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Phytol Anti-oxidative and Anti-inflammatory Effects in Hydrogen Peroxide Challenged Human PMBCs Involves NFκB Pathway

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ABSTRACT: Anti-oxidant and anti-inflammatory activities of phytol were investigated in human PBMCs challenged with the oxidative stress using H_2O_2. The OH ion released from H_2O_2 is a potent ROS that burden the cellular antioxidants, results excess of active oxygen radicals and stimulate the synthesis of inflammatory mediators. NFκB activation plays important role in establishing and increasing the inflammatory response as well as production of more ROS.

Cells were treated with 100μM H_2O_2 and various concentration of phytol. The cellular antioxidants and NO were estimated. The expression of inflammatory genes IL6, TNFα, IL1β, COX2 and iNOS was determined using QRTPCR and protein expression for IL6, TNFα, IL1β and PGE2 was studied using ELISA. NFκB activation was studied by estimating IKKβ and IκBα phosphorylation level.

Addition of H_2O_2 to the cultured cells results reduced level of antioxidants SOD, GPx and catalase, while higher level of CAT and NO were observed. Phytol treatment results restoration of these variables in dose dependent manner. Similarly H_2O_2 treatment results higher expression of IL6, TNFα and IL1β both at mRNA and proteins level. It also has results elevated gene expression for COX2 and iNOs, increased synthesis of PGE2 and activation of NFκB. Phytol co treatment to the cells results significant lowering of the inflammatory mediators and NFκB activation. Understanding the mechanism, Phytol results in its therapeutic effects is a challenge in its recruitment as a potential drug. In the present study, NFκB activation is studied in conjunction with the inflammatory cytokines that can induce its activation, in turn their expression is stimulated by the products of NFκB pathway cyclically. The results of present study shown that phytol can alleviate the H_2O_2 induced oxidative stress and inflammatory response, and prevent the NFκB from activation.

Keywords: Phytol, H_2O_2, PBMCs, Oxidative stress, inflammation, NFκB.

Abbreviation: PBMCs; peripheral blood mononuclear cells, H_2O_2; hydrogen per oxide, SOD; superoxide dismutase, CAT; catalase, GPx; glutathione peroxidase, IL-6; Interleukin-6, IL-1β; interleukin -1 beta, TNF α; tumor necrosis factor alpha, COX2; cyclooxygenase-2, iNOS; Induced able nitric oxide synthase, PGE2; prostaglandin E-2, NFκB; Nuclear Factor kappa-light-chain-enhancer of activated B cell.

I. INTRODUCTION

Inflammation is an imperative and highly integrated homeostatic response to any insult or interference to the normal physiological conditions and processes. These responses can be mounted against exogenous agents like infectious microbes or injury to the cells or extracellular components [1]. Following damaging events the immune system evokes protective events that eliminate damaging elements , remove the debris and initiate repair and resolved, however, a continuum of stimulation resulting from the actual damaging elements, products from the damaging process or immune cells can contributes to the progression of many diseases. Although the course of inflammation followed by a broad systemic response involving circulating cells of the immune system, but the extent and persistence is determined by the local changes at the tissue level, including activation of resident and circulating immune cells, release of inflammatory cytokines and production of reactive radicals, establishing the oxidative stress [2].

Oxidative stress an old known pathophysiological phenomenon which has attracted a greater interest for those who trying to understand the development of chronic diseases and their mechanism of tissue destruction and imparting their debilitating effects, the diseases like diabetes, arthritis, neurodegeneration, CVDs, cancers etc. Another interesting known fact is that all of these diseases in past were found to be linked somehow to inflammation. Either these thought to be the result of inflammatory process or stimulate the inflammatory process. With the current knowledge we know that both of these processes i.e. oxidative stress and inflammation are very closely related to each other [3]. Both ROS and their products can induce the inflammatory response with activation of NFκB, and increase production of pro-inflammatory cytokines like IL1β, IL18, IL1, IL6 and TNFα [4, 5]. However the

immune cells stimulated in response to these pro inflammatory cytokines are capable of releasing large amount of oxidative radicals, while NFκB, IL6 and IL1β can stimulate the production of ROS. These mechanisms intercalate the oxidative stress and inflammation in self-perpetuating vicious cycle where one escalates the other.

