



Cytotoxic, Anti-Inflammatory and Adipogenic Effects of Inophyllum D, Calanone, Isocordato-oblongic acid, and Morelloflavone on Cell Lines

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Abstract – This paper reports *in vitro* cytotoxic, anti-inflammatory and adipocyte differentiation with adipogenic effects of coumarins inophyllum D (**1**) and calanone (**2**), and a chromanone carboxylic acid namely isocordato-oblongic acid (**3**) isolated from *Calophyllum symingtonianum* as well as a biflavonoid morelloflavone (**4**) isolated from *Garcinia prainiana* on MCF-7 breast adenocarcinoma RAW 264.7 macrophages and 3T3-L1 preadipocytes cells, respectively. The cytotoxicity study on MCF-7 cell was conducted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Meanwhile, the study of anti-inflammatory effects in RAW 264.7 macrophages and adipogenic effects on 3T3-L1 pre-adipocytes were conducted through nitrite determination assay and induction of adipocyte differentiation, respectively. In the cytotoxicity study, inophyllum D (**1**) was the only compounds that exhibited significant cytotoxic effect against MCF-7 cell with IC₅₀ of 84 µg/mL. Further, all by inhibiting the compounds have shown anti-inflammatory effects in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages of nitrite concentration with production. In addition, the compounds also exhibited adipogenic effects on 3T3-L1 pre-adipocytes by stimulating lipid formation. Thus, this study may provide significant input in discovery of the potential effects cytotoxic, anti-inflammatory and adipogenic agents.

Keywords – Cytotoxic, Anti-inflammatory, Adipogenic

Introduction

Generally, plants produce vast and diverse organic compounds. According to Hanson,¹ natural products refer to organic compounds that are synthesized by living organisms and they can be divided into three vast categories. The first category is primary metabolites which found in all plants and vital for metabolic functions. Meanwhile, the second category is important for cellular structures which consist of high molecular weight polymeric materials such as cellulose, proteins and lignins. The third category of naturally occurring compounds is secondary metabolites. Plant secondary metabolites specifically are usually distributed differentially among limited range of

species and they usually do not involve directly in growth and development.² The secondary metabolites mainly promote biological effects on other organisms or in other words influence ecological interactions between the plants and their environment.^{1,2} Due to these interesting biological activities possessed by secondary metabolites, they have been the major sources for development of pharmaceutical products against many diseases over the past century and continue to play the irreplaceable role up until today.³

The search for new alternative therapeutic approaches on many current dreaded diseases, such as cancers, antiinflammatory and diabetes diseases has becoming the most prominent study among the researchers throughout the world. Until recently, nature has continuously been regarded as the most valuable reservoir of therapeutic agents against wide range of diseases in modern drug discovery and development. As part of the study in

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searching bioactive secondary metabolite from plant, we have successfully isolated few compounds from different plant species. Thus, in this study, the compounds inophyllum D (**1**), calanone (**2**), isocordato-oblongic acid (**3**) and morelloflavone (**4**), and, isolated from *Calophyllum symingtonianum* and *Garcinia prainiana* have been evaluated for their potential bioactivities, especially cytotoxic, anti-inflammatory and adipogenic effects. To the best of our knowledge, there is no previous report of the compounds on their bioactivity on MCF-7, RAW 264.7 and 3T3-L1 cell lines.

Experimental

Materials – Dulbecco's modified eagle media (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (pen-strep), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Gibco (Invitrogen Corporation, USA). Griess reagent, Oil red O, insulin and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich, USA; dimethylsulfoxide (DMSO), isopropanol and ethanol from Thermo Fisher Scientific, USA; and 3-isobutyl-1-methyl-xanthine (IBMX) and dexamethasone from Calbiochem, Germany.

