International Journal of Academic Health and Medical Research (IJAHMR)
ISSN: 2643-9824
Vol. 9 Issue 9 September - 2025, Pages: 58-67

Chemical Analysis and Anticancer Potential of Phenolic Compounds Derived from *Ehretia asperula* Zoll. & Mor.

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Abstract: *Ehretia asperula Zoll. & Mor, known medicinally in Vietnam, has been utilized in folk medicine, particularly in treating cancer. Our goal is to isolate, characterize, and assess the cytotoxicity of boned E. asperula leaves to a set of E. asperula cytotoxicity. E. asperula leaves were dried, then extracted using methanol, and the dried methanol extract was fractioned using a set of organic solvents based on their increasing polarity. The most active solid phase from the column was the ethyl acetate fraction where column chromatographies on silica gel and Sephadex LH-20 were performed. The elucidation of the molecules was performed using NMR, and mass spectrometry. The cells on which the cytotoxic activity was tested include Hep-G2 (liver), LU-1 (lung), MCF-7 (breast), and He La (cervical), using the sulforhodamine B (SRB) assay. The cytotoxicity tests on E. asperula leaves indicated that the leaves are no active cancer therapeutic options. Focusing on the phenolics, 4 were isolated and identified as: Caffeic acid (Ed 3.2), Methyl caffeate (Ed 5.5), Rosmarinic acid (Ed 17.3), Methyl rosmarinate (Ed 11.4). Out of all, Caffeic acid and Rosmarinic acid were the only ones that exhibited some activity. Methyl caffeate and Methyl rosmarinate showed stronger cytotoxic activities against Hep-G2, HeLa, and MCF-7 cell lines with respect to their IC50 values ranging from 2.83 to 9.32 µg/mL. Methyl caffeate demonstrated better results compared to Methyl rosmarinate among all of the three active cell lines. The results suggest there is some kind of structure-activity relationship since the methyl esterification of the free carboxylic acid group substantially increased the cytotoxicity of the compounds. This could be attributed to the increased lipophilicity associated with the methyl group which enhances cellular permeation. More mechanistic studies and in vivo experiments are needed for Methyl caffeate and Methyl rosmarinate as they appear to be valuable lead candidates.*

Keywords: Ehretia asperula, Natural products, Cytotoxicity, Caffeic acid, Rosmarinic acid, Structure-activity relationship

I. Introduction

The use of natural products from plants has served as a crucial and reliable source of new drugs, especially in oncology, for a long time. Vietnam, with its rich biodiversity and its long tradition of herbal medicine, has a vast and largely unexplored reservoir of potential medicinal plants whose therapeutic properties can be investigated. Many modern pharmaceuticals, including most anti-cancer drugs, are directly obtained from, or inspired by, natural products. Guided chemical research through bioactivity is a very effective way to arrive at active principles from natural sources.

Ehretia asperula Zoll. & Mor, more commonly called "Xa den" is currently classified within the family Boraginaceae. This plant is a type of climbing shrub with brittle stems and can be found in the mountainous woodlands of provinces Son La, Ha Nam, Quang Ninh and Hoa Binh in Vietnam. In traditional Vietnamese medicine, a plethora of the plant is used, especially stems and leaves, for complaints of boils and inflammation, and even conditions of the liver. In more recent history, the plant has been popularized due to the inconceivable effectiveness in the treatment and management of malignant tumors. This in turn led to a plethora of scientific studies. *E. asperula* has been researched in Vietnam and other countries for its chemical and biological components. Le The Trung and co-workers' studies from 1999 and Nguyen Huy Cuong in the year 2008 studied the plant's anti-cancer properties and its chemical constituents. Such studies claim to have found a plethora of compound classes: friedelan and lupan type triterpenoids, as well as certain alkaloids and phenolic compounds. It has been shown, however, that there is a significant difference between the expected activity of the separated compounds and their actual results, and this suggests that further insight and systematic studies should be conducted.

Although these initial studies exist, an in-depth examination of phenolic compounds pertaining to the ethyl acetate fraction of *E. asperula*, along with an evaluative focus on their cytotoxic potential, including detailed structure activity relationship studies, remains largely untapped. Thus, the goal of the current investigation is to isolate the chemical constituents of the leaves of *E. asperula* and elucidate their chemical structures, and detailed evaluation of their in vitro cytotoxic activity against a range of human cancer cell lines, thus adding to the scientific knowledge regarding this important medicinal plant.

II. Materials and Methods

2.1 Plant Material

The collection of the leaves of *E. asperula* Zoll. & Mor was done in Jun 2024 at Bai Rong Hamlet in Thuong Tin, Kim Boi, Phu Tho Province, Vietnam. It was authenticated by Dr. Nguyen Xuan Phuong of the Institute of Ecology and Biological Resources, which also has the deposited template for future reference.

2.2 General Experimental Procedures

In the Institute of Chemistry, VAST, the BRUKER Avante 500 Spectrometer was used with the collected data on spectroscopy, which includes one- and two-dimensional NMR spectra (1H-NMR at 500 MHz and 13C-NMR at 125 MHz). From the Institute of Marine Biochemistry, VAST, on the Agilent 1260 series Single Quadrupole LC-MS system, I obtained the data for the High-Resolution Mass Spectra. In column chromatography, silica gel (0.040-0.063 mm) along with the reversed phase silica gel (YMC, 30-50 µm) was used. The TLC is performed on the aluminum plates, which are used as the support for the pre-coated silica gel 60 F254 and the RP-18 F254 (Merck, Germany).

