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Potent phytoceuticals cocktail exhibits anti-inflammatory and antioxidant activity on LPS-triggered RAW264.7 macrophages *in vitro*

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A R T I C L E I N F O

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A B S T R A C T

Chronic inflammatory conditions, which include respiratory diseases and other ailments, are characterized by persistent inflammation and oxidative stress, and represent a significant health burden, often inadequately managed by current therapies which include conventional inhaled bronchodilators and oral or inhaled corticosteroids in the case of respiratory disorders. The present study explores the potential of Vedicinals®9 Advanced, a polyherbal formulation, to mitigate LPS-induced inflammation and oxidative stress in RAW264.7 mouse macrophages. The cells were pre-treated with Vedicinals®9 Advanced, followed by exposure to LPS to induce an inflammatory response. Key experimental outcomes were assessed, including nitric oxide (NO) and reactive oxygen species (ROS) production, as well as the expression of inflammatory and oxidative stress-related genes and proteins. Vedicinals®9 Advanced significantly reduced LPS-induced NO and ROS production, indicating strong anti-inflammatory and antioxidant properties. Additionally, the formulation downregulated the LPS-upregulated mRNA expression of pro-inflammatory cytokines, such as TNF-α and CXCL1, and oxidative stress markers, including GSTP1 and NQO1. Furthermore, Vedicinals®9 Advanced downregulated the LPS-induced protein expression of the chemokines CCL2 and CCL6, the LPS co-receptor, CD14, and the pro-inflammatory cytokines G-CSF and IL-1β. These findings highlight the potential of Vedicinals®9 Advanced as a therapeutic option for managing CRDs and other inflammatory conditions. The formulation's ability to simultaneously target inflammation and oxidative stress suggests it may offer advantages over existing treatments, with potential for broader application in inflammatory diseases.

1. Introduction

Chronic respiratory diseases (CRDs) are a growing burden amongst society and impact around 34 % of Australians [1]. Globally, chronic respiratory diseases have impacted nearly 454.6 million people in 2019, with chronic obstructive pulmonary disease (COPD) alone accounting for 3.3 million deaths [2].

CRDs encompass asthma, COPD, lung cancer, and more. The increasing prevalence of respiratory diseases highlights the need for alternative solutions for their treatment and management. Key pathophysiological features of CRDs include oxidative stress and inflammation [3,4]. These are two core contributing factors leading to the progression of various diseases ultimately hindering the quality of life of individuals. Despite there being numerous treatments available on the market for inflammatory conditions, many of them can exhibit unwanted side effects or be ineffective for long-term treatment,

Abbreviations: LPS, Lipopolysaccharide; CD14, Monocyte differentiation antigen CD14; TNF-α, Tumor necrosis factor-alpha; IL-1β, Interleukin-1 beta; G-CSF, Granulocyte colony stimulating factor; CCL2, Chemokine Ligand 2; CCL6, Chemokine Ligand 6; CXCL1, CXC chemokine Ligand; NO, Nitric Oxide; ROS, Reactive Oxidative Species.

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highlighting the need for alternative solutions [5]. In this context, the use of plant-derived compounds embedded with wide-spectrum biological activities represents a promising avenue in the identification of novel therapeutic agents characterized by improved efficacy and reduced adverse effects compared to conventional therapies [6,7].

Besides playing a pivotal role in the development of CRDs, inflammation represents an important underlying pathophysiological feature of numerous other diseases affecting organs such as pancreas, heart, kidney, brain, liver, intestine, reproductive system, and others [8]. Physiologically, inflammation represents one of the primary protective mechanisms activated by the immune system to prevent external factors such as invading pathogens and noxious chemicals to cause damage to the affected tissue [9]. However, when this mechanism fails at neutralizing the damaging stimulus, the inflammation becomes uncontrolled until a new, pathologic homeostatic state is reached termed chronic inflammation [10].

A key contributing factor that has been seen to play a role in the development of inflammation is lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria which is universally known for its potency in activating macrophages ultimately triggering inflammatory responses [11]. Exposure to airborne LPS is associated with cytokine production and can ultimately lead to pathophysiological features characteristic of CRDs such as airway inflammation. Studies have found that chronic exposure of LPS can also result in infiltration of neutrophils as well as pulmonary injury [12]. LPS binds to the toll-like receptor 4 (TLR-4) on the surface of immune cells such as macrophages, and it triggers the production of a series of pro-inflammatory cytokines and chemokines which, in turn, activate and orchestrate the complex mechanisms, involving multiple cell types leading to inflammation [13]. Typical inflammatory mediators secreted upon LPS exposure include the interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [14], and the chemokines C-X-C motif ligand 1 (CXCL1) [15], chemokine ligand 2 (CCL2) [16], and CCL6 [17]. Other LPS-induced factors which further contribute to the inflammatory process, particularly within the respiratory system, include the protein cluster of differentiation 14 (CD14) [18] and granulocyte-colony stimulating factor (G-CSF) [19]. Furthermore, another hallmark of inflammation is the activation of the inducible nitric oxide synthase (iNOS) pathway, resulting in the production of nitric oxide (NO), which in turn reinforces inflammation [20].

Another fundamental hallmark of inflammatory processes, which develops secondarily to inflammation, is oxidative stress, consisting in an imbalance between the secretion and detoxification of reactive oxygen species (ROS) resulting in an accumulation of ROS in the affected tissue, further reinforcing inflammation and tissue damage [21,22].

Glutathione S-transferase Pi 1 (GSTP1) is an enzyme found to play a crucial role in the regulation of detoxification of ROS [23,24]. This enzyme has been identified in various studies for its role in protecting various types of epithelial cells from oxidative stress [23,25]. NAD(P)H dehydrogenase (quinone)1, also known as, NQO1 is another enzyme involved in the reduction of inflammatory and oxidative mediators [26,27]. Various studies exploring LPS-induced inflammatory models similarly found NQO1 to play a crucial role in protecting different types of cells from LPS-induced inflammation [28–30].

