**MASTER'S THESIS**

# Purification and structure analysis of mammosphere-forming inhibitors derived from *Cynanchum auriculatum*

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**February, 2017**

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**Hong-Yuan Deng**

(Supervised by Professor Dong-Sun Lee)

A thesis submitted in partial fulfillment of the requirement

for the degree of Master of Biotechnology

February, 2017

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **$CHCl_3$** | Chloroform |
| **MeCN** | Acetonitrile |
| **EtOAc** | Ethyl Acetate |
| **HPLC** | High Performance Liquid Chromatography |
| **mAU** | milli (absorbance unit) |
| **MeOH** | Methanol |
| **Min** | minute |
| **MS** | mass spectrometry |
| **NMR** | Nuclear Magnetic Resonance |
| **ODS** | Octadecyl-silica |
| **P. TLC** | Preparative Thin Layer Chromatography |
| **Rpm** | Rotations per Minute |
| **RP-HPLC** | Reversed-Phase High Performance Liquid Chromatography |
| **$SiO_2$** | Silicon Dioxide |
| **TLC** | Thin layer chromatography |
| **$T_R$** | Retention time |
| **UV** | Ultraviolet |

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# ABSTRACT

Many studies have supported the existence of cancer stem cells (CSCs) as a subpopulation of cancer cells. CSCs are drug-resistant and radiation-resistant cancer cells to be responsible for tumor progress, maintenance and recurrence of cancer, and metastasis. The breast CSCs inhibitor that inhibits cancer stem cell derived from MCF-7 cells was isolated from the root parts of *C. auriculatum*. The ethyl acetate fraction obtained from methanol extracts exhibited the outstanding inhibitory effect on mammospohere formation. From the ethyl acetate fraction, a new compound named DH was purified through Silica gel column chromatography, ODS open column chromatography, Sephadex LH-20 column chromatography, and preparative thin-layer chromatography. DH-1 and DH-2 were purified using reversed-phase high performance liquid chromatography. Compound DH-1 and DH-2 are steroidal glycoside called kidjoranin-3-O-β-D-cymaropyranoside and Caudatin-3-O-β-D-cymaropyranoside. We purified CSCs inhibitor from *C. auriculatum.* Thus, the isolated compounds exhibited a strong anti-CSCs activity, which may contribute to the interpretation of the pre-pharmacological and clinical effects of *C. auriculatum*.

**Keywords:** *Cynanchum auriculatum*; Mammospheres formation assay; cancer stem cells (CSCs); active compound; structure analysis. Kidjoranin-3-O-β-D-cymaropyranoside; Caudatin-3-O-β-D-cymaropyranoside.

# INTRODUCTION

The roots of *Cynanchum wilfordii* and *Cynanchum auriculatum* have been widely used as traditional herbal medicines in Eastern Asia. *C. wilfordii* is an ingredient for tonic herbal drugs and it shows pharmaceutical benefits against tumors, antioxidants, vascular diseases, and diabetes mellitus [1-5]. In China, *C. auriculatum* has been used as a tonic agent having the activities of anti-tumor, gastroprotective, antidepressant, and anti-aging [6-9]. Cynanchum species have long been used in traditional chinese medicine (TCM). This genus comprises of about 200 species in Asclepiadaceae family and is distributed worldwide, including east Africa, the Mediterranean region, the tropical zone of Europe, and the subtropical and temperate zones of Asia. There are 53 species and 12 varieties are native to southwestern region of China. Among them, 16 species, including *C. auriculatum, C.atratum, C.glaucescens, C.bungei, C.chekiangense, C.vincetoxicum, C. saccatumi, C. inamoenum, C. mongolicum, C. otophyllum, C. paniculatum, C. stauntonii, C. decipines, C. wallichii, C. wilfordii,* and *C. versicolor,* are high medicinal value [10, 11]. Phytochemical studies of Cynanchum species have resulted in the isolation of numerous biologically active C21 steroidal compounds. Most of them possess a variety of pharmacological actions, e.g. antitumor, antiaging, antidepressant, antifungal, and antiviral activities, and also appetite suppressing ability [10]. However, in Korea, the human consumption of *C. auriculatum* is still not approved due to its safety concerns. Thus, only the *C. wilfordii* is registered in Korean Herbal Pharmacopoeia [8, 9]. A number of

studies have indicated that the compounds derived from medicinal herbs play a crucial role in the anti-cancer activity and have been developed as a new anti-cancer drugs [12,13].

Interestingly, anti-tumorigenesis activities of *Cynanchum auriculatum* have not been fully understood in many studies in vitro and in vivo, so this study has revealed on the reaction mechanism of this plant [14,15], yet the *Cynanchum auriculatum* studies on cancer stem cell has not been explored. Moreover, because cancer stem cells (CSCs) have been identified as rare cell populations in many cancers, including leukemia and solid tumors. Accumulating evidence has suggested that CSCs are capable of self-renewal and differentiation into various types of cancer cells. Thus, CSCs are thought to be responsible for cancer initiation, progression, metastasis, recurrence and drug resistance [16]. Therefore, in this study, we investigate anti-CSCs activity in human breast cancer stem cells (BCSCs) as a tool to isolate the active compound of natural product from *Cynanchum auriculatum* [17,18].

In our going studies for discovering new anti-CSCs drugs using traditional herbal medicine plant, a 100% MeOH extracts from the root parts of *Cynanchum auriculatum* showed a potent anti-CSCs activity against human breast cancer stem cells. In this study, a detailed chemical and biological investigation of the 100% MeOH extracts has led to the isolation of a new two compounds by activity-guided chromatographic separation. As a result, these findings support the use of DH-1 and DH-2 for chemoprevention of breast cancer stem cells. DH-1 and DH-2 were purified and named as Kidjoranin-3-O-β-D-cymaropyranoside and Caudatin-3-O-β-D-cymaropyranoside.

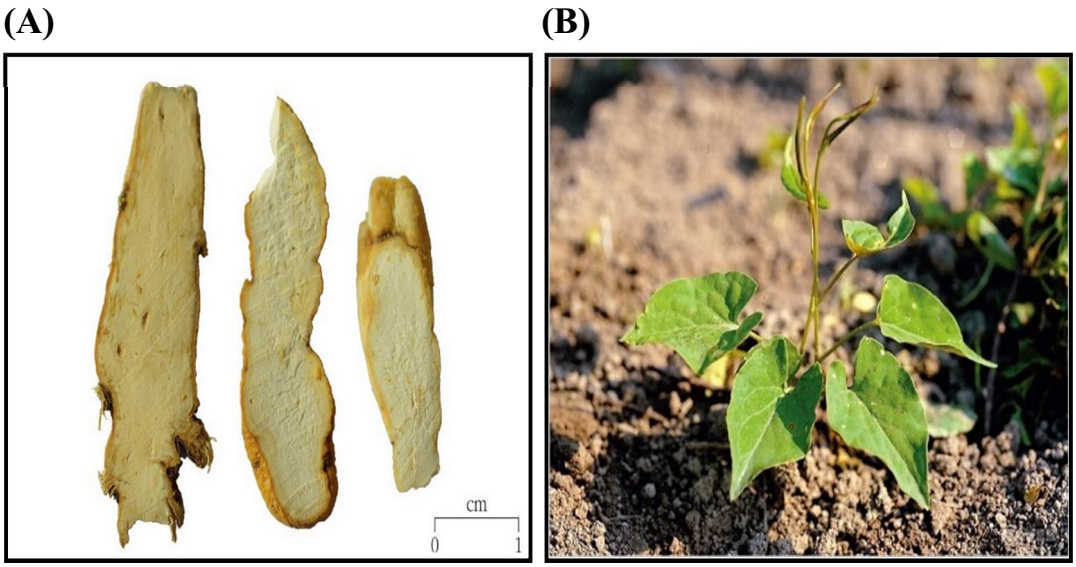



**Figure 1. The Photography of roots and aerial parts on *Cynanchum auriculatum.*** (A)Roots of *Cynanchum auriculatum.* (B) Aerial parts on *Cynanchum auriculatum.*

# MATERIALS AND METHODS

## 1. General Experimental Procedures.

Evaporated sample was obtained using rotary evaporator (Hei-VAP Value Digital, Heidolph, Germany). For collecting and measuring the purity of DH-1 and DH-2，HPLC analysis was carried out using Shimadzu ODS column, LC-20A series, Fraction collector F-10, Shim-pack GIS PREP-ODS, 10 x 250mm, 10 μm $C_{18}$ particle size (Tokyo, Japan). Silica gel preparative TLC (PTLC) glass plates (Kiesel gel 60 $F_{254}$, 20 x 20cm, 1000 microns, Merck, Germany) before HPLC were used to separate the active band. Thin layer chromatography (TLC) analysis was performed on Silica gel 60 $F_{254}$ (20 x 20cm, Merck, Germany) plates. Silica gel (Kiesel gel 60, 0.063-0.200mm, 70-230 mesh ASTM, Merck, Germany) were used for column chromatography (25 x 340mm, 100g) as a stationary phase, and reverse-phase ODS gel (YMC, ODS-A, pore size 120Å, particle size 10μm) were used for open column chromatography. Sephadex LH-20 gel (LH20 100-100G, Sigma-Aldrich, co., Sweden) was used for gel filtration in column chromatography (20 x 400mm). The spots were detected and captured under UV radiation ($UV_{254nm}$ and $UV_{365nm}$). All other chemicals and solvents were of analytical grade, and used without further purification. MCF-7 human breast cancer cell line was used in the mammosphere formation assay as a bioassay-guided chromatographic fractionation and isolation in each step for this whole experiment. The compound was analyzed by liquid chromatography-mass spectrometry（Jeol, Japan）.

**2. Plant Material.**

The root parts of *C. auriculatum* were obtained from cultured *C. auriculatum*.