From decades the symptomatic and preventive treatment of inflammatory diseases revolve around the drugs grouped as NSAIDs like aspirin, diclofenac, pyroxicametc, However long term use of these drugs develop a number of complications which by themselves have debilitating effects sometime severe than the ailment for the treatment of which these have been prescribed [6]. Therefore the treatment of such chronic degenerative inflammatory diseases requires discovering new, effective and safe therapeutic agents. Since ancient times man has utilized herbs and plant not only as food source but their parts or extract have been utilized for the treatment of health ailments and even today these are used as source of therapeutic agents. Their beneficial effects are attributed to the phytochemicals with strong antioxidant and anti-inflammatory activities.

Phytol an open chained diterpene alcohol, occur in nature abundantly as part of chlorophyll molecule and as precursor to the Vitamin E and Vitamin K. Due to its fragrance used in cosmetics, toiletries, cleaner and detergents[7]. Pharmacological studies have reveled wide spectrum of medicinal effects of phytol and its metabolites. Islam et al., in 2015 have reviewed the medicinal effect and pharmacological activities of phytol in detail [8]. Phytol have been tested for its cytotoxic, anti-tumorigenic, anti-nociceptive, anti-oxidant, anti-inflammatory and anti-cancer effects both in vivo, in vitro and ex vivo models [9]. In addition phytol is considered quite nontoxic, in rats a high LD50 of 10,000 mg/kg have been reported by McGinty and Letizia [10]. It has been reported that phytol results anti-inflammatory effects by reducing the oxidative stress and cytokine production in mice [11]. Phytol was reported to exertanti-arthritic effects by reducing the IL6 and TNFα in the synovial fluid and IL6 and COX2 inhibition in the spinal tissues [12]. In silico studies have revealed that phytol can bind with IL6, TNFα and NFκB, tha interaction might be responsible for various anti-inflammatory and anti-oxidant effects that are exerted by phytol [13, 14]. Further studying the phytol interaction with the components of the inflammatory and antiproliferative pathway may fill the gaps in our understanding of the mechanism that phytol uses to exert its various therapeutic effects and its utilization as a remedy for various ailments. In the present study we describe that in human PMBCs, phytol can induce anti-oxidative effects, reduce the inflammatory response and this involve the NFκB pathway, specifically results the reduction of IKKβ and IκBα phosphorylation.

II. METHODS AND MATERIAL

A. PMBC Isolation and Culture

Blood from apparently healthy donors was drawn in EDTA tubes. PMBC were isolated from the blood using Ficoll-Paque Plus (Sigma-Aldrich) per manufacturer instructions. The mononuclear cells obtained from the gradient were washed twice with PBS (Invitrogen) and re-suspended in RPMI1640 (Invitrogen), added with penicillin 100U/ml (Invitrogen), streptomycin 100mg/ml (Invitrogen) and FBS 10% (Invitrogen), incubated at 37°C under 5% CO_2.

B. Treatment Scheme

Cells were platted ($1X10^6$) in wells for 24 hours, were treated with 1µM, 5µM and 25µM of Phytol (Sigma-Aldrich) in absence or presence of H_2O_2 100 µM. All the treatments were carried out in triplicate.

C. Cell Viability Assay

Cellular viability was determined by MTT (methylthiazoletetrazolium) assay as per method described by Wu *et al.,* 2007 [15]. Briefly MTT solution (5mg/ml) was added to the wells containing treated cultured cells to achieve final concentration 0.5 mg/ml and further incubated for 4 h at 37°C. After incubation media was exchanged with acidified DMSO, after 10min incubation, absorbance was taken at 540nm.

D. Antioxidant Enzyme Assay

Activity of antioxidant enzymes SOD, CAT, GPX and GSH were performed following protocols described by Kakkar *et al.* (1984) [16], Chance and Maehly (1955) [17], Paglia, D.E. and Valentine (1967) [18], Moron *et al.* (1979) [19] respectively.

E. Nitric Oxide assay

Following treatment, culture mediums from subsequent wells were mixed with equal volume of Griess reagent, incubated for 15min. Absorption was recorded at 550nm. The readings were measured against sodium nitrate standard curve.

F. Enzyme Linked Immunosorbent Assay

The cells were cultured and treated as mentioned above; at the end of incubation the medium was aspirated and stored at -700C. Subsequently was used for estimation of IL6, TNFα, TGFβ and PGE2 using human ELISA kit (R&D systems, USA). Sensitivity of ELISA kits for IL6, TNFα, TGFβ and PGE2 were 0.7pg/ml, 5.5pg/ml, 1.0pg/ml and 41.4pg/ml respectively.

