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Rosmarinic Acid Content and Antioxidant Activity in *Ehretia asperula* Zollinger et Moritzi Cell Suspension Cultures

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ABSTRACT

Background: *Ehretia asperula* Zollinger et Moritzi is a medicinal tree abundant in rosmarinic acid, the primary phenolic component, with numerous beneficial biological properties, including antioxidant, antibacterial, antiviral, anti-allergy, anti-arthritis, asthma and anti-cancer.

Methods: The cell suspension cultures of *E. asperula* Zollinger et Moritzi derived from the leaf *in vitro* callus were established in liquid B5 medium added with 0.4 mg/L NAA, 0.1 mg/L BA and 45 g/L glucose. After four weeks, 50 mg/L of chitosan was given to the cell suspension cultures to stimulate rosmarinic acid (RA) production after a 48-hour treatment period. RA content was analyzed using the HPLC and spectrophotometry method after four weeks and 48 hours of chitosan treatment. In addition, the antioxidant capacity of the extracts from *E. asperula* Zollinger et Moritzi was also tested using DPPH free radical scavenging assay.

Result: The RA content and antioxidant capacity of *E. asperula* Zollinger et Moritzi' extracts from the leaf *in vitro* > callus derived from the leaf *in vitro* > field-grown leaf > biomass of cell suspension cultures. These results suggested a strong correlation between RA concentration and antioxidant capacity. The use of *E. asperula* Zollinger et Moritzi cell suspension cultures with chitosan as an elicitor for RA production and evaluation of antioxidant activity is presented in this study for the first time. Our results suggest that cell suspension cultures and others may be a good source of RA, an antioxidant compound.

Key words: Antioxidant, Cell suspension cultures, *Ehretia asperula* zollinger et moritzi, Rosmarinic acid.

INTRODUCTION

In the 1840s, Zollinger and Moritzi reported the first description of *E. asperula* Zollinger et Moritzi, a medicinal plant belonging to the Boraginaceae family (Riedl, 1997). This plant is native to China, Myanmar, Thailand and Vietnam. It is mainly found in Vietnam's northern highland regions, particularly in the province of Hoa Binh (Tram *et al*., 2021). Traditional medicine has made extensive use of its aerial parts to treat a variety of diseases, especially to prevent liver disease, diabetes, hypertension, jaundice and acne (The Asia Foundation, 2012). Studies indicated that *E. asperula* Zollinger et Moritzi extracts had cytotoxic activity against HepG2, MCF-7, MDA-MB-231 and Hela cells (Nguyet *et al*., 2018; Tuyen *et al*., 2022) and could resist retinal cell death (R28) (Le *et al*., 2021).

Phenolic compounds are significant antioxidants with chemopreventive activities such as antioxidant, anticancer, antimutagenic and anti-inflammatory properties (Kumar *et al*., 2023). Their antioxidant capacity depends on the amount and location of their hydroxyl groups, which can quickly transfer H^+ to reactive oxygen species (Wafa, 2024). One of the main phenolic compounds in *E. asperula* Zollinger et Moritzi is rosmarinic acid (RA) (Le *et al*., 2021).

Species in the Lamiaceae and Boraginaceae family have significant concentrations of RA. Recently, RA has been attracted because of its beneficial biological properties, including antioxidant, anti-inflammatory, anti-tumor, anti-allergic, antimicrobial, antiviral and cardioprotective properties (Kim *et al*., 2015; Nadeem *et al*., 2019). In addition to being obtained from wild plants, RA has been biosynthesized *in vitro*, such as in callus of *Zataria multiflora* Boiss (Françoise *et al*., 2007), hairy roots of *Agastache foeniculum* (Pursh) Kuntze (Nourozi *et al*., 2016), *Lepechinia caulescens* (Vergara-Martínez *et al*., 2021) and *Agastache rugosa* (Yeo *et al*., 2023), cell suspension cultures of *Satureja khuzistanica* (Sahraroo *et al*., 2018), shoots of *Melissa officinalis* L. (Vanda *et al*., 2019), roots of *Ocimum basilicum* L. (Biswas, 2020) and *Origanum dictamnus* L. (Sarropoulou *et al*., 2023).