Vedicinals®9 Advanced is a preparation containing a mixture of herbal active compounds such as quercetin, baicalin, glycyrrhizin, epigallocatechin-gallate, piperine, hesperidin, curcumin, luteolin, and rutin. In a previous study, we have reported the potent *in vitro* anticancer activity of the Vedicinals®9 preparation on A549 human lung adenocarcinoma cells [31]. The present study explores the therapeutic potential of Vedicinals®9 Advanced in alleviating oxidative stress and inflammation induced using lipopolysaccharide (LPS), *in vitro*, in RAW264.7 mouse macrophages. The present study provides evidence that Vedicinals®9 Advanced preparation showed potent anti-inflammatory and antioxidant effects by significantly reducing the LPS-induced production of NO and ROS. Mechanistically, Vedicinals®9 Advanced significantly reduced the LPS-induced overexpression of mRNA encoding for the genes GSTP1, NQO1, CXCL1 and TNF-α, as well as the LPS-induced overexpression of CCL2, CCL6, CD14, G-CSF and IL-1β.

The results of the present study highlight the therapeutic potential of Vedicinals®9 Advanced against numerous diseases whereby chronic inflammation and oxidative stress play an important role, with potential application across all CRDs and, in general, numerous inflammatory ailments.

2. Methods

2.1. Cell culture and treatment with Vedicinals®9 advanced

RAW264.7 cells were cultured using Dulbecco's Modified Eagle Medium (DMEM, Merck Australia) which was supplemented with 5 % fetal bovine serum (FBS, Merck Australia), 100 unit/mL penicillin, and 100 μg streptomycin (Pen-Strep, Merck Australia). The cells were kept in 37°C incubation in a humidified incubator with 5 % CO_2 and were routinely checked for mycoplasma contamination. The Vedicinals®9 Advanced formulation was provided and manufactured by Vedicinals India Private Limited, Pune, Maharashtra, India, and it was dissolved at the indicated working concentration in supplemented DMEM immediately before use.

2.2. MTT Cell viability assay

In order to assess the cell viability of the RAW264.7 cells when exposed to Vedicinals®9 Advanced, and to identify the maximum concentration of the test item to be used in the successive experiments without impacting cell viability, an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was conducted as reported previously [32]. The Vedicinals®9 Advanced preparation was tested at concentrations of 0.0625, 0.125, 0.25, 0.5, and 1 % v/v. The cells were seeded into a 96 well plate at a density of 10,000 cells/well and left to attach overnight. Successively, the treatment was added to the appropriate wells and returned to incubation for a further 24 hours. Finally, the MTT solution (Merck Australia) was added to each well as per the manufacturer's instructions and incubated at 37°C for 4 hours before removing the supernatant. The formazan crystals produced by the reaction were resuspended using 100 μL of dimethyl sulfoxide (DMSO, Merck Australia). The reading was conducted using TECAN Infinite plate reader with an absorbance of 570 nm which was then converted to a percentage and graphed.

2.3. ROS fluorescence-based quantitation

The quantitation of the ROS produced was performed as reported previously [33]. Briefly, 10000 RAW264.7 cells/well were plated in a black 96-well plate and allowed to incubate overnight before beginning treatment. The cells were exposed to Vedicinals®9 Advanced at final concentrations of 0.0625 % and 0.125 % v/v for 1 hour. This was followed by a 24-hour exposure to 1 μg/mL LPS. Afterward, 10 μM 2′, 7′-Dichlorofluorescin Diacetate (DCF-DA, Merck Australia) was added to each well, and the plate was incubated for 30 minutes. Fluorescence intensity was then measured using the FLUOstar Omega plate reader with excitation at 488 nm and emission at 525 nm.

2.4. ROS imaging

The imaging of the intracellular ROS produced was performed as reported in a previous study [33]. RAW264.7 cells (100000/well) were grown on a cover slip placed in a 6-well plate and left to attach overnight. The cells were initially exposed to Vedicinals®9 Advanced at final concentrations of 0.0625 % and 0.125 % v/v for 1 hour. This was followed by a 24-hour exposure to 1 μg/mL LPS. After incubation, the cells were rinsed twice with PBS and then incubated with 10 μM DCF-DA for

30 minutes. The cells were rinsed again with PBS, and fluorescence images were taken at × 20 magnification using a fluorescence microscope (Zeiss Axio Imager Z2, Oberkochen, Germany).

2.5. NO measurement - Griess reagent assay

A Griess reagent assay was used determine the levels of NO produced by the cells [34], similarly to what reported in a previous study [35]. RAW264.7 cells were seeded at a density of 100000 cells/well into a 6 well plate and incubated overnight to attach. The cells were initially exposed to Vedicinals®9 Advanced at final concentrations of 0.0625 % and 0.125 % v/v for 1 hour. This was followed by a 24-hour exposure to 1 μg/mL LPS. A 1:1 ratio of DMEM to Gries Reagent (Merck Australia) was added and the readings were conducted using TECAN infinite M1000 plate reader at an absorbance of 540 nm, after incubating the Griess reagent for 15 minutes. For absolute quantification of the levels of NO, the results were compared against those of a standard $NaNO_3$ calibration curve as per the manufacturer's instructions.

2.6. mRNA expression studies – RT-qPCR

For the assessment of the effect of Vedicinals®9 on mRNA expression, qPCR was conducted using the following protocol, as reported in a previous study [36].

2.6.1. Cell treatment and harvesting

The RAW264.7 cells were seeded into a 6-well plate at 100,000 cells/well. The following day, each well was treated with 0.125 % v/v Vedicinals®9 for an hour, followed by 24 hours incubation with LPS, as reported in the previous subsections. After treatment, the cells were placed on ice and washed in triplicate with ice-cold phorphate-buffered saline (PBS, Merck Australia). The cells were then lysed in 500 μL TRI Reagent (Merck Australia) and the cell lysate was stored at −80°C until RNA extraction.

