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Research Article

Desmodium triflorum Mitigates Palmitate-Induced mTOR Activation and Oxidative Stress in HepG2 Cells

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Abstract

Non-alcoholic fatty liver disease (NAFLD) remains a predominant etiology of chronic hepatic disease, accompanied by no definitive pharmacological therapies currently available. This study investigates the antisteatotic potential of *Desmodium triflorum* (*Grona triflora*) ethanolic extract (ET-DT) in palmitic acid (PA) induced HepG2 cell model of hepatic steatosis. Phytochemical profiling confirmed the presence of alkaloids, phenols, and flavonoids in ET-DT. Palmitic acid exposed HepG2 cells exhibited reduced viability (assessed via 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay), elevated lactate dehydrogenase (LDH) leakage, and increased reactive oxygen species ROS, all of which were mitigated by ET-DT co-treatment (25 µg/mL and 50 µg/mL). ET-DT restored glutathione levels and superoxide dismutase activity, indicating enhanced antioxidant capacity. A reduction in lipid accumulation was observed in a dose-dependent manner by Oil Red O staining, while Sirius Red staining demonstrated decreased collagen deposition in ET-DT-treated groups. Reverse transcriptase PCR analysis showed that ET-DT suppressed PA-induced upregulation of mammalian target of Rapamycin (mTOR) gene expression. These findings suggest that *Desmodium triflorum* extract ameliorates PA-induced steatosis through antioxidant, antifibrotic, and mTOR pathway modulation, highlighting its therapeutic potential for NAFLD management.

Keywords: *NAFLD; Desmodium triflorum; NAFLD; HepG2; Palmitic Acid; LDH Leakage; mTOR*

Abbreviations

NAFLD: Non-Alcoholic Fatty Liver Disease; ET-DT: *Desmodium triflorum* Ethanolic Extract; PA: Palmitic Acid; mTOR: Mammalian Target of Rapamycin; NASH: Non-Alcoholic Steatohepatitis; HSCs: Hepatic Stellate Cells; SCD-1: Stearoyl-CoA Desaturase 1

Introduction

Non-alcoholic fatty liver disease (NAFLD) is acknowledged as the most common chronic liver condition globally, impacting around 25 -30% of the population and acting as a principal factor in chronic liver disease, with greater incidence in males compared to females

[1,2]. Non-alcoholic fatty liver disease is characterized by the accumulation of over 5% fat in hepatocytes in the absence of substantial alcohol intake or secondary etiologies, including viral infections or pharmacological interventions [3]. The global prevalence of NAFLD has shown a steady increase over time, with estimates ranging from 26% in earlier studies to over 38% in recent years [1, 4]. This rising trend imposes a significant economic burden globally and is closely linked to type 2 diabetes, obesity and metabolic syndrome [5,6]. Non-alcoholic fatty liver disease encompasses a spectrum of hepatic disorders ranging from uncomplicated hepatic steatosis to non-alcoholic steatohepatitis (NASH), which may advance to cirrhosis, fibrosis and hepatocellular carcinoma [7,8]. HepG2 cells, derived from human hepatocarcinoma cell line, are extensively utilized as *in vitro* model for the investigation of liver biology and metabolism due to their ability to mimic several key functions of primary hepatocytes, including protein synthesis and drug metabolism [9]. palmitic acid (PA), a saturated fatty acid comprising 16 carbon atoms, has been associated with the pathogenesis of NAFLD. Palmitic acid exposure induces lipotoxicity and intracellular accumulation of lipid in HepG2 cells [10], disrupting lipid metabolism by upregulating genes involved in *de novo* lipogenesis (e.g. fatty acid synthesis) and down regulating genes responsible for oxidation of fatty acid (e.g. peroxisome proliferator-activated receptor alpha). This imbalance contributes to hepatic steatosis, oxidative stress, inflammation, and lipid peroxidation-hallmarks of NASH [11,12]. Notwithstanding its increasing clinical importance and prevalence, there is presently no sanctioned pharmacological intervention to NAFLD. A promising therapeutic strategy involves reducing oxidative stress, inflammation and accumulation of hepatic lipid. Plant-derived extracts have emerged as valuable resources for developing novel pharmaceuticals due to their long-standing use in traditional medicine and demonstrated hepatoprotective properties [13]. Among these, *Grona triflora* (syn. *Desmodium triflorum*), a herb used in Ayurveda and Chinese medicine, has shown potential hepatoprotective effects against PA-induced damage, making it a candidate for further investigation in NAFLD management [14]. *Desmodium triflorum*, has a documented history of traditional use in managing liver disorders, supported by preliminary evidence of hepatoprotective properties attributed to its rich phytochemical profile [15].

Objectives of the Study

The aim of present investigation is to evaluate the efficacy of ethanolic extract from *Desmodium triflorum* (ET-DT) in mitigating pathophysiological features of NAFLD, specifically focusing on its effects on lipid accumulation, inflammation, and oxidative stress. Additionally, we aimed to elucidate underlying mechanisms by which ET-DT alleviates PA-induced NAFLD, exploring potential pathways involved in lipid metabolism regulation and cellular protection. Investigation of this traditional medicinal plant in ameliorating NAFLD has been previously unexplored is the distinguishing feature of this study.

