THE CYTOTOXIC ACTIVITY OF LIGNANS AND MONOTERPENOIDS FROM THE MALE *CARICA PAPAYA* FLOWERS ON A549 LUNG CANCER, HEP3B LIVER CANCER, AND MCF-7 BREAST CANCER CELL LINES

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(Received: October 03, 2023; Revised: November 28, 2023; Accepted: November 30, 2023)

Abstract - Two lignans (lariciresinol (**1**) and dehydrodiconiferyl alcohol (**2**)), and four monoterpenoids (vitexoid (**3**), 6-hydroxy-2,6-dimethyl-2,7-octadienoic acid (**4**), 6-hydroxy-2,6-dimethyloct-7-enoic acid (**5**), 2,6-dimethylocta-2,7-diene-1,6-diol (**6**)) from the male *Carica papaya* flowers are conducted a cytotoxic activity for lung cancer cells (A549), liver cancer cells (Hep3B), and breast cancer cells (MCF-7). The results, lignan (**1**), and monoterpenoid (**3**), (**6**) exhibited medium cytotoxic activity on these cancer cells with IC_{50} from 34.73±1.55 to 66.12±5.60 µg/mL. Lignan (**2**) displayed medium cytotoxic activity on MCF-7, Hep3B with IC_{50} 60.85±4.43, 62.32± 5.03 µg/mL, respectively, and weak cytotoxic activity on A549 with IC_{50} 70.99±7.26 µg/mL. Monoterpenoids (**4**), and (**5**) showed medium cytotoxic activity on MCF-7 with IC_{50} 54.15±5.89, 67.49±2.41 µg/mL, respectively, and weak cytotoxic activity on A549, and Hep3B with IC_{50} from 72.25±3.13 to 86.03±7.57 µg/mL. Ellipticine was a positive control.

Keywords - A549 lung cancer cells; Hep3B liver cancer cells; lignan; MCF-7 breast cancer cells; monoterpenoid.

1. Introduction

Carica papaya L., belonging to the Caricaceae family, is considered a valuable nutraceutical fruit plant. It is native to tropical America and is commonly known as Papaya in English, Papita in Hindi, and Erandakarkati in Sanskrit. Papaya is widely planted in the delta provinces, along the rivers, and on alluvial soils in Vietnam. *Carica papaya* contains phytochemicals such as phenolics, alkaloids, glucosinolates, carotenoids, ascorbic acid, acid amines, and enzymes [1]. Papaya has the advantage of being easy to grow, producing fruits quickly, and high productivity, and many parts of the papaya tree, such as leaves, barks, fruits, latex, roots, flowers, and seeds, are used for different medicinal purposes. Because of the uses of medicinal plants, many research projects have focused on determining this plant's chemical composition and biological activity, mainly the parts of the female papaya [2-5]. However, the parts of the male papaya still need to be studied [6-8].

Male *Carica papaya* flowers have many pharmacology activities, including cytotoxicity. Local people in Quangnam-Danang, in particular, and Vietnam, in general, have used male *Carica papaya* flowers to treat pharyngitis, cough, bronchitis, hoarseness, or hearing loss in adults and children. In addition, male *Carica papaya* flowers have long been used to support the treatment of lung cancer, liver cancer, and breast cancer [9]. However, only a few cytotoxic activity studies have been done on their male flower extracts [10-12]. There have been no published on the cytotoxic activity of compounds from the male *Carica papaya* flowers.

According to the screening test results on the cytotoxic activity of some extracts from the male papaya flowers from Quangnam-Danang on lung cancer cells (A549), liver cancer cells (Hep3B), and breast cancer cells (MCF-7) of our researcher's group [10], the cytotoxic activity on these cancer cells of compounds from the male *Carica papaya* flowers were tested. This study aimed to determine the cytotoxic activity on cancer cells of A549, Hep3B, MCF-7 of lignans, and monoterpenoids isolated from the male *Carica papaya* flowers in Quangnam - Danang.

2. Materials and methods

2.1. Plant materials

The male *Carica papaya* flowers were collected at Quangnam - Danang, Vietnam in December 2016. Its scientific name was identified by botanist Ngo Van Trai (Vietnam National Institute of Medicinal Materials), Nguyen The Anh, and Ho Ngoc Anh (Institute of Chemistry). A voucher specimen No. DD001 was deposited at the Herbarium of the Institute of Chemistry, Vietnam Academy of Science and Technology.

