Research Article

Anti-Inflammatory Activity of Extraction and Bioaccessible Fraction of *Albizia Lebbeck* (L.) Benth. on Interleukin-1β-Induced Inflammation in Human Retinal Pigment Epithelial (ARPE-19) Cells

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ABSTRACT

Age-related macular degeneration (AMD) is a common eye disease that affects older adults and is the leading cause of vision loss. The major cause of AMD is inflammation of retinal pigment epithelium (RPE) cells. *Albizia lebbeck* (L.) Benth. leaves contain various phytochemicals that have several biological attributes, especially anti-inflammatory properties. This study examined the anti-inflammatory effect of extraction and bioaccessible fraction of *A. lebbeck* leaves (ALE and BFA, respectively) on human retinal pigment epithelial ARPE-19 cells that were stimulated for inflammation by interleukin-1β (IL-1β). ARPE-19 cells were pre-treated with ALE or BFA for 1 h before inducing with 0.1 ng/ml IL-1β for another 24 h and then induced with IL-1β for 24 h. Our results show that pretreatment with ALE and BFA significantly reduced the secretion of pro-inflammatory mediators (IL-6, IL-8, and MCP-1) without toxicity ($p < 0.05$). These findings suggest that ALE can protect human retina cells from inflammatory induction and thus AMD development. The portion of *A. lebbeck* leaves that underwent simulated digestion retained its ability to protect ARPE-19 cells from inflammatory induction. However, further investigation into the impact of *A. lebbeck* leaves on AMD is warranted, including studies involving both experimental animal models and human subjects.

Keywords: *Albizia lebbeck* (L.) Benth; Age-related macular degeneration; Inflammation; Interleukin-1β; ARPE-19 cells

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บทความวิจัย

ฤทธิ์ต้านการอักเสบของสารสกัดและส่วนที่ผ่านกระบวนการย่อยภายในร่างกายของใบตะคึกต่อเซลล์จอประสาทตามนุษย์ที่เหนี่ยวนำให้เกิดการอักเสบด้วยอินเตอร์ลิวคิน-1เบต้า

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บทคัดย่อ

ภาวะจอประสาทตาเสื่อมเป็นโรคตาที่พบบ่อยซึ่งส่งผลกระทบต่อผู้สูงอายุและเป็นสาเหตุสำคัญของการสูญเสียการมองเห็น การอักเสบของเซลล์จอประสาทตาเป็นสาเหตุสำคัญของการเกิดภาวะจอประสาทตาเสื่อม ใบตะคึกมีสารพฤกษเคมีที่มีคุณสมบัติทางชีวภาพหลากหลาย โดยเฉพาะคุณสมบัติในการต้านการอักเสบ การศึกษานี้จึงมีวัตถุประสงค์เพื่อตรวจสอบฤทธิ์ต้านการอักเสบของสารสกัดและส่วนที่ผ่านกระบวนการย่อยภายในร่างกายของใบตะคึก (ALE และ BFA ตามลำดับ) ต่อเซลล์จอประสาทตามนุษย์ที่เหนี่ยวนำให้เกิดการอักเสบ เซลล์จอประสาทตาจะถูกเลี้ยงด้วย ALE และ BFA เป็นเวลา 1 ชั่วโมง จากนั้นเหนี่ยวนำด้วย IL-1β เป็นเวลา 24 ชั่วโมง การศึกษานี้พบว่าเซลล์จอประสาทตาที่ถูกเลี้ยงด้วย ALE และ BFA สามารถลดระดับของ IL-6, IL-8 และ MCP-1 ได้อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) และไม่เป็นพิษต่อเซลล์ งานวิจัยนี้แสดงให้เห็นว่าใบตะคึกสามารถปกป้องเซลล์จอประสาทตาของมนุษย์จากการกระตุ้นให้เกิดการอักเสบซึ่งเป็นสาเหตุของการเกิดภาวะจอประสาทตาเสื่อมได้ และส่วนที่ผ่านกระบวนการย่อยภายในร่างกายของใบตะคึกก็ยังคงความสามารถป้องกันการอักเสบของเซลล์จอประสาทตาได้ อย่างไรก็ตามควรมีการศึกษาผลของใบตะคึกต่อภาวะจอประสาทตาเสื่อมโดยศึกษาในสัตว์ทดลองและในมนุษย์ต่อไป

