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# *Cocus nucifera* milk enhances liver cell viability and down-regulates nuclear hormone receptor RXR-α but not PPAR-α expression in high dose

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## ABSTRACT

The use of coconut milk in daily diet often worried consumers as they believe this high fat food product leads to increase risk of acquiring obesity and cardiovascular diseases. However, in contrast to common believe, several studies showed that coconut fat might be capable of improving the cardiovascular function and atherosclerotic conditions. In order to justify the actual health role of coconut milk, this study was designed to investigate the potential molecular effect of coconut milk in modulating human lipid metabolism using the popular *in vitro* surrogate of liver – human hepatocarcinoma HepG2 cell line. The cytotoxic effect of coconut milk against HepG2 cell viability was first studied by using MTT assay. Coconut milk treatment was shown to improve liver cell viability in a dose dependent manner. To access the influence of coconut milk on the transcriptional response of lipid activated nuclear receptor (namely PPAR-α, and RXR-α) in HepG2 cell line, Real-Time Reverse Transcription PCR was performed on the total mRNA sample extracted from the coconut milk treated cell specimen. Besides that, Western blotting was also used to analyse the protein expression. The Real-Time PCR results revealed that the increasing amount of coconut milk negatively influenced the expression of both PPAR-α and RXR-α mRNA by approximately 27% and 36%, respectively. Meanwhile, no significant results were obtained from the Western assay. In a nutshell, this study showed that the application of coconut milk did not alter the cell viability at all tested concentration, while the normal lipid metabolism was only maintained when it was administered at low dose.



## KEYWORDS

*Cocus nucifera*;
Cell viability;
RXR-α;
PPAR-α;
Gene expression.

## INTRODUCTION

Lipid homeostasis is a dynamic process which requires a delicate balance between lipid anabolism (lipogenesis), catabolism (oxidation), as well as its uptake and secretion by the liver[1]. The maintenance of this process is of top priority since any metabolic deregulation of this compound could often lead to the manifestation of several life-threatening diseases, e.g. metabolic syndrome, obesity, diabetes, atherosclero-

sis, and other cardiovascular complications[2]. In order to achieve a steady input and output of fatty compounds by the liver, the lipid level in human body is constantly monitored by a molecular network consisting of multiple lipid sensing nuclear receptors[3].

These receptors, reacting under the presence of their respective ligands, will in turn elicits a series of effectors' activities – such as lipid transport, storage and elimination – which are important in facilitating the surveillance of lipid equilibrium within the body. These lipid sensitive receptors plays an essential role in lipid metabolism and are able to response to a large amount of extrinsic ligands from the dietary, drugs, or environmental sources[4,5]. Popular examples of such lipid activated nuclear receptors include peroxisome proliferator activated receptors (PPARs), and its heterodimeric partner retinoid X receptors (RXRs), etc[4].

Coconut, scientifically term as *Cocos nucifera*, is a tropical crop commonly seen along the coastal region of Southeast Asia and Melanesia. Locals often utilised coconut milk in cooking cultural cuisine[6]. According to CODEX-STAN 240 report, coconut milk is a high fat food product which contains at least 10% saturated fat component[7]. Based on diet-heart hypothesis, this high lipid feature automatically labelled coconut milk as an unhealthy food source as it might contribute to the increasing risk of metabolic diseases such as atherosclerosis, obesity, diabetes, and more. This claim has raise concern among the consumers and caused most of them to minimise or even avoid the use of such product in daily diet.

Despite being a common food ingredient, past research on coconut milk is fairly limited. The lack of solid support shows that the use of coconut milk is condemned solely base on general perception, and investigational approaches that can be used to clarify this probable misconception are urgently needed. As a matter of fact, a product with similar fat/lipids constituents to the coconut milk compound, the virgin coconut oil, has shown promising effect in improving the lipid profile and slowing atherosclerotic progression[8,9]. Furthermore, the use of diet-heart hypothesis might not be entirely reliable in accessing the health effects of coconut fat. This is because instead of the quantity, it is the quality of the fat intake that will dictate the physiological risks of the food compound[10].