2.2. Chemicals and equipment

NMR spectra were recorded on a Bruker 500 MHz spectrometer. Column chromatography (CC) was performed using a silica gel (Kieselgel 60, 230-400 mesh, Merck) or RP-18 resins (150 µm, YMC), thin layer chromatography using a pre-coated silica gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck).

Methanol, *n*-hexane, methylene chloride, ethyl acetate, acetone, *n*-butanol, distilled water, sulforhodamine B (SRB), dimethyl sulfoxide (DMSO), trichloroacetic acid, acetic acid, tris(hydroxymethyl)aminomethane, ellipticine get analytical standards.

Tested cell lines: A549 (lung cancer), MCF-7 (breast cancer), and Hep3B (liver cancer) produced by J.M. Pezzuto, University of Hawaii, USA, and Jeanette Maier, University of Milan, Italy.

2.3. Extraction and isolation

The dried powdered male *Carica papaya* flowers (5 kg) were extracted three times with methanol using a sonicator to yield 300 g of dark solid residue. This extract was suspended in water and successively partitioned with *n*-hexane, methylene chloride (MC), ethyl acetate (EA),

and *n*-butanol to obtain corresponding *n*-hexane (54 g), MC (52 g), EA (20 g), and *n*-butanol (70 g) residues.

The MC residue was roughly separated on a silica gel column, eluting with methylene chloride/methanol (0-100% volume of methanol) to give 5 fractions CPMC1-CPMC5. Fraction CPMC2 (4.0 g) was chromatographed on a silica gel column, eluting with methylene chloride/methanol (20/1, v/v) to give 4 smaller fractions CPMC2A-CPMC2D. Fraction CPMC2B (0.8 g) was loaded on a silica gel column and eluted with methylene chloride/ethyl acetate (2/1, v/v) to give compounds (**1**) (5 mg) and (**2**) (2.5 mg). Compound (**3**) (7 mg) was isolated from fraction CPMC2A (1.2 g) using reverse phase C-18 (RP-18) column and acetone/distilled water (2/1, v/v) as an eluent. Fraction CPD4 (3.5 g) was separated on a silica gel column, eluting with methylene chloride/methanol (20/1, v/v) to give three fractions CPMC4A-CPMC4C. Fraction CPMC4A was repeatedly chromatographed on a silica gel column, eluting with methylene chloride/ethyl acetate (4/1, v/v) to give four fractions CPMC4A1-CPMC4A4. Compounds (**4**) (10 mg) and (**5**) (8 mg) were isolated from fraction CPMC4A4 using silica gel column and methylene chloride/methanol (10/1, v/v) as eluent. Fraction CPMC4C was separated on RP-18 column eluting with methanol/distilled water (2/1, v/v) and then purified on a silica gel column eluting with methylene chloride/acetone (3/1, v/v) to give compound (**6**) (7 mg).

2.4. Evaluation of the cytotoxic activity of compounds

The tested cell lines were grown as monolayers in DMEM (Dulbecco's Modified Eagle Medium) with an accompanying composition of 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES và 1.0 mM sodium pyruvate, plus 10% fetal bovine serum-FBS (Gibco). Cells were cultured after 3-5 days in an acetic acid/methanol buffer at a ratio (1:3) and cultured at 37ºC, 5% CO_2.

The *in vitro* cytotoxic activity test method is certified by the National Cancer Institute - NCI as a standard cytotoxic test to screen and detect substances capable of inhibiting the growth of cancer cells or killing cancer cells under *in vitro* conditions. The cytotoxic activity of the compounds was determined by the Monks method [13]. The test was carried out to determine the total cellular protein content based on the optical density (OD) measured when the protein composition of the cells was stained with sulforhodamine B (SRB). The calculated OD value is directly proportional to the amount of SRB attached to the protein molecule, so the more cells, the larger the OD value. The test is carried out under the following specific conditions:

The reagent (10 μL) mixed in 10% DMSO (in sterile distilled water) was introduced into the wells of the 96-well plate to have a screening concentration of 100 μg/mL. The active reagent is determined IC_{50} using a concentration range of 100, 20, 4, 0.8 μg/mL. Each concentration of test sample is performed in 3 wells.

Trypsinizing experimental cells to leave cells and counting in the counting chamber to adjust the density ($3x10^4$ cell/mL) to suit the experiment.

Add the appropriate number of cells (190 μL of medium) to the test wells and let them grow for 3-5 days.

