International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 12-07-2021, Accepted: 27-07-2021, Published: 12-08-2021
Volume 6, Issue 4, 2021, Page No. 676-681

Ameliorative effect of glycyrrhetinic acid in fatty acid induced steatosis of HepG2 cells

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Abstract
Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the important lifestyle disorders found across the globe and its prevalence is growing rapidly. There is no Food and Drug Administration (FDA) approved remedy for the treatment of Non-Alcoholic Fatty Liver Disease (NAFLD) and there is growing interest on the botanicals. This study aims to explore the anti-NAFLD effect of glycyrrhetinic acid in fatty acid induced steatosis of HepG2 cells. Glycyrrhetinic acid was isolated from the stems of *Glycyrrhiza glabra* L., and its structure was confirmed using spectroscopic studies. The anti-NAFLD effect of glycyrrhetinic acid was evaluated in fatty acid induced steatotic HepG2 cells. The Nuclear Magnetic Resonance (NMR) assignments of glycyrrhetinic acid were in agreement with previously published literature. The MTT assay showed that the compound did not possess any toxicity upto 100 µM. The treatment with glycyrrhetinic acid significantly lowered intracellular lipid accumulation and the leakage of transaminases from HepG2 cells. The efficacy of glycyrrhetinic acid was comparable with that of fenofibrate, the positive control drug. Further detailed investigations on this compound might yield some useful clues for the better management of NAFLD.

Keywords: fatty liver, traditional medicine, *Glycyrrhiza glabra*, HepG2 cells

Introduction
Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the important lifestyle disorders found across the globe and its prevalence is growing rapidly with the increase of obesity, diabetes, insulin resistance (Benedict and Zhang, 2017; Kumar *et al*., 2021) [3, 34]. It affects the obese and diabetic patients easily than the normal adults; it has been reported in about 76% of patients with type 2 diabetes and about 80% among obese population (Le *et al*., 2017) [19]. NAFLD is a progressive disease, starts from simple steatosis with no clinical symptoms, through nonalcoholic steatohepatitis, fibrosis, cirrhosis, liver failure and death (Chalasani *et al*., 2012) [8]. Worldwide incidence of NAFLD was estimated to be 24-25% among the general public (Bedogni, 2005) [2]. Another meta-analysis indicated that the prevalence of NAFLD in Asia as higher (52.34 per 1000) than that of the west (28 per 1000) (Younossi *et al*., 2016) [31]. In India, the prevalence of NAFLD was estimated between 4-5% (Sarin and Maiwall, 2012; Lange *et al*., 2021) [17].

Management of NAFLD is usually done by treating the hepatic manifestations as well as the associated comorbidities like obesity and type 2 diabetes mellitus (Chalasani *et al*., 2018) [7]. To date, there is no Food and Drug Administration approved remedy for the treatment of NAFLD. Various drugs like insulin sensitizers, lipid lowering agents, etc. were repurposed for the treatment of NAFLD (Singh *et al*., 2017) [26]. Lifestyle interventions like diet modifications, improved physical activity and weight loss are generally recommended for the management of NAFLD in clinical scenario (Chalasani *et al*., 2018; Semmler *et al*., 2021) [7, 32]. There is a growing interest on the botanicals for the treatment of NAFLD; these botanicals through different pathways like antioxidants, insulin sensitizing, lipid lowering, renin-angiotensin system blocking, anti-inflammation, etc., exert anti-NAFLD effect (Xiao *et al*., 2013) [29]. Preclinical and clinical experiments indicated the anti-NAFLD effect of many phytochemicals (Bagherniya *et al*., 2018; Dai *et al*., 2021) [1, 35].

