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Phytochemical Analysis, Antioxidant, Antifungal Activity, and Genotoxicity of Essential Oil and Solvent Extracts of *Tetraclinis articulata* leaves from Northern Morocco

Amina Benoutman [1,*], El Hadi Erbiai [1,2], Hajar Ettakifi [1], Mounir Legssyer [1], Nadia Handaq [1,6], Hanane Makrane [1], Rabah Saidi [1] , Zouhaire Lamrani [1], Manuela Pintado [3], Eugénia Pinto[4,5], Joaquim C. G. Esteves da Silva [2], Abdelfettah Maouni [1]

[1] Biology, Environment, and Sustainable Development Laboratory, ENS, Abdelmalek Essaadi University, Tetouan 93000, Morocco; amina.benoutman@etu.uae.ac.ma (A.B.); elhadi.erbiai@etu.uae.ac.ma (E.H.E.); hajar.ettakifi@etu.uae.ac.ma (H.E.); mlegssyer@uae.ac.ma (M.L.); n.handaq@uae.ac.ma (N.H.); h.makrane@uae.ac.ma (H.M.); r.saidi@uae.ac.ma (R.S.); zlamrani@uae.ac.ma (Z.L.); amaouni@uae.ac.ma (A.M.);
[2] Chemistry Research Center (CIQUP), Institute of Molecular Sciences (IMS), Department of Geosciences, Environment and Spatial Planning, Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal ; jcsilva@fc.up.pt (J.C.G.E.d.S.);
[3] CBQF-Center for Biotechnology and Fine Chemistry, Associated Laboratory, School of Biotechnology, Portuguese Catholic University, 4169-005 Porto, Portugal; mpintado@ucp.pt (M.P.);
[4] Laboratory of Microbiology, Biological Sciences Department, Faculty of Pharmacy, University of Porto (FFUP), 4050-313 Porto, Portugal; epinto@ff.up.pt (E.P.);
[5] CIIMAR-Interdisciplinary Center of Marine and Environmental Research, University of Porto, 4450-208 Matosinhos, Portugal;
[6] Biological Sciences Department, Faculty of Sciences, Moulay Ismail University of Meknes, B.P.11201, Avenue Zitoune, Meknes, Morrocco;
* Correspondence: amina.benoutman@etu.uae.ac.ma (A.B.);

Scopus Author ID -

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Abstract: *Tetraclinis articulata* is a medicinal plant commonly known in Morocco as" Araar Tree ". Various parts of *T. articulata* are traditionally used for various therapeutic purposes. In this context, the present study aims to evaluate the antioxidant, antifungal, and genotoxicity of methanolic (TaLME), acetonic (TaLAE) extracts, and essential oil (TaLEO) from *Tetraclinis articulata* leaves growing in northern Morocco. This was carried out through chemical analysis, bioactive substance determination, and essential oil and extract testing. The bioactive substances determined by the spectrophotometric method showed a higher content of phenols and flavonoids in TaLME. The Liquid chromatography-mass spectrometry (LC-MS) analysis of phenolic compounds revealed protocatechuic acid as predominant in TaLME, followed by paraben acid and syringic acid, and in TaLAE, *p*-hydroxybenzoic was the principal compound, followed by protocatechuic acid and vanillic acid. The gas chromatography-mass spectrometry (GC-MS) analysis of TaLEO (yield of 0.77 %) revealed the presence of 22 compounds (95.80 %) with main components (%) α-pinene (29.62), bornyl acetate (21.45), and camphor (16.80). Biological activities were evaluated: antioxidant efficiency using four different methods (DPPH, ABTS, FRAP, ORAC), deoxyribonucleic acid (DNA) protection, genotoxicity test, and antifungal activity against five pathogenic fungi (*Aspergillus fumigatus*, *Candida albicans*, *Epidermophyton floccosum*, *Trichophyton rubrum*, *and Microsporum canis*). According to the results, the extracts and EO had significant antioxidant and antifungal activity against the dermatophytes. The genotoxicity test exhibited that the *T. articulata* extracts and EO were bereft of any mutagenic activity. Overall, this study demonstrates that *Tetraclinis articulata* plant essential oil and extracts can represent an intriguing potential source of natural compounds that can be helpful for future investigations.

Keywords: *Tetraclinis articulata*; biochemical composition; essential oil; phenols; flavonoids; antioxidant activity; antifungal activity; genotoxicity test.

1. Introduction

In recent years, there has been an increased interest in the search for natural molecules that are now thought to be effective treatments or preventative measures for complex diseases [1]. Several researchers are often interested in the therapeutic effects of a natural compound. The natural substances of medicinal plants have multiple uses in many industrial sectors, such as pharmaceuticals, agri-food, and cosmetics [2]. A large part of the public is again turning to incorporating natural molecules with original chemical and biological characteristics in their formulations. Presently, there is an increasing benefit to using naturally occurring substances. Essential oils from aromatic and medicinal plants are among the natural substances derived from plants showing signs of biological activity [3]. Numerous studies have focused on the chemical and pharmaceutical properties of aromatic and medicinal plants, which are a source of secondary metabolites with the potential to treat a range of pains [4]. The essential oils from diverse plants are known to possess antimicrobial and antioxidant activity [5,6].

Reactive oxygen species (ROS) are known to have both positive and negative effects on biological systems. The physiological function of numerous cellular responses demonstrates the positive effects of ROS. Contrarily, ROS can harm various cellular constituents at high concentrations, including lipids, proteins, and nucleic acids [7]. Therefore, they may have participated in the development of many illnesses, such as inflammation, cancer, immune disorders, and cardiovascular and pulmonary diseases [8].

Natural antioxidants from medicinal plants are of interest because several synthetic antioxidants have been suggested to prevent and treat specific diseases, but their toxicity has resulted in dangerous side effects in their use [9]. Based on the effects of natural antioxidants on oxidative stress associated with certain illnesses, considerable effort and research have been made [10]. The antioxidant properties of essential oils from various aromatic plants have been extensively researched [11,12]. Some *Tetraclinis* species from various nations, including Morocco, Algeria, and Tunisia, and their essential oils and extracts, have been shown to have antioxidant activity against free radicals [13–15].