คำสำคัญ: ตะคึก, ภาวะจอประสาทตาเสื่อม, การอักเสบ, อินเตอร์ลิวคิน-1เบต้า, เซลล์จอประสาทตามนุษย์ชนิด ARPE-19

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INTRODUCTION

Age-related macular degeneration (AMD) is a prevalent eye disease that is the leading cause of vision loss among the elderly population. The number of AMD cases is predicted to increase by approximately 100 million between 2020 and 2040, primarily due to the exponential growth of the global aging population[1, 2]. AMD is a degenerative eye condition affecting the macula, which is a part of the retina. Clinically, AMD can be divided into two major forms: dry AMD (atrophic or non-exudative AMD) and wet AMD (neo-vascular exudative AMD). However, dry and wet AMD can occur in one person as dry AMD can progress into wet AMD. This progression can lead to a loss of central vision, which can hinder the daily life of older persons[3-5]. Consequently, protecting against or delaying the progression of AMD is crucial to reducing vision loss risk. Although AMD development is complex, the main cause of AMD progression is inflammation[6] of the retinal pigment epithelium (RPE) cells in the macula region of the retina. RPE cells are located between the Bruch's Membrane (BM) and photoreceptors, and they play a crucial role in the visual cycle. These cells function as the out blood-retinal barrier (BRB), responsible for transporting ions, nutrients, and metabolites through lateral plasma membrane junctions. They also transport and store retinoids to maintain the visual cycle. In addition, RPE cells function as phagocytes by engulfing the degenerated tips of the outer segments of photoreceptors. These cells are constantly exposed to visible light, have high levels of local oxygen and a high metabolic rate, and exist in a reactive species (RS)-rich environment[7-9]. Moreover, RPE cells regulate immune and inflammatory responses to stress in the retina[10]. Since inflammation plays a significant role in AMD development, inflammatory mediators released by lymphocytes and macrophages that infiltrate the posterior segment of the eye can trigger the dysfunction of RPE cells[11-14]. Numerous studies have investigated how RPE cells interact with pro-inflammatory cytokines, such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β). These cytokines can attach to particular receptors on RPE cells, which greatly increases the expression of other cytokines and chemokines, namely, interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1)[15-18]. IL-6 is a cytokine produced by activated RPE cells. It stimulates vascular endothelial cells to produce chemokines, such as IL-8 and MCP-1, which attract different types of white blood cells to the sites of inflammation. IL-8 serves as a chemoattractant for white blood cells, particularly neutrophils, eosinophils, and T cells, guiding them to the site of inflammation. It is produced in various cell types, including epithelial cells, monocytes, and macrophages. MCP-1 is a chemokine that plays a crucial role in inflammation in the human retinal pigment epithelium by activating several intracellular signal transduction pathways that upregulate the expression of adhesion molecules and are pro-inflammatory[19]. Previous research has indicated that levels of inflammation in serum or ocular tissue are altered during the development and progression of exudative AMD[20-22]. Consequently, an appealing strategy for protecting against AMD may involve specific

biological compounds or substances for anti-inflammation in RPE cells. *Albizia lebbeck* (L.) Benth., commonly known as lebbeck or Ta-kuk (in Thai), belongs to the family Fabaceae/Mimosaceae. It is typically cultivated in regions with tropical or sub-tropical climates[23]. Previous studies have reported that *A. lebbeck* has various biological benefits, such as anti-inflammatory, antioxidant, anticancer, and antibacterial capabilities[24]. Numerous phytochemicals have been found in *A. lebbeck*, including carotenoids, flavonoids, tannins, and saponins. The leaves of this plant have been traditionally used for treating various eye diseases, such as night blindness, helminthiasis conjunctivitis, and corneal opacity[23]. In addition, recent studies have shown that certain phytochemicals present in the young leaves of *A. lebbeck* have potent anti-inflammatory properties that can relieve inflammation in RPE cells. Additionally, previous studies have demonstrated that *A. lebbeck* leaf extract may protect human microglial HMC3 cells against neurotoxicity caused by glutamate[25]. Despite this extensive research, no study has yet demonstrated the effect of *A. lebbeck* leaves on inflammatory induction in human retinal cells that cause AMD development. Consequently, to our knowledge, this investigation is the first attempt to evaluate the anti-inflammatory effects of extraction and the bioaccessible fraction of *A. lebbeck* leaf extract on inflammatory induction in human retinal pigment epithelium ARPE-19 cells with IL-1β.