Materials and Methods

The plant *D. triflorum*, commonly known as creeping tick trefoil or three-flower beggar weed, is a member of Fabaceae family widely scattered over tropical and subtropical areas, including India. For this study, *D. triflorum* was collected from various locations in and around Thiruvananthapuram, Kerala, India, during its peak growth season. The plant was verified by a taxonomist from the Department of Botany, University of Kerala, and a voucher specimen was kept in the herbarium of the Department with a voucher number of KUBH 11592 (Figure 1).

Figure 1: *Desmodium triflorum plant.*

Description of the plant-*Desmodium triflorum*

Family: Fabaceae

Vernicular name : Malayalam-Nilamparanda, Hindi-Kudaliya, Sanskrit-Tripadi.

Extraction of plant

10g of shade dried *D. triflorum* leaves wereblended in a clean glass container with 200 mL of 70% ethanol. The mixture was vigorously agitated for 24h to facilitate the extraction of bioactive compounds. Following this maceration process, the mixture was gently pressed to separate the liquid extract from the plant residue. A rotary evaporator was then used to evaporate the resultant ethanolic extract at lower pressure to remove excess solvent and obtain a concentrated extract. For subsequent studies, the dried ET-DT was used. To prepare a stock solution, dissolve 1 mg of ET-DT in 1 mL of 0.01% dimethyl sulfoxide (DMSO). To guarantee sterility and remove any particulate matter, pass the sample solution through a 0.22 µm Millipore syringe filter.

Cell lines

Hepatocellular carcinoma (HepG2) cells were procured from National Centre for Cell Sciences (NCCS) in Pune, India. Dulbecco's Modified Eagle Medium (DMEM; GIBCO, USA) was utilized for cell cultivation, which was supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate, and an appropriate antibiotic solution to ensure sterility. In a 25 cm^2 tissue culture flask, the cell line was kept at 37°C in a humid environment with 5% CO_2. Once the cells reached confluency, they were trypsinized using 0.25% trypsin-EDTA solution to detach them from the culture flask. The isolated cells were subsequently reseeded at a density of 1×10^4 cells/mL for subsequent investigations. Regular sub culturing and media changes were performed to ensure optimal growth and viability of the HepG2 cells

Phytochemical analysis

The phytochemical composition of the ethanolic extract was analyzed using the following methodologies.

Alkaloids

To detect alkaloids Dragendorff's method was employed [16]. Prepare a modified Dragendorff's reagent by dissolving 8g of $Bi(NO_3)_3 \cdot 5H_2O$ in 20 mL of HNO_3, then mix with 2.72g KI in 50 mL water. Allow the mixture to stand until KNO_3 crystals are formed. Discard the supernatant, and adjust the volume to 100 mL using water. The precipitate was treated with Na_2CO_3 to liberate the alkaloids, which were then extracted with ether. For the alkaloid test, mix 0.5 mL of the alcoholic extract solution with 2.0 mL of HCl. Subsequently add 1.0 mL of the prepared reagent to the acidic medium. The rapid emergence of an orange-red precipitate signified the existence of alkaloids.

Flavonoids

To detect flavonoids, 0.5 mL alcoholic extract solution was combined with 5-10 drops of dilute hydrochloric acid (HCl) and a pinch of magnesium chloride ($MgCl_2$). The mixture was heated, a dirty brown or reddish-pink tint indicated the presence of flavonoids [17].

Phenols

Ferric Chloride Test was employed to assess phenol content. Mix 2 mL of the alcoholic extract solution with 2 mL of distilled water and few drops of 10% aqueous ferric chloride ($FeCl_3$) solution. Presence of phenol was indicated by the appearance of a green or blue color [18].

Induction of PA toxicity and ET-DT co-administration

To induce toxicity in HepG2 cells, 20 µM of PA was added and left to incubate for four hours. Following the induction of PA toxicity, treat the cells with fresh medium containing different concentrations of the ethanolic extract of ET-DT. The cells were incubated at 37°C in a 5% CO_2 atmosphere for additional 24 hours.

In vitro hepatoprotective effect of ET-DT co-administration on PA-exposed HepG2 cells using MTT assay

The MTT assay was conducted to assess cell viability. Following 1X phosphate buffered saline (PBS) wash, add 30 µL MTT solution to the cell culture. Incubate the cells for 3 hours at 37°C. After removing MTT solution with PBS, add 200 µL of DMSO to solubilise formazan crystals. Incubated the cells for 30 minutes at room temperature, allowed the color to develop. Cell debris was removed by centrifugation for 2 minutes. Measured the optical density with a spectrophotometer at 540 nm. Determine the viability of cells using the formula:

$$\text{Percentage viability} = \frac{Mean\ OD\ samples}{Mean\ OD\ control} X100$$

In vitro hepatoprotective effect of ET-DT co-administration on PA exposed HepG2 cells using Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase leakage is a critical indicator of severe and irreversible cell damage. For LDH assay, cell free supernatant collected from culture plates that had been treated with different ET-DT concentrations (6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL) was utilized. The mixture for assay consisted of 2.7 mL of potassium phosphate buffer, 0.1 mL of 6 mM NADH solution, and 0.1 mL of sodium pyruvate solution, which were thoroughly mixed. The LDH activity could be determined by measuring the reduction in optical density at 340 nm.

