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The fruiting body extracts of Antrodia cinnamomea exert significant anti-breast cancer cell activities

得獎獎項

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關鍵字：牛樟芝(Antrodia cinnamomea)、人類乳癌細胞

作者簡介

「追求卓越，成功自然就會找上門」是商廣明—我奉行的座右銘，喜好出外旅遊增廣自己的世界觀。自從進入建國中學數理資優班後，便對自己動手做實驗產生了濃厚的興趣，從檢索文獻到實驗設計，付諸實行再改善實驗，追求書本上沒有的新知識，心中滿足感便油然而生，僅管這其中難免會碰到挫折、失敗，但這不正是在追求卓越時會面臨的困難，我相信只要不斷地努力、不斷的克服，最後終將邁向成功。

我是蘇子軒，目前就讀建國中學二年級數理資優班，在專題研究時間培養了對做生物實驗的興趣。從學習操作，到自行思考、設計各個實驗的安排，讓我對這方面的領域有更深的了解，同時在找尋文獻時增進不少相關生物知識。其中可能面臨實驗失敗等不同的挫折，但都無礙於我秉持一股熱忱，找出先前的錯誤並加以修正，然後繼續實驗，讓我們的研究更完整、更好。

摘要

牛樟芝(Antrodia cinnamomea)是台灣特有的原生藥用真菌，長期被民間認為具有治療癌症、腹瀉、高血壓或保肝的效果。本研究的目的是探討牛樟芝子實體乙醇萃取物對人類乳癌細胞的抑制效用，乳癌是全世界女性最常見的癌症之一，我們透過比較不同培養時期三個月(AC-3)、六個月(AC-6)及九個月(AC-9)子實體之化學指紋圖譜，並測試它們抑乳癌細胞活性之差異。結果顯示，培養三個月的牛樟芝子實體 AC-3 抽出成份對乳癌細胞的生長抑制作用最佳；而利用西方墨點法探討牛樟芝對細胞週期或凋亡相關蛋白質的變化，推測 AC-3 可影響乳癌細胞的細胞週期調控，並誘發細胞凋亡。未來我們將進一步深入研究牛樟芝子實體 AC-3 之抗癌作用機制，並研究對乳癌細胞轉移作用的影響，找出牛樟芝子實體中主要的抗乳癌活性成分。

Abstract

Antrodia cinnamomea(AC) is a precious medicinal fungus that is endemic to Taiwan. It has been used anecdotally as a remedy for cancers, diarrhea, hypertension, and hepatoprotection. The objective of this study is to investigate the pharmacological function of fruiting body extracts of AC against human breast cancer cells. Breast cancer is the most common and life threatening cancer disease in women worldwide. We observed that the total ethanolic extracts of AC fruiting bodies could dose-dependently inhibit proliferation of T47D human breast cancer cells. We also compared cytotoxic activity of the extracts of AC fruiting bodies cultured for three (AC-3), six (AC-6), and nine (AC-9) months, respectively. Among the tested extracts, the magnitude on inhibiting T47D cell proliferation is AC-3 > AC-6 ≥ AC-9. Furthermore, the effect of AC-3 extracts on important marks involved in cell-cycle and apoptosis in cancer cells was investigated using Western blotting. We hypothesize that AC-3 is able to modulate cell-cycle machinery as well as inducing apoptosis in T47D cancer cells. The chemical fingerprints of the AC-3, AC-6, and AC-9 extracts were established and compared using HPLC. In our future study, we will elucidate the mechanistic insights of AC-3 extracts and its derived active compounds on suppressing breast cancer cell activity and migration.

目錄

摘要……iv

ABSTRACT……v

目錄……1

壹、前言……2

一、背景介紹與動機……2

二、研究目的……3

貳、研究流程……4

參、研究材料與方法……5

一、牛樟芝……5

二、人類乳癌細胞株……5

三、牛樟芝抽出物之置備……5

四、高效液相層析法(HPLC)……6

五、Time-lapse microscopy……7

六、Cytotoxicity assay……7

七、西方墨點法(western blot)……8

肆、研究結果……9

伍、討論……11

陸、結論……11

柒、未來展望與應用……12

捌、參考文獻……13

玖、附圖與附表……15

壹、 前言

一、 背景介紹與動機

1. 人類乳癌細胞

乳癌是全世界女性最常見的癌症之一，一年全世界約有 50 萬人死於乳癌。在歐美國家，乳癌一直是進年排名第一之女性癌症，平均每四名女性癌症患者中就有一個罹患的是乳癌，而在台灣位居女性癌症死亡率第四名。因此，找尋有效治療乳癌或預防期轉移或復發的方法與手段顯得特別重要。

乳癌細胞種類複雜，可依其是否具有某些重要受體，例如動情素受體(Estrogen Receptor, ER)、黃體素受體(Progesterone Receptor, PR)、人類表皮生長因子受體 II(Human Epidermal Growth Factor Receptor 2, HER2)等的有無，而加以分類。其中，細胞膜上有動情素受體的乳癌細胞(ER+)，例如人類 T47D 乳癌細胞可受雌激素影響其生長，而 MDA-MB-231 則為 triple negative[ER(-), PR(-), HER2(-)]之乳癌細胞。

2. 牛樟芝(*Antrodia cinnamomea*)

牛樟芝(*Antrodia cinnamomea*)，一種台灣特有的藥用真菌，長期被視為治療或預防肝癌、腹瀉、高血壓與保肝等疾病的保健食品或民俗藥。因其僅能寄生於牛樟木(*Cinnamomum kanehirai*)上生長，而牛樟樹又是國家保育級植物，因而十分稀有與珍貴，故在台灣與部分亞洲國家，牛樟芝的售價高可達每公斤一萬元美金(S.Y. Wang et. al, 2011)。

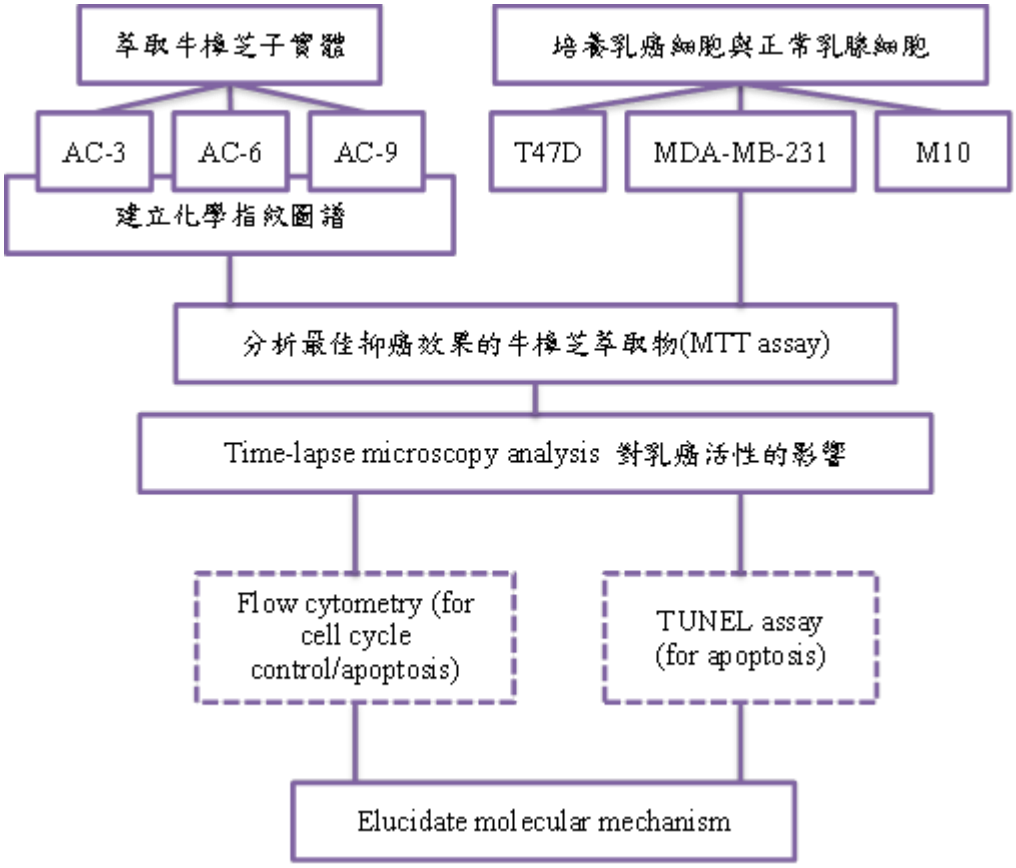
整個牛樟芝菌株分為菌絲體(Mycelia)與子實體(Fruiting body)兩主要部份。目前相關研究大多使用菌絲體作為材料，因為其價格便宜且較易培養，但亦被認為其藥效較差。

二、 研究目的

本研究的目的是探討並比較牛樟芝不同培養時期子實體的乙醇萃取物對人類乳癌細胞生長的抑制效果，並探討其的抑癌乳癌細胞活性的可能作用機制。

1. 比較牛樟芝不同培養時間的子實體乙醇萃取物對人類乳癌細胞造成的影響。
2. 探討牛樟芝子實體乙醇萃取物抑制人類乳癌細胞生長的作用機制。
3. 建立牛樟芝子實體萃取物之化學指紋圖譜及其中可能之活性成份。

貳、 研究流程


萃取牛樟芝子實體
培養乳癌細胞與正常乳腺細胞
AC-3
AC-6
AC-9
T47D
MDA-MB-231
M10
建立化學指紋圖譜
分析最佳抑癌效果的牛樟芝萃取物(MTT assay)
Time-lapse microscopy analysis 對乳癌活性的影響
Flow cytometry (for cell cycle control/apoptosis)
TUNEL assay (for apoptosis)
Elucidate molecular mechanism

參、 研究材料與方法

一、 牛樟芝(Antrodia cinnamomea)

牛樟芝子實體樣品為與國內實驗林研究站的研究人員經由合作方式所提供，培養方法如文獻所報導(S.-Y. Wang et. al, 2011)。牛樟芝子實體培養在牛樟木上分別於三個月(AC-3)、六個月(AC-6)、九個月(AC-9)後採收。

二、 人類乳癌細胞株

1. 人類乳癌細胞株 T47D(ER+)與 MDA-MB-231(ER-)由 ATCC 購得。

   T47D 培養於 RPMI-1640+10%FBS(Fetal Bovine Serum)

   MDA-MB-231 培養於 DMEM/F12+10%FBS。

2. 正常人類乳腺細胞株 M10 由 ATCC 購得。

   M10 培養於 MEM+10%FBS。

以上細胞株培養基均有分別加入 penicillin (100/mL)與 streptomycin (100μg/mL)。細胞均培養於 37^{o}C 及含 5%CO_2 之恆溫箱中。細胞株均定期進行 mycoplasma RT-PCR 實驗的檢測，以確保細胞株為遭受細菌感染。

三、 牛樟芝抽出物之置備

1. 牛樟芝子實體以液態氮(liquid nitrogen)急速冷凍後，快速搗碎。
2. 將粉末置於 10 倍體積的乙醇(95% ethanol)中萃取三小時。
3. 以 5500r.p.m.離心 3 分鐘，上清液取出後備用。

4. 牛樟芝粉末再回懸浮於乙醇溶液中，反覆萃取三次。

5. 收集三次的上清液以 0.22μm 的濾紙過濾並進行減壓濃縮先去除以醇，再以冷凍乾燥機乾燥過夜。

四、 高效液相層析法(HPLC)

1. 將牛樟芝的乙醇萃取物曲適量溶於甲醇(10mg/mL)。

2. HPLC 條件主要參考(S.-Y. Wang et. al, 2011)：

   (1) Column: A Luna C18(2) column (250x10.0 mm; Phenomenex, Torrance CA).

   (2) Solvent systems: H_2O (A), methanol (B) and acetonitrile (C).

   (3) Solvent gradient profile:

Time	**0-5 min**	**5-95 min**	**95-105 min**	**105-115 min**
A: B: C	40:30:30 (isocratic)	40:30:30 to 10:10:80 (linear gradient)	10:10:80 to 0:0:100 (linear gradient)	0:0:100 (isocratic)
Flow rate	0.5mL/min	0.5mL/min	1.0mL/min	1.0mL/min

五、 Time-lapse microscopy

1. 將 60mm 培養盤塗上一層 fibronectin(25μg/mL)，並靜候一小時。

2. 將細胞種在培養盤上，24 小時後再處理藥品(AC-3, 50μg/mL)。

3. 在顯微鏡(Zeiss Axiovert 200M microscope equipped with an environmental chamber with phase-contrast optics)下每半小時照一張圖，連續 24 小時。

4. 數據分析

六、 Cytotoxicity assay

1. 對乳癌細胞之毒性測試主要是參考(Huang et. al, 2010)
2. 將要測量的乳癌細胞株種到 96 孔盤($1x10^5$cell/well)，培養隔夜至細胞貼伏孔盤底部。
3. 以正常乳腺細胞株 M10 為對照組，測試 AC 萃取液是否對正常細胞具毒性。
4. 根據實驗設計於細胞培養基中加入不同濃度及種類的牛樟芝乙醇萃取物(AC-3, AC-6, AC-9)。
5. 以常用之化療藥(Tamoxifen, Paclitaxel)為對照組。
6. 藥物處理 24 小時與 48 小時。
7. 將培養液去除後，加入 100μL/well 的 3-(4,5-dimethylthiazol-2-yl)-2,5-diphemyl tetrazolium bromide (MTT)溶液(5mg/mL)。
8. 待反應四小時候，去除含 MTT salt 的培養液，並每孔加入 100μL DMSO，震蕩 1-2 分鐘後測 ELISA reader OD_{570} 之吸光值。
9. 殘存活細胞(viable cell)的計算公式：

   Viable cells(%)：[OD570(treated cell culture)/OD570(control, untreated cell cultured)x100]

七、西方墨點法(Western blot analysis)

1. 收集分別以 AC-3(50μg/mL)及 Vehicle(0.5% DMSO)處理 24 小時與 48 小時的細胞株，並萃取蛋白質(total cellular proteins) (Chiang et. al,

2005).

