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Influence of α-Phellandrene on Oxidative stress in high Glucose induced insulin resistant 3T3-L1 adipocytes

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ABSTRACT

Hyperglycaemia induced oxidative stress and insulin resistance in adipose tissue impairs adipogenesis causing disturbed glucose homeostasis and dyslipidemia. Phytochemicals with antioxidant properties are gaining importance in combating oxidative stress associated metabolic disorders. The role of α-phellandrene, a monoterpene presents in the essential oil of various medicinally important spices on adipogenesis under hyperglycaemic and insulin resistant 3T3-L1 adipocytes remains unclear. Mature 3T3-L1 adipocytes were exposed to glucose (25mM) and insulin (0.6nM) to induce insulin resistance. These IR-3T3-L1 adipocytes were treated with α-phellandrene (65μM) and its effect on mitochondrial membrane potential (MMP), reactive oxygen species (ROS), activities of enzymic antioxidants superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidise (GP_X), Glutathione-s-transferase (GST), Glutathione reductase (GR) and reduced glutathione (GSH) and lipid peroxidation were studied. Further the binding affinities of α -phellandrene with Nrf2-Keap1 was also analysed by docking studies. The standard drug rosiglitazone (0.1 μM) was taken as positive control. In IR-3T3-L1 adipocytes, the endogenous antioxidant levels were significantly reduced with increases in ROS and lipid peroxidation products. On treatment with α-phellandrene, the activities of SOD, CAT, GP_X, GST, GR and GSH were significantly improved with decreases in intracellular ROS and lipid peroxidation. In silico analysis revealed that α-phellandrene binds with Nrf2 (-5.3 kcal/mol) causing its activation. From this we conclude that α-phellandrene enhanced antioxidant defences in high glucose induced insulin resistant 3T3-L1 adipocytes.

Keywords: 3T3-L1 adipocytes cells; α -phellandrene; Antioxidant enzymes; ROS; Nrf2-Keap.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycaemia that arises due to defective β-cell function and insulin resistance. Genetic, environment and lifestyle factors are significant determinants of DM. This disorder impairs the functionality of various organs leading to acute and chronic complications [1]. According to International Diabetes Federation report of 2019, approximately 463 million diabetic cases are reported globally and the number is anticipated to increase to 578 million by 2040 [2]. The most common form of diabetes is type 2 which is characterised by insulin resistance in target organs such as liver, skeletal muscle and adipose tissue.

Adipose tissue is a complex endocrine organ that serves as a 'master regulator' of systemic energy homeostasis [3]. Initially it was considered as reservoir of free fatty to support energy demands during fasting conditions. Recently it has been found that adipose tissue secretes a wide array of biologically active components that play a pivotal role in insulin sensitivity, food intake and inflammation [4]. Under hyperglycaemic condition, reactive oxygen species (ROS) diminish insulin sensitivity in adipocytes leading inflammation and dyslipidemia. It has been well-established that excessive production of ROS with decline in endogenous antioxidant level is implicated in pathogenesis and complications of diabetes mellitus.

α-Phellandrene (α-PA) is a cyclic monoterpene present in the essential oils of *Matricaria chamomilla L., Zingiber officinale Rosco., Schinus mole, Schinus terebinthifolius Raddi and artemisia feddei* etc. Several studies have documented the hypolipidemic, anticancer and anti-inflammatory properties of α-PA [5]. In this context, the present study was designed to evaluate the effect of α-PA on oxidative stress markers in high glucose induced insulin resistant 3T3-L1 adipocytes (IR-3T3-L1).

MATERIAL AND METHODS

Chemicals:

α-Phellandrene, Dulbecco's modified Eagle medium (DMEM), Foetal bovine serum (FBS) and antibiotic-antimycotic solution, insulin and 3-isobutyl methylxanthine (IBMX), were purchased from Sigma Aldrich Pvt.Ltd, India. All other reagents and chemicals used were of analytical grade.

