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STUDY ON CHEMICAL COMPOSITION AND INHIBITIONAL EFFECT ON CANCER CELL DEVELOPMENT OF *Mallotus apelta* (LOUR.) MÜLL. –ARG., FROM THE EUPHORBIACEAE FAMILY

SUMMARY OF CHEMISTRY DOCTORAL THESIS IN ORGANIC CHEMISTRY

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INTRODUCTION

The natural and climatic conditions, characterized by diversity across regions, have endowed Vietnam with a rich plant ecosystem. Additionally, Vietnam is one of the countries with an ancient tradition of traditional medicine, utilizing a variety of herbs in disease treatment and health enhancement. In recent decades, there has been a growing trend among scientists worldwide to deeply research medicinal plants and animals to discover natural compounds with high biological activity for use in the production of medicines or functional foods to serve life.

The plant M. apelta has long been used in traditional medicine to treat various ailments. Recent studies have shown that some compounds in M. apelta have particularly interesting biological activities. For instance, malloapelta B, isolated from the leaves by Vietnamese scientists, has shown anti-cancer potential through strong inhibition of NF-κB activation. Another compound, malloapeltic acid, isolated from the roots, exhibits anti-HIV properties. This suggests the immense potential of unknown compounds in M. apelta for supporting and treating intractable diseases. Practical experience indicates the need for comprehensive studies to isolate all compounds from this species and thoroughly test their biological activities. Therefore, I chose the topic: "Study on the chemical composition and inhibitional effect on cancer cell development of *Mallotus apelta* (Lour.) Muell.-Arg. Family Euphorbiaceae."

Objectives of the thesis:

- To deeply investigate the chemical composition of *Mallotus apelta* in Vietnam.
- To study the cytotoxic effects of the isolated compounds on cancer cells.

The thesis includes:

1. Isolation of 21 compounds from the leaves of Mallotus apelta in Vietnam using chromatography methods.
2. Determination of the chemical structures of the isolated compounds, based on modern spectral methods.
3. Evaluation of the anti-cancer activity of the isolated compounds against ovarian, prostate, breast, and colorectal cancer cells. Study of the mechanisms of cytotoxicity in breast and prostate cancer cells.

New contributions of the thesis:

1. Isolation and structural determination of 14 new compounds: malloapelta C and D (**MA1a**, **MA1b**, **MA2a**, **MA2b** - 2 pairs of enantiomers), malloapelta

E - H (**MA3-MA6)**, malloapelta I and II (**MA7** and **MA8)**, malloapelta J-L (**MA9-MA11)**, and malloflavoside (**MA12)**.

2. The isolated compounds **MA1-MA8** and malloapelta B were found to have strong inhibitory effects on cancer cell growth. Compounds **MA2, MA3,** and malloapelta B were found to inhibit the growth of TOV-21G ovarian cancer cells through apoptosis and inactivation of the NF-κB factor. Compound **MA8** was found to be cytotoxic to breast and prostate cancer cell lines, MCF-7 and PC-3, through ANO1 inhibition pathways.

CHAPTER 1. OVERVIEW

1.1. Introduction to the *Mallotus* genus

The genus *Mallotus* (Ba bet) is a relatively large genus in the family Euphorbiaceae, comprising about 150 species, scattered across the tropical regions of South Asia and Southeast Asia. Species of the genus Mallotus are usually shrubs or small trees. They grow in primary forests below 1000 meters above sea level. In Vietnam, statistics show that there are about 40 species of the genus *Mallotus*, including 7 endemic species

1.2. Botanical characteristics of the *Mallotus* genus

An overview reveals that 315 compounds have been isolated and structurally identified from this genus, with the most common being phenolic compounds and terpenoids (79 compounds each), followed by flavonoids (53 compounds), benzopyrans and coumarins (51 compounds), phloroglucinols (36 compounds), and the remainder being steroids and other compounds (28 compounds). Species of the genus Mallotus possess a variety of biological activities such as anti-cancer, anti-inflammatory, antioxidant, antibacterial, antifungal, immunomodulatory, and antiviral properties.

1.3. Introduction to *Mallotus apelta* species

The species *Mallotus apelta* (Lour.) Muell.-Arg, commonly known as bùm bụp or bông bet, is a shrub or small tree ranging from 1 to 6 meters in height, typically growing wild at elevations of 700 meters above sea level. The plant is widely distributed in southern China and Southeast Asian countries, including Vietnam. An overview indicates that globally, 51 compounds have been isolated from M. apelta, including 29 benzopyrans and coumarins, 3 flavonoids, 5 phenolic compounds, 7 triterpenoids, 6 diterpenoids, and 1 other compound.

CHAPTER 2. SUBJECTS AND METHODS

2.1. Research subjects

The leaves of *Mallotus apelta* (Lour.) Mull.Arg. were collected in Ngoc Thanh, Phuc Yen, Vinh Phuc, Vietnam (21°22'35.4" N + 105°43'23.9" E) in August 2018 and identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The specimen (**MA180**8) is preserved at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology.

2.2. Research method

2.2.1. Isolation of compounds

The methods used include Thin Layer Chromatography (TLC), Column Chromatography (CC), and High-Performance Liquid Chromatography (HPLC).

2.2.2. Structure determination

The methods used include high-resolution mass spectrometry, nuclear magnetic resonance (NMR) spectroscopy, electronic circular dichroism spectroscopy, theoretical CD spectrum calculation methods, and polarimetry ($[\alpha]_D$)

2.2.3. Bioactivity assessment

The methods used include MTT, MTS, and CCK-8 assays, YFP fluorescence signal analysis, short-circuit current measurement, intracellular calcium concentration measurement, protein content evaluation, Western blot, cell migration assays, caspase-3 activity evaluation, and statistical analysis methods.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Isolation of compounds

The dried leaves of M. apelta (5.0 kg) were powdered and extracted with methanol (3 times × 8 liters) using ultrasound equipment (at 50°C, each time for 4 hours). The combined extracts were filtered through filter paper, and the solvent was evaporated under reduced pressure to yield 568 g of methanol extract residue. This residue was dissolved in 4.0 liters of distilled water, and liquid-liquid partitioning was performed using dichloromethane to obtain a dichloromethane residue (MAL1, 250.0 g).

The MAL1 fraction (120.0 g) was chromatographed on a silica gel column using a gradient elution with n-hexane - acetone solvent systems (40:1, 20:1, 10:1, 5:1, 1:1, v/v) to yield five subfractions: MAL1A (15.0 g), MAL1B (23.0 g), MAL1C (15.0 g), MAL1D (25.0 g), and MAL1E (50.0 g).

The aqueous layer (MAL2) was separated from the organic solvents and then chromatographed on a Diaion HP-20 column using water to remove polar components. The elution was subsequently performed with increasing concentrations of methanol in water (25, 50, 75, and 100%, v/v) to obtain four subfractions: MAL2A to MAL2D. MAL2C was further chromatographed on silica gel using a dichloromethane - methanol solvent system (20:1, 10:1.5:1, 1:1, v/v) to yield four smaller subfractions: MAL2C1 (500 mg), MAL2C2 (700 mg), MAL2C3 (1.2 g), and MAL2C4 (1.0 g).

