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Original Research

Ferulic acid modulates ultraviolet-B radiation mediated inflammatory signaling in human dermal fibroblasts

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ABSTRACT:

Ultraviolet B (UVB 290-320 nm) participate in the development of the cutaneous inflammatory response which includes a cascade of events that involves increased expression of cyclooxygenase-2 (COX-2), release of tumor necrosis factor-alpha (TNF-α) and other inflammatory cytokines. Peroxisome proliferator-activated receptors (PPARα/γ) are considered to be potential targets for photo protection because they inhibit UVB mediated inflammatory responses. In this study, we investigated the effect of ferulic acid on UVB-radiation induced expression of TNF-α and COX-2 in human dermal fibroblasts (HDFa). Further, the action of ferulic acid on PPARα/γ activation and its binding interaction with these proteins were analyzed by induced fit docking. We found that onetime UVB exposure (19.8 mJ/cm^2) showed significantly increased the expressions of COX-2 and TNF-α in HDFa after 4 h post-irradiation when compared to the control cells. Ferulic acid pretreatment for 30 min before UVB exposure prevented UVB-induced overexpression of these inflammatory markers. It has also been found that ferulic acid activates PPARα/γ expressions in HDFa. Further, induced fit docking analysis showed that there was a greater binding interaction of ferulic acid with PPARγ than PPARα. Thus, ferulic acid exhibits beneficial effects against UVB-induced inflammatory responses probably through down-regulating COX-2 and TNF-α expressions and activating PPARα/γ agonists.

Keywords:
Ultraviolet B radiation, Ferulic acid, Human dermal fibroblasts, Inflammatory markers, Photoprotection

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INTRODUCTION

Epidemiological studies have showed that ultraviolet (UV) radiation exposure mediates several damaging effects that include melanoma and non-melanoma skin cancers (Afaq and Santosh, 2012). Although UVB radiation (280–320 nm) be a small portion of sun light that reaches the earth, it is considered to be a most deleterious agent because it can penetrate the skin to a depth of 160–180 μm and alters the skin architecture (Chilampalli *et al.,* 2011; Gregoris *et al.,* 2011). UVB is a strong pro-inflammatory agent with profound effects on skin in part through its ability to stimulate cytokine production. UVB exposure leads to activation of many cytokines such as cyclooxygenase-2 (COX-2), tumor necrosis factor-a (TNF-α) and interleukin-6 (IL-6), (Alexia *et al.,* 2003; Kondo *et al.,* 1993). These cytokines support the development of the UVB-induced cutaneous inflammatory responses that is observed in the skin as sunburn and erythema (Kondo 1999).

Peroxisome proliferator-activated receptor (PPARa/γ) regulates inflammatory signaling and cytokine activation in different experimental systems (Zhang *et al.,* 2004). PPARs belong to the nuclear receptor super family, a family of ligand activated transcriptional factors and it consists of three isotypes (PPARα, δ and γ). PPARs function as ligand dependent transcription factors and can heterodimerize with retinoid X receptors and then bind to PPAR-responsive elements (PPRE) in target gene promoters, which usually leads to transcriptional activation. Moreover, PPARs inhibits inflammatory gene expression in experimental models (Ricote *et al.,* 1998). Previous studies provide strong evidence for the role of PPARα/γ in controlling inflammation and suggest their potential as therapeutic targets for inflammatory diseases (Kim *et al.,* 2012).

Dietary phytochemicals offer exciting platforms for the management of UV related disorders. Dietary phytochemicals modulate UVB radiation-mediated damages by their antioxidant, anti-inflammatory and immunomodulatory actions (Ramachandran and Prasad 2008). Ferulic acid (3-methoxy-4-hydroxycinnamic acid) is a naturally occuring phenolic compound derived from the phenylpropanoid pathway. It is commonly abundant in fruits, vegetables and Cereals. (Prasad *et al.,* 2011).

