



## Cinnamaldehyde prevents high fat diet (HFD) induced obesity mediated gene transcription through AMPK activation

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### ABSTRACT

The present study was evaluated to determine if Cinnamaldehyde could attenuate HFD induced obesity in wistar rats through AMPK activation. The rats were randomly divided into five groups: Normal; HFD; Orlistat (OR) treated at 50mg/kg with HFD; Cinnamaldehyde (CA) treated at 40mg/kg and 80mg/kg with HFD. Western blot and RT PCR in white adipose tissue (WAT) was carried out. HFD treated group of rats stimulated lipid synthesis related genes like, PPAR- $\gamma$ , aP2, ACC via inhibiting AMPK activation. CA and OR (standard anti-obesity drug) treatment showed up regulated the levels of pAMPK and its substrate pACC. AMPK signalling is responsible for the inhibition of adipocyte differentiation and fat accumulation. AMPK inhibit the activity of ACC through phosphorylation. mRNA expression of PPAR- $\alpha$  was up regulated in both CA and OR treated groups of rats. PPAR- $\gamma$ , aP2, ACC mRNA expression were down regulated in both CA and OR treated rats in a dose dependent manner. These results suggest that CA ameliorates HFD induced obesity by down regulating the expression of lipogenesis related genes via AMPK activation in the WAT. Thus, these results provide molecular basis for the future research involved in the signalling pathways by which CA modulates lipid synthesis.

**Keywords:** AMPK, White Adipose Tissue, Gene Expression, High Fat Diet, Immuno Blot

### INTRODUCTION

Obesity is a multifactorial abnormality that has a genetic basis but requires environmental influences to manifest. Numerous epidemiological studies and clinical trials have examined the roles of lifestyle (e.g. physical inactivity) and dietary factors (e.g. fat, carbohydrates, proteins and minerals) in obesity prevention and weight control. In the past decade, the body of studies on gene environment interactions has also grown rapidly. The recent epidemic of obesity along with the increasing spread of Western type lifestyles worldwide is a good illustration of the concept of gene pool of a certain population; it seems that dramatic changes in lifestyle and dietary habits have played a role in triggering the recent surge of excessive adiposity.

In obese condition, the pancreas is affected and its function of secreting insulin is altered leading to insulin resistance that is developed due to the increased deposition of fats in adipose tissue and non adipose tissue, leading to either cell death or cell dysfunction [1, 2].

Adiposity is therefore synonyms with obesity. Adipose tissue is deposited fat is more active than peripheral subcutaneous fat. The two main types of adipose tissue present in mammals are white adipose tissue (WAT) and brown adipose tissue (BAT). White adipose tissue is the main constituent of adipose tissue is used as substrate of energy when required. Excessive accumulation of white adipose results in obesity and its associated disorders.

White adipose tissue plays a key role in regulating whole body energy metabolism. Several genetic and pharmacological studies indicate that an increase in FA oxidation capacity in adipocytes leads to the attenuation of

adiposity and improvement of obesity induced metabolic disorders. Brown adipose tissue is responsible for expenditure of energy for cold adaption due to non oxidative phosphorylation

Several genetic and pharmacological studies indicate that an increase in FA oxidation capacity in adipocytes leads to the attenuation of adiposity and improvement of obesity-induced metabolic disorders. Peroxisome proliferator activated receptors (PPARs) regulate the expression of various important genes involved in lipid and glucose metabolism [3].

