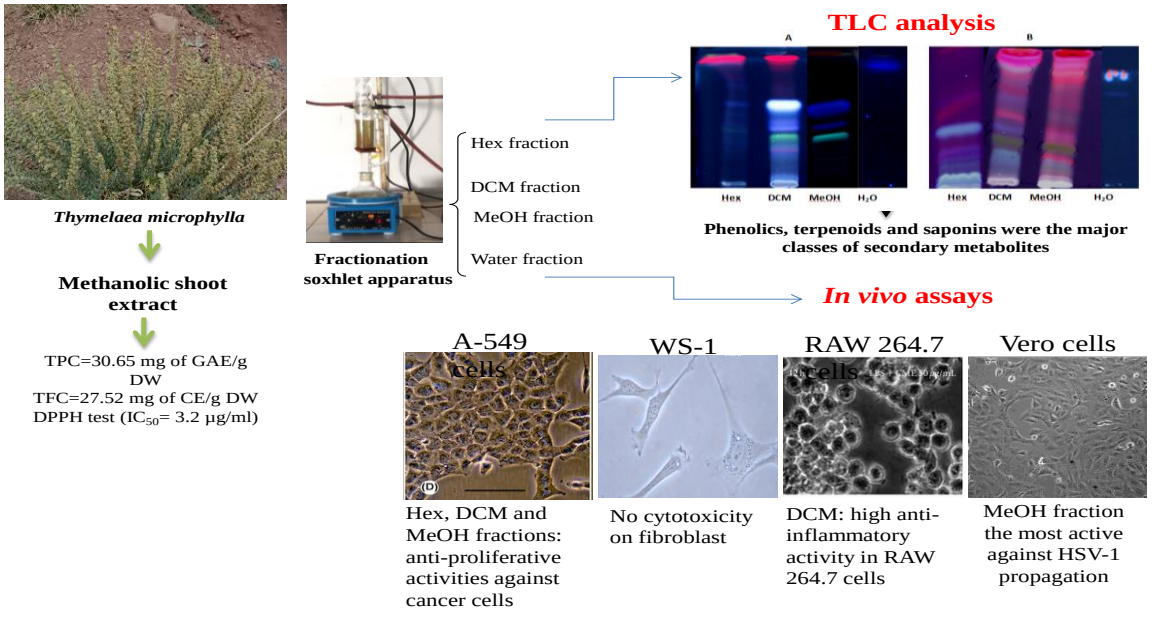
Rim BEN MANSOUR[1], Feten CHAOUACHI[1,2], Hanen FALLEH[1], André PICHETTE[2], Jean LEGAULT[2], Riadh KSOURI[1,*]

[1] Laboratoire des Plantes Aromatiques et Médicinales, Centre de Biotechnologie de Borj-Cédria (CBBC), BP 901, 2050 Hammam–Lif, Tunisia
[2] Laboratoire LASEVE, Université du Québec à Chicoutimi, 555, Boulevard de l'Université, Chicoutimi, Québec, Canada G7H 2B1
[*] Corresponding author : Tél.: +216 79325855; Fax: +216 79 325638; E-mail address: ksouririadh@gmail.com

Highlights

- *Thymelaea microphylla* is an edible and medicinal species with a powerful antioxidant activity
- *T. microphylla* fractions exhibited prominent anti-proliferative activities against cancer cells especially A-549 cells
- *T. microphylla* fractions showed no cytotoxicity on normal fibroblast WS-1.
- *T. microphylla* methanolic fraction was the most active on the herpes virus and on its adsorption.
- Phenolics, terpenoids and saponins were the major classes of secondary metabolites in *T. microphylla* fractions

Graphical Abstract



Abstract
Thymelaea microphylla is an edible and medicinal species that exhibits high antioxidant activity and phenolic content. Moreover, four fractions (hexane, dichloromethane, methanol and water) were tested towards their *ex-vivo* antioxidant, anticancer, anti-inflammatory and antiviral effects. Dichloromethane fraction had marked protection against cell oxidative stress (IC_{50} = 1.5 µg/mL). Hexane, Dichloromethane and Methanol fractions exhibited prominent anti-proliferative activities against cancer cells especially A-549 cells, with very low IC_{50} values of 0.13, 0.018 and 0.5 µg/mL, respectively (50, 101 and 14 higher than Etoposide). Moreover, the three fractions showed no cytotoxicity on normal fibroblast WS-1. DCM exhibited high anti-inflammatory activity in RAW 264.7 cells. Theses fractions were also tested on herpes (HSV-1). MeOH fraction was the most active acting both directly on the virus and on its adsorption. Phenolics, terpenoids and saponins were the major classes. These findings demonstrate that *T. microphylla* is a very promising source of selective anticancer molecules.

Keywords: *Thymelaea microphylla*; ORAC test; anti-inflammatory activity; anti-proliferative capacity; antiviral ability.

Abbreviations
DCFH-DA: 2',7'-dichlorofluorescin-diacetate**; DPPH**:2,2-Diphenyl-1-picryl hydrazyl; **ROS**: Reactive Oxygen Species; **MTT**: 3-(4,5-diméthylethiazol-2-yl)-2,5-diphényltetrazoluim bromide; **TPC**: Total Phenolic Content; **TLC**: thin layer chromatography; **LPS**: lipopolysaccharide; **ORAC**: Oxygen radical absorbance capacity;