Ferulic acid recovers the antioxidant cell defense system and stimulates cytoprotective enzymes due to its resonance-stabilized phenoxy radical structure such as phenolic nucleus and unsaturated side chain (Picone *et al.,* 2009). Ferulic acid can block the penetration of UV radiation into the epidermis. This sunscreen ability of ferulic acid can reduce UV-induced erythema (Saija *et al.,* 2000; Oresajo *et al.,* 2008). Moreover, ferulic acid could exert beneficial therapeutic effects of free radical-related syndromes such as neurodegenerative disorders, cancer, cardiovascular diseases and diabetes (Barone *et al.,* 2009). Recently, we found that ferulic acid inhibits UVB mediated ROS generation, TBARS levels and apoptosis in human dermal fibroblasts (Kanagalakshmi and Prasad, 2014). In the present study, we examined the beneficial effect of ferulic acid on the UVB mediated inflammatory responses by targeting PPARα/γ agonists in human dermal fibroblasts.

MATERIALS AND METHDOS

Chemicals

HDFa cells were procured from Invitrogen Bioservices, India. Low Serum Growth Supplement, fetal bovine serum (FBS), human epidermal growth factor, fibroblast growth factor, heparin, trypsin-EDTA and were obtained from Invitrogen Bioservices, India. Ferulic acid, monoclonal antibodies anti-TNFα, anti-COX-2, β-actin anti-mouse and goat anti-mouse IgG-HRP polyclonal antibody were purchased from Sigma chemical Co., St. Louis, MO, USA. Bovine serum albumins (BSA), radio immune precipitation assay (RIPA) buffer were purchased from Himedia, Mumbai. All other analytical

grade chemicals, solvents and reagents were purchased from SD Fine Chemical, Mumbai.

Culturing human skin fibroblasts

HDFa cells were maintained at 37°C under 5% CO_2 condition in medium-106 supplemented with 2% v/v fetal bovine serum, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 µg/ml heparin and antibiotics. The HDFa cells were cultured to grow for 7 days to obtain the maximum confluence for experiments. Then, HDFa cells were harvested using trypsin-EDTA, subcultured and the remaining cells were used for photo protection experiments (Ramachandran *et al.,* 2010).

Study design

Cultured fibroblasts were divided into four groups as follows:

Group 1: Normal fibroblasts without any treatment;

Group 2: Normal fibroblasts with 40 µg/mL of FA;

Group 3: UVB-irradiated fibroblasts;

Group 4: UVB-irradiated fibroblasts pretreated with 40 µg/mL of FA.

Treatment of the HDFa cells

Thirty minutes before UVB exposure, 40 µg/mL of ferulic acid was added to the HDFa cells. Trypan blue dye exclusion test was carried out to find out the toxicity and suitability of 40 µg/mL of ferulic acid for photoprotection studies. Before UVB exposure, the HDFa cells were washed once with PBS solution. Mock-irradiated HDFa showed no viability changes over the 30 min period of incubation (HDFa cells were maintained at 37°C under 5% CO_2 condition in medium-106 supplemented with 2% v/v fetal bovine serum, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 µg/ml heparin and antibiotics. The HDFa cells were cultured to grow for 7 days to obtain the maximum confluence for experiments. Then, HDFa cells were harvested using trypsin-EDTA, subcultured and the remaining cells were used for photoprotection experiments (Ramachandran *et al.,* 2010).

Irradiation procedure

HDFa cells were washed twice with PBS and UVB irradiated in a thin layer of medium without FBS. A battery of TL 20 W/20 fluorescent tubes (Heber Scientific, Chennai, India) was used as UVB source, which possess a wavelength range of 290–320 nm, peaked at 312 nm, and with an intensity of 2.2 mW/ cm^2 for 9 min. The total UVB radiation exposure was 19.8 mJ/ cm^2, with an average value of 1.52 × 10−3 mJ/ cell. Immediately after UVB exposure, the HDFa cells were kept at 37°C for 4 h at in 5% CO_2 environment. Irradiated HDFa cells were then washed with PBS, and transferred to sterile centrifuge tubes for biochemical analysis (Kanagalakshmi and Prasad 2014).