Determination of effects of ET-DT on antioxidant markers

Preparation of cell lysate

After being trypsinized in a Trypsin-EDTA solution, cells were transferred to Eppendorf tubes and centrifuged for 5 minutes at 5,000 rpm. Discard the supernatant, reconstituted the pellet in 200 µL of lysis buffer comprising 0.1M Tris, 0.2M EDTA, 2M NaCl, and 0.5% Triton X-100. The samples were incubated at 40°C for 20 minutes to facilitate cell lysis. The resulting cell lysate was used for further analyses.

Lipid peroxidation

Lipid peroxidation predominantly impacts polyunsaturated fatty acids, results in the formation of malondialdehyde (MDA) upon breakdown. To assess lipid peroxidation, combine 50 µL of cell lysate with 500 µL of 70% ethanol and 1 mL of 1% thiobarbituric acid (TBA). The tubes were submerged in a boiling water bath for duration of 20 minutes. Add 50 µL of acetone to each tube after it had cooled to room temperature; absorbance was measure at 535 nm using a spectrophotometer [19].

Reduced glutathione (GSH)

The accumulation of oxidized glutathione (GSSG) due to continuous ROS production, including lipid peroxides, H_2O_2, and OH, leads to decreased GSH levels, serving as a precise indicator of the cell's oxidative state. To measure GSH levels, 0.5 mL of phosphate buffer, 1.3 mL of distilled water, 1 mL of cell lysate, and 0.2 mL of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were combined, thoroughly mixed. Measure the absorbance at 420 nm using a spectrophotometer. GSH levels were evaluated by comparing them to a standard curve of reduced glutathione [20].

Superoxide dismutase (SOD)

As an antioxidant in cells exposed to oxygen, SOD is vital to survival. The reaction mixture consisted of 50 mM phosphate buffer, 45 µM methionine, 5.3 mM riboflavin, and 84 µM potassium ferric cyanide. To this mixture, 50 µL of cell lysate was added. Incubate the tubes for 10 minutes at 25°C, and measure the absorbance at 600 nm and calculated the SOD activity [21].

Oil red O staining

To evaluate accumulation of lipid in HepG2 cells undergone PA exposure and co-administered with ET-DT, culture the cells in a 24-well plate at a density of $1x10^5$ cells/well. Cells were exposed to PA and treated with varying concentrations of ET-DT for 24 hours. After three rounds of ice-cold PBS washing, the cells were fixed for 30 minutes with 4% paraformaldehyde. After fixation, the cells underwent three rounds of washing before being stained with Oil Red O for 15 minutes at room temperature in order to visualize lipid droplets. Washing with PBS helped to remove unbound stains. Each sample was then treated with DMSO to extract the Oil Red O. The OD of the samples was measured at 510 nm using a spectrophotometer after shaking for 5 minutes at room temperature [22].

Measurement of ROS generation using dichlorodihydrofluorescein diacetate (DCFH-DA) staining

To quantify intracellular ROS activity, following PBS washing, cells were treated with 50 µL of DCFH-DA, a dye that reacts with peroxyl, hydroxyl, and other ROS. Incubate the cells for 30 minutes to allow the dye to penetrate and react with ROS. Wash with PBS to remove extra dye, use a fluorescent microscope to record fluorescence. Fluorimeter was employed to quantify the fluorescence intensity, which was expressed in arbitrary units, with excitation at 470 nm and emission at 525 nm (ab113851 DCFH-DACelular ROS Detection Assay Kit For measurement of ROS).

Assessment of collagen deposition by Sirius red staining

Sirius red staining method was employed to assess collagen deposition. In order to bind collagen fibers, stain the cells with 0.1% Sirius Red in saturated picric acid for 1 hour. Morphological images were obtained to visualize collagen distribution. Extensive washing with 0.01 M HCl was performed to remove unreacted stain. After dissolving the stained collagen in 0.1 M NaOH, the mixture was centrifuged for 5 minutes at 7,000 rpm. Measure the absorbance at 530 nm to quantify collagen content. Similar procedures were applied to control cells for comparison (Picro- Sirius Red Stain Kit for Collagen).

Relative expression of mTOR gene

Reverse transcriptase polymerase chain reaction (RT-PCR) was employed to analyze how ET-DT affected the expression of the mTOR gene in PA-induced HepG2 cells. This technique allowed for the quantification of mRNA levels to determine how ET-DT influences mTOR gene expression in response to PA exposure.

Primer design

Using Roche primer designing software, the primer pairs for mTOR were created with the following specifications: GC content: 50 to 55%; primer length: 20 to 23 nucleotides; and melting temperature (Tm): around 60°C. The designed primers were depicted in the table 1.

Oligo name	Forward		Reverse	
	Sequence (5' ->3')	Tm	Sequence (5' ->3')	Tm
mTOR gene	GCTTGATTTGGTTCCCAGGACAGT	61.4°C	GTGCTGAGTTTGCTGTACCCATGT	59.4°C

Table 1: *Primer details.*

RNA isolation, cDNA synthesis and agarose gel electrophoresis

RNA isolation kit was used to extract total RNA from cells in accordance with manufacturer's instructions. Reconstitute dried RNA pellet in TE buffer to facilitate downstream processing. Perform cDNA synthesis using commercial kit and a thermal cycler. The reverse transcription reaction involved temperature cycles of 25°C for 5 minutes, 42°C for 30 minutes, and 95°C for 2 minutes. The Thermoscientific amplification kit was used for this process.