1. Introduction
Cancer is a multifaceted disease that represents one of the leading causes of mortality in the world. While great efforts have been made over the past decades to improve the available therapeutic options, cancer still remains a major cause of disease and death in most countries. Lung cancer, a highly invasive, rapidly metastasizing and prevalent cancer, is the top killer cancer in both men and women in the United States of America (USA) and worldwide (Lemjabbar-Alaoui et al., 2015). Besides, colorectal cancer is the third most common cancer in the world and its incidence continues to rise every year. New therapeutic options to treat cancers are a high priority for most of the pharmaceutical companies. Considerable research activity is devoted to the discovery of more potent treatments, while minimizing their toxic side effects. However, most anticancer agents display a narrow therapeutic window due to their lack of selectivity against cancer cells. The ultimate goal of cancer chemotherapy is the development of selective drugs that can kill malignant tumour cells or render them benign without affecting normal cells (Khazir et al., 2014). Moreover, it was accepted that cancer was always associated with inflammatory responses as well as a decrease of endogenous antioxidant capacities and an overproduction of ROS (Uttara et al., 2009). Inflammation played a crucial role in cancer progress. Thus, there is an over-whelming need to develop new anticancer and anti-inflammatory chemo-preventative agents that are both effective and safe. One practical approach to this problem is the use of terrestrial plants as a platform for drug development. Plants have been used as a source of traditional medicine and pharmaceutical drugs for centuries. Medicinal plants are source of active natural products, in particular, polyphenols which are considered chemo preventive agents because they can quench or prevent the formation of ROS and reactive nitrogen species. Particularly, medicinal plants native to extreme climatic conditions, including halophytic and xero-halophytics ones have been found to exhibit high bioactive phytochemicals content. In fact, having suffered a strong selective pressure, these plants have, unlike glycophytes, adaptive mechanisms to tolerate

salinity and drought thanks to a successful system consisting of significant levels of biomolecules (Oueslati et al., 2012). A large flora of plants of Thymelaeaceae family particularly the *Thymelaea* genus are used as natural remedy to treat otitis, diabetes, rheumatism, inflammation of the prostate and uterus cancer (Gmira et al., 2007). However, studies on the biological activities of the genus are scares and limited mostly to *Thymelaea hirsuta* which showed several biological activities such as hypoglycemic, antidiabetic, anticancer and antioxidant properties (Kawano et al., 2007). *Thymelaea microphylla* is commonly called "Methnane" in Tunisia and is common in arid and desert pastures. It is endemic species of North Africa and is traditionally used for the treatment of inflammation and hypertension (Le Foc'h, 1983). Besides, it is used to treat various cutaneous conditions such as erysipelas, skin cancer, abscess and pimples. The decoction of the leaves of this species is known to be useful in cases of infertility and as a purgative. The traditional medicinal use of this plant is not based on scientific research and few data dealing with its phytochemical composition and biological activities are available. Flavonoids, bis-coumarin, lignin, essential oil (Noman et al., 2015) and triterpenoids were identified from this species. Some studies showed *in vitro* antioxidant (Djeridane et al., 2010) and antibacterial activities of this species from Algeria. Nevertheless, the anticarcinogenic, anti-inflammatory and antiviral activities of edible *T. microphylla* species are still unexplored and there is no scientific evidence which supports their use in the literature. Therefore, this study aimed firstly to quantify phenolic compounds and to screen the *in vitro* antioxidant power of crude extract. The obtained results promote as to evaluate for the first time the *ex*-vivo antioxidant, anti-inflammatory, anticancer and anti-viral capacities of *T. microphylla* fractions on human colon and lung cancer cells lines and murine macrophages. Chemical composition of fractions by Thin Layer Chromatography (TLC) was determined.

2. Materials and Methods

2.1. Chemical and reagents

Folin–Ciocalteu reagent, sodium carbonate anhydrous (Na_2CO_3), gallic acid, sodium nitrite solution ($NaNO_2$), aluminum chloride hexahydrate solution ($AlCl_3$, $6H_2O$), vanillin, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid, iron (III) chloride anhydrous ($FeCl_3$), and ascorbic acid were purchased from Fluka (Buchs, Switzerland). Butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA), sodium hydroxide (NaOH), β-carotene, linoleic acid were purchased from Sigma- Aldrich (GmbH, Sternheim, Germany). Sulfuric acid (H_2SO_4) and Kalium-hexacyanoferrat (III); $K_3Fe(CN)_6$ were obtained from Merck (Darmstadt, Germany). Fluorescein sodium salt (FL), 2',7'-dichlorofluorescin-diacetate (DCFH-DA), 2',7'-dichlorofluorescin (DCFH), 2',7'-dichlorofluorescein (DCF), tert-butyl hydroperoxide (t-BuOOH), 6-hydroxy-2,5,7, 8-tetramethyl-2-carboxylic acid (Trolox), 2,2-azobis (2-amidino-propane) dihydrochloride (AAPH) were all purchased from Sigma–Aldrich (Oakville, ON).

2.2. Plant material and preparation of crude extract

Shoots of *T. microphylla* were collected in March 2019 from Ras Jdir (35°48' N, 10°09' E, 133 km from Tunis, semi-arid climate). The plant material was identified at the Biotechnology center of the Technopark of Borj Cedria by Pr Abderrazek SMAOUI. Voucher specimens (RT-CBBC-59) was deposited in the herbarium of the Laboratory of Aromatic and Medicinal Plants (LPAM). For quantification, the air-dried and finely ground shoots (2.5 g) were extracted by magnetic stirring with 25 ml of 80% aqueous methanol for 30 min. Extracts were kept for 24 h at 4°C, and then filtered through a Whatman N°4 filter paper. Samples were stored at 4°C until analysis.

2.3. Fractions preparation

For biological activities powdered shoots (200g) were then extracted in a soxhlet apparatus using several solvents with higher polarity hexane, dichloromethane, methanol and water. Afterwards, the extracts were filtered, after that, the solvent of the combined extract was evaporated under reduced pressure using rotary vacuum evaporator, shoot extracts were freeze- dried and the residue was reconstituted in DMSO before testing.

2.4. Cell culture

The human lung carcinoma A-549 (ATCC #CCL-185), colon adenocarcinoma DLD-1 (ATCC #CCL-221) and murine macrophage RAW 264.7 (ATCC #TIB-71) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA). The A-549, DLD-1cell lines were grown in Minimum Essential Medium with Earle's salts, while the RAW 264.7 cell line was grown in Dulbecco's modified Eagle's medium (Mediatech Cellgro®, Herndon, USA). Both media were supplemented with10% fetal calf serum (Hyclone, Logan, USA) for (A-549 and DLD-1), but with 20% fetal calf serum for RAW 264.7solution of vitamins (1×), sodium pyruvate (1×), non-essential amino acids (1×), penicillin (100 IU) and streptomycin (100 µg/ml) (Mediatech Cellgro®). Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2.

