Supporting Information

***Rec. Nat. Prod.* 18:5 (2024) 538-543**

Chemical Composition and Evaluation of the Antibacterial, Synergistic Antibacterial, Antioxidant and Cytotoxic Activities of the Essential Oil of *Macrothelypteris torresiana* (Gaudich.) Ching

Xiangyi Li[1], Shu Qiu[1], Shiyu Song[1] and Pengxiang Lai[*1,2]

[1] *Sdu-Anu Joint Science College, Shandong University, Weihai, 264209, P. R. China*
[2] *Marine College, Shandong University, Weihai, 264209, P. R. China*

Table of Contents	Page

Experimental Section

S1: *Extraction and chemical characterization of MT-EO*

The aerial parts of *M. torresiana* (Figure S1) were hydrodistilled for 3 h using Clevenger equipment to extract the essential oil. The oil was dried using anhydrous sodium sulfate and kept at 4 °C until analysis.

The GC/FID analysis was performed using the Agilent 7890A gas chromatographer, which uses an HP-5MS 30 m × 0.25 mm silica column and 0.25 m fixed phase. The Agilent 7890A was equipped with an Agilent 5975C mass detector and a non-polar HP-5MS column for GC/MS analysis. The oven temperature was initially set at 60 °C, maintained for 1 minute, then increased to 230 °C at a rate of 5 °C per minute and held for 14 minutes. The injection of helium carrier gas was performed at a flow rate of 1.3 mL/min with a split ratio of 50:1. The injector and the ion source were programmed at temperatures of 250 °C and 240 °C,

respectively. The conditions for mass spectrometry were as follows: electron impact ionization mode at 70 electron volts with a mass range of 50-550 m/z. MT-EO component was identified using a comparative analysis of its mass spectrum with those present in the NIST and Wiley libraries, as well as through a comparison of its retention index (relative to C_7-C_{30} n-alkanes) with the standard RI provided in the literature [1-4]. Additionally, the chromatogram profile of MT-EO is shown in Figures S2 and S3.

S2: *Antibacterial activity of MT-EO*

The antibacterial capabilities of MT-EO were assessed against four bacterial strains using established methods [5]. In order to ascertain the MIC of MT-EO, the bacterial cultures were diluted to a concentration of 10^6 CFU/mL and mixed with various twofold dilutions of MT-EO that were prepared in a 2% dimethylsulfoxide solution. These mixtures were then transferred to a 96-well plate. The MIC of MT-EO was identified as the lowest concentration to inhibit bacterial growth after incubation of the plate at 37 °C for 24 hours, using TTC as an indicator.

To determine the MBC of MT-EO, 100 µL of a colorless solution from the MIC plates were transferred onto MH agar and kept for another 24 hours in incubators at 37 °C. The MBC was determined to be the lowest concentration of MT-EO where no bacterial growth appeared on the agar plates.

S3: *Synergistic effect of MT-EO with traditional antibiotics*

The antibacterial interaction of MT-EO combined with conventional antibiotics was assessed using the checkerboard method in 96-well plates based on the results obtained from the antibacterial activity assay [6]. In summary, a series of 50 µL of twofold serial dilutions of MT-EO was added sequentially along the rows, while the antibiotic was diluted sequentially along the columns of a 96-well microtitre plate, respectively. The concentration in the wells varied from 1/64 to 4 times the MIC. Then, 100 µL of standardized bacterial inoculum, approximately 1×10^6 CFU/mL, was introduced into each well and incubated at 37 °C for 24 hours. The FICI, representing the synergistic effects of MT-EO and antibiotics, was calculated with the following formula:

$$\text{FICI} = \frac{\text{MIC of EO in combination}}{\text{MIC of EO alone}} + \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}}$$

If FICI ≤ 0.5, the results were regarded as synergistic [7].

Figure S1: *Cytotoxicity of MT-EO*

Four cancerous human cell lines (HCT-116, A-549, HepG2, and MCF-7) and one normal human cell line LO2, were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 µnit/mL streptomycin, and 100 units/mL penicillin.

The cytotoxic effects of MT-EO were assessed using an MTT-based assay [8]. In 96-well microtitre plates, cells were incubated for 24 hours at a density of 5×10^4 cells per well. The cell lines were subsequently exposed to varying concentrations of MT-EO for 24, 48, and 72 h. MTT was added to all wells and left for 4 hours to produce formazan. The formazan was dissolved in 150 µL of DMSO. The measurement of absorbance was conducted at 570 nm. Cell

growth in the control group, which received neither essential oil nor doxorubicin treatment, was considered to be at 100%. The results are quantified as IC_{50} values, which represent the concentration at which cell growth is inhibited by 50%. The selectivity indices were calculated by dividing the IC_{50} values (48 h) of LO2, indicative of non-cancerous cells, by the IC_{50} values (48 h) of the cancer cells.