Material and Methods

Chemicals and reagents

DMEM/F-12 powder was obtained from Life Technologies Corporation (Grand Island, NY 14072, USA). Fetal Bovine Serum (FBS) was purchased from PAN Biotech (South America). Penicillin-Streptomycin Solution was acquired from Caisson Laboratories (Smithfield, UT 84335, USA). Trypsin-EDTA (Life Technologies Corporation, Grand Island, NY 14072, USA). DMSO was obtained from Sigma-Aldrich (St. Louis, MO 63103, USA). MTT was purchased from Bio Basic Inc. (Markham, ON L3 R 8 T4, Canada). ELISA kits were acquired from BioLegend Way (San Diego, CA 92121, USA).

Sample preparation

The young leaves of *A. lebbeck* were collected from Srinakarin Dam in Kanchanaburi province, Thailand. The edible parts were blanched for two minutes in hot water at a ratio of 1:10 (w/v) before air-drying, following the procedures of Phoraksa et al.[25], and then homogenized in an electric blender. Thereafter, the samples underwent freeze-drying and then homogenized again using an electric blender. The dried powder of young leaves *A. lebbeck* was wrapped in aluminum foil, vacuum-sealed, and kept at -20 °C.

Sample extraction

The powder samples were extracted using hexane in a 1:15 (w/v) ratio. For extraction, each sample was mixed on a vortex and shaken at room temperature, then incubated for 16 h. Thereafter, the sample was sonicated in an ultrasonic bath (Hettich Zentrifugen, Rotina 38, North America) and centrifuged at 4100 g for 10 mins (Hettich® Instruments, Rotina 38R, Germany), repeating the process twice. The supernatant was combined and evaporated at 40 °C until dry. The extract of *A. lebbeck* leaves

(ALE) was then stored at -20 °C until use. The yield of ALE was 3.44 ± 0.14%.

In vitro simulated digestion

Simulated gastrointestinal digestion was conducted using the methods previously described by Ferruzzi et al.[26] and Chitchumroonchokchai et al.[27]. In the gastric phase, 0.8 g of freeze-dried *A. lebbeck* were homogenized with 20 ml of 120mM NaCl in a 50 ml polypropylene tube using a homogenizer (model T25D, Germany). The pH was then adjusted to 2.0 ± 0.1 with 1M HCl. Pepsin (40 mg/ml in 100mM HCl) was added (2 ml), and the volume was adjusted to 40 ml with 120mM NaCl. The tube was then blanked with nitrogen gas, tightly capped, sealed with parafilm, and incubated in a shaking water bath for 1 h at 37ºC and 95 rpm. In the small intestinal phase, pH was adjusted to 6.0 ± 0.1 with 1M sodium bicarbonate ($NaHCO_3$). Crude bile extract (40 mg/ml in 100mM $NaHCO_3$) (3 ml), pancreatin (10 mg/ml in 100mM $NaHCO_3$) (2 ml), and lipase (5 mg/ml in 100mM $NaHCO_3$) (2 ml) were added to the reaction tube. The pH was then increased to 6.5 ± 0.1 with 1 M $NaHCO_3$. The tube was filled with nitrogen gas, tightly capped, sealed with parafilm, and put in a shaking water bath for 2 h at 37ºC and 95 rpm. After the simulated small intestinal phase of digestion was completed, the sample solution underwent centrifugation (Becton Dickinson Dynac Centrifuge in Sparks, MD, U.S.A.) at 4,000g for 1 h at room temperature. This process was done to isolate the aqueous fraction or supernatant. A control digestion without *A. lebbeck* was also conducted to determine any cytotoxic effects of digested compounds in the aqueous fraction. After centrifugation, the supernatant was filtered using a Millipore Corp. 0.22 µm pore size polytetrafluoroethylene (PTFE) membrane to obtain the fraction with mixed micelles or bioaccessible fraction of *A. lebbeck* (BFA).

ARPE-19 cells

The human retinal pigment epithelial (ARPE-19) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in the DMEM/F-12 supplement with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) and 1% (v/v) of penicillin/streptomycin solution (100x). The cells were incubated at 37 °C in a humidified environment of 5% CO_2.

