

## Beneficial effects of tropical fruit-derived polyphenols against lipid-mediated stress *in vitro*

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### Abstract

Fatty liver formation is a consequence of hyperlipidemia, associated with an imbalance between lipid uptake and its metabolism. Persistent lipid load in the liver cells results in lipid-mediated toxicity or lipotoxicity which leads to the pathogenesis of several metabolic diseases. Recent studies suggest that activation of autophagic pathway might be useful to remove excess hepatic fat. Dietary polyphenols present in fresh fruits and vegetables are able to induce autophagy apart from their well-known anti-oxidative property. Our preliminary observation suggests that fruit-derived polyphenols might be effective to prevent hepatic fat accumulation by inducing autophagy. Total phenolic contents from dragon fruit and mangosteen have been used to address the beneficial properties of these two tropical fruits. Results showed that these polyphenols protect cells against lipotoxicity and oxidative stress. Expression levels of several key genes that are involved in lipid metabolism and energy expenditure were upregulated in presence of these polyphenols. This preliminary study suggests that the underutilized tropical fruits might be useful to develop functional foods, especially those containing beneficial phenolics.

*Index Terms:* Autophagy, glucotoxicity, hepatocyte, lipotoxicity, polyphenols

### 1. Introduction

Functional foods are receiving increased attention due to the fact that apart from nutrients, they contain a number of active ingredients that are beneficial for human health. The incident of non-communicable diseases are increasing and becoming one of the main concerns for health care sector and policymakers. The rising epidemic of obesity, obesity-related cardiovascular diseases (CVDs), and cancers has prompted the search for novel and feasible approaches to tackle the situation. High prevalence of these diseases in Asia concerns immediate attention from both healthcare and economic perspectives. One promising alternative therapeutic area comprises functional foods. In this regard, the bioactive properties of fresh fruits and vegetables in

preventing metabolic diseases are being investigated extensively. Tropical fruits and vegetables are often underutilized and their beneficial properties are either underestimated or not investigated enough to convince the consumers to use them as a means of maintaining good health. Polyphenols are the known beneficial phytochemicals present in fruits and act as an important determinant of their nutritional quality.<sup>1</sup> Polyphenols, with at least two phenol subunits, are the main classes of phenols found in plants along with their bioactive properties. According to the number of phenol rings, polyphenols are grouped into phenolic acids, stilbenes, flavonoids, tannins and lignans.<sup>1</sup> The health benefits of polyphenols are well established, especially as antioxidants. Consumption of polyphenol-rich foods has shown

to increase antioxidant capacity in plasma with reduced oxidative stress in liver. Several studies have reported that polyphenols help to prevent the development of cancers, CVD, diabetes, neurodegenerative diseases<sup>2</sup>, chronic obstructive pulmonary disease<sup>3</sup>, lung diseases<sup>4</sup>, osteoporosis<sup>5,6</sup>, adverse effect on aging<sup>7-9</sup> and protect skin damages from UV radiation.<sup>10</sup> The anti-carcinogenic properties of polyphenols are partly due to their ability to scavenge free radicals and ROS. Polyphenols present in green tea, such as catechin or epigallocatechin-3-gallate (EGCG), protects from obesity, insulin resistance, hypertension and obesity-related fatty liver diseases.<sup>11-13</sup> All these beneficial properties of polyphenols are believed to explain the “French paradox”, the lower CVD occurrence in France despite having relatively higher saturated fat intake and it is believed that this is because of consumption of higher dietary polyphenols.<sup>14</sup> Tropical fruits contain a wide number of polyphenols that have been found to prevent several cellular abnormalities which often lead to the development of cardiovascular and metabolic diseases. Polyphenolic compounds such as phenolic acids, flavonoids and lignans can modulate carbohydrate and lipid metabolism, reduce hyperglycaemia and insulin resistance, thus preventing long-term diabetes complications.<sup>15-17</sup>

In hyperglycaemia, chronic elevated level of plasma glucose damages insulin-targeted tissues and induces insulin resistance due to glucose-mediated toxicity or glucotoxicity. Elevated level of glucose efflux through constitutive glucose transporters into the cell overwhelms the mitochondrial electron transport chain which often results in the generation of reactive oxygen species (ROS) and induces cell death. Chronic hyperglycaemia causes type II diabetes (T2D) which contributes to the progression of long-term complications in vital organ such as blood vessels, heart, nerves, eyes and kidneys. In prolonged hyperglycaemia, pancreatic  $\beta$ -cells are often subjected to glucolipotoxicity which leads to the development of type I diabetes. T2D is often associated with obesity where individuals suffer from high level of circulatory lipids, a condition