2.5. Cells and virus for anti-herpes activity

The African Green Monkey Kidney cells (Vero cells ATCC: CCL81) were grown in monolayer culture with M199 medium supplemented with 10% fetal calf serum (Hyclone, Logan, USA), solution of vitamins (1×), sodium pyruvate (1×), non-essential amino acids (1×), penicillin (100 IU) and streptomycin (100 µg/mL). Herpes simplex virus type 1 (HSV-1; 15577 strain) was purchased from the American Type Culture Collection (ATCC, Rockville, MD).

2.6. Phenolic content analysis

2.6.1. Analysis of total phenolic content

Total phenolic content of the shoot extracts (80% aqueous methanol) was determined using Folin-Ciocalteu reagent slightly modified by Dewanto et al. (2002) using gallic acid as a standard. An aliquot of diluted sample extract was added to 0.5 ml of distilled water and 0.125 ml of the Folin–Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before addition of 1.25 ml of 7% Na_2CO_3. The solution was then adjusted with distilled water to a final volume of 3 ml and mixed thoroughly, and held for 90 min at ambient temperature. After incubation in dark, the absorbance at 760 nm was recorded. Total phenolic content of plant parts was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg $GAEg^{-1}$ DW) through the calibration curve with gallic acid. All samples were analyzed in triplicates.

2.6.2. Analysis of flavonoïd content

Total flavonoid content was measured using a colorimetric assay developed by Dewanto et al. (2002). An aliquot of diluted sample or standard solution of (+)-catechin was added to 75 µl of $NaNO_2$ solution (7%), and mixed for 6 min, before adding 0.15 ml $AlCl_3$ (10%). After 5 min, 0.5 ml of 1 M NaOH solution was added. The final volume was adjusted to 2.5 ml, thoroughly mixed, and the absorbance of the mixture was determined at 510 nm. Total flavonoid content was expressed as mg (+)-catechin equivalent g^{-1} DW (mg CE g^{-1} DW), through the calibration curve of (+)-catechin (0–400 µg ml^{-1} range). All samples were analyzed in triplicates.

2.6.3. Assessment of total condensed tannin content

The proanthocyanidins were determined by spectrophotometer method (Sun et al., 1998). Three millilitres of 4% methanol vanillin solution and 1.5 ml of concentrated H_2SO_4 were added to 50 µl of suitably diluted sample. The mixture was allowed to stand for 15 min, and the absorbance was measured at 500 nm against solvent as a blank. All samples were analyzed in triplicates.

2.7. Assessment of antioxidant activities

2.7.1. Total antioxidant capacity

Total antioxidant capacity of methanolic extract was evaluated through the assay of the green phosphate/Mo5þ complex according to the method described by Prieto et al. (1999). An aliquot (0.1 ml) of samples was combined with 1 ml of reagent solution (0.3 N sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Methanol (80%) was used instead of sample for the blank. The tubes were capped and incubated in a boiling water bath for 90 min. Then, the samples were cooled to room temperature and the absorbance was measured at 695 nm against blank. Antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE g1DW). All samples were analyzed in triplicate.

2.7.2. DPPH· radical scavenging activity

Radical scavenging activity of the extract was measured using the radical DPPH˙, according to the method of Hanato et al. (1988). Extracts (2 ml) were added to 0.5 ml of 0.2 mM MeOH DPPH˙ solution. After an incubation period of 30 min at room temperature, the absorbance was determined against a blank at 517nm. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the regression equation prepared from the concentration of the extracts and the inhibition percentage. BHT was used as a positive control. Samples were analyzed in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation:

Radical scavenging activity (RSA)% = $[(A_0-A_1)/A_0] \times 100$ where A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min.

2.7.3. Determination of reducing power

The ability of extracts to reduce iron (III) was assessed by the method of Oyaizu (1986). The dried shoot extract (50–400 µg/ml) in 1 ml of the corresponding solvent was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$; 10 g /100 ml), then the mixture was incubated at 50°C for 20 min. After incubation, a total of 2.5 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction then that was centrifuged at 650*g* for 10 min. The supernatant (2.5 ml) was then mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride solution and the absorbance was measured at 700nm.

2.7.4. β-Carotene bleaching test (BCBT)

A modification of the method described by Koleva et al. (2002) was employed. β- Carotene (2 mg) was dissolved in 20 ml chloroform and to 4 ml of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40°C and 100 ml of oxygenated ultra-pure water was added, then the emulsion was vigorously shaken. Sample extract and reference compounds (BHT and BHA) were prepared in ethanol. An aliquot (150 µl) of the β-carotene: linoleic acid emulsion was distributed in each of the wells of 96-well microtitre plates and ethanolic solutions of the test samples (10 µl) were added. Three replicates were prepared for each of the samples. The microtitre plates were incubated at 50°C for 120 min, and the absorbance was measured at 470 nm. Readings of all samples were performed immediately (t = 0 min) and after 120 min of incubation. The antioxidant

activity (AA) of the extracts was evaluated in term of β-carotene blanching using the following formula:
AA (%) = $[(A_0-A_1)/A_0] \times 100$ where A_0 is the absorbance of the control at 0 min and A_1 is the absorbance of the sample at 120min .The results are expressed as IC_{50} values (µg/ml).

2.7.5. ORACFL assay

The procedure was modified from the method described by Ou et al. (2001). Briefly, the ORAC assay was carried out on a Fluoroskan Ascent Fl™ plate reader (Labsystems). Trolox was used as a control standard. The experiment was conducted at 37.5°C and pH 7.4, with a blank sample in parallel. Trolox standards (12.5–100 µM), fluorescein (4.19µM), and AAPH solutions were prepared prior to use in phosphate buffer (75 mM, pH 7.4), blanks solutions in triplicate were also prepared using corresponding solvents to serve as controls, then 15µl of shoot extracts and blanks were placed in the well of a Costar 3631 assay plate (Corning Incorporated, Corning, NY) and fluorescein (75µl) was added to each well, subsequently, AAPH (15µl) was added. The plate was top read at excitation and emission wavelengths of 485 and 530nm, respectively, at 37.5°C. The fluorimeter was programmed to record the fluorescence of fluorescein every 30s after addition of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH). The final results were calculated by comparing the net areas under the fluorescein decay curves between the blank and the samples. ORAC values were expressed in micromoles of Trolox equivalents (TE) per milligram (µmol TE/mg).