Tetraclinis articulata is a medicinal plant commonly known in Morocco as the "Araar tree". It's a monoecious, resinous species with evergreen foliage that is a member of the Cupressaceae family [16,17]. *T. articulata* is endemic to North Africa and covers around 1 million hectares in the three countries of the Maghreb (Morocco, Algeria, and Tunisia) [18,19]. In Morocco, forests cover approximately 566.000 hectares and are divided into 6 main biogeographical units: the rif, oriental, eastern middle atlas, valleys of the central plateau and the western plateau, western middle atlas, and high atlas [20]. The various parts of this species has been used traditionally for a variety of therapeutic purposes, including the treatment of respiratory and intestinal infections [21], hypertension, diabetes [22], and gastrointestinal pain [23]. Some studies have reported a bioactivity evaluation of the essential oils and extracts of *T. articulata*. Furthermore, other studies have demonstrated this tree's essential oil's antioxidant, antibacterial, antifungal, and anti-inflammatory properties [24–26].

The purpose of this study was to valorize the plant species from the region of northern Morocco and extend the knowledge of their chemical compositions, bioactive compound

contents, and biological activities. To achieve this goal, this study investigated the methanolic and acetonic extracts and essential oil of *T. articulata* through their chemical compositions using gas chromatography-mass spectrometry (GC-MS), phenolic compounds characterization by Liquid chromatography-mass spectrometry (LC–MS), phenolics and flavonoids determination by spectrophotometric method, antioxidant evaluation by four different methods (DPPH, ABTS, FRAP, ORAC), DNA protection), antifungal activity against five pathogenic fungi and finally their mutagenic activity by genotoxicity testing method.

2. Material and Methods

2.1. Standards, reagents, and culture medium.

2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) and 2-Diphenyl-1-picrylhydrazyl (DPPH) were bought from Alfa Aesar (Ward Hill, MA, USA) and Sigma (Darmstadt, Germany), respectively. Potassium ferricyanide ($C_6N_6FeK_3$), Trichloroacetic acid ($C_2HCl_3O_2$) were purchased from (Sigma, Darmstadt, Germany). Phenolic standards (paraben acid, ferulic acid, vanillic acid, cinnamic acid, p-coumaric acid, p-hydroxybenzoic acid, gallic acid, protocatechuic acid, syringic acid), Folin–Ciocalteu phenol reagent, catechin, L-ascorbic acid, sodium hydroxide, sodium nitrite, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), alkane standard (C8-C37), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, aluminum chloride, and ethyl acetate were purchased from Merck KGaA (Darmstadt, Germany). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), DMSO, Fluorescein ($C_{20}H_{12}O_5$) and bromophenol blue was obtained (from Sigma, Darmstadt, Germany). Hexane, n-hexane, and acetone were from CABLO ERBA Reagent, S.A.S (Val de Reuil Cedex, France). RPMI-1640 broth medium and Sabouraud dextrose agar (SDA) were obtained from from Biochrom AG (Berlin, Germany) and Bio-Mèrieux (Marcy L'Etoile, France), respectively. All other chemicals and solvents, including methanol, were bought from Honeywell in St. Muskegon, Michigan, in the United States.

2.2. Plant harvesting.

The *T. articulata* tree leaves were harvested in October 2018 in a *Tetraclinis* forest belonging to the SIBE (Site of Biological and Ecological Interest) in Tetouan Province in northern Morocco (35°50'068" N, 5°42'79" W, 220 m of altitude, thermo-Mediterranean vegetation level, a subhumid bioclimatic level at temperate winter, and siliceous substrate). The plant was determined based on the Manual of Identification of Vascular Plants (Practical Flora of Morocco) [27]. The plant leaves have been weighed and reduced into small pieces, allowed to dry naturally at room temperature for 20 days on paper protected from light and moisture, and then ground up and conserved in dark glass bottles.

2.3. Preparation of extracts and essential oils.

One gram of fine dried leaves powder was added to 20 mL of methanol or acetone and extracted at room temperature under continuous shaking (150 revolutions per minute) for 24 hours, and then the extracts were filtered through Whatman filter paper N° 4. The residue was re-extracted twice, according to the authors Erbiai *et al*. [28]. The combined extracts were evaporated at 40°C. The dried extracts were weighed and stored at -81°C for further use. The

essential oil was extracted from the *T. articulata* leaves using hydrodistillation by a Clevenger-type apparatus [29]. The oil was obtained after four hours of mixing 100 g of leaves and 500 mL of distilled water. For further chemical analysis, the TaLEO was conserved at 4°C in amber bottles.

2.4. Determination of total phenolics content (TPC) and total flavonoids content (TFC).

The TPC was determined by the Folin-Ciocalteu (FC) procedure used by Erbiai *et al*. [28]. A quantity of 100 µL of plant extract (1 mg/mL) was mixed with 500 µL of the freshly prepared FC reagent and 400 µL of Na_2CO_3 (7.5 %). Using a UV-Vis Spectrophotometer, the absorbance at 760 nm was measured after the mixture had been incubated at 40°C for 20 minutes compared to a blank. TPC values were determined based on the calibration curve of gallic acid (500–15.62 µg/mL). The results were expressed in micrograms of gallic acid equivalents (GAE) per gram of dry extract (DE) or essential oil (EO).

The TFC of *T. articulata* was determined using a method used by Erbiai *et al*. [28]. A mix of 250 µL of the plant extract (1 mg/mL), 1250 µL of distilled water, and 75 µL of $NaNO_2$ (5%) were incubated for 5 min. After that, 150 µL of $AlCl_3$ (10%) was added to the mixture. 275 µL of distilled water and 500 µL of NaOH (4%) were added after 6 min of incubation. The absorbance was measured at 510 nm using distilled water as a blank. TFC values were calculated using the calibration curve of quercetin (500–15.62µg/mL), and the results were expressed as µg quercetin equivalents (EQ)/g of DE or EO.

2.5. LC-MS analysis of phenolic compounds.

The phenolic compounds of *Tetraclinis articulata* leaves were analyzed using the conditions of Erbiai *et al*. [30] and HPLC equipment. Briefly, the phenolic extracts were conducted by liquid chromatography-mass spectrometry. The peaks were detected at 280nm, using an Acclaim™ 120 reverse phase C18 columns (3µm 150 × 4.6mm) at 35°C. The composition of the mobile phase was 100% acetonitrile and 1 % acetic acid. According to the UV-Vis spectra, the phenolic compounds in the samples were characterized and identified by their retention times and mass spectra compared with commercial standards. Based on the areas of the peaks recorded at 280nm, the quantification was performed by comparing them to calibration curves derived from the standard of each compound. The results were expressed in µg per gram of DE.

