Research article

Evaluation of Uttroside B, a potent bioactive from *Solanum nigrum* Linn, as a candidate drug molecule against non-alcoholic fatty liver disease

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ABSTRACT

Introduction and Aim: The incidence of non-alcoholic fatty liver disease is increasing steadily across the global population. NAFLD may progress to the more serious non-alcoholic steatohepatitis (NASH), a condition that can subsequently advance to fibrosis, cirrhosis, and in many cases, to hepatocellular carcinoma (HCC). There are currently no drugs approved by the FDA for the treatment of NAFLD. We previously reported the remarkable therapeutic potency of Utt-B, a saponin isolated in our lab, from the leaves of *Solanum nigrum* Linn (*S. nigrum*), against hepatocellular carcinoma (HCC). In the current study, we have investigated the therapeutic efficacy of Utt-B against NAFLD, which eventually leads to NASH.

Materials and Methods: HepG2 cells were used for *in vitro* experiments. MTT assay, Oil Red O staining and Immunoblotting were used to evaluate the hepatoprotective and therapeutic effects of Utt-B against NAFLD.

Results: Utt-B treatment effectively reduced lipid droplet accumulation within HepG2 cells, demonstrating its potential in mitigating fat deposition associated with NAFLD. Utt-B activated AMPK signaling, leading to the down-regulation of FASN, a key enzyme regulating lipogenesis, suggesting its ability to modulate pathways involved in lipid metabolism.

Conclusion: Our results highlight Utt-B as a promising therapeutic agent for metabolic liver disorders, including NAFLD and NASH, warranting further exploration of the molecule in clinical settings.

Keywords: NAFLD; NASH; lipogenesis; AMPK; liver disease.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), also known as metabolic dysfunction-associated steatotic liver disease (MASLD), is a clinical condition characterised by excessive hepatic accumulation of fat within the liver in individuals with minimal to no alcohol consumption. This condition represents a significant health concern, highlighting the intricate interplay between metabolic factors and liver health (1).

NAFLD spans a spectrum from the less severe non-alcoholic fatty liver (NAFL) to the more critical non-alcoholic steatohepatitis (NASH), in which hepatic steatosis is accompanied by lobular inflammation and apoptosis, culminating in the heightened risks of fibrosis and cirrhosis. The global spread of unhealthy lifestyles, characterized by sedentary habits and overnutrition, is fostering an escalating prevalence of non-alcoholic fatty liver disease (NAFLD) across diverse age groups, including children and the elderly. Sedentarism and overnutrition serve as catalysts for the progression of hepatic steatosis, potentially leading to inflammatory responses, fibrosis, and other complications (2). Elevated consumption of glucose, fructose, and saturated fats can trigger hepatic de novo lipogenesis and provoke subclinical inflammation in adipose and liver tissues, consequently inducing insulin resistance in adipose tissues, liver, and skeletal muscles. Contributing further to the pathogenesis of NAFLD are, factors such as hyperglycemia, heightened pro-inflammatory cytokines, ceramide signaling, and dysregulated adipokine secretion from adipose tissues (3).

Projections from modelling studies suggest that by 2030, approximately 10% of the global population may be affected by diabetes, while nearly half of the United States population is expected to be obese (4). The prevalence of adult non-alcoholic fatty liver disease (NAFLD) in India, has been estimated to be between 6.7% and 55.1% (5). Despite the escalating numbers of individuals affected by fatty liver and its consequential impact on health and lifestyle, there is currently a lack of FDA-approved drugs for disease management. The present approach to managing NAFLD involves lifestyle modifications along with the administration of Vitamin E and anti-diabetic medications.

