Adhatoda Vasica: a potential ayurvedic intervention against COVID-19 associated impaired immune response and hypoxia-inflammation phenotype

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Research Article

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Abstract

Background: The importance of hypoxia inducible factor-1 α (HIF-1α) stabilization in uncontrolled infection and inflammation is widely accepted. Several inhibitors of HIF signalling are in clinical trials for malignancy, ischemia and inflammatory diseases. Increased hypoxia is being reported to be an important modifier for several pathological features of COVID-19 such as impaired immunity, hyper-inflammation, thrombosis, lung injury and sepsis.

Methods: In this study we tested the effect of whole aqueous extract *Adhatoda Vasica* (AV), that our group has shown to have anti-hypoxic and anti-inflammatory effects, on various outcomes of hypoxic response. Effects of AV were assessed in preclinical mouse models of pulmonary fibrosis, bacterial sepsis and siRNA induced hypoxia-thrombosis phenotype. Therapeutic relevance of AV in current pandemic were also examined through transcriptome and molecular docking analysis.

Results: Oral administration AV extract attenuated the increased levels of airway inflammation, collagen content, transforming growth factor-β1 (TGF-β1), IL-6, HIF-1α and improved the overall survival rate in bleomycin treated and Cecum Ligation and Puncture (CLP) induced mice. AV treatment also rescued the prolyl hydroxylase domain 2 (*phd2)* siRNA induced HIF-1α and associated blood coagulation phenotypes in mice. Transcriptome analysis of lungs of AV treated naïve mice reveal downregulation of hypoxia, inflammation, TGF-β1 and angiogenesis and upregulation of adaptive immunity related genes. These genes and pathways show opposite expression in transcriptome of BALF and PBMCs of SARS-CoV2 infected patient. Molecular docking of AV constituents presents in extract reveal many molecules with low binding energy (≤ -8) to multiple SARS-CoV2 and host target proteins that are relevant in viral entry and replication.

Conclusion: Our results provide a scientific rationale for this ayurvedic herbal medicine in ameliorating the anti-inflammatory and anti-HIF-1α effect for potential use in management of COVID19 patients.

Introduction

Increased alveolar hypoxic response levels are inevitable consequences of many respiratory disorders such as chronic obstructive pulmonary disease and pulmonary fibrosis [1,2]. The key player of cellular response to hypoxia is the hypoxia-inducible factor (HIF)-1α and its regulatory protein, the prolyl hydroxylase domain (PHD)-2 enzyme [3]. The induction of HIF-1α is considered to be pro-inflammatory. It leads to transcriptional activation of essential genes implicated in airway remodelling and inflammation, such as vascular endothelial growth factor, transforming growth factor-1b, inducible nitric oxide synthase, interleukin -17 (IL-17), and IL-6 [3,4]. Thus, it is not just a consequence of diseases, elevated tissue/cellular hypoxia actively participates in exaggerating the inflammatory response contributing to progressive lung damage/injury.

HIF-1α also plays pivotal role in infection, especially in promoting viral and bacterial replication [5]. In the present COVID-19 pandemic by the severe acute respiratory coronavirus (SARS-CoV2) the role of hypoxia response in inducing severe lung inflammation and other outcomes has been one of the most highlighted observation [6–8]. Clinically, the interaction of the host and SARS-CoV2 is broadly described in three stages: first, asymptomatic state; second, a non-severe symptomatic state characterized by upper airway and conducting airway response; third, severe respiratory symptomatic state with the presence of hypoxia, acute respiratory distress syndrome (ARDS) and progression to sepsis [7]. During incubation and non-severe state, a specific humoral and cell-mediated adaptive immune response is required to eradicate the virus and prevent disease progression to a severe state. Thus, strategies to boost immune responses at this stage are undoubtedly important [7,9]. However, defective immune response causes further accumulation of immune cells in the lungs, progressing to aggressive production of a proinflammatory cytokine such as IL-6, TNF-Alpha resulting in an influx of immune cells and cytokines that damage the airways/ lung architecture. This vast release of cytokines by the immune system in response to the viral infection and/or secondary infections causes severe inflammation, endothelial dysfunction, sepsis and multi-organ damage [7,9,10]. In addition, recent research also report coagulation abnormalities in severe COVID-19 cases [11]. The relation of hypoxia-coagulation is well known, where we and others also showed the crucial role of hypoxic response in the form of thrombosis and bleeding susceptibility through HIF-1α and vWF axis [12,13]. Thus, medicinal agents that possess immune-boosting and anti-hypoxic effects could hold a promise for a better therapeutic option to preclude the SARS-CoV2 infection and severity.

