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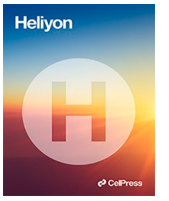

A fatty acid-rich fraction of an endolichenic fungus *Phoma* sp. suppresses immune checkpoint markers via AhR/ARNT and ESR1

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ABSTRACT

Lung cancer has the highest mortality rates worldwide. The disease is caused by environmental pollutants, smoking, and many other factors. Recent treatments include immunotherapeutics, which have shown some success; however, the search for new therapeutics is ongoing. Endolichenic fungi produce a whale of a lot of secondary metabolites, the therapeutic effects of which are being evaluated. Here, we used a crude extract and subfractions of the endolichenic fungus, *Phoma* sp. (EL006848), isolated from the *Pseudevernia furfuracea*. It was identified the fatty acid components, palmitic acid, stearic acid, and oleic acid, exist in subfractions E1 and E2. In addition, EL006848 and its fatty acids fractions suppressed benzo[*a*]pyrene (an AhR ligand)-induced expression of PD-L1 to inhibit the activity of multiple immune checkpoints. E2 subfraction, which had a higher fatty acid content than E1, downregulated expression of AhR/ARNT and several human transcription factors related to ESR1. Moreover, E2 showed a strong inhibitory effect on STAT3 expression and mild effect on NF-kB activity. These results suggest that fatty acids extracted from an endolichenic fungus can exert strong immunotherapeutic effects.

Abbreviations: AhR, Aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; ATF3, activating transcription factor 3; ATF4, activating transcription factor 4; BaP, benzo[a]pyrene; CREBBP, CREB binding protein; DMSO, Dimethyl sulfoxide; EGR1, early growth response 1; ELF, endolichenic fungi; ESR1, estrogen receptor 1; FOXG1, forkhead box G1; GC-MS, gas chromatography/mass spectrometry; GATA3, GATA binding protein 3; HVEM, TNF receptor superfamily member 14; HSP90, Heat stock protein 90; HNF4A, hepatocyte nuclear factor 4 alpha; LC-MS, liquid chromatography/mass spectrometry; ICOSL, Inducible T Cell Costimulator Ligand; ITS, internal transcribed spacer; MFI, Mean; Fluorescence Intensity, PDA; potato dextrose agar, PD-L1; programmed death-ligand 1, POU2AF1; POU class 2 homeobox associating factor 1, MYF5; myogenic factor 5, NFATC2; nuclear factor of activated T cells 2, NFATC3; nuclear factor of activated T cells 3, NFATC4; nuclear factor of activated T cells 4, NF-kB; nuclear factor kappa B, SP1; Sp1 transcription factor, SP2; Sp2 transcription factor, STAT3; signal transducer and activator of transcription 3, TBP, TATA-box binding protein; TLC, thin layer chromatography; 4-1BBL, TNF superfamily member 9; VISTA, V-set immunoregulatory receptor; VTCN1, V-set domain containing T cell activation inhibitor 1.

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1. Introduction

Lung cancer is the veriest widespread cancer worldwide, with poor survival rate [1]. Lung cancer and other lung disorders develop and progress as a result of inflammation. Causes are varied, but environmental pollutants play a role in disease pathogenesis [2]. Surgical resection is an forceful treatment for non-small cell lung cancer; however, because nearly half of lung cancer diagnoses are made at an advanced stage, it is not always feasible [3]. Therefore, in addition to surgery, chemotherapy and targeted therapies for receptor tyrosine kinases, immunotherapy in combination with therapy or as a basic therapy is on the agenda to improve the survival of lung cancer [4,5].

Immune checkpoint inhibitors have transformed treatment of non-small lung cancer cell over the past 10 years [6]. Cancer immunotherapy mainly aims to suppress the expression of immune checkpoints that inhibit antitumor immune effects or to block the interaction between antigen-presenting tumor cells and antigen-receiving immune cells [7]. Although these immune check points are very diverse, the Galectin-9, HVEM, VISTA, 4-IIBL, VTCN1 and PD-L1 we used in this study can be given as examples. In particular, PD-L1 is defined as a valuable therapeutic as an immune checkpoint. PD-L1 expression in host immune cells and tumor cells has important clinical implications against PD-L1/PD-1 blocking therapy in cancer patients. Monoclonal antibodies can improve the antitumor effects and survival rates of immune checkpoint PD-1/PD-L1 blockade [8,9]. Antibodies used are pidilizumab, pembrolizumab and nivolumab against PD-1, atezolizumab, durvalumab and avelumab against PD-L1, and ipilimumab and tremelimumab against CTLA-4. In clinical studies, the combination of chemotherapy and immunotherapeutics has been evaluated for survival benefits in lung cancer, and the results have been reported as promising. The combination of pembrolizumab and chemotherapy or atezolizumab and chemotherapy has been approved by the FDA for first-line therapy [10]. However, despite the progress of targeted therapies and treatments such as immune checkpoint inhibition, the success rates remain unsatisfactory. Therefore, it is important to develop new therapeutics and additional treatments.