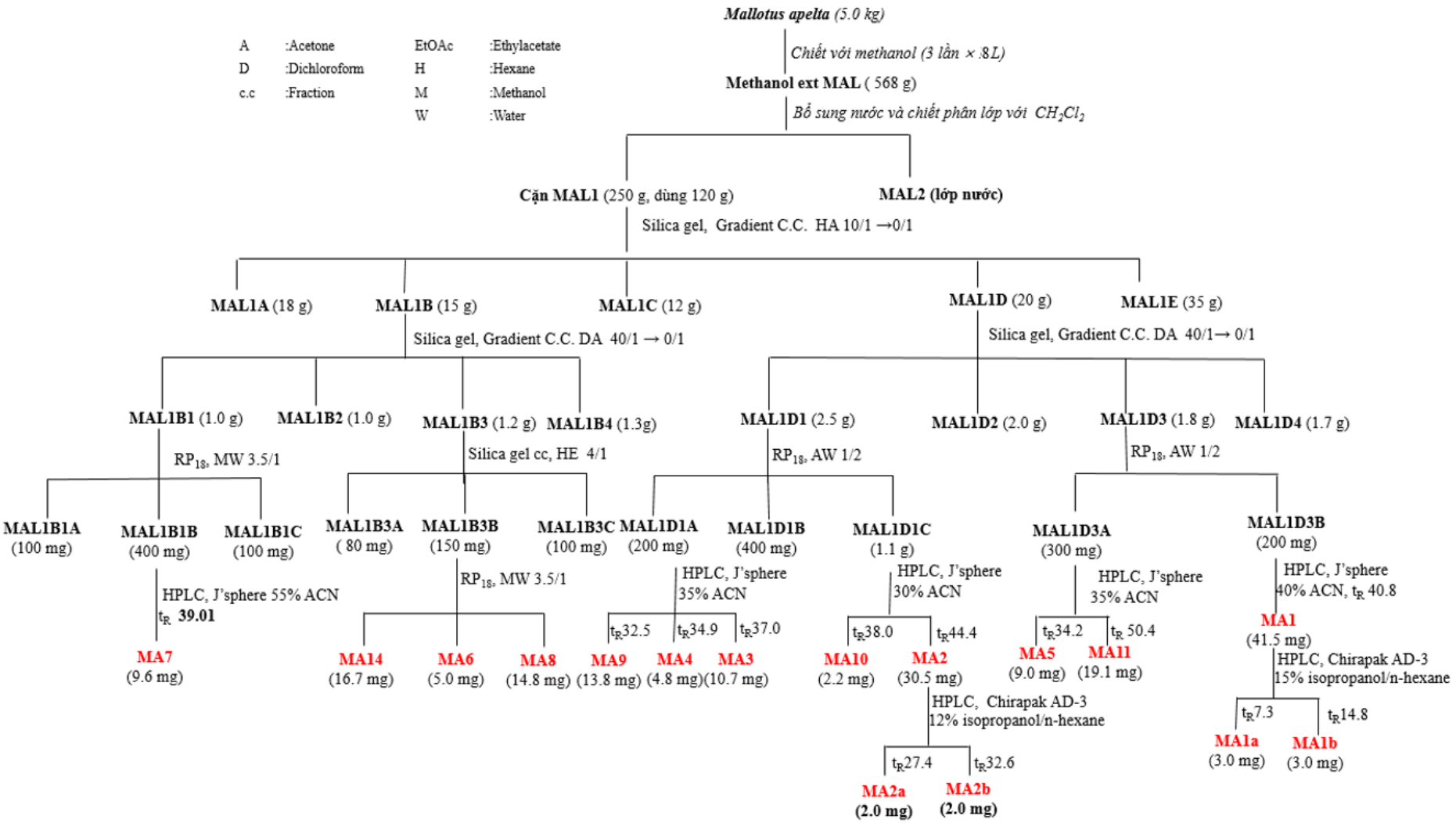

Figure 3.1. **Diagram for isolating compounds from *M. apelta*, MAL1 fraction**

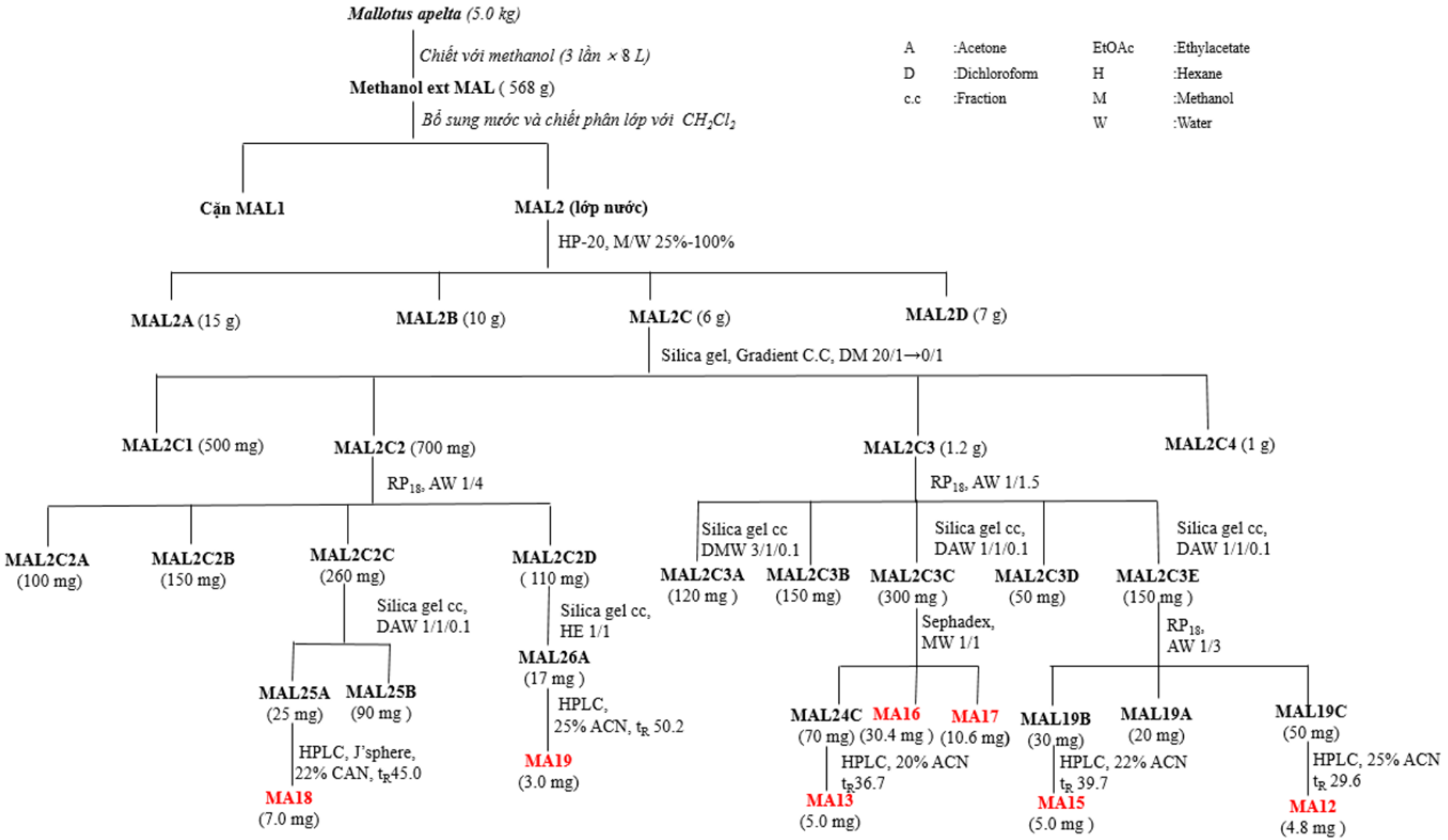

Figure 3.2. **Diagram for isolating compounds *from M. apelta*, MAL2 fraction**

3.2. Physical properties and spectral data of the compounds

3.2.1. Compound MA1: Malloapelta C (New compound)

White amorphous powder.

Molecular formula: $C_{18}H_{24}O_6$, M = 336.

Polar rotation $[\alpha]_D^{25}$ = 0,0 (*c* 0,1 $CHCl_3$)

(**MA1a** $[\alpha]_D^{25}$ = +21,1 (*c* 0,05 $CHCl_3$), **MA1b** $[\alpha]_D^{25}$ = -20,3 (*c* 0,05 $CHCl_3$))

HR-ESI-MS at *m/z* 337,1646 $[M+H]^+$. Theoretical calculation for formula $[C_{18}H_{25}O_6]^+$, 337,1646.

^{1}H-NMR ($CDCl_3$): 3.74 (dd, 3.0, 8.5, H-3), 4.25 (d, 3.0, H-4), 6.12 (s, H-6), 1.32 (s, H-11), 1.32 (s, H-12), 6.29 (dq, 2.0, 16.0, H-2′), 6.58 (dq, 7.0, 16.0, H-3′), 1.87 (dd, 2.0, 7.0, H-4′), 3.51 (s, H-4-OMe), 3.76 (s, H-5-OMe), 3.89 (s, H-7-OMe), 1.97 (d, 8.5, H-3-OH),

^{13}C-NMR ($CDCl_3$): 77.6(C-2), 70.5(C-3), 74.6(C-4), 161.4(C-5), 88.5(C-6), 158.6(C-7), 111.0(C-8), 151.6(C-9), 102.2(C-10), 24.2(C-11), 23.3(C-12), 194.8(C-1′), 134.3(C-2′), 145.2(C-3′), 18.1(C-4′), 57.3(C-4-OMe), 55.9(C-5-OMe), 55.8(C-7-OMe)

3.2.2. Compound MA14: Malloapelta B

White amorphous powder.

Molecular formula: $C_{17}H_{20}O_4$, M = 288.

^{1}H-NMR ($CDCl_3$): 5.45 (d, 10.5, H-3), 6.58 (d, 10.5, H-4), 6.03 (s, H-6), 1.34 (s, H-11), 1.34 (s, H-12), 6.38 (d, 17.0, H-2′), 6.69 (dd, 17.0, 6.5, H-3′), 1.91 (d, 6.5, H-4′), 3.77 (s, H-5-OMe), 3.84 (s, H-7-OMe),

^{13}C-NMR ($CDCl_3$): 76.5 (C-2), 127.1 (C-3), 116.7 (C-4), 156.5 (C-5), 88.0 (C-6), 158.1 (C-7), 111.7 (C-8), 151.9 (C-9), 104.2 (C-10), 27.7 (C-11), 27.5 (C-12), 194.2 (C-1′), 134.8 (C-2′), 144.8 (C-3′), 18.1 (C-4′), 55.9 (C-5-OMe), 55.6 (C-7-OMe)