2. 蛋白質利用 Bradford dye-bindery(Bio-rad)方法定量後，即可進行 12% SDS-PAGE analysis(30μg/well)。

3. 將在 SDS gel 上之蛋白質轉印(transfer)到 PVDF membrane (Millipore)上。

4. 將 blot 以含 3%的脫脂奶粉的 Blocking buffer (10mM Tris, pH 7.5, and 100mM NaCl, TBS)中 30min 後，以 TBST 清洗，並重複三次於振盪器上洗。

5. 以 1:2000 稀釋比例的一次抗體於室溫震盪反應過夜。

6. 以 TBST 清洗後，以二抗抗體反應 2 小時。

7. 最後以冷光液(Amersham)呈色反應後，以 X-ray film 顯影呈色。

肆、 研究結果

一、 不同培養時期之牛樟芝子實體乙醇萃取物之效率及指紋圖譜分析成分

利用乙醇萃取之 AC-3、AC-6 以及 AC-9 之總粗抽出物效率分別為 8.27%、9.27%及 6.58%。

我們參考相關文獻的 HPLC 條件進行實驗，分析三個不同培養時期的牛樟芝子實體 AC-3, AC-6, AC-9 的化學圖譜成分差異如 **Fig. 3** 所示，培養時間愈久，牛樟芝子實體所含的成分(peaks)愈多；圖譜經與文獻比較，peak 之分布相類似。其中在 AC-3 之兩個主要的化合物為去氫齒孔酸(dehydroeburicoic acid)和去氫硫色多孔菌酸(dehydrosulfurenic acid)。此次成分分析結果有助於我們日後活性研究的探討。

二、 牛樟芝子實體乙醇萃取物對人類乳癌細胞的移動性影響

我們將AC-3處理過後的T47D放置在顯微鏡下，每半小時拍攝一張，連續拍攝 24 小時，與沒有處理 AC-3(DMSO as control)的樣品比較，**Fig. 4** 顯示，在處理 AC-3 的樣品中，每顆 T47D 細胞的平均移動距離較沒有處理 AC-3 的來的短($p<0.05$)，而在象限圖中，也以有處理 T47D 的樣品移動距離較短。另外，就細胞的形態看來，有處理 AC-3 的細胞在分裂的能力上相對弱於對照組，故由以上結果可知，AC-3 對 T47D 在增生(proliferation)與移動(migration)上皆有一定的抑制作用。

三、 牛樟芝子實體乙醇萃取物對人類乳癌細胞的生長抑制效果

利用 MTT assay，我們檢測 AC-3、AC-6、AC-9 對人類乳癌細胞的生長抑制率，結果如 **Fig. 5**。**A1**、**A2** 分別顯示了三個細胞株在加入AC-3 24、48 小時後，不同濃度下的細胞存活率，在 50μg/mL 劑量下可抑制 46%、37%T47D 之增生；**B1**、 **B2** 為 AC-6 對三個細胞株的生長抑制率，**C1**、

C3 則為 AC-9。我們可以得知，比照人類正常乳腺細胞 M10，牛樟芝子實體對 T47D 細胞有較好的抑制增生作用。AC-3、AC-6、AC-9 中，又以 AC-3 的抗癌效果最佳。

此外，從 **Fig. 5 A1、A2** 中，可觀察到濃度在 50 μg/mL 後的細胞存活率已無太大差異，故接下來實驗我們都選用 50 μg/mL 的 AC-3，濃度，進一步探討其對 T47D 細胞的抑制作用機制等。

四、 牛樟芝子實體 AC-3 乙醇萃取物造成人類乳癌細胞與細胞週期及凋亡有關蛋白質的含量改變

我們利用西方墨點法檢測 AC-3 在濃度 50 μg/mL 下，處理 24 和 48 小時對 T47D 細胞的數個細胞週期(cell cycle)和細胞凋亡(cell apoptosis)相關蛋白質的表現變化。結果如 **Fig. 6** 所示。數個週期素(cyclin)和週期素激酶(cyclin-dependent kinase, Cdk)出現明顯變化。其中 Cdk4、Cyclin D3、Cyclin E，在加入 AC-3 後 48 小時，表現量大幅下降，顯示 AC-3 可影響人類乳癌細胞中的細胞週期調控。

此外，我們可以觀察到 PARP (poly (ADP-ribose) polymerase)與 cleaved-PARP 的表現量亦出現明顯變化。PARP 在 DNA 修復上占有重要地位，若發生細胞凋亡(apoptosis)，caspase (cysteine-aspartic protease)將被活化水解切割 PARP 蛋白質，是故觀察 PARP cleavage form 的表現量有助於探討是否發生細胞凋亡(apoptosis)。我們發現 cleaved-PARP 在 T47D 加入 AC-3 的 48 小時後明顯增加，顯示 AC-3 應該可誘發 T47D 細胞凋亡。

這些初步的觀察結果將會進一步利用流式細胞儀(flow cytometry)與 TUNEL 分析來進一步確認，此外，其他重要參與乳癌細胞活性或凋亡之重要的訊息傳遞因子也將利用西方墨點法進一步分析是否受到 AC-3 調控。

伍、 討論

根據實驗結果顯示，AC-3 對 T47D 比對 MDA-MB-231 乳癌細胞有較好的生長抑制效果。根據 HPLC 的圖譜顯示(**Fig. 3**)，在 AC-3 的圖譜中可知去氫齒孔酸(dehydroeburicoic acid)和去氫硫色多孔菌酸(dehydrosulfurenic acid)為兩個主要化合物相對含量最多(**Fig. 3**)。去氫齒孔酸在目前以發表的文獻研究中指出可誘導神經膠原瘤細胞凋亡，而去氫硫色多孔菌酸則可對白血病和胰臟癌產生細胞毒性，但皆並未有針對乳癌細胞生長抑制的相關資料，並且在牛樟芝乙醇萃取物中也還未被證實會對癌細胞產生毒殺作用，而是否經由去氫齒孔酸和去氫硫色孔菌酸來抑制人類乳癌細胞的活性，也尚未被證實，這些值得進一步探討。

此外，我們在進行西方墨點法的實驗時，發現若干個細胞週期相關的蛋白質表現出現明顯變化，例如 Cdk4、Cyclin D3、Cyclin E，這些蛋白質在細胞週期中主要參與在 phase G1 及 S，是故推測 AC-3 可能造成對 T47D 細胞造成 G1/S arrest，此待進一步研究確定。

陸、 結論

本研究目前成果顯示人工栽培的牛樟芝子實體並不需要長時間培養，亦即三個月的培養即可達最顯著之抑制乳癌細胞活性的效果，此項研究發現將可降低未來開發人工牛樟芝子實體為抗癌產品之生產成本與重要參考資訊。

柒、 未來展望與應用

根據我們的實驗結果，我們認為 AC-3 對 T47D 可能會產生 Cell cycle arrest，因此，我們已經著手進行，AC-3 對 T47D 在 6, 12, 24, 36, 48 小時的影響，流式細胞儀可以用來觀察隨著時間的增長，cell cycle 的改變，再找出合理的解釋後，也將使用 Western blot 的技術，驗證受到改變的蛋白質。

另外，細胞凋亡(apoptosis)也有可能造成癌細胞生長遭到抑制的原因之一，為了驗證 T47D 是否會因為 AC-3 造成細胞凋亡，首先，我們將利用 TUNEL(Terminal transferase dUTP Nick End Labeling)技術，初步檢查是否會因為 DNA 的斷裂，在螢光顯微鏡下看到 dUTP 與 Nick 結合後的螢光亮點，若是，則我們可以再利用 Western blot 找出真正造成細胞凋亡的機制。

而現今對於癌症的研究中，細胞的移動(motility)與細胞骨架(skeleton)也是很熱門的議題，以細胞的移動性而言，would-healing assay 可以觀察 AC-3 對 T47D 的移動性影響，此外，time-lapse microscopy 的分析也可以告訴我們 AC-3 影響的程度如何。而細胞骨架的研究方法，則可使用倒立共軛焦顯微鏡(confocal)觀察染色後的細胞骨架是否有形態上的改變。

總體來說，在確立以及瞭解 AC-3 對 T47D 的生長抑制機制後，可以藉由高液相層析儀把化合物分離，找出對 T47D 影響的主要化合物，希望可以提供下一個乳癌化療藥開發的可能性。

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玖、 附圖與附表

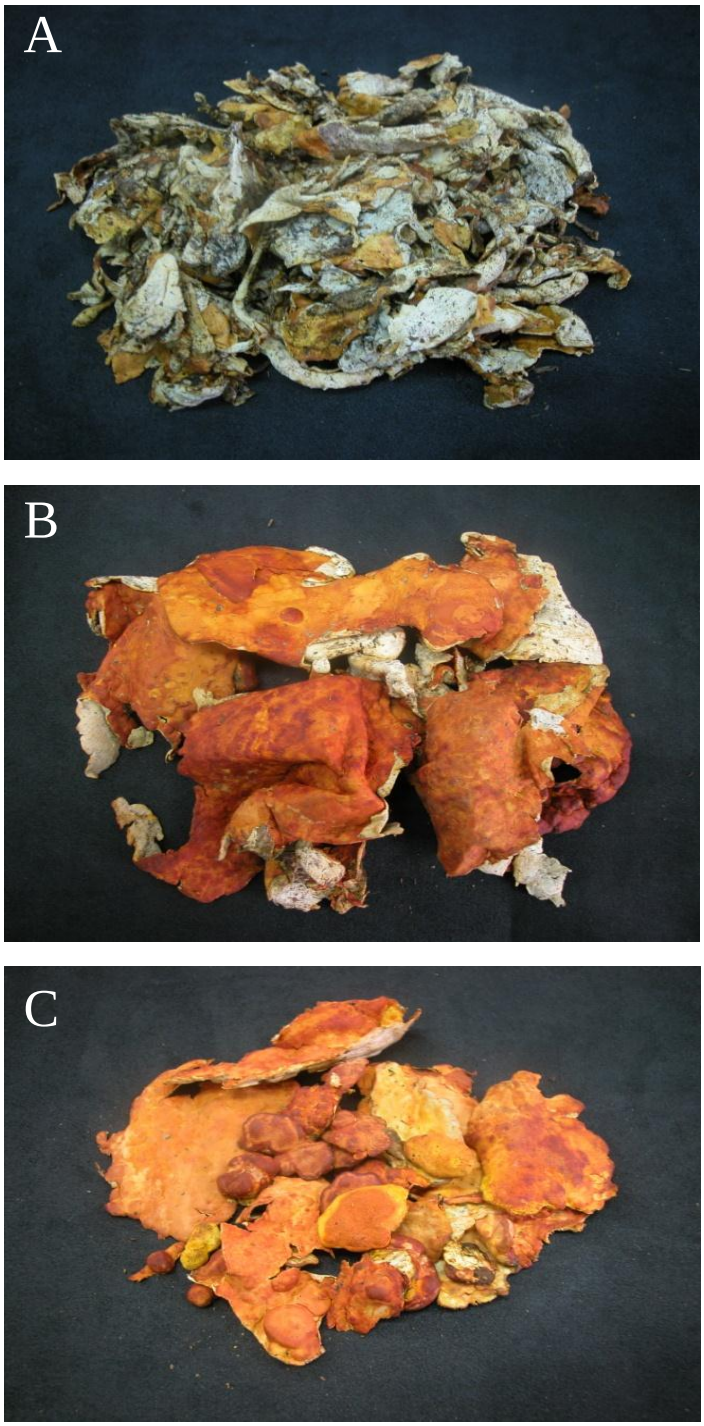


Figure 1. Morphological observation of *A. cinnamomea*, which were grown on *Cinnamomum kanehirai* for 3(A), 6(B), and 9(C) months

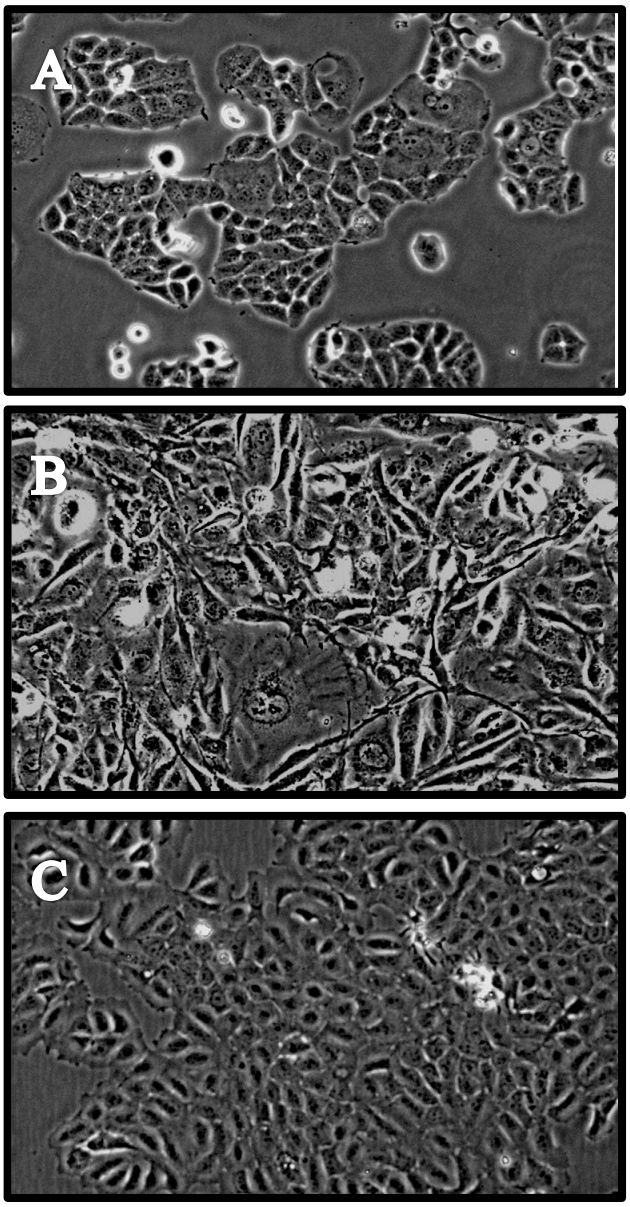


Figure 2. Morphological observation of T47D (A), MDA-MB-231 (B), and M10 (C). 10x under microscopy