**3. Extraction and characterization of DH-1 and DH-2 from *C. auriculatum*.**

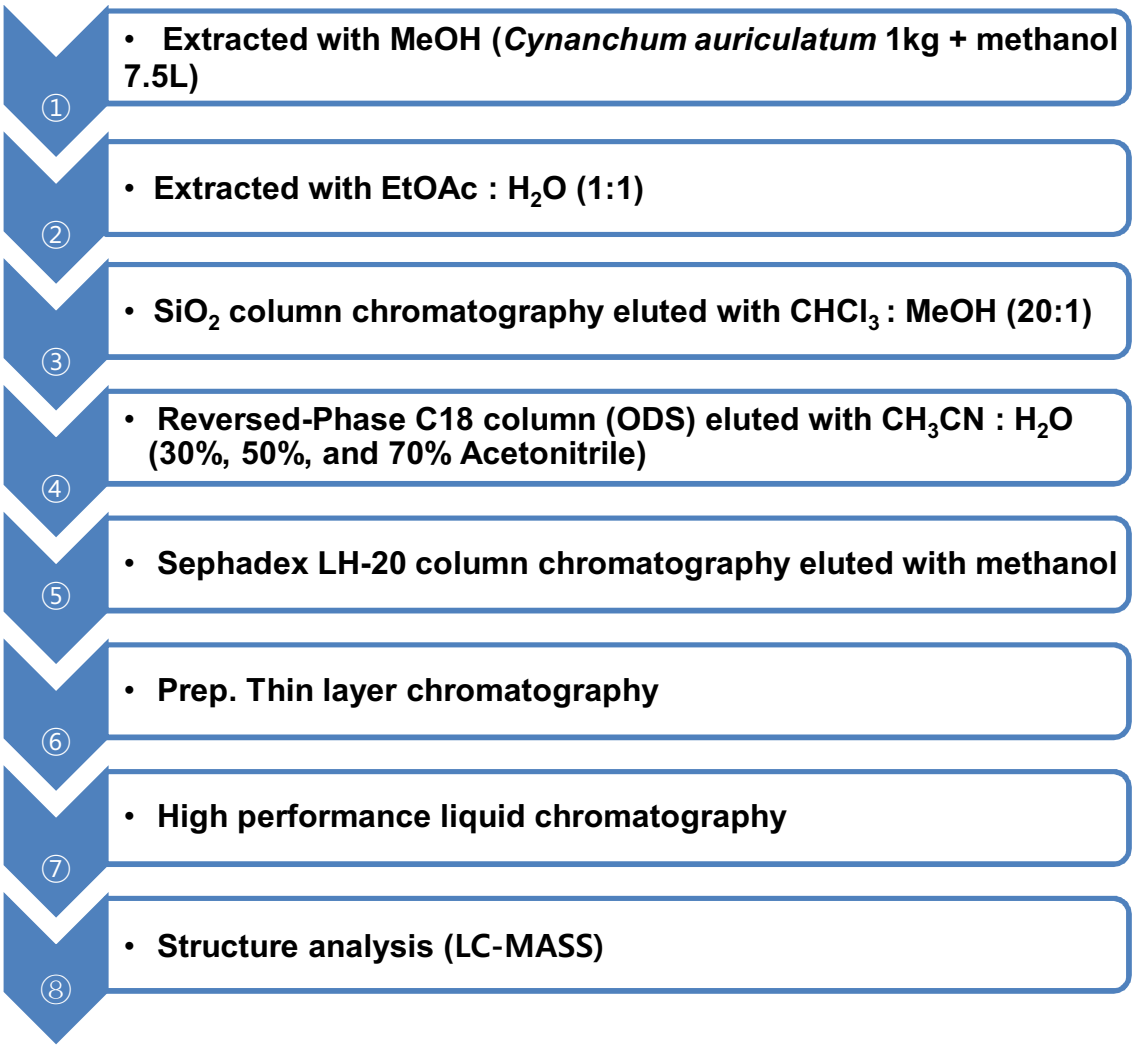



**Figure 2. Overall of purification procedure of mammosphere-forming inhibitors derived from *C. auriculatum*.**

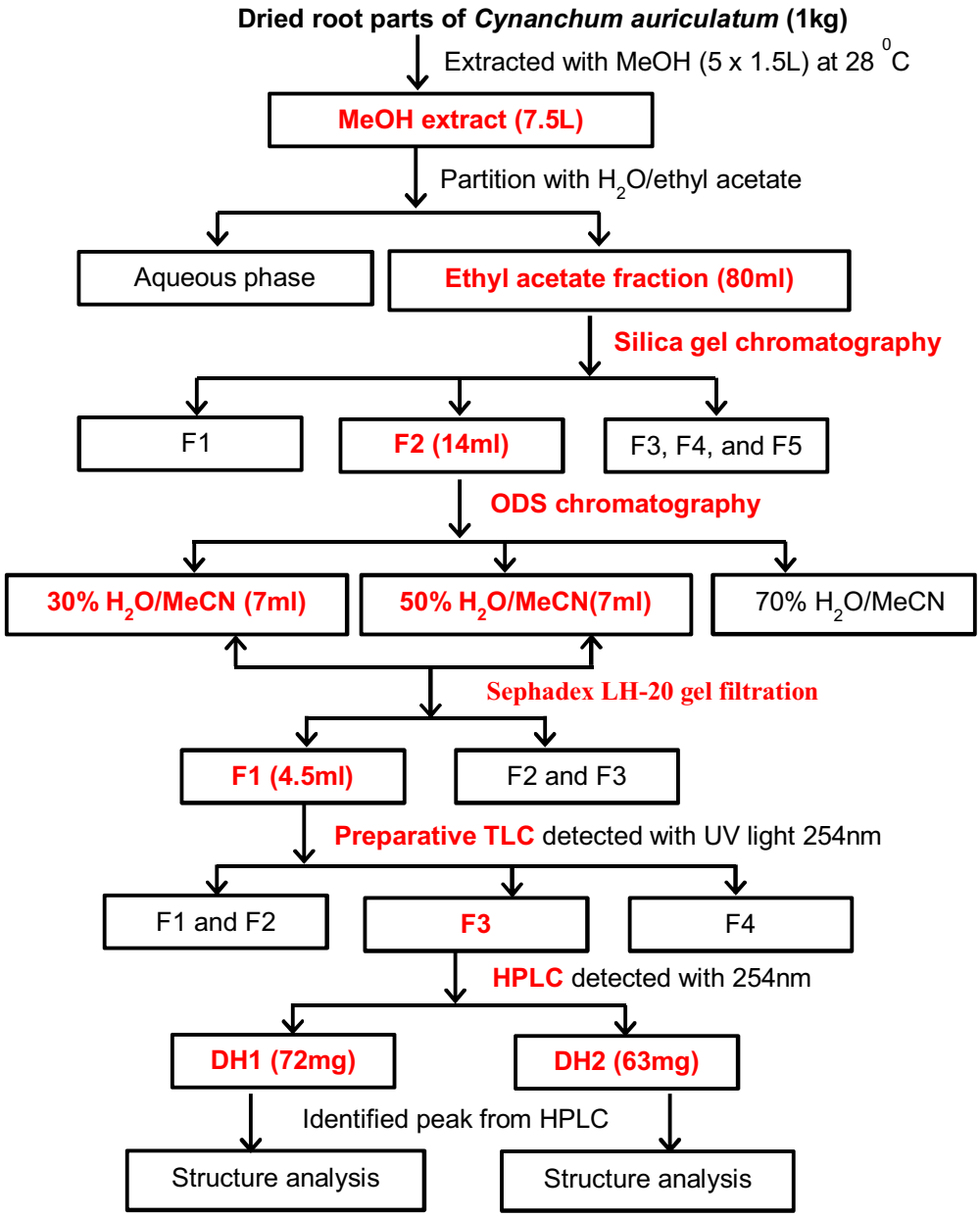



**Figure 3. A flowchart depicting the fractionation of methanol extracts from the aerial parts of *C. auriculatum*.** The *C. auriculatum* roots (1.0 kg) were extracted with MeOH at 28 °C shaking overnight. After evaporation of the solvent, The *C. auriculatum*/methanol extraction suspended in distilled water was

partitioned with ethyl acetate successively 1:1 (v/v) in a separating funnel. The ethyl acetate fraction was further sequentially subjected in silica gel column chromatography. The silica gel fraction F2 was subsequently fractionated by reversed-phase ODS open column chromatography with solvent gradient 30%, 50%, and 70% of acetonitrile in distilled water. The 30% and 50% eluted-sample from ODS were further subjected onto gel filtration Sephadex LH-20, and possibly lead to achieve three subfractions, respectively. The Sephadex LH-20 gel filtration fraction F1 was further separated in Prep TLC, which gave a dark band through the detection of $UV_{254nm}$. A target band (active band，F3) was collected and injected in HPLC. The identified peak was collected by using $UV_{254nm}$ light detection. Finally, the isolated compounds were analyzed by liquid chromatography-mass spectrometry.

### 3.1. Preparation of methanolic crude extracts.

The dried and grinded plant material (1kg) of *C. auriculatum* was extracted with 100% methanol (MeOH) (1.5L x 5) in 2800 mL Erlenmeyer flasks at 28$^{o}$C, 200 rpm for 24 hours in the shaking incubator. After shaking overnight, whole sample were divided into 500 mL plastic bottle and centrifuged at 3500 rpm for 10 min at 5 $^{o}$C. The methanol-extracted supernatant was decanted and filtered through a filter paper (Advantec, 100 circles, No. 2, 185mm), and all the solvent extracts were dried under vacuum rotary evaporator to get dried crude *C. auriculatum* extract at 40 $^{o}$C to 45 $^{o}$C under reduced pressure, which was subjected to further fractionation and bioassay.

### 3.2. Fractionation of the methanolic extracts by means of liquid-liquid partitioning.

Methanol-extracted sample of *C. auriculatum* with distilled water (150 mL) was concentrated to remove all methanol and resuspended in 200 mL of distilled water and sequentially partitioned successively with equal volumes of ethyl acetate (EtOAc) in a separating funnel (1:1 v/v) ratio. The lower aqueous phase was discarded and the top ethyl acetate phase was retained and screened for possible anti-CSCs activity. All the solvent phase extracts were dried under vacuum rotary evaporator to get dried crude *C. auriculatum* extracts at 40 $^{o}$C to 45 $^{o}$C under reduced pressure. The ethyl acetate-soluble fraction was suspended in 80 mL of methanol for a stock solution and subjected for further fractionation and bioassay.

### 3.3. Isolation and purification of active substances in the bioactive ethyl acetate fraction by bioassay-guided fractionation.

**Silica gel ($SiO_2$) column chromatography:** 10 mL (from stock 80 mL) of the bioactive in the ethyl acetate fraction was subjected to normal silica gel column chromatography on a column (silica gel 60, 70-230 mesh ASTM, Merck Co., 100g gel/column). The column (25 x 340mm) was eluted with a solvent system of chloroform: methanol ($CHCl_3$: MeOH = 20:1, v/v) to yield 5 subfractions. All fractions were evaporated to dryness and stocked with MeOH 1.5 mL for further bioassay and fractionation. On the other hand, in each fraction were observed by

TLC plates (10 cm x 10 cm, covered with silica gel, Merck 60 $F_{254nm}$). Individual fraction was spotted on the TLC plate, and developed in the solvent system ($CHCl_3$: MeOH = 20:1, v/v) to investigate the active fraction.

**Reversed-Phase C18 (ODS) gel columns chromatography fractionation:** The F2 fraction from silica gel was further purified by open column chromatography with Reversed-Phase C18 gel. The sample was reconstituted in methanol and stored for overnight; then centrifuged at 13,000 rpm, 4 $^{o}$C for 10 min. The aqueous solution parts were dried under vacuum rotary evaporator; then dissolved and eluted in a solvent system of 30% of acetonitrile in water. Then, the following solvent system was used to remove the sample by washing ODS gel with a solvent system 50% of acetonitrile in water, 70% of acetonitrile in water. All of the eluents compounds were individually concentrated in vacuum and resuspended in 1.5 mL of methanol. Finally, individual fraction was tested in the mammospheres formation assay to examine the active compounds in order to confirm the target fraction.

**Purification by gel filtration Sephadex LH-20**: The active fraction of 30% and 50% acetonitrile sample that reconstituted with methanol was centrifuged at 13,000 rpm, 4 $^{o}$C for 10 min, and further purified by column chromatography (20 x 400mm) with gel filtration Sephadex LH-20. The column was eluted with 100% methanol to yield three subfractions of the impure mixed compounds. All of the

three subfractions were independently concentrated in vacuum and resuspened in 1 mL of methanol. The single fraction was tested in the mammospheres formation assay to examine the active ingredient. Therefore, F1 were obtained. Finally, F1 were combined and dried to remain only 1 mL for further purification.

### 3.4. Identification of active components by Preparative TLC using $UV_{254nm}$ fluorescence detection.

Preparative TLC was used to separate the gel filtrated sample. The 500 μL sample (from 4.5 mL stocked) was loaded onto the preparative thin layer chromatography (P. TLC) glass plates (20 x 20 cm) covered with Silica gel (60, F254; 1 mm thick) and developed in the solvent gradient of $CHCl_3$: MeOH (20:1, v/v) using a TLC glass tank pre-saturated with the mobile phase and each plate was developed to a height of about 26 x 30 cm chamber. After development, plates were removed and dried; the positions of varied compounds were visualized by fluorescence under UV radiation ($UV_{254nm}$ and $UV_{365nm}$). Individual band was separately removed by scraping off the silica gel from the glass using surgery knife and collected in the 15 mL conical tube. The 6 mL of methanol was added to purify each compound from the gel. Then, the mixture of gel and solvent was vortexed, filtered and recovered by centrifugation. Hence, the eluents were concentrated in vacuum to give individual band and stocked in 1 mL of methanol for further bioassay and HPLC fractionation.

### 3.5. HPLC analysis.

Liquid chromatography analysis was operated using Shimadzu ODS column, LC-30A/20A/10Avp/10A-series Solvent Delivery Module (Pump), Detector, and Fraction Collector (Tokyo, Japan); and it is also possible to connect and control the SPD-M20A/M30A Photodiode Array Detector from the system controller as a 4-wavelength detector via the network. Chromatographic separations were conducted using Shim-pack GIS PREP-ODS, 10 x 250 mm, 10 μm $C_{18}$ particle size column. The mobile phase was composed of two solvents: $H_2O$ (A) and MeCN (B) as a linear gradient elution program was applied as follows: 0-80% B (0-20 min), 80%-100% B (20-30 min), 100-100% B (30-40 min), 100-80% B (40-50 min), 80-0% B (50-60 min). The flow rate was maintained at a 2.5 mL $min^{-1}$, and temperature of the column was set at room temperature.

Briefly, samples from preparative TLC of F3 was performed by HPLC. The sample reconstituted in methanol was filtered through a 0.45-μm Millipore filter. Then, the sample injection volume was set up in 500 μL, respectively, and the UV detection was performed at 220 and 254nm. However, the elutes were on-line monitored with UV detector and peak fractions were collected according to the chromatogram. The DH-1 and DH-2 compound was collected and concentrated to dryness; then resuspended in methanol for further bioassay.

### 4. Mammosphere formation assay.

Human breast cancer cells, MCF-7 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-7 cells were grown in Dulbecco's Modified Essential Medium (DMEM; Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone). MCF-7 cells were maintained at 37°C in a humidified incubator with 5% $CO_2$. Cells were plated at a density of $1x10^6$ cells in 10 cm culture dish.

Anti-CSCs activity was qualitatively evaluated by mean of the mammosphere formation assay technique. A cellular bioassay was used to culture breast cancer cell lines as tumorspheres assay from STEMCELL Technologies. The MCF-7 human breast cell line was seeded in 6-well plate at a density of 50,000 cells in 2 mL of completed medium per well, but for 24-well plate (500 μL of completed medium/well) (see the components of MammoCult in Table. 1), and the purified-sample from each fraction was added into the plate and cultured at 37°C in a humidified atmosphere containing 5% $CO_2$ for 7 days before imaging and counting. After one week of culturing, the cell size activity was observed under a microscope and photographed.

**5. Structure analysis of purified DH-1 and DH-2.**

The 1D were recorded on FT-NMR spectrometer (JEOL, Tokyo, Japan). The $^1H$ (600 MHz) and $^{13}C$ (600MHz) chemical shift were expressed in ppm and were referenced at methanol D3 for proton and carbon. The structure analysis of sample

were done by using 2D NMR containing Correlation spectroscopy (COSY), Heteronuclear multiple-bond correlation spectroscopy (HMBC), and Heteronuclear single-quantum correlation spectroscopy (HSQC).