G. Real Time PCR

Total RNA was extracted using Trizol reagent (Invitrogen Corp.). The first strand was synthesized using the RevrtAddTM (Thermo Scientific) first strand cDNA synthesis kit. Primers for IL6, TNFα, IL1β, COX2 and INOs were designed using primer3. qRTPCR was performed as described previously by Zai *et al.,* 2019 [20].

H. Western Blotting

Cells lysate was prepared in the presence of protease and phosphatase inhibitor cocktail. Western blotting for IKKβ and IκBa was performed as described previously by Maryam *et al.,* 2019 [21].

I. Statistical Analysis

The Static was performed using Excell 2010 and GraphPad Prism 5 software, Mean and SD were calculated, the results were analyzed using one-way anova using Bonferroni as post test ($p<0.05$, CI 95%), significant different were indicated from control and H_2O_2 only treated group.

III. RESULTS

A. Cell Viability Assay

The effect of phytol on cellular viability was determined on human PBMCs utilizing MTT assay. Different concentrations of phytol (1µM, 5µM, 25µM, 50µM and

100µM) were used to treat the cells for 24hrs. A cellular viability >80% was observed up to 25µM of phytol (Fig. 1) while the highest concentration of 100µM given cellular viability >50%. The first three doses have been selected to study the antioxidant and anti-inflammatory effects of phytol.

B. Anti-oxidant Enzymes

Cellular treatment with H_2O_2 result significantly reduced level of SOD and GPx. Although it was observed that phytol treatment has restored these enzymes level in dose dependent manner, but only co treatment with 25µM phytol has results a significant change in comparison to H_2O_2 treated group.

A non-significant increase in the level of CAT enzyme was observed in the H_2O_2 treated cells and a similar non-significant dose dependent restoration of enzyme level was observed with phytol co treatment in different concentration.

C. GSH Level

Significantly reduced level of GSH level was observed in H_2O_2 treated cells when compared to the control group. A non-significant increase in the GSH level was observed when the cells were co treated with the 1µM and 5µM phytol, while 25µM concentration of phytol results a significant change in comparison to H_2O_2 treated cells (Table 2)

Table 1: Primer sequence for RT.

S. No.	Name	Sequence	Product Size
1.	**Beta Actin**		
	Forward	AGAGCTACGAGCTGCCTGAC	177
	Reverse	TGTTGGCGTACAGGTCTTTG	
2.	**IL1 Beta**		
	Forward	TCCAGGAGAATGACCTGAGC	111
	Reverse	GTGATCGTACAGGTGCATCG	
3.	**IL6**		
	Forward	GGCAGAAAACAACCTGAACC	116
	Reverse	CCTCAAACTCCAAAAGACCAG	
4.	**TNF Alpha**		
	Forward	TCTCTCTAATCAGCCCTCTGG	99
	Reverse	CCTCAGCTTGAGGGTTTGC	
5.	**COX2/PTGS2**		128
	Forward	CTAGAGCCCTTCCTCCTGTG	
	Reverse	GGGGATCAGGGATGAACTT	
6.	**iNOS**		129
	Forward	CATGTCTGGGAGCATCACC	
	Reverse	CTCTTGGGTCTCCGCTTCTC	

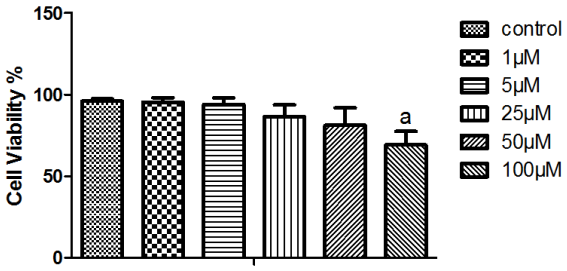

Fig. 1. The effect of Phytol treatment on human PBMCs viability; graph showing Mean±SD values; superscript 'a' indicating significance difference with control group.

D. NO production

Effects of phytol on NO production were investigated in PBMCs, H_2O_2 treatment results significant increase in the NO production. Phytol treatment results in dose dependent manner a significant decrease NO production in comparison to H_2O_2 treated cells (Table 2). The observation may be secondary to the effects of phytol on the iNOS expression (Table 2).