Three types of PPARs have been identified: alpha, gamma and delta (beta). PPAR- alpha serve as cellular receptor for fibrates, a class of drugs used in the treatment of dyslipidemia [4]. Fibrates effectively decreased serum triglycerides and raises serum HDL- cholesterol levels. PPAR- $\alpha$  induces the expression of the fatty acid transport protein and fatty acid translocase, proteins that facilitate the transport of free fatty acids across the cell membrane. PPAR- $\alpha$  also directly increases the transcription of enzymes of the peroxisomal  $\beta$ -oxidation pathway such as long chain acyl-coA synthetase or acyl-coA oxidase and is the rate limiting enzyme in the peroxisomal  $\beta$ -oxidation pathway. PPAR- $\alpha$  has the ability to reduce energy storage, mainly through degradation of fatty acid synthesis in the adipose tissue and liver [5]. This indicates that PPAR- $\alpha$  is a core modulator of peroxisomes and mitochondrial  $\beta$ -oxidation during the fatty acid synthesis and tissue lipid metabolism and its act as a strong anti- inflammatory agent. PPAR- $\gamma$  is present mainly in insulin target organs such as liver, adipose tissue and skeletal muscle [6]. PPAR- $\gamma$  is expressed at high levels in adipose tissue and is a central regulator of adipocyte gene expression and differentiation. PPAR  $\gamma$ , a lipogenic transcription factor predominantly expressed in adipose tissue, plays an important role in the regulation of adipocyte differentiation, lipid synthesis, and glucose homeostasis [7]. Lipogenic transcription factors regulate the gene expression of enzyme involved in lipid synthesis like, aP2 [8]. Adipocyte specific fatty acid binding protein (aP2) functions in fatty acid import, storage and export [9]. The lipid binding proteins are a family of intracellular with 15kDa proteins capable of high affinity selective binding of hydrophobic ligands such as fatty acids, bile salts and retinoids. Several tissues including adipose, liver, intestine, heart and brain express high levels of lipid binding proteins.

Acetyl CoA carboxylase (ACC) catalyzes the carboxylation of acetyl CoA to malonyl CoA, the rate limiting step in fatty acid synthesis. Malonyl CoA ireregulation may induce insulin resistance and obesity [10]. Acetyl-CoA carboxylase (ACC) is mainly involved in lipid biosynthesis, can be phosphorylated and inactivated by AMPK [11]. AMP- activated protein kinase (AMPK) is a serine- threonine kinase and it is activated followed by rise in the intercellular AMP: ATP ratio [12]. AMPK plays a key role in the regulation of energy homeostasis and stimulates the  $\beta$ -oxidation of fatty acids in mitochondria for lipid utilization [13]. AMPK activation requires phosphorylation at Thr172 of AMPK $\alpha$  subunit and it acts on metabolic enzymes and transcription factors [14]. One of the first proteins identified as a target of AMPK was acetyl-coA carboxylase (ACC) which synthesizes malonyl-coA from acetyl-coA and is a key enzyme of the lipogenic pathway [11].

In peripheral tissues and the central nervous system, AMPK controls the energy by responding to nutritional and hormonal signals and altering the metabolism according to eating behaviour and energy consumption [15]. Recently, AMPK has emerged as a therapeutic target for the metabolic diseases such as obesity and type2 diabetes mellitus [16]. AMPK inhibits the activity of ACC through phosphorylation. Under normal conditions, inhibition of ACC by AMPK through phosphorylation leads to lower the malonyl CoA content and a subsequent fall in fatty acid synthesis and raise in mitochondrial fatty acid oxidation. Inactivation of ACC by AMPK helps to promote fatty acid utilization, leading to oxidation of fat in muscle tissue.

Many anti-obesity drugs act by ameliorating the fat absorption by inhibiting lipid breakdown in intestine- eg. orlistat or reducing the appetite by increasing the satiety and altering the central nervous system- eg, sibutramine and rimonabant [17]. However, these drugs produce severe cardiac and psychiatric side effects. Therefore, in recent years, plant based herbal drugs are used for the treatment of obesity [18]. Therefore, many studies have been performed to search and develop the new anti-obesity drugs or dietary supplements through the use of medicinal compounds that could minimize the side effects [19, 20]. Cinnamaldehyde (CA) is a pungent compound and is mainly isolated from the *Cinnamomum verum* and *Cinnamomum Cassia*. Various researchers found that CA act as anti-diabetic, anti-inflammatory, anti-oxidant [21], anti-cancer, anti-septic and act as a flavouring agent in the icecreams, candy, chewing gums, beverages and other food stuffs [22, 23]. Our previous study indicated that, CA was found to be good antioxidant activity and also inhibit visceral adipose tissue accumulation, leptin levels.

This study was planned to investigate that CA can suppress the lipid synthesis and promote fatty oxidation in the process of fat metabolism by studying the mRNA expression levels of lipid metabolism related genes in adipose tissue. The genes like, AMPK, ACC, PPARs and aP2 involved in the regulation of fatty acid synthesis and oxidation

was investigated. Therefore, the aim of this study was to distinguish the effects of the CA with high fat diet on the transcription levels of AMPK, ACC, PPARs and ap2 in white adipose tissue.