We have recently shown an extract of *Adhatoda Vasica* (AV); an ayurvedic medicine possesses robust anti-hypoxic properties and can reduce severe airway inflammation induced by an augmented hypoxic response in treatment-resistant asthmatic mice [14]. The anti-HIF-1α effect of AV also restores the cellular hypoxia-mediated loss of mitochondrial morphofunction *in vitro* [14]. As a follow-up we evaluated AV's usefulness in other severe lung pathologies, where hypoxia signalling is pertinent. With hypoxia assuming relevance in COVID19 during this time we further tested it potential in potentially limiting inflammatory outcomes and viral replication using whole-genome expression and molecular docking analysis respectively.

Methods

Preparation of plant extract and LC-MS fingerprinting:

Adhatoda Vasica (AV) was collected from Delhi-NCR region, India in the flowering season (November to March). Water extract of plant (leaves, twigs and flowers) was prepared according to classical method described for rasakriya in *Caraka Samhita* [15]. The process for the formulation involved preparation of decoction condensation and drying as described in earlier study [16]. Chemical fingerprinting of prepared AV extract was carried out by LC-MS at CSIR-CDRI, Lucknow, India; in two independent experiment.

Animals

The study was designed and performed following guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by Institutional Animal Ethics Committee of CSIR-Institute of Genomics & Integrative Biology (IGIB), New Delhi, India. The BALB/c and C57BL/6 male mice (8-10 weeks old) were bred under the pathogen-free condition. They were acclimatized to animal house environment one week before starting the experiments at CSIR-IGIB, New Delhi, India and maintained according to guidelines of CPCSEA. All the surgical procedures were performed under sodium pentobarbital anaesthesia and maximum efforts are taken for minimum suffering of animals.

Grouping and treatment of mice

Mice were mainly divided into two groups as Vehicle and treatment according to the experiment. In case of Cecum ligation puncture (CLP) experiment (n = 5-9), BALB/c mice were divided in Vehicle (distil water and 10% ethanol, oral) and CLP (mice underwent CLP surgery). CLP mice subdivide in CLP+Cyclo A (Cyclosporin A treated CLP mice) and CLP+AV-D2 (Adhatoda extract treated CLP mice) group. For CLP experiment, treatment of AV (130mg/kg dissolved in distilled water, oral) or Cyclo A (Cyclosporin A, 15mg/kg dissolved in 10% ethanol, orall) was started two days (48hours) before CLP and was continued till the mice survives after CLP (figure 1). In that some mice (*n*=3-4) from each group were sacrificed after 20 hours of CLP to assess lung histology and cytokine levels. Similarly, in bleomycin fibrosis model (n = 5), C57BL/6 mice were divided in Vehicle (i.e. Sham), Bleo (bleomycin treated) and Bleo+AV-D2 (AV 130mg/kg treated Bleo mice). In that AV treatment was done from day 18 to 21, as shown in the schematic (figure 1A). Bleomycin (3.5 U/kg of mice) was given intratracheally to isoflurane-anesthetised C57BL/6 mice on day 0 of the protocol (figure 1) to induce fibrotic changes in mice as described previously [17]. For transcriptomic research (n = 4-5), BALB/c mice divided into Vehicle (distil water, oral) and Adhatoda Vasica (AV) extract group. AV group is further subdivided into two according to its dose: AV-D2 (Adhatoda Vasica extract 130 mg/kg, dissolved in distilled water, oral) and AV-D4 (Adhatoda Vasica extract 260 mg/kg, dissolved in distilled water, oral) as described previously [14]. Distil water or AV (130mg/kg or 260mg/kg) treatment was given to mice by oral gavage for consecutive four days as represented in the figure. In PHD2 siRNA induced hypoxia model (n = 4-5) BALB/c mice were divided in scrambled siRNA (Scrm siRNA), prolyl hydroxylase domain-2 siRNA (PHD2 siRNA) and AV-D4 treated PHD2 siRNA group (PHD2 siRNA+AV-D4) group. AV-D4 dose (260 mg/kg, dissolved in distilled water, oral) given for four consecutive days and 90µg siRNA (Sigma) administered intranasally which dissolved in ultrapure DNase and RNAse free water with in-vivo jetPEI as the transfection reagent (Polyplus Transfection, France) to isoflurane-anesthetised mice on day 1, 3 and 5th of the protocol.