Another 96-well plate without reagent but with cancer cells (190 μL) was prepared in 3 columns for day 0 control. After 01 hour, the day 0 control plate cells were fixed with trichloroacetic acid-TCA. The day 0 plate was a separate experimental plate. The experimental procedure of the day 0 control plate was similar to the reagent test plate.

After the growth phase in a CO_2 incubator, cells were fixed to the bottom of the well with TCA for 30 minutes and stained with SRB for 1 hour at 37°C. Discard the SRB, and the test wells were washed three times with 5% acetic acid and allowed to dry in air at room temperature.

Finally, use 10 mM tris(hydroxymethyl)aminomethane solution to dissolve the bound SRB and stain the protein molecules, put on a plate shaker, shake gently for 10 minutes, and use the ELISA Plate Reader (Bio-Rad) to read the results of the color content of SRB dyes through the absorption spectrum at 515-540 nm. The percentage of cells that are inhibited (%) in the presence of reagents will be determined through the following formula:

$$\%\ Cell\ inhibited = 100\% - \frac{OD\ (reagents) - OD\ (day\ 0)}{OD\ (negative\ control) - OD\ (day\ 0)}$$

The tests were repeated 3 times to ensure accuracy. Ellipticine (Sigma-Aldrich, USA) at concentrations of 10 μg/mL, 2 μg/mL, 0.4 μg/mL, and 0.08 μg/mL was always used as a positive control. DMSO 10% was used as a negative control. The IC_{50} value (concentration that inhibits 50% of growth) was determined using TableCurve 2Dv4 computer software (System Software Inc., San Jose, California, USA).

The tests were conducted at the Institute of Biotechnology, Vietnam Academy of Science and Technology.

3. Results and discussions

3.1. Chemical structure of compounds

The chemical structure of two lignans (**1**), (**2**) and four monoterpenoids (**3**)-(**6**) (Figure 1) was determined by NMR spectra and compared with the reported data. Spectroscopic data of compounds (**1**)-(**6**):

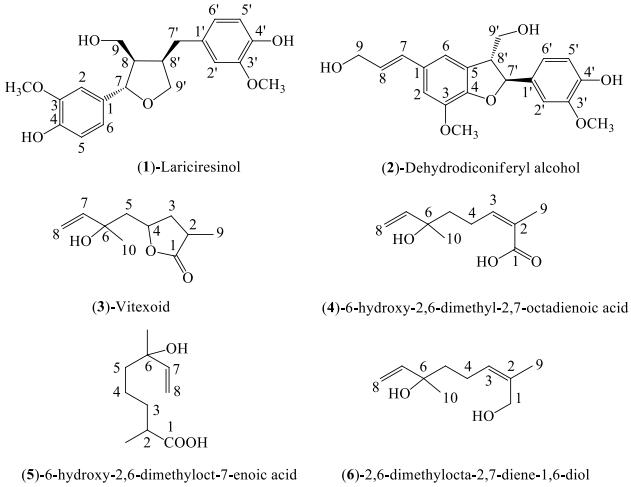

Figure 1. *Chemical structure of compounds (1)-(6)*

Lariciresinol (**1**): White amorphous powder. ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 6.87 (1H, d, *J* = 1.5 Hz, H-2), 6.88 (1H, d, *J* = 8.0 Hz, H-5), 6.81 (1H, dd, *J* = 1.5, 8.0 Hz, H-6), 4.79 (1H, d, *J* = 7.0 Hz, H-7), 2.41 (1H, m, H-8), 3.92 (1H, H_a-9), 3.78 (1H, H_b-9), 3.89 (s, 3-OMe), 6.69 (1H, br s, H-2′), 6.84 (1H, d, *J* = 8.5 Hz, H-5′), 6.70 (1H, br d, *J* = 8.5 Hz, H-6′), 2.92 (1H, dd, *J* = 5.0, 13.5 Hz, H_a-7′), 2.56 (1H, dd, *J* = 10.5, 13.5 Hz, H_b-7′), 2.74 (1H, m, H-8′), 4.06 (1H, dd, *J* = 6.5, 8.5 Hz, H_a-9′), 3.75 (1H, dd, *J* = 6.5, 8.5 Hz, H_b-9′), 3.87 (s, 3′-OMe). ^{13}C-NMR (125 MHz, $CDCl_3$), δ (ppm): 134.8 (C-1), 108.3 (C-2), 146.7 (C-3), 145.1 (C-4), 114.2 (C-5), 118.8 (C-6), 82.9 (C-7), 52.6 (C-8), 61.0 (C-9), 56.0 (C_3-O2111Me), 132.3 (C-1′), 111.2 (C-2′), 146.6 (C-3′), 144.1 (C-4′), 114.4 (C-5′), 121.2 (C-6′), 33.4 (C-7′), 42.5 (C-8′), 72.9 (C-9′), 56.0 (C_3′-OMe) [14].

