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Amendment of antioxidant andanti-inflammatory properties by a Quinozoline structured Alkaloid fraction using endothelial cells for augmenting cell migration process.

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Abstract

The effects of ALK-F AV (Alkaloid fraction of *Adathodavasica*) on lipopolysaccharide (LPS) stimulated oxidative damage in cultured human umbilical vein endothelial cells (HUVECs) were studied. The protective effects were assessed using MTT and measuring the generation of reactive oxygen species (ROS). The pro-angiogenic activity of ALK-F AV was tested using in vitro Matrigel tube forming assay and Western Blot. Transwell assay and scratch assay observed the effects of ALK-F AV on the migration of human umbilical HUVECs. Our results proved that ALK-F AV facilitated the proliferation of HUVEC, which was otherwise suppressed by exposure to LPS. ALK-F AV reduced LPS-induced inflammatory mediators and facilitated the establishment of a capillary network. Taken together, the present data indicate that ALK-F AV has attenuated the vascular injury induced by LPS. Thus, promoting angiogenesis through a process that extends beyond the direct delivery of angiogenic factors by up-regulating MMPs expression in endothelial cells.

Keywords: Lipopolysaccharide (LPS); Human umbilical vein endothelial cells (HUVECs); Reactive oxygen species (ROS); *Adathodavasica;* Endothelial cells; Matrix Metalloproteinases (MMPs); Matrigel; Pro angiogenic factor.

1. Introduction

Diabetes is a chronic metabolic disorder with the blunted inflammatory syndrome in addition to uplifted plasma levels of the pro-inflammatory cytokines such as IL-6 and TNF-α, a primary cause for inflammation and oxidative damage, both of which play a pivotal role in endothelial and cardiovascular dysfunction [1-3]. The main mediator in maintaining the vascular tone and its homeostasis is nitric oxide. There are three enzyme isoforms producing NO, endothelial nitric oxide synthase(eNOS), inducible nitric oxide synthase(iNOS) and neural nitric oxide synthase (nNOS) through the conversion of L-arginine amino acid into L-citrulline [4]. Lipopolysaccharide is a bacterial cell wall constituent that can trigger the infiltration of immune cells towards the inside compartment of the blood vessel and increases the vasculature. A combined model of HUVECs and LPS can portray a suitable system for inflammation and oxidative stress damage. ROS plays a vital role in the reduction of antioxidant potency and increases DNA loss and the underlying reason for many diseases such as cardiovascular, inflammatory, diabetic, neurological, and lipid peroxidation [5]. ROS performs a substantial character in organ injury such as brain and uterus since the uplifted value of ROS is associated with a reduction in the overall antioxidant ability and a notable increase in DNA disruption.

Nonetheless, several studies have found that under hyperglycemic conditions NO vigorously acts with superoxide anion ($O2^{\bullet-}$) to form peroxynitrite($ONOO^-$), a highly potent oxidizing molecule that diffuses across phospholipid membranes, leading to a decline in substratum nitrosylation and bioavailability of nitric oxide [6–8].

In addition, such TLR4-activation-mediated production and increase of cytokines and chemokines can stimulate cell migration and proliferation, and can also increase the expression of the matrix metalloproteases-2 (MMP-2) and matrix metallo-proteases-9 (MMP-9) which are involved in the degradation of extracellular matrix [9-12]. MMP-2 can promote endothelial cells migrating from the original blood vessels to the outer periphery and forming new blood vessels by degrading the basement membrane and remodeling extracellular matrix. MMP-9 was positively correlated with the expression of VEGF, suggesting the MMP-9 level might play important roles in angio-genic process [13].

In this study, we collectively evaluated the anti-inflammatory. And antioxidantefficacy of quinazoline structured Aalkaloid fraction of *Adhatodavasica* (ALK-F AV) was evaluated on Human umbilical vein endothelial cells usingLPS induced model. Our findings were corroborative through the suppression of inflammatory mediators and regulation of the expression of key pro-inflammatory cytokines such as TNF-α, IL-6, iNOS, eNOS, MMP-9, and MMP-2 [14].

