



***Bulbophyllum drymoglossum* aqueous extract modulates immunometabolism and oxidative stress in porcine alveolar macrophages**

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Objective: This study evaluated the regulatory effects of *Bulbophyllum drymoglossum* aqueous extract (BDAE) on oxidative stress, mitochondrial function, and metabolic reprogramming in porcine alveolar macrophages.

Methods: BDAE was prepared through aqueous extraction, and its chemical composition was characterized through gas chromatography–mass spectrometry analysis. Cytotoxicity and antioxidant properties of the extract were evaluated in NIH/3T3 fibroblasts and 3D4/31 porcine alveolar macrophages using cell viability assays, flow cytometry, and fluorescence imaging techniques. Mitochondrial function was assessed via the measurement of mitochondrial mass and membrane potential by MitoTracker Red and JC-1 staining methods, respectively. The metabolic effects were examined through flow cytometric analysis of glucose uptake and lipid accumulation. Additionally, gene expression levels related to fatty acid metabolism and mitochondrial respiratory chain complexes were quantified using quantitative real-time polymerase chain reaction analysis.

Results: BDAE exhibited low cytotoxicity and effectively reduced intracellular reactive oxygen species under both basal and inflammatory conditions. It maintained mitochondrial membrane potential and prevented phorbol 12-myristate 13-acetate (PMA)-induced mitochondrial dysfunction. BDAE reversed PMA-induced metabolic alterations by restoring glucose uptake, enhancing the expression of genes involved in fatty acid β -oxidation, lipogenesis, and mitochondrial respiratory complexes, and attenuated lipid accumulation in macrophages. The bioactivities were associated with the extract's abundance of carbohydrate derivatives and polyols.

Conclusion: BDAE shows significant antioxidative and metabolic regulatory effects in macrophages, suggesting its potential as a natural bioactive compound for modulating immunometabolism and oxidative stress in animal health. Additional *in vivo* validation and mechanistic studies are necessary to advance its application in livestock production.

Keywords: Aqueous Extract; *Bulbophyllum drymoglossum*; Immunomodulation; Mitochondrial Membrane Potential; Reactive Oxygen Species

INTRODUCTION

Oxidative stress and inflammatory dysregulation in immune cells are critical factors that affect animal health and productivity, especially in livestock facing environmental and metabolic stresses. Macrophages, key mediators of innate immunity, respond dynamically through alterations in reactive oxygen species (ROS) generation, mitochondrial function, and energy metabolism. Excessive or chronic oxidative stress can impair mitochondrial integrity and activate inflammatory cascades, highlighting the need for modulators that restore redox balance and metabolic homeostasis [1–3].

Natural bioactive compounds derived from plants are increasingly recognized as promising modulators of immune cell function and oxidative stress, presenting safer and effective alternatives to conventional synthetic drugs [4]. *Bulbophyllum drymoglossum*, an orchid species with a history of ethnomedicinal use, contains diverse metabolites such as carbohydrate derivatives and polyols known to support cellular bioenergetics and antioxidant defenses [5]. However, despite these properties, the effects of *Bulbophyllum drymoglossum* aqueous extract (BDAE) on macrophage redox regulation, mitochondrial function, and metabolic reprogramming remain insufficiently understood [4,6].

This study seeks to fill these gaps by characterizing the chemical composition of BDAE and evaluating its regulatory effects on intracellular ROS production, mitochondrial membrane potential, and metabolic pathways involved in glucose and lipid utilization in porcine alveolar macrophages (3D4/31). Using phorbol 12-myristate 13-acetate (PMA) to induce oxidative stress, we further investigate BDAE's ability to modulate immune cell bioenergetics and maintain mitochondrial health [7–9]. Additionally, innate immune activation profiling via morpho dynamic analysis provides deeper insight into BDAE's immunomodulatory potential [10,11]. By integrating chemical profiling, cellular functional assays, and advanced immune activation evaluation, this work delivers valuable insights into BDAE as a natural immunometabolic regulator [12,13]. These findings hold significant implications for the development of plant-based therapeutics aimed at enhancing animal health, promoting sustainable livestock production, and addressing inflammatory and oxidative stress-related conditions within veterinary biotechnology.

MATERIALS AND METHODS

Preparation of *Bulbophyllum drymoglossum* aqueous extract

Fresh *Bulbophyllum drymoglossum* plants were collected, rinsed with deionized water (dH₂O), and cut into 2–3 cm segments. A total of 100 g plant material was extracted in 300 mL dH₂O at 110°C for 15 min using an autoclave (WAC-60; Daihan Scientific). The extract was filtered through a 0.2 µm sterile cellulose acetate filter (Minisart; Sartorius) and stored at –80°C. The final concentration of BDAE was determined by dry weight measurement following lyophilization.

Cell culture and treatment

Porcine alveolar macrophages (3D4/31; ATCC CRL-2844) and mouse embryonic fibroblasts (NIH3T3; ATCC CRL-1658) were maintained at 37°C in a 5% CO₂ humidified incubator. 3D4/31 cells were cultured in a 4:6 (v/v) mixture of DMEM (10-013-CVR; Corning) and RPMI-1640 (10-040-CVR; Corning), while NIH3T3 in DMEM only. All media

contained 10% heat-inactivated fetal bovine serum (FBS; TMS-013; Merck Millipore) and 1% penicillin-streptomycin (LS202-02; Welgene). For anti-inflammatory studies, cells were pretreated for 12 h with various BDAE concentrations prior to stimulation with 2 nM PMA (P1585-1MG; Sigma-Aldrich) in fresh medium containing the same BDAE.