Coconut milk is no doubt a heavy source of saturated fatty acid, but majority of its fat content is constituted by a unique sub-group of lipid known as medium chain length fatty acid (MCFA). Unlike other saturated fatty acid, MCFA is metabolised immediately once it reaches the liver, and thus it will not form any lipid deposits on blood vessels or adipose tissues[11]. As a result of that, consumption of coconut fat with MCFA, even in high quantity, might not raise significant alteration to the normal cardiovascular function or lipid homeostasis. Several studies have shown that coconut oil supplement is not only capable of protecting the cardiovascular system from atherogenic progression, but it is also able to improve the conditions of obese individuals[12,13].

Nevertheless, authorities such as American Heart Association and the National Heart Foundation in USA, who hold strongly on the diet-heart hypothesis, remain cautious of the use of coconut fat in cooking. Their belief is supported by few studies, whereby coconut oil was found to be inferior of other vegetable dietary oils – e.g. menhaden oil, olive oil, and palm oil – in terms of protecting cardiovascular function[14,15]. Another study also pointed out that coconut oil exhibit great atherogenic potentials, and this risk potential of coconut oil can only be reduced when it is blended with rice bran oil or sesame oil, both of which are rich in unsaturated fatty acid[16]. Judging by such controversy, this project was designed to provide a preliminary insight regarding the actual health effects of coconut milk on human body, which is to focus on the influence of this lipid-rich food compound towards lipid metabolism. In order to achieve this objective, PPAR-α and RXR-α were chosen as the target genes since these receptors play a key role in the regulation of the lipid metabolic pathways.

PPAR-α is an important homeostatic agents that acts to initiate the breakdown of excessive fatty acid[17]. This active role of PPAR-α in lipid catabolism has made it a potential therapeutic target against atherosclerotic progression[18]. RXR-α, on the other hand, is a pleotropic heterodimer[19] that act as an essential partner of PPAR-α activation. Without the dimerization with RXR-α, the binding efficiency of PPAR-α onto the target genes promoter region will be less efficient[20]. Henceforth, the probable molecular effect of *Cocos nucifera* milk on lipid metabolism (particularly on the catabolic

reaction) was deduced based on the transcriptional and translational response of these two genes in HepG2 cells.

## EXPERIMENTAL

### Cell culture maintenance

The *in vitro* surrogate of liver used in this study was the hepatocellular carcinoma HepG2 cell line obtained from American Type Cell Culture (ATCC). HepG2 samples were grown by using minimum essential medium (MEM) supplemented with 2.0 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate, 1X MEM non-essential amino acid solution (Gibco, USA), 2.2 mg/ml sodium bicarbonate (QRëC, New Zealand), and 10% (v/v) foetal bovine serum (FBS; Biowest, France). The cells were maintained in a humid incubator with 5% (v/v) $CO_2$ at 37ºC. Cell culture medium was replenished every two to three days to ensure optimum cell growth.

### Coconut milk

Ultra high temperature (UHT) processed coconut milk (Ayam Brand Santan) was purchased from local retail store and used directly for cell treatment without further modification.

### Cell viability assay

The viability of HepG2 cells following the treatments by using commercialised coconut milk was assessed by using MTT Cell Proliferation Assay Kit (ATCC, USA). Cells with 80% confluency were trypsinised, resuspended with full medium, and plated accordingly (approximately 1 X $10^5$ of cells per well) in a flat-bottomed tissue culture graded 96 well plate. After 24 hours, the cells were treated with different concentration of coconut milk (1, 5, 10%). Untreated cells were used as a control for this assay, with its viability was regarded as 100%. All samples were incubated in 5% $CO_2$ incubator at 37°C for 24 hours. Subsequently, the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to each well and the cells were incubated for another 2 to 4 hours until the emergence of intracellular purple precipitate was observed. Detergent was then added, and the cells were left in the dark (room temperature) for 2 more hours. Finally, the absorbance in each well was read at 570 nm in the Infinite M200 microplate reader (Tecan, Switzerland). All experiments were carried out in triplicates and repeated twice.