2.6. GC-MS analysis of essential oil.

TaLEO was chemically analyzed by gas chromatography (GC) (Trace 1300 GC; Thermo Fisher Scientific, Waltham, MA, USA) coupled to a mass spectrometry (MS) system (ISQ single quadrupole mass spectrometer; Thermo Fisher Scientific). The gas chromatography-mass spectrometry system (GC-MS) was equipped with a TG5-MS capillary column (60m × 0.25mm i.d.; 0.25µm film thickness) containing a nonpolar stationary phase (5% phenyl, 95% dimethylpolysiloxane). Splitless injection mode (1:10) was used to set the injection and detector temperatures at 300°C [31]. The temperature programming was between 40°C and 200°C at a rate of 6°C/min. Helium was used as the carrier gas at a flow rate of 1.2mL/min. The following MS parameters were used: an ion source temperature of 300°C, an electron ionization mass spectrum set at 70eV, and a mass range of 50 to 650amu. The compounds were characterized by the determination of the retention index (RI) relative to those

of a homologous alkane series (C_8–C_{20} and C_{21}–C_{40}) and matching the recorded mass spectra to those stored in the spectrometer database NIST 2014 (National Institute of Standards and Technology) and PubChem Libraries [29]. NIST MS Search 2.2 Library 2014 was used to analyze the data, and Software Thermo XcaliburTM 2.2 SP1.48 was used to operate the data acquisition.

2.7. Determination of the antioxidant activities.

The antioxidant properties of TaLME, TaLAE, and TaLEO were evaluated using four different methods, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC).

DPPH radical scavenging assay: Using the technique described by Awa *et al*. [32], the scavenging activity of TaLME, TaLAE, and TaLEO was assessed with small modifications. Briefly, 2.5mL of methanolic solution of the free radical DPPH (0.4%) was added to 100µL of sample or acid ascorbic at each concentration (250–7.81µg/mL). The absorbance values were measured using a spectrophotometer at 517nm after 30 minutes of incubation. All trials were performed in triplicate. The DPPH scavenging activity was determined by the calculation of the percentage of inhibition using the following equation (1) :

$$\%\ \text{of inhibition} = \langle(\mathbf{A}_{\text{DPPH}} - \mathbf{A}_{\text{S}})|\mathbf{A}_{\text{DPPH}}\rangle \times 100 \qquad (1)$$

Where $\mathbf{A}_{\text{DPPH}}$ is the absorbance of DPPH solution, $\mathbf{A}_{\text{S}}$ is the absorbance of the sample. The IC_{50} (concentration inhibiting the reduction of 50% of the free radical) of each sample was subsequently calculated from the equation, which determines the percentage of inhibition as a function of the concentration of the sample.

ABTS radical scavenging assay: The $ABTS^{\bullet+}$ free radical-scavenging activity was identified as previously reported by Miguel *et al*. [33] with small modifications. The $ABTS^{\bullet+}$ radical cation was produced by reacting an aqueous solution of ABTS (7 mM) with potassium persulfate (2.45mM) for 16 hours in the dark at room temperature. An absorbance of 0.700 at 734nm was obtained by diluting the ABTS solution with methanol. Then, 1450µL of $ABTS^{\bullet+}$ solution and 50µL of the sample were combined, and after 6 minutes, the absorbance was measured at 734nm. The following equation (2) was used to determine the scavenging activity of samples:

$$\%\ \text{of inhibition} = \langle(\mathbf{A}_{\text{ABTS}} - \mathbf{A}_{\text{S}})|\mathbf{A}_{\text{ABTS}}\rangle \times 100 \qquad (2)$$

Where $\mathbf{A}_{\text{ABTS}}$ is the absorbance of the ABTS solution, $\mathbf{A}_{\text{S}}$ is the absorbance of the sample. The extract concentration providing IC_{50} was calculated from the graph of the scavenging effect percentage against extract concentration in the solution.

FRAP assay: Reducing power was calculated based on the method described by Jabri-Karoui *et al*. [34] with minor modifications. Briefly, 1000µL of the plant extracts or ascorbic acid (1mg/mL) was mixed with 2500µL of sodium phosphate buffer (0.2M, pH 6.6) and 2500µL of potassium ferricyanide (1%). After being incubated at 50°C for 20 minutes, the mixture solution was added to 2500 µL of trichloroacetic acid (10%) and centrifuged at 3000rpm for 10 minutes. 2500µL from the upper layer of the solution was mixed with 2500µL of distilled water and 500µL of $FeCl_3$ (0.1%). Absorbance was measured at 700nm. The results are expressed by IC_{50} (µg/mL).

ORAC assay: The ORAC test was carried out following the method utilized by Contreras *et al*. [35] with small modifications. The final reaction mixture was 200µL and was conducted in 75mM phosphate buffer (pH 7.4). A sample at various concentrations (0.0125 at

0.0025mg/mL), 20µL of Trolox (10 at 80µM), 60µL of AAPH, and 120µL of fluorescein are present in each well of a 96-well black polystyrene microplate. Daily preparations of AAPH and Trolox solutions were made, and fluorescein was diluted from a stock solution (1.17mM) in 75mM phosphate buffer (pH 7.4). The excitation and emission wavelengths were set at 485nm and 528nm, respectively. For 80 minutes, the fluorescence was recorded at 1 min intervals. This experiment was carried out using a multidetection plate reader (Synergy H1, Vermont, USA) controlled by Gen5 Biotek software 3.04. The ORAC values were expressed as µmol of the sample's Trolox equivalents (TE)/g.

2.8. Analyses of DNA degradation.

The rate of calf thymus DNA degradation was assessed using *Tetraclinis articulata* essential oil, methanolic and acetonic extracts: antioxidant (AO) and prooxidant (PO), on both assays with and without $FeCl_3$ (Iron), using the method previously described by Silva *et al*. [36]. In sterile distilled water, the DNA solution was prepared at 0.25mg/mL and stored for 7 days at 4°C in the dark with daily homogenization. In PBS-7.4, a stock solution of essential oil and methanolic and acetonic extracts was prepared at a concentration of 1mg/mL. The volumes of the various components were cited as follows in both assays: AO assay with iron and without 200µL of DNA with 10µL of FeCl3, 50 or 400µL of H_2O_2 (7.01M), various sample volumes (400, 300, 200, 100, 80, 60, and 40 µL), along with PBS phosphate buffer to bring the overall volume to 1000µL. The positive control (no degradation) was a PBS-adjusted DNA solution, either with or without iron.

An agarose gel electrophoresis was performed after the sample was incubated for 1h at 37°C in the dark. To achieve this, loading buffer (1:4) was added to each sample, and 10 µL were then transferred to an agarose solution (0.75%) gel that had been made with TAE buffer and GreenSafe Premium at a concentration of 0.03µL/mL. After that, 1.25 hours of electrophoresis at 150mV were performed, gels were examined using a molecular imager (GelDOC XR), and Image Lab Software v5.1 (BioRad, Hercules, California, USA) was used to process the resulting image.