Cell viability and proliferation assay

Cell viability and proliferation were assessed using a WST-8 assay kit (QM2500; BIOMAX). Cells were incubated with 10% (v/v) WST-8 solution for 2 h, and the absorbance at 450 nm was measured with a FilterMax F3 microplate reader (Molecular Devices). Cell proliferation was evaluated via trypan blue exclusion and manual cell counting with a hemocytometer.

Gas chromatography–mass spectrometry analysis of *Bulbophyllum drymoglossum* aqueous extract

BDAE composition was characterized by gas chromatography–mass spectrometry (GC-MS; 7890B GC/5977B MSD; Agilent Technologies) with an HP-5MS column (30 m×0.25 mm, 0.25 µm). Helium was used as the carrier gas at 1.0 mL/min. The oven temperature was held at 60°C for 2 min, raised at 10°C/min to 300°C, then held for 10 min. Injector, transfer line, and ion source were maintained at 250°C, 280°C, and 230°C, respectively. 1 µL of derivatized sample (BSTFA+1% TCMS, 70°C, 30 min) was injected in splitless mode. EI mass spectra (70 eV, m/z 50–600) were acquired. Compounds were identified using the NIST/EPA/NIH library (ver. 2.3), accepting reverse similarity >700. Relative abundance: peak area/total ion chromatogram [14,15].

Flow cytometry

ROS were quantified after incubation with 1 µM H₂DCFDA (Sigma-Aldrich) for 30 min at 37°C. Mitochondrial parameters were assessed using Mito Tracker Red (100 nM; Thermo Fisher Scientific) and JC-1 dye (2 µM; Thermo Fisher Scientific), respectively. Following staining, cells were washed with PBS and analyzed by flow cytometry (Guava easyCyte; Merck Millipore). A total of 3,000–5,000 events per sample were acquired, and data analysis was performed using FlowJo software ver. 10.6.2 (Tree Star).

Staining for lipid droplets

Lipid droplets were quantified using Nile Red staining. Cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature, and then washed three times with PBS. Cells were then stained with a 1 mg/mL Nile Red (72485-100MG; Sigma-Aldrich) solution in PBS for 15 minutes. After washing twice with 1 mL of PBS, the solution was completely removed. Fresh PBS was added and images were taken using a fluorescence microscope. Imaging

was performed with a Leica DMi8 microscope, and lipid content was quantified densitometrically using LAS X software and Adobe Photoshop CC 2024.

Glucose uptake assay

Cells were treated with 50 μ M 2-NBDG (Cayman Chemical) for 30 min, washed with PBS, and analyzed by flow cytometry. Analysis was performed with FlowJo 10.6.2.

Quantitative real-time polymerase chain reaction

Total RNA was extracted (TRIzol; Invitrogen), reverse-transcribed (WizScript cDNA Kit; Wizbiosolutions), and subjected to quantitative real-time polymerase chain reaction (qRT-PCR; SYBR Green; BioFACT) using a StepOnePlus System (Applied Biosystems). Expression was normalized to β -actin using the $2^{-\Delta\Delta Ct}$ method (Table 1).

Cellytics analysis of innate immune activation

Innate immune responses were quantified using the Cellytics platform (MetaImmuneTech) equipped with label-free lens-free shadow imaging technology (LSIT). After activation (IL-2, IL-12, PMA, ionomycin), the following parameters were analyzed: Polarization and Protrusion Dynamics (PPD), Weighted Shape Metric SD (WSM_SD), and Composite Structural Parameter (CSP = PPD $\times$ WSM_SD). The innate immunity index (I^3) was calculated as (CSP_ASC/CSP_Vehicle) $\times$ 100. All data were service-provided (Supplement 1) [16].

Statistical analysis

Results are expressed as mean $\pm$ standard deviation ($n\geq 3$). Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison using GraphPad Prism v10.2.3. p-values: ns, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

RESULTS

Cellular safety and chemical characterization of *Bulbophyllum drymoglossum* aqueous extract

To evaluate the cellular compatibility and compositional profile of BDAE, a series of *in vitro* cytotoxicity assays and chemical analyses were performed. Fresh *Bulbophyllum drymoglossum* plants were subjected to aqueous extraction at 110°C for 15 minutes, and the resulting BDAE was prepared as described (Figure 1A). Viability assays were conducted in NIH/3T3 fibroblasts and porcine alveolar macrophages (3D4/31) following 12-hour exposure to a concentration range of BDAE (0.00009–0.09 μ g/mL). No significant reduction in cell viability was observed in NIH/3T3 fibroblasts at any tested dose, while a modest but statistically significant decrease was noted in 3D4/31 cells at the highest concentration (**** $p<0.0001$) (Figure 1B). Four-day proliferation assays indicated that BDAE treatment did not alter cell growth kinetics compared to the vehicle control for either cell type, confirming that BDAE is not cytotoxic under these conditions (Figure 1C). Compositional analysis by GC-MS demonstrated that BDAE is primarily comprised of carbohydrate derivatives and polyols, with D-allose (30.07%), myo-inositol (23.62%), and sucrose (17.24%) as major constituents, and additional metabolites such as β -D-glucopyranose, D-fructose, and D-mannitol (Figures 1D, 1E). Minor levels of fatty acid derivatives, including octadecanoic, eicosanoic, docosanoic, and tetracosanoic acids, were detected. The predominance of these metabolites suggests that BDAE may modulate cellular energy metabolism and redox homeostasis [5]. Collectively, these results demonstrate that BDAE shows minimal cytotoxicity in both fibroblast and macrophage cell models at the tested concentrations and possesses a diverse metabolite profile, thereby

