

# Bioactive Compound Bisacurone in the Turmeric Extract (Turcuron) Prevents Non-alcoholic Fatty Liver Disease by Reduction of Lipogenesis

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## ABSTRACT

**Objectives:** The current study was carried out to demonstrate potential role of Turcuron, a standardized turmeric extract containing 8% Bisacurone to protect against non-alcoholic fatty liver disease (NAFLD). **Methods:** HepG2 cell lines exposed to oleic acid (OA) and high-fat diet-induced hepatic steatosis in mice were used as experimental models to explore the hepatoprotective effects of Turcuron. The cytotoxicity was quantitatively assessed by MTT assay. Lipid accumulation and intracellular triglyceride level were evaluated by oil red O staining. Expressions of SREBP-1c and PPAR- $\gamma$  proteins in the HepG2 cells and in HFD fed mice liver were examined by western blot. **Results:** Turcuron suppressed fatty acid synthesis and subsided the oleic acid-induced lipid accumulation in HepG2 cells. Moreover, Turcuron also decreased lipid accumulation in the liver of HFD-fed mice compared with the control group. The expression of lipogenic genes involved in hepatic lipid metabolism SREBP-1c and PPAR- $\gamma$  were decreased both in the HepG2 cells and in the liver of Turcuron supplemented mice. These results suggest that Turcuron may actively ameliorate non-alcoholic fatty liver disease. **Conclusion:** The findings of the present study provide corroborative scientific evidence that Turcuron may actively ameliorate non-alcoholic fatty liver disease.

**Key words:** NAFLD, Bisacurone, SREBP-1, Oil- O red, HFD.

## INTRODUCTION

Suffering from chronic liver disease is a considerable cause of morbidity and mortality around the world.<sup>1</sup> In recent years Non-alcoholic Fatty Liver Disease (NAFLD) has been considered as most important liver disease characterized by accumulation of fat and triglyceride in the liver and cytoplasm of hepatocytes more than 5-10% by weight.<sup>2</sup> The accumulation of lipid-derived metabolites that can activate inflammatory and ROS generating pathways can cause lipotoxicity. Besides, it has been reported that oxidative stress, mitochondrial upset, modulations in lipid metabolism and inflammation could be closely connected with the advancement of NAFLD.<sup>3-5</sup> NAFLD depicts measure of damage ranging from hepatocellular

steatosis leads to myriad complications like steatohepatitis, fibrosis and irrevocable cirrhosis leads to hepatocellular carcinoma. NAFLD is commonly connected with diseases like cardiovascular, obesity and type 2 diabetes disease and metabolic syndrome. Recent investigations have demonstrated that prevalence of some form of NAFLD is up to 2.8-30% in general population, whereas patients with obesity and diabetes mellitus have up to 75% of NAFLD.<sup>6-8</sup>

Currently there is no medically affirmed treatment for NAFLD, NAFLD is considered reversible through altering lifestyle by enhanced physical activity and weight loss. However, in practice this is difficult approach to achieve for most of the patients. Therefore, look

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for safe nutraceuticals with high prophylactic and/or therapeutic efficacies have been received mounting interest and numerous types of these agents have been recommended for the medication of NAFLD/ NASH.<sup>9</sup> Recently, several studies have showed that polyphenols rich berries, can offer benefits in ameliorating and avoiding metabolic syndrome related alterations in obesity and lipid ailments.<sup>10-12</sup>

Turmeric (*Curcuma longa*) is a perennial herb which belongs to the family (*Zingiberaceae*) has been conventionally used as a family treatment for a variety of diseases. There are several active ingredients such as curcumin, bisdemethoxycurcumin and demethoxycurcumin, phenolic acids and polysaccharides have been present in *C. longa*,<sup>13,14</sup> and several pharmacological activities have been reported about this genus for instance antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, anticancer, hepatoprotective, hypoglycaemic properties and are mainly attributed to the presence of curcumin.<sup>15-21</sup> Bisacurone is one of the biologically active compounds of *C. longa* reportedly. There have been several studies that have investigated the effectiveness of bisacurone.<sup>22-24</sup> In the current research work, we designed to evaluate the suppression effect of Turcuron (turmeric extract standardized to 8 % bisacurone) on steatosis, by using *in vitro* hepatic cell steatosis model and high fat diet-fed NAFLD induced model mice. Further, we have attempted to reveal the basic mechanism.