### Treatments and total protein/mRNA extraction

HepG2 cells cultured in 25 $cm^2$ tissue culture flasks were allowed to grow until they reached 70% confluence. The initial medium was removed from the flasks and the cells were washed twice with phosphate buffered saline (PBS; Amresco, USA). Cells were starved with 0.5% FBS for 2 hours prior to treatments. To test the effect of coconut milk on lipid activated nuclear receptors, the pre-starved cells were subjected to stimulation with different amounts of coconut milk [0, 1, 5, and 10% (v/v)] for another 24 hours. After the treatments, total cellular RNA as well as the total protein components of the cell samples were extracted by using Tri Reagent® LS according to the manufacturer's instruction. Solubilised total cellular RNA was then used for downstream investigation (Real Time RT-PCR), or otherwise stored in –80ºC freezer if not of immediate usage; while solubilised protein sample was used for Western blot analysis, or otherwise stored in -20ºC if not of immediate usage.

### Real time reverse transcription polymerase chain reaction (Real time RT PCR)

The target mRNAs were amplified through Bio-Rad MyiQ™ Single-Colour Real-Time PCR Detection System by applying the corresponding primer pairs as listed in the following: PPAR-α forward primer – 5'-CCGTTATCTGAAGAGTTCCTG-3'; PPAR-α reverse primer – 5'-GTTGTGTGACATCCCGACAG-3'[21]; β-actin forward primer – 5'-TCACCCTGAAGTACCCCATC-3'; and β-actin reverse primer – 5'-CCATCTCTTGCTCGAAGTCC-3'[21]; RXR-α forward primer – 5'-GCTGGAATGAGCTGCTCATC-3'; RXR-α reverse primer – 5'- GGTACTTGTGCTTGCAGTAG-3'[22]. The reaction was performed by using the Bio-Rad I-Script One Step RT-PCR Kit. The reaction mixture was assembled on ice according to the composition recommended by the manufacturer. Relative quantification method was then used to measure the mRNA expression of PPAR-α and RXR-α by normalising the

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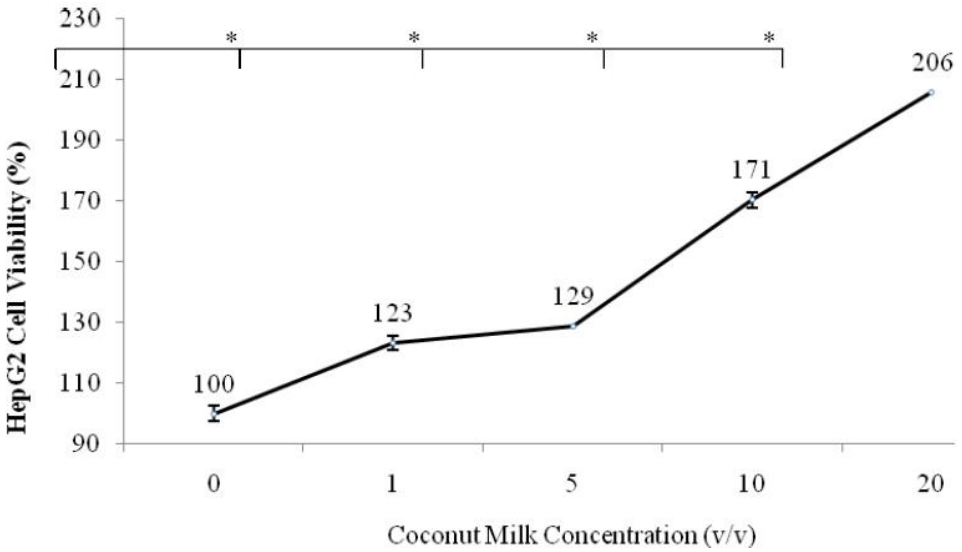


**Figure 1 : Line graph that depicts the coconut milk effect on HepG2 cells viability. *$p < 0.05$ represent statistically significant change from control (untreated HepG2 cells).**

target genes expression against the housekeeping gene, β-actin. This was achieved by utilising the delta-delta $C_T$ method and the calculated expression of target genes mRNA were presented in fold-changes as compare to untreated control (fold-value equals to 1.00).