Glycyrrhetinic acid is a pentacyclic triterpenoid related to β-amyrin obtained from the hydrolysis of Glycyrrhizin, which was obtained from the herb liquorice (*Glcyrrhiza glabra* L.). *G. glabra* belongs to the family Fabaceae and it was imported into India from Persia, Asia Minor and other central Asian countries for centuries. Glycyrrhizin is the active compound found in this plant which is nearly 50 times sweeter than that of sucrose and its sweetness is perceptible even in 1:20,000 dilution (Hayashi and Sudo, 2009) [13]. It is traditionally used as flavouring and masking agent for the bitter drugs like aloe and quinine. It is effective in the treatment of peptic ulcer and also has expectorant properties (Jiang *et al*., 2020) [15]. On hydrolysis, Glycyrrhizin yielded glycyrrhetinic acid, which is the biologically active metabolite of Glycyrrhizin (Negishi *et al*., 1991) [23]. Glycyrrhetinic acid has many pharmacological properties with antiviral, antifungal, antiprotozoal, and antibacterial activities (Langer *et al*., 2016) [18]. Its anticancer (Lallemand *et al*., 2011) [16], anti-inflammatory (Finney and Somers, 1958) [11] and hepatoprotective effects (Cao *et al*., 2017; Chen *et al*., 2014; Jeong *et al*., 2002; Mahmoud and Al Dera, 2015) [6, 10, 14, 21] of glycyrrhetinic acid were highly explored. This study aims to explore the anti-NAFLD effect of glycyrrhetinic acid in fatty acid induced steatosis of HepG2 cells.

Methodology
Isolation of glycyrrhetinic acid
Stems of *Glycyrrhizaglabra*wereprocured from the local market and its botanical identity was confirmed by the taxonomist at Entomology Research Institute, Loyola College, Chennai. The plant material was cleaned thoroughly and made into a coarse powder.Isolation of glycyrrhetinic acid was done accordance with the method of

Rajpal (2011). Briefly, coarsely powdered stems (1 kg) were added with 3 L of water and boiled for one hour. The contents were filtered through Whatman #3 filter paper under reduced pressure and the process was repeated for two more times with the marc. The filtrates were combined and cooled to 4 °C; 20% sulphuric acid was slowly added with the filtrate for the precipitation of crude glycyrrhizin (65 g).Crude glycyrrhizin was refluxed in 10% hydrochloric acid for one hour; the contents were poured on ice and extracted with methylene dichloride (3 x 100 mL). The organic layer was washed with water, dried over anhydrous Na2SO4 for the yield of crude glycyrrhetinic acid (40 g). Crude glycyrrhetinic acid was packed over a silica gel flash column (Buchi) and eluted with the gradients of chloroform and methanol. Repeated crystallizations with methanol yielded pure glycyrrhetinic acid (99.0%, 28 g).

Spectrascopy

Fourier transform infrared (FT-IR) spectrum was recorded on a Perkin-Elmer grating spectrometer (Spectrum Two) in KBr disc. ^{1}H and ^{13}C NMR were taken on Bruker instrument at 500 and 125 MHz, respectively in $CDCl_3$ with TMS as the internal standard. Chemical shifts values are given in δ scale.

Bioassays

Chemicals

Dulbecco Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and Trypsin were procured from Gibco (India); Fenofibrate, MTT, ORO, sodium palmitate and sodium oleate were purchased from Sigma (India). Antibiotic mixture was purchased from Hi-Media chemicals (India). Transaminase assay kits were purchased from Agappe diagnostics (India).All other chemicals were purchased from Qualigens.

Cell culture

HepG2 cells obtained from National Center for Cell Sciences (India) were cultured in DMEM. The cells were maintained in a humidified 5% CO_2 incubator at 37 °C. The cells used for the experiments were at about 75% confluence. Dimethyl Sulfoxide (DMSO) at the concentration of 0.01% along with DMEM medium was used as vehicle control.

Fixation of dose for *in vitro* assays

The cell viability was measured by MTT assay (Borenfreund *et al*., 1988). The cells were seeded into 96 well-plates at a density of 3 x 10^4 cells/well. Then, the cells were treated with the test materials at the concentration of 12.5–200 µMfor 24 h. After 24 h, the spent media were removed; 50 µL of MTT (5 mg/mL) was added to each well, and the plates were incubated at 37 °C for 3 h. The MTT solution was removed and discarded; then 100 µL of DMSO was added to each well. The absorbance in each well was measured at 492 nm using microplate absorbance reader. The percentage viability was calculated using the following formula:

$$\text{Percent cell viability} = \frac{A\ 492\ \text{nm (test material)}}{A\ 492\ \text{nm (control)}} \times 100$$

Evaluation of anti-NAFLD effect of glycyrrhetinic acid

HepG2 cells were seeded in 24 well plates at a density of 4 x 10^4 cells/well and incubated overnight. After incubation the spent medium was exchanged with fresh medium containing palmitate-oleate with or without fenofibrate/glycyrrhetinic acid. The lipid accumulation was measured by ORO staining (Toppo *et al*., 2017) [27]. The levels of transaminases were estimated in the spent media using commercial diagnostic kits.