PD-L1/PD-1 interaction inhibits the activation of T cells, including the production of cytokines. It has been reported that PD-L1 levels can be induced by exposure to pollutants such as cigarette consumption or BaP (one of the chemical in the tobacco products), and BaP regulates PD-L1 levels through AhR [11]. In patients with a smoking history, PD-L1/PD-1 blocking therapy provides clinical benefits and demonstrates good survival [12,13]. In addition, PD-L1 expression can be regulated by multiple cellular processes and signaling pathways. Interferon gamma, epidermal growth factor and transforming growth factor β can upregulate PD-L1 levels by activating signaling pathways [11,14,15]. Transcription regulators, loosing of tumor suppressor gene functions, epigenetic modifications and microRNAs are among the factors that modulate PD-L1 expressions [16–18].

A variety of biologically active natural metabolites are produced by endophytic fungi, which live inside the internal tissues of plants. Interest in endophytic fungi increased after the anticancer drug paclitaxel (Taxol) was derived from the endophytic fungus *Taxomyces andreanae* [19]. Lichen thalli enable a symbiotic relationship with the fungus; these symbionts are called endolichenic fungi. Previous studies have examined the anticancer activity of endolichenic fungi and their secondary metabolites [20–22]. Endolichenic fungi are known to produce several fatty acid metabolites [23,24]. Indeed, an extract from the endolichenic fungus *Phoma* sp. contained oleic acid, palmitic acid, and stearic acid as the major components. Studies show that fatty acid nanoparticles can be used as adjuvants to promote nano absorption [25,26].

Here, we examined the inhibitory effects of a crude extract from the endolichenic fungus *Phoma* sp., as well as those of obtained subfractions with a high fatty acid component, on immune checkpoint molecules and transcriptional factors, including PD-L1, in lung cancer cells. Our results show that fatty acid-rich fraction affects the expression of multiple immune checkpoints, via transcriptional regulators, resulting in anticancer, immunomodulatory, and anti-inflammatory effects.

2. Materials and methods

2.1. Sample collection, isolation, and identification of the endolichenic fungus

Lichen specimen collected in 2019 during the field trips in Mt. Uludağ, Bursa, Türkiye, organized by Prof. Sesal from Dept. of Plant Diseases and Microbiology, Marmara University, Istanbul, Türkiye. Endolichenic fungi used in this study were isolated from *Pseudevernia furfuracea* with the surface sterilization method [27]. Voucher specimens has been deposited at the Korean Lichen and Allied Bioresource Center (KOLABIC) in the Korean Lichen Research Institute (KoLRI), Sunchon National University, Korea (https://cc.aris.re.kr/kolabic).

First of all, endolichenic fungus was cultured on PDA (potato dextrose agar) (BD Difco, Sparks, MD, United States). As already

Table 1
EL006848 ITS sequence information.

Nucleotide sequence of EL006848
TTATGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAGAGTGTAAAAATGTACTTTTTGGACGTCGTCGTTATGAGTGCAAAGCGCGAGATGT ACTGCGCTCCGAAATCAATACGCCGGCTGCCAATTGTTTTGAGGCGAGTCTACACGCAAAGGCGAGACAAACACCCAACACCAAGCAGAGCTTGAAGGTAC AAATGACGCTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCG CATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAGTTTTTTCAGACGCTGATTGCAACTACAAAGGGTTTA AGTTTTGTCCAATCGGCGGGCGAACCCACCGAGGAAACGAAGGTACTCAAAAGACATGGGTAAGAGATAGCAGGCAAAGCCTACAACTCTAGGTAATGATC CTTCCGCAGGTTCACCTACGGAAACCTTGTTACGA

shown, fungus internal transcribed spacer sequencing was carried out [20,28]. After that, DNA was extracted from EL006848 cultured using a DNeasy Plant Mini Kit following the manufacturer's protocols (Qiagen, Hilden, Germany). Amplify the ITS (internal transcribed spacer) region of rDNA with standard primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) [29] and LR5 (5′-ATCCTGAGGGAAACTTC-3′) [30]. EL006848 was identified as *Phoma* sp. and based on ITS sequence analysis based on BLAST searches of the GenBank database showed 99.82% similarity to *Phoma* sp. (GenBank Accession No. MK299419.1). The ITS sequence of EL006848 was given in Table 1.

2.2. Fungal culture, preparation of the extract and fractions

PDA (Potato dextrose agar, BD Difco, Sparks, MD, United States) powder was prepared into distilled water, after that mixed to dissolve the powder and autoclaved. Following the autoclave, PDA solution was transferred right into the petri dishes after being cooled to 55–60 °C. Then, ELF mycelia grown on agar were transferred in liquid culture media (PDB (potato dextrose broth)) in 500 mL Erlenmeyer flasks and 3–4 weeks of incubation at 25 °C in a shaking incubator (150 rpm). After that, each flask was then filled with 200 mL of ethyl acetate (EtOAc), and it was shaken vigorously for about 2 h. The filtrate and mycelia from each culture were then separated by filtering. By allowing the filtrate to stand in a separating funnel, the filtrate was divided into layers that were water-soluble and EtOAc-soluble. By using a rotary evaporator to evaporate EtOAc to dryness while maintaining a vacuum, 8.1 g of the crude extracts of EL006848 were obtained. For use in experiments, the crude extract was dissolved in 100% DMSO [31].