### Western blot

Total protein sample was first subjected to vertical separation by using SDS-PAGE. Subsequently, separated proteins on the PAGE-gel were transferred to PVDF membrane (Millipore, USA) by using Mini Trans-Blot® System (Bio-Rad, USA). After the transfer, the membrane was immersed into the blocking buffer containing bovine serum albumin (BSA; Bio Basic, Canada). Following the blocking process, the PVDF membrane was first exposed to the primary antibody [PPAR-α (Santa-Cruz Biotechnology, USA); RXR-α (Cell Signalling Technology, USA)], and then the HRP-linked secondary antibody (Santa-Cruz Biotechnology, USA) in sequential steps. It is important to note that the membrane was washed with TBST buffer [50 mM Tris (Promega, USA), 150 mM NaCl (Millipore, USA), 0.1% Tween 20 (Amresco, USA)] thrice after each antibody treatment. Lastly, HRP substrate (Millipore, USA) was applied to the membrane and the chemiluminescence emitted by the targeted protein band was visualised under FluorChem FC2® Imager (Alpha Innotech Corporation, USA). Band intensity was analysed by utilising the imager software.

### Statistical analysis

Results obtained from all experiments were presented as means ± standard deviation (SD) of at least three replicates. The mean values of these replicates were then compared by using Student's *t* test (two sided) in the SPSS Statistics 21 software (IBM, USA). A value of $p < 0.05$ was considered statistically significant.

## RESULTS

### Cell viability tests

As indicated in Figure 1, coconut milk treatment improved the viability of HepG2 cells. HepG2 cells treated with increasing concentration of coconut milk [0, 1, 5, 10 and 20% (v/v)] exhibited an increase in cell viability in a dose dependent manner – from 100%, to 123%, 129%, 171% and lastly, 206%.

### Coconut milk down-regulates PPAR-α mRNA expression

This dose response test showed that the administration of increasing doses of coconut milk gradually reduced the mRNA expression of PPAR-α. As shown in Figure 2(a), the nuclear receptor's mRNA expression declined from 1.18-fold at 1% (v/v) to 0.90-fold at 5% (v/v), 0.80-fold at 10% (v/v), and 0.86-fold at 20% (v/v) of coconut milk. Despite the slight fluctuation, SPSS analysis showed that these changes were not statistically significant, suggesting that the expres-