2.Material and methods

2.1 Cell culture

HUVECs were bought from Himedia Laboratories (Mumbai, Maharashtra, India). The cells were seeded in HUVECs expansion medium with 10 % fetal bovine serum(Gibco NY, USA)

at 37°C in 5% CO_2 incubator. The HUVECs between 3-5 passages were subjected to ALK-F AV (1ng -10µg/mL) and LPS (1 µg/mL) for 24 h**.**

2.2 Cell viability assay

Reduction of MTT (3-[4, 5-dimethylthiazol- 2-yl]-2, 5-diphenyltetrazolium bromide) was used in checking the cell viability. Cell seeding was done on a 96- well plate and after the 24 h of incubation, it was then treated with the ALK-F AV (1ng -10µg) and were incubated again for another 24 h. Afterof 4 h of incubation,10 µl of MTT solution (5 mg / ml in PBS) was added to every well . Later, the medium was aspirated to dissolve the insoluble purple formazan crystals with the solubilizing agent (DMSO). The absorbance was measured at 570nm .The absorbance of the ALK-F AV treated cells and the control cells were used to determine the percentage of cell viability [15].

% Cell viability = (O.D. of test / O.D. of control) *100

2.3 Measurement of cytokines by ELISA

The LPS induced expression of the pro-inflammatory cytokines TNF-α and IL-6 and the action of ALK-F AV in the regulation of expression were estimated using Standard ELISA Kit protocol (Sigma-Aldrich (USA). The absorbance was detected at 450nm with the use of a microplate reader (Thermo Scientific, CA, USA) [16].

2.4 Estimation of the antioxidant enzyme activity

The activities of superoxide dismutase (SOD) , Catalase (CAT) and glutathione peroxidase (GPx) were assessed using protocols assay kit (Cayman Chemical, USA). The enzyme activities were then normalized with control [17,18].

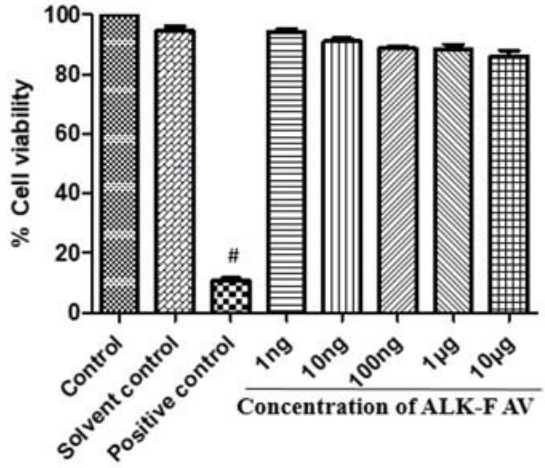
2.5RNA isolation and qPCR

Total RNA was isolated using Trizol (Himedia, Mumbai, India) method. The concentration and also the purity of the isolated RNA were spectrophotometrically determined at 260 and 280nm respectively. 2µg of total RNA was transcribed to a single stranded cDNA using Reverse transcription method and also the qPCR analysis was performed using aliquots of the cDNA samples and SYBR Green PCR Master Mix. The gene expression of iNOS, eNOS, MMP-9,2 and and GAPDH (standard) were analyzed employing a BIO-RAD CFX96 Real-Time PCR System (C1000 Touch thermal cycler). The $2^{-\Delta\Delta CT}$ method was employed to investigate the fold increase. All reactions were performed in triplicates [19-22].