## EXPERIMENTAL SECTION

### Animal Model

An animal model that mimics the human counterpart is essential for preclinical evaluation of new treatment modalities for obesity. Wistar albino male rats weighing about 120-150g were used for the experiment. Animals were kept in animal house at a temperature of 25°C and 45-55% relative humidity with 12 hours each of dark/light (day and night) cycles. Animals were fed pellet diet and water ad – libitum. The experimental protocol was approved by the animal ethical committee (IAEC No. SU/BRULAC/RD/006/2013).

### Administration Modality

All the rats were divided randomly into following 5 groups of 6 animals each and acclimatized to the laboratory environment for 1 week before the experiment. Group I: rats were fed with normal or standard diet for 8 weeks. Group II: (Obesity induced rats) - rats were fed with high fat diet (HFD- Research diet, USA) for 8 weeks. Group III: (HFD + Orlistat) - rats with HFD and Orlistat (50mg/kg body weight) simultaneously for 8 weeks orally. Group IV: (HFD + CA) - rats were fed with Cinnamaldehyde (40mg/Kg body weight) and HFD simultaneously for 8 weeks orally. Group V: (HFD + CA) - rats were fed with Cinnamaldehyde (80mg/Kg body weight) and HFD simultaneously for 8 weeks orally. The Normal diet and HFD control rats were treated with vehicle (Corn Oil) only. The body weights of different groups of rats were weighed for a period of eight weeks. After 8 weeks period, the rats were sacrificed. The white adipose tissue, pancreas and spleen were collected and frozen in liquid nitrogen, and stored at -80°C until analyzed.

### Reverse Transcription- Polymerase Chain Reaction (RT- PCR)

#### RNA Extraction

White Adipose tissue frozen at -80°C were stored in liquid nitrogen for transportation and 1ml of TRIZOL was added per 100 mg of tissue. The tissues were ground and incubated for 5 min at room temperature. To this, 2 ml of chloroform was added, shaken vigorously for 15 sec and placed on ice at 4°C for 5 min and centrifuged at 12000 rpm for 15 mins at 4°C. Aqueous phase was taken and equal volumes of isopropanol was added for a further 15 mins of centrifugation at 15000 rpm for 10 mins at 4°C to precipitate the RNA. The RNA sample was subsequently vortexed gently and quantified before storing at -80°C.

1mg of total RNA isolated from control and treated samples mixed with premix containing oligo (dT) primer and DEPC treated water to a final concentrations of 20µl and incubated at 45°C for 60 min. The reaction was stopped by heat inactivation at 95°C for 5 min. Subsequently, cDNA was amplified with gene specific primers using the Taq PCR master mix (Qiagen, Germany). The 20µl amplification mixture contained cDNA. Taq PCR master mix containing MgCl<sub>2</sub>, each gene primer and water. After a 15 min preincubation at 94°C, PCR amplification was performed for 35 cycles under the following conditions: 30s of denaturation at 94°C, 30s of annealing at 60°C and 1 min of extension at 72°C. The PCR products were separated using agarose gel electrophoresis and visualised, documented by using quantity one software (BioRad, USA). For amplification of the target genes, the following primers used are GAPDH (57°C, 35 cycles), ACC (60°C, 35 cycles), PPAR-α, PPAR-γ (60°C, 35 cycles), ap2 (60°C, 35 cycles) for the experimental group (Table 1). The relative expression levels of target genes were normalized using GAPDH as an internal control.

### Western Blot

The tissue samples were homogenized in 10 volumes of 20mM Tris Hcl P<sup>H</sup> (7.4) containing 5mM EDTA and 10 mM β- mercapto ethanol using a homogenizer and centrifuged at 10.000 rpm for 30min at 4°C. The supernatants were used for analyses. These proteins were separated by 12% SDS PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane, blocked overnight with 5% non fat dried milk in PBS-T at 2-8°C, reacted with mouse monoclonal primary antibodies (AMPK, pAMPK, ACC, pACC, PPAR-γ and aP2) and incubated overnight at 4°C and washed. The membrane was washed three times with TBST, incubated further with alkaline phosphatase conjugated with goat anti-mouse antibody or anti-rabbit antibody at room temperature for 2 hours. After reaction with horse radish peroxidase conjugated with goat antimouse antibody, the immune complexes were visualised by using the chemiluminescence ECL plus detection reagents following the manufacturer's procedure (Amersham bioscience). Antibody to AMPK, pAMPK, ACC, pACC, PPAR-γ, aP2 and GAPDH and secondary anti-mouse HRP were purchased from santa Cruz biotechnology.

