

## Original Article

# Variations in chemical constituents and bioactivities of essential oils within *Elatostema stewardii* Merr. revealed by GC-MS-based metabolomics

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## ABSTRACT

*Elatostema stewardii* Merr., an endemic species in China, is valued for its medicinal and food uses, yet its chemical compositions and biological activities remain unexplored. This study aims to analyze the essential oils (EOs) obtained from different plant parts (leaves, stems, and roots) of *E. stewardii* by gas chromatography-mass spectrometry (GC-MS)-based metabolomics. Collectively, 96 EO constituents were identified, primarily consisting of fatty acids (trace-61.56%), diterpenes (3.47-32.39%), alkanes (7.79-16.32%), and ketones (1.46-12.00%). Major constituents included *n*-hexadecanoic acid (trace-60.58%), 2-methyloctadecane (4.63-12.31%), phytol (0.19-12.00%), and 6,10,14-trimethyl-2-pentadecanone (1.39-9.92%). Multivariate analyses principal component analysis (PCA) and partial least squares-discriminant component analysis (PLS-DA) revealed significant EO chemical variations in plant parts, identifying 15 key differential constituents such as *n*-hexadecanoic acid, 3,5,11,15-tetramethyl-1-hexadecen-3-ol, and linoleic acid. Bioactivity assays demonstrated significant differences among the EOs, with the leaf-derived EO exhibiting the strongest cytotoxicity (IC<sub>50</sub>: 36.58-69.23 μg mL<sup>-1</sup>), anti-inflammatory effects (IC<sub>50</sub>: 47.03 μg mL<sup>-1</sup> for NO inhibition), and antioxidant capacity (IC<sub>50</sub>: 0.08, 0.33 mg mL<sup>-1</sup> for ABTS, DPPH scavenging, respectively; FRAP: 2.95 mmol Fe<sup>2+</sup>/mL). Correlation analysis showed that compounds like β-ionone-5,6-epoxide and neophytadiene were positively associated with these bioactivities. These findings highlight substantial chemical and bioactivity differences among *E. stewardii* EOs, identifying the leaf EO as a promising pharmaceutical candidate and demonstrating the effectiveness of GC-MS-based metabolomics in investigating *Elatostema* EOs.

## 1. Introduction

Natural products like essential oils (EOs) are complex secondary metabolites naturally synthesized by aromatic plants, comprising a diverse array of compounds, including alkanes, ketones, monoterpenes, and sesquiterpenes [1]. Recently, EOs have received significant attention for their wide-ranging biological effects, such as anticancer, anti-inflammatory, and antioxidant potential [2-4]. Owing to their versatile bioactivities, EOs are widely utilized across the pharmaceutical, cosmetic, and food industries, underscoring their pivotal role in health and wellness [1]. Current studies have increasingly concentrated on the chemical compositions and bioactivities of plant-derived EOs, given their potential to serve as safe and effective natural agents for therapeutic and nutritional applications [5,6]. In particular, EOs derived from medicinal and edible plants have attracted growing interest due to their promising applications in various health-related fields [7].

*Elatostema stewardii* Merr., a perennial herb species (24-40 cm) belonging to the Urticaceae family, is endemic to China and predominantly distributed in provinces such as Jiangxi, Fujian, and Hunan [8]. Based on our field surveys and literature reviews, *E. stewardii* possesses notable medicinal and dietary value [8-10]. Traditionally, the whole plant of *E. stewardii* is used to promote blood circulation, reduce inflammation, and treat fractures, while its stems

and leaves are employed to relieve cough [8,9]. In addition, the stems and leaves, characterized by their distinctive aroma and flavor, are consumed as wild vegetables or used as ingredients in meat dishes [10]. Despite these traditional applications, the chemical compositions and pharmacological properties of *E. stewardii* remain underexplored, thereby hindering its broader development and sustainable utilization. Although previous studies have identified compounds such as phytol, neophytadiene, and linoleic acid in the EOs of *Elatostema* species like *E. laetevirens* and *E. umbellatum* [11], the bioactivities of EOs from *Elatostema* plants have not yet been investigated.

Metabolomics based on gas chromatography coupled with mass spectrometry (GC-MS) has emerged as an effective approach for comprehensive profiling of EO metabolites [12]. This approach, when integrated with multivariate analysis techniques, including principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), enhances the comparative evaluation of EO compositions across different samples, including diverse plant parts, and enables identification of key differential compounds [13]. For instance, Tang *et al.* utilized GC-MS and PLS-DA analysis to reveal the volatile compositions of rhizomes and root tubers derived from *Curcuma longa*, identifying 20 significant discriminative metabolites [13]. However, to our knowledge, GC-MS combined with multivariate analysis has not yet been conducted within the *Elatostema* genus.

In this study, we employed GC-MS-based metabolomics to analyze the EO metabolite profiles from the roots, stems, and leaves of *E.*

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*stewardii*. Furthermore, we conducted a comparative evaluation of cytotoxic, anti-inflammatory, and antioxidant effects of these EOs. The relationships between their volatile constituents and biological activities were also examined. This research provides new insights into the chemical diversity and bioactivity potential of *E. stewardii* EOs, contributing to their potential applications in pharmaceutical and functional food applications.

## 2. Materials and Methods

### 2.1. Chemicals and reagents

For EO extraction, the ultra-pure water was produced by a Milli-Q apparatus (Millipore Corporation, Billerica, MA, USA), and the anhydrous sodium sulphate ( $\text{Na}_2\text{SO}_4$ ) was produced by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). For GC-MS analysis, HPLC-grade *n*-hexane was sourced from CNW Technologie (Düsseldorf, Germany), and the saturated alkanes standard covering  $\text{C}_7$  to  $\text{C}_{40}$  was obtained from Sigma-Aldrich (St. Louis, MO, USA). In bioactivity assays, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), cisplatin (DDP), NG-monomethyl-L-arginine (L-NMMA), penicillin/streptomycin mixture, and Griess Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium/nutrient mixture F-12 (DME/F-12), Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), and phosphate buffer saline (PBS) were produced by Wuhan Pricella Biotechnology Co., Ltd (Wuhan, China). Cell Counting Kit-8 (CCK-8) was acquired from Beyotime Biotechnology Co., Ltd (Shanghai, China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), iron chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), iron (II) sulfate hexahydrate ( $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ ), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), and analytical methanol and ethanol were obtained from Macklin (Shanghai, China). Acetate buffer (pH 3.6, 300 mM) was produced by Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China), while ascorbic acid (VC), potassium persulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ), and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were obtained from Aladdin (Shanghai, China).

