Methods Article

Progress in Drug Discovery & Biomedical Science

HH PUBLISHER

A reliable and reproducible assay for determining the effect of natural product on macrophages lipid uptake and cholesterol efflux: A case study of maslinic acid

Bee Kee Ooi[1], Nafees Ahemad [2], and Wei Hsum Yap[1*]

[1]School of Biosciences, Taylor's University, Subang Jaya, Selangor, Malaysia.
[2]School of Pharmacy, Monash University Malaysia, Petaling Jaya, Selangor, Malaysia

Abstract : Macrophage foam cell formation represents a key feature that contributes to the development of atherosclerotic lesions. Assessment of cardioprotective natural compounds targeting macrophage foam cell formation processes including lipid uptake and cholesterol efflux could lead to the identification of potential lead compounds for development into novel anti-atherosclerotic drugs. In this case study, maslinic acid, a natural product was used to study the effect on lipid uptake and cholesterol efflux in THP-1-derived macrophages. Oil red O (ORO) staining and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled oxidized low-density lipoprotein (Dil-labeled oxLDL) uptake assays were performed to determine lipid uptake by macrophages while cholesterol efflux was assessed using 3-hexanoyl-NBD labeled cholesterol. ORO-stained images were further analyzed using ImageJ analysis software to determine intracellular lipid droplets accumulation and flow cytometric analysis of median fluorescence intensity were obtained to quantify Dil-labeled oxLDL uptake by macrophages. Meanwhile, 3-hexanoyl-NBD labeled cholesterol uptake and efflux from THP-1-derived macrophages were characterized. The fluorescence intensity values obtained from the medium and cell lysates were used to determine the cholesterol efflux. The results have shown that incubation with maslinic acid suppressed oxLDL-induced macrophage foam cell formation which may be contributed from its effect in reducing lipid uptake and enhancing cholesterol efflux. In conclusion, the optimized ORO staining, Dil-labeled oxLDL uptake, and fluorescent-labeled cholesterol efflux assays provide reproducible and reliable results for assessment of foam cells formation.

Keywords: Low-density lipoprotein (LDL); Oil red O (ORO), 1,1′-Dioctadecyl-3,3,3,3′-tetramethylindocarbocyanide perchlorate (Dil); 3-hexanoyl-NBD labeled cholesterol

Received: 30th July 2019
Accepted: 28th August 2019
Published Online: 10th September 2019

***Correspondence**: Yap Wei Hsum, School of Biosciences, Taylor's University, Subang Jaya, Selangor, Malaysia; weihsum.yap@taylors.edu.my

Citation: Ooi BK, Ahemad N, and Yap WH. A reliable and reproducible assay for determining the effect of natural product on macrophages lipid uptake and cholesterol efflux: A case study of maslinic acid. Prog Drug Discov Biomed Sci 2019; 2(1): a0000031

Introduction

Foam cell formation represents one of the early hallmarks of atherosclerotic lesion, which may result from imbalanced lipid uptake and impaired cholesterol efflux in macrophages. Modified low-density lipoprotein (LDL) is thought to act as an inflammatory factor in evoking atherosclerosis[1,2]. LDL within the intima layer may undergo modifications, including oxidation, glycation, desialylation, and acylation. The modified LDL is recognized by macrophages scavenger receptors[3-6]. In contrast, the efflux of cholesterol in macrophages can be mediated through reverse cholesterol transporters or by a passive aqueous diffusion process[6,7]. Therapeutic intervention targeting foam cell formation which focuses on decreasing lipid uptake while enhancing cholesterol efflux in macrophages are expected to retard atherosclerosis progression.

LDL is a spherical shaped particle with a diameter of 18 - 25 nm and has a density range of 1.019 - 1.063 g/ml[8,9]. It can be obtained from commercial suppliers or isolated from human plasma by ultracentrifugation methods[10-12]. In this experiment, LDL was isolated from human plasma by density gradient ultracentrifugation using Beckman Coulter Optima L-100XP with an SW40 Ti swinging-bucket rotor, and followed by overnight dialysis at 4°C. Two conventional methods including Oil red O (ORO) staining[13] and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled oxidized low density lipoprotein (Dil-labeled oxLDL) uptake assay[13,14], were used to assess foam cell formation in macrophages. Meanwhile, several methods have been used to study cholesterol efflux from macrophages. Radioisotope-labeled cholesterol method has been traditionally used for measuring cholesterol efflux. However, this method is labor-intensive, and radioisotope has short shelf-life after preparation. Therefore, fluorescent-labeled cholesterol (3-hexanoyl-NBD labeled choles-

terol) was chosen as an alternative method for studying cholesterol efflux in this study[15,16].