2.6.2. RNA extraction and quantification

The RNA was extracted using a phenol-chloroform extraction as reported previously. [33] Briefly, 125 μL of chloroform (Merck Australia) was added to each sample in TRI Reagent, pulse vortexed, and then left to incubate for 10 minutes at room temperature. After incubation, the samples were centrifuged at 12,000 g for 15 minutes at 3°C. The aqueous layer was carefully transferred to new vials and precipitated with 250 μL of ice-cold isopropanol (Merck Australia), pulse vortexed and left to incubate for a further 10 minutes at room temperature and then centrifuged at 12,000 g for 10 minutes at 3°C. The supernatant was removed, and the RNA pellet was washed twice with 500 μL ethanol 75 % (Merck Australia). After each wash, the sample was vortexed and centrifuged at 8000 g for 5 minutes at 3°C. The ethanol was then aspirated, and the pellet was left to dry before being resuspended in 20 μL of nuclease free (NF)water.

The RNA concentrations and quality were quantified using a NanoDrop spectrophotometer. Samples with a concentration $\geq$ 100 ng/μL, a 260/280 ratio $\geq$ 1.8 and a 260/230 ratio $\geq$ 2.0 were used for the reverse transcription. The samples were then stored at −80°C.

2.6.3. Reverse transcription

The cDNA was synthesized from 800 ng of RNA after DNase treatment using the DNase I kit (Merck Australia), following the manufacturer's instructions. The reaction mixture included random hexamer primers (500 ng/μL, Thermo Fisher Scientific), dNTPs (10 mM, Thermo Fisher Scientific), M-MLV reaction buffer (1 ×, Merck Australia), and dithiothreitol (100 mM, Thermo Fisher Scientific). The reverse transcription process involved the steps indicated in Table 1, performed using a Mastercycler nexus GSX1 thermal cycler (Eppendorf, Hamburg, Germany).

Table 1
Reverse transcription thermal cycling steps.

	Temperature (°C)	Time (minutes)
Denaturing	65	10
Annealing	25	10
Reverse Transcription	37	50
Reverse Transcriptase inactivation	70	15

2.6.4. Real-time qPCR

Real-time qPCR was conducted with 16 ng of cDNA, iTaq Universal SYBR Green supermix (1 ×, BioRad), and 5 μM of both forward and reverse primers on a CFX96 real-time PCR detection system (BioRad). The thermal cycling conditions were as follows: initial polymerase activation and cDNA denaturation at 95 ºC for 30 seconds; 40 cycles of denaturation at 95 ºC for 15 seconds, followed by annealing/extension at 60 ºC for 30 seconds, with plate readings taken after each cycle. Forward and reverse primers were obtained from Sigma-Merck, as listed in Table 2. The 2^-ΔΔCt method was used to determine the relative fold change in mRNA expression, with the gene encoding hypoxanthine phosphoribosyltransferase 1 (Hprt) serving as the housekeeping control.

2.7. Protein expression study - mouse XL cytokine array

The effect of Vedicinals®9 Advanced on the LPS-induced changes in the expression of inflammatory and oxidative stress markers was assessed with a Proteome Profiler Mouse XL Cytokine array kit purchased R&D Systems, Australia, as reported in a previous study [37]. The RAW267.4 cells were seeded into a 6 well plate at a density of 100,000 cells/well and treated, following overnight attachment, with Vedicinals®9 Advanced (0.125 % v/v) for one hour followed by 24 hours exposure to LPS, as described in a previous section. The cells were then placed on ice and washed twice with ice-cold PBS prior to being lysed with 500 μL radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher Scientific, Australia) supplemented with protease inhibitor cocktail (Merck Australia). After 15 minutes of incubation on ice, the cell debris was then removed through centrifugation (14,000 RPM/4°C/15 minutes).

By utilising a bicinchoninic acid (BCA) assay kit (Merck Australia), the protein content of each sample was quantified following the manufacturer's instructions. Successively, 300 μg proteins per sample were hybridized on the Proteome Profiler Mouse XL Cytokine array kit, and the membranes were processed and imaged following the manufacturer's instructions. The membranes were imaged using a Chemidoc system (BioRad), and the densitometric analysis was performed with FIJI Image J software.

2.8. Statistical analysis

The results represented are repeated in triplicate and are expressed as mean ± SEM. Statistical analysis was performed with one way ANOVA test, followed by Tukey's or Dunnett's multiple comparison tests. using the GraphPad Prism software (version 9.3). a p-value < 0.05 for pairwise comparisons was deemed statistically significant.

3. Results

3.1. Effect of Vedicinals®9 Advanced on RAW264.7 cell viability

To assess the effect of the Vedicinals®9 Advanced formulation on the viability of RAW264.7 cells, and to identify the maximum concentration to be used for treatment without significantly impacting the cells' viability, an MTT assay has been performed exposing the cells to increasing concentrations of Vedicinals®9 Advanced for 24 hours (Fig. 1). The viability of RAW264.7 cells was not significantly impacted upon treatment with concentrations of Vedicinals®9 Advanced up to

Table 2
List and sequence of primers used in RT-qPCR.

Protein name	Gene name	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
CXCL1	Cxcl1	AAAGATGCTAAAAGGTGTCC	GTATAGTGTTGTCAGAAGCC
TNF-α	Tnf	CTATGTCTCAGCCTCTTCTC	CATTTGGGAACTTCTCATCC
GSTP1	Gstp1	CTAATGCCATCTTGAGACAC	CTTACCATTCTCATAGTTGGTG
NQO1	Nqo1	CCTTTCCAGAATAAGAAGACC	AATGCTGTAAACCAGTTGAG
HPRT1	Hprt	AGGGATTTGAATCACGTTTG	TTTACTGGCAACATCAACAG

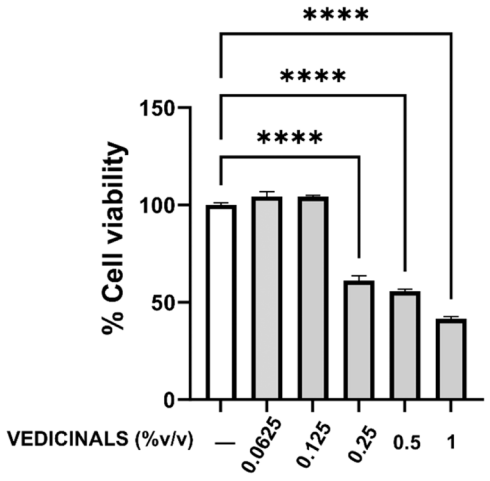

Fig. 1. Effect of Vedicinals®9 Advanced formulation on the viability of RAW264.7 cells. RAW264.7 cells were exposed to increasing concentrations of Vedicinals®9 Advanced formulation (ranging between 0.0625 % and 1 % v/v) for 24 hours. After treatment, the cell viability was quantified through MTT assay. Absorbance values were normalized as percentage compared to the untreated control group. Data are represented as mean ± SEM, n = 3. Statistical analysis: One-Way ANOVA. ****: $p < 0.0001$vs untreated control group.