Cell Culture and differentiation:

The 3T3-L1 preadipocytes were purchased from NCCS, Pune, India. The cells were cultured in growth medium containing DMEM with normal glucose enriched with 10% FBS, 100 units/mL of penicillin and 100µg/ml of streptomycin in 5% CO_2 at 37°C. After confluence, the cells were placed in differentiation medium containing dexamethasone (1µM), IBMX (0.5 mM) and insulin (1µg/ml) in DMEM containing 10% FBS. The differentiation medium was replaced every 2 days until mature adipocytes were formed.

Establishment of IR-3T3-L1adipocyte cell model:

Mature 3T3-L1 adipocytes were exposed to high glucose (25mM) with 0.6nm/L of insulin for 24 hrs to convert to IR-3T3-L1 adipocytes [6]. The residual glucose in control group and model group were evaluated to establish to onset of insulin resistance.

MTT Viability Assay:

The Cell viability was determined by MTT assay. Briefly, the 3T3-L1 adipocytes were cultured in 96 well plates and treated with α-PA (10-100 µM) for 24 hrs. Subsequently, cell viability was determined by incubation with MTT solution in DMEM at 37 ^{0} C for 4 hrs, followed by dissolving the violet-formazan crystals with 100 µL DMSO. The absorbance at 570 nm was recorded [7].

Experimental design:

Mature adipocytes and IR-adipocytes were grouped as follows:[8]

Group1: Normal Control: Mature 3T3-L1 adipocytes were exposed to normal glucose (5mM) with 0.6nm/L of insulin for 24 hrs.

Group 2: Diabetic Control (IR-3T3-L1 adipocytes)

Group 3: IR-3T3-L1 adipocytes with α-PA: IR-3T3-L1 adipocytes were treated with α-PA (65 µM) for 24 hrs.

Group 4: IR-3T3-L1 adipocytes with Rosiglitazone: IR-3T3-L1 adipocytes were treated with standard drug rosiglitazone (0.1µM) for 24 hrs.

Mitochondrial membrane potential (ΔΨm):

The changes in the MMP of IR-3T3-L1 cells treated with α-PA were assessed by previously mentioned method. The cells were incubated with JC-1 (10 µM) for 20 min at 37 °C and the fluorescence were analysed spectrophotometrically at 579 nm(excitation) and 644 nm (emission) wavelengths [9].

Determination of Intracellular ROS:

The intracellular ROS levels were quantified by using a DCFH-DA fluorescent dye method [10]. The IR-3T3-L1 and α-PA treated cells were washed twice with phosphate-buffered saline and incubated with DCFH-DA (25 µM) for 1 h in 5% CO_2-humidified atmosphere at 37°C. The cells were then washed twice with PBS and the intensity of fluorescence was measured at 485 nm (excitation) and 530 nm (emission) wavelengths.

Measurement of antioxidant and Lipid peroxidation Markers:

The cell lysates of IR-3T3-L1 and the α-PA treated adipocytes were assayed for the activities of enzymatic antioxidant (SOD, CAT, GP_X, GST and GR) and for the levels of GSH and lipid peroxidation products as per the previously described procedures [11-17].

In silico docking studies:

The interactions between α-PA and target proteins Nrf2-Keap1 (PDB ID: 4ZY3) was analysed using AutoDock (V. 4.0) in Pyrx software. The docking analyses of α-PA were visualized by Biovia discovery studio visualiser 2020. The molecular interaction was calculated based on the binding energy (kcal/mol) [18].

Statistical Methods:

Experimental values are expressed as mean ± standard deviations of three experiments. Data were analysed by using one-way ANOVA. The level of $P \leq 0.05$ was used as statistical significance. All calculations were done using the SPSS 26.0 version.