Dehydrodiconiferyl alcohol (**2**): White amorphous powder. ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 6.87 (1H, s, H-2), 6.90 (1H, s, H-6), 6.57 (1H, d, *J* = 16.0 Hz, H-7), 6.25 (1H, dt, *J* = 6.0, 16.0 Hz, H-8), 4.31 (2H, d, *J* = 6.0 Hz, H-9), 3.91 (s, 3-OMe), 6.93 (1H, br s, H-2′), 6.88 (1H, d, *J* = 9.0 Hz, H-5′), 6.91 (1H, br d, *J* = 9.0 Hz, H-6′), 5.58 (1H, d, *J* = 7.5 Hz, H-7′), 3.63 (1H, m, H-8′), 3.97 (1H, H_a-9′), 3.92 (1H, H_b-9′), 3.87 (s, 3′-OMe). ^{13}C-NMR (125 MHz, $CDCl_3$), δ (ppm): 130.9 (C-1), 110.6 (C-2), 144.5 (C-3), 148.2 (C-4), 128.1 (C-5), 114.8 (C-6), 131.4 (C-7), 126.5 (C-8), 63.9 (C-9), 56.0 (C_3-OMe), 132.9 (C-1′), 108.8 (C-2′), 146.7 (C-3′), 145.8 (C-4′), 114.4 (C-5′), 119.5 (C-6′), 88.3 (C-7′), 53.6 (C-8′), 64.0 (C-9′), 56.0 (C_3′-OMe) [15].

Vitexoid (**3**): Colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 2.67 (1H, m, H-2), 2.03 (1H, m, H_a-3); 2.10 (1H, m, H_b-3), 4.74 (1H, m, H-4), 1.78 (1H, dd, *J* = 3.0, 11.5 Hz, H_a-5), 2.03 (1H, dd, *J* = 8.0, 15.5 Hz, H_b-5), 5.93 (1H, dd, *J* = 10.5, 17.0 Hz, H-7), 5.14 (1H, dd, *J* = 1.5, 10.5 Hz, H_a-8), 5.33 (1H, dd, *J* = 1.5, 17.0 Hz, H_b-8), 1.27 (3H, d, *J* = 7.0 Hz, H-9), 1.33 (3H, s, H-10). ^{13}C-NMR (125 MHz, $CDCl_3$), δ (ppm): 179.34 (C-1), 33.83 (C-2), 36.60 (C-3), 75.75 (C-4), 47.06 (C-5), 72.62 (C-6), 144.03 (C-7), 112.86 (C-8), 15.84 (C-9), 28.74 (C-10) [16].

6-hydroxy-2,6-dimethyl-2,7-octadienoic acid (**4**): Colorless oil. ^{1}H-NMR (500 MHz, DMSO-d_6), δ (ppm): 6.62 (1H, m, H-3), 2.21 (2H, m, H-4), 1.48 (2H, t, *J* = 8.0 Hz, H-5), 5.86 (1H, dd, *J* = 11.0, 17.5 Hz, H-7), 4.97 (1H, dd, *J* = 2.0, 11.0 Hz, H_a-8), 5.16 (1H, dd, *J* = 2.0, 17.5 Hz, H_b-8), 1.70 (3H, s, H-9), 1.16 (3H, s, H-10). ^{13}C-NMR (125 MHz, DMSO-d_6), δ (ppm): 169.34 (C-1), 127.83 (C-2), 141.52 (C-3), 23.08 (C-4), 40.61 (C-5), 71.29 (C-6), 145.77 (C-7), 111.17 (C-8), 12.22 (C-9), 27.67 (C-10) [17].