Isolation of inophyllum D (1), calanone (2), and isocordato-oblongic acid (3) – Isolation of inophyllum D (**1**), calanone (**2**), and isocordato-oblongic acid (**3**) were isolated from *Calophyllum symingtonianum* according to Aminudin et al.⁴

Isolation morelloflavone – The dried and powdered of stem bark of *Garcinia prainiana* (350 g) was extracted by soxhlet extractor for 18 hours with *n*-hexane (2.5 L) and methanol (2.5 L) successively at room temperature. Concentration of each solvent under reduced pressure afforded a crude *n*-hexane extract (13 g) and crude methanol extract (21 g) as a sticky brown liquid. The methanol extract (10 g) was submitted to vacuum liquid chromatography (VLC) on silica gel using gradient of chloroform-ethyl acetate 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and ethyl acetate, furnishing 9 fractions. Fraction 7 (150 mg) was applied to silica gel chromatography column eluting with chloroform-methanol (99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 91:9, 90:10) led to the isolation of biflavanoids characterized as morelloflavone (**3**, 21 mg).⁵

Cell Lines – 3T3-L1 pre-adipocyte and RAW 264.7 macrophage cell were obtained from American Type Culture Collection (ATCC), USA. Meanwhile, MCF-7 breast adenocarcinoma cell line was obtained from courtesy of Prof. Masa-Aki Ikeda of Tokyo Medical and Dental University, Japan.

Cell Culture – Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, and 2% Penstrep. The cells were maintained at 37°C in 5% CO₂ humidified air.

Cytotoxicity Assay – This test was carried out on MCF-7, RAW 264.7 and 3T3-L1 cells to determine the cytotoxic activity of the compounds against these cell lines. The cytotoxic activity or indication of cell viability on RAW 264.7 and 3T3-L1 cells was further analysed to determine the suitable dosage of the compounds for nitrite determination test and adipocyte differentiation respectively. Assay was performed according to previously described method.⁶ The cells were seeded into 96-well plate treated with the samples with varying concentrations (3.125 - 100 µg/mL) for 24 hours. Then, media was removed and cells were washed once with PBS and then 20 µL of MTT solution (5 mg/mL) was added to each well. The cells were further incubated for 4 hours at 37 °C in a humidified 5% CO₂ incubator. Then, 100 µL DMSO was added to each well and the absorbance was measured at a wavelength of 570 nm and reference wavelength of 630 nm with a microplate reader. Results were expressed as percentage of cell viability as compared with control group. By extrapolation from the linear regression of the data of cytotoxic effects against MCF-7 cell, the 50% reduction in cell number relative to control or IC₅₀ was established.

Nitrite Determination Assay – RAW 264.7 cells were seeded at density of 2×10^5 cells/mL in 24-well culture plate and pre-incubated with and without a range of five different suitable doses concentrations of the compounds (2.5 - 50 µg/mL) for 1 hour in a humidified 5% CO₂ incubator at 37 °C. Then, the treatment and control wells were added with 1 µg/mL lipopolysaccharides (LPS) and re-suspended. Meanwhile, the negative wells contained only DMEM complete medium. The plate was further incubated for 15 hours at 37 °C in a humidified 5% CO₂ incubator. 50 µL of each medium supernatant was then mixed with 50 µL Griess reagent (1:1) (40 mg/mL) and incubated at room temperature for 10 minutes. The absorbance at 540 nm was measured and the results were compared with sodium nitrite (NaNO₂) standard curve to determine the nitric oxide (NO) production.⁷

Adipocytes Differentiation (Adipogenesis) – 3T3-L1 preadipocytes cells were seeded into 24-well plate at a density of 2×10^4 cells/mL and grown to confluent. Two days post confluent (Day 0) cells were induced to differentiate using differentiation cocktail (complete DMEM, 0.5 mM IBMX, 0.25 µM dexamethasone, 1 µg/mL insulin). To examine compounds effect on adipogenesis, insulin