3.3. Structure determination of isolated compounds

3.3.1. Compound MA1: Malloapelta C (New compound)

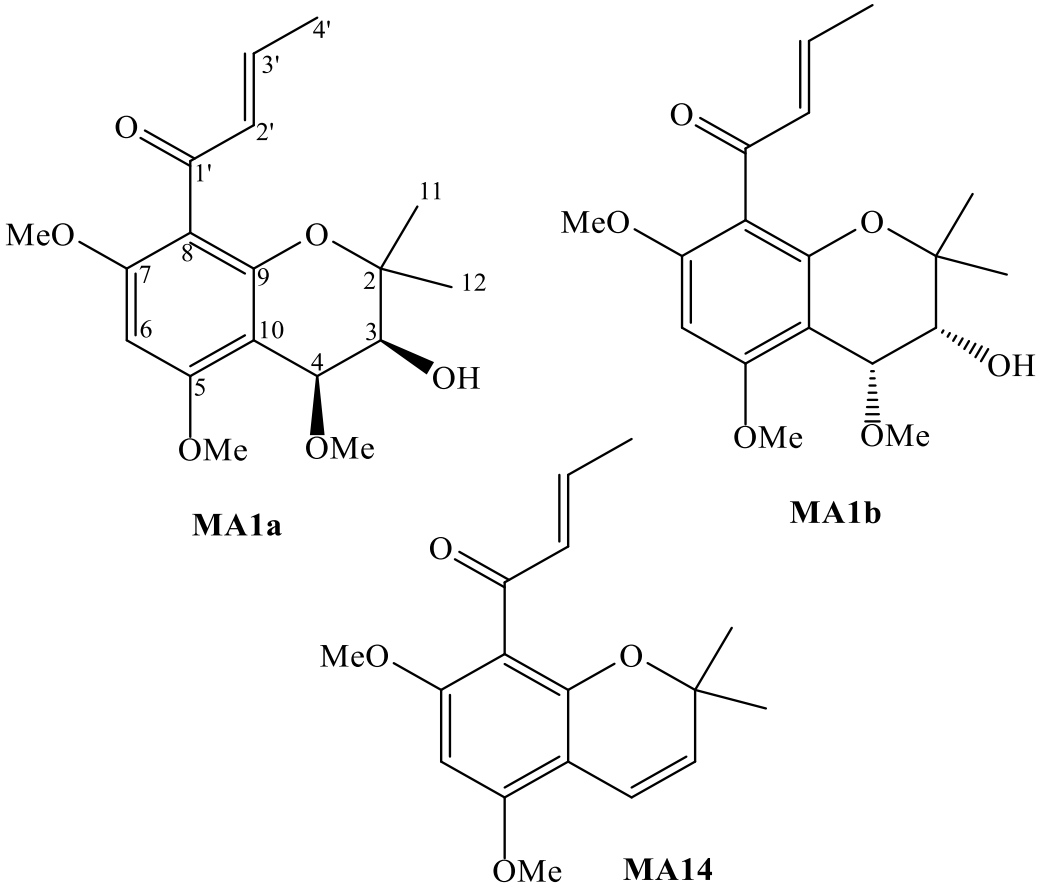

Figure.3.3. Chemical structures of the two enantiomers MA1a and MA1b and the reference compound

The compound **MA1** was obtained as a white amorphous powder, and its molecular formula was determined to be $C_{18}H_{24}O_6$ based on the HR-ESI-MS spectrum at m/z 337.1646 $[M+H]^+$ (Theoretical calculation for formula $[C_{18}H_{25}O_6]^+$, 337.1646). The ^{1}H-NMR (measured in $CDCl_3$) showed signals of an aromatic proton at δ_H 6.12 (s), two olefinic protons *at* δ_H 6.29 (1H, dq, *J* = 2.0, 16.0 Hz) và 6.58 (1H, dq, *J* = 7.0, 16.0 Hz), two oxymethines at δ_H 3.74 (1H, dd, *J* = 3.0, 8.5 Hz) and 4.25 (1H, d, *J* = 3.0 Hz), three methoxy groups at δ_H 3.51, 3.76 and 3.89 (each 3H, s), three methyl group at δ_H 1.87 (3H, dd, *J* = 2.0, 7.0 Hz) và 1.32 (6H, s). The 13C-NMR and HSQC spectra of **MA1** showed signals of 18 carbons, including a carbonyl at δC 194.8, six carbons not directly bonded to protons at δC 77.6, 102.2, 111.0, 151.6, 158.6, and 161.4, five methine carbons at δC 70.5, 74.6, 88.5, 134.3, and 145.2, and six methyl carbons at δC 18.1, 23.3, 24.2, 57.3, 55.8, and 55.9. The analysis of the ^1H-NMR and ^{13}C-NMR spectra suggested that the structure of **MA1** is similar to **MA14** (malloapelta B), with the substitution of the double bond at C-3/C-4 by hydroxyl and methoxy groups. This is based on HMBC correlations between H-11/H-12 (δH 1.32) and C-2 (δC 77.6)/C-3 (δC 70.5), H-3 (δH 3.74) and C-2 (δC 77.6)/C-10 (δC 102.2)/C-11 (δC 24.2)/C-12 (δC 23.3), between H-4 (δH 4.25) and C-2 (δC

77.6)/C-5 (*δ*C 161.4)/C-9 (*δ*C 151.6)/C-10 (*δ*C 102.2), and between the methoxy group (*δ*H 3.51) and C-4 (*δ*C 74.6). HMBC correlations from H-6 (*δ*H 6.12) to C-5 (*δ*C 161.4)/C-7 (*δ*C 158.6)/C-8 (*δ*C 111.0)/C-10 (*δ*C 102.2), and from methoxy groups (*δ*H 3.76 and 3.89) to C-5 (*δ*C 161.4) and C-7 (*δ*C 158.6) indicated the positions of two methoxy groups at C-5 and C-7. The large coupling constant between H-2' and H-3' (J = 16.0 Hz) indicated the E configuration of the double bond of the 1-oxobut-2-enyl group. Furthermore, the position of this group at C-8 was determined based on the HMBC correlation from H-2' (*δ*H 6.29) to C-8 (*δ*C 111.0). The small coupling constant between H-3 and H-4 (J = 3.0 Hz) suggested a cis configuration for H-3 and H-4 of the pyran ring. Thus, the structure of **MA1** was determined to be (3S*,4S*)-8-[1-oxobut-2(E)-enyl]-4,5,7-trimethoxy-2,2-dimethylchroman-3-ol and named malloapelta C. However, the optical rotation of **MA1** (~ 0) and the CD spectrum of **MA1** showed no Cotton effect, suggesting it is racemic. Subsequently, HPLC (chiral column: Chirapak AD-3, ID 4.6 mm × 250 mm length) eluted with 15% isopropanol/n-hexane separated the two enantiomers [**MA1a**: [α]D = +21.1 (c 0.05, CHCl3) and **MA1b**: [α]D = -20.3 (c 0.05, $CHCl_3$)]. The calculated CD spectrum of **MA1(**3S,4S) showed a positive Cotton effect at 200-220 nm, matching the experimental CD spectrum of **MA1a**. Similarly, the experimental CD spectrum of **MA1b** showed a negative Cotton effect at 200-220 nm, matching the calculated CD spectrum of **MA1(**3R,4R). Therefore, the absolute configurations of **MA1a** and **MA1b** were determined to be (3S,4S)-malloapelta C and (3R,4R)-malloapelta C, respectively.

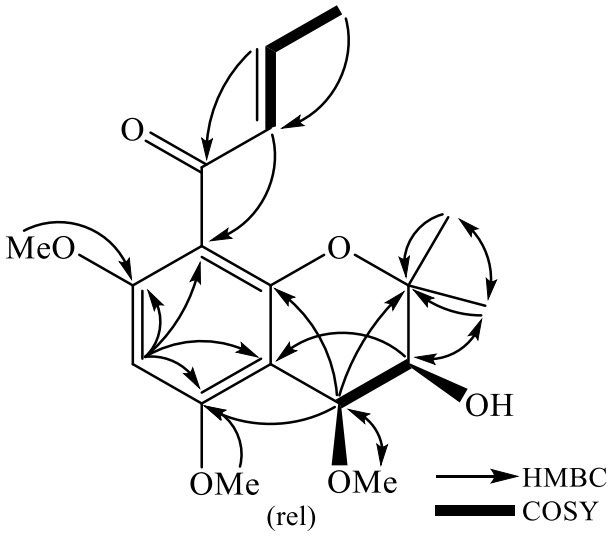

Figure 3.4. COSY keys and HMBC interactions of MA1

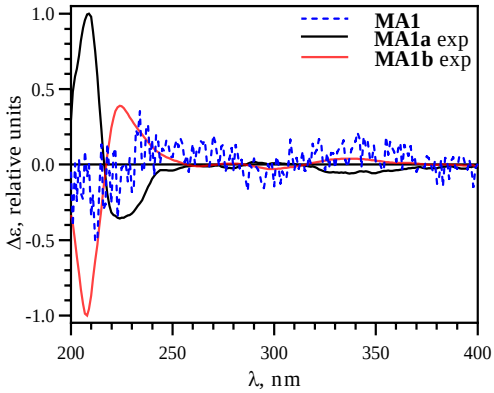

Figure 3.5. CD spectra of MA1 and enantiomers (MA1a and MA1b).

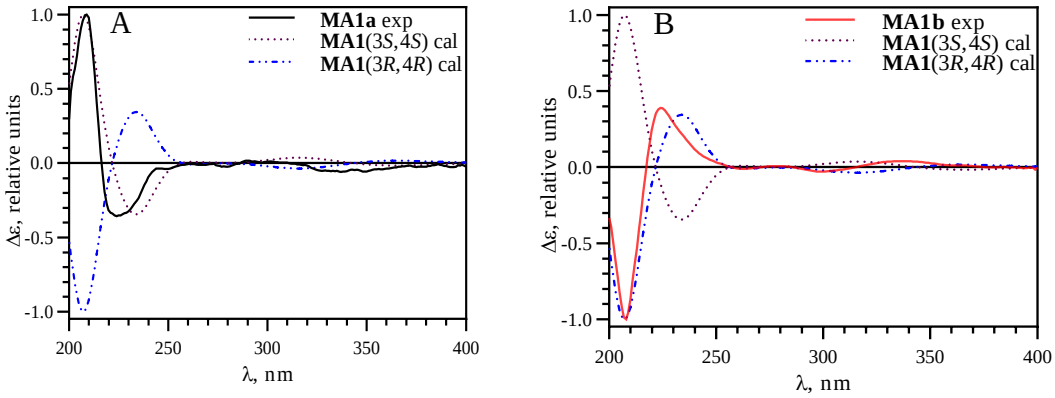

Figure 3.6. Experimental CD spectra MA1a (A), MA1b (B) and calculated CD spectra of compounds MA1 (3S,4S) and (3R,4R).