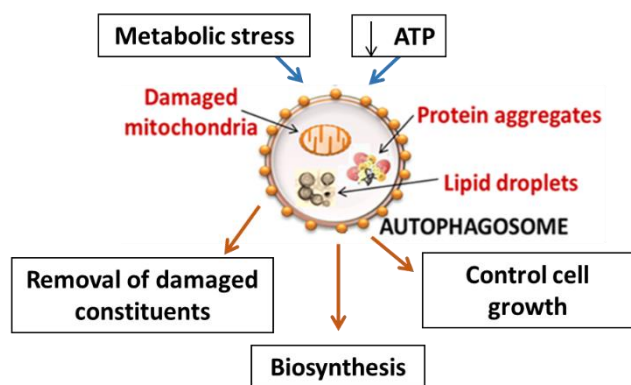
also known as hyperlipidaemia. A consequence of hyperlipidaemia is the ectopic fat deposition where lipids accumulate in non-adipose tissue and impair cellular function. Excess lipid accumulation in peripheral tissues including pancreatic  $\beta$ -cells and liver results in lipotoxicity, further leads to insulin resistance and T2D.<sup>18-20</sup>

The term “glucolipotoxicity” is widely used to describe the combined effects of glucotoxicity and lipotoxicity. Removal of damaged organelles, metabolizing excess lipids and cellular storage by catabolic processes reduce glucolipotoxicity. Among the well described phenomenon, autophagy is believed to be most effective catabolic process that helps to reduce intracellular fat and other lipid metabolites that exert lipotoxicity; while removal of damaged organelles and detoxifying free radicals reduce glucotoxicity.

Recycling and reusing of cellular metabolites are a well-conserved adaptive response in the living beings during unfavourable condition. Autophagy is such a mechanism that degrades unnecessary components to help maintain cellular energy needs during stress conditions (**Figure 1**). Stress conditions such nutrient deprivation, mitochondrial damages and metabolic stresses are known to induce autophagy.<sup>21-24</sup> Autophagy is necessary for embryonic development as this process maintains cellular homeostasis by eliminating misfolded proteins, unwanted or damaged organelles.<sup>25,26</sup> This process was initially thought to be adaptive in nature. However, recent advancement in science suggests that this recycling process could be beneficial to prevent lipid accumulation in the cells. The role of autophagy in lipid metabolism has thus gained attentions for the interplay between autophagy and metabolic syndromes.

Several recent findings suggest that autophagy is also involved in intracellular lipid break down to provide free fatty acids, a process known as lipophagy.<sup>27,28</sup> Sinha et al (2013) reported that hepatic lipid can be removed by inducing autophagy, suggesting an interesting role of this degradative process in preventing lipid accumulation in cells.<sup>29</sup> Bioactive food

constituents such as polyphenolics, triterpenoids, isothiocyanates, selenium as well as vitamins C, D, E and K2 were found to be inducers of autophagy.<sup>30</sup> These compounds are abundant in functional foods that induced autophagy and are listed in human diet for a long time. The best examples are tea, coffee, cocoa, red wine and nuts. The beneficial properties of these food ingredients are well known, however the molecular mechanism behind this beneficial effect remains to be illustrated.



**Figure 1.** The autophagic process is an adaptive response, initiated mainly by stressed conditions.

A recent animal study indicated that caffeine significantly reduces hepatosteatosis and concurrently increase autophagy and lipid uptake in lysosomes. The lipolytic actions of caffeine via autophagy may contribute to its ameliorative effects on NAFLD.<sup>31</sup> This suggests that induction of autophagy might be effective strategy to prevent lipid accumulation or lipid-mediated disorders. Nowadays, natural supplements and synthetic drugs that induce autophagy are widely developed for therapeutic purposes<sup>32-34</sup> and individual plant-derived polyphenols have been studied as potential therapeutic agents to treat metabolic syndromes.<sup>35</sup>

In this regard, we have used two tropical fruit-derived polyphenols which are known to induce autophagy and speculated that glucose and lipid-mediated toxicity might be mitigated in our experimental *in vitro* model.

## 2. Experimental approach

### Extraction of polyphenol

Total polyphenolic content was extracted from *H. undatus* and *G. mangostana* using the standard liquid-liquid extraction protocol. Freshly harvested fruit samples were lyophilized and extracted with methanol-water (60:40, v/v), at a ratio of solute to solvent of 1:10, at 35°C for one hour. The solvent was separated by centrifugation at 4°C, 2000 g for 10 minutes and then concentrated using a Buchi Rotavapor R-200 (Buchi, Switzerland) at 50°C. Extracts were filtered using 0.45µM nylon membrane filter (Agilent, Germany) to obtain clear hydrophilic solution. The filtrate was stored at -20 °C.