Clotting and bleeding time assay, blood collection and platelet measurement

Tail bleeding was measured as described previously [13]. Briefly, anesthetized mice's tail amputated with a sharp scalpel, and bleeding time was then determined by monitoring the duration of animal tail bleeding until it ceased and was kept in a prone position and immersed in PBS. Clotting time measured by the capillary tube method. The mice's tail was cleaned with 70% alcohol and punctured with a 1ml syringe needle. Filled two capillary tubes with free-flowing blood from the puncture site after wiping the first drop of blood. Stop clock started and capillary tubes were broken to see whether a thin fibrin stand formed between two broken ends. After fibrin stand is observed, clotting time measured from the average of two capillary tubes. For platelet measurement, blood obtained by cardiac puncture and collected in EDTA coated MiniCollect tubes (Greiner Bio-One Gmbh, kremsmünster, Austria) as described [13]. The whole blood was used to measure total and of active Platelet count and was carried out through flow cytometry using FACSCalibur (BD Biosciences, USA). Briefly, diluted whole blood (1:4) in PBS was incubated with APC conjugated anti-CD62P (eBioscience Inc, San Diego, CA, USA) and FITC

conjugated anti-CD41 (eBioscience Inc, San Diego, CA, USA) for 15 min. Matched fluorescein-conjugated isotype control antibodies were used simultaneously for staining for comparison. The activity was compared using CellQuest Pro software (BD Biosciences, USA).

Bronchoalveolar lavage fluid collection and histopathology

Bronchoalveolar lavage fluid (BAL) was collected by instilling 1 ml PBS into the tracheotomised airway and recovered BAL fluids were processed to get total leukocyte count, as described previously (). For lung histology, the lungs were excised and fixed in 10% buffered formalin. The fixed, paraffin-embedded tissues cut into 5um sections and either stained with hematoxylin and eosin (H&E) to asses inflammation or Masson's trichome (MT) staining to assess collagen content.

Cecum ligation puncture (CLP) procedure

Mice were anesthetised by injecting intraperitoneally a solution of 1:1 ketamine (75mg/kg) and xylazine (15mg/kg). The abdomen was shaved, and the peritoneum area was disinfected betadine solution followed by wiping with a 70% alcohol. Under aseptic conditions, a 1 cm midline incision was made, and the cecum carefully exposed with the adjoining intestine. The cecum was then tightly ligated with a 3.0 Mersilk (PROLENE, 8680G; Ethicon) sutures at the base and punctured once with a 19-gauge needle on the same side of the cecum. A small amount of stool extruded to ensure patency of the puncture sites. The cecum then returned to the peritoneal cavity, and the wound was closed with 3.0 Mersilk sutures. Control mice (i.e. Sham), the cecum was exposed out and then returned to the peritoneum without ligation or puncture. Mice were resuscitated by injecting subcutaneously 1 ml of pre-warmed 0.9% saline solution using a 25G needle. After surgery, animals placed immediately to a cage with exposure to a heating lamp of 150W until they recovered from the anaesthesia. The recovery time is from 30 min to 1 hour. Mice were monitored every 12 hours for survival or euthanised after 20 hours (n=3) for measurement of cytokines while they were fed with their regular diet and water. Two independent experiments recorded the mortality of mice after CLP surgery.

TGF-b, IL-6,IFN-g, HIF-1a and vWF measurement

The levels of TGF-b, IL-6, IFN-g (BD, USA), HIF-1a (R&D, USA) and vWF (USCN, China) were measured in lung tissue homogenate or in plasma of the mice by sandwich ELISA, as per manufacturer's protocol.

RNA isolation and whole transcriptome analysis:

Total RNA was isolated from mouse lung tissue treated with AV (AVD2 and AV-D4, n=5) or distilled water (vehicle, n=4) using the RNeasy Plus Mini Kit (Qiagen, CA, USA) following the manufacturer's protocol. For genome-wide expression analysis, the Affymetrix GeneChip MTA 1.0 array was used according to the manufacturer's instruction. For each sample, 250 ng of RNA was quantified and hybridized to microarray chips following a series of consecutive steps described in the protocol. After hybridization, microarray chips are then scanned using an Affymetrix GCS 3,000 scanner (Affymetrix, CA, USA) and the signal values are further evaluated using the Affymetrix® GeneChip™ Command Console software. Raw data automatically extracted using the Affymetrix data extraction protocol in the Affymetrix GeneChip® Command Console® Software (AGCC). CEL file import, mRNA level, all analysis, and export of the results were all performed using Affymetrix® Expression Console™ software. A comparative study between the vehicle and the AV treated samples done by using fold-change and p-value, genes considered to the differentially expressed by applying the criteria of significance p-value less than or equal to 0.05.

Functional enrichment and Connectivity map analysis

For functional analysis, we used Enrichr (amp.pharm.mssm.edu) tool. For pathway and gene ontology analysis, we examined gene enrichment in Cellular Compartment, Biological Processes, BioPlanet, Wiki, KEGG human pathway and gene set enrichment was considered if P-value less than 0.05 in Enrich r tool. For connectivity map (CMap) analysis, differentially expressed genes ranked

according to fold change and list of top 150 up and down-regulated genes compatible with the CMAP data signatures was used to query the connectivity using clue.io touchstone database.