2.7.6. Antioxidant cell assay using 2',7'-dichlorofluorescin-diacetate (DCFH-DA)

Antioxidant activity was evaluated using the DCFH-DA assay as described by Legault et al. (2003), with some modifications. Briefly, Human Skin Fibroblast cells were plated in 96 microwell plates at 10,000 cells per well and incubated for 48h at 37°C and 5% CO_2. The cells were washed with 150 µl Hank's balanced salt solution (HBSS) at pH 7.4 and incubated for 30 min with 100 µl HBSS (pH 7.4) containing 5 µM DCFH-DA. The cells were then washed again with 150 µl HBSS. To assess antioxidant activity, the cells were incubated either with a growing concentration of extract from *T. microphylla*, trolox or quercetin, in the absence or presence of 200 µM *t*ert-butylhydroperoxide (*t*BH). Fluorescence was measured after 1h and 4h on the automated 96-well plate reader (Fluoroskan Ascent FL™, Labsystems) using an excitation wavelength of 485nm and an emission wavelength of 530nm.

2.8. Cytotoxicity assay

Exponentially growing cells were plated at a density of 5×10^3 cells per well in 96-well microplates (Costar, Corning Inc.) in 100 µl of culture medium and were allowed to adhere for 16h before treatment. Then, 100 µl of increasing concentrations of extract dissolved in the appropriate solvent (DMSO) were added. The final concentration of solvent in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. The cells were incubated for 48h in the absence or in the presence of extract. Cytotoxicity was assessed using the resazurin reduction test. Fluorescence was measured on an automated 96-well Fluoroskan Ascent Fl™ plate reader (Labsystems) using an excitation wavelength of 530nm and an emission wavelength of 590nm. Cytotoxicity was expressed as the concentration of extract inhibiting cell growth by 50% (IC_{50}).

2.9. Measurement of anti-inflammatory activity by nitrite quantification

Exponentially growing cells were plated in 24-well microplates (BD Falcon) at a density of 2×10^5 cells per well in 400 µl of culture medium and were allowed to adhere overnight. Cells were then treated or not with positive control N (G)-nitro-l-arginine methyl ester (L-NAME), or increasing concentrations of extracts dissolved in the appropriate solvent, and incubated at

37°C, 5% CO_2 for 24 h. The final concentration of solvent in the culture medium was maintained at 0.5% (v/v) to avoid solvent toxicity. Cells were then stimulated with 100µg/ml lipopolysaccharide (LPS). After 24 h, cell-free supernatants were collected and stored at −80°C until NO determination using the Griess reaction (Green et al., 1990) with minor modifications. Briefly, 100 µl aliquots of cell supernatants were incubated with 50µl of 1% sulphanilamide and 50µl of 0.1% N-1-naphtylethylenediamine dihydrochloride in 2.5% H_3PO_4 at room temperature for 20 min. Absorbance at 540nm was then measured using an automated 96-well Varioskan Ascent plate reader (Thermo Electron) and the presence of nitrite was quantified by comparison with an $NaNO_2$ standard curve.

2.10. Antiviral activity

Cells and viruses were incubated with acyclovir and various extracts (100 µg/ml) at different stages during the viral infection cycle in order to determine the antiviral mode of action (Koch et al., 2008). (A) For virus pretreatment assay, the virus suspension was incubated in a medium containing different concentrations of the extracts for one hour at 37°C prior to infection of Vero cells. (B) The cell pre-treatment assay was performed with cell monolayers that were pre-treated with the extracts or positive control acyclovir for one hour at 37°C prior to virus inoculation. (C) For analysing the antiviral inhibition during the adsorption period, HSV-1 was mixed with the drug and immediately added to the cells, incubated for one hour at 37°C. (D) The effect of extracts during the replication period was tested by adding the compounds to the overlay medium after the infection. The wells overlaid with medium without test sample were used as the controls. Cells were fixed with formaldehyde and stained with crystal violet and subsequently lysis plaques were counted.

2.11. Qualitative phytochemical analysis

The identification of major chemical groups of the plant extracts was carried out by thin layer chromatography (TLC) on silica gel (60 F2SJ glass plates, 250 µm layer thickness, Silicycle, Québec. Canada). An aliquot of *T. microphylla* extract in methanol solution (1 mg/mL, 10 µL) was directly deposited (as bands) onto the TLC plates. TLC plates were developed in a presaturated solvent chamber with ethyl-acetate, acetic acid/formic acid/water (50:10:10:25) as developing reagents until the solvent front reached 1 cm from the top of plates. The developed TLC plates were then removed from the chamber, and allowed to air-dry for 30 min, followed by spraying with 2-aminoethyldiphenylborinate/ polyethylene glycol (NPPEG) to reveal the flavonoids and phenolic acids. For the detection of terpenoids, we use chloroform/methanol/water (26:14:3) as mobile phase. After that, TLC plates were sprayed with 5% H_2SO4 and heated at 110 °C for 5 min. For the detection of steroidic saponins, plates were migrated in a mixture of solvents (chloroform/methanol) (25:1) and sprayed with 5% H_2SO4 followed by 20% vanillin. All these plates were visualized by inspection of the plates under visible light. Each TLC plate was also monitored under UV light at 254 and 366 nm. The retention factor of these compounds was determined.