Cell viability of ALE in ARPE-19 cells

The MTT assay was used to investigate the cell viability of ARPE-19. ARPE-19 cells were seeded (100,000 cells per well) in 48-well plates for 24 h. After rinsing the cells with DMEM-F12, they were treated with DMEM-F12 containing ALE at 5, 10, 25, 50, and 100 µg/ml concentrations. Following incubation for 24 h, the old media was removed, and the cells were rinsed with phosphate buffer saline (PBS). Cells were added with MTT solution (0.5 mg/ml in PBS) and then incubated at 37°C for 3 h. The MTT solution was then discarded, and the formazan crystal was dissolved by adding dimethyl sulfoxide (DMSO). Finally, optical density at 540 nm was measured by a microplate reader (BioTek® Instruments, Vermont, USA). The experiments were carried out in triplicate. Data were expressed as the percentage of cell viability relative to the control cells group. The control group was treated with DMEM-F12 containing DMSO at a concentration of 0.2%. For further analysis, we selected the highest concentration of

ALE that showed no statistically significant difference in cell viability compared to the control group.

Effect of ALE and BFA on ARPE-19 cells induced inflammation with IL-1β

ARPE-19 cells were seeded in 48-well plates at a density of 100,000 cells per well. After 24 h of incubation, the cells were rinsed with DMEM-F12 and incubated with DMEM-F12 containing the ALE at 1, 10, and 100 µg/ml of BFA diluted 1:3 (v/v) with serum-free media for 1 h. Thereafter, 0.1 ng/ml of IL-1β was added to each well and incubated for 24 h. The culture medium was then analyzed to determine the biomarkers (IL-6, IL-8, and MCP-1) for the anti-inflammatory properties of ALE and BFA. The cells were evaluated for cell viability using MTT assay.

ELISA test

After the respective experimental time, a culture medium was used to determine the amount of IL-6, IL-8, and MCP-1. For this assay, we followed the instructions provided by the manufacturer. To measure IL-6, IL-8, and MCP-1 levels, we coated capture antibodies in 96-well plates and left them to incubate overnight at 4°C. We then rinsed the plates using a solution of PBS containing 0.05% (v/v) of Tween-20 and incubated them with 1% (w/v) BSA in PBS for 60 minutes. Thereafter, we incubated human IL-6, IL-8, and MCP-1 standards or culture medium at room temperature. Following this step, we added biotinylated detecting antibodies and incubated them for 60 mins at room temperature. To facilitate immune complex detection, we reacted the solution with the streptavidin and horseradish peroxidase tetramethylbenzidine system. We stopped the reaction by adding 2M of H_2SO_4. Finally, we measured the levels of pro-inflammatory cytokines at an absorbance of 450 nm using a microplate reader.

Statistical analysis

Data were presented as mean ± standard deviation (SD), calculated from at least three independent experimental procedures. Statistical analysis was conducted using a one-way variance analysis. Duncan's post-hoc test was used to compare the differences between groups using SPSS version 19. A *p*-value of less than 0.05 was considered statistically significant.

Results

Effect of ALE on the viability of ARPE-19 cells

Before research can be conducted, the toxicity of ALE on ARPE-19 cells must be assessed. The cells were incubated with ALE at various concentrations (5, 10, 25, 50, and 100 µg/ml) for 24 h, while a control group was exposed to DMSO at a concentration of 0.2% in DMEM-F12 Results indicated that all concentrations of ALE were not toxic to ARPE-19 cells (**Fig. 1**). Consequently, the maximum concentration of ALE at 100 µg/ml was chosen for the next investigation.