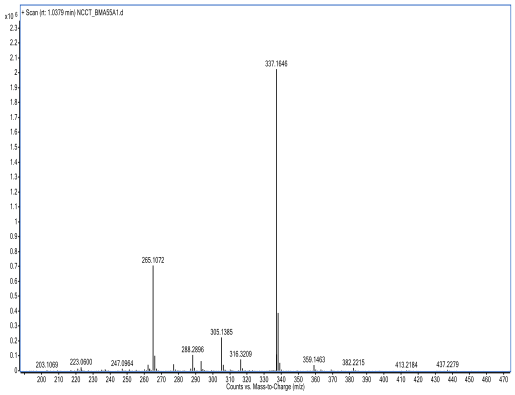

Figure 3.7. HR-ESI-MS spectrum of MA1

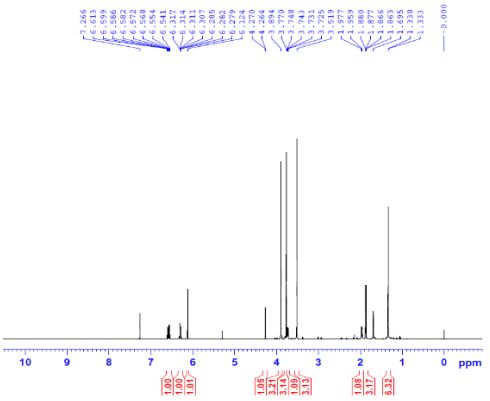

Figure3.8. 1H-NMR spectrum of MA1

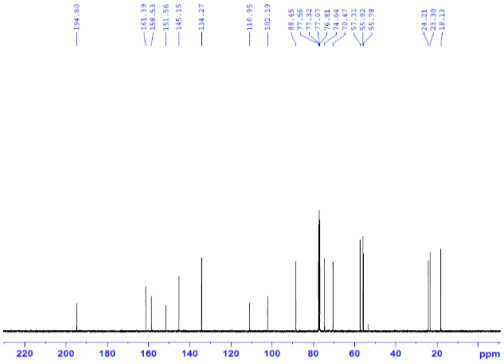

Figure 3.9. 13C-NMR spectrum MA1

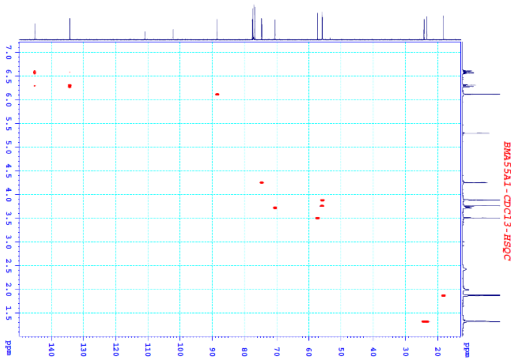

Figure 3.10. HSQC keys of MA1

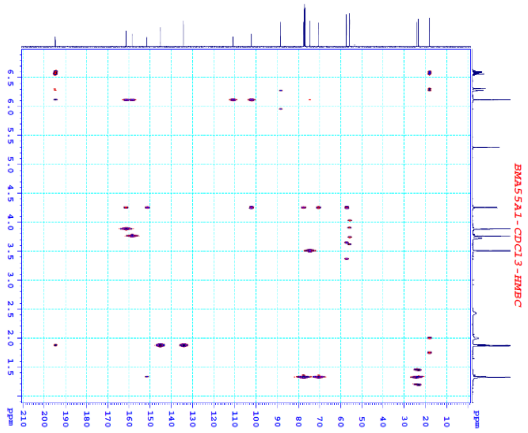

Figure 3.11. HMBC keys of MA1

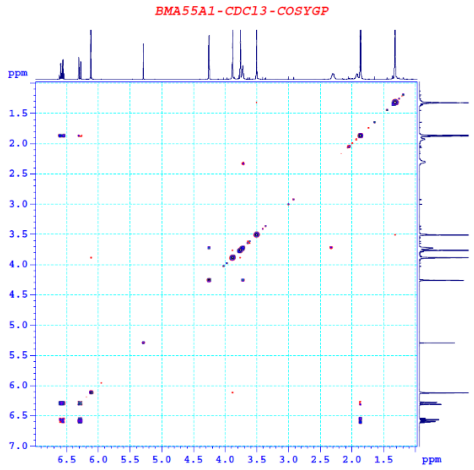

Figure 3.12. COSY keys of MA1

3.3.2. Compound MA14: Malloapelta B

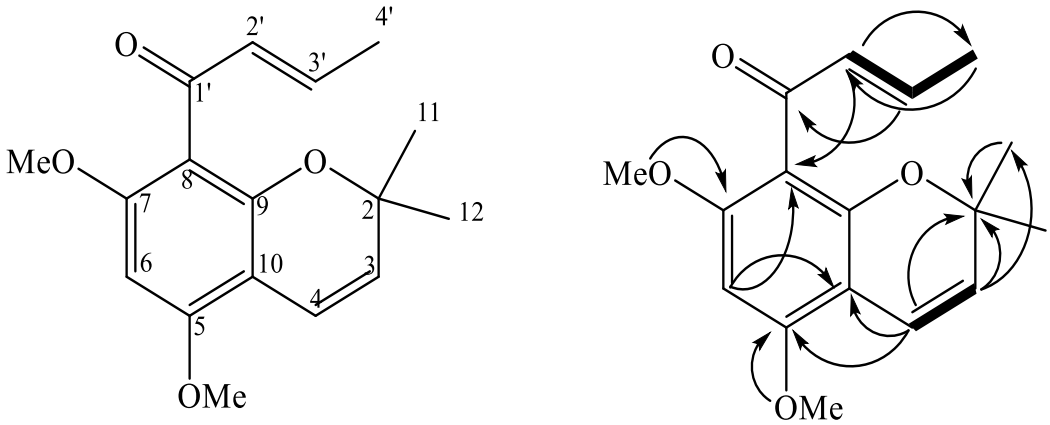

Figure 3.13. Structure and HMBC interactions of MA14

The compound **MA14** was obtained as a white amorphous powder. The HR-ESI-MS spectrum provided a molecular formula of $C_{17}H_{20}O_4$, with m/z 289.1441 [M+H]+ (calculated for C17H21O4: 289.1440). The 1H-NMR spectrum (in CDCl3) showed signals for two methoxy groups at δH 3.86 (6H, s), two tertiary methyl groups

at δH 1.34 (6H, s), one secondary methyl group at δH 1.91 (3H, d, J = 6.5 Hz), and five olefinic protons [δH 6.03 (1H, s), 5.45 (1H, d, J = 10.5 Hz), 6.58 (1H, d, J = 10.5 Hz), 6.38 (1H, d, J = 17.0 Hz), and 6.69 (1H, dq, J = 17.0, 6.5 Hz)] as shown in the table below. The 13 C-NMR spectrum revealed the presence of a carbonyl group (δC 194.2), five quaternary olefinic carbons (δC 104.2, 111.7, 151.9, 156.5, and 158.1), five olefinic methine carbons (δC 127.1, 116.7, 88.0, 134.8, and 144.8), three methyl groups (δC 27.5, 27.7, and 18.1), and two methoxy groups (δC 55.6 and 55.9). The chemical shift at δC 76.5 (s) suggested that this carbon atom is bonded to oxygen. This was based on HMBC correlations between H-11/H-12 (δH 1.34) and C-2 (δC 76.5)/C-3 (δC 127.1), between H-3 (δH 5.45) and C-2 (δC 76.5)/C-10 (δC 104.2)/C-11 (δC 27.7)/C-12 (δC 27.5), and between H-4 (δH 6.58) and C-2 (δC 76.5)/C-5 (δC 156.5)/C-9 (δC 151.9)/C-10 (δC 104.2). HMBC correlations from H-6 (δH 6.03) to C-5 (δC 156.5)/C-7 (δC 158.1)/C-8 (δC 111.7)/C-10 (δC 104.2), and from methoxy groups (δH 3.51) to C-5 (δC 156.5)/C-7 (δC 158.1) indicated the positions of two methoxy groups at C-5 and C-7. Comparison of the NMR spectral data of compound **MA14** with malloapelta B showed complete agreement. Thus, compound **MA14** was identified as malloapelta B.

