Thesis for the Degree of Doctor of Philosophy

# Computational Screening of Ginseng Saponins and Efficacy Analysis of Active F2, Rh1, Rf and PPT Ginsenosides on 3T3-L1 Adipocyte

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Kyung Hee University

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By

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A dissertation Submitted to the Graduate School of Biotechnology of

Kyung Hee University in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

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## ABBREVIATIONS

| | |
|---|---|
| PPD | Protopanaxadiol |
| PPT | Protopanaxatriol |
| WAT | White adipose tissue |
| BAT | Brown adipose tissue |
| BMI | Body mass index |
| WHR | Waist-to-hip circumference ratio |
| WC | Waist circumference |
| CVD | Cardio vascular disease |
| PPARγ | Peroxisome proliferator-activated receptor-gamma |
| GLUT4 | Glucose transporter 4 |
| KLF5 | Kruppel-like factor 5 |
| AMPK | Adenosine monophosphate-activated protein kinase |
| FAS | Fatty acid synthesis |
| PEPCK | Phosphoenol pyruvate carboxykinase |
| LPL | Lipoprotein lipase |
| HFD | High-fat diet |
| LFD | Low-fat diet |
| PDB | Protein data bank |
| LGA | Lamarckian genetic algorithm |
| PASS | Prediction of activity spectra for substances |

| | |
|---|---|
| Pa | Probabilities of activity |
| Pi | Probabilities of inactivity |
| Ps | Picosecond |
| Ns | Nenosecond |
| ADMET | Absorption, distribution, metabolism, excretion, and toxicity |
| MD | Molecular dynamic |
| RMSD | Root mean square deviation |
| RMSF | Root mean square fluctuation |
| IBMX | Isobutylmethylxanthine |
| DMEM | Dulbecco's modified eagle's medium |
| ATCC | American type culture collection |
| BCS | Bovine calf serum |
| AB | Antibiotic |
| DM | Differentiation medium |
| DMSO | Dimethyl sulfoxide |
| aP2 | Adipogenic protein |
| DS | Discovery studio |
| SE | Standard error |
| PNLIP | Pancreatic triacylglycerol lipase |
| PL | Pancreatic lipase |

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## ABSTRACT

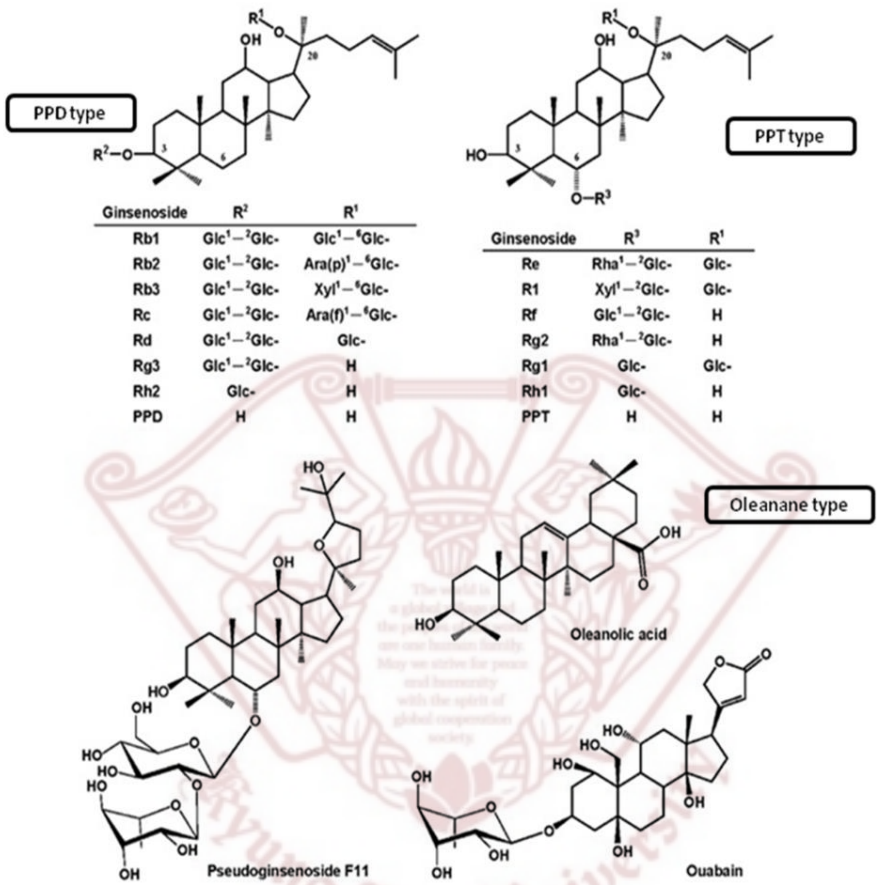
Obesity is identified as an excess accumulation of body fat. It is a chronic medical disease that can lead to several other diseases. It has become necessary to discover methods to reduce obesity in order to maintain a healthy life. Until now, several drugs have been shown to treat obesity. But due to various side effects researchers have focused on natural resources to treat obesity. Among many medicinal herbs, *Panax ginseng* is one of the most popular oriental medicinal plants that has been used for long time in order to treat several diseases including obesity. Therefore in our study we have also focused on *Panax ginseng* derived saponins called ginsenosides to treat obesity. For our study, at first from 128 ginsenoside saponin based on the literature, the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties, to cover important PPT and PPD saponins and availability, 15 ginsenosides (F2, Rg3, Rb1, Rb2, Rc, Rd, Rg1, Rg2, Rh2, Ro, Re, Ck, Rf, Rh1, PPT) were chosen for docking simulation. To find out there binding interaction with major genes involved in adipogenesis and obesity. It was seen that among them 6 ginsenosides (F2, Rg3, Re, Ck, Rh1, Rf) possessed bond formation with PPARγ, the major transcriptional factor for adipogenesis. Since PPT ginsenoside is an important saponin, we further checked docking interaction of PPT ginsenoside with Pancreatic Lipase (PL) that is involved in fat break down in intestine. After docking simulation PPT was found to have binding affinity with PL. Therefore we next carried out experimental investigations to see there efficacy in cell level. But among them compound K, Re and Rg3 already has there experimental evidences on 3T3-L1 cell line. So, we performed experiments using other compounds. 3T3-L1 is an adipocyte cell line that is commonly used for studying obesity in cell level. From our experimental results it was seen that, the selected ginsenosides were not toxic to the cell from lower (1 μM) to higher (100μM) concentrations. Then we checked the oil red o staining to see the lipid accumulated by the cells and after that we quantified the amount of lipid in cells. The results shown to decrease the amount of lipid accumulated by the cells dose dependently when they were treated with ginsenosides. Finally we checked the expression of adipogenic genes. They were seen to be inhibited when checked using RT-PCR and qRT-PCR. That proves that the process of adipogenesis was blocked. Therefore from our computational

screening and experimental evidences it was proved that F2, Rh1 Rf and PPT ginsenoside may be useful compounds to be used as an anti-obese drug.

# I. GENERAL INTRODUCTION

Adipocytes are essential to maintain energy balance and mediate numerous factors involved in immunological responses, vascular diseases, and appetite regulation (Gregoire *et al.*, 1998; Gregoire *et al.*, 2001; Nawrocki *et al.*, 2005). In order to maintain health and prevent diseases adipocyte regulation is necessary. Changes in adipocyte size and number often involve a complex interplay between the differentiation and proliferation of pre-adipocytes (Gregoire *et al.*, 2001). Adipose tissue stores lipid in the form of triglycerides and cholesterol esters within the lipid droplets representing specialized organelles inside the adipocytes. These are highly active endocrine cells that regulate varieties of physiological functions (Mahalia *et al.*, 2007). Impaired adipogenesis may result in obesity or insulin resistance and diabetes (Bays *et al.*, 2004), and so the prevention of metabolic diseases has become an issue of consideration to suppress and maintain a healthy life. Studies have found that bioactive natural compounds may be potent agents for curing various diseases, and so natural products that modulate protein expression have attracted attention of researchers (Surh *et al.*, 2003).

The *Panax ginseng* Meyer plant has a long history in Asian countries and is extensively used. It has a wide range of beneficial effects. The main active components in ginseng are ginsenosides, which are generally assumed to be involved in the pharmacological actions such as lipid metabolism (Attele *et al.*, 1999). They can be classified into three groups according to their aglycone structures: 20(S)-protopanaxadiol (ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Rg3), 20(S)-protopanaxatriol (ginsenosides Re, Rg1, Rg2, Rh1) (Helms *et al.*, 2004), and oleanene (ginsenoside Ro) (Fig. 1.1). In a diabetic ob/ob mouse model, ginsenosides showed anti-hyperglycemic and anti-obese activity (Xie *et al.*, 2005). Its extracted and isolated ginsenosides also showed anti-hyperglycemic and anti-obesity properties in diabetic rodents (ob/ob and KKAy mice) (Chung *et al.*, 2001). There are many more studies regarding ginseng and obesity currently underway, both in animal models and at the cellular level.




| Ginsenoside | $R^2$ | $R^1$ |
|---|---|---|
| Rb1 | $Glc^1-{}^2Glc$- | $Glc^1-{}^6Glc$- |
| Rb2 | $Glc^1-{}^2Glc$- | $Ara(p)^1-{}^6Glc$- |
| Rb3 | $Glc^1-{}^2Glc$- | $Xyl^1-{}^6Glc$- |
| Rc | $Glc^1-{}^2Glc$- | $Ara(f)^1-{}^6Glc$- |
| Rd | $Glc^1-{}^2Glc$- | Glc- |
| Rg3 | $Glc^1-{}^2Glc$- | H |
| Rh2 | Glc- | H |
| PPD | H | H |

| Ginsenoside | $R^3$ | $R^1$ |
|---|---|---|
| Re | $Rha^1-{}^2Glc$- | Glc- |
| R1 | $Xyl^1-{}^2Glc$- | Glc- |
| Rf | $Glc^1-{}^2Glc$- | H |
| Rg2 | $Rha^1-{}^2Glc$- | H |
| Rg1 | Glc- | Glc- |
| Rh1 | Glc- | H |
| PPT | H | H |

**Fig. 1.1.** Structure of ginsenosides.

For the study, at first from 128 ginsenosides based on the literature, the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties, to cover important PPT and PPD saponins and availability, 15 ginsenosides (F2, Rg3, Rb1, Rb2, Rc, Rd, Rg1, Rg2, Rh2, Ro, Re, Ck, Rf, Rh1, PPT) were chosen for docking simulation. To find out there binding interaction with major genes involved in adipogenesis and obesity. It was seen that among them 6 ginsenosides (F2, Rg3, Re, Ck, Rh1, Rf) possessed bond formation with PPARγ, the major transcriptional factor for adipogenesis. Since PPT ginsenoside is an important saponin, we further checked docking interaction of PPT ginsenoside with Pancreatic Lipase (PL) that is involved in fat break down in intestine. And found PPT to have binding affinity with PL. Therefore next experimental investigations were carried out to see there efficacy in cell level. But among them compound K, Re and Rg3 has there experimental evidences on 3T3-L1 cell line. So, we performed experiments using other compounds (Fig. 1.2).

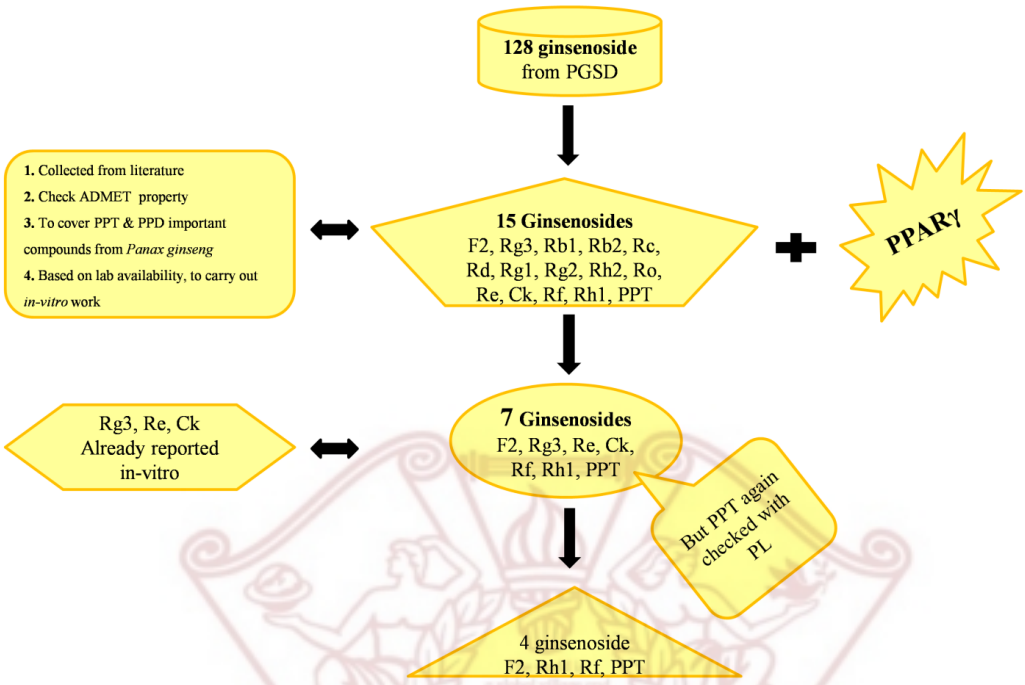



**Fig. 1.2.** Selection of *Panax ginseng* saponins.

## II. REVIEW OF LITERATURE

### 2.1. Biology of adipose tissue

Adipose tissue is loose connective tissue composed of adipocytes, obtained from lipoblasts. Its main role is to collect energy in the form of lipids (Kershaw *et al.,* 2004). Two types of adipose tissue exist: white adipose tissue (WAT) and brown adipose tissue (BAT). The formation of adipose tissue is controlled in part by the adipose gene. Adipose tissue was first discovered by the Swiss naturalist Conrad Gessner in 1551 (Cannon *et al.,* 2008). In mammals, there are two main depots of WAT located in subcutaneous and intra-abdominal. In obesity, intra-abdominal fat accumulation is strongly associated with the development of related diseases but the accumulation of subcutaneous fat exhibits no correlation (Stephane *et al.,* 2012). WAT through the process of differentiation forms the matured adipocytes by storing lipid droplets into it (Fig. 2.2).

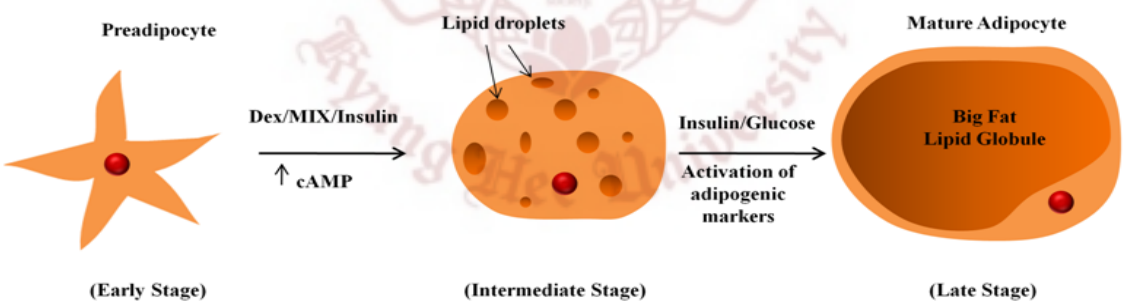



**Fig. 2.2.** Conversion of pre-adipocyte to adipocyte through the process of differentiation.

BAT is involved in thermogenesis and is positioned in discrete pockets in the paravertebral, supraclavicular and periadrenal sites (Frontini *et al.,* 2010). A widely used process to measure the body fat of a person is the body mass index (BMI), which is the ratio of weight in kilograms to height in meters squared ($kg/m^2$). It has been found to be correlated with morbidity and mortality risk in many populations (Willett *et al.,* 1999). Abdominal obesity can be quantified using simple parameters such as waist circumference (WC) and the waist-to-hip circumference ratio (WHR). BMI, WC and WHR are highly interrelated and it is believed that a combination of these parameters may be used to identify people at risk of Cardio Vascular Disease (CVD) better than any of them individually (Ardern *et al.,* 2003; Janiszewski *et al.,* 2007; Meisinger *et al.,* 2006).

The process of differentiation known as adipogenesis is synchronized by an enlarged network of transcription factors that co-ordinate the expression of hundreds of proteins responsible for the formation of mature fat cell phenotypes. At the core of this network are two main regulators of adipogenesis, PPARγ and C/EBPα, which supervises the entire terminal differentiation process. PPARγ, in particular is believed to be the master regulator of adipogenesis; without it, precursor cells are unable to express any known aspect of the adipocyte phenotype (Rosen *et al.,* 1999).

Conversely, cells deficient in C/EBPα are susceptible to adipocyte differentiation; however, these C/EBPα-deficient cells are insulin resistant (El-Jack *et al.,* 1999; Wu *et al.,* 1999). Once the adipogenesis program begins, a transcriptional cascade starts to induce the expression of metabolic genes and adipokines associated with the adipocyte phenotype, such as fatty acid-binding protein 4 (FABP4; also known as aP2), glucose transporter 4 (GLUT4) also known as SLC2A4), leptin and adiponectin; this is known as the terminal differentiation stage (Lefterova *et al.,* 2009; Hwang *et al.,* 1997; Rosen *et al.,* 2006) (Fig. 2.3). Ginsenoside or its extracts are shown to act on these genes and alternate the differentiation.

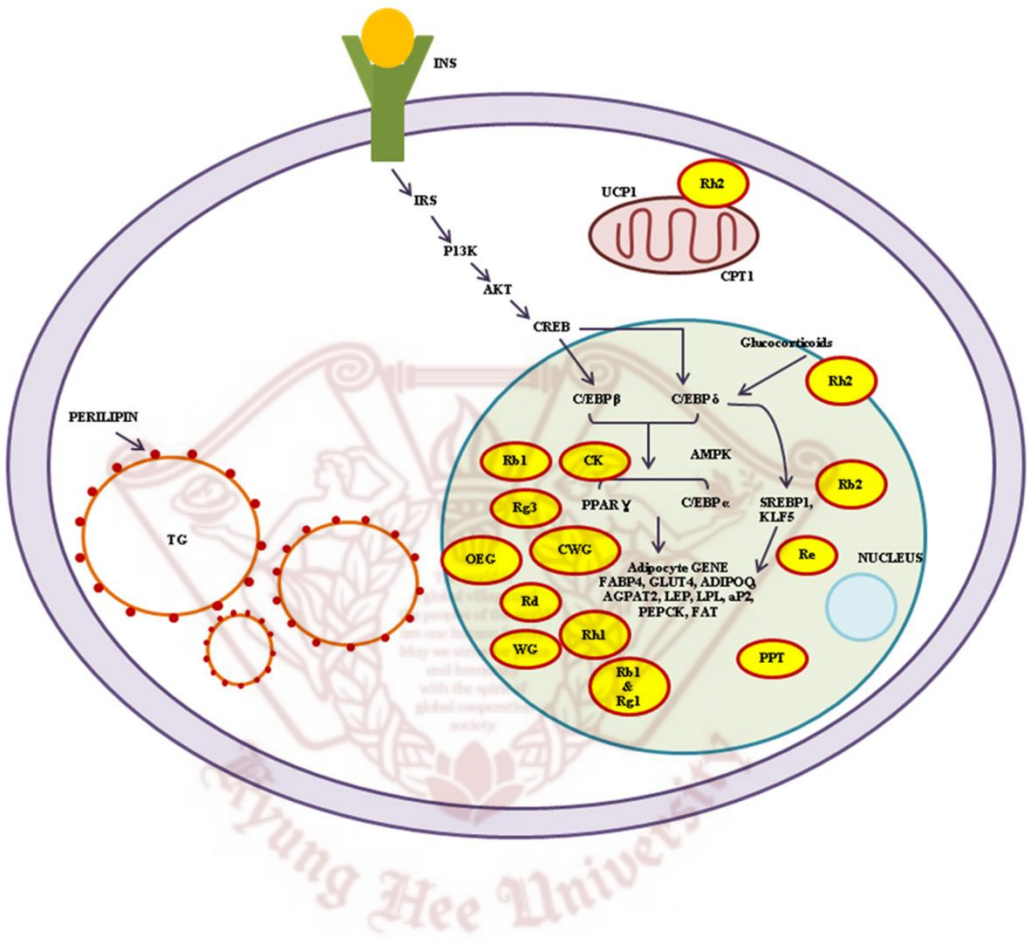



**Fig. 2.3.** Ginsenosides involved in adipogenesis pathway and their interaction with the proteins involved in the pathway.

## 2.2. Obesity and ginseng mechanisms

Ginseng is a plant that belongs to the Araliaceae family and *Panax genus*. The two main types of ginseng are *Panax ginseng* Meyer (Asian ginseng) and *Panax quinquefolius* (American ginseng). Korean ginseng is again divided into red and white ginseng depending on the processing method of the root (David *et al.,* 2011). Twenty-eight studies on diabetic mice and rats from 7 research centers (in 6 different nations) represented that both Korean ginseng (*Panax ginseng*) and American ginseng (*Panax quinquefolius*) are functional in anti-hyperglycemic supplements, putatively acting via improvements in insulin secretion, insulin sensitivity, islet protection, obesity reduction, antioxidation, energy expenditure, and fat absorption (Zhanxiang *et al.*, 2012). To study obesity 3T3-L1, 3T3-F422A, 1246, Ob1771, TA1 and 30A5 cell lines are chosen mostly because they are unipotent preadipocytes and they can either remain as preadipocytes or be converted to adipose tissue. They are ideal for studying molecular events related to the conversion of preadipocytes into adipocytes (James *et al.,* 2000).

Three classes of transcription factors have been found that directly influence this conversion: PPARγ, C/EBPs, and the basic helix–loop–helix family (ADD1/SREBP1c) (Evan *et al.,* 2000). PPARγ is highly expressed in adipose tissue. Activation of PPARγ generates adipocyte differentiation and lipid accumulation by altering numerous genes regulating adipogenesis, lipid uptake and lipid metabolism. Notably, PPARγ void cells cannot differentiate into adipocytes, and the adipose specific ablation of PPARγ produces adipocyte hypocellularity and reduced adiposity (Laplante *et al.,* 2003). Most of the studies of ginseng and obesity have been completed at the cellular level using the 3T3-L1 mouse cell line (Table 2.1).