Table 1. Lists of primers used to perform qPCR

Target gene	Primer sequences (5'–3')	
	Forward	Revers
<i>β-actin</i>	GACATCAAGGAGAAGCTGTGC	TGAAGGTAGTTTCGTGGATGC
<i>FASN</i>	GTGGAGGTGCGCCAGATACT	CCTCGTGGGATGTGGGAGTC
<i>ACACB</i>	CAAGGTCCTGGGCAGAGAGG	ATGATCGGGACTGGGCTGTG
<i>ATGL</i>	CCTTCACCGTCCGCTTGC	TGTCTGCTCCTTTATCCACCG
<i>ACLY</i>	TTGGAGCAGACGGGCAAAGA	CAGCTTTCCACGCCGCTTTGA
<i>SUCLG1</i>	CAGGGCACCTTTCATAGCCA	CCTTTCCCTGGAGTGTTCC
<i>CPT1A</i>	CAAGATAGCGGCCGAAAAGC	GATAATCGCCACGGCTCAGA
<i>CD36</i>	TATCGTGCCATCCTCTGGC	AAACATCCCCACACCAACACT
<i>FFAR2</i>	CGGCCCTTGACGGAGTGAT	AAGAGGACGAGGCACAGCTC
<i>ACADVL</i>	GCGGTGAATCATGCTGCTAA	GTGGATCCCTGGTCCATGTT
<i>ACADL</i>	GCGTGGCTTATGACTGTGTG	ACTCGGGCATCCACATAAGC
<i>NDUFB8</i>	GGGGTGAACCGATACACTGG	CGAAACCGGAGAGGTGCTTA
<i>Uqcrc1</i>	ATGACCAGTGTCAGCAGTG	TAGAAGCGCAGCCAGAACAT
<i>COX4I1</i>	TTCGCCGAGATGAACAGGAG	GGGCCGTACACATAGTGCTT
<i>ATP5F1A</i>	GGGTGTCAGGGGCTATCTTG	TTTTTCCAACAGGGCTTGG

qPCR, quantitative polymerase chain reaction.

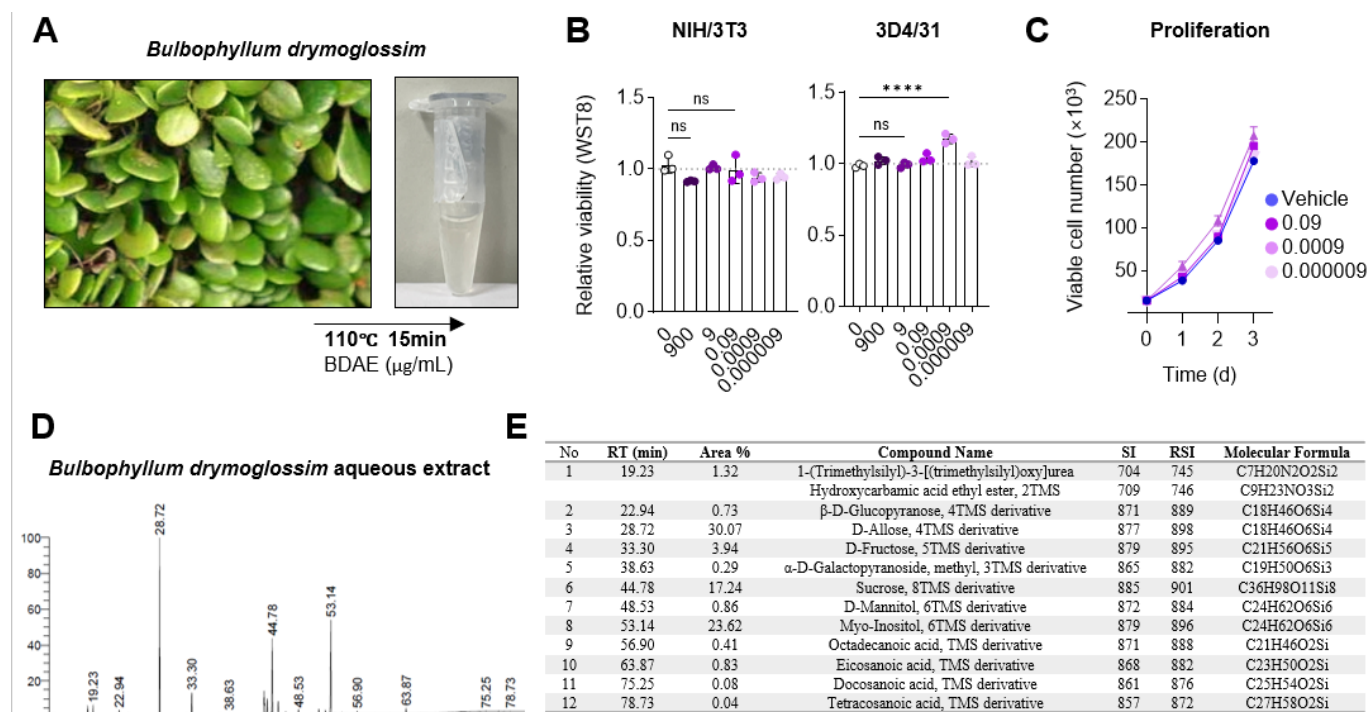


Figure 1. Effects of *Bulbophyllum drymoglossum* aqueous extract (BDAE) on cell viability, proliferation, and chemical composition. (A) Representative images of *Bulbophyllum drymoglossum* leaves and aqueous extract (BDAE) preparation by high-temperature extraction (110°C, 15 min). (B) Relative viability of NIH/3T3 fibroblasts and porcine alveolar macrophages (3D4/31) after 24 h exposure to increasing concentrations of BDAE, measured using the WST-8 assay. ns, not significant; **** $p < 0.0001$ versus vehicle control. (C) Effects of BDAE on cell proliferation in 3D4/31 cells during 3 days of culture with indicated extract concentrations. (D) Representative GC-MS total ion chromatogram of BDAE, showing major metabolite peaks. (E) Table of major compounds in BDAE as identified by GC-MS, including retention time, area percentage, compound names, similarity index (SI), and molecular formula. GC-MS, gas chromatography–mass spectrometry.

supporting its suitability for further biological evaluation in line with Animal Bioscience reporting standards.