S4: *Antioxidant capacity of MT-EO*

The antioxidant capacity of MT-EO was evaluated by quantifying its ability to scavenge DPPH and ABTS radicals, and its ferric-reducing antioxidant power (FRAP) [9].

The assay for DPPH radical scavenging activity was conducted using a modified well plate assay. In a 96-well plate, 50 µL of different concentrations of essential oil and 150 µL of DPPH solution (0.05 mg/mL) were added to each well, except for the blank test wells. A 200 µL methanol solution was added to the control test well. The solutions were incubated for 6 h in the dark after shaking the solutions for a minute. The samples were analyzed for absorbance at 517 nm with a 96-well microplate reader. Butylated hydroxytoluene (BHT) and Trolox served as the standard reference materials. The percentage of DPPH radical scavenging was calculated using the following formula:

Scavenging (%) = $[1 - (A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100$

The absorbance of A_{sample} is represented by the sample; the absorbance of A_{blank} is represented by the blank; and the absorbance of $A_{control}$ is represented by the control.

$ABTS^{\bullet+}$ scavenging assay: A stock solution containing 7 mM ABTS and 2.45 mM potassium persulfate was prepared and allowed to stand for 16 hours at room temperature in the dark. ABTS solution was diluted with PBS to approximately 0.700 (±0.02) absorbance at 734 nm. MT-EO was diluted to appropriate concentrations in methanol. 150 µL of ABTS solution and 50 µL of different concentrations of MT-EO were mixed in a 96-well plate. The absorption rate was determined at 734 nm after 30 minutes of incubation in the dark at room temperature. The same formula used for DPPH was also used to calculate the scavenging effect of $ABTS^{\bullet+}$.

To conduct the FRAP test, the FRAP reagent was prepared by combining acetate buffer (0.3 M, pH 3.6), TPTZ solution (10 mM) in HCl (40 mM), and $FeCl_3$ (20 mM) at a ratio of 10:1:1. Subsequently, FRAP reagent (180 µL) was mixed with 20 µL of MT-EO (at concentrations varying from 0.075 to 0.5 mg/mL) in a 96-well plate and incubated at 37 °C for 30 minutes in the dark. A volume of 180 µL of the FRAP reagent was combined with 20 µL of methanol, which served as the blank. The absorbance was measured at 593 nm. The iron-reducing antioxidant capacity of MT-EO using Trolox was measured using a calibration curve created by linear regression. The antioxidant capacity was calculated in µmol Trolox/g using the Trolox equivalent antioxidant capacity method.

S5: *Statistical analysis*

The data was analyzed using IBM SPSS version 29.0 and GraphPad Prism 9.0. Results were considered statistically significant at $p < 0.05$ using the Student's t-test. Every experiment

was performed in triplicate.

References

[1] R.P. Adams (2017). Identification of essential oil components by gas chromatography/mass spectrometry. Texensis Publishing. Gruver, Texas.

[2] N.R. Andriamaharavo (2014). Retention Data NIST Mass Spectrometry Data Center, NIST Mass Spectrometry Data Center.

[3] P.J. Linstrom and W.G. Mallard (2014). NIST Chemistry WebBook, NIST Standard Reference Database Number 69. (http://webbook.nist.gov).

[4] M. Kulić, D. Drakul, D. Sokolović, J. Kordić-Bojinović, S. Milovanović and D. Blagojević (2023). Essential oil of *Satureja montana* L. from Herzegovina: assessment of composition, antispasmodic, and antidiarrheal effects, *Rec. Nat. Prod*. **17**, 536-548.

[5] Clinical and Laboratory Standards Institute (2012). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *Approved standard M07-A9*. National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, USA.

[6] P. Bellio, L. Fagnani, L. Nazzicone and G. Celenza (2021). New and simplified method for drug combination studies by checkerboard assay, MethodsX **8**, 101543.

[7] G. Hogg, J. Barrand and C. Webb (1998). In-vitro activity of the combination of colistin and rifampicin against multidrug-resistant strains of *Acinetobacter baumannii*, *J. Antimicrob. Chemother*. **41**, 494-495.

[8] E. İnal, E.O. Nath, M. Abudayyak, Ş. Ulusoy, H.A. İnan, M. Çiçek and M. Kartal (2023). Chemical composition of different parts of the *Vitex agnus-castus* L. essential oils and their *in-vitro* cytotoxic activities. *Rec. Nat. Prod*. **17**, 904-917.