**Table 1**. Mammospohere formation assay using tumorsphere derived from MCF-7 cells for 6-wells or 24- wells plate.

| Components | Amount |
|---|---|
| Mammocult basal medium | 9 ml |
| Mammocult proliferation supplements | 1 ml |
| Heparin | 10 µl |
| Hydrocortisone | 5 µl |
| Penicillin/Streptomyces | 100 µl |
| Total volume | 10.115 ml |

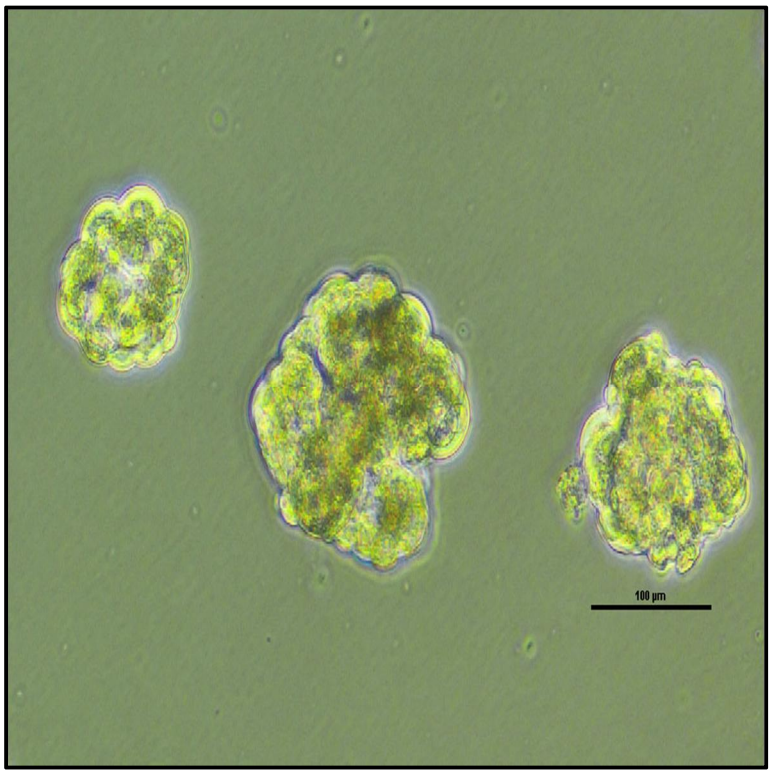



**Figure 4. Mammospheres derived from MCF-7 breast cancer.**

# RESULTS

## 1. Purification of active substances from *C. auriculatum*.

### 1.1. The anti-mammosphere-forming activity of solvent-partitioning fractions.

To identify the active substances exerting the anti-mammosphgere-forming activity, *C. auriculatum* was partitioned with ethyl acetate organic solvents as given the overall and briefly scheme (Fig. 2 and Fig. 3). The anti-mammosphgere-forming activity was evaluated by using mammospheres formation assay to examine each fraction (Fig. 4). It was shown that the ethyl acetate-soluble fraction presented a very high anti-mammosphere-forming activity. Further to isolate active substance, substances from *C. auriculatum* was sorted out the mobility of the compounds on the TLC plate with various types of solvent conditions (chloroform: methanol =10:1, 20:1, and 30:1, v/v) by spotting and developing in solvent-saturated chamber. TLC plates were dried and detected under UV light (254 and 365nm) (Fig.5). As a result, the TLC plate was shown the best separation in chloroform: methanol= 20:1 for further purified the elements, respectively (Fig. 5B).

### 1.2. The anti-mammosphere-forming activity of fractions obtained from ethyl acetate aqueous fraction.

The active parts in the ethyl acetate fraction were separated by silica gel column chromatography and yielded 5 subfractions (F1 to F5) (Fig. 6A). Then, each fraction was further analyzed by TLC with UV light to visible and capture the

TLC plate (Fig. 6B). Last, the anti-mammosphere-forming activity of each fraction was evaluated by the mammospheres formation assay (Fig. 6C). F2 sample significantly inhibited mammosphere-formation activity. Thus the fraction was consequently subjected for further purification. This result can be assumed through the inverted microscope. As a result, F2 was convinced to be further investigated in the next step.

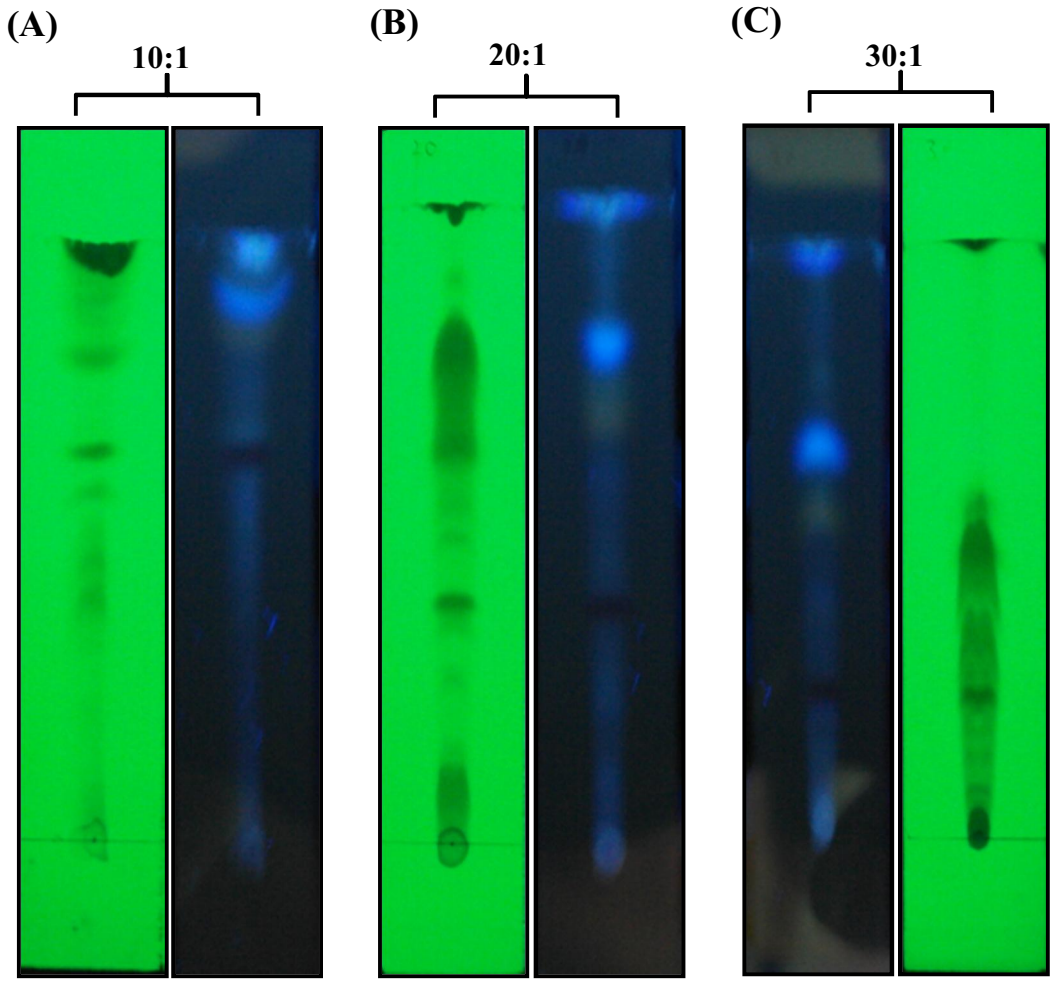



**Figure 5. Thin layer chromatography analysis of ethyl acetate extraction from *C. auriculatum*.** Ethyl acetate: water equally (1:1, v/v)–extracted samples from *C. auriculatum* were analyzed by spotting on silica gel 60 $F_{254}$ TLC plates. TLC plates were developed in solvent-saturated chamber after the spotting of samples. The chromatogram was dried and detected under a UV lamp with wavelength $UV_{254nm}$ and $UV_{365nm}$. (A) Chloroform: methanol=10:1; (B) Chloroform: methanol=20:1; (C) Chloroform: methanol=30:1.

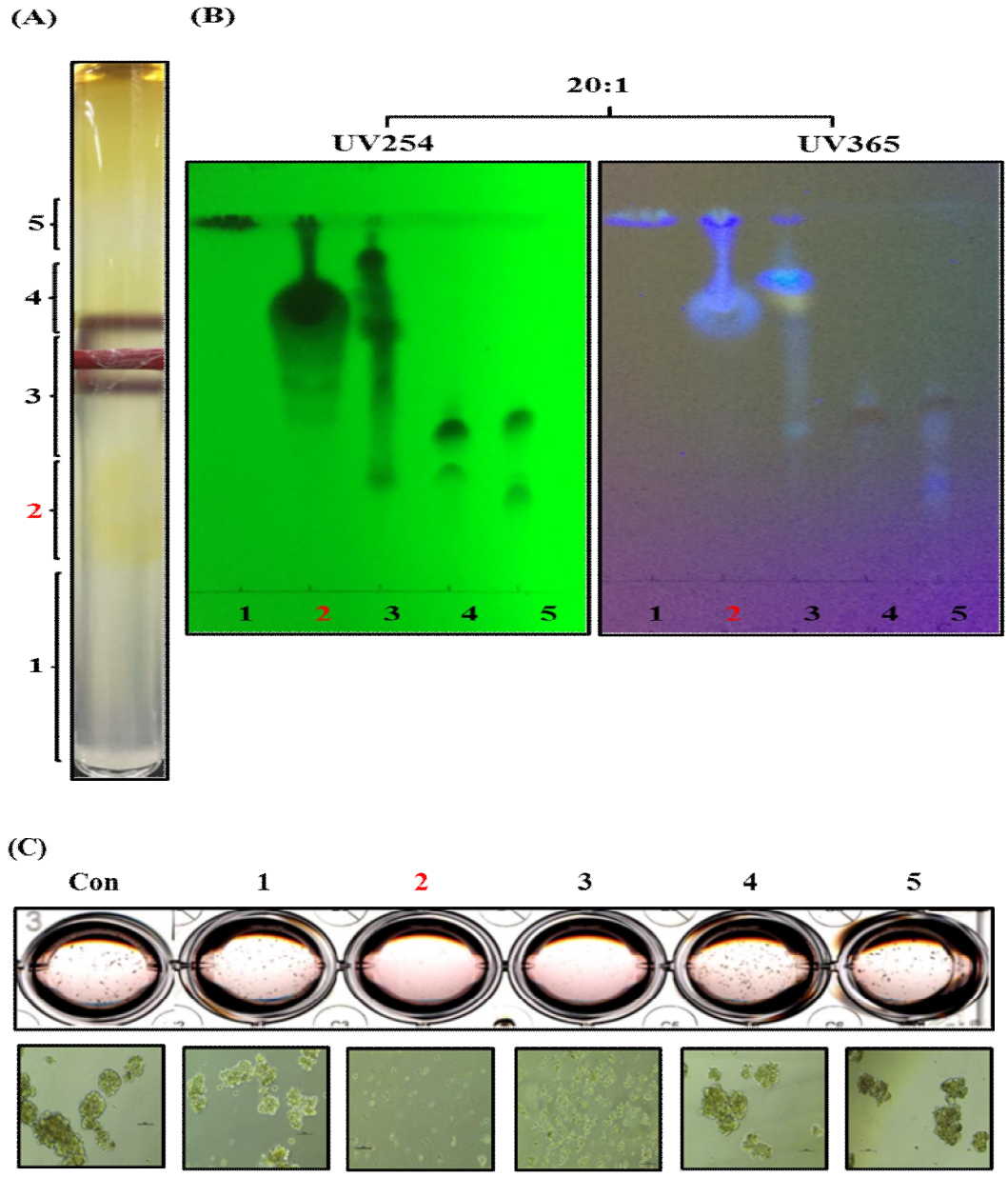



**Figure 6. The purification procedure of mammosphere-forming inhibitors derived from *C. auriculatum* using $SiO_2$ gel chromatography.** (A) Sample was isolated by using $SiO_2$ gel chromatography with a solvent mixture $CHCl_3$: MeOH = 20:1 (v/v) to elute fraction sample and were obtained to 5 subfractions. (B) TLC plates analysis of the purified sample. Each of the eluate fractions was spotted on TLC plate and developed in solvent-saturated chamber in $CHCl_3$: MeOH = 20:1 (v/v). The TLC plates were dried; then, the spots on TLC plates

were analyzed and detected under a UV lamp. (C) The eluates (F1 to F5) were dried up and reconstituted with methanol. Mammospheres formation assay using MCF-7 cells. After a week incubation (5% $CO_2$, humidified incubator at 37°C, 7 days). Photograph of mammosphere using inverted microscope was checked.

**1.3. Open ODS column chromatography of the anti-mammosphere-forming activity using the F2 compound.**

ODS open column chromatography (C18) was employed to investigate the most active compound. F2 was concentrated to dryness in order to remove methanol, and dissolved in the mixture solvents in water and acetonitrile (30%), which gave a yield as depended on the percentage of the solvent system. Purified components were classified the target activity with a relatively to purity, the fraction was further figured out by TLC. One among all, 30% and 50% acetonitrile sample was consisted of the activity. Furthermore, in order to confirm the identical spot from TLC, mammosphere formation assay was used to discover the bioactivity materials. As shown in Fig. 7A, three fractions (30% acetonitrile, 50% acetonitrile and 70% acetonitrile) were shown. However, 30% and 50% acetonitrile sample was existed significantly inhibited activity on breast cancer stem cell as bioactive-guided assay, indicating that the identical spot from TLC plate was assigned to be the best candidate for further isolation. (Fig. 7A)

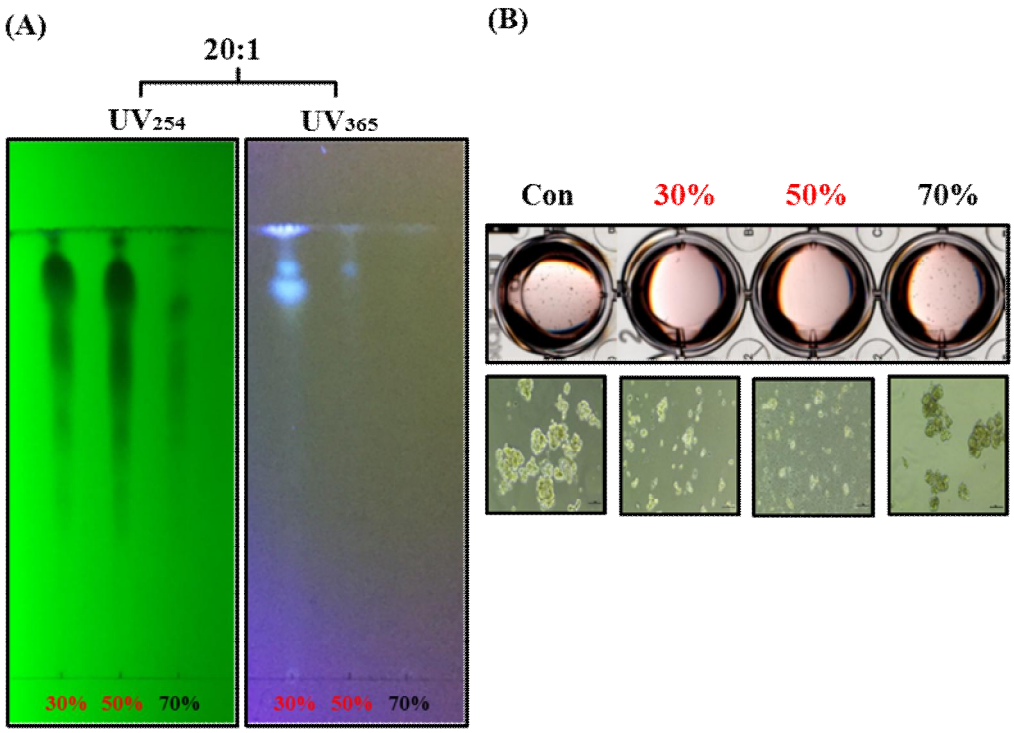



**Figure 7. The Purification procedure of mammosphere-forming inhibitors in *C.auriculatum* using Reversed-Phase $C_{18}$ (ODS) gel chromatography.** (A) ODS open column chromatography (C18) eluted with $CH_3CN$: $H_2O$ (30%, 50% and 70% of $CH_3CN$) using the partial-purified sample. Thin-layer chromatography analysis of the partial-purified sample from ODS resin, purified-sample was spotted and developed in chloroform: methanol (20:1, v/v). UV light was used to detect the pigments. (B) Mammosphere formation assay using MCF-7 and photograph of mammosphere using inverted microscope.