**Histopathological Analysis**

Pancreas and spleen were removed from the animals and fixed in buffered solution of 10% formalin, washed, dehydrated, cleared and embedded in paraffin. Then, the specimens were processed into 5 µm sections for light microscopic examination using hematoxylin and eosin stain.

**Statistical Analysis**

The data were expressed as mean ± SD. All statistical analysis was performed using SPSS 20.0 statistical software (IBM, USA). Significant differences among the treatment groups were analysed by variance (One way ANOVA) followed by least significant difference (LSD) test. Results were considered to be statistically significant at P values < 0.05. Graphs for this study were plotted using graph pad prism version 6.02.

**RESULTS**

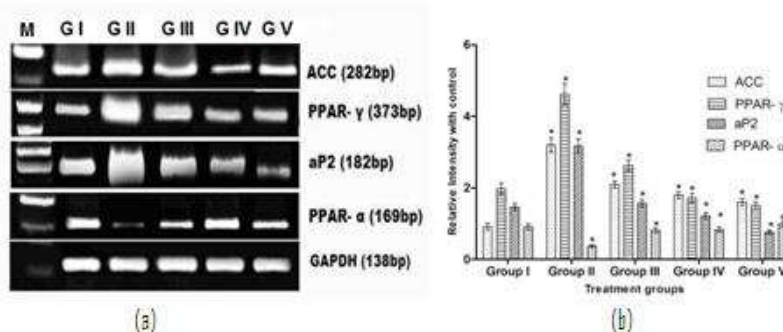
**mRNA gene expression levels of WAT in RT-PCR**

HFD fed rodents appear to be the best model of visceral obesity syndrome, because obesity pathogenesis in humans with insulin resistance [24]. This study demonstrated that ingestion of CA can prevent HFD induced visceral WAT accumulation. Table 1 shows the Sequences of primers used in RT-PCR analysis. To understand the mechanism involved in the effects of CA on lipid metabolism, mRNA expression levels of lipogenesis related genes in WAT were investigated. Fig 1 shows the HFD induced obese rats had up regulated mRNA expression levels of adipocyte markers such as PPAR-γ, aP2, ACC in the WAT. As shown in Fig 1. ACC, PPAR-γ, aP2 were down regulated in CA and OR treated rats when compared to those in HFD fed rats in a dose dependent manner. As shown in Fig 1, CA affect the expression of PPAR-γ, ACC and aP2 which are all associated with TG synthesis. The mRNA expression level of the lipolysis related PPAR-α gene was down regulated in HFD fed rats as compared with the normal group of rats. The expression level of the lipolysis related PPAR-α gene was up regulated in CA and OR treated rats as compared with the HFD rats.

Table 1: Sequences of primers used in RT- PCR analysis

Gene	Forward Primer (5'- 3')	Reverse Primer (5'- 3')
PPAR- γ	CTGTTATGGGTGAAACTCTGGGAG	ATAGGCAGTGCATCAGCGAA
PPAR- α	CCCTGAACATCGAGTGTCTGA	CTTGCCAGAGATTTGAGGTCTCT
aP2	AGATTGAAGCTGCCAGGGCTTAT	TCAGGAGGGTCTCATAGAA
ACC	CCTCCGTCAGCTCAGATACA	TTTACTAGGTGCAAGCCAGACA
GAPDH	AAGCTGTGGCGTGATGGCCG	TGGGCCCTCAGATGCCTGCT

Figure 1: The effects of Cinnamaldehyde on mRNA gene expression in white adipose tissue (WAT). Representative bands (a) and relative changes (b) of PPAR-γ, ACC, aP2, PPAR-α mRNA expression by RT- PCR



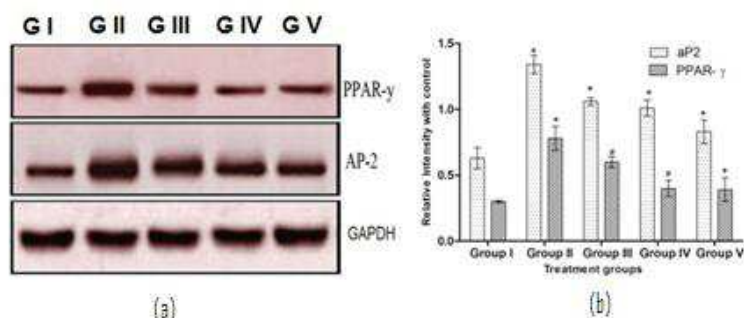
Values are mean±SD (n=3). Statistical significance: p < 0.001\*. Group II compared with Group I and Group III, IV, V compared with Group II.