The crude extract (318.4 mg) was subjected to a flash chromatography system equipped with a silica gel Redisep® Rf 12 g flash column (Teledyne ISCO, USA), eluted with the step-wise gradient of MeOH in CH_2Cl_2 (0-10-20-100%, v/v) at a flow rate of 8 mL/min. The eluents were pooled into 10 fractions (E1–E10) as shown in Fig. 1.

2.3. NMR and gas chromatograph-mass spectrometry (GC-MS) analysis

A Bruker Avance III HD 500 spectrometer (Bruker BioSpin, Billerica, MA, USA) was used to obtain NMR spectra. For GC-MS analysis, fractions E1 and E2 underwent methylation via a method described in Ref. [32]. Pentadecanoic acid (C15:0) was added into the methylation mixture as an internal standard. Fatty acid methyl esters of fractions E1 and E2 were analyzed using gas chromatography (Trace 1310, Thermo Scientific, Waltham, MA, USA) hyphenated with a single quadrupole mass spectrometry (ISQ LT, Thermo Scientific) using electron ionization as an ion source. Samples (1.0 μL) were analyzed using a J&W DB-5MS column (60 m × 0.25 mm, 0.25 μm, Agilent Technologies, Santa Clara, CA, USA). Helium was used as a carrier gas at a flow rate of 1.5 mL/min. The GC oven was held at 50 °C for 2 min before being raised to 180 °C using a linear gradient of 5 °C/min. The temperature was held for 8 min at 180 °C, then successively increased to 210 °C at 2.5 °C/min and 325 °C at 5 °C/min. Then, it was held for 10 min, which gave a total run time of 81.0 min. The MS transfer line temperature was 280 °C and the split ratio was 30.0. the MS data was acquired in a scan mode with a range of 35–650 Da.

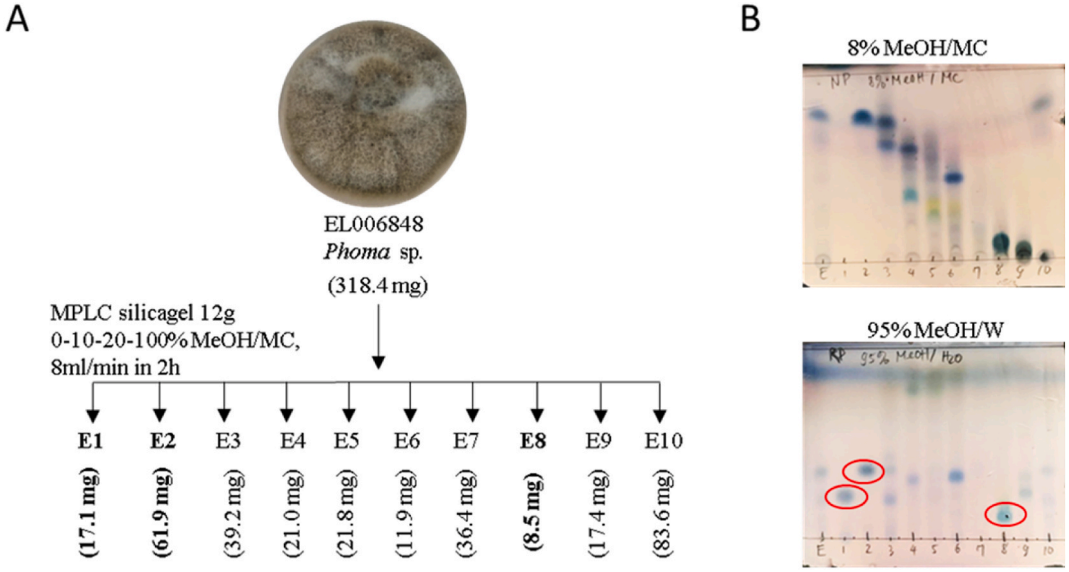


Fig. 1. Endolichenic Fungus, *Phoma* sp. extract produce secondary metabolites. (A) Image of the endolichenic fungus, EL006848, belonging to *Phoma* sp. isolated from the lichen *Pseudevernia furfuracea.* The eluents were pooled into 10 fractions (E1–E10) (B) Crude extract was subjected to a flash chromatograph with MeOH.

2.4. Cell culture

This study used of the H1975 (non-small cell lung cancer) cell line. The H1975 cell was cultivated in RPMI with 10% fetal bovine serum and 1% penicillin-streptomycin solution from GenDepot in Katy, Texas, in the United States. Cells were cultured in 5% CO2 at 37 °C in a humid environment [33].

2.5. Cell viability assay

In DMSO, extract and fractinons were dissolved (Sigma-Aldrich, St. Louis, MO, United States). On 96-well plates, cells were seeded at a density of $3x10^3$ cells/well, allowed to proliferate for the duration of the next day, and then treated with crude extract or sub-fraction for 48h. Following incubation, each well received 15 μL of the MTT reagent (Sigma-Aldrich) before the samples underwent an additional 4 h of incubation. The formazan crystals were dissolved in 150 μL DMSO after the supernatants had been removed after 4 h. Using Gen5 (v2.03.1) software on a microplate reader, absorbance was measured at 570 nm (BioTek, Winooski, VT, United States) [34].