### 2.2. Plant materials

The whole plant of *E. stewardii* was collected from Lushan City of Jiangxi Province with GPS coordinates 1073 m (above sea level), 115°59'05.50" E, 29°32' 51.67" N. Plant identification was conducted by Xingxing Chen from Lushan botanical garden, Jiangxi Province. The voucher specimens (LGB-ES-01) were stored in the herbarium at Lushan Botanical Garden. The collected plant materials were then manually separated into root (ES-R), leaves (ES-L), and stems (ES-S). The plant materials were air-dried, crushed into fine powder, and filtered through a 40-mesh screen. The powdered materials were maintained at -20°C before additional analysis.

### 2.3. EO collection

EO extractions of pulverized samples from different plant parts of *E. stewardii* were processed by hydrodistillation in a Clevenger extraction system, following the protocol outlined in the Chinese Pharmacopoeia [14]. Approximately 200 g of pulverized material of each plant part was placed in a round-bottom flask (2000 mL) containing 1000 mL of ultrapure water and distilled for 6 h. The EO extraction for each sample was performed four times. Subsequently, the resulting EOs were dehydrated using anhydrous  $\text{Na}_2\text{SO}_4$  for 24 h and maintained at 4°C until further chemical and bioactivity assessments. EO yield, expressed in percentage, was computed as the oil weight in grams relative to the dry sample weight in grams (w/w, %).

### 2.4. Metabolite characterization by GC-MS-based metabolomics

The chemical constituents of *E. stewardii* EOs were detected by a GC-2030 equipment combined with a QP2020 NX mass spectrometer (Shimadzu, Kyoto, Japan). Each EO sample was diluted with *n*-hexane to a final concentration of 1.0 mg mL<sup>-1</sup>. Equal volumes of 30  $\mu\text{L}$  from

each sample were pooled to create a quality control (QC) group. For analysis, 5  $\mu\text{L}$  of the analyte was loaded chromatographically separated on a DB-5 MS column (30 m  $\times$  250  $\mu\text{m}$ , 0.25  $\mu\text{m}$  film thickness; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas, flowing at a rate of 1 mL min<sup>-1</sup>. The front inlet purge flow was set to 3 mL min<sup>-1</sup>. The column temperature program began at 50°C (held for 1 min), increased to 310°C at a rate of 8°C min<sup>-1</sup>, and was kept at 310°C for 11.5 min. The ion source, transfer line, and injection port temperatures were maintained at 230°C, 280°C, and 280°C, respectively. Mass spectrometric data were detected using the mode of electron impact (EI) at 70 eV, operating in full-scan mode across an *m/z* range of 50-500 with a scan speed of 12.5 spectra per second. Analysis of raw data, such as baseline correction, alignment, peak picking, deconvolution, and integration, was conducted using Leco Chroma TOF software (V4.3X). Linear retention indices (LRIs) of the EO components were determined by referencing *n*-alkanes ( $\text{C}_7$ - $\text{C}_{40}$ ). Identification of EO components relied on matching mass spectra with the NIST23 database, alongside comparison of LRIs with published data [15] and the NIST Chemistry WebBook (<https://webbook.nist.gov/chemistry/>). The relative abundance of each determined metabolite was represented by the ratio of individual peak area to the total ion chromatogram (TIC) area.

### 2.5. Cytotoxicity evaluations

Cytotoxicity effects of EOs samples were assessed using the CCK-8 assay on three human cancer cell lines: A549 (lung), HeLa (cervical), and HGC-27 (gastric). A549 and HeLa cells were cultured in DME/F-12 medium, whereas HGC-27 cells were maintained using RPMI 1640 medium. All media were supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin, and cultures were kept at 37°C with 5% CO<sub>2</sub>. EO samples were weighed and dissolved in DMSO to prepare stock solutions at a concentration of 100 mg mL<sup>-1</sup>. The culture medium was replaced with EO solutions at a final concentration of 100  $\mu\text{g}$  mL<sup>-1</sup> in triplicate after 24 h of incubation. DDP (5  $\mu\text{g}$  mL<sup>-1</sup>) and 0.1% DMSO served as the positive and negative controls, respectively. The supernatant was discarded after 48 h of treatment, then 100  $\mu\text{L}$  of 10% (v/v) CCK-8 reagent was added to each well and incubated for 2-3 h. The absorbance at 450 nm was recorded using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA). The EO samples, showing an inhibition rate exceeding 50%, were further analyzed to estimate their half-maximal inhibitory concentration (IC<sub>50</sub>).

### 2.6. Anti-inflammatory determination

The effect of anti-inflammation was performed using the RAW 264.7 murine macrophages induced by LPS [16]. RAW 264.7 cells at a density of 8  $\times$  10<sup>4</sup> per well were placed into 96-well plates and maintained for 6 h in DMEM medium enriched with 10% FBS and 1% penicillin/streptomycin, at 37°C with 5% CO<sub>2</sub>. Stock solutions of EOs at 100 mg mL<sup>-1</sup> were prepared by weighing and dissolving them in DMSO. After removing the medium, EO solutions (100  $\mu\text{g}$  mL<sup>-1</sup>) were dispensed at 100  $\mu\text{L}$  per well in triplicate. L-NMMA (12.4  $\mu\text{g}$  mL<sup>-1</sup>) was used as a positive control, while 0.1% DMSO was employed as a negative control. After 24 h of treatment, 50  $\mu\text{L}$  of supernatant from each well was combined with equal volumes of Griess reagents A and B (50  $\mu\text{L}$  each) to determine nitric oxide (NO) production by measuring absorbance at 540 nm. For assessing the cytotoxic effects of EOs, 10% CCK-8 solution (100  $\mu\text{L}$ ) was added after the removal of the remaining supernatant. EOs that inhibited NO production by 50% without exhibiting toxicity toward RAW 264.7 cells were subjected to IC<sub>50</sub> value determination.

### 2.7. Determination of antioxidant potential

#### 2.7.1. DPPH radical scavenging evaluation

The antioxidant activity of *E. stewardii* EOs was tested based on their capacity to scavenge DPPH radicals. A fresh DPPH solution at a concentration of 0.1 mM was created using methanol. Each EO sample was dissolved in DMSO and serially diluted to five concentrations. In a 96-well plate, 20  $\mu\text{L}$  of each EO dilution was mixed with 180  $\mu\text{L}$  of DPPH solution in triplicate. VC and BHT, dissolved in DMSO, served as

positive controls, while the group containing 20  $\mu\text{L}$  of DMSO and 180  $\mu\text{L}$  of DPPH solution was utilized as the negative control. Incubated in darkness at 37°C for 30 min, the mixtures were subsequently measured for absorbance at 517 nm, and  $\text{IC}_{50}$  values were calculated for each EO.