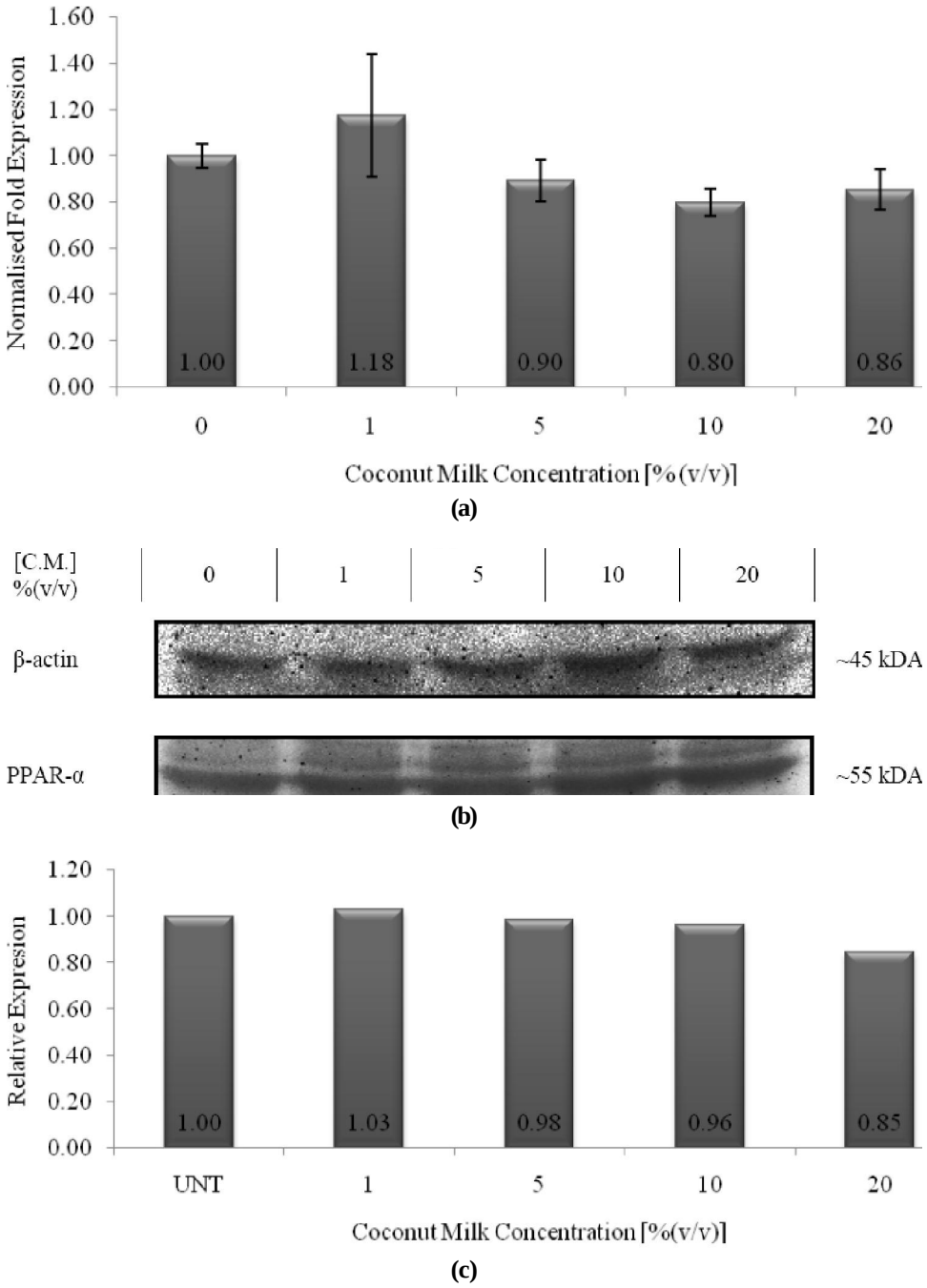


**Figure 2 : (a) The mRNA expression of PPAR-α in coconut milk dose response test. The fold expression lies on the bottom of each bar was normalised to β-actin and relative to the untreated HepG2 cells. (b) Chemilimuninescence band observed after Western blotting. (c) The protein expression of PPAR-α in coconut milk dose response test. The value placed at the bottom of each bar signifies the protein band intensity that was normalised to β-actin and relative to the untreated HepG2 cells.**

sion of PPAR-α are not heavily affected by the administration of coconut milk. Consistent with the mRNA result, there was no significant alteration found in the protein expression [Figure 2(c)].