E. Anti-Inflammatory Effects

Anti-inflammatory effects of different concentration of phytol were studied by observing the changes in the expression of inflammatory genes and synthesis of inflammatory proteins in the H_2O_2 treated cells. Expression of mRNA encoding cytokines IL6, TNFα, IL1β, COX2 and iNOs and protein expression of IL6, TNFα, IL1β and PGE2 were analyzed. In PMBCs the treatment with H_2O_2 results significantly higher expression for the IL6, TNFα, IL1β, COX2 and iNOs in comparison to control group (Fig. 2).

Table 2: The effect of Phytol treatment on anti-oxidants and nitrite in H_2O_2 treated human PBMCs.

	Control	H_2O_2	H_2O_2+Phytol (1µM)	H_2O_2+Phytol (5µM)	H_2O_2+Phytol (25µM)	Phytol (25µM)
SOD (U/mg Protein)	6.22±0.52^{b}	3.467±0.2^{a}	3.82±0.18^{a}	4.427±0.52	5.787±0.86^{b}	5.933±0.25^{b}
CAT (U/mg Protein)	23.06±2.71	34.09±5.18	35.14±6.35	28.51±3.26	25.8±2.11	23.57±3.17
GPx (U/mg Protein)	17.84±1.67^{b}	8.53±1.23^{a}	9.097±0.43^{a}	11.5±1.7^{a}	15.07±1.5^{b}	18.34±1.17^{b}
GSH (nM/mg Protein)	14.88±1.71^{b}	9.097±0.72^{a}	10.56±1.45	13.61±1.93	16.51±1.69^{b}	17.6±1.35^{b}
Nitrite (µM)	5.1±0.39^{b}	44.79±3.92^{a}	38.99±3.37^{a}	26.83±2.26$^{a\,b}$	10.67±2.14$^{a\,b}$	6.02±0.25^{b}

Mean±SD; surperscrip 'a' and 'b' indicant the significant difference from control and H_2O_2 treated group respectively.

