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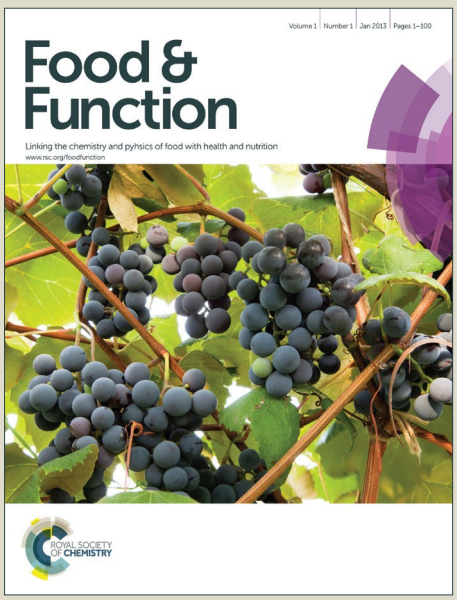

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Averrhoa carambola L. Peel Extract Suppresses Adipocyte Differentiation in 3T3-L1 Cells

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ABSTRACT: Obesity is associated with an increased risk of many chronic diseases. Recently, a growing body of evidence has shown that phytochemicals may inhibit adipogenesis and obesity. In this study, we report for the first time, the ability of *Averrhoa carambola* L. peel extract commonly know as starfruit (SFP) to effectively suppress adipocyte differentiation in 3T3-L1 preadipocytes and therefore, address it as a potential candidate to treat obesity and its related disease. (−)-epicatechin was identified as the bioactive compound likely responsible for this suppression. As the genetic expression studies revealed that the adipogenic activity of SFP extract was due to the simultaneous downregulation of the C/EBPα and PPARγ as well as the upregulation of PPARα receptor genes, a detailed computational docking studies was also elucidated to reveal the likely binding mode of (−)-epicatechin to the receptor of interest, accounting for the likely mechanism that results in the overall suppression of adipocyte differentiation.

KEYWORDS:

Averrhoa carambola, 3T3-L1 cells, adipocyte differentiation, PPARγ, PPARα, (−)-epicatechin

INTRODUCTION

Obesity is defined as an abnormal or excessive fat accumulation in adipose tissues and it is a condition that is often triggered by multiple environmental and genetic factors. It is strongly associated with metabolic abnormalities, cardiovascular diseases as well as several types of cancers.[1] The prevalence of obesity in both developed and developing countries has become significantly prominent and its growth has become a major challenge for health care professionals to combat.[2, 3] Considering the rising problem of obesity in the present society, many forms of treatment have been established to combat this disease.[4] While the basis of the treatment involves the promotion of healthy physical regime in a form of well-designed physical exercises, this alone could not be used to fully treat an obese patient. The prescription of weight-loss medications is certainly required to complement this treatment, in order to effectively produce results amongst obese patients. Some of the common weight-loss prescriptions include synthetic drugs or supplement such as Lorcaserin (Belviq) as well as Phentermine-topiramate (Qsymia).[5] However, these treatments do have significant risks and repercussions along with it. The use of these synthetic medications has been found to result in adverse side effects that consequently affect therapy compliance and patient's quality of life. [6, 7]

Considering the rising risks and the negative implications of synthetic drugs on obese patients, a host of complementary and alternative supplementary treatments, such as the use of natural sources are therefore required.[8] In a recent article published by our group, we have reviewed and evaluated a series of studies previously done internationally on the recently discovered ability of phytochemicals extracted from fruit-waste components to inhibit a fundamental process responsible for

obesity.[9]

Adipogenesis, is the process through which preadipocytes are differentiated into adipocytes, where excess energy can be effectively stored in a form of triglycerides.[10] It is believed that the ability to regulate this process is crucial to prevent and improve obesity and its related metabolic syndrome.[11] Adipogenesis is mainly controlled by the coordination of activated peroxisome proliferator activated receptors (PPAR) and CCAAT-enhancer binding protein (C/EBP) transcription factors which modulate the expression of adipogenic genes.[10, 12, 13] Considering the fact that PPARs play essential roles in fatty acid metabolism, they are considered as the major drug targets in treating obesity and obesity-related disease.[14] PPARα reduces energy storage and is considered as a core modulator in the fatty acid synthesis, fatty acid oxidation and lipid metabolism.[15, 16] Activation of PPARα has been shown to be beneficial for treating obesity and its related diseases.[17, 18] PPARγ and C/EBPα on the other hand, are two fundamental transcriptional regulators which act cooperatively during adipocyte differentiation.[19] As a master regulator of adipogenesis induced during adipocyte differentiation, PPARγ is proven to be essential and adequate for this process.[1] Upon ligand binding activation, PPARγ regulates target genes containing a PPAR-response element (PPRE) in their promoters such as lipoprotein lipase (LPL) and adipocyte Protein 2 (aP2). [12, 20]

Averrhoa carambola L. commonly known, as star fruit is widely cultivated in tropical and semitropical regions especially in Southeast Asia. Traditionally, *Averrhoa carambola* L. is regarded as a medicinal plant and its leaves and flesh have been used to treat many diseases, including various skin disorders, headaches, vomiting, coughing, diarrhea, fever and diabetes.[21-23] In addition to this, Shui and coworkers

have also reported that star fruit has been found to be a good source of natural antioxidants and can effectively scavenge free radicals due to the presence of high amounts of polyphenols within the chemical composition of its extract.[24] However, while star fruit has been a known medicinal source for traditional medical treatment, the one component that has often been neglected by researchers would be its peel component. In a recent comparative study conducted by Matsusaka and coworkers, it has been found that the ethanolic extract of the thin-waxy peel of star fruit have the most abundant antioxidants and contain remarkably 16 times more polyphenolic content than the juice and flesh.[25]

Taking these reports into consideration, this study investigated for the first time the adipocyte differentiation effect of the extracts of *Averrhoa carambola* L. peel, commonly known as star fruit peel (SFP), on 3T3-L1 preadipocytes, its inhibition potential of lipid accumulation on 3T3-L1 preadipocytes to mature adipocytes during adipogenesis as well as its genetic role leading to the overall inhibition of lipid accumulation. The active compound responsible for this adipogenesis inhibition has been identified to be a polyphenolic compound, (−)-epicatechin of which, its utility prospective as a supplement or drug candidate in treating obesity was also further explored via binding mode analysis through computational modeling and molecular docking.