## Coconut milk down-regulates RXR-α mRNA expression in a dose dependent manner

As compared to PPAR-α, coconut milk treatment yielded a decreasing pattern of RXR-α mRNA expression in a dose dependent manner [Figure 3(a)]. The transcript level was slightly up-regulated to 1.07-fold at 1% (v/v), but the level decreased into 0.83-fold, 0.72-fold and 0.68-fold at 5%, 10%, and 20% (v/v)

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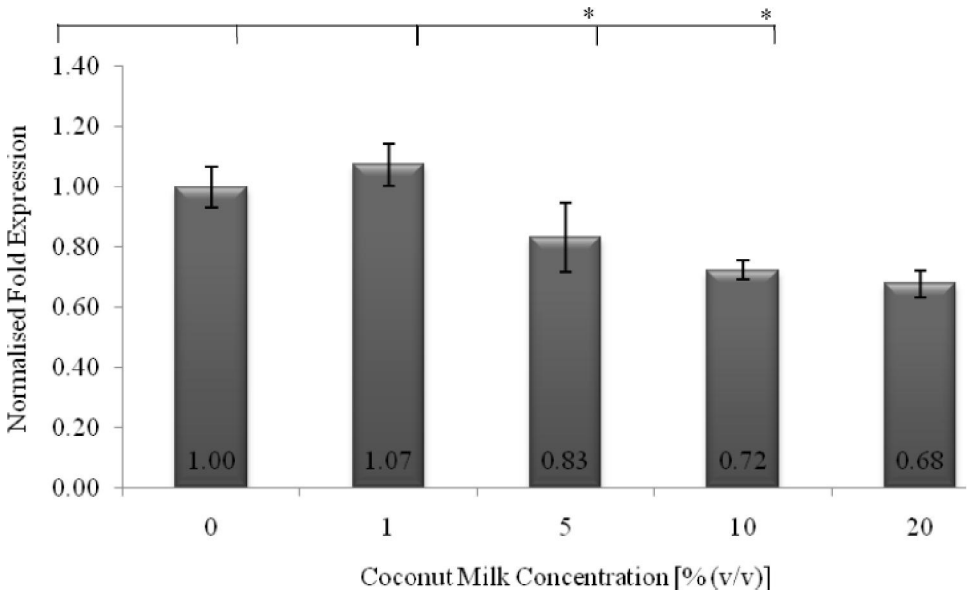
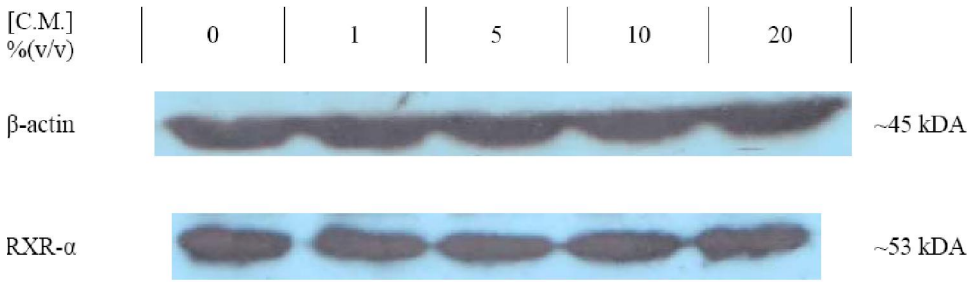
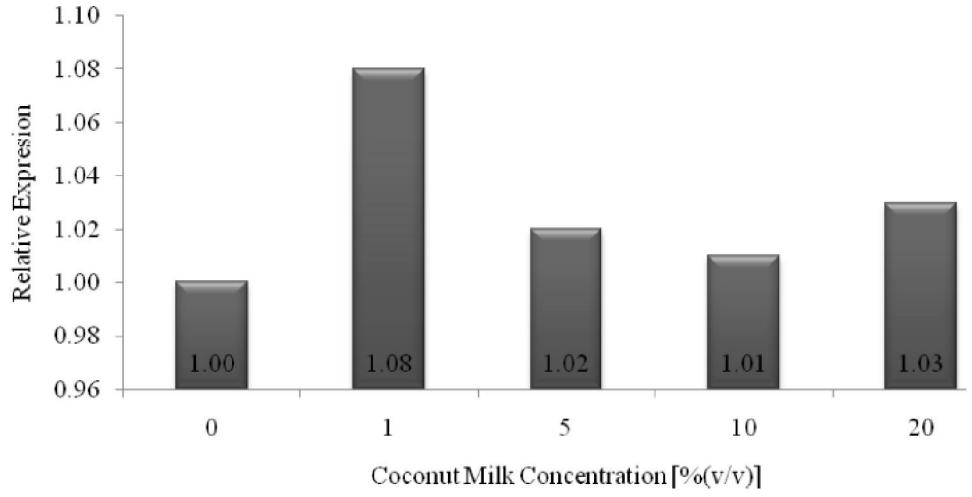