### 2.7.2. ABTS radical cation scavenging assay

The antioxidant capacity of *E. stewardii* EOs was further assessed using the ABTS radical cation decolorization assay. To prepare a working solution, 7 mM ABTS was reacted with 2.45 mM  $\text{K}_2\text{S}_2\text{O}_8$  and kept in the dark for 12-16 h before use. The absorbance of the ABTS $\cdot^+$  solution was set to  $0.70 \pm 0.02$  at 734 nm by dilution with ethanol. In 96-well plates, 20  $\mu\text{L}$  of each EO sample was seeded in triplicate and mixed with 180  $\mu\text{L}$  of the ABTS $\cdot^+$  solution. VC and BHT, dissolved in DMSO, served as positive controls, while a well containing 20  $\mu\text{L}$  of DMSO and 180  $\mu\text{L}$  of the ABTS $\cdot^+$  solution was used as the negative control. The samples were kept at 37°C away from light for 30 min, followed by absorbance measurement at 734 nm. The  $\text{IC}_{50}$  values for each EO were determined based on their respective inhibition rates.

### 2.7.3. Evaluation of ferric ion reducing antioxidant activity (FRAP).

To further evaluate the antioxidant ability of *E. stewardii* EOs, a ferric ion reducing antioxidant activity (FRAP) assay was carried out. A newly made FRAP reagent was obtained by blending 300 mM acetate buffer (pH 3.6), 10 mM TPTZ dissolved in 40 mM HCl, and 20 mM  $\text{FeCl}_3$  in a 10:1:1 (v/v) ratio. In a 96-well microplate, 20  $\mu\text{L}$  of each EO (final concentration: 0.5 mg  $\text{mL}^{-1}$ ) was added to 180  $\mu\text{L}$  of the FRAP reagent. VC and BHT, dissolved in DMSO at a concentration of 10  $\mu\text{g mL}^{-1}$ , served as positive controls, while a well containing 20  $\mu\text{L}$  of DMSO and 180  $\mu\text{L}$  of FRAP solution was employed as a negative control. The reaction was performed in triplicate at 37°C in the dark for 30 min, after which absorbance was measured at 595 nm. A standard curve was generated using  $\text{FeSO}_4$  solutions at concentrations of 1000, 500, 250, 125 and 62.5  $\mu\text{M}$ . The FRAP values of the test EOs were expressed as  $\mu\text{mol Fe}^{2+}/\text{mL}$ .

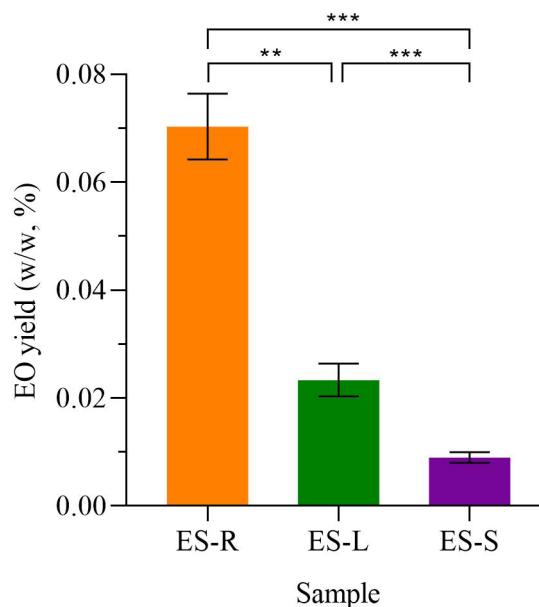
## 2.8. Statistical analysis

Analysis of variance (ANOVA) was conducted via IBM SPSS Statistics (IBM SPSS Inc., Chicago, IL, USA). PCA and partial least-squares discriminant analysis (PLS-DA) were carried out on Soft Independent Modeling of Class Analogy (SIMCA, version 14.1) (MKS Umetrics AB, Umeå, Sweden). The PLS-DA model was validated by a permutation test with 200 permutations. Differential EOs were identified based on an ANOVA  $p$ -value below 0.05 and a PLS-DA variable importance in projection (VIP) score exceeding 1.5 [17]. For biological activity assessments, assays were conducted in triplicate. Statistical significance was determined by one-way ANOVA followed by Tukey's test in SPSS, with  $p$ -values < 0.05 considered statistically significant.  $\text{IC}_{50}$  values were determined, and the heatmap was generated by GraphPad Prism 8.01 (San Diego, CA, USA).

## 3. Results and Discussion

### 3.1. EO yield

The EO content from different plant parts of *E. stewardii* varied notably, ranging from 0.009% (w/w) to 0.070% (Figure 1). The highest EO content was found in the roots (0.070%), followed by the leaves (0.024%) and stems (0.009%), suggesting that the root tissue is the most abundant source of EOs in *E. stewardii*. Previous studies reported EO yields of 0.0016% and 0.0018% from the aerial parts of *E. laetevirens* and *E. umbellatum*, respectively [11], which are comparable to the combined yield obtained from the leaves and stems of *E. stewardii* in the present study. Statistical analysis revealed highly significant differences in EO yields among all sample pairs ( $p < 0.001$  or  $p < 0.01$ ) (Figure 1), indicating the organ-specific variability in EO biosynthesis and accumulation within *E. stewardii*. These pronounced differences in EO yields across plant organs are consistent with observations in other aromatic and medicinal species, including *Laportea aestuans* [18] and *Cuminum cyminum* [19]. This study, to our knowledge, was



**Figure 1.** EO yield from different plant parts of *E. stewardii*. (ES: *E. stewardii*; R: roots; L: leaves; S: stems.) Asterisks above the columns indicate statistical differences in EO yield between plant parts (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

the first to investigate EO yield differences across various plant parts of *Elastostema* species. Although *E. stewardii* is relatively abundant in some regions of China, such as Jiangxi Province, its low EO yield may limit large-scale exploitation. Previous studies have shown that EO yields of a given plant species can be significantly influenced by multiple factors, including extraction methods [20], harvesting time [21], and environmental conditions such as soil type, temperature, and humidity [22]. Therefore, future studies are warranted to optimize extraction procedures (e.g., microwave-assisted or supercritical  $\text{CO}_2$  extraction) and cultivation practices to enhance EO yield and support the sustainable utilization of *E. stewardii*.