SARS-CoV-2 transcriptome meta-analysis

We obtained the raw RNA sequencing data from SARS-CoV-2 patients Broncho-alveolar lavage fluid (BALF) and peripheral blood mononuclear cells (PBMC) from the authors [18] and analysed them inhouse. Functional enrichment and gene set enrichment was done as described above using the differentially expressed genes. The results were then compared with AV transcriptome data to find intersections between gene ontologies, enriched gene sets, and connectivity map perturbations between upregulated genes of BALF, PBMC and downregulated genes of AV and between downregulated genes of BALF, PBMC and upregulated genes of AV.

Molecular docking

The complete genome sequence of the novel SARS-CoV-2 virus was obtained from the National Centre for Biotechnology Information (NCBI) nucleotide database (NC_045512.2). The available 3D crystal structures of all the target proteins such as 3CLpro, PLpro, RdRp, S-protein, ACE2 and JAK2 were taken from protein data bank [19]. Others structures (NSP4, NSP7, NSP8, NSP9, NSP13, NSP14, NSP15 and NSP16, and TMPRSS2) were built using homology modeling with suitable templates using Swiss model [20] and I-TASEER web-servers [21]. The active regions of the proteins were identified by COACH meta-server and the results were compared with results from CASTp web server [22]. The anti-COVID-19 activity of the compounds extracted from the Adhatoda vasica were investigated using Molecular Docking studies using Schrodinger suite (Maestro) [23] and AutoDock vina packages [24]. In Schrodinger suite, all the target proteins were prepared using protein preparation wizard that included optimization followed by minimization of heavy atoms of proteins. The energy minimized 3D structures of all the ligands were prepared using LigPrep. The best pose of ligands that fit well in the protein cavity was carried out using OPLS3 force field with Glide package in Extra Precision mode (XP) mode. According to the size of binding cavity of the proteins, the coordinates x, y and z of the grid box were chosen with the grid resolution of 1 Å for calculations using AutoDock vina package.

Statistical analysis

Statistical significance determined by one-way analysis of variance and analysis was done using GraphPad Prism software. In the case of mice experiment, all data represent mean ± SEM; n= 3-10 in each group and significance denoted by *p <0.05, **p <0.01, ***p <0.001. p-value > 0.05 is considered non-significant (NS). Significance of the survival study determined by Log-rank (Mantel-Cox) test using GraphPad Prism software.

Results

AV treatments inhibits the bleomycin induced pulmonary fibrosis features as well as increased HIF-1α levels in mice

To test the effect of AV treatment on lung fibrosis, bleomycin treated mice were orally administered with AV (130mg/kg, AV-D2) as shown in figure 1A. We observed a significant increase in TGF-β1 and HIF-1α levels in bleomycin (Bleo) treated mice lung compared to control-Sham mice, which decreased after AV-D2 treatment (figure 1B, C). Masson's trichrome staining showed a marked increase in collagen deposition in Bleo mice lungs compared to Sham mice (figure 1D). AV-D2 treatment reduces this increased collagen deposition in Bleo mice (figure 1D).

AV ameliorates the hallmarks of lung inflammation and injury in mice model of sepsis

Mice that underwent CLP (Cecal ligation puncture) surgery show significant increases in IL-6 levels, whereas IFN-g was decreased in lung homogenate after 20 hours of surgery compared to sham mice (figure 1F, G). AV pre-treatment restored the levels of both cytokines in mice lungs but Cyclo-A (a positive control) pre-treatment reduced only IL-6 levels in mice (figure 1F, G). Besides, histological analyses showed that CLP went mice lung sections stained with haematoxylin and eosin (H&E) had increased inflammation and blood exudation (figure 1H). Pre-treatment of AV-D2 and Cyclo A to CLP mice seems effective in reducing these lung histological changes (figure 1H). CLP

surgery also leads to a significant decrease in mice survival rate compared to sham group (figure 1I). Treatment of Cyclo-A or AV-D2 to CLP mice significantly increases their survival rate compared to CLP untreated mice (figure 1I). In CLP+Cyclo A and CLP+AV-D2 group, the mice survival rate after 24 hours is 66.6 and 44.4 %, respectively (figure J).Though, in both group's survival rate is 33.33% at the end of 142 hours of CLP surgery (figure J).