RESULTS AND DISCUSSION

Effects of α-PA on cell viability:

The survival rate of 3T3-L1 cells treated with various concentrations of α-PA (10–100 µM) was greater than 95% as shown in the figure 1. The IC_{50} value of α-PA was found to be 65µM.

Effect of α-PA on MMP in 3T3-L1 Cells:

Several studies highlight the link between mitochondrial dysfunction and insulin resistance. MMP and intracellular ATP levels reflect the functioning ability of mitochondria. In our study, we observe that in IR-3T3-L1 adipocytes, the levels of MMP were significantly decreased when compared to normal control. On exposure to α-PA (65 µM) for 24 hrs, the MMP levels were significantly improved (Figure 2). Chun-Lin et al reported that treatment of linalool improved the MMP levels in insulin resistant 3T3-L1 adipocytes [19].

Role of α-PA and **intracellular ROS levels:**

In IR-3T3-L1 adipocytes, the ROS level was significantly increased by 98.02%, when compare to the normal control. Exposure with α-PA (65 µM) for 24 hrs significantly decreased ROS levels by 37.88% (Figure 3). Chronic hyperglycaemia, autooxidation of glucose and activation protein kinase C were associated with exaggerated production of ROS which affects the normal functioning of adipocytes. Under physiological conditions, the by-product of electron transport system represents the major source for ROS. However, in mitochondrial dysfunction especially in insulin resistant condition, decreases in MMP levels is associated with increased mitochondrial ROS production and damage. Further, complex I and III on the mitochondrial electron transport chain act as major generators of intracellular ROS under insulin resistance conditions. Agents that improve MMP levels significantly decrease ROS by ameliorating mitochondrial function and improve insulin sensitivity [20].

Influence of α-PA on antioxidant levels:

Antioxidants are considered as potential therapeutic agents in the treatment of oxidative stress associated diseases. They sequester free radicals/chelate metal ions and prevent the deleterious effects of oxidation by ROS in human tissues. Thus, antioxidants prevent the initiation of lipid peroxidation. A decrease in the activities of Superoxide dismutase (SOD) (50.77%), Catalase (CAT) (65.96%), Glutathione peroxidase (GP_x) (63.72%), Glutathione-s-transferase (GST) (48.48%) and glutathione reductase (GR) (58.55%) were observed in IR-3T3-L1 adipocytes. Treatment with α-PA **(**65µM) significantly improved the antioxidant levels to near normal. Similarly in IR-3T3-L1 adipocytes, the levels of intracellular GSH were found to be significantly decreased. However, treatment with α-PA (65 µM) for 24 hrs, improved GSH levels (Table 1). Rosiglitazone showed similar effects to that of α-PA. Recent studies show that isopulegol, a dietary monoterpene decreased ROS by upregulating the intracellular antioxidant levels in adipocytes of high fat/streptozotocin induced diabetic rats [21].

Role of α-PA on Lipid peroxidation:

Lipids are the primary targets of ROS and the generated hydroperoxides have toxic effects on cells *per se* and through degradation to highly toxic hydroxyl radicals that propagates the free-radical pathway. The increase in lipid peroxidation is associated with decline in endogenous antioxidant defence mechanisms. Lipid peroxidation affects cellular structures and is responsible for the development of secondary chronic complications such as atherosclerosis and neural disorders in diabetes mellitus. In IR-3T3-L1 adipocytes, the levels of lipid peroxidation products (TBARS) were found to be increased (51.69%) when compared to normal control cells. A significant reduction (38.57%) in TBARS levels were observed in α-PA treated group (Figure 4). Priyanka *et al.,* reported that curcumin reduced TBARS level in 3T3-L1 adipocytes [22].