### 3.2. EO characterization by GC-TOF-MS analysis

The compositions of EOs from three plant parts of *E. stewardii* were first uncovered using GC-MS, identifying a total of 96 constituents, which accounted for 70.37% to 86.83% of the total EO content. The 30 most abundant EO compositions have been listed in Table 1, with the complete dataset provided in Table S1. These annotated EOs were classified into 11 classes, with the most prominent being fatty acids (trace-61.56%), diterpenes (3.47-32.39%), alkanes (7.79-16.32%), ketones (1.46-12.00%), and sesquiterpenes (2.60-6.22%) (Table S1). Several compound classes, including fatty acids, alkanes, ketones, and sesquiterpenes, are also commonly found in many aromatic plants, such as *Ruta graveolens* [23]. In the ES-R sample, fatty acids were the most abundant metabolites with a relative abundance of 61.56%, followed by alkanes (7.79%), diterpenes (3.47%), esters (2.83%), and sesquiterpenes (2.60%). In the ES-S group, fatty acids (24.00%), diterpenes (22.44%), and alkanes (16.32%) were predominant, along with notable levels of ketones (6.44%) and sesquiterpenes (4.34%). In contrast, the ES-L sample was dominated by diterpenes (32.39%), ketones (12.00%), alkanes (11.55%), and sesquiterpenes (6.22%) (Table S1). Analysis of individual metabolites revealed both similarities and differences among the three EO profiles. In the ES-R EO, the dominant constituents were *n*-hexadecanoic acid (60.58%), 2-methyloctadecane (5.67%), squalene (4.44%),  $\alpha$ -*epi*-7-*epi*-5-eudesmol (1.60%), and 6,10,14-trimethyl-2-pentadecanone (1.39%). In the ES-S sample, *n*-hexadecanoic acid (23.45%) and 2-methyloctadecane (12.31%) were also predominant, followed by 3,5,11,15-tetramethyl-1-hexadecen-3-ol (9.40%), phytol (8.58%), and 6,10,14-trimethyl-2-pentadecanone (6.40%). The abundant constituents in the ES-L sample included phytol (12.00%), neophytadiene (10.30%), 6,10,14-trimethyl-2-pentadecanone (9.92%), isophytol (8.84%), and 2-methyloctadecane (4.63%) (Table 1, Table S1). Although some predominant EOs, such as

**Table 1.** Main EO compositions (%) identified from different plant parts of *E. stewardii*.

No. <sup>a</sup>	Compound <sup>b</sup>	LRI <sup>c</sup>	LRI <sup>d</sup>	EO sample <sup>e</sup>		
				ES-R	ES-S	ES-L
73	<i>n</i> -Hexadecanoic acid	1987	1984	60.58	23.45	tr
14	2-Methyloctadecane	1863	1867	5.67	12.31	4.63
56	Phytol	2115	2116	0.19	8.58	12.00
65	6,10,14-Trimethyl-2-pentadecanone	1844	1847	1.39	6.40	9.92
53	Isophytol	1948	1949	0.47	3.35	8.84
51	Neophytadiene	1838	1836	0.79	0.77	10.30
55	3,5,11,15-tetramethyl-1-hexadecen-3-ol	2087		tr	9.40	0.50
96	Squalene	2814	2815	4.44	0.39	0.17
36	Acorenone B	1702		0.20	3.10	1.56
34	$\alpha$ - <i>epi</i> -7- <i>epi</i> -5-Eudesmol	1668		1.60	0.25	1.33
88	2,4-Ditert-butyl-6-nitrophenol	1681		0.30	2.09	tr
50	4,8,12,16-Tetramethylheptadecan-4-olide	2351		0.80	0.25	1.13
7	Hexadecane	1600	1600	0.45	0.64	1.07
8	Cyprane	1647		0.42	0.68	0.89
63	$\beta$ -Ionone epoxide	1487	1473	tr	tr	1.64
19	Heptacosane	2700	2700	0.04	0.40	1.14
74	linoleic acid	2145	2131	0.98	0.56	tr
90	2,4,2',4'-Tetramethyl-biphenyl	1713		0.27	0.64	0.55
15	Nonadecane	1900	1900	0.37	0.55	0.53
54	$\alpha$ -Kaurene	2071	2082	0.85	0.16	0.44
87	2,4-Di- <i>tert</i> -butylphenol	1510	1512	0.21	0.12	0.90
94	3-hexadecylthiophene	2157		tr	0.24	0.94
17	Pentacosane	2500	2500	tr	0.54	0.56
39	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	1589		0.38	0.35	0.34
21	Nonacosane	2900	2900	0.04	0.12	0.85
43	Methyl palmitate	1925	1927	0.57	0.33	0.07
92	2,3-Dimethylantracene	2056		0.21	0.49	0.20
75	2-Isopropyl-5-methyl-1-heptanol	1299		0.28	0.28	0.26
89	3,4-Diethyl-1,1'-biphenyl	1693	1692	0.14	0.32	0.37
83	4,6,8-Trimethyl-1-nonene	1317		0.28	0.28	0.25

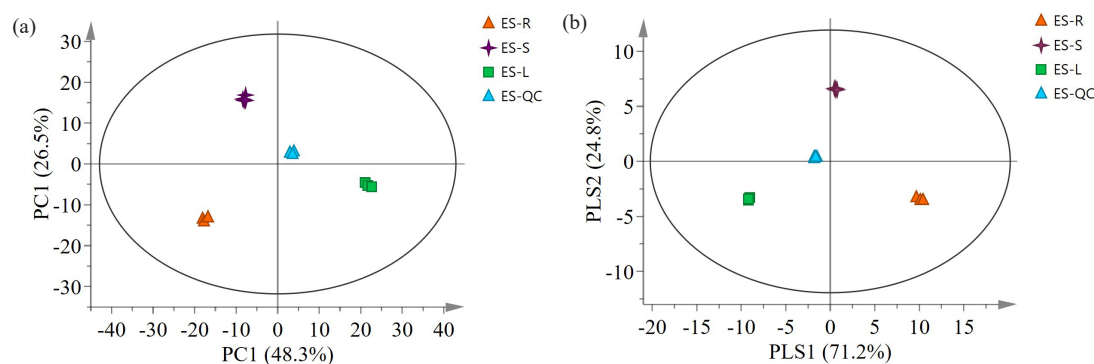
<sup>a</sup>The number was consistent with that in Table S1.<sup>b</sup>Compounds were characterized via comparison of linear retention indexes and MS spectra.<sup>c</sup>Linear retention index (LRI) on the DB-5 MS column, determined experimentally by referencing a homologous series of C<sub>n</sub>-C<sub>40</sub> *n*-alkanes.<sup>d</sup>Linear retention index (LRI) on the DB-5/DB-5 MS column, obtained from the literature or retrieved from the NIST Chemistry WebBook database.<sup>e</sup>ES: *E. stewardii*, R: roots, L: leaves, S: stems; tr: trace compounds (< 0.01%).

neophytadiene, phytol, and isophytol, were also abundant in EOs of *E. laetevirens* and *E. umbellatum* [11], key constituents like *n*-hexadecanoic acid and 2-methyloctadecane characterized in this study were not detected in these two species, indicating significant interspecific variations in EO compositions within *Elatostema* species.