## MATERIALS AND METHODS

### Chemical and reagents

All the chemicals and reagents were purchased from Sigma Aldrich, Himedia, SRL India and USA. Antibodies have been purchased from Santa Cruz Biotechnology, USA.

### Sample preparation

Turcuron a standardized extract containing 8.0% Bisacurone from turmeric rhizome (Figure S1) was obtained from the Department of Phytochemistry, Vidya Herbs Pvt. Ltd. (Bangalore, Karnataka, India) and dissolved in 0.1% DMSO at the appropriate concentrations.

### Cell culture

HepG2 cell lines obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) added with fetal bovine serum (10%)

penicillin/ streptomycin (1%) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>

### Cell viability

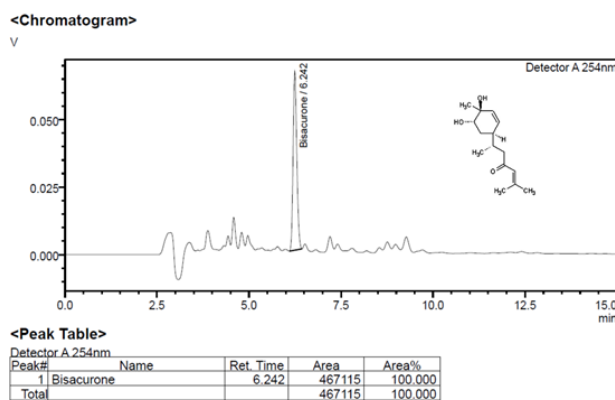
Effects of Turcuron and oleic acid (OA) on cell viability was determined by a colorimetric assay that used 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) as described previously.<sup>25</sup> In Brief, HepG2 cells (2.5×10<sup>3</sup> cells/well) were incubated in 96- well microplate with varying concentrations of Turcuron (50, 100, 150, 200, 300 and 400 µg/mL) and OA (0.1-5.0µM) for 24 h. By the end of the incubation, the cell culture medium was decanted and 100µl of fresh medium containing 5mg/ mL MTT was added to the cells and kept it for 4h incubation at 37°C. Later, 100µL of dimethyl sulphoxide (DMSO) was dispensed and allowed to stand at room temperature for 10 min for colour formation. The O.D was read at 570 nm using microplate reader. Cell viability was expressed as percentage of decrease in cell number compared to control (non-treated) cells.

### Induction of steatosis in HepG2 cells by OA

HepG2 cells (0.8×10<sup>5</sup> cells/well ) were grown in a 6-well plate, after 24 h cells were incubated with and without Turcuron (150 and 250µg/mL) in the presence or absence of 2.5mM OA and incubated an additional 24h. In the end, the intracellular lipid accumulation was observed by staining with oil red O. Furthermore, lipid content was determined by addition of 100µL of isopropanol (100%) to each well and agitated for 10 min at room temperature. Finally, samples were measured spectrophotometrically at 510 nm.<sup>26</sup>

### Oil red O staining

To carry out Oil red o staining, the cells were washed 2-3times with physiological saline and fixed in 4% para



**Supplementary Figure S1: LCMS chromatogram and chemical structure of bisacurone claimed to contain in the turmeric extract.**

formaldehyde for 10 min. subsequently plates were washed additionally 2-3 times with saline. Finally, the cells were stained using 0.5% Oil Red O in isopropanol for 30 min, further cells were washed properly with distilled water to remove the unbound dye for optical microscopic observation.

### Western blot analysis

To examine the effects of Turcuron on OA induced steatosis, the total proteins of HepG2 cells and liver tissue homogenates were extracted using lysis buffer followed by centrifugation at 10,000 rpm for 30 min at 4°C. The concentration of protein were determined by Bradford assay. The proteins of equal amount were resolved on 10% polyacrylamide gels and transferred onto PVDF membrane, subsequently the membranes were blocked using 5% skimmed milk solution and then incubated with primary antibodies at 4°C for overnight. The blots were washed with TBST and TBS buffer for three times. Then blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody following incubation membranes were washed again two times with TBST and TBS buffer. The transferred proteins were pictured with a chemiluminescence system.