0.125 % v/v (Fig. 1). Treatment with higher concentrations of 0.25 %, 0.5 %, and 1 % v/v resulted in significant reductions in the cells' viability of 38.9 %, 44.4 %, and 58.5 %, respectively (Fig. 1). The non-toxic concentrations 0.0625 % and 0.125 % v/v were therefore used for the subsequent functional and mechanistic experiments.

3.2. Vedicinals®9 Advanced reduces LPS-induced ROS production

A DCF-DA assay was performed to assess the antioxidant activity of Vedicinals®9 Advanced. RAW264.7 cells were pre-treated with 0.0625 % or 0.125 % v/v Vedicinals®9 Advanced for one hour, followed by 24 hours exposure to 1 μg/mL LPS. The results of this assay are shown in Fig. 2. LPS treatment resulted in a significant 43.4 % increase in ROS production compared to the untreated control (Fig. 2a). Pre-treatment with 0.0625 % Vedicinals®9 Advanced did not exert any significant effect on LPS-induced ROS production (Fig. 2a), while pre-treatment with 0.125 % Vedicinals®9 Advanced induced a significant 13.4 % reduction in ROS-levels compared to the LPS only-treated group (Fig. 2a). The cell imaging data obtained through fluorescence microscopy show a similar trend (Fig. 2b).

3.3. Vedicinals®9 Advanced reduces LPS-induced NO production

To assess the anti-inflammatory activity of Vedicinals®9 Advanced, the concentration of secreted NO was measured using a Griess reagent assay. RAW264.7 cells were pre-treated with 0.0625 % or 0.125 % v/v Vedicinals®9 Advanced for one hour, followed by 24 hours exposure to 1 μg/mL LPS. The results of this assay are shown in Fig. 3. Treatment with LPS resulted in a significant 145.5-fold increase in the levels of secreted NO (Fig. 3). Pre-treatment with Vedicinals®9 Advanced at concentrations of 0.0625 % and 0.125 % v/v significantly reduced the levels of LPS-induced NO by 32.4 % and 64.7 %, respectively, compared to the LPS only-treated group (Fig. 3).

3.4. Vedicinals®9 Advanced downregulates the expression of LPS-induced pro-inflammatory genes

The expression of the pro-inflammatory genes *Cxcl1* (encoding for

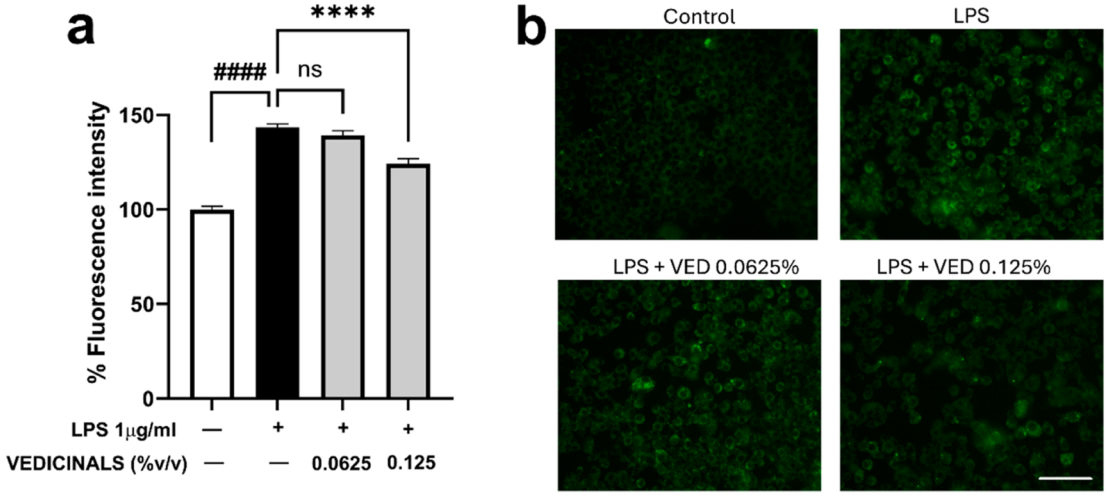

Fig. 2. Vedicinals®9 Advanced reduces LPS-induced ROS production in RAW264.7 cells. RAW264.7 cells were exposed to 0.0625 % or 0.125 % v/v Vedicinals®9 Advanced formulation for one hour, followed by co-incubation with 1 μg/mL LPS for further 24 hours. After treatment, the intracellular ROS levels were quantified and imaged using DCF-DA. (a) ROS quantitation. Absorbance values were normalized as percentage compared to the untreated control group. (b) ROS Imaging at the fluorescence microscope. Images were taken at 20X magnification. Scale bar = 100 μm. Data in (a) are represented as mean ± SEM, n = 3. Statistical analysis: One-Way ANOVA. ns: not significant; ####: $p < 0.0001$ vs untreated control group; ****: $p < 0.0001$ vs LPS-treated group.