AV treatment inhibits hemostatic outcomes of hypoxia induced by PHD2 siRNA in mice

Next, to test whether AV anti- HIF-1α effects also prevents the hypoxia-induced blood coagulation phenotype [13], we treated BALB/c mice with AV-D2 (130 mg/kg) and AV-D4 (260 mg/kg) concentration (figure 2A). Oral administration of AV-D2 and AV-D4 to naïve mice does not cause any significant change in body weight, and lung and liver histological architecture (figure 2B, C), indicating its non-toxic nature. In the case of hemostasis parameter, treatment of AV-D4 dose to naïve healthy BALB/c mice causes a decrease in total as well as activated platelet count. Still, it does not affect mice tail bleeding time (figure 2D, E). To confirm the above-observed effect of AV-D4 on blood parameters, we induce cellular hypoxia in mice by specific PHD2 siRNA treatment (figure 2F), as described previously [13]. PHD2 siRNA treatment leads to a significant decrease in blood clotting and tail bleeding time (figure 2G). It also causes an overall increase in total and activated platelet count in mice blood (figure 2H). These changes induced by PHD2 siRNA are associated with an increase in blood HIF-1α and vWF levels indicating the platelet aggregation development (figure 2I, J). Interestingly, AV-D4 treatment to PHD2 siRNA mice causes a significant reversal of blood coagulation phenotype in terms of mice's blood clotting time, platelet count (both total and active) and vWF levels (figure 2 G-I). These effects of AV are associated with the reversal of the increased blood HIF-1α levels (figure 2J). However, AV treatment does not affect the mice's bleeding time, which was reduced after PHD2 siRNA treatment (figure 2G).

Modulation of immune response and hypoxia pathway genes: Revealed from lung transcriptome of AV treated mice

Lung transcriptomic analysis showed an upregulation of 1258 genes after AV-D4 treatment and 375 gens after AV-D2 treatment in naive mice. While, 1133 genes in AV-D4 and 262 genes in AV-D2 were downregulated, compared to Vehicle (distilled water) treated mice. We observed enrichment of pathways like IL-2 signaling, T cell signaling, T cell-mediated immunity, natural killer cell-mediated cytotoxicity, Haematopoietic cell lineages in the AV-D4 up-regulated genes. Similarly, biological processes like neutrophil activation and degranulation, neutrophil-mediated immunity, immune response regulation, and cellular defense response was enriched in AV-D4 up-regulated genes (figure S1A). In AV-D2 up-regulated genes, pathways relevant in mitochondria, T cell signaling, cytotoxic T cell-mediated immune response are enriched (figure S1A). Whereas, pathways like collagen biosynthesis, extracellular matrix organization, TGF beta regulation, hypoxia, and associated inflammatory MAPK-signaling significantly enriched in both AV-D4 and AV-D2 downregulated genes (figure S1A). Overall, it indicates that AV treatments favour the expression of genes that are important in immunity and adaptive immune response and inhibitory to genes involved in hypoxia associated angiogenesis, fibrosis, and inflammatory cascade.

Since the above observations made in independent experiments pointed towards AV's role in hypoxia- inflammation- coagulation- sepsis axis, we thought it would be worthwhile to investigate the repurposing possibility of AV against SARS-CoV2 infection. We examined transcriptome profiles of BALF and PBMC specimens of SARS-CoV2 patients recently published by Xiong et al [18]. Analysis of differentially expressed genes in BALF and PBMC of SARS-CoV2 patients shows that the genes which are up-regulated during infection are related to viral transcription, viral replication, and viral process (figure S1B). Besides, pathways like TGF-beta signaling, VEGFR pathway, IL-6 signaling, and HIF-1 signaling are significantly enriched in a set of up-regulated genes of BALF or PBMC or both samples (figure S1B). Whereas downregulated genes are related to pathways like adaptive immune system, IL-2 signaling, mitochondrial relevant function, T cell signaling, local inflammatory response, and processes related to neutrophil activation/degranulation, neutrophil-mediated immunity, and interferon-gamma signaling (figure S1B).

These findings show that the pathways and processes enriched in SARS-CoV2 infected individuals overlap with those of AV gene expression but in the opposite direction. Overall, it was observed that functional enrichment patterns in the up-regulated genes of SARS-CoV2 BAL and PBMC samples were seen in down-regulated genes of AV treated mice and vice-versa, suggesting a possible therapeutic application of AV in preventing or reversing the SARS-CoV2 induced transcriptional changes.

Towards this analysis of 'common genes' between AV and SARS-CoV2 i.e. the genes that are up-regulated by AV treatment, but the same were down-regulated during SARS-CoV2 infection or vice-versa, shows pathways/processes such as immune response, fibrosis, hypoxia

response, platelet activation, and aggregation are enriched (figure 3A, S2). In the case of AV treatment, shared immune function-related genes are up-regulated (figure 3, S2) and hypoxia, fibrosis, and hemostasis related genes are down-regulated (figure 2, S2), compared to SARS-CoV2 BAL and PBMC samples as shown in the figure 3A and detailed in figure S2A. These findings supported with the increased levels of immune cells in bronchoalveolar lavage fluid (BAL) of mice treated with AV, compared to vehicle-treated mice (figure S2F).