In most cases of the studies, ginseng or its single saponin has been shown to inhibit the expression of PPARγ resulting in the inhibition of adipogenesis, which can combat obesity (Park *et al.,* 2008; Hwang *et al.,* 2007; Mohammad *et al.,* 2011; Hwang *et al.,* 2009; Kim *et al.,* 2007; Wan *et al.,* 2013; Dongmin *et al.,* 2012). 20 (*S*)-protopanaxatriol (PPT), one of the ginsenoside metabolites which includes Re, Rf, Rg1, Rg2 and Rh1, has been shown to increase PPARγ transactivation activity in a dose dependent manner, at the concentration of 10 μM. It increased activation by 5.5 fold which shows activity similar to troglitazone, a well known PPARγ agonist.

PPT induces adipogenesis by increasing the expression of PPARγ target genes such as aP2, LPL and PEPCK (Han *et al.,* 2006). Another study showed that treatment of 3T3-L1 adipocytes at concentration 10 μM of ginsenoside Rb1 resulted in increased expression of PPARγ2, C/EBPα and increased lipid accumulation about 56 %, which accelerated adipocyte differentiation (Wenbin *et al.,* 2007). Ginsenoside Rb2 is found to be an important component in the development of drugs for lowering lipid levels. As the experimental groups of high fatty acid conditions were supplemented with 10 μg/ml of ginsenoside Rb2, it significantly reduced TAG level by 4~47 % at different intervals of time. Along with lowering TAG levels in 3T3-L1 adipocytes stimulated the expression of SREBP and leptin mRNA (Kim *et al.,* 2009).

Table 2.1. Effect of ginseng on different molecular targets related to obesity on 3T3-L1 cell line

| Materials | Dose | Molecular mechanism | Reference |
|---|---|---|---|
| Ginsenoside 20(S) protopanaxtriol | 10 µM for 7 days | Activation of PPARγ<br>↑ aP2 (7.7x), LPL (8.9x), PEPCK (3.9x), GLUT-4 | (Han *et al.*, 2006) |
| Ginsenoside Rb1 | 10 µM for 6 days | Promotes adipogenesis<br>↑ PPARγ2 (4.5x), C/EBPα (4.7x), aP2 (2.2x) | (Wenbin *et al.*, 2007) |
| Ginsenoside Rb2 | 10 µg/ml | Lowers cholesterol & triacylglycerol<br>↑ SREBP (2.6x), Leptin (4.2x), FAS, GPDH | (Kim *et al.*, 2009) |
| Red ginseng extract, triol, diol, and less polar ginsenosides | 10 µM, 50 µM, 100 µM for 6 days | Ginsenosides digested *in vitro* have an inhibitory effect on lipid accumulation. Rg3 and Rk1 more effective. | (Kim *et al.*, 2009) |
| *Panax ginseng* extract PGE {high Rc content} | 1 µg/ml and 10 µg/ml during differentiation | Increases adiponectin expression<br>↑ Acrp30 (46±14.3 % & 58.2±16.8 %) | (Chia *et al.*, 2011) |
| Ginsenoside Rh2 | 0.01 µM, 0.1 µM, & 1 µM 24 hours treatment | Activation of glucocorticoid receptors, Inhibition of proliferation at 1 µM by 13 % Rh2 acts like dexamethasone to induce differentiation | (Niu *et al.*, 2009) |

| | | | |
|---|---|---|---|
| Ginsenoside Rb1 & Rg1 (93.9 % & 96.8 % Purity) | 20 µM during differentiation | Improved glucose homeostasis by suppressing adipogenesis in adipocytes<br>↓ PPARγ, C/EBPα, aP2 (slightly decreased) | (Park *et al.*, 2008) |
| Ginsenoside Rh2 | 20 µM, 40 µM for 8 days | Inhibition of adipogenesis<br>↑ UCP-2, CPT-1, ↓ PPARγ (2.0x, 1.3x) | (Hwang *et al.*, 2007) |
| Cultivated wild ginseng (CWG) | 50, 100, 200, 250, 500, 1,000 µg/ml for 8 days | Cell cycle blocked at sub-G1 phase at 9 %, 16.52 % & 20.82% at concentration 250, 500 & 1000 µg/ml<br>↓ PPARγ, C/EBPα | (Mohammad *et al.*, 2011) |
| Ginsenoside Rg3 | 20 µM, 40 µM for 6 days | Involved in the PPARγ and AMPK signaling pathways<br>↓ PPARγ (0.7x), ↑ AMPK<br>(3hr treatment 1.2x & 2.5x) | (Hwang *et al.*, 2009) |
| Ginsenoside Rd | 80 µM during differentiation | Adipocyte differentiation inhibition<br>↓ PPARγ (AMPK) activation was involved | (Kim *et al.*, 2007) |
| Ginsenoside Rh1 | 50 µM, 100 µM for 2 days with DMI | Potentially inhibited adipogenesis<br>↓PPAR-γ, C/EBP-α, FAS, aFABP | (Wan *et al.*, 2013) |
| Compound K | 0.05 µM, 0.5 µM, & 5 µM for 2 days | Suppresses adipogenesis<br>↓PPAR-γ, C/EBP-α, Leptin, aP2 | (Dongmin *et al.*, 2012) |

When ginsenosides were *in vitro* digested with artificial gastric and intestinal fluids (10 µM, 50 µM and 100 µM) it showed an inhibitory effect on lipid accumulation in 3T3-L1 adipocytes, and it was also shown that among Rg3, Rk1, Rg5 the less polar ginsenoside that is Rg3, significantly decreased the lipid content in differentiated 3T3-L1 adipocytes (Kim *et al.*, 2009). Ginsenoside Rh2 at low concentrations (0.01 & 1 µM) has the ability to induce adipogenic differentiation by activating glucocorticoid receptors (GR) that regulates lipid metabolism through promoting lipogenesis in adipose tissue but treatment with Rh2 at higher concentration (1 µM) for 24 hr showed an inhibition of the proliferation by 13 % (Niu *et al.*, 2009).

Again, Rb1 and Rg1 in 3T3-L1 adipocytes activated PKA dependent cAMP pathway and suppressed intracellular triglyceride accumulation. The increase of insulin stimulated glucose uptake with ginsenosides (20 µM) and exendin-4 was less than that of Rosiglitazone by 1.8 fold (Park *et al.*, 2008). Two recent studies with ginsenoside Rh1 and compound K on 3T3-L1 showed to be effective during adipogenesis process (Wan *et al.*, 2013; Dongmin *et al.*, 2012).

Other than cell lines, animal models such as humans, rats or mice are also used to check the efficacy of single ginsenosides or its crude extracts (Table 2.2). In a current study, researchers aimed to determine the effects of Korean red ginseng (KRG) in obese women. Subjects were instructed to take 12 capsules of 500 mg KRG for a total of 6g extract, three times a day and to show that the effects of KRG on obesity differ depending on the gene involved. Comparisons of subjects before and after taking KRG showed significant improvements in terms of weight, body mass index (BMI), waist-hip ration (WHR), daily food intake (FI), and the Korean-style questionnaire on the quality of life (KOQOL). KRG group (9.09 %) displayed >5 % weight loss, while none of those in the placebo group showed comparable weight loss (Kwon *et al.*, 2012).

Table 2.2. Effects of ginseng on obesity-related parameters in animal studies

| Materials | Subject Type | Dose | Result | Reference |
|---|---|---|---|---|
| *Human Model* | | | | |
| Korean red ginseng (KRG) | Fifty obese women recruited and randomized received KRG (n=24) or placebo (n=26) | 12 capsules of 500 mg for a total of 6 g extract, three times a day for 8 weeks | In the KRG group BMI, WHR, FI, KOQOL, and AST showed significant decrease after treatment. Where in this group (9.09 %) played >5 % weight loss. | (Kwon *et al.*, 2012) |
| *Mice Model* | | | | |
| Korean white ginseng extracts (KGE) | Four-week-old female (HFD)-induced obese ICR mice | four diets for 8 weeks: 1: LFD, containing 4 % fat (LFD group); 2: HFD, containing 40 % fat (HFD group); 3: HFD supplemented with 0.8 % KGE (HFD + 0.8 KGE group);4: | KGE may delay the absorption of dietary fat via the inhibition of pancreatic lipase; 1,000 mg/kg KGE significantly inhibited the elevation of plasma TG levels up to 2x after oral administration of a lipid emulsion. | (Lee *et al.*, 2010) |

| | | | | |
|---|---|---|---|---|
| | | HFD supplemented with 1.6 % KGE (HFD + 1.6KGE group). | | |
| Wild ginseng (WG; *Panax ginseng*) | Nine-week old male leptin-deficient (B6.VLepob, 'ob/ob') mice | For 4 weeks daily WG 100 mg/kg and WG 200 mg/kg | Significant reduction in body weight from 0.5 to 1x was seen compared to control, blood glucose was decreased from control 60 % to 20 % and 40 %, also upregulated PPAR-γ, GLUT4, LPL and IR mRNA expression in tissues. | (Yun *et al.*, 2004) |
| *Panax ginseng* berry | Male adult C57BL/Ks db/db mice and their lean littermates | For 12 consecutive days, mice received intraperitoneal injections of *Panax ginseng* berry extract at 150 mg/kg body wt. | After 10 d body weight reductions up to 2 g. Also reduction in blood glucose levels 66 mg/dL in db/db mice . | (Xie *et al.*, 2002) |
| Wild ginseng ethanol extract (WGEE) | Male 5-week-old ICR mice | 250 mg/kg or 500 mg/kg of WG for an 8-week period | WGEE improved insulin resistant 55 % and 61 %. Also decreased White and brown adipocyte diameters 62 % and 46 % compared to HFD. WG 500 inhibited TG, TC LDL-C and free fatty acid at 15 %, 32 %, 50 % and 32 %. | (Yun *et al.*, 2004) |

| | | | | |
|---|---|---|---|---|
| Ginseng saponin extract (GSE) | 8 week-old male Balb/c mice | A group of five mice fed a chow diet containing 3 % (w/w) GSE and a group of Nine mice fed a HFD containing 3 % GSE | Dietary supplements of ginseng saponins administered to male Balb/c mice decreased 2 ~ 3 g body weight .Dose-dependently pancreatic lipase activity reduced 20 ~ 80 % of the control. | (Karu *et al.*, 2007) |
| *Rat Model* | | | | |
| Crude saponin (CS) of Korean red ginseng (KRG) | Three week old male Sprague-Dawley (SD) rats fed a high-fat (HF) diet. | Received daily intraperitoneal injections of CS at 200 mg/kg or saline at 1 ml/kg for 3 weeks. | Possessed anti-obesity effects in rats fed an HF diet by reducing body weight (20~30 %), CS reduced fat mass 10~70 % depending on region of fat. Also regulated serum leptin and NPY expression in the hypothalamus when obesity is induced with a HF diet. | (Kim *et al.*, 2005) |
| Ginsenoside Rb1 | Adult male Long-Evans rats | Rb1 in saline at doses of 0, 2.5, 5, 10, or 20 mg/kg, intraperitoneal Rb1 at a dose of 10 mg/kg | HFD-induced obese rats had reduced food intake (upto 8 g) and body weight (40x) compared to saline group and increased energy expenditure (0.5x in light). Also decreased fasting blood glucose and improved glucose tolerance to a greater extent than the paired feeding in HFD-induced obese rats. | (Ye *et al.*, 2010) |

A dose of 1000 mg/kg Korean ginseng extract which contains several ginsenosides at different percentages such as (0.40 %) Rg1, (1.28 %) Re, (0.19 %) Rf, (2.15 %) Rb1, (2.09 %) Rc, (1.80 %) Rb2, (0.92 %) Rd, (0.25 %) Rg2, (0.07 %) Rg3 and (0.02 %) Rh1 inhibited the elevation of plasma triglyceride levels compared to the lipid emulsion alone given to female ICR 4 week old mice (Lee *et al.*, 2010). Wild ginseng when administered to ob/ob mice at doses of 100mg/kg and 200 mg/kg lost body weight and also had a decreased blood sugar level. It regulated mRNA expression, increased peroxisome proliferator-activated receptor-γ (PPAR-γ) and lipoprotein lipase (LPL) in adipose tissue, and also increased glucose transporter 4 (GLUT4) and insulin receptors (IR) in skeletal muscle and the liver (Mollah *et al.*, 2009).

Carbon dioxide supercritical fluid extract (SFE) of ginseng in which panaxynol and panaxydol were found to be the major components had significant effects on weight loss in the C57BL/6J strain mice. The average starting weight of mice fed a high-fat diet was 35.0±1.6 g, but after 10 weeks, the average weight of mice fed SFE-supplemented high-fat diets was between 24.5±1.4 and 25.9±1.3 g. The largest anti-obesity effect for SFEs was found at 50 °C. At both 200 and 300 bars obtained at 50 $^{o}$C, showed the most profound anti-obesity effect. Both showed ~15 % and ~30 % weight loss compared to the normal diet group and high fat diet group (Woo *et al.*, 2011).

One study showed that an oil extract of ginseng (OEG) included in the diet significantly lowered body weight, visceral fat accumulation, feed efficiency, plasma TG, and hepatic and white epididymal adipocyte size, as well as expression of PPARγ in the liver and adipose tissue of 6 weeks old Male C57BL/6J mice. The enzyme activity was reduced by 20~77 % compared to the CON group in the presence of OEG at the concentration of 0.1~100.0 mg/mL. Dietary OEG improved obesity-related parameters in the blood, liver, and adipose tissue in a mouse model and suppressed obesity induced by a HF diet by regulating lipid metabolism and modulating PPARγ protein expression (Kim *et al.*, 2011).

PD-type and PT-type saponins from Red Ginseng have been shown to have anti-obesity effects in Male 4-week-old Sprague-Dawley rats fed a HF diet by reducing their body weight, food consumption and fat storage. The rats received daily intraperitoneal injections of PD or PT

at 50 mg/kg for 3 weeks. They were also effective in regulating serum lipid and leptin and hypothalamic NPY and CCK expression. In contrast to the High Fat fed rats, those treated with PD and PT showed significantly suppressed elevation of the serum leptin concentration by 68 % and 53 % respectively. Comparing all the other experiments carried out in this study it could be said that, PD-type saponins had more potent anti-obese properties than PT-type saponins, indicating that PD-type saponins are the major contributors to ginseng's anti-obesity properties (Kim *et al.,* 2009).

Korean red ginseng Extracts consist of ginsenoside Rb1, 7.9 g/kg; -Rb2, 2.8 g/kg; -Rc, 3.0 g/kg; -Rd, 1.1 g/kg; -Re, 4.1 g/kg; -Rf,1.1 g/kg; -Rg1, 3.6 g/kg; -Rg3, 0.5 g/kg; and other minor ginsenosides and components. At the dosage of 30 g/kg of KRGE compared to the HFD fed mice the level of leptin decreased 20 fold, insulin decreased 2 fold and adiponectin increased 2 fold. All of which have important functions in obesity and metabolic diseases in specific pathogen free male C57BL/6J mice (4 weeks old, 18~20 g). HFD not only elevated serum leptin and insulin levels to 260 % and 216 %, respectively, but also lowered adiponectin to 38 %. KRGE effectively regulated abnormal lipid metabolism induced HFD and a KRGE dose of 10 g/kg diet results in a potent lipid metabolic effect. Thus, KRGE prevents HFD-induced obesity (Song *et al.,* 2012). Studies also suggested that ginseng saponins may be good candidates for a natural dietary supplement that reduces weight by delaying intestinal absorption of dietary fat through the inhibition of pancreatic lipase activity (Karu *et al.,* 2007). The combination of 0.5 % mulberry leaf water extract, 0.5 % Korean red ginseng, and 0.5 % banaba leaf water extract fed at 0.5 % of the diet significantly increased insulin sensitivity and improved hyperglycemia, possibly by regulating PPAR-mediated lipid metabolism in 4-week-old C57BL/KsJ-db/db, male (Park *et al.,* 2005).

Many studies of American ginseng, which is often used in western countries, have also been carried out. In one study, American ginseng (*Panax quinquefolius*) extract (GE) containing Re as the most abundant ginsenoside potently inhibited cell proliferation, affected the cell cycle, and reduced lipid acquisition by 13 % and 22 %. Adiponectin increased expression in 3T3-L1 cells at the concentrations 20.2 $\mu$g/ml and 40.3 $\mu$g/ml (Yeo *et al.,* 2011).

Another study using American ginseng was done on female ICR strain mice (3-week old) and male Wistar King strain rats (6-week old). Where 1 % or 3 % of crude saponins were isolated from the stems and leaves of *Panax quinquefolium* and where ginsenosides Rb1, Rb2, Rc and Rd were identified, decreased adipose tissue weight was present. It also prevented high fat diet induced fat storage in adipose tissue by inhibiting the intestinal absorption of dietary fat through the inhibition of pancreatic lipase activity and inhibited elevation of plasma triglycerol (Liu *et al.,* 2008). The effects of protopanaxdiol (PDG) and protopanaxatriol (PTG) isolated from the leaves of American ginseng on porcine pancreatic lipase activity have been studied *in vitro* on female Kunming mice, 3 weeks of age. The protopanaxatriol type of ginsenosides contained ginsenosides Rg1 and Re. The protopanaxatriol type of ginsenosides contained ginsenosides Rb1, Rc, Rb2, Rb3 and Rd by HPLC. Where the lipase inhibitor PDG was significantly active in preventing and ameliorating obesity, fatty liver and hyper-triglyceridemia in mice fed a high-fat diet at the concentration of 0.25~1 mg/ml. This suggests that the anti-obesity effects might be a primary reason for the inhibition of pancreatic lipase activity in mice. Thus, PDG may be useful in the prevention and treatment of obesity and hyper-triglyceridemia in humans (Rui *et al.,* 2010).

Another study demonstrated that oral administration of American ginseng berry juice (AGBJ) in which the main constituents were ginsenosides Re (0.88 mg/g), Rb2 (0.77 mg/g), Rb3 (2.90 mg/g), and Rd (0.39 mg/g). It significantly reduced high blood glucose levels and body weight in ob/ob mice. The animals were given daily berry juice 0.6 mL/kg or vehicle for 10 consecutive days. But the mechanism behind the activity was not clear (Xie *et al.,* 2007). *Panax notoginseng* saponins (PNS) was given to the KKAy mice at the dosage of PNS 50 mg/kg and other group PNS 200 mg/kg respectively and all the animals received daily intraperitoneal injections for 30 days consecutively. It showed improved glucose tolerance significantly in a type 2 diabetes KKAy gene mouse model after a 12-day treatment. On the 30$^{th}$ day, the serum insulin resistance index and triglyceride levels of the PNS-treated group decreased significantly, and the development of glomerular lesions was also significantly inhibited (Yang *et al.,* 2009).

Aqueous fractions extracted from Radix Ginseng, Radix Rehmanniae, Radix Puerariae, Radix Asparagi, Cortex Phellodendri and Radix Scutellariae have been investigated for their effects on lipolysis, as measured by the glycerol release in 3T3-L1 differentiated adipocytes cell culture. Water-soluble substances from Radix Ginseng, Radix Rehmanniae and Radix Puerariae decreased lipolysis in basal and isoproterenol stimulated 3T3-L1 adipocytes (Hong *et al.*, 2002). So many studies are being reported considering ginseng and obesity.

Therefore, the aim was designed to first screen ginsenosides from *Panax ginseng* using *in-silico* screening to find out more active components. And then to carry out works in experimental level using adipocyte cell line (Fig. 2.4). So that from the study some useful compounds may be found thoese may be considered as like drug in order to treat obesity.

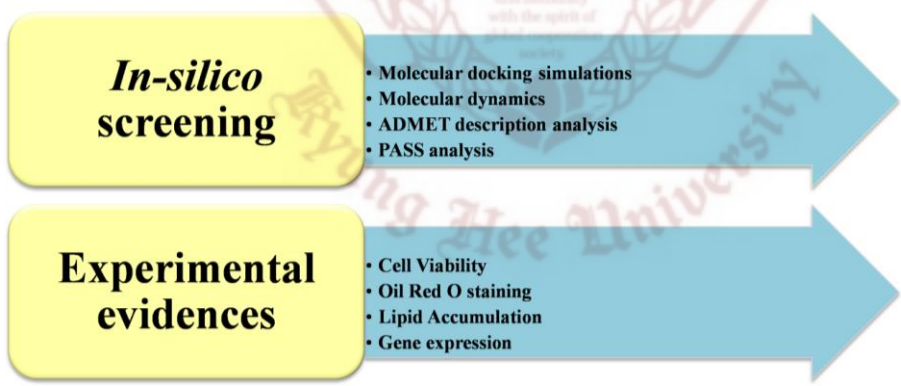



**Fig. 2.4.** Experimental work outline for obesity.

## III. Ginsenoside F2 possesses anti-obesity activity via binding with PPARγ and inhibiting adipocyte differentiation in the 3T3-L1 cell line

### 3.1. Abstract

*Panax ginseng* Meyer has been shown to be effective in mitigating various diseases. Protopanaxadiols (PPD) and protopanaxatriols (PPT), which are the main constituents of ginseng, have been shown to impact obesity. Therefore we selected several important ginsenosides to perform our docking study and determine if they had binding affinity with the peroxisome proliferator activated receptor gamma (PPARγ), which is a major transcription factor in adipocytes. Among them, only a few ginsenosides demonstrated binding affinity with PPARγ. Other then ginsenoside F2 rest of them was previously reported by the researchers in experimental study in case of obesity cell line 3T3-L1 adipocyte. In few recent studies, it was reported that F2 has protective effects on malignant brain tumors as well as anti cancer activity in breast cancer. Therefore, we felt it was important to focus on F2 when considering obesity. Our study focused on this ginsenoside and analyzed its impact on 3T3-L1 adipocytes. Following the molecular interaction studies, further experimental studies were carried out and demonstrated that ginsenoside F2 when treated with different doses reduces the level of lipid accumulated by the 3T3-L1 cell line during adipogenesis. Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR results showed reduction in PPARγ and perilipin gene expression levels compared to that of differentiated adipocytes without any treatment. So considering the binding with a major adipocyte transcription factor and the performed experiments, we suggest that ginsenoside F2 may reduce obesity via the inhibition of adipogenesis in the 3T3-L1 cell line.