3.RESULTS

3.1 Assessment of cell viability using MTT assay

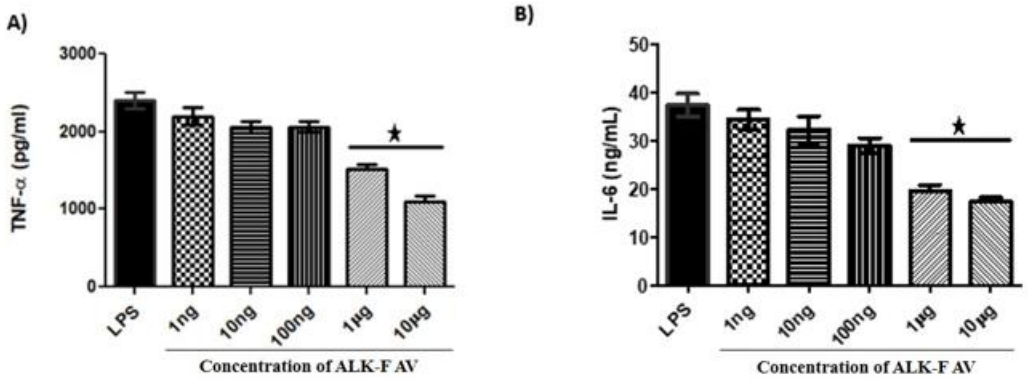
MTT reduction was used to access the cytotoxic effect of ALK-F AV HUVECs. The cell viability in the presence of the ALK-F is represented (Fig.1). The results showed that ALK-F of AVdid not alter the cell viability even at the highest concentration (10µg) and were taken for further studies.



Assessment of cell viability in presence ALK-F AV (1ng to 10µg/ml) in HUVECs. Methanol was used as vehicle and 0.01% triton x-100 used as positive control. Statistical evaluation was done by one way ANOVA followed by Duncan's multiple range test (DMRT) and expressed as mean ± S.D in triplicates of three independent experiments. $^{\#}$, $P < 0.01$ as compared with control, *, $P < 0.05$ as compared with control.

3.2 Effect of ALK-F AVin the expression of pro-inflammatory cytokines using ELISA analysis

The down regulation of pro-inflammatory cytokines (TNF-α and IL-6) which was stimulated by LPS was carried out by ALK-F AV (Fig 2).There was a significant decrease ($p<0.05$) in the expression of these pro-inflammatory cytokines in the highest tested concentrations (1µg and 10µg) of ALK-F AV (Fig.2).

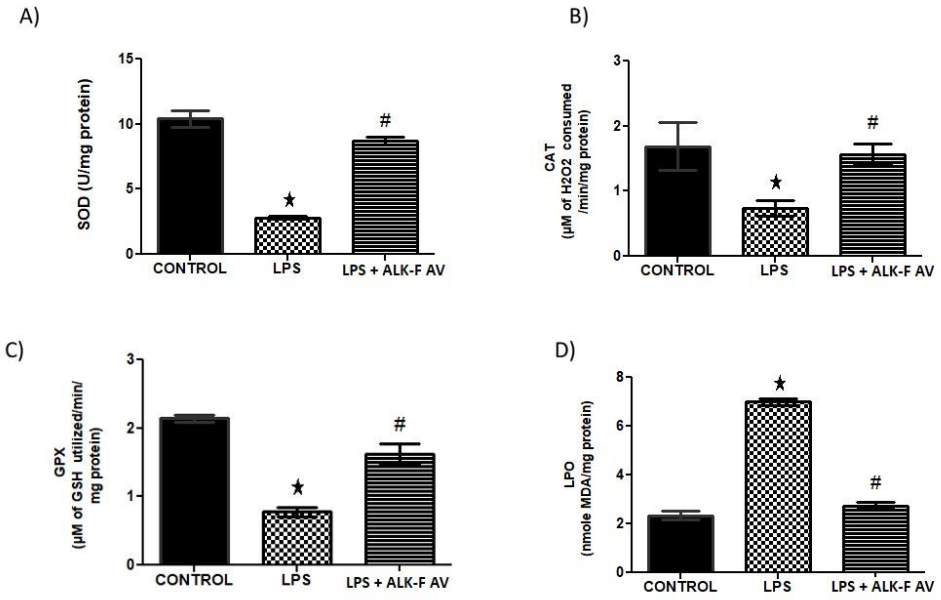


Effect of ALK-F AV on in TNF-α and and IL-6 expression in LPS treated HUVECs. (A) TNF-α and (B) IL-6 cytokines were measured using enzyme-linked immunosorbent assay (ELISA) on the supernatants of treated cells. HUVECs were treated with 1 ng/mL to 10 µg/ml of ALK-F AV

for 1 h, and continuously incubated with LPS (1 µg/ml) for an additional 18 h. Statistical evaluation was done by one way ANOVA followed by Duncan's multiple range test (DMRT) and presented as mean ± SD in triplicates of three independent experiments. *, $P < 0.05$ as compared with LPS.