**1.4. Separation of the anti-mammosphere-forming activity compound from 30% and 50% acetonitrile elutes using Sephadex LH-20 column.**

The highest inhibition of 30% and 50% acetonitrile sample was further purified by using a gel filtration column chromatography onto a Sephadex LH-20 column; pre-equilibrated with 100% methanol and eluted with the same solvent. There were three subfractions obtained, and each fraction was isolated by amount-dependence (Fig.8A). Individual fraction was analyzed by TLC for further investigation of the active fraction based on the spot as described from previous sessions in C18 ODS open column chromatography. Single fraction was spotted on TLC plate and developed in the solvent gradient system of chloroform: methanol (20:1, v/v). The plate was dried and detected under UV light 254nm and 365nm (Fig. 8B). The band was determined under UV light 254nm with consisting of dark spot on the upper side near the front line in F1. To be assured the dark spot as revealed at the beginning by TLC on its anti-mammosphere-forming activity, mammosphere formation assay was engaged. As a result, the mammosphere formation assay defined that the F1 was subsisted of the target band through its highest activities (Fig. 8C) In short, the identical components from gel filtration chromatography (F1) were further purified for an anti-mammosphere-forming activity inhibitor.

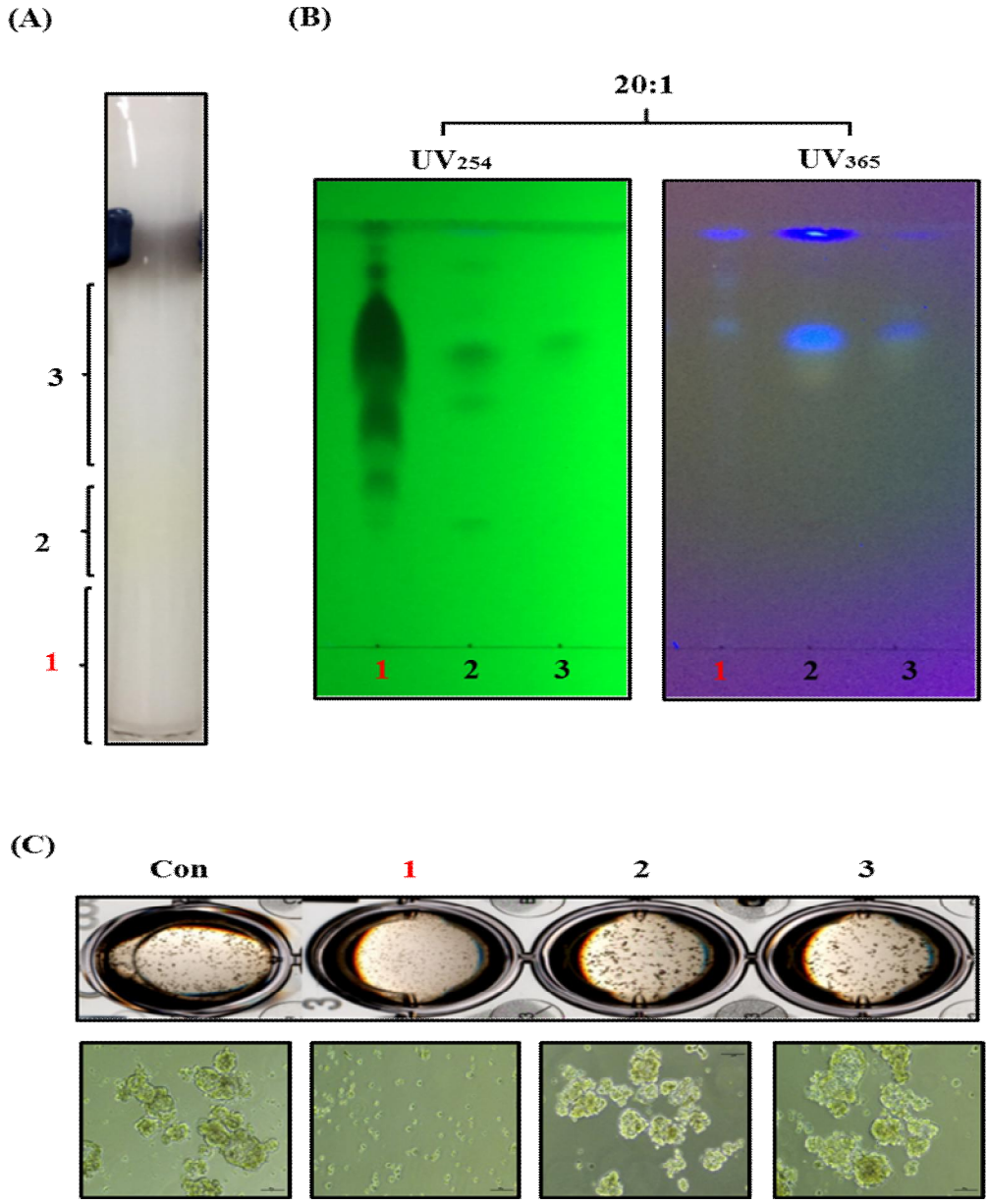



**Figure 8. The Purification procedure of mammosphere-forming inhibitors in *C. auriculatum* using Sephadex LH-20 gel chromatography.** (A) Sephadex LH-20 column chromatography eluted with methanol. (B) Thin-layer chromatography analysis of the eluted fractions of *C. auriculatum* which was developed in chloroform/methanol (20:1, v/v). (C) Mammosphere formation assay using MCF-7 cells and photogrph of mommonsphere using partial-purified sample.

**1.5. Isolation of the anti-mammosphere-forming activity compounds from compounds separated using Preparative TLC.**

In order to separate successfully these mixtures of active compound using preparative TLC, a biphasic solvent system was selected on the basis of TLC analysis. Therefore, the preparative TLC separation of Sephadex LH-20-purified fraction was first performed in a diverse solvent system of chloroform and methanol by gradually decreased down the polarity of the solvent from 10:1, 20:1 and 30:1 (v/v) (Data not shown). Thus, the solvent mixture formed by chloroform added with methanol ($CHCl_3$: MeOH = 20:1, v/v) was shown the best separation of the target band and led to isolate into 4 subfractions (Fig. 9A). Then, F3 seemed to contain the most identical spot respectively, which displayed the highest inhibition of anti-mammosphere-forming activity according to TLC analysis with UV light 254nm follow previous observation (Fig. 9B). Individual fraction was alternatively assayed on cell in one types of concentration (10 μL from stock 1 mL in methanol); the activities were exposed after a week. Therefore, F3 was found as the anti-mammosphere-forming activities compare to negative control without treatment. so it means that the identical compound which was notified in the TLC as describe above was presented the investigation active target compound, this may indicate that this compound was consisted of to be further isolated. As a result, only active component from F3 could be a potential for further purification.

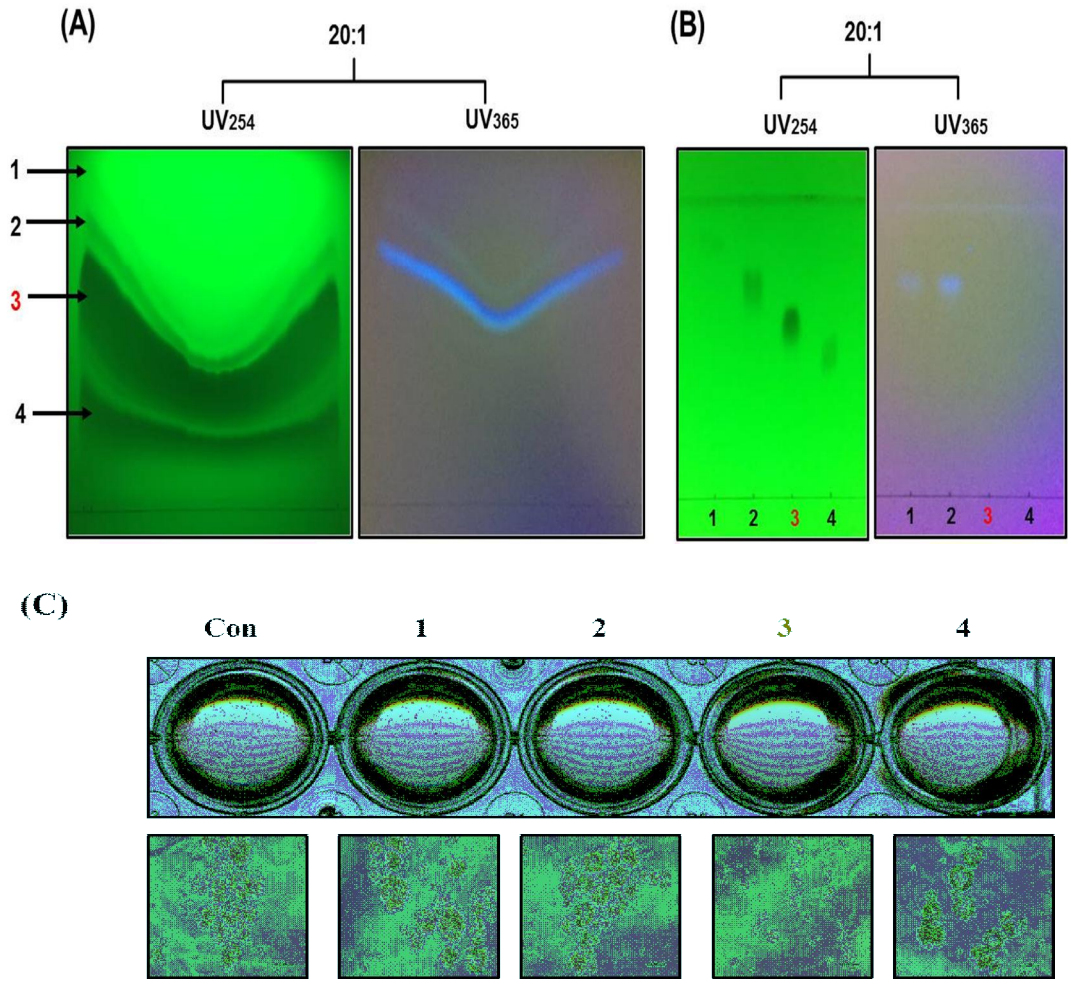



**Figure 9. Purification procedure of mammosphere-forming inhibitors in *C. auriculatum* using a preparative thin layer chromatography.** (A) Preparative Thin-Layer Chromatography of F1 fractions in Sephadex LH-20 gel chromatography. The 4 subfractions were analyzed after the development with chloroform and methanol (20:1, v/v); detected under UV light 245 and 365 nm. (B) TLC analysis of the Prep-TLC bands after scraped and purified sample. Individual band was spotted and developed in chloroform:methanol (20:1, v/v). The plate was dried and visualized under UV light 254 and 365 nm. (C) Mammosphere formation assay using MCF-7 cells and photogrph of mommosphere. Individual band from purified samples were done in the assay.