### Experimental animals

Male C57Bl/6 mice (12-week-old) were purchased from Biogen, Bengaluru, India (Reg No. 971/PO/RcBiBt/S/2006/CPCSEA). The animals were housed in temperature (22±3°C) and humidity (30-70 %) controlled rooms with 12h light/dark cycle. For seven days of acclimatization period, all mice were maintained on commercial diet and tap water *ad libitum*. The experimental protocol has been approved by the Institutional Animal Ethics Committee of Vidya Herbs Pvt. Ltd. Bangalore. India. With the protocol number VHPL/PCL/IAEC/07/19.

### Experimental design

12-week-old C57Bl/6 male mice were randomized as follows: Group 1 mice fed a normal diet were maintained as normal control. Group 2-4 were fed a high fat diet (D12451 Research Diet, USA) for 14 weeks. From week 9 to till the end of week 14 (total 6 weeks), mice in (group 3 and 4) were administered (o.p) (with Turcuron 100 mg/kg and 200 mg/kg b.w. respectively. Turcuron was prepared in 10 % Tween 20 and administered to the respective group of mice through oral gavage. The daily feed consumption was monitored. Weekly changes in the body weight were recorded. At the end of experimental period, all the

mice were euthanised. Blood was collected from each animal by cardiac puncture and the serum separated for analysis of cholesterol, triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) using a biochemical analyser (Randox RX Imola, Co Antrim, UK). Liver was excised, weighed and homogenised in tissue lysis buffer for further analysis.

### Histopathological examination

The liver histological changes were examined using hematoxylin and eosin (HE) staining and Oil red O staining. Briefly, liver tissue was fixed in formalin (4%), dehydrated with ethanol, paraffin-embedded subsequently sliced into 4 µm width. The slides were stained with H&E and Oil red O and observed for liver histomorphology using an inverted microscope.

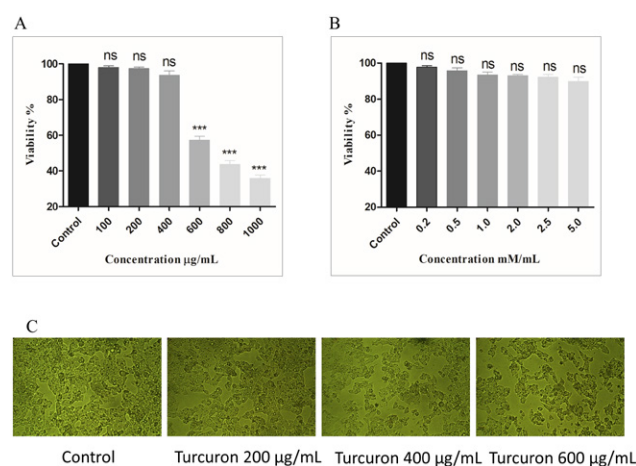
### Statistical analysis

One-way analysis of variance (ANOVA) as well as Tukey's test were used to analyse the data. Using GraphPad Prism (Version 5.0). Data are expressed as mean ± standard deviation.

## RESULTS

### Effect of Oleic acid (OA) and Turcuron on viability of HepG2 cells

To assess the effects of OA and Turcuron on viability of HepG2 cells, MTT assay has been carried out. As demonstrated in Figure 1(A), the data suggest that concentrations of 0.1 -0.4mg/mL of Turcuron did not have any adverse effect on viability of HepG2 cells.



**Figure 1: Cell viability effect of Turcuron in HepG2 cells. (A) HepG2 cells were treated with various concentration of Turcuron (100-1000µg/mL) and (B) HepG2 cells were stimulated with different concentration of oleic acid (0.2-5.0 mM) for 24 h, cell viability was determined by MTT assay. (C) Bright field microscopy image of Turcuron untreated and Turcuron treated HepG2 cells.**

Turcuron treatment with 0.6mg/ml for 24h resulted in a significant inhibition of cell growth. On the other hand, HepG2 cells were incubated with 0.2–5 $\mu$ M (OA) for 24h to induce lipid accumulation, yet this action did not result in cytotoxicity (Figure 1B).