6-hydroxy-2,6-dimethyloct-7-enoic acid (**5**): Colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 2.48 (1H, m, H-2), 1.68 (1H, m, H_a-3), 1.43 (1H, m, H_b-3), 1.38 (2H, m, H-4), 1.53 (2H, m, H-5), 5.90 (1H, dd, *J* = 11.0, 17.0 Hz, H-7), 5.20 (1H, d, *J* = 17.0 Hz, H_a-8), 5.04 (1H, d, *J* = 11.0 Hz, H_b-8), 1.18 (3H, d, *J* = 7.0 Hz, H_2-Me), 1.28 (3H, s, H_6-Me). ^{13}C-NMR (125 MHz, $CDCl_3$), δ (ppm): 181.0 (C-1), 39.1 (C-2), 33.9 (C-3), 21.5 (C-4), 42.0 (C-5), 73.2 (C-6), 145.0 (C-7), 111.8 (C-8), 16.9 (C_2-Me), 27.8 (C_6-Me) [18].

2,6-dimethylocta-2,7-diene-1,6-diol (**6**): Colorless oil. ^{1}H-NMR (500 MHz, CD_3OD), δ (ppm): 3.92 (2H, s, H-1), 5.41 (1H, dt, *J* = 1.0, 7.0 Hz, H-3), 2.09 (2H, m, H-4), 1.56 (2H, m, H-5), 5.94 (1H, dd, H-7), 5.06 (1H, dd, *J* = 1.5, 11.0 Hz, H_a-8), 5.23 (1H, dd, *J* = 1.5, 17.5 Hz, H_b-8), 1.66 (3H, s, H-9), 1.27 (3H, s, H-10). ^{13}C-NMR (125 MHz, CD_3OD), δ (ppm): 68.96 (C-1), 135.88 (C-2), 126.83 (C-3), 23.36 (C-4), 43.05 (C-5), 73.79 (C-6), 146.26 (C-7), 112.06 (C-8), 13.62 (C-9), 27.60 (C-10) [19].

3.2. Evaluation of the cytotoxic activity of compounds

The results of evaluating the cytotoxic activity of two lignans (**1**), (**2**) and four monoterpenoids (**3**)-(**6**) from the male *Carica papaya* flowers on tested three cell lines: A549 (lung cancer), MCF-7 (breast cancer), and Hep3B (liver cancer) are presented in Table 1.

Table 1. *The cytotoxic activity of compounds (1)-(6)*

Compound	IC_{50} (µg/mL)		
	A549	**MCF-7**	**Hep3B**
Lignan			
(**1**)	50.80±5.93	66.12±5.60	62.62±5.49
(**2**)	70.99±7.26	60.85±4.43	62.32±5.03
Monoterpenoid			
(**3**)	54.63±3.67	34.73±1.55	60.37±3.71
(**4**)	78.98±4.64	54.15±5.89	72.25±3.13
(**5**)	86.03±7.57	67.49±2.41	77.77±5.52
(**6**)	49.06±2.82	38.28±1.53	54.07±2.85
Ellipticine	0.43±0.04	0.37±0.03	0.50±0.04

Ellipticine: The positive control, which acts stably in the experiment.

Lignan (**1**), monoterpenoid (**3**), and (**6**) exhibited medium cytotoxic activity on these cancer cells with IC_{50} from 34.73±1.55 to 66.12±5.60 µg/mL. Lignan (**2**) displayed medium cytotoxic activity on MCF-7, Hep3B with IC_{50} 60.85±4.43, 62.32±5.03 µg/mL, respectively, and weak cytotoxic activity on A549 with IC_{50} 70.99±7.26 µg/mL. Monoterpenoids (**4**), and (**5**) showed medium cytotoxic activity on MCF-7 with IC_{50} 54.15±5.89, 67.49±2.41 µg/mL, respectively, and weak cytotoxic activity on A549, and Hep3B with IC_{50} from 72.25±3.13 to 86.03±7.57 µg/mL. Ellipticine was a positive control.

Lignans exhibit many biological activities such as antioxidant, anti-inflammatory, anti-cardiovascular, anti-viral, and anticancer [20]. Two lignans (**1**), and (**2**) have been isolated from the male *Carica papaya* flowers. These lignans inhibit all three cancer cell lines A549, MCF-7, and Hep3B with IC_{50} from 50.80±5.93 to 70.99±7.26 µg/mL. According to previous studies, lignan (**1**) was isolated from the genus *P. villosa*. It was reported to be toxic to PC3 prostate cancer cells, NB4 leukemia, KB carcinoma, and B16 melanoma cells under *in vitro* testing conditions [21]. Besides, lignan (**1**) inhibits cell proliferation and induces apoptosis in Hep-G2 liver cancer cells with IC_{50} 205 µg/mL after 48 hours [22]. Lignan (**2**) was isolated from *Cucurbita moschata* and had anti-fat and anti-obesity properties in 3T3-L1 cells and mouse embryonic fibroblasts [23]. In addition, lignan (**2**) can also be effective in treating osteoporosis by regulating the bone formation process through activating estrogen receptors, according to research by W. Lee et al. [24].