### Cell culture and treatments

Human liver carcinoma cells, HepG2 (ATCC HB-8065) have been used in this study as an *in vitro* model. The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 10U/ml penicillin-streptomycin, 100 mM sodium pyruvate, and 100 X MEM non-essential amino acids solution (Gibco, New York, USA). Cells were cultured in tissue culture flasks or dishes kept in a water jacket incubator at 37°C and in the presence of 7.5% carbon dioxide (CO<sub>2</sub>).

### Cell proliferation assay (MTS)

Cell viability was measured by MTS reagents, (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega, Wisconsin, USA) according to the manufacturer's protocol. 5000 cells per well were plated in 96-well plates containing 100 µl of culture medium and treated with food dyes as indicated in the figure legends. To analyze cell viability, 20 µl of the MTS solution was added to each well and the plates were incubated at 37°C in a humidified (5% CO<sub>2</sub>) incubator until it developed desired color. Absorbance of the reaction mix was measured at OD490 nm using a microplate reader (Varioskan Flash Multimode Reader, Thermo Scientific, USA).

### Oil Red O staining

To detect intracellular lipid accumulation, the cells were cultured in 24-well plates according to the experimental conditions indicated in the figure

legends. After treatment, the cells were washed with 1X PBS and then fixed with 10% formalin for one hour at room temperature. The cells were then washed twice with distilled water followed by 60% isopropanol for 5 minutes at room temperature and let it dry at room temperature. Once dried, the cells were stained with 0.21% Oil Red O solution (Sigma-Aldrich, Missouri, USA) for 10 minutes. The cells were then washed with distilled water for 4 times and dry at room temperature. 100% isopropanol was added to each well and incubated for 10 minutes under gentle shaking to elute lipid bound dye. The intensity of oil-red-o measured using a microplate reader at OD 500 nm.

#### *Extraction of RNA from cultured cells*

The cultured cells were washed with sterile PBS twice before lysed in 1.5 ml of QIAzol Lysis Reagent (Qiagen, Germany) and incubated at room temperature for 5 minutes. The lysate was transferred into 2 ml microfuge tubes. 300  $\mu$ l of chloroform was added into each tube and centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatant was transferred to a new microfuge tube and mixed with 0.75 ml isopropanol. The RNA was precipitated at room temperature for 10 minutes followed by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was discarded and RNA pellet was washed once with 75% ethanol.

#### *Real Time PCR*

1<sup>st</sup> strand cDNA was synthesised using the QuantiTect Reverse Transcription Kit (Cat. No. 205311, Qiagen) following manufacturer's protocol. Briefly, 2.5  $\mu$ g of total RNA was mixed with 2  $\mu$ l of gDNA buffer and top up to 14 $\mu$ l with RNase-free water. After 2 minutes incubation at 42°C, the sample mix was chilled on The reverse-transcription master mix was added and the reaction volume was brought up to 20  $\mu$ l. This reaction mix was incubated at 42°C for 30 minutes and finally the reverse transcriptase was inactivated by heating the samples at 95°C for 3 minutes. The prepared cDNA was used as a template for real time PCR using QuantiFast SYBR® Green RT-PCR Kit (Cat. No. 204154, Qiagen, Germany) following manufacturer's

protocol. Briefly, 1  $\mu$ l of 1st strand cDNA, 0.5  $\mu$ l of each set of primers and 10 $\mu$ l of QuantiFast SYBR® Green RT-PCR Master Mix were mixed and topped up to a total reaction volume of 20 $\mu$ l with DNase free water. The cDNA was amplified and quantified using Eco Real-Time PCR System (Illumina, California, USA).

#### *Acridine orange staining*

27  $\mu$ g/ $\mu$ l acridine orange solution (Sigma-Aldrich) was dissolved in PBS as suggested. 48 hours before treatment, one coverslip was placed in each well of a 6 well plate and cells were cultured at 37°C in a humidified (5% CO<sub>2</sub>) incubator. After treated with polyphenols (75  $\mu$ M and 150  $\mu$ M) for 72 hours, cells were stained with acridine orange solution. Each sample was mixed gently and images were captured immediately using fluorescence microscope (Nikon AZ100). Images were taken according to the recommended filter for green and red fluorescence and then merged using *Image J* software.