### Effect of Turcuron on lipid accumulation in OA induced hepatic steatosis

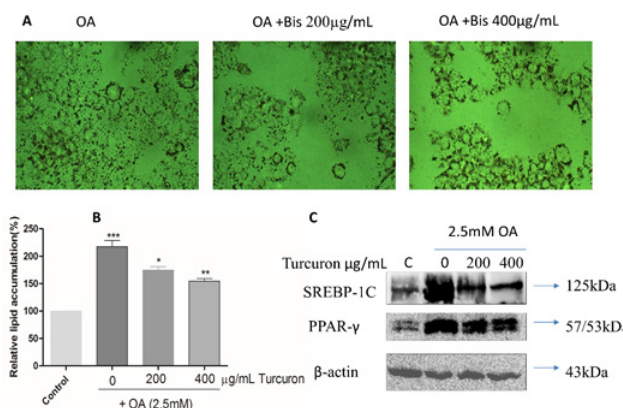
OA was employed to stimulate accumulation of lipids subsequently cells were treated with Turcuron for 24h. Lipid accumulation was estimated by Oil Red O staining. Oleic acid could induce lipid accumulation in HepG2 cell lines and these cells were incubated with isopropanol to release oil droplets. In our study, interestingly we observed that Turcuron treatment significantly ( $P < 0.05$ ) reduced lipid accumulation (Figure 2A and B).

### Turcuron Inhibits the Lipogenic protein Expression

As shown in Figure 2C. Increased levels of lipogenic factors SREBP-1C and PPAR- $\gamma$  have been associated with Oleic acid acid stimulation. Our western blot results clearly demonstrated that the treatment with Turcuron resulted in the significant decrease of SREBP1C and PPAR- $\gamma$ , the expression of SREBP1C and PPAR- $\gamma$  were detected by western blot.

### Turcuron mitigates body weight gain in HFD-fed Mice

The HFD-fed mice showed significantly higher body weight throughout the treatment period ( $p < 0.001$ ).



**Figure 2: Effects of Turcuron on OA induced lipid accumulation in HepG2 cells. (A)** Lipid accumulation was measured by staining with Oil-red-O (B) Lipid accumulation was quantified by measuring the optical density of the extracted dye at 510 nm. Results are the mean  $\pm$  SEM from three independent experiments. \*  $p < 0.05$  compared with the OA group (C) HepG2 cells were treated with 2.5 mM oleic acid (OA) for 24 h to induce lipid accumulation, followed by Turcuron treatment (200 and 400 $\mu$ g/mL) for 24 h, transcription factors associated with lipogenesis were detected by western blot.

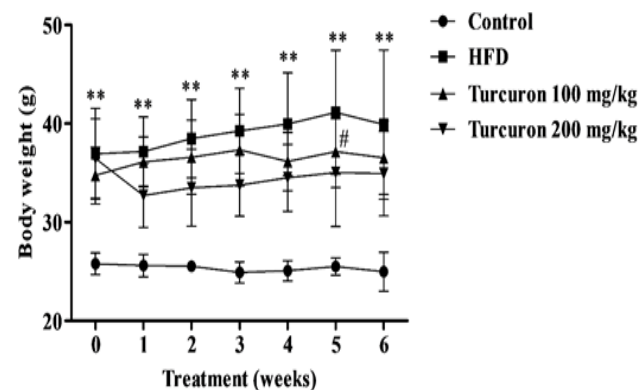
Turcuron treatment did not significantly alter the body weight of HFD mice. The body weight changes in mice during the treatment is as shown in Figure 3.

### Turcuron ameliorates the serum levels of biochemical markers in HFD-Fed Mice

The influence of Turcuron on liver function parameters in HFD-fed mice are presented in Figure 4. HFD-fed mice showed a significantly elevated levels of serum TC, AST, ALT and ALP compared to control ( $p < 0.05$ ). Turcuron treatment dose dependently reduced the serum levels of these biochemical markers of NAFLD. The improvement in serum biochemical measures was significant at 200 mg/kg dose of Turcuron. Serum TG level was considerably elevated in HFD group compared to control animals. Turcuron administration to HFD mice decreased the TG level, however the not significant.