Western blot analysis for pro-inflammatory markers expression

Western blot analysis was carried out for TNFα and COX-2 expressions in ferulic acid plus UVB-irradiated HDFa. The results were normalized to β-actin gene expression. Treated HDFa cells were washed with PBS and detached using 0.25% trypsin/EDTA solution. Cell suspensions were centrifuged and the pellets were lysed with an ice-cold lysis RIPA buffer containing a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA) for 30 min. The lysate was centrifuged at 4°C at 13,000 rpm for 10 min and the supernatant was used to determine protein concentration using Nanodrop 2000 (Thermo Scientific, USA). Cell extracts containing 50 µg of proteins were subjected to electrophoresis on 12% SDS-PAGE gel and transferred to a PVDF membrane using transblot semi-dry apparatus (Biorad, USA). PVDF membranes were blocked with non-fat milk (5% (w/v) for 6 h and then incubated overnight with TNFα and COX-2 antibodies (Sigma-Aldrich, USA), in blocking solution at 37°C. Then the membranes were washed with TBST thrice with 10 min interval and incubated with secondary antibody (diluted 1:2000) in

blocking solution for 2 h at 37°C. Then, the PVDF membranes were washed with TBST thrice with 10 min interval and the developed bands were detected using a DAB solution. The images were acquired by Image Studio software (LI-COR, USA) (Ramachandran *et al.,* 2012).

RNA isolation and real-time quantitative PCR.

The total RNA was extracted from the HDFa cells using RNeasy Mini kit (Qiagen, USA) as per the protocol recommended by the manufacturer. The mRNA expression of PPARα/γ in HDFa cells was determined using real-time PCR, as described previously (Sharma and Katiyar, 2010). RNA purification and quantity was analyzed by nanodrop 2000 (Thermo Scientific, USA). Experiments were run in triplicate to confirm amplification integrity. Manufacturer-synthesized primer pairs were used to measure the mRNA expression level of PPARs. PCR cyclic condition 25°C for 10 min; 42°C for 50 min; 75°C for 15 min were used for cDNA synthesis. The cyclic condition used for amplification was 95°C for 2 sec; 55°C for 15 sec; and 68°C for 20 sec as prescribed by the primer's manufacturer. The expression levels of genes were normalized to 18S mRNA expression level. The cyclic threshold (Ct) for positivity of real-time PCR was determined based on negative controls.

Molecular docking

Molecular docking was performed on Red Hat Enterprise Linux EL‑5 workstation using Maestro (Schrodinger LLC 2009, USA). GLIDE‑5.5 searches were performed for understanding docking interactions between ferulic acid and PPARα/γ. All molecular modeling was carried out using OPLS‑AA (Optimized Potential Liquid Simulation for All Atom) force field (Glide, 2009). PyMOL (DeLano WL, 2002) software employed for the analysis of hydrogen bond interactions. Hydrophobic interactions were analyzed between protein and ligand using Ligplot software (Wallace AC, 1995).

Ligprep 2.3 module (Schrödinger, USA) was employed for ferulic acid preparation. The three dimensional crystal structure of PPAR α/γ (PDB Id: 1K7L/ PDB Id: 3DZY) and Cox-2 (PDB Id: 6COX) were downloaded from the Protein Data Bank (PDB) (http://www.rcsb.org). Protein preparation wizard of Schrodinger's was used for PPARα/γ and COX-2 preparation. Non-hydrogen atoms were minimized until the average root mean square deviation reached default value of 0.3Å. Sitemap 2.3 was used to understand binding site in the ligand binding domain (LBD) of the PPARα/γ and COX-2 (Schrodinger Suite 2009).

Induced fit docking (IFD) was performed to predict ferulic acid binding modes and structural movements in the LBD region of PPARα/γ and COX-2 using Glide and Prime modules. The prepared proteins were loaded in the workstation and the Grid values were calculated about 20 Å in order to cover all the active site amino acids. The Vander Waal's radii of nonpolar amino acids and ligand atoms were scaled by a default value of 0.50. About 20 conformational images were created and analyzed for the best conformational pose based on the docking score and glide energy.