### 3.3. Metabolite variations among EO samples

As shown in Table 1 and discussed above, notable variations in both chemical classes and individual metabolites were observed among the EO samples from different parts of *E. stewardii*. To globally visualize these metabolite differences, all detected molecules were analyzed using PCA and PLS-DA methods (Figure 2). The PCA plot yielded cumulative R<sup>2</sup>X and Q<sup>2</sup> values above 0.5 (Table S2), indicating good model reliability [24]. PLS-DA analysis revealed R<sup>2</sup>Y (cum) and Q<sup>2</sup> (cum) values over 0.9, and permutation testing with 200 permutations yielded a negative Q<sup>2</sup> intercept (Table S2, Figure S1), confirming the robustness of the established model. Both PCA and PLS-DA plots exhibited clear separations among the three sample groups (Figure 2), underscoring significant differences in EO compositions across plant parts of *E. stewardii*. These findings are consistent with previous reports, such as in *Hypericum scabrum*, where distinct EO profiles were also found between plant parts [25]. The observed variations may result from tissue-specific biosynthetic pathways, which are affected by genetic, developmental, and environmental conditions [26]. Our results supported the observation that plant part-specific variations in EO profiles are common across different species, highlighting the complexity and specificity of EO production in *E. stewardii*.

The integration of VIP scores from PLS-DA analysis and *p*-values obtained from ANOVA assay enables the identification of key compounds that contribute significantly to biochemical variations observed between samples [17]. In the present work, we adopted this approach to characterize discriminative compounds among EO samples of *E. stewardii*. Metabolites with VIP values exceeding 1.5 and *p*-values < 0.05 [17] were considered as the most significant differential molecules, accounting for the chemical variations observed across the EOs from different plant parts. The analysis identified 15 discriminative metabolites such as diterpenes, fatty acids, ketones, and sesquiterpenes. Notably, *n*-hexadecanoic acid exhibited the highest VIP value (8.54), followed by linoleic acid (6.57), 3,5,11,15-tetramethyl-1-hexadecen-3-ol (5.97), 2-methyloctadecane (5.21), and neophytadiene (4.35) (Table 2). This study, to our knowledge, firstly reported the application of multivariate analysis to identify variations in EO constituents in *Elatostema* species. Among these discriminative compounds, several metabolites, such as *n*-hexadecanoic acid, neophytadiene, and phytol, are known for their biological activities, including anti-inflammatory, antioxidant, cytotoxic, and neuropharmacological properties [27-29]. Variations in the abundance of these bioactive compounds may partially account for differences in the therapeutic potential of *E. stewardii* EOs. These compounds may serve as potential chemical markers for distinguishing and authenticating EO products or raw plant materials derived from specific plant parts of *E. stewardii*.

**Figure 2.** Metabolite variations of EOs from different plant parts of *E. stewardii* were analyzed by (a) PCA and (b) PLS DA. ES: *E. stewardii*; R: roots; L: leaves; S: stems; QC: quality control.

**Table 2.** Differential EO constituents among different plant parts of *E. stewardii*.

No. <sup>a</sup>	Compound	LRI <sup>b</sup>	LRI <sup>c</sup>	Class	VIP value <sup>d</sup>	p-value <sup>e</sup>
73	<i>n</i> -Hexadecanoic acid	1987	1984	Fatty acids	8.54	< 0.001
74	Linoleic acid	2145	2131	Fatty acids	6.57	0.001
55	3,5,11,15-Tetramethyl-1-hexadecen3-ol	2087		Diterpenes	5.97	< 0.001
14	2-Methyloctadecane	1863	1867	Alkanes	5.21	< 0.001
51	Neophytadiene	1838	1836	Diterpenes	4.35	< 0.001
56	Phytol	2115	2116	Diterpenes	4.12	0.001
53	Isophytol	1948	1949	Diterpenes	3.22	< 0.001
65	6,10,14-Trimethyl-2-pentadecanone	1844	1847	Ketones	3.20	< 0.001
36	Acorenone B	1702		Sesquiterpenes	2.88	< 0.001
96	Squalene	2814	2815	Others	2.80	< 0.001
88	2,4-Ditert-butyl-6-nitrophenol	1681		Others	2.72	< 0.001
78	<i>cis</i> -13-Docosenamide	2790		Amides	2.49	< 0.001
34	$\alpha$ - <i>epi</i> -7- <i>epi</i> -5-Eudesmol	1668		Sesquiterpenes	2.13	< 0.001
63	$\beta$ -Ionone epoxide	1487	1473	Ketones	1.80	< 0.001
50	4,8,12,16-Tetramethylheptadecan-4-olide	2351		Esters	1.62	< 0.001

<sup>a</sup>The number was consistent with that in Table S1.

<sup>b</sup>Linear retention index (LRI) on the DB-5 MS column, determined experimentally by referencing a homologous series of C<sub>7</sub>-C<sub>30</sub> *n*-alkanes.

<sup>c</sup>Linear retention index (LRI) on the DB-5/DB-5 MS column, sourced from the literature or retrieved from the NIST Chemistry WebBook database.

