



Synthesis and in-vitro biological assessment of 4-phenyl-1h-1,2,3-triazole in 3T3-L1 adipocytes

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ABSTRACT

Screening of new therapeutic agents with anti-lipogenic and anti-adipogenic properties have attended much prominence in the last many decades. Due to their structural diversity and chemical versatility, heterocyclic compounds have emerged as promising agents for the development of new therapeutics against various diseases. Thus, present study aims to synthesise phenyl group containing 1, 2, 3 triazole compound and screening for anti-adipogenic, anti-lipogenic and antioxidant activity. Spectroscopic characterisation confirms the synthesis of 4-phenyl-1H-1,2,3-triazole derivative. Antioxidant studies revealed a significant increase in the activities of Superoxide Dismutase (SOD) and Catalase (CAT), along with elevated levels of reduced glutathione (GSH), suggesting enhanced cellular antioxidant defence. While, a non-significant difference was noted between treated and untreated cells for adipocyte differentiation studies, including Oil-Red-O staining and triglyceride estimation. Furthermore, gene expression analysis of Lipoprotein Lipase (LPL) revealed a non-significant decrease ($P > 0.05$), indicating that the compound does not significantly affect LPL expression at the transcriptional level. In conclusion, though the studied compound does not exhibit anti-adipogenic and anti-lipogenic activities, it shows notable antioxidant property. These findings suggest that compound may exert its biological activity through modulation of oxidative stress but not through modulation of adipogenic transcriptional factors.

Keywords: Heterocyclic compounds, 4-phenyl-1H-1,2,3 triazole, antioxidant studies, Oil Red O staining, anti-adipogenic activities.

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Introduction

Obesity is emerging as a major public health challenge worldwide. As per the recent statistics, in United States 40.3% prevalence was reported in adult population, among which 41.3% and 39.2 % prevalence was contributed by women and men respectively [1]. It has also been assumed that by the year 2030 one in every two persons would have obesity and associated complication [2,3]. In India, a sudden rise of 30.5% of obese people has been forecasted [3]. This, worldwide statistics exhibits the the upcoming burden of obesity and its associated complications. Obesity is a disorder mainly characterised by adipose tissue expansion, energy imbalance, hyperplasia, alterations in lipid metabolism, hypertrophy and chronic low-grade inflammation [4]. Adipose tissue is not only important for lipogenesis and lipolysis but also manage various physiological aspects like body mass, weight, vascular functions, insulin sensitivity etc. In mammals, adipose tissues are broadly classified as white adipose tissue (WAT) and brown adipose tissue (BAT) [4]. WAT is mostly present in visceral depot (mesenteric, pericardial, omental) and at subcutaneous depot, and thus responsible for overweight and obesity, conversely,

BAT is rich in mitochondria and thus responsible for heat generation. Altered metabolism responsible to rise adipocyte number and volume contributing overweight and obesity [5]. Moreover, conversion of preadipocytes into mature lipid containing adipocyte is strictly modulated by peroxisome proliferator-activated receptor gamma (PPAR γ), sterol regulatory element-binding protein 1c (SREBP-1c) and CCAAT/enhancer-binding protein alpha (C/EBP α) types of transcription factors for lipid uptake and storage by targeting other genes [5]. Among these genes, expression of lipoprotein lipase (LPL) exhibited its own significance in lipid metabolism. LPL enzyme hydrolyses circulating triglycerides rich lipoproteins such as very-low-density lipoproteins (VLDL) and chylomicrons into free fatty acids and glycerol, facilitating the uptake and storage of fatty acids in adipose tissue [6,7]. Thus, LPL is a rate limiting enzyme for lipid accumulation in adipocytes. Altered gene expression and activity of LPL have been reported for dyslipidaemia and metabolic abnormalities, signifying its importance as a molecular target in obesity research [8]. Heterocyclic compounds contain one or more other atoms such as nitrogen, oxygen, sulphur etc. other than carbon; forming a heteroatom ring structure [9].