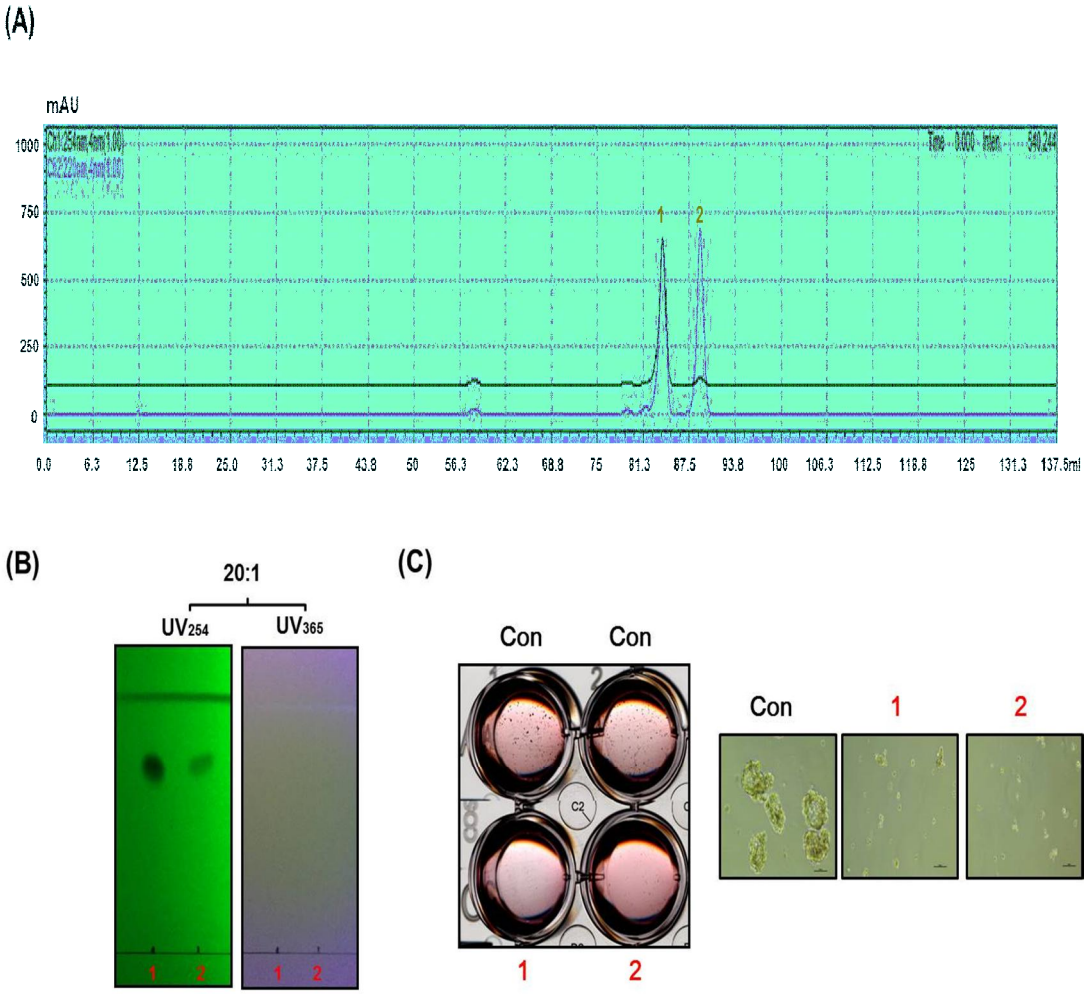



**Figure 10. Major fractions collected using High Performance Liquid Chromatography in two wavelength. Samples were collected based on 254 and 220nm wavelength.** (A) HPLC chromatogram was detected in $UV_{220nm}$ and $UV_{254nm}$. Preparative TLC fraction was fractionated by reversed-phase HPLC: mobile phase, two solvents: A, $H_2O$ and B, MeCN with a linear gradient as follows: 0-80% B (0-50ml), 80-100% B (50-75ml), 100%-100% B (75-100ml); absorbance was monitored at 254 nm with an elution program at 2.5 mL $min^{-1}$ and 2 subfractions (DH-1 and DH-2) were obtained; however, the major two peak were collected and identified as DH-1and DH-2. (B) HPLC purified-samples were analyzed by TLC with pre-coated silica gel and developed with

($CHCl_3$:MeOH=20:1, v/v). The spots were detected with UV light. (C) Mammosphere formation assay using MCF-7 cells and photograph of mommonsphere. These were assay was used to investigate the active compound from HPLC purified-sample.

### 1.6. Purification of active substances in partial purified sample using Preparative HPLC.

The biological active components of F3 were found to be the highest quantity of designing component with TLC established; therefore, this fraction was targeted for isolation of anti-mammosphere-forming compounds. The chromatogram effluents were pooled together based on TLC analysis and on-line chromatogram. Further scanning, two types of the HPLC profile were used to investigate the peaks, detected with 220 and 254 nm. Consequently, two main peaks were obtained through the UV detection of 254nm and 220nm (Fig. 10A). The purity of isolated compound, designed as DH-1 and DH-2, TLC was achieved (Fig. 10B). As a result, either UV light 254 or 365 nm detected, the plate was consisted of a single spot, so this means the isolate compound (DH-1 and DH-2) might be pure enough to be further studied. However, to be sure for the isolated compound of the DH-1 and DH-2 was the active compound that have been investigated, mammosphere assay and photograph of mommonsphere were used in order to confirm the target compound (Fig. 10C). Therefore, the HPLC isolated compounds know as DH-1 and DH-2 was obtained the activity against CSCs cells compare to negative control,

this demonstrated that the purified sample could be further study of structure identification by NMR (Fig, 11 and Fig. 19).

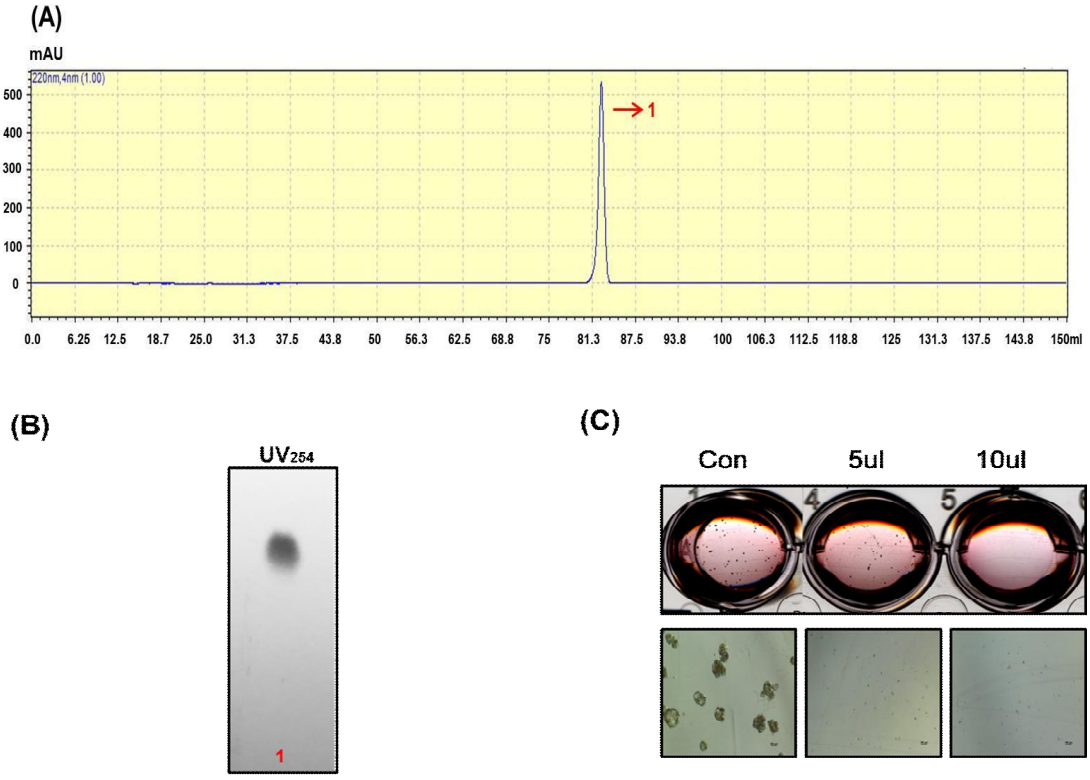



**Figure 11. The peak 1 (DH-1) was fractionated and collected using High Performance Liquid Chromatography.** HPLC condition: mobile phase, two solvents: A, $H_2O$ and B, MeCN with a linear gradient as follows: 0-80% B (0-50ml), 80-100% B (50-75ml), 100% B (75-100ml); absorbance was monitored at 254 nm with elution program at 2.5 mL $min^{-1}$. (A) Chromatogram of the purified-sample from RP-HPLC with UV light 254 nm detected; the sample was eluted with MeCN: $H_2O$ =100% (TR=32.5 min). (B) TLC analysis of the RP-HPLC purified-sample. The purified-sample was spotted on TLC plate and developed in the solvent-saturated chamber with a solvent system $CHCl_3$: MeOH = 20:1 (v/v). TLC plate was dried and detected under UV lamp 220 nm. (C) Mammosphere formation assay using breast cancer, MCF-7 cells and photograph of

mommonsphere. This assay was used to investigate the active compound from RP-HPLC purified-sample, designed as DH-1.