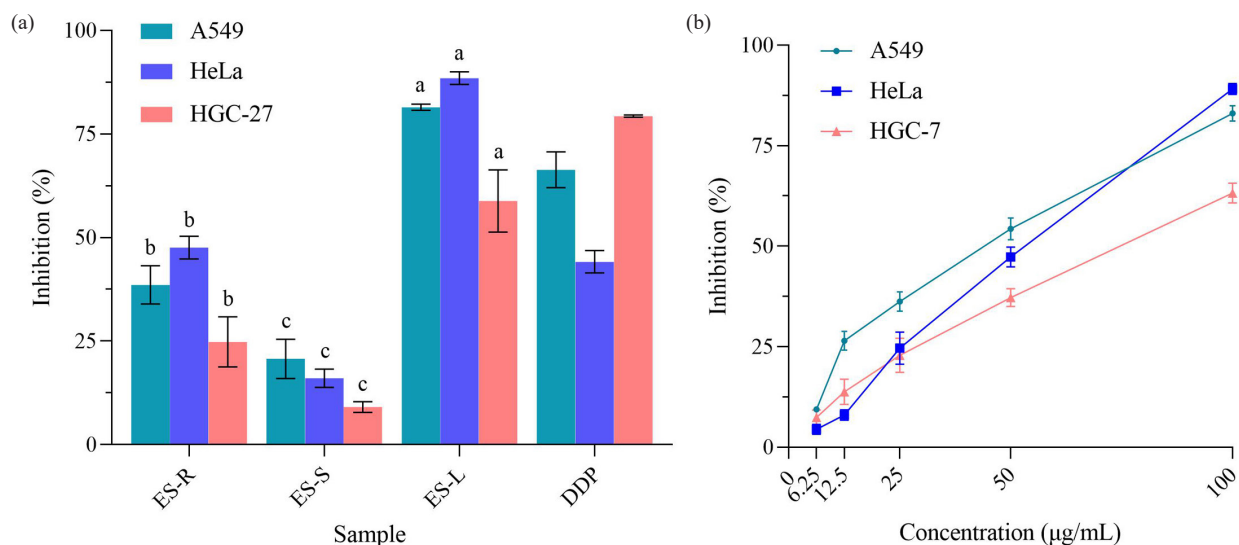
<sup>d</sup>VIP values: variable importance in the projection values obtained from the PLS-DA model.

<sup>e</sup>p-values are obtained from ANOVA analysis.

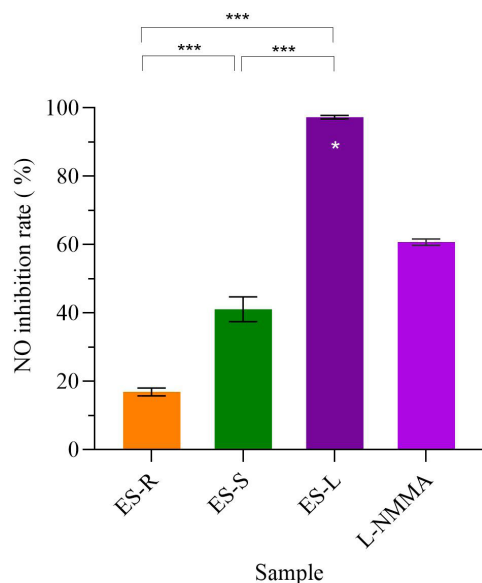
### 3.4. Cytotoxicity assays

Significant variations in EO metabolites among the three plant parts of *E. stewardii* were observed (Figure 2), indicating potential differences in their bioactivities. The cytotoxicity of the EOs from *E. stewardii* was comparatively evaluated on A549, HeLa, and HGC-27 cancer cell lines. The findings revealed marked differences in cytotoxicity, with growth inhibition rates ranging from 9.05% to 88.50% at a test concentration of 100  $\mu\text{g mL}^{-1}$  (Figure 3a). Across the samples, the EO derived from leaves (ES-L) exhibited the strongest activity, with inhibition rates of 88.47%, 88.5%, and 58.84% for A549, HeLa, and HGC-27 cells, respectively. In contrast, EOs from roots (ES-R) and stems (ES-S) showed moderate to weak activity, with inhibition rates below 50% (Figure 3a). These findings partly support the traditional consumption of *E. stewardii* leaves and stems as vegetables with potential nutraceutical value. Statistical analysis confirmed significant differences in bioactivity

among these EO samples ( $p < 0.05$ ). The positive control, DDP, exhibited potent inhibition at 5  $\mu\text{g mL}^{-1}$ , validating the effectiveness of the bioactivity evaluation system. Given that ES-L EO displayed over 50% inhibition, its IC<sub>50</sub> value was further determined, yielding 36.58  $\pm$  1.81, 48.42  $\pm$  2.87, and 69.23  $\pm$  2.86  $\mu\text{g/mL}$  for A549, HeLa, and HGC-27 cells, respectively, in a dose-dependent manner (Figure 3b). Although cytotoxicity has been previously reported in *Elatostema* species such as *E. papillosum* [30], this study was the first to explore the antiproliferative activity of volatiles from *Elatostema* species. Our findings highlight *E. stewardii* leaf EO as a promising candidate for the discovery of anticancer drugs. These *in vitro* results indicate that future *in vivo* studies, including pharmacokinetic evaluations, are warranted to characterize the absorption, distribution, and bioavailability of the active EO, considering factors such as sex and disease model-specific drug behavior [31,32].



**Figure 3.** Cytotoxicity of EO samples from different parts of *E. stewardii* against three cancer cell lines. (a) Cytotoxic effects of three EO samples at 100  $\mu\text{g mL}^{-1}$  and cisplatin (DDP) at 5  $\mu\text{g mL}^{-1}$ . (b) Cytotoxic effects of ES-L EO at five different concentrations. Different lowercase letters (a, b, c) in Figure 3(a) indicate statistically significant differences between samples ( $p < 0.05$ ) within the same cancer cell line, while samples sharing the same letter are not significantly different. (ES: *E. stewardii*, R: roots, L: leaves, S: stems).



**Figure 4.** NO inhibition by EOs from different parts of *E. stewardii*. EO samples and L-NMMA were tested at 100  $\mu\text{g mL}^{-1}$  and 12.4  $\mu\text{g mL}^{-1}$ , respectively. (ES: *E. stewardii*; R: roots; L: leaves; S: stems.) The asterisk above the columns indicates statistically significant differences in NO inhibition between plant parts (\*\*\*) ( $p < 0.001$ ). The asterisk (\*) in the ES-L column denotes the significant cytotoxic effect of ES-L EO on RAW 264.7 cells at 100  $\mu\text{g mL}^{-1}$ .