These heteroatomic structures defines the reactivity, electronic distribution and overall, three-dimensional shape of the molecule and hence, provide higher biological advantageous properties over pure carbon-based cyclic rings. These compounds have been reported for the formation of core structure of the drugs by favouring various bondings including hydrogen, π - π , and electrostatic interactions with proteins, enzymes, and nucleic acids. Therefore approximately 90% of newly discovered pharmaceutical compounds focuses on the heterocyclic rings [10,11]. For example, heterocycles like triazoles and thiazoles have been prodigiously studied for their antimicrobial and anticancer activities, while nitrogen-based pyridines and imidazole reported significant for cardiovascular and CNS drugs [12]. Study of such versatile heterocyclic compounds as LPL activity modulator may represents a rational strategy to identify novel chemical ring structures for adipogenesis and metabolic homeostasis. Previous studies reported that phenyl substituents in a chemical drug enhances hydrophobic interactions with enzyme active sites, while the triazole moiety reported to contribute strong binding through polar interactions that may modulate lipid metabolism [13]. Therefore, this study focuses on the synthesis of phenyl and triazole based derivative and its potential to inhibit the lipid biosynthesis in 3T3L1 cells.

Materials and Methods

Synthesis of heterocyclic compounds (4-phenyl-1H-1,2,3-triazole derivative)

The compound was synthesised as per the method described by Ledade *et al* [14].

Characterisation of heterocyclic compounds

Synthesised compound was characterised using ^1H NMR technique, ^{13}C NMR and the HRMS (High-Resolution Mass Spectrometry). HRMS was determined utilizing the Waters LCT Premier XETOF ARE-047 apparatus.

Cell Line Maintenance

The effect of the synthesised heterocyclic compound was evaluated on mouse embryonic fibroblast cells 3T3L1. Cells were purchased from National Centre for Cell Science (NCCS) Pune, India and maintained using Dulbecco's Modified Eagle Medium (DMEM) (Himedia) with 10% Fetal Calf Serum (FCS)/ Fetal Bovine Serum (FBS) (Himedia) with 1% Penicillin and streptomycin (Himedia) using 5% CO₂ at 37°C in CO₂ incubator (Sartorius). Experiments were done on 2nd-passaged cells.

Cell Viability assay

Cell viability of 3T3L1 cells was performed using MTT assay as per the method described by Kumar *et al* [15] with and without the synthesised compound (treated and untreated), in triplicates. The IC₅₀ (Half Maximal Inhibitory Concentration) value was calculated using percent cell inhibition and using Graph Pad Prism software version 11.1.0 (224).

Adipocyte differentiation assay

The adipocyte differentiation protocol was performed as per the method given by Kwak *et al* [16]. Protocol was performed in triplicate with (treated) and without (untreated) the synthesised compound.

Oil-Red-O staining

After post induction, differentiated cells were stained using Oil-Red-O stain as per the method described by Durge *et al*. [17].

For quantification of stained lipid, dye was eluted using 100% isopropanol for 15 minutes with gentle shaking and absorbance was measured at 520 nm.

Lipid estimation

Lipid estimation was done using a standard triglyceride assay kit (Yumizen CR-RTRG0923).

Antioxidant Assay

All standard methods were used to determine enzymatic and non-enzymatic antioxidants. Concentration of non-enzymatic antioxidant glutathione was estimated as per the method given by Beutler *et. al*. [18], enzymatic activity of catalase was determined as per the method given by Aebi *et. al*. [19] and superoxide dismutase activity was assayed by Markland and Markland *et al*. [20]

Quantitative Real Time (RT) PCR

Differentiated cells were washed twice with PBS and RNA was isolated using TRIZOL reagent (Invitrogen CA, USA) as per the standard isolation procedure. PCR reaction was performed (Thermo Scientific Applied Biosystem Quant Studio 1 Real Time PCR) in a final volume of 20 μl consisting of 2 μl cDNA, dNTP 1 μl , 100 nmol primers, LPL (F: 5' TCCAAGGAAGCCTTTGAGAA 3'; R: 5'- CCATCCTCAGTCCCAGAAAA 3'), β -actin was endogenous control (F: 5-ACCGTGAAAAGATGACCCAG-3' R: 5'-TACGGATGACAACGTACAC-3' and 10 μl power up SYBR green master mix (Applied Biosystem-A25741). Amplification was performed with initial incubation at 95 °C for 10 minutes and then 40 cycles repeating 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec. The resultant amplicons were electrophoresed using 1% agarose gel, stained with ethidium bromide and imaged using a Gel Doc imaging system (Biorad, USA) [21,22].

Statistical Analysis

All statistical analysis were done using Med Calc and Graph Pad Prism statistical software (version 11.1.0 (224). Student "t" test was used to compare grouped data. Data was represented in Mean $\pm$ SD. P < 0.05 was considered as a significant level.