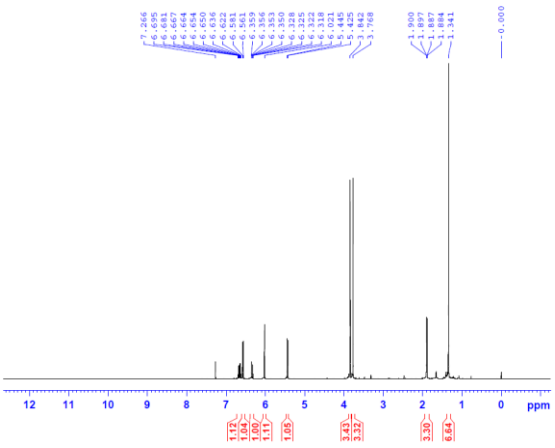

Figure 3.14. ^{1}H-NMR spectrum of MA14

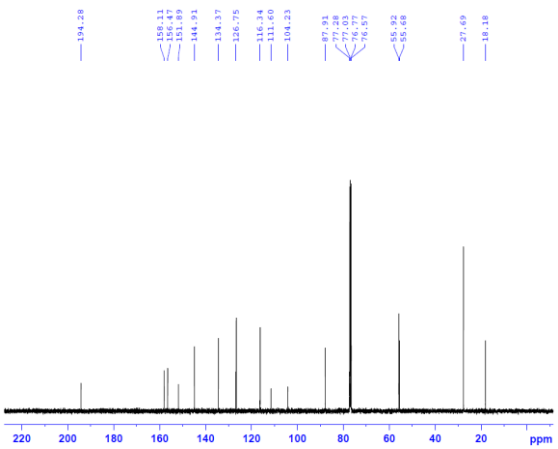

Figure 3.15.^{13}C-NMR spectrum of MA14

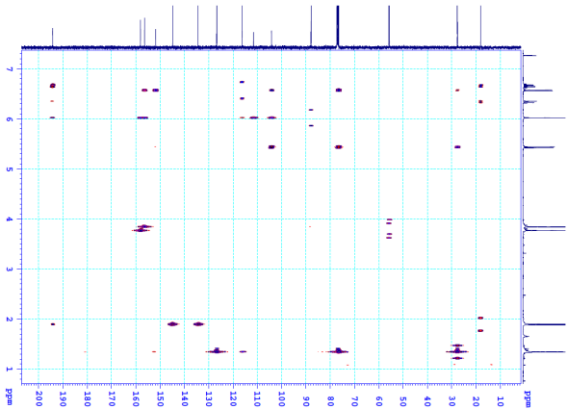

Figure 3.16. HMBC keys of MA14

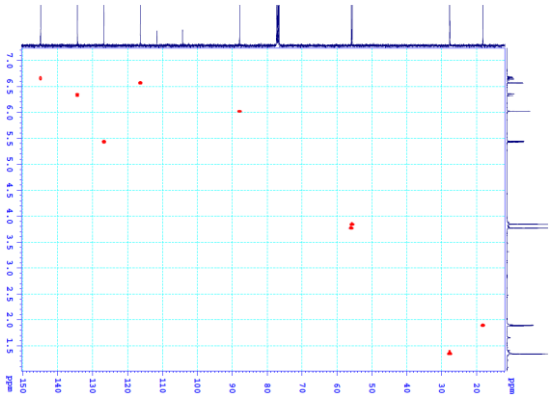

Figure 3.17. HSQC keys of MA14

3.4. Conclusion on compounds isolated from *M. apelta.*

Thus, 21 compounds have been isolated (**MA1a**, **MA1b**, **MA2a**, **MA2b**, **MA3-MA19)**, including 14 chromenes (**MA1-MA11, MA14)**, 5 flavonoids (**MA12, MA13, MA15-MA17)**, and two other compounds (**MA18, MA19)** from M. apelta. Among them, 14 are new compounds and 7 are known compounds.

Table 3.1. The compounds were isolated and determined from M. apelta

STT	**Compound**	**Tên gọi**
1	Two new compound **MA1a** and **MA1b**	(+)-malloapelta C (**MA1a**) (-)-malloapelta C (**MA1b**)
2	Hai New compound **MA2a** and **MA2b**	(-)-malloapelta D (**MA2a**) (+)-malloapelta D (**MA2b**)
3	New compound **MA3**	malloapelta E
4	New compound **MA4**	malloapelta F
5	New compound **MA5**	malloapelta G
6	New compound **MA6**	malloapelta H
7	New compound **MA7**	malloapelta I
8	New compound **MA8**	malloapelta II
9	New compound **MA9**	malloapelta J
10	New compound **MA10**	malloapelta K
11	New compound **MA11**	malloapelta L
12	New compound **MA12**	malloflavoside
13	Compound **MA13**	apigenin 6-*C*-*β*-D-xylopyranosyl-8-*C*-*α* -L-arabinopyranoside
14	Compound **MA14**	malloapelta B
15	Compound **MA15**	schaftoside
16	Compound **MA16**	apigenin-7-*O*-*β*-D-glucoside
17	Compound **MA17**	apigenin 7-*O*-*β*-D-apiofuranosyl (1→2)-β-D-glucopyranoside
18	Compound **MA18**	blumenol C glucoside
19	Compound **MA19**	acantrifoside E

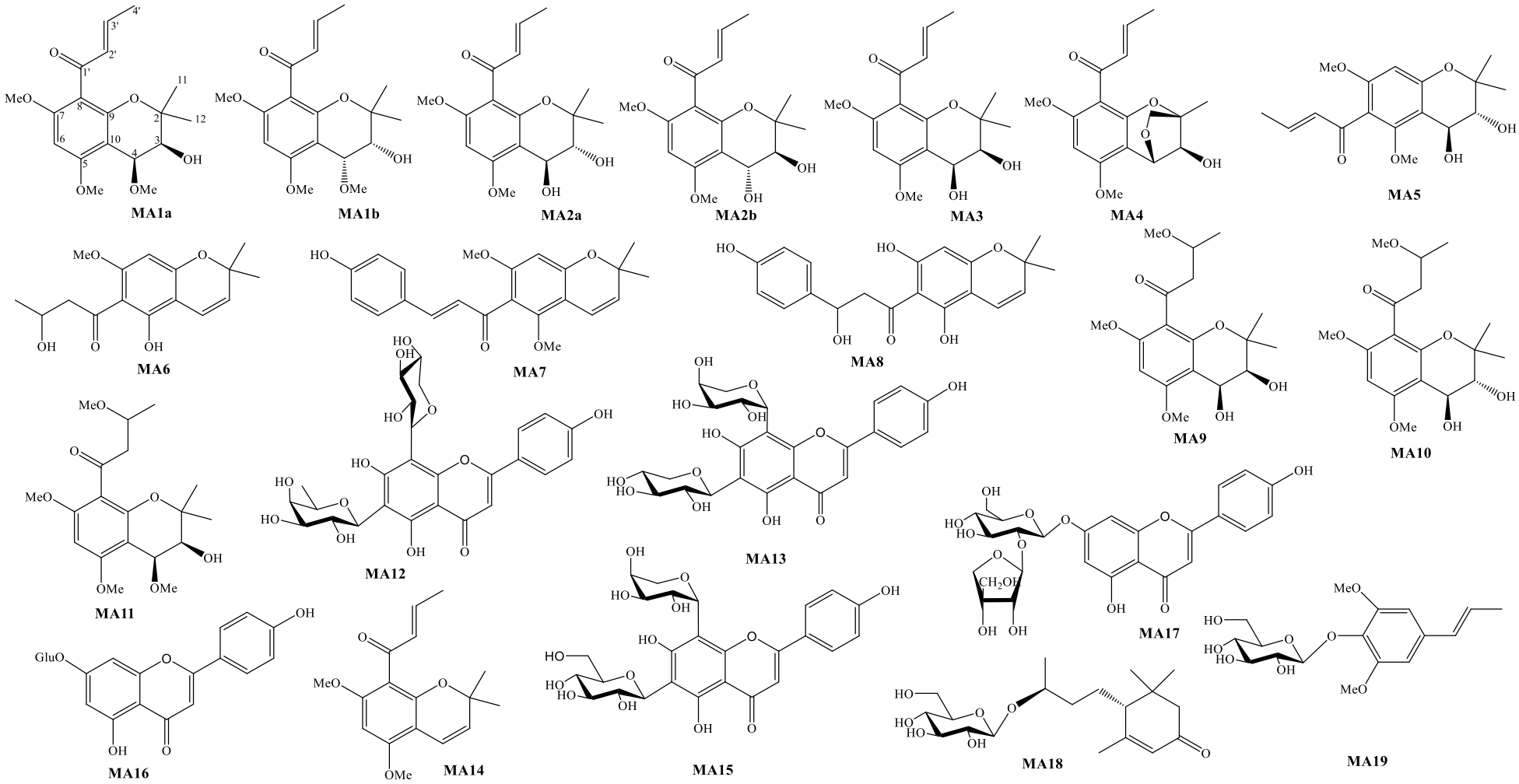

Figure 3.18. Structures of compounds isolated from M. apelta

3.5. Biological activity of compounds

3.5.1. Evaluation and screening of the cancer cell growth inhibitory activity of isolated compounds.

Firstly, the compounds were screened for cytotoxic effects on cancer cells in two cell lines, PC-3 and MCF-7. PC-3 and MCF-7 cells were treated with the compounds at a concentration of 30 μM for 48 hours, and the percentage of viable cells was determined using the MTS assay. The results showed that up to 9 compounds exhibited cytotoxic effects on cancer cells greater than 50% (i.e., the percentage of viable cells was less than 50%), including **MA1-MA8** and **MA14.** These compounds had effects on both PC-3 and MCF-7 cell lines. Among them, **MA8** had the best effect, with the survival rates of the two lines being 1.9±0.02% and 10.0±0.09%, respectively.

The IC_{50} values of **MA1-MA8** and **MA14** were further tested on four cell lines: PC-3, MCF-7, TOV-21G, using carboplatin and capecitabine as positive controls. The results showed that, for the PC-3 cell line, the compounds with the strongest inhibitory effects were ranked as **MA14>MA2>MA3>MA4>MA8>MA7>MA6>MA1.** For the MCF-7 cell line, the order was **MA14>MA3>MA4>MA8>MA2>MA7>MA1>MA5>MA6.** For the HT-29 cell line, the decreasing order was **MA14>MA3>MA2>MA7>MA4>MA1>MA8>MA6>MA5.** For the TOV-21G cell line, as **MA7** and **MA8** were not tested, the order was **MA2>MA14>MA3>MA1>MA5>MA4>MA6.**

Thus, it can be seen that the compounds **MA14, MA2,** and **MA3** have very low IC_{50} values across all four cancer cell lines, with values less than 5 μM. Additionally, the compounds **MA4, MA7,** and **MA8** all showed good anticancer activity against the three cell lines PC-3, MCF-7, and HT-29 with IC_{50} values less than 10 μM.