Natural products (bioactive compounds) have been proven to be a great source of novel drug leads and therapeutic agents for the prevention and treatment of various diseases. There are many studies reporting that cardioprotective natural compounds can alleviate atherosclerotic plaque formation[17-19]. Maslinic acid, a natural pentacyclic triterpene compound found in numerous medicinal plants has been shown to exhibit antioxidant and anti-inflammatory properties[20,21]. These evidences suggest that maslinic acid could be a potential natural compound capable of protecting against foam cells formation.

In this study, a rapid and direct assay for assessing the effect of natural products such as maslinic acid on macrophages lipid uptake and cholesterol efflux was developed and optimized (Figure 1). The present work generated reproducible and reliable results.

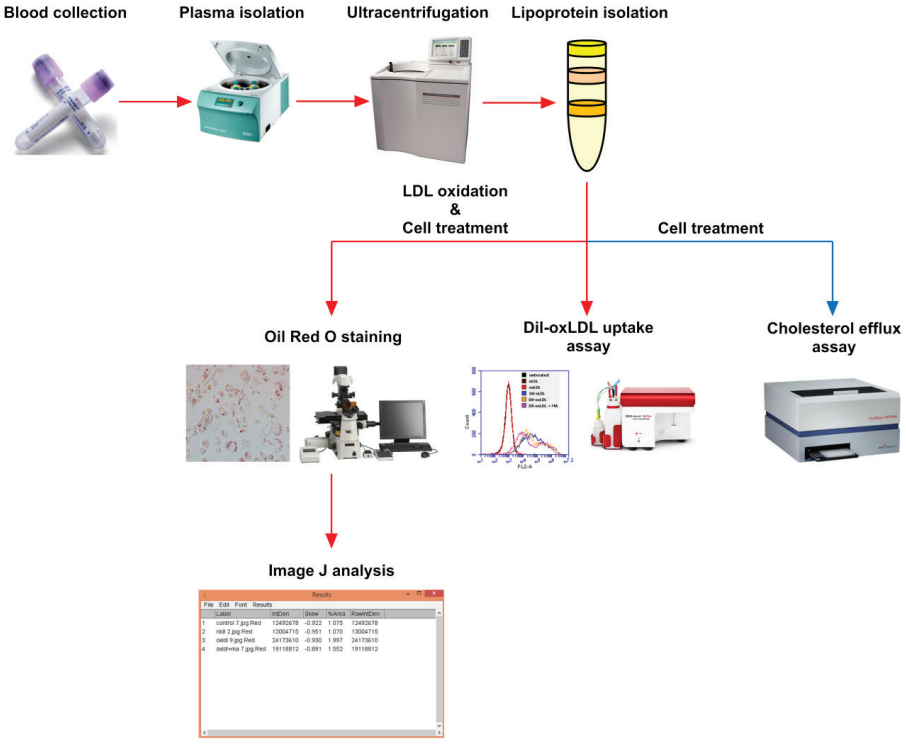


Figure 1: Schematic flow chart of the protocol on LDL isolation, assessment of lipid uptake and cholesterol efflux in THP-1 macrophages. Red arrow indicates the involvement of LDL oxidation; blue arrow indicates the absence of LDL oxidation.

Method details

Isolation of low-density lipoprotein (LDL) from human plasma

Materials

- Fasting peripheral venous blood samples preserved in BD Vacutainer® K2 EDTA tubes
- Centrifuge
- 15 mL and 50 mL Falcon tubes
- Solid potassium bromide (KBr)
- KBr solution (density=1.21 g/mL) by dissolving 31.7 g of KBr in 100 mL of deionized water containing 0.05% EDTA
- KBr solution (density=1.063 g/mL) by dissolving 9.03 g of KBr in 100 mL of deionized water containing 0.05% EDTA
- KBr solution (density=1.006 g/mL) by dissolving 1.01 g of KBr in 100 mL of deionized water containing 0.05% EDTA
- 13.2 mL polyallomer ultracentrifuge tube
- Ultracentrifuge tube rack
- Beckman Coulter Optima L-100XP
- SW40 Ti swinging-bucket rotor
- Pur-A-Lyzer maxi dialysis kit
- Phosphate-buffered saline (PBS)
- 0.2 µm Millipore CA membrane
- Pierce BCA protein assay kit containing BCA Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide), BCA Reagent B (4% cupric sulfate) and bovine serum albumin (BSA) standard ampules (2 mg/mL)
- 1.5 mL microcentrifuge tubes

Procedures

1. Collect blood samples from volunteers who have

read and signed the patient information sheet and consent form (Inclusion criteria: 18 years old and above).