This study describes the establishment of *E. asperula* Zollinger et Moritzi cell suspension cultures to obtain RA from biomass. It compared RA content in cell biomass, callus, field-grown and *in vitro* plants. The antioxidant activity of these materials was tested *in vitro* using the DPPH test.

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MATERIALS AND METHODS

Plant material

The dark green leaves of field-grown *E. asperula* Zollinger et Moritzi plants (6-year-olds) were used to extract RA (Fig 1A). The leaves *in vitro* were used for callus induction and RA extraction (Fig 1B).

Cultures of callus and cell suspension

The leaves *in vitro* were cultivated on Gamborg (B5) medium supplied with 0.4 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 mg/L BA (benzyl adenine) and 30 g/L glucose to create callus. Every five weeks, friable callus was subcultured.

To establish a suspension of cells, 1 g fresh weight (FW) of friable callus from the second subculture was transferred into liquid B5 medium (20 mL) added with 30 g/L glucose, 0.4 mg/L NAA (naphthalene acetic acid) and 0.1 mg/L BA. on a shaker fixed at 90 rpm.

After three weeks, cell suspensions were transferred to a B5 liquid medium supplemented with 45 g/L glucose, 0.4 mg/L NAA and 0.1 mg/L BA. After four weeks, 50 mg/L of chitosan was added to the medium to stimulate RA production in 48 hours.

The cultures were conducted in dark condition, at a temperature of 25±2°C and humidity of 70±2%.

The study was carried out from October 2021 to December 2022 in the laboratory of Thu Dau Mot University, Vietnam.

Analysis of RA content

After the treatment with chitosan, the biomass of cell suspension cultures was harvested and dried at 50°C and RA content was determined.

Extraction of RA

One gram of dry powders, including field-grown plant leaves, *in vitro* leaves, callus and biomass from cell suspension cultures (Fig 2), were mixed with 12 mL of 50% ethanol and extracted for three hours at 70°C and 200 rpm using a magnetic stirrer (Tram *et al*., 2022). The extracts were filtered and the solutions were retained to analyze RA.

Determination of RA

Spectrophotometry analysis of RA

200 µL extract solutions were mixed with 4.6 mL ethanol and 200 µL of a 0.5 M zirconium oxide chloride solution. After five minutes, a spectrophotometer was used to measure the reaction mixture's absorbance at 362 nm. Equation y = 0.0224x - 0.0332 (R^2 = 0.9992) was used to calculate the concentration of RA, using RA (Sigma-Aldrich) at a concentration range of 2-40 µM as a standard (Öztürk *et al*., 2010).

HPLC analysis of RA

Before injection into the HPLC system for RA analysis, extract solutions were passed *via* a 0.45 ìm membrane filter. The injected volume was 20 µL.

The HPLC column was C18 (4,6 × 250 mm, 5 µm, Shim-pack GIST, Japan). The mobile phase flow rate for RA was maintained at 1 mL/min at 30°C and its detection wavelength was 280 nm. The values for retention time (RT) were 10 minutes. The isocratic conditions were 80% A and 20% B. Mobile phase A consisted of methanol and mobile phase B consisted of water containing 0.1% acetic acid (Adham, 2015).

The calibration curve was prepared by plotting concentration versus an area with the equation y = 4377.6x - 4932.9 (R^2 = 0.9993).

Analysis of antioxidant capacity (DPPH assay for scavenging free radicals)

After filtering through filter papers, ethanolic extracts were evaporated at 50°C to dryness. The extracts were subjected to a DPPH free radical scavenging test, utilizing the methodology described in prior research (Marinova and Batchvarov, 2011). The extracts were dissolved in absolute alcohol in a series of concentrations: 2000, 1000, 500, 250, 125 and 62.5 µg/mL. Ascorbic acid was the positive control and absolute alcohol was the negative control. The test of the DPPH free radical scavenging assay was presented in Table 1. After 30 minutes of dark, room-temperature incubation, the mixture's absorbance at 517 nm was determined. The following formula was used to determine the percentage of inhibition of DPPH free radicals:

$$\% \text{ Inhibition} = \left[1 - \frac{\text{Abs (sample)}}{\text{OD (control)}}\right] \times 100$$

(Hung *et al*., 2014).

Analytical statistics

Using Statgraphics Centurion XV software, the data were statistically processed with a 5% significance level. The mean and standard deviation of each triple experiment are displayed in all experimental data.