### 3.2. Introduction

Obesity is identified as an excess accumulation of body fat, which may be due to over eating, low physical activity, or environmental or genetic conditions. It is a chronic medical disease that can lead to several other diseases, including diabetes, heart disease, stroke, arthritis, and even some cancers. It is predicted that obesity will reach to an alarming level in the near future if it is not considered a vital issue. Therefore, it has become necessary to discover methods to reduce obesity in order to maintain a healthy life. Until now, several drugs have been shown to treat obesity. However, many of them have later been found to cause various side effects. Thus, to avoid unwanted side effects, researchers have driven their attention to naturally available compounds that may possess anti-obesity effects (Yun *et al.,* 2010).

Among the many medicinal herbs, *Panax ginseng* is one of the most popular oriental medicinal plants that have been used for thousands of years in China, Korea, Japan, and many other countries. Ginseng saponins, also known as ginsenosides are being regarded as the principal components responsible for the pharmacological activities of ginseng. They possess multiple pharmacological actions linked to several diseases that include the central nervous system (CNS), cardiovascular, endocrine, and immune systems. These ginsenosides are divided into two parts which are PPD and PPT. Where PPD types include Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2 and PPT types includes Re, Rf, Rg1, Rg2 and Rh1(Attele *et al.,* 1999; Gillis *et al.,* 1997). Researchers have already focused on ginsenosides to investigate their impact on adipocytes, which are the building blocks of obesity for example, Rb1, Rh1, Rg3, Rd, Rh2 (Wenbin *et al.,* 2007; Gu *et al.,* 2013; Hwang *et al.,* 2009; Kim *et al.,* 2007; Hwang *et al.,* 2007). Among the PPD or PPT type ginsenosides, we considered 12 saponins (Rb1, Rb2, Rg1, Rg2, Rg3, Rc, Rd, Re, Ro, Rh2, Ck and F2) to carry out the molecular docking study, and then for further investigation only those ginsenosides were selected that demonstrated an interaction with the targeted protein by the formation of hydrogen bonds. After the molecular docking studies some of the ginsenosides possessed binding affinity with the protein but some did not. Among the selected ginsenosides, it was discovered that all had experimental evidences with the 3T3-L1 adipocyte cell line, except for ginsenoside F2. Ginsenoside F2 was produced by the hydrolysis of protopanaxadiol type saponin mixture by various glycosides (Ko *et al.,* 2007). It has been reported that F2 could be a new potential chemotherapeutic drug for glioblastoma multiforme

(GBM) treatment by inhibiting the growth and invasion of cancer (Shin *et al.,* 2012). Another report suggested that F2 initiates an autophagic progression in breast cancer stem cells (CSCs), where treatment with an inhibitor of autophagy enhanced F2-induced cell death (Mai *et al.,* 2012). From the studies it was obvious that F2 was an important saponin of ginseng and it should also be considered to study on other diseases. Therefore we chose F2 to find out its activity on obesity.

### 3.3. Materials and Methods

#### 3.3.1. Compounds fishing and molecular interaction studies

A computational method of molecular docking used for identification of novel molecules from natural products is an effective approach (Ma *et al.,* 2011). In order to identify molecular interactions between PPARγ and ginsenosides from the Korean *Panax ginseng* medicinal plant, this study was proposed. The X-ray crystal structure of PPARγ (PDB ID: 2ATH) (Sohn *et al.,* 2011) was obtained from the Protein Data Bank (PDB) (Berman *et al.,* 2000) with a co-crystalized compound of Rosiglitazone. The resolution of PPARγ was 2.28 Å. Initially, the co-crystalized ligand was extracted from the PDB environment and considered as being without ligand. A total of 12 ginsenosides (Rb1, Rb2, Rg1, Rg2, Rg3, Rc, Rd, Re, Ro, Rh2, Ck, and F2) were obtained from our own in-house *Panax ginseng* saponins database. The PPARγ residues were considered as those binding Rosiglitazone. Previous studies also confirmed that TYR473, HIS449, SER289 and HIS323 residues are important for PPARγ inhibition (Sohn *et al.,* 2011). In case of PPARγ, water molecules were removed, hydrogen atoms were added, and then the complex was optimized using the Autodock wizard (Goodsell *et al.,* 1996; Jones *et al.,* 1997; Rarey *et al.,* 1996). The molecular docking program Autodock 4.2.3 was used for identification of binding possibilities between PPARγ and ginsenosides. This program was followed by Lamarckian genetic algorithm (LGA) (Morris *et al.,* 1998) for producing a more accurate binding model (Park *et al.,* 2006; Badry *et al.,* 2003). In addition, the known PPARγ inhibitor Rosiglitazone, which is commercially available, was re-docked to verify the model reproducibility. The selected crucial amino acids of PPARγ were considered to perform docking simulation. The detailed docking simulation was described in our previous experiment (Sathishkumar *et al.,* 2012) and the results were analyzed by Chimera 1.6.1.

Furthermore, the computational program PASS was used for predicting biological activity spectrum based chemical structure formula (Langunin *et al.,* 2000; Langunin *et al.,* 2010). This technology was employed with our selected ginsenosides for identifying the type of biological activity present related to obesity. In addition, this method produced a list of biological activities, along with probability of activity (Pa) and probability of inactivity (Pi) values.

### 3.3.2. Experimental study

#### Reagents

Insulin and Isobutylmethylxanthine (IBMX) were purchased from Wako (Japan). Dexamethasone and Oil Red O staining reagents were purchased from Sigma Chemical Co. (MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Welgene (USA). Newborn calf serum was obtained from Gibco (USA), and antibiotic solution was purchased from GenDEPOT (USA). Ginsenoside F2 product (No. A0449) was purchased from Chengdu Must Bio-Technology Co., Ltd (Wuhou, Chengdu, China) with the purity of ≥ 98 % by HPLC.

#### Cell culture

Mouse embryo fibroblast 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC; VA, U.S.A.) and incubated in DMEM, containing 10 % BCS and 1 % AB, at 37 °C with a 5 % $CO_2$ atmosphere. To induce differentiation three days after confluence, which was considered day 0, the pre-adipocytes were cultured in the differentiation medium (DM), which contained: DMEM, 10 % BCS, 1 % AB and DMI (1 µM dexamethasone, 0.5 mM IBMX and 10 µg/ml insulin) from day 0 to day 3. Subsequently, the medium was switched to growth medium containing DMEM with 10 % BCS and 10 µg/ml insulin which was replaced on days 3, 5, and 7. The medium contained ginsenoside F2 from day 0 at the concentrations of 10 µM, 50 µM and 100 µM.

**MTT assay**

Cells were cultured in 96 well plates at a density of $1 \times 10^4$ cells/ 100 µl / well. After an 18 hour incubation, cells were replaced in DMEM containing 10 % BCS and ginsenoside F2 at concentrations of 10 µM, 50 µM and 100 µM for 48 hours. Subsequently, MTT regent was added to the medium and allowed to incubate for 4 hours at 37 ℃. All liquid was removed from the wells and 100 µl of DMSO was added to each well and then incubated for another hour. Finally, the resultant OD was measured at 570 nm using an ELISA reader.

**Oil-Red O staining**

Cells were cultured in 24 well plates for differentiation. Oil Red O staining was performed on day 8 of differentiation. Cells were washed with phosphate-buffered saline (PBS), and fixed with 10 % formalin for one hour. The cells were then washed with 60% isopropanol and stained with Oil Red O solution for 10 min. Excess stain was washed away several times with sterile water and cells were dried for imaging. Finally, the stained lipid droplets of the Oil Red O solution were dissolved using 100 % isopropanol and quantified via absorbance at 520 nm.

**Reverse Transcriptase –Polymerase Chain Reaction (RT-PCR)**

Cells were differentiated and on day 8 total RNA was extracted from the cells with or without treatment of ginsenoside F2. A total RNA extraction kit (Intron Biotechnology, Korea) was used for this purpose. cDNA synthesis for PPARγ and perilipin was done with 1 µg of total RNA using a kit (Thermo Scientific, Lithuania). Primer sequences for the markers are as follows: PPARγ, 5'-ATGGGTGAAACTCTGGGAGATT-3' for forward, 5'-AGCTTCAATCGGATGGTTCTT-3' for reverse, Perilipin, 5’-GATCGCCTCTGA ACTGAAGG-3’ for forward, 5’-CCTCTGCTGAAGGGTTATCG-3’ for reverse, Beta actin, 5’-ATGAAGTGTGACGTTGACATCC-3’ for forward, 5’-CCTAGAAGCATTTGCGGTGC ACGATG-3’for reverse. The reaction conditions were as follows: denaturation at 94 ℃ for 30 sec; annealing at 60 ℃ (PPARγ and Beta actin), 55 ℃ (Perilipin) ; and extension at 72 ℃ for 1

min. Thirty cycles were carried out and the resulting PCR products were electrophoresed on a 1 % agarose gel and visualized with Image J software.

**Real Time Polymerase Chain Reaction**

Real time reverse transcription PCR (qRT-PCR) was performed using a real-time rotary analyzer (Rotor-Gene 6000; Corbet Life Science, Australia) and 1 µg of cDNA in a 10µl reaction volume, using SYBR® Green SensiMix Plus Master Mix (Quantace, England), with gene specific primers. PPARγ, 5'-ATGGGTGAAACTCTGGGAGATT-3' for forward, 5'-AGCTTCAATCGGATGGTTCTT-3' for reverse, perilipin 5'-GATCGCCT CTGAACTGAAGG-3' for forward, 5'-CTTCTCGATGCTTCCCAGAG-3' for reverse, beta actin, 5'-ATGAAGTGTGACGTTGACATCC-3' for forward, 5'-CCTAGAAGCATTTGCGG TGCACGATG-3'for reverse. The PCR conditions for each of the 40 cycles were 95 °C for 10 sec, 60 °C for 10 sec, and 72 °C for 20 sec. Beta actin was used for comparison purposes.

**Statistical analysis**

All data are presented as mean ± standard error (S.E.) and all experiments were independently performed three times. The mean values of the treatment groups were compared with untreated groups using Student's t-test. Statistical significance was assigned at * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$.

**3.4. Results and Discussion**

**Molecular interaction results of PPARγ with ginsenosides**

Molecular docking studies were carried out with the 12 ginsenosides and the adipocyte protein, PPARγ, which has the most notable function of adipose tissue develpoment. It is responsible for the responses of other major genes involved in mature adipocyte formation (Farmer *et al.,* 2005) and adipogenesis is associated with the stimulation of PPARγ. All the results were revalidated using Xscore program (Table 3.1). Analysis of the docking result suggested that Ginsenoside F2 formed one hydrogen bonds with Tyr473 yielding a binding

affinity of -9.31 kcal/mol at the distance of 3.36 Å. The oxygen atom of the active site residue Tyr473 formed the bonds with the oxygen atom present in ginsenoside F2. Some more hydrogen bonds were formed with different amino acid residues which are Glu471, Pro467, Gln470 and Asp475.

Table 3.1. Binding affinity prediction of PPARγ with ginsenoside F2, Ck, Rg3 and Re

| Protein | Ginsenoside | Hydropho-bic pair Score (pKd) | Hydropho-bic match score (pKd) | Hydropho-bic surface score (pKd) | Predicted mean binding affinity (pKd) | Predicted binding energy (kcal/mol) |
|---|---|---|---|---|---|---|
| PPARγ | F2 | 6.52 | 7.84 | 6.12 | 6.83 | -9.31 |
| | Ck | 8.40 | 9.22 | 8.15 | 8.59 | -11.72 |
| | Rg3 | 9.69 | 9.68 | 8.38 | 9.25 | -12.61 |
| | Re | 9.56 | 10.24 | 9.26 | 9.69 | -13.21 |

The docking result of Compound K showed the formation of two hydrogen bonds with a binding affinity of -11.72 Kcal/mol. The nitrogen group of the amino acid active site residue His323 formed a bond with the O-H group of the glucose molecule in the compound K at the distance of 2.72 Å. Another Hydrogen bond was formed with the amino acid active site residue Ser289 and oxygen atom of compound at 2.7 Å distance. One more hydrogen bond was formed with Cys285 amino acid residue.

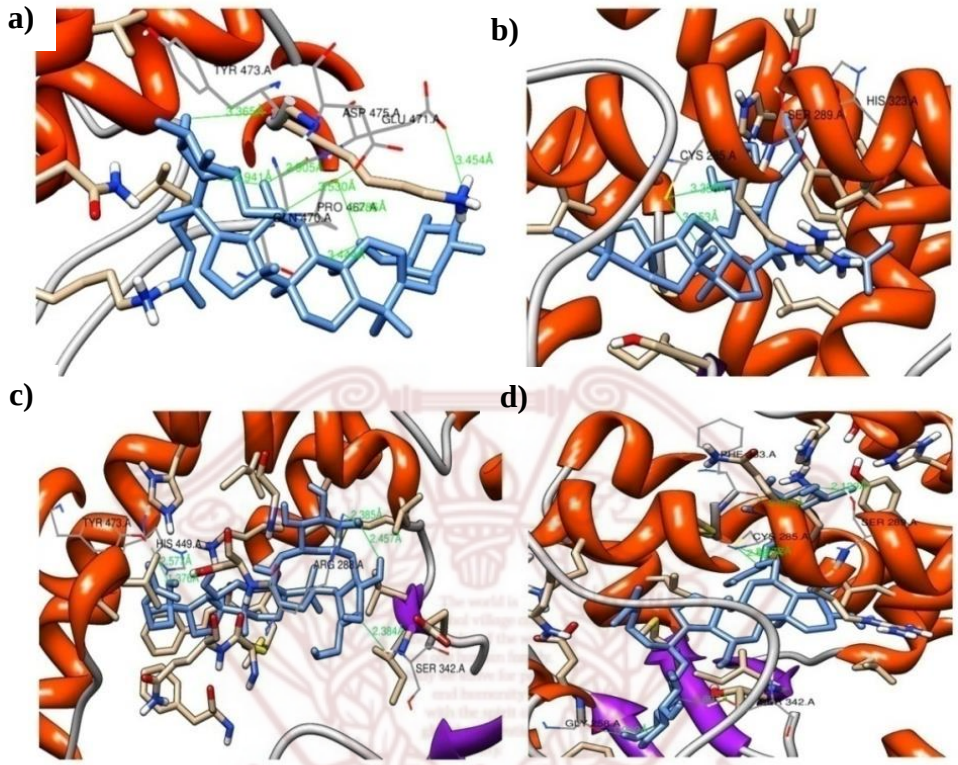



**Fig. 3.1.** Ginsenosides and amino acid residue hydrogen bond formation. Complex structures of a) F2, b) Ck, c) Rg3 and d) Re with the PPARγ active site residue after bond formation.

The docked result showed that ginsenoside Rg3 formed two hydrogen bonds, which interacted with the oxygen atom of the amino acid yielding a binding affinity of -12.61 Kcal/mol where the active site residues were Tyr473 at the distance of 2.57 Å and His449 at the distance of 1.37 Å. Rg3 also formed some more hydrogen bond with Arg288, Ser342 residues respectively.

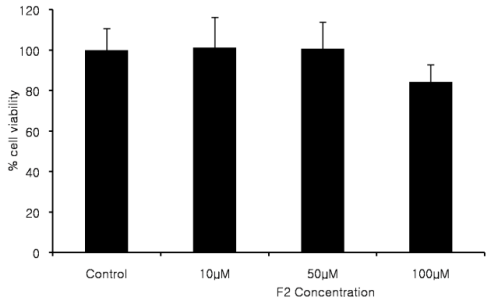
Analysis of the ginsenoside Re docking result showed that one hydrogen bond was formed with the Ser 289 active site residue with binding energy -13.21 Kcal/mol at the distance of 2.21 Å. Several hydrogen bonds were formed other than the active sites that are Gly258, Ser342, Cys285 and Phe363. As the control compound Rosiglitazone showed interactions at four active site residues Ser289, His323, His449, and Tyr473, resulting in a binding affinity of -10.0 kcal/mol. From the analyzed docking result described above suggested that among the 12 ginsenosides evaluated, only 4 ginsenosides which are F2, Ck, Rg3 and Re formed hydrogen bonds in active side residues of PPARγ having binding affinity. The obtained ginsenosides complex structures of F2, Ck, Rg3 and Re docking interactions, including the hydrogen bond formation, can be visualized in the Fig. 3.1. The docking simulation of PPARγ with these four ginsenosides resulted in the formation of hydrogen bonds at their active sites with good binding affinity. Among them compound K, Re and Rg3 already has there experimental evidences on 3T3-L1 cell line. It was shown that they worked as anti-obese and anti–diabetic compounds. Ck in 3T3-L1 cell line inhibited PPARγ that resulted in inhibition of adipocyte differentiation. Where else Re and Rg3 demonstrated the evidence of glucose uptake in mature 3T3-L1 cells. Ginsenoside Rg3 was studied on PPARγ that proved to work as antiobese compound in 3T3-L1 cell line (Park *et al.,* 2012; Lee *et al.,* 2011; Hwang *et al.,* 2006).

Therefore, this manuscript shows that, although we selected 12 ginsenosides at the beginning from that considering the compounds having binding interaction we selected only F2 for experimental studies, which may be used for PPARγ inhibition to confirm our Insilco hypothesis. In addition, the computational program PASS was used for predicting biological activity spectrum. It shows all 4 ginsenosides supported probability of active Pa >0.7 as the transcription factor inhibitor. These results were described in Table 3.2.

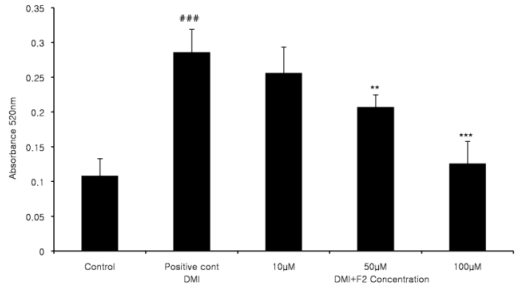
Table 3.2. Predicted biological activity (Pa > 0.7) of the selected ginsenosides from *Panax ginseng*

| Biological activity | Compounds | Probability of activity (Pa) |
|---|---|---|
| Transcription factor inhibitor | Ginsenoside F2 | 0.728 |
| Transcription factor inhibitor | Compound K | 0.742 |
| Transcription factor inhibitor | Ginsenoside Rg3 | 0.736 |
| Transcription factor inhibitor | Ginsenoside Re | 0.718 |

**A)**




**C)**




**B)**

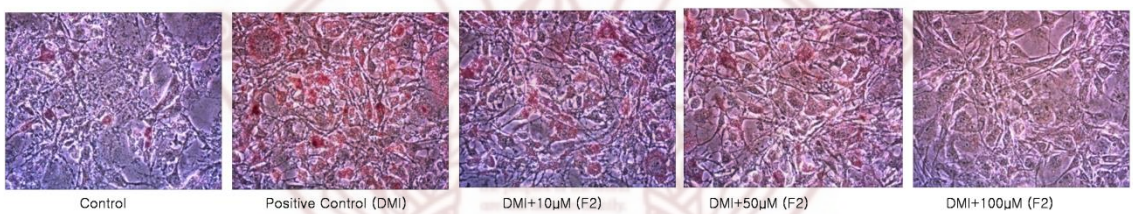



**Fig. 3.2.** The effect of ginsenoside F2 on 3T3-L1 adipocyte differentiation. **(A)** The measurement of the cytotoxic activity of compound F2 at different concentration was done by MTT assay after treatment for 48 hours. **(B)** Lipid content was visualized by Oil Red O staining on day 8 of differentiation at the concentrations of 10, 50, and 100 μM **(C),** followed by lipid content evaluation by absorbance measurement.

**Cell viability**

To investigate whether ginsenoside F2 was toxic to cells, we first performed a cell viability assay at different ginsenoside F2 concentrations: 10 µM, 50 µM, and 100 µM for 48 hours. The results showed that at the noted concentrations, F2 was not remarkably toxic to the 3T3-L1 cell line (Fig. 3.2A). Therefore, further experiments were carried out considering the same F2 concentrations.

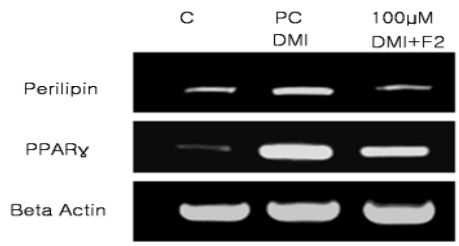
**Inhibition of lipid content by Oil Red O staining**

Next Oil red O staining assay was done to examine the efficacy of ginsenoside F2 on the adipogenesis of 3T3-L1 cells, which suggested that ginsenoside F2 could potentially inhibit the adipogenesis process. Oil Red O staining and the triglyceride content were measured. From the stained pictures it is clearly visible that the cells accumulated lipid in the DMI supplemented wells, while the cells receiving DMI along with ginsenoside F2 contained less amount of lipid in a dose dependent manner (Fig. 3.2B). To measure triglyceride content, the collected stained lipid was measured. Ginsenoside F2, at the concentrations of 50 and 100 µM accumulated less lipid content by 27 % and 55 %, respectively, compared to that of the differentiated positive control (Fig. 3.2C). This result conveys that, at these concentrations, the adipocyte differentiation process was blocked.

**Down-regulation of PPARγ and Perilipin gene expression and quantification**

Finally to evaluate adipocyte-specific gene expression, we selected two genes, PPARγ and perilipin, that closely related with the lipid contents inside adipocytes. Perilipin is a lipid droplet associated protein (Kovsan *et al.*, 2007) and the occurrence of these proteins in tissues denotes triacylglycerol metabolism. Perilipin is located directly on the surface layer of every differentiating 3T3-L1 adipocyte, and surrounds the core triacylglycerol of intracellular lipid droplets of these adipocytes that may be in culture, or sourced from white and brown adipose tissue (Blanchette *et al.*, 1995). Thus it was clear that these two markers play separate, but crucial roles in adipogenesis, and the expression of these two proteins was investigated and mRNA expression was quantified. 3T3-L1 cells were differentiated and treated, then the expression profile of the adipocyte-specific genes were investigated by RT-PCR and quantitative real time PCR. DMI treated adipocytes demonstrated increased marker expression compared to the normal control group, which did not receive DMI and ginsenoside. PPARγ and perilipin expressions were down-regulated when treated with ginsenoside F2, which also contained DMI (Fig. 3.3A). Treatment with ginsenoside F2 reduced the mRNA levels of PPARγ and perilipin by 95 % and 89 % respectively (Fig. 3.4B). Therefore gene expression studies suggest that adipogenesis was inhibited by blocking the associated genes via ginsenoside F2 treatment.