2. Centrifuge the blood samples at 2700 rpm for 15 minutes at room temperature to separate the red blood cells and plasma.
3. Carefully transfer the plasma into 50 mL falcon tube.
4. Perform second centrifugation at 3300 rpm for 10 minutes to remove any residual intact blood cells that were carried over from the first centrifugation.
5. The plasma can be subjected to ultracentrifugation immediately by following the subsequent steps or stored at -80°C until further usage.
6. Adjust the plasma density to 1.35 g/mL with solid KBr, according to Radding and Steinberg formula[22] prior to density gradient ultracentrifugation.

$$X = \frac{V(df-di)}{1-(0.312 \times df)}$$

Whereby:

X	=	mass of KBr (g)
V	=	volume of plasma (mL)
df	=	final density/desired density
di	=	initial density
0.312	=	partial specific volume of KBr

7. A discontinuous gradient was constructed by carefully layering 3.5 mL of plasma into a 13.2 mL polyallomer ultracentrifuge tube, followed by 2 mL of 1.21 g/mL KBr solution, 4 mL of 1.063 g/mL KBr solution and 2 mL of 1.006 g/mL KBr solution.
8. Place the tubes into the chilled SW40 Ti swinging-bucket rotor.
9. Ultracentrifuge the samples at 40,000 rpm for 6 hours at 4°C.
10. Thereafter, carefully remove the tubes from the rotor.
11. Using a marker pen, label the individual lipid fractions (the yellow-orange fraction indicates the LDL fraction).
12. Transfer the LDL fractions into 15 mL falcon tubes.
13. Measure the density of LDL fraction prior dialysis process.
14. Dialyze the LDL fraction using the Pur-A-Lyzer maxi dialysis kit (Sigma-Aldrich, USA) in dark for 24 hours at 4°C against 3 changes of 1 L of 0.01 M PBS.
15. Filter the LDL fraction using 0.2 µm Millipore CA membrane.
16. Determine the protein concentration of LDL fraction using Pierce BCA protein assay kit and aliquot the LDL fractions into 1.5 mL microcentrifuge tubes.
17. Store the LDL fractions in -80°C until further analysis.

Copper sulfate ($CuSO_4$)-induced LDL modification and foam cell formation (Oil red O staining)

Materials

- Phosphate-buffered saline (PBS)
- Copper sulfate ($CuSO_4$)
- 1.5 mL microcentrifuge tubes
- 37°C CO_2 incubator
- 24 well tissue culture plate
- Phorbol 12-myristate-13-acetate (PMA)
- Maslinic acid stock solution (21mM) in DMSO
- 10% paraformaldehyde
- 60% isopropanol
- Deionized water (ddH_2O)
- Oil Red O stock (ORO) and working solution: Dissolve 0.5 g ORO powder in 80 mL of 100% isopropanol. Adjust the volume to 100 mL with isopropanol after 24 hours incubation at 56˚C in the water bath. Prepare the fresh working solution by diluting the pre-warmed stock solution at 60˚C with deionized water in 6:4 (v: v) ratio. Allow the working solution to stand at room temperature for 10 minutes and then filter it using 0.2 µm Millipore CA membrane before use.