The biological activity exhibited by monoterpenoids includes analgesic, anti-inflammatory, anticonvulsant, antidepressant, anti-Alzheimer, anti-Parkinsonian, antiviral, and antibacterial (anti-tuberculosis) effects [25]. Four monoterpenoids (**3**)-(**6**) have been isolated from the male *Carica papaya* flowers. These monoterpenoids show all three cancer cell lines A549, MCF-7, and Hep3B with IC_{50} from 34.73±1.55 to 86.03±7.57 µg/mL. The research by J. Wu et al. have published on the ability of monoterpenoid (**3**) to inhibit the proliferation of HeLa cervical cancer cells [16].

According to the literature search up to the time of the study, this is the first publication on the cytotoxic activity on tested three cell lines: A549 (lung cancer), MCF-7 (breast cancer), and Hep3B (liver cancer) of lignans (**1**), (**2**), and monoterpenoids (**3**)-(**6**) from the male *Carica papaya* flowers.

4. Conclusions

In summary, all the lignans and monoterpenoids isolated from the male *Carica papaya* flowers exhibited cytotoxic activity against tested cancer cell lines (A549, MCF-7, Hep3B) under *in vitro* conditions. Lignan (**1**), monoterpenoid (**3**), and (**6**) exhibited medium cytotoxic activity on these cancer cells with IC_{50} from 34.73±1.55 to 66.12±5.60 µg/mL. Lignan (**2**) displayed medium cytotoxic activity on MCF-7, Hep3B with IC_{50} 60.85±4.43, 62.32±5.03 µg/mL, respectively, and weak cytotoxic activity on A549 with IC_{50} 70.99±7.26 µg/mL. Monoterpenoids (**4**), and (**5**) showed medium cytotoxic activity on MCF-7 with IC_{50} 54.15±5.89, 67.49±2.41 µg/mL, respectively, and weak cytotoxic activity on A549, and Hep3B with IC_{50} from 72.25±3.13 to 86.03±7.57 µg/mL with ellipticine was a positive control. It is the first publication on the cytotoxic activity of these tested cell lines of two lignans (**1**), (**2**), and monoterpenoids (**3**)-(**6**) isolated from the male *Carica papaya* flowers.

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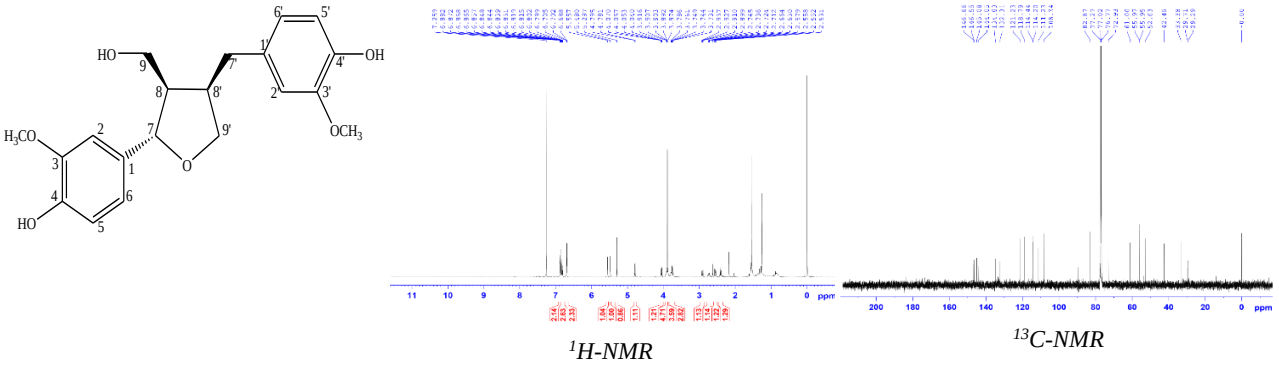
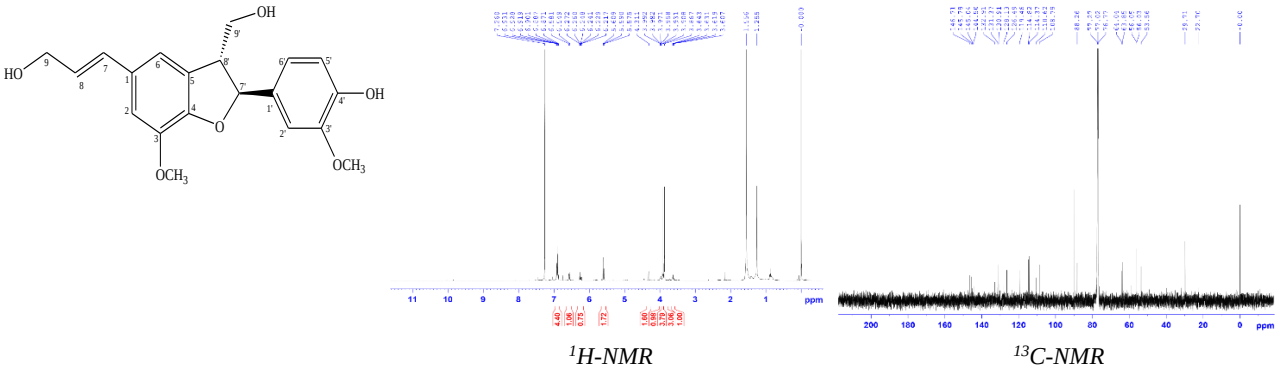
SUPPLEMENTAL MATERIAL