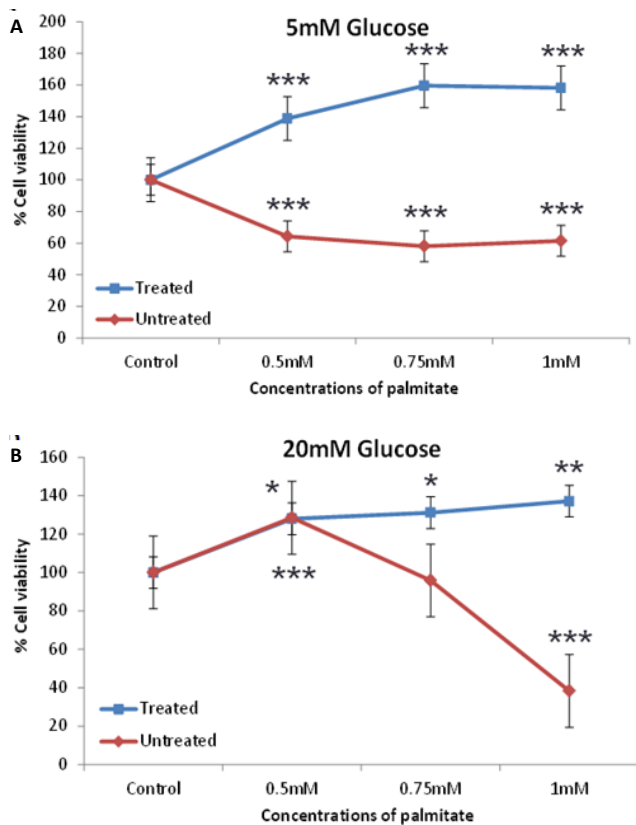
#### *Statistical analysis*

The data were expressed as mean  $\pm$  standard error (SE) for all experiments, except for the results for gene expression levels that were presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using SPSS Statistic 22.0 (IBM, USA). One-way analysis of variance (ANOVA) and post hoc Dunnett's test were applied in all independent experiments to compare values between control and treated groups. The values depicting  $p < 0.05$  were considered as statistically significant.

### **3. Results and Discussion**

To mimic the physiological condition of hyperlipidaemic condition, cells were cultured in in saturated fat (palmitate) containing medium. The percentage of cell viability is presented in **Figure 2**. MTS assay was performed after treating the cells with palmitate either in presence of extracted polyphenols (treated) or without polyphenols (untreated). The data showed a clear opposite trend in cell survival rate between treated and untreated groups. Cells that were treated with polyphenols (blue line) showed a pro-survival characteristic that increased gradually along with

the increase of palmitate concentrations. On the other hand, untreated cells (red line) underwent cell death that was also increased with the increase of palmitate concentrations. Compared to 20mM glucose (*Figure 2B*), the effect of polyphenol on viability was more obvious in 5mM glucose containing medium (*Figure 2A*). In presence of 20mM glucose and palmitate, cell viability was remarkably increased in presence of polyphenols (*Figure 2B*), suggesting the protective role of these polyphenols against glucose and lipid mediated stresses.



**Figure 2.** Polyphenols affect cell viability at different palmitate and glucose concentrations. 2000 cells/well was distributed before 48 hours of incubation and the percent of cell survivability was measured by MTS assay. Values are mean  $\pm$  SE of four replicates. Polyphenols (75 $\mu$ M) significantly increased cell viability, while cell survivability in the untreated group is significantly decreased. (\* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$  vs control)