According to the calculations made using the equations, the results are expressed as a percentage of inhibition of DNA band degradation (3) and a percentage of DNA degradation (4) for AO and PO assays, respectively:

$$\text{Inhibition of DNA degradation } (\%) = [(I_{sample} - I_{background})/I_{DNA\ solution}] \times 100 \quad (3)$$

$$\text{DNA degradation } (\%) = 100 - [(I_{sample} - I_{background})/I_{DNA\ solution} \times 100] \quad (4)$$

Where I_{sample} is the intensity of each sample band, $I_{background}$ is the intensity of the background, measured beside the control bands, and $I_{DNA\ solution}$ is the intensity of the intact DNA solution. A triplicate of each test was performed.

2.9. Genotoxicity.

Salmonella Typhimurium TA98 with and without metabolic activation (S9) was used in the Ames test [37] to test the mutagenicity of TaLME, TaLAE, and TaLEO of *T. articulata* at a concentration of 100µg/mL. Positive controls included 2-aminoanthracen and daunomycin, and negative controls included DMSO 4% and water.

2.10. Evaluation of antifungal activity.

Five fungi were used in this study: *Aspergillus fumigatus* ATCC 46645 and *Candida albicans* ATCC 10231 from the American Type Culture Collection-ATCC. *Epidermophyton floccosum* FF9, *Trichophyton rubrum* FF5, and *Microsporum canis* FF1 from clinical strains.

The antifungal activity of *T. articulata* was evaluated using the broth microdilution technique, which has been previously described by Erbiai *et al*. [30] and suggested by the Clinical and Laboratory Standard Institute (CLSI) (M38-A2, filamentous fungi; M27-A3, yeasts). The methanolic, acetonic extract and the essential oil were dissolved in dimethyl sulfoxide (DMSO), which was then serially diluted in RPMI 1640 medium to the required concentrations (The final solution prepared must contain no more than 2% DMSO). In a 96-well plate were distributed 100µL of each dilution. The inoculum of yeasts (cells) and filamentous fungi (spores) was prepared using physiological water, and the standards used were spore count for filamentous fungi and spectrophotometric method for yeasts, respectively. Inoculum was diluted in RPMI until the desired concentration and 100µL were added to each well. The concentration that prevented any discernible growth (100%) was determined to be the minimum inhibitory concentration (MIC) after incubation at 36ºC for 48 hours for *Aspergillus fumigatus* and *Candida albicans* and at 26ºC for 5-7 days for dermatophytes. To evaluate the minimum fungicidal concentration (MFC) 10µL were taken from the MIC determination's wells without turbidity and inoculated into sabouraud dextrose agar petri dishes. The lowest concentration of the fungicidal agent that completely prevented growth under the incubation conditions was known as the minimum fungicidal concentration. Controls include sterility with RPMI-1640, growth with RPMI-1640 medium plus DMSO (1.0%) and fungi suspension, quality using *Candida krusei* ATCC 6258 and carried out with voriconazole;

2.11. Statistical analysis.

Values were expressed as mean standard deviation (SD) for each test performed in triplicate. Using GraphPad Prism 8.0.1 software (San Diego, CA, USA), a one-way analysis of variance (ANOVA) was used to determine the statistical significance of the data, and then a posthoc Tukey's multiple comparison tests were performed with a = 0.05 significance level.

3. Results and Discussion

3.1. Extraction yield and total phenolics and flavonoids contents.

The extraction yield, TPC, and TFC values of the essential oil and organic extracts of *T. articulata* leaves are shown in Table 1.

Table 1. Extraction yield and total phenolics and flavonoid contents of essential oil (TaLEO) and methanolic and acetonic extracts (TaLME, TaLAE) of *T. articulata* leaves.

Plant Extracts	TaLEO	TaLME	TaLAE
Extractive yield (%)	0.77 ± 0.02	32.70 ± 1.49	24.04 ± 0.98
Total phenolics (µg GAE/g of DE or EO)	5.03 ± 0.32	125.75 ± 9.33	113.63 ± 0.89
Total flavonoids (µg EQ/g of DE or EO)	1.18 ± 0.01	78.26 ± 0.25	61.51 ± 11.68

The value of TaLEO extraction yield was 0.77%, which is almost similar to the studies reported by Toumi *et al*., with values of 0.78% 0.75% in the region of Ouled Mimoun and El Haçaiba, respectively, and Barrero *et al*. with the value of 0.70% [38,39]. However, our yield was higher than the previous works [23,24,38,40–42]. During a study carried out on the leaves

of *T. articulata*, the content obtained for different extraction times was variable: 0.06% (3 hours), 0.22% (4 hours), 0.35% (5 hours), and 0.61% (6 hours). For organic extract yield, the results showed that methanolic extract was given a value higher than acetonic one with a percentage equal to 32.70 and 24.04, respectively. Compared to the literature on the same species, our values of TaLME and TaLAE extraction yield are significantly higher than those reported by Sliti *et al*. [43]; based on their findings, 50% and 80% of methanolic extract yields ranged from 14 %to 19.1% depending on the study periods (January, April, November). The difference in the yields may be due to certain factors such as the climate, the nature of the soil, the harvest and extraction period, and sometimes the use of different extraction equipment [24].

The Folin-Ciocalteu reagent and the gallic acid standard calibration curve were used to determine the total phenolic content based on the absorption values of the various extract solutions. As shown in Table 1, There was a significant amount of phenolic compounds in all three extracts, whereas the highest TPC obtained was in TaLME (125.75µg GAE/g of DE, followed by TaLAE (113.63µg GAE/g of DE), while, the essential oil was observed to have the lowest TPC (5.03µg GAE/g of EO). Compared to our results, El Guiche *et al*. [44] reported a lower TPC content for the methanolic extract (34.71µg GAE/mg DM), as well as Sliti *et al*. [43] determined a lower content for both 50% and 80% methanolic extracts. However, Djouahri *et al*. [45] found that ethanolic (70%) and methanolic (100%) extracts from cones of *T. articulata* contained a significant amount of TPC with a value of 185.18 and 156.63 mg GAE/mg extract, respectively, which were noted to be higher than our samples. El Jemli *et al*. [46] also found significant TPC content (175.67µg GAE/mg edw) in aqueous extracts from *T. articulata* leaves compared to ours.

Regarding the TFC, the results in Table 1 reveal that the methanolic extract had the highest flavonoid content in comparison with the acetonic extract and essential oil with the values of 78.26, 61.51µg EQ/g of DE, and 1.18µg EQ/g of EO, respectively. Compared to previous studies [46], the aqueous and methanolic extracts contained lower TFC concentrations with 11.78µg QE/mg edw and 37.14µg QE/mg DM, respectively. Further, Ben Jemia *et al*. [14] demonstrated that 80 % aqueous acetone extract contained a higher content of TFC (17.97mg CE/g DW).