Results

Synthesis and characterisation of heterocyclic compound (4-phenyl-1H-1,2,3-triazole derivative)

Post-synthesis, 4-phenyl-1H-1,2,3-triazole was obtained as a solid off-white material with (97%) yield, indicating the efficiency of the solvent-free ball-milling approach. Spectral data for the compound 4-phenyl-NH-1,2,3-triazole was found as:

(off-white solid (yield = 97%), ^1H NMR (CDCl₃, 500 MHz): 14.47 (1H, bs), 7.92 (1H, s), 7.83 (J = 8.0 Hz, 2H, d), 7.43 (J = 10.0 Hz, 2H, t), 7.34 (J = 9.0 Hz, 1H, t); ^{13}C NMR (CDCl₃, 125 MHz): 147.2, 129.0, 128.8, 127.6, 126.7, 126.1; HRMS: m/z [M+H]⁺ calcd. for C₈H₇N₃: 146.0709; found: 146.0720). The ^1H NMR spectrum (CDCl₃, 500 MHz) showed a broad singlet at δ 14.47 ppm corresponding to the proton (NH) of the triazole ring, confirming the formation of the 1H-tautomer. A characteristic singlet at δ 7.92 ppm was showed a triazole C-H proton, while the phenyl ring containing aromatic protons exhibited as two doublets at δ 7.83 ppm (J = 8.0 Hz, 2H) and multiplets at δ 7.43–7.34 ppm (3H), consistent with a monosubstituted phenyl group. The Six distinct signals at δ 147.2, 129.0, 128.8, 127.6, 126.7, and 126.1 ppm was showed by the ^{13}C NMR spectrum (CDCl₃, 125 MHz), representing the aromatic carbons and triazole.

However, the formation of heterocyclic ring was confirmed by the downfield signal at δ 147.2 ppm which was corresponded to the triazole carbon attached to nitrogen. Also, HRMS analysis showed a molecular ion peak at m/z 146.0720 $[M+H]^+$, which is in closure to the value of 146.0709 for $C_8H_7N_3$, favouring the molecular formula of the synthesized compound. Thus, 1H NMR, ^{13}C NMR, and HRMS data confirmed the formation of 4-phenyl-1H-1,2,3-triazole compound (Fig. 1).

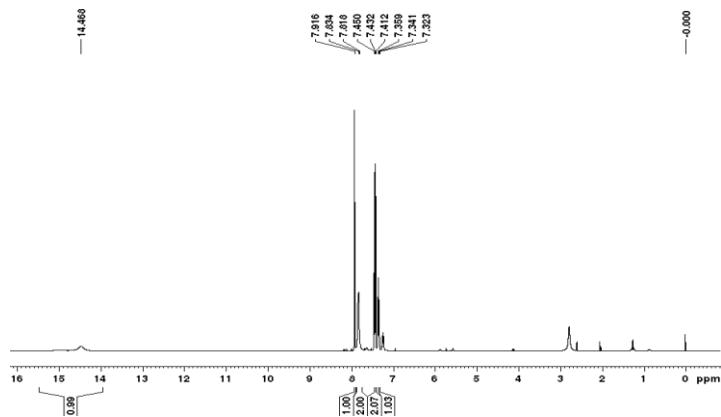


Figure 1 1H NMR of 4-phenyl-NH-1,2,3-triazole.

Cell viability assay and calculation of IC_{50} value

The effect of synthesised compound was assessed for cell viability using the MTT assay in 3T3L1 cells. As shown in the Fig. 2, the vehicle control group showed nearly 100% cell viability which was gradually decreases upon increasing compound concentration (5-100 $\mu g/mL$). A significant decrease in cell viability was reported when cells were treated with 5 $\mu g/mL$ (65.77 ± 2.72), 12 $\mu g/mL$ (54.15 ± 3.09), 25 $\mu g/mL$ (49.12 ± 4.72), 50 $\mu g/mL$ (41.49 ± 2.17) and 100 $\mu g/mL$ (31.86 ± 1.56) when compared to vehicle control (100.01 ± 0.01). These results were used to find percent inhibition of the cells and used to generate a dose-dependent curve (figure 2b), which showed IC_{50} value 5.7 $\mu g/mL$ with a correlation coefficient (R^2) of 0.97, indicating a good fit of the model. This compound concentration was used for the further studies.