**Protein Expression**

We confirmed in western blot analysis, whether CA and OR activates AMPK via phosphorylation in the adipose tissue. As shown in Fig 3, CA treated rats had significantly higher protein expression levels of phosphorylated AMPK and ACC. AMPK plays a key role in regulating carbohydrate and lipid metabolism and a potential medicinal target for treatment of metabolic diseases. The activation of AMPK leads to raised fatty acid oxidation and ketogenesis along with the simultaneous inhibition of TG, lipogenesis, cholesterol synthesis and glucose production [25]. As shown in Fig 2. PPAR-γ, aP2 were down regulated in CA and OR treated rats when compared to those in HFD fed rats in a dose dependent manner. Treatment with CA and OR stimulate the phosphorylation of AMPK and leads to inhibition of ACC. ACC is highly expressed in lipogenic tissues and is regulated at the transcriptional and translational levels [26]. These results suggest that CA may prevent the development of obesity and hyperlipidaemia

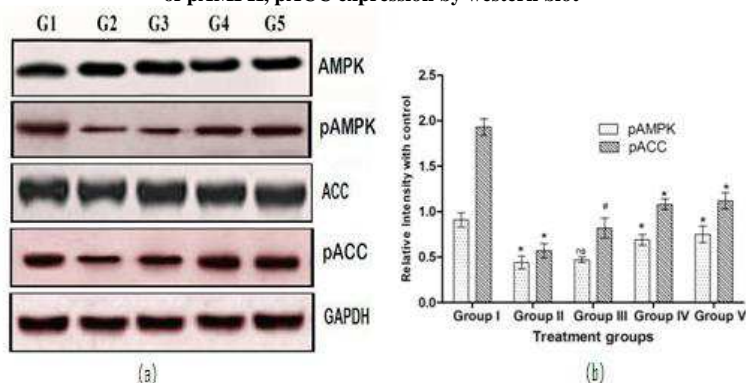
in HFD induced rats by down regulating lipogenic genes and promotion of fatty acid oxidation or lipolysis related genes in WAT.

**Figure 2: Effect of Cinnamaldehyde on protein expression in the white adipose tissue. Representative bands (a) and relative changes (b) of PPAR-γ, aP2 expression by western blot**



Values are mean±SD (n=3). Statistical significance:  $p < 0.001^*$ ,  $0.01^\#$ . Group II compared with Group I and Group III, IV, V compared with Group II.

**Figure 3: Effects of Cinnamaldehyde on protein expression in the white adipose tissue. Representative bands (a) and relative changes (b) of pAMPK, pACC expression by western blot**

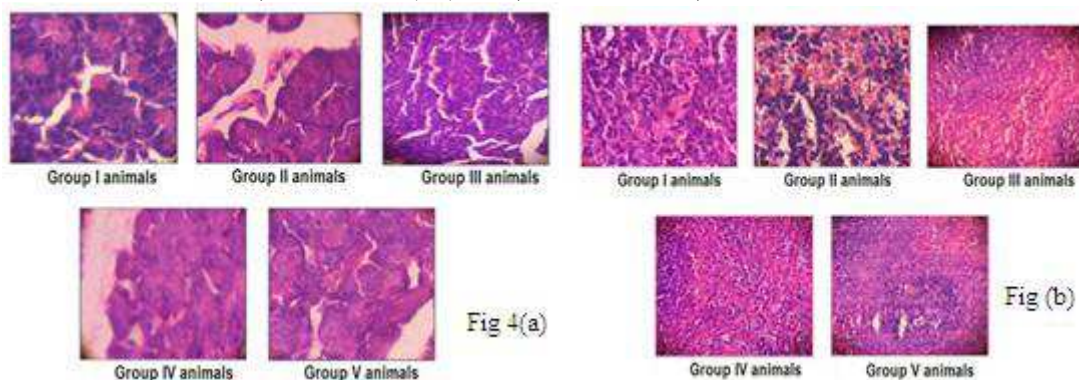


Values are mean±SD (n=3). Statistical significance:  $p < 0.001^*$ ,  $0.01^\#$ , ns-Non significant. Group II compared with Group I and Group III, IV, V compared with Group II

**Morphological Changes in Pancreas and Spleen**

Throughout the experiment, weight of pancreas was higher in the HFD induced group of rats than that in the normal group of rats. HFD induced group of rats had normal acini surrounded by increased fatty tissue. G III and G IV animals showed normal pancreatic acini with peripancreatic increase in fatty tissue Fig 4(a). The treatment group (GV) of rats shows normal acini similar to that of normal group of rats. Spleen histological analysis revealed no significant differences between all the groups, as shown in Fig 4 (b).