Procedures

1. Dilute LDL sample to a concentration of 2 mg/mL in PBS prior to the oxidation process.
2. Incubate LDL with 50 µM (final concentration) of $CuSO_4$ at 37˚C in dark for 24 hours.
3. Determine the extent of LDL oxidation using thiobarbituric acid-reactive substances (TBARS) assay.
4. To assess the effect of maslinic acid on foam cell formation, seed THP-1 cells in 24 well tissue culture plate (3×10^5 cells/ml) and induce THP-1 differentiation with 100 ng/ml PMA for 72 hours.
5. Resazurin assay was performed to select maslinic acid concentration at sub-cytotoxic level. Maslinic acid showed cytotoxicity effect on PMA-differentiated THP-1 macrophages at 50 µM onwards. Therefore, 20 µM was selected for the following experiment (Appendix 1).
6. Incubate the PMA-differentiated THP-1 macrophages with either native low-density lipoprotein (nLDL) or oxLDL (100 µg/mL) in the presence or absence of 20 µM maslinic acid for 24 hours. Four treatment groups are indicated as below:
    - Untreated
    - Treatment with nLDL only
    - Treatment with oxLDL only
    - Treatment with oxLDL and maslinic acid

7. After 24 hours of treatment, aspirate the medium and gently rinse the cells twice with PBS.
8. Fix the cells with 10% paraformaldehyde in PBS for 30 minutes.
9. Rinse the cells twice with PBS and followed by 60% isopropanol for 15 seconds.
10. Stain the cells with filtered ORO solution for 30 minutes in dark.
11. Destain the cells with 60% isopropanol for 15 seconds.
12. Wash the cells twice with ddH_2O, 1 minute each and always cover the cells with ddH2O.
13. Capture the macrophage-derived foam cells (positively ORO-red stained) via Eclipse-Ti inverted microscope with a camera.
14. Analyze the stained intracellular lipids in THP-1 macrophages by using ImageJ analysis software[23].
15. Integrated density data representing the extent of lipid droplets accumulation was recorded.

Dil-labeled oxidized LDL uptake assay (Analysis by flow cytometry)

Materials

- Phosphate-buffered saline (PBS)
- 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) stock solution (3 mg/mL) in DMSO
- 1.5 mL microcentrifuge tubes
- 37°C CO_2 incubator
- Copper sulfate
- 24 well tissue culture plate
- Phorbol 12-myristate-13-acetate (PMA)
- Maslinic acid stock solution (21mM) in DMSO
- 1× trypsin
- Accuri C6 flow cytometer

Procedures

1. Incubate 1.5 mg/mL of LDL with 0.15 mg/mL (final concentration) of Dil at 37˚C in dark for 24 hours.
2. Incubate Dil-labeled LDL with 50 µM (final concentration) of $CuSO_4$ at 37˚C in dark for 24 hours.
3. To assess the effect of maslinic acid on uptake of Dil-labeled oxLDL, seed THP-1 cells in 24 well tissue culture plate (3×10^5 cells/ml) and induce THP-1 differentiation with 100 ng/ml PMA for 72 hours.
4. Incubate the PMA-differentiated THP-1 macrophages with nLDL, oxLDL, Dil-labeled nLDL or Dil-labeled oxLDL (50 µg/mL) in the presence or absence of 20 µM maslinic acid for 4 hours. Six treatment groups of macrophages consist:
    - Untreated
    - Treatment with nLDL only (negative control)
    - Treatment with oxLDL only (negative control)
    - Treatment with Dil-nLDL only
    - Treatment with Dil-oxLDL only
    - Treatment with Dil-oxLDL and maslinic acid
5. Aspirate the medium and gently rinse the cells thrice with ice-cold PBS containing 2 mg/mL BSA.
6. Detach the cells with 0.5 mL of 1× trypsin and harvest the culture.
7. Centrifuge the cells at 4000 rpm for 5 minutes at 4˚C.
8. Wash the cells twice with ice-cold PBS and resuspend the cells with 200 µL of ice-cold PBS.
9. Analyze the cells at a slow flow rate (14 µL/min, 10 µm core) using Accuri C6 flow cytometer equipped with a 488 nm laser.
10. Set up a dot plot with FL2 on the x-axis and cell count on the y-axis to analyze the gated population of living THP-1 macrophages, recording 10,000 events for each sample.
11. Record the median fluorescence intensity (MFI) values.

Cholesterol efflux assay

Materials

- 24 well tissue culture plate
- 37°C CO_2 incubator
- Phorbol 12-myristate-13-acetate (PMA)
- Maslinic acid stock solution (21mM) in DMSO
- 3-hexanoyl-NBD labeled cholesterol
- Phosphate-buffered saline (PBS)
- RPMI-1640 medium
- Fetal bovine serum (FBS)
- Lysis buffer
- FLUOstar OPTIMA fluorimeter

Procedures

1. To assess the effect of maslinic acid on cholesterol efflux, seed THP-1 cells in 24 well tissue culture plate (3×10^5 cells/ml) and induce THP-1 differentiation with 100 ng/ml PMA for 72 hours.
2. Pre-treat PMA-converted THP-1 macrophages in

the presence or absence of 20 µM maslinic acid for 24 hours at 37˚C.