For PCR amplification, the initial denaturation was set at 95°C for 30 seconds, followed by annealing for 30 sec and extension at 72°C for 5 minutes. The cycle was executed 30 times, concluding with a final extension phase of 15 minutes at 72°C. Subject the amplified product to agarose gel electrophoresis using 50 volts for 30 minutes. Gel documentation software was used to capture, visualize and analyse the size and intensity of the gel.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software 5.01 (GraphPad Software, Inc., San Diego, CA). The data were represented as mean ± SD (n = 3) and analyzed by one-way analysis of variance (ANOVA) and $P < 0.05$ was considered significant.

Results and Discussion

Nonalcoholic fatty liver disease is a metabolic disorder that is distinguished by the accumulation of excessive lipids in the liver, which is not linked to alcohol consumption [23]. It affects approximately 25% of the global population and is strongly correlated with obesity, insulin resistance, and dyslipidemia. The disease spectrum includes simple fatty liver and NASH, which can progress to fibrosis, hepatic inflammation, cirrhosis, liver failure, and cardiovascular complications. Nonalcoholic fatty liver disease is caused by a complex interaction among behavioral, environmental, and genetic elements. Early stages involve imbalances in lipid production and breakdown, leading to triglyceride accumulation in hepatic cells. Principal factors encompass inflammation, oxidative stress, insulin resistance, and mitochondrial malfunction [1-3].

D. triflorum, a plant native to the Himalayas and parts of Asia, has been conventionally utilized in Ayurvedic and Tibetan medicine for numerous ailments. Recent studies highlight its therapeutic properties, including antibacterial, antiepileptic, antifungal, and radioprotective activities [24]. Ethanolic extract of *D. triflorum* was prepared using standard methods and confirmed to contain alkaloids, flavonoids, and phenols. Hepatoprotection was validated through MTT assay and LDH leakage test, demonstrating its protective effects against PA-induced toxicity.

Phytochemical screening of ET-DT

Phytochemical screenings of ET-DT indicated the presence of phenolic compounds and flavonoids, which are known for their potent antioxidant and hepatoprotective properties. The qualitative analysis confirmed the presence of alkaloids, flavonoids, and phenols (Table 2). These findings align with previous studies that highlight phenols and flavonoids as major bioactive compounds contributing to the therapeutic potential of medicinal plants [25,26].

Phytochemicals	Present/ Absent
Alkaloids	++
Phenol	+++
Flavonoids	+++

Table 2: *Phytochemical screening of D. triflorum.*

(+): Trace amount present; (++): Moderate amount present; (+++): Abundant amount present; (-): Absent.

In vitro hepatoprotective effect of ET-DT co-administration on PA-treated HepG2 cells using 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay

The hepatoprotective effect of ET-DT was evaluated by MTT assay on HepG2 cells exposed to PA. Palmitic acid treatment significantly reduced cell viability to 44.91%, indicating severe cytotoxicity. However, co-administration of ET-DT at a concentration of 25 µg/mL

restored cell viability to 87.69%, demonstrating its protective effect against PA-induced damage. These results confirm the efficacy of ET-DT in mitigating lipid toxicity and oxidative stress in hepatic cells. Further studies were conducted using a concentration of 25 µg/mL based on these findings. Morphological changes induced by different concentrations of ET-DT were captured using an epi-fluorescent microscope linked to a Pro5 CCD camera (Figure 2). Table 3 summarizes the percentage of viable cells across different treatment conditions.

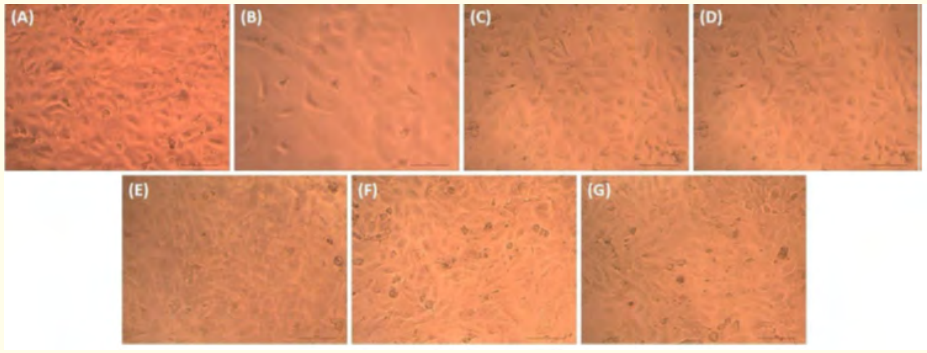

Figure 2: *Photomicrographs of PA exposed cultured HepG2 cells with ET-DT co-administration. (A) Cells that have not been treated (B) Cells treated with PA and those co-administered with varying quantities of ET-DT (C) 6.25 (D) 12.5 (E) 25 (F) 50 and (G)100 µg/mL.*