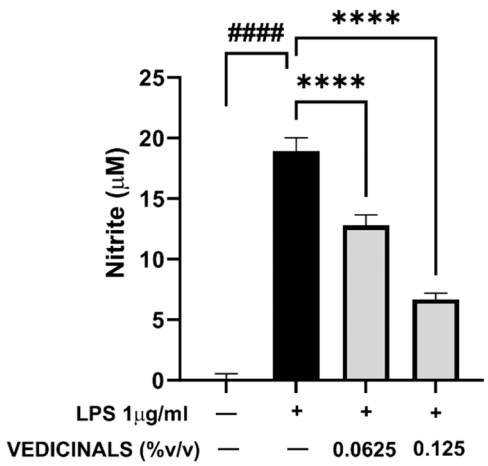

Fig. 3. Vedicinals®9 Advanced reduces LPS-induced NO production in RAW264.7 cells. RAW264.7 cells were exposed to 0.0625 % or 0.125 % v/v Vedicinals®9 Advanced formulation for one hour, followed by co-incubation with 1 μg/mL LPS for further 24 hours. After treatment, the secreted NO levels were quantified using the Griess reagent. Data are represented as mean ± SEM, n = 3. Statistical analysis: One-Way ANOVA. ####: $p < 0.0001$ vs untreated control group; ****: $p < 0.0001$ vs LPS-treated group.

CXCL-1) and *Tnf* (encoding for TNF-α) was measured through qPCR. RAW264.7 cells were pre-treated with 0.125 % v/v Vedicinals®9 Advanced for one hour, followed by 24 hours exposure to 1 μg/mL LPS. The results of this assay are shown in Fig. 4. Exposure to LPS triggered a significant 28.5-fold increase in *Cxcl1* mRNA levels and a significant 12.3-fold increase in *Tnf* mRNA levels compared to the untreated control (Figs. 4a and 4b, respectively). Treatment with 0.125 % Vedicinals®9 Advanced significantly reduced the expression of *Cxcl1* by 47.9 % (Fig. 4a) and the expression of *Tnf* by 75.8 % (Fig. 4b) compared to the LPS only-treated group.

3.5. Vedicinals®9 Advanced downregulates the expression of LPS-induced antioxidant genes

The expression of the pro-inflammatory genes *Gstp1* (encoding for GSTP-1) and *Nqo1* (encoding for NQO1) was measured through qPCR. RAW264.7 cells were pre-treated with 0.125 % v/v Vedicinals®9 Advanced for one hour, followed by 24 hours exposure to 1 μg/mL LPS. The results of this assay are shown in Fig. 5**.** Exposure to LPS triggered a significant 10.4-fold increase in *Gstp1* mRNA levels and a significant 20.5-fold increase in Nqo1 mRNA levels compared to the untreated control (Figs. 5a and 5b, respectively). Treatment with 0.125 % Vedicinals®9 Advanced significantly reduced the expression of *Gstp1* by 47.9 % (Fig. 5a) and the expression of *Nqo1* by 75.8 % (Fig. 5b) compared to the LPS only-treated group.

3.6. Vedicinals®9 Advanced downregulates the expression of LPS-induced proteins related to inflammation

The relative expression of a series of proteins involved in inflammation has been measured using a Proteome Profiler Mouse XL Cytokine Kit. RAW264.7 cells were pre-treated with 0.125 % v/v Vedicinals®9 Advanced for one hour, followed by 24 hours exposure to 1 μg/mL LPS. The results of this experiment are shown in Fig. 6. Exposure to LPS resulted in a significant increase in the expression of CCL2 (13.1-fold, Fig. 6a), CCL6 (29.0-fold, Fig. 6b), CD14 (1.2-fold, Fig. 6c), G-CSF (13.1-fold, Fig. 6d), and IL-1β (31.7-fold, Fig. 6e) compared to the untreated control. Treatment with 0.125 % v/v Vedicinals®9 Advanced reversed this trend, as it significantly reuced the expression of the aforementioned proteins compared to the LPS only-treated group. In particular, the expression of CCL2 was reduced by 79.0 % (Fig. 6a), the expression of CCL6 was reduced by 47.7 % (Fig. 6b), the expression of CD14 was reduced by 53.5 % (Fig. 6c), the expression of G-CSF was reduced by 37.4 % (Fig. 6d), and the expression of IL-1β was reduced by 30.7 % (Fig. 6e).

4. Discussion

Chronic inflammatory diseases include conditions such as CRDs, cardiovascular diseases, cancer, diabetes, and neurodegenerative disorders, and are now recognized as the most significant cause of death globally, accounting for over 50 % of all deaths worldwide [38]. Common pathophysiological processes underlying chronic inflammatory diseases include chronic inflammation and oxidative stress [39,40]. Chronic inflammation consists of a complex, multifactorial process characterized by the excessive production of inflammatory mediators which, in turn, recruit immune system cells to the inflamed site [38,41]. This process is often triggered by exposure to environmental irritants such as, in the case of inflammatory processes underlying CRDs,

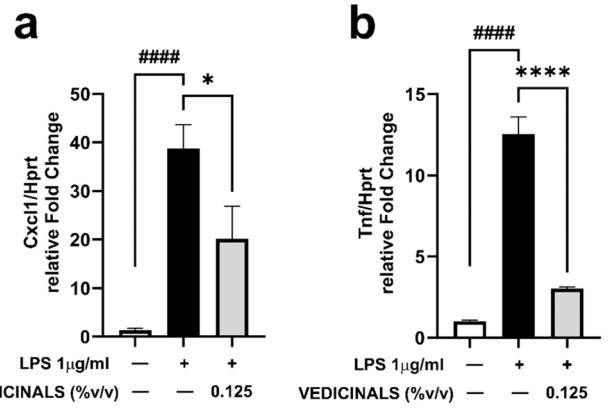

Fig. 4. Vedicinals®9 Advanced reduces the LPS-induced expression of proinflammatory genes. RAW264.7 cells were exposed to 0.125 % v/v Vedicinals®9 Advanced formulation for one hour, followed by co-incubation with 1 μg/mL LPS for further 24 hours. After treatment, the relative expression of the genes *Cxcl1* (a) and *Tnf* (b) was measured *via* RT-qPCR. Data are represented as mean ± SEM, n = 3. Statistical analysis: One-Way ANOVA. ####: $p < 0.0001$ vs untreated control group; *: $p < 0.05$ vs LPS-treated group; ****: $p < 0.0001$ vs LPS-treated group.