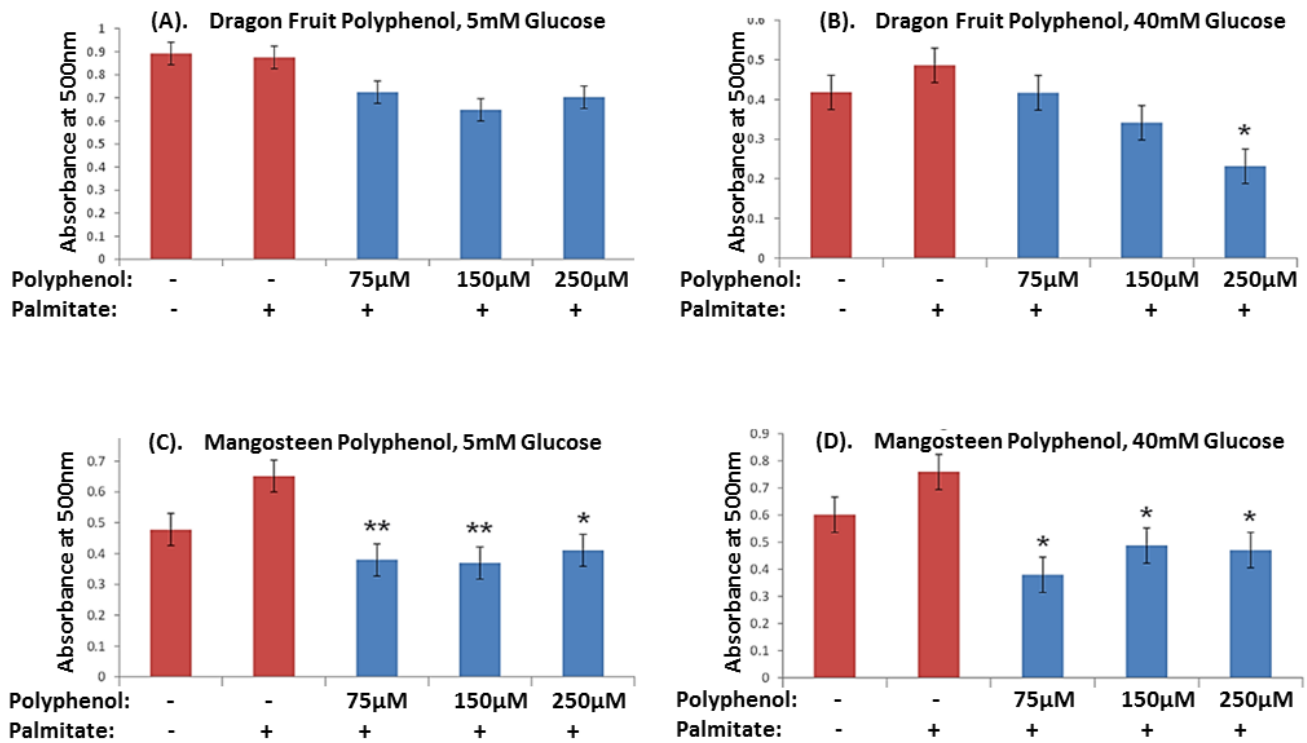
Induction of autophagy in the form of lipophagy (that selectively degrades cellular lipids) is known to reduce metabolic stresses by removing hepatic lipid droplets. Hence, the amount of lipid accumulated in these cells in hyperlipidemic

condition was measured to explain the previous initial observation (as shown in *Figure 2*). Lipid accumulation was evaluated by oil-red-o (ORO) staining in presence of palmitate along with polyphenols at different concentrations. Interestingly, intra cellular lipid level was reduced in all experimental units (*Figure 3*). Dragon fruit derived polyphenols are effective in preventing lipid accumulation in hyperglycaemic condition (*Figure 3B*) while mangosteen derived polyphenols significantly reduced lipid accumulation at all different polyphenol concentrations in both hypoglycaemic and hyperglycaemic conditions (*Figure 3C* and *Figure 3D*).

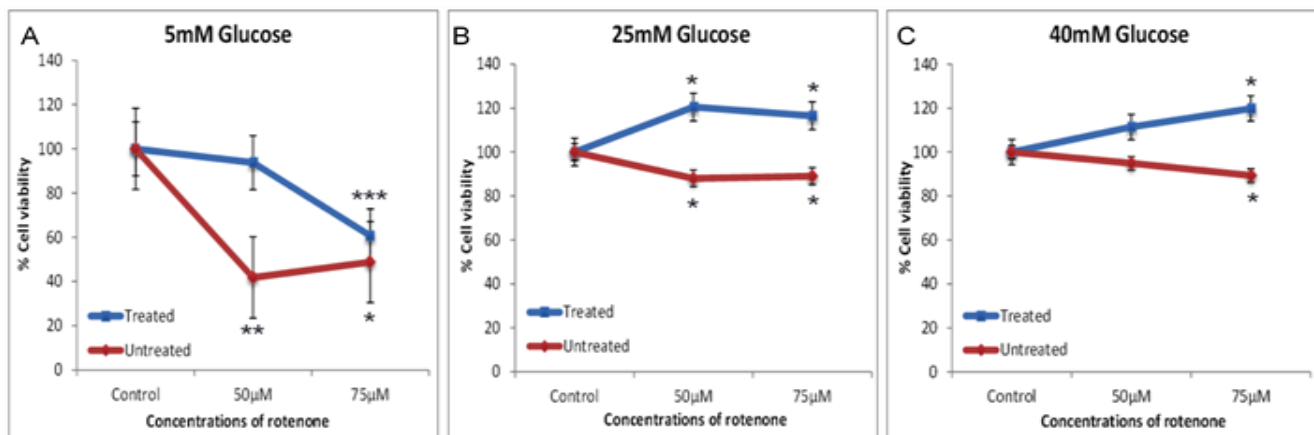
To confirm the pro-autophagic properties of these polyphenols, cells were treated with rotenone to impair mitochondrial function and cell survivability was measured. Rotenone increases reactive oxygen species (ROS) generation by blocking oxidative phosphorylation and subsequently inhibiting mitochondrial respiration.<sup>36</sup> When mitochondria are affected, cells that undergo extensive autophagy manage to survive.