[9] X. Su, Y. Gao , Y. Xiang , P. Lai and X. Xing (2019). Chemical composition and biological activities of the essential oil from *Aristolochia fordiana* Hemsl. *Rec. Nat. Prod*. **13**, 346-354.

Figure S2: The aerial parts of *M. torresiana*

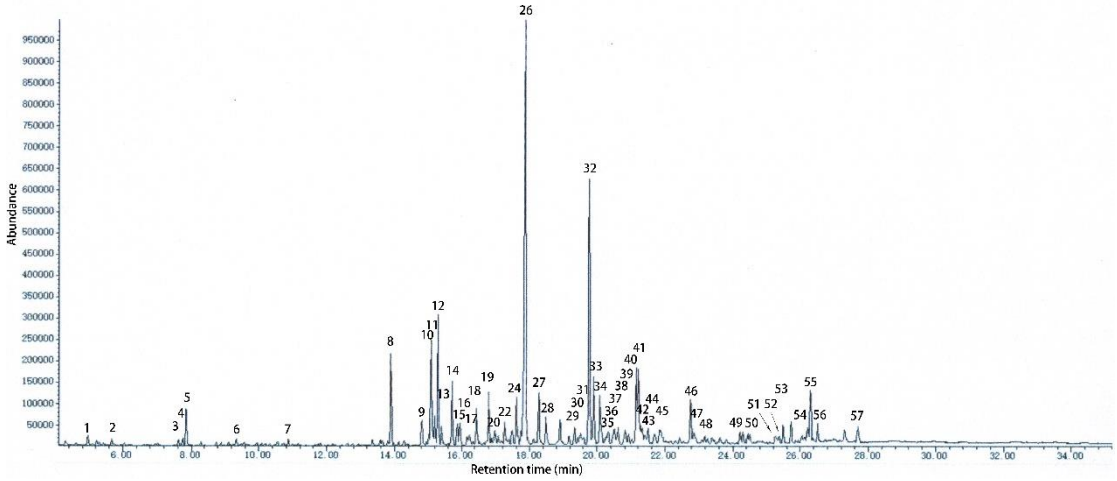

Figure S3: GC/MS Chromatogram of the *M. torresiana* essential oil

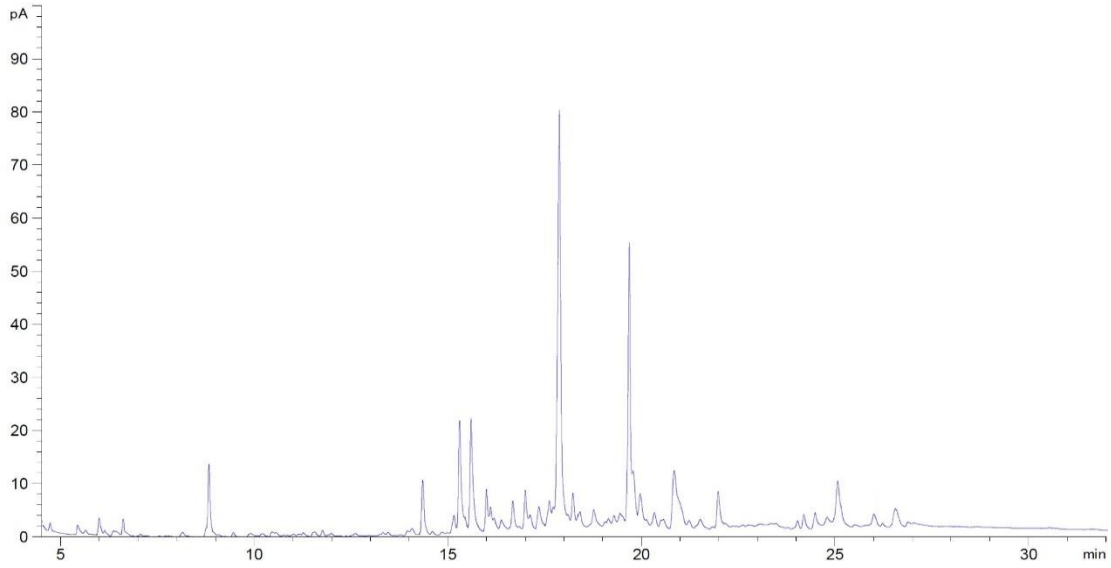

Figure S4: Gas chromatogram of the *M. torresiana* essential oil