Statistics

All the data generated in this study were presented as mean ± SD. One-way ANOVA followed by Tukey's HSD (SPSS, version 11.5) were used to assess the statistical significance and the data were considered significant at $P \leq 0.05$.

Results and Discussion

Structural elucidation of glycyrrhetinic acid

Glycyrrhetinic acid (Figure 1) was obtained as white amorphous powder. It was analysed for $C_{30}H_{46}O_4$. IR (KBr) ν_{max}3436 (br), 2949, 2925, 2888 (lactone carbonyl), 1700 (C=O stretching), 1662, 1618 (olefinic), 1457, 1384, 1324 (C-O stretching), 1176, 1153, 1024, 984 and 916 (olefinic) cm^{-1} (Figure 2); ^{1}H NMR (δ_H $CDCl_3$, 400 MHz): 7.26 (s, 1H, COOH), 5.26 (s, 1H, H-12), 3.25 (dd, 1H, H-3), 2.81 (ddd, 1H, H-1), 2.35 (s, 1H, H-9), 2.19 (dd, 1H, H-18), 2.01 (m, 1H, H-15), 1.98 (m, 1H, H-21), 1.98 (dd, 1H, H-19), 1.82 (ddd, 1H, H-16), 1.66 (m, 1H, H-2), 1.65 (m, 1H, H-7), 1.62 (m, 1H, H-2'), 1.59 (m, 1H, H-19'), 1.45 (m, 1H, H-6), 1.42 (m, 1H, H-6'), 1.41 (m, 1H, H-7'), 1.37 (m, 1H, H-22), 1.34 (s, 3H, H-26), 1.32 (m, 1H, H-22'), 1.25 (m, 1H, H-21'), 1.22 (m, 1H, H-16'), 1.17 (s, 3H, H-24), 1.14 (s, 3H, H-27), 1.13 (s, 3H, H-6), 1.04 (m, 1H, H-15'), 0.98 (s, 3H, H-29), 0.97 (m, 1H, H-1'), 0.80 (m, 3H, H-15), 0.83 (m, 3H, H-16), 0.68 (m, 1H, H-5) (Figure 3); ^{13}C NMR (δ_H $CDCl_3$, 125 MHz): 200.28(C-11), 181.18(C-30), 169.25(C-13), 128.49 (C-12), 78.84 (C-3), 61.81(C-9), 54.94(C-5), 48.28(C-18), 45.47 (C-8), 43.79 (C-20), 43.2(C-14), 40.9(C-19), 39.14(C-1), 37.72(C-4), 37.09(C-22), 32.76 (C-10), 31.77 (C-7), 31.98 (C-17), 30.93 (C-21), 28.55(C-29), 28.45(C-23), 28.10(C-28), 27.29(C-2), 26.49(C-16), 26.41(C-15), 23.42(C-27), 18.69(C-26), 17.49 (C-6), 16.38 (C-25) and 15.59 (C-24) (Figure 4). The NMR assignments were in agreement with previously published literature (Chaturvedula *et al*., 2014) [9].

Toxicity of glycyrrhetinic acid to HepG2 cells

MTT assay has been used in many cell based testing systems for the evaluation of cell viability, proliferation and the toxicity of the test materials. Yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was converted into purple formazan crystals by the activity of NAD(P)H-dependent oxidoreductase present in live cells (Mosmann, 1983) [22]. The formation of purple formazan crystals can be correlated with the presence of more live cells in the test system and its concentration is measured by spectrophotometric method. In our study, the concentration of formazan crystals were not significantly lowered by the treatment with glycyrrhetinic acid upto 100 µM concentration (Figure 5). It indicated the lack of toxicity of glycyrrhetinic acid upto 100 µM; linear regression analysis indicated the LD_{50} value of glycyrrhetinic acid as 337.32 (R^2 - 0.761).