The effects of polyphenols on cell viability during rotenone-induced oxidative stress were evaluated by MTS assay as shown in *Figure 4*. The data indicated that rotenone significantly reduced cell survivability in the absence of polyphenol supplementation (red line). In contrast, cells that were treated with polyphenols exhibited higher survival rate compared to control in 25mM and 40mM glucose (*Figure 4B* and *Figure 4C*). This data suggest that apart from nullifying oxidative stress, polyphenol-induced autophagy might help the cells to survive better when their mitochondria are affected by recycling cellular reserves. Autophagy may also help these cells to remove damaged mitochondria and thus increases cell survivability.

To confirm the presence of autophagic vesicles, cells were stained with acrydine orange. In neutral pH, acrydine orange produces green fluorescence, while it results orange-red fluorescence in presence of acidic autophagic vesicles. As



**Figure 3.** The effects of polyphenols on cellular lipid accumulation were measured by ORO staining. Absorbance was taken after 72 hours incubation. Values are mean ± SE of three replicates. Both types of polyphenols reduced the amount of lipid accumulated in the hepatocytes, however mangosteen derived polyphenols is more effective than dragon fruit-derived polyphenols. (\*P<0.05, \*\*P<0.001)

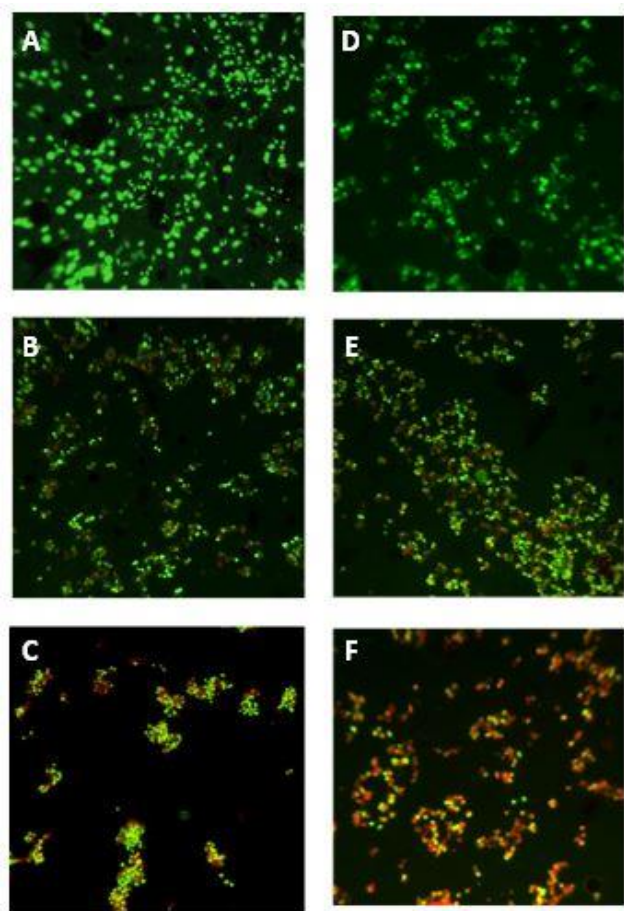


**Figure 4.** Polyphenols influence cell growth at different concentrations of glucose and rotenone. 2000 cells/well were distributed and treated with polyphenols and rotenone accordingly. MTS reagents were added after 48 hours incubation and absorbance were taken at the second hour. Values are mean ± SE of four replicates. In 25 mM and 40 mM glucose, polyphenols significantly protect cell from oxidative stress. (\*P<0.05, \*\*P<0.001, \*\*\*P<0.0001)

illustrated in **Figure 5**, due to the formation of autophagosomes, cells that were treated with polyphenols exhibited the presence of acidic autophagic vesicles and their number was highest in 150 µM polyphenol treated cells.

To elucidate the beneficial effects of these polyphenols, we have investigated the expression pattern of genes involved in lipid metabolism upon exposing the cells to different concentrations of polyphenols (**Figure 6**). mRNA expression level of peroxisome proliferator-activated

receptors alpha (PPAR- $\alpha$ ), peroxisome proliferator-activated receptors gamma coactivator-1 alpha (PGC-1 $\alpha$ ), uncoupling protein 1 (UCP1), and sterol regulatory element binding protein 2 (SREBP-2) were determined by Real-Time PCR (**Figure 6**). These are the key genes involved in glucose and lipid metabolism and energy expenditure in the liver. The mRNA expression level of all these genes (SREBP-2, PPAR- $\alpha$ , PGC-1 $\alpha$ , and UCP1) was significantly up-regulated in the samples treated with 250 $\mu$ M of experimental polyphenols (**Figure 6**).