Methanol was the best solvent for extracting and determining bioactive compounds when we compared the extraction yields, total phenolics content, and total flavonoids content between TaLEO, TaLME, and TaLAE.

3.2. Phenolic compounds by LC-MS analysis.

The phenolic compounds identified and quantified in methanolic (80%) and acetonic (80%) extracts of *T. articulata* leaves are shown in Table 2.

Table 2. Phenolic compounds identified and quantified in the methanolic (TaLME) and acetonic (TaLAE) extracts of *T. articulata* leaves (µg/g of DE).

Peaks	Phenolic compounds	TaLME	TaLAE
1	Gallic acid	2.82	3.69
2	Protocatechuic acid	27.76	24.75
3	*p*-Hydroxybenzoic acid	-	26.59
4	Vanillic acid	5.14	9.57
5	Syringic acid	7.36	3.40
6	*p*-Coumaric acid	0.50	3.59
7	Ferulic acid	3.48	6.81

Peaks	Phenolic compounds	TaLME	TaLAE
8	Paraben acid	12.06	8.29
9	Cinnamic acid	3.00	3.79

Among all the compounds analyzed, protocatechuic acid showed the highest amount with a value of 27.76µg/g of DE, followed by paraben acid with a content of 12.06µg/g of DE, syringic acid with a content of 7.36µg/g of DE, and the absence of p-hydroxybenzoic acid for TaLME extract. While p-hydroxybenzoic acid was the major compound with a value of 26.59µg/g of DE, followed by protocatechuic acid with a content of 24.75µg/g of DE, vanillic acid with a value of 9.57µg/g of DE, and paraben acid with the content of 8.29µg/g of DE for TaLAE extract. The compound found with the lowest concentration was p-coumaric acid (0.50µg/g of DE) in TaLME. The absence of the p-hydroxybenzoic acid in the methanolic extract is probably due to several factors, including sampling, processing, storage, and the type of solvent used in the extraction, all of which could have an impact on the number of active compounds in our plant, since the extract may contain traces of residual solvent, by the interference of the solvent with the bioassay [47]. In a previous work by Djouahri *et al*. [45], gallic acid was found to be the major phenolic compound in cones methanolic extract (100%) of *T. articulata* from Algeria with the value of 1.98 ± 0.20µg/g of dry matter. In the same context, the chromatographic profile of *T. articulata* revealed The existence of several phenolic acids and associated substances, some of which are known to have interesting biological effects like antioxidant, insecticidal, and cytotoxicity [14,47–49].

3.3. Chemical composition of T. articulata essential oil by GC-MS analysis.

The GC-MS chromatogram of *T. articulata* essential oil is shown in Figure 1, and it reveals the presence of 22 peaks corresponding to 22 different chemical compounds, which are presented in Table 3 with their percentage, retention time, and retention index.

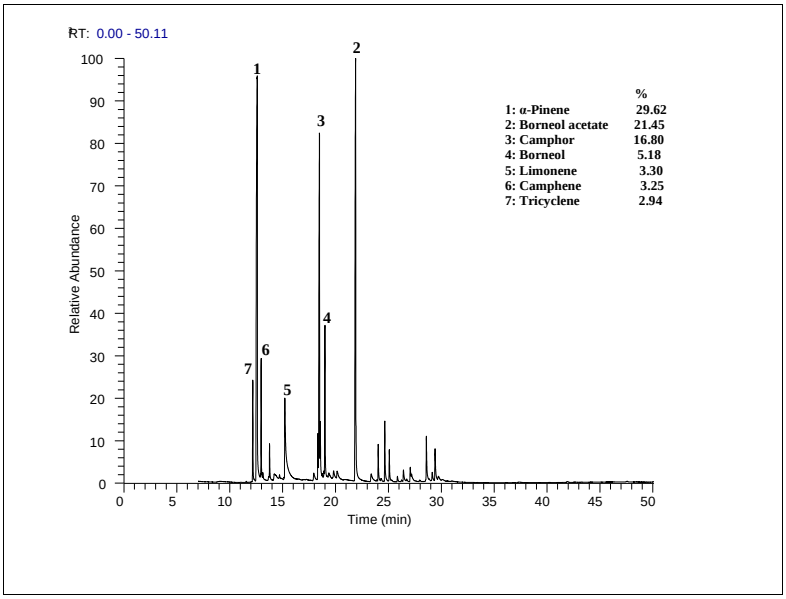

Figure 1. GC-MS chromatographic profile of *T. articulata* essential oil leaves.

Table 3. Chemical composition percentage of *T. articulata* essential oil (%) with values of retention time (RT) and retention indexes (RI).

Chemical compound	RT	RI	%
Tricyclene	12.17	930	2.94
α-Pinene	12.60	940	29.62
Camphene	12.97	960	3.25

Chemical compound	RT	RI	%
Limonene	15.20	1031	3.30
Isopinocarveol	18.33	1138	1.41
Camphor	18.48	1144	16.80
Camphene hydrate	18.56	1150	1.62
Pinocarvone	18.91	1164	0.37
Borneol	19.00	1165	5.18
Myrtenal	19.82	1196	0.38
Borneol acetate	21.90	1285	21.45
α-Copaene	24.03	1374	1.15
Isoledene	24.65	1377	1.84
Caryophyllene	25.08	1418	1.02
Humulene	25.85	1452	-
Germacrene D	26.42	1470	0.52
α-Murolene	26.73	1495	0.18
α-Amorphene	27.06	1498	0.64
Caryophyllene oxide	28.59	1582	1.63
Humulene hypoxide II	29.14	1606	0.63
Cubenol	29.41	1642	1.40
Cadinol	29.73	1660	0.47
Total			95.8