^{1}H-NMR ^{13}C-NMR

SM 1. *^{1}H-NMR and ^{13}C-NMR spectra of (**1**)-Laricresinol*



^{1}H-NMR ^{13}C-NMR

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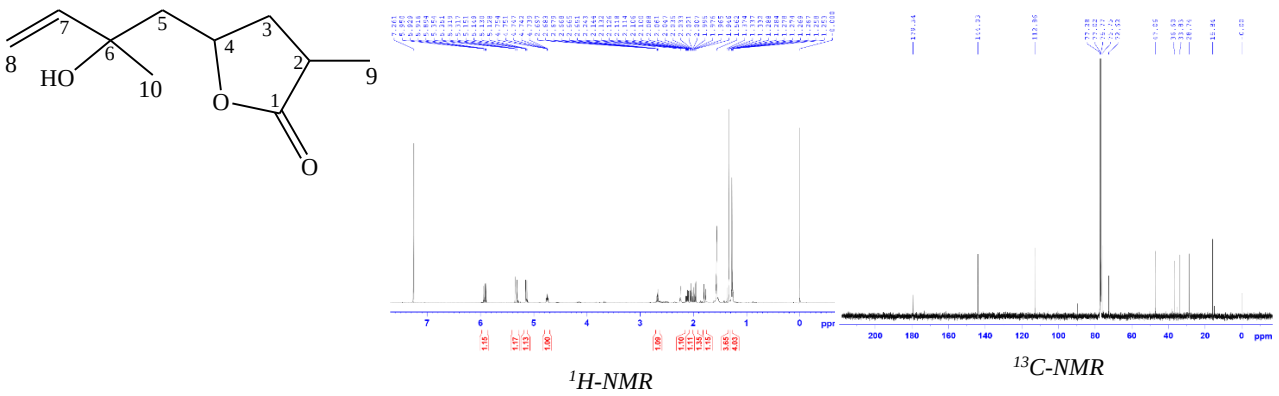
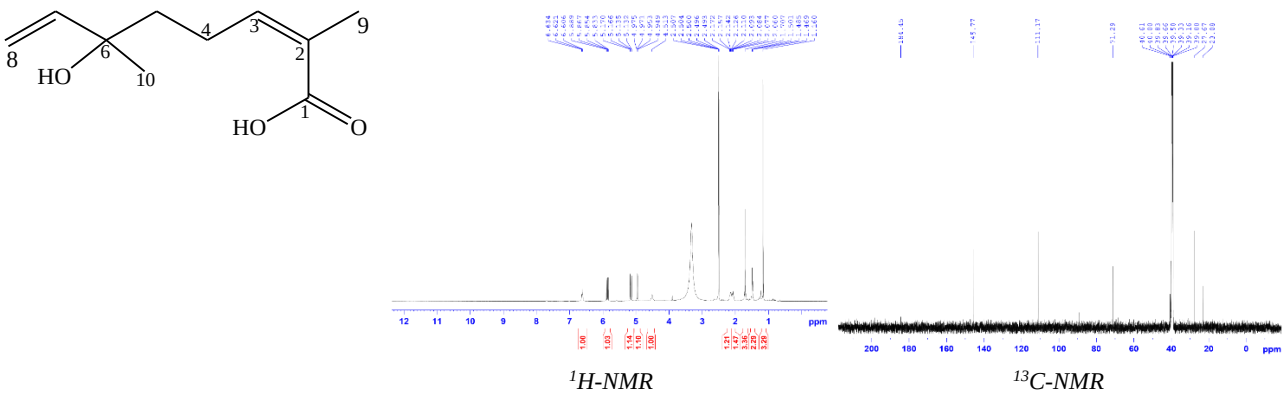