MATERIALS AND METHODS

Reagents and Chemicals. Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, Cell Counting Kit-8 (CCK-8, 96992), Oil Red O, dimethyl sulfoxide (DMSO), formic acid, dichloromethane (DCM), acetonitrile (ACN) and (−)-epicatechin were purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal Bovine

Serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM) were obtained from GIBCO (Grand Island, NY, USA). TRIzol reagent, Purelink RNA mini kit and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). IScript cDNA synthesis kit was purchased from Bio-Rad (Hercules, CA, USA). GoTaq colorless master mix was purchased from Promega (Madison, WI, USA). Primers were synthesized by 1st Base Pte Ltd (Singapore, Singapore). Analytical grade ethanol and methanol were purchased from Merck (Damstadt, Germany).

Extraction of *Averrhoa carambola* L. peel. 20 pieces of unripe (green) star fruit were purchased from local wet market. The fruits were washed and peeled. The peels were further cut into smaller pieces and stored in -80 ℃ freezer (72 hrs). The frozen samples were then lyophilized (72 hrs, -80 ℃). Dried peels were ground into fine powder using blender. 53.0 g of crude powder was extracted with 50% aqueous ethanol (1500 mL) in a water bath (30 ℃) for 24 hrs. The mixture was filtered through Whatman no.4 filter paper, and the filtrate condensed by rotary evaporation. The condensed filtrate was further lyophilized (72 hrs, - 80 ℃) to obtain brown solids (21.2 g) and stored in -80 ℃ freezer until use. The crude extraction yield is exceptionally high with an average percentage of 40% however this has been further validated experimentally in triplicates.

Silica Gel Chromatography. 5.0 g of crude extract (brown solid) was re-suspended in methanol for further extraction. The clear solution of the methanol phase was separated by filtration. The methanol insoluble part was collected as a single fraction (Fraction 15). The homogeneous methanol extract was concentrated under vacuum before subjecting it to further separation via silica gel chromatography with a sequential gradient elution of DCM:MeOH:formic acid from 100:0:2 to 0:100:2, to yield 14 fractions labelled as Fraction 1-14 respectively. The fractions were then

condensed and lyophilized into powder form and stored in -80 ℃ freezer until used for analysis.

HPLC Analysis. Waters 2695 separation mode equipped Waters 2996 photodiode array detector covering 210-400 nm range were used for data analysis. The separation was performed on an Agilent Zorbax SB-C18 column 5 micrometer, 4.6 x 150 mm) with 0.1% of formic acid (A) and methanol (B) as the mobile phase. The column temperature was room temperature, and the injection volume was 80 μL. The gradient elutions were as follows: 0% B/100% A was increased linearly to 100% B/ 0% A over 100 minutes, held isocratic for 15 minutes, decreased to initial conditions over 15 minutes and held isocratic for 5 minutes. The flow rate was 1.0 mL/min

LC-MS Analysis. ThermoFinnigan LCQ Fleet MS which was equipped with Thermo Accela LC was used for LC-MS data collection. 0.1% of formic acid in water as solvent A. 0.1% of formic acid in ACN as solvent B. The column was flushed with 5% of B for one minute, and then increases the percentage of B from 5% to 95% over 5 minutes. Keep 95% for another 2 minutes and decrease to 5% over 1 minute. Flow rate 0.25mL/min. The full scan mass spectra from m/z 50-2000 were acquired with both negative mode and positive mode. 10 µL of respective extracts with concentration of 0.5mg/mL, dissolved in water were injected into LC-MS for analysis.

Cell Viability Assay. Cell viability was performed using Cell Counting Kit-8 (CCK-8, Sigma Aldrich) according to the manufacturer's protocol. Briefly, 5000 cells were seeded into each well of the 96-well plate and cultured in 100 µL DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2 for 24 hrs. Then various concentrations of star fruit peel extract were added in the plate to achieve indicated final concentration. The cells were

then incubated in a humidified incubator at 37°C with 5% CO_2 for 48 hrs. 10 μL of CCK-8 was then added into each well and the plate was incubated in dark for another 4 hrs. The absorbance was measured at 450 nm with a Synergy4 Multifunction Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA).

Adipocyte Differentiation of 3T3-L1 Cells. Adipocyte differentiation procedure was described previously.[26] Generally, 3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2 to confluence (2000 cells/cm^2). Confluent cells were maintained in DMEM supplemented with 10% FBS for 24 hrs. Post-confluent cells (Day 0) were then induced to adipocyte differentiation with a hormonal cocktail containing DMEM supplemented with 10% FBS, 2.5 μg/mL insulin, 0.5 μM dexamethasone, and 100 μM IBMX. After 48 hrs (Day 2), the medium was then replaced with DMEM containing 10% FBS and 2.5 μg/mL insulin. After another 48 hrs (Day 4), medium was changed to fresh DMEM containing 10% FBS. Every 48 hrs thereafter, medium was replaced with DMEM containing 10% FBS. On day 8, the cells were harvested for analysis. Star fruit peel extract and (−)-epicatechin were added into DMEM to achieve the indicated concentrations for the entire adipogenic period.

Oil Red O Staining. 3T3-L1 cells differentiated for 8 days were washed twice with PBS (pH 7.4) and then fixed with 3.7% formalin (20 min). The cells were washed twice with distilled water and stained with freshly diluted Oil Red O solution (six parts of 0.5% Oil Red O stock solution and four parts of H_2O) with gentle agitation (60 min). Excess stain was removed with 60% aqueous ethanol, and then cells were washed three times with distilled water before observation under an Olympus CKX41 phase contrast microscope. For quantitative determinations of

accumulated lipid in cells, Oil Red O staining was eluted with isopropanol. The amount of dye eluted was quantified by spectrophotometer at 513 nm.

RNA Extraction and Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was extracted from 3T3-L1 cells using TRIzol reagent and Purelink RNA mini Kit on day 8. Reverse transcription was performed with Iscript cDNA synthesis kit, according to the manufacturer's instructions. PCR reactions were performed using the GoTaq colourless master mix and Takara TP600 thermal cycler. The thermal cycle conditions were: after heating at 95°C for 5 min, PCR amplification was done with 40 cycles of 95°C for 30 sec, respective annealing temperature for 30 sec, 72°C for 30 sec, followed by a terminal extension at 72°C for 7 min. Primers used for PCR was shown in Table 1. The PCR products were subjected to electrophoresis on 2% agarose gels stained with ethidium bromide. The bands were scanned by a UV scanner; GAPDH was employed as the internal control.