3.5.2. Evaluation of the mechanism of cancer cell death of highly active compounds through apoptosis and inactivation of nuclear factor NF-κB.

The process of inhibiting cell growth and viability showed a dose-dependent effect at 72 hours after incubation with compounds **MA1-MA5** and **MA14,** with IC_{50} values ranging from 1.62-4.02 μM, demonstrating strong cytotoxic effects on TOV-21G cancer cells. Among these, compounds **MA2, MA3,** and **MA14** exhibited significantly higher cytotoxic effects on human cancer cells compared to the others. However, previous studies have shown that compound **MA14** does not display cytotoxicity on RAW264.7 macrophage cells up to 30 μM. Therefore, compounds

MA2, MA3, and **MA14** were evaluated for their cytotoxic activity against ovarian cancer cells through apoptosis and NF-κB factor inactivation.

3.5.2.1. Effects of compounds MA2, MA3, MA14 on apoptosis factors

To determine the level of apoptosis induced by compounds **MA2, MA3,** and **MA14** on ovarian cancer cell lines, the ability to activate the apoptotic signaling pathway was assessed using Western blot analysis with corresponding antibodies.

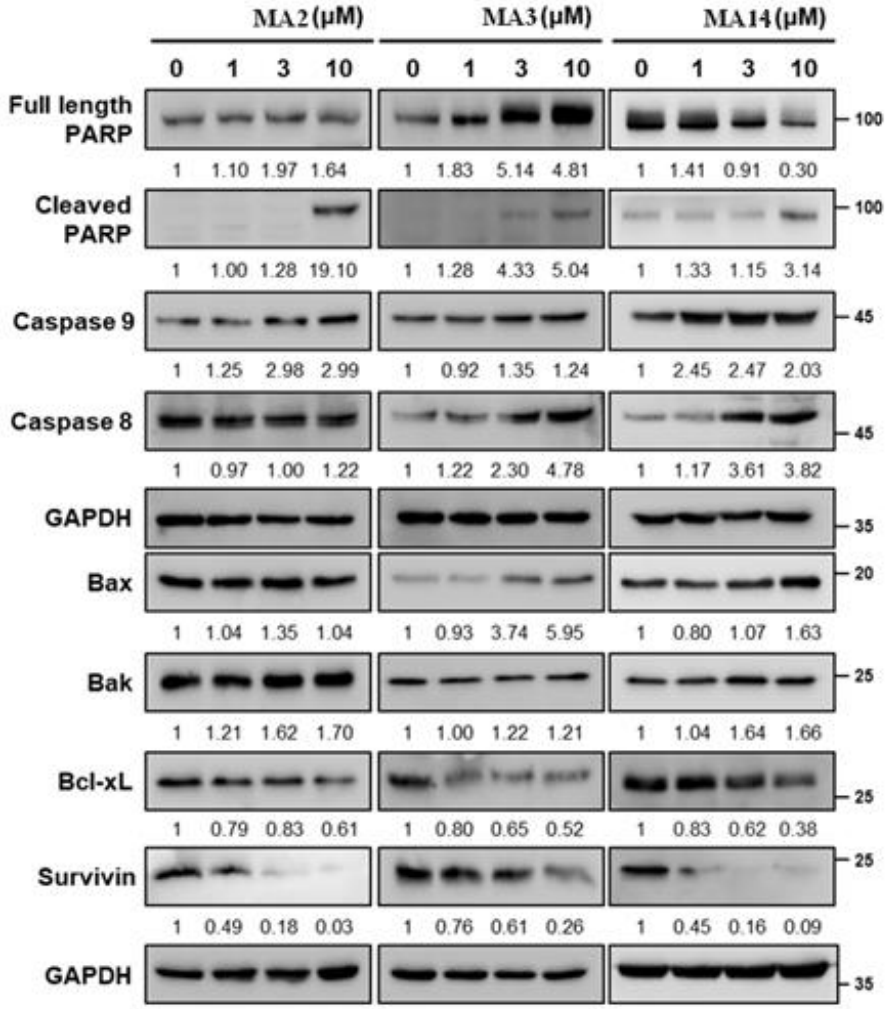

Figure 3.19. Effects of compounds MA2, MA3, MA14 on apoptosis factors

The results showed that treating TOV-21G cells with the three compounds activated the pro-apoptotic proteins Bak and Bax while reducing the expression of the anti-apoptotic proteins Bcl-xL and survivin. Moreover, these compounds increased the expression levels of poly (ADP-ribose) polymerase (PARP), caspase 9, and caspase 8, indicating that these compounds induce both intrinsic and extrinsic apoptosis. Therefore, it can be concluded that these compounds exhibit anticancer effects through the apoptosis pathway.

3.5.2.2. Effects of MA2, MA3, MA14 on the NF-κB signaling pathway

Compound **MA14** is known as an inhibitor of LPS-induced NF-κB activation [124]. Therefore, compounds **MA2** and **MA3** were tested alongside **MA14** to determine their potential effects on the NF-κB signaling pathway.

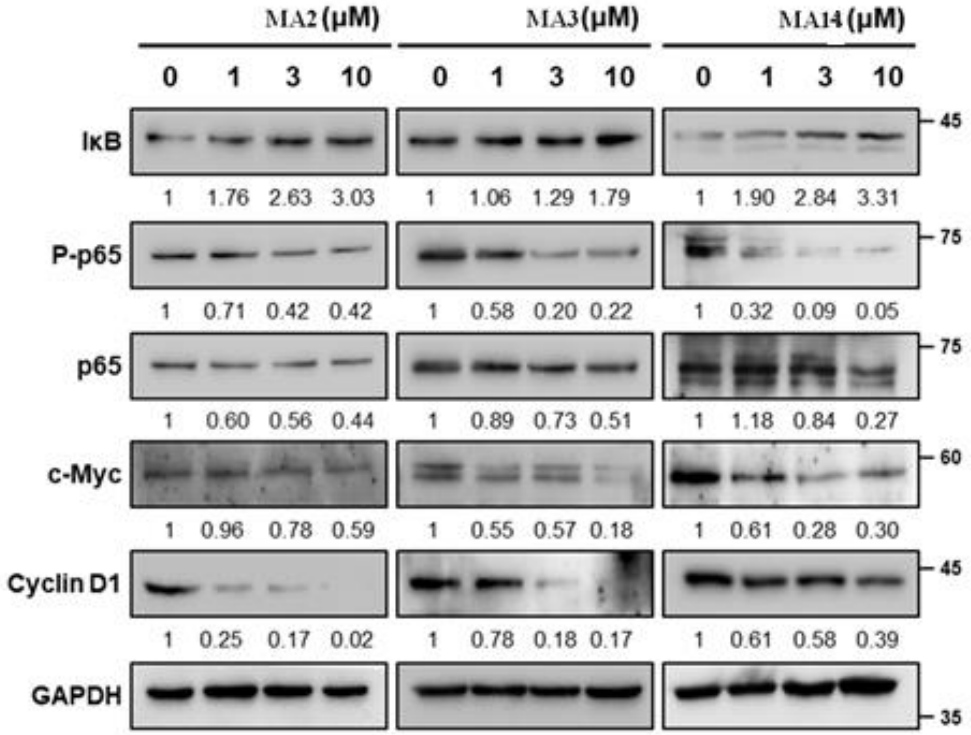

Figure 3.20. Effects of MA2, MA3, MA14 on the NF-κB signaling pathway

The results showed that compounds **MA2** and **MA3** reduced the levels of phosphorylated NF-κB and total NF-κB, while increasing the levels of IκB, the inhibitor of NF-κB. The NF-κB target genes, c-myc and cyclin D1, were reduced when TOV-21G cells were treated with **MA2** and **MA3.** On the other hand, the NF-κB signaling pathway has been reported to be involved in cell growth, apoptosis, inflammation, and tumor formation. Therefore, these findings suggest the potential application of compounds **MA2, MA3,** and **MA14** in cancer and inflammatory disease treatment through the modulation of NF-κB.

3.5.3. Effects of MA2, MA3, MA14 on the NF-κB signaling pathway

3.5.3.1. Identification and characterization of ANO1 inhibitors

The inhibitory effects of compounds **MA7, MA8, MA14, MA16, MA18,** and **MA19** on ANO1 were evaluated using the YFP assay in FRT cells stably expressing human ANO1 (Figure 3.21). The results showed that both compounds **MA7** and **MA8** exhibited ANO1 inhibitory effects greater than 99% at a concentration of 25 μM, as indicated by the gradual reduction and eventual loss of fluorescence signal intensity over time after the addition of ATP (Figure 3.21A). The expression of ANO1 in FRT, FRT-ANO1, and PC-3 cell lines was examined by Western blot, and the results showed that only the specific band for ANO1 was identified in the FRT-ANO1 and

PC-3 cell lines. Notably, in the PC-3 cell line, compound **MA8** reduced ANO1 expression more significantly than **MA7,** and the level of ANO1 expression decreased in cells treated with **MA7** or **MA8** compared to the control group. At a concentration of 10 μM, **MA8** significantly inhibited ANO1 expression compared to the control group ($P < 0.01$). At the same concentration, there was no statistically significant difference between the **MA7**-treated samples and the control group (Figure 3.21C).