3.3 Effect of ALK-F AVon anti-oxidant activity

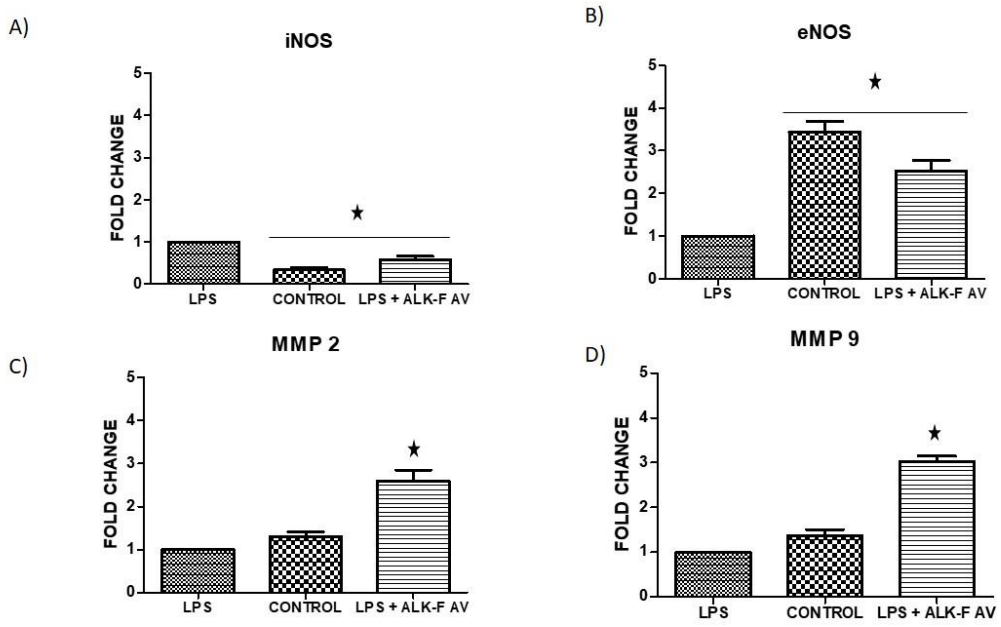
Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) production, the product of lipid peroxidation and the data showed that ALK-F AV significantly decreased the MDA production in HUVECs. To examine the effects of ALK-F AV on the intracellular antioxidant system, SOD, CAT, GST andGPx activities were measured. The results revealed that LPS induction decreases enzymatic activities which were later lessened in the ALK-F AV treated cells (Fig. 3A-D).



Effect of ALK-F AV on antioxidant enzyme activity and lipid peroxidation. A) SOD, B) CAT, C) GPx and D) LPO levels in HUVECs. Statistical evaluation was done by one way ANOVA followed by Duncan's multiple range test (DMRT) and presented as mean ± SD in triplicates of three independent experiments. *, $P < 0.05$ as compared with LPS.

3.4Effect of ALK-F AVon gene expression using Real-time PCR

LPS elicited the response of iNOS and was restrained by ALK-F AV (2.9 fold). There was an increase in eNOS, MMP-9 and MMP-2 mRNA expression levels in LPS treated cells at 10µg of ALK-F AV (2.5, 3.2 and 2.6fold). These results suggest that transcription of LPS induced iNOS can be down-regulated, where eNOS, MMP-9, and MMP-2 can be upregulated by ALK-F AV (Fig. 8).