2.6. Quantitative reverse-transcription PCR

On a 6-well plate, H1975 were plated at a density of 2 x 10^5 cells/well and grown overnight. Crude extract or DMSO were used to treat the cells. Using RNAiso Plus, total RNA was extracted from H1975 cells (TaKaRa, Otsu, Japan). cDNA was produced from 1 μg of RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, United States). SYBR Green was used for qPCR (Enzynomics, Seoul, Korea). On a CFX instrument, qRT-PCR experiments and analysis were carried out (Bio-Rad, Hercules, CA, United States) [35].

2.7. PD-L1 surface protein expression

On a 6-well plate, H1975 were plated at a density of 2 x 10^5 cells/well and grown overnight. Then, 4 h before being exposed to benzo[*a*]pyrene (1 μM), cells were treated with crude extract and sub-fractions. Following this, the cells were incubated for 48 h. Following incubation, cells collected in PBS by trypsin digestion. After that three rounds of washing, cell pellets were resuspended in 2% BSA and a 100 μL aliquot was utilized for anti-PD-L1 antibody (Cell signaling, #13684) labeling at 4 °C for 30 min in the dark. Cell was utilized with secondary antibody anti-Rabbit IgG (H + L) (Cell signaling, Alexa flour 488 conjugate, #4412) at 4 °C for 30 min in the dark after being washed twice with FACS buffer. The labeled cells were examined by flow cytometry using Cytoflex (Beckman Coulter Life Sciences, Indianapolis, IN, United States) and CytExpert 2.0.0.152 software [22].

2.8. Western blotting

Cells treated with crude extract or E2 for 48 h, washed twice with cold PBS, and lysed in lysis buffer. After membranes incubated with primary antibodies (PD-L1, AhR, HSP90, STAT3, NF-KB (Cell signalling)) and secondary antibodies (Thermo Fisher Scientific), after that bands detected with using an Immobilon Western Chemiluminescent HRP Substrate Kit (Merck Millipore, Germany), and luminescence imaging. Band densities were evaluated with Multi-Gauge 3.0 and standardized to GAPDH or α-tubulin levels in each sample. The values are expressed in densitometric units that are arbitrary and relate to the signal intensities [36].

2.9. Reporter assay

4 ng of Renilla-luc (pRL-TK) plasmid and 100 ng/well of reporters (STAT, NF-kB) were transfected into HEK293T cells in a 24-well plate. After 24 h incubation, cells were treated with indicated concentration of crude extract, E2, E8 or DMSO for 24 h incubation at 37 °C in 5% CO2. Luciferase activity was measured and normalized against Renilla activity to control for differences in the transfection efficiency [34].

2.10. String

The Search Tool database for Retrieval of Interactive Genes (http://string-db.org) was employed to forecast probable protein-protein interactions between gene candidates.

2.11. Correlation analysis and survival analysis

GEPIA (Gene Expression Profiling Interactive Analysis, RRID:SCR_018294, http://gepia.cancer-pku.cn/) was extracted to estimate the differential expression level between targest genes in LUAD (lung adenocarcinoma) patients [37]. The Pearson Coefficient was used to analyze the expression correlation between the two genes. The log2 of the transcript per million is known as log2(TPM). Hazard ratio (HR) and survival curve for the indicated gene and ESCC cohort were determined by log-rank test and Kaplan-Meier method on LUAD. The p value < 0.05 was noted statistically significant.

2.12. RT^2 profiler™ PCR array

The expression of transcriptional factor genes in sub-fraction E2-treated cells were evaluated using the Human Transcriptional Factors RT^2 Profiler™ PCR Array (Qiagen, SA Biosciences, Valencia, CA, USA). We measured the expression of 84 important genes in accordance with the manufacturer's instructions. Based on the RT-PCR cycle threshold values, fold changes in mRNA expression were