These 22 compounds represented 95.80% of the analyzed essential oil, and their identification was based on a comparison with the retention indices in the database of the National Institute of Standards and Technology (NIST2014). These important chemical compositions detected in the essential oil of the *T. articulata* tree growing in northern Morocco were predominated by the following substances: α-pinene (29.62%), bornyl acetate (21.45%), camphor (16.80%), borneol (5.18%), limonene (3.30%), and camphene (3.25%) (Figure 1, Table 3). This result was observed to be partly different from the one reported by Barrero *et al*. [39] of the same sample collected near our collection site (Tetouan region), which showed that camphor (19.1%), bornyl acetate (16.5%), borneol (9.6 %) and caryophyllene oxide (3%) were the main components detected. In other studies from Morocco, one from Khenifra [23] and another one from Khemisset [24], were found bornyl acetate (38.54-30.74%) and α-pinene (6.71-23.54%) as the major chemical compounds identified in their TaLEOs. Similar to these results from Khenifra and Khemisset regions, a previous work from Algeria by Chikhoune *et al*. [13] noted that bornyl acetate (40.2–59.2%) and α-pinene (19.8–24.9%) were dominant in TaLEO from Hammam Melouane and Tipaza regions, respectively. However, Herzi *et al*. [40] from Tunisia cited α -pinene (24.9%) as the most abundant constituent, which was in agreement with our findings, without detecting bornyl acetate in their hydrodistillation extract of *T. articulata* leaves. *T. articulata* has been used in traditional medicine for centuries because of the many biological properties that make it an excellent medicinal plant. It is known for its antioxidant, anti-inflammatory, and cytotoxic properties. Interestingly, *T. articulata* EO contains a wide range of compounds; several of these compounds are known for their anti-inflammatory activity, such as α-pinene, which could attain 32%, and limonene attain 9% in *T. articulata* EO [50–52]. In addition, chemical composition plays a key role in the antioxidant activity of *T. articulata* extracts since a change in composition can affect the activity rate. The study obtained by Herzi *et al*. [40] indicated that supercritical fluid extraction extracts demonstrated high antioxidant activity in DPPH and ABTS assays, with α-pinene (up to 31.32%), linalool acetate (up to 18.18%), alloaromadendrene (up to 11%) and γ-caryophyllene (up to 6%) were the major compounds. In general, the difference in the percentage and the predominance of the detected molecules from one region to another may be due to many

factors, including geographic, microclimatic, and edaphic conditions of the collection site [28], as materials and conditions of the sample preparation and characterization.

3.4. Antioxidant activity.

The antioxidant activities of TaLME, TaLAE, and TaLEO were determined spectrophotometrically using four different assays: DPPH, ABTS, FRAP, and ORAC activity. The reason for choosing several methods to detect antioxidant activity is due to the difference in the mechanisms that the samples perform against free radicals, and this activity may be affected by several other factors. The results of these methods were expressed as the percentage of inhibition. Trolox was used as a reference antioxidant for ABTS and ORAC assays, while ascorbic acid was used for DPPH and FRAP assays. Using the linear equation for these assays, the IC_{50} value was calculated and presented in Table 4.

Table 4. The antioxidant capacity of the studied *T. articulata* essential oil (TaLEO), methanolic (TaLME), and acetonic (TaLAE) extracts.

Extracts	DPPH (IC_{50}; µg/mL)	ABTS (IC_{50}; µg/mL)	FRAP (IC_{50}; µg/mL)	ORAC (µmol TE/g of extract)
TaLME	10.30 ± 0.01	0.40 ± 0.02	18.33 ± 0.00	15779.58 ± 42.58
TaLAE	27.10 ± 0.01	2.00 ± 0.13	36.00 ± 0.00	6362.30 ± 18.16
TaLEO	60.00 ± 0.00	220.00 ± 0.01	50.00 ± 0.00	6726.37 ± 103.64
Ascorbic Acid	3.64 ± 0.01	-	5.40 ± 0.00	-
Trolox	-	0.1±0.05	-	-

Concerning the DPPH assay, the *T. articulata* extracts and essential oil were capable of reducing the stable DPPH (purple-colored radical) into DPPH-H (yellow-colored radical). The TaLME had the strongest free radical-scavenging activity with an IC_{50} value of 10.3µg/mL, while the lowest capacity to reduce DPPH was observed in TaLEO with IC_{50} equal to 60µg/mL. TaLME, TaLAE, and TaLEO were less effective than ascorbic acid, a synthetic antioxidant (IC_{50} = 3.64µg/mL). Compared with the literature, our results were superior to those of El Jemli *et al*. [53] (IC_{50} = 12.05 ± 0.24mg/mL) who studied TaLEO leaves in Marrakech. In addition, Djouahri [54] found that methanol crude extracts collected from the alwestern region of Algeria had significantly lower DPPH scavenging activity with IC_{50} equal to 28.55mg/mL. The aqueous extract antioxidant activity obtained from *T. articulata* leaf infusions revealed that DPPH scavenging activity has an IC_{50} = 27.38µg/mL, almost similar to our results obtained for acetonic extract [46].

Regarding the ABTS test, our findings revealed a significant activity. The IC_{50} values were found to be ranged from 0.4 ± 0.2 µg/mL to 220 ± 0.01µg/mL. It can be noticed that the superior ABTS effect was observed in the methanolic extract, followed by the acetonic extract, whereas the lowest effect was observed in the essential oil. However, our results are lower than the reference antioxidant (Trolox IC_{50} = 0.1 ± 0.05µg/mL). Our results were highest to El Jemli *et al*. [53], which measured the antioxidant activity of EO obtained from *T. articulata* leaves (8.90 mg/mL). Additionally, the antioxidant effect of the aqueous extract obtained from *T. articulata* leaf infusions showed that ABTS activity had an IC_{50} equal to 32.92µg/mL lower than our finding [46].

The finding revealed a remarkable reducing power for the extracts and essential oil with IC_{50} values ranging from 18.33 to 50µg/mL. The TaLME had an important activity with IC_{50} = 18.33µg/mL, followed by acetonic extract with IC_{50} = 36µg/mL, while the lowest effect was observed in TaLEO with IC_{50} equal 50µg/mL. Moreover, the synthetic antioxidant, ascorbic

acid, showed higher activity (IC_{50} = 5.40µg/mL) than our results. As a result of their study, El Jemli *et al*. [53] provided lower results than our study, which measured the reducing power activity of EO obtained from *T. articulata* leaves, and they found IC_{50} equal to 0.15 mg/mL. In their study, Djouahri [54] reported that methanol crude extracts had a lower reducing power activity than our methanolic extract, with an IC_{50} of 500µg/mL. Similarly, the aqueous extract obtained from *T. articulata* leaf infusions indicated that reducing power activity had an IC_{50} = 47.12µg/mL [46].

Regarding the ORAC assay of the investigated medicinal plant, we found that the values ranged from 6362.30 to 15779.58µmol TE/g of extract. The highest ORAC value was found in the methanolic extract (15779.58µmol TE/g of extract), followed by essential oil (6726.37) and acetonic extract (6362.30). To our knowledge, this type of activity is used for the first time.

3.5. Agarose gel electrophoresis for DNA protection.

The DNA degradation capabilities of H_2O_2 and H_2O_2 in the presence of iron cations are shown in Figure 2. The methanolic extract showed an inhibition percentage of DNA oxidation above 100% for the AO without iron. This indicates that the tested concentrations of methanolic extract were able to inhibit peroxide-induced DNA cleavage, which is an oxidative process. In some cases, inhibitions of DNA degradation exceeded 100%; this suggests an interaction between the extracts and the DNA molecule, which increases fluorescence. Moreover, the acetonic extract and essential oil revealed little to no protective effect.