2.3 Extraction and Isolation

Ground to a powder and dried, the 5.8 kg *E. asperula* leaves have also been macerated with methanol for 3 days and then with Methanol for 3 days more. The process will then be repeated 3 more times for maximum compound extraction. The weighed (320 grams) crude extract Methanol extracts collectively and afterwards, under a reduced pressure condition blocked their spouts. The extract was then mixed with a certain amount of water which was diluted. The extraction was then done with the liquid in a sequence of n-hexane and then ethyl acetate. Based upon the ethyl acetate gated preliminary isolations, further purification was done. Sample ethyl acetate fractions which were someplace (~ 34.47 grams) are used for predated glowing, indicating the possession of a large quantity of active compounds.

A silica gel column of the ethyl acetate portion with column chromatography and a D/M gradient elution system was divided into six portions. The D/M 5:1 system helped separate the column into sub-fractions of Ed 2.1 and Ed 2. The sub-fraction Ed 2.3 was further purified by reversed-phase YMC column chromatography in a 1:2 M/W mix and 37.5 mg of compound Ed 3.2 was isolated from the mixture. The compound Ed 5.5 was isolated in 7 mg through sub-fraction Ed 2.1, which underwent silica gel column chromatography of H/D/M 5:15:1 wherein the fraction Ed 2.4 was isolated. This was purified by silica gel in a D/A 5:1 mixture and then Reversed Phase YMC column with M/W 1:1.

Fraction Ed 1.3 was isolated during the third column chromatography using a D/A 5:1 gradient, yielding fraction 7.3. Further, fraction Ed 7.3 was column chromatographed over silica gel (D/M, 2:1) to obtain fraction 16.4, which after underwent a subsequent column chromatography over silica gel (D/M, 3:1) to provide fraction 17.1. The final purification was performed on a reversed-phase YMC column using a mobile phase of methanol (M) to water (W) in a 1:3 ratio in which 51 mg of compound Ed 17.3 was recovered. Moreover, fraction Ed 7.2 was column chromatographed over silica gel (D/M, 7:1) to obtain fraction 8.3. After that, compound 11.4 was obtained in 100 mg after performing column chromatography over silica gel (D/M 10:1) and reversed-phase YMC column (M/W, 1:1). The structural elucidation of isolated compounds was performed using advanced spectroscopy and literature comparison.

2.4 Cytotoxicity Assay

Evaluating the in vitro cytotoxicity of the isolated compounds included the testing of four human cancer cell lines: Hepatocellular carcinoma (Hep-G2), human lung adenocarcinoma (LU-1), human breast adenocarcinoma (MCF-7), and HeLa cervical cancer cells. The method of cytotoxicity testing that was used was the sulforhodamine B (SRB) assay which is a cytotoxicity method that colorimetrically quantifies cell survival. In this method, the quantity of the protein-bound SRB dye corresponds to the quantity of the viable cells.

Standard procedures were used to culture the cell lines in the respective media. The compounds were prepared in DMSO and subsequently diluted to a final test concentration with the culture medium. The control wells consisted of solely DMSO that acted as the negative control and a known cytotoxic drug (e.g. Ellipticine) as the positive control. Following a 72-hour incubation period, the cells were then fixed and stained with the SRB dye and the absorption was taken at 515-540 nm on the ELISA reader. The percent cell survival (CS%) was then calculated and the half-maximal inhibitory concentration (IC50) value was calculated from the dose-response curve for each active compound.

III. Results

3.1 Isolation and Structure Elucidation of Chemical Constituents

Through sequential column chromatography on silica gel and reversed-phase stationary phases, four pure compounds were isolated from the ethyl acetate fraction of Ehretia asperula. The structures of these compounds were elucidated by extensive spectroscopic analysis, including NMR and MS data, and by comparing the data with those reported in the literature.

- **Caffeic acid** (Ed 3.2): This compound was obtained as a white amorphous powder. The 1H-NMR spectrum showed two doublets at δ 7.55 (d, J=16.0 Hz, H-3) and δ 6.24 (d, J=16.0 Hz, H-2), which are characteristic of a trans-olefinic double bond.1 Three aromatic protons at δ 7.06 (d, J=2.0 Hz, H-5), δ 6.95 (dd, J=2.0,8.0 Hz, H-9), and δ 6.80 (d, J=8.0 Hz, H-8) indicated a 1,3,4-trisubstituted aromatic ring. The spectroscopic data was in excellent agreement with those reported for caffeic aci1.
- **Methyl caffeate** (Ed 5.5): What else could be its 1H-NMR spectrum characteristic? The profound shifts accompanying the inequivalence created by the trans configurations are diagnostic of trans-olefins and 1,3,4trisubstituted aromatics …. and possessing evidentiary propionyl signals. The trans-olefinic and 1,3,4-trisubstituted aromatic proton signals spanning the olefinic and propionyl moieties were the focus of profound shifts accompanying inequivalence created by the olefin-cis configurations. The 13C-NMR spectroscopy coupled aligned with the signal at 52.0 supporting a methoxy group. The latter serves as terminal functionality in the methyl group and indicative of the methyl ester as evidenced by the methyl caffeate. The distinct lack of the recovering aromatic, simply. Antiport circulation as primary attributes of the inequivalence shattered the forward the point of recovering the caffeate which underwent methylation to produce the methyl caffeate moiety, and hence the confirmation.
- **Rosmarinic acid** (Ed 17.3): This compound was obtained as a dark yellow powder. Two distinct sets of aromatic proton signals were observed, one of which exhibited features of a 1,3,4-trisubstituted benzene ring, and the other was associated with a 1,3,4-trisubstituted aromatic moiety characterized additionally by a oxymethine proton, a trans-olefinic double bond, and a methylene group. The 13C-NMR revealed to me two carbonyl signals and a total of eighteen carbon signals which is complete consistent with the proposed structure of rosmarinic acid. The complete spectroscopic data correlated with literature values of rosmarinic acid.
- **Methyl rosmarinate** (Ed 11.4): Isolated as a dark yellow powder, the spectral data for this compound were also suggestive of a derivative of rosmarinic acid. What stood out was a singlet at δ 3.72 in the 1H-NMR which corresponded to a signal at δ 52.7 in the 13C-NMR spectrum. This data set also suggested a methyl ester group was present. The 2D HMBC spectrum confirmed the bound structure between the methyl proton signal at δ 3.72 and the ester carbonyl carbon at δ 172.2, and so the structure is confirmed as methyl rosmarinate.