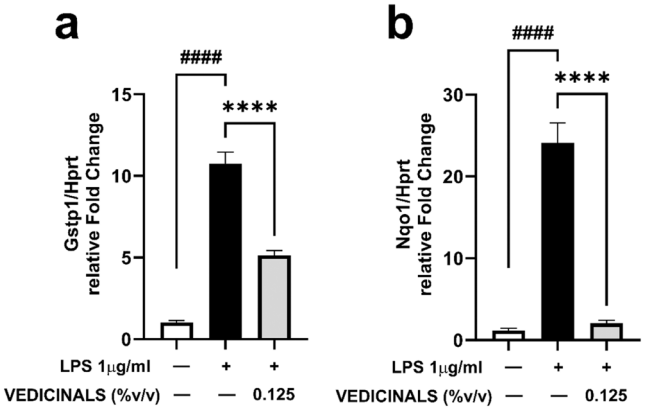

Fig. 5. Vedicinals®9 Advanced reduces the LPS-induced expression of antioxidant genes. RAW264.7 cells were exposed to 0.125 % v/v Vedicinals®9 Advanced formulation for one hour, followed by co-incubation with 1 μg/mL LPS for further 24 hours. After treatment, the relative expression of the genes *Gstp1* (a) and *Nqo1* (b) was measured *via* RT-qPCR. Data are represented as mean ± SEM, n = 3. Statistical analysis: One-Way ANOVA. ####: $p < 0.0001$ vs untreated control group; ****: $p < 0.0001$ vs LPS-treated group.

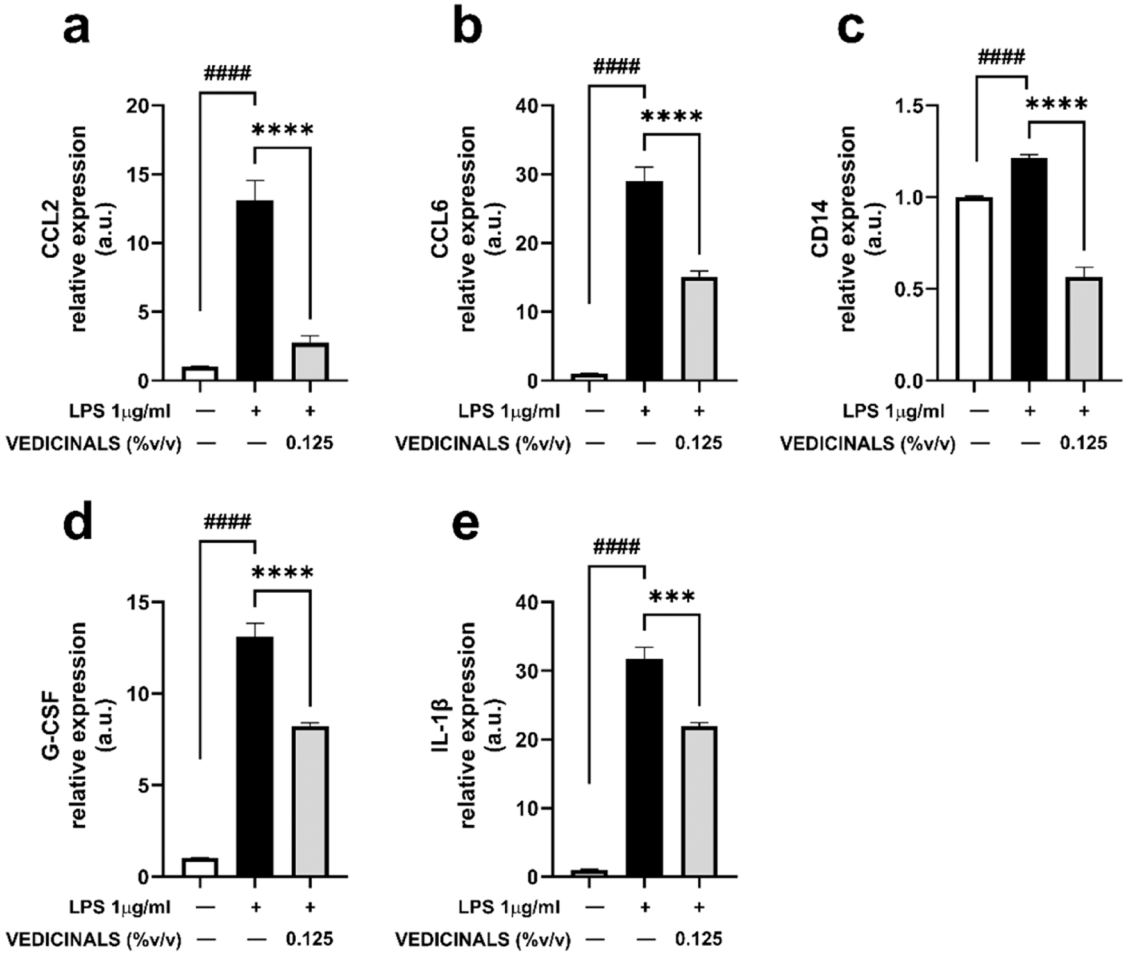

Fig. 6. Vedicinals®9 Advanced reduces the LPS-induced expression of proteins mediating inflammation. RAW264.7 cells were exposed to 0.125 % v/v Vedicinals®9 Advanced formulation for one hour, followed by co-incubation with 1 μg/mL LPS for further 24 hours. After treatment, the relative expression of the proteins CCL2 (a), CCL6 (b), CD14 (c), G-CSF (d), and IL-1β (e) was measured *via* Proteome Profile Mouse XL Cytokine Array Kit. Data are represented as mean ± SEM, n = 4. Statistical analysis: One-Way ANOVA. ####: $p < 0.0001$ vs untreated control group; ***: $p < 0.001$ vs LPS-treated group, ****: $p < 0.0001$ vs LPS-treated group.