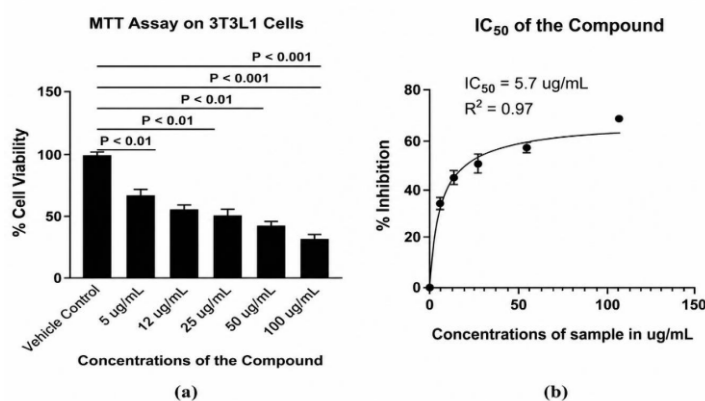


Figure 2: Cell viability assay of studied compound on 3T3L1 cells (a). Significant differences were shown between vehicle control untreated cells (0 $\mu g/mL$), and cells treated with 5 $\mu g/mL$, 12 $\mu g/mL$, 25 $\mu g/mL$, 50 $\mu g/mL$ and 100 $\mu g/mL$. IC_{50} graph (b) for the studied compound (IC_{50} value reported as 5.7 $\mu g/mL$).

Oil Red O staining of 3T3 L1 cells

In preliminary examination for the anti-adipogenic activity, the effect of synthesised compound in 3T3L1 adipocyte cell differentiation was evaluated using Oil-Red-O staining procedure after 12 days of induction. As shown in Fig. 3, both control (untreated) (a, b and c) and cells treated with the compound (d, e and f) showed intracellular lipid accumulation, suggesting a successful cell differentiation. However, similar intensity and distribution of red-stained lipid droplets throughout the flask suggesting somewhat similar lipid accumulation in untreated and treated cells. The extent of lipid droplet formation appeared consistent across all samples, suggesting that the compound did not markedly affect adipocyte differentiation under the experimental conditions. These results suggest that the synthesised compound does not significantly inhibit lipid accumulation in 3T3-L1 cells, as assessed by Oil-Red-O staining.

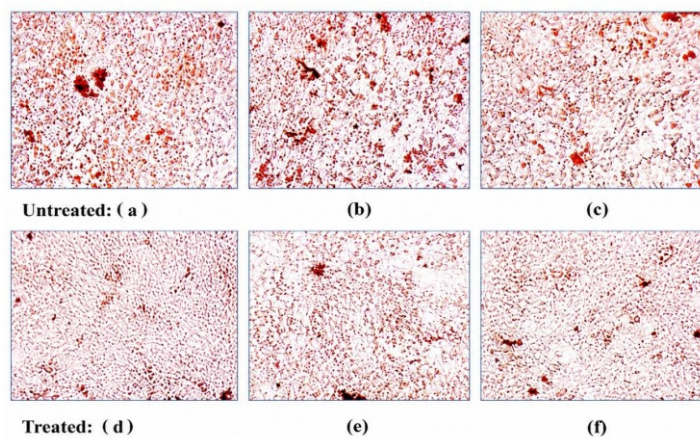


Figure 3: Adipocyte differentiation in 3T3-L1 cells analysed by Oil Red O staining after 12 days. Untreated cells (Control; a, b and c) showing lipid accumulation. Compound-treated cells (d, e and f) also showing lipid accumulation. Images were captured at 10X magnification.

Estimation of Lipid

Oil-Red-O staining results were further validated by estimation of stained lipid and by direct quantification of triglycerides from the cells. Stained lipids with Oil-Red-O stain were estimated using isopropanol exhibited a non-significant ($P > 0.05$) level of lipids between untreated (100.02 ± 0.01) and treated cells (110.29 ± 8.30), indicating that the compound does not significantly influence lipid accumulation as measured by dye retention (Fig. 4a). Similarly, direct estimation of intracellular triglyceride levels demonstrated comparable lipid content between untreated and treated groups (Fig. 4b). Although a marginal increase in triglyceride levels was observed in treated cells (101.33 ± 8.32) as compared to untreated cells (92.00 ± 8.00), the difference remained statistically non-significant ($P > 0.05$). Overall, both methods indicate that the synthesised compound does not significantly alter lipid accumulation in differentiated 3T3-L1 cells. These findings corroborate that under the studied conditions the compound does not exert a definite significant effect on adipocyte lipid storage.