We have previously reported that uttroside B (Utt-B), a saponin isolated from the leaves of *Solanum nigrum* Linn (*S. nigrum*) in our lab, shows remarkable potency

against HCC, while being pharmacologically safe, under *in vivo* conditions (6-8). This innovation has secured patent recognition from the USA (US20190160088), Canada (3,026,426), Japan (JP2019520425), South Korea (KR1020190008323), and Europe (EP3463382). Moreover, Utt-B received the 'orphan drug' designation from the US FDA, against liver cancer. *S. nigrum*, has been extensively used in ayurveda and naturopathy as an immunomodulator and for curing various liver diseases (9, 10). These factors inspired us to explore whether Utt-B exhibits therapeutic efficacy against NASH.

In the current study we have investigated whether Utt-B, besides being an excellent anti-HCC agent, can be effectively used in therapeutic intervention against NAFLD, using an in vitro lipogenesis model. Specifically, we have attempted to assess whether Utt-B can manifest a hypolipidemic effect, contributing to the reduction of hepatic fat accumulation in the in vitro lipogenesis model system.

MATERIALS AND METHODS

Chemicals

Isolation and purification of Utt-B were done as reported previously (6). Important cell culture reagents such as Foetal Bovine Serum (GIBCO; 10270-106), Dulbecco's Modified Eagle Medium (DMEM; GIBCO, 12800-017), and streptomycin sulfate (GIBCO, 11860-038) were purchased from Invitrogen Corporation (Grand Island, NE, USA). MTT reagent (D0801) was obtained from TCI Chemicals (India) Pvt. Ltd. (Chennai, India). Antibodies against Vinculin (9508S), phospho-AMPKα (2531S) and FASN (3180) were obtained from Cell Signaling Technologies (CST; Beverly, MA, USA). Antibody against AMPKα (74461) was obtained from Santa Cruz Biotechnology (SC; Santa Cruz, CA, USA). All other chemicals were procured from Sigma Chemicals (St. Louis, MO, USA), unless otherwise mentioned.

Cell culture

The liver cancer cell line, HepG2 was procured from ATCC and cultured in DMEM supplemented with 10% Foetal Bovine serum.

Preparation of free fatty acid mixture

Free fatty acid (FFA) mixture was prepared as described previously (11). 100 mM of palmitate was prepared by dissolving 25.64mg of palmitic acid in 0.1M NaOH at 70°C. Simultaneously, a 10% BSA solution was prepared by dissolving FFA-free BSA in DMEM medium, at 55°C, in an adjacent water bath. The FFA mixture was added to the 10% FFA-free BSA solution at 55°C, and this was vortexed vigorously for 10s followed by an incubation of 10 min at 55°C. The FFA/BSA solution was cooled to room temperature and subjected to sterilization through filtration using a syringe filter with a pore size of 0.45 μm. The solution was stored at -20°C.

Oil red O staining

Oil Red O (ORO) staining of cells was performed as described previously, but with slight modifications (12). At 24h post palmitic acid (PA)-treatment, HepG2cells were rinsed thrice with PBS and subsequently fixed with 4% paraformaldehyde for duration of 30 min. Following fixation, the cells were washed twice with iced PBS and then treated with 0.5% ORO for 15 min. The cells were then rinsed several times with PBS, following which, the stained cytoplasmic neutral lipids were visualized and imaged under DMi8 Inverted Fluorescence Research Microscope with DMC 2900 Digital Camera.

In vitro assays

MTT assay and Immunoblotting were performed as previously described (13).

Statistical analysis

Data analysis was performed using GraphPad Prism software Version 8 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was assessed using one-way ANOVA. $p < 0.05$ was considered as statistically significant. The error bars represent ± SD and are indicative of variability observed across three independent experiments.

RESULTS

As our previous studies revealed a superior therapeutic index for Utt-B, compared to cancer cells of other origins (6), we hypothesized that the compound may be regulating lipid metabolism, which directly correlates with the proliferation of liver cancer cells. To verify this, we manipulated a predominant condition of lipid deposition associated with non-alcoholic fatty liver disease (NAFLD) using an in vitro model, in which HepG2 cells were subjected to varying concentrations of the saturated free fatty acid, palmitic acid (PA). The deposition of lipid droplets within the cells was assessed through Oil Red O staining, which revealed maximal deposition in cells treated with PA at a concentration of 3mM. However, treatment with 3mM PA resulted in severe toxicity towards HepG2 cells and hence could not be selected for further studies (Fig. 1).