### Turcuron reduces liver weight and hepatic steatosis in HFD-Fed Mice

The HFD-fed mice showed considerable increase in the liver weight compared to control animals ( $p < 0.05$ ). 200 mg/kg (b.w) Turcuron treatment significantly reduced the liver weight of HFD mice (Figure 5A). The effect of Turcuron on liver weight was observed to be dose dependent. Hepatic steatosis is characterised by excess lipid accumulation in the form of lipid droplets (macrovesicular or microvesicular) in hepatocytes. We examined the intrahepatic TG content to determine whether Turcuron affected HFD-stimulated liver steatosis, intrahepatic Tryglyceride (TG) content elevated in HFD mice in comparison to the normal group. However, Turcuron restored the TG contents to normal levels in mice as compared to HFD-fed mice (Figure 5B). Further, the semi-quantitative evaluation of



**Figure 3: Effect of Turcuron on the body weight during the treatment. The values are expressed as mean $\pm$ SD ( $n=6$ ). The data were analysed by one-way ANOVA followed by Tukey's test. \*\* $p < 0.01$  vs. control group, # $p < 0.05$  vs. HFD group.**

histopathological abnormalities associated with NAFLD revealed that Turcuron significantly reduced the steatosis and ballooning ( $p < 0.01$ ) in HFD mice. There was also a considerable decrease in the lobular inflammation after Turcuron treatment. Overall Turcuron significantly reduced the NAFLD activity score in the liver of HFD-mice (Figure 5C).

### Turcuron reduces the hepatic expression of lipogenic and inflammatory protein in HFD model mice

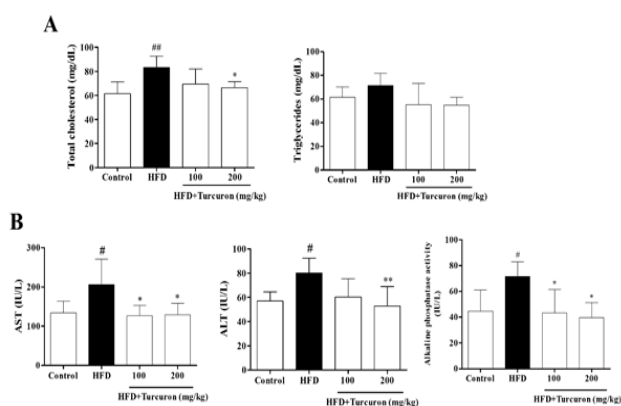
We have carried out western blot analysis to investigate the expression of transcription factors that play key role in hepatic lipid accumulation. HFD-fed mice showed up regulation of PPAR $\gamma$  in comparison with control group. However, Turcuron administration markedly

decreased the expression of adipogenic proteins in HFD-mice (Figure 6A). Further, the expression of hepatic inflammatory proteins was measured by western blotting (Figure 6B). There was a noticeable increase in the levels of NF-kB, Cox-2 and TNF- $\alpha$  in HFD-fed mice compared to normal group. However, Turcuron treatment demonstrated an appreciable decrease in the hepatic expression of NF-kB, Cox-2 and TNF- $\alpha$  proteins.