Gene expression of (a) iNOS, b) eNOS, c) MMP-9 and d) MMP-2 in HUVEC's. The reactions were prepared according to the standard protocol for one step KAPA SYBR® FAST qPCR Kit Master Mix (2X). Statistical evaluation was done by one way ANOVA followed by Duncan's multiple range test (DMRT) and data were expressed as mean ± S.D of three independent experiments in triplicates. *, $P < 0.05$ as compared with LPS.

4.Discussion

The endotoxin Lipopolysaccharide (LPS) was utilized during this investigation to influence the inflammatory endothelial response in HUVECs [23,24]. Endothelial damage and repair studies were deliberated by LPS due to its character of increasing cell permeability and impairing the endothelial barrier, which is a necessary step within the initiation stage of inflammation [25]. Our studies have also reported high level expression of pro-inflammatory chemokines ,TNF-α and IL-6 under LPS stimulation in HUVECs.

Although ALKF-AV influence on eNOS and iNOS expression in HUVECs remains elusive. The results of this study have shown that ALKF-AV can increase the expression of eNOS and reduce the expression of iNOS induced by LPS.The pillar findings in our current study include (i) Lipopolysaccharide-induced a rise in endothelial cell monolayer permeability due to the excessive formation of nitric oxide, leading to coupling with superoxides resulting in the emergence of peroxynitrite (ONOO–). ii) LPS also raised iNOS mRNA and decreased eNOS mRNA expression; iii) ALKF-AV significantly inhibited LPS induced iNOS expression and also increased eNOS mRNA expression in HUVECs.

The most interesting finding from this study is that ALKF-AV differentially controls NO formation from eNOS and iNOS in HUVECs. Studies show that eNOS provides a gentle, diverse source of NO, while iNOS generates enormous quantities of NO [26]. There is substantial evidence that nitrative stress persuades the reaction from iNOS between ROS and NO in a rate-limiting manner of diffusion that plays a causative role in tissue damage. Large quantities of NO provided by iNOS deftly get reacted with the radical superoxide for the formation of potent oxidant peroxynitrite. NO remains highly reactive radical, and the cytotoxic radical ONOO- is generated by oxygen-free radicals which could wreck the cellular functions [27]. Present results indicate that ALKF-AV increases eNOS production to exert the angiogenic effect of NO, thereby contributing to its protective effect [28]. On the opposite hand, ALKF-AV prevents iNOS expression caused by the pathophysiological condition and thus protects tissues from nitrative stress.

Lipid peroxidation occurred by excessive ROS gives the product MDA and is commonly used as an oxidative stress biomarker. The escalated level of MDA is correlated with increased levels of oxygen-free radicals, to which polyunsaturated fatty acids present in cell membranes causes lipid peroxidation. But the enzymatic antioxidant defense system is acting as a natural defender upon lipid peroxidation[29].

In this context, SOD, Peroxidase, GPX and GSH are vital elements of the antioxidant defense system, where inhibition of this mechanism ends in increased vulnerability to free radical cellular damage. As per this research, oxidative stress was increased by LPS as shown by MDA overproduction and reduced antioxidant enzymes like SOD, Peroxidase, GPX, and GSH. When the endogenous antioxidant factors were limited in the cell apoptosis was found to be persuaded by oxidative stress.

Therefore protection of those endogenous antioxidant activities could preserve the HUVECs. ALKF-AV was found to significantly decrease MDA, NO levels, increase SOD, Peroxidase, GPX and GSH compared with LPS group.