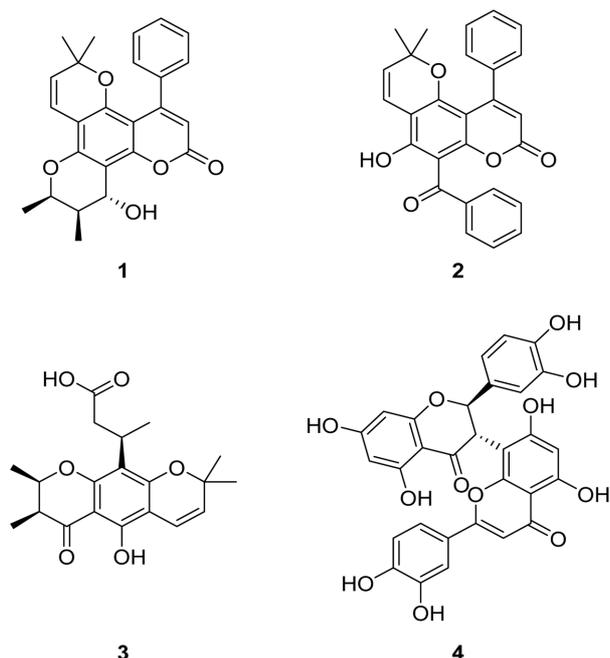


Fig. 1. Structure of tested compounds.

was replaced with various compounds' concentration WITH effect of the compounds on adipogenesis, insulin was replaced with various concentration of the compounds. After two days (Day 2), medium was replaced with maintenance media (complete DMEM, 1 $\mu\text{g}/\text{mL}$ insulin).⁸ Adipocyte differentiation was both analysed qualitatively and quantitatively after ten days (day 10) differentiation stimulation. Cells were washed with PBS three times and fixed with 10% formalin for one hour at room temperature. The cells were then washed once again with PBS and stained with freshly diluted and filtered Oil Red O solution (three parts of 0.6% Oil Red O in isopropanol and two parts of water) for another one hour. Further, the cell were washed twice with distilled water and observed under microscope for qualitative analysis. For quantitative analysis, stained cells were dissolved with isopropanol and then absorbance was measured at 520 nm.

Statistical Analysis – Results for the cytotoxic, anti-inflammatory and adipogenic effects were presented as mean \pm standard error or standard deviation which interpreted using Microsoft Office Excel 2013®.

Result and Discussion

Cytotoxicity Effects of the Compounds on MCF-7 breast adenocarcinoma cell – The evaluation of the cytotoxic effects of inophyllum (1), isocordato-oblongic acid (2), calanone (3) and morelloflavone (4) was done by

using MTT assay. MTT assay was used to measure the cell viability of MCF-7 cell line after exposure to a range of concentration of the compounds for 24 hours. The concentrations were 3.125 to 100 $\mu\text{g}/\text{mL}$ of each of the compounds. DMSO was used as a control of the assay, at a final tested concentration 0.1% of DMSO and lower (the same percentage of DMSO concentration in 100 to 3.125 $\mu\text{g}/\text{mL}$ of compound concentrations) did not affect cell viability significantly on MCF-7 cells (data not shown). Meanwhile, the group of cells which were not exposed to any of treatment represented as the control group of the assay and was considered as 100% of viable cells. Results were expressed as percentage of cell viability as compared with the control group.

Based on the results showed in Figure 2 (A, B and C), all range of the tested concentration of isocordato-oblongic acid (2), calanone (3) and morelloflavone (4) showed no obvious inhibition trend on cell viability of MCF-7 cells. The IC_{50} can't be extrapolated and tested concentration of the assay showed more than 80% of percentage of cell viability. On the other hand, inophyllum D (1) (Fig. 2D) showed cytotoxic effects with an obvious inhibition trend on cell viability percentage with IC_{50} value obtained was 84 $\mu\text{g}/\text{mL}$. In the evaluation of cytotoxicity study, the presence of cytotoxic effects of inophyllum D (1) against other cancer cells has been reported.⁹ It was reported that inophyllum D (1) showed a weak cytotoxic effects against Raji cells and it was suggested that the compound may possess a potential cancer chemopreventive property. However, the cytotoxic effect of inophyllum D (1) was much lower as compared to the cytotoxic effect of currently available anti-cancer agents against MCF-7 human breast cancer, doxorubicin. Previously, it was reported that the IC_{50} of doxorubicin against MCF-7 human breast cancer was 0.417 $\mu\text{g}/\text{mL}$.¹⁰