In order to determine the hypolipidemic effect of Utt-B *in vitro*, it was important to identify concentrations of PA and Utt-B that exert minimal cytotoxicity on HepG2 cells. Hence, we checked the cytotoxicity imparted by different combinations of varying concentrations of PA and Utt-B and observed a dose-dependent increase in cytotoxicity with an increase in the concentration of PA while treatment with 100 nM of Utt-B did not alter the cytotoxicity induced by PA (Fig 2 A-D). Cell viability assay indicated that over 70% of the cells remain viable on treatment with

0.1mM PA and that co-treatment with 100 nM Utt-B does not alter the viability of PA-treated HepG2 cells (Fig 2E). Hence, we selected the combination of 0.1mM PA and 100nM Utt-B for further studies.

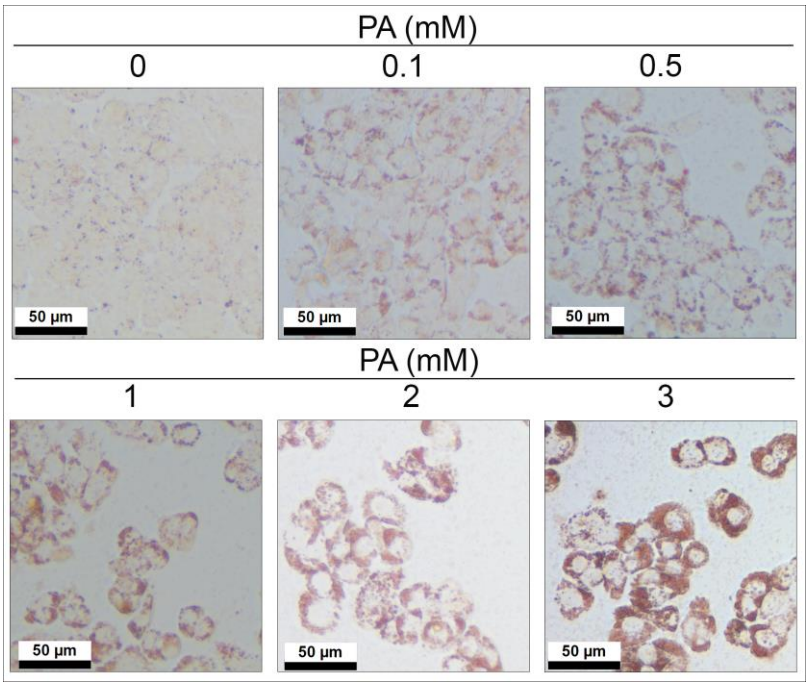

Fig. 1: PA induces a dose-dependent accumulation of lipid droplets in HepG2 cells: HepG2 cells were subjected to treatment with increasing concentrations of PA to induce lipid accumulation