3.2 Cytotoxic Activity of Isolated Compounds

The cytotoxic activity of the four isolated compounds was evaluated against four human cancer cell lines using the SRB assay. The results are summarized in Table 1.

Table 1: Cytotoxic Activity of Isolated Compounds

Compound	ID	Hep-G2 (IC_{50}, µg/mL)	LU-1 (IC_{50}, µg/mL)	HeLa (IC_{50}, µg/mL)	MCF-7 (IC_{50}, µg/mL)
Caffeic acid	Ed 3.2	Not Active	Not Active	Not Active	Not Active
Rosmarinic acid	Ed 17.3	Not Active	Not Active	Not Active	Not Active
Methyl caffeate	Ed 5.5	2.83	Not Active	3.38	4.40
Methyl rosmarinate	Ed 11.4	8.28	Not Active	9.32	5.64

According to Table 1, Caffeic acid and Rosmarinic acid did not show cytotoxic activity against any of the tested cell lines. On the other hand, their methyl ester derivatives, Methyl caffeate and Methyl rosmarinate, exbhibited remarkable cytotoxic activity. Methyl caffeate exhibited very strong activity against Hep-G2, HeLa, and MCF-7 cell lines with IC50 2.83, 3.38, and 4.40 µg/mL, respectively. Methyl rosmarinate also exhibited activity against the same cell lines although at higher concentrations with IC50 8.28, 9.32, and 5.64 µg/mL, respectively. None of the four compounds demonstrated activity against the LU-1 lung.

IV. Discussion

Every study comes with its findings, and this one is no exception, particularly with the newfound structure-activity relationship (SAR) with the isolated phenolic compounds, which serve as the highlight of this study. Perhaps the most remarkable finding is the disparity of cytotoxic activity of the methyl esters (methyl caffeate and methyl rosmarinate) versus the parent acids (caffeic and rosmarinic).1 The parent acids, unlike the methyl esters, are reportedly inactive against a majority of cancer cell lines, which is concerning, owing to the claims made regarding the abundant evidence that supports their lethality to a majority of cancer cell lines.

This difference highlights the structural change of the free carboxylic acid being esterified. A free carboxylic acid group (−COOH) is highly polar and can undergo ionization to form a carboxylate anion (−COO−) relatively easily, especially at the physiological pH. Due to the polarity of the compound, it becomes hydrophilic, and hence, the capacity to permeate through the lipophilic cell barrier and enter the cytoplasm is highly restricted. Thus, it is very plausible that the Caffeic acid and Rosmarinic acid... By contrast, the methyl ester group (−COOCH3) is non-ionizable and is also a polar molecule. This methyl esterification increases the lipophilicity of the entire molecule. Thus, it is more probable that this increased biological activity is attributed to the increased lipophilicity of the molecule. More lipophilic molecules partition into and diffuse more readily across the lipid bilayer of the cell membrane and are, therefore, more likely to interact with, cellular targets to elicit cell death. This serves as the clear illustration of the relationship between the lipophilicity of a molecule and its biological activity.

The active compounds also show a difference in potency which is the case with Methyl caffeate and Methyl rosmarinate in the Hep-G2, HeLa, and MCF-7 cell lines. These two compounds have a difference in structure, which could explain the different potency. Methyl caffeate is much smaller than Methyl rosemarinate, which is a dimer-like structure. Methyl rosmarinate's bigger size with the increase in hydroxyl groups with respect to Methyl caffeinate negatively influences potency. The bigger size may restrict diffusion across the cell membrane or be binding to a target protein's active site. Also, the extra hydroxyl groups in Methyl rosmarinate increase polarity which decreases the lipophilic character provided by the methyl ester. As a results lipophilc compounds, such as Methyl caffeinate, will have more activity as they are less polar than Methyl rosmarinate. This indicates that this type of compounds may exhibit a certain balance that the optimum could be lipophilicity, molecular size, and a low number of polar functional groups in respect of maximal cytotoxic activity.