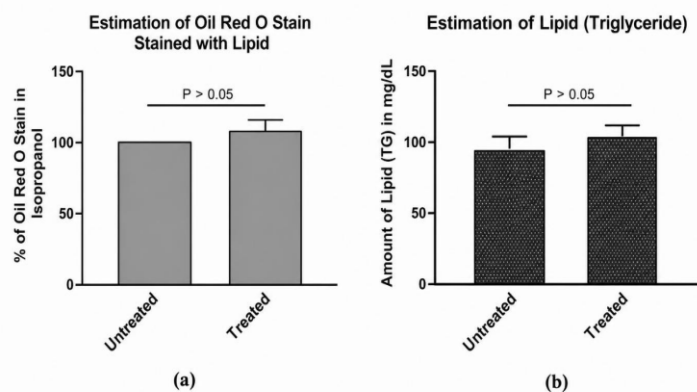


Figure 4: Quantitative estimation of lipid accumulation in differentiated 3T3-L1 cells. (a) Indirect measurement of lipid content using Oil Red O staining, expressed as percentage of extracted stain in isopropanol. (b) Direct estimation of intracellular triglyceride (TG) levels (mg/dL). Cells were treated with the synthesized compound compared with untreated cells. Data are presented as Mean $\pm$ SD ($n = 3$). No statistically significant difference was observed between the studied groups ($P > 0.05$).

Determination of antioxidant activity

The antioxidant potential of the synthesized compound was evaluated in 3T3-L1 cells by measuring the activities of key antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), and reduced glutathione (GSH). As shown in Fig. 5 a, catalase enzyme activity was significantly increased in treated cells (1.7543 ± 0.21) as compared to untreated cells (1.1876 ± 0.13) ($P < 0.05$), suggesting enhanced detoxification of hydrogen peroxide. Likewise, an increased activity of the superoxide dismutase enzyme (Fig. 5 b), was observed in treated cells (6.3926 ± 0.71) as compared to untreated cells (1.4080 ± 0.29) with a highly significant increase ($P < 0.001$), suggesting improved dismutation of superoxide radicals. Also, the level of reduced glutathione (Fig. 5 c) was significantly increased in treated cells (0.01801 ± 0.002) as compared to untreated cells (0.01227 ± 0.001) ($P < 0.05$), suggesting an overall enhancement of cellular antioxidant capacity and improved redox status. Succinctly, these results suggest that the synthesized compound significantly enhances both enzymatic (CAT and SOD) and non-enzymatic (GSH) antioxidant defence mechanism in 3T3-L1 cells, demonstrating its potential to alleviate oxidative stress.

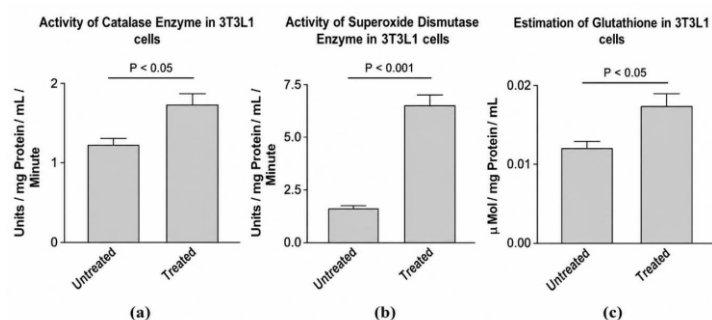


Figure 5: Assay of activities of Catalase (a), SOD (b) and estimation of GSH (c) in treated and untreated cells. Data are presented as Mean $\pm$ SD ($n = 3$). Statistical significances are represented by P value.