**A)**




**B)**

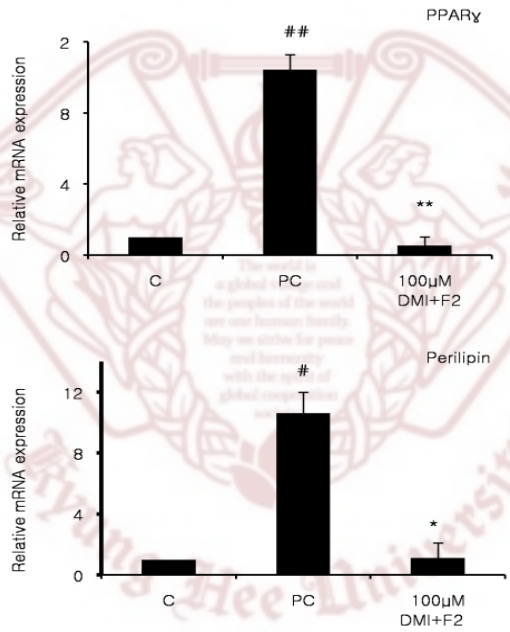



**Fig. 3.3.** Effect of ginsenoside F2 on adipocyte markers. At day 8 of differentiation total RNA was isolated. Control groups were treated with the normal medium, while the positive control received differentiation medium (DMI). Treated groups received differentiation medium with different F2 concentration (DMI+F2), while expression levels were evaluated by RT-PCR and visualized via gel (**A**). Gene expression levels were quantified using qRT-PCR and results analyzed (**B**). Presented data were statistically significant $^{\#}p<0.05$ compared with the normal control group and positive control, $*p<0.05$ compared with DMI treated positive control and DMI and F2 treated group.

Obesity is associated with the accumulation of excess fat in adipocytes. The process of adipogenesis includes morphological changes, interruption of cell growth, expression of many lipogenic enzymes, and large scale of lipid accumulation (Rosen *et al.,* 2000). Therefore, blocking this process will help inhibit the formation of mature fat cells. In order to inhibit adipocyte differentiation, other researchers have also shown interest in blocking PPARγ (Kim *et al.,* 2007; Hwang *et al.,* 2007; Farmer *et al.,* 2005). In our study, we started with 10 µM F2 to demonstrate its efficacy on adipocyte differentiation, while 100 µM ginsenoside F2 nearly blocked the differentiation process.

### 3.5. Conclusion

Molecular interaction studies describe the first 12 ginsenosides selected, but among them, only four ginsenoside had hydrogen bond formation with the active side residues of the adipogenic protein. From those selected four ginsenosides three of them were previously discussed so we chose ginsenoside F2 for our experimental investigation. F2 had good binding affinity with PPARγ, the major transcription factor for adipocyte differentiation. Therefore the confirmation of the insilico hypothesis experimental studies were carried out demonstrated that ginsenoside F2 possesses anti-obesity activity. The invitro analysis clearly showed that F2 treated cells contained fewer lipids compaired to not treated cells and also down-regulated the adipocyte markers, which successfully inhibited adipocyte differentiation. These evidences supports that ginsenoside F2 derived from *Panax ginseng* might be used to prevent obesity with the aid of its anti-obesity activity.

In conclusion it can be stated that, this was the first study to show PPARγ inhibitory mechanism with molecular interactions that includes experimental evidences of ginsenoside F2 on the 3T3-L1 adipocyte cell line and molecular level.

## IV. Inhibitory activity of PPARγ by ginsenoside Rh1 analyzed via computational screening and experimental investigations

### 4.1. Abstract

Peroxisome proliferator activated receptor gamma (PPARγ) is a major transcription factor. It is over expressed during the process of adipogenesis and PPARγ stimulates adipogenic specific proteins, leading to several diseases including obesity. Natural products are of great choice to treat obesity and *Panax ginseng* Meyer has been an important option. Ginsenoside Rh1, a protopanaxatriol ginsenoside has shown to be effective in various diseases. Therefore we investigated the activity of Rh1 in obesity. Initially, computational screening was applied to identify the pharmacokinetic properties of ginsenoside Rh1 using the ADMET method, then molecular interaction was assessed on the ginsenoside Rh1 and PPARγ using the molecular docking method. The results showed that ginsenoside Rh1 had a strong hydrogen bond interaction with PPARγ active site. In order to further verify this complex stability, molecular dynamic simulation was performed. To confirm the *in silico* screening cell cytotoxic assay was performed at different concentrations, the gene expression levels of adipogenic proteins were measured using RT-PCR and qRT-PCR. In summary, our findings suggest that ginsenoside Rh1 can be a useful source of PPARγ and some more adipogenic-related genes down-regulation by blocking the process of adipogenesis in the 3T3-L1 cell line.

## 4.2. Introduction

In recent years, obesity has been one of the utmost public health problems. It is a chief risk factor for serious metabolic diseases that may increase the risk of premature death. Obesity originates from an imbalance in energy consumption and energy expenditure that eventually results in the pathological growth of adipocytes (Jou *et al.,* 2010). Adipocytes are the key cellular components of fatty tissue. Excess fat is accumulated in adipocytes as excessive amounts of lipids (triglycerides), resulting in prominent triglyceride content in plasma and tissues such as liver and muscle, where it directs the pathological dysfunction of these tissues (Tang *et al.,* 1999; Fruhbeck *et al.,* 2001). Fat accumulation and adipocyte differentiation are linked with the occurrence and development of obesity (Jeon *et al.,* 2004). Adipocyte differentiation is related to the coordinated regulation of gene expression. Peroxisome proliferator-activated receptor gamma (PPARγ), subtype of PPARs, is a ligand-dependent transcription factor of the nuclear hormone receptor superfamily (Wilson *et al.,* 1997). PPARγ is a crucial transcription factor in adipogenesis (Tontonoz *et al.,* 1994) and over expression of PPARγ intensifies adipogenesis in vitro (Tontonoz *et al.,* 1994). These results in the expression of adipocyte-specific genes, such as adipocyte fatty acid-binding protein (aP2), lipoprotein lipase (LPL), leptin, adiponectin and fatty acid synthase (FAS) (Cristancho *et al.,* 2011; Gregoire *et al.,* 1998), finally leading to morphological changes and lipid accumulation within the cells.

Researchers have long been searching for a treatment for obesity. Regardless of the short-term benefits of treating obesity with drugs, medication-induced weight loss is often allied with harmful side effects, and weight gain resumes when the medications are discontinued (Abdollah *et al.,* 2003), As a result, the use of naturally obtained compounds may be a good choice in the treatment of various diseases, including obesity. *Panax ginseng* is widely used in oriental societies as an important medicinal plant. There have been many reports that ginseng has various pharmacological effects on the central nervous system, as well as the endocrine, immune, and cardiovascular systems (Jin *et al.,* 2009; Attele *et al.,* 1999; Lu *et al.,* 2009). Ginsenosides (steroidal glycosides), found in the extracts of different species of ginseng, are the major active constituents responsible for these pharmacological properties of ginseng. Based on their chemical structure, ginsenosides are classified into three main groups: protopanaxadiol

(PPD), protopanaxatriol (PPT), and oleanolic saponins. Both animal and cell level studies have enticed researchers to investigate the efficacy of these ginsenosides, which have been shown to act as anti obese compounds (Dong *et al.*, 2012), In the present study, computational investigations were performed to determine if ginsenoside Rh1 has any molecular interaction with the major transcription factor of the adipocyte PPARγ and also to determine the changes in gene expression level in the 3T3-L1 cell line with treatment with ginsenoside Rh1.

## 4.3. Materials and Methods

### 4.3.1. Compounds screening and molecular interaction studies

Computer-aided identification of compounds from natural products is an effective approach for the development of novel inhibitors (Ma *et al.*, 2011). The aim of the present study was to identify molecular interactions between PPARγ and ginsenoside Rh1 from Korean *Panax ginseng*. The X-ray crystal structure of PPARγ (PDB ID: 2ATH) (Sohn *et al.*, 2011) was collected from the Protein Data Bank (PDB) (Berman *et al.*, 2000) with a co-crystalized compound of Rosiglitazone. The resolution of PPARγ was 2.28 Å. Initially, the co-crystalized ligand was extracted from the PDB condition and considered to be without ligand. In addition, the known PPARγ inhibitor (Rosiglitazone) was re-docked with PPARγ to confirm their binding mode reproducibility. Ginsenoside Rh1 was obtained from our own in-house *Panax ginseng* saponins database. The crucial active PPARγ residues were considered to be those binding to the known inhibitor (Rosiglitazone). Earlier studies have established that Tyr473, His449, Ser289 and His323 residues are vital for PPARγ inhibition (Sohn *et al.*, 2011). In the case of PPARγ, water molecules were removed from their complex structures, and hydrogen atoms were added. The molecular docking program Autodock 4.2.3 was used for identification of binding possibilities between PPARγ and ginsenoside Rh1 (Goodsell *et al.*, 1996; Jones *et al.*, 1997; Rarey *et al.*, 1996). This program followed the Lamarckian genetic algorithm (LGA) (Morris *et al.*, 1998) to produce a more precise binding mode (Park *et al.*, 2006; Badry *et al.*, 2003). The selected essential amino acids of PPARγ were considered to perform the docking simulation. The selected molecular optimization and docking simulation parameters were

described in our previous reports (Sathishkumar *et al.*, 2012) and similarly the results of the analysis were analyzed by DS3.5.

### 4.3.2. Molecular dynamic

Molecular dynamic (MD) simulations were performed using the Gromacs (version 4.6) software package, (Spoel *et al.*, 2005) with the Gromacs96 43a1 force field (Christen *et al.*, 2005) and a single-point-charge (expanded) (SPC-E) water model (Ljungh *et al.*, 1996). The apo form of PPARγ (without ligand) and obtained complexes of PPARγ with known inhibitor and ginsenoside Rh1 were used to perform the MD simulations. The known inhibitor and ginsenoside Rh1 topology files and force field parameters were generated using the Dundee PRODRG2 server (Schuttelkopf *et al.*, 2004). The system was neutralized by adding the appropriate counter ions ($Na^+$ or $Cl^-$), and conjugated gradient algorithms were used for energy minimization in order to reduce the close contact between the ginsenoside Rh1 and PPARγ protein. The LINCS algorithm was applied for all bond constraints (Hess *et al.*, 1997). Simulations were performed in the NPT ensemble at a temperature of 300 K. Finally, a 10 ns MD simulation was carried out for ginsenoside Rh1 with the PPARγ protein, and snapshots were saved every 2 ps. In the analysis, GROMACS 4.6 subroutines including *g_rms* and *g_rmsf* were used to analyze root mean square deviation (RMSD) and root mean square fluctuation (RMSF), respectively. All dynamic simulations were performed using an Intel® 2.93 GHz Xenon®CPU 5679 with 64-bit RHEL 6 server.

### 4.3.3. ADMET and PASS descriptors prediction

Pharmacokinetics is a fundamental scientific discipline that underpins applied therapeutic. Therefore ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties were calculated for ginsenoside Rh1. The procedure used to carry out this experiment followed our previous papers (Karpagam *et al.*, 2013; Sathishkumar *et al.*, 2013). Furthermore, the computational program PASS was used for predicting possible biological activity based on chemical structure (Lagunin *et al.*, 2000; Lagunin *et al.*, 2010). This tool was employed for the ginsenoside Rh1 structure to identify the possible biological activity scores related to obesity

(Fayeza *et al.*, 2014). In addition, this method produced a list of biological activities from the ginsenoside Rh1 structure, along with probability of activity (Pa) and probability of inactivity (Pi) values.

### 4.3.4. Experimental study

#### Reagents

Insulin and Isobutylmethylxanthine (IBMX) were purchased from Wako (Japan). Dexamethasone was purchased from Sigma Chemical Co. (MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Welgene (USA), Newborn calf serum was obtained from Gibco (USA), and antibiotic solution was purchased from GenDEPOT (USA). Ginsenoside Rh1 (Product no.A0240) in powder form was purchased from Chengdu Must Bio-Technology Co., Ltd and had a purity ≥98 % by HPLC (Wuhou, Chengdu, China).

#### Cell culture

Mouse embryo fibroblast 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC; VA, U.S.A.) and incubated in DMEM, containing 10 % BCS and 1 % AB, at 37 °C with a 5 % $CO_2$ atmosphere. To induce differentiation three days after confluence, which was considered day 0, the pre-adipocytes were cultured in the differentiation medium (DM), which contained: DMEM, 10 % BCS, 1 % AB and DMI (1μM dexamethasone, 0.5 mM IBMX and 10 μg/ml insulin) from day 0 to day 3. Afterward, the medium was switched to growth medium containing DMEM with 10 % BCS and 10 μg/ml insulin on day 3, 5, and 7. The medium contained ginsenoside Rh1 from day 0 at concentrations of 50 μM, and 100 μM.

#### MTT assay

Cells were cultured in 96 well plates at a density of $1 \times 10^4$ cells/ 100 μl / well. After an 18 hour incubation, cells were replaced in DMEM containing 10 % BCS and ginsenoside Rh1 at concentrations of 10 μM, 50 μM and 100 μM for 48 hours. Afterward, MTT regent was added to the medium and allowed to incubate for 4 hours at 37 °C. All liquid was removed from the

wells, to which 100 µl of DMSO was then added incubated for another hour. Finally, the resultant OD was measured at 570 nm using an ELISA reader.

**Oil-Red O staining**

Cells were cultured in 24 well plates for differentiation. Oil Red O staining was performed on day 8 of differentiation. Cells were washed with phosphate-buffered saline (PBS), and fixed with 10 % formalin for one hour. The cells were then washed with 60 % isopropanol and stained with Oil Red O solution for 10 min. Excess stain was washed away several times with sterile water and cells were dried for imaging. Finally, the stained lipid droplets of the Oil Red O solution were dissolved using 100 % isopropanol and quantified via absorbance at 520 nm.

**Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)**

Cells were differentiated and, on day 8, total RNA was extracted from the cells with or without treatment with ginsenoside Rh1. A total RNA extraction kit (Intron Biotechnology, Korea) was used for this purpose. cDNA synthesis for PPARγ, Perilipin and aP2 was performed with 1 µg of total RNA using a kit (Thermo Scientific, Lithuania). Primer sequences for the markers were as follows: PPARγ, 5'-ATGGGTGAAACTCTGGGAGATT-3' for forward, 5'-AGCTTCAATCGGATGGTTCTT-3' for reverse, Perilipin, 5’-GATCGCCTCTGAACTG AAGG-3’ for forward, 5’-CCTCTGCTGAAGGGTTATCG-3’ for reverse, aP2, 5’-AAAGACAGCTCCTCCTCGAAGGTT-3, for forword, 5,-TGACCAAATCCCCATTTACGC-3, for reverse, Beta actin, 5’-ATGAAGTGTGACGTTGACATCC-3’ for forward, 5’-CCTAGAAGCATTTGCGGTGCACGATG-3’for reverse. The reaction conditions were as follows: denaturation at 94 °C for 30 sec; annealing at 60 °C (PPARγ, aP2 and Beta actin), 55 °C (Perilipin) ; and extension at 72 °C for 1 min. Thirty cycles were carried out, and the resulting PCR products were electrophoresed on a 1 % agarose gel and visualized with Image J software.

**Real-Time Polymerase Chain Reaction**

Quantitative real time PCR (qRT-PCR) was performed using a real-time rotary analyzer (Rotor-Gene 6000; Corbet Life Science, Australia) and 1 µg of cDNA in a 10 µl reaction volume, using SYBR® Green SensiMix Plus Master Mix (Quantace, England), with gene specific primers:- PPARγ, 5'-ATGGGTGAAACTCTGGGAGATT-3' for forward, 5'-AGCTTCAATCGGATGGTTCTT-3' for reverse, Perilipin 5'-GATCGCCTCTGAACTG AAGG-3' for forward, 5'-CTTCTCGATGCTTCCCAGAG-3' for reverse, aP2, 5'-AAAGACAGCTCCTCCTCGAAGGTT-3, for forword, 5,-TGACCAAATCCCCATTTACGC-3, for reverse, Beta actin, 5'-ATGAAGTGTGACGTTGACATCC-3' for forward, 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'for reverse. The PCR conditions for each of the 40 cycles were 95 °C for 10 sec, 60 °C for 10 sec, and 72 °C for 20 sec. Beta actin was used for comparison purposes.

**Statistical analysis**

All data are presented as mean ± standard error (S.E.), and all experiments were independently performed three times. The mean values of the treatment groups were compared with untreated groups using Student's t-test. Statistical significance was assigned *at* * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$.

## 4.4. Result and Discussion

### 4.4.1. Molecular interaction study

Docking studies were performed on ginsenoside Rh1 (Fig. 4.1A) and adipocyte protein PPARγ. Based on the interaction the AutoDock result was analyzed. The accuracy of the AutoDock result was confirmed by considering the lowest binding free energy and the hydrogen bonds between the PPARγ and ginsenoside Rh1 (Fig. 4.1B) The docking result of ginsenoside Rh1 showed the formation of one hydrogen bond with a binding affinity of -12.12 kcal/mol. With the use of a potential recalculation program XSCORE version 1.3 the docking score was revalidated shown in Table 4.1. The nitrogen group of the amino acid of the active site residue His449 formed a bond with the O-H group of the glucose molecule in the ginsenoside Rh1. The hydrogen bond distance was 2.13 Å. His449 is considered to be an important active site for protein ligand interaction (Yushma *et al.*, 2011; Bruning *et al.*, 2007).

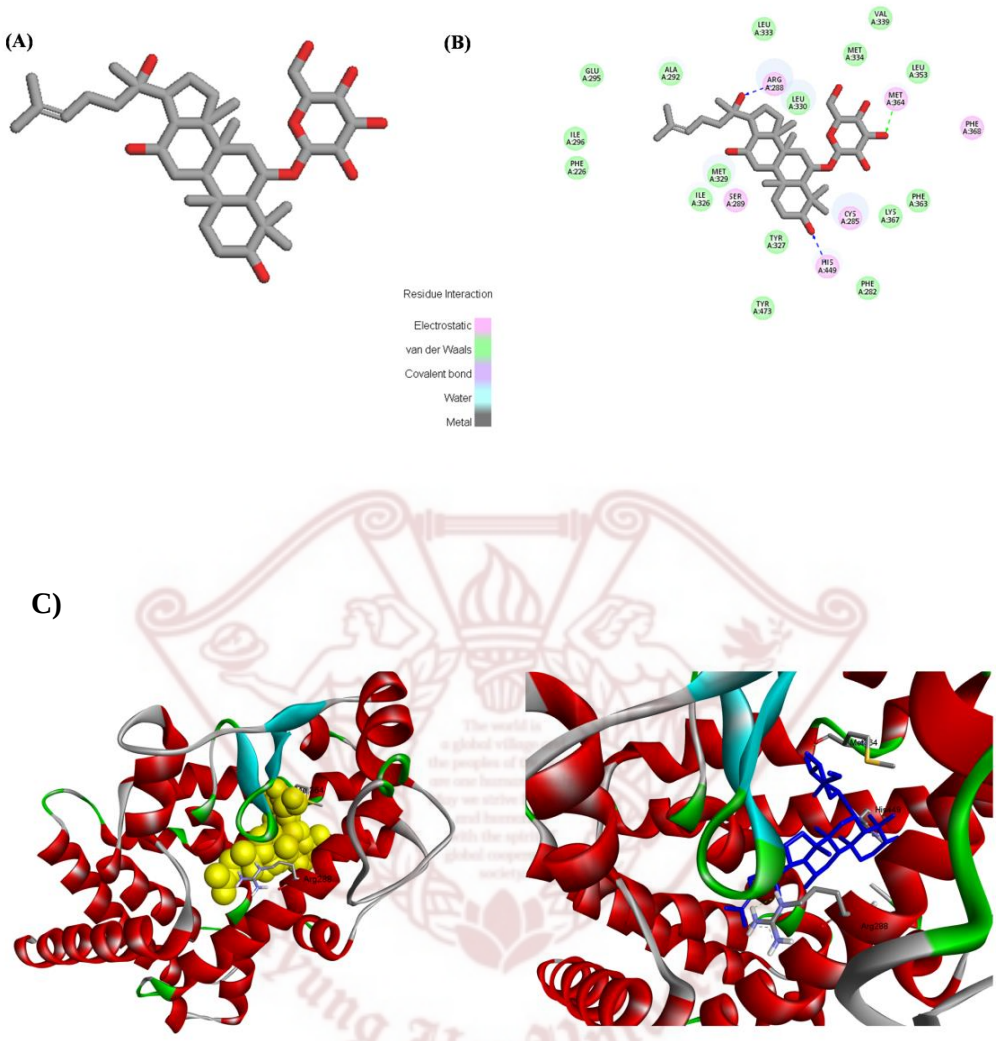



**Fig. 4.1. (A)** Ginsenoside Rh1 chemical structure **(B) g**insenoside Rh1 and protein amino acid complex structure **(C)** 3D structure of the formed bond between Rh1 & protein.

Table 4.1. Binding affinity prediction of PPARγ with ginsenoside Rh1

| Protein | Ginsenoside | Hydrophobic pair score (pKd) | Hydrophobic match score (pKd) | Hydrophobic surface score (pKd) | Predicted mean binding affinity (pKd) | Predicted binding energy (kcal/mol) |
|---|---|---|---|---|---|---|
| PPARγ | Rh1 | 8.89 | 9.45 | 8.31 | 8.88 | -12.12 |

The protein also formed an electrostatic bond with the active site Ser289 and a Van Der Waals bond with the active site Tyr473. Additional two more hydrogen bond Arg288 and Met 364 were formed. In the figure given as supplementary file shows the compound docked at the active site where the alpha helix are red colored, the compound is shown in color blue. Active site is mentioned in color black with 3 letter code and residue numbers. H-bond is marked with green dotted lines. The control compound Rosiglitazone showed interactions at four active site residues, Ser289, His323, His449, and Tyr473, (Sohn *et al.*, 2011) resulting in a binding affinity of -10.0 kcal/mol. From the obtained ginsenosides complex structures of Ginsenoside Rh1 docking interactions, including the hydrogen bond formation, 3D pictures are given for better understanding of the bond formation (Fig. 4.1C). The hydrogen bonds formed and the corresponding bond lengths are illustrated. Based on these results, ginsenoside Rh1 was found to possess binding affinity with PPARγ.