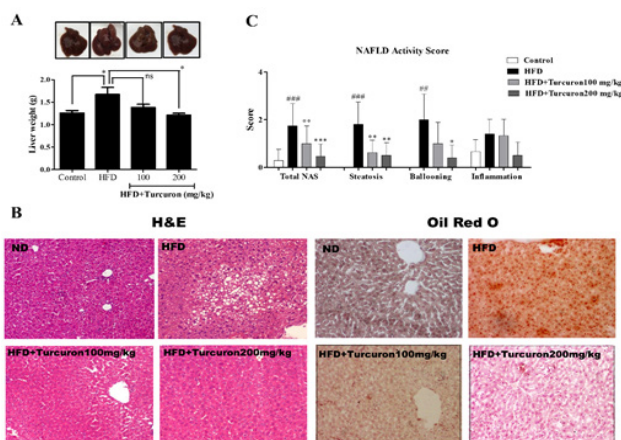
### DISCUSSION

NAFLD is a serious health complication involving steatohepatitis, steatosis, fibrosis and cirrhosis.<sup>27</sup> Several drugs have been developed to subside NAFLD. In addition, due to side effects of these drugs, research has focussed on new treatments, such as using bioactive compounds from natural resources to develop treatments for NAFLD.<sup>28,29</sup> Several researches have been reported the importance of medicinal to identify new effective compounds that could prevent hepatic lipogenesis and their possible application used in the treatment of NAFLD.<sup>30,31</sup> OA elicited accumulation of lipid in HepG2 cell line has been realistically known model for NAFLD study. As reported in earlier studies an unsaturated fatty acid, oleic acid, was used to induce steatosis in HepG2 cells<sup>32,33</sup> to investigate the therapeutic benefit of Turcuron by elucidating the underlying mechanism of action.

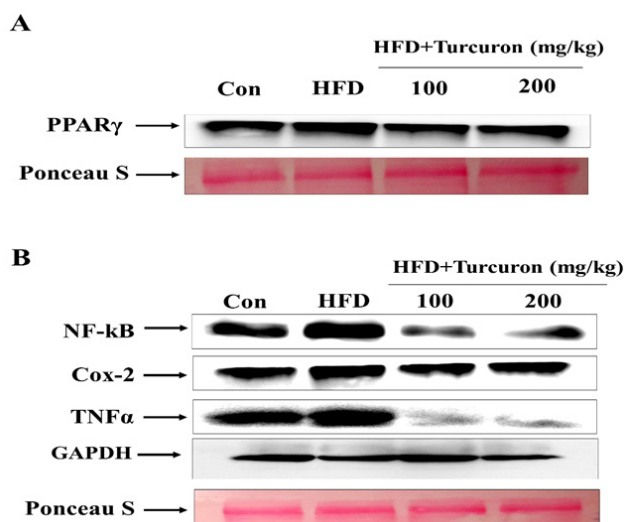
Treatment of HepG2 cells with OA results in the lipid accumulation and can be easily visualized by Oil Red O staining technique using phase contrast



**Figure 4: Effect of Turcuron on serum biochemical markers of liver function in C57Bl/6 mice. Values are mean $\pm$ SD ( $n=6$ ). # $p < 0.05$  vs. control group; \* $p < 0.05$  and \*\* $p < 0.01$  vs. HFD group.**



**Figure 5: Effect of Turcuron on liver weight and histology. (A) Mean liver weights of treatment groups, (B) Representative histopathological analysis of the liver by H&E staining and Oil red O staining (magnification: 100X), (C) Semi-quantitative evaluation of non-alcoholic fatty liver disease (NAFLD) in C57Bl/6 mice. Values are expressed as mean $\pm$ SD. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. HFD group. HFD; High fat diet.**



**Figure 6: Effect of Turcuron on hepatic expression of vital proteins in HFD-mice. Effect of Turcuron on the expression of PPAR $\gamma$  (A) and regulation of inflammatory marker proteins in the liver (B). The protein bands were detected by western blot analysis.**

microscopy. This technique is accomplished by staining of intracellular lipid droplets by Oil Red O. In addition, extraction of stain and measurement of optical density that is proportional to intracellular lipid content was performed using the colorimetric procedure. Therefore, visualization and quantification of OA-induced HepG2 cell steatosis may act as a valuable model to study the pathogenesis of NAFLD. The results of our study clearly demonstrated that non cytotoxic dose of Turcuron effectively subsided intracellular lipid accumulation in dose dependent manner. Further we examined whether the Turcuron treatment improves lipogenesis linked with NAFLD through the management of the transcription factors expressions implicated in metabolism of lipid. SREBP-1 and PPAR- $\gamma$  are important transcription factors strongly linked in the regulation of lipogenic genes and key to lipid synthesis, respectively.<sup>34</sup> These transcription factors were upregulated by oleic acid treatment. Our results clearly indicated that treatment with Turcuron on OA-induced hepatic lipogenesis in HepG2 cells inhibited TG, cholesterol synthesis and subsided accumulation of cellular lipid by inhibiting expression of SREBP-1c and PPAR- $\gamma$ , our results are consistent with previous published reports.<sup>35,36</sup>