Bulbophyllum drymoglossum aqueous extract attenuates intracellular reactive oxygen species generation in macrophages

The effect of BDAE on intracellular ROS production was evaluated in porcine alveolar macrophages (3D4/31 cells) using flow cytometry and fluorescence imaging. Dose-dependent analysis showed that increasing concentrations of BDAE markedly suppressed basal ROS levels, with significant reductions observed at 0.09 μg/mL and 0.009 μg/mL, as determined by H₂DCFDA fluorescence (Figure 2A; ** $p < 0.01$, **** $p < 0.0001$). To assess BDAE's effect under inflammatory conditions, cells were pretreated with BDAE and subsequently stimulated with PMA, a potent ROS inducer. PMA treatment greatly increased ROS levels compared to control and vehicle groups. Notably, BDAE pretreatment substantially attenuated PMA-induced ROS generation, as confirmed by a significant decrease in H₂DCFDA mean fluorescence intensity (Figure 2B; ** $p < 0.0001$, *** $p < 0.001$).

Fluorescence microscopy corroborated these findings, showing elevated DCF fluorescence in PMA-treated cells and

marked suppression in the BDAE+PMA group (Figure 2C). Quantification of DCF-positive cells revealed that co-treatment with BDAE significantly reduced the proportion of ROS-high cells compared to PMA alone (* $p < 0.0001$). Taken together, these findings indicate that BDAE exhibits potent antioxidant properties, significantly reducing both basal and PMA-induced oxidative stress in macrophages.

Bulbophyllum drymoglossum aqueous extract preserves mitochondrial mass and membrane potential in macrophages

The impact of BDAE on mitochondrial integrity was assessed in 3D4/31 macrophages using flow cytometric analysis of mitochondrial mass and membrane potential. Dose-dependent evaluation with MitoTracker Red staining revealed that BDAE treatment maintained mitochondrial mass, with only the highest concentration (0.0009 μg/mL) showing a modest but statistically significant reduction compared to control; lower concentrations did not significantly alter mitochondrial content (Figure 3A, * $p < 0.05$). Assessment of mitochondrial membrane potential using the JC-1 dye demonstrated that PMA stimulation caused a marked decrease in the JC-1 red/green fluorescence ratio, indicative of mitochondrial depolar-