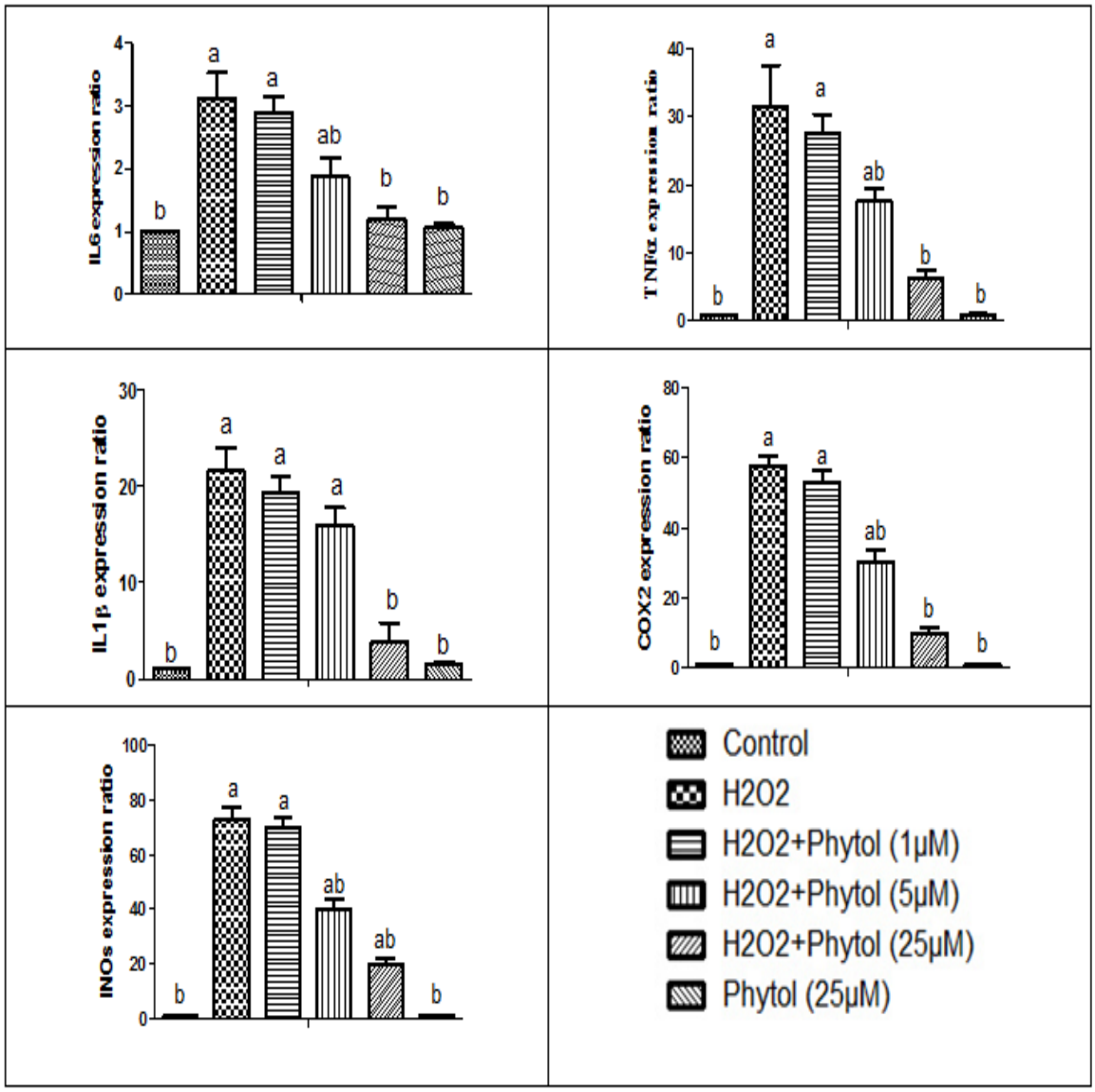

Fig. 2. The effect of Phytol treatment on inflammatory genes expression in H_2O_2 treated human PBMCs; graph showing Mean±SD values; superscript 'a' and 'b' indicating significance difference with control group and H_2O_2 treated group.

The treatment with phytol results suppression for these gene expression significantly. For IL6 and TNFα a significant difference was observed with 5 and 25μM of phytol, while for IL1β the significant difference observed at 25μM of phytol, compared to H_2O_2 treated cells. Expression of genes for COX2 and iNOs were also decrease significantly when cells were co treated with 5 and 25μM phytol. No significant difference was observed for these genes expression when the H_2O_2 treated cells were co administered with 1μM of phytol.

The H_2O_2 treatment to cells was also results a significant increase in the expression of proteins for IL6, TNFα, IL1β and PGE2 (Fig. 3). Co treatment with all three concentrations of phytol results significantly reduced synthesis of IL6 and PGE2 protein synthesis in comparison to the H_2O_2 treated cells. A similar significant reduction in the comparison of H_2O_2 treated group was observed for the synthesis of TNFα and IL1β proteins with 5 and 25μM of phytol, no significant difference observed for 25μM phytol only.

F. NFκ-B Activation

It was observed that H_2O_2 treatment result a significant higher level of IKKβ and IκBα phosphorylation resulting activation of NFκ-B complex. Phytol at all treatment concentration results a significant reduction in the IKKβ and pIκBα level when compared to the H_2O_2 treated cells (Fig. 4).