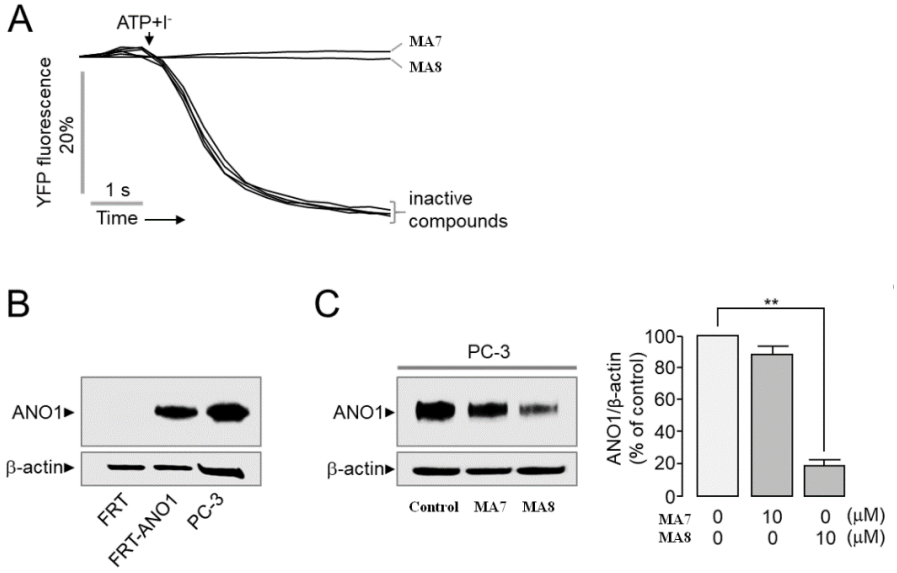

Figure 3.21. Detection of substances that inhibit ANO1.

Compounds **MA7, MA8,** and **MA14** inhibited the viability of PC-3 cells, with IC_{50} values of 8.89 ± 0.17, 7.29 ± 0.20, and 1.60 ± 0.05 respectively, compared to the positive control, capecitabine. Among these compounds, **MA8** significantly inhibited the activity of the ANO1 channel, reduced the expression level of the ANO1 protein in cells, and decreased the viability of PC-3 cells. Therefore, **MA8** was selected for further study.

3.5.3.2. *Selective inhibition of ANO1 by MA8*

To investigate the inhibitory effect of **MA8** on the ANO1 chloride channel, apical membrane currents were measured in FRT cells expressing human ANO1. The results are shown in Figure 3.22. The results indicated that compound **MA8** significantly inhibited the ANO1 chloride current activated by ATP, with an IC_{50} of 2.64 μM. To observe the effect of **MA8** on the calcium signaling pathway, FRT cells were loaded with the fluorescent calcium indicator, Fluo-4. Pretreatment with **MA8** did not significantly alter the increase in intracellular calcium levels induced by ATP. To evaluate the effect of **MA8** on other chloride channels, apical membrane currents were measured for the cystic fibrosis transmembrane conductance regulator (CFTR) in FRT cells expressing human CFTR. The results showed that **MA8** exhibited minimal inhibitory effect on CFTR channel activity at a concentration of 30 μM, indicating selective inhibition of ANO1.

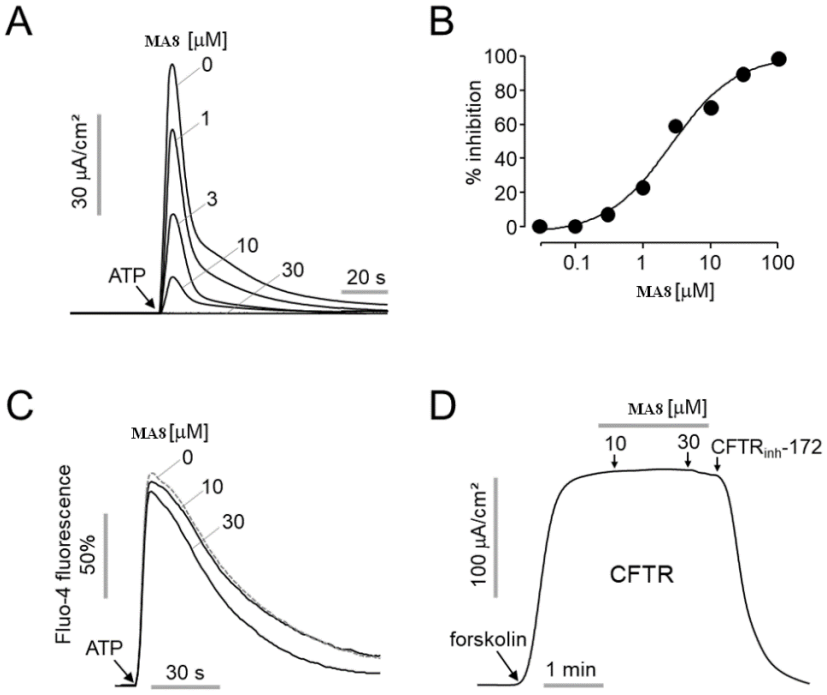

Figure 3.22. Selective inhibition of ANO1 by MA8

3.5.3.3. Inhibitory effects of MA8 on cell growth and migration in PC-3 and CAL-27 cells.

Previous studies have shown that ANO1 blockers inhibit cell growth in metastatic prostate cancer cells and squamous cell carcinoma cells. In this study, **MA8** significantly reduced the expression of the ANO1 protein in PC-3 and CAL-27 cell lines, with the degree of ANO1 reduction inversely proportional to the concentration of **MA8.** The greatest reduction in ANO1 expression was observed at a concentration of 10 μM for both PC-3 and CAL-27 cell lines (Figure 3.23 A and B). Additionally, **MA8** significantly decreased cell viability in PC-3 and CAL-27 cells and mildly affected viability in ANO1 KO cells (Figure 3.23 C and D). Importantly, **MA8** did not affect the viability of ANO1 KO PC-3 cells at a concentration of 3 μM, indicating its cytotoxic effect is significant on PC-3 cells expressing ANO1. **MA8** also markedly reduced the viability of CAL-27 cells expressing ANO1, but not in ANO1 KO CAL-27 cells.

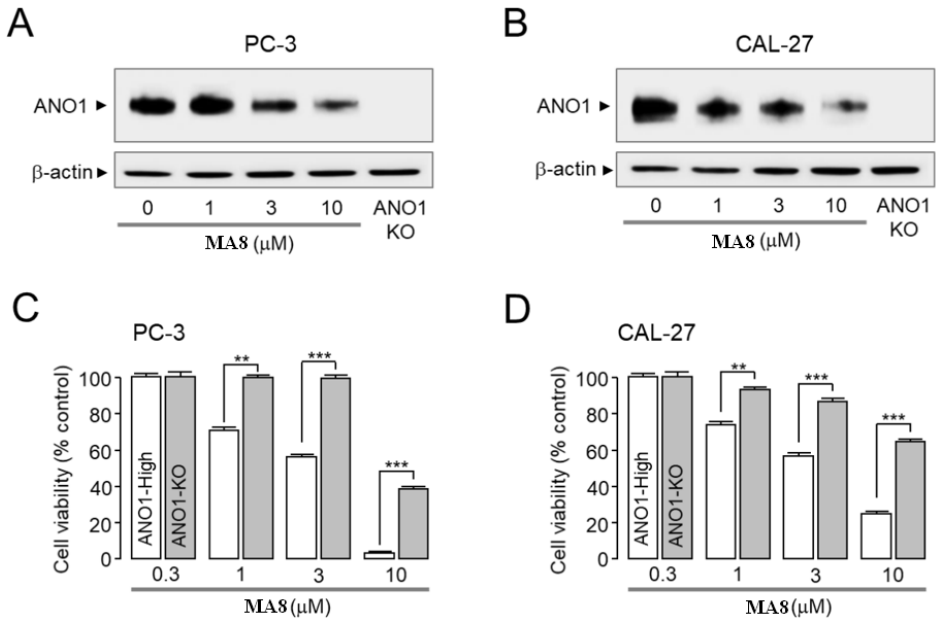

Figure 3.23. Effect of MA8 on the expression level of ANO1 protein and cell viability in PC-3, CAL-27 cells.

To determine whether **MA8** inhibits cancer cell migration, a wound healing assay was conducted on PC-3 and CAL-27 cell lines. The results showed that at concentrations of 1, 3, and 10 μM, **MA8** significantly inhibited cell migration. Specifically, **MA8** inhibited PC-3 cell migration by 31.3%, 68.0%, and 88.3% respectively, and CAL-27 cell migration by 31.8%, 64.0%, and 87.5% respectively.

3.5.3.4. Effect of MA8 on increased Caspase-3 activity and PARP cleavage in PC-3 and CAL-27 cells

The pharmacological inhibition of ANO1 protein induces apoptosis in various cancer cells. To assess whether **MA8** induces apoptosis in PC-3 and CAL-27 cells with high ANO1 expression, the effects of **MA8** on caspase-3 activity and PARP cleavage were observed in these cells. The results showed that **MA8** significantly increased the population of cells positive for caspase-3 in both PC-3 and CAL-27 cell lines (Figure 3.24 A and B). The activity of caspase-3 was markedly increased by **MA8** in a dose-dependent manner in both PC-3 and CAL-27 cells, and this increase in caspase-3 activity induced by **MA8** was completely inhibited by AC-DEVD-CHO, a specific caspase-3 inhibitor (Figure 3.24 C and D). Additionally, **MA8** treatment significantly increased the cleavage of PARP-1 in PC-3 and CAL-27 cells.