### 3.5. Anti-inflammatory activity

The whole plant of *E. stewardii* has traditionally been used to treat inflammation in China; however, its anti-inflammatory effects have not been previously reported. In this study, EO samples from *E. stewardii* were detected for their anti-inflammatory potential by calculating NO production in LPS-induced RAW 264.7 macrophages. At 100  $\mu\text{g mL}^{-1}$ , the leaf-derived EO (ES-L) exhibited the highest inhibition of NO production (97.32%), followed by ES-S (41.08%) and ES-R (16.9%). The positive control, L-NMMA (12.4  $\mu\text{g mL}^{-1}$ ), showed significant inhibition, demonstrating the assay system's reliability (Figure 4). However, cytotoxicity analysis revealed that ES-L EO at 100  $\mu\text{g mL}^{-1}$  induced over 30% growth inhibition in RAW 264.7 cells, suggesting that the observed NO suppression may be partly due to cytotoxic effects rather than specific anti-inflammatory activity. To ensure a more accurate assessment, the EO concentration was reduced to 50  $\mu\text{g mL}^{-1}$ , a non-toxic dose for RAW 264.7 cells. At this concentration, ES-L EO still demonstrated a notable NO inhibition rate of 52.76%, with an  $\text{IC}_{50}$  value of 47.03  $\mu\text{g/mL}$ . Even though previous studies have reported the anti-inflammatory potential of EOs from other *Urticaceae* species, such as *Urtica dioica* [33], this study was the first to explore the anti-inflammatory activity of EOs from *Elatostema* plants. Our findings indicate that EOs from *E. stewardii*, particularly the leaf EO, possess promising anti-inflammatory potential. These results not only support the ethnomedicinal uses of *E. stewardii* for inflammatory remedies but also underscore the broader therapeutic potential of *Elatostema* species for future pharmacological exploration. Since environmental factors such as altitude, temperature, and soil composition, as well as plant growth stages, can significantly influence EO composition and bioactivity [22], future studies involving samples from multiple habitats and developmental stages are needed to further validate these findings.

### 3.6. Antioxidant capacity

Eos from various plant species have demonstrated promising antioxidant activity, emphasizing the importance of investigating plant-derived EOs for their antioxidant potential [34]. In the current research, the antioxidant capacity of EOs derived from different parts of *E. stewardii* was evaluated using DPPH, ABTS, and FRAP assays. The results highlighted significant differences in antioxidant activity

**Table 3.** Antioxidant capacity of EOs from different plant parts of *E. stewardii*.

Samples	$\text{IC}_{50}$ value ( $\text{mg mL}^{-1}$ )		FRAP value ( $\mu\text{mol Fe}^{2+}/\text{mL}$ )
	DPPH assay	ABTS assay	
ES-L	0.33 $\pm$ 0.01 <sup>c</sup>	0.08 $\pm$ 0.002 <sup>c</sup>	0.30 $\pm$ 0.008 <sup>a</sup>
ES-S	4.54 $\pm$ 0.15 <sup>b</sup>	1.38 $\pm$ 0.07 <sup>b</sup>	0.08 $\pm$ 0.004 <sup>c</sup>
ES-R	6.12 $\pm$ 0.23 <sup>a</sup>	2.21 $\pm$ 0.28 <sup>a</sup>	0.06 $\pm$ 0.002 <sup>d</sup>
VC	0.005 $\pm$ 0.0001 <sup>d</sup>	0.006 $\pm$ 0.0001 <sup>d</sup>	0.14 $\pm$ 0.004 <sup>b</sup>
BHT	0.019 $\pm$ 0.001 <sup>d</sup>	0.007 $\pm$ 0.0001 <sup>d</sup>	0.049 $\pm$ 0.001 <sup>e</sup>

VC and BHT in the FRAP assay were tested at 10  $\mu\text{g mL}^{-1}$ . EO samples for FRAP assay were tested at 0.5  $\text{mg mL}^{-1}$ . Lowercase letters (a-e) indicate statistically significant differences ( $p < 0.05$ ) between samples within the same assay, where values sharing the same letter are not significantly different. (ES: *E. stewardii*, R: roots, L: leaves, S: stems.)

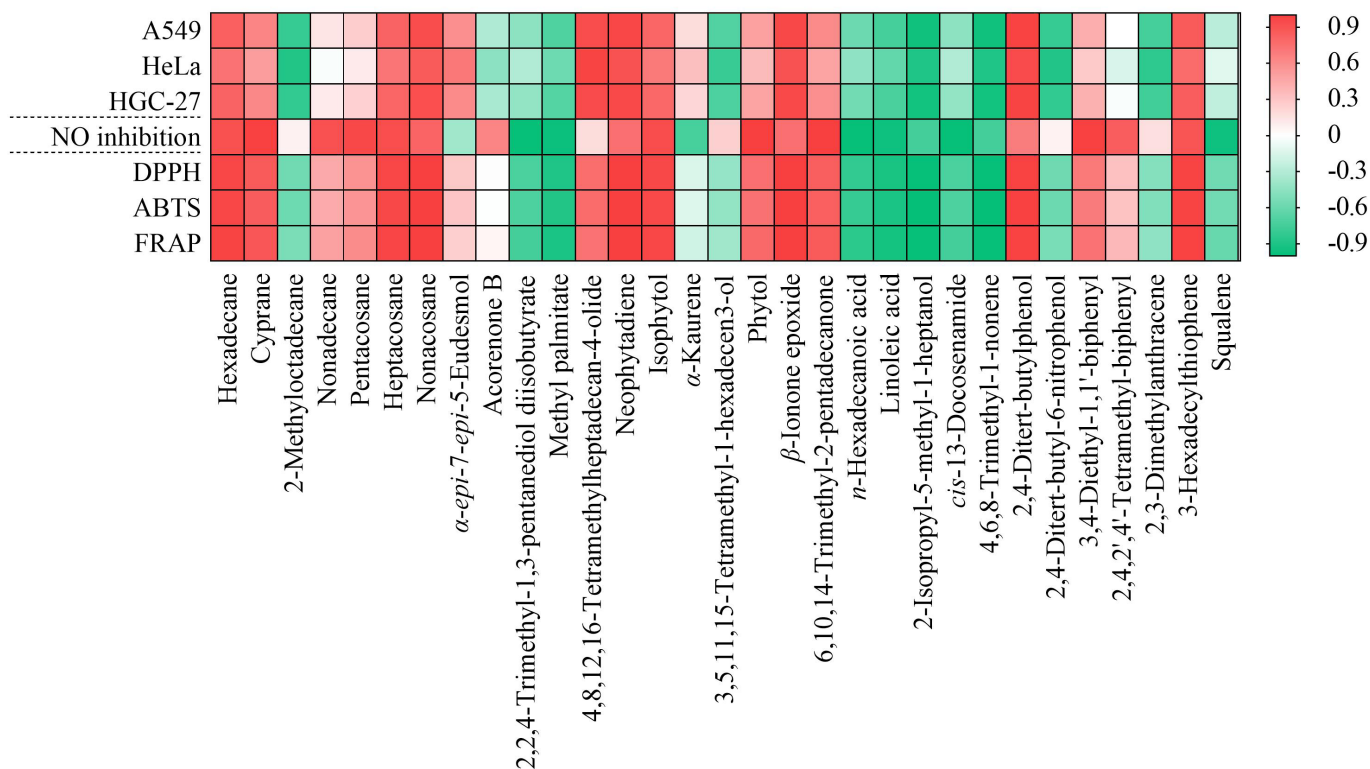
among the EOs ( $p < 0.05$ ). Among them, ES-L EO exhibited the greatest activity, with  $\text{IC}_{50}$  values of 0.33  $\text{mg mL}^{-1}$  and 0.08  $\text{mg mL}^{-1}$  in DPPH and ABTS radical scavenging assays, respectively, and a FRAP value of 0.30  $\mu\text{mol Fe}^{2+}/\text{mL}$  at a concentration of 0.5  $\text{mg mL}^{-1}$ . ES-S and ES-R EOs also showed antioxidant activity, with DPPH and ABTS  $\text{IC}_{50}$  values varying between 1.38 and 6.12  $\text{mg mL}^{-1}$  and FRAP values between 0.06 and 0.08  $\mu\text{mol Fe}^{2+}/\text{mL}$  (Table 3). The positive controls, VC and BHT, exhibited significant antioxidant activity in the DPPH, ABTS, and FRAP assays, confirming the effectiveness of our evaluation system. Previous studies have reported strong antioxidant effects in non-volatile extracts from *Elatostema* species, such as *E. papillosum* and *E. rugosum* [35,36]. For example, the methanol extract of *E. papillosum* leaves significantly inhibited DPPH radicals, with an  $\text{IC}_{50}$  of 32.35  $\pm$  0.68  $\mu\text{g mL}^{-1}$  [36]. Nevertheless, this research was the first to uncover the antioxidant capacity of EO extracts from *Elatostema* taxa. Our findings suggest that the leaf EO of *E. stewardii* may serve as a promising resource for antioxidant agent discovery, showing superior activity compared to EOs from its stems and roots.