bacterial LPS [42]. One of the prominent inflammatory mediators whose levels rise during chronic inflammation is NO, which is produced by tissue resident macrophages and whose production is stimulated by LPS *via* an increase in the expression and function of the iNOS [43]. LPS activates the inflammatory cascade upon binding to its receptor, toll-like receptor 4 (TLR4). This interaction, and the subsequent downstream signal transduction activation, is supported by the LPS co-receptor CD14 [18,44,45]. Besides NO, LPS exposure triggers the expression of several pro-inflammatory mediators, which include the cytokines TNF-α, IL-1α and IL-1β, commonly recognised as the principal pro-inflammatory cytokines [46,47], G-CSF [19,48], as well as chemokines such as CXCL1, CCL2, and CCL6, which in turn recruit more cells of the immune system to the site of inflammation [15,17,49–51]. Occurring concomitantly with the production of inflammatory mediators and the recruitment of immune system cells, oxidative stress consists in an unbalance between the production and the detoxification of ROS. When this happens, ROS accumulate within the cells causing cell damage, contributing to declining organ function and increased mortality [21,52]. As a mechanism of defence from the excessive ROS production, cells usually increase the expression and function of detoxifying enzymes such as GSTP1 and NQO1 [23,24,26,27], which can therefore be considered biomarkers of oxidative stress in this context.

In the quest for the development of innovative therapeutic option to treat inflammation and oxidative stress underlying chronic inflammatory disorders, plant extracts and plant-derived bioactive compounds, commonly referred to as phytoceuticals, represent a powerful source of new potential treatments, as numerous phytoceuticals are renowned for their wide-spectrum anti-inflammatory and antioxidant activities [6,7].

In the present study, we have tested the *in vitro* antioxidant and anti-inflammatory activity of VEDICINALS®9 Advanced, a polyherbal preparation containing nine phytoceuticals (curcumin, rutin, luteolin, piperine, epigallocatechin gallate, glycyrrhizin, hesperidin, rutin, and baicalin). In a previous report, we have shown the promising *in vitro* anticancer activity of a similar polyherbal preparation, which significantly decreased human lung adenocarcinoma cells proliferation, migration, and metastatic capacity [31]. Here, we show that the VEDICINALS®9 Advanced preparation significantly counteracts LPS-induced inflammation and oxidative stress *in vitro* in RAW264.7 mouse macrophages at a concentration of 0.125 % v/v. From a functional perspective, we demonstrated that pre-treatment with VEDICINALS®9 Advanced resulted in the significant reduction of the LPS-induced production of both ROS and NO. This finding is in agreement with numerous reports showcasing that several single components of the VEDICINALS®9 Advanced preparation have strong antioxidant and anti-inflammatory activity, mediated by the suppression of ROS and NO production. These include, among others, rutin [53,54], curcumin [55], glycyrrhizin [56], and quercetin [57]. Mechanistically, we employed qPCR and protein array to determine the molecular pathways impacted by VEDICINALS®9 Advanced that lead to the observed anti-inflammatory and antioxidant activities. With regards to oxidative stress, the observed reduction in ROS production in LPS-treated cells exposed to 0.125 % VEDICINALS®9 Advanced was concomitant to a corresponding

reduction of the expression of the mRNAs encoding for the detoxifying enzymes GSTP1 and NQO1. To the best of our knowledge, no reports in literature show that any of the phytoceutical components of VEDICINALS®9 Advanced reduce the expression of these two enzymes. Interesting, quercetin and curcumin were shown to inhibit the activity of GSTP1 [58,59]. However, considering the fact that the increase in the expression of these two enzymes is triggered by elevated ROS levels [26,27], we hypothesize that the observed reduction of the expression of GSTP1 and NQO1 mRNAs is secondary to the reduction in ROS levels achieved by VEDICINALS®9 Advanced.

With regards to the mechanism behind the anti-inflammatory activity of VEDICINALS®9 Advanced, we demonstrated that this polyherbal preparation significantly reduced the LPS-induced overexpression of (i) the pro-inflammatory cytokines TNF-α (at the mRNA level), IL-1β, and G-CSF (at the protein level); (ii) the chemokines CXCL1 (at the mRNA level), CCL2, and CCL6 (at the protein level); and (iii) the LPS co-receptor CD14 at the protein level.

TNF-α and IL-1β are among the main pro-inflammatory cytokines released by macrophages upon exposure to LPS, and they function as initiators of the innate immune response [47,60].

Our finding that VEDICINALS®9 Advanced reduces the expression of these two cytokines agrees with several reports showing that single components of the polyherbal formulation exert their anti-inflammatory activity through the inhibition of these cytokines. Baicalin, for example, was shown to counteract the pro-inflammatory activity of LPS in RAW264.7 macrophages by reducing the production of NO, TNF-α, and IL-1β among other factors, protecting mice with severe LPS-induced endotoxemia [61]. Similarly, in another report, baicalin was shown to repress the LPS-induced expression of TNF-α and IL-1β *in vitro* in LPS-treated HT-29 intestinal cells [62]. In this context, baicalin was also shown to significantly decrease the expression of the cytokine G-CSF [63] and of CD14 [64] in LPS-stimulated RAW 264.7 macrophages. Glycyrrhizin, a component of licorice extract, was also shown to inhibit the expression of TNF-α and IL-1β in an *ex vivo* LPS-stimulated whole blood model [65], and it significantly reduced TNF-α expression in a mouse model of LPS-induced acute lung injury ALI, protecting mice from LPS-induced damage [66]. Another phytoceutical known to reduce the LPS-induced production of IL-1β and TNF-α as part of its anti-inflammatory activity is epigallocatechin gallate, which reduced the expression of these cytokines both *in vivo*, on an LPS-induced mouse ALI model, and *in vitro* in LPS-stimulated RAW264.7 cells [67]. Similarly, piperine was recently shown to reduce the expression of IL-1β and TNF-α in LPS-stimulated RAW-264.7 macrophages [68]. Other phytoceuticals that have been reported to reduce the LPS-induced expression of TNF-α and IL-1β in various models include curcumin [69–71], hesperidin [72,73], quercetin [74–76], luteolin [77–79], and rutin [80,81].