RESULTS

Ferulic acid inhibits UVB-induced TNF-α and COX-2 expressions in HDFa

Western blot analysis shows that there was an overexpression of TNF-α and COX-2 in the UVB exposed HDFa (Figure 1). It indicates inflammatory responses in HDFa cells as compared with control HDFa cells. TNF-α and COX-2 expression levels were significantly down-regulated in ferulic acid pretreated plus UVB irradiated HDFa (Figure 1).

Ferulic acid activates PPARα/γ mRNA expression in HDFa

Quantitative Real Time-PCR analyses were adopted to analyze the activation of PPARα/γ mRNA expression in ferulic acid and/or UVB-irradiated HDFa

cells (Figure 2A,B). In this study mRNA levels of PPARα/γ were down-regulated in UVB-exposed HDFa. Whereas, FA treatment prevented the UVB induced loss of PPARα/γ and up-regulated these mRNA expression in HDFa cells.

Molecular docking with PPARα/ γ

Ferulic acid was docked against PPARα (PDB code: 1K7L). The glide energy score, docking score and hydrogen bond interactions of PPARα with its cocrystallized ligand 2-(1-methyl-3-oxo-3-phenyl-propylamino)-3-{4-[2-(5-methyl-2-phenyl-oxazol -4-yl)-ethoxy]- phenyl}-propionic acid was shown in the table. 1. The energy score of PPARα with cocrystallized ligand was -86.02 (kcal/mol) and with ferulic acid was -39.31 (kcal/mol). PPARα has a common hydrogen bond interaction (Tyr 464 and Ser 280) with co-crystallized ligand and ferulic acid. The amino acid residues such as Phe 273, Cys 276 and Ile 354 showed hydrophobic bond interactions with ferulic acid and the co-crystallized ligand (Figure 3).

The energy score of PPARγ with ligand retinoic acid was -49.18 (kcal/mol) and with the ferulic acid it was -40.44 kcal/mol. PPARγ possess a common hydrogen bond interaction (Arg 316) with retinoic acid and ferulic acid. The amino acid residues such as Ala 272, Ile 268, Leu 326, Leu 309, Phe 313 and Ile 310 showed hydrophobic interactions with ferulic acid and retinoic acid. The aminoacid Ala 271 was interacting with ferulic acid through hydrogen bonding and interacting with retinoic acid through hydrophobic interaction (Figure 4).

Molecular docking with COX-2

The energy score of COX-2 with the co-crystallized ligand 1-Phenylsulfonamide-3-

Table. 1. Induced fit docking results of PPARα, PPARγ and Cox-2 with their cocrystallized ligands and ferulic acid. Induced-fit docking was carried out using Schrodinger software. Ferulic acid interacts with PPARα, PPARγ and COX-2 through hydrogen bonding and hydrophobic interactions. Ferulic acid possess greater binding interaction with PPARγ (glide energy -40.44) than PPARα (glide energy 39.31) when compared with their corresponding cocrystallized ligands.

Protein	Compound/Ligand	Docking Score (Kcal/mol)	Glide Energy (Kcal/mol)	Hydrophobic Bond Interactions	Hydrogen-Bond Interactions	Distance between donor and acceptor (A°)
PPARα	2-(1-methyl-3-oxo-3-phenyl-propylamino)-3-{4-[2-(5-methyl-2-phenyl-oxazol-4-yl)-ethoxy]- phenyl}-propionic acid	-16.22	-86.02	Gln 277, Ile 272, Ile 339, Phe 273, Leu 254, Cys 275, Leu 347, Met 355, Leu 347, Met 330, Val 332, Phe 351, Leu 321, Ile 354, Cys 276	Tyr 464 (O-H-O) His 440 (N-H-O) Ser 280 (O-H-O)	3.02 2.98 2.72
	Ferulic acid	-8.15	-39.31	Phe 273, Cys 276, Leu 460 and Ile 354	Tyr 464 (O-H-O) Ser 280 (O-H-O) Tyr 314 (O-H-O)	2.88 2.74 2.79
PPARγ	Retinoic Acid	-13.93	-49.18	Phe 346, Cys 432, Ile 324, Ile 310, Phe 313, Leu 326, leu 309, ile 268, ala 272, Ala 271.	Ala 327 (N-H-O) Arg 316 (N-H-O) Arg 316 (N-H-O) Gln 275 (N-H-O)	3.15 2.88 2.86 2.62
	Ferulic acid	-7.75	-40.44	Ala 272, Ile 310, Phe 313, Leu 309, Cys 269, Ile 268, Leu 326	Asn 306 (O-H-O) Ala 271 (O-H-O) Arg 316 (N-H-O)	2.74 2.69 3.04
Cox-2	1-Phenylsulfonamide-3-Trifluoromethyl-5-Parabromophenylpyra-zole	-11.68	-62.09	Val 349, Ser 530, Ala 527, Gly 526, Val 523, Ala 516, Leu 352, Ser 353	Tyr 355 (O-H-N) His 90 (N-H-O) Gln 192 (O-H-O) Phe 518 (N-H-O)	3.08 3.31 3.25 3.32
	Ferulic acid	-7.95	-37.82	Trp 387, Phe 518,Leu 384, Ser353 and Val 523	Tyr 385 (O-H-O) His 90 (N-H-O) Leu 352 (O-H-O)	3.22 2.70 2.86