Furthermore, in the presence of iron cations (AO with iron), iron reacts with H_2O_2 via Fenton reactions. Consequently, hydroxyl and peroxyl radicals are produced, which are much more aggressive to the DNA molecule than peroxide alone [36]. However, iron is also found in several biologically relevant tissues [55], so including this data may also provide valuable insight into the overall efficacy of the extract.

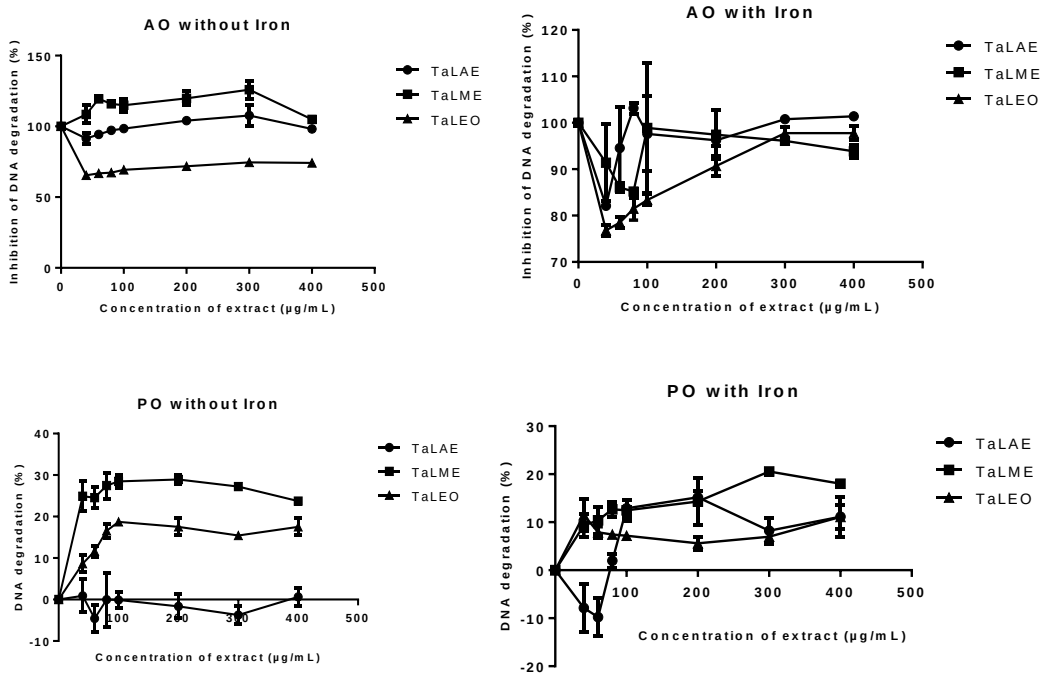

Figure 2. Evaluation of DNA degradation inhibition (%) and (%) degradation DNA in antioxidant (AO) and prooxidant (PO) tests at various concentrations of *T. articulata* essential oil (TaLEO) and extracts (TaLME, TaLAE).

Regarding the AO with iron, the concentration of 80 µg/mL was capable of fully protecting DNA (100%) for acetonic extract compared to methanolic extract and essential oil, which showed a very small protective effect (Figure 2).

The acetonic extract had no prooxidant effect on the PO without iron. According to the results, DNA did not degrade at concentrations between 60 and 300µg/mL (negative values for DNA band degradation). Nevertheless, significant DNA band degradation was observed for both the methanolic extract and the essential oil, contributing to the prooxidant effect. For the PO with iron, concentrations between 40 and 60µg/mL demonstrate no DNA degradation for acetonic extract; in contrast, the methanolic extract and essential oil degraded the DNA bands (Figure 2).

Benoutman *et al*. [56] evaluated the ability of the essential oil, methanolic, and acetonic extracts of *Thymus capitatus* to prevent oxidant-induced DNA damage. They found that AO without iron of methanolic extract can protect the DNA from degradation at a concentration ranging from 300 to 400µg/mL, and the AO with iron revealed that methanolic and acetonic extract were capable of fully protecting DNA (100%) at 200µg/mL. On the other hand, at concentrations between 150 and 400µg/mL, the PO without iron had no prooxidant effect in the acetonic extract. Meanwhile, for the PO with iron, the DNA bands were degraded by all extracts. Compared to our results, the AO without iron of TaLME revealed inhibition of DNA oxidation above 100% at all concentrations, and the TaLAE at 80µg/mL could fully protect DNA in the AO with iron. For the PO, the TaLAE can protect the DNA in the 40 to 60µg/mL concentration. In general, at low concentrations, the phenolic compounds typically function as antioxidants. Still, at high concentrations, they can undergo self-oxidation and transform into prooxidants, which results in the production of semiquinones and superoxide radicals [57,58]. Further, Silva *et al*. [36] demonstrated that ascorbic acid, known for its potent antioxidant properties, could also act as a prooxidant in some concentrations, leading to the significant degradation of DNA bands. Overall, extracts in iron-containing systems appeared to interact less with DNA than in systems containing only DNA and extracts. Since iron is oxidized to iron II in the presence of air, resulting in electron capture, this reduction may oxidize some components of the extract, resulting in a loss of reactivity.

3.6. Genotoxicity.

To ascertain whether metabolic activation could cause the mutagenicity, essential oil and extracts were tested both with and without the S9 fraction and, therefore, whether the extracts could be used as food. Using the 2.5-folding rule, the results were considered positive or negative compared to the respective solvent control. Using the strain of *Salmonella typhimurium* (TA98), 100µg/mL of each plant extract was added, both with and without metabolic activation (S9). Table 5 lists the number of revertant colonies per plate.

Table 5. Genotoxicity of methanolic, acetonic extracts and essential oil of *T. articulata* (TaLME, TaLAE, and TaLEO) against *Salmonella typhimurium* TA98 (+S9) and bacterial strain TA98 (−S9).