Binding affinities of α-PA with Nrf2-keap1:

The Nrf2-Keap1 pathway is the major protective response to oxidative stress conditions. Under physiological conditions, Keap1 tightly regulates the activity of the transcription factor Nrf2 by targeting it for ubiquitination and proteasome-dependent degradation. In response to stress, an intricate molecular mechanism facilitated by sensor cysteines within Keap1 allows Nrf2 to escape ubiquitination, accumulate within the cell, and translocate to the nucleus, where it can promote its antioxidant transcription program. Recent advances have revealed that Keap1 contains multiple stress sensors and inactivation modalities, which together allow diverse cellular functions such as oxidative stress and dysregulated autophagy to regulate Nrf2 activity.

In docking studies, we found that α-PA interacts with Cys 583, Lys 565, Arg 551, Pro 549, Asp585, Ser 592, Val 594, Asn 532 and Met 580 residues of Keap1 with a binding energy of -5.3 kcal/mol and prevents its interaction with Nrf2 (Figure 5). This interruption in Nrf2-Keap1 interaction potentially activates Nrf2 causing its translocation to nucleus to upregulate the antioxidant mechanisms. Recent studies show that resveratrol inhibits Nrf2-Keap1 activation and improves antioxidant activities [23].

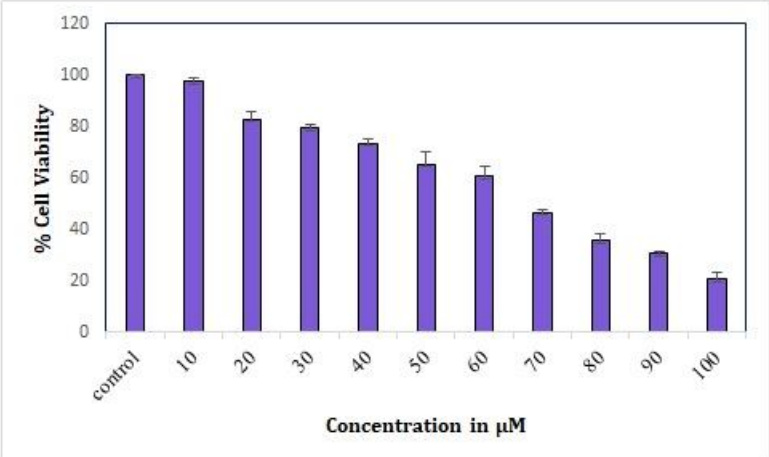

Figure 1: Effects of α-PA on cell viability. Values are shown as the mean ± SD (n = 3). P< 0.001 vs control.