Molecular Docking. The induced-fit docking (IFD) protocol in Maestro suite was carried out to predict the binding of PPARα (PDB ID: 2REW)[27] with (−)-epicatechin. The overall procedure has four stages: Briefly, during Stage 1 initial softened-potential Glide docking is performed on a vdW scaled-down rigid receptor;[28-30] a scaling of 0.5/0.5 was set for receptor/ligand vdW radii, respectively. The top 20 poses for each test ligand was retained. In Stage 2, receptor sampling and refinement was performed on residues within 5.0 Å of each ligand for each of the generated ligand:protein complexes. The side-chains, as well as the backbone and ligand, underwent subsequent energy minimizations. Stage 3 involved re-docking the test ligands into their respective 20 structures that are within 30.0 kcal/mol of their lowest energy structure. Finally, the ligand poses were scored in Stage 4 using a combination of Prime and GlideScore scoring functions in which the top ranked pose

for each ligand was chosen as the final result. The SP scoring function was used in all docking stages. Visualization of the protein-ligand complexes was done with the Visual Molecular Dynamics (VMD) software.[31]

Statistical Analysis. Data are expressed as means ± SEM. Differences were considered statistically significant when the *p* value was less than 0.05, assessed using an unpaired Student's *t*-test.

RESULTS

Effect of Star Fruit Peel Extract on the Cellular Lipid Accumulation in Differentiating 3T3-L1 Preadipocyte Cells. In this study, the effect of SFP crude extract on cellular lipid accumulation in differentiating 3T3-L1 preadipocytes was determined using Oil Red O staining, which indicates intracellular triacylglycerol (TG) accumulation. SFP extract suppressed TG accumulation in a dose-dependent manner as evidenced by the decrease in the size and the number of lipid droplets (Figure 1A), as well as the total quantification of lipid contents relative to the TG accumulation in the positive control group (Figure 1B). At a concentration of 1000 mg/L, SFP extract was shown to inhibit most of adipocyte differentiation. We also measured the cytotoxicity of SFP extract against 3T3-L1 cells. Exposure to SFP extract (200 – 1000 mg/L) placed no significant effect on 3T3-L1 cell viability compared to that of untreated cells (Figure 1C). This is a good indication suggesting that the suppression of TG accumulation initially observed at a dose-dependent manner was due to inhibitory effect of components present in SFP extract and not due to its toxicity.

Effect of Star Fruit Peel Extract on Adipocyte-Specific Gene Expression. Adipocyte differentiation and adipogenesis involve a series of programmed changes in gene expression.[11] During adipocyte differentiation, transcription factors such as

C/EBPs and PPARs are involved in regulating the expression of many adipogenic proteins, including LPL and aP2.[24, 32-34] To determine whether the inhibition of adipogenesis by SFP extract was due to an SFP extract-mediated alteration in the differentiation program, the mRNA expression of a number of adipocyte-specific genes was analyzed via RT-PCR analysis. As shown in Figure 2, RT-PCR revealed that SFP extract significantly decreased the mRNA expression of PPARγ and C/EBPα, the two master adipogenic transcription factors, as well as upregulate PPARα in a dose-dependent manner during adipocyte differentiation in response to treatment with SFP extract.

The apparent alterations in mRNA levels of adipocyte-specific genes suggest that SFP extract attenuates adipocyte differentiation, at least partially, by simultaneous downregulation of PPARγ and C/EBPα as well as by the upregulation of PPARα.

Identification of Bioactive Fractions and Relevant Molecules of Star Fruit Peel Extract. In order to further investigate how the bioactive phytochemical(s) in SFP extract play a role in the suppression effect of adipogenesis, the identification of the bioactive compound is first crucial. SFP extract was subjected to elaborate separation and isolation via filtration and silica column chromatography using methanol and dichloromethane as the mobile phase to yield 15 separate fractions (F1~F15). In order to identify the active fraction responsible for the anti-adipogenic effect initially observed in the SFP crude extract, each of these 15 fractions were subjected to adipocyte differentiation assay on 3T3-L1 preadipocyte cells (Figure 3). Out of the 15 fractions, only one fraction (F12) showed a significant inhibition in TG accumulation and this suggests that it is the only fraction that contain a significant amount of the bioactive compound responsible for the SFP extract's initial

suppression on adipogenesis. Cytotoxicity of F12 was further determined using Cell Counting Kit-8 under exact condition when testing for the cytoxicity of crude SFP and no cytotoxicity was observed in 3T3-L1 cells with a co-incubation of F12 (data not shown).

To better understand the chemical composition of the bioactive fraction, F12 was analyzed by reverse phase HPLC followed by LC-MS of which, two groups of major peaks (Figure 4A) with retention time 23.6 min and 32.8 min were observed with a m/z ratio of $[M+H]^+$ = 291.14 and $[M+H]^+$ = 827.25 respectively. Considering that the m/z ratio of the compound present in the second major peak (peak 2) to posses a molecular weight greater than 500, the compound is not likely able to fit into the binding pocket of any target receptors and therefore we hypothesized that the bioactive compound lies likely within peak 1 with m/z ratio of 291.14. As further purification and isolation of F12 was not possible to separate the 2 major peaks due to its initial trace amount, alternative way of identification and confirmation of the active compound was used.

With reference to a study conducted by Shui et. al on the antioxidants capacity of star fruit, they have identified (−)-epicatechin and (+)-catechin as the primary polyphenolic components present in star fruit extract.[24] As our results show that one of the major peaks contain the exact $[M+H]^+$ ratio as (−)-epicatechin and (+)-catechin, a co-injection with the pure compounds were done to confirm the presence and absence of the respective compounds in F12. As pure (+)-catechin has a retention time that falls out of the range of the two major peaks identified in F12, this compound was not considered for further analysis (Figure 4D). (−)-epicatechin however, when run under the same HPLC condition reflected a retention time that was similar to the first major peak in F12 (Figure 4D). A co-injection of pure (−)-epicatechin with F12 at

various volumes of 20% (Figure 4B) and 80% (Figure 4C) respectively indicates a similar change in the desired peak area confirming the presence of (−)-epicatechin in F12 (Figure 4E). For further confirmation if (−)-epicatechin was indeed the bioactive compound responsible for the anti-adipogenic activity observed in SFP crude extract, adipogenesis assay of the pure compound (−)-epicatechin was done on 3T3-LI preadipocytes.