Concentration of ET-DT (µg/mL)	**Percentage of viability** **Values represented as mean ± SD, number of replicates = 3**
Control	100
Palmitic acid	44.91 ± 1.25
6.25	54.38 ± 4.01
12.5	61.94 ± 3.87
25	87.69 ± 3.62
50	64.75 ± 2.95
100	49.55 ± 2.45

Table 3: *Invitro hepato protective effect of ET-DT in relation to the viability percentage on HepG2 cells exposed to PA.*

In vitro hepatoprotective effect of ET-DT co-administration on PA-exposed HepG2 cells using LDH leakage assay

Assay of LDH leakage is an extensively employed methodology for evaluation of plasma membrane damage as well as cell death, serving as a biomarker for cytotoxicity evaluations. To assess the hepatoprotective properties of ET-DT against PA-induced cytotoxicity in HepG2 cells, we conducted an LDH leakage assay.The results indicated a substantial increase in the release of LDH in cells subjected to PA treatment in comparison with untreated controls, indicating significant cell membrane damage. However, in cells co-administered with ET-DT, LDH leakage was significantly reduced compared to PA-treated cells. The protective effect was found to be dose-dependent, ie; lower levels of LDH leakage were observed at higher ET-DT concentrations (Table 4). These findings suggest that ET-DT effectively mitigates PA-induced cytotoxicity by preserving cell membrane integrity.

Treatment	LDH Leakage (U/L) Values represented as mean ± SD, number of replicates = 3	% Reduction in LDH Leakage
Control	100 ± 5	-
PA (20 µM)	250 ± 10	-
PA + ET-DT (6.25 µg/mL)	180 ± 8	28
PA + ET-DT (12.5 µg/mL)	150 ± 6	40
PA + ET-DT (25 µg/mL)	120 ± 5	52
PA + ET-DT (50 µg/mL)	100 ± 4	60
PA + ET-DT (100 µg/mL)	80 ± 3	68

Table 4: *Effect of ET-DT on LDH leakage in PA-treated HepG2 cells.*

Effect of ET-DT co-administration on antioxidant markers

Lipid peroxidation

Historically, cell death was attributed to the formation of reactive metabolites binding to cellular proteins. However, an alternative hypothesis suggests that liver injury is primarily caused by P450-derived oxidant stress and lipid peroxidation. Although lipid peroxidation may not be a direct mechanism of cell death, it serves as a reliable marker for ROS generation [27]. Therefore, mitigating mitochondrial oxidant stress could be a viable therapeutic strategy for addressing hepatotoxicity. Recent studies have highlighted the biological significance of lipid peroxidation products, not only in the pathogenesis of liver diseases but also as valuable clinical biomarkers [28,29]. To assess lipid peroxidation, a colorimetric method using MDA as a standard was employed. The results indicated a substantial increase in MDA levels in cells subjected to PA treatment in comparison to untreated control cells. However, co-administration of ET-DT resulted in a substantial decrease in MDA production, indicating reduced lipid peroxidation in PA-exposed cells when treated with ET-DT (Table 5). This finding suggests that ET-DT effectively mitigates oxidative stress by reducing lipid peroxidation, thereby offering hepatoprotective benefits.

Reduced glutathione

Reduced glutathione, a tripeptide and thiol-based reducing agent, plays a critical role in regulating redox processes. It is integral to cellular mechanisms that determine survival, apoptosis, and necrosis, while also influencing the activity of transcription factors and signal transduction molecules. In HepG2 cells subjected to PA, the levels of GSH were significantly diminished compared to untreated control cells. However, co-administration of ET-DT notably increased the levels of GSH in comparison to PA-exposed cells, as illustrated in table 5.

Super oxide dismutase

Oxidative stress, a concomitant pathogenic process, is increasingly acknowledged as a critical factor in the onset and advancement of liver damage. Superoxide dismutase, an essential antioxidant defense mechanism that neutralizes superoxide anions, was the first characteristic studied in this context. The current research assessed the impact of co-administering ET-DT on SOD levels in HepG2 cells treated with PA. The findings indicated that the levels of SOD in PA-treated cells were significantly lower than those in untreated controls. However, co-administration of ET-DT significantly elevated SOD levels relative to PA-exposed cells, as depicted in table 5.

Determination of lipid accumulation in PA-exposed HepG2 cells co-administered with ET-DT using Oil Red O staining

Neutral lipids are specifically stained with Oil Red O, but it does not bind to biological membranes. The introduction of PA into cells leads to a substantial increase in lipid droplet formation in cytoplasm. When these lipid droplets accumulate to high levels, they can become

toxic, causing apoptosis and necrosis if their concentration exceeds a certain threshold. Despite the potential for lipotoxicity caused by PA in HepG2 cells [30], there have been limited studies addressing this issue, and few attempts have been made to understand the effects of lipid build up in cells. Quantitative analysis and morphological examination have shown an elevation of intracellular lipid levels in cells exposed to PA. However, co-administration of ET-DT resulted in a decrease in lipid accumulation in cells exposed to PA. Oil Red O staining was used both qualitatively (Figure 3) and quantitatively (Table 5) to assess lipid composition and confirm lipid accumulation. Red lipid droplets in PA-treated groups had notably larger size and content than in the normal group.