CMap analysis identified the list of compounds whose expression pattern matches with AV and could be repurposed against SARS-CoV2 infection

To identify similarities and differences in the gene expression pattern of AV with other FDA-approved drugs and bio-actives, we mapped the transcriptomic signature of AV using Connectivity Map (CMap) database. A positive score in CMap analysis indicates a similar expression pattern of AV with compared compounds gene expression signature, whereas a negative score indicates an opposite pattern. We also queried the SARS-CoV2 BAL gene signature with CMap and observed that the compounds which are connected positively with AV have a negative score with SARS-CoV2 (figure 4A) transcriptome data. Noteworthy is the HDAC inhibitor 'Apicidin' which has high positive connectivity with AV gene signature(+91.76 score) (figure 4A) and a negative connectivity (-79.09 score)with SARS CoV2 BALF signature. (figure). Apicidin has recently been shown to bind to viral non-structural protein 5 (nsp5) protein and features in the list of potential drugs which could be repurposed against SARS-CoV2 infection [25].

Molecular docking predicts AV could bind SARS-CoV2 and host target proteins

Chemical components present in *Adhatoda Vasica* (table S1, S2) were examined by molecular docking analysis with SARS-CoV2 and host proteins. Tables 1 and S3 represent the docking results of the constituents of *Adhatoda vasica* with key target proteins of SARS-CoV-2 virus. The trend in the docking results obtained from Schrodinger suite of packages is almost similar to those of AutoDock vina package. For brevity and further analysis, results from Schrodinger package are only considered. It is observed from the results that all the flavonoids derivatives are found to have high negative binding affinity than quinazoline alkaloids analogues. Specifically, the three compounds namely Luteolin-6,8-di-C-glucoside, Luteolin-6-C-glucoside-8-C-arabinoside and Luteolin-6,8-di-C-arabinoside have higher docking score values (-7.45 kcal/mol for PLpro, -9.43 for S-protein, -15.25 for NSP14, -11.12 for TMPRSS2, -9.82 for ACE2), (-11.59 kcal/mol for 3CLpro, -10.60 for RdRp) and (-10.86 kcal/mol for NSP16, -13.82 for JAK2) respectively (table 1). The key residues of target proteins that contribute more for binding with the compounds of *Adhatoda vasica* are shown in Table S4. The compound Luteolin-6-C-glucoside-8-C-arabinoside makes cation-π interaction with residue (His 41) of 3CLpro with higher affinity -11.59 kcal/mol (figure 4B). The π-π stacking interaction between the Luteolin-6,8-di-C-glucoside and residues (Phe 426 and Phe 506) of NSP14 protein enhances the binding affinity to -15.25 kcal/mol when compared to other compounds as shown in figure 4C and other interaction plots are represented in Supporting material figure S3.

Table 1: Binding affinity of compounds of *Adhatoda Vasica* with different target proteins of SARS-CoV-2 virus in kcal/mol using Schrodinger XP Glide package.

Compounds	3CLpro	PLpro	RdRp	S-pro	NSP14	NSP16	ACE2	TMPRSS2	JAK
Choline	-2.04	-2.304	-3.02	-2.403	-1.903	-2.721	-6.246	-2.314	-1.769
Betaine	-2.638	-2.599	-3.449	-2.775	-3.109	-2.732	-6.079	-3.328	-2.851
Vasicinol	-4.523	-2.542	-2.941	-3.187	-5.557	-3.848	-3.546	-3.591	-7.001
Adhavasicinone	-4.291	-2.715	-2.963	-3.42	-5.246	-3.147	-3.702	-3.011	-7.132
Linarinic_acid	-4.547	-3.161	-3.486	-3.449	-6.048	-4.593	-3.539	-4.26	-6.181
Vasicine	-4.027	-2.835	-1.961	-2.843	-5.313	-3.292	-3.026	-3.473	-5.973
Vasicinolone	-4.098	-2.787	-3.627	-3.207	-5.939	-4.303	-3.53	-4.371	-7.249
5_methoxyvasicine	-4.009	-3.234	-3.72	-2.676	-5.199	-1.982	-3.155	-2.73	-5.08
Vasicine_glycoside	-6.387	-3.692	-3.803	-4.239	-9.359	-5.857	-3.964	-6.069	-8.374
Vasicinone	-3.762	-2.416	-3.062	-3.007	-5.152	-1.35	-2.884	-4.59	-7.027
Luteolin_6_8_di_C_glucoside	-9.913	-7.451	-9.574	-9.429	-15.251	-9.894	-9.823	-11.124	-12.28
Luteolin_6C_glucoside_8C_arabinoside	-11.59	-7.161	-10.6	-6.864	-14.993	-9.519	-6.449	-9.442	-13.46
Kaempferol_3_O_rutinoside	-8.879	-6.889	-7.518	-6.606	-12.022	-7.604	-5.177	-6.888	-11.7
Apigenin_6C_glucoside_8C_arabinoside	-10.57	-6.194	-7.23	-5.865	-13.67	-8.532	-5.762	-6.918	-11.67
Luteolin-6_8-di-C-arabinoside	-9.978	-7.082	-9.778	-6.324	-12.728	-10.86	-7.249	-7.768	-13.82
Luteolin_6C_glucoside	-10.8	-5.599	-8.126	-5.991	-10.659	-8.749	-5.576	-7.688	-9.974
Apigenin-6_8-di-C-arabinoside	-11.04	-6.062	-8.711	-6.011	-12.173	-9.899	-4.378	-7.286	-10.77
Apigenin_6C_glucoside	-8.789	-4.01	-7.647	-5.016	-9.825	-8.23	-4.18	-6.826	-8.35
Luteolin-6-C-arabinoside	-9.046	-4.262	-8.744	-6.631	-10.316	-10.578	-4.268	-7.116	-10.83
Quercetin_3_O_glucoside	-8.119	-6.111	-8.233	-7.416	-10.757	-7.54	-5.078	-7.827	-12.49
Apigenin-8-C-arabinoside	-5.679	-5.185	-6.216	-6.15	-9.971	-6.764	-4.922	-5.119	-10.77