(−)-Epicatechin Suppresses Lipid Accumulation in 3T3-L1 Cells. To investigate the potency of (−)-epicatechin that has been identified to be present within the bioactive fraction (F12) of SFP extract, adipogenesis assay of the pure compound at various concentrations (0 to 500 μM) were done on 3T3-L1 preadipocytes cells. Cytotoxicity of (−)-epicatechin was first determined using Cell Counting Kit-8 and as shown in Figure 5C, no significant effect on the proliferation of 3T3-L1 cells was observed even in the presence of (−)-epicatechin at high concentrations of up to 500 μM. Adipogenesis assay indicated that treatments of (−)-epicatechin significantly decreased lipid accumulation compared to its DMSO-treated positive control cells upon differentiation for 8 days. Subsequent Oil Red O staining and quantification showed that this suppression effect on lipid accumulation was dose-dependent. Lipid accumulation was reduced when treated with higher concentrations between 50-200 μM (−)-epicatechin as compared to the positive control while no significant reduction was observed when treated with 10 μM of (−)-epicatechin (Figure 5B). Thus, our results confirmed the suppression effect of (−)-epicatechin on adipogenesis in 3T3-L1 cells. As the only bioactive fraction isolated from crude SFP extract exhibits a major peak that shares the same retention time and m/z ratio of (−)-epicatechin and cross referencing to a paper that has identified the presence of this compound in star fruit, we therefore conclude that the bioactive compound in SFP extract responsible for the

reduction of lipid accumulation in 3T3-L1 cells is mainly due to the presence of (−)-epicatechin.

Molecular Docking of (−)-Epicatechin Binding to PPARα. After establishing the bioactive compound responsible for the anti-adipogenesis activity in SFP extract, elucidating the molecular mechanism between (−)-epicatechin and the target protein initially identified for the suppression activity via RT-PCR was then investigated. The upregulation of PPARα was of particular interest, as we believe that it is due the binding of (−)-epicatechin to this target protein that consequently results in the overall suppression of adipogenesis. Details of the predicted binding mode of (−)-epicatechin towards PPARα were presented in Figure 6. As we can see, the (−)-epicatechin in the predicted binding complex occupied the U-shaped ligand-binding pocket in a very similar fashion with the synthetic fibrate GW735 and BMS-631707 (Figure 6B). Among them, GW735 is a high-affinity ligand for PPARα[35] and BMS-631707[27, 36] is a PPARα/γ dual agonist. Comparison of the binding modes of these molecules in the binding structures may provide insights into the binding properties of (−)-epicatechin with PPARα. An overlay of bound (−)-epicatechin, GW735 and BMS-631707 in their configurations is shown in Figure 6B. The (−)-epicatechin dihydroxyl phenol group is located in the similar position and orientation as the GW735 carboxylate and the BMS-631707 carboxylate group. The two hydroxyls on the benzene ring are oriented toward the AF-2 helix and held in place through a network of hydrogen bonds with Y464 (Figure 6D). The same network of hydrogen bonds with the AF-2 helix occurs in the binding of ligands to PPARα and rosiglitazone in the ternary complex with PPARγ.[37] The conservation of this network of hydrogen bonds is believed to be crucial for ligand-mediated activation of these receptors. As a result, the binding of (−)-epicatechin may shift the equilibrium toward the active

configuration of PPARα via direct stabilization of the AF-2 helix through hydrogen bond with (−)-epicatechin. Moreover, the chromene group in (−)-epicatechin projects deeper into the cavity formed by helices H3 and H10 and form hydrogen bonds with the backbones of A454 and V270, thus enabling (−)-epicatechin to be firmly bound to PPARα (Figure 6D). Understanding the binding mode of (−)-epicatechin onto the target receptor puts things into greater perspective as of how the bioactive compound plays a significant role leading to the promotion of adipogenesis inhibition.

DISCUSSION

Overall, the initial set of crude SFP bioactivity data showed promising inhibition potential of adipogenesis of 3T3-L1 cells of over 80% and 100% inhibition at 500mg/L and 1000 mg/L concentration of crude peel extract respectively (Figure 1B) in comparisons to its flesh component that have been previously reported by Niwano et al.[38] It is with this set of data that we proceeded on with further isolation and purification to identify the likely bioactive compound responsible for peel extract's bioactivity. Our study identified that among the many polyphenols that are known to be present in *Averrhoa carambola,* the peel component of this fruit contains a useful polyphenol known as (−)-epicatechin, that is potentially useful in reducing lipid accumulation and adipocyte differentiation in 3T3-L1 cells. This study also further investigated how the SFP crude extract was able contribute to an overall reduction in lipid accumulation via analyzing the possible mechanism of the crude extract on adipocyte-specific genes at mRNA expression level. It was found that the inhibition of 3T3-L1 adipocyte differentiation was mainly due to the simultaneous downregulation of the expression of C/EBPα and PPARγ, and this is consistent to a study that has already investigated the effect of (−)-epicatechin in tea on protein

expression via western blotting.[39] Cross-referencing to a similar study conducted by Kim et al, they further highlighted that (−)-epicatechin also decreases the transcription FoxO1- the forkhead transcription factor class O1 involved in adipocyte differentiation- via PI3K (phosphoinositide 3-kinase)/Akt and MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular signal regulated kinase)] pathways.[40] However, the effect of (−)-epicatechin in the upregulating PPARα adipogenic genes as revealed in our study was not discussed in either paper, which made this observation particularly interesting. In a computational study conducted by Kersten et al and Fruchart et al respectively, they discovered how the binding of polyphenols to PPARα can lead to its activation and subsequently resulting in the sequential regulation of gene expressions involved in cellular lipid uptake and oxidation.[17, 18] It is therefore believed that the similar phenomena is happening in SFP extract whereby, the identified bioactive compound (−)-epicatechin binds and activates the PPARα receptor, particularly through hydrogen bonds formed between the hydroxyl groups of (−)-epicatechin and the critical residues in the ligand binding pockets mainly Y464 and H440 in the AF-2 helix of PPARα, thereby accounting for the overall suppression in adipogenesis via elevation of fatty acid oxidation and lipolysis.[41] Although we believe that further investigation is required to confirm the binding activity of (−)-epicatechin to PPARα, the computational docking for now would suffice to support our existing experimental observation. Further studies such as utilizing luciferase activity to analyze the promoter binding activity could be done in the future to draw a solid conclusion of its binding relationship.[40]

Identification of (−)-epicatechin as a bioactive compound responsible for the inhibition of lipid accumulation and anti-adipogenic activity in the peel component of

star fruit has never been reported previously and we believe that this could potentially be a significant breakthrough to future studies in discovering more sources to treat obesity and its related diseases; as (−)-epicatechin is known to be widely available not only in tea[39, 40] but also in various fruits and vegetables such as blackberry, broad bean, apple and black grape.[42, 43] In summary, the data that we have presented have shown the capacity of star fruit peel's beneficial effect in treating obesity and obesity-related diseases. Although further investigation is required to study the physiological relevance of these results, the identification of the bioactive compound and the molecular relationship of the relevant active compounds to target protein receptors elucidated, are useful in highlighting the potential of SFP extract as a possible candidate for alternative source of treatment for obesity and its related diseases in the near future.