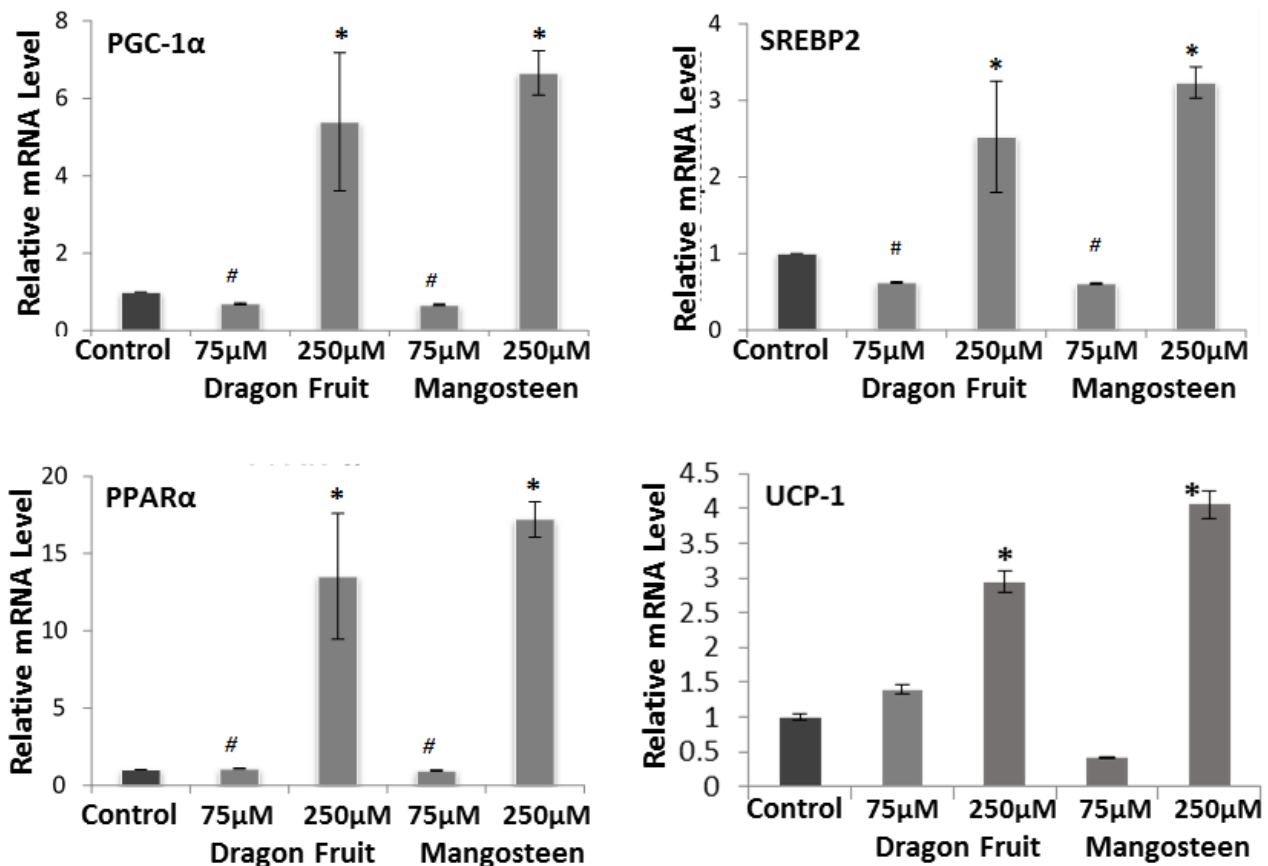


**Figure 5.** Polyphenols induced massive autophagy in hepatic cancer cells. Microphotographs were captured using a fluorescence microscope (30X magnification). A, B, C: control, 75  $\mu$ M, and 150  $\mu$ M polyphenol, respectively in 5mM glucose. D, E, F: the same group of cells in 40 mM glucose.