RESULTS AND DISCUSSION

E. asperula Zollinger et moritzi cell suspension cultures

Cell suspension cultures were started using the friable callus of *E. asperula* Zollinger et Moritzi (Fig 3A). Single cells and small clumps were present in the dark yellow cell suspensions after four weeks of culture (Fig 3B).

RA in *E. asperula* Zollinger et moritzi cell suspension cultures

This study used spectrophotometry and HPLC methods to determine the RA content in extract samples from *E. asperula* Zollinger et Moritzi.

In Table 2, RA is a phenolic compound with significant content in the tested extracts. *In vitro* leaves had the highest concentration, followed by callus and field-grown leaves and the lowest concentration was in cell suspension cultures. RA content in the extracts measured by the HPLC

method was higher than that measured by the spectrophotometry method.

DPPH test for scavenging free radicals in the extracts

The results showed that the extracts created yellowish solutions on a purple background, demonstrating antioxidant activity in the DPPH experiment (Fig 4).

Table 1: Test of DPPH free radical scavenging assay.

Sample	Sample solution (mL)	Absolute alcohol (mL)	DPPH solution (mL)
Blank	0	4.0	0
Control	0	3.5	0.5
Sample	0.5	3.0	0.5

A comparative analysis of the antioxidant activity of the extracts from leaves of field-grown and *in vitro E. asperula* Zollinger et Moritzi plants, callus and biomass of cell suspension cultures was presented in Fig 5.

The inhibition percentage of DPPH free radical scavenging by leaves *in vitro* and callus extracts was provided as 90.3% and 90.99% at the concentration of 2000 ìg/mL, which have more potent antioxidant activity than the extracts of field-grown leaves (82.24% at the concentration of 2000 µg/mL) or biomass of cell suspension cultures (65.53% at the concentration of 2000 µg/mL) (Fig 5).

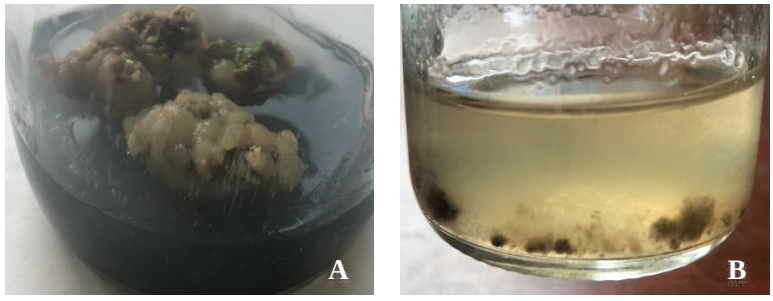
From the standard curve showing the linear correlation between the concentration of the sample test and the value of % inhibition of DPPH free radicals (Fig 5), the DPPH inhibition IC_{50} value of the experimental extracts was

(A): Field-grown *Ehretia asperula* Zollinger et Moritzi plants; (B): *Ehretia asperula* Zollinger et Moritzi plants *in vitro.*

Fig 1: Plant materials.

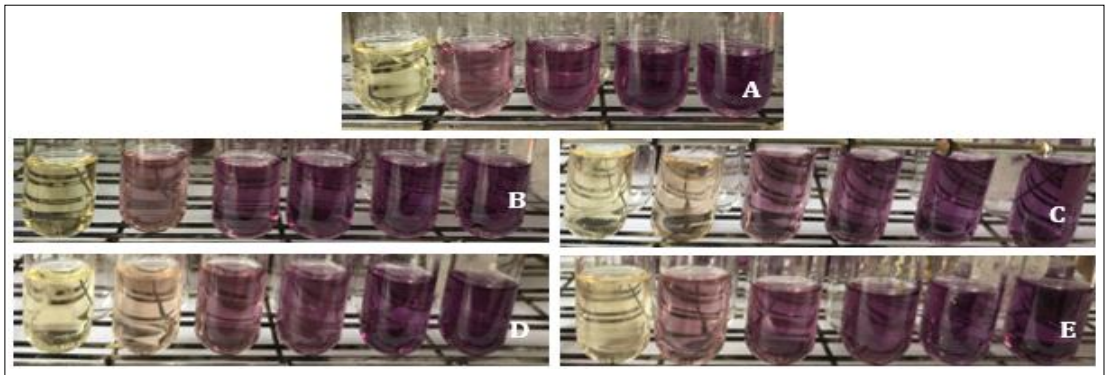
(A): Dried leaves of field-grown plants; (B): Dried leaves of plants *in vitro*; (C): Dried callus; (D): Dried biomass of cell suspension cultures.