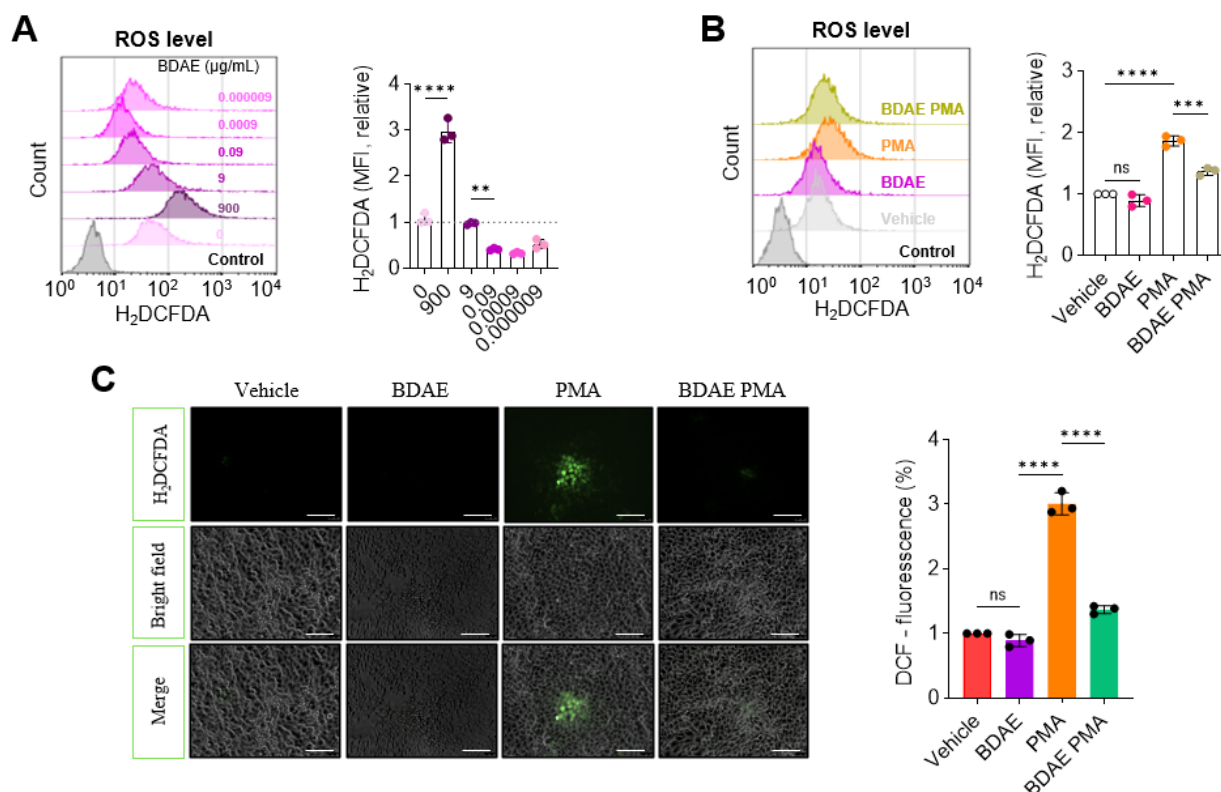


Figure 2. Modulation of reactive oxygen species (ROS) levels by BDAE in porcine alveolar macrophages. (A) Flow cytometry histograms and quantification of intracellular ROS (H₂DCFDA fluorescence) after treatment with increasing concentrations of BDAE. Data is shown as mean fluorescence intensity (MFI) relative to control. (B) Effect of BDAE pretreatment on PMA-induced ROS production. Histograms and quantification (MFI, relative to control) illustrate suppression of PMA-stimulated ROS by BDAE. (C) Representative fluorescence images and quantification of DCF-positive cells following treatment with vehicle, BDAE, PMA, or BDAE+PMA. Upper: H₂DCFDA fluorescence; middle: bright field; lower: merged images. The scale bar represents 50 μm. Right: percentage of DCF-positive fluorescence per group. Data represent mean±SEM. ns, not significant; ** p<0.01; *** p<0.001; **** p<0.0001 versus indicated groups. BDAE, *Bulbophyllum drymoglossum* aqueous extract; PMA, phorbol 12-myristate 13-acetate; SEM, standard error of the mean.

ization and dysfunction. In contrast, cells pretreated with BDAE prior to PMA exposure exhibited a significantly higher JC-1 ratio compared to the PMA-only group, suggesting partial preservation of mitochondrial membrane potential (Figure 3B, * p<0.05, ** p<0.01). Altogether, these results show that BDAE helps maintain mitochondrial mass and prevents PMA-induced mitochondrial membrane depolarization in macrophages, underscoring its protective effect on mitochondrial function under oxidative stress conditions.

***Bulbophyllum drymoglossum* aqueous extract modulates glucose uptake and cellular energy metabolism in macrophages**

The metabolic effects of BDAE were investigated in 3D4/31 macrophages. Flow cytometric glucose uptake assays using 2-NBDG demonstrated that PMA stimulation significantly reduced glucose uptake, whereas BDAE pretreatment alleviated this effect, leading to a moderate restoration of glucose uptake capacity (Figure 4A, * p<0.05). Gene expression analysis via quantitative RT-PCR revealed that PMA drastically sup-

pressed mRNA levels of genes involved in fatty acid uptake and β-oxidation (CPT1A, CD36, FEAR2, ACADVL, ACADL), while BDAE pretreatment substantially restored their expression (Figure 4B, * p<0.05 to **** p<0.0001). Similarly, BDAE reversed PMA-induced downregulation of lipid synthesis and lipolysis genes, including FASN, ACACB, ATGL, ACLY, and SUCLG1 (Figure 4C). Notably, BDAE also restored the expression of genes encoding mitochondrial respiratory chain complexes I–V (NDUFB8, uqcrc1, COX4I1, ATP5F1A), with statistical significance observed in most targets (Figure 4D). Lipid content staining using Nile Red indicated that PMA-induced lipid accumulation was markedly attenuated by BDAE pretreatment, as reflected by reduced fluorescence intensity compared to the PMA group (Figure 4D, *** p<0.001, **** p<0.0001). These findings indicate that BDAE counteracts the metabolic reprogramming induced by inflammatory stimuli, supporting energy metabolism, mitochondrial function, and preventing excessive lipid accumulation in activated macrophages [17,18].