Effect of glycyrrhetinic acid on intracellular lipid accumulation of HepG2 cells

Various *in vitro* models for NAFLD have been reported in the literature; fatty acid induced steatosis of HepG2 cells is one of the widely used models of NAFLD. In this model also various fatty acids and their combinations were tried and their effect on intracellular lipid accumulation, lipotoxicity and viability of the cells were evaluated. These studies indicated that the administration of a saturated fatty acid (eg: palmitic acid) lowered the viability of cells, while the administration of unsaturated fatty acid (eg: oleic acid) not affected the viability but increased the steatosis in the cells (Gomez-Lechon *et al*., 2007`) [12]. Hence, the models using both palmitic and oleic acid were found superior than that of other models (Ricchi *et al*., 2009) [25]. In this study, the accumulation of fat into the HepG2 cells was estimated using Oil Red O stain. Oil Red O stain is a diazo dye used to stain neutral triglycerides and lipids; lipid droplets appear red in colour after staining wi```th an absorbance maxima at 518 nanometers (Lee *et al*., 2019) [20]. Our results clearly indicated that the treatment with fenofibrate and glycyrrhetinic acid significantly lowered the Oil Red O stain. It indicated that the accumulation of lipids in the HepG2 cells was also reduced (Figure 6).The microphotographs of the cells also indicated the reduction of Oil Red O stain by the treatment (Figure 7).

Effect of glycyrrhetinic acid on transaminase level of HepG2 cells```

Hepatic steatosis increased the influx of fatty acids into the hepatocytes, which in turn causes increased lipid accumulation within the cells (Vidyashankar *et al*., 2013) [28]. Within the hepatocytes, these fatty acids are then converted into triglycerides, which may either be stored as lipid droplets or undergo oxidation. Increased fatty acid influx during steatosis caused increased accumulation of fatty acids within the cells; it caused increased generation of reactive oxygen species, which results in oxidative damage and subsequent cell death (Bradbury, 2006) [5]. Transaminases are the enzymes found only in the hepatocytes and the increased cell death due to the accumulation of fatty acids elevated the levels of transaminases in the spent media (Yang *et al*., 2019) [30]. Our study also clearly represented the elevated transaminase levels in the spent media (Figures 8 & 9) and the treatment with glycyrrhetinic acid significantly lowered the leakage of transamainases.

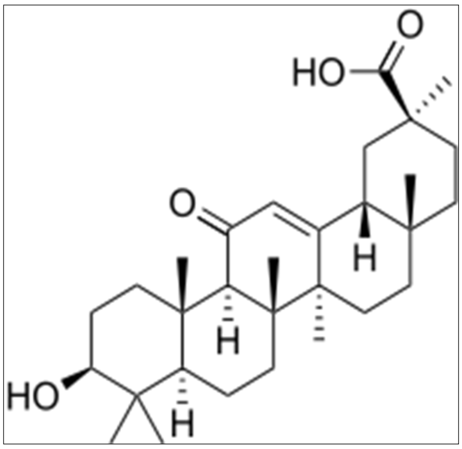

Fig 1: Structure of glycyrrhetinic acid isolated from the stems of *Glycyrrhiza glabra*