Gene expression analysis

Oil-Red-O staining and lipid estimation findings were further validated using LPL gene expression study. LPL is a key enzyme which modulates lipid metabolism; hence the present study targets this gene. Agarose gel electrophoresis of the amplified cDNA (Fig. 6 a) revealed somewhat similar band intensities of LPL gene expression in both untreated and treated cells. β -actin was used as an endogenous control to ensure equal loading and normalization. The similarity in band intensity suggests that the compound did not markedly alter LPL gene expression at the transcriptional level. This observation was further supported by the quantitative analysis of gene expression (Fig. 6 b). The fold change in LPL gene expression in treated cells (1.19 ± 0.30) showed a slight decrease compared to untreated cells (1.29 ± 0.50); however, this reduction was not statistically significant ($P > 0.05$). Overall, both qualitative (agarose gel) and quantitative (fold change analysis) results indicate that the studied compound does not significantly modulate LPL gene expression.

Discussion

Mass analysis and Spectroscopic studies showed successful synthesis of the 4-phenyl-1H-1,2,3-triazole compound. A broad-spectrum singlet at δ 14.47 ppm corresponding to the NH proton of the triazole ring, which is in accordance with previously reported studies stated a downfield NH signal is due to a strong intramolecular hydrogen bonding and aromatic stabilization^{13,23}.

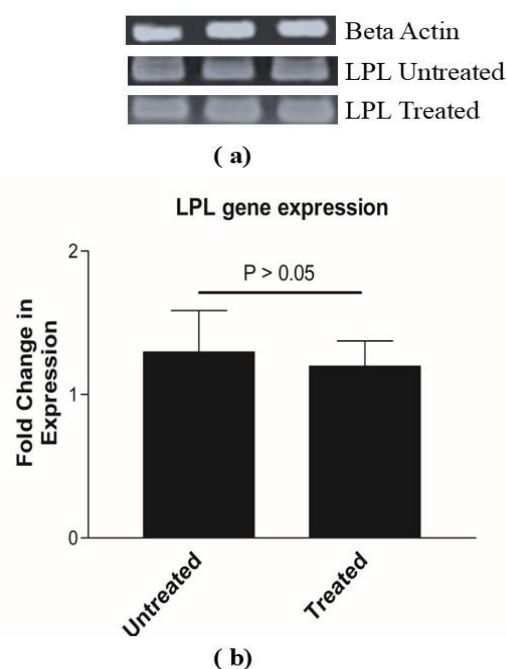


Figure 6: Gene expression of LPL gene in 3T3L1 cells. (a): Agarose gel picture of expressed gene in untreated and treated cells. β -actin was taken as an endogenous control. (b): Fold change in expression was compared between untreated and treated cells. Data are presented as Mean $\pm$ SD ($n = 3$). Statistical significances are represented by P value.

C-H proton is a basic feature of the 1H-1,2,3-triazole molecule which is attributed by δ 7.92 ppm²⁴ while a monosubstituted phenyl ring is in consistent with the phenyl substituted triazoles²⁵. Six distinct carbon signals are revealed by the ¹³C NMR spectrum.

Such carbon resonances associated with multiple nitrogen atoms are often considered as the unique characteristics of the heterocyclic compounds^{13,26}. Further, the HRMS data confirming the correct molecular structure and high purity of the synthesised compound. These findings strongly corroborate with the previously reported studies on 1,2,3-triazole derivatives, highlighting distinct spectroscopic properties and structural stability. To test biological functions of the synthesised compounds such confirmed structure is essential and reliable [13, 23-25]. The MTT assay was performed to determine IC 50 value of the synthesised compound using dose response curve. The relatively good correlation coefficient ($R^2 = 0.97$) supports the reliability of the dose-response curve. The observed IC 50 value may associate with moderate to potent biological properties as per the previously reported study [26]. Increased oxidative stress, redox imbalance, altered enzyme activities and modulation of the associated pathways triggered by the cytotoxic and anti-proliferative effect of the drugs; causes decreased cell viability [24, 27-29]. The effect of synthesised compound on 3T3L1 cells using Oil-Red-O stain was evaluated and no major significant decrease in cellular lipid staining was noted between treated and untreated groups, as reported in previous studies [31]. This finding indicating that under the testing conditions the synthesised compound does not markedly alter the adipocyte differentiation. This result was further supported by the quantification of stained lipids using isopropanol and direct triglyceride estimation. Results showed that, as compared to the untreated control cells the treated cells did not produce a statistically significant change in lipid accumulation. In 3T3L1 cells, to determine intracellular lipid accumulation during adipocyte differentiation Oil-Red-O stain is widely used; as it specifically binds only with the triglyceride [32]. Adipocyte differentiation is a complex process regulated by multiple transcription factors like PPAR γ , SREBP-1c, C/EBP α etc. Adipokine genes responsible for the modulation of lipid metabolism are regulated by these transcription factors. Although lipid levels remained unchanged in this study, it is possible that the studied compound modulates specific molecular targets rather affecting the overall lipid storage, showing that lipid accumulation, adipogenesis and metabolic enzyme regulation may occur through independent cellular mechanisms [33,34]. Both Oil-Red-O stain quantification and triglyceride estimation between treated and untreated cells, confirming that the compound does not significantly alter lipid accumulation. Similar observations where compound influence metabolic gene expressions, adipogenesis and enzyme activities independently without altering lipid accumulation was reported previously [33]. Therefore, a non-significant change in lipid accumulation between treated and untreated cells suggests that the synthesised compound may not alter the morphological differentiation of adipocytes, but could still influence some other underlying metabolic/ enzymatic processes such as redox biology/antioxidant potential. In this study, key antioxidant enzymes are targeted to evaluate antioxidant potential of the synthesised compound. Mitochondrial dysfunctions generate free radicals responsible for the oxidative stress, which leads to DNA damage, inactive enzymes etc. and finally cell death. Enzymatic and non-enzymatic antioxidants prevent this cell damage by scavenging free radicals via specific mechanisms. Among them, dismutation of superoxide radical through SOD into hydrogen peroxide and glutathione-based reactive oxygen species (ROS) detoxification using enzymes like glutathione peroxidase (GPX) and glutathione reductase (GR) play significant role in combating