Anti-inflammatory Effects of the Compounds on RAW 264.7 Macrophages – The cytotoxic effects of the selected flavonoids on RAW 264.7 cells were first measured using the MTT assay (Fig. 3) for possible cytotoxic effect of compounds towards cell line and determination of suitable dosage concentration in subsequent nitrite determination assay. Based on the results, the concentrations of 10 to 50 $\mu\text{g}/\text{mL}$ of isocordato-oblongic acid (2), calanone (3) and morelloflavone (4) were chosen for tested concentrations in nitrite determination since the viability of the cells was not affected after 24 hours of treatment in indicated concentration. Meanwhile, inophyllum D (1) exerted significant cytotoxic effect in dose dependent manner. The concentration of 25, 50 and 100 $\mu\text{g}/\text{mL}$ were excluded for the following nitrite determination test

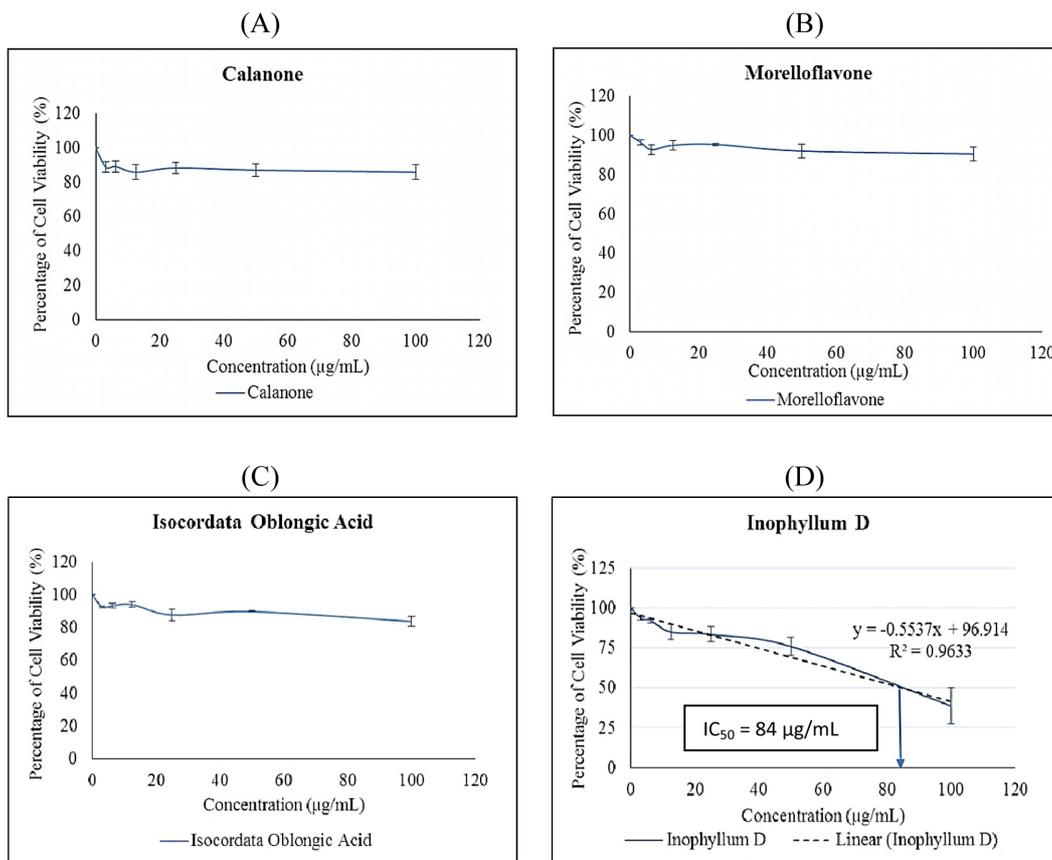


Fig. 2. Cytotoxic effects of selected flavonoids on MCF-7 cells by MTT assay. Results are presented as mean \pm SD of triplicate of three experiments. (A) Calanone (B) Morelloflavone (C) Isocordato-oblongic acid (D) Inophyllum D.