Parameter assessed	Control	PA treatment	PA+ ET-DT treatment
MDA (µM/mg protein)	0.63 ± 0.005	0.89 ± 0.002**	0.70 ± 0.01*
GSH (µM/mg protein)	8.0 ± 1.17	3.9 ± 1.03*	7.4 ± 1.61**
SOD (Enzyme activity in U/mL)	1.80 ± 0.02	0.45 ± 0.07*	1.72 ± 0.07**
Lipid Deposition (Absorbance at 510 nm)	0.09 ± 0.01	0.26 ± 0.02*	0.08 ± 0.04*

Table 5: *Effect of ET-DT on lipid peroxidation and lipid deposition, values are expressed as mean ± SD (n = 3). Statistical analysis was performed using GraphPad Prism Software 5.01. Data were analyzed by one-way ANOVA. *P < 0.01, **P < 0.001 compared to control.*

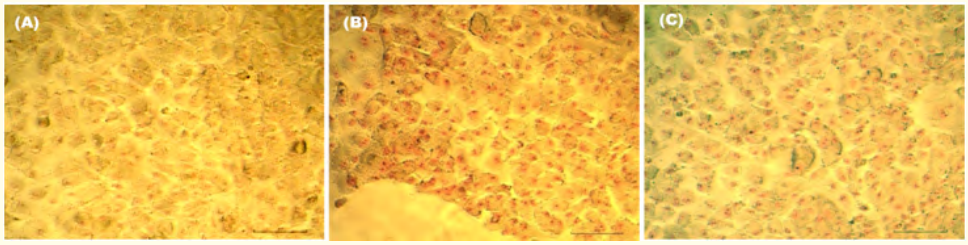

Figure 3: *Photomicrographs illustrating in vitro accumulation of lipids in HepG2 cells treated with PA co-administered with ET-DT using Oil Red O Staining method; (A) Cells that have not been treated (B) PA treated cells (C) PA treated cells co-administered with ET-DT.*

It is already reported by Ramachandran., *et al*. 2014, that decrease in intracellular antioxidant enzymes and proteins accounts to increased hepatotoxicity [31] and we determined the effects of ET-DT administration in modulating the antioxidant markers. Oxidative stress plays a significant role in NAFLD pathogenesis by reducing intracellular antioxidant enzymes like SOD and GSH. Lipid peroxidation caused by ROS further exacerbates cellular damage. Palmitic acid exposure significantly reduced GSH and SOD levels in HepG2 cells while increasing lipid peroxidation. Co-administration of ET-DT effectively restored GSH levels, reduced lipid peroxidation, and elevated SOD activity, confirming its hepatoprotective action. Palmitic acid exposure in HepG2 cells can lead to increased oxidative stress and cellular damage, as SOD plays a critical role in protecting cells against oxidative stress. Targeting SOD, GSH and other antioxidant molecules may offer a potential therapeutic strategy for prevention and treatment of NAFLD and other oxidative stress related diseases. Abnormal lipid accumulation is a hallmark of NAFLD. Palmitic acid-treated HepG2 cells exhibited increased intracellular lipid content due to triglyceride buildup [32,33]. Oil Red O staining revealed substantial lipid droplet formation in PA-treated cells, which was markedly reduced upon ET-DT co-administration. This reduction highlights the ability of ET-DT to mitigate lipotoxicity.

Measurement of ROS generation using the DCF-DA staining technique

Reactive oxygen species are commonly detected during apoptotic cell death, which is triggered by alterations in the intracellular microenvironment. In this study, the generation of intracellular ROS in HepG2 cells subjected to PA treatment and the effect of ET-DT

co-administration on ROS production were analyzed, visualized, and quantified based on fluorescence intensity (AU). The investigation revealed that PA-treated cells exhibited a maximum fluorescence intensity of 17,061.89 AU, indicating high ROS production. In contrast, cells co-administered with ET-DT showed a significantly reduced fluorescence intensity of 2,910.28 AU, demonstrating a marked decrease in ROS levels under these conditions. These findings confirm the ability of ET-DT to mitigate ROS generation in PA-exposed cells (Figure 4 and 5).

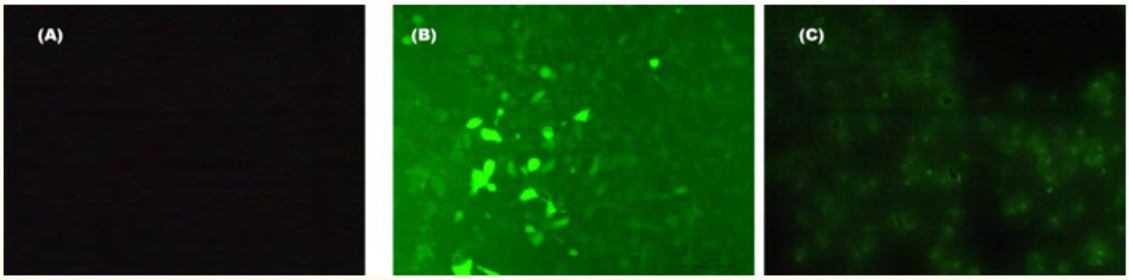

Figure 4: *DCFH-DA staining for the determination of ROS generation; (A) Cells that have not been treated (B) PA treated cells (C) PA treated cells co-administered with ET-DT.*