3. Incubate the cells with 1 µg/ml 3-hexanoyl-NBD labeled cholesterol (without FBS) for an additional 6 hours.
4. Wash the cells twice with PBS.
5. Incubate the cells with RPMI-1640 (with 10% FBS as lipid acceptor) for 4 hours.
6. Collect the fluorescence-labeled cholesterol in the supernatant medium as well as the cell lysates (lysed using lysis buffer from Thermo Fisher Scientific, USA)
7. Detect the fluorescence intensity of the labeled cholesterol at 485 nm excitation/ 520 nm emission filter set using FLUOstar OPTIMA fluorimeter.
8. Calculate the rate of cholesterol efflux by following formula: Cholesterol efflux=cholesterol in medium/ (cholesterol in medium +cholesterol in cells)

Method validation

Isolation of low-density lipoprotein (LDL) from human plasma

To confirm the identity of the isolated LDL fraction, several assays were carried out to determine its density and electrophoretic mobility. The experimental results showed that the isolated LDL fraction was in the density range of 1.019 - 1.063 g/mL. Apart from density, lipoproteins can be classified according to their electrophoretic mobility as alpha (HDL), pre-beta (VLDL) and beta (LDL)[24,25]. Based on Figure 2, the electrophoretic mobility of isolated LDL fraction was observed in the beta region.

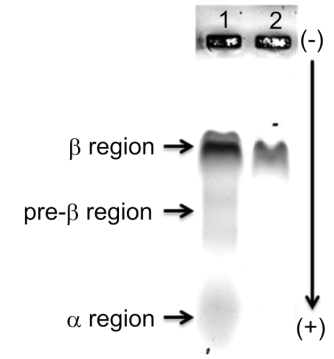


Figure 2. Electrophoretic mobility patterns. Lane 1 indicates human plasma; and Lane 2 indicates LDL fraction.

Foam cell formation (Oil red O staining) and inhibitory effects of maslinic acid

Oil red O staining is a lychrosome and it is commonly used for detection of neutral triglycerides and lipids within cultured cells and tissues[13,26]. Based on Figure 3A, exposure of THP-1 macrophages to oxLDL for 24 hours resulted in an increased accumulation of the Oil red O-stained intracellular lipid droplets. This cell morphology indicates the characteristic appearance of foam cells formation. Meanwhile, co-incubation of oxLDL with maslinic acid resulted in an evident reduction of intracellular lipid accumulation in THP-1 macrophages. The captured images of ORO-stained THP-1 macrophages were analyzed for their accumulated intracellular lipids using ImageJ software. Statistical analysis (Figure 3B) shown that maslinic acid treatment have resulted in significantly lower lipids accumulation, compared to the oxLDL treatment group.

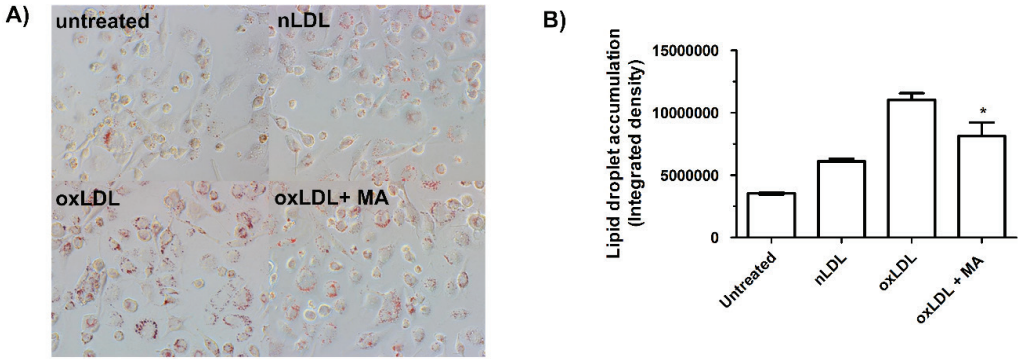


Figure 3. Effects of maslinic acid on oxLDL-induced THP-1 macrophage foam cell formation. A) Representative ORO-stained images and B) mean integrated density of untreated THP-1 macrophages, THP-1 macrophages treated with either nLDL, oxLDL, or oxLDL and maslinic acid. Each bar represents the mean ± standard deviation from three independent experiments. One-way ANOVA with a post hoc Tukey correction was used to compare the different means. A value of $p < 0.05$ was considered statistically significant. * denotes statistical difference compared to cells treated with oxLDL alone.