ACKNOWLEDGEMENT

D. Zhang was supported by COS Collaborative Research Grant M4080928 in Nanyang Technological University, and also thanks the NTU HPC for support and resources.

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Table 1. Primer Sequences used for PCR

gene	forward primer	reverse primer	annealing temperature (° C)
PPARα	AGGCTGTAAGGGCTTCTTTCG	GGCATTTGTTCCGGTTCTTC	54
PPARβ	TCCATCGTCAACAAAGACGGG	ACTTGGGCTCAATGATGTCAC	55
PPARγ	CGCTGATGCACTGCCTATGA	AGAGGTCCACAGAGCTGATTCC	57
C/EBPα	CGCAAGAGCCGAGATAAAGC	CACGGCTCAGCTGTTCCA	56
aP2	CATGGCCAAGCCCAACAT	CGCCCAGTTTGAAGGAAATC	54
LPL	ATCGGAGAACTGCTCATGATGA	CGGATCCTCTCGATGACGAA	55
GAPDH	GGTGAAGGTCGGTGTCAACG	CAAAGTTGTCATGGATGACC	51

Figure caption

Figure 1. Effect of star fruit peel extract on adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was performed with or without treatment of 1000 mg/L, 500 mg/L, 100 mg/L, 10 mg/L star fruit peel extract for the whole adipogenic period. On day 8, cells were stained with Oil Red O. (A) Observation of cell after Oil Red O staining under phase contrast microscopy 40X objective (lane 1), 10X objective (lane 2), 4X objective (lane 3) or naked eye (lane 4). (B) Quantitation of lipid accumulation determined by Oil Red O. (C) Cytotoxicity of star fruit peel extract measured by cell viability assays. *p<0.05 compared with positive control. **p<0.01 compared with positive control.

Figure 2. Effect of star fruit peel extract on the expression of adipogenic genes. Adipocyte differentiation was performed with or without treatment of 1000 mg/L, 500 mg/L, 100 mg/L, 10 mg/L star fruit peel extract for the whole adipogenic period. On day 8, cells were harvested and mRNA expressions were evaluated by RT-PCR. The bands were analysed using ImageQuant TL (version 7.0). Negative: negative control; Positive: positive control (without treatment of star fruit peel extract). Three replicates were used to represent the error bars. *p<0.05 compared with positive control, **p <0.005 compared with positive control.

Figure 3. (A) TLC spots of fractions 6-12 after separation via silica column chromatography. (B) Effect of star fruit peel extract fractions on adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was performed with or without treatment of 100 mg/L each fraction for the whole adipogenic period. On day 8, cells were stained with Oil Red O. Observation of cell after Oil Red O staining under phase contrast microscopy 40X objective (lane 1), 10X objective (lane 2) or naked eye (lane 3) objective. F1 (1.5 mg) and F3 (1.2 mg) were excluded from

analysis as the amount is too small.

Figure 4. HPLC analysis of Fraction 12 (F12). (A) HPLC trace of SFP extract fraction 12. (B) HPLC spectrum of SFP fraction 12 with 20% co-injection of (-)-epicatechin. (C) HPLC spectrum of SFP fraction 12 with 50% co-injection of (-)-epicatechin. (D) HPLC spectrum of pure (+)-catechin and (-)-epicatechin overlapped with one another (E) Overlayed HPLC spectrum of SFP fraction 2 extract, highlighting the difference in peak area of peak 1, when co-injected at different volumes, 20% and 50% respectively.

Figure 5. Effect of (−)-epicatechin on adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was performed with or without treatment of 200 μM, 100 μM, 50 μM, 10 μM (−)-epicatechin for the whole adipogenic period. On day 8, cells were stained with Oil Red O. (A) Observation of cell after Oil Red O staining under phase contrast microscopy 40X objective (lane 1), 10X objective (lane 2), 4X objective (lane 3) or naked eye (lane 4). (B) Quantitation of lipid accumulation determined by Oil Red O. (C) Cytotoxicity of (−)-epicatechin measured by cell viability assays. *p<0.05 compared with positive control. **p<0.01 compared with positive control.

Figure 6. Predicted PPARα–(−)-epicatechin binding structures. (A) The final snapshot of the 3-ns MD simulation of PPARα–(−)-epicatechin complex. The PPARα backbone is represented by the yellow ribbon, and (−)-epicatechin is represented with vdw and is color coded as follows: carbon, cyan and oxygen, red. (B) Superposition of the structures of GW735 (green) and BMS-631707 (mauve) bound to PPARα. (C) Chemical structures of the compounds described in (B). (D) Hydrogen bonds formed by the (−)-epicatechin and the surroundings are indicated as green dotted lines.