Fig 2: Extract materials.



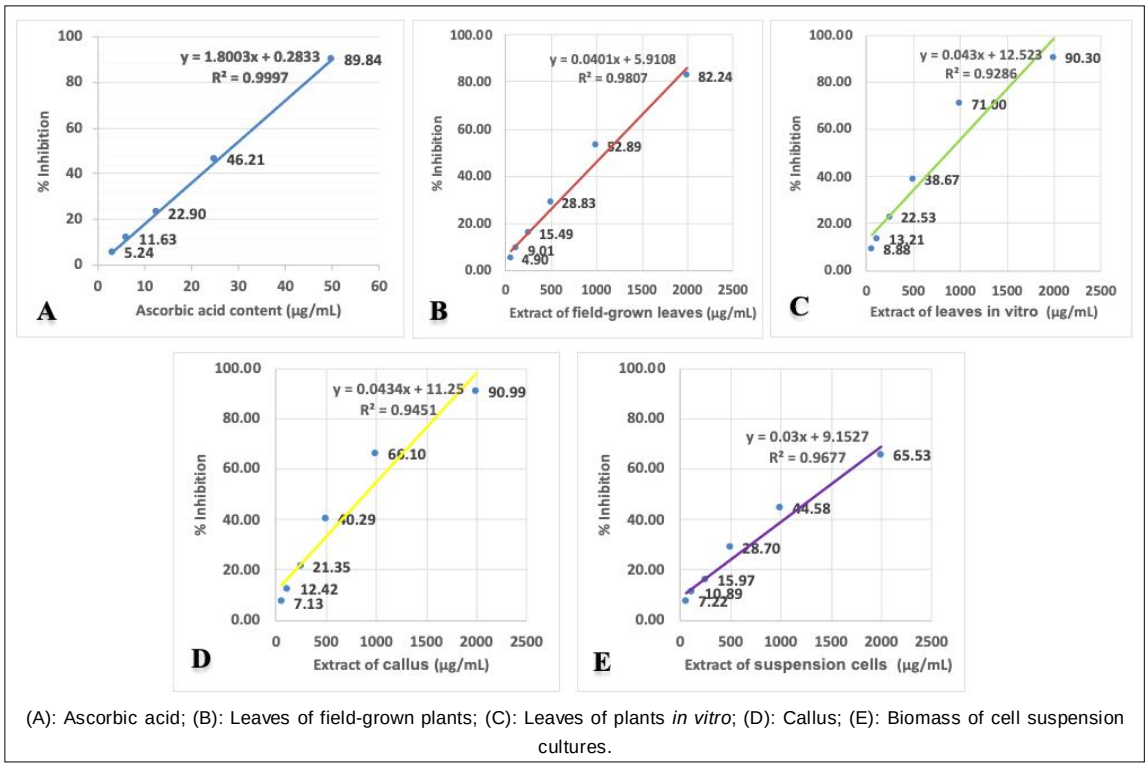
(A): Friable callus; (B): Cell suspensions cultures.

Fig 3: The formation of *Ehretia asperula* zollinger et moritzi cell suspension cultures.



(A): Ascorbic acid; (B): Leaves of field-grown plants; (C): Leaves of plants *in vitro*; (D): Callus; (E): Biomass of cell suspension cultures.

Fig 4: DPPH color changes induced by the extracts.



(A): Ascorbic acid; (B): Leaves of field-grown plants; (C): Leaves of plants *in vitro*; (D): Callus; (E): Biomass of cell suspension cultures.

Fig 5: Inhibition percentage of DPPH free radical by the extracts.

Table 2: RA content in the extracts of *Ehretia asperula* zollinger et moritzi.