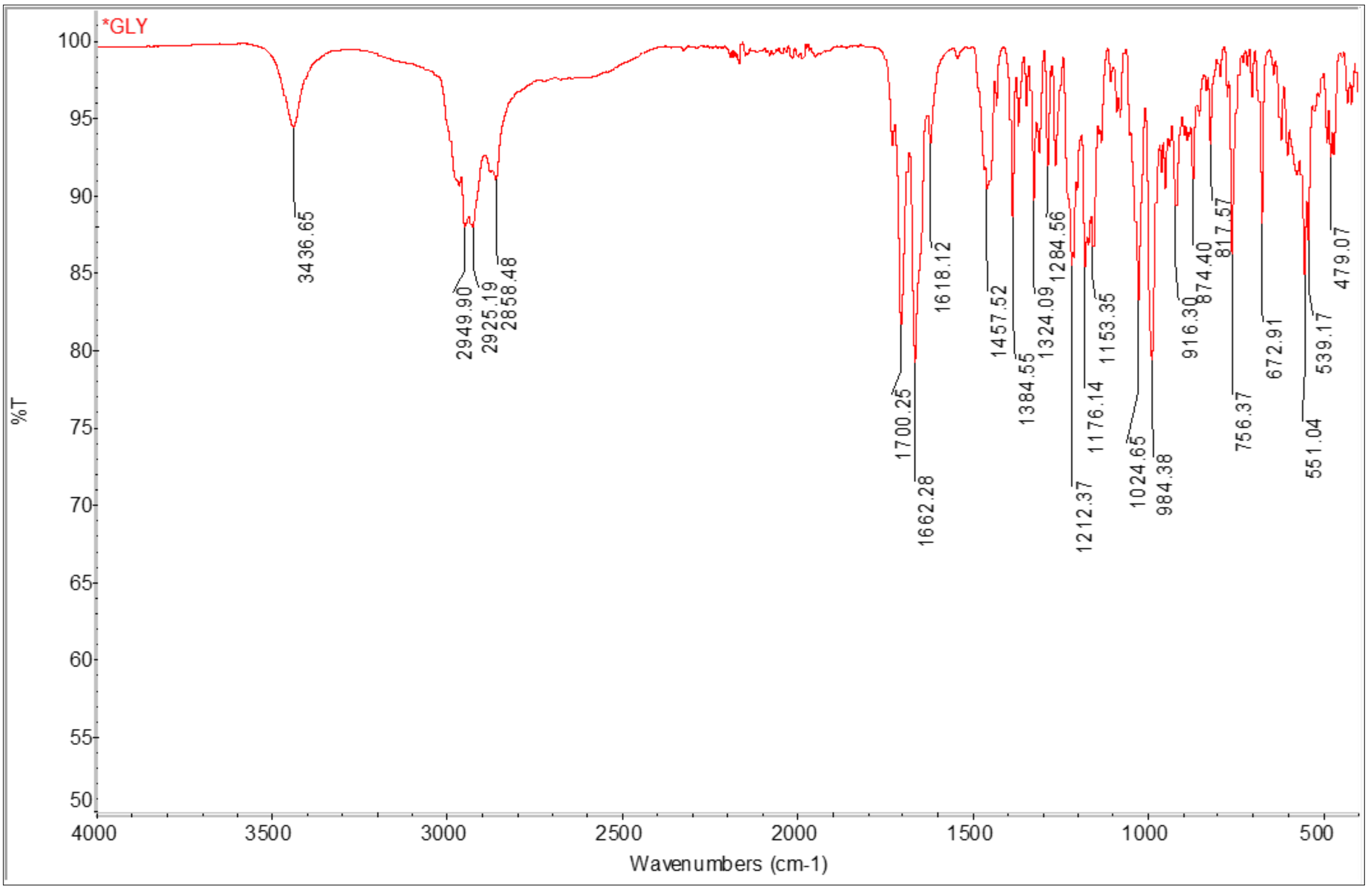

Fig 2: FT-IR spectrum of glycyrrhetinic acid isolated from the stems of *Glycyrrhiza glabra*