3. Results and Discussion

3.1. Phytochemical analysis and antioxidant activity of crude extract

3.1.1. Total phenolic, flavonoid and tannin contents

Results regarding the total polyphenols, flavonoids and proanthocyanidin contents of *T. microphylla* shoot extract are shown in Table 1. Aqueous methanol shoot extract exhibited an important amount of polyphenols (30.65 mg of GAE/g DW) followed by flavonoids (27.52 mg of CE/g DW) while tannin content was low (1.1 mg of CE/g DW). These results demonstrate that *T. microphylla* is rich on phenolic compounds. Moreover, the phenolic and flavonoids content found in this study are higher than that reported by Djeridane et al. (2010)

in the shoots of Algerian *T. microphylla* whose aqueous methanol extract exhibited phenolic and flavonoids contents of 10 mg of GAE/g DW and 2 mg of rutin equivalent/g DW, respectively. Other studies on the *Thymelea* genus reveals that aqueous extract obtained from *T. hirsuta* shoots as well as from the different organs were rich on total phenolic and flavonoids (Ouda et al., 2014).

Table 1. Total polyphenols, flavonoids and condensed tannins contents and antioxidant activities of aqueous methanolic shoot extract of *T. microphylla* and authentic standards (BHT and Ascorbic acid).

	Total phenols content (mg GAE/g DW)	Flavonoid content (mg CE/ g DW)	Tanin content (mg CE/g DW)	Total antioxidant capacity (mg AE/g DW)	DPPH test IC_{50} (µg/ml)	Reducing power EC_{50} (µg/ml)	*β*-Carotene bleaching assay IC_{50} (µg/ml)
Shoots extract	30.65 ± 0.51	27.52 ± 0.44	1.1 ± 0.13	57.96 ±0.15	3.2 ±0.31	360 ±0.45	318.3 ±0.12
BHT	-	-	-	-	11.5 ±0.10	-	75 ±0.11
Ascorbic acid	-	-	-	-	-	37.33 ±0.40	-

3.1.2. Assessment of antioxidant activity

Since there is a definite role of free radicals in the pathogenesis of several diseases (Uttara et al., 2009), the antioxidant capacities of *T. microphylla* were also studied. Antioxidant evaluation of *T. microphylla* 80% methanol extract was conducted using DPPH, total antioxidant activity, reducing power and β-carotene linoleate system assays (Table 1). The results indicated that *T. microphylla* displayed strong radical scavenging activity (IC_{50}= 3.2 µg/ml) which was more powerful than that of the positive control BHT (IC_{50} = 11.5 µg/ml). Radical scavenging power of Tunisian *T. microphyla* was higher than that of the Algerian species reported by Djeridane et al. (2010) (IC_{50} = 19 µg/ml). Besides, our results showed that *T. microphylla* exhibited significant total antioxidant activity (58.0 mg GAE/g DW) and moderate reducing power (EC_{50} value of 360.0 µg/ml) and β-carotene bleaching (IC_{50} = 318.0 µg/ml) effects. From these results, it is clear that *T. microphylla* exhibited a greatest antioxidative activities especially radical scavenging one which correlates with high phenolic and flavonoid contents.

These useful *in-vitro* results prompted us to investigate for the first time the *ex-vivo* bioactivities of *T. microphyla* including antioxidant, anti-inflammatory, anticancer and antiviral ones. Moreover, a successive extraction using solvent with different polarities was used in order to fractionate *T. microphyla* extract and understand the relationship between the biological activity and the fraction.

3.1.3. TLC characterization of T. microphylla fractions

The four *T. microphylla* fractions showed characteristic TLC chromatograms (Figure 1). After chromatographic separation and chemical derivation with NP/PEG reagent, phenolic acids were detected as bright blue zones in UV light at 365 nm while flavonoids showed orange-yellow with distinct yellowish orange fluorescences under the same conditions (Wagner and Bladt, 1996) TLC chromatograms of DCM and methanol fractions were characterized by the predominance of phenolic compounds (Figure 1A). Interestingly an intense spot characteristic of phenolic acid (bright blue spot) at retarding factor (Rf) 0.6 was detected in DCM and MeOH. Additional phenolic acids (Rf 0.5 and 0.45) were present in DCM. Flavonoids (yellow florescence, Figure 1A) were present in DCM and MeOH also. Previous studies on Algerian *T. microphylla* reported the predominance of hydroxycinnamic and hydroxybenzoic acids derivatives in the ethyl acetate fraction (Djeridane et al., 2010). Besides, flavonoid

aglycones and glucosides as well as biflavonoids, lignans and coumarin were identified in ethyl acetate and chloroform extracts of this species.

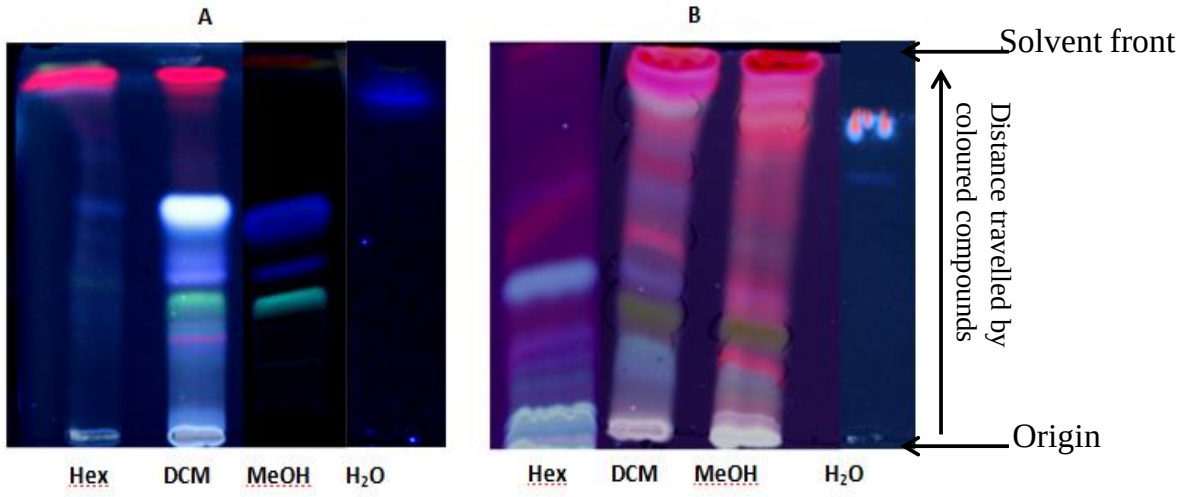


Figure 1. TLC of *T. microphylla* fractions. (A) elution with chloroform/methanol/water (26:14:3) detection under UV light at 365nm after spraying of NP/PEG reagent. (B), elution with chloroform/methanol (25:1) detection under UV light at 365 nm after spraying with 5% H_2SO_4 followed with 20%vanillin.