Effect of ALE and BFA on pro-inflammatory mediator secretion in ARPE-19 cells induced inflammation by IL-1β

L-6, IL-8, and MCP-1 were used as biomarkers for ARPE-19 cell-induced inflammation[28]. ARPE-19 cells were treated with ALE at 1, 10, and 100 µg/ml, or BFA diluted 1:3 (v/v) with serum-free media for 1 h before inducing inflammation with IL-1β 0.1 ng/ml for 24 h. Results indicated that ARPE-19 cells induced

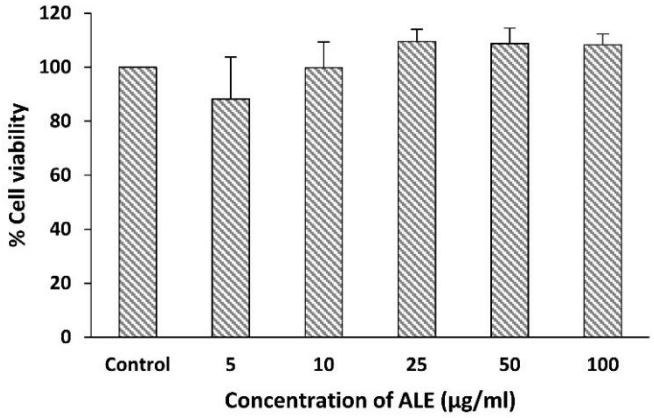

Figure 1. Effect of ALE on the viability of ARPE-19 cells. Cells were incubated with ALE at 5, 10, 25, 50, and 100 µg/ml for 24 h. The viability of the cell was assessed by MTT assay. Graphs represent an average of cell viability as mean ± SD values (n = 3).

with 0.1 ng/ml of IL-1β for 24 h significantly increased the secretion of IL-6, IL-8, and MCP-1 compared to the control group ($p < 0.05$) (**Figs. 2A and 2B**). Pre-incubation with ALE could significantly reduce levels of IL-6, IL-8, and MCP-1 when compared to ARPE-19 cells treated with IL-1β alone ($p < 0.05$), depending on the amount of ALE (**Fig. 2A**) without toxicity (**Fig. 3A**). In addition, the pre-treatment of BFA (diluted 1:3 (v/v) with serum-free media) could significantly decrease levels of IL-6, IL-8, and MCP-1 when compared to ARPE-19 cells treated with IL-1β alone ($p < 0.05$) (**Fig. 2B**) without toxicity (Fig. 3B). These results demonstrated that ALE could protect ARPE-19 cells from inflammatory induction in a dose-dependent manner without toxicity. Moreover, the parts of *A. lebbeck* leaves that went past *in vitro* simulated digestion could still protect ARPE-19 cells from inflammatory induction without toxicity.

Discussion

Dietary phytochemicals are of increasing interest due to their potential to decrease the risk factors for several chronic diseases and provide multiple health benefits. Previous studies have shown that *A. lebbeck* leaves have an anti-inflammatory effect. An aqueous fraction of digested *A. lebbeck* leaves, containing carotenoids (e.g., α-carotene, β-carotene, and lutein) as well as flavonoids (e.g., luteolin, kaempferol, quercetin, and apigenin) significantly reduced levels of TNF-α and IL-6 and the expression of COX-2[29]. A study conducted by Meshram et al. reported that the leaves of *A. lebbeck* could protect rats from hind paw edema induced by carrageenan[30]. Moreover, another study found that *A. lebbeck* leaves extracted with hexane contained several phytochemicals, including lutein, quercetin, luteolin, and kaempferol, that could protect human microglial HMC3 cells from neurotoxicity induced by glutamate[25]. However, these substances need to perform their biological functions in humans