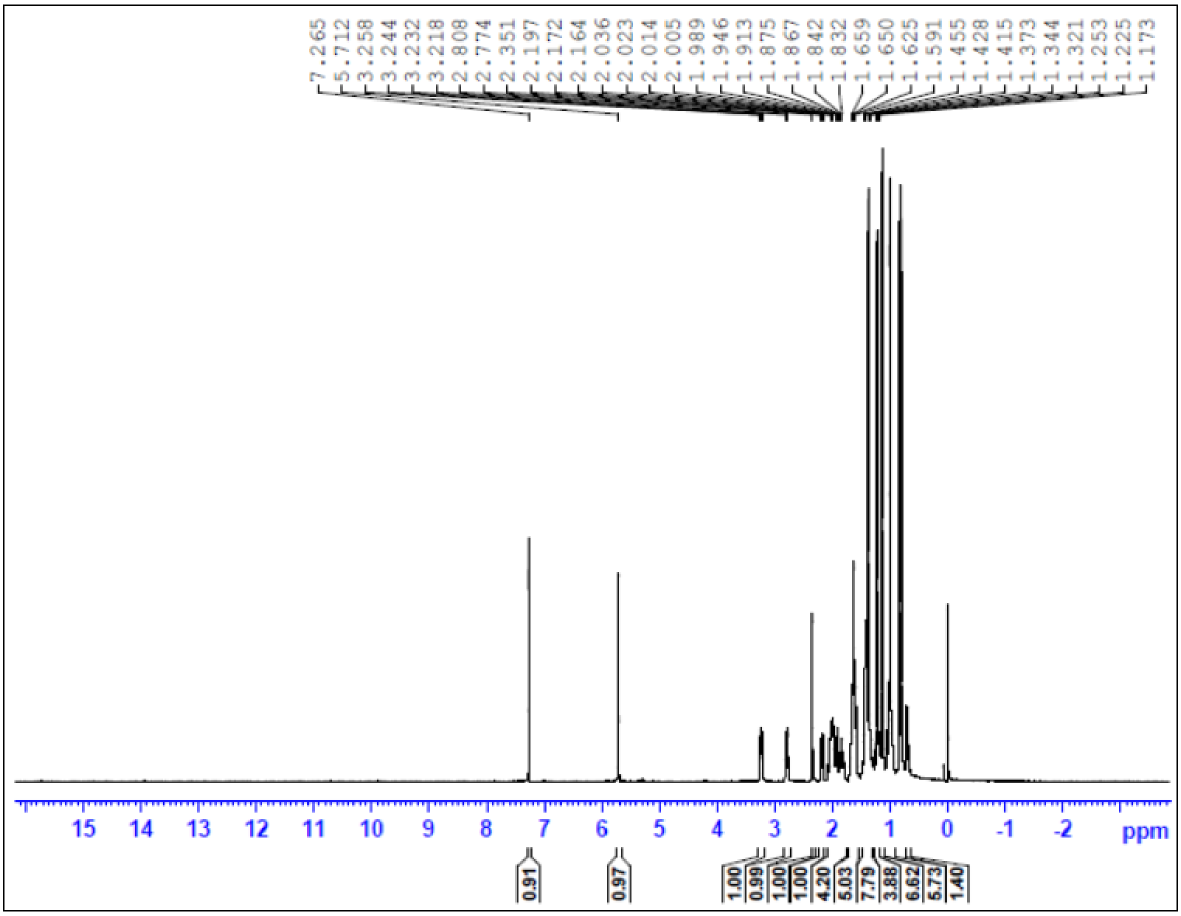

Fig 3: ^{1}H NMR spectrum of glycyrrhetinic acid isolated from the stems of *Glycyrrhiza glabra*