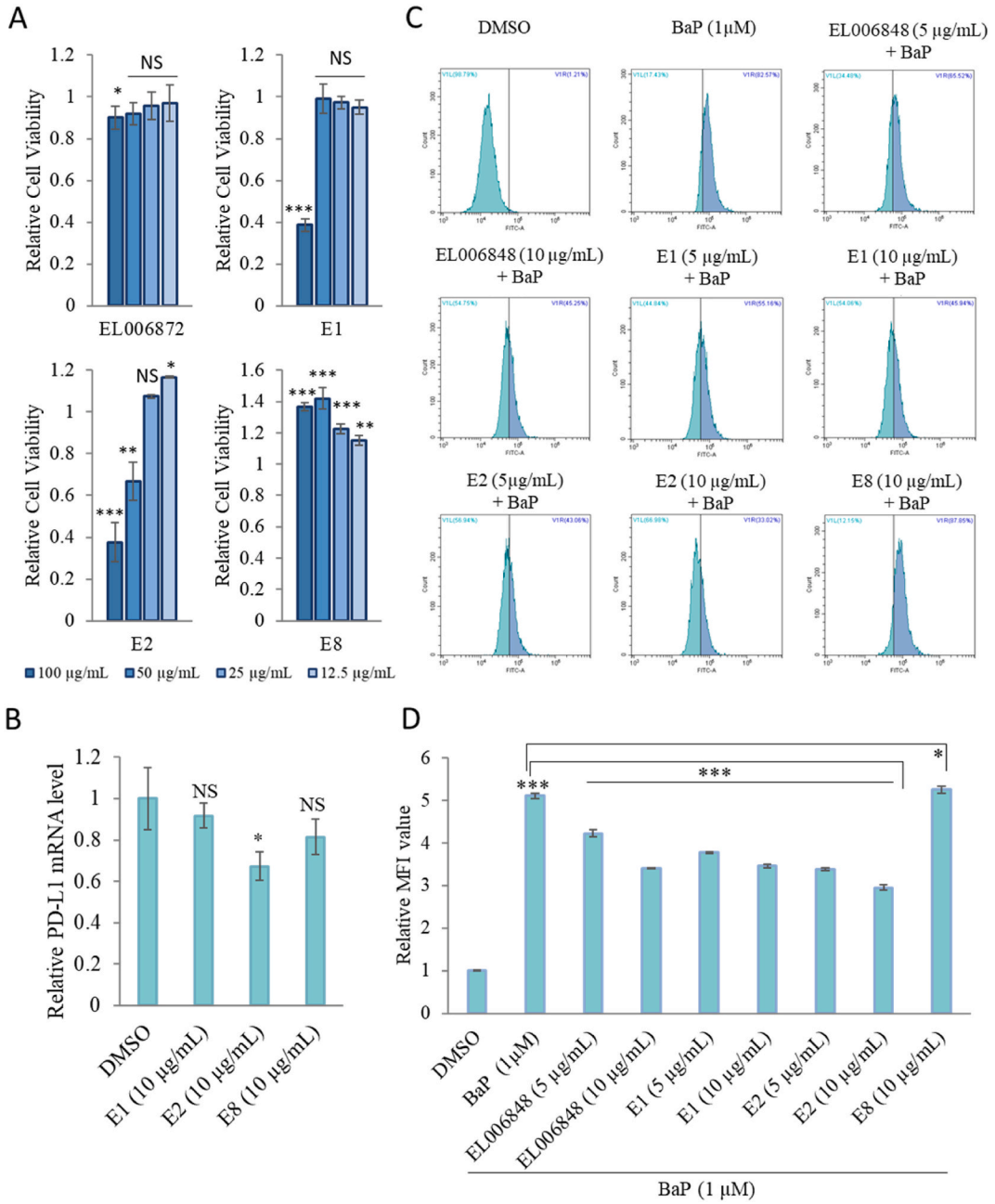


Fig. 2. Ability of the EL006848 crude extract and fractions E1 and E2 to inhibit PD-L1. (A) Effect of the EL006848 crude extract and fractions E1, E2 and E8 on the viability of H1975 cells. Dimethyl sulfoxide was used as a control. Data are presented as the mean ± S.D. (standard deviation); *p < 0.05; **p < 0.01; ***p < 0.001; NS, no significant compared with DMSO. (B) H1975 cells were treated for 48 h with E1, E2, or E8 (10 µg/mL) and expression of PD-L1 mRNA was measured by qRT-PCR. Data represent the mean ± S.D. (standard deviation): *p < 0.05; **p < 0.01; ***p < 0.001 (compared with DMSO-treated H1975 cells). (C, D) Effect of the EL006848 extract, and fractions E1, E2, and E8 on surface expression of PD-L1 by H1975 cells exposed to BaP (1 µM) for 48 h. Treatments (crude extract, E1, E2 and E8) were administered 4 h before BaP (1 µM) exposure. MFI: mean fluorescence intensity. Data are presented as the mean ± S.D. (standard deviation); n = 3. *p < 0.05; **p < 0.01; ***p < 0.001; NS, no significant difference compared with the BaP-treated group or between the indicated groups.

calculated. The validity of the experiment was demonstrated by a heat map and scatter plot of the test versus control samples [38].

2.13. Statistical analysis

Means and standard deviation (SD) are used to express data. Version 12.5 of Sigma Plot was used to conduct all statistical analyses. To examine the statistical significance between two groups, the Student's t-test was employed.

3. Results

3.1. The crude extract and subfractions E1 and E2 of Phoma sp. EL006848 suppress expression of PD-L1

The viability of the H1975 cell line was tested in the presence of the total EtOAc extract and its subfractions to determine the concentrations to be used for further experiments. We found that the crude extract and the subfractions were non-toxic at a concentration of 12.5 μg/mL (Fig. 2A). Next, we examined the effects of fractions E1, E2, and E8 on expression of PD-L1 mRNA. E2 downregulated expression of PD-L1 mRNA (Fig. 2B). To further validate the results, we checked the ability of the crude extract, and the E1, E2, and E8 (at concentrations of 5 and 10 μg/mL) to inhibit expression of benzo[*a*]pyrene-induced PD-L1 surface protein. The results confirmed that crude extract and fractions E1 and E2 inhibited expression of PD-L1 surface protein, with E2 showing the greatest inhibition. Fraction E8 had no inhibitory effect (Fig. 2C and D). Thus, fraction E2 contained the highest concentration of the major bioactive components.