IL-6 plays a serious role in the recruitment of monocytes at inflammatory sites. Endothelial cells can be activated by TNF-α to convey other inflammatory cytokines, which might harm the vascular integrity irreversibly [30]. We observed that ALKF-AV inhibited the development of TNF-α and IL-6, persuaded by LPS. The assembly of ROS has been induced by LPS, which might subsequently promote transcription factor, NF-Kβ which has a chief role in the inflammatory response and to control the degree of pro-inflammatory cytokines [31]. The expression of adhesion molecules and cytokines were modulated by LPS, and these alterations have a major part in the pathogenesis of inflammatory reactions which has been noted from several studies [32]. LPS-treated HUVECs release ROS, and therefore the cytokine induction in HUVECs may well be explained partially by a mechanism involving ROS and NF-κB activation. Hence the effects of ALK-F AV on the activation of pro-inflammatory cytokines caused by LPS

were evaluated. LPS in HUVECs had elevated levels of proinflammatory-related chemokines TNF-α and IL-6; which was ameliorated by ALK-F AV treatment in an incredibly dose-dependent manner which may be manifested from the results.

During wound healing, endothelial cells secrete MMP2, MMP-9 and vascular endothelial growth factors, thus stimulating the vascular endothelial cells proliferation, migration and accelerating neo-vascularization [33-36]. Matrix metalloproteinases can promote endothelial cell migration from existing blood vessels to the outer periphery to form new blood vessels by degrading the basement membrane and remodelling the extracellular matrix. When HUVECs were cultured on basement membrane-like materials, the endothelial cells would be arranged in a straight line soon and formed tubes [37]. Among these, MMP-2 and MMP-9 have been stressed due to their type IV collagenase activity is well accepted on the period of angiogenesis. The results thus obtained reveal the sequential suppression efficacy of more than one inflammatory cytokine by ALK-F AV. Therefore ALK-F AV has proven for its modulatory action in the regulation of inflammatory mediators and stabilizes angiogenesis using the LPS-induced model.

Conclusion

Under routine situations, the detailed regulation of intracellular ROS was executed by using numerous anti-oxidant assessment. But excessive ROS generation through intrinsic or extrinsic stimuli might also lead to cell homeostasis imbalance which in long run causes damage or dysfunction. Our experimental effects showed that once LPS stimulation with superior endothelial cellular pro-angiogenic factors, ALK-F AV reduced NO and iNOS expressions via upregulating MMP-2/-9 expression and gelatinolytic activity which can bebeneficial in the prevention or remedy of the inflammatory diseases. Thus ALK-F AV may be a potential target compound for the treatment of inflammatory diseases that gave us insights into the new idea of promoting angiogenesis through up-regulating the expression of MMP in ECs.

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DISCLOSURE STATEMENT

This research did not receive any grant from funding agencies.

CONFLICT OF INTEREST

No potential conflict of interests.

AUTHOR'S CONTRIBUTION

All the Authors have equal contribution towards conducting experiments, draft preparation, study consultation, writing, data handling. Corresponding author, Dr.Amala Reddy has

contributed towards consolidating study materials, experiments design, study validation, supervision and data analysis.

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Abbreviations

iNOS	-	Inducible nitric oxide synthase
eNOS	-	endothelial nitric oxide synthase
MMP-9	-	Matrix metalloprotease 9
MMP-2	-	Matrix metalloprotease 2
ROS	-	Reactive oxygen species
VEGF-α	-	Vascular endothelial growth factor
MTT	-	3-[4, 5-dimethylthiazol- 2-yl]-2, 5-diphenyltetrazolium bromide
ECM	-	Extracellular matrix
SOD	-	Superoxide dismutase
CAT	-	Catalase
GPx	-	Glutathione peroxidases
GSH	-	Glutathione
ALK-F AV	-	Alkaloid fraction of *Adathodavasica*
RT-PCR	-	Real time polymerase chain reaction
LPS	-	Lipopolysaccharide
WHO	-	World health organisation
NSAID’s	-	Nonsteroidal anti-inflammatory drugs
NO	-	Nitric oxide
DCFH-DA	-	Dichloro-dihydro-fluorescein diacetate
RIPA	-	Radioimmunoprecipitation assay buffer
TNF- α	-	Tumour necrosis factor
IL-6	-	Interleukin
PCR	-	Polymerase chain reaction
GAPDH	-	Glyceraldehyde 3-phosphate dehydrogenase
TLR4	-	Toll like receptor