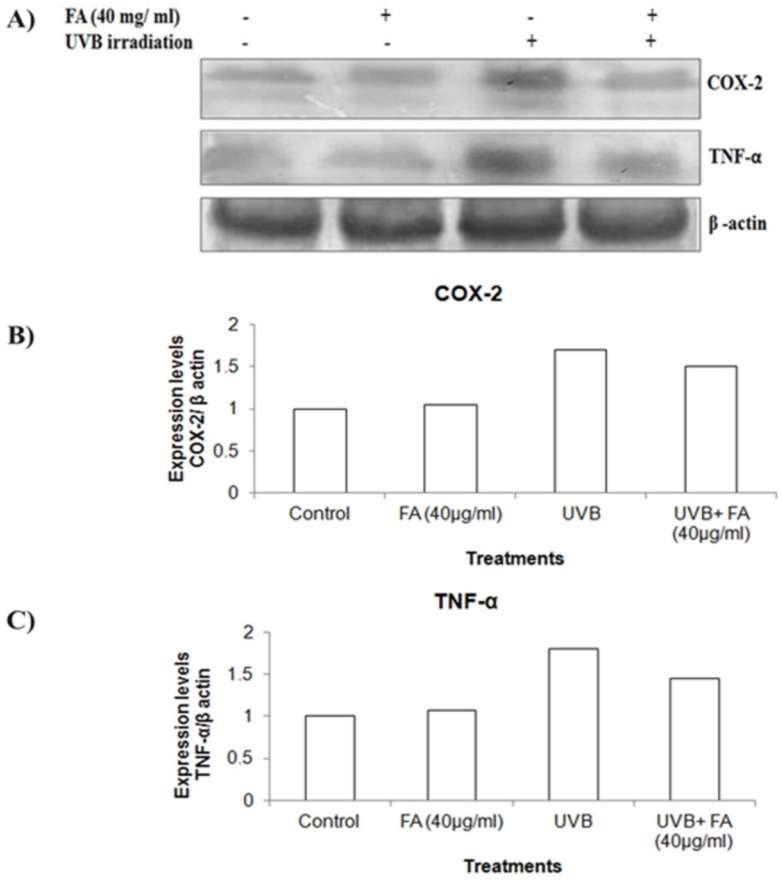

Figure 1. Effect of ferulic acid on UVB-induced activation of TNFα and COX-2 in HDFa cells. HDFa cells were exposed to UVB (19.8 mJ/cm^2) with or without ferulic acid for 30 min. Cells were harvested at 4 h after UVB exposure, and the expression of TNFα and COX-2 were analyzed by Western blot. ferulic acid treatment down-regulated these protein expressions when compared with UVB control group. The graph represents the quantification results normalized to β-actin levels.