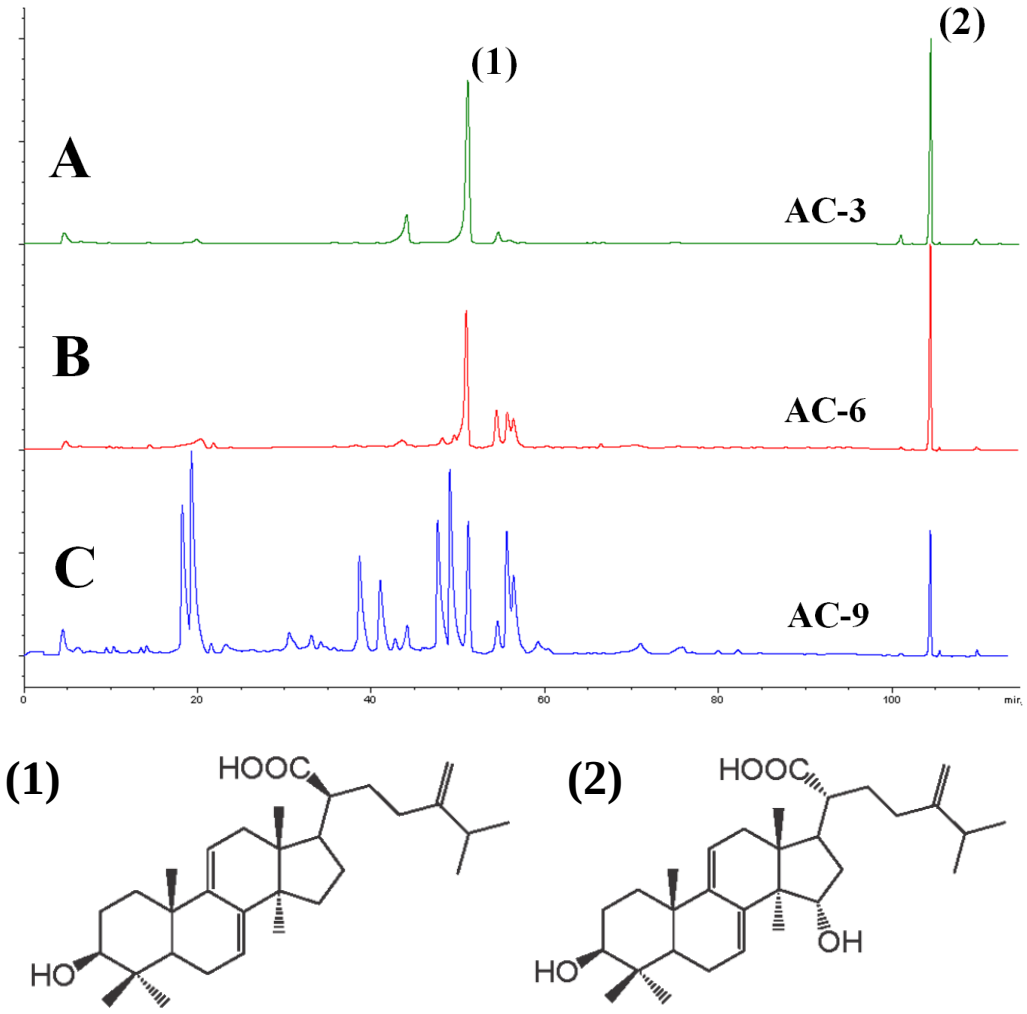


Figure 3. HPLC profiling of index compounds of ethanolic extracts of *A. cinnamomea* fruiting body (AC-3, AC-6, AC-9). Two major compounds in AC-3, dehydroeburicoic acid(1) and dehydrosulfurenic acid(2), are observed.(chemical structure shown)

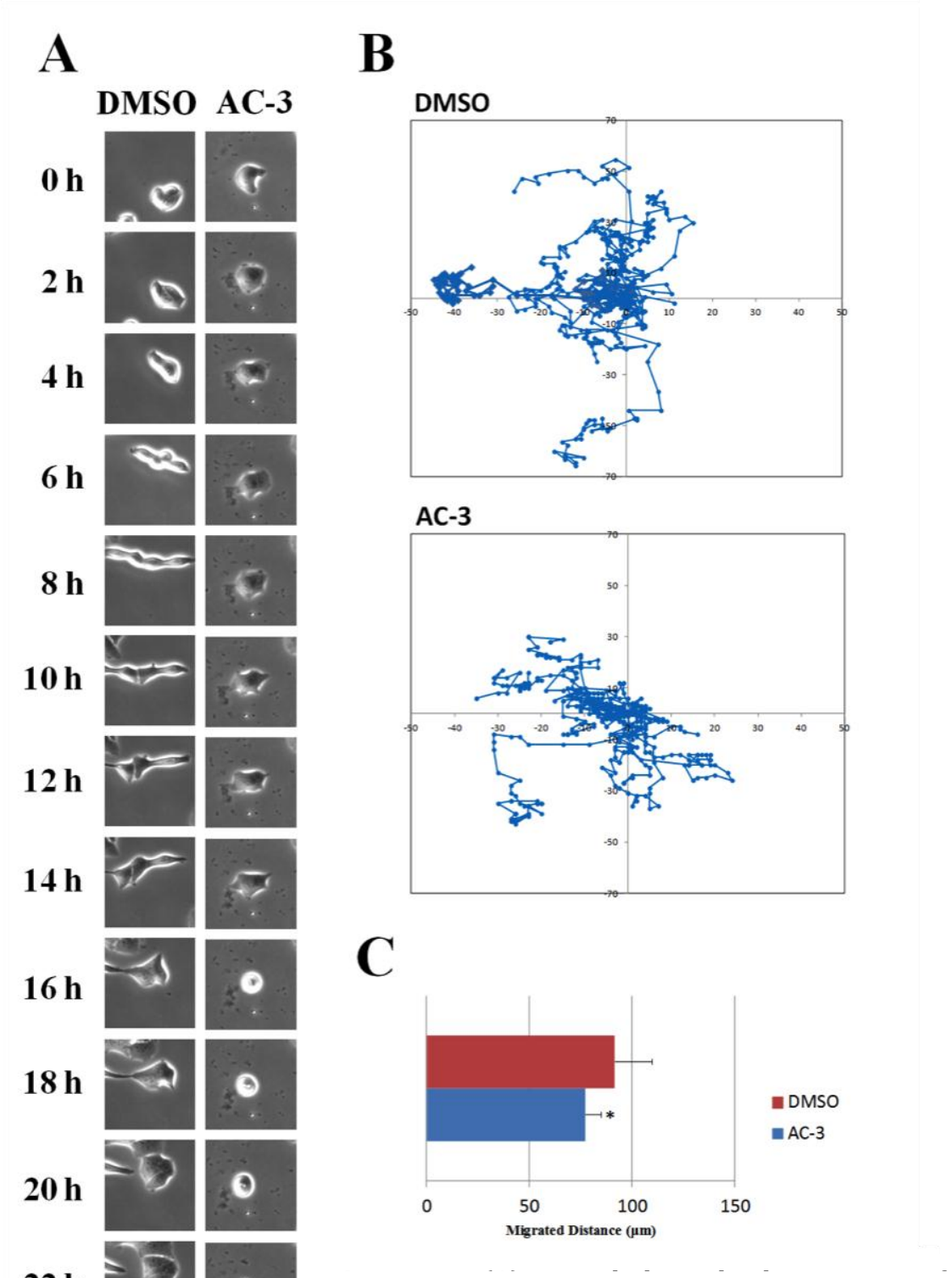
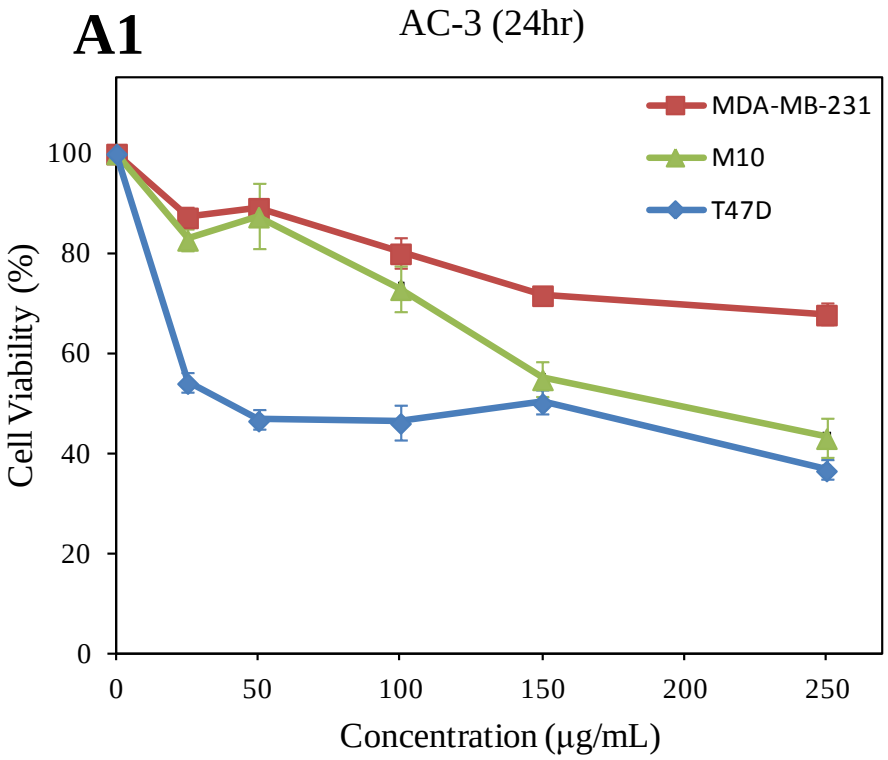
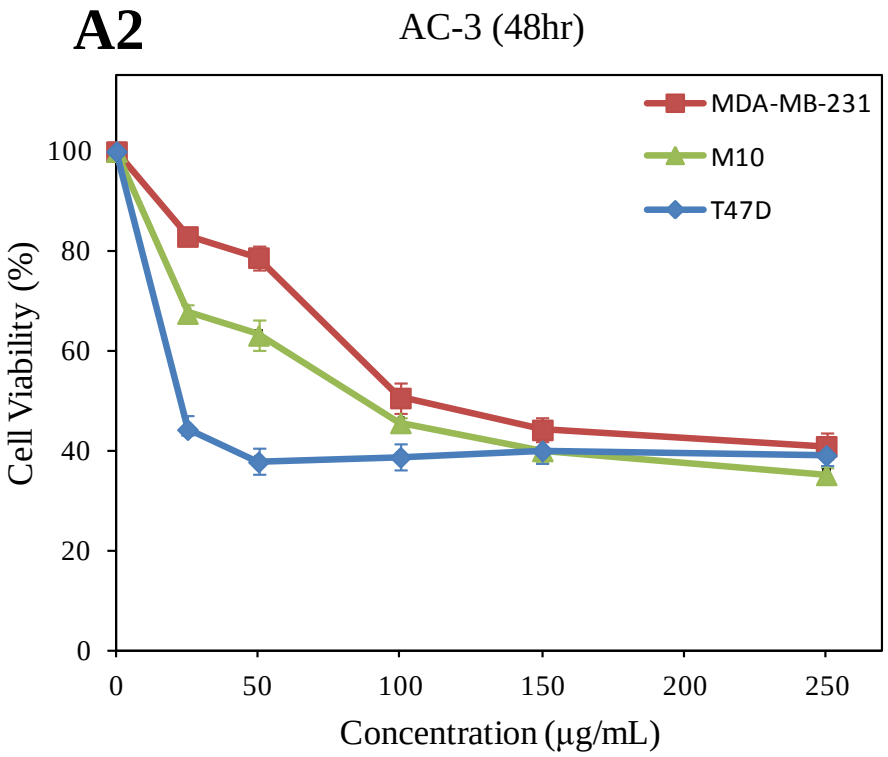


Figure 4. (A) Morphological observation of T47D treated with DMSO or AC-3 under time-lapse microscopy every 2 h. (B) The movement of each T47D cell treated with DMSO or AC-3 (C) The average migrated distance of of T47D treated with DMSO or T47D

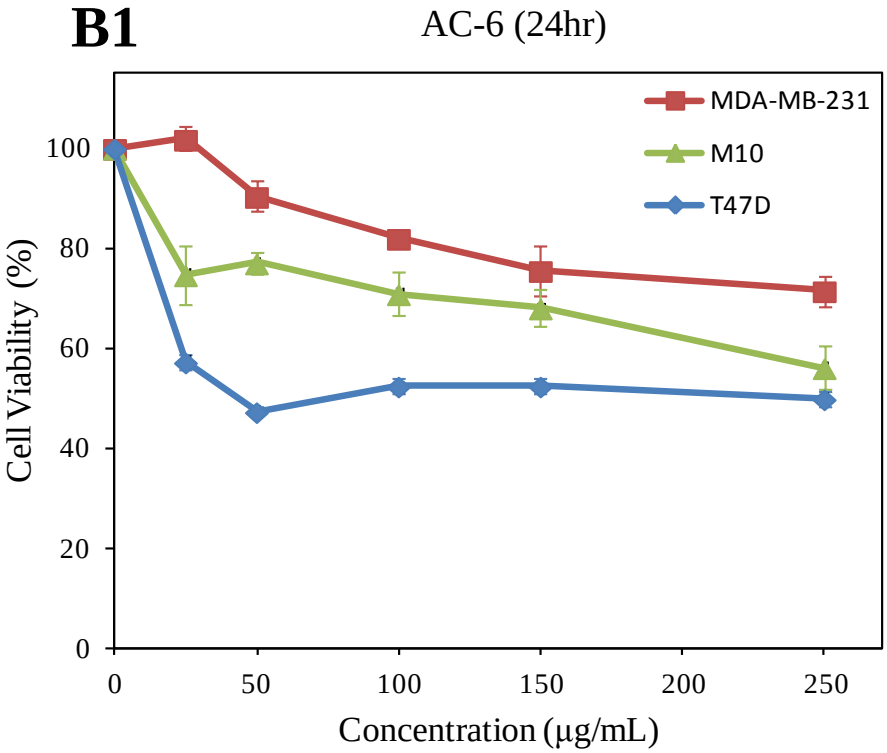
A1


AC-3 (24hr)
MDA-MB-231
M10
T47D
Cell Viability (%)
0
20
40
60
80
100
0
50
100
150
200
250
Concentration (µg/mL)

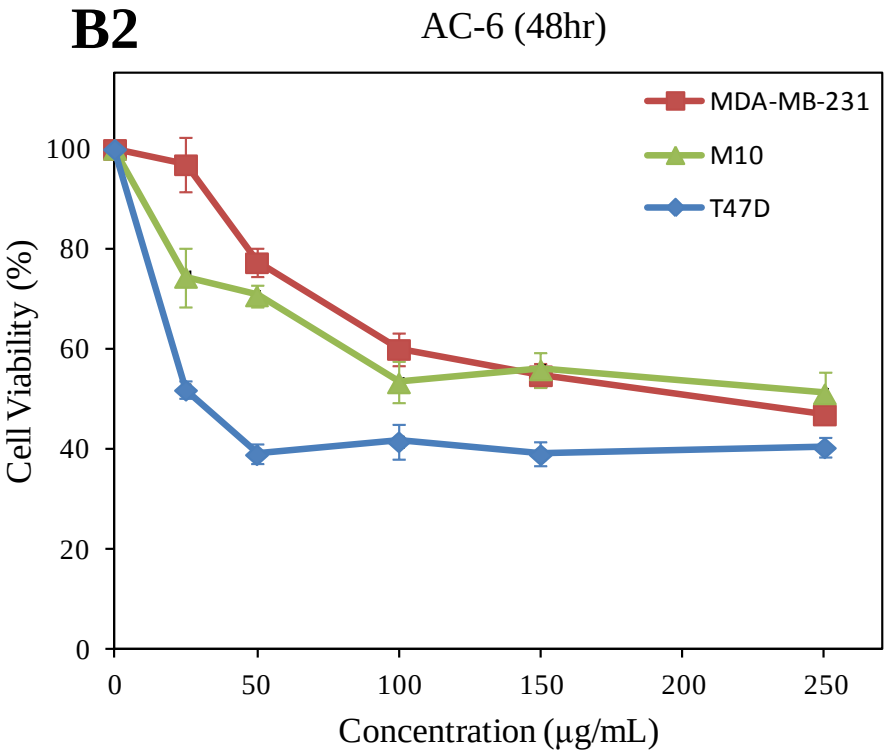
A2


AC-3 (48hr)
MDA-MB-231
M10
T47D
Cell Viability (%)
0
20
40
60
80
100
0
50
100
150
200
250
Concentration (µg/mL)