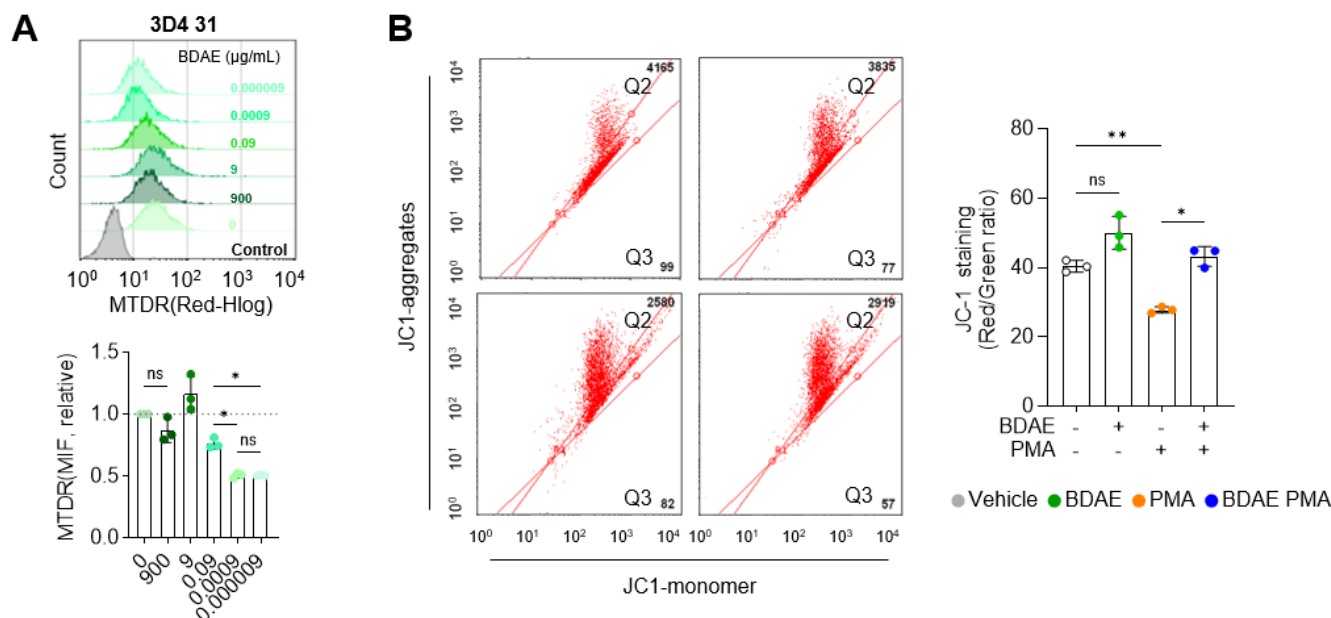


Figure 3. Effects of BDAE on mitochondrial integrity and membrane potential in porcine alveolar macrophages. (A) Flow cytometry histograms and quantification of Mito Tracker Deep Red (MTDR) fluorescence showing mitochondrial mass in 3D4/31 cells after treatment with increasing concentrations of BDAE. Data are shown as mean fluorescence intensity (MFI) relative to control. (B) Representative flow cytometry plots of JC-1 staining for mitochondrial membrane potential. Q2 represents healthy cells with high red (aggregate) fluorescence, Q3 indicates cells with low membrane potential. Quantification (right) shows the ratio of JC-1 red aggregates to monomers under the indicated conditions. Data represent mean $\pm$ SEM. ns, not significant; * $p < 0.05$; ** $p < 0.01$ versus indicated groups. BDAE, *Bulbophyllum drymoglossum* aqueous extract; PMA, phorbol 12-myristate 13-acetate; SEM, standard error of the mean.

Schematic summary of the protective effects of *Bulbophyllum drymoglossum* aqueous extract against phorbol 12-myristate 13-acetate-induced macrophage stress

A conceptual summary is presented to illustrate the integrated effects of BDAE in porcine alveolar macrophages (3D4/31) under inflammatory stress (Figure 5). Upon stimulation with PMA, macrophages experience increased oxidative stress, mitochondrial dysfunction, and excessive lipid accumulation, as evidenced by elevated ROS generation (DCF-DA), impaired mitochondrial membrane potential (JC-1), and increased lipogenesis (Nile Red) [19,20]. Pre-treatment with BDAE exerts protective effects by attenuating PMA-induced oxidative stress, maintaining mitochondrial function, and reducing both lipogenesis and lipotoxicity. These collective actions demonstrate that BDAE mitigates key features of stress and inflammation at the cellular level, supporting its potential as a modulator of macrophage metabolic and oxidative responses.

DISCUSSION

This study comprehensively characterized the bioactive properties of BDAE and elucidated its regulatory effects on oxidative stress, mitochondrial function, and metabolic reprogramming in porcine alveolar macrophages [19]. Our findings demonstrate that BDAE exerts dual roles in cellular redox

regulation by inducing mild ROS generation at low to moderate concentrations, while providing robust antioxidant protection under inflammatory conditions, thus maintaining cellular homeostasis.

GC-MS analysis revealed that BDAE predominantly contains carbohydrate derivatives such as D-allose, myo-inositol, and sucrose, alongside minor fatty acid components [5]. These metabolites are well recognized for supporting cellular energy metabolism and redox balance, which likely underpin BDAE's ability to modulate intracellular ROS and enhance antioxidant defenses [21]. Notably, the biphasic ROS response marked by elevated ROS levels at higher doses but suppression under PMA-induced oxidative stress suggests a hermetic effect that enhances macrophage adaptive resilience without inducing cytotoxicity.

Further, BDAE preserved mitochondrial membrane potential and mitigated dysfunction induced by PMA-triggered oxidative stress, as evidenced by partial restoration of the JC-1 red/green fluorescence ratio [22]. This mitochondrial protection is crucial to prevent downstream inflammatory cascades and metabolic disturbances. The concurrent downregulation of mitochondrial respiratory chain genes supports normalization of disrupted mitochondrial bioenergetics following inflammatory insult.

Metabolic analyses indicate that BDAE effectively inhibits PMA-stimulated enhancements in glucose uptake and fatty