### 4.4.2. Structure alteration and stability estimation by molecular dynamic

Molecular dynamic simulations were conducted to access the structural stability of PPARγ and their complexes. For this study, the apo forms of PPARγ and complex along with known inhibitor (PPARγ-Control, Rh1-Control) were selected based on their molecular interaction and ADMET screening. The MD simulation of known inhibitor (Rosiglitazone) with PPARγ was used for comparison with ginsenoside complexes. The obtained MD results were analyzed on the basis of the root mean square deviation (RMSD) and root mean square fluctuation (RMSF) values. The RMSD values in Fig. 4.2A of ginsenoside Rh1 complexes including the apo form (PPARγ) – (PPARγ-CTRL) (PPARγ -Rh1) reached equilibrium around 1.96 ns, 2.38 ns, and 2.07 ns respectively. The RMSD results clearly showed that, after reaching equilibrium for PPARγ - Rh1, the complexes were stable throughout the entire simulation up to 10000 ps (10 ns). Furthermore, the root mean square fluctuations (RMSF) of the apo forms of PPARγ and their complexes were analyzed for local protein mobility. The RMSF values of C α atoms against the residue numbers for each molecule during the 10000 ps trajectory data are displayed in Fig. 4.2B. Analysis of the RMSF values indicates that only a few atoms of the PPARγ complexes showed fluctuation compared to the native structure, and less deviation was observed in the active site residues. These results show that PPARγ complexes experience less fluctuation compared to the native structures. These molecular simulation results clearly indicate that the binding orientation of Rh1 with PPARγ did not affect the protein stability during the simulation time period.

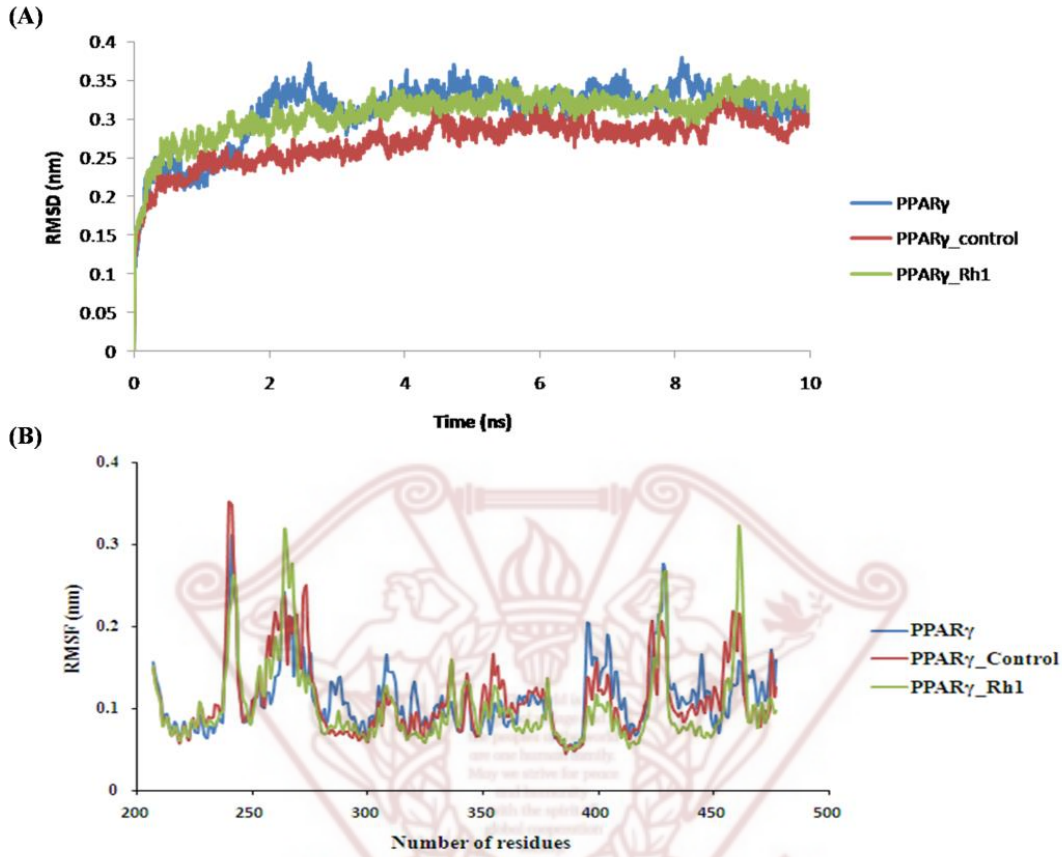



**Fig. 4.2.** Molecular dynamic trajectory analysis during 10 ns. **(A)** Root mean square deviation (RMSD) of values of free pancreatic lipase and pancreatic lipase complexes, **(B)** Root mean square fluctuations (RMSF) plots of C α atoms against number of residue.

Table 4.2. ADMET results of selected ginsenoside Rh1 with pharmacokinetic properties

| Ginsenoside | MW | Solubility Level | QP (%)# | Qplog Khsa# | CYP2D6 inhibition * | Hepato-toxicity * | QPlog Po/w# | Lipinski Rule of 5 violations |
|---|---|---|---|---|---|---|---|---|
| Rh1 | 638.8 | -4.8 | 49.4 | 0.1 | 0.2 | 0 | 2.6 | 2 |

MW: Molecular weight accepted range 130-725

Aqueous solubility (Solubility level) (accepted range: -6.5-0.5)

QP (%): Percentage of human oral absorption in GI (acceptable range: <25 % is poor and >80 % is high).

QPlogKhsa: Serum protein binding (acceptable range: -1.5/1.5).

CYP2D6 inhibition: 0 is Non-inhibitor and 1 is inhibitor.

Hepatotoxicity: 0 is non-toxic and 1 is toxic.

QPlogPo/w: Octanol/water partition coefficient (acceptable range -0.2 to 6.5).

Lipinski rule of 5 violations: Maximum is 4 violations.

# Predicted values using Qikprop program in Schrödinger.

* Predicted values using ADMET descriptors in Discovery Studio 2.5.

### 4.4.3. ADMET and PASS analysis of ginsenoside Rh1

Ginsenoside Rh1 was further evaluated for its drug-like behavior using physicochemical and pharmacologically relevant descriptors that were calculated using the Qikprop module available from the Schrödinger program. Properties such as molecular weight, human oral absorption in the gastrointestinal tract (QP (%): acceptable range: <25 % is poor and >80 % is high), serum protein binding (QPlogKhsa: acceptable range -1.5 to 1.5), CYP2D6 inhibition probability (0 = non-inhibitor and 1 = inhibitor), and octanol/water partition coefficient (QPlogPo/w: acceptable range -0.2 to 6.5) were measured for selected ginsenoside molecules. In

addition, toxicity prediction is a very important task in natural product research, (Mohan *et al.*, 2007) thus we predicted hepatotoxicity descriptors (0 = non-toxic and 1 = toxic) for selected ginsenoside molecules using the ADMET module accessible from DS 3.5. The detailed results of predicted ADMET values of each ginsenoside with acceptable ranges are described in Table 4.2.

Table 4.3. Predicted biological activity (Pa) and inactivity (Pi) of ginsenoside Rh1 *Panax ginseng*

| Pa | Pi | Activity |
|---|---|---|
| 0.920 | 0.002 | Cholesterol antagonist |
| 0.597 | 0.005 | Transcription factor inhibitor |
| 0.557 | 0.016 | Antidiabetic |
| 0.498 | 0.004 | Cholesterol synthesis inhibitor |
| 0.446 | 0.029 | Antihypercholesterolemic |
| 0.290 | 0.142 | Lipid metabolism regulator |
| 0.052 | 0.008 | Cholesterol absorption inhibitor |

In addition, the computational program PASS was used for predicting the biological activity spectrum. From the PASS results of Table 4.3, ginsenoside Rh1 supported the probability of active Pa >0.7 as a transcription factor inhibitor, antidiabetic, cholesterol antagonist , cholesterol synthesis inhibitor, antihyper cholesterolemic , cholesterol absorption inhibitor and lipid metabolism regulator which all factors are directly or indirectly related to fat as well as obesity.

### 4.4.4. Experimental Study

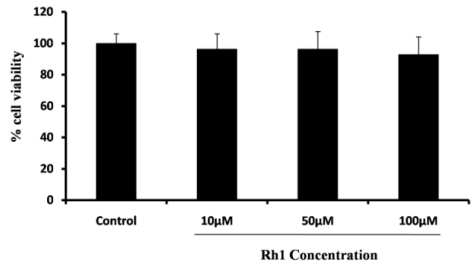
**Cell viability**

To investigate whether ginsenoside Rh1 was toxic to cells, we performed a cell viability assay at different ginsenoside Rh1 concentrations of 10 µM, 50 µM, and 100 µM for 48 hours. The results showed that at the noted concentrations, Rh1 was not significantly toxic to the 3T3-L1 cell line (Fig. 4.3A).

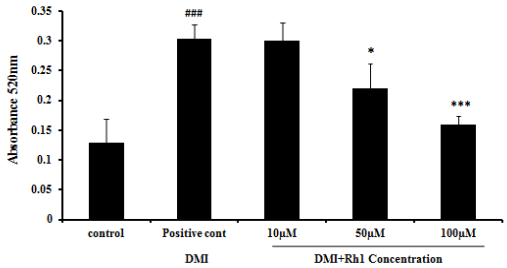
**Inhibition of lipid content by Oil Red O staining**

Next Oil red O staining assay was done to examine the efficacy of ginsenoside Rh1 on the adipogenesis of 3T3-L1 cells, which suggested that ginsenoside Rh1 could potentially inhibit the adipogenesis process. Oil Red O staining and the triglyceride content were measured. From the stained pictures, it is clearly visible that the cells accumulated lipid in the DMI supplemented wells, while the cells receiving DMI along with ginsenoside Rh1 contained less amount of lipid in a dose dependent manner (Fig. 4.3B). To measure triglyceride content, the collected stained lipid was measured. Ginsenoside Rh1, at the concentrations of 50 and 100 µM accumulated less lipid content, compared to that of the differentiated positive control. This result conveys that, at these concentrations, the adipocyte differentiation process was blocked (Fig. 4.3C).

**A)**




**C)**




**B)**

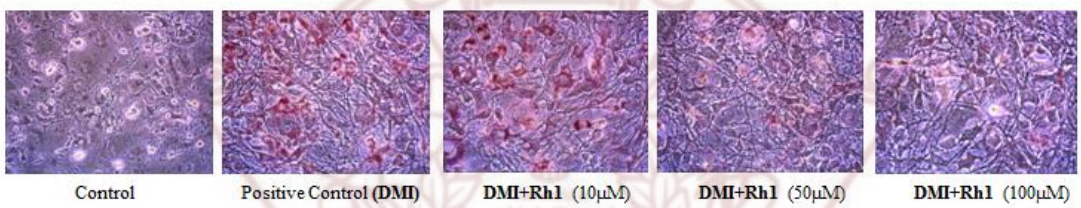



**Fig. 4.3.** The effect of ginsenoside Rh1 on 3T3-L1 adipocyte differentiation. **(A)** The measurement of the cytotoxic activity of compound Rh1 at different concentration was done by MTT assay after treatment for 48 hours. **(B)** Lipid content was visualized by Oil Red O staining on day 8 of differentiation at the concentrations of 10, 50, and 100 μM **(C),** followed by lipid content evaluation by absorbance measurement.

**Inhibition of aP2, PPARγ, Perilipin by ginsenoside Rh1**

Finally, to evaluate adipocyte-specific gene expression, we selected the adipocyte specific gene aP2, transcriptional factor PPARγ and perilipin. Adipogenic protein (aP2) is directly related to the process of adipogenesis (Teoman *et al.*, 2000). Perilipin is closely related to the lipid contents of adipocytes. Perilipin is a lipid droplet associated protein (Kovsan e al., 2007) and the occurrence of these proteins in tissues denotes triacylglycerol metabolism. Perilipin is located directly on the surface layer of every differentiating 3T3-L1 adipocyte and surrounds the core triacylglycerol of intracellular lipid droplets of these adipocytes that may be in culture or sourced from white and brown adipose tissue (Blanchette *et al.*, 1995). Thus, it was clear that these markers all play separate but crucial roles in adipogenesis, and the expression of these proteins were investigated, and mRNA expression was quantified. 3T3-L1 cells were differentiated and treated, and the expression profile of the adipocyte-specific genes was investigated by RT-PCR and quantitative real time PCR. DMI treated adipocytes demonstrated increased marker expression compared to the normal control group, which did not receive DMI and ginsenoside Rh1. Expression of PPARγ, perilipin and aP2 were down-regulated when it was treated with ginsenoside Rh1, which also contained DMI (Fig. 4.4A). Treatment with ginsenoside Rh1 reduced the mRNA levels of PPARγ, perilipin and aP2 (Fig. 4.4B). Therefore gene expression studies suggest that adipogenesis was inhibited by blocking the associated genes via ginsenoside Rh1 treatment.

Obesity is a disease of high concern. Several drugs and medications have been being suggested to treat obesity but have not been shown great success. Adipogenesis is an important factor in the treatment of obesity. Adipogenesis is initiated by the production of the key transcription factor PPARγ, which is responsible for inducing the expression of adipocyte specific genes (Rosen *et al.*, 2000). Therefore, we choose this protein for the current study.

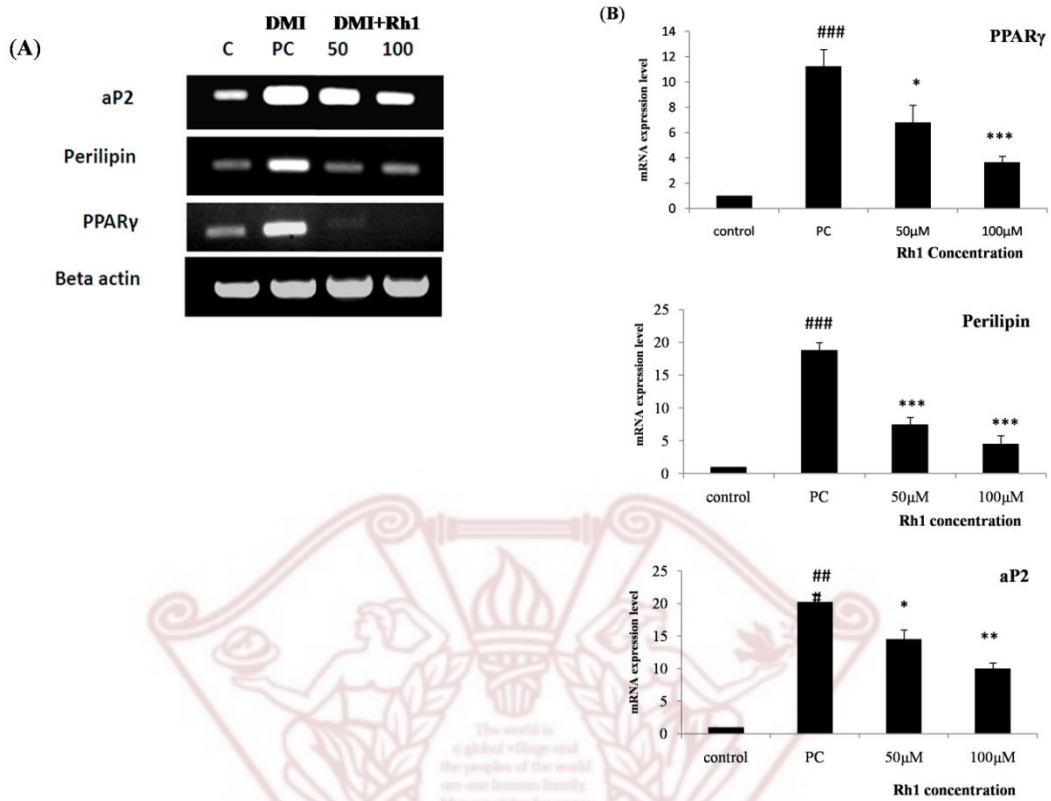



**Fig. 4.4.** Effects of ginsenoside Rh1 on adipocyte markers were evaluated. At day 8 of differentiation total RNA was isolated. Control groups were treated with the normal medium, while the positive control received differentiation medium (DMI). Treated groups received differentiation medium with different Rh1 concentration (DMI+Rh1), while expression levels were evaluated by RT-PCR and visualized via gel (**A**). Gene expression levels were quantified using qRT-PCR and results analyzed (**B**). Presented data were statistically significant $^{###}p<0.0005$ compared with the normal control group and positive control, $*p<0.05$, $**p<0.005$ and $***p<0.0005$ compared with DMI treated positive control and DMI with Rh1 treated groups.

## 4.5. Conclusion

It was already predicted by the experimental studies that ginsenoside Rh1 has anti obese activity (Wan *et al.,* 2013). But we further investigated computational screening as well as some additional experiments to find out more impacts of ginsenoside Rh1 on adipocyte and its markers. Our molecular docking result shows that ginsenoside Rh1 forms a hydrogen bond with the PPARγ active site His449 with a strong binding energy. Additionally two more bonds were formed at other active site residues, Ser289 and Tyr473. Next, a molecular dynamic simulation was performed to verify the stability of the protein and the interaction of their complexes. Here, the PPARγ apo form and complex with Rosiglitazone and Rh1 were used as the starting material. The conformational variations of PPARγ were analyzed for 10000 ps simulation time. The RMSD of the starting and final structures indicated that the complex was stable after reaching an equilibrium state. Therefore, the molecular systems were well behaved. This stability confirms the strong hydrogen bond and other electrostatic and van der Waals interactions, which were further supported by the RMSF values as a function of the residue fluctuations. Furthermore, our ADMET results showed that Rh1 has acceptable pharmacokinetic properties. Rh1 has favorable aqueous solubility and oral absorption in the human gastro intestinal tract, and it has no hepatotoxicity. Next, our PASS datas show that ginsenoside Rh1 supports several properties that can be useful in reducing obesity-related diseases, including transcriptional factor inhibitor, antidiabetic, cholesterol related factors and lipid metabolism. All of these factors are closely related to reducing obesity and achieving control over other associated diseases. Furthermore our cytotoxic results suggest that ginsenoside Rh1 does not result in toxicity to cells. Finally, the gene expression results clearly indicated that ginsenoside Rh1 inhibits the expression of markers at 50 μM and 100 μM and also inhibits adipogenesis. Ginseng and its saponins have been used for thousands of years to treat a number of diseases. The results of the computational investigations and experimental evidences in the present study support the conclusion that, ginsenoside Rh1 may possess drug like properties that can be used to treat obesity.

## V. *In silico* screening of ginsenoside Rf as PPARγ inhibitor and its *in vitro* investigation as anti-obese compound on 3T3-L1 adipocyte

### 5.1. Abstract

Adipocytes, which are the main cellular component of adipose tissue, are the building blocks of obesity. The nuclear hormone receptor PPARγ is a major regulator of adipocyte differentiation and development. Obesity, which is one of the most dangerous yet silent diseases of all time, is fast becoming a critical area of research focus.In this study, we initially aimed to investigate whether the ginsenoside Rf, a compound that is only present in *Panax ginseng* Meyer, interacts with PPARγ by molecular docking simulations. After we performed the docking simulation the result has been analyzed with several different software programs, including Discovery Studio, Pymol, Chimera, Ligplus and Pose View. All of the programs identified the same mechanism of interaction between PPARγ and ginsenoside Rf, at the same active site. To determine the drug like and biological activities of ginsenoside Rf, we calculates its ADMET and Pass properties. Considering the results obtained from the computational investigations the in vitro experiments were focused on. Since the docking simulations predicted the formation of structural bonds between ginsenoside Rf and PPARγ, we also investigated whether any evidence for these bonds could be observed on the cellular level. These experiments revealed that ginsenoside Rf treatment of 3T3-L1 adipocytes down regulated the expression levels of PPARγ and perilipin, and also decreased the amount of lipid accumulated at different doses. The ginsenoside Rf appears to be promising compound that could prove useful in anti-obesity treatments.

## 5.2. Introduction

The global prevalence of obesity has been increasing at a dreadful rate, with both developed and developing countries affected by this disease. In 2005, the World Health Organization predicted that at least 400 million adults worldwide were obese; moreover, this number is estimated to double in roughly ten years. In Western countries, which generally are also high income countries, obesity has typically been associated with a high calorie diet and a low level of physical activity; however, low and middle income countries are increasingly dealing with these burdens as well (http://www.who.int/dietphysicalactivity/publications/facts/obesity/en/print.html). In combination with heavy smoking or drinking, obesity has been found to be associated with several chronic medical conditions and a limited quality of life (Sturm, 2002). In a systematic review of the economic burden of obesity worldwide, Withrow and colleagues (Withrow and Alter, 2011) concluded that obesity added for 0.7~2.8% of the total health care expenses of a given country; moreover, obese people incurred 30% higher medical costs compared with people of normal weight. Obesity is a complex metabolic disorder, and is related to a higher risk of many important human diseases, including type 2 diabetes, hypertension, cardiovascular disease, stroke and certain types of cancer such as colorectal, breast and prostate cancer (Cao, 2007). Therefore, the development of effective anti-obesity drugs is crucial for treating obesity and for reducing the risk of its associated disorders. For years, researchers have been searching for drugs or naturally-derived compounds that can effectively treat obesity. However, satisfactory results have yet to be achieved; thus, intense research is still focused on the identification of anti obesity agents. *Panax ginseng* of the Araliaceae family is one of the most beneficial of Asian plants. Since ancient times, ginseng has been used as a curative drug and as a health tonic (especially the dried ginseng root) in countries such as Korea, Japan and China. At present times, ginseng has also been used in a variety of commercial health products worldwide, including ginseng capsules, soups, drinks and cosmetics. Chemical studies of ginseng have revealed that it contains saponins, antioxidants, peptides, polysaccharides, fatty acids, alcohols and vitamins. Saponins, which are also known as ginsenosides, are broadly believed to be the most highly bioactive compounds in ginseng (Paek *et al.,* 2005). Based on their chemical structure, ginsenosides are generally divided into two groups: protopanaxadiol (PD) and protopanaxatriol (PT). The PD includes ginsenosides such as Rb1, Rb2, Rc, Rd, Rg3, Rh2 and Rh3; for these ginsenosides, group the sugar moieties are

attached to the 3-position of the dammarane-type triterpine. However, the sugar moieties of the PT are attached to the 6-position of the dammarane-type triterpine; these ginsenosides include compounds such as Re, Rf, Rg1, Rg2 and Rh1 (Seo *et al.*, 2005). Ginsenosides, either as crude or single saponins, were thought for years to be responsible for most pharmacological actions of ginsengs (Kaku *et al.*, 1975). The ginsenoside Rf which is a ginseng saponin found only in *Panax ginseng* (Chan *et al.*, 2000), is a steroid-like compound harboring several sugar moieties that has also been shown to exert protective effects against several diseases (Yue *et al.*, 2010; Wen *et al.*, 2014). Therefore, in this study we investigated whether this ginsenoside interacts with peroxisome proliferator activated receptors gamma (PPARγ) which is the major transcriptional factor of adipocyte and is mainly present in adipose tissue (Desvergene and Wahli, 1999). We performed a molecular docking simulation and then validated this result with five different software programs; each program predicted the same mechanism of bond formation at the same active site. Next, we characterized ginsenoside Rf pharmacologically by determining its absorption, distribution, metabolism, excretion and toxic (ADMET) properties and its prediction of activity spectra for substances (PASS). To validate these observations experimentally and to observe the outcome of ginsenoside Rf binding to PPARγ, we carried out *in-vitro* experiments in which 3T3-L1 adipocytes were treated with ginsenoside Rf. These experiments revealed that ginsenoside Rf was effective in reducing lipid accumulation. Moreover, reverse transcription polymerase chain reaction (RT-PCR) and quantitative real time polymerase chain reaction (qRT-PCR) analyses also revealed that ginsenoside Rf treatment of 3T3-L1 adipocytes downregulated the expression of PPARγ and perilipin. The *in-silico*, ADMET, PASS and experimental results presented here indicated that ginsenoside Rf may be an applicatory compound to ameliorate obesity.