Dil-labeled oxidized LDL uptake assay (Fluorescence-activated cell sorting)

1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) is a lipophilic carbocyanine dye. It can diffuse laterally into the lipid bilayers through their alkyl chains. It is commonly used to label oxLDL to quantify the uptake of DiI-labeled oxLDL in human endothelial cells[13,26,27,28]. As shown in Figure 4A and 4B, the median fluorescence intensity (MFI $1.0510^5 \pm 7.4510^3$) indicated that 50 µg/mL of Dil-labeled oxLDL can be up taken and aggregated by THP-1 macrophages. THP-1 macrophages incubated with both Dil-labeled oxLDL and 20 µM of maslinic acid have shown a significant reduction in uptake of Dil-labeled oxLDL (MFI $7.3610^4 \pm 1.7010^4$). Negative controls including untreated THP-1 macrophages and THP-1 macrophages incubated with nLDL or oxLDL alone were also included.

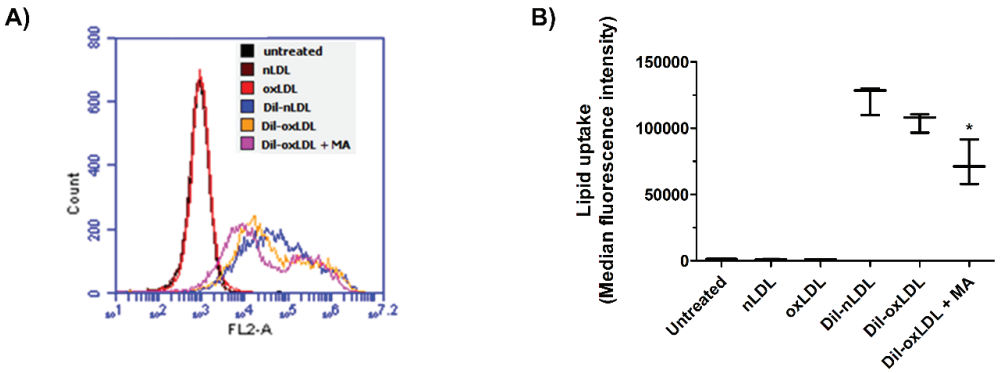


Figure 4. Effect of maslinic acid on DiI-labeled oxLDL uptake in THP-1 macrophages as determined by flow cytometry analysis. A) Representative flow cytometric histogram analysis and B) the median fluorescence intensity of DiI-oxLDL uptake by THP-1 macrophages. Each bar represents the mean ± standard deviation from three independent experiments. One-way ANOVA with a post hoc Tukey correction was performed. A value of $p < 0.05$ was considered statistically significant. * denotes statistical difference compared to cells treated with DiI-oxLDL alone.

Cholesterol efflux assay

3-hexanoyl-NBD cholesterol, a fluorescently-labeled cholesterol, as a probe for the measurement of cholesterol efflux in macrophages[29]. As shown in figure 5, it was shown that maslinic acid increased cholesterol efflux by 9.46 ± 1.382 as compared to the untreated group. It indicated that maslinic acid can counteract the pathogenesis of atherosclerosis via promoting cholesterol efflux from the macrophage. It is postulated that maslinic acid may target the passive diffusion as well as active cholesterol efflux mechanism which mediated by ATP-binding cassette (ABC) transporters and scavenger receptor class B type I (SR-BI) [30-32].

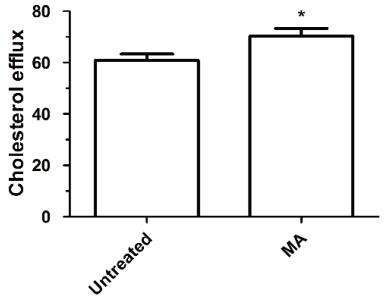


Figure 5. Effect of maslinic acid on cholesterol efflux from THP-1 macrophages. Each bar represents the mean ± standard deviations of three independent experiments. Independent sample test was run to compare the different means. A value of $p < 0.05$ was considered statistically significant. * denotes statistical difference compared to untreated cells.