We have used human liver cell line (HepG2) in order to assess the beneficial properties of these fruit-derived polyphenols. Liver is the central organ of metabolism and hepatocytes are special

cells that deal with high level of glucose and lipids in hyperlipidemia and hyperglycemia. Glucolipototoxicity often affects these cells that causes insulin resistance, cellular damage due to xenotoxic stress, and induces cell death. All these cellular events are associated with non-alcoholic fatty liver disease (NAFLD) and its advanced form such as NASH or cirrhosis. Inhibition of ROS and intracellular lipids is a proposed alternative strategy to combat this situation. Both glucose metabolism and lipid peroxidation generate ROS, excess amount of this metabolic byproduct eventually induces cellular stress or cell death. Polyphenols are known antioxidants and are believed to be effective in protecting cells from glucolipototoxicity, though the precise mechanism is not well illustrated. Apart from anti-oxidative property, certain polyphenols are believed to be efficient inducers of autophagy. This self-degradative catabolic process might help to remove defective mitochondria and other cellular metabolites such as lipid droplets during hyperglycemia and hyperlipidemia. Autophagy deficient mice developed hepatomegaly due to increased TAGs and cholesterol accumulation.<sup>37</sup> Autophagy (mainly lipophagy) exerts its protective effects by breaking down lipids to produce ATP, promote metabolism of toxic lipid molecules and prevent energy depletion which results in apoptosis or necrosis during mitochondrial impairment.<sup>38</sup>

In this experiment, cells treated with polyphenols exhibited higher viability in presence of palmitic acid (**Figure 2**), suggests that polyphenols might protect these cells from glucolipototoxicity by inhibiting ROS as well as inducing autophagy. This was further supported by staining the cells with lipid-specific oil-red-o staining. Mangosteen derived polyphenol (**Figure 4C** and **Figure 4D**) is more effective to clear hepatic lipid than dragon fruit derived polyphenol (**Figure 4A** and **Figure 4B**). Mangosteen is rich in xanthenes, a class of polyphenolic compounds, which is responsible for its bioactive properties. Previous studies reported that mangosteen-derived xanthenes can inhibit cell growth through induction of autophagy by suppressing mTOR pathway. This suggests that the reduction of hepatic lipid accumulation could



**Figure 6.** mRNA expression of indicated genes detected by Real-Time PCR. Data are expressed as mean  $\pm$  SD of three replicates. DF: polyphenol from dragon fruit; MNG: mangosteen polyphenol. (\* $P < 0.001$ , \*\* $P < 0.0001$ ).

be due to the induction of autophagy by xanthenes or other similar polyphenols present in these fruits. On the other hand, hydroxycinnamates are the main phenolic compound in dragonfruits<sup>39</sup>. So far, their role in autophagy has not yet been illustrated. However, based on this study, both polyphenols are able to induce autophagy as detected by staining acrydine orange (**Figure 5**).

Mitochondria are most vulnerable organelles in glucolipotoxicity that often leads to decrease cellular ATP level which leads to necrosis or triggers mitochondria-mediated (intrinsic) apoptotic cell death. Autophagy, in form of mitophagy, plays a protective role in cells with defective mitochondria by recycling and removing the damaged mitochondria from cytoplasm. As stated earlier, autophagic process helps to protect cells from such stress which we have observed in our experiment. To confirm this speculated phenomenon, we have treated cells with rotenone,

a known mitochondrial poison that impairs ATP production<sup>40</sup>, a situation that occurs during glucolipotoxicity. High survival rate in the rotenone-treated cells in the presence of polyphenol was most probably attributed to autophagy (mitophagy and lipophagy). The result supports the hypothesis that pro-autophagic property of polyphenols improves cell survivability by recycling cellular organelles or cellular reserves.

Finally, the expression pattern of certain genes involved in lipid metabolism and induction of autophagy was investigated. SREBP2, involved in cholesterol metabolism, was upregulated in all experimental units upon treating cells with polyphenols. Existing evidences suggest that SREBP2 contributes to the activation of autophagy inducing genes<sup>41</sup> and SREBP2 knockout mice exhibited decreased autophagosome formation and along with reduced



LC3 expression, a known molecular marker of autophagy.<sup>42</sup> Similarly, expression level of PGC-1 $\alpha$  and its downstream targets such as PPAR $\alpha$  and UCP1 were significantly high in cells treated with these extracted polyphenols. Expression of these genes induces mitochondrial biogenesis and fatty acid oxidation that ultimately helps to reduce cellular lipid level. Anti-obesity drugs often target this pathway, such as upregulating UCP1 to accelerate uncoupling mitochondrial respiration, leading to the elevated heat production by dissipating oxidation energy.<sup>43</sup>

## 5. Conclusion

This study is a part of our ongoing research project where we are investigating the molecular mechanisms that drive the beneficial effects of phytonutrients, especially polyphenols that are present in our tropical fruits. Though the findings need to be confirmed using *in vivo* or other *in vitro* models, this preliminary observation suggests that increased consumption of these tropical fruits might be beneficial to our local population who are at high risk of developing obesity or obesity-related disorders. Amongst these two fruits, total phenolic contents from mangosteen are beneficial for releasing hepatic lipid load and mediating lipid metabolism. Further *in vivo* studies are necessary to determine the bioactive compounds that exert these beneficial effects.

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