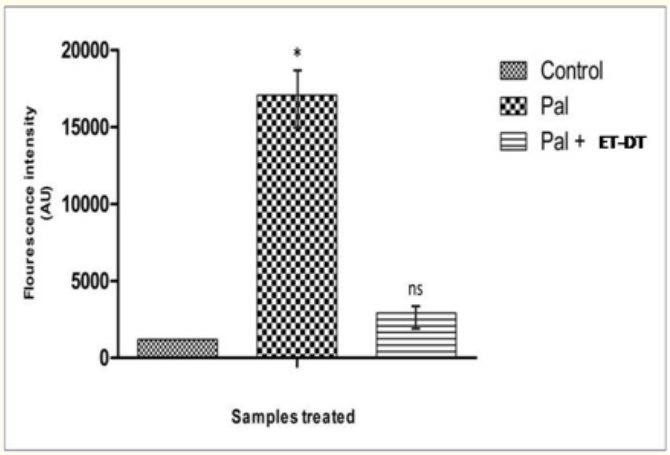

Figure 5: *Graphical depiction illustrating ROS production, Experiments were done in triplicates and results represented as mean ± SD. One-way ANOVA and Dunnett's tests were performed to analyze data. *p < 0.001 compared to control.*

Reactive oxygen species are chemically reactive entities involved in cellular signaling but can cause oxidative stress when overproduced [34]. PA exposure significantly increased ROS generation in HepG2 cells, as evidenced by DCFH-DA staining. ET-DT co-administration greatly reduced ROS levels, demonstrating its antioxidant efficacy.

Collagen deposition using Sirius Red staining

Exposure of HepG2 cells to PA has been associated with increased collagen levels [35], a consequence of liver damage and inflammation. This process triggers a fibrotic response in hepatic cells, marked by the accumulation of extracellular matrix constituents, including

collagen, in hepatic tissue [36]. Sirius Red staining was employed to evaluate collagen levels in PA-treated cells, as this method effectively highlights collagen deposition. The findings revealed that PA administration significantly increased collagen accumulation, indicating an enhanced fibrotic response. However, co-administration with ET-DT notably reduced collagen deposition, suggesting that ET-DT may mitigate PA-induced fibrosis (Figure 6). This reduction in collagen levels highlights the potential protective role of ET-DT against lipotoxicity and fibrosis caused by PA exposure.

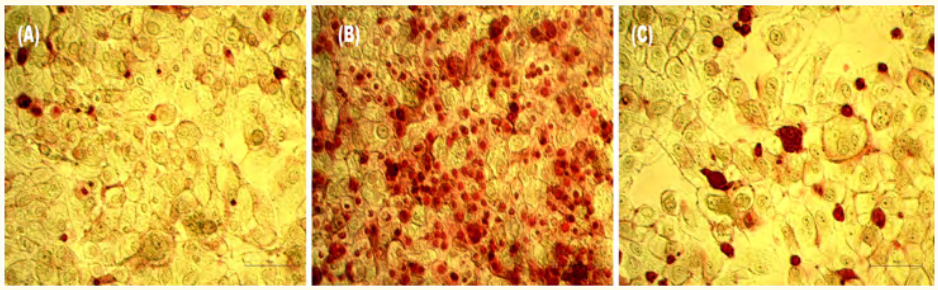

Figure 6: *Sirius Red staining; (A) Cells that have not been treated (B) PA treated cells (C) PA treated cells co-administered with ET-DT.*

Collagen accumulation is a critical marker of fibrosis progression in NAFLD. Palmitic acid-induced lipotoxicity triggers inflammatory responses that activate hepatic stellate cells (HSCs), leading to collagen deposition [37]. Sirius Red staining confirmed increased collagen levels in PA-treated HepG2 cells. ET-DT co-administration significantly reduced collagen deposition, suggesting its potential to prevent fibrosis progression.

Relative expression study of mTOR in PA-exposed HepG2 cells co-administered with ET-DT using RT PCR analysis

The expression of mTOR is influenced by oxidative stress, often leading to its elevated levels in response to oxidative injury. This upregulation is considered part of a cellular feedback mechanism that regulates oxidative stress and the inflammatory response [38,39]. To explore the potential therapeutic role of mTOR in preventing NAFLD, HepG2 cells were subjected to oxidative stress through PA exposure. The expression of mTOR was then analyzed using RTPCR and compared with cells treated with ET-DT. The results indicated a substantial increase in mTOR expression in PA-treated cells when juxtaposed with normal HepG2 cells. However, co-administration of ET-DT led to a substantial reduction in mTOR expression, indicating its potential to mitigate PA-induced oxidative stress and inflammation. These findings highlight the regulatory impact of ET-DT on the mTOR pathway under conditions of oxidative stress.