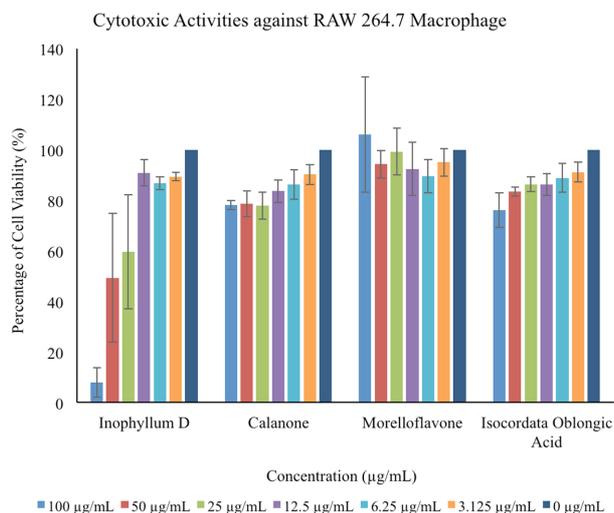


Fig. 3. Cytotoxic effects of selected flavonoids on RAW 264.7 cells by MTT assay. Results are presented as mean \pm SD of triplicate of three experiments.

because of the significant reduction in the cell viability of treatment concentration against control group shown in those concentrations. The chosen suitable dose for ino-

phyllum D (**1**) was in range of 2.5 to 20 $\mu\text{g/mL}$.

In nitrite determination assay, the inhibition of nitric oxide (NO) production can be evaluated by using Griess reaction. The assay is a rapid and simple method that can be used to determine the NO production by measuring the concentration of nitrite, a stable oxidized product of NO in cell culture media.¹¹ The reduction in NO production indicates the anti-inflammatory activities in the cells. Based on the Figure 4 (C and D), the treatment of isocordato-oblongic acid (**3**) and inophyllum D (**1**) showed a dose dependent suppression of NO production. Meanwhile, Figure 4 (A and B) also clearly showed that calanone (**2**) and morelloflavone (**4**) suppressed the level of NO production in LPS-stimulated RAW 264.7 macrophages as compared to the LPS-stimulated cells group without any treatment.

In nitrite determination assay, anti-inflammatory activities can be evaluated by measuring the inhibition of nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) in LPS stimulated RAW 264.7 cells which have been treated with the compounds. iNOS is highly