In our study, *T. microphylla* fractions were analyzed for their steroids and terpenoids content also. The plates were air dried and visualized by spraying with vanillin-sulfuric acid spray followed by heating. The results indicated that Hexane, DCM and MeOH fractions were rich on monoterpenes, triterpenes and steroids detected as blue, pink and purple spots (Figure 1B). Presence of triterpenoids, phytosterols (β-sitosterol and β-sitosterol-3-O-glucoside, dihydroxylated monoterpene and monoterpene glucosides (Kerbab et al., 2014) were highlighted in *T. microphylla* solvent extracts.

3.1.4. In-vitro and ex- vivo antioxidant activity in T. microphylla shoot fractions

Results regarding the antioxidant capacity of different extracts (hexane, dichloromethane, methanol and water) of *T. microphylla* are shown in Table 2. *In-vitro* antioxidant activity of shoot was assessed using ORAC assay. The ORAC values ranged from 0.08 to 6.23 µmol TE/mg. Among the fractions, DCM had the highest antioxidant activity, with ORAC value of 6.23 µmol TE/mg, followed by MeOH (4.84 µmol TE/mg). However, Hex and H_2O extracts showed weak activities. ORAC value of dichloromethane fraction was comparable to that of positive control quercetin (7.34 µmol TE/mg). In addition, it appears that the ORAC values in the present study were stronger than those reported in the literature (Silva et al., 2007). Since the ORAC assay follows a hydrogen atom transfer mechanism (the H of the polyphenols neutralizes the radicals formed), the results suggest that DCM and MeOH may contain potent antioxidants that have the ability to scavenge toxic peroxyl radical and thus possess radical chain breaking antioxidant activity by H-atom transfer (Ou et al., 2001).

Table 2. Oxygen radical absorbance capacity (ORAC) values expressed as Trolox equivalents per gram and antioxidant cell assay expressed as IC_{50} (µg/ml) of *T. microphylla* fractions and authentic standards (Trolox and Quercetin).

Fractions	ORAC value (µmol Trolox Equivalent/mg DW)	IC_{50} (µg/ml)
Hex	0.081 ± 0.01	156 ± 54
DCM	6.23 ± 0.19	1.5 ± 0.3
MeOH	4.84 ± 0.26	15 ± 0.3
H_2O	0.82 ± 0.04	15 ± 2
Trolox	0.24 ± 0.01	0.24 ± 01
Quercetin	7.34 ± 0.40	0.69 ± 0.06

Values are mean±S.D. of three replications.

The antioxidant activity of *T. microphylla* fractions was also assessed *ex-vivo* using a cellular based-assay (Legault et al., 2003). DCF assay was performed with Human Skin Fibroblast cells (WS1). Results showed that DCM exhibited marked capacity to inhibit the *t*BH-induced oxidation of DCFH with an IC_{50} value of 1.5 µg/ml (Table 2), followed by water and methanol fractions which inhibited this oxidation with an IC_{50} value equal to 15 µg/ml. However, inhibition of DCFH oxidation by hexane fraction was low (IC_{50} value = 156 µg/ml). In comparison, the IC_{50} values of standard antioxidant and phenolic compounds were 0.2 and 0.7 µg/ml for Trolox and quercetin, respectively. This result indicates that DCM significantly inhibits ROS production and thus exhibits the ability to protect cells from oxidative stress. To the best of our knowledge, this is the first report on the cellular antioxidant property of *T. microphylla.*

In the current study, among the tested fractions, DCM possesses strong *in-vitro* and *ex-vivo* antioxidant capacity which could be attributed to the presence of active antioxidant compounds. TLC analysis confirms the presence of high quantities of phenolic compounds mainly phenolic acids as well as triterpene in DCM fraction. Phenolic acids were found to greatly reduce free radical damage in cell systems exposed to oxidative stress by attenuating hydroxyl and peroxyl radical generations, protein oxidation and lipid peroxidation (Kanski et al., 2002). Besides, several tested triterpenoids have been found to protect cell against free radical stress by inhibiting significantly ROS formation and thus may be protective agents for diseases associated with increased amounts of ROS.

3.2. Evaluation of *T. microphylla* fractions cytotoxicity against tumor cell lines

In this study, the cytotoxicity of shoot fractions was evaluated against human skin fibroblast cell lines WS1, colon carcinoma cell lines DLD-1, and lung carcinoma cell line A-549. The IC_{50} values obtained are shown in Table 3. Etoposide was used as a positive control with IC_{50} values ranging from 2 to 39 µM. Interestingly, DCM displayed remarkable activity against the two tested cancer cell lines and these activities were even higher than that of the positive control. In fact, DCM inhibited A-549 and DLD-1 cells proliferation with IC_{50} values of 0.018 and 0.5 µg/ml, respectively. These activities were 101 and 1.8 folds higher than that of positive control Etoposide which displayed IC_{50} values of 3.1µM (1.8 µg/ml) and 1.5 µM (0.9 µg/ml) against A-549 and DLD-1, respectively. Hex and MeOH fractions exhibited very powerful activities, also, especially against A-549 cells. They exhibited IC_{50} equal to 0.13 and 0.0397 µg/ml, respectively, which were 14 and 49.3 folds higher than that of positive control. Moreover, Hex fraction was active against DLD-1 (IC_{50} equal to 1.5 µg/ml) while MeOH displayed moderate activity against the later cell line. Water fraction was found to be moderately active against both tumor cell lines with IC_{50} values of 74 and 40 µg/ml against A-549 and DLD-1 cells respectively. Likewise, there were significant differences in DCM

cytotoxic effects on A-549 and DLD-1 cells which may highlight a specific cytotoxic mechanism that is possibly used by the fraction to seize the cell growth of A-549 cell line.
The findings of the current study revealed effective cytotoxic effects on A-549 and DLD-1 cells by *T. microphylla* fractions. No previous studies have investigated the cytotoxic effect of *T. microphylla* extracts. Moreover, in the current study, the positive control, Etoposide was cytotoxic against normal WS-1 cells with IC_{50} value of 39 µM (23µg/ml) Interestingly, DCM, MeOH and Hex fractions exhibit no cytotoxicity (IC_{50} > 200µg/ml) except for water fraction which was cytotoxic at low concentration (Table 3). These data indicate that *T. microphylla* exert a highly selective anticancer activity. There is a need to find new chemical agents able to differentiate between normal and cancerous cells.