B1


AC-6 (24hr)
MDA-MB-231
M10
T47D
Cell Viability (%)
100
80
60
40
20
0
0
50
100
150
200
250
Concentration (µg/mL)

B2


AC-6 (48hr)
MDA-MB-231
M10
T47D
Cell Viability (%)
100
80
60
40
20
0
0
50
100
150
200
250
Concentration (µg/mL)

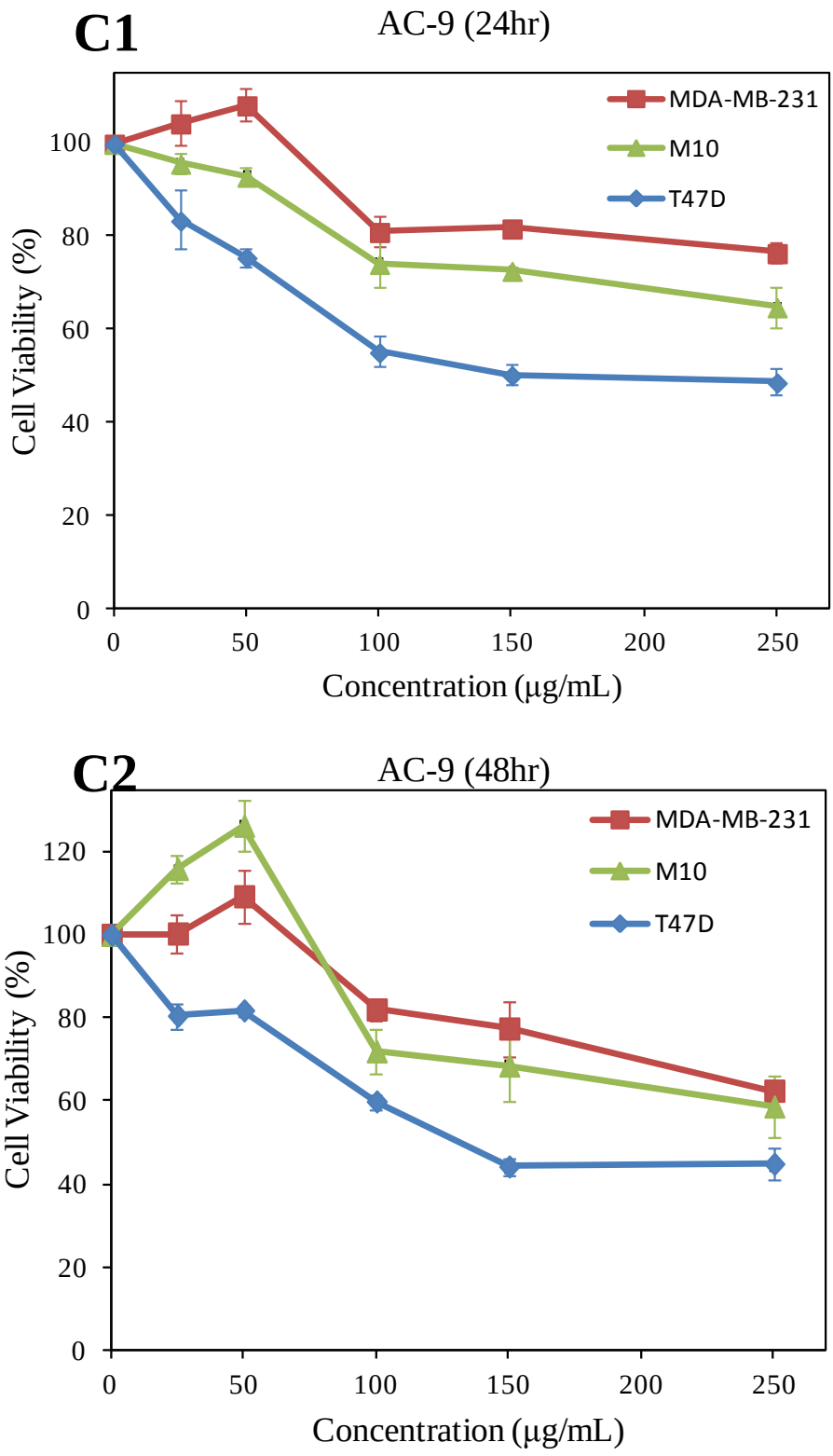


Figure 5. Effects of ethanol extracts of *A. cinnamomea* fruiting bodies cultured for 3 (A), 6 (B), and 9 (C) months n cell proliferation of T47D cells were examined by MTT assay. The extracts were dissolved in 0.5% DMSO. Cells were treated with 0, 25, 50, 100, 150, and 250 µg/mL of *A. cinnamomea* for 24 (1) and 48 (2) h.

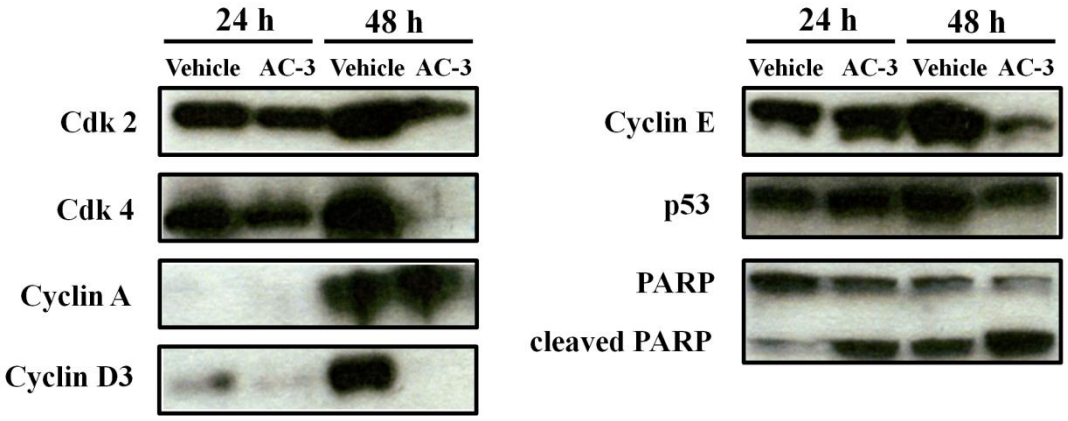


Figure 6. Western blotting of key biomarkers involved in cell arrest or cell apoptosis in T47D cells treated with vehicle or AC-3. (50 μg/mL at times indicated).

Novel Bioactivities and Mechanistic Insights of the Medicinal Fungus *Antrodia cinnamomea* against Human Breast Cancer Cells

Abstract

Antrodia cinnamomea (AC) is a precious medicinal fungus endemic to Taiwan, which has been used anecdotally as a folk remedy for cancer diseases, diarrhea, hypertension, and hepatoprotection in Asian countries. Breast cancer is one of the top life threatening diseases in women worldwide. The objective of this study was to provide science-based evidences for the bioefficacy of fruiting body extracts of AC against human estrogen receptor positive (ER+) T47D breast cancer cell activities. The ethanolilc extracts from 3-month-cultured AC fruiting bodies, designated AC-3E, observed with most significant activity against T47D cells among the tested extracts from different growth stages were employed for the pharmacological activities and molecular mechanisms investigations *in vitro* and in xenograft BALB/c nude mouse system. AC-3E were observed to attenuate *in vitro* and *in vivo* proliferation, migration, and tumor growth of T47D breast cancer cells by deregulating signaling pathway PI3K/Akt/mTOR and cell-cycle mediators, and induction of apoptosis. AC-3E were also functioned as an anti-angiogenic agent against blood vessel branching and microvessel formation *ex vivo* and *in vivo*. Chemical fingerprinting and cytotoxicity assay results indicate that the lanostane-type triterpenoid dehydroeburicoic acid (**2**) is the major bioactive compound in AC-3 extracts, which can serve as the index compound for quality control of the traditional folk medicine. This is the first report to demonstrate that AC extracts exert similarly potent activity to that of current estrogen antagonism drug tamoxifen against ER dependent T47D breast cancer cells.

Introduction

Breast cancer is one of the top life-threatening diseases in women worldwide. Current cancer therapies, including endocrine therapy, chemotherapy, and targeted therapy, play an important role in the treatment of breast cancer; however, drug resistance, severe side effects, and high recurrent rate or metastatic tumors are remained palliative to breast cancer patients. Systemic tamoxifen (endocrine) treatment, for instance, could cause tamoxifen-stimulated tumor growth or drug resistance (Schafer et al., 2000). The press need for development of new therapeutic or preventive agent for breast cancer has spurred the search for phytomedicines or phytoagents with novel molecular mechanisms.

Antrodia cinnamomea (AC) (known as Niu-Chang-Chih in Chinese medicine) is a precious medicinal fungus endemic to Taiwan and has been popularly used as a folk medicine in various disorders. Recently, therapeutic effect of AC fruiting body power against chronic alcohol consumption induced liver damage in rats was reported (Huang et al., 2010) and other hepatoprotective activities of fruiting body extracts or of mycelium from solid or submerged cultivation were reviewed (Ao et al., 2009; Geethangili and Tzeng, 2011). The anti-inflammatory activities of mycelium or fruiting body extracts of AC through inhibition of nitric oxide and proinflammtory cytokines (e.g., TNF-α, IL-1β) production, and inducible nitric oxide synthase and COX-2 expression in lipopolysaccharide-stimulated macrophages or in microglia cell line (Geethangili and Tzeng, 2011).

Several reports on anti-cancer activities of fruiting body extracts of *A. cinnamomea* have shown that total AC extracts prepared by organic solvents such as methanol, chloroform, ethanol or ethylacetate can exhibit HepG2, PLC/PRF/5 liver cancer cells (Hsu et al., 2007), colon, Jurkat or prostate cancer cell lines (Rao et al., 2007), or leukemia HL60 cells (Hseu et al., 2004), through regulation of Bcl-2 family proteins, activation of caspases or NF-κB protein. In the previous reports regarding *A. cinnamomea* against human breast cancer cell activities, most of the studies focused on studying fermented culture broth of AC against triple negative MDA-MB-231 cell activity (Yang et al., 2012). The mode of action can be through inhibiting the expression of COX-2 and inducing cell cycle arrest or apoptosis in MDA-MB-231

cells *in vitro* or in xenograft mouse model (Hseu et al., 2007; Hseu et al., 2008), or through inhibiting MAPK signaling pathway (Yang et al., 2011). So far, only the fermented culture broth of AC was reported to induce apoptosis in estrogen receptor positive (ER+) breast cancer MCF-7 cells (Yang, H. L. et al., 2006), little or none of the reports concern the bioactivity and mode of action of the fruiting body extracts of AC against ER+ breast cancer cells either MCF-7 or T47D cells.

Because approximately 70% of breast cancer patients are diagnosed as ER+ cancer, and the current ER antagonistic drug (tamoxifen) shows drug resistance or side effects (Ring and Dowsett, 2004), this study thus aimed to investigate the pharmacological activity and the underlying modes of action of the fruiting body extract of *A. cinnamomea* against human ER+ breast cancer cells T47D. As a criterion of quality control of the precious folk medicine, the chemical fingerprint and active constituent(s) in the *A. cinnamomea* extract were characterized.

Materials and Methods

Cell lines and culture conditions

T47D (American Type Culture Collection, ATCC, Manassas, VA, USA), a human mammary ductal carcinoma cell line, was grown in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Life Technologies, Grand Island, NY, USA); H184B5F5/M10 (ATCC), a human mammary epithelial cell line, was cultured in minimum essential medium (MEM; Life Technologies). All cell lines were cultured in specific media supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin in a humidified 5% CO2 incubator at 37°C.

Antordia cinnamomea fruiting bodies extract preparation

A. cinnamomea fungus was grown on aromatic tree *Cinnamomun kanehirai* Hayata at the Experimental Forest Station, National Taiwan University and authentificated by Dr. Wen-Wei Hsiao. The fruiting bodies of *A. cinnamomea* were harvested at the growing stages at 3-month, 6-month, or 9-month, and designated AC-3, AC-6, and AC-9, respectively. The fruiting body samples were lyophilize-dried and powdered by pestle in liquid nitrogen. Fruiting bodies powder (10 g) of *A. cinnamomea* was extracted with ten times volume of 95% ethanol at room temperature for 3 h and repeated twice of extraction. The extracts were collected by centrifugation and the total ethanolic extracts (2.3 g, 1.9 g, and 1.5 g) designated AC-3E, AC-6E, and AC-9E were concentrated in a rotary evaporator and lyophilized.

Chemicals and reagents

Dimethyl sulfoxide (DMSO), tamoxifen citrate (Tam), 3-(4,5-cimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI) and fibronectin were purchased from Sigma–Aldrich. Carboxymethylcellulose (CMC), Tween-20, Primary antibodies against phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), p-Akt, janus kinase 1 (Jak1), signal transducer and activator of transcription 3 (Stat3), p38 MAPK, E-cadherin, poly ADP ribose polymerase (PARP) (Cell Signaling Technology), actin, estrogen receptor alpha (ERα), mammalian target of rapamycin (mTOR) (Chemicon, Millipore), and p-p21 (Cayman Chemical) were used. All other antibodies in this study

were from Santa Cruz Biotechnology.

Experimental animals

Female BALB/c nude mice (4-week-old) obtained from National Laboratory Animal Center, Taipei, Taiwan were given a standard laboratory diet and distilled water *ad libitum* and kept on a 12 h light/dark cycle at 22 ± 2 °C under specific pathogen-free conditions. All animal work will be done in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC), Academia Sinica, Taiwan.

Cell proliferation assay

In cell proliferation assay, T47D and M10 cells were seeded and grown in 96-well plates at 1 × 104 cells/well overnight. Cells were treated for 48 h with vehicle (0.5% DMSO or 0.5% ethanol), AC-3E, AC-6E, AC-9E or Tam. Cell viability was measured by MTT-based colorimetric assays according to *Huang et al.* (2010).

Time-lapse microscopy analysis

T47D cells were seeded and allowed to adhere overnight in RPMI-1640 medium containing 10% FBS on a 60-mm-diameter culture plate coated with 10 μg/mL fibronectin. AC-3E (50 μg/mL), Tam (30 μM) and vehicle were added at the beginning of time-lapse. The time-lapse experiments were conducted on an inverted Zeiss Axiovert 200 M microscope equipped with an environmental chamber with phase-contrast optics (images taken every 30 min). An average of 15 cells were randomly selected to calculate the cells migrated distances and trajectories within 24 h, using the object-tracking application of Metamorph software (Molecular Devices).

Confocal microscopy

For F-actin staining, T47D cells were fixed with 4% paraformaldehyde after treated with AC-3E or Tam. Cells were then permeabilized with PBS containing 0.2% Triton X-100, and rinsed with PBS. Fixed cells were incubated in buffer with rhodamine-phalloidin for 30 min. The nuclei were stained with DAPI. The cellular staining on actin in T47D cells was viewed and capture on a Zeiss LSM 510 META laser scanning confocal microscope.