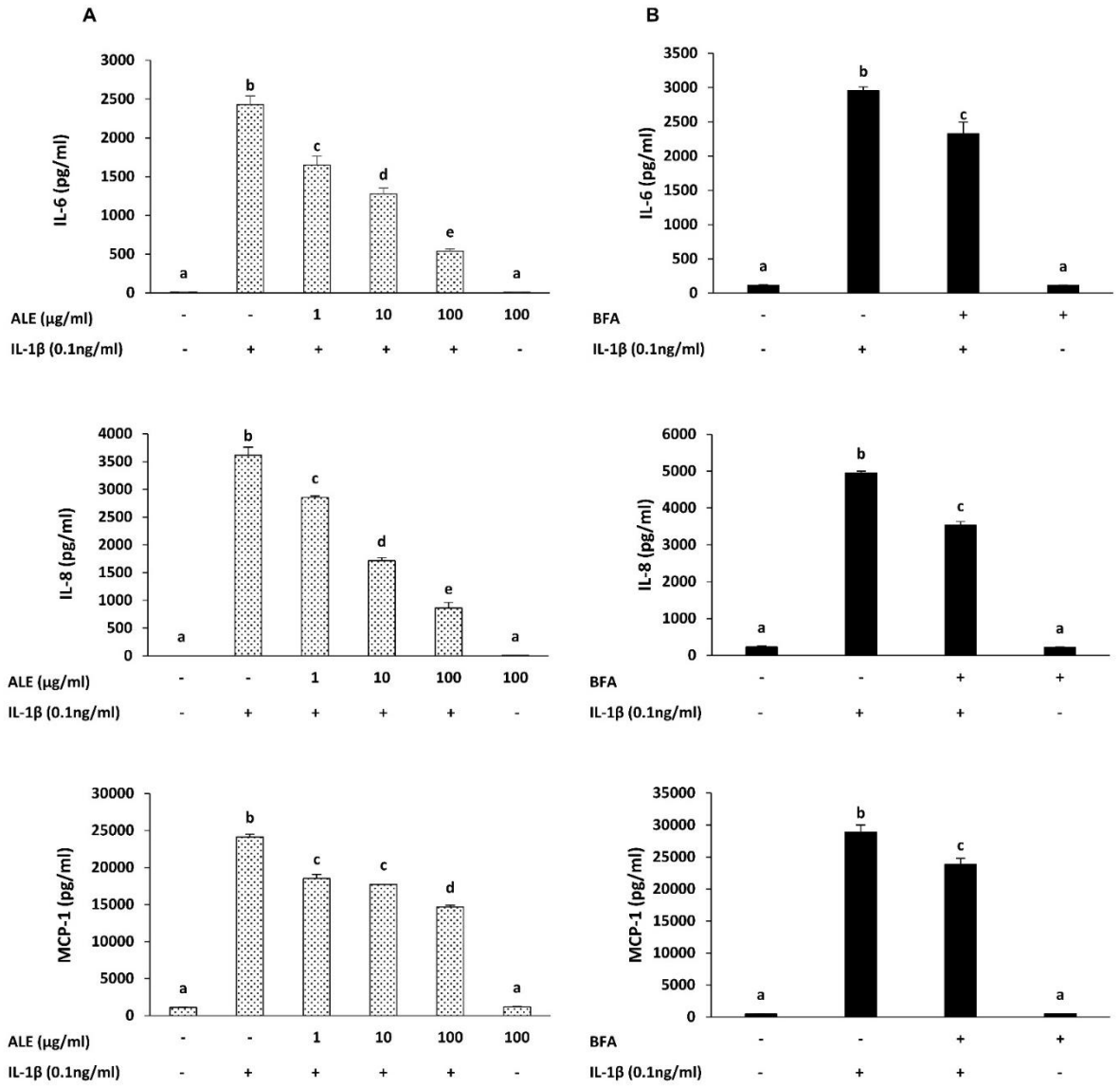

Figure 2. Effect of ALE and BFA on pro-inflammatory mediator secretion in ARPE-19 cells induced by IL-1β. Cells were incubated with 1, 10, and 100 µg/ml of ALE (A) or BFA (B) for 60 mins prior to induction with IL-1β at 0.1 ng/ml for 24 h. Negative controls were cells treated with 0.2% DMSO and bioaccessible fraction without ALE for ALE and BFA, respectively. IL-6, IL-8, and MCP-1 levels in culture media were determined by sandwich ELISA. Results are reported as mean ± SD values (n = 3). Different superscript letters (a-e) showed significant differences at $p < 0.05$.