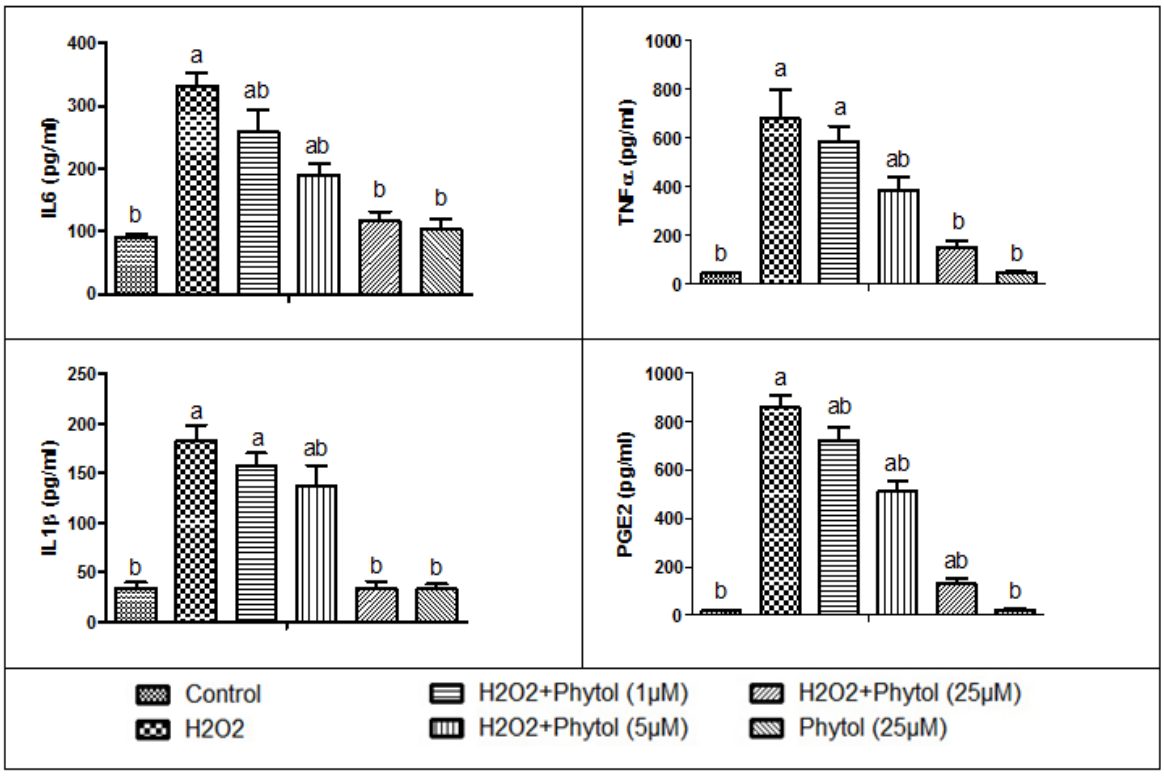

Fig. 3. The effect of Phytol treatment on synthesis of pro inflammatory cytokines and PGE2 in H_2O_2 treated human PBMCs; graph showing Mean±SD values; superscript 'a' and 'b' indicating significance difference with control group and H_2O_2 treated group.