cellular oxidative stress. In this study, increased activities of catalase, superoxide dismutase (SOD), and elevated levels of reduced glutathione (GSH) demonstrated significant enhanced antioxidant defence mechanisms in 3T3-L1 cells. The observed increase in SOD activity indicates enhanced dismutation of superoxide radicals into hydrogen peroxide, while the increased activity of catalase is possibly due to detoxification of this hydrogen peroxide. This coordinated activities of SOD and catalase may reduce intracellular oxidative stress [35,36]. The significant increase in intracellular GSH levels may reduce ROS via glutathione dependent enzymes. The elevated level of the GSH suggests that the compound may favour redox balance to prevent cell damage from oxidative stress [37]. Thus, the studied compound significantly favours both enzymatic and non-enzymatic antioxidant defence mechanisms. Previous studies, suggesting the similar results of the triazole derivatives via modulation of cellular defence systems for enhanced antioxidant properties [38]. As in this study, only key antioxidants are studied, further studies on antioxidant mechanisms and validations are required.

Lipoprotein Lipase (LPL) enhance lipid uptake and storage in adipocytes by hydrolysing circulating triglycerides into free fatty acids. Present study exhibited a non-significant LPL gene expression in treated and untreated cells. This is possibly due to the fact that, under experimental condition the synthesised compound does not directly activate the transcriptional factor like (PPAR γ), SREBP1c and (C/EBPs), regulating the key lipogenic and adipogenic genes [39]. A biological activity of a drug/compound chiefly relies on its ability to modulate enzyme activity, post-transcriptional regulation and alternative metabolic targets rather than direct gene expression changes. Conversely, previous studies have been shown that triazole-based derivatives exhibit significant anti-lipase activity, suggesting their potential role in modulating lipid metabolism pathways [40]. However, the synthesised heterocyclic compound with 4-phenyl-1H-1,2,3-triazole derivative could not exhibit such findings, possibly due to varied triazole structure and as multiple other parallel pathways contribute to the lipid homeostasis.

Conclusion

Techniques like ¹H NMR, ¹³C NMR, and HRMS showed successful synthesis of the compound via a solvent-free ball-milling approach. A significant enhanced activities of antioxidants like SOD, catalase, and GSH suggesting a potential antioxidant property of the compound. Moreover, lipid accumulation studies, including Oil Red O staining and triglyceride estimation in treated and untreated cells demonstrated a non-significant difference, indicating that the compound is unable to inhibit lipid accumulation. These findings were further validated by the LPL gene expression analysis. A non-significant expression of LPL gene showed that the compound does not significantly modulate LPL gene expression at the transcriptional level, although the synthesised 4-phenyl-1H-1,2,3-triazole derivative demonstrated antioxidant potential, it does not lower lipid accumulation. Thus, further studies are required to elucidate the potential role of this compound on lipid homeostasis.

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