The release of chemoattractant cytokines, or chemokines, is another fundamental process which contributes to the LPS-induced activation of the immune response by recruiting, to the site of inflammation, different effector cells of both the innate and adaptive immune system. These chemokines include CCL, CCL6, and CXCL1 among others [16,17,51]. In this context, the inhibition of the LPS-induced production of these three chemokines achieved with VEDICINALS®9 Advanced represents another important mechanisms through which the preparation exerts its strong anti-inflammatory activity. From this point of view, our results are in line with several recent reports showcasing the ability of different components of VEDICINALS®9 Advanced to reduce the expression of these cytokines. In particular, quercetin was reported to reduce CCL2 expression in both macrophages and microglial cells stimulated by LPS [82], and to reduce CXCL1 expression in LPS-stimulated mouse-derived myeloid dendritic cells and plasmacytoid dendritic cells [83]. Baicalin inhibited CCL2 expression in LPS-induced macrophages, protecting rats from experimental cerebral ischemia [84], and significantly reduced CXCL1 levels in a mouse model of osteoarthritis [85]. Glycyrrhizin was also shown to inhibit CCL2 production in polymorphonucleate cells [86] and to reduce CSCL1 expression in an experimental colitis mouse model [87]. Furthermore, piperine reduced CCL2 expression in a cerulein-induced mouse model of chronic pancreatitis [88] and to downregulate CXCL1 expression in IL-1β-induced primary human dermal fibroblasts [89]. Finally, curcumin was reported to inhibit 5-fluorouracil-induced overexpression of CXCL1 in the colonic mucosa of mice [90], and to dose-dependently reduce LPS-induced CCL2 expression in C6 rat astrocytoma cells [91]. To the best of our knowledge, no report in literature shows that any of the phytoceutical components of VEDICINALS®9 Advanced downregulates the expression of the chemokine CCL6. The results of the present study are summarized in Fig. 7, which also represents a graphical abstract for the manuscript.

In summary, the results of this study underscore the promising therapeutic potential of VEDICINALS®9 Advanced as a potent modulator of LPS-induced oxidative stress and inflammation, specifically targeting key molecules like NO and ROS, and mechanistically downregulating the expression of the cytokines TNF-α, IL-1β, and G-CSF, the chemokines CCL2, CCL6, and CXCL1, and the LPS co-receptor CD14. By influencing these critical mediators, VEDICINALS ®9 Advanced addresses core pathophysiological processes associated with chronic inflammatory diseases, particularly chronic respiratory diseases (CRDs). This suggests that VEDICINALS ®9 Advanced could be a valuable treatment option not only for CRDs but also for a broader spectrum of conditions where inflammation and oxidative stress are central, such as cardiovascular diseases, neurodegenerative disorders, and metabolic syndromes.

However, the results of the present study are not exempt from limitations. Firstly, our investigation focused on a polyherbal formulation, and while the results are promising, future studies should be performed aimed at isolating and identifying the specific active compounds responsible for the observed effects. Additionally, the study was conducted *in vitro*, using LPS-stimulated RAW264.7 mouse macrophages, which, while informative, does not fully replicate the complexity and multicellular *milieu* of the *in vivo* environment. To counteract this limitation, further research should explore the effects of VEDICINALS®9 Advanced in animal models of inflammation and oxidative stress to better understand its potential therapeutic impact. Moreover, the study did not examine the long-term effects or the potential for the development of resistance, which are crucial factors in the clinical application of anti-inflammatory agents. Despite these limitations, the findings reported in the present study provide a solid foundation for future studies aimed at exploring the broader therapeutic potential of VEDICINALS®9 Advanced.

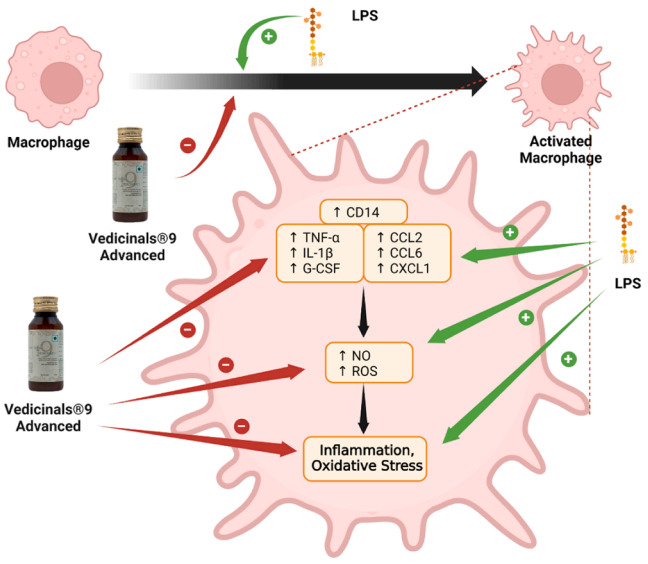

Fig. 7. Summary of the findings of the present study Figure created with BioRender at www.biorender.com.

5. Conclusions

The findings reported in the present study reveal the multifaceted therapeutic potential of VEDICINALS®9 Advanced in managing chronic inflammatory diseases by targeting the interplay between oxidative stress and inflammation. Despite the limitations highlighted, this research serves as a proof-of-concept, demonstrating the efficacy of VEDICINALS®9 Advanced in modulating key inflammatory and oxidative pathways. These results lay a strong foundation for future investigations into the application of this polyherbal preparation in chronic diseases, including but not limited to, CRDs, cardiovascular disorders, and other inflammation-driven conditions. The study not only opens avenues for further research into the individual components of the formulation but also emphasizes the need for further *in vivo* investigations to fully elucidate the therapeutic potential of VEDICINALS®9 Advanced in complex disease models.

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CRediT authorship contribution statement

Prakash Salunke: Writing – review & editing, Resources, Funding acquisition. **Joachim Gerlach:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Sofia Kokkinis:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Tammam El-Sherkawi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Kamal Dua:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Jessica Katrine Datsyuk:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Gabriele De Rubis:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Keshav Raj Paudel:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors would declare the following interest: The VEDICINALS®9 Advanced formulation we have tested is a product of VEDICINALS® International and more information about it can be found in their official website. https://www.vedicinals.com/vedicinals-9/.

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