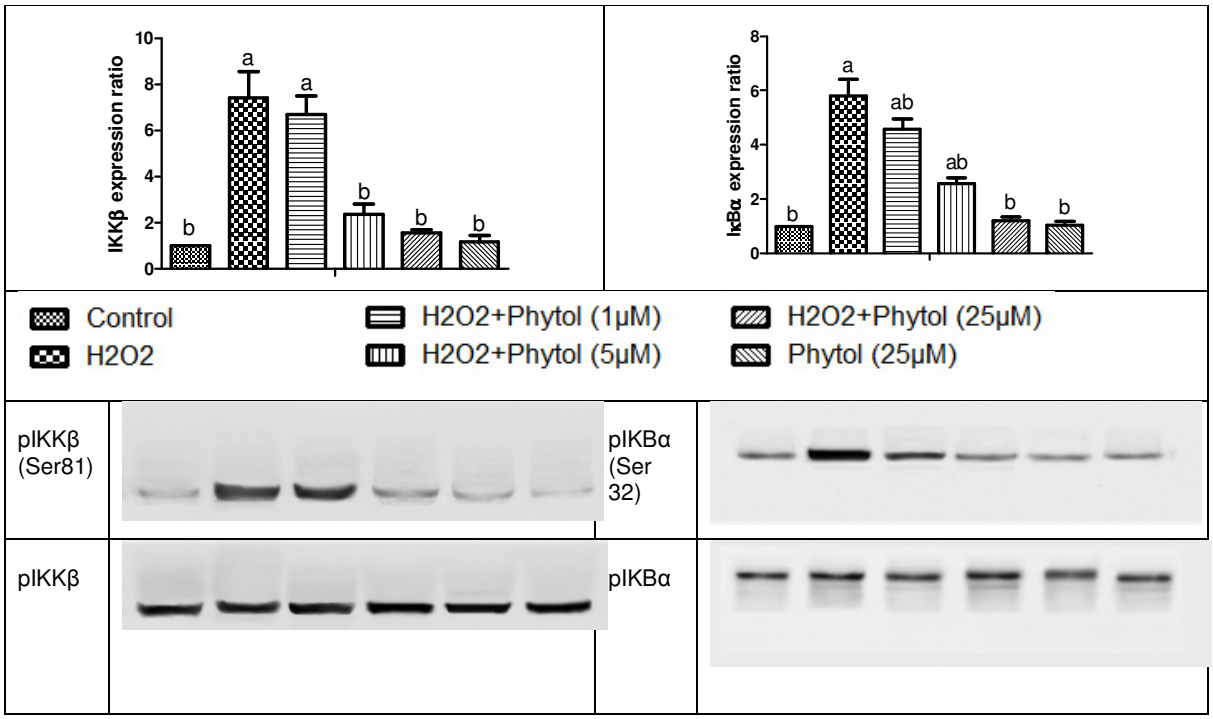

Fig. 4. Phytol treatment effects on the NFκB pathway activation, inhibition of IKKβ and IKBα phosphorylation; (A) Graph representing relative expression as Mean±SD. Significance difference with control and H_2O_2 treated groups indicated with superscript 'a' and 'b' respectively; (B) Western blot, L1, L2, L3, L4, L5 and L6 representing control, H_2O_2 + Phytol (1µM), H_2O_2 + Phytol (5µM), H_2O_2 + Phytol (25µM), Phytol (1µM).

IV. DISCUSSION

Reactive oxygen species are the consequence of metabolic reactions of the cells and in small amounts they contribute to the normal physiological activities of the cell. The cellular internal milieu control is equipped with anti-oxidant molecules and enzymes that keep these oxidants limited. Any disruption to the balance either because of higher production or depletion of antioxidants may lead to oxidative stress. The excess oxidative radicals are capable of reacting virtually any of the biomolecules and results damage which thought to play important role in the development of many chronic debilitating diseases. Prolonged excessive production of ROS plays central role in the development of inflammation [22]. The development of chronic inflammatory conditions is might be the main mode of action which is behind the development of damage and chronic debilitating diseases by oxidative stress [3].

In the present study H_2O_2 was used to develop the imbalance in the ROS control and induction of the inflammatory genes. H_2O_2 is an endogenous non radical ROS, mitochondrial respiration produce superoxide ion which is converted to H_2O_2 by the action of superoxide dismutase. H_2O_2 results oxidative damage by releasing hydroxyl radical that is one of the most potent ROS and can damage all of the three major biomolecules i.e. lipids, proteins and DNA. The damaged products of these reactions serve as DAMPs and can stimulate the immune system. The excess of ROS can induce many redox sensitive transcription factors including NFκB, that is implicated in the induction of ROS induced inflammation [23].

GSH is one of the most potent and abundant antioxidant and is responsible for most of the antioxidant capacity of the body. GSH reduces hydrogen peroxide, the reaction largely catalyzed by GSH peroxidase, and oxidized to GSSG, which can be restored as GSH by the enzyme GSSG reductase in presence of NADPH. When the presences of excessive radical overwhelm this restoration capability the cell actively excrete GSSG and this may result the depletion of GSH [24]. This oxidant/antioxidant disturbed balance also results decrease activity levels of Glutathione peroxidase [25]. It has been reported that the activity of antioxidant enzymes is controlled differentially [26]. Increased level of catalase activity was observed with H_2O_2 treatment; this might be compensatory to higher level of oxidants. Moreover, H_2O_2 and its products of lipid peroxidation are capable of mediating the catalase gene expression [27]. The activity level of SOD enzyme was reduced following the treatment with H_2O_2. SOD catalyze the superoxide (SO_2^-) dis-mutation to H_2O_2 and O_2, however the protein is prone to be oxidized by hydrogen peroxide at histidine amino acids which results the conformational changes and results loss of enzymatic activity [28].

It was observed in this study that the addition of phytol restored the cellular GSH level, enzymatic activity level of GSHPx and SOD significantly; however, the level of catalase remained slightly raised in comparison to control. This antioxidant effect of phytol may attributed to its ROS scavenging capability as the hydrogen of its allylic alcoholic group can scavenge oxidants efficiently [9] and phytol was found to possess good in-vitro radical scavenging activity when tested against DPPH and ABTS [29].

ROS like H_2O_2, superoxide and cytokines can induce the production of nitric oxide [30]. Oxidative stress, hypoxia and cytokines like TNFα, IL1β also leads to the induction of iNOS which plays important role in inflammatory processes. The chronic inflammations like arthritis found to be associated with increased circulating levels of nitric oxide, while the treatment with iNOS inhibitors found to improve the conditions. NO is cytotoxic and may lead to apoptosis in the producing cells as well as neighboring cells and thus lead to tissue damage [31]. Camila *et al.,* (2013) [32] have demonstrated that phytol can inhibit in vitro production of nitric oxide. We have shown here that phytol can attenuate the in vivo H_2O_2 induced NO production with down regulation of iNOS gene, this indicate its antioxidant effects and its application may found helpful in many chronic conditions where NO production suppression is required.