### 3.7. Correlation between EO constituents and bioactivities

The bioactivity findings highlight significant variations in the bioactivities of EOs derived from different plant parts of *Elatostema*, emphasizing the significance of exploring the correlation between EO constituents and observed activities to identify potential bioactive molecules. To gain deeper insights into the connections between biological activities and EO compositions in *E. stewardii*, Pearson's correlation analysis was performed, considering variations across plant parts. Key metabolites, including differential constituents and the top 30 most abundant EOs, were selected to generate a heatmap.

The results revealed that EO constituents were linked to bioactivities through both positive and negative correlations (Figure 5). Several compounds, such as  $\beta$ -ionone-5,6-epoxide, neophytadiene, and phytol, showed positive associations with the cytotoxic, anti-inflammatory and antioxidant effects observed in *E. stewardii* EOs (Figure 5).  $\beta$ -Ionone and its analogs are recognized as potential candidates for anticancer therapy [37]. Extracts containing neophytadiene have demonstrated significant growth inhibition in cancer cells like A549, with  $\text{IC}_{50}$  values less than 10  $\mu\text{g mL}^{-1}$  [38], and have demonstrated efficacy in both *in vitro* and *in vivo* models of LPS-induced inflammation [39]. Additionally, phytol has been renowned for its cytotoxic, antioxidant, and anti-inflammatory properties [40]. The higher relative abundance of these metabolites in ES-L EO compared to the other two samples (Table 1) may partly explain why this EO exhibited the strongest biological activity.

In contrast, some metabolites, such as *n*-hexadecanoic acid, linoleic acid, and 4,6,8-trimethyl-1-nonene, showed strong negative effects. Although *n*-hexadecanoic acid is recognized for its cytotoxic, anti-inflammatory, and antioxidant capacity [29,41,42], it exhibited strong negative associations in this study, possibly due to antagonistic interactions with other EO metabolites. This study represents the first exploration of associations between EO constituents and biological activity in *Elatostema* plants. The findings provide valuable insights for



**Figure 5.** Heatmap showing Pearson correlation analysis of selected metabolites and bioactivities in *E. stewardii* EOs. The values on the right side of the figure represent the positive (>0) and negative (<0) relationships between metabolites and biological activities.

further research into the mechanisms underlying *Elatostema* EO effects and the identification of pharmacologically active agents.

#### 4. Conclusions

This study offers the first systematic exploration of the composition and bioactivity of EOs from *E. stewardii*, an endemic Chinese species with significant medicinal and food value. GC-MS analysis identified a diverse array of compounds, fatty acids, diterpenes, alkanes, and ketones. Multivariate analyses, including PCA and PLS-DA, revealed distinct chemical profiles among EOs from different plant parts (leaves, stems, and roots), with key differential constituents such as *n*-hexadecanoic acid and linoleic acid contributing to these variations. Bioactivity assays demonstrated substantial differences in cytotoxic, anti-inflammatory, and antioxidant effects among the three EOs, with the leaf EO exhibiting the most potent activities, followed by the stem and root EOs. These findings partly support the traditional medicinal and dietary uses of *E. stewardii*. Correlation analysis further indicated that certain metabolites exerted both positive and negative influences on bioactivity, with some metabolites, such as  $\beta$ -ionone-5,6-epoxide and neophytadiene, making strong positive contributions. Overall, our study highlights the pharmaceutical potential of *E. stewardii* EOs, particularly the leaf EO, and provides a basis for the sustainable utilization and further advancement of *Elatostema* species. Additionally, our findings demonstrate the efficacy of GC-MS-based metabolomics in profiling and evaluating *Elatostema* EOs.

#### CRedit authorship contribution statement

**Fengke Lin:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization, Funding acquisition. **Zihao Liu:** Data curation. **Yuqing Liu:** Data curation. **Meiyi Niu:** Data curation. **Rui Zhao:** Data curation. **Zhongxin Duan:** Data curation. **Yunan Hu:** Data curation. **Binsheng Luo:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

#### Declaration of competing interest

There are no conflicts of interest.

#### Declaration of generative AI and AI-assisted technologies in the writing process

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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#### Supplementary data

Supplementary material to this article can be found online at [https://dx.doi.org/10.25259/AJC\\_712\\_2025](https://dx.doi.org/10.25259/AJC_712_2025).

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