Extracts	RA (mg/g DW)	
	Spectrophotometry	HPLC
Field-grown leaves	40.19±2.06^{b}	89.37±17.86^{c}
Leaves *in vitro*	45.75±2.58^{a}	310.77±11.89^{a}
Callus	40.57±1.27^{b}	153.08±9.18^{b}
Biomass of ell suspension cultures	22.39±0.20^{c}	74.64±6.83^{c}

*Means followed by different superscript letters in the same column present significant differences (P≤0.05).

Table 3: IC_{50} values of plant extracts for DPPH free radical scavenging activity.

Extracts	Ascorbic acid	Field-grown leaves	Leaves *in vitro*	Callus	Biomass of cell suspension cultures
IC_{50} (µg/mL)	27.62	1099.52	871.54	892.86	1361.64

interpolated (Table 3). The leaves *in vitro* extracts had the strongest anti-radical activity (IC_{50} = 871.54 µg/mL) in the extracts. However, ascorbic acid's IC_{50} value (27.62 µg/mL) was higher than the *E. asperula* Zollinger et Moritzi extracts.

In Table 2, RA contents in *E. asperula* Zollinger et Moritzi extracts were high. The lowest RA concentration in the biomass of cell cultures was due to a portion of RA dissolved in the liquid medium (Tram *et al*., 2022). In particular, the RA contents recorded by the HPLC method were higher than those measured by spectrophotometry. The HPLC method is susceptible, has good quantitative ability and a higher level of accuracy than the spectrophotometry method. The RA content of the extract from field-grown leaves reached 8.94%, equivalent to the RA content from *E. asperula* Zollinger et Moritz leaves extracted with 50% ethanol (9.95%), as reported by Ly (2016). The RA content in *E. asperula* Zollinger et Moritzi cell suspension cultures (74.64 mg/g DW) was equivalent to that in *Thymus lotocephalus* shoot cultures (78.57 mg/g DW) (Gonçalves *et al*., 2019), higher in *Dracocephalum moldavica* L. cell suspension cultures (27.2 mg/g DW) (Weremczuk-Jeżyna *et al*., 2017).

About the assessment of antioxidant activity, the tested extract samples had antioxidant capacity equivalent to the extracts from *in vitro E. asperula* Zollinger et Moritzi plants grown under a fluorescent lamp (IC_{50} = 1101.10 µg/mL) in the experiment of Tram *et al.* (2018). In particular, the extract from the biomass of cell suspension cultures had a lower antioxidant capacity than others, possibly due to some biologically active substances (especially RA) being secreted from the cells during growth and dissolved in the culture medium (Tram *et al*., 2022).

The experimental extract samples of *E. asperula* Zollinger et Moritzi also had an antioxidant capacity equivalent to or higher than the antioxidant capacity of some previously studied herbs, such as *Allamanda neriifolia* root, stem and leaf extracts with IC_{50} values of 713.44, 1397.24 and 936.86 µg/mL, respectively (Hung *et al*., 2014), extracts from *Sauropus androgynus*, *Polyscias fruticosa*, *Portulaca oleracea* L. with IC_{50} values of 993.85, respectively; 2110.08 and 2835.33 µg/mL (Mai *et al*., 2017).

A strong negative correlation was found between the RA contents in the experimental extracts and the IC_{50} values (r = -0.934 for the spectrophotometric method, r = -0.756 for the HPLC method). RA plays an essential role in the bioactivity of *E. asperula* Zollinger et Moritzi extracts. The antioxidant activity of RA is well known due to its four phenolic hydrogens and two catechol (1,2-dihydroxy benzene) rings (Bhatt *et al*., 2013). A significant correlation between total phenolic contents (especially RA contents) from the cultures *in vitro* and antioxidant activity has been reported in many previous studies (Shiga *et al*., 2009; Samarakoon *et al*., 2016; Weremczuk-Jeżyna *et al*., 2017; Gonçalves *et al*., 2019).

CONCLUSION

These results suggested obtaining a large RA concentration is possible when using *E. asperula* Zollinger et Moritzi cell suspensions, callus, plantlet *in vitro* or field-grown leaves. Therefore, *E. asperula* Zollinger et Moritzi cell suspensions is a good candidate for *in vitro* RA biosynthesis. In addition, the DPPH assay of the *E. asperula* Zollinger et Moritzi extracts has significant *in vitro* radical scavenging potential, suggesting that it can protect cells from oxidative stress.

Conflict of interest

There is no conflict of interest.

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