A slight imbalance in oxygen metabolism intermediates can start a vicious cycle for the establishment of chronic inflammatory conditions, where ROS induces the production of pro inflammatory cytokines and these are capable of inducing the production of more of the ROS. This cascade leads to the local tissue damage as well as systemic inflammatory response. Transcription factor NFκB is always associated with redox induced inflammatory response and plays a central role by controlling expression of pro inflammatory cytokines and enzymes like IL6, TNFα and COX2 [33, 34].

Our results shown that phytol inhibit the activation of NFκB and reduce the expression of pro-inflammatory cytokines like IL6, TNFα, IL1β and of PGE2 synthesis with lowering the expression of COX2.

In unchallenged cells the NFκB remains localized in the cytoplasm bound with its specific inhibitory proteins IκBα. Cellular damage products (both PAMPs and DAMPs), oxidative radicals including H_2O_2 and cytokines can induce the phosphorylation of IκB kinases (IKKα and IKKβ). These active kinases, mainly IKKβ, results phosphorylation of IκBs and sequestration of NFκB to the nucleus, where it serves as transcription factor for the pro inflammatory genes [35].

The assessment of inflammatory cytokines including IL6, TNFα and IL1β was performed at gene expression level using QRTPCR and at protein expression level using ELISA. Raised levels of these cytokines were observed atin both experiments with H_2O_2 treatment. Expression for the COX2 and INOs genes was also remained elevated with the H_2O_2 addition. The raised level of these inflammation mediators was found to be associated with the presence of higher level of phosphorylated species of IKKβ and IκBα, both were remained elevated with H_2O_2addition.

Our findings are in confirmation with Carla *et al.,* 2018 [36], and Pomari *et al.,* 2014 [37] and several of studies where H_2O_2 has reported to activate NFκB and results induction of pro inflammatory cytokines. These pro inflammatory cytokines are potent mediator of immune response and tissue damage, while PGE2 is considered the main mediator of debilitating conditions in chronic diseases like rheumatoid arthritis, while safe inhibition of COX2 synthesis to eliminate PGE2 is yet a target to achieve.

In phytol treated cells the level of phosphorylated IKKβ and pIκBα were reduced in dose dependent manner. This NFκB induction inhibition may be responsible for the subsequent reduction in the expression of inflammation inducers. A significant dose dependent reduction in the expression of IL6, TNFα, IL1β and

COX2 genes was observed with the phytol administration. Phytol has been reported by Silva *et al.,* 2014 [11] to reduce LPS induced pro-inflammatory cytokines and PGE2 levels in mice.

Diterpenes like phytol were reported to have anti-inflammatory and anti-redox activities, while many of the naturally occurring terpenoids were reported as inhibitor of NFκB. These can inhibit the activation at various levels i.e. by interfering the phosphorylation of IκBα, translocation of the complex to the nucleus or it's binding to the DNA. ROS scavenging and RNS inhibitory activity of phtyol might be responsible for the correction of redox state in cells treated with H2O2 and help in keeping the NFκB in inactive state or it directly interfere with the activators of NFκB activation process like other diterpenes. Solomon Rotimi *et al.,* (2014) [38] has reported a strong in-silico interaction between phytol and p50 subunit of the complex which may interfere with its DNA binding capacity and serves as active transcription factor.

V. CONCLUSION

In conclusion the result of this study shown that phytol a diterpene has antioxidant and anti-inflammatory effects. The anti-inflammatory effects of phytol are exerted by its interference to the NFκB activation pathway. This inference may be results with the scavenging of oxidative radicals which are strong activator of NFκB and/or its direct inhibitory effects on activator of the NFκB or activated complex itself. Our results indicate that phytol can alleviate the redox (H_2O_2) induced oxidative stress and inflammatory response by inhibiting the activation of NFκB complex. However further investigation of phytol interaction with the NFκB complex, its activator and inhibitor may leads to a better understanding of anti-inflammatory effects exerted by phytol.

VI. FUTURE SCOPE

Further elucidation of the precise mechanism of NFκB inhibition by phytol may provide the blueprint for the development of phytol based therapeutics for the treatment of many inflammatory and debliitatory diseases, where NFκB activation plays key role in the development of pathological response.

Conflict of Interest: There is no conflict of interest.

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