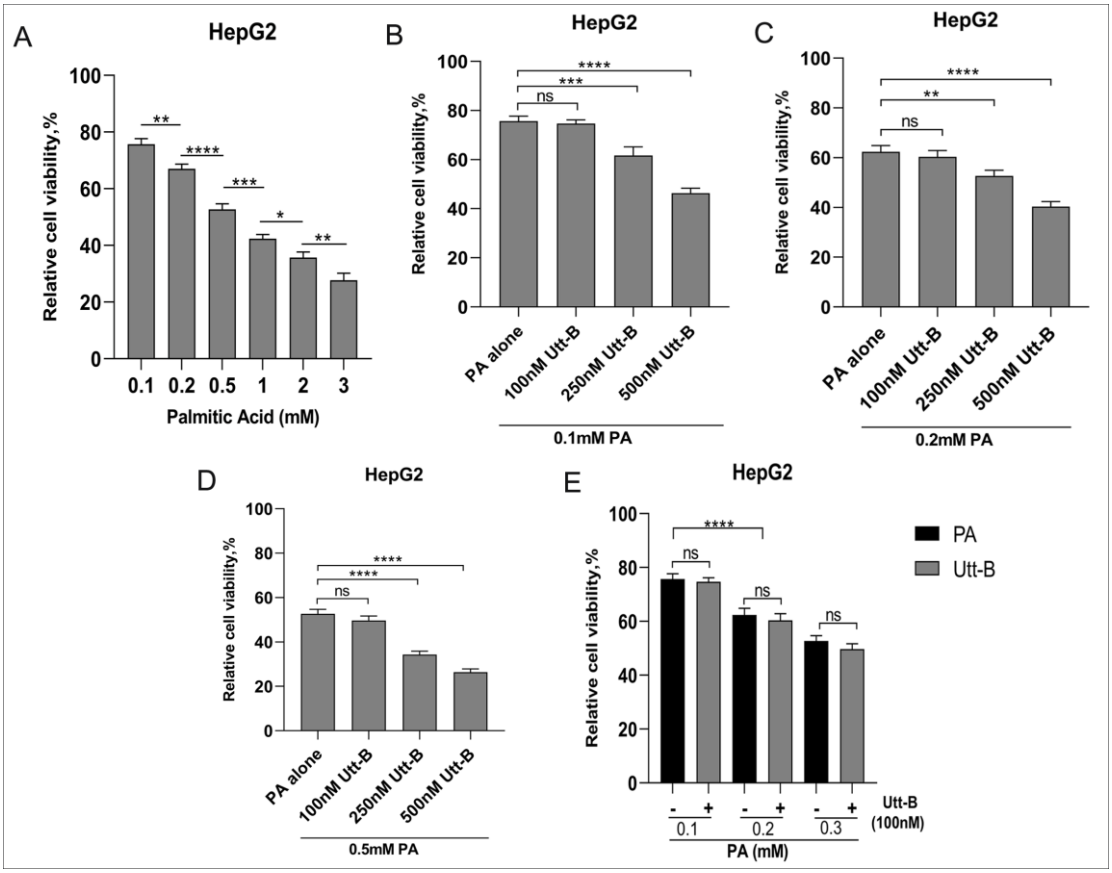

Fig. 2: Cytotoxicity assay of HepG2 cells treated with PA and Utt-B: PA treatment induces dose-dependent cytotoxicity in HepG2 cells **(A)**; HepG2 cells treated with varying concentrations of Utt-B and 0.1mM PA **(B)**, 0.2mM PA **(C)** and 0.5mM PA **(D);** Treatment with 100nM Utt-B does not alter to cytotoxicity induced by PA in HepG2 cells **(E);** One-way Anova was used for statistical analysis, ****p≤0.0001.

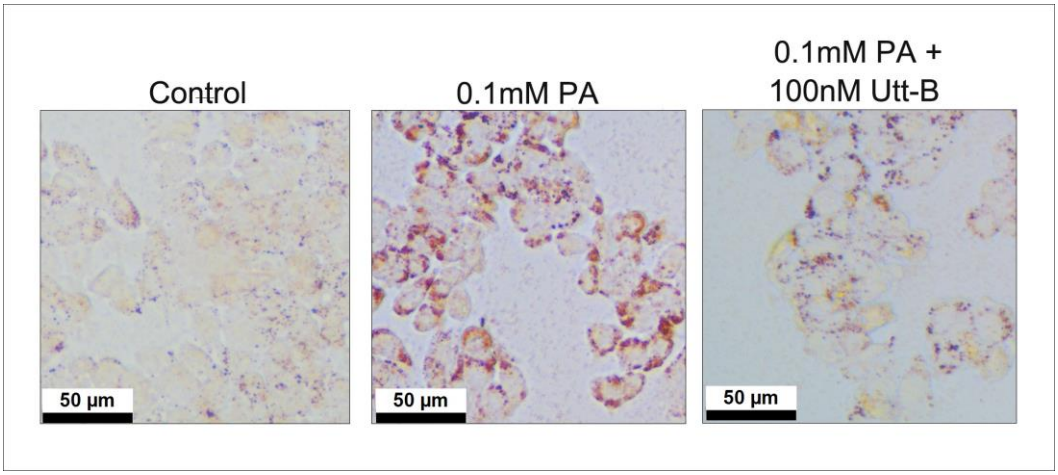

Fig. 3: Utt-B exhibits hypolipidemic effect *in vitro*: Utt-B reduces the accumulation of lipid droplets in HepG2 cells treated with PA