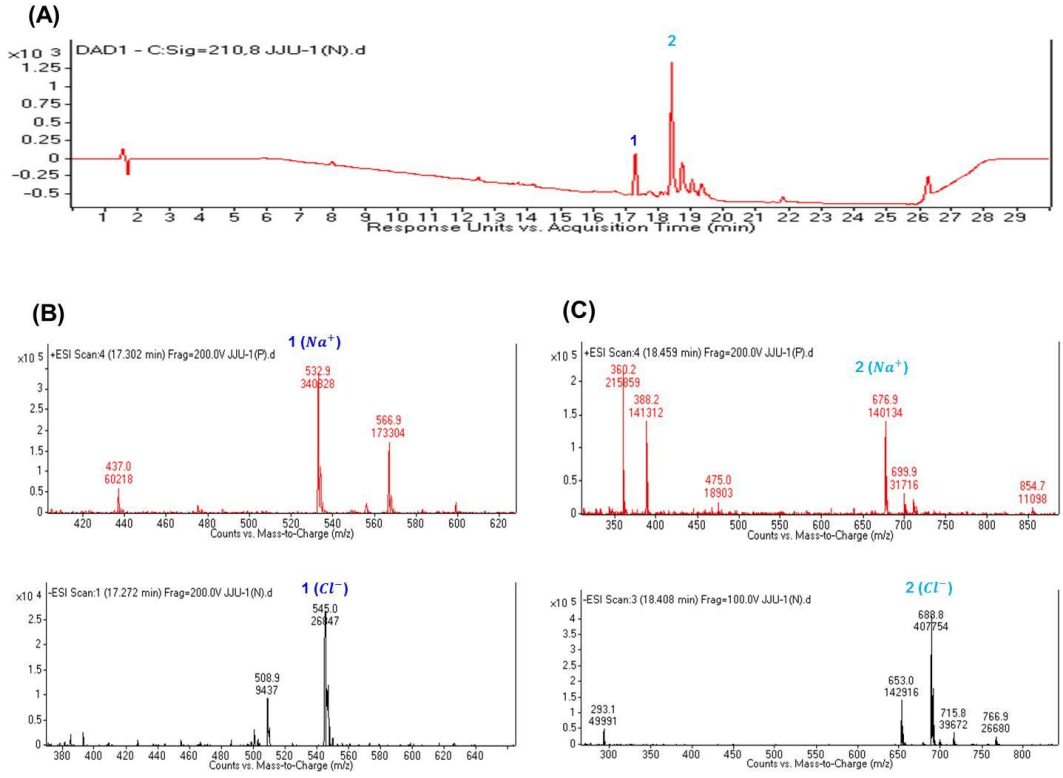



**Figure 12. The peak 1 (DH-1) was fractionated and analyzed using LC-MASS.**

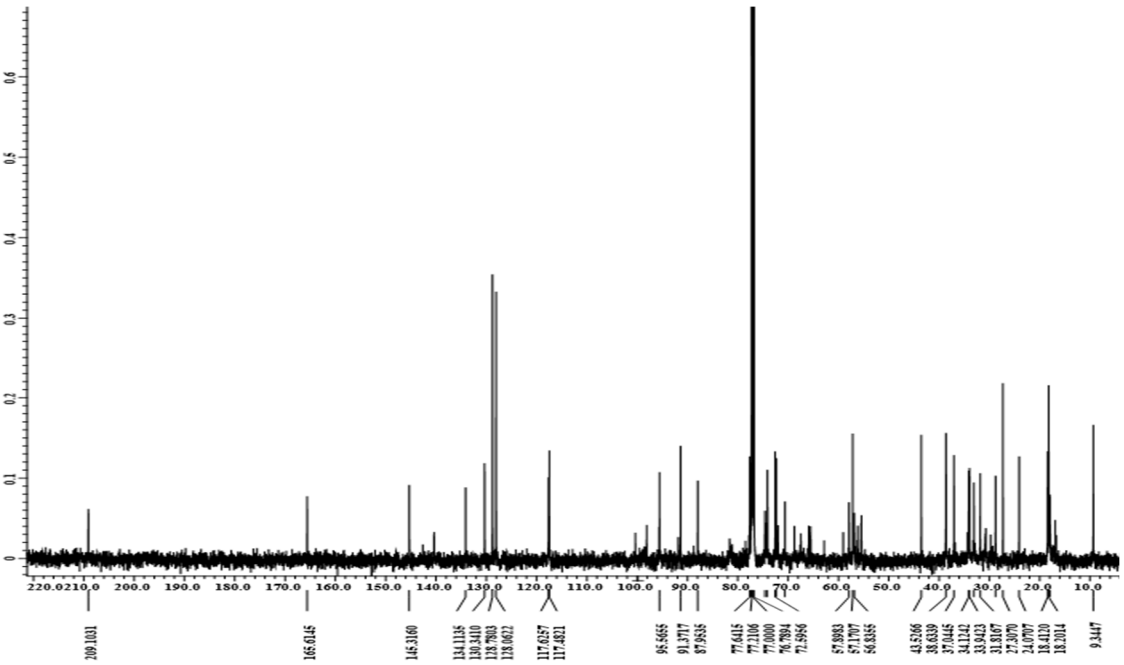



**Figure 13. 13C-NMR spectrum of compound DH-1(2).**

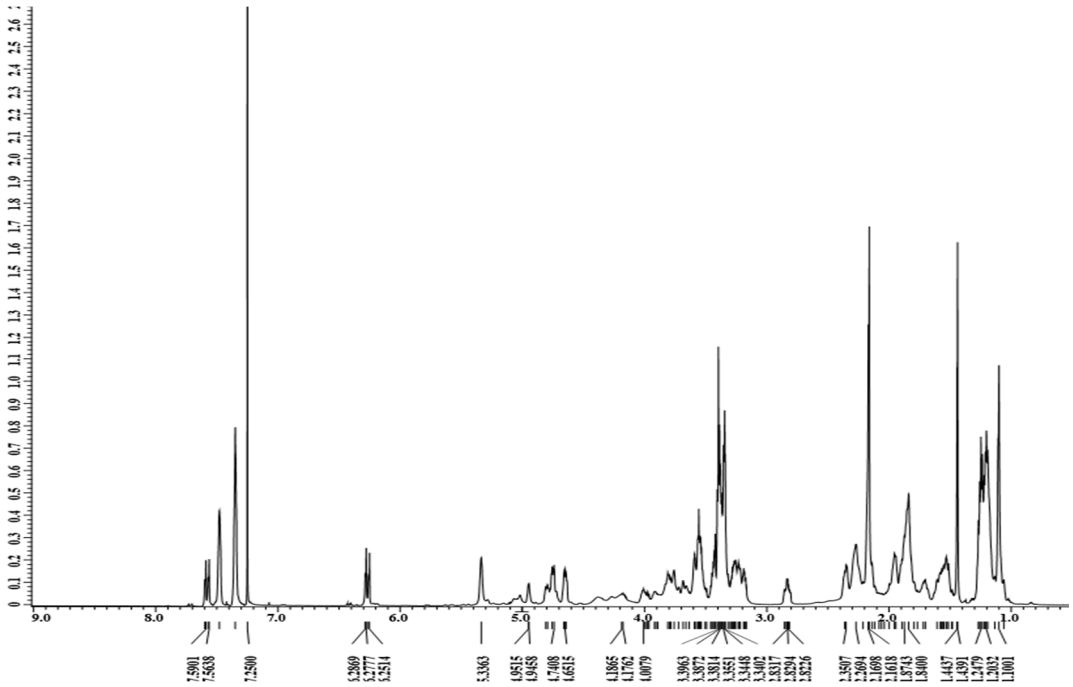


**Figure 14. 1H-NMR spectrum of compound DH-1(2).**

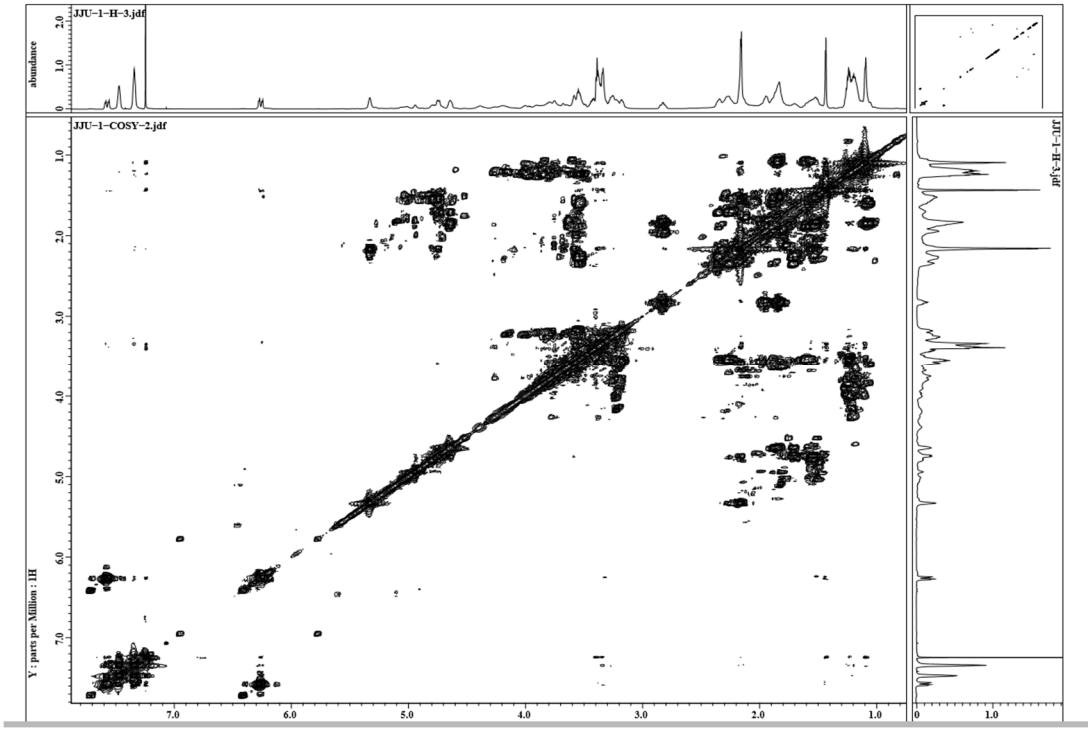



**Figure 15. 1H-1H COSY spectrum of compound DH-1(2).**

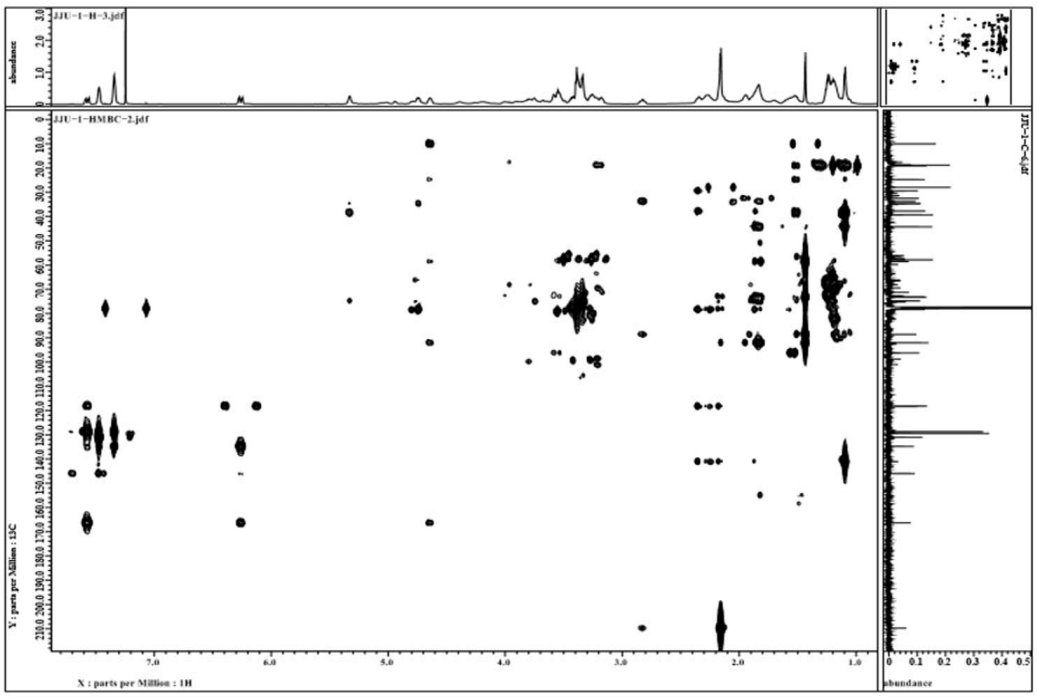



**Figure 16. 1H-13C HMBC spectrum of compound DH-1(2).**

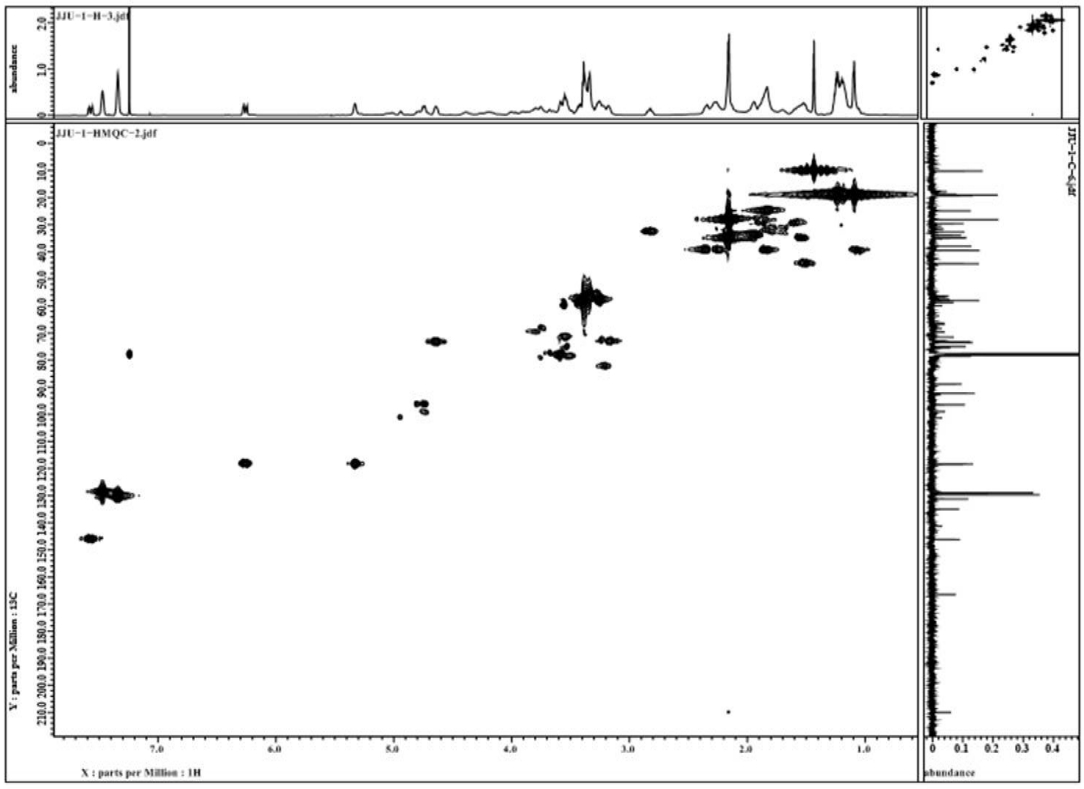
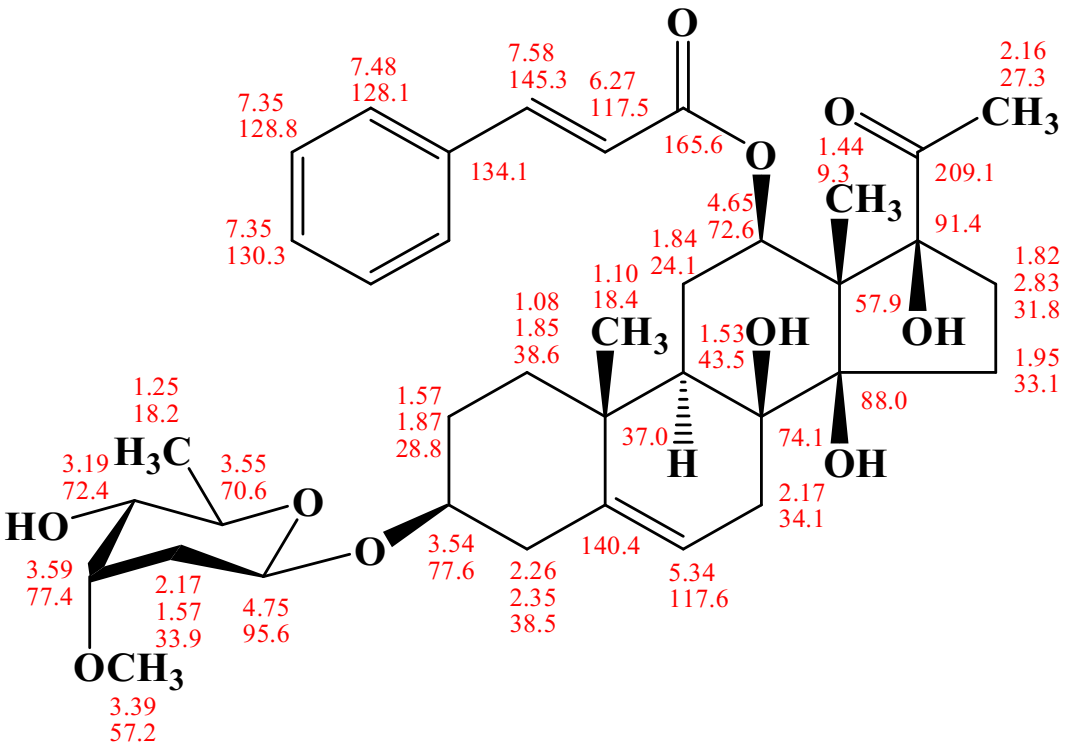