Extracts solvent	Dose/Plate µg/mL	TA98 (+ S9)	TA98 (- S9)
TaLME	100	15.50 ± 3.50	21.00 ± 02
TaLAE	100	11.00 ± 01	25.50 ± 4.50
TaLEO	100	27.00 ± 04	28.50 ± 3.50
DMSO 4 %	100	9.00 ± 01	17.00 ± 01
Water	100	11.50 ± 0.50	16.50 ± 1.50
2-Aminoanthracen	10	1232 ± 28	-
Daunomycin	06	-	481.50 ± 3.50

To determine whether a sample is mutagenic, we used the 2.5-folding rule: Without metabolic activation (-S9), values below 23 and 29 colonies per plate for samples diluted in DMSO 4 % or an aqueous solvent, respectively, were deemed non-mutagenic; in the presence of metabolic activation (+S9), values inferior to 41.25 and 42.5 colonies per plate in DMSO 4 % and an aqueous solvent, respectively, were deemed non-mutagenic. Therefore, the results reported in Table 5 reveal that methanolic and acetonic extracts and essential oil of *Tetraclinis articulata* were bereft of any mutagenic activity.

3.7. Antifungal activity.

The MICs and MFCs of TaLME, TaLAE, and TaLEO from leaves of *T. articulata* obtained by the broth microdilution method are shown in Table 6.

Table 6. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the *T. articulata* methanolic and acetonic extracts (TaLME, TaLAE), and essential oil (TaLEO).

Fungal Strains	TaLEO (µL/mL)	TaLME (mg/mL)		TaLAE (mg/mL)	
	MIC	MIC	MFC	MIC	MFC
***Candida albicans* ATCC 10231**	≥20.00 ± NA	3.57 ± 1.24	8.00 ± 2.45	0.88 ± 0.30	5.00 ± 0.00
***Aspergillus fumigatus* ATCC 204305**	20.00 ± 0.00	5.00 ± 0.00	12.00 ± 6.78	3.44 ± 1.21	10.00 ± 0.00
***Trichophyton rubrum* FF5**	2.50 ± 0.00	0.90 ± 0.31	1.88 ± 0.63	0.79 ± 0.27	1.25 ± 0.00
***Microsporum canis* FF1**	2.50 ± 0.00	0.86 ± 0.30	0.88 ± 0.30	0.71 ± 0.21	1.00 ± 0.30
***Epidermophyton floccosum* FF9**	2.50 ± 0.00	0.79 ± 0.27	1.75 ± 0.61	1.10 ± 0.27	1.75 ± 0.61

The TaLME and TaLAE were able to inhibit the growth of all fungi. However, MIC and MFC were lower for TaLAE (MIC 0.71–3.44mg/mL and MFC 1.00–10mg/mL) than for TaLME (MIC 0.79–5mg/mL and MFC 0.88–12mg/mL). Dermatophytes were most susceptible, *M. canis* the most susceptible for the TaLAE, and *E. floccosum* for the TaLME.

The TaLEO also inhibited the growth of all fungal strains studied at concentrations ranging from 2.5 to ≥20µL/mL; the lowest inhibition concentration was observed for dermatophytes (MIC = 2.5µL/mL).

Comparing our results to the antifungal effect of *T. articulata* EO against *C. albicans*, using the direct contact method [59], there was a significant difference between their MIC (268 µg/mL) and ours (≥20 µL/mL). Moreover, Ibrahim *et al*. [60] revealed that *T. articulata* EO could inhibit *C. albicans* at a MIC that inhibits 90% of the growth of 0.659µL/mL (our results are for MIC that inhibits 100% of growth). Several factors may affect the activity of a plant substance, including the extraction process and the amount of active substances. In addition, it should be remembered that *T. articulata* contains α-pinene, bornyl acetate, caryophyllene, camphor, linalool acetate, borneol, limonene, and camphene as the dominant constituents in many studies of EO of different organs of *T. articulata* in different countries. These compounds have known antimicrobial properties [13,14,40,61–65].

Many other studies have shown that *T. articulata* has antifungal properties. Karima *et al*. examined the antifungal activity of *T. articulata* aqueous extracts in vitro, and they found that at 1500 ppm, these extracts significantly reduced *Pyrenophora teres* mycelia growth by 87.05% [66]. Furthermore, *T. articulata* EO was investigated for its antifungal activity against *Fusarium spp*. *Aspergillus flavus*, and *Aspergillus niger* by using direct contact [67]. It was found that *Aspergillus niger* and *Aspergillus flavus* were inhibited at 61.63% and 64.44% respectively. At the same time, *Fusarium spp*. was completely inhibited by the concentration of oil at 20 µL/mL. According to Boubaker and colleagues [68], the essential oil extracted from the aerial part of *T. articulata* produced antifungal activity in vitro against *Geotrichum citri-*

aurantii, *Penicillium italicum*, and *Penicillium digitatum*. The MIC for the spore germination of all fungi was 4.0 µL/mL. Bourkhiss *et al*. [69] evaluated the antifungal activity of sawdust oil against *Aspergillus niger*, *Penicillium parasiticus*, and *Trametes pini*. A 1/1000 v/v inhibitory activity was found. The antifungal effect of *Tetraclinis articulata* oil was also studied against five phytopathogenic fungi: *Botrytis cinerea*, *Fusarium avenaceum*, *Fusarium oxysporum*, *Fusarium culmorum*, and *Fusarium solani*. As a result, all tested fungi were inhibited, with inhibitions ranging from 25.36% to 71.17%. It was observed that the preparation had a strong antifungal effect against *Botrytis cinerea* (71.77%) [70].

Tetraclinis articulata has interesting antifungal properties. These effects were influenced by several of several variables, including the collection site, the type of extract, methodology, and secondary metabolites that inhibited fungal growth.

4. Conclusions

As part of the valorization of Moroccan flora, our goal was to investigate the chemical composition and biological activities of aromatic and medicinal plants from northern Morocco, which are widely used in traditional medicine. One of these plants is *Tetraclinis articulata*, whose leaves are known for their pharmacological properties. To achieve our goal, The purpose of the current study is to investigate the chemical composition, identification of phenolic compounds, total phenols, flavonoid contents, the antioxidant, antifungal activity, and genotoxicity test of *T. articulata* essential oil, and methanolic and acetonic extracts. The phytochemical study on the essential oil of *T. articulata* showed the presence of a majority of compounds, such as α-pinene, bornyl acetate, and camphor, besides other components. Further, the characterization of phenolic compounds by LC-MS of methanolic extract indicated that protocatechuic acid was predominant in TaLME, followed by paraben and syringic acid. Moreover, the *p*-hydroxybenzoic was the major compound in TaLAE, followed by protocatechuic acid and vanillic acid. Total phenols and flavonoids of the methanolic extract were discovered to be higher than those of the acetonic extract and essential oil. In addition, all extracts (TaLME, TaLAE, and TaLEO) showed considerable biological activities, while the genotoxicity test revealed that the extracts and essential oil were devoid of any mutagenic activity. These results suggest that this work can prove that plants are very interesting reservoirs for future research.

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Conflicts of Interest

The authors declare no conflict of interest.

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