## 5.3. Materials and Methods

### 5.3.1. Docking and screening

Molecular docking is a commonly and frequently applied technique in drug design, due to the ability of this technique to predict the specific positioning of a ligand in the active site of a protein (Plewczynski *et al.,* 2011). For any sorts of biological process interactions between biomolecules are elemental are fundamental. Therefore we performed molecular docking to simulate the interaction of ginsenoside Rf from *Panax ginseng* with PPARγ. To this end, the molecular docking program Autodock 4.2.3 was used (Goodsell *et al.,* 1996; Jones *et al.,* 1997; Rarey *et al.,* 1996). The structure of ginsenoside Rf was obtained from our in-house database of *Panax ginseng* saponins structures. The crystal structure of PPARγ, at 2.28 Å resolution was obtained from the Protein Data Bank (PDB) (PDB ID: 2ATH) (Berman *et al.,* 2000; Sohn *et al.,* 2011) this structure is a co-crystallization of PPARγ in complex with one of its ligands, Rosiglitazone. To obtain the structure of PPARγ alone the co-crystallized ligand was removed from PDB structure; to confirm the reproducibility of the binding of PPARγ to Rosiglitazone, the ligand was re-docked into PPARγ. The residues found to interact with Rosiglitazone, a known inhibitor of PPARγ, were considered crucial residues in the active site of PPARγ. Previous studies have demonstrated that residues Tyr473, His449, Ser289 and His323 are crucial for the inhibition of PPARγ (Berman *et al.,* 2000). Water molecules were removed from the PPARγ structure, and hydrogen atoms were added. To more precisely identify the binding mode between PPARγ and ginsenoside Rf the Lamarckian genetic algorithm (LGA) was also performed (Morris *et al.,* 1998; Park *et al.,* 2006; Badry *et al.,* 2003). In molecular docking simulation between PPARγ and Rf, the essential amino acids were selected. The molecular optimization and docking simulation parameters used have been previously described (Sathishkumar *et al.,* 2012). To validate the docking interaction the simulation output was analyzed with Discovery Studio (DS) 3.5, Chimera, Pymol, Pose View and Ligplus.

### 5.3.2. Prediction of ADMET and PASS prediction

Pharmacokinetic studies of a potential drug generally involve determining its ADMET properties. This determination is particularly important, since it has been estimated that more

than 50 % of all potential drugs fail during clinical trials due to their inadequate ADMET properties (Konstantin *et al.,* 2005). Improvements in Computational studies and in drug the discovery process have enabled the identification of several pharmacologically active compounds, which must be optimized and also undergo pre-clinical ADMET evaluations. It is extremely important to determine the ADMET properties of a compound before clinical trials; thus, we determined the ADMET properties for ginsenoside Rf using methods previously described (Karpagam *et al.,* 2013; Sathishkumar *et al.,* 2013). In addition, we also used another computational program, PASS, to predict the possible biological activities of ginsenoside Rf based on its chemical structure (Langunin *et al.,* 2000; Langunin *et al.,* 2010). In particular, we determined the possible biological activity scores related to obesity for ginsenoside Rf (Fayeza *et al.,* 2014). This approach yielded a list of potential biological activities mediated by ginsenoside Rf, along with their associated probabilities of activity (Pa) and probabilities of inactivity (Pi).

### 5.3.3. Experimental Study

#### Materials

Ginsenoside Rf was obtained from the Kyung Hee University Ginseng Research Bank (Yongin, South Korea) in powdered form, and was determined to be ≥95 % pure by HPLC. Insulin and isobutylmethylxanthine (IBMX) were obtained from Wako (Japan), dexamethasone was purchased from Sigma Chemical Co. (MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Welgene (USA). Newborn calf serum was purchased from Gibco (USA), and antibiotic solution was purchased from GenDEPOT (USA).

#### Cell culture and differentiation

3T3-L1 preadipocytes were obtained from the American Type Culture Collection (ATCC; VA, USA). Cells were cultured in DMEM, supplemented with 10 % BCS and 1 % AB, at 37 °C in a 5 % $CO_2$ atmosphere. Differentiation was induced three days after confluence was reached (defined as day 0) by culturing cells in differentiation medium (DM) from day 0 to day

3. The DM contained DMEM, 10 % BCS, 1 % AB, and DMI (1μM dexamethasone, 0.5 mM IBMX, and 10 μg/ml insulin). Cells were additionally fed with growth medium containing DMEM, 10 % BCS and 10 μg/ml insulin on days 3, 5, and 7. In all experiments, the medium also contained Rf at a concentration of either 10 μM or 100 μM from day 0 until the day of the experiment.

**MTT assay**

Cells were plated in 96-well plates at a density of $1 \times 10^4$ cells/well. After 24 hours, the medium was replaced with DMEM containing 10 % BCS and ginsenoside Rf at a concentration of 1 μM, 10 μM, 50 μM, or 100 μM; cells were then incubated for 48 hours. After this incubation, 20 μl of MTT reagent was added to the medium, and cells were incubated for 4 hours at 37 °C. The medium was then replaced with 100 μl DMSO, and the cells were incubated for another hour. Finally, the resultant ODs at 570 nm were measured with an ELISA reader

**Oil-Red O staining**

Cells were differentiated in 24-well plates; on day 8, Oil Red O staining was performed. Cells were fixed with 10 % formalin for one hour, washed with 60 % isopropanol, and then stained with Oil Red O solution for 1hour. Excess stain was removed by washing the cells with sterile water, and cells were then dried for imaging. Finally, lipid droplets were solubilized in 100 % isopropanol and quantified by determining the resultant absorbances at 520 nm.

**RNA preparation and RT-PCR**

Cells were differentiated either in the presence or absence of ginsenoside Rf; on day 8, total RNA was extracted using a total RNA extraction kit (Intron Biotechnology, Korea). cDNA was then synthesized from 1 μg of total RNA with a cDNA synthesis kit (Thermo Scientific, Lithuania). PCR amplification was then performed using the following gene-specific primers: PPARγ;forward, 5'-ATGGGTGAAACTCTGGGAGATT-3';reverse, 5'-AGCTTCAATCG GATGGTTCTT-3';perilipin;forward, 5’-GATCGCCTCTGAACTGAAGG-3’; reverse, 5’-CTTCTCGATGCTTCCCAGAG -3’;beta-actin; forward, 5’-ATGAAGTGTGACGTT GACATCC-3’; reverse, 5’-CCTAGAAGCATTTGCGGTGCACGATG-3’. Thermocycling

conditions were as follows: denaturation at 94 °C for 30 sec, annealing at 58 °C (PPARγ) or 60 °C (perilipin and beta-actin), and extension at 72 °C for 1 min; 28 of these cycles were carried out. The resultant PCR products were electrophoresed on a 1 % agarose gel and visualized with Image J software.

**qRT-PCR**

qRT-PCR was performed using real-time rotary analyzer (Rotor-Gene 6000; Corbet Life Science, Australia) and 1 µg of cDNA in a 10µL reaction volume. SYBR® Green SensiMix Plus Master Mix (Quantace, England), was used with the following gene specific primers: PPARγ; forward, 5'-ATGGGTGAAACTCTGGGAGATT-3';reverse, 5'-AGCTTCAATCGGATGGTT CTT-3'; perilipin; forward, 5'-GATCGCCTCTGAACTGAAGG-3'; reverse, 5'-CTTCTCGATGCTTCCCAGAG-3';beta-actin; forward, 5'-ATGAAG TGTGACGTTGACATCC-3'; reverse, 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'. Thermocycling parameters were 95 °C for 10 sec, 60 °C for 10 sec, and 72 °C for 20 sec. Beta-actin was used as an internal control gene.

**Statistical analysis**

All experiments were performed independently three times: data are presented as means ± standard error (SE). Mean values were compared between the treated groups and the untreated groups using Graph Pad *t-test* quick calc version 6.04. Statistical significance is designated by the following symbols: * $p < 0.05$; ** $p < 0.005$; and *** $p < 0.0005$.

## 5.4. Results and Discussion

**Molecular interaction study**

A molecular docking simulation of the interaction between ginsenoside Rf and PPARγ was carried out with Autodock; this interaction was also then analyzed with DS3.5, Pymol, Chimera, Ligplus, and Pose View. The accuracy of the AutoDock result was confirmed by the

observation that it yielded the lowest binding free energy out of all possible docking positions, in addition to assigning hydrogen bonds between PPARγ and ginsenoside Rf.

The docking simulation between ginsenoside Rf and PPARγ predicted the formation of two hydrogen bonds at residues Ser289 and His323 in the active site of PPARγ; this simulation also predicted a binding affinity of -2.9 kcal/mol. Both His323 and Ser289 are considered to be important residues for ligand interaction in the active site of PPARγ (Yushma *et al.,* 2011; Bruning *et al.,* 2007). The high level of agreement between the results from the five different programs supports the mechanism of interaction between ginsenoside Rf and PPARγ, in addition to the specific bonds formed between ginsenoside Rf and PPARγ. The predicted docking interactions of ginsenoside Rf with PPARγ, including the hydrogen bonds of these interactions, are shown in Fig. 5.1(A-E).

The structure obtained with DS 3.5 predicted that the hydrogen atoms of PPARγ Ser289 and His323 form a bond with the oxygen atom of ginsenoside Rf. Additionally, two hydrogen bonds are formed between PPARγ Phe282 and Met364 and ginsenoside Rf.

Similarly, the interaction simulated by Pose View predicted the formation of a bond between the hydrogen atoms in the Ser289 and His323 residues of PPARγ and the oxygen atom of ginsenoside Rf. In addition, Pose View predicted the formation of hydrogen bonds between PPARγ Met348 and Met364 and ginsenoside Rf.

The Ligplus simulation also predicted hydrogen bond interactions between the Ser289 and His323 residues of PPARγ and the oxygen atom in ginsenoside Rf. In agreement with the other simulations, Pymol also predicted hydrogen bonds between the PPARγ active site residues Ser289 and His323 and ginsenoside Rf, in addition to hydrogen bond formation between PPARγ Phe282 and Met364 and ginsenoside Rf. The Chimera program also yielded the same hydrogen bond formations, between PPARγ Ser289 and His323 and the oxygen atom of ginsenoside Rf. In addition, Chimera predicted additional hydrogen bonds between PPARγ Phe282 and Met364 and ginsenoside Rf. The control compound Rosiglitazone showed interactions at four active site residues: Ser289, His323, His449, and Tyr473 (Sohn *et al.,* 2011). These interactions resulted in a binding affinity of -10.0 Kcal/mol. Based on these results, ginsenoside Rf was also predicted to exhibit a good binding affinity with PPARγ.

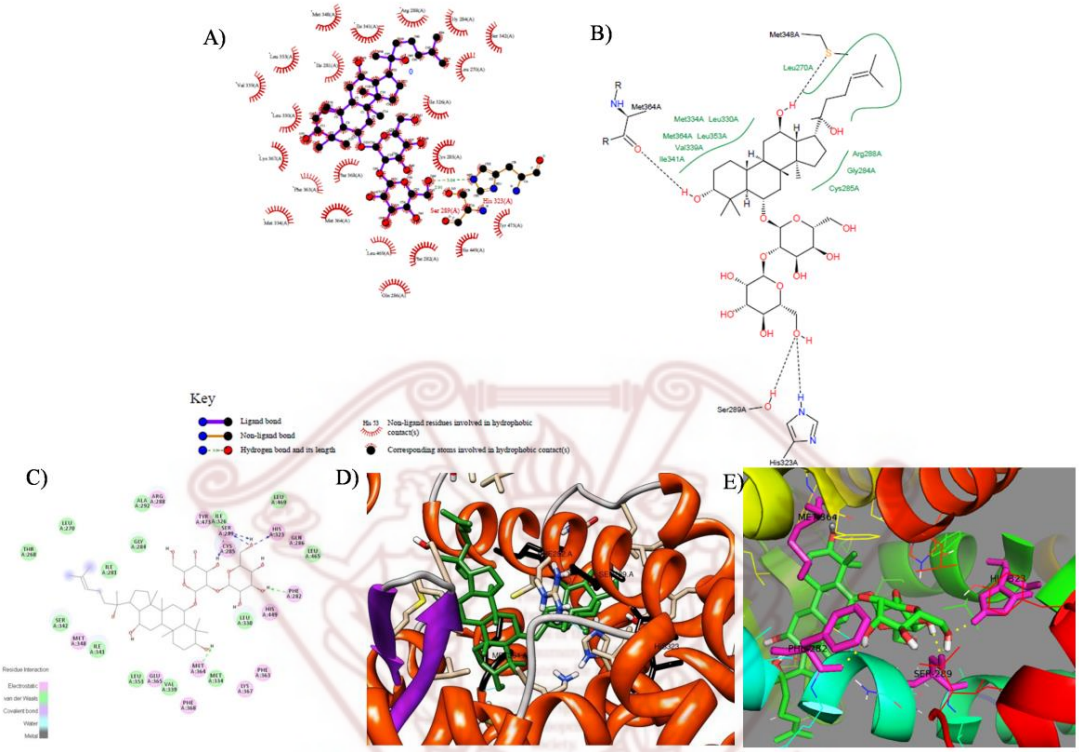



**Fig. 5.1.** Ginsenoside Rf and PPARγ amino acid residue hydrogen bond formation at the active site visualized by **A)** Lig Plus **B)** Pose View **C)** DS 3.5 **D)** Chimera **E)** Pymol.

**ADMET and PASS analysis**

Using the Qikprop module in the Schrödinger program, the physiochemical and pharmaceutically properties of ginsenoside Rf were determined. These properties included molecular weight, human oral absorption in the gastrointestinal tract, serum protein binding, CYP2D6 inhibition probability, and octanol/water partition coefficient. Since toxicity prediction is also very important in natural prodict research (Mohan *et al.*, 2007), we also predicted the hepatotoxicity descriptors for ginsenoside Rf using the ADMET module in DS3.5. The detailed results of the predicted ADMET values for ginsenoside Rf, along with their acceptable ranges are, given in Table 5.1. Next, the computational program PASS was used to predict the biological activity spectrum of ginsenoside Rf. This analysis suggested that ginsenoside Rf could exhibit activity (Pa >0.7) as a transcription factor inhibitor, an antidiabetic agent, a cholesterol antagonist, a cholesterol synthesis inhibitor, an antihypercholesterolemic agent, a cholesterol absorption inhibitor, and a lipid metabolism regulator. All of these functions are directly or indirectly related to fat, as well as to obesity (Table 5.2).

Table 5.1. ADMET results of selected ginsenoside Rf with pharmacokinetic properties

| Ginse-noside | MW | Solubility Level | QP (%)# | Qplog Khsa# | CYP2D6 inhibition* | Hepato-toxicity* | QPlog Po/w# | Lipinski Rule of 5 violations |
|---|---|---|---|---|---|---|---|---|
| Rf | 640.8 | -5.3 | 49.4 | 0.2 | 0.3 | 0 | 2.9 | 2 |

MW: Molecular weight accepted range 130-725

Aqueous solubility (Solubility level) (accepted range: -6.5-0.5)

QP (%): Percentage of human oral absorption in GI (acceptable range: <25 % is poor and >80 % is high).

QPlogKhsa: Serum protein binding (acceptable range: -1.5/1.5).

CYP2D6 inhibition: 0 is Non-inhibitor and 1 is inhibitor.

Hepatotoxicity: 0 is non-toxic and 1 is toxic.

QPlogPo/w: Octanol/water partition coefficient (acceptable range: -0.2 to 6.5).

Lipinski rule of 5 violations: Maximum is 4 violations.

# Predicted values using Qikprop program in Schrödinger.

* Predicted values using ADMET descriptors in Discovery Studio 2.5.

Table 5.2. Predicted biological activity (Pa) and inactivity (Pi) of ginsenoside Rf

| Pa | Pi | Activity |
|---|---|---|
| 0.907 | 0.003 | Cholesterol antagonist |
| 0.605 | 0.005 | Transcription factor inhibitor |
| 0.565 | 0.015 | Antidiabetic |
| 0.480 | 0.004 | Cholesterol synthesis inhibitor |
| 0.391 | 0.039 | Antihypercholesterolemic |
| 0.239 | 0.177 | Lipid metabolism regulator |
| 0.049 | 0.011 | Cholesterol absorption inhibitor |

**Experimental study**

To investigate whether ginsenoside Rf exhibited any cytotoxicity, cell viabilities were determined after 48 hours in the presence of various concentrations of ginsenoside Rf, including 1 μM, 10 μM, 50 μM, and 100μM. ginsenoside Rf was not found to be significantly cytotoxic to 3T3-L1 cells, even at the highest concentration tested (100 μM the result shown in Fig. 5.2A).

**Oil Red O staining & Lipid accumulation**

Oil Red O staining was performed to examine the extent of lipid accumulation in 3T3-L1 adipocytes undergoing adipogenesis, either in the presence or absence of ginsenoside Rf. Visualization of Oil Red O-stained cells clearly revealed the accumulation of lipids in DMI-treated cells, whereas cells treated with DMI in the presence of ginsenoside Rf accumulated lipids to a lesser extent (Fig. 5.2B). To quantify the triglyceride contents of the cells, the absorbance of the solubilized Oil Red O was measured for each treatment. Cells differentiated in the presence of ginsenoside Rf (10 and 100 μM) contained 14 % and 45 % less lipid, respectively, compared with positive control differentiated cells (Fig. 5.2C). This result suggests that ginsenoside Rf treatment inhibits adipocyte differentiation.

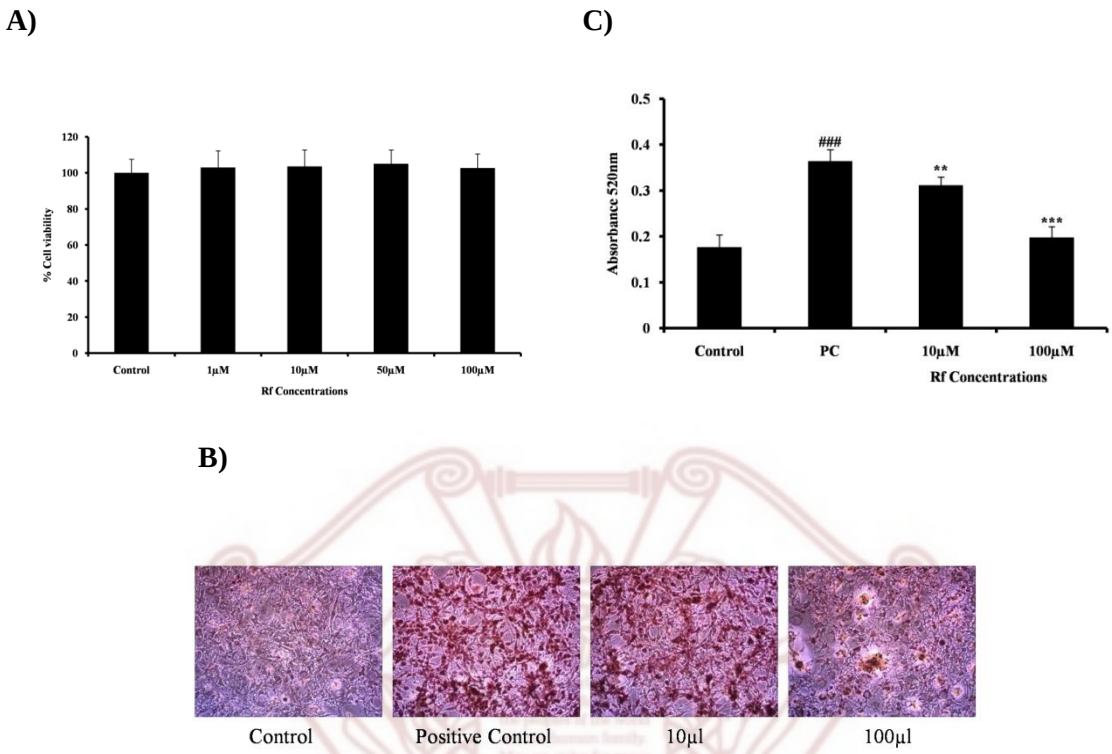



**Fig. 5.2.** Cytotoxicities of different concentrations of ginsenoside Rf on 3T3-L1 adipocytes (A). Effects of ginsenoside Rf (10 and 100 μM) on 3T3-L1 adipocyte differentiation visualized by Oil red O staining (B) and quantification of lipid (C).