The disagreement between our results for Rosmarinic acid regarding an earlier study is something that needs to be said. Unlike our compound (Ed 17.3) which showed no activity against Hep-G2 cells, a previous study noted Rosmarinic acid as having activity against the same cell line with an IC50 value of 5 µg/mL. Some reasons could explain this difference in results. To begin with, in the previous study, the Rosmarinic acid had been isolated from the Ehretia longiflora plant, whereas our compound is from Ehretia asperula. Disparate plant species, no matter how close taxonomically, can produce entirely different secondary metabolites, or even slight changes in stereochemical configuration changes that can be profound in their biological activity. Different results can also be attributed to aspects like the methodology changes, the specific subclone of Hep-G2 utilized and even the duration of the assay. This reproducibility problem highlights another problem regarding natural product research. The problem of different laboratories.

Research conducted in the future ought to concentrate on identifying the exact action mechanism for both Methyl caffeate and Methyl rosmarinate. What type of cell death is induced, be it apoptotic, through cell cycle arrest, or through the silencing of other key signaling pathways, is crucial for advancement of these compounds as drug candidates. Also, it would be informative to extend the investigation to a wider variety of cancer cell lines, as well as assessing their in vivo efficacy and toxicity in animal models. This would be the animal to complete their therapeutic profile. Elicitng other ester derivatives and other close analogs may also remain useful in further refining the established SAR for increased cytotoxic activity.

V. Conclusion

The leaves of *E. asperula* Zoll. and Mor that were used in this study were found to contain four phenolic compounds; Caffeic acid, Methyl caffeate, Rosmarinic acid, and Methyl rosmarinate, all of which were isolated and identified. Biological evaluation showed a interesting and relative structure activity relationship in which methyl-esterification of the carboxylic acid moiety was deemed a central factor in the exhibited activity. The non-esterified acids were inert, but their methyl esters exhibited significant cytotoxicity towards the Hep-G2, HeLa, and MCF-7 human cancer cell lines.

Of the compounds tested, Methyl caffeate was the most active and this is possibly related to the ideal combination of lipophilicity and molecular size. The results of this study add to the phytochemical databank of E. asperula, and at the same time, indicate that Methyl caffeate and Methyl rosmarinate can be considered as lead compounds meriting further investigation as antitumor agents. Research on their mechanistic pathways as well as their activity in living organisms should be pursued.

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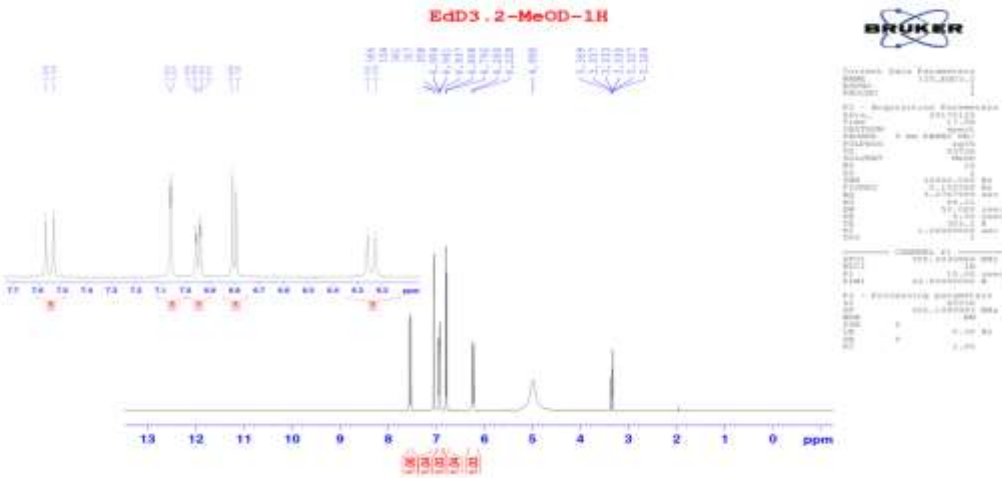
Appendix

Spectroscopic Data

Caffeic acid (Ed 3.2):

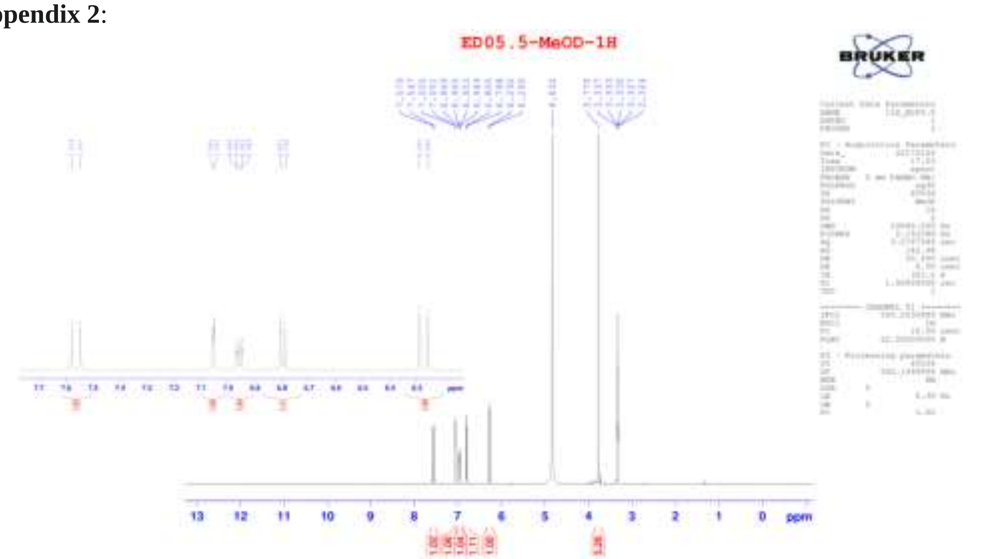
- Structure:
  1H-NMR (500 MHz, CD3OD): δ 7.53 (1H, d, J=16.0 Hz, H-3), 7.05 (1H, d, J=1.5 Hz, H-5), 6.96 (1H, dd, J=8.0,1.5 Hz, H-9), 6.79 (1H, d, J=8.0 Hz, H-8), 6.22 (1H, d, J=16.0 Hz, H-2).
- 13C-NMR (125 MHz, CD3OD): δ 169.0 (C-1), 148.3 (C-7), 145.8 (C-6), 145.6 (C-3), 126.7 (C-4), 121.7 (C-9), 115.3 (C-8), 114.3 (C-5), 113.9 (C-2).
- **Appendix 1**:



NMR spectrum: 1H-NMR (CD3OD, 500 MHz) off Ed 3.2.

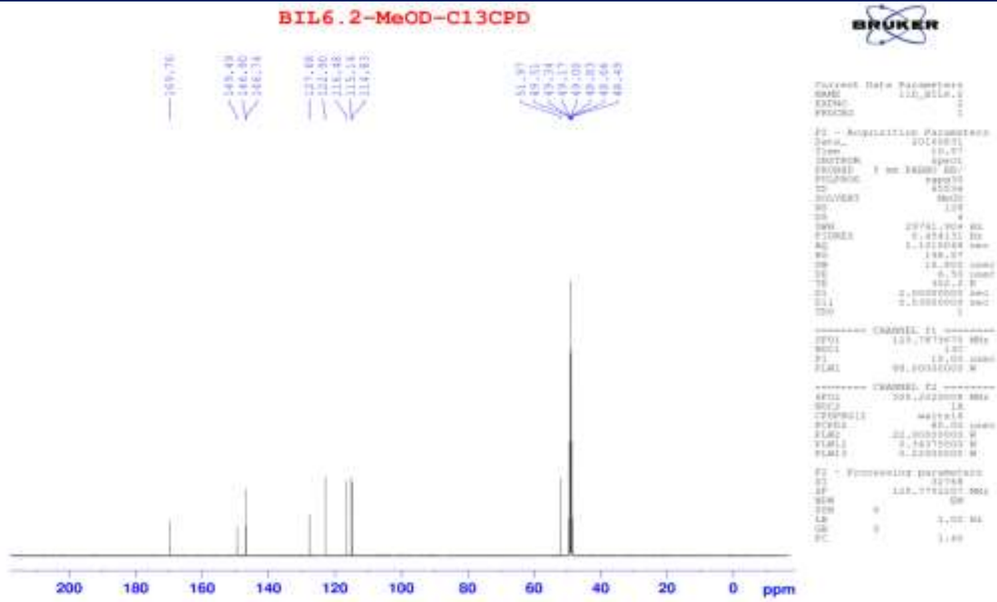
Methyl caffeate (Ed 5.5):

- **Structure:**
- 1H-NMR (500 MHz, CD3OD): δ 7.53 (1H, d, J=15.5 Hz, H-3), 7.03 (1H, d, J=2.0 Hz, H-5), 6.94 (1H, dd, J=8.0,2.0 Hz, H-9), 6.78 (1H, d, J=8.0 Hz, H-8), 6.25 (1H, d, J=15.5 Hz, H-2), 3.75 (3H, s, −OCH3).
- 13C-NMR (125 MHz, CD3OD): δ 168.6 (C-1), 148.4 (C-7), 145.8 (C-6), 145.7 (C-3), 126.6 (C-4), 121.8 (C-9), 115.3 (C-8), 114.0 (C-5), 113.7 (C-2), 50.8 (−OCH3).
- **Appendix 2**:



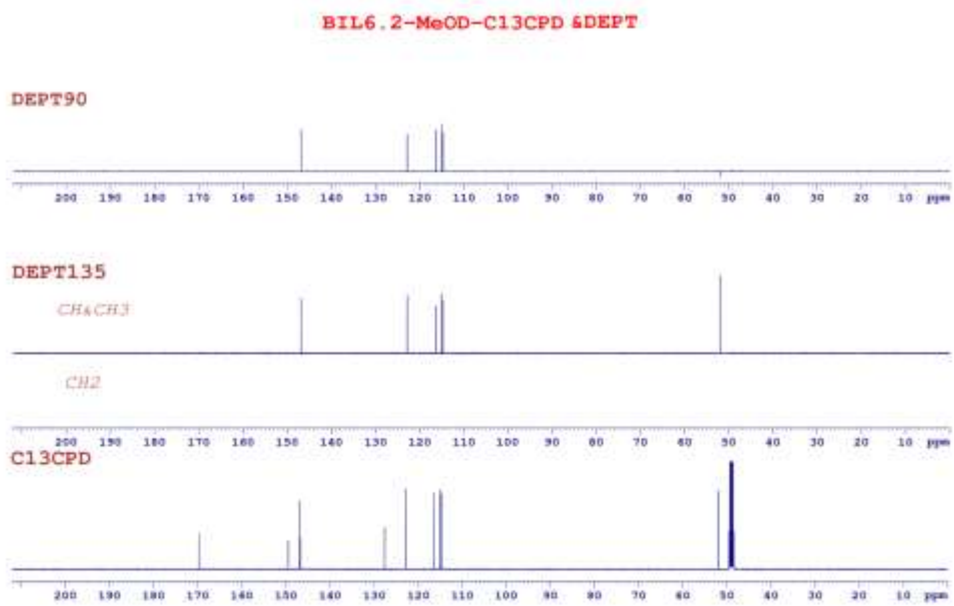
Spectrum 1H-NMR (CD3OD, 500 MHz) off Ed 5.5.