Wound-healing assay

T47D cells were seeded in a two-side chamber at 1 × 106 cells/mL and allowed to adhere overnight. Chamber was removed and then treated with indicated concentrations of AC-3E. Photos were taken under microscopy every 24 h to observe the cell migration.

Transwell migration assay

T47D cells (1 × 105) were seed into a transwell chamber (Millipore) containing serum-free medium and indicated concentrations of AC-3E or Tam for migration assay. The chambers were put into a 24-well plate in which each well covers with 500 μL of medium containing 10% FBS, and incubated for 96 h. Cells on the upper surface of the chamber were scraped by a cotton swab, and the remaining cells were fixed and stained with DAPI (1 μg/mL). Migrated cells in the lower surface of the chamber were counted at 100× magnification by inverted fluorescence microscopy.

TUNEL assay

T47D cells (3 × 104) were seeded and allowed to adhere overnight on coverslip coated with 0.1% (w/v) poly-L-lysine (Sigma). Cells were treated with vehicle or AC-3E and Tam for 48 h. Cells were fixed using 4% formaldehyde/PBS at 4°C for 25 min. After TdT enzyme reaction and PI staining (10 μg/mL) following the DNA Fragmentation assay kit (ApoAlert®, Clontech), cover slips were sealed overnight in the dark. Apoptotic cells were visualized with green fluorescence using a standard fluorescence filter set (520 ± 20 nm). PI-stained cells exhibited red fluorescence when viewed at > 620 nm.

Western blot analysis

T47D cells (1×106) were grown in 10 cm dish overnight and starved in serum-free medium for 24 h. After treated with AC-3E at different time points, proteins were harvested and resolved by 5-15% gradient SDS-PAGE, and electrophoretically transferred to PVDF membranes, which were then blocked in 3% w/v skimmed milk with specific primary antibodies overnight (4°C) and then incubated with secondary antibodies for 3 h at room temperature. Enhanced chemiluminescence detection

reagents (ECL; Millipore) were used to visualize positive reactive protein band by exposure to chemiluminescence light film.

Matrigel™ encapsulated chorioallantoic membrane (CAM) assay

Chick embryo CAM assays were performed by modification of previously published method (Kim et al., 1998). Fertilized eggs were placed into an incubator as soon as embryogenesis started and kept under constant humidity at 37°C. On the 7th day, a window was made under aseptic conditions on the eggshell. An aliquots (50 μL) of Matrigel ™ (BD Biosciences, Bedford, MA) containing VEGF (50 ng/mL) supplemented with AC-3E or Tam were mixed well on ice, pre-gelated at 37°C, and then placed onto the CAM. The opening was closed with a cellophane tape and incubation went on until the day of the experiment. Capillary tube formation in the CAM was readily analyzed by the images taken at the 3rd day after grafting.

Inhibition of T47D tumor growth in BALB/c nude mice

Mice were divided into groups of seven and were implanted with 17β-estradiol pellets (0.72 mg, release over 60 days; American Innovative Research) one day before (day -1) the mammary fat pad region of mice was injected with T47D cells (5 × 106 cells/200 μL PBS) (day 0). At day 7, the mice carried tumor at size of 48 - 55 mm3 were randomized and assigned into five groups. AC-3E and Tam were dissolved in the vehicle (10% ethonal, 81% CMC and 9% tween-20). AC-3E (50 mg/kg body weight, AC-3E−50; or 100 mg/kg body weight, AC-3−100) and Tam (30 mg/kg body weight, Tam−30) were given orally (*p.o.*) every other day starting on day 7. Tam−30&AC-3E−50 alternate group were *p.o.* with Tam−30 on day 7 and AC-3E−50 on day 9 and alternate for the following treatment days. The PreAC-3E−50 group of mice was *p.o.* pretreated with AC-3E−50 for seven doses before tumor cell implanted on day 7. The sham control and tumor control mice were *p.o.* with vehicle every other day. The tumor volume (V) was measured by two diameters with calibers every other days for five weeks and calculated by the formula $V = \{(D + d)/2\}3$, where D and d were the larger and smaller diameters, respectively (Chung et al., 2006). At the end of the study, mice were killed by cervical dislocation. Tumors were removed, fixed with 10% buffered formalin, embedded in paraffin, and then examined visually and microscopically for growth and of tumor cells. Tumor specimen were collected and

subjected to pathological and immunohistochemical examinations.

Identification of index compounds

The total ethanolic AC-3 extract was separated by HPLC using the Agilent 1100 HPLC system equipped with a UV detector. A Luna RP C18 column (250 × 4.6 mm; Phenomenex, Torrance CA) was employed with three solvent systems, H2O (A), MeOH (B) and acetonitrile (C). The gradient elution profile was as follows: 0-5 min, A:B:C = 40:30:30 (isocratic); 5-95 min, A:B:C = 40:30:30 to A:B:C = 10:10:80 (linear gradient); 95-105 min, A:B:C = 10:10:80 to A:B:C = 0:0:100 (linear gradient); 105-115 min, 100% C (isocratic); the flow rate was 0.5 mL/min at 0-95 min; 1.0 mL/min at 95 to 115 min; and the detector wavelength was set at 254 nm. The structures of two major compounds, namely dehydrosulfurenic acid (**1**) and dehydroeburicoic acid (**2**), were elucidated using various spectral analyses and also confirmed with previously published article (Lin et al., 2011).

Standard calibration curves (peak area *vs.* concentration) of the two candidate index compounds, dehydrosulfurenic acid and dehydroeburicoic acid, were obtained at a range of compound concentrations, 25, 50, 100, 250, 500 and 1000 μg/mL. The peak area of the two compounds in the chromatogram of AC-3 extract (1 mg/mL) was defined by HPLC analysis, and their contents in the extract were calculated based on the quantity calibrated from the respective standard calibration curves.

Statistic analysis

Data are all expressed as mean ± standard deviation. ANOVA was used to determine the significant differences between different conditions. $p < 0.05$ is considered statistically significant.

Results

AC-3 extracts (AC-3E) inhibit T47D cell proliferation

The anti-cancer cell proliferation activity on T47D (ER+) cells of the fruiting bodies extracts of *A. cinnamomea* grown at stage 3-month (AC-3E), 6-month (AC-6E), and or 9-month (AC-9E) were compared using MTT assay. Tamoxifen (Tam), an estrogen antagonistic drug, was used as a reference control in this study. The result in Fig. 1 shows that, at 48 h treatment, the magnitude of cell cytotoxicity against T47D cells is AC-3E > AC-6E > AC-9E, with the IC50 of 52.7 μg/mL, 122.6 μg/mL, 141.9 μg/mL, respectively. Furthermore, AC-3E exhibited less toxicity on normal human mammary epithelial cell M10 than that of AC-6E and AC-9E at its IC50 value for T47D. The IC50 of of Tam treatment on T47D cells was 30 μM, however, a higher toxicity of Tam on normal mammary cell M10 were detected at 24 μM. This study was then focused on addressing the anti-T47D breast cancer effects of AC-3E.

AC-3E attenuate T47D cell migration

Time-lapse microscopy was used to capture the kinetic characteristics of T47D cell morphology changes, proliferation, and migration under the treatment of vehicle (0.5% DMSO), AC-3E (50 μg/mL), and Tam (30 μM) for 24 h. The images were continuously captured from 0 to 24 h and the representative images taken every 4 h are shown in Fig. 2. The AC-3E and Tam treated cells were all rounded-up in their phenotype with no further proliferation observed compared to the vehicle-treated cells (Fig. 2A). A typical cell cytokinesis was captured at 12 h in vehicle treated T47D cells, but not in the AC-3E or Tam treated cells. Trajectories of T47D cells migration within 24 h under different treatments were monitored. The most significant cell dispersion area was seen in the vehicle-treated cells, and relative small and restricted area was revealed in AC-3E or Tam treated cells. In consistent with the observation of cell trajectories, the migrated distances of T47D cells were found shorter with AC-3E (92 μm; $p < 0.05$) and Tam (120 μm; $p < 0.05$) treatment than that of the vehicle-treated (172 μm) cells (Fig. 2C).

Confocal microscopy in couple with immunofluorescent staining with actin was employed to observe the dynamic of stress fiber formation in T47D cells. In

comparison with control cells, fluorescence intensity (red) indicated that polymerized actin was greatly reduced in AC-3E and Tam treated cells (Fig. 2D). AC-3E and Tam treatment attenuated stress fiber formation may be attributed to their deteriorate effect on T47D cell migration.

The horizontal and vertical migration of cancer cell is important for metastasis. The Boyden transwell migration assay revealed that AC-3E at 10 and 25 μg/mL and Tam at 15 μM inhibited migration of T47D cells (Fig. 3A). Furthermore, AC-3E and Tam also significantly inhibited T47D cell migration shown in would-healing assay (Fig. 3B). These results suggest that AC-3E may reduce the ability of T47D cells to invade basement membrane barriers.

AC-3E induce DNA damage and apoptosis in T47D cells

Induction of cell and DNA damages in T47D cells was investigated using TUNEL assay and PI staining. Fig. 4 shows that AC-3E dose-dependently increased DNA and cell damages as revealed by positively TUNEL stained (green fluorecein-dUTP) and PI stained (red FITC) cells with 48 h treatment of AC-3E, suggesting that AC-3E caused apoptosis in T47D cells. In contrast, AC-3E did not cause M10 cell damage at high concentration (50 μM) treatment as no fluorescence intensity was detected.

AC-3E modulate protein expression involved in cell-cycle mediation or cell migration and invasion

Western blot analysis shows that AC-3E at 50μg/mL inhibited the protein expression of several cell cycle mediators, including Cdk 4, Cdk 6, cyclin B1, cyclin D3, Rb, and p-Rb in a time dependent manner (Fig. 5A), indicating that cell cycle machinery in T47D cells were disturbed by AC-3E treatment.

Fig. 5B reveals that the protein expression of Rac1 and RhoA, two of the small Rho GTPase proteins involved in mediating actin dynamics in motile cells and during cell morphogenesis (Hall and Nobes, 2000) were inhibited. In addition, E-cadherin, functioning as invasion suppressor, and β-catenin protein, an oncoprotein, were observed to be up-regulated and down regulated, respectively, in AC-3E treated cells time dependently (Fig. 5B).

AC-3E regulate key proteins involved in cell growth, proliferation, apoptosis, or

inflammation

Overactivation of PI3K/Akt/mTOR pathway has been reported to reduce cancer cell apoptosis and allowing proliferation in cancer cells (Carraway and Hidalgo, 2004). Previous study also suggested that estrogen through ER induces cell survival by activating PI3K/Akt pathway (Bratton et al., 2010; Ahmad et al, 1999). In this study, Western blotting results show that the protein levels of ERα, key molecules involved in PI3K/Akt pathway, such as PI3K, p-Akt, Akt, mTOR, MDM2 were all suppressed in AC-3E treated cells time dependently compared to the vehicle control cells (Fig. 5C). Moreover, Jak1, Stat3, NF-κB, p38 and COX-2 proteins involved in cell inflammation and carcinogensis were also deregulated by AC-3E in T47D cells.

AC-3E inhibit ex vivo CAM angiogenesis

Matrigel™ encapsulated CAM assay was employed to investigate whether AC-3E can inhibit blood microvessel formation. As the data shown in Fig. 6, VEGF induced branching of blood vessels and increased micro-vessel formation in the vehicle control. AC-3E and Tam were dose-dependently reduced the number of branching blood vessels ($p < 0.05$) (Fig. 6), indicating both AC-3E and Tam possess anti-angiogenic activity.

AC-3 suppress T47D mammary tumor growth in xenograft mouse model with reduction of proliferation, angiogenesis and macrophage infiltration

We compared the AC-3E, Tam, and the Tam&AC-3E alternate treatment effect on cancer prevention or therapeutic efficacy in BALB/c nude mice. The experimental design and protocol for evaluating *in vivo* anti-breast cancer effect of AC-3E is shown in Fig. 7A. Mice were pretreated *p.o.* with 50 mg/kg AC-3E (PreAC-3E−50) for 7 days before tumor cells implantation on day 0, or *p.o.* treated with 50 or 100 mg/kg AC-3E (AC-3E−50 and AC-3E−100), 30 mg/kg Tam (Tam−30), or Tam−30 and AC-3E−50 alternate treatment (Tam−30&AC-3E−50) every two days till the end of the experiments at day 35. The mean tumor volume (n = 6) was significantly reduced without showing deteriorate effect on mice body weight in all test groups (Fig. 7B and C). The inhibition (%) on tumor size were 90%, 89%, 81%, 80%, 75%, respectively, in AC-3E−100, PreAC-3E−50, AC-3E−50, Tam−30, and Tam−30&AC-3E−50 groups, as compared to the tumor control group. The data demonstrate that AC-3E have

comparable or superior effect to that of endocrine drug tamoxifen on suppressing T47D mammary tumor growth.

Immunohistochemical staining results showed that proliferation index Ki67, endothelial cell marker CD31, and macrophage marker F4/80 were all significantly reduced in tested mice (Fig. 7E), indicating that AC-3E treatment suppressed T47D cell proliferation, tumor angiogenesis, and macrophages infiltration.

Chemical fingerprinting of AC-3E and identification of major bioactive compound

HPLC and various spectral analyses were employed to establish the LC profile and major chemical constituents present in the AC-3E for establishing chemical fingerprints of the bioactive extracts. As shown in Fig. 8A, two major compound peaks are seen in the LC profile separated using a RP C18 HPLC column, and the structure and name of both compounds were elucidated as dehydrosulfurenic acid (**1**) and dehydroeburicoic acid (**2**). The content of compound **1** and **2** in AC-3E was determined as 42.4% and 41.7%, respectively, by the respective calibration curves established in this study. The cytotoxic effects of dehydrosulfurenic acid (**1**) and dehydroeburicoic acid (**2**) on T47D and M10 cell lines was further examined using MTT assay. The results in Fig. 8B demonstrate that compound **1** did not have toxic effect to both breast cancer cell and normal cell lines, whereas, compound **2** exerted similar anti-T47D cell activity to that of AC-3E, with little toxic effect to M10 cells. The results indicate that dehydroeburicoic acid (**2**) is major bioactive compound in AC-3 extracts.

Discussion

This report presents the novel bioactivities of *A. cinnamomea* extracts attenuated *in vitro* and *in vivo* proliferation, migration, and tumor growth of estrogen receptor positive T47D human breast cancer cells by deregulating PI3K/Akt signaling pathway and cell-cycle mediators, inducing apoptosis, and inhibiting tumor angiogenesis *in vivo*. This is the first to demonstrate that AC extracts exert similarly potent activity to that of current estrogen antagonism drug tamoxifen against estrogen receptor dependent T47D breast cancer cells.