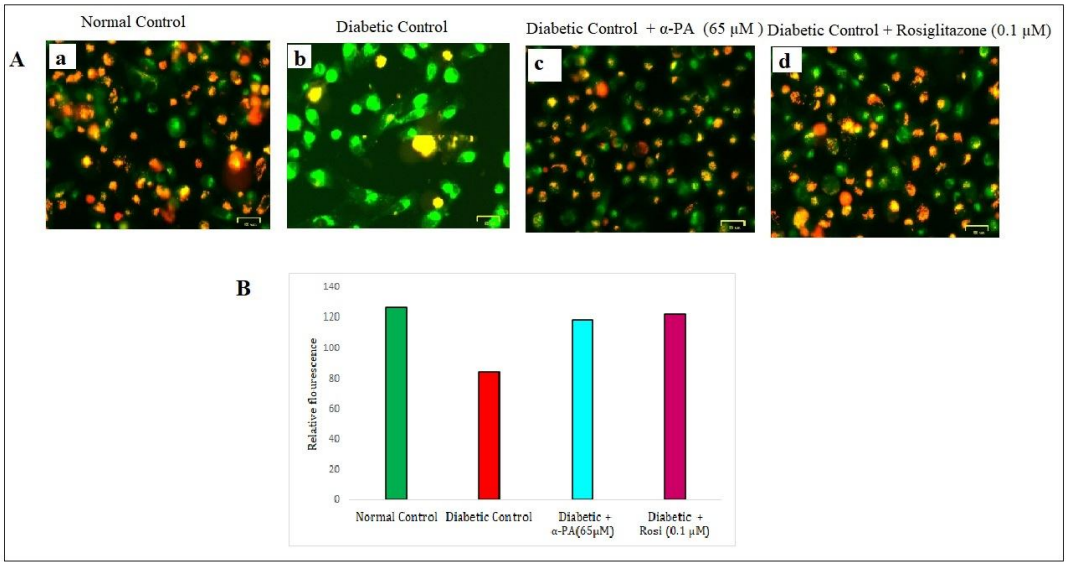

Figure: 2:(A) Effects of α-PA on MMP in experimental adipocytes. (**B**) The relative fluorescence intensity of MMP in experimental cell lines

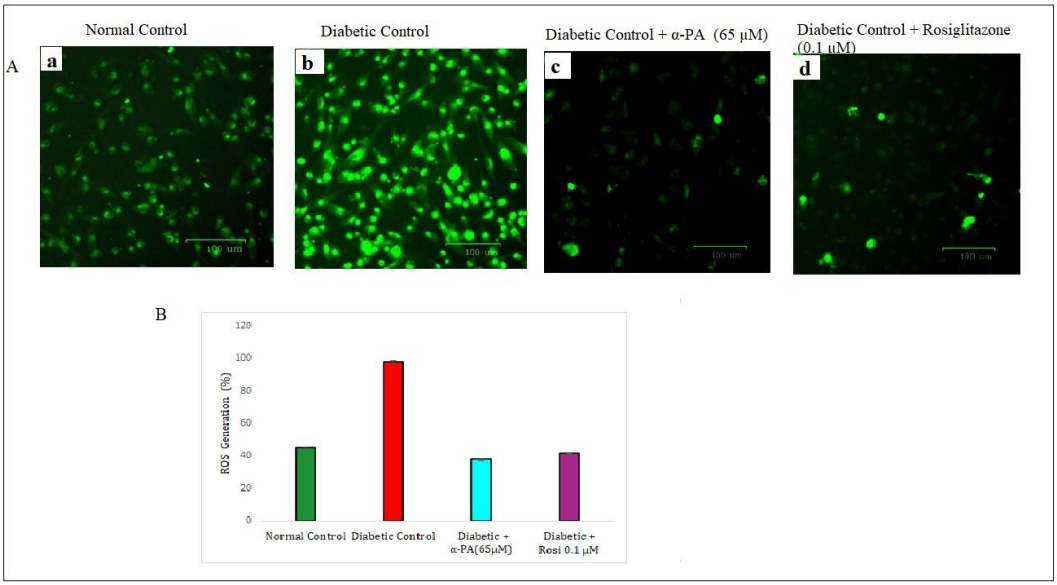

Figure 3 (A): Effects of α-PA on intracellular ROS content in cells treated with DCFH-DA dye. **(B)** Effects of α-PA on intracellular ROS as expressed in percentage. Data are expressed as mean ± SD (n = 3).

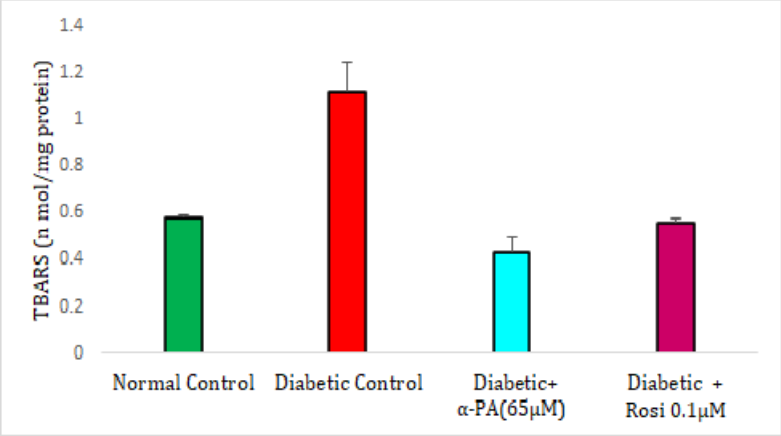

Figure 4: Effect of α-PA on TBARS levels in experimental cell lines. Values are means, with SD (n = 3). Mean value is significantly different from the control cells (P < 0.001).