Table 3. Cytotoxic activity of *T. microphylla* fractions against human lung carcinoma cells (A549), colorectal adenocarcinoma cells (DLD-1) and normal skin fibroblasts (WS1).

Fractions	A-549	DLD-1	WS-1
Hex	0.13±0.02	1.5±0.2	>200
DCM	0.018±0.001	0.5±0.2	>200
MeOH	0.037±0.005	119±72	>200
H_2O	74±0.5	40±7	0.31±0.05
Etoposide	0.0018±0.005	0.009±0.001	0.022±0.08

Etoposide was used as positive control. Values are mean±S.D. of three replications.
IC_{50} values (µg/ml).

The overall data suggests that *T. microphylla* especially DCM and Hex fractions have a significant influence on tumour cell viability and targeted colon and lung carcinoma cell lines, indicating the presence of powerful cytotoxic and selective compounds. These molecules could likely be bioactive terpenoids. Kawano et al. (2007) reported strong antimelanogenesis activity in B16 melanoma cells by *T. hirsuta* from Tunisia which was attributed to diterpenes daphnanes action. One of the possible mechanisms responsible of phenolic compounds is their antioxidant action. It was found that the scavengers of ROS including alkylperoxyl radical (ROO•) may play an important role in cancer prevention (Sawa et al., 1999). On the other hand, Haddad et al. (2006) demonstrated that different flavonoids exerted their antiproliferative effects at lower doses in prostate cancer cells compared to normal prostate cells.

3.3. Evaluation of the anti-inflammatory activity of *T. microphylla* extracts on LPS-activated RAW 264.7 macrophages

During the inflammatory process, various inflammatory mediators, including nitric oxide (NO), prostaglandins and adhesion molecules are closely associated with the classical symptoms of inflammation such as pain, heat, redness, swelling, and loss of function. NO is generated from amino acid L-arginine by the enzymatic action of inducible NO synthase (iNOS) which is stimulated during inflammation by bacterial endotoxins (e.g., lipopolysaccharide) and cytokines (Korhonen et al., 2005).
The anti-inflammatory activity of *T. microphylla* fractions was investigated by measuring their capacities to inhibit NO cellular generation in stimulated RAW 264.7 macrophages which could be typical indicator for anti-inflammmatory activity. Moreover, the cytotoxic effect of the fractions was evaluated on macrophages using resazurin to ensure that the anti-inflammatory activity was not due to cytotoxicity. Below 90% macrophages survival, the NO concentration was not considered to be significant. As shown in Figure 2**,** DCM fraction provided potent anti-inflammatory activity without any cytoxicity. Dichloromethane fraction inhibited NO release in a dose-dependent manner, and inhibition percentages varied from

22.9% at 1.25 µg/ml to 100% at 160 µg/ml, with an IC_{50} value of 16 µg/ml. In comparison, L-NAME, positive control, significantly inhibited the NO release by 82% at 250 µM (67.4 µg/ml). Regarding the other fractions, hexane and methanol displayed interesting anti-inflammatory effect inhibiting NO production by 63.8 and 44.2% at 20 µg/ml. Nevertheless, these fractions become cytotoxic at higher concentrations. Water fraction was toxic even at low concentration and thus was not been considered.

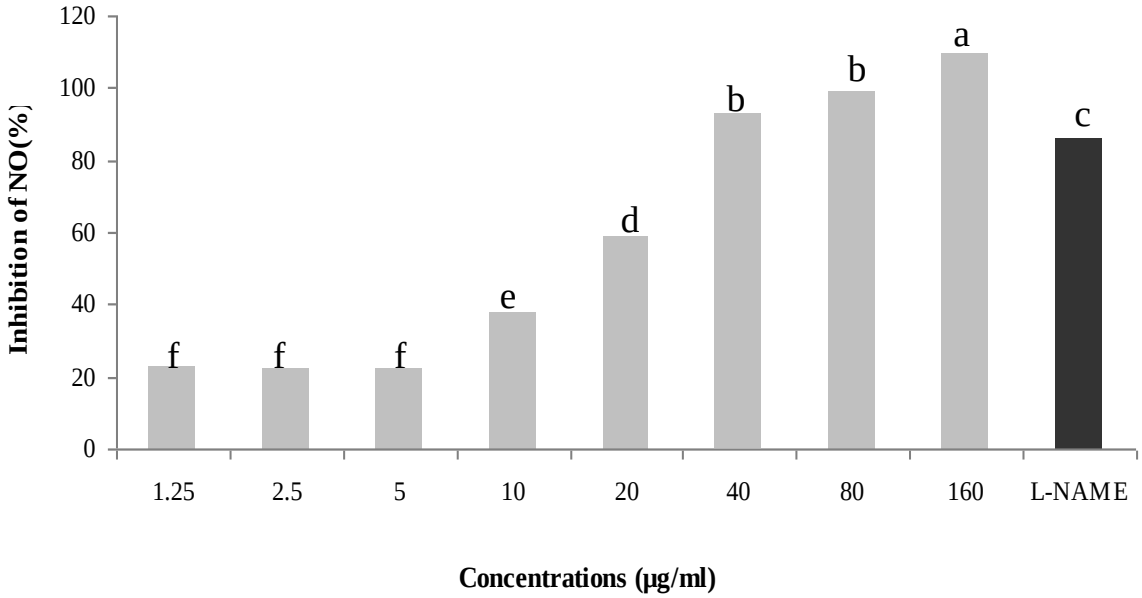


Figure 2. Inhibitory effect on NO production of dichloromethane fraction from *T. microphylla* in LPS-induced RAW 264.7 macrophages. *Values with different superscripts (a, f) are significantly differents at P<0.05.*