**Gene expression levels of PPARγ and Perilipin**

Finally, the gene expression levels of PPARγ and perilipin were determined. PPARγ is the main transcription factor driving adipogenesis (Desvergene and Wahli, 1999), whereas perilipin is a lipid droplet-associated protein (Kovsan *et al.,* 2007). The upregulation of these genes generally signifies increased triacylglycerol metabolism. Perilipin is found on the surface of every differentiated adipocyte and also envelopes the core triacylglycerols in intracellular lipid droplets; these observations hold true in both transformed adipocytes and also in primary adipocytes derived from white and brown adipose tissue (Blanchette *et al.,* 1995). These two markers have been determined to play distinct, yet necessary, roles in adipogenesis; thus, the mRNA expression levels of these two genes were quantified. To this end, 3T3-L1 cells were differentiated, treated with ginsenoside Rf, and the expression profiles of PPARγ and perilipin were investigated by RT-PCR and quantitative real-time PCR. This analysis revealed that the expression levels of PPARγ and perilipin were increased in DMI-treated adipocytes compared with control cells, which did not receive either DMI or Rf. On the other hand, the expression levels of PPARγ and perilipin were downregulated when cells were treated with ginsenoside Rf in the presence of DMI (Fig. 5.3A). Interestingly, treatment with ginsenoside Rf at concentrations of 10 and 100 μM reduced the mRNA levels of PPARγ by 8 % and 52 %, and the mRNA levels of perilipin by 14 % and 65 %, respectively (Fig. 5.3B). These results support the hypothesis that treatment with ginsenoside Rf inhibits adipogenesis, and indicate that the mechanism may involve the downregulation of key mediators of adipogenesis.

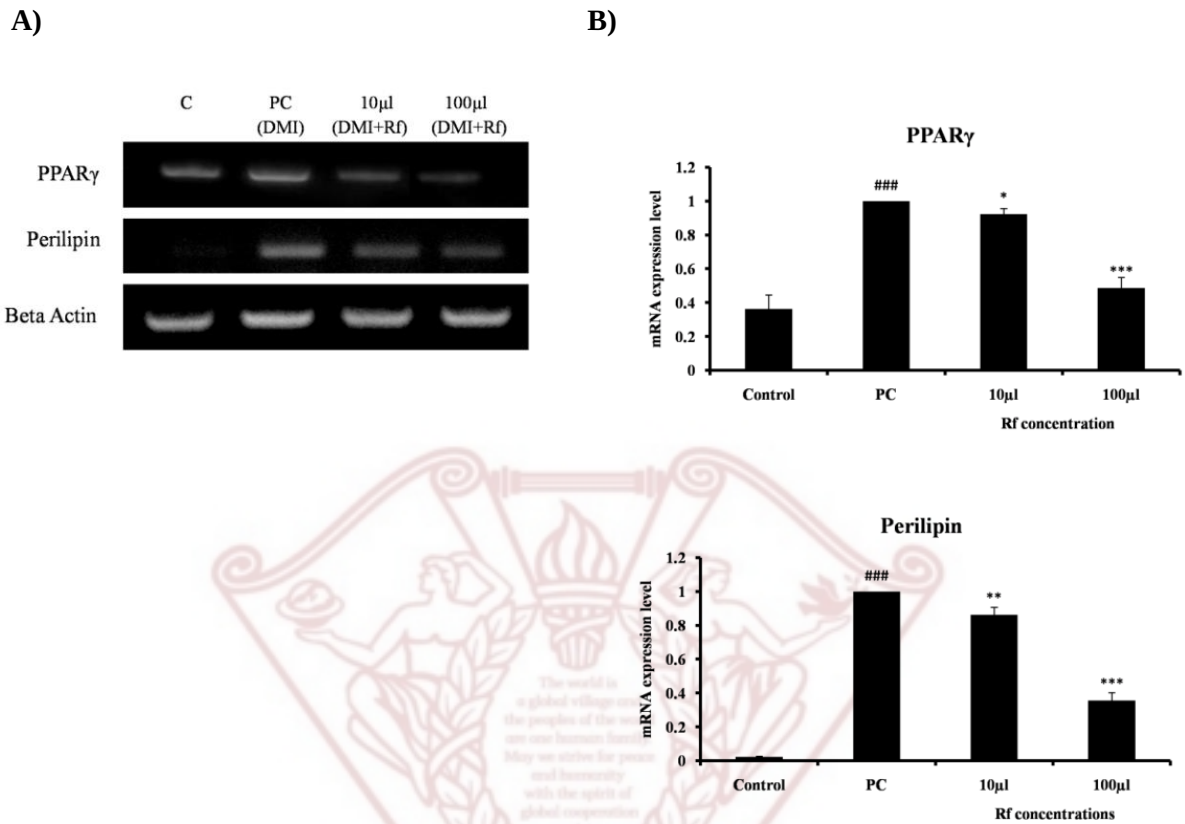



**Fig. 5.3.** Effect of ginsenoside Rf on adipocyte genes. At day 8 of differentiation total RNA was isolated. Control groups were treated with the normal medium, while the positive control received differentiation medium (DMI). Treated groups received differentiation medium with different ginsenoside Rf concentration (DMI+Rf), while expression levels were evaluated by RT-PCR and visualized via gel (A). mRNA expression levels were quantified using qRT-PCR and results analyzed (B). Presented data were statistically significant $^{###}p<0.0005$ compared with the normal control group and positive control, $*p<0.05$ $**p<0.005$ and $***p<0.0005$ compared with DMI treated positive control and DMI and ginsenoside Rf treated group at different concentrations.

## 5.5. Conclusion

The prevalence of obesity has increased dramatically worldwide, and it is now considered to be one of the leading global health risks. Moreover, obesity also causes several other health problems such as diabetes, heart disease, and some forms of cancer. Medicinal compounds derived from plants can interact extremely efficiently with biological systems, since they are obtained directly from nature. Thus, plants are considered to be important sources for the identification of novel drug candidates. In this study, we used an automated docking program to generate a model of ginsenoside Rf docked with PPARγ, and observed that ginsenoside Rf was indeed docked closely in the active site of PPARγ in this model. The results of this docking simulation were confirmed by several different docking programs; based on this agreement, we determined the ADMET properties of ginsenoside Rf. The ADMET values indicate that ginsenoside Rf has the potential to be a suitable drug. Moreover, the PASS results also suggest that ginsenoside Rf can perform useful biological activities. Since our computational studies indicated that ginsenoside Rf could be useful as a biologically-active, drug-like compound, we next endeavored to test the effects of ginsenoside Rf in *in vitro* assays using 3T3-L1 adipocytes. We first confirmed that Rf was not cytotoxic at the concentrations used in our assays. Lipid accumulation assays of treated adipocytes revealed that ginsenoside Rf-treated adipocytes exhibited lower levels of intracellular lipids compared with untreated adipocytes, as assessed by both visualization and quantification of absorbances. Moreover, we found that ginsenoside Rf treatment of adipocytes downregulated the mRNA levels of PPARγ and perilipin, as assessed by both RT-PCR and qRT-PCR. Cumulatively, the computational and experimental data presented here suggest that ginsenoside Rf, a ginsenoside obtained from *Panax ginseng*, may help attenuate obesity by interacting with PPARγ and inhibiting adipogenesis. However, further studies in animal models will be required to assess the true potential of ginsenoside Rf as an anti-obesity drug.

## VI. *In silico* screening of ginsenoside PPT ginsenoside and its inhibitory activity on pancreatic lipase

### 6.1. Abstract

Pancreatic triacylglycerol lipase (PNLIP), also known as pancreatic lipase (PL), is primary lipases that are critical for triacylglyceride digestion in human. In this study, we tried to find out the efficacy of protopanaxatriol (PPT) ginsenoside as an inhibitory agent for pancreatic lipase. A PL inhibitor may play a role to decrease the gastrointestinal absorption of fats and tend to excrete it as feces rather than being absorbed to be used as a source of caloric energy. This process may help for reducing weight. Therefore, at first with the application of molecular docking simulation it was figured out whether PPT and pancreatic lipase possesses binding interaction. Later molecular dynamic simulation was done to see the structural changes as well as the stability of the lipase with or without binding with the compound. Next the absorption, distribution, metabolism, excretion and toxicity (ADMET) of PPT were analyzed to find out its drug like properties. And then another computer program, prediction of activity spectra for substances (PASS) was used to see the biological activity and pharmacological effects of PPT. Finally, we checked the cytotoxic effect of different concentrations of PPT on 3T3-L1 adipocyte cell line that is an ideal cell model used to study on obesity at cellular level. Gene expression result using RT-PCR and qRT-PCR has shown to inhibit pancreatic lipase at 100 μM concentration.

## 6.2. Introduction

Obesity, one of the most common physical predicament now a days and is considered a probable risk factor for the prosperousness of a modern country. Obesity is the reason of a mass of devastating diseases, including insulin resistance and type 2 diabetes, lipid profile disorders, osteoarthritis, hyperuricemia, malignancies, and cardiovascular diseases which includes hypertension, coronary heart diseases, and stroke (Arbeeny, 2004; Cairns, 2005; Gurevuch *et al.,* 2009). It is predicted that by 2015, more than 1.5 billion people will be over-weight, and that at least 2.6 million annual deaths can be associated to obesity (WHO 2010 whoint/features /factfiles/obesity/en/). Pancreatic lipase (PL) are the primary lipases excreted by the pancreas, and is responsible for breaking down dietary lipids into unesterified fatty acids (FAs) and monoglycerides (MGs). The unesterified FAs and MGs will link with bile salt, cholesterol, and lysophosphatidic acid (LPA) to form micelles. Once absorbed by the intestines, it will be resynthesized to triacylglycerides and stored within the lipid cells as a primary source of energy for the human body. As consuming too much dietary lipids means excessive calorie intake, targeted inhibition of PL may reduce caloric intake and have implications in weight control (Mukherjee, 2003; Thomson *et al.*, 1997; Shi and Burn, 2004). *Panax ginseng* is an admired agricultural material used in many traditional medicinal therapies. Nowadays the use of this plant has been increased and the applications are focused on increasing resistance to physical, chemical and biological stress and boost general vitality (Kiefer and Pantuso, 2003). Ginsenosides, also known as ginseng saponins are the molecular components responsible for the activities of ginseng. The primitive structure of ginsenosides formed of a gonane steroid nucleus with 17 carbon atoms arranged in four rings. The typical biological reaction for each ginsenoside is credited to the difference in the type, position and number of sugar moieties attached by a glycosidic bond at C-3 and C-6 (Liu *et al.,* 2001). They are of three main types: protopanaxadiols (Rb1, Rb2, Rg3, Rh2), protopanaxatriols (Rg1, Rg2, Re, F1) and oleanolic acid derivatives (Yue *et al.,* 2007). Consequently, current work was devoted to evaluate *Panax ginseng* potential PL inhibitory activity as well as its biologically available protopanaxatriol (PPT). Initially, we used computer-aided molecular docking of PPT into the binding pocket of PL to reach preliminary conclusions about PPT/PL binding energetic. Next, molecular dynamic simulation was performed to find out the conformational changes of the complex structure of protein with or with out ligand. And then, the absorbtion, distribution, metabolism, excretion

and toxicity (ADMET) properties along with prediction of activity spectra of substances (PASS) was analyzed to find out the biological and pharmacological properties of PPT. Eventually, the docked active compounds were tested *in vitro* using 3T3-L1 adipocyte cell line against PL to identify their inhibition potential. And for that purpose we checked the cytotoxic activity of PPT on 3T3-L1 adipocyte to find out its toxic effect on cell level at different concentrations. And finally reverse transcription polymerase chain reaction (RT-PCR) and quantitative real time polymerase chain reaction (qRT-PCR) was done. From which it was clearly evaluated that pancreatic lipase possessed inhibitory activity on adipocyte differentiation via blocking adipogenesis.

## 6.3. Materials and Methods

### 6.3.1. Docking experiment

The 2D structure of PPT was collected from our internal database of *Panax ginseng* saponins structures which was drawn using ChemSketch (http://www.acdlabs.com (Advanced Chemistry Development, Inc. Toronto, Ontario, Canada). Then it was reformed to 3D by importing into Accelrys Discovery Studio 3.5 (DS3.5) (DS, http://www.accelrys.com; Accelrys, Inc. San Diego, CA, USA). The 3D form of PL was found from the Protein Data Bank (PDB code: 1LPB) with resolution; 2.46 Å (Egloff *et al.,* 1995). Using the DS visualizer templates for protein residues hydrogen atoms were entered to the protein and water molecules was removed. Docking simulation was carried out using the advanced automated docking program AutoDock Vina 4.0. This program is used for drug discovery, molecular docking, virtual screening, offering multi-core capability, high performance, enhanced accuracy, and ease of use (Trott and Olsen, 2010). In order to obtain the solo structure of PL the co-crystallized ligand was deleted from PDB structure; to verify the reproducibility of the binding of PL to Orlistat, a known inhibitor of PL, the ligand was re-docked into PL. The residues found to collaborate with Orlistat, were considered vital residues in the active site of PL. Previous studies have demonstrated that residues Ser152, His263, Asp176 are crucial for the inhibition of PL (Egloff *et al.,* 1995). These active sites are considered to be the most favorable region for docking simulations. The energy minimization was performed to PL protein and in depth docking

parameters were described in our previous study (Sathishkumar *et al.*, 2012). The best molecular interaction was determined based on binding direction of PL key residues and their corresponding binding affinity count. The results were imported and analyzed using Ligplot+ version 1.4.5.

### 6.3.2. Prediction of ADMET and PASS prediction

Pharmacokinetic studies of a probable drug commonly involve deciding its ADMET properties. While it has been projected that more than 50 % of all possible drugs fail during clinical trials due to their unsuitable ADMET properties this determination is principally important (Konstantin *et al.*, 2005). Advancements in computational studies and discovery process in drug have enabled the recognition of several pharmacologically active compounds, which must be optimized and also go through pre-clinical ADMET evaluations. Therefore we can easily identify that it is essential to verify the ADMET properties of a compound before clinical trials and was evaluated for PPT following our previous methods (Sathishkumar *et al.*, 2013). In addition, another computational program, PASS, was used to predict the possible biological activities of PPT based on its chemical structure (Lagunin *et al.*, 2000; Lagunin *et al.*, 2010). We specifically determined the possible biological activity scores of PPT related to obesity. These applications proposed list of potential biologically active properties mediated by PPT, along with their affiliated probabilities of activity (Pa) and probabilities of inactivity (Pi) (Fayeza *et al.*, 2014).

### 6.3.3. Molecular dynamic

Gromacs (version 4.6) software package was used for molecular dynamic (MD) simulations, (Spoel *et al.*, 2005) with the Gromacs96 43a1 force field (Christen *et al.*, 2005) and a single-point-charge (expanded) (SPC-E) water model (Ljungh and Moran *et al.*, 1996). For the simulation apo form of PL (without ligand) and obtained complexes of PL with Orlistat and PPT were used. The Orlistat and PPT topology files and force field parameters were produced using the Dundee PRODRG2 server (Schuttelkopf and Van 2004). In order to neutralize the system the appropriate counter ions ($Na^+$ or $Cl^-$) were added, and for energy minimization

conjugated gradient algorithms were used to reduce the close contact between the PPT and PL protein. The LINCS algorithm was used for all bond constrictions (Hess *et al.*, 1997). Simulations were performed in the NPT ensemble at a temperature of 300 K. Finally, a 10 ns MD simulation was carried out for PPT with the PL protein, and snapshots were saved every 2 ps. For the analysis, GROMACS 4.6 subroutines including *g_rms* and *g_rmsf* were used to evaluate root mean square deviation (RMSD) and root mean square fluctuation (RMSF), respectively. All dynamic simulations were carried out using an Intel® 2.93 GHz Xenon®CPU 5679 with 64-bit RHEL 6 server.

### 6.3.4. Experimental study

#### Reagents

Insulin and Isobutylmethylxanthine (IBMX) were purchased from Wako (Japan). Dexamethasone was purchased from Sigma Chemical Co. (MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Welgene (USA), Newborn calf serum was obtained from Gibco (USA), and antibiotic solution was purchased from GenDEPOT (USA). PPT ginsenoside was purchased from Chengdu Must Bio-Technology Co., Ltd (Wuhou, Chengdu, China) with the purity of ≥ 98 % by HPLC.

#### Cell culture and differentiation

3T3-L1 cells at the condition of preadipocytes were received from the American Type Culture Collection (ATCC; VA, USA) and were cultured in DMEM, supplemented with 10 % BCS and 1 % AB, at 37 °C in a 5 % $CO_2$ atmosphere. Three days after reaching confluence, differentiation was induced by culturing cells in differentiation medium (DM) and considering the time period as day 0 to day 3. The DM contained DMEM, 10 % BCS, 1 % AB, and DMI (1 µM dexamethasone, 0.5 mM IBMX, and 10 µg/ml insulin). Next, cells were fed with growth medium containing DMEM, 10 % BCS and 10 µg/ml insulin on days 3, 5, and 7. Through out this differentiation procedure one set of well received PPT at a concentration of 100 µM from day 0 until the day of experiment.

**MTT assay**

3T3-L1 Cells were plated in 96-wells at a density of $1 \times 10^4$ cells/well. After 18 hours, the cells were treated with DMEM containing 10 % BCS and PPT at a concentration of 1 µM, 10 µM, 50 µM, or 100 µM; and incubated for 48 hours. Next, 20 µl of MTT reagent was added to each well and again incubated for 4 hours at 37 °C. After this incubation period the medium was replaced with 100 µl DMSO, and in this condition cells were kept for another hour. Finally, the resultant OD was measured at 570 nm using an ELISA reader.

**Reverse Transcriptase –Polymerase Chain Reaction (RT-PCR)**

Cells were differentiated and on day 8 total RNA was extracted from the cells with or without treatment of PPT. A total RNA extraction kit (Intron Biotechnology, Korea) was used for this purpose. cDNA synthesis for pancreatic lipase was done with 1 µg of total RNA using a kit (Thermo Scientific, Lithuania). Primer sequences for the markers are as follows: Pancreatic lipase, 5'-CTGGGAGCAGTAGCTGGAAG-3' for forward, 5'-AGCGGGTGTTGATCTGTGC-3' for reverse, Beta actin, 5'-ATGAAGTGTGACGTTGACATCC-3' for forward, 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'for reverse. The reaction conditions were as follows: denaturation at 94 °C for 30 sec; annealing at 60 °C and extension at 72 °C for 1 min 20 sec. Thirty two cycles were carried out and the resulting PCR products were electrophoresed on a 1 % agarose gel and visualized with Image J software.

**Real Time Polymerase Chain Reaction**

Real time reverse transcription PCR (qRT-PCR) was performed using a real-time rotary analyzer (Rotor-Gene 6000; Corbet Life Science, Australia) and 1 µg of cDNA in a 10 µl reaction volume, using SYBR® Green SensiMix Plus Master Mix (Quantace, England), with gene specific primers. Pancreatic lipase, 5'-CTGGGAGCAGTAGCTGGAAG-3' for forward, 5'-AGCGGGTGTTGATCTGTGC-3' for reverse, Beta actin, 5'-ATGAAGTGTGACGTTG ACATCC-3' for forward, 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'for reverse. The

PCR conditions for each of the 40 cycles were 95 °C for 10 sec, 60 °C for 10 sec, and 72 °C for 20 sec. Beta actin was used for comparison purposes.

**Statistical analysis**

All data are presented as mean ± standard error (S.E.) and all experiments were independently performed three times. The mean values of the treatment groups were compared with untreated groups using Student's *t*-test. Statistical significance was assigned at $* p < 0.05$, $** p < 0.005$, $*** p < 0.0005$.

**6.4. Result and Discussion**

**Molecular interaction studies**

Molecular docking is an important application to observe the accurate prediction of protein-ligand interaction geometries at molecular level. In our study, this was performed for PL with PPT and Orlistat as the control ligand for validation using AutoDock Vina. The specific active site residues were kept flexible, along with the ligand. The interaction results of docking score values of control (Orlistat) and PPT along with binding energy and Van Der Waals bonds formed are listed in Table 6.1. PPT interacts with PL residues of Ser152, His263, Asp176 with a binding affinity of -7.9 kcal/mol Fig. 6.1. The potential binding orientation of the PL important active site Ser152 residue with PPT involved one hydrogen bond formation with OH group of PL. Similarly, the His263 residue was involved in one hydrogen bond interactions, while the Asp179 residue had one hydrogen bond interaction. In addition, Van der Waals interaction was formed by Leu213, Tyr114, Phe215, Ile209, Pro180 and Phe77 residues, strengthening the protein-ligand interaction. Orlistat as control was also found to form hydrogen bond formation at the same active site of PL with the binding affinity of -6.7 kcal/mol. The comparison between control and compound interaction with protein after molecular docking simulation shows that PPT and PL had good binding effinity.

Table 6.1. Control and PPT ginsenoside bond formation and Binding energy with PL

| ompound | H-Bond Interaction | Van Der Waals Interactions | Energy |
|---|---|---|---|
| Control (Orlistat) | **Ser152, His263, Asp176** His151, Gly76 | Ala260, Arg256, Trp252, Ile78, Leu264, Asp79, Ala259, Tyr114, Phe215, Ile209, Pro180, Phe77 | -6.7 |
| PPT (1LPB) | **Ser152, His263, Asp176** | Leu213, Tyr114, Phe215, Ile209, Pro180, Phe77 | -7.9 |

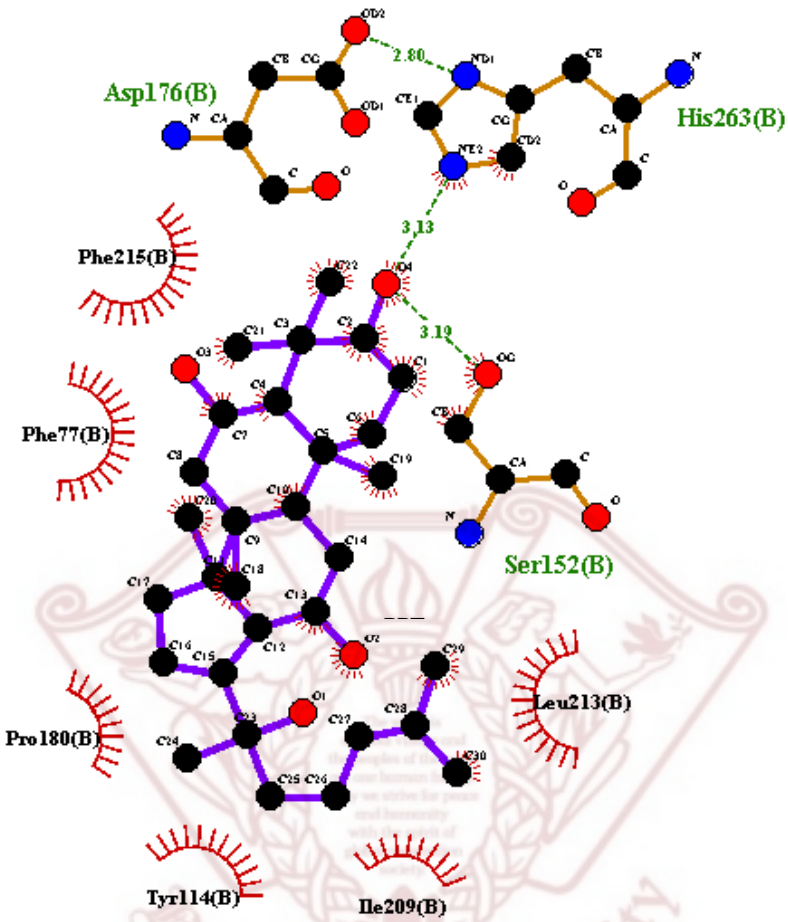



**Fig. 6.1.** Complex structure of ginsenoside PPT and Pancreatic Lipase (PL) interaction at active site of amino acid.

**ADMET and PASS analysis**

Using the Qikprop module in the Schrödinger program, the physiochemical and pharmaceutical properties of PPT were determined. The properties those detected are molecular weight, human oral absorption in the gastrointestinal tract, serum protein binding, CYP2D6 inhibition probability, and octanol/water partition coefficient. Since prediction of toxicity is an important factor in natural product research (Mohan *et al.*, 2007), we also revealed the hepatotoxicity descriptors for PPT using the ADMET module in DS3.5. The predicted ADMET values for PPT along with their acceptable ranges are, given in Table 6.2.