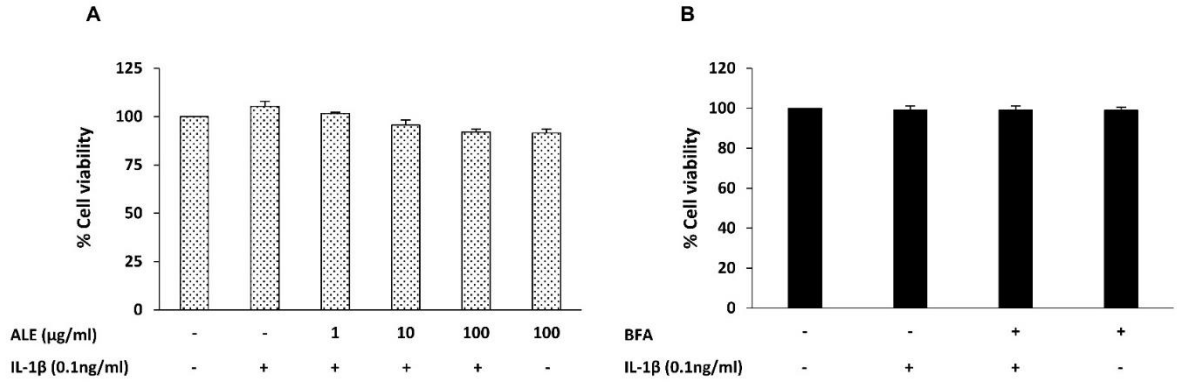

Figure 3. Effect of ALE and BFA on the viability of ARPE-19 cells induced by IL-1β. Cells were incubated with 1, 10, and 100 µg/ml of ALE (A) or BFA (B) for 60 mins prior to induction with IL-1β at 0.1 ng/ml for 24 h. Negative controls were cells treated with 0.2% DMSO and bioaccessible fraction without ALE for ALE and BFA, respectively. The viability of the cell was assessed by MTT assay. Graphs represent an average of cell viability as mean ± SD values (n = 3).

after digestion for them to be effective. Our study examined the anti- inflammatory effect of extraction and the bioaccessible fraction of *A. lebbeck* on the ARPE- 19 cell- induced inflammation. IL- 6, IL- 8, and MCP- 1 were used as inflammation biomarkers for ARPE- 19 cell-induced inflammation. This investigation showed that pre- treatment of ALE and BFA suppressed the secretion of IL- 6, IL- 8, and MCP- 1 from IL-1β- induced ARPE- 19 cells. Moreover, we found that neither ALE nor BFA was lethal to the cells (Fig. 3) or affected the formation of pro-inflammatory mediators in non-induced cells (Fig. 2). IL- 1β could activate RPE cells to secrete cytokines *in vitro*, according to reported findings by Koraneeyakijkulchai et al. that IL-1β activates the expression of IL-6, IL-8, MCP-1, and ICAM-1 in RPE cells by triggering MAPK signaling pathways and NF-κB transcription factors[31]. The variety of phytochemicals from *A. lebbeck* leaves extracted with hexane was confirmed to have anti- inflammatory activities. *In vivo* and *in vitro* studies on ocular tissue have shown that lutein and zeaxanthin have significant anti-inflammatory potential[31-33]. The low prevalence of early and late AMD was associated with high lutein and zeaxanthin-rich diets. In addition, further research on ARPE- 19 cells verified that lutein and zeaxanthin had anti-inflammatory properties[34-36]. As a natural compound, quercetin could protect retinal cells from oxidative damage by activating the transcription factor Nrf2/ NRF1 which stimulates the antioxidant system and prevents oxidative injury in the retina. Studies conducted on animals have shown that quercetin has a strong protective effect[37, 38]. Additionally, quercetin has the potential to protect human RPE cells from oxidative stress by inhibiting pro-inflammatory mediators and mitochondrial apoptotic pathways[39]. Fisetin and luteolin have displayed potential anti- inflammatory properties, while also providing protective benefits against oxidative stress- induced cell death in RPE cells[40]. Kaempferol has a protective effect

against retinal light injury in mice[41]. Consequently, our study shows that *A. lebbeck* leaves might be an alternative food supplement to reduce or slow the progression from dry to wet AMD. However, further studies are required to determine the exact mechanisms of *A. lebbeck* leaf extract in IL-1β-induced ARPE-19 cells.

Conclusion

Our study showed that the extraction and the bioaccessible fraction of *A. lebbeck* have anti-inflammatory effects in human retinal cells induced inflammation which causes AMD development by reducing the production of IL-6, IL-8, and MCP-1 without toxicity. This finding suggests that consuming *A. lebbeck* leaves daily may help to preserve and improve eye health. Even though the *in vitro* evidence supports the beneficial effects of *A. lebbeck* leaves on ocular health, more research *in vivo* on patients with inflammation- induced ocular damage is necessary to understand their biological functions. Moreover, further investigation into the impact of *A. lebbeck* leaves on AMD is warranted, including studies involving both experimental animal models and human subjects.

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Conflict of Interest

The authors declare no conflict of interest.

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