(a)




(b)




(c)

**Figure 3 : (a) The mRNA expression of RXR-α in coconut milk dose response test. The fold expression lies on the bottom of each bar was normalised to β-actin and relative to the untreated HepG2 cells. *$p < 0.05$ represent statistically significant change from control (untreated HepG2 cells). (b) Chemilimuninescence band observed after Western blotting. (c) The protein expression of RXR-α in coconut milk dose response test. The value placed at the bottom of each bar signifies the protein band intensity that was normalised to β-actin and relative to the untreated HepG2 cells.**

coconut milk treatment, respectively. Despite the significant decrease in mRNA expression, again there are no apparent changes observed in the intensity of protein bands. This might be due to the delayed or slower

translational response of RXR-α gene.

## DISCUSSION

Cytotoxic effect was absent in cells treated with different concentrations of coconut milk. Instead, an induction of proliferative capacity was observed following the administration of the milk compound. In a relevant study by Thomas and colleagues (2008) using tender coconut water showed that the coconut compound was non-cytotoxic and relatively efficient in maintaining the viability of BHK-21/C13 (baby hamster kidney fibroblasts) cell line, which they attributed this feature to the presence of multiple essential nutrients (e.g. proteins, amino acids, vitamins and minerals) within the coconut water[23]. However, since coconut water and coconut milk are of different entity, it is remained to be elucidated on whether these two compounds possess overlapping effects on the viability of living cells.

As mentioned above, past research on coconut milk is fairly limited. Little is known regarding the actual metabolic effects of this food compound. Recently, a product with similar fat/lipids constituents as the coconut milk compound, the virgin coconut oil (VCO), was found to promote the cardiovascular function instead. Administration of VCO to Sprague-Dawleys rats reduced all lipid and lipoprotein parameters in the test subject whilst up-regulate the level of the good cholesterol – high density lipoprotein cholesterol (HDL-C)[9]. This result is consistent with a clinical studies conducted by Norton et al. (2004)[8], in which subjects who consume up to 50% of coconut oil out of the total dietary fat did not have any significant changes in their total cholesterol and low density lipoprotein cholesterol (LDL-C, often called bad cholesterol) levels. Shockingly, the constant consumption of coconut oil actually led to a remarkable raise in the HDL level[8]. Besides the improvement of the lipid profile, VCO was also shown to be anti-atherogenic. Nevin and Rajamohan (2008) highlighted the decrease in thrombotic risk factors such as platelets, fibrin and fibrinogen, and factor V in VCO-fed rat, which in consequent will decrease the tendency of blot clot (anti-thrombotic) and plaque formation (anti-atherosclerotic)[12]. This group also concluded that the polyphenolic antioxidant fraction of VCO was capable of limiting the oxidation of LDL – one of the priming steps in atherosclerotic formation – both *in vitro* and *in vivo*[9,12].

PPAR-α is a well studied nuclear transcription factor derived from a coding region located at chromosome 22 – 22q12-13.1[24]. This gene encode a nuclear protein with 468 amino acid long that contains 5 functional domains, which are designated as domain A/B, C, D, E, and F[25]. As compared to other PPAR isoforms, the binding pocket of PPAR-α (domain F) is slightly more lipophilic, which provides the nuclear receptor a larger binding capacity to various endogenous and synthetic ligands[26]. Natural ligands of PPAR-α include different types of fatty acid and fatty acid derivaties which are either introduced from external food source or generated through internal metabolic pathways[27,28]. Once activated, the nuclear-localised PPAR-α will act to enhance the expression of multiple genes involve in the hydrolysis of its own ligands[17]. This allows the lipid sensing receptor to maintain a balance between energy input and output through beta oxidation of fatty acid, and such catabolic feature allow the utilisation of this receptor in tackling different metabolic diseases that affect normal lipid metabolism.