3.2. The active fraction E2 contains a mixture of fatty acids

For chemical investigation, fractions E1 and E2 were subjected to NMR spectroscopic analysis. Because the ^{1}H NMR spectra of E1 and E2 suggested that both fractions contained fatty acids (Fig. 3C and D), we conducted additional GC-MS analysis after derivatization of fatty acids to methyl esters [19,33]. The GC-MS results for E1 and E2 were qualitatively similar, despite differences in the relative quantities between peaks (Fig. 3B). Three major chromatographic peaks were identified as methyl esters of palmitic acid (**1**, C16:0),

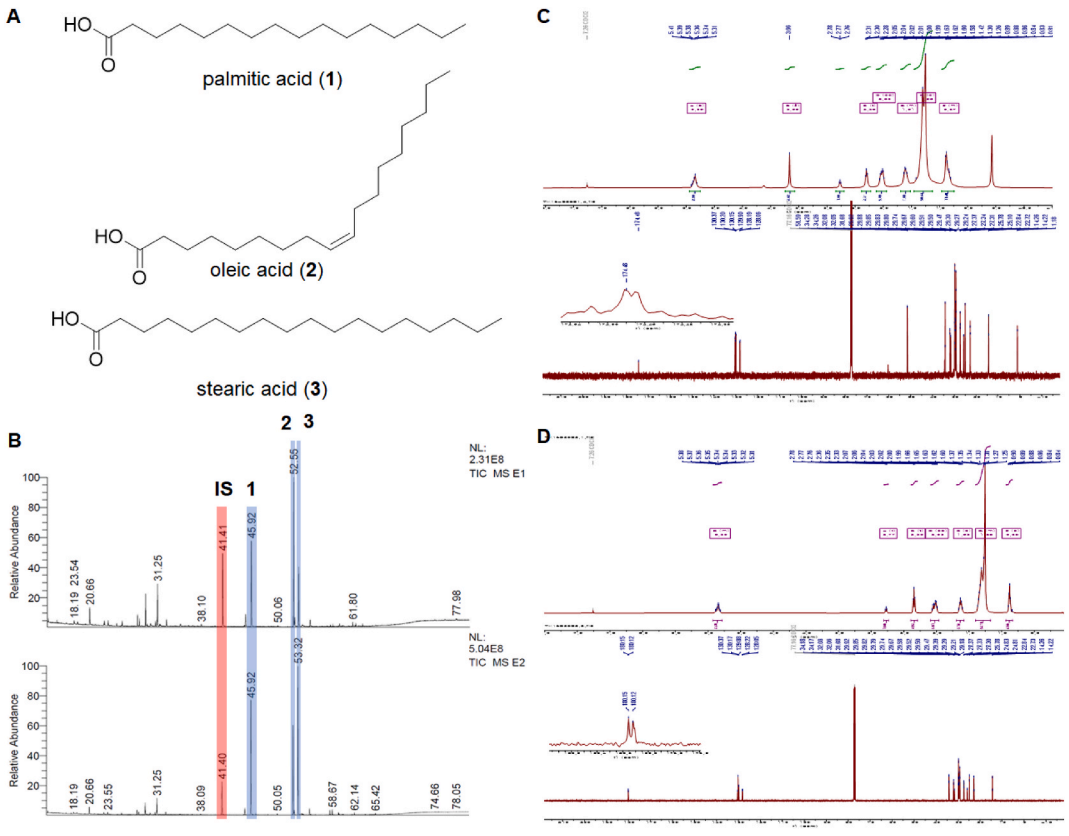


Fig. 3. Chemical investigation of fractions E1 and E2. (A) Chemical structures of the identified fatty acids. (B) GC-MS total ion current (TIC) chromatograms of the methyl ester-derivatized E1 and E2. The chromatographic peaks of the methyl esters of **1**–**3**, and the internal standard (IS), are marked. (C) The ^{1}H and ^{13}C NMR spectra of fraction E1 (in $CDCl_3$). (D) The ^{1}H and ^{13}C NMR spectra of fraction E2 (in $CDCl_3$).

oleic acid (**2**, C18:1, *cis*-9), and stearic acid (**3**, C18:0) by spectral matching against the NIST 11 library, followed by confirmation using reference standards (Fig. 3A). However, the difference in the TLC R_f values for **1** and **2** led us to suppose that these two fractions possess qualitatively different chemical compositions. The ^{13}C NMR spectrum of E1 showed carbonyl carbon signals at δ_C 174.5 ppm, while that of E2 showed resonances at δ_C 180.1 ppm (Fig. 3C and D), suggesting that fraction E1 was a mixture of methyl esters of **1**, **2**, and **3**, while E2 comprises intact **1**, **2**, and **3**. The presence of a strong carbon signal at δ_C 51.6 ppm in the ^{13}C NMR spectrum of E1 supported