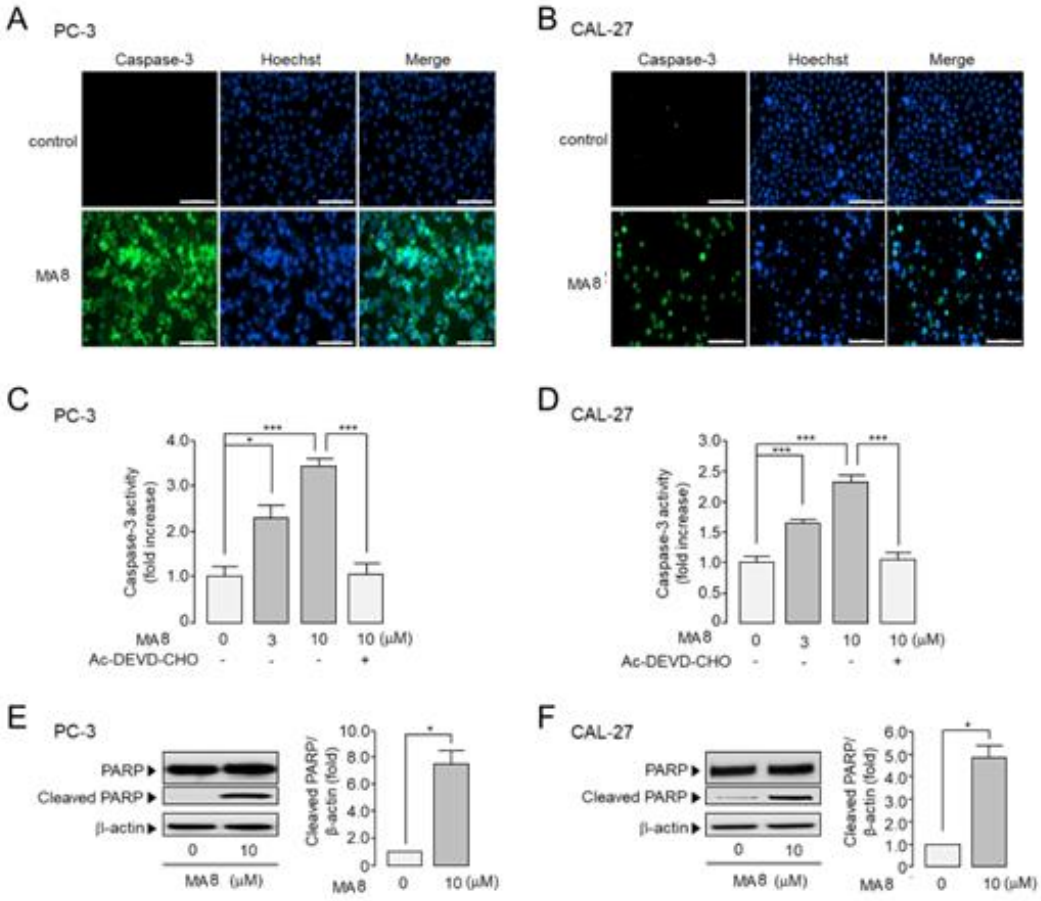

Figure 3.24. Effect of MA8 on caspase-3 activity and PARP cleavage in PC-3, CAL-27 cells.

Previous reports have indicated that ANO1 inhibitors can modulate cancer progression by reducing ANO1 expression in various types of cancer cells. ANO1 inhibitors have been shown to decrease cell viability in breast cancer, squamous cell carcinoma of the head and neck, and esophageal squamous cell carcinoma (ESCC) by inhibiting the activity of Ca^{2+}/calmodulin-dependent protein kinase II and the expression of epidermal growth factor receptor (EGFR) growth factor receptor. Moreover, overexpression of ANO1 promotes tumor development by activating the AKT/SRC/ERK1/2 signaling pathway through EGFR intermediates or the Ras-Raf-MEK-ERK1/2 signaling pathway. Therefore, ANO1 represents a potential therapeutic target in cancer treatment, and the use of ANO1 inhibitors may yield promising results in reducing the growth of malignant tumors.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

From *Mallotus apelta* leaves harvested in Ngoc Thanh, Phuc Yen, Vinh Phuc province, after soaking, extraction, and isolation using chromatographic methods and modern spectroscopic techniques, 21 chemical compounds have been identified and their structures determined:

- 14 new compounds: (+)-malloapelta C (**MA1a**) and (-)-malloapelta C (**MA1b**), (-)-malloapelta D (**MA2a**) and (+)-malloapelta D (**MA2b**), malloapelta E (**MA3)**, malloapelta F (**MA4)**, malloapelta G (**MA5)**, malloapelta H (**MA6)**, malloapelta I (**MA7)**, malloapelta II (**MA8)**, malloapelta J (**MA9)**, malloapelta K (**MA10)**, malloapelta L (**MA11)**, and malloflavoside (**MA12)**. Specifically, HPLC with a chiral column was used to separate four chiral compounds: **MA1a**, **MA1b**, **MA2a**, and **MA2b** from racemic mixtures. Absolute configurations were confirmed using experimental CD spectroscopy combined with computational methods.
- 7 known compounds: apigenin 6-C-β-D-xylopyranosyl-8-C-α-L-arabinopyranoside (**MA13)**, malloapelta B (**MA14)**, schaftoside (**MA15)**, apigenin-7-O-β-D-glucoside (**MA16)**, apigenin 7-O-β-D-apiofuranosyl (1→2)-β-D-glucopyranoside (**MA17)**, blumenol C glucoside (**MA18)**, and acantrifoside E (**MA19)**.

The inhibitory effects on cancer cell growth of all isolated compounds from Mallotus apelta were studied. The results are as follows:

- Compounds **MA14, MA2,** and **MA3** showed strong anticancer activity against PC-3, MCF-7, HT-29, and TOV-21G cell lines with IC_{50} values < 5 µM. Additionally, **MA4, MA7,** and **MA8** exhibited good anticancer activity against PC-3, MCF-7, and HT-29 with IC_{50} < 10 µM.
- Compounds **MA2** and **MA3** were found to inhibit the growth of TOV-21G ovarian cancer cells through apoptosis induction and NF-κB inactivation, evidenced by increased expression of Bak and pro-apoptotic Bax, decreased expression of anti-apoptotic Bcl-xL, and survivin inhibition. **MA2** and **MA3** also reduced NF-κB phosphorylation and total NF-κB levels, along with downregulating NF-κB target genes c-myc and cyclin D1.
- Both **MA7** and **MA8** demonstrated > 99% inhibition of ANO1 in FRT cells at 25 µM concentration. Notably, **MA8** showed stronger reduction of ANO1 expression in PC-3 cells compared to **MA7.** This correlates with findings from chloride channel inhibition and calcium signaling pathway studies. **MA8** at 10 µM was also found to reduce proliferation and migration in PC-3

and CAL-27 cells, based on experiments with ANO1 KO cells. Additionally, **MA8** significantly increased caspase-3 activity and PARP cleavage, indicative of programmed cell death processes.

RECOMMENDATIONS

Both compounds **MA7** and **MA8** have shown to inhibit the growth and migration processes in PC-3 and CAL-27 cells by reducing ANO1 expression. Additionally, **MA8** enhances caspase-3 activity and PARP cleavage, promoting programmed cell death. Therefore, further studies on the mechanism of action of compound MA8 are needed to discover its potential application in cancer treatment.

LIST OF PUBLISHED WORKS RELATED TO THE THESIS

1. **Nguyen Hoang Anh**, Duong Thi Hai Yen, Nguyen The Cuong, Bui Huu Tai, Pham Hai Yen, Pham The Chinh, Pham Van Cuong, Nguyen Hoai Nam, Phan Van Kiem, Su Hyeon Cho, Seon Ju Park, Nguyen Xuan Nhiem, *Three new chromanes and one new flavone C -glycoside from Mallotus apelta,* Journal of Asian Natural Products Research, 2023, 25, 18-26.
2. Yohan Seo, **Nguyen Hoang Anh**, Yunkyung Heo, So Hyeon Park, Phan Van Kiem, Yechan Lee, Duong Thi Hai Yen, Sungwoo Jo, Dongkyu Jeon, Bui Huu Tai, Nguyen Hoai Nam, Chau Van Minh, Seung Hyun Kim, Nguyen Xuan Nhiem, Wan Namkung *Novel ANO1 Inhibitor from Mallotus apelta Extract Exerts Anticancer Activity through Downregulation of ANO1*. International Journal of Molecular Sciences, 2020. 21, DOI: 10.3390/ijms21186470.
3. Phan Van Kiem, Nguyen Xuan Nhiem, **Nguyen Hoang Anh**, Duong Thi Hai Yen, Nguyen The Cuong, Bui Huu Tai, Pham Hai Yen, Nguyen Hoai Nam, Chau Van Minh, Pham The Chinh, Yu Hyun Jeon, Seon Ju Park, Seung Hyun Kim, So Hee Kwon, *Enantiomeric chromene derivatives with anticancer effects from Mallotus apelta,* Bioorganic Chemistry, 2020, 104, 104268.
4. **Nguyen Hoang Anh**, Seon Ju Park, Do Thi Trang, Duong Thi Hai Yen, Bui Huu Tai, Pham Hai Yen, Phan Van Kiem, Pham The Chinh, Nguyen Xuan Nhiem, *Genus Mallotus (Euphorbiaceae): A review on traditional medicinal use, phytochemistry and biological activitie,* Vietnam Journal of Science and Technology, 2022, 60, 141-174.