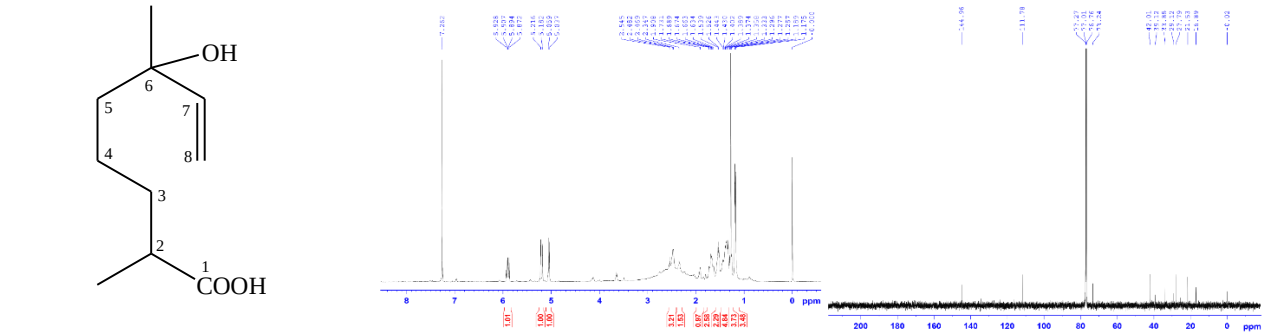
^{1}H-NMR ^{13}C-NMR

SM 3. *^{1}H-NMR and ^{13}C-NMR spectra of (**3**)-Vitexoid*



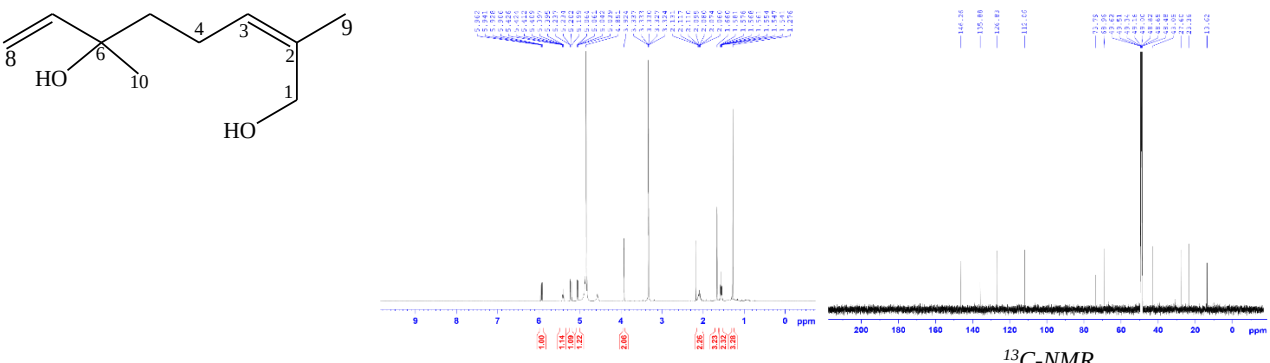
^{1}H-NMR ^{13}C-NMR

SM 4. *^{1}H-NMR and ^{13}C-NMR spectra of (**4**)-6-hydroxy-2,6-dimethyl-2,7-octadienoic acid*



^{1}*H-NMR* ^{13}*C-NMR*

SM 5. *^{1}H-NMR and ^{13}C-NMR spectra of (**5**)-6-hydroxy-2,6-dimethyloct-7-enoic acid*



^{1}*H-NMR* ^{13}*C-NMR*

SM 6. *^{1}H-NMR and ^{13}C-NMR spectra of (**6**)-2,6-dimethylocta-2,7-diene-1,6-diol*