**Fig 4: Effects of CA on pancreas (a) and spleen (b) morphology. ND, normal diet group- GI; High fat diet (HFD) induced obese group-II; HFD+ Orlistat (OR) - GIII; HFD+CA 40- GIV; HFD+CA 80- GV**



**DISCUSSION**

Our previous study showed that CA administration leads to weight loss; reduce insulin resistance and hypercholesterolemia [27]. Obesity is a well recognized risk factor for type 2 diabetes, especially when combined with other known metabolic disorders and reduction of the metabolic disorders through weight management has

been an important therapeutic goal. A variety of metabolic disorders are highly associated with insulin resistance which is caused by genetic and environmental factors including obesity and physical fitness [28].

In adipose tissue AMPK regulates lipogenesis and lipolysis, decreases the fatty acid uptake and triglyceride synthesis and increases the fatty acid oxidation. AMPK is a heterotrimeric serine/ threonine kinase that is widely expressed in a variety of organs, including liver, brain, skeletal muscle and adipose tissue. Many adipocyte derived hormones such as leptin, adiponectin, reduce fat mass via activation of AMPK in adipocytes [29]. In the present study, we investigated the protective effects of CA against HFD induced rats and found that CA prevented HFD induced obesity via AMPK activation. One of the classical therapeutic targets of the system is ACC, which catalyze the key regulatory steps in fatty acid synthesis. In the present study, higher levels of phosphorylated AMPK and ACC were seen in CA and OR treated rats as compared with HFD fed rats. This result may elucidate the mechanism by which CA promotes fatty acid oxidation, enhancing insulin sensitivity and inhibiting TG accumulation in a dose dependent manner. CA and OR resulted in a significant suppression of PPAR- $\gamma$  expression. PPAR- $\gamma$  mainly found in Adipose tissue, are key adipogenesis and lipogenesis transcription factors [30]. Among the PPAR isomers  $\alpha$ ,  $\beta$  and  $\gamma$ , PPAR- $\gamma$  is the major transcription factor linked to adipocyte differentiation and is found almost abundant in adipose tissue. PPAR- $\gamma$  plays a crucial role in the manifestation of the mature adipose phenotype. PPAR- $\gamma$  is also expressed in low levels in liver. Modulations of AMPK and PPAR- $\gamma$  signalling might be responsible for the recovery of obesity with CA. Based on these results, we conclude that CA stimulated fatty acid  $\beta$ -oxidation via AMPK activation and ACC inactivation, and increased the expression of lipolysis related gene (PPAR-  $\alpha$ ). Stimulation of PPAR- $\alpha$  promotes uptake, utilization, and catabolism of fatty acids by up regulation of genes involved in fatty acid transport, fatty binding and activation, peroxisomal and mitochondrial fatty acid  $\beta$ -oxidation [31].

aP2 (adipocyte protein 2) is a carrier protein for fatty acids that is primarily expressed in adipocyte and macrophages. aP2 is also called fatty acid binding protein 4 (FABP4). Blocking this FABP4 protein either through genetic drugs has been the possibility of treating heart disease, diabetes, asthma, obesity, and fatty liver disease [32]. CA and OR treatment in HFD fed rats significantly down regulated the mRNA levels of lipogenesis related genes such as PPAR- $\gamma$ , aP2, ACC in the white adipose tissue and enhanced the expression of PPAR- $\alpha$ .

## CONCLUSION

Our results clearly implicate that CA is a potentially safe and effective plant derived compound for the treatment of obesity via the stimulation of AMPK activation in the HFD induced obesity rats. CA treatment also stimulates the expression of PPAR- $\alpha$  in WAT. Simultaneous treatment of CA in HFD fed rats; the expression patterns of PPAR- $\gamma$ , ACC, aP2 were down regulated as similar to that normal group of rats. Thus, these findings indicate that CA acts as a potent anti-obesity agent.

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