**Figure 17. 1H-13C HMQC spectrum of compound DH-1(2).**




**Kidjoranin-3-O-β-D-cymaropyranoside ($C_{37}H_{50}O_{10}$; 654)**

**Figure 18. Molecular structure of compound DH-1(2).**

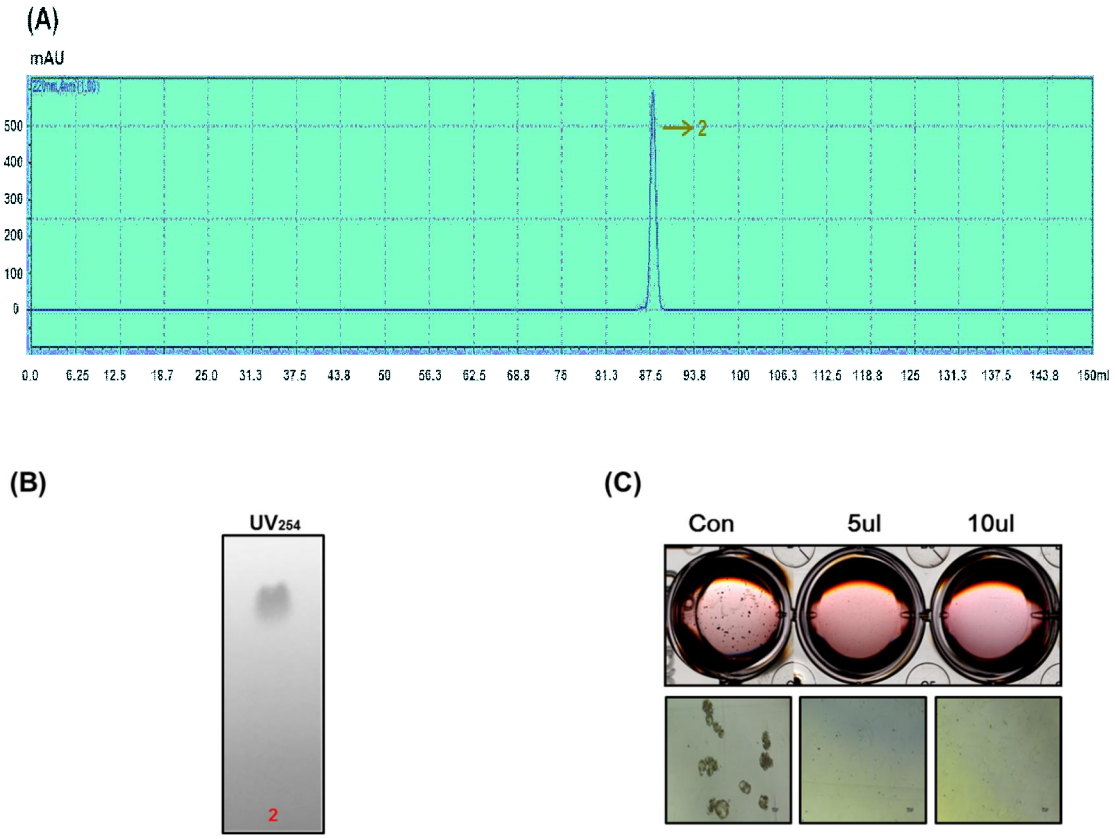



**Figure 19. The peak 2 (DH-2) was fractionated and collected using High Performance Liquid Chromatography.** HPLC condition: mobile phase, two solvents: A, $H_2O$ and B, MeCN with a linear gradient as follows: 0-80% B (0-50ml), 80-100% B (50-75ml), 100% B (75-100ml); absorbance was monitored at 254 nm with elution program at 2.5 mL $min^{-1}$. (A) Chromatogram of the purified-sample from RP-HPLC with UV light 254 nm detected; the sample was eluted with MeCN: $H_2O$ =100% (TR=35 min). (B) TLC analysis of the RP-HPLC purified-sample. The purified-sample was spotted on TLC plate and developed in the solvent-saturated chamber with a solvent system $CHCl_3$: MeOH = 20:1 (v/v). TLC plate was dried and detected under UV lamp 220 nm. (C) Mammosphere formation assay using MCF-7 cells and photograph of mommonsphere. This assay

was used to investigate the active compound from RP-HPLC purified-sample, designed as DH- 2.