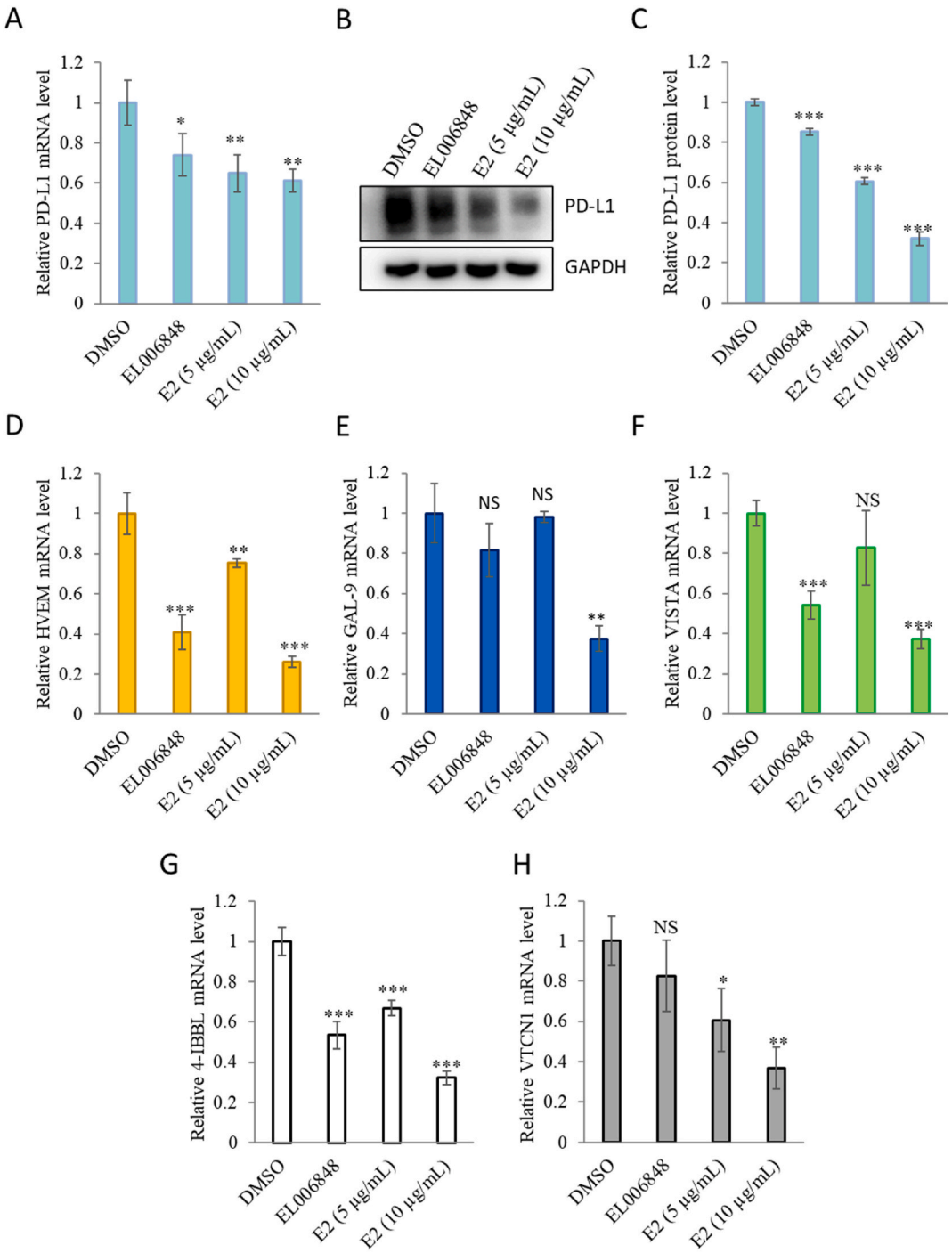
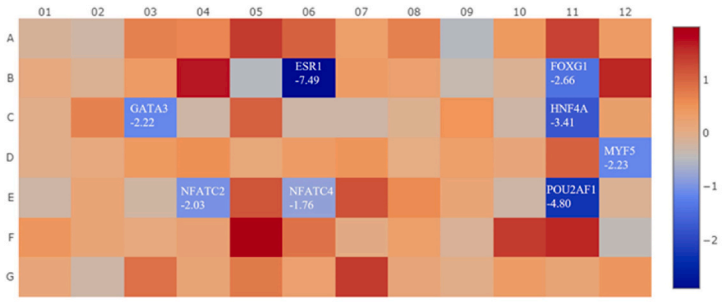
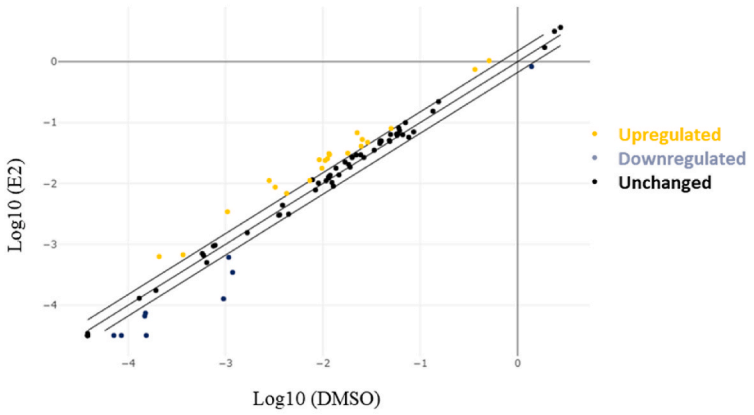


Fig. 4. Fractions of EL006848 containing fatty acid components downregulate expression of multiple immune checkpoint molecules. H1975 cells were treated with crude extract (10 μg/mL) or E2 (5 μg/mL and 10 μg/mL) for 48 h, and then analyzed by qRT-PCR or Western blotting. (A) Expression of PD-L1 mRNA, (B, C) total PD-L1 protein, and (D) HVEM, (E) Galectin-9, (F) VISTA, (G) 4-IIBL, and (H) VTCN1 mRNA. Data are presented as the mean ± SD, $n = 3$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS, no significant compared with the DMSO-treated group.