Anti-inflammatory activity includes molecules that have different mechanisms of action, including antioxidant activity. Taken together, the superiority of DCM as an inhibitor of pro-inflammatory factor NO production in RAW 264.7 cells associated to its efficiency to inhibit cellular oxidation in the *ex-vivo* test (it showed high cellular antioxidant activity with IC_{50} value of 1.5 µg/ml) suggest that the anti-inflammatory activity could be linked at least in part, to the presence of antioxidant compounds; phenolics. Indeed, several phenolic compounds have been reported to possess anti-inflammatory activity and to be beneficial in the treatment of chronic inflammatory diseases associated with overproduction of NO (Jiang and Dusting, 2003). Recently, chlorogenic acids derivatives have been isolated from *T. microphylla*. Chlorogenic acid has been found to significantly inhibit the production of various inflammatory markers including NO in lipopolysaccharide (LPS)-stimulated murine RAW macrophages without any cytotoxicity (Hwang et al., 2014). Moreover, DCM activity could be mediated by terpenoids and steroids.

3.4. Anti-herpes activity

The potential antiviral effect of different *T. microphylla* fractions was determined, *in vitro*, against Herpes Simplex Virus type 1. HSV-1 is very common infection in the population. Actually, there are some treatments as acyclovir and docosanol. Extracts effect at different stage of viral infection was evaluated. The inhibitory effect was determined following the addition of extracts at different times during viral infection. To identify the step at which replication might be inhibited, cells were infected with HSV-1 after its incubation with plant extract, pretreatment of the virus with the extract prior to infection and addition of the extract

during or after adsorption period. In all experiments untreated virus infected cells were used as control. Results were expressed as the percentage of plaques lysis (Table 4). Acyclovir was used as positive control. The results show that all the tested fractions protected cells against HSV-1 herpes virus by various mechanisms of actions. When virus was pretreated with fractions at concentration of 100 µg/ml before cells infection (Treatment A), viral infectivity was greatly inhibited indicating that the four fractions interacted directly with HSV-1 to inhibit virus infection. The highest direct effect of fractions on HSV-1 was found with MeOH (88% inhibition of lysis plaque) followed by H_2O (63% inhibition of lysis plaque). Moreover, MeOH, Hex and DCM fractions were found to be able to protect cells against virus infection (Treatment B) as well as to inhibit infection via an adsorption inhibition (Treatment C), while water was inactive. Among the tested fractions, MeOH exhibited strong protection activity (75% inhibition of lysis plaque). Besides, this fraction effectively protected against virus adsorption. Altogether, these results showed that the fractions did not inhibit virus replication (treatment D), however, MeOH fraction was strongly active protecting cells against influenza virus by various mechanisms of actions such as acting either directly on the virus and on its adsorption. To the best of our knowledge, it is the first time that antiviral activity studies are reported for *T. microphylla*. Probably, the active compounds in MeOH fraction may protects the host cell infection by interacting with the surface glycoproteins receptors of the viral envelope which are necessary for adsorption or entry of the virus into host cells. Moreover, active compounds may induce the disintegration of the entire HSV particles and the solubilization of the virus envelope (Alvareza et al., 2009). As described above, *T. microphylla* MeOH fraction was rich on phenolics and terpenoids which suggest that these compounds could be responsible, in part, of the anti-herpes activity. Medini et al. (2014) showed that the antiviral activities in halophyte *Limonium densiflorum* may be related to the presence of polyphenolic compounds. A number of phenolic acids such as caffeic and chlorogenic acids showed an antiviral activity against HSV-1. In addition, various flavonoids including quercetin are known for their virucidal action and their direct inactivation of HSV-1 (Khan et al., 2005). Moreover, the presence of terpenoids can explain the direct effect of *T. microphylla* fractions on HSV-1. In particular, daphnane-type diterpenes, characteristics of Thymelaeaceae family, were found to exert potent antiviral capacity.

Table 4. Effect of *T. microphylla* fractions on HSV-1 propagation in Vero cells.

	Plaque lysis number					
	Control	Acyclovir	Hex	DCM	MeOH	H_2O
Treatement A	8±3	3±1	5±1	5±1	1±0	3±1
Treatement B	8±4	6±2	4±2	4±1	2±2	9±1
Treatement C	8±3	5±4	7±2	6±2	3±2	12±5
Treatement D	5±4	1±1	9±3	10±5	6±2	8±4

Data are represented as the mean of three independent experiments including their standard deviation values

4. Conclusion

Results suggest that Tunisian *T. microphylla* may serve as an excellent lead for the development of anticancer agents for lung cancer particularly A-549 lung carcinoma. It is an accessible source of natural antioxidants, anti-inflammatory and antiviral compounds also, with considerable health benefits (***Patent was deposited, N°*** TN2016/0334).

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution Statement

Rim BEN MANSOUR. Experimentation, data curation, writing the original manuscript; **Feten CHAOUACHI**. Contribution to investigation, data curation (*in vivo* assays); **Hanen FALLEH**. Contribution to data curation and writing paper **André PICHETTE**. Contribution to data curation, contribution to supervision and writing paper; R.BS.), **Jean LEGAULT**. Contribution to conceptualization and to resources. **Riadh KSOURI**. contribution to data curation, supervision, writing paper.

ORCID

Rim Ben Mansour: 0000-0002-1951-0938
Feten CHAOUACHI: 0000-0001-6460-8777
Hanen FALLEH: 0000-0002-6765-803X
André PICHETTE 0000-0002-6760-1463
Jean LEGAULT 0000-0001-6292-6798
Riadh KSOURI: 0000-0001-60042585

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