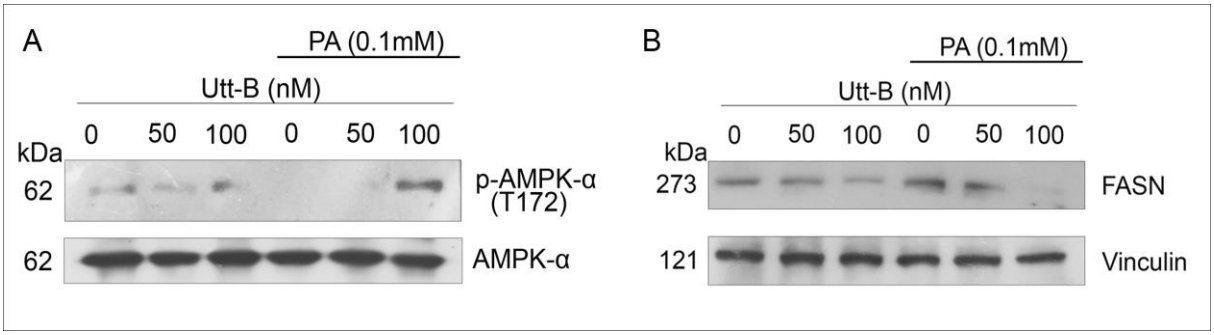

Fig. 4: Utt-B modulates AMPK signalling to regulate lipogenesis: Utt-B induces the phosphorylation of AMPK-α **(A)** and down-regulates the expression of fatty acid synthase in HepG2 cells treated with PA **(B).**

To evaluate the potential of Utt-B in mitigating lipid droplet deposition, HepG2 cells were co-treated with 0.1mM PA and 100nM Utt-B. This co-treatment led to a significant reduction in lipid droplet staining within the cells, indicative of the potential hypolipidemic effect of Utt-B, *in vitro* (Fig. 3).

Most metabolic disorders, including non-alcoholic fatty liver disease (NAFLD), are intricately linked to dysregulations in the phosphorylation of AMPK-α (7). Hence, we investigated whether exposure of 0.1mM PA, the concentration we selected for our combinatorial studies with Utt-B, to HepG2 cells can result in a reduction in the phosphorylation levels of AMPK-α, mimicking a condition aligning with the characteristic patterns observed in various metabolic disorders. Interestingly, treatment with 0.1mM PA completely abolished the phospho-AMPK-α in HepG2 cells, while Utt-B reinstated the phospho-AMPK-α at T172, in PA-treated HepG2 cells (Fig 4A), indicating its efficacy to protect against metabolic disorders.

Next, we checked whether the activation of AMPK-α through Utt-B-induced phosphorylation exerts downstream effects on lipogenesis. For this, we examined the expression levels of fatty acid synthase (FASN), a pivotal enzyme governing the rate-limiting step of lipogenesis, in cells treated with different concentrations of Utt-B and PA, either alone or in combination. We found that treatment with PA up-regulates the expression of FASN, while Utt-B down-regulates the enzyme, which leads to reduced deposition of lipids. Interestingly, while PA-treatment led to an up-regulation in the expression of FASN, treatment with Utt-B exhibited a dose-dependent down-regulation in the PA-induced upregulation of FASN, which highly (Fig 4B) correlates with the reduction in lipid deposition as observed in the Oil Red O staining (Fig 3). Taken together, these results strongly support our hypothesis that Utt-B may be regulating lipid metabolism in the liver and suggest modulation of lipogenesis via AMPKα phosphorylation as the potential mechanism through which Utt-B accomplishes this function.