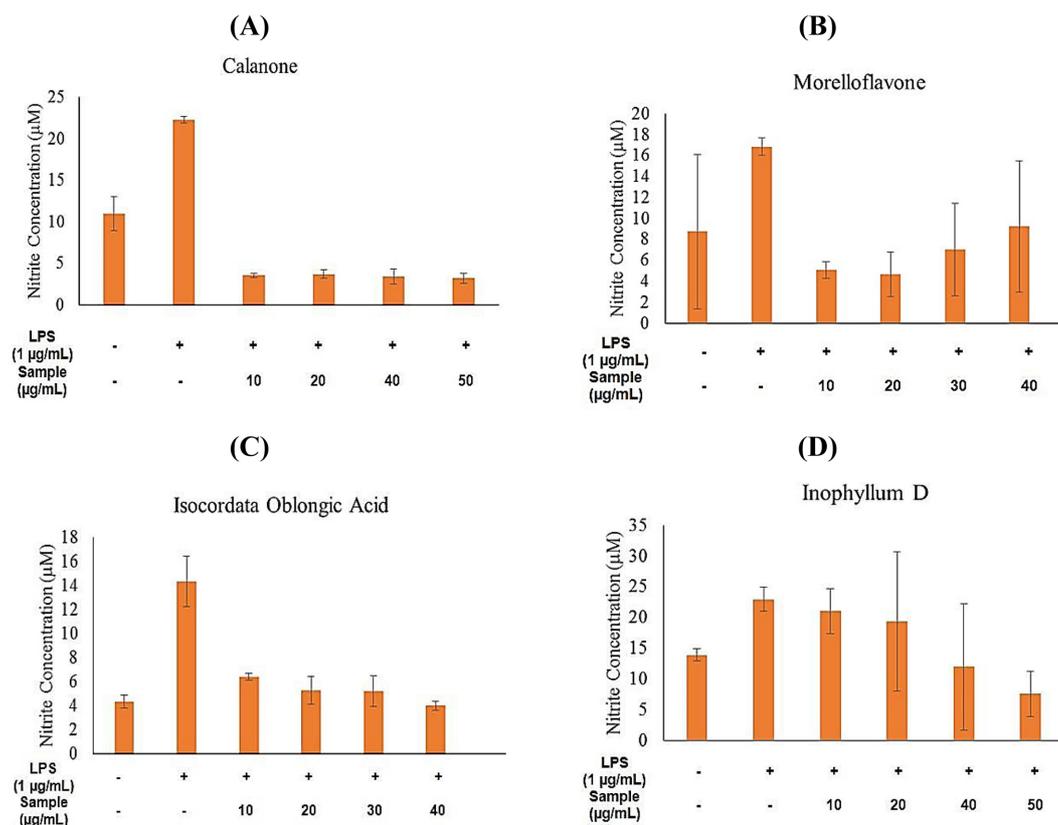


Fig. 4. Anti-inflammatory effects of selected flavonoids on RAW 264.7 cells by nitrite determination test. The data shown were nitrite concentration against experimental groups for (A) Calanone (B) Morelloflavone (C) Isocordato-oblongic Acid (D) Inophyllum D. Results are presented as mean \pm SD of triplicate from one independent experiment.

associated with the inflammation mechanism.¹² iNOS is present in macrophages and also in many other cells such as endothelial, hepatocytes, cardiac myocytes and respiratory epithelial cells. Briefly, tissue macrophage plays an important role in cellular and humoral immune responses. In response to stimuli such as pathogens or tissue damage, tissue macrophages are initially activated by the classical pathway which was designed to destroy and eliminating the foreign agent and dead tissues. One of the mechanisms that occur at the site of inflammation is an increase in iNOS expression and activation. iNOS in macrophage can be induced by a number of different mediators of inflammation such as interleukin-1, tumor necrosis factor, and interferon- γ as well as by bacterial endotoxin. When induced, iNOS produces NO. NO produced in activated macrophages is a short-lived, miscible and free radical gas product with several functions at the site of inflammation.¹³

It was clearly shown that all the compounds suppressed the level of NO in LPS-stimulated RAW 264.7 cell. It was suggested that the suppressed level of NO was due to the suppression of iNOS activities. Theoretically, NO is

produced when the activity of iNOS enzyme in the macrophage is activated by stimuli (LPS) induction.¹³ In this study, it can not be assumed that the suppression of iNOS activities by the compound probably due to the suppressive effects of iNOS expression level in LPS-stimulated RAW 264.7 macrophage since this study did not proceed to the examination of the level of iNOS enzyme expression by western blotting or examination of mRNA expression levels of iNOS by reverse transcriptase polymerase chain reaction (RT-PCR). It was also not clearly understood that the inhibitory activity of inflammation was due to direct inhibition of iNOS enzyme activity that can be measured using cell homogenate, based on the conversion of [3 H] arginine to [3 H] citruline, or due any other indirect possibilities.¹⁴ The possible mechanism of the compounds in producing anti-inflammatory was unknown and cannot be described in detail in this current study.