A



B



C

Symbol	Fold Change
AR	0.90
ARNT	0.85
CEBPG	0.70
E2F1	0.94
ELK1	0.70
ESR1	**0.13**
FOS	0.78
FOXA2	0.93
FOXG1	**0.38**
GATA3	**0.45**
GTF2B	0.84
HAND1	0.83
HAND2	0.83
HDAC1	0.95
HNF1A	0.83
HNF4A	**0.29**
MYF5	**0.45**
MYOD1	0.83
NFATC1	0.85
NFATC2	**0.49**
NFATC4	**0.57**
PAX6	0.83
POU2AF1	**0.21**
PPARA	0.93
SMAD9	0.91
STAT1	0.75
STAT3	0.82

D

Symbol	Fold Change
ATF3	2.68
ATF4	2.04
EGR1	3.28
CREBBP	2.55
NFATC3	2.26
TBP	2.69
SP1	2.68
SP3	3.03

E

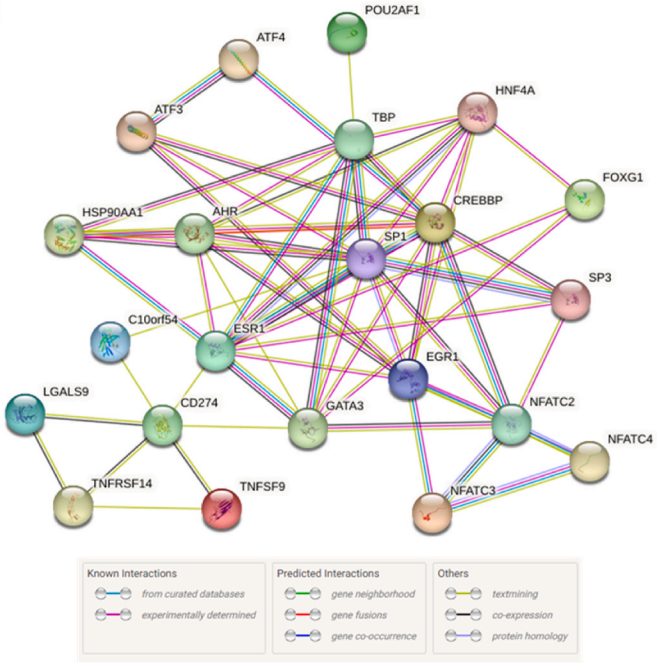


Fig. 5. E2 modulates expression of transcriptional regulators. H1975 cell line was treated with E2 or DMSO and then subjected to the RT2 profiling kit protocol. (A) Heat map of the Human Transcriptional Regulators RT^2 Profiler™ PCR Array. (B–D) Up and downregulated transcriptional regulators. (E) Genes network analysis by STRING.

this suggestion. Thus, we hypothesized that the mixture of **1**, **2**, and **3** was bioactive, whereas the methyl esters were much less potent.

3.3. E2 containing fatty acid components inhibits multiple immune checkpoint molecules

We used fraction E2, which is richer in fatty acid components than E1, for future experiments. First, we confirmed downregulation of PD-L1 mRNA by the crude extract and E2 (Fig. 4A). We then analyzed PD-L1 total protein levels from H1975 cell protein lysate by western blotting after cells exposure to the crude extract (10 μg/mL) and E2 (5 μg/mL and 10 μg/mL). The crude extract and E2 downregulated PD-L1 protein level (Fig. 4B and C). The crude extract inhibited expression of HVEM, VISTA, and 4-1BBL mRNA, while Galectin-9 and VTCN1 had no inhibitory effect. Fraction E2 downregulated expression of HVEM, VISTA, 4-1BBL, Galectin-9, and VTCN1 mRNA in a dose-dependent manner. Taken together, these results suggest that the crude extract and fractions containing fatty acid components have potential inhibitory effects on multiple immune checkpoints (Fig. 4D–H).

3.4. E2 regulates expression of ESR1-related transcriptional regulators

To identify the mechanisms regulated by E2, we performed pathway-oriented gene expression analysis using the Human Transcriptional Regulators RT^2 Profiler™ PCR Array. Fig. 5A and B shows up- and downregulated transcription-regulatory genes in the presence of 10 μg/mL E2 (compared with the control group). Fig. 5C shows a large list of downregulated genes, with ESR1, FOXG1, GATA3, HNF4A, MYF5, NFATC2, NFATC4, and POU2AF1 being downregulated (fold change <0.60). Transcriptional regulators ATF3, ATF4, EGR1, CREBBP, NFATC3, TBP, SP1, and SP2 were upregulated (Fig. 5D). Based on the results of the Human Transcription Regulators RT^2 Profiler™ PCR Array scan, we screened for any association between ICPs, AhR, and HSP90, and genes that were upregulated or downregulated. The results revealed a network of interrelated genes affected by E2 (Fig. 5E). Since ESR1 was the best