Table 6.2. ADMET results of selected ginsenoside PPT with pharmacokinetic properties

| Ginse-noside | MW | Solubility Level | QP (%)# | QplogK hsa# | CYP2D6 inhibition* | Hepato-toxicity* | QPlog Po/w# | Lipinski Rule of 5 violations |
|---|---|---|---|---|---|---|---|---|
| PPT | 474.7 | -6.2 | 100 | 1.0 | 0.356 | 0 | 5.0 | 1 |

MW: Molecular weight accepted range 130-725

Aqueous solubility (Solubility level) (accepted range: -6.5-0.5)

QP (%): Percentage of human oral absorption in GI (acceptable range: <25 % is poor and >80 % is high).

QPlogKhsa: Serum protein binding (acceptable range: -1.5/1.5).

CYP2D6 inhibition: 0 is Non-inhibitor and 1 is inhibitor.

Hepatotoxicity: 0 is non-toxic and 1 is toxic.

QPlogPo/w: Octanol/water partition coefficient (acceptable range: -0.2 to 6.5).

Lipinski rule of 5 violations: Maximum is 4 violations.

# Predicted values using Qikprop program in Schrödinger.

* Predicted values using ADMET descriptors in Discovery Studio 2.5.

Next, using another computational program PASS we predicted the biological activity spectrum of PPT. The outcome of this analysis suggested that PPT could exhibit activity (Pa >0.7) as a transcription factor inhibitor, an antidiabetic agent, a cholesterol antagonist, a cholesterol synthesis inhibitor, an antihypercholesterolemic agent, a cholesterol absorption inhibitor, and a lipid metabolism regulator shown in Table 6.3. These factors are important since they are directly or indirectly related to fat, as well as to obesity.

Table 6.3. Predicted biological activity (Pa) and inactivity (Pi) of PPT ginsenoside

| Pa | Pi | Activity |
|---|---|---|
| 0.718 | 0.007 | Cholesterol antagonist |
| 0.702 | 0.004 | Transcription factor inhibitor |
| 0.311 | 0.077 | Antidiabetic |
| 0.569 | 0.002 | Cholesterol synthesis inhibitor |
| 0.289 | 0.060 | Antihypercholesterolemic |
| 0.412 | 0.088 | Lipid metabolism regulator |

**Structure alteration and stability estimation**

Molecular dynamic simulations were operated to identify the structural stability of PL and their complexes. In order to go through this study, the apo forms of PL and complex along with known inhibitor (PL-Control, PPT-Control) were chosen based on their molecular interaction and ADMET analysis. The MD simulation of known inhibitor (Orlistat) with PL was used for comparison with PPT ginsenoside complex. The output MD result was analyzed on the basis of the root mean square deviation (RMSD) and root mean square fluctuation (RMSF) values. The RMSD values in Fig. 6.2A of PPT complexes including the apo form (PL) (PL-CTRL) (PL-PPT) attained equilibrium around 2.96 ns, 2.8 ns, and 3.92 ns respectively. The RMSD results showed that, after reaching equilibrium for PL-PPT, the complexes were stable till the end of the simulation, which is up to 10000 ps (10 ns). Furthermore, the apo forms of PL and their complexes were analyzed for local protein mobility to get the root mean square fluctuations (RMSF). In Fig. 6.2B The RMSF values of C α atoms against the residue numbers for each molecule during the 10000 ps trajectory are shown. The values indicate that only a few atoms of the PL complexes fluctuated compared to the native structure, and less deviation was seen in the active site residues. And these suggest that PL complexes undergo less fluctuation compared to the native structures. Therefore the molecular simulation results clearly indicate that the binding orientation of PPT with PL did not possess impact on the protein stability during the simulation time period.

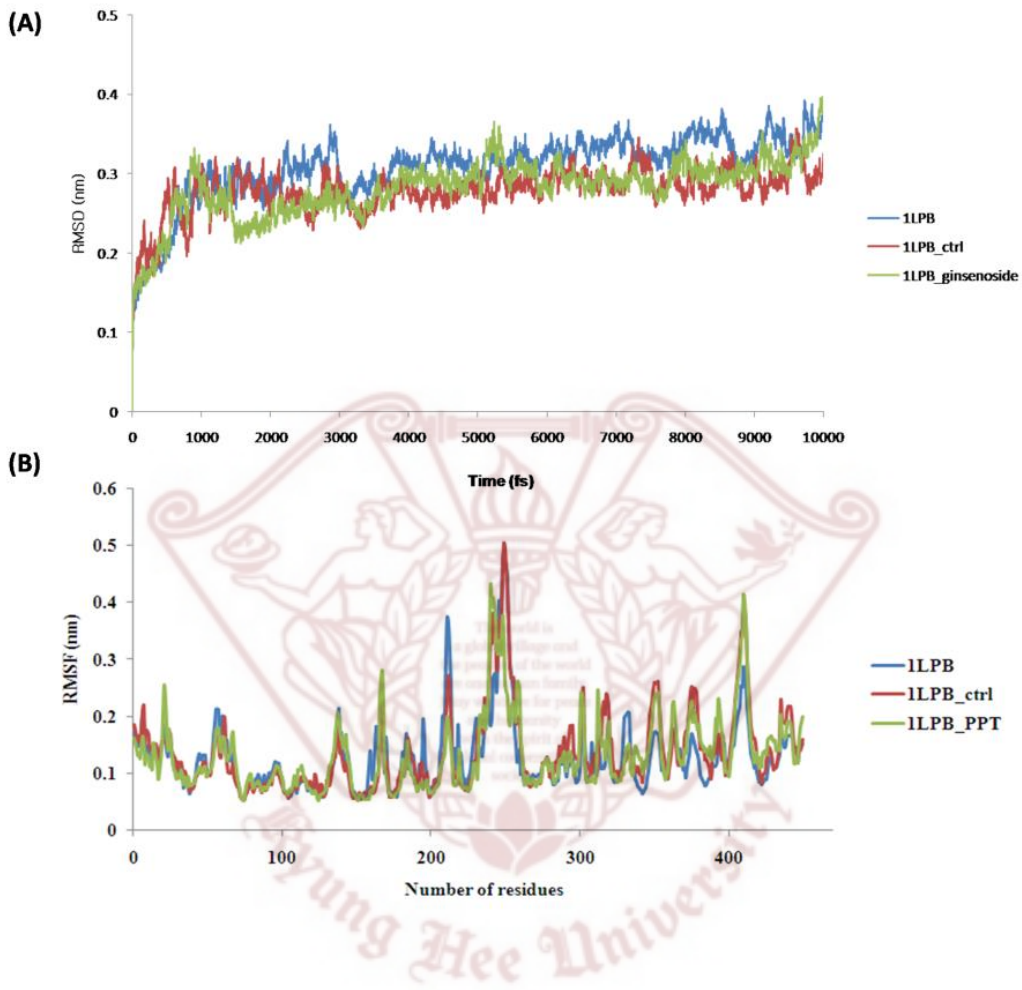



**Fig. 6.2.** Molecular Dynamic trajectory analysis during 10ns **(A)** Root mean square deviation (RMSD) of values of free pancreatic lipase and pancreatic lipase complexes, **(B)** Root mean square fluctuations (RMSF) plots of cα atoms against number of residue.

***In vitro* analysis**

To identify whether PPT exhibited cytotoxic effect, MTT assay was done after 48 hours of treatment with various concentrations (1 µM, 10 µM, 50 µM, and 100 µM) of PPT. From the result it was observed that PPT was not significantly toxic to 3T3-L1 cells even at the treated highest concentration, shown in Fig. 6.3.

Later we also carried out gene expression analysis to know the condition during adipogenesis at 100 µM concentration of PPT. From the RT-PCR result it could be observed that compared to the control group the DMI treated positive control gene expression was stimulated while the group with PPT treatment has shown to inhibit expression Fig. 6.4A. Same in qRT-PCR analysis the mRNA expression of positive control was increased compared to the normal control group and the other group which received DMI as well as PPT had reduced mRNA expression level shown in Fig. 6.4B.

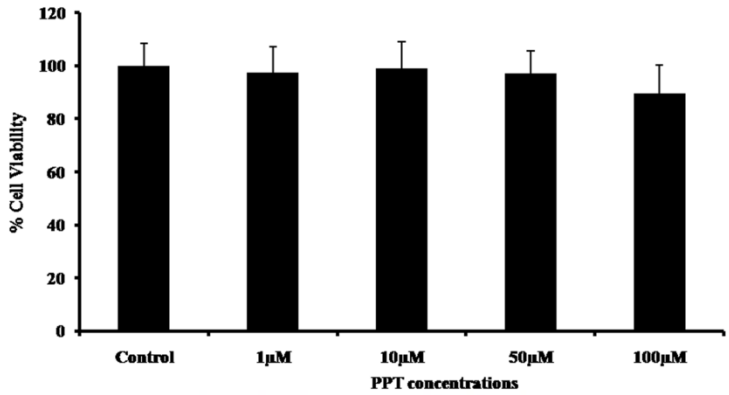



**Fig. 6.3.** The cytotoxic effect of different concentrations of PPT.

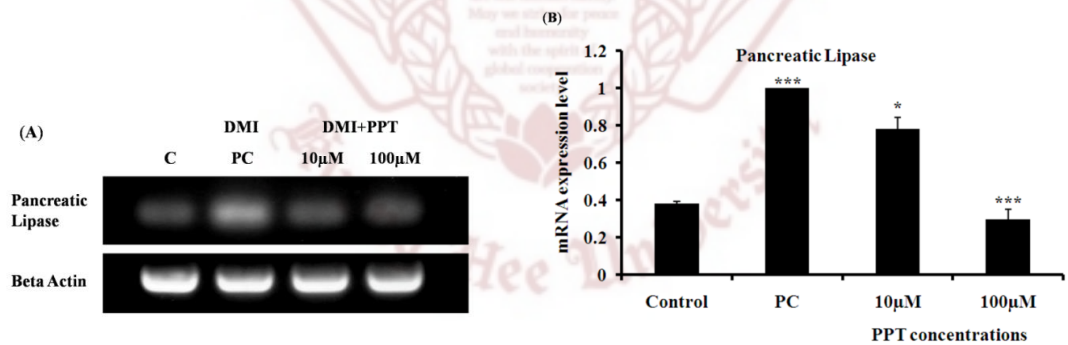



**Fig. 6.4.** Gene expression analyses. Control cells were treated with normal medium, whereas positive control cells received differentiation medium (DMI). Treated groups received differentiation medium with 100 µM concentrations of PPT (DMI+PPT). The expression levels of various genes were evaluated by RT-PCR; amplification products were visualized by gel electrophoresis (**A**). Quantification of mRNA expression levels by qRT-PCR (**B**).

## 6.5. Conclusion

Current evaluations in understanding the pathophysiology of the disease process have opened up new ways to diagnose and evolve novel therapies to fight against obesity, among these various enzymes involved in lipid metabolism provide provocative targets in the development of antiobesity agents. Lipid metabolism is carefully balanced to control homeostasis (Foster and Cummings *et al.,* 2006; Mukherjee, 2003). When the balance is disturbed, obesity or dyslipidemia is caused along with a range of severe metabolic diseases. One of the most essential techniques to balance obesity is to promote inhibitors of PL. Natural products established from traditional medicinal plants and microbial sources have always brought up valuable options for the improvements of new types of therapeutics. Therefore we selected PPT ginsenoside, one class of saponin of the famous medicinal plant *Panax ginseng* to find out its activity on pancreatic lipase. In one study, it was suggested that PPT may be of naturally derived PPARγ agonists, which commits insulin-sensitizing effects of PPARγ through regulating lipid and glucose metabolism in 3T3-L1 adipocyte. From the study, it was considered that PPT might enhance insulin resistance by lowering lipotoxicity in muscle and liver through storing more lipids in adipocyte and enhancing insulin sensitivity through increase of GLUT4 expression in adipocyte, which provides antidiabetic effects (Kyu *et al.,* 2006). Since our investigation provides the idea about bond formation and interaction between PPT and PL there was a probability of PPT to have impact on this lipase and may possess efficacy on adipocyte. And so we performed some investigations *in vivo.* First we checked the cytotoxic effect of PPT from which we found it not to be toxic at different treated concentrations. Later on from our RT-PCR and qRT-PCR analysis it was proved that PPT inhibited the expression of pancreatic lipase. Therefore considering all the works carried out our hypothesis suggests that PPT may be an important component that possesses binding interaction with pancreatic lipase structure and has biological and pharmacological properties to act as a drug like compound. During cell level analysis PPT did not contain toxicity at high concentrations and also inhibited pancreatic lipase activity. It also suggested that further studies related to *in-vivo* trials may be done to consider it as a drug like component for the utility as anti-obesity.

## VII. GENERAL CONCLUSION

Obesity drags a person in the high risk category for many diseases, including type 2 diabetes, cardio vascular problems, and even different types of cancer. In modern society, obesity has reached epidemic proportions and is thus a critical topic to study. From the reported studies and also from our work, it can highlighted that ginseng may work as an anti-obese agent. Some ginsenosides have been shown to improve glucose tolerance showing the efficacy as an anti-diabetic. Still more studies of the adipogenesis pathways should be done focusing on different pathways and markars that may be related with adipogenesis and obesity. For example; Perilipin, Kruppel-like factor 5 (KLF5) genes are considered a key regulator of adipocyte differentiation (Philip *et al.*, 2004; Yumiko *et al.*, 2005). It is vital to notice these markers. We may notice that ginseng and its single components have shown to be an important medicinal plant to treat obesity. Adipocytes are the source of treatment with various ginsenosides. Most of the studies have focused on the transcriptional regulator of genes. Studies on humans are not sufficient to get enough of an idea about its actual impact on obesity. Further studies will lead to a better understanding of the relevant markers and develop improved treatments. However, all the ginsenosides do not pose the anti obese effect. Some ginsenosides, for example Rb1 and Rh2, have been shown to cause obesity at the cellular level (Wenbin *et al.*, 2007; Niu *et al.*, 2009). In these cases, how the genes are involved should be studied. The earlier studies did not report any notable side effects when ginseng was administered in animal models at the maximum level of 500 mg/kg 3 times a day. Some ginsenosides have also been shown to effect on glucose levels, and thus may function as an anti-diabetic agent (Park *et al.*, 2008; Yun *et al.*, 2004; Ye *et al.*, 2010). Further studies and works on MAPK and cAMP like pathways are being suggested to focus on, related to adipogenesis. Proteins located in nuclear membrane or lipid surface can be studied more that may be blocked by ginsenosides to inhibit the adipocytes being matured fat cells. Reports from our studies suggested that ginsenoside F2, Re, CK, Rg3, Rh1, Rf shows molecular interaction with PPARγ and from the experimental outputs of ginsenoside F2, Rh1, Rf its been shown to inhibit the protein expression and therefore works as an anti-obese compound. Computational studies of PPT ginsenoside shows that it may have drug like activity via interaction with pancreatic lipase that and PPT may act as an inhibitory compound on pancreatic lipase

expression, that may inhibit from breaking down of fat and consequently prohibitibg absorption of fat in the intestine.

Finally, from the studies carried out it can be concluded that, computational screening may be essential to discover drug like component from natural resources to treat obesity or other diseases and then prove their level of efficacy by checking them in cell level or animal models.

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## 국문초록

**Docking system에 의한 인삼사포닌의 선발 및 F2, Rh1, Rf, PPT의 비만에 대한 효과**

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비만은 체지방의 과잉 축적이 원인이며, 여러 가지 다른 질병으로 이어질 수있는 만성 질환이다. 이에 따라 건강한 삶을 유지하기 위해 비만을 감소시키는 방법을 발견하는 것이 필요 시 되고 있다. 지금까지 몇 가지 약물이 비만을 치료하는 것으로 보고됐다. 그러나 다양한 부작용으로 인해 최근 비만연구는 약용 식물에 초점을 맞추고 있다. 그 중 많은 약용식물 가운데, 고려인삼(*Panax ginseng*)은 비만 등 여러 질병을 치료하기 위해 오랫동안 사용되어 온 가장 인기 있는 약용 식물 중 하나이다. 따라서 본 연구에서는 비만을 치료할 수 있는 고려인삼의 약리효능 물질인 진세노사이드(Ginsenosides)에 초점을 맞추고 있다. 본 연구팀은 실험을 수행하기 전 선행연구로서 문헌에 따라 128 가지의 진세노사이드(Ginsenosides)를 가상스크리닝(*In-silico*)을 통해 흡수, 분포, 대사, 배설 및 독성(ADME/T)을 확인하였다. ADMET 의 결과값을 통해 15 가지의 진세노사이드 (F2, Rg3, Rb1, Rb2, Rc, Rd, Rg1, Rg2, Rh2, Ro, Re, CK, Rf, Rh1, PPT)를 선별하였다. 위 진세노사이드는 도킹 시뮬레이션(Docking simulation)을 통해 비만의 특정 단백질과 상호 결합력을 계산하였다. 그 결과 6 가지의 진세노사이드(F2, Rg3, Re, Ck,

Rh1, Rf) 가 PPARγ 라는 단백질과의 상호결합을 나타냈다. 그리고 진세노사이드 PPT 가 중요한 사포닌이기 때문에, 우리는 소장에서 지방 브레이크 다운에 관여하는 췌장 리파제 (PL)과 진세노사이드 PPT 의 도킹 상호 작용을 추가로 조사했다. 도킹 시뮬레이션 결과 진세노사이드 PPT 는 PL 과의 상호결합을 나타냈다. 따라서 우리는 세포 수준에서 실험을 실시했다. 하지만 그 중 CK, Re, Rg3 는 이미 3T3-L1 세포 라인에 대한 실험이 보고되었다. 그래서, 본 연구는 다른 진세노사이드(F2, Rh1, Rf, PPT)을 사용하여 실험을 수행 하였다. 3T3-L1 은 일반적으로 비만연구에 사용되는 지방 세포 세포주이다. 우리의 실험결과는 다음과 같이 보여준다. 낮은농도(1 μM)에서 높은농도(100μM)까지의 선정된 진세노사이드(ginsenodies)는 세포에 유해한 물질이 아니다. 그리고 우리는 oil red o staninig 실험을 통해 세포 속에 액체가 축적되는 것을 확인하고, 세포 속에 축적된 액체 양을 나타냈다. 그 결과는 세포에 축적된 액체들의 양이 진세노사이드에 의하여 감소하였다. 마지막으로 우리는 지방세포유전자의 발현을 확인하였다. RT-PCR 과 qRT-PCR 을 사용한 바 유전자 발현을 억제하는 것으로 보인다. 이것은 지방세포화 진행과정을 막는 것으로 입증된다. 그리하여 컴퓨터 스크린과 증명된 실험결과로부터 F2, Rh1, Rf 그리고 PPT 진세노사이드는 비만치료제로 유용하게 사용되어질 수 있다.

**ACKNOWLEDGEMENT**

In the loving memories of my father

Md. Sirajuddowlla (1951-2013)

First of all, I would like to express my deepest gratitude to the almighty Allah for the limitless blessings on me to accomplish this work successfully. I am really thankful for the enomous blessing that almighty has bestowed upon me not only during my PhD period but also throughout my life.

This dissertation would have not been possible without the guidance and help of several individuals who in anyway contributed and extended their valuable assistance in preparing and completing this dissertation.

I have great pleasure to place on record my deepest gratitude and heart felt thanks to my advisor and the chairman of my dissertation committee, Prof. Deok-Chun Yang, Professor and Head of the Laboratory of Hanbang Bio, Kyung Hee University (KHU), Korea for his sustained interest, expert guidance, contructive criticism and constant encouragement throughout the course of investigation. Additionally, Professor Yang also impresses me by his highly professional attitude in reaserach, which is necessary to learn and follow in the future.

I am immensely grateful to the members of the dissertation committee, Prof. Se-Young Kim, Prof. Tong Ho Kang, Prof. Yeon Ju Kim and Prof. Jin-Kyu Chang for their support and valuable advises throughout the study.

I sincerely thank all the members of the laboratory of Hanbang Bio, for their kind help and support throughtout the study, which includes special thanks to my friends Dr. Sri Renuka Devi Balusamy and Shadi Rahimi. I personally thank Prof. Yeon Ju Kim for the incessant help, valuable suggestions, kind advice and efforts taken in guiding during various stages of the study.

I greatly acknowledge the help and encouragement from all the members of my team, especially Dr. Natarajan Sathishkumar for his great effort and advice during my study.

I cannot forget the memories of each of the moments during my stay at KHU. I am very much thankful to my international friends in Korea for their affection and help provided throughout the study also I am very much thankful to all my Korean friends I have met for their friendly behavior and endless helps.

I would like to mention that word seen inadequate to show my gratitude to my beloved father Md. Sirajuddowlla, who has always been my inspiration for doing PhD but sadly I do not have him in this world to show my success. But I know my acheivements will make him proud. I am also greatful to my mothers for her all time support. Heartfelt thanks to my family and friends in Bangladesh for all the supports I received throughout my life.

Finally but most importantly, I would like to thank my husband Muhammad Kamrul Hasan for his help, support, encouragement and love, without which this journey of my PhD would never have been possible.

I also acknowledge the favours of numerous persons who, though not been individually mentioned here, have all directly or indirectly contributed to this work.