Trifluoromethyl-5-Parabromophenylpyrazole was -62.09 kcal/mol and with ferulic acid the energy score was found to be -37.82 kcal/mol. COX-2 posses a common hydrogen bond interaction (His 90) with co-crystallized ligand and Ferulic acid. The amino acid Leu 352 was interacting with ferulic acid through hydrogen bonding and interacting with co-crystallized ligand through hydrophobic interaction. The amino acid such as Ser 353 and Val 523 shows hydrophobic bond interactions with ferulic acid and the co-crystallized ligand (Figure 5).

DISCUSSION

UVB (280–320 nm) causes acute inflammatory skin damages including erythema, production of pro-inflammatory mediators and infiltration of inflammatory cells (Lee *et al.,* 2013). Previous studies have demonstrated that UVB-induced oxidative stress plays a critical role in the induction of proinflammatroy cytokines including TNF-α expression (Ramachandran *et al.,* 2012). TNF-α can promote both DNA damage and activation of nuclear factor kB (NF- kB) inducing the formation of sunburned cells and leading to photodamage in the skin (Muthusamy and Piva, 2010). In the present study, TNF-α was over expresed in the UVB irradiated HDFa cells when compared to control cells. The pre-administration of anti-inflammatory agents was found to be an effective strategy for preventing UVB-irradiation induced skin inflammation. In this

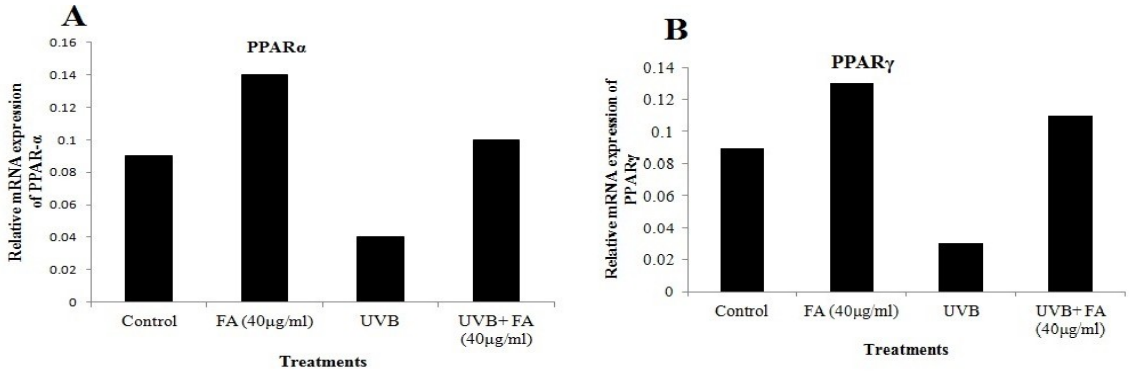

Figure 2. Ferulic acid activated UVB-induced PPARα/γ mRNA expression in cultured human dermal fibroblasts. Total RNAs were prepared after UV irradiation and subjected to real-time PCR. A), mRNA expression of PPARα. B), mRNA expression of PPARγ.

study, ferulic acid treatment diminished the UVB-induced overexpression of TNF-α in HDFa.

Previous studies have indicated that UVB mediated ROS over production and TNFα activation resulted in cPLA2 synthesis and increasing the formation of arachidonic acid. This overproduction of arachidonic acid can be converted to eicosonoids, forming multiple prostaglandins through the COX-2 pathway and ultimately leading to skin cell death. The expression of COX-2 has been used as inflammatory marker for evaluating UVB irradiation induced skin inflammation. In this report, we observed that UVB exposure increased