Discussion

Adhatoda Vasica or Vasa has been extensively used in Ayurveda for treating a wide range of inflammatory and respiratory conditions [26]. Even in modern clinical practice, it is recommended for strong bronchodilatory and antitussive effects [26,27]. Also, AV's active ingredients and their derivates such as Bromhexine , Ambroxol are effective against various respiratory ailments like asthma, COPD, and tuberculosis [26]. However, none of the studies have so far studied the effect of AV on molecular markers of asthma/inflammation phenotype in its aqueous extract form prescribed in Ayurveda. For the first time, we have shown that the anti-asthmatic effect of AV is not only limited to acute asthmatic features but it also alleviates the severe airway inflammation in steroid-nonresponsive asthmatic mice [14]. We show that the AV extract causes inhibition of HIF-1α (key transcription factor in hypoxia) by increasing the expression of its negative regulator, PHD2, thereby modulates hypoxic response, which forms the basis for its diverse therapeutic effects [14]. Further, AV treatment to lung epithelial cells has also been shown to reduce hypoxia's downstream impact such as mitochondrial dysfunction [14]. In this study we examined anti-hypoxic effects of AV in other hypoxia-inflammation prevalent conditions.

Our study shows that AV could reverse the pulmonary fibrosis (PF) pathological features in the bleomycin mice model (figure 1). HIF-1α stabilization is observed in many cell types of PF lungs and causes increases in collagen synthesis, fibrosis, TGF-β1, VEGF levels and proliferation of fibroblasts [1,28]. AV treatment reduces the increased expression of HIF-1α protein in bleomycin mice lungs and attenuates increased TGF-β1 and collagen content in mice lungs (figure 1A-D). These preliminary results substantiate the hypoxia modulating effect of AV in chronic lung disease conditions such as fibrosis.

Considering the anti-inflammatory role of AV, we also determined the effect of AV in severe life-threatening inflammatory conditions, i.e., sepsis, using most commonly used cecal ligation and puncture (CLP) mediated polymicrobial sepsis model (figure 1E). AV treatment reduces the increased IL-6 levels in mice, an important proinflammatory cytokine frequently associated with severe inflammatory conditions like sepsis (figure 1F). AV treatment is also able to alleviate the increased inflammatory, blood exudates as well as reduction of CLP induced mortality rate in mice (figure 1H-J). Interestingly, we observed a decrease in IFN-g levels upon CLP in mice, which was restored after AV treatment (figure 1G). Our observation with the IFN-g level in CLP mice is not in line with previous studies [29], and it is likely due to the differences in the time of measurement after CLP. However, our results are similar to the clinical findings that support adjuvant IFN-g immunotherapy concept to improve the host immune response against infection [30,31].

Hypoxia or HIF-1α activation is also thought to be a possible link between sepsis and thrombosis [32]. We observed that higher concentration of AV could reduce the increased platelet levels in naive mice (figure 2D). Further, mice treated with AV also mitigates the HIF-1α induced thrombotic complications and significantly prevents the decrease in blood clotting time and reduces the increased levels of platelets, vWF, and HIF-1α in PHD2 siRNA treated mice (figure 2F-J). These results suggest that the anti-hypoxic effect of AV could also prevent the development of hypercoagulable state in response to hypoxia.