We observed that AC-3E could significantly attenuate T47D migration through the assay results of live video microscopy monitoring, wound healing and transwell invasion assay (Fig. 2 and 3). The anti-T47D cell migration effect of AC-3E is likely through deregulation of actin remodeling by RhoA and Rac1 GTPase (Sepp and Auld, 2003) on the basis of our confocal microscopy and Western blotting results (Fig. 2 and Fig. 5). On the other hand, E-cadherin and β-catenin proteins involved in epithelial-mesenchymal transition, which is essential for cancer cell migration, were also regulated by AC-3E treatment. E-cadherin acts as an invasion suppressor of various epithelial malignancies, including breast cancer (Hirohashi, 1998), while, β-Catenin, a multifunctional protein, mediates cancer cells to be more motile and invasive (Hayashida et al., 2005). The up-regulation and down-regulationon E-cadherin and β-catenin, respectively, by AC-3E present the novel anti-T47D breast cancer cell activity of the extracts.

The PI3K/Akt signaling pathway is known to involve in cell proliferation. While PI3K is activated, the expression of Akt increased, and then causes apoptosis resistance or cell cycle promotion. Previous study also indicated that ERα can induce estrogen dependent MCF-7 cell survival through activating PI3K/Akt pathway (Bratton et al., 2010). We observed in this study that AC-3E treatment resulted a time-dependent, down-regulation of ERα protein expression, and a series of proteins involved in PI3K/Akt pathway, such as PI3K, Akt, mTOR in estrogen dependent T47D breast cancer cells (Fig. 5), suggesting that AC-3E inhibited T47D cell proliferation via blockage of ERα/PI3K/Akt-mediated signaling pathway. The result may imply that AC-3E can function as antagonistic agent to estrogen.

The janus kinase (Jak)/signal transducer and activator of transcription 3 (Stat3) pathway is an important mediator of cell inflammation and carcinogenesis. Activated by growth factor or cytokine receptors, Stat3 up-regulates several oncogenic factors, for instance, NFκB and COX-2, which are important modulator of inflammation, angiogenesis, and metastasis (Yu et al., 2009). The expression of Jak1, Stat3, NFκB, and COX-2 proteins were reduced in AC-3E treated T47D cells (Fig. 5), suggesting that the anti-breast cancer cell activity of AC-3 extracts, may in part through de-regulating Jak/Stat3 pathway.

Together, schematic mechanisms of AC-3 extracts to exert anti-breast cancer cell activities are proposed. Firstly, AC-3 extracts inactivate ERα, and subsequently blockade the PI3K/Akt/mTOR proliferating and survival pathway, which, in turn, inhibits cell cycle progression and induces cell apoptosis. AC-3 extracts may also de-regulate Jak/Stat3 pathway, resulted in inhibiting COX-2 expression. On the other hand, AC-3 extracts exhibit remarkable anti-cancer cell migration activity through suppressing actin remodeling and stress fiber formation, and epithelial-mesenchymal transition via down-regulation of the expression of Rac1, RhoA, and β-catenin, and up-regulation of E-cadherin (Fig. 9).

Accumulating evidences indicate that angiogenesis is an essential process for tumor cell growth, expansion. The *ex vivo* chick chorioallatonic membrane (CAM) assay results demonstrate the profound anti-angiogenic activity of AC-3E comparable to that of tamoxifen, as microvasculature was markedly reduced (Fig. 6). Moreover, the endothelial cell marker protein CD31 was observed abundantly present in the T47D tumor specimen, indicating tumor angiogenesis (Fig. 7). Notably, AC-3E treatment showed profound effect on suppressing the level of CD31 proteins. The highly proliferative status of T47D cells (highly expressed proliferating marker Ki67) in tumor specimen (tumor control) was observed which was also significantly inhibited in AC-3E treated groups. Notably, our T47D tumor xenograft data reveal that among the five treatment groups, the AC-3E−100 and PreAC-3E−50 treatment showed most significant suppression on tumor growth with 90% and 89% ($p < 0.01$), indicating the novel antitumor activity of AC-3 extracts. Together, AC-3 extracts can be employed as a doublebarreled approach to treat human breast cancer by attacking both the cancer cells as well as tumor associated blood vessel cells.

Several forms of compounds, including terpenoids, flavonoids, polysaccharide, polyacetylenes, benzoquinone derivatives have been identified from mycelium culture or fruiting body of *A. cinnamomea*, and some of the compounds' bioactivities have been reported (Yang et al., 2012; Geethangili and Tzeng, 2011; Lin et al., 2011). Camphorataimide B, for instance, isolated from AC mycelium culture was claimed to inhibit the cell growth, cell cycle progression of and tumor growth of triple negative human breast cancer MDA-MB-231 cells (Lin et al., 2012). Antrocin isolated from AC fruiting bodies could lead to MDA-MB-231 cell death through Akt/mTOR pathway. Given that, little information are concerned the anti-ER+ breast cancer cell activity. In this study, two lanostane-type triterpenes, namely dehydrosulphurenic acid (**1**) and dehydroeburicoic acid (**2**), were identified and quantified from AC-3 fruiting body extracts. Recently, both compounds were reported to exert *in vitro* anti-inflammatory and anti-insecticidal activities (Geethangili and Tzeng, 2011). Dehydrosulphurenic acid was found to cause cell death in human leukemic U937 cells and lead to apoptosis in pancreatic BxPC3 cells (Chen et al., 2009). Dehydroeburicoic acid could induce calcium- and calpain-dependent necrosis in human U87MG glioblastomas (Deng et al., 2009) and leukemia cell apoptosis (Du et al., 2012). In this study, we found only dehydroeburicoic acid (**2**) exhibiting toxic effect to ER positive breast cancer cell T47D. The compound dehydroeburicoic acid is thus served well a role as the index and bioactive compound in the AC-3 extracts. The specific modes of action of dehydroeburicoic acid on breast cancer cells may be warrant for further investigation.

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Figure Legends

Fig. 1. Cell viability of T47D and M10 cells treated with AC-3E, AC-6E, AC-9E, or Tam at indicated concentrations.

Fig. 2. Kinetic characteristics of T47D cell proliferation and motility in the presence of AC-3E and Tam. Video frame of representative cells were taken by time-lapse microscopy (A). Fifteen cells were randomly chosen to monitor the cell trajectories (B) and migrated distances (C). Immunofluorescence confocal microscopy (D). ($p < 0.01$)

Fig. 3. Effect of AC-3E against T47D cell migration examined by Boyden transwell assay (A) and wound-healing assay (B).

Fig. 4. TUNEL assay and PI-staining of T47D cells treated with indicated concentrations of AC-3E or Tam.

Fig. 5. Western blot analysis of protein expression profile related to (A) cell-cycle mediation, (B) cell migration, and (C) cell growth, proliferation, and apoptosis in AC-3E−treated T47D cells.

Fig. 6. The anti-angiogenesis effect of AC-3 was examined using chorioallantoic membrane (CAM) assay.

Fig. 7. Effects of AC-3E and Tam on the growth of breast tumor in T47D xenograft model. Each group contained six BALB/c female nude mice with an average body weight of 16 g. (A) Experimental design and protocol. (B) Tumor growth curve and volume in each treatment group. Treatment-to-control (T/C) ratio are shown. (C) Mean body weight of each treatment group. (D) Representative photograph of dissected tumors of all tested groups. (E) Representative images of immunohistochemical staining of tumor specimen with Ki67, CD31, and F4/80: positive Ki67 *(reddish brown)*, positive CD31 *(red)*, positive F4/80 *(green)*, and DAPI staining for nucleus *(blue)*.

Fig. 8. (A) Chemical fingerprinting of AC-3E and quantification of two major compounds using HPLC analysis. (B) Cytotoxicity assay of compounds 1 and 2 on T47D and M10 cells.

Fig. 9. Summary of proposed molecular mechanisms of AC-3E to exert anti-breast cancer activities.

Figure 1

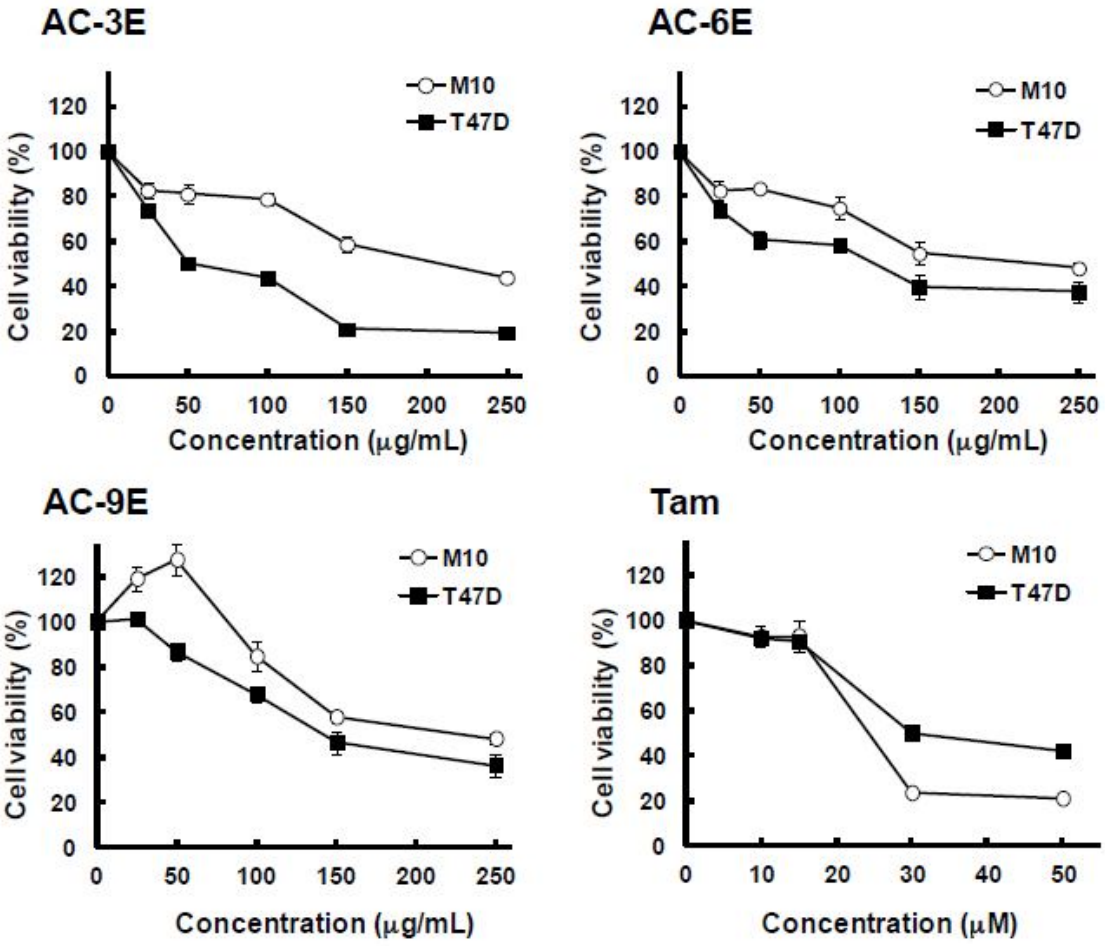

AC-3E
M10
T47D
Cell viability (%)
Concentration (μg/mL)
AC-6E
M10
T47D
Cell viability (%)
Concentration (μg/mL)
AC-9E
M10
T47D
Cell viability (%)
Concentration (μg/mL)
Tam
M10
T47D
Cell viability (%)
Concentration (μM)

Figure 2

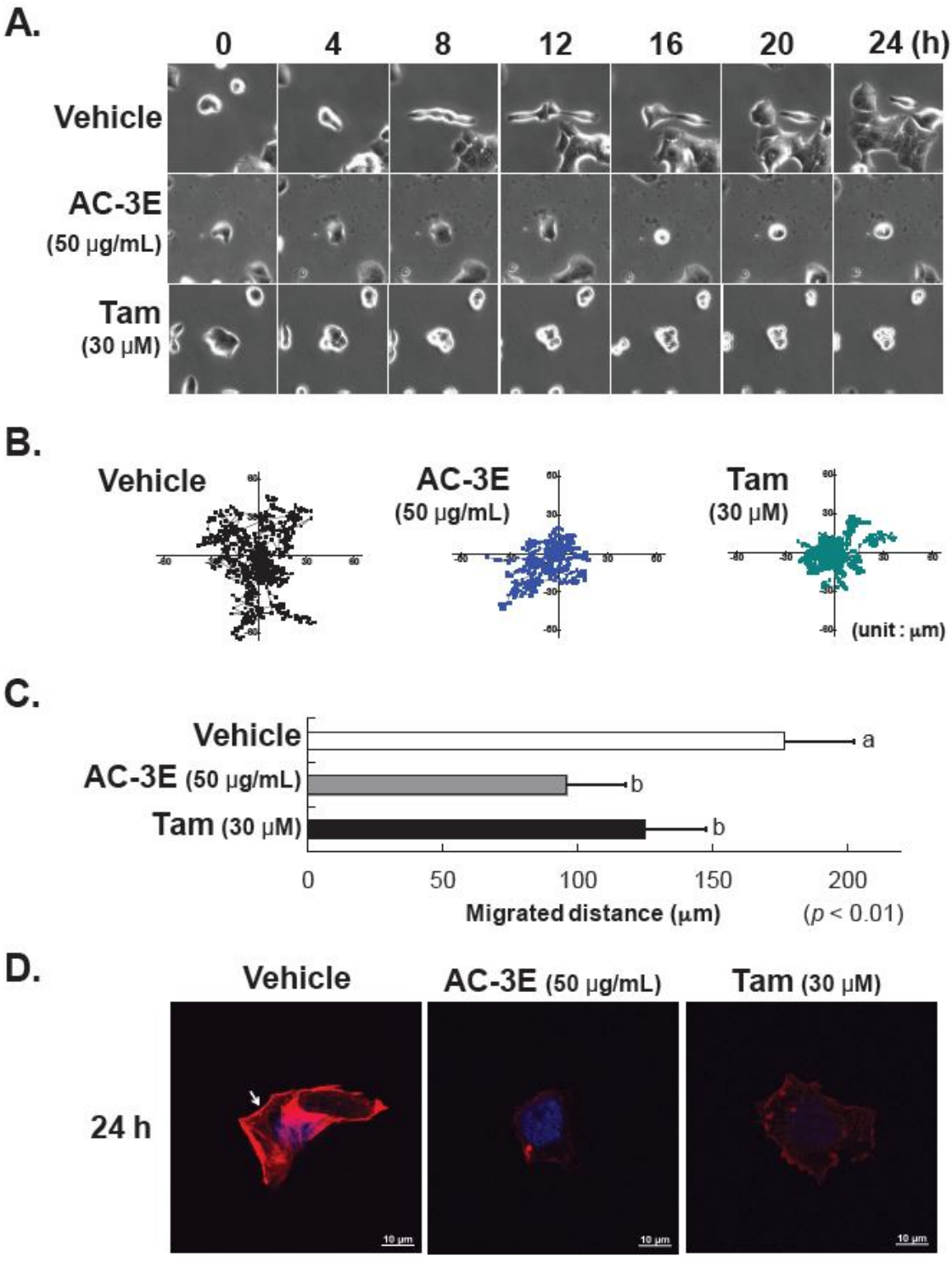

A.
0
4
8
12
16
20
24 (h)
Vehicle
AC-3E
(50 μg/mL)
Tam
(30 μM)
B.
Vehicle
AC-3E
(50 μg/mL)
Tam
(30 μM)
(unit : μm)
C.
Vehicle
AC-3E (50 μg/mL)
Tam (30 μM)
a
b
b
0
50
100
150
200
Migrated distance (μm)
(p < 0.01)
D.
Vehicle
AC-3E (50 μg/mL)
Tam (30 μM)
24 h
10 μm
10 μm
10 μm