In addition, we have carried out experiments in high fat diet induced NAFLD in mice.<sup>37</sup> The body weight of HFD-mice increased significantly compared to control group following a 14-week diet consumption. Also, there was significant increase in the liver weight due to accumulation of lipids and development of fatty liver. HFD-induced hepatosteatosis was confirmed by the histological changes in liver. A 6-week treatment with Turcuron at 100 and 200 mg/kg b.w. markedly improved the hepatosteatosis, fatty liver and lipid accumulation. This result was confirmed by the improvement in the serum levels of liver function parameters. Turcuron dose-dependently ameliorated the NAFLD in HFD-fed mice. Hepatic changes of NAFLD such as steatosis, ballooning and inflammation were pronounced in HFD-mice. Turcuron treatment significantly lowered these pathological changes in the liver, resulting in reduced NAS. Further, Turcuron attenuated the elevated levels of serum non-specific enzymes AST and ALT. Turcuron exerted its liver protective effect without having significant influence on the body weight loss. We have demonstrated the changes in the hepatic expression of PPAR $\gamma$  following Turcuron treatment in HFD-mice. High fat diet-fed mice have increased expression of PPAR $\gamma$  and develop hepatic steatosis<sup>38</sup> Further over expression of PPAR $\gamma$  led to adipogenic steatosis in mice.<sup>39</sup> In agreement with this, HFD-fed mice of present study showed a significant increase in

the PPAR $\gamma$  expression. However, Turcuron treatment reduced the protein expression to a large extent.

An increased hepatic inflammation is one of the major characteristics of NAFLD.<sup>40</sup> There exists a close link between NAFLD, insulin resistance and inflammation.<sup>41</sup> Activation of NF- $\kappa$ B, a hallmark of inflammation is associated with the progression of NAFLD. In our study, we found a marked elevation of NF- $\kappa$ B expression in HFD group compared to control mice, including a clear upregulation of Cox-2 and TNF- $\alpha$ . Turcuron treatment mitigated the hepatic inflammation by downregulating these inflammatory markers in the liver of HFD mice. Our findings are consistent with earlier reports.<sup>42</sup>

## CONCLUSION

Our findings demonstrated that Turcuron exhibited a possible preventive impression against hepatic steatosis which we have proposed was based on the mechanism of lipogenesis. Turcuron treatment could significantly restore the liver function, histopathological characteristics, improves hepatic fat accumulation and decreases pro-inflammatory cytokines. Turcuron could be a promising standardized extract to prevent NAFLD. Although additional investigation is necessary to validate further.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

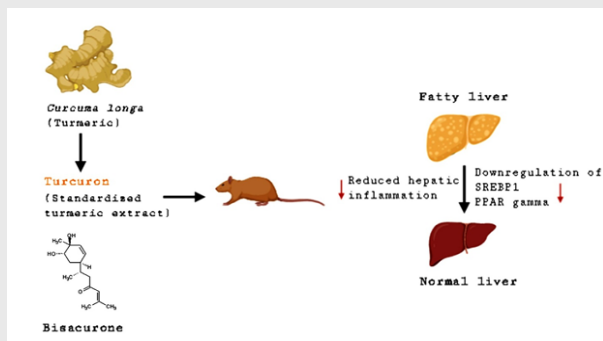
**OA:** Oleic acid; **MTT:** 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide; **HFD:** High fat diet; **NAFLD:** Non-alcoholic fatty liver disease; **PPAR $\gamma$ :** **SREBP1:** COX2; NF $\kappa$ b- $\alpha$ .

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## PICTORIAL ABSTRACT



## SUMMARY

In summary, the present study showed that Turcuron effectively decreased lipid accumulation in OA-induced steatosis with reduced expression of hepatic lipid metabolic gene in liver cells. In HFD-induced obese mice, Turcuron lessened, serum ALT and hepatic TG and gain in liver weight. Additionally, decreased levels of lipogenesis genes expression including PPAR- $\gamma$ , SREBP-1. Furthermore, Turcuron down regulated the expression levels of NF- $\kappa$ B, Cox-2 and TNF- $\alpha$  in the livers of HFD fed mice. These results are explained together, Turcuron could be efficient enough to thwart hepatic steatosis by inhibiting lipogenesis and could be a worthwhile for NAFLD treatment.

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