Adipogenic Effects of the Compounds on 3T3-L1 Pre-adipocyte – The compounds were also tested on its effect on 3T3-L1 cell line using MTT assay. Based on the results (Fig. 5), the three concentrations of 10, 25, and 50

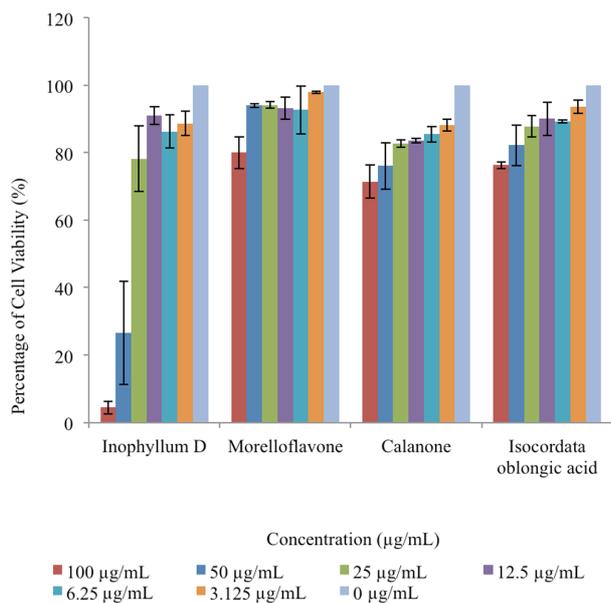


Fig. 5. Cytotoxic effects of selected flavonoids on 3T3 L1 pre-adipocyte. Results are presented as mean ± SD of triplicates of two repeated experiments.

µg/mL of isocordato-oblongic acid, calanone and morelloflavone were chosen for tested concentrations in adipocyte differentiation since the viability of the cells was not affected after 48 hours of treatment. In contrast, inophyllum D exerted a significant cytotoxic effect in dose dependent manner. The concentration of 50 and 100 cell µg/mL were excluded for the subsequent assay because of the significant reduction in the cell viability of treatment concentration against control group shown in those concentrations. The chosen suitable dosages for inophyllum D were 5, 15 and 25 µg/mL.

In adipocyte differentiation, the compounds were examined whether inducing or inhibiting differentiation of 3T3-L1 pre-adipocyte into mature adipocyte within 10 days post differentiation stimulation. Accumulation of lipid droplets indicated the adipogenic effects of the compounds. At day 10, the cells were stained with Oil Red O staining to observe lipid accumulation in differentiated cells. Further quantification of lipid accumulation was done by measuring the absorbance of Oil Red O extracted from the cells using isopropanol with a microplate reader.

Hence, it is expected that by targeting the regulation of adipogenesis, abnormal energy regulation and insulin insensitivity that occur in diabetes and obesity-mediated metabolic syndrome can be overcome. The process involved complex signalling events between several transcription factor families such as the CCAAT/enhancer binding protein

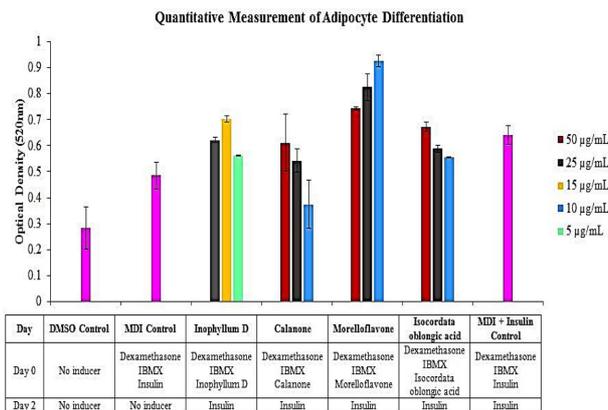


Fig. 6. Effects of selected flavonoids on 3T3-L1 differentiation. All values were presented as mean ± SD indicating a measure of the dispersion of a set of data from its means from one independent experiment with three replications.