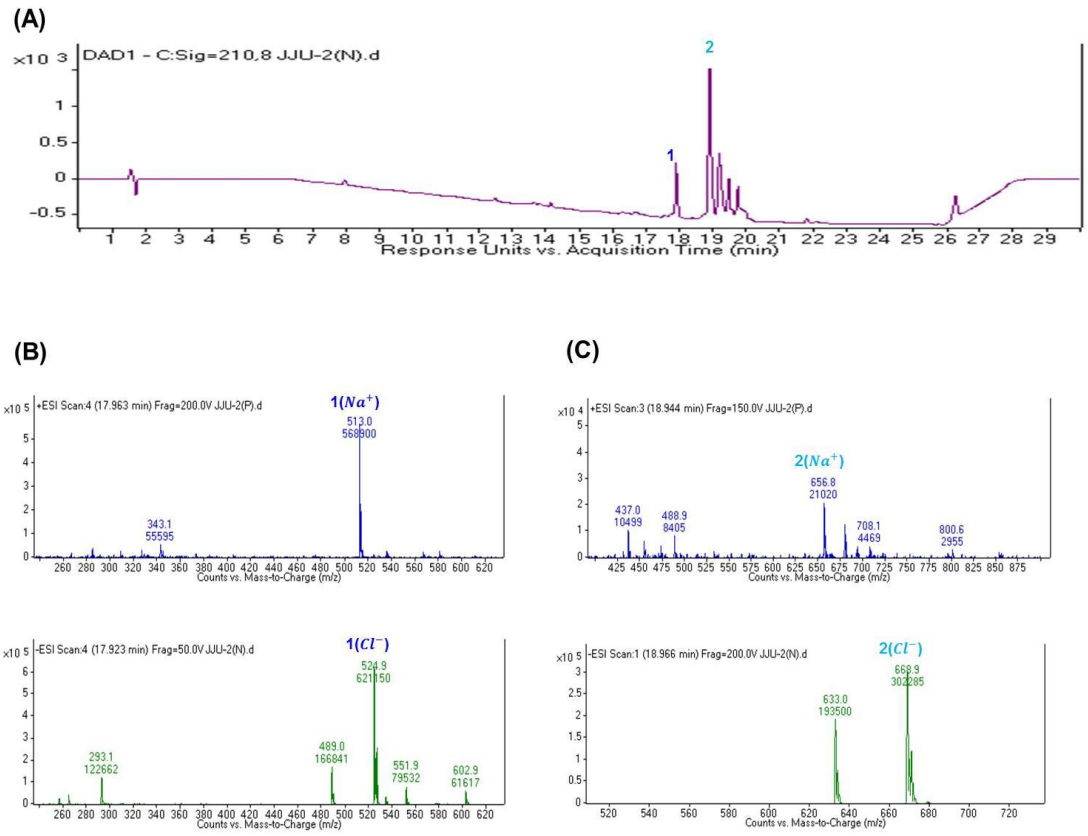



**Figure 20. The peak 2 (DH-2) was fractionated and analyzed using LC-MASS.**

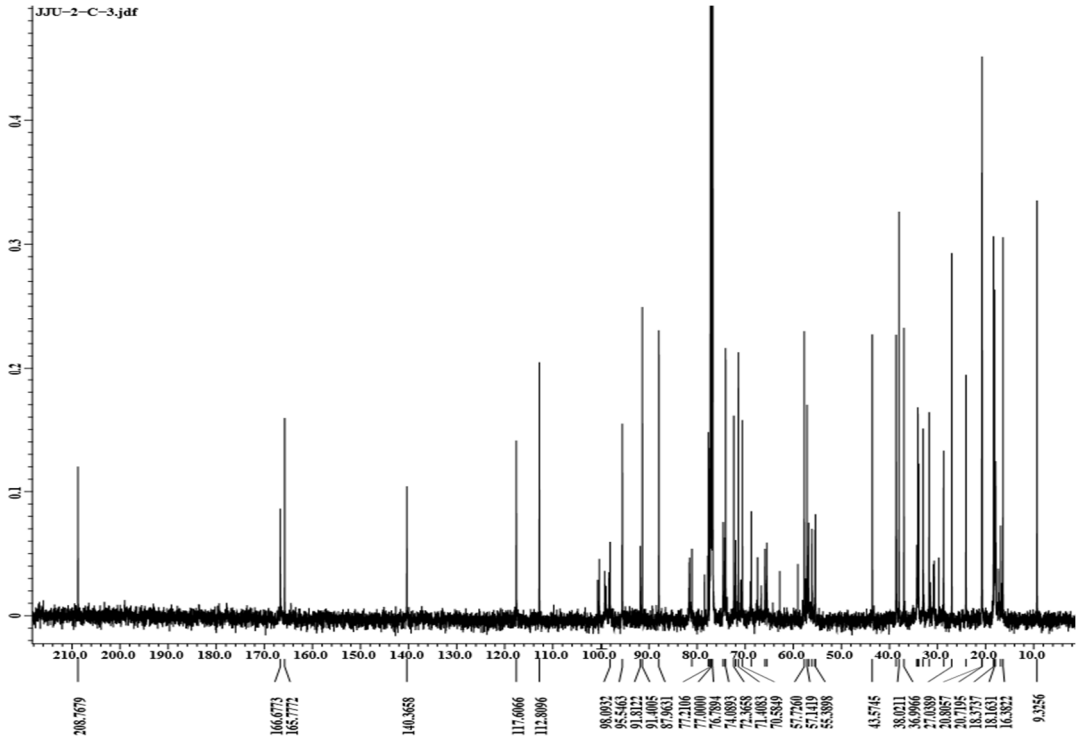



**Figure 21. 13C-NMR spectrum of compound DH-2(2).**

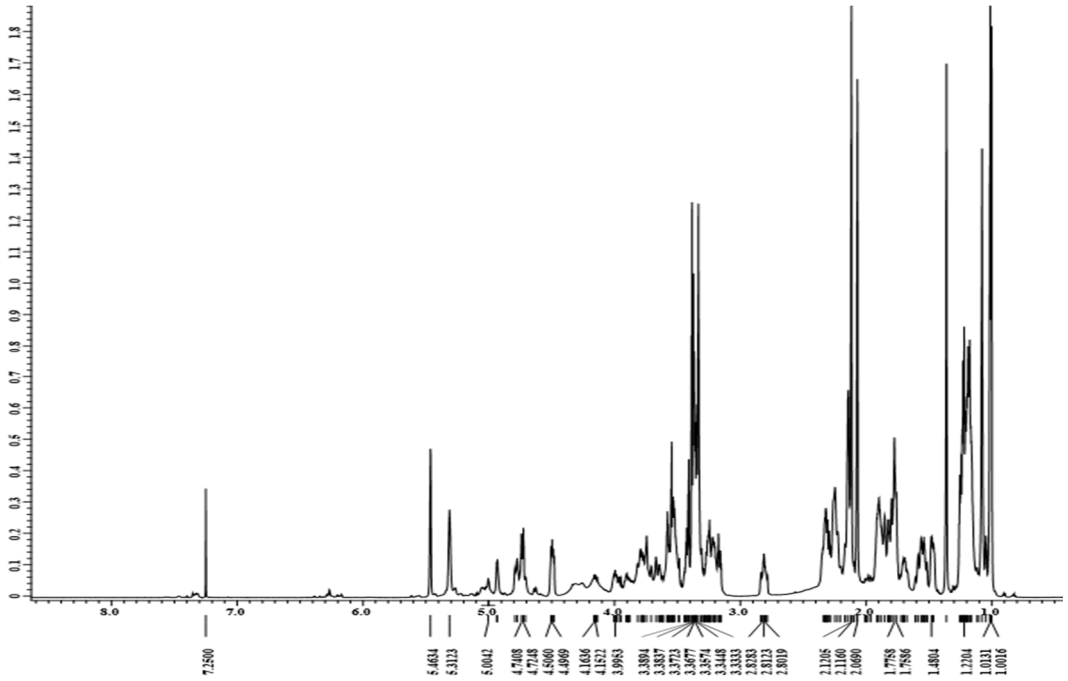


**Figure 22. 1H-NMR spectrum of compound DH-2(2).**

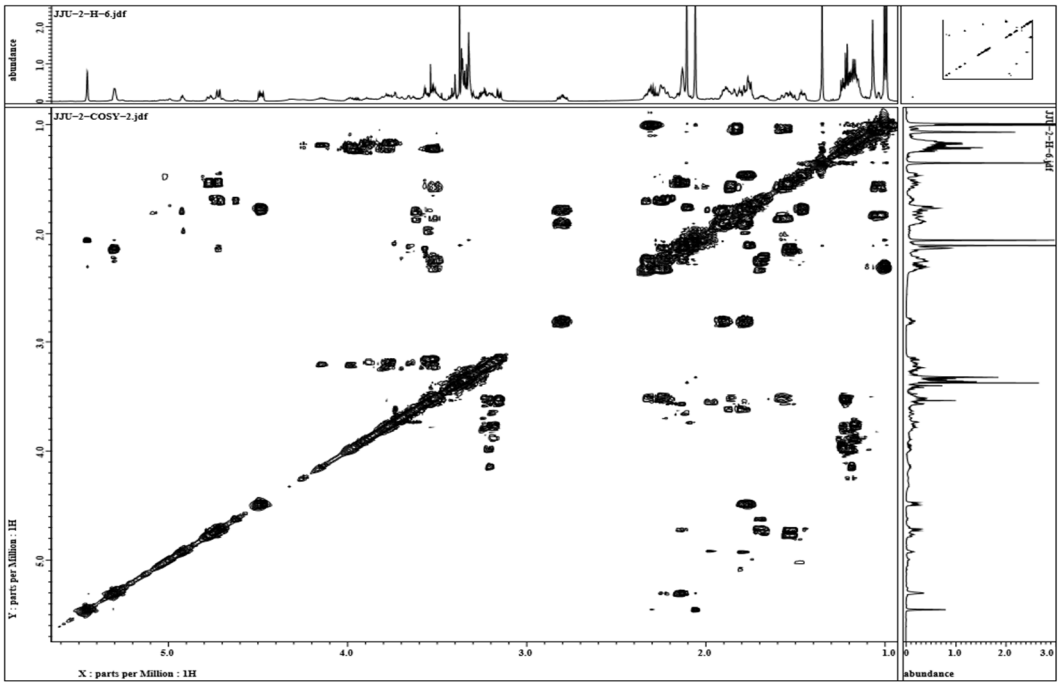



**Figure 23. 1H-1H COSY spectrum of compound DH-2(2).**

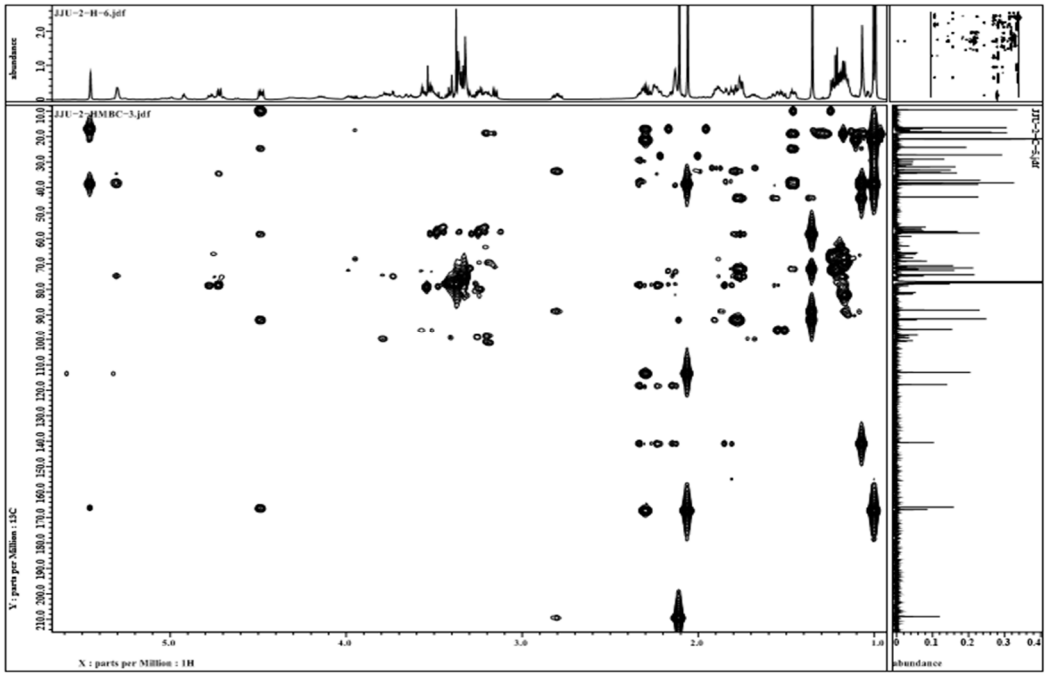



**Figure 24. 1H-13C HMBC spectrum of compound DH-2(2).**

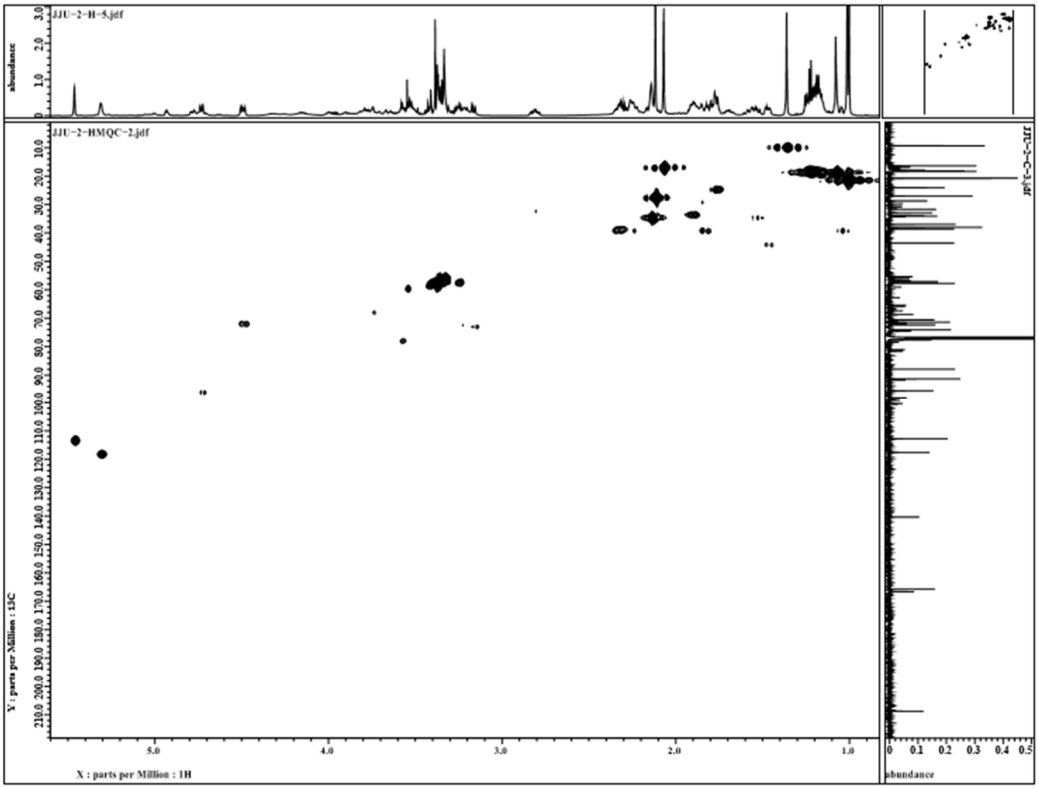
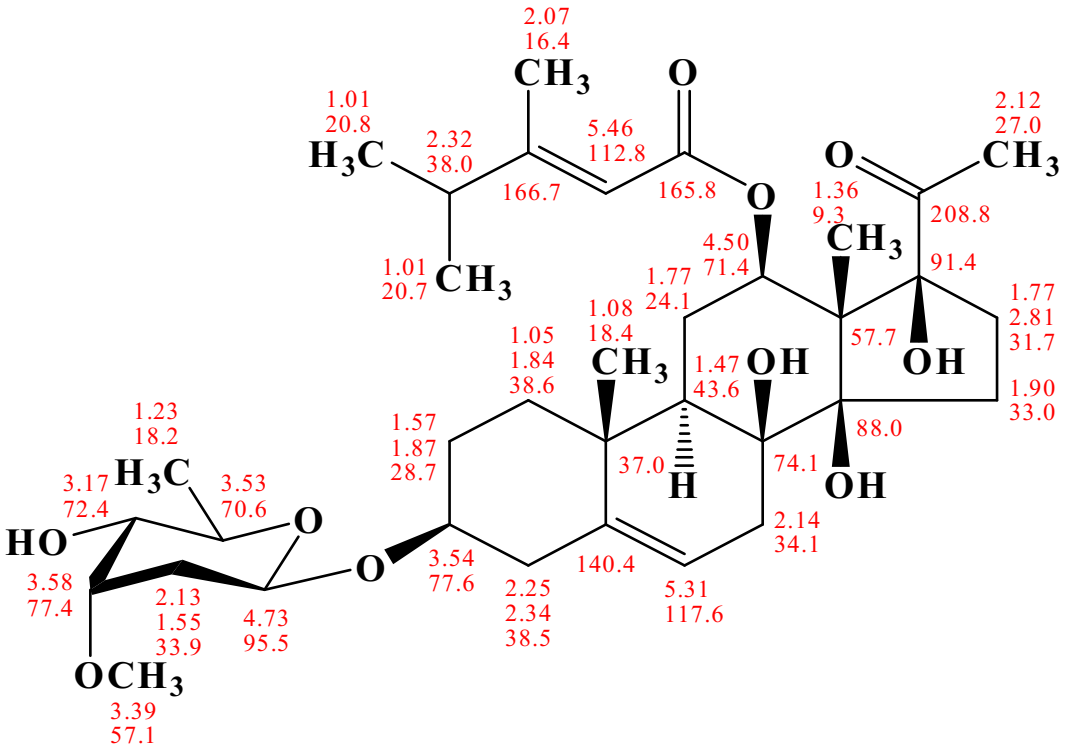



**Figure 25. 1H-13C HMQC spectrum of compound DH-2(2).**




**Caudatin-3-O-β-D-cymaropyranoside; ($C_{35}H_{54}O_{10}$; 634)**

**Figure 26. Molecular structure of compound DH-2(2).**

**2. Structure analysis of DH-1 and DH-2 using 1-, 2-NMR, and Mass spectrophotometry.**

Using optimized HPLC condition, Compound DH-1 showed several peaks containing one peak, number 2. Molecular weights of Peak 2 are 676.9, sodium type and 688.8 chloride type. Molecular weights of DH-1 is 654 (Figure 12). Molecular structure of DH-1 was analyzed with C-NMR, H-NMR, 2D NMR (H-H COSY, H-C HMBC, H-C HMQC), LC-Mass (Figure 13-17). DH-1 is kidjoranin-3-*O*-β-*D*-cymaropyranoside, $C_{37}H_{50}O_{10}$ (Figure 18). Using optimized HPLC conditiom, Compound DH-2 showed several peaks containing one peak, number 2. Molecular weights of Peak 2 are 656.8, sodium type and 668.9 chloride type. Molecular weights of DH-2 is 634 (Figure 20). Molecular structure of DH-2 was analyzed with C-NMR, H-NMR, 2D NMR (H-H COSY, H-C HMBC, H-C HMQC), LC-Mass (Figure 21-25). DH-2 is caudatin-3-*O*-β-*D*-cymaropyranoside, $C_{35}H_{54}O_{10}$ (Figure 18). We purified mammosphere-forming inhibitors and show molecular structure as kidjoranin-3-*O*-β-*D*-cymaropyranoside and caudatin-3-*O*-β-*D*-cymaropyranoside.

# DISCUSSION

General stem cells have the ability to differentiate into many different cells. However, abnormal stem cells will develop into abnormal cell populations. These abnormal stem cells are CSCs. These CSCs share many characteristics with normal stem cells, including self-renewal and differentiation [18]; and CSCs can survive in a nutrient- and oxygen- deficient environment [19] and have drug resistance, they are considered as one reason that cancer cannot be completely cured. The existence of CSCs has been implicated in many tumor types including breast cancer. The presence of CSCs, either intrinsic or therapy induced, has been attributed to the progression and poor prognosis in breast cancer patients [18-19].

The root of *Cynanchum auriculatum* is a famous traditional tonic in the Oriental System of Medicine and has been used for nourishing the blood and prolonging life. In recent years, the antitumor activity of the root of *C. auriculatum* has attracted much attention and C-21 steroidal glycosides were considered as its major active components. In the present study, two known C-21 steroidal glycosides were isolated from the root tuber of *Cynanchum auriculatum* Royle ex Wight. The in vitro antitumor activities of the C-21 steroidal glycosides obtained in this work were tested on the cell lines MCF-7 assay [20].

Taken together, our finding result would be valuable for exploiting the potential of *Cynanchum auriculatum* extraction (CAE) in the therapy of breast malignancy. The anti-CSCs activity effects of CAE and its component on human breast cancer stem cells will provide an evidence to develop a chemotherapeutic

drug against breast cancer cell. However, In addition, the anticancer activity of CAE to determine the details mechanism of action of these compound in vitro and in vivo is needed to be examined in the future.

# CONCLUSION

CSCs are drug-resistant and radiation-resistant cancer cells to be responsible for tumor progress, maintenance and recurrence of cancer, and metastasis. The breast mammosphere-forming inhibitors of MCF-7 cells was isolated from the aerial parts of *Cynanchum auriculatum* extraction (CAE). The ethyl acetate fraction obtained from methanol extracts exhibited the outstanding inhibitory effect on mammospohere formation. From the ethyl acetate fraction, a compound named DH-1 and DH-2 was purified through Silica gel column chromatography, ODS open column chromatography, Sephadex LH-20 column chromatography, preparative thin-layer chromatography, and reverse phase high-performance liquid chromatography. The DH-1 and DH-2 isolated compound is highly effective against tumoresphere cell tested, and could be used in the control of cancer cell. Thus, the isolated compounds exhibited a strong anti-CSCs activity, which may contribute to the interpretation of the pre-pharmacological and clinical effects of CAE. DH-1 and DH-2, mammosphere-forming inhibitors show molecular structure are steroidal glycosides or C21 steroids from Cynanchum as kidjoranin-3-*O*-β-*D*-cymaropyranoside and caudatin-3-*O*-β-*D*-cymaropyranoside.

The phytochemical studies on C21 steroid constituents from Cynanchum species are initially motivated by their widespread clinical use in Chinese folk medicines. Modern pharmacological investigations of these constituents indicate their immense potential in the treatment of various diseases as antitumor, antifungal, antiaging, antiviral, Na+/K+-ATPase inhibitory, appetite suppressant agents. Among various types of C21 steroid constituents, caudatin and kidjoranin

derivatives in *C. auriculatum* exhibit most remarkable pharmacological activity. Studies of these two types of derivatives highlight the existence of various potential leads to develop new anticancer agents. Although these derivatives have shown various biological activities, the mechanisms of actions have not been fully elucidated. This is probably due to the lack of comprehensive research on the structure-activity relationship of these compounds. Therefore, there are needs to be bridge in this field in order to exploit the full medicinal potential of C21 steroid constituents. In future research, emphasis can be stressed on identifying the rules governing the association between the structure and activity of these compounds, and elucidating the unknown mechanism of action for the development of therapeutically and clinically potential drugs.

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# ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who helped me during the writing of this thesis. During my academic life at Jeju National University, I have a very learning environment and accommodation until I complete my degree. Firstly I would like to express the deepest appreciation to my Professor Dong-Sun Lee, who has the attitude and the substance of a genius: he continually and convincingly conveyed a spirit of adventure in regard to research, as excitement in regard to teaching. Without his guidance and persistent help this thesis would not have been possible. Moreover, to Dr. Hack Sun Choi, I do appreciate your patience, encouragement, and professional instructions during my thesis. Finally, to my laboratory researchers, Kang Jiyoung, Kim Jihyang, Kim Sulim have assisted me in the experiment. As well as, In addition, I would like to thank my wife Zhu Kehua, my family, and all my friends and family to help me through the difficulties continue to support encourage me.