Summary

In summary, these simple and cost-effective assays can be used in assessing the effect of natural products on foam cell formation. In this case study, maslinic acid was shown to suppress foam cells formation by reducing oxLDL uptake as well as marginally promoting cholesterol efflux in macrophages.

Ethics Statement

The experiment was approved by Taylor's University Ethics Committee.

Conflict of Interest

The authors declared that there is no conflict of interest.

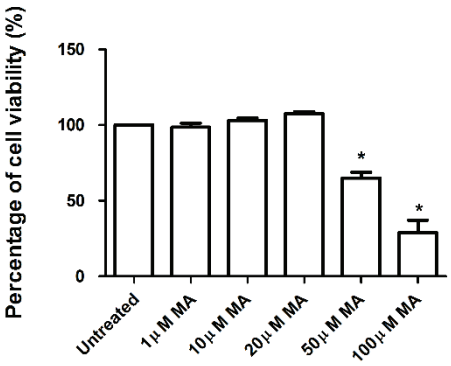
Acknowledgments

This work was supported by the Ministry of Education (MOE) Fundamental Research Grant Scheme (FRGS/ 1/ 2015/ SKK08/ TAYLOR/ 03/ 1) awarded to W-HY.

References

1. Levitan I, Volkov S & Subbaiah PV. Oxidized ldl: Diversity, patterns of recognition, and pathophysiology. Antioxid Redox Signal 2010; 13(1): 39-75.
2. Maiolino G, Rossitto G, Caielli P, *et al.* The role of oxidized low-density lipoproteins in atherosclerosis: The myths and the facts. Mediators Inflamm 2013; 2013: 13.
3. Nagy L, Tontonoz P, Alvarez JGA *et al*. Oxidized ldl regulates macrophage gene expression through ligand activation of PPARγ. Cell 1998; 93(2): 229-240.
4. Moore KJ & Freeman MW. Scavenger receptors in atherosclerosis. Arterioscler Thromb Vasc Biol 2006; 26(8): 1702-1711.
5. Stephen SL, Freestone K, Dunn S, *et al.* Scavenger receptors and their potential as therapeutic targets in the treatment of cardiovascular disease. Int J Hypertens 2010; 2010.
6. Ooi BK, Goh BH & Yap WH. Oxidative stress in cardiovascular diseases: Involvement of Nrf2 antioxidant redox signaling in macrophage foam cells formation. Int J Mol Sci 2017; 18(11): 2336.
7. Ghosh S. Macrophage cholesterol homeostasis and metabolic diseases: Critical role of cholesteryl ester mobilization. Expert Rev Cardiovasc Ther 2011; 9(3): 329-340.
8. Chapman MJ, Laplaud PM, Luc G, *et al.* Further resolution of the low density lipoprotein spectrum in normal human plasma: Physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifugation. J Lipid Res 1988; 29(4): 442-458.
9. Murtola T, Vuorela TA, Hyvönen MT, *et al.* Low density lipoprotein: structure, dynamics, and interactions of apoB-100 with lipids. Soft Matter 2011; 7(18): 8135-8141.
10. Griffin BA, Caslake MJ, Yip B, *et al.* Rapid isolation of low density lipoprotein (ldl) subfractions from plasma by density gradient ultracentrifugation. Atherosclerosis 1990; 83(1): 59-67.
11. Vieira OV, Laranjinha JA, Madeira VM, *et al.* Rapid isolation of low density lipoproteins in a concentrated fraction free from water-soluble plasma antioxidants. J Lipid Res 1996; 37(12): 2715-2721.
12. Dong J, Guo H, Yang R, *et al.* Serum ldl- and hdl-cholesterol determined by ultracentrifugation and HPLC. J Lipid Res 2011; 52(2): 383-388.
13. Xu S, Huang Y, Xie Y, *et al.* Evaluation of foam cell formation in cultured macrophages: An improved method with oil red o staining and diI-oxLDL uptake. Cytotechnology 2010; 62(5): 473-481.
14. Zhou YD, Cao XQ, Liu ZH, *et al.* Rapamycin inhibits oxidized low density lipoprotein uptake in human umbilical vein endothelial cells via mTOR/NF-κB/LOX-1 pathway. PloS one 2016; 11(1): e0146777.
15. Song W, Wang W, Wang Y, *et al.* Characterization of fluorescent NBD-cholesterol efflux in THP-1-derived macrophages. Mol Med Rep 2015; 12(4): 5989-5996.
16. Song YS, Lee SH, Park BH, *et al.* Cholesterol efflux monitoring in macrophage form cells by using fluorescence lifetime imaging. SPIE 2015.