In the course of our study, we realize that the effects of AV on phenotypic features of the lung and systemic inflammation could also prove beneficial for the present pandemic situations. In SARS-CoV2 infection, the presence of elevated hypoxia response seems to be a consequence of hyper-inflammation that contributes to disease severity [6,7]. We relate the therapeutic relevance of AV for the above-observed effects against severe patho-phenotypes associated with the critical stage of COVID-19, characterized by severe lung inflammation, hypoxemia, angiogenesis, sepsis, and altered coagulation profile [6,8–11]. Therefore, the anti-hypoxic property of AV would be advantageous to attenuate the critical inflammatory stage of COVID-19. Indeed, our transcriptome results show that the innate immune system-related gene ontology terms and pathways critical to adaptive immune response are enriched in overexpressed genes of AV treatment, whereas same processes and pathways were under expressed in COVID-19 patients transcriptome data (figure 3A, S1). On the other hand, genes that are significantly under-expressed in response to AV treatment but were over-expressed in COVID-19 patient's transcriptome are enriched in functional annotations like collagen biosynthesis, angiogenesis, TGF-β regulation, fibrosis, hypoxia pathways, and platelet activation and aggregation (figure 3A, S1). These results coincide with our biochemical observations in mice and corroborate with results of our pilot-study. Based on these results, we speculate that the treatment with AV could not only help to induce the early-stage antiviral effect but also mitigates the late-stage complications or severe impact of the hypoxia-inflammation axis in COVID-19 subjects.

Furthermore, CMap analysis identifies the positive connectivity of AV with other potential drugs/compounds which recently predicated for SARS-CoV2 prevention such as Apicidin (figure 4A). The HDAC2 inhibitor, Apicidin, was predicted to interact with CoV nsp5. Therefore, we screened chemical components of AV identified through liquid chromatography against SARS-CoV2 and host target proteins (table S1, S2). We observed quinazoline as well as O and N glycosides efficiently binds with SARS-CoV2 spike protein (Spro), 3C-like proteinase (3CLpro, main protease, NSP5), papain-like proteinase (PLpro), RNA dependent RNA polymerase (RdRp), and other non-structural proteins (nsps) (table 1 and S3). We observed AV constituents interact with host protein such as ACE2, TMPRSS2, and JAK which promote the entry and further inflammatory consequence of virus infection (table 1 and S3). SARS-CoV-2 is a single-stranded RNA virus. More than half of the 5′ genome virus encodes for two polyproteins (replicase), namely pp1a and pp1ab. These two proteins are cleaved into 16 nsps, including RdRp by two essential viral proteases, 3CLpro and PLpro. This process makes the formation of the replication complex of SARS-CoV-2. The 3′ end of the genome encodes four essential structural proteins, spike (S), envelope (E), matrix/membrane (M), and nucleocapsid (N), along with a set of accessory proteins. SARS-CoV-2 gains entry into the host cell by binding the viral S protein (Spro) to the host ACE2 receptor. The S protein is cleaved into S1 and S2 by a cell-derived protease. S1 binds to ACE2, and S2 is activated by the host serine protease TMPRSS2 and results in membrane fusion. Once inside, SARS-CoV-2 hijacks the host machinery to transcribe, replicate, and translate its RNA genome and structural proteins before being reassembled, encapsulated, and exocytosed from the cell [33]. Thus, strong binding of AV constituents with the above virus and host proteins indicates its potential role in preventing viral entry, replication, and, therefore, its infection. Transcriptomic analysis also shows reduced expression of genes like IFITM1 and IFITM6, which plays important role viral defence.

Conclusion

Treatment of *Adhatoda Vasica* extract shows the profound effect on the immunity-inflammation axis in such a way that it would be relevant in the initial and later phases of SARS-CoV-2 infection. It shows the multi-dimensional effect at the genome, molecular, cellular, and phenotype level to mitigate the hypoxia-hyperinflammation features (figure 5). Our study also highlights the likely impact of AV against hypoxia predominant lung inflammation, fibrosis, sepsis, and blood coagulation symptoms. Moreover in-silico analysis also

predict AV role in preventing viral entry and replication, and there it would not only useful in later stages if COVID-19 but also useful in early stages of viral infection. Therefore, it would be worthwhile to test AV on COVID-19 patients. This is a unique study of its kind, wherein we could validate the multi-dimensional application of an ayurvedic medicine in complex diseases through systemic integration of Ayurveda knowledge with experimental and bioinformatic tools of science while retaining the original and multi-component nature of the herb.

Declarations

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The transcriptome data from this study have been submitted to the Gene Expression Omnibus (GEO) under the accession number: GSE156759

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Contributions

A.G. designed and performed the experiment, analysed the results and wrote the paper. D. D. performed CMap analysis and interpreted the results. S. K. performed molecular docking analysis. L.P., B.R.P. and V.J. contributed to animal model experiments. V.A. performed transcriptomic analysis. R.R . contributed in figure and schematic representation. V.S. designed, performed, interpreted the molecular docking analysis and wrote the paper. B.P. conceptualized the study, provided AV, quality control information, discussion, designed the experiment, analysed the results and wrote the paper. A.A. and M.M. designed experiments, analysed and discussed the results and wrote the paper. All the authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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