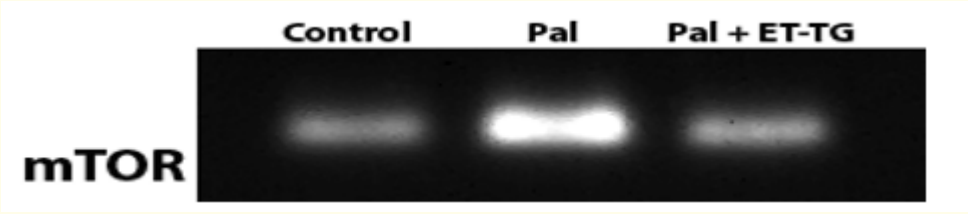

Figure 7: *Photomicrograph of agarose gel illustrating gene expression profile of mTOR gene.*

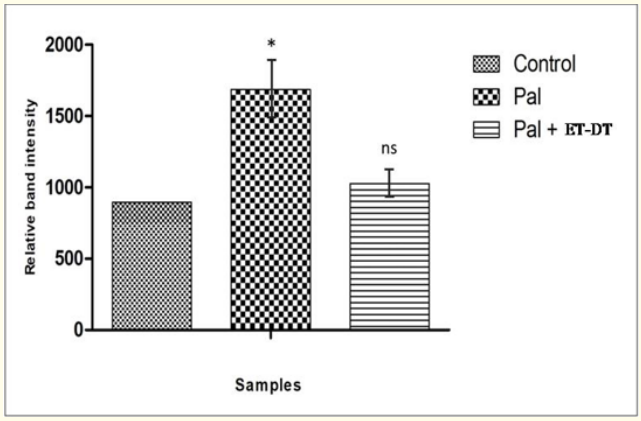

Figure 8: *Graphical representation illustrating the expression levels of mRNA of mTOR. Experiments were done in triplicates and results represented as mean ± SD. One-way ANOVA and Dunnett's tests were performed to analyze data. *p< 0.001 compared to control.*

Figure 8 illustrates the relative expression levels of mTOR mRNA in HepG2 cells under different conditions, including untreated cells, cells exposed to PA, and cells co-administered with ET-DT. The graphical representation provides a visual comparison of how ET-DT affects mTOR mRNA expression in presence of PA-induced oxidative stress. The mTOR is a key regulator of lipid metabolism as well as oxidative stress responses. Rapamycin reduces the expression of SREBP target genes involved in liver lipid metabolism and acetyl-coenzyme A carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD-1) levels. Inactive endoplasmic reticulum precursors of these enzymes require proteolysis to translocate to the nucleus, a process regulated by mTORC1. Human fatty liver tissue samples show a strong correlation between STING/mTORC1 activation, liver inflammation, and liver cell lipid droplets. Targeting STING/mTORC1 may be a novel way to reduce fatty acid accumulation in liver cells and chronic inflammation in fatty livers [40,41]. mTOR was found to be upregulated in HepG2 cells treated with PA, as demonstrated by mRNA analysis [42]. mTOR overexpression was detected in HepG2 cells treated with PA due to oxidative injury. ET-DT co-administration reduced mTOR expression levels significantly, indicating its ability to modulate mTOR signaling pathways and mitigate PA-induced hepatotoxicity.

Overall, the findings underscore the hepatoprotective potential of ET-DT against PA-induced toxicity through antioxidant modulation, reduction in lipid accumulation as well as oxidative stress markers, inhibition of collagen deposition and regulation of mTOR expression. The outcomes of this study suggest that ET-DT possesses the potential to be a promising therapeutic agent in the management of NAFLD and its associate complications.

Conclusion

Nonalcoholic fatty liver disease is a condition that is defined by the accumulation of hepatic fat in individuals who do not consume alcohol. It is influenced by a variety of factors, while the mechanisms driving NAFLD pathogenesis remain incompletely understood. It is clear that cellular stress, mitochondrial dysfunction, and inflammation play critical roles in its progression, leading to cellular damage and apoptosis. Scientific investigations have highlighted the therapeutic potential of *D. triflorum* in treating various conditions. This study sought to investigate the hepatoprotective effects of ET-DT against PA-induced toxicity in HepG2 cells. The extract was found to contain alkaloids, phenols, and flavonoids. Hepatoprotection was validated using cell viability and LDH leakage assay. Palmitic acid exposure

significantly increased LDH leakage while ET-DT co-administration reduced it. Antioxidant enzyme analysis confirmed that ET-DT restored reduced glutathione levels and superoxide dismutase activity in treated groups. Oil Red O staining demonstrated a substantial accumulation of lipids in PA-treated cells that was significantly reduced by ET-DT co-administration. Similarly, ET-DT decreased ROS generation induced by PA exposure. Collagen deposition was assessed using Sirius Red staining, revealing increased collagen levels in PA-treated cells that were mitigated by ET-DT co-administration. RT PCR analysis showed upregulation of mTOR expression in PA-treated cells; however, ET-DT co-administration effectively downregulated mTOR expression, suggesting a potential mechanism for its hepatoprotective effects. In conclusion, this study highlights the significant hepatoprotective benefits of ET-DT against PA-induced toxicity through its antioxidant properties, reduction of lipid accumulation and ROS generation, inhibition of collagen deposition, and modulation of mTOR expression. These findings underscore the therapeutic potential of ET-DT for managing NAFLD.

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Conflict of Interest

Authors declare that there exists no conflict of interest.

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