- **Appendix 3**:



Spectrum 13C-NMR (CD3OD, 125 MHz) off Ed 5.5.

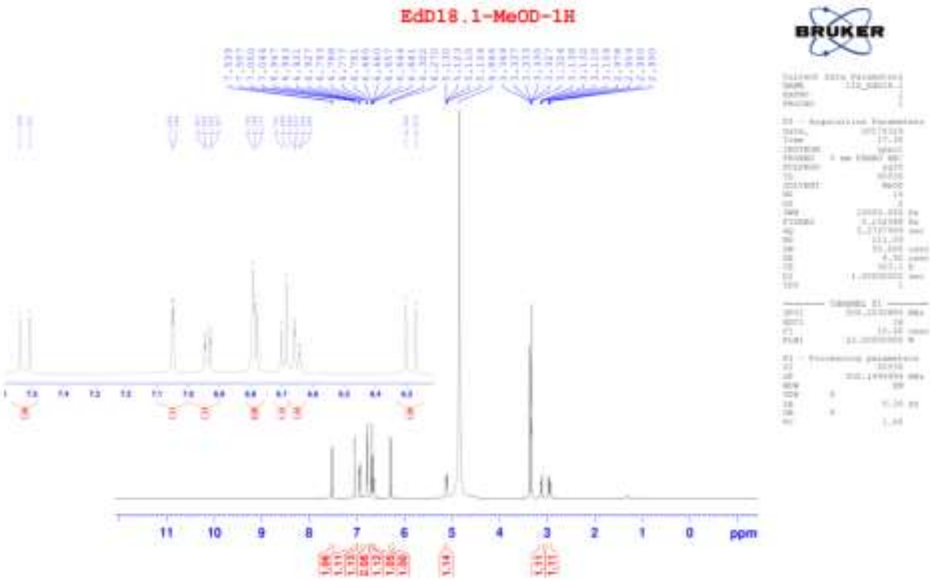
- **Appendix 4**:



Spectrum DEPT off Ed 5.5.

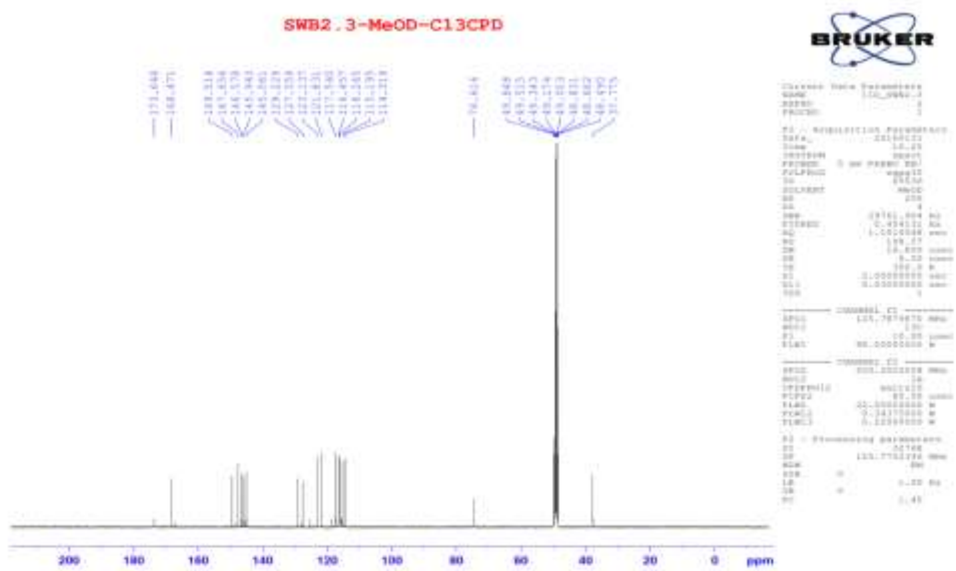
Rosmarinic acid (Ed 17.3):

- **Structure:**
- 1H-NMR (500 MHz, CD3OD): δ 7.52 (1H, d, J=15.5 Hz, H-3), 7.04 (1H, d, J=2.0 Hz, H-5), 6.91 (1H, dd, J=8.0,2.0 Hz, H-9), 6.75 (1H, d, J=8.0 Hz, H-8), 6.70 (1H, d, J=2.0 Hz, H-5'), 6.69 (1H, d, J=8.0 Hz, H-8'), 6.57 (1H, dd, J=8.0,2.0 Hz, H-9'), 6.26 (1H, d, J=15.5 Hz, H-2), 5.19 (1H, dd, J=10.0,3.5 Hz, H-2'), 3.06 (1H, dd, J=14.5,5.5 Hz, H-3'a), 3.00 (1H, dd, J=14.5,5.5 Hz, H-3'b).
- 13C-NMR (125 MHz, CD3OD): δ 174.3 (C-1'), 169.4 (C-1), 149.5 (C-7), 145.7 (C-3), 145.0 (C-6'), 144.9 (C-7'), 143.2 (C-6), 130.4 (C-4'), 128.2 (C-4), 122.2 (C-9'), 121.4 (C-9), 117.6 (C-5'), 116.3 (C-8'), 116.1 (C-8), 115.8 (C-2), 115.3 (C-5), 77.8 (C-2'), 38.9 (C-3').
- **Appendix 5**:



Spectrum 1H-NMR (CD3OD, 500 MHz) off Ed 17.3.