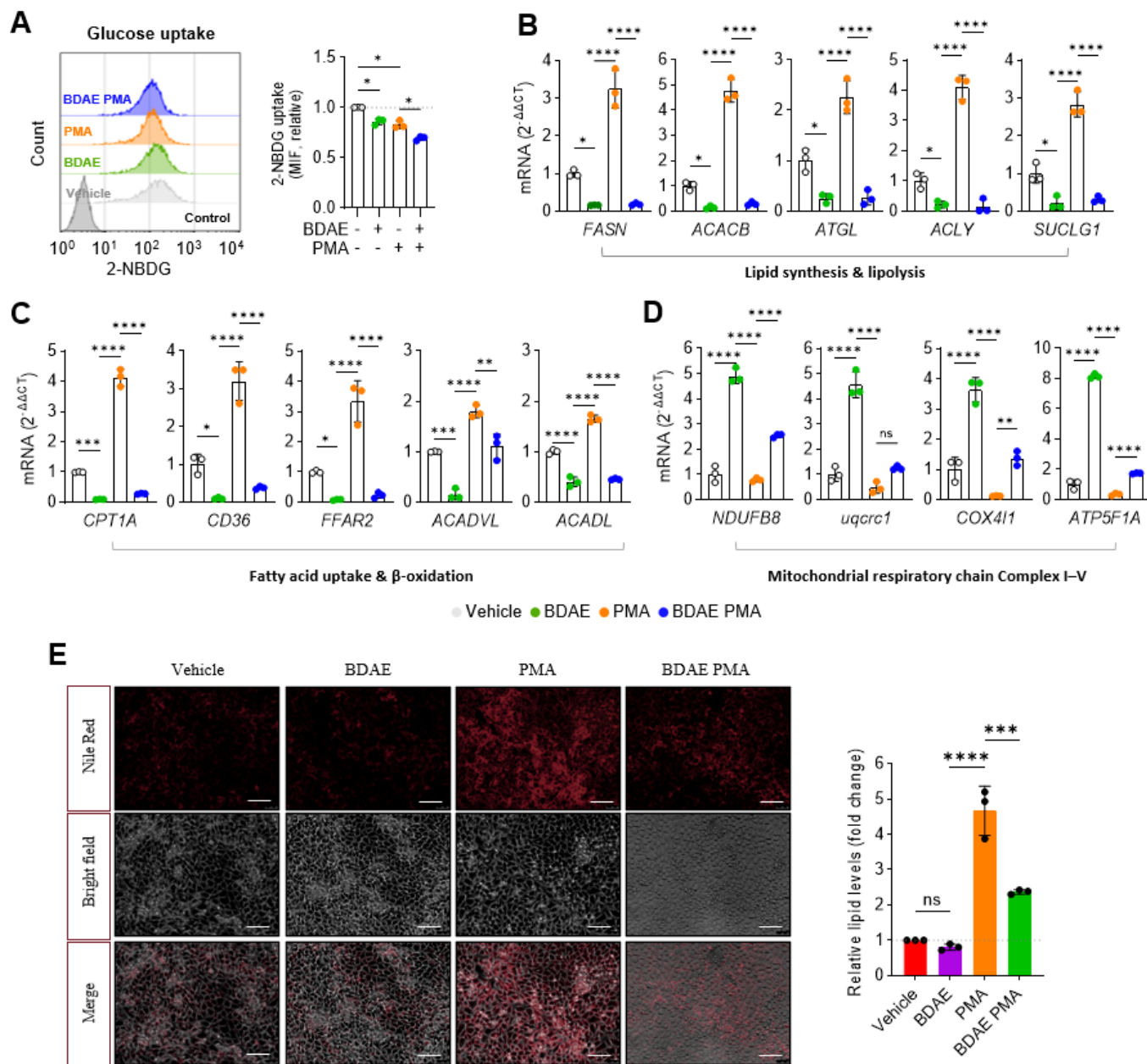


Figure 4. BDAE suppresses metabolic reprogramming in PMA-stimulated porcine alveolar macrophages. (A) Flow cytometry histograms and quantification of 2-NBDG uptake (glucose uptake) in 3D4/31 cells under indicated treatments. (B) qPCR analysis of mRNA expression for genes related to fatty acid uptake and β -oxidation (CPT1A, CD36, FFAR2, ACADVL, ACADL). (C) mRNA expression of genes involved in lipid synthesis and lipolysis (FASN, ACACB, ATGL, ACLY, SUCLG1). (D) mRNA levels of mitochondrial respiratory chain complex subunits (NDUFB8, UQCRC1, COX4I1, ATP5F1A). (E) Representative images and quantification of intracellular neutral lipid droplets visualized by Nile Red staining, with corresponding bright-field and merged images. The scale bar represents 50 μ m. The bar graph shows fold change in lipid content relative to control. Data are presented as mean $\pm$ SEM. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ versus indicated groups. BDAE, *Bulbophyllum drymoglossum* aqueous extract; PMA, phorbol 12-myristate 13-acetate; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean.

acid metabolism pathways, including critical genes involved in β -oxidation and lipogenesis. Reduction of intracellular neutral lipid droplets upon BDAE treatment points to its protective role against lip toxicity, a known contributor to chronic inflammation and cellular dysfunction [23]. Through coordinated regulation of energy substrate utilization and maintenance

of mitochondrial integrity, BDAE broadly modulates macrophage metabolic reprogramming a critical determinant of inflammatory phenotypes and immune responses [19,24].

Complementary data using NK-92 cells demonstrated enhanced innate immune activation following BDAE exposure, as reflected by increased CSP and Innate Immunity Index (I^3)