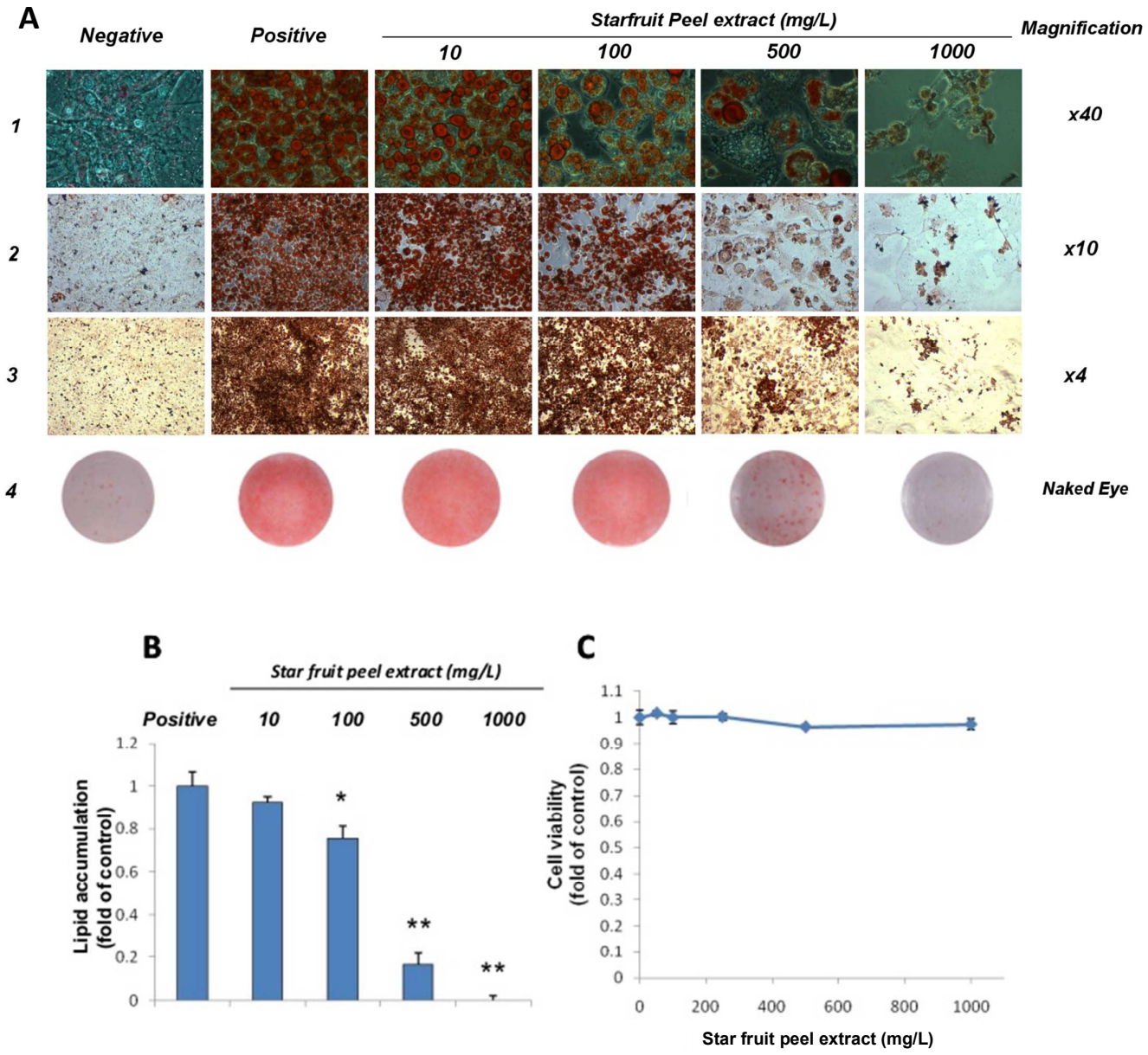


Figure 1

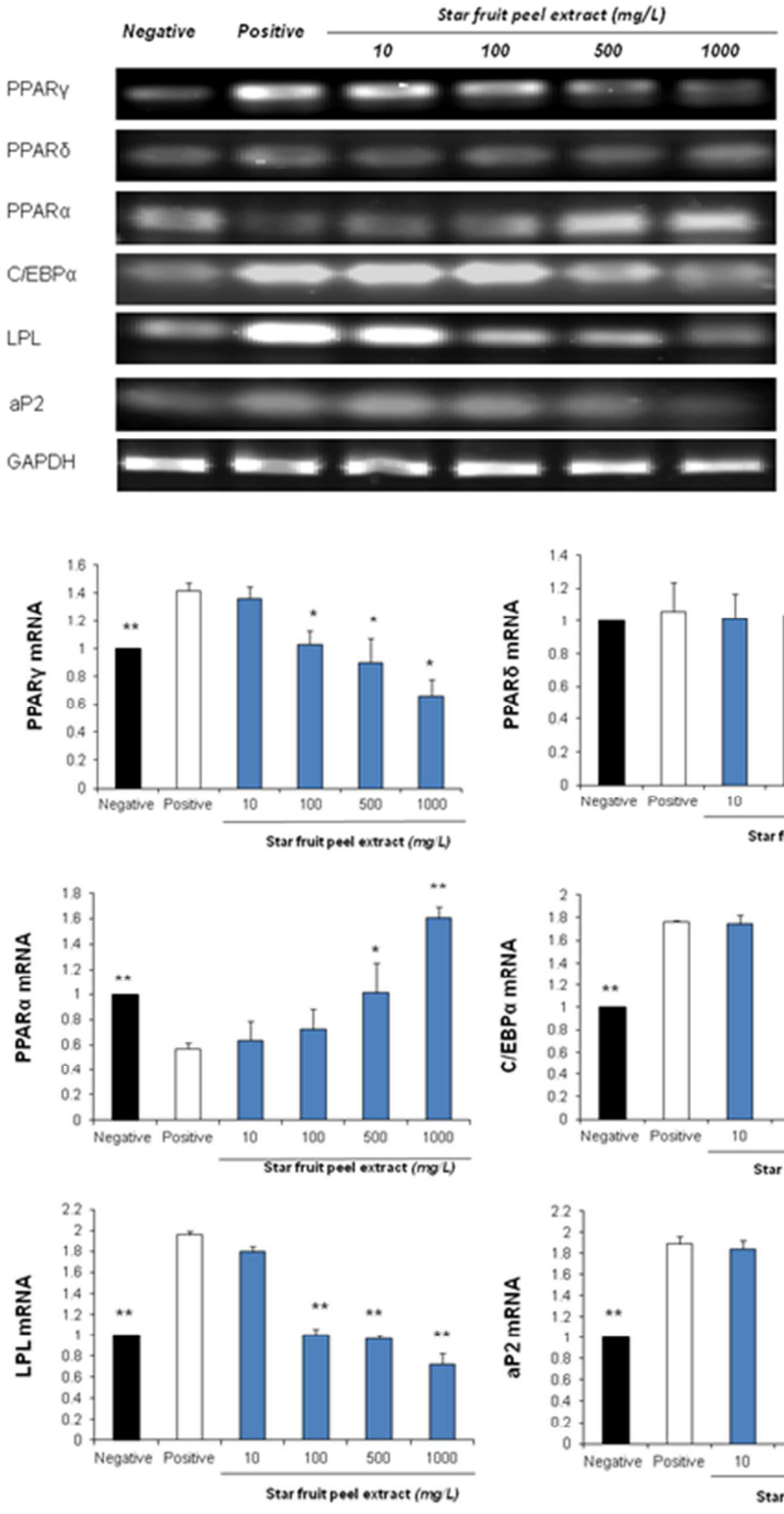
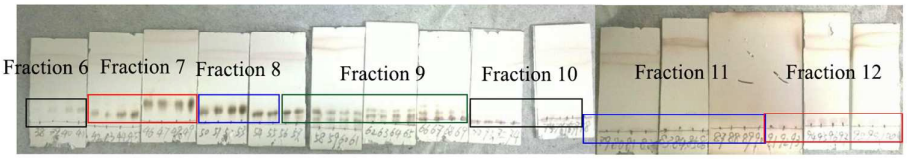