Figure 3

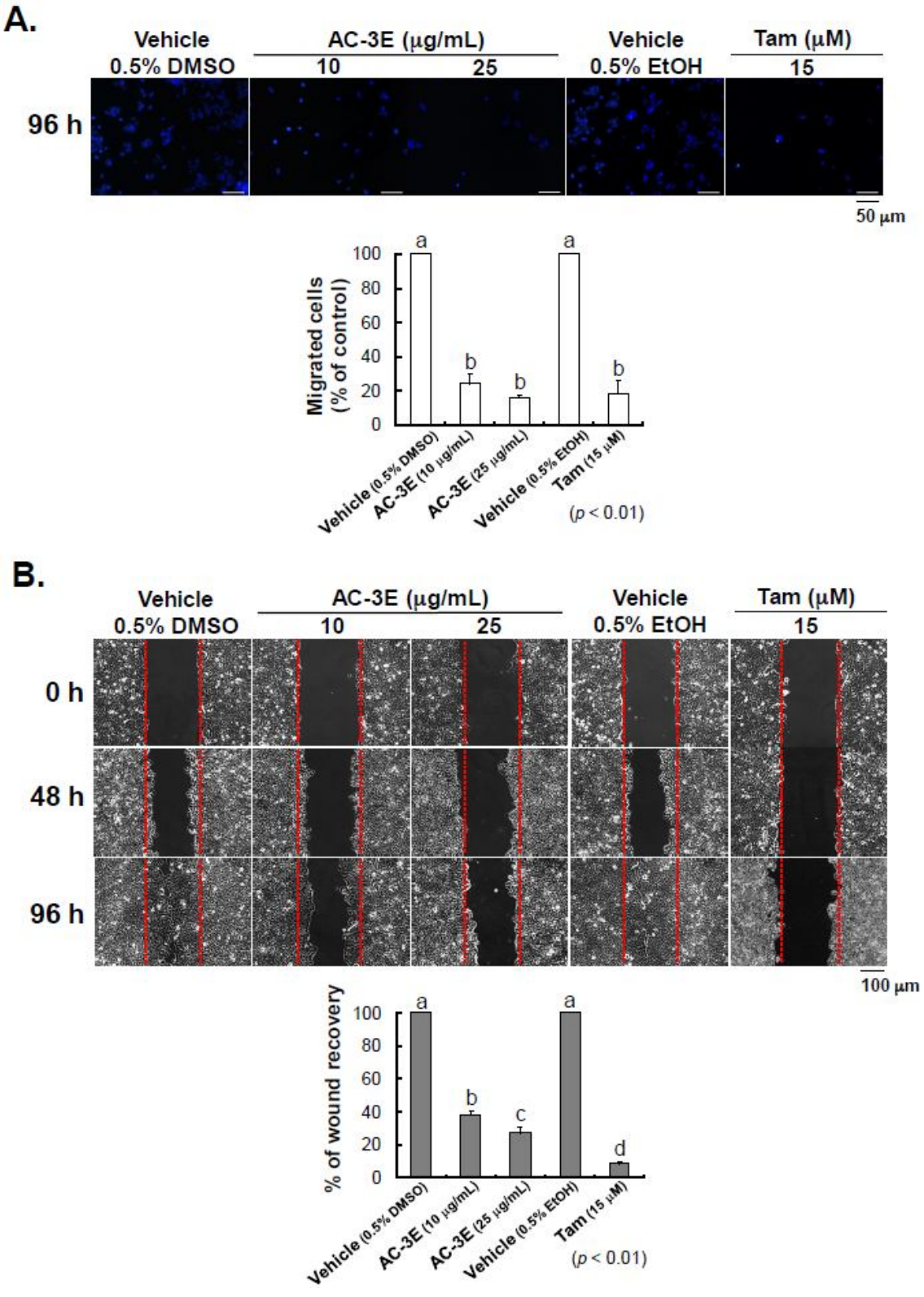

A.
Vehicle
0.5% DMSO
AC-3E (μg/mL)
10
25
Vehicle
0.5% EtOH
Tam (μM)
15
96 h
50 μm
Migrated cells
(% of control)
100
80
60
40
20
0
a
b
b
a
b
Vehicle (0.5% DMSO)
AC-3E (10 μg/mL)
AC-3E (25 μg/mL)
Vehicle (0.5% EtOH)
Tam (15 μM)
(p < 0.01)
B.
Vehicle
0.5% DMSO
AC-3E (μg/mL)
10
25
Vehicle
0.5% EtOH
Tam (μM)
15
0 h
48 h
96 h
100 μm
% of wound recovery
100
80
60
40
20
0
a
b
c
a
d
Vehicle (0.5% DMSO)
AC-3E (10 μg/mL)
AC-3E (25 μg/mL)
Vehicle (0.5% EtOH)
Tam (15 μM)
(p < 0.01)

Figure 4

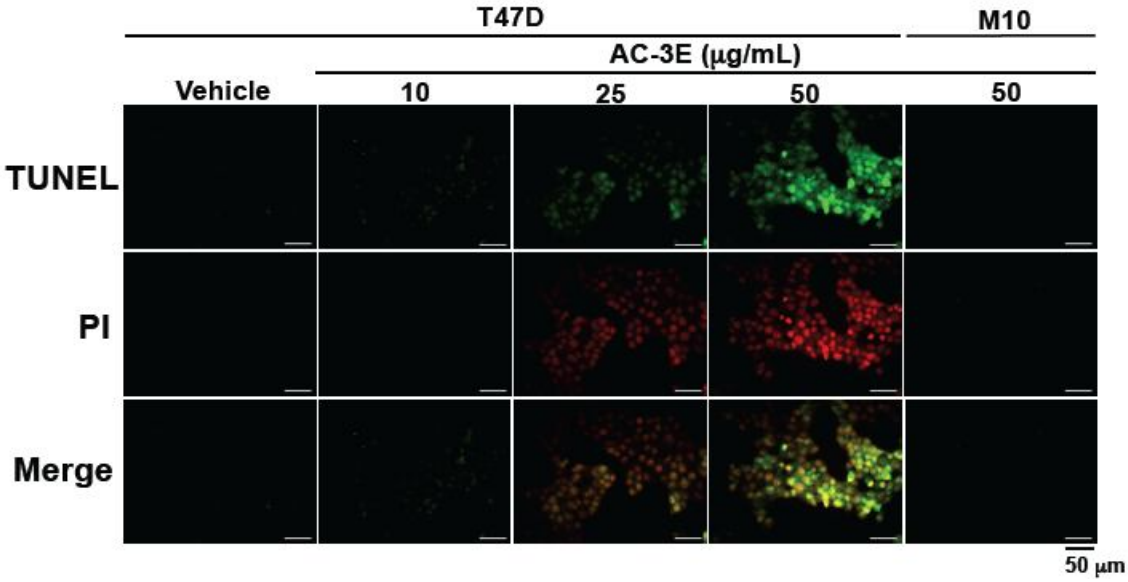


Figure 5

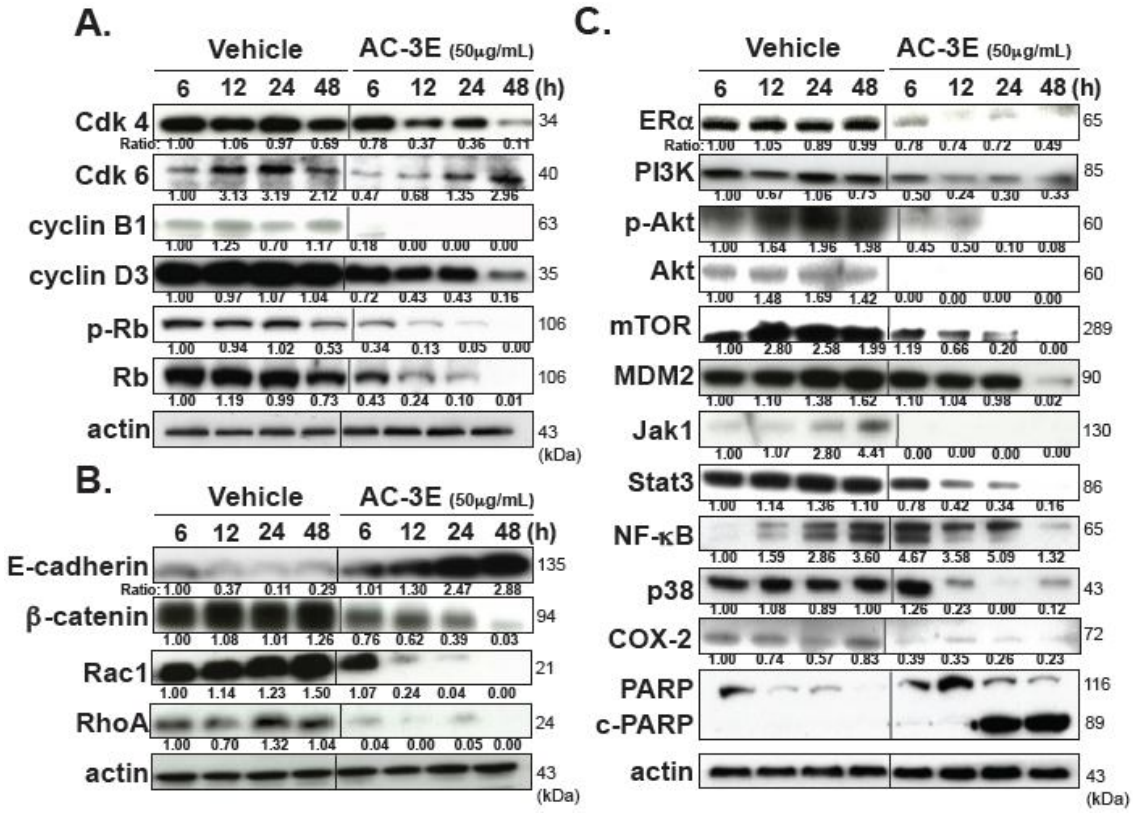

A.
Vehicle
AC-3E (50μg/mL)
6 12 24 48 6 12 24 48 (h)
Cdk 4
Ratio: 1.00 1.06 0.97 0.69 0.78 0.37 0.36 0.11
34
Cdk 6
1.00 3.13 3.19 2.12 0.47 0.68 1.35 2.96
40
cyclin B1
1.00 1.25 0.70 1.17 0.18 0.00 0.00 0.00
63
cyclin D3
1.00 0.97 1.07 1.04 0.72 0.43 0.43 0.16
35
p-Rb
1.00 0.94 1.02 0.53 0.34 0.13 0.05 0.00
106
Rb
1.00 1.19 0.99 0.73 0.43 0.24 0.10 0.01
106
actin
43
(kDa)
B.
Vehicle
AC-3E (50μg/mL)
6 12 24 48 6 12 24 48 (h)
E-cadherin
Ratio: 1.00 0.37 0.11 0.29 1.01 1.30 2.47 2.88
135
β-catenin
1.00 1.08 1.01 1.26 0.76 0.62 0.39 0.03
94
Rac1
1.00 1.14 1.23 1.50 1.07 0.24 0.04 0.00
21
RhoA
1.00 0.70 1.32 1.04 0.04 0.00 0.05 0.00
24
actin
43
(kDa)
C.
Vehicle
AC-3E (50μg/mL)
6 12 24 48 6 12 24 48 (h)
ERα
Ratio: 1.00 1.05 0.89 0.99 0.78 0.74 0.72 0.49
65
PI3K
1.00 0.67 1.06 0.75 0.50 0.24 0.30 0.33
85
p-Akt
1.00 1.64 1.96 1.98 0.45 0.50 0.10 0.08
60
Akt
1.00 1.48 1.69 1.42 0.00 0.00 0.00 0.00
60
mTOR
1.00 2.80 2.58 1.99 1.19 0.66 0.20 0.00
289
MDM2
1.00 1.10 1.38 1.62 1.10 1.04 0.98 0.02
90
Jak1
1.00 1.07 2.80 4.41 0.00 0.00 0.00 0.00
130
Stat3
1.00 1.14 1.36 1.10 0.78 0.42 0.34 0.16
86
NF-κB
1.00 1.59 2.86 3.60 4.67 3.58 5.09 1.32
65
p38
1.00 1.08 0.89 1.00 1.26 0.23 0.00 0.12
43
COX-2
1.00 0.74 0.57 0.83 0.39 0.35 0.26 0.23
72
PARP
116
c-PARP
89
actin
43
(kDa)

Figure 6

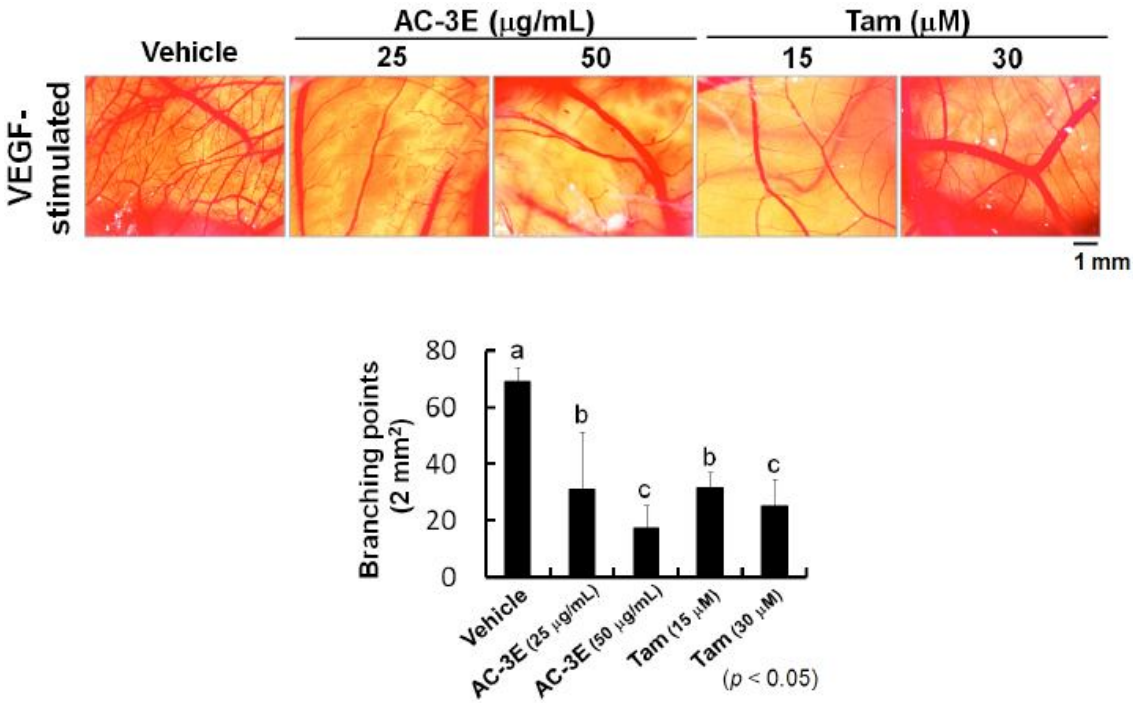

AC-3E (μg/mL)
Tam (μM)
Vehicle
25
50
15
30
VEGF-stimulated
1 mm
Branching points (2 mm²)
80
60
40
20
0
a
b
c
b
c
Vehicle
AC-3E (25 μg/mL)
AC-3E (50 μg/mL)
Tam (15 μM)
Tam (30 μM)
(p < 0.05)