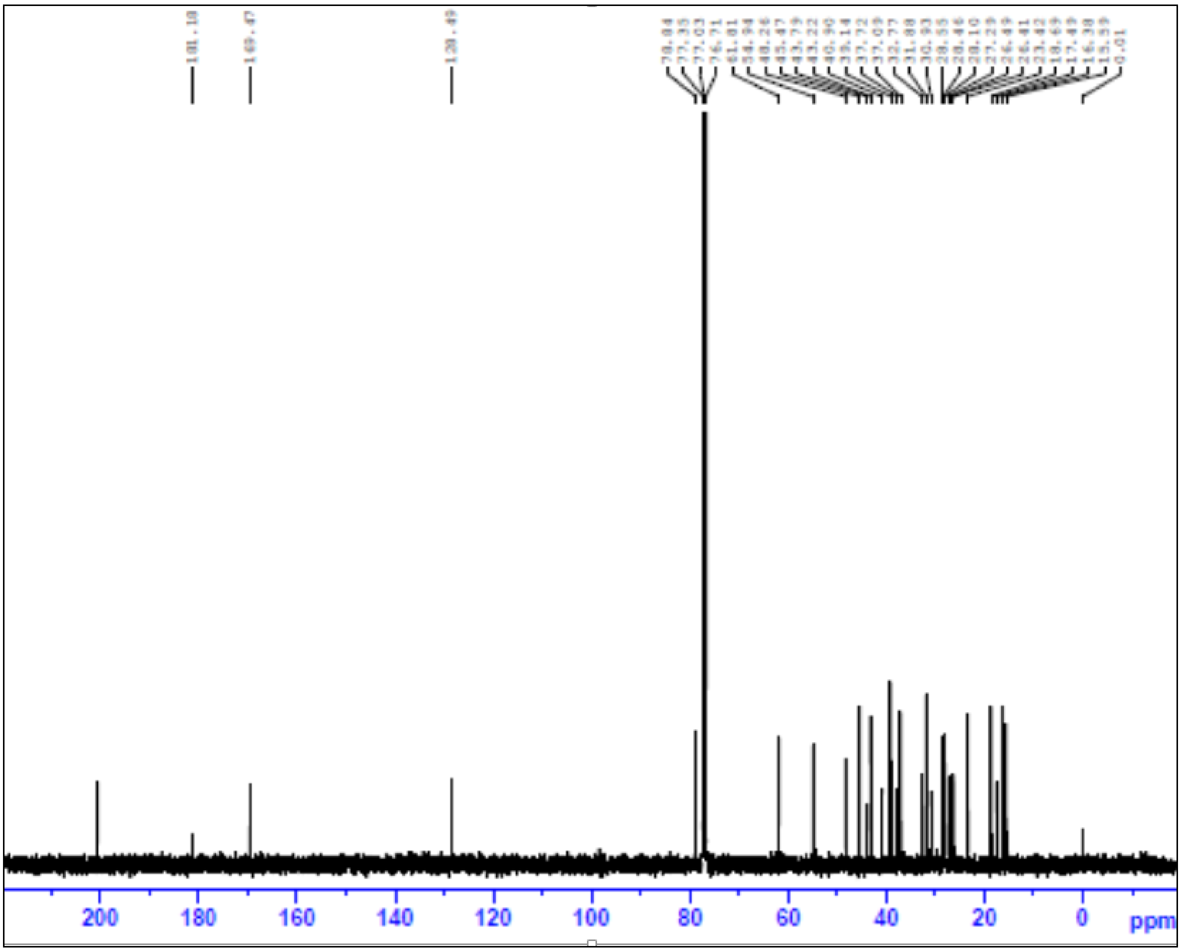

Fig 4: ^{13}C NMR spectrum of glycyrrhetinic acid isolated from the stems of *Glycyrrhiza glabra*

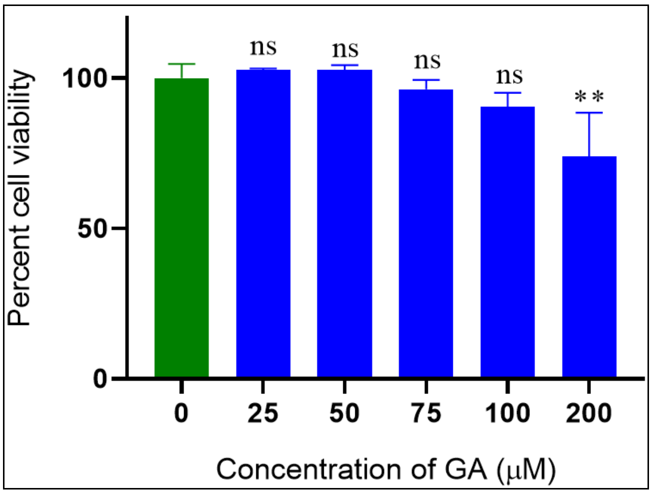

Fig 5: Effect of glycyrrhetinic acid on the viability of HepG2 cells after 24 hours of treatment