Figure 2

(A) The homogeneous methanol extract was subjected to further separation via silica gel chromatography with a sequential gradient elution of DCM:MeOH:formic acid from 100:0:2 to 0:100:2, to yield 14 fractions



(B)

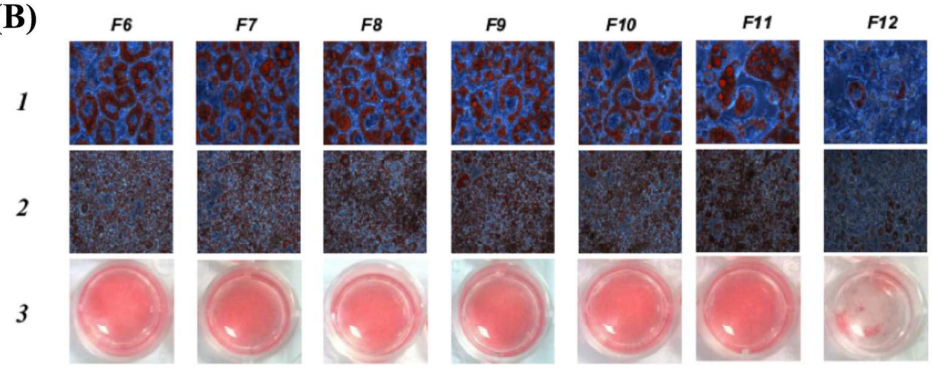


Figure 3

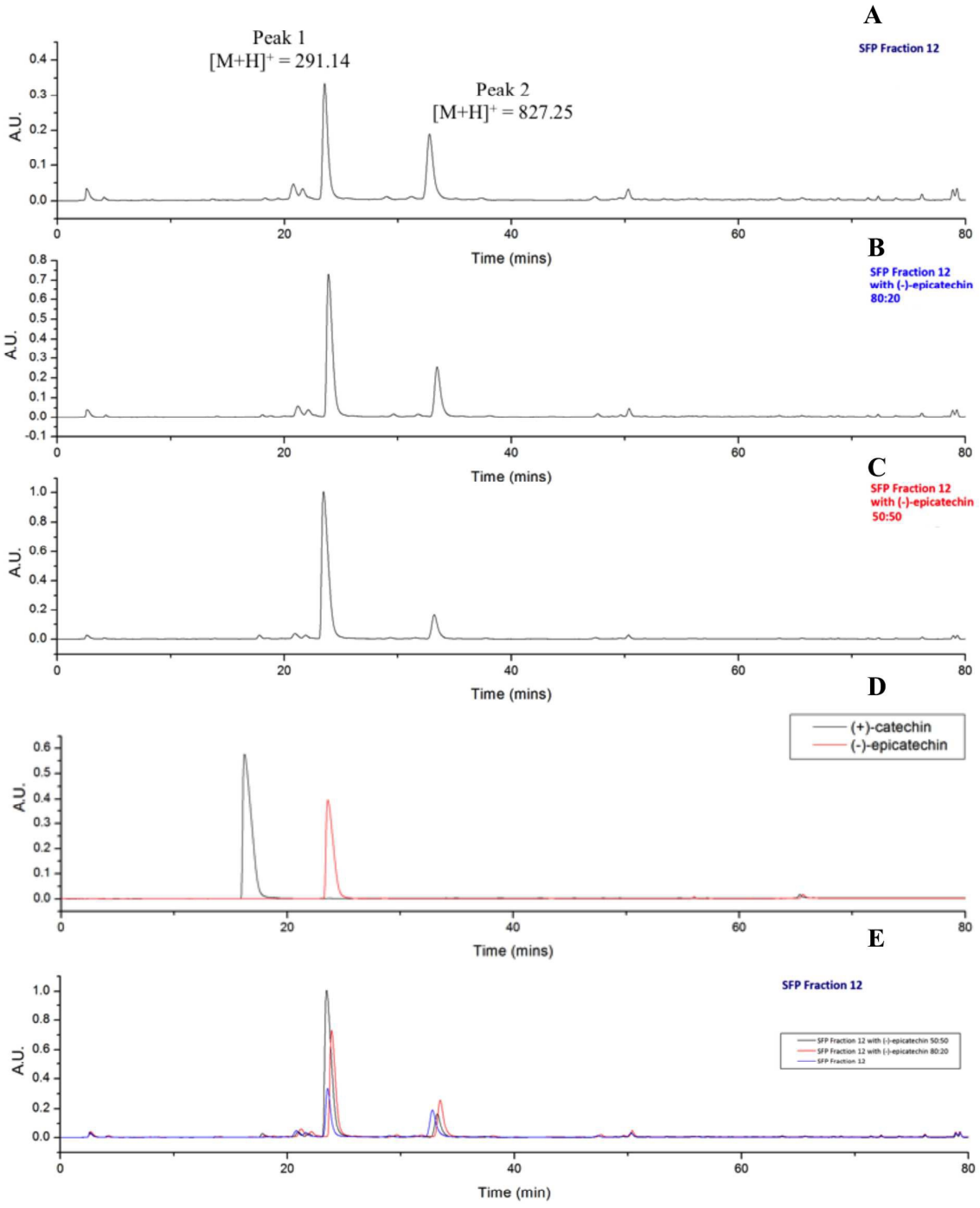


Figure 4

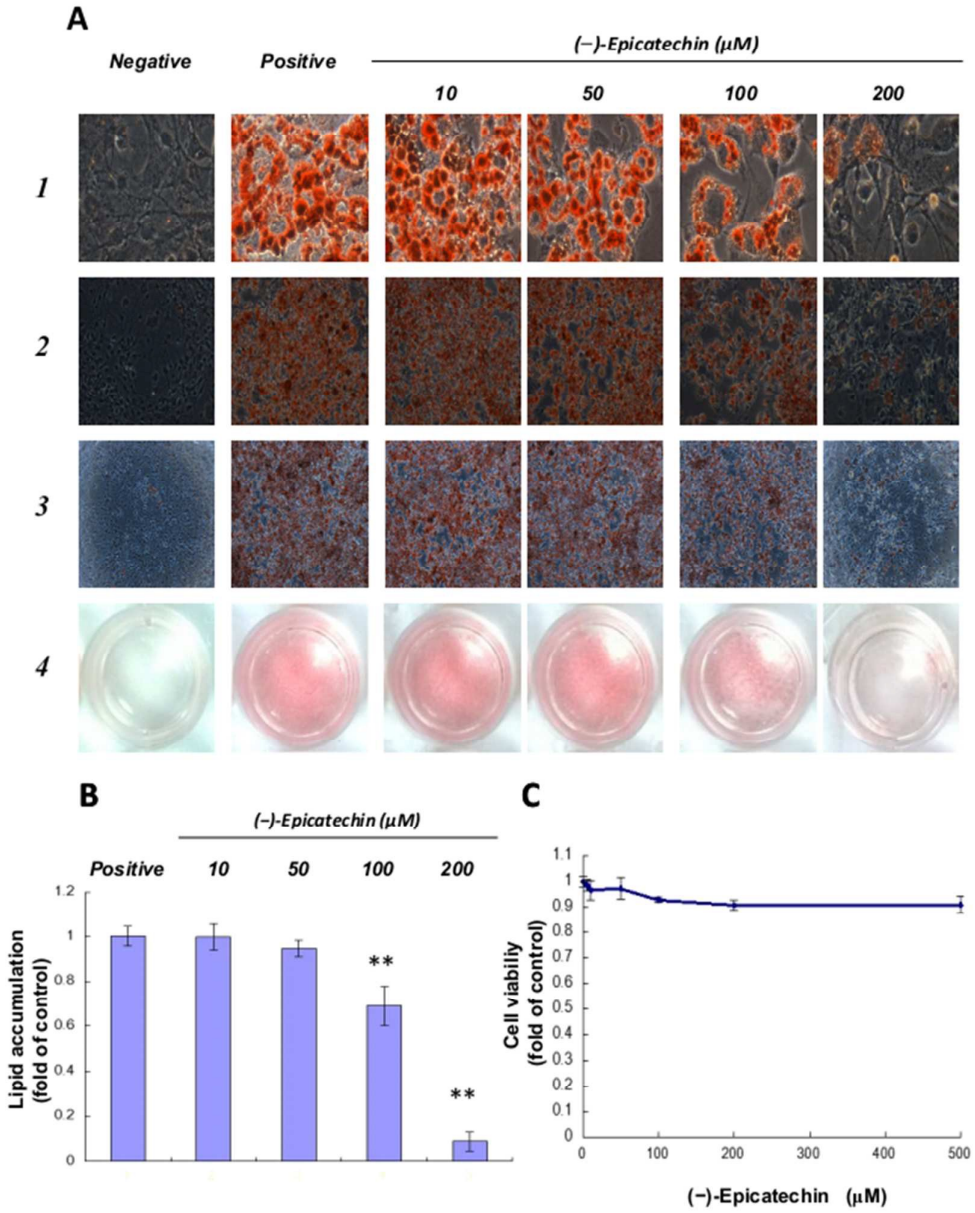


Figure 5

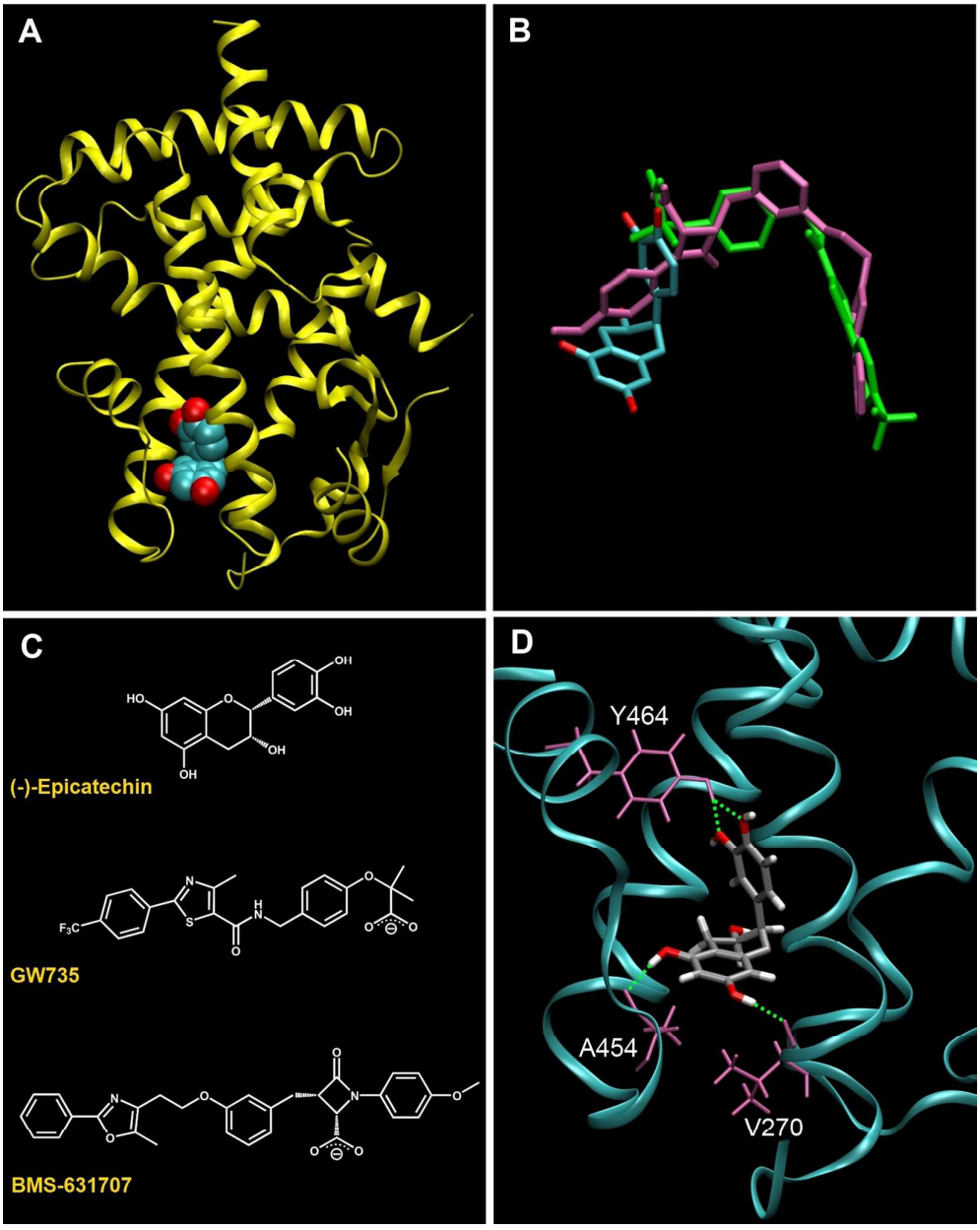


Figure 6

For Table of Contents Only