Figure 7

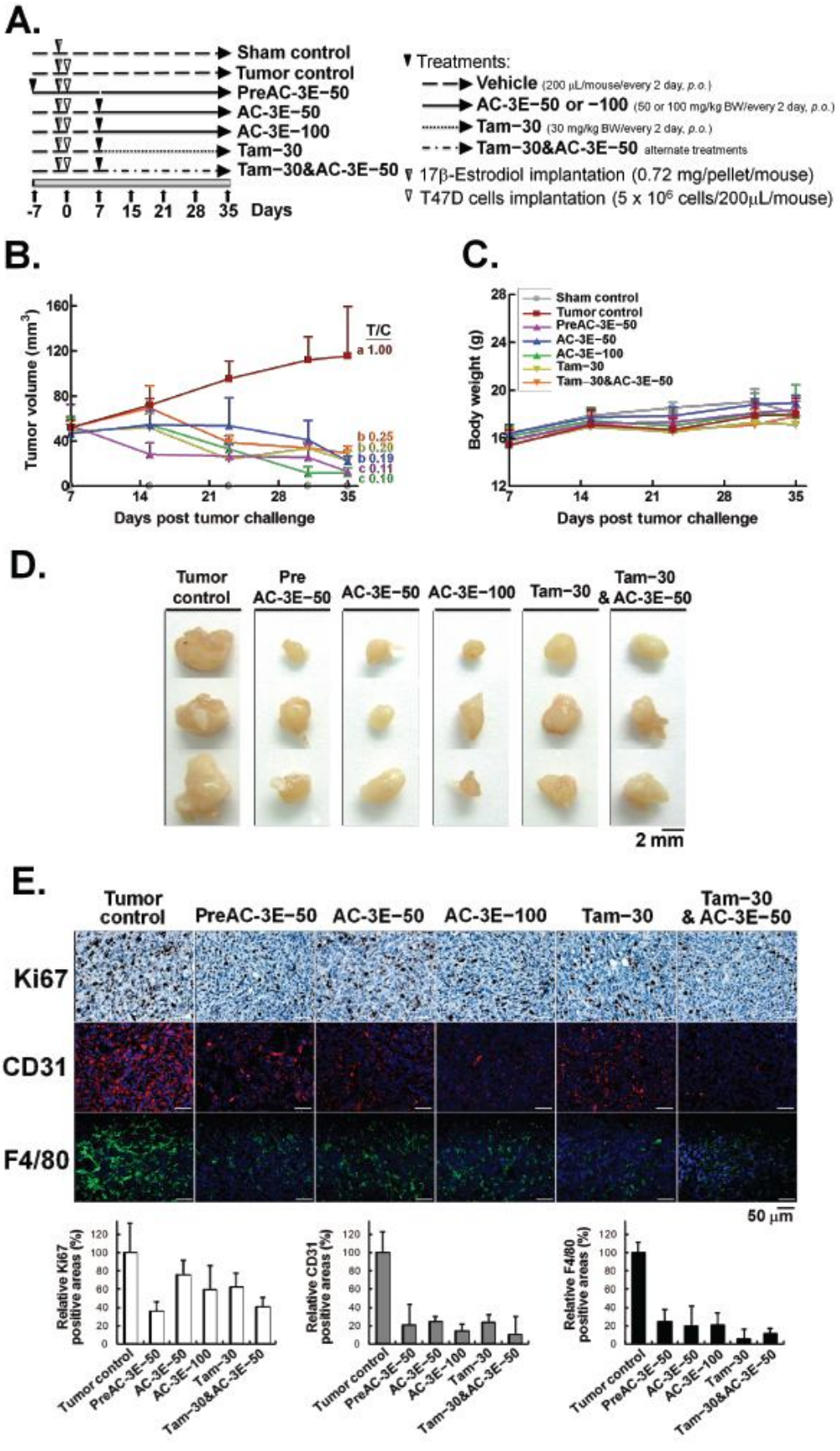

A.
Sham control
Tumor control
PreAC-3E-50
AC-3E-50
AC-3E-100
Tam-30
Tam-30&AC-3E-50
-7 0 7 15 21 28 35 Days
Treatments:
Vehicle (200 μL/mouse/every 2 day, p.o.)
AC-3E-50 or -100 (50 or 100 mg/kg BW/every 2 day, p.o.)
Tam-30 (30 mg/kg BW/every 2 day, p.o.)
Tam-30&AC-3E-50 alternate treatments
17β-Estrodiol implantation (0.72 mg/pellet/mouse)
T47D cells implantation (5 x 10^6 cells/200μL/mouse)
B.
Tumor volume (mm3)
T/C
a 1.00
b 0.25
b 0.20
b 0.19
c 0.11
c 0.10
Days post tumor challenge
C.
Body weight (g)
Sham control
Tumor control
PreAC-3E-50
AC-3E-50
AC-3E-100
Tam-30
Tam-30&AC-3E-50
Days post tumor challenge
D.
Tumor control
Pre AC-3E-50
AC-3E-50
AC-3E-100
Tam-30
Tam-30 & AC-3E-50
2 mm
E.
Tumor control
PreAC-3E-50
AC-3E-50
AC-3E-100
Tam-30
Tam-30 & AC-3E-50
Ki67
CD31
F4/80
50 μm
Relative Ki67 positive areas (%)
Relative CD31 positive areas (%)
Relative F4/80 positive areas (%)
Tumor control
PreAC-3E-50
AC-3E-50
AC-3E-100
Tam-30
Tam-30&AC-3E-50

Figure 8

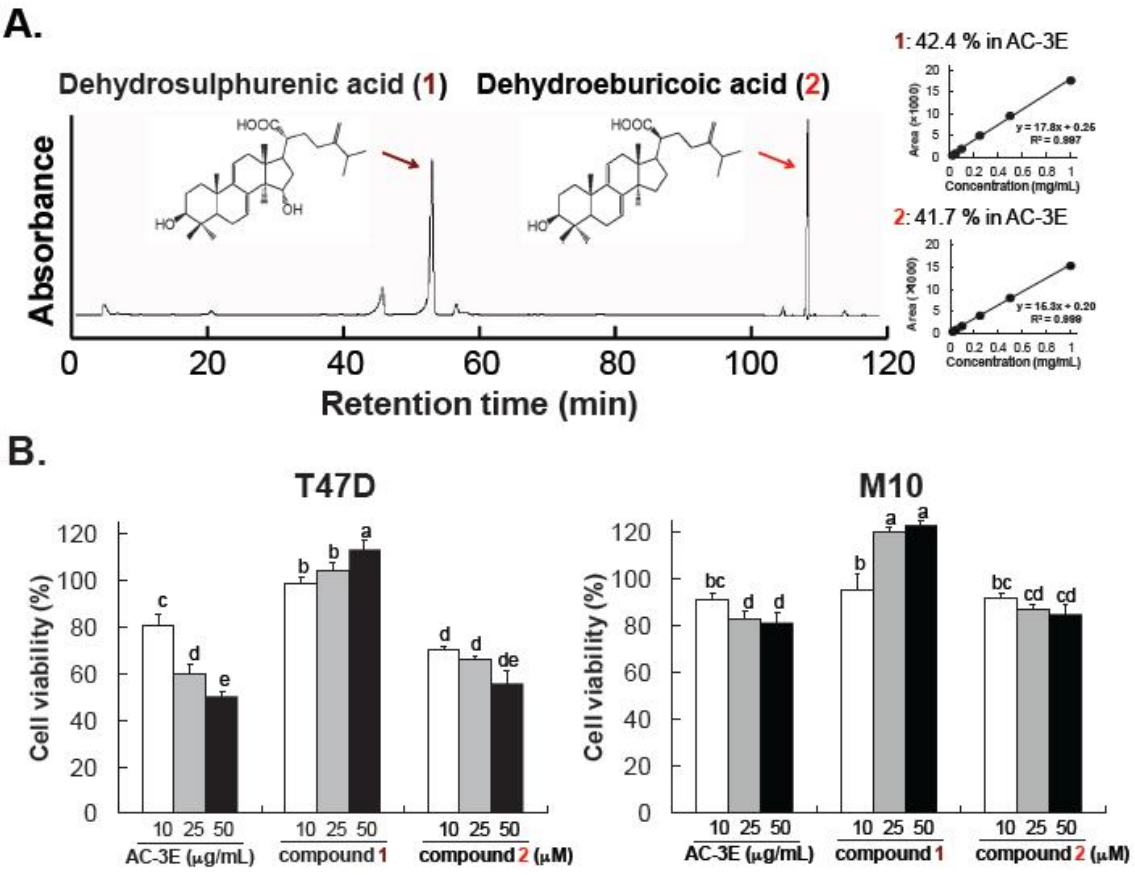
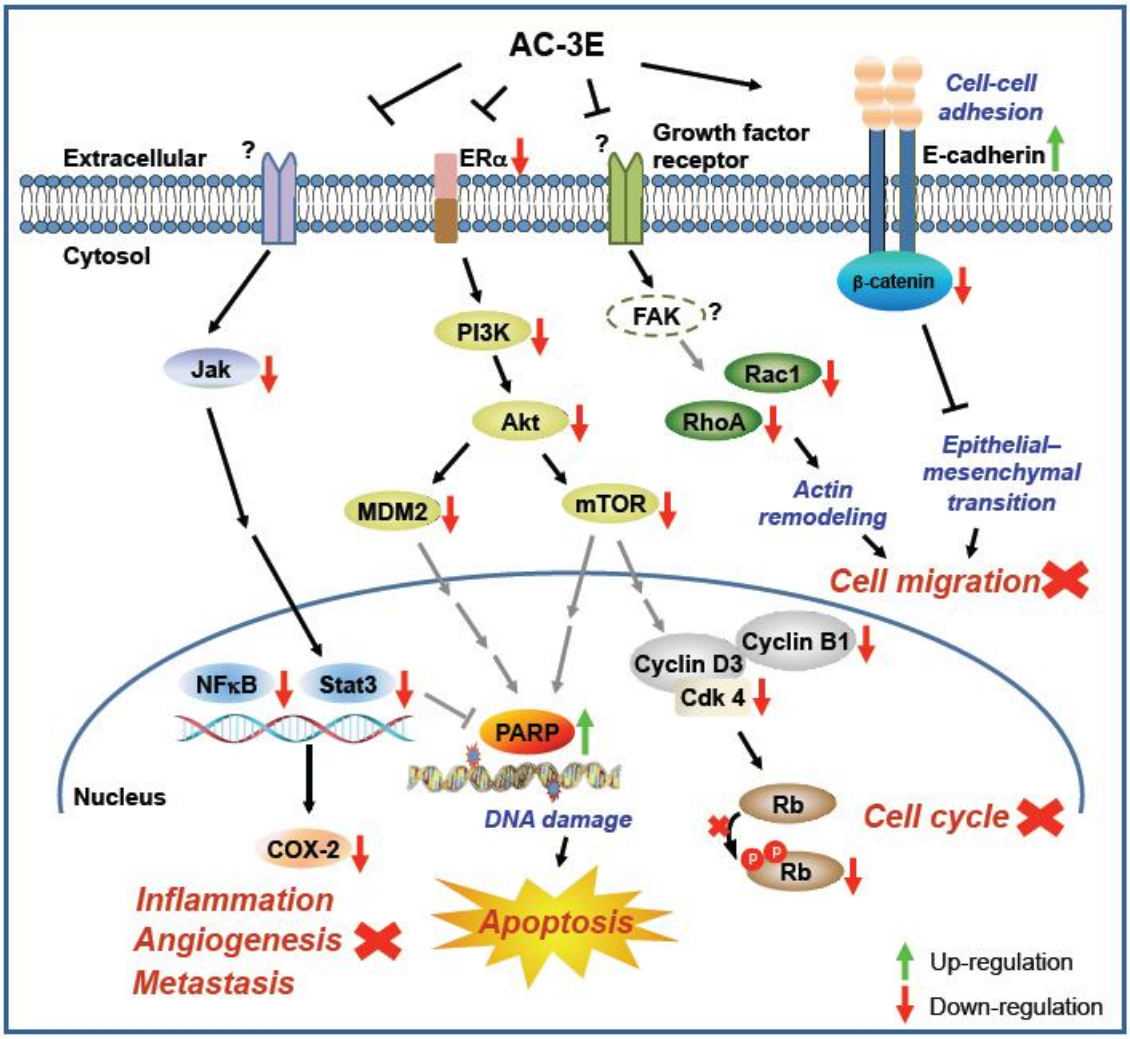

A.
Dehydrosulphurenic acid (1)
Dehydroeburicoic acid (2)
Absorbance
Retention time (min)
1: 42.4 % in AC-3E
2: 41.7 % in AC-3E
Concentration (mg/mL)
B.
T47D
M10
Cell viability (%)
AC-3E (μg/mL)
compound 1
compound 2 (μM)

Figure 9


AC-3E
Extracellular
Cytosol
ERα
Growth factor receptor
Cell-cell adhesion
E-cadherin
β-catenin
Jak
PI3K
FAK
Rac1
RhoA
Akt
MDM2
mTOR
Actin remodeling
Epithelial–mesenchymal transition
Cell migration
Cyclin B1
Cyclin D3
Cdk 4
NFκB
Stat3
PARP
DNA damage
Nucleus
Rb
Cell cycle
COX-2
Inflammation
Angiogenesis
Metastasis
Apoptosis
Up-regulation
Down-regulation

評語

1、已有初步之 data，應給予鼓勵。

2、但實驗利用 AC-3 之萃取物做實驗，並非用純化之 peak(1) or (2) Fig3.建議用純化之 dehydroeburicoic acid 重複實驗，才能突顯實驗之新穎性。

3、在不同之 cell line 之 IC_{50} 應取得。

4、除 cell line 之 IC_{50} 之 Viability 之測試外，應做 cell migration and invasion assay by Transwell method .而非 time-lapse microscopy.