In fact, a group of synthetic drug known as fibrates (e.g. fenofibrate and gemfibrozil) are popularly used in treating dyslipidemic patient[29]. Multiple studies further revealed that aside of re-establishing a healthier lipid profile with decrease LDL and circulating triglyceride levels, these agonists also exhibit cardiovascular protective function[30]. Majority of researchers now generally believed that inflammatory response plays a more definitive role in atherosclerotic development as compared to hyperlipidemia[31]. Since the activation of PPAR-α could effectively reduced the inflammatory response[32], administration of PPAR-α is, in theory, capable of slowing the atherogenic progression in the vascular system. This theory was proven with *in vivo* study, where the administration of synthetic PPAR-α ligand GW7647 was shown to reduce the formation of atherosclerotic lesion and foam cells in LDLR null mice[33].

Since VCO retains most of the hydrophobic compounds originally found in coconut milk[34], it was hypothesised earlier in this project that the biological benefits of VCO could also be replicated by using coconut milk. It is especially interesting if coconut milk

displays similar anti-atherogenic function as shown by VCO. Since PPAR-α activation leads to cardio-protective and lipid lowering effects, it was expected that the application of coconut milk (with potential anti-atherogenic effect) would enhance the expression of PPAR-α mRNA. However, results obtained from this project showed that the expression of this gene was not drastically altered even when 20% (v/v) coconut milk was administered. This might suggest that coconut milk does not convey its metabolic effect, either good or bad, entirely through PPAR-α. Nevertheless, coconut milk did up-regulate the expression of PPAR-α to 1.18-fold at low dose [1%, (v/v)] and subsequently down-regulated it to 0.86-fold when higher dosage [20%, (v/v)] was used, although the results were not statistically significant. These changes in expression implied that the consumption of coconut milk at an appropriate, low amount could most likely induce the breakdown of fatty acid by a small degree, and vice versa.

The docking of PPAR-α on the promoters of its target genes (peroxisome proliferator response element, PPRE) is strongly dependent on the heterodimerisation with its pleotropic partner RXR-α, since this response element only bind efficiently to PPAR:RXR heterodimer but not PPAR monomer/homodimer[27]. Such interaction allows the retinoid receptor to share certain characteristic with PPAR-α, such as its anti-atherogenic feature. This is proven when the activation of RXR-α by its agonists was shown to promote the attenuation of atherosclerotic formation in ApoE null mice[19]. Surprisingly, the down-regulation of RXR-α was more drastic as compared to PPAR-α when higher dosages of coconut milk [10 and 20% (v/v)] were used. Since RXR-α also heterodimerise with other lipid activated nuclear receptors such as other PPAR isomers and liver X receptors (LXRs)[35,36], the down-regulation of RXR-α could share a common pattern with nuclear factors other than PPAR-α, which further strengthened the deduction that coconut milk might modulates its health effects through other nuclear receptors.

## CONCLUSION

In conclusion, coconut milk administration does not yield any cytotoxic effect towards the model system used in this project. Coconut milk only down-regulated the expression of RXR-α significantly when higher dosage of coconut milk was used but not PPAR-α. This, in other words, means that coconut milk will only affect the catabolism of fatty acid when consumed in high concentration. More research will be needed to conclude the actual health effects of this food product not only in lipid metabolism, but also other metabolic pathways such as glucose metabolism and inflammatory response. Whether or not this lipid-rich product will lead to the increase risk of metabolic diseases can only be affirmed by using further experiments, such as those involve the use of *in vivo* model.

## ACKNOWLEDGEMENT

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