DISCUSSION

Fatty liver disease occurring as a result of metabolic dysfunctions (non-alcoholic/metabolic dysfunction associated fatty liver disease) is characterised by the excessive accumulation of lipids within hepatocytes. While early-stage NAFLD is generally asymptomatic, it bears the potential to progress into non-alcoholic steatohepatitis (NASH) due to inflammatory processes and liver cell injuries, and further develop to cirrhosis and eventual liver failure (14). In the present study, we investigated the hypolipidemic properties of Utt-B, a phyto saponin isolated in our lab from the methanolic extract of the leaves of *Solanum nigrum* Linn (6). Our study employed an *in vitro* model of fatty liver, inducing lipid droplet deposition in liver cancer cells. Utt-B-treatment resulted in a notable reduction in lipid

droplet accumulation within the cells, underscoring its efficacy in mitigating fat deposition, a pivotal characteristic associated with NAFLD. Even though our previous studies have established the therapeutic efficacy of Utt-B against hepatocellular carcinoma (6-8), this is the first study reporting it as an effective therapeutic agent against NAFLD.

NAFLD is strongly linked to metabolic syndrome, a cluster of risk factors encompassing obesity, insulin resistance, hypoglycemia, hypertension, and abnormal triglyceride accumulation (15). Central to metabolic processes, AMPK emerges as a pivotal player, with dysregulations in its activation leading to numerous metabolic disorders. The multifaceted role of AMPK in lipid metabolism is of particular significance in this context. It exerts regulatory control over crucial processes such as lipogenesis and the *de novo* synthesis of cholesterol and triglycerides. Moreover, AMPK facilitates lipid catabolism by promoting the β-oxidation of fatty acids (16, 17).

We have previously reported that Utt-B induces autophagy by activating AMPKα through phosphorylation at T172 (7). In our current investigation, we observed that treatment with PA leads to a reduction in phosphorylation of AMPKα in HEPG2 cells, paralleling the diminished AMPK activity seen in metabolic disorders. Conversely, Utt-B treatment triggered AMPK phosphorylation, activating the kinase. This implies a potential role for Utt-B in modulating the down-stream pathways of AMPK signalling, particularly in the regulation of lipid metabolism.

AMPK activation has been reported to impede the activation of sterol regulatory element binding protein 1c (SREBP1c), a key player in the synthesis of lipogenic enzymes such as acetyl co-A carboxylase1 (ACC) and FASN. Inhibition of SREBP-1c further inhibits its downstream enzymes ACC and FASN, thereby decelerating the process of lipogenesis (18). In our study also, it was noted that in congruence with the AMPK activation, Utt-B induced a simultaneous down-regulation of FASN, the pivotal and rate-limiting enzyme involved in lipogenesis, in a dose-dependent manner, attesting the hypolipidemic effect of Utt-B.

These observations not only enhance our comprehension regarding the pharmacological profile of Utt-B but also underscore the possibility of its versatile pertinence in addressing various hepatic conditions. This positions Utt-B as a promising candidate drug for further exploration in the context of metabolic liver disorders.

CONCLUSION

As a condition marked by the aberrant accumulation of fat in the liver, NAFLD represents a precursor to the more severe non-alcoholic steatohepatitis (NASH), which, in turn, can progress to advanced fibrosis or cirrhosis with an elevated risk to develop hepatocellular carcinoma (HCC). The absence of FDA-approved drugs for the treatment of both NAFLD and NASH highlights the urgency for innovative therapeutic strategies to address these conditions. The findings of our study collectively imply that Utt-B may harbour hepatoprotective properties through the regulation of lipogenesis. This research lays the groundwork for further investigations into the development of Utt-B as a drug for managing NAFLD and NASH.

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CONFLICT OF INTEREST

The authors declare no competing interest.

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