17. Gonzlez-Reyes S, Guzmn-Beltrn S, Medina-Campos ON *et al.* Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats. Oxid Med Cell Longev 2013; 2013: 14.
18. Yang D, Xiao CX, Su ZH, *et al.* (-)-7(S)-hydroxymatairesinol protects against tumor necrosis factor-α-mediated inflammation response in endothelial cells by blocking the MAPK/NF-κB and activating Nrf2/HO-1. Phytomedicine 2017; 32: 15-23.
19. Choi ES, Yoon JJ, Han BH, *et al*. Ligustilide attenuates vascular inflammation and activates Nrf2/HO-1 induction and, NO synthesis in HUVECs. Phytomedicine 2018; 38: 12-23.
20. Huang L, Guan T, Qian Y, *et al.* Anti-inflammatory effects of maslinic acid, a natural triterpene, in cultured cortical astrocytes via suppression of nuclear factor-kappa B. Eur J Pharmacol 2011; 672(1): 169-174.
21. Yap WH, Khoo KS, Ho ASH, *et al.* Maslinic acid induces HO-1 and NOQ1 expression via activation of Nrf2 transcription factor. Biomed Prev Nutr 2012; 2(1): 51-58.
22. Radding CM & Steinberg D. Studies on the synthesis and secretion of serum lipoproteins by rat liver slices. J Clin Invest 1960; 39(10) 1560-1569.
23. Yap WH, Phang SW, Ahmed N, *et al.* Differential effects of sPLA2-GV and GX on cellular proliferation and lipid accumulation in HT29 colon cancer cells. Mol Cell Biochem 2018; 447(1): 93-101.
24. Rudel LL, Greene DG & Shah R. Separation and characterization of plasma lipoproteins of rhesus monkeys (Macaca mulatta). J Lipid Res 1977; 18(6): 734-744.
25. Cox R & García-Palmieri M. Cholesterol, triglycerides, and associated lipoproteins. in Walker, H, Hall, W & Hurst, J (eds.), Clinical methods: the history, physical, and laboratory examinations. 1990; 3rd, Boston: pp. 153-160.
26. Shi H, Mao X, Zhong Y, *et al.* Lanatoside C promotes foam cell formation and atherosclerosis. Sci Rep 2016; 6: 20154.
27. Hossain E, Ota A, Karnan S, *et al.* Lipopolysaccharide augments the uptake of oxidized LDL by up-regulating lectin-like oxidized LDL receptor-1 in macrophages. Mol Cell Biochem 2015; 400(1): 29-40.
28. Luo Y, Duan H, Qian Y, *et al.* Macrophagic CD146 promotes foam cell formation and retention during atherosclerosis. Cell Res 2107; 27: 352-372.
29. Chen CY, Shyue SK, Ching LC, *et al.* Wogonin promotes cholesterol efflux by increasing protein phosphatase 2B-dependent dephosphorylation at ATP-binding cassette transporter-A1 in macrophages. J Nutr Biochem 2011; 22(11): 1015-1021.
30. Meurs I, Out R, Van Berkel TJ, *et al.* Role of the ABC transporters ABCA1 and ABCG1 in foam cell formation and atherosclerosis. Future Lipidol 2008; 3(6): 675-687.
31. Yu XH, Fu YC, Zhang DW, *et al*. Foam cells in atherosclerosis. Clin Chim Acta 2013; 44: 245-252.
32. Schumacher T & Benndorf RA. ABC transport proteins in cardiovascular disease-a brief summary. Molecules 2017; 22(4): 589.



Appendix

Appendix 1: Cytotoxic effects of maslinic acid on PMA-differentiated THP-1 macrophages. Values represent means ± SD of three independent experiments performed in triplicates. Statistical significance was determined by One-way ANOVA followed by a Tukey's test were compared to the control group. * represents $p < 0.05$ compared to untreated cells.