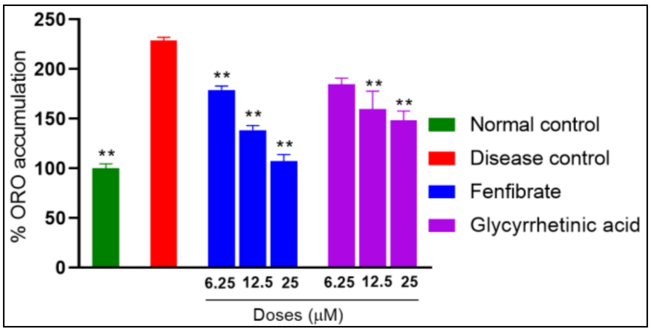

Fig 6: Effect of glycyrrhetinic acid on intracellular lipid accumulation of palmitate – oleate induced steatosis in HepG2 cells after treatment for 24 hours

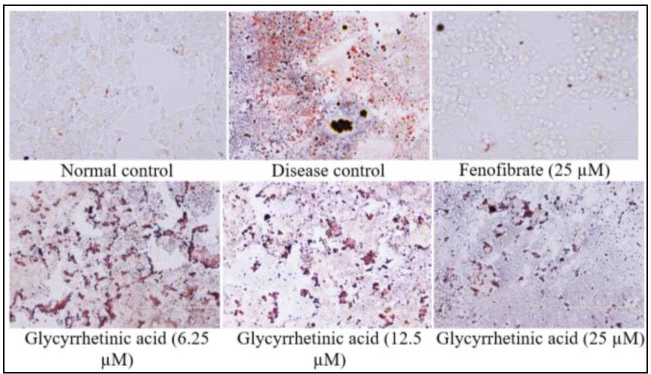

Fig 7: Some representative microphotographs of palmitate – oleate induced steatotic HepG2 cells after treatment for 24 hours

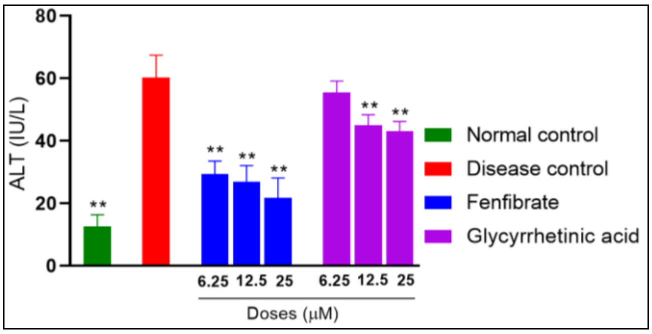

Fig 8: Effect of glycyrrhetinic acid on the level of alanine transaminase leakage from palmitate – oleate induced steatosis in HepG2 cells after treatment for 24 hours

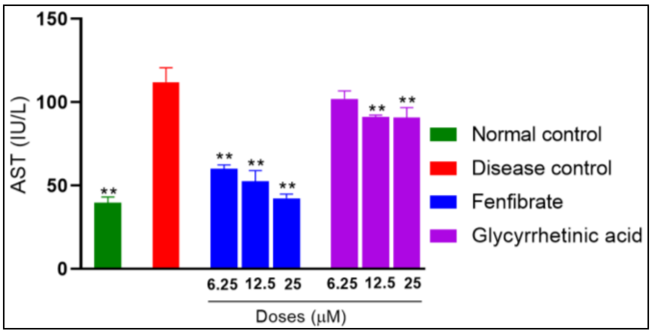

Fig 9: Effect of glycyrrhetinic acid on the level of aspartate transaminase leakage from palmitate – oleate induced steatosis in HepG2 cells after treatment for 24 hours

Conclusion

Glycyrrhetinic acid is one the major metabolite of *Glycyrrhizaglabra*, one of the important traditional medicinal plants of India. In this study, the therapeutic effect of glycyrrhetinic acid on the cellular model of NAFLD was demonstrated. The efficacy of glycyrrhetinic acid was comparable with that of fenofibrate, the positive control drug. Further detailed investigations on this compound might yield some useful clues for the better management of NAFLD.

Acknowledgements

The authors are grateful to the management of Entomology Research Institute and Loyola College for providing necessary facilities for this project.

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