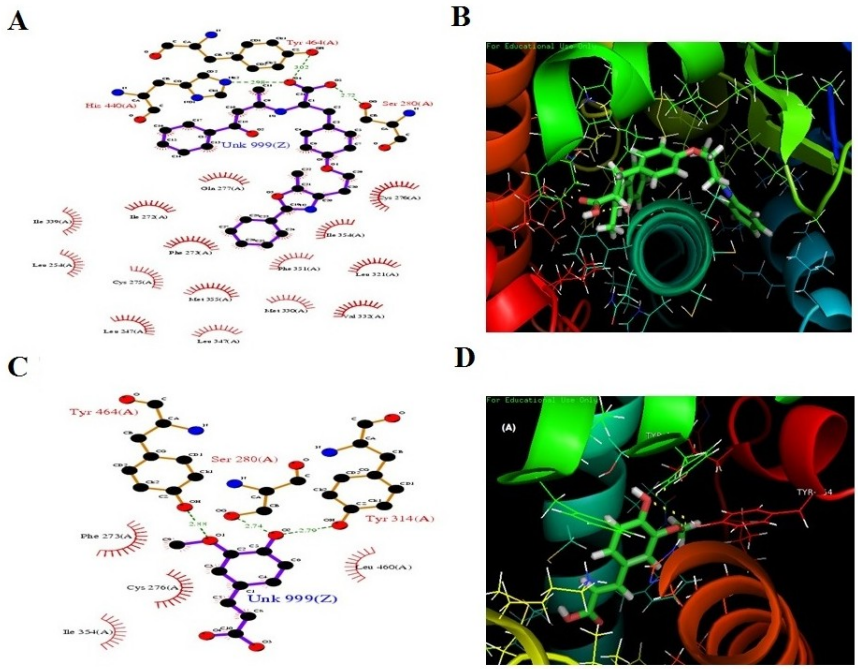

Figure 3. Binding interaction of ferulic acid and cocrystallized ligand with PPARα. Ligplot image showing hydrogen bonding and hydrophobic interactions of PPARα with its cocrystallized ligand (A) and ferulic acid (C). Pymol view of LBD region of PPARα with cocrystallized ligand (B) and ferulic acid (D). Ferulic acid has similar binding sites as compared with the cocrystallized ligand on the LBD region of PPARα.

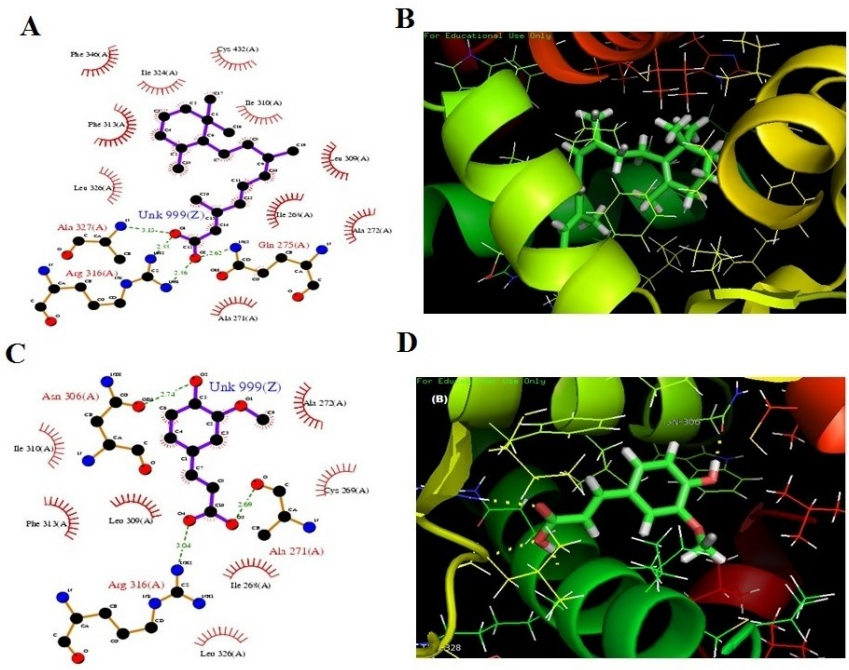

Figure 4. Binding interaction of ferulic acid and retinoic acid with PPARγ. Ligplot image showing hydrogen bonding and hydrophobic interactions of PPARγ with its cocrystallized ligand retinoic acid (A) and ferulic acid (C). Pymol view of LBD region of PPARγ with cocrystallized ligand (B) and ferulic acid (D Ferulic acid has similar binding sites as compared with the cocrystallized ligand on the LBD region of PPARγ.