(C/EBP), peroxisome proliferator-activated receptor (PPAR) and sterol regulatory element-binding protein-1c (SREBP-1c) families.¹⁵

In this experiment, the compounds were examined for their activities whether inducing or inhibiting differentiation of pre-adipocyte into mature adipocyte. Accumulation of lipid droplets indicated the adipogenic effects of the compounds. The results showed that calanone isocordato-oblongic acid, and inophyllum D exhibited enhanced adipogenic effects (Fig. 6). As the concentration of the compounds increased, the lipid accumulation also increased. As compared to the positive control of this experiment (MDI + insulin), the increasing dose-dependent lipid accumulation in the treated cell groups with these three compounds demonstrated that they potentially exhibit the activities of mimicking insulin action in adipogenesis. In inophyllum D, the lipid accumulation at 25 µg/mL was shown to be decreased slightly as compared to the intermediate concentration of inophyllum D (15 µg/mL) yet still higher as compared to low concentration treatment (5 µg/mL). Thus, due to the greater lipid accumulation in both high and intermediate concentration treatment in comparison to the low concentration treatment, it was suggested that inophyllum D treatment exhibited an enhanced property of adipocyte differentiation as the concentration increases. It was also suggested that the slight decreased in lipid accumulation was due to the small effect of cytotoxic activities on the 3T3-L1 pre-adipocyte since the cell viability showed the percentage of cell viability at 25 µg/mL was slightly reduced (78.18%). Hence, it also suggested that the best concentration of inophyllum D in enhancing adipocyte differentiation was at the intermediate concentration of 15 µg/mL. The lipid

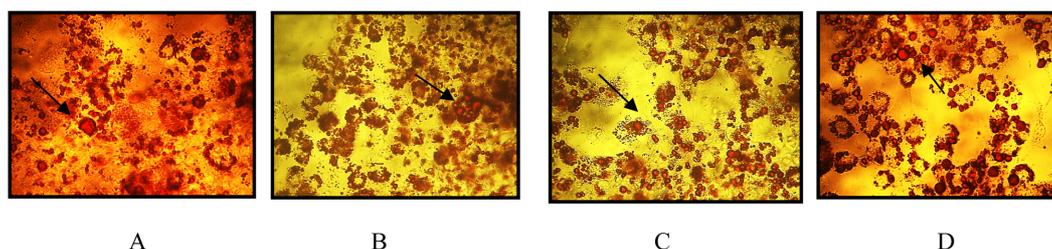


Fig. 7. Adipogenic effect of inophyllum D (A), calanone (B), morelloflavone (C) and isocordato-oblogic acid (D) at 25 $\mu\text{g}/\text{mL}$ on 3T3-L1 adipocyte. The cells were stained with Oil red O at 10 days after induction (400 \times magnification). Arrows indicated the presence of lipid droplet.

accumulation of adipogenic effect could be observed (Fig. 7). By using this visual observation, it was clearly observed the adipogenic effect of the compound on 3T3-L1 adipocytes.

In contrary, morelloflavone showed a significant decrease in lipid accumulation in the increasing dose-dependent manner. The diminished effect of adipogenesis of morelloflavone may suggest that morelloflavone potentially exhibits anti-obesity properties at higher concentration. According to the paper reviewed by Yun,¹⁶ a potential treatment of obesity can be from naturally-occurring compounds that specifically promote inhibitory effect on adipocyte differentiation. It was emphasized that the potential anti-obesity properties may exhibit in the higher concentration of morelloflavone (higher than tested concentrations).

Inophyllum D was the only compound that exhibited significant cytotoxic effect against MCF-7 cell with IC_{50} of 84 $\mu\text{g}/\text{mL}$. Further, all the compounds have shown anti-inflammatory effects in LPS-induced RAW 264.7 macrophages with inhibition of nitrite concentration as compared to the positive control. Last but not least, all the compounds in the range of tested concentrations exhibited potential property of activities. Mimicking the insulin adipogenic and lipid formation inhibitor.

Acknowledgments

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