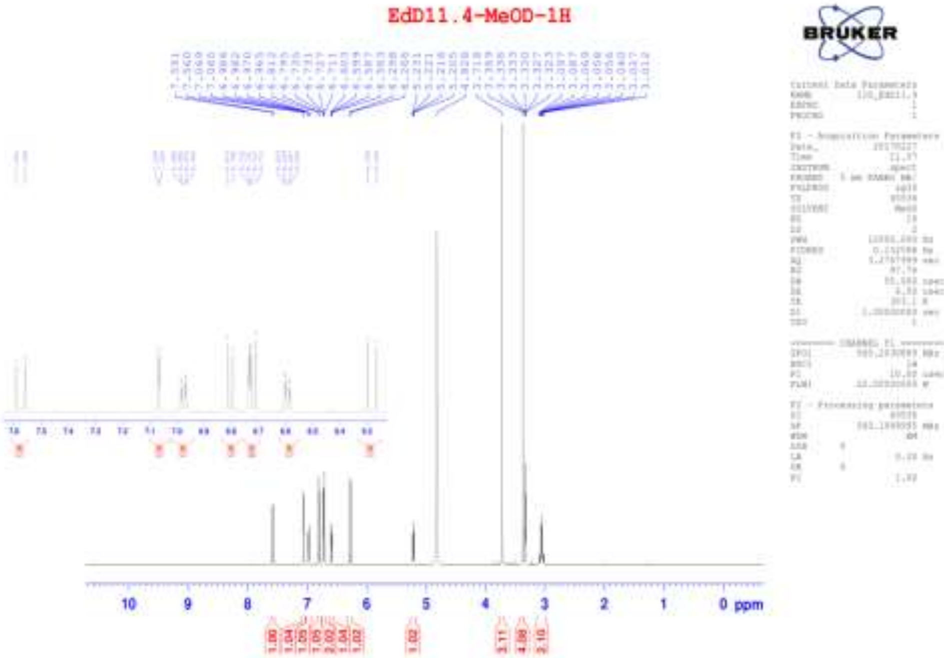
- **Appendix 6**:



Spectrum 13C-NMR (CD3OD, 125 MHz) off Ed 17.3.

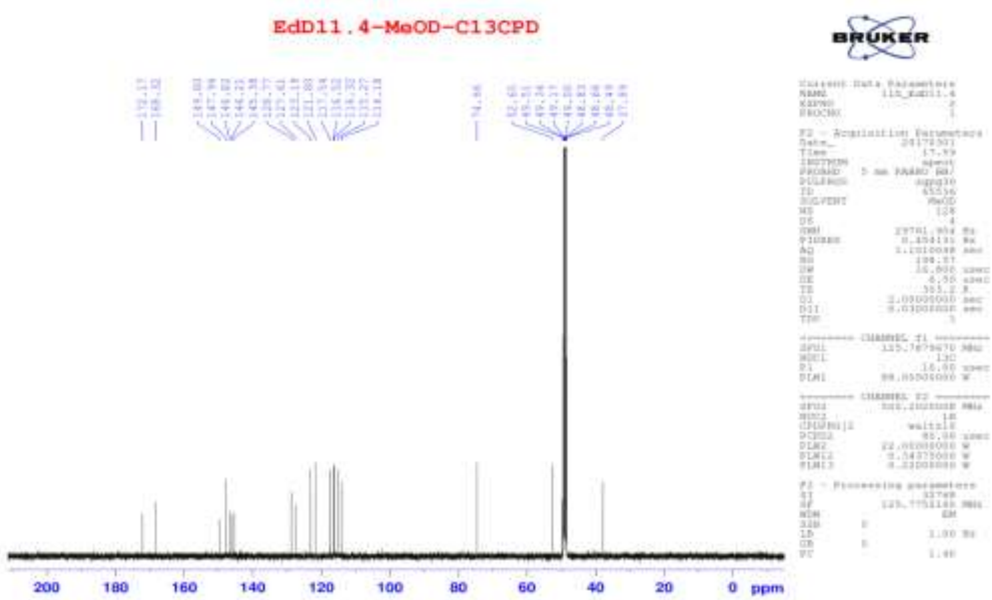
Methyl rosmarinate (Ed 11.4):

- **Structure:**
- 1H-NMR (500 MHz, CD3OD): δ 7.52 (1H, d, J=15.5 Hz, H-3), 7.04 (1H, d, J=2.0 Hz, H-5), 6.91 (1H, dd, J=8.5,2.0 Hz, H-9), 6.75 (1H, d, J=8.5 Hz, H-8), 6.71 (1H, d, J=2.0 Hz, H-5'), 6.64 (1H, d, J=8.0 Hz, H-8'), 6.61 (1H, dd, J=8.0,2.0 Hz, H-9'), 6.27 (1H, d, J=15.5 Hz, H-2), 5.11 (1H, dd, J=7.5,5.0 Hz, H-2'), 3.72 (3H, s, −OCH3), 3.06 (1H, dd, J=14.5,5.5 Hz, H-3'a), 3.00 (1H, dd, J=14.5,5.5 Hz, H-3'b).
- 13C-NMR (125 MHz, CD3OD): δ 172.1 (C-1'), 168.2 (C-1), 150.1 (C-7), 148.6 (C-3), 147.3 (C-6), 146.1 (C-6'), 145.7 (C-7'), 128.4 (C-4'), 127.5 (C-4), 123.3 (C-9), 122.8 (C-9'), 117.9 (C-5'), 116.9 (C-8'), 116.2 (C-8), 115.3 (C-5), 114.2 (C-2), 74.9 (C-2'), 52.8 (−OCH3), 38.0 (C-3').
- **Appendix 7**:



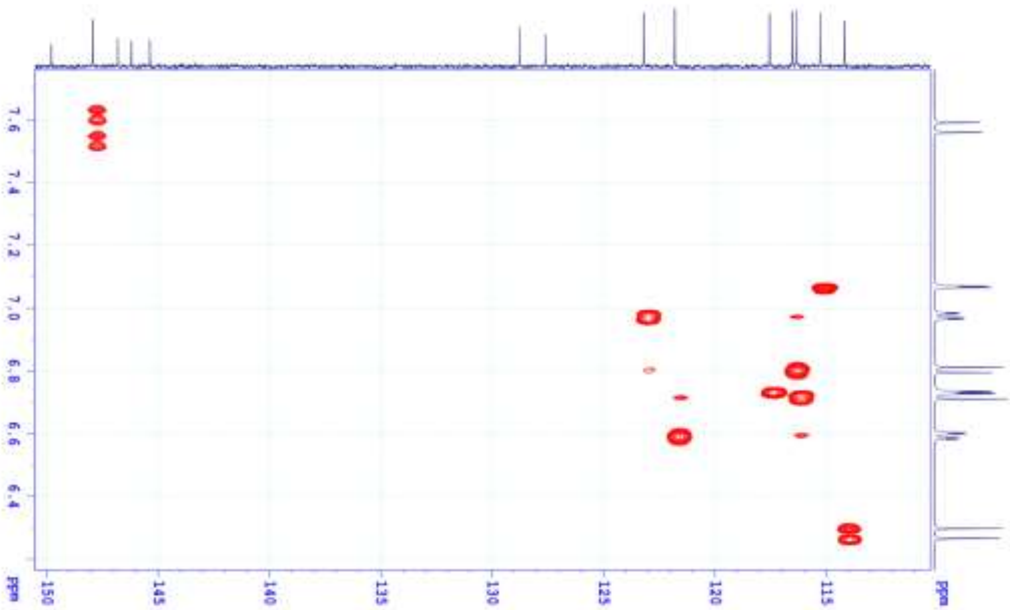
Spectrum 1H-NMR (CD3OD, 500 MHz) off Ed 11.4.

- **Appendix 8**:



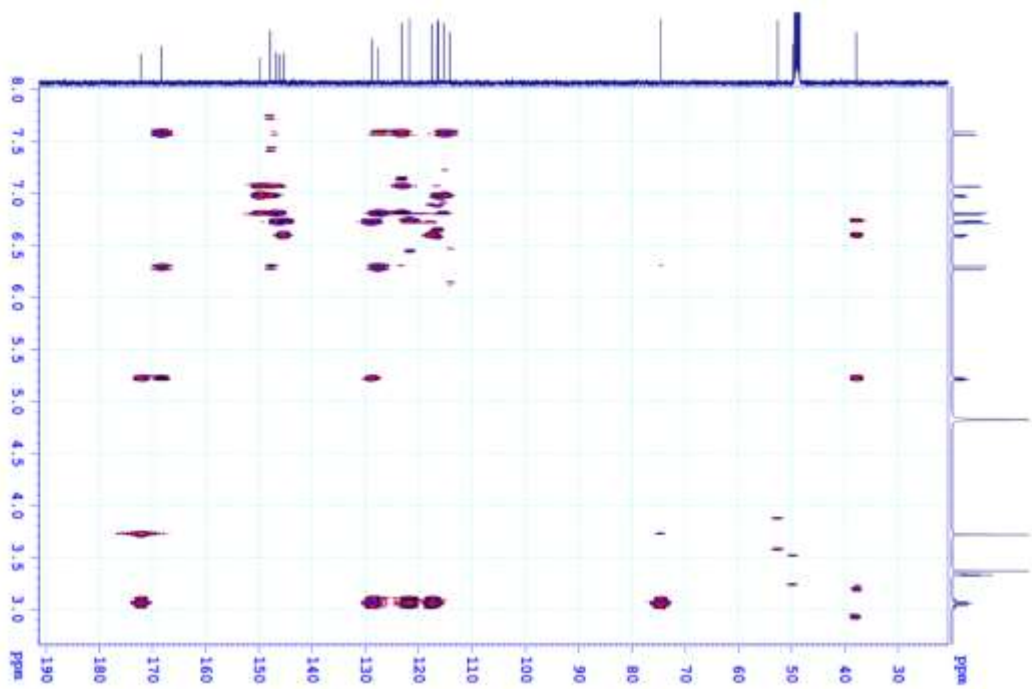
Spectrum 13C-NMR (CD3OD, 125 MHz) off Ed 11.4.

- **Appendix 9**:



Spectrum HSQC two-dimensional off Ed 11.4.

- **Appendix 10**:



Spectrum HMBC two-dimensional off Ed 11.4.