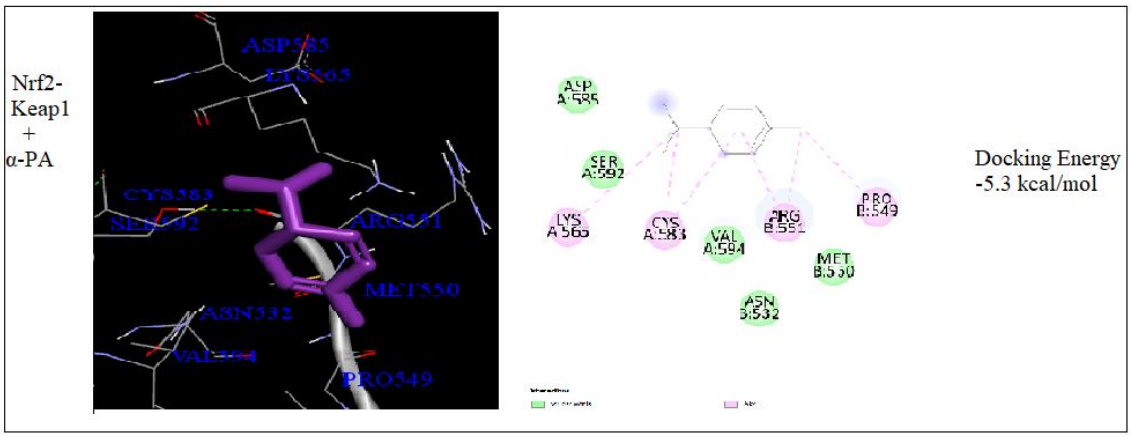

Figure:5: The molecular interaction between α-PA with the target proteins Nrf2- Keap1.

Table 1: Effect of α-PA on enzymic and non-enzymic antioxidant in experimental cell lines. Values are expressed as mean ± SD (n=3). P<0.001 significant difference from control.

GROUPS	SOD (Units/mg Protein)	CAT (Units/mg Protein)	GPX (nmol/mg protein)	GST (nmol/mg protein)	GR (U/mg Protein)	GSH (nmol/mg protein)
Normal Control	6.46 ± 0.36[a]	4.26 ± 0.32[a]	9.29 ± 0.33[a]	1.32± 0.04[a]	5.26 ± 0.43[a]	7.25±0.05[a]
Diabetic Control	3.28 ± 0.24[b]	2.81 ± 0.14[b]	5.92 ±0.11[b]	0.64 ±0.02[b]	3.08 ±0.30[b]	4.40±0.04[b]
Diabetic + α-PA (65µM)	4.39 ± 0.28[c]	3.55 ± 0.29[c]	7.25 ± 0.35[c]	0.94 ±0.03[c]	4.05±0.07[c]	6.03±0.06[c]
Diabetic +Rosiglitazone (0.1nM/ml)	5.33 ± 0.26[d]	4.03 ± 0.11[c]	8.04 ± 0.07[c]	1.11 ±0.06[d]	4.55±0.27[c]	6.84±0.05[d]

CONCLUSION

From this, we conclude that α-PA ameliorated mitochondrial membrane potential, curtailed intracellular ROS and enhanced endogenous antioxidants by activating Nrf2-Keap1 signalling in IR-3T3-L1 adipocytes. Thus, α-PA is effective in the management of oxidative stress associated alterations in IR-3T3-L1 adipocytes.

CONFLICT OF INTEREST

The authors declare no potential conflict of interests with respect to publication of this article.

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