COX-2 protein expression in HDFa and ferulic acid pretreatment restored the UVB induced expression of COX-2 in HDFa. The inhibition of COX-2 expression by ferulic acid may be caused by its phenol function, which is associated with decreased anti-radical activity (Lee *et al.,* 2013). The hydrogen-donating ability of ferulic acid was previously reported to correlate with COX-2 inhibitory activity (Hirata *et al.,* 2005). Previously, we showed ferulic acid scavenges free radicals and restored UVB induced oxidative events (Prasad *et al.,* 2007). Molecular docking results show that ferulic acid directly interacts with COX-2 protein.

It was reported that expression and activation of PPARγ blocks inflammation were induced by cytokine production (Hirsch 2003, Blanquart 2003, Grimble 2002). PPARs regulate important cellular functions, including cell differentiation, proliferation, and inflammation. In this study, ferulic acid was found to be an activator of PPARγ and subsequently alleviates UVB induced inflammatory markers expression. PPARγ possess a small polar and a hydrophobic residue in the LBD that form hydrogen bonds and hydrophobic binding interactions with ferulic acid and the cocrystallized ligand. Arg 316 present in the LBD region of PPARγ was a common amino acid that interacts with both ferulic acid and the cocrystallized ligand. Ferulic acid also activates PPARα and the amino acid Tyr 464 and Ser 280 were the common amino acids that are interact with ferulic acid and the cocrystallized ligand. Further, induced fit docking analysis showed that there was a greater binding interaction of ferulic acid with PPARγ than PPARα. The binding interaction of these proteins are due to increase in polarity, number of OH groups present in the LBD, position of the OH group in the

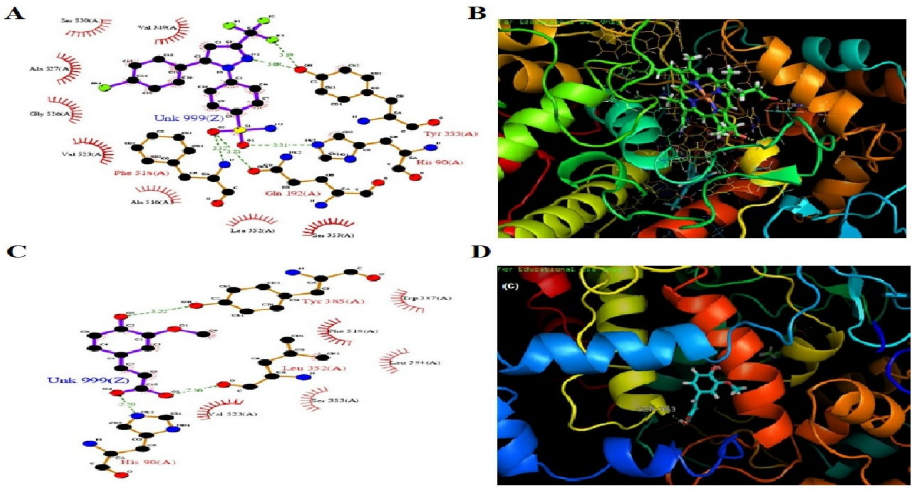

Figure 5. Binding interactions of ferulic acid and the cocrystallized ligand (1-Phenylsulfonamide-3-Trifluoromethyl-5-Parabromophenylpyrazole) with COX-2. Ligplot image showing hydrogen bonding and hydrophobic interactions of COX-2 with its cocrystallized ligand retinoic acid (A) and ferulic acid (C). Pymol view of interaction of LBD region of COX-2 with cocrystallized ligand (B) and ferulic acid (D). Ferulic acid has similar binding sites as compared with the cocrystallized ligand on the LBD region of COX-2.

ferulic acid, based on the steric effect/inductive effect of the ferulic acid. Ligand exposure is also considered to be one of the reasons for strong binding interaction. A recent study by Waku *et al.* (2009) shows that ligand interact with LBD region and thereby activating PPARγ conformations. Covalent interactions stimulate alteration of the side-chain network nearby created covalent bond to create diverse transcriptional strengths. Thus, ferulic acid exhibits beneficial effects against UVB-induced inflammatory responses probably through down-regulating COX-2 and TNF-α expressions and activating PPAR α/γ agonists.

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