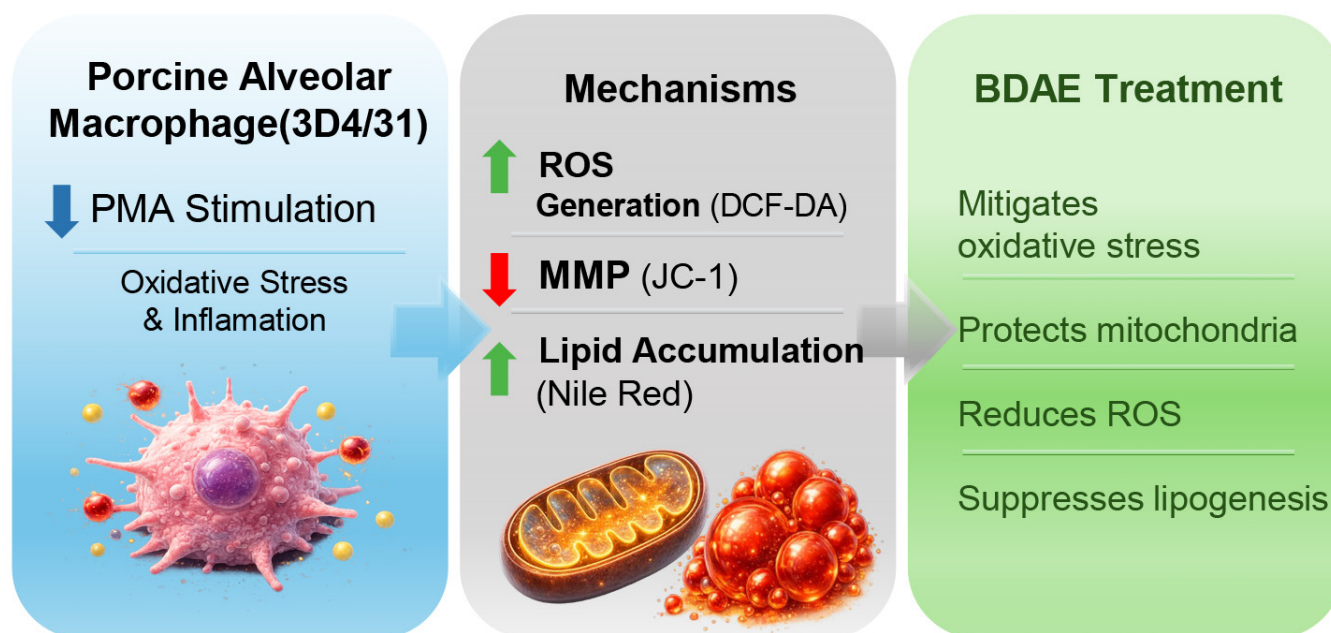


Figure 5. Schematic summary of the protective effects of *Bulbophyllum drymoglossum* aqueous extract (BDAE) in porcine alveolar macrophages. Exposure to PMA induces oxidative stress in 3D4/31 cells, leading to increased ROS generation, mitochondrial dysfunction, and lipid accumulation. BDAE treatment alleviates PMA-induced oxidative stress, preserves mitochondrial function, reduces ROS production, and suppresses lipogenesis and lip toxicity. Overall, BDAE confers cytoprotective and anti-inflammatory effects under stress conditions, as depicted in the schematic. PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species.

under ASC stimulation (Supplement 1). These results suggest that BDAE may improve immune vigilance and functional heterogeneity, reinforcing its immunomodulatory capacity alongside the macrophage data [25–28].

Collectively, these findings highlight BDAE as a multifunctional natural compound capable of balancing oxidative stress, safeguarding mitochondrial function, modulating metabolic homeostasis, and promoting immune activation [29,30]. From a biotechnological perspective, the integration of chemical, metabolic, redox, and immunological data supports the potential of BDAE as a candidate to modulate macrophage function and control inflammatory oxidative damage [31]. Its ability to fine-tune mitochondrial health and redox status without compromising cell viability establishes its promise for applications in animal health and management of inflammation-related disorders.

This work benefits from a rigorous multi-layered approach combining chemical profiling, cellular functional assays, and advanced immune phenotyping [32,33]. Nevertheless, further *in vivo* studies are essential to confirm these protective effects under physiological conditions and to evaluate systemic benefits and safety in livestock. Additionally, mechanistic investigations into molecular targets and signaling pathways mediating BDAE's effects will strengthen its translational potential.

CONSLUSION

This study demonstrated that BDAE effectively regulates oxidative stress, preserves mitochondrial function, and modulates metabolic reprogramming in porcine alveolar macrophages [29]. By modulating intracellular ROS levels in a biphasic manner, BDAE supports redox homeostasis at low to moderate doses while providing antioxidant protection against inflammatory oxidative stress [34,35]. It maintains mitochondrial membrane potential and mitigates dysfunction caused by oxidative challenges [36,37]. Additionally, BDAE inhibits inflammation-associated metabolic alterations by suppressing glucose uptake, fatty acid β -oxidation, lipogenesis, and lipid accumulation. These effects are likely driven by its rich composition of carbohydrate derivatives and polyols, which contribute to its bioenergetic and antioxidative properties. Furthermore, BDAE enhances innate immune activation, underscoring its broader immunomodulatory potential. Together, these findings highlight BDAE as a promising natural bioactive compound for regulating immunometabolism in animal health, with potential therapeutic and preventive applications in sustainable livestock production. Future studies should aim to validate these effects *in vivo* and clarify the underlying molecular mechanisms.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHORS' CONTRIBUTION

Conceptualization: Kim SJ.
 Data curation: Park EH, Kim J.
 Formal analysis: Kim J.
 Methodology: Park EH, Kim J.
 Software: Park EH, Kim J.
 Validation: Park EH, Kim J.
 Investigation: Park EH, Kim J.
 Writing - original draft: Park EH, Kim J, Kim SJ.
 Writing - review & editing: Park EH, Kim J, Kim SJ.

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Not applicable.

SUPPLEMENTARY MATERIAL

Supplementary file is available from: <https://doi.org/10.5713/ab.25.0638>

Supplement 1. BDAE enhances ASC-induced morphological activation in NK-92 cells quantified by Cellytics analysis.

DATA AVAILABILITY

Upon reasonable request, the datasets of this study can be available from the corresponding author.

ETHICS APPROVAL

Not applicable.

DECLARATION OF GENERATIVE AI

During the preparation of this manuscript, the authors used Perplexity (<https://www.perplexity.ai/>), an artificial intelligence tool developed by Perplexity AI, Inc. (California, USA), solely to assist with language refinement and correction of grammar and style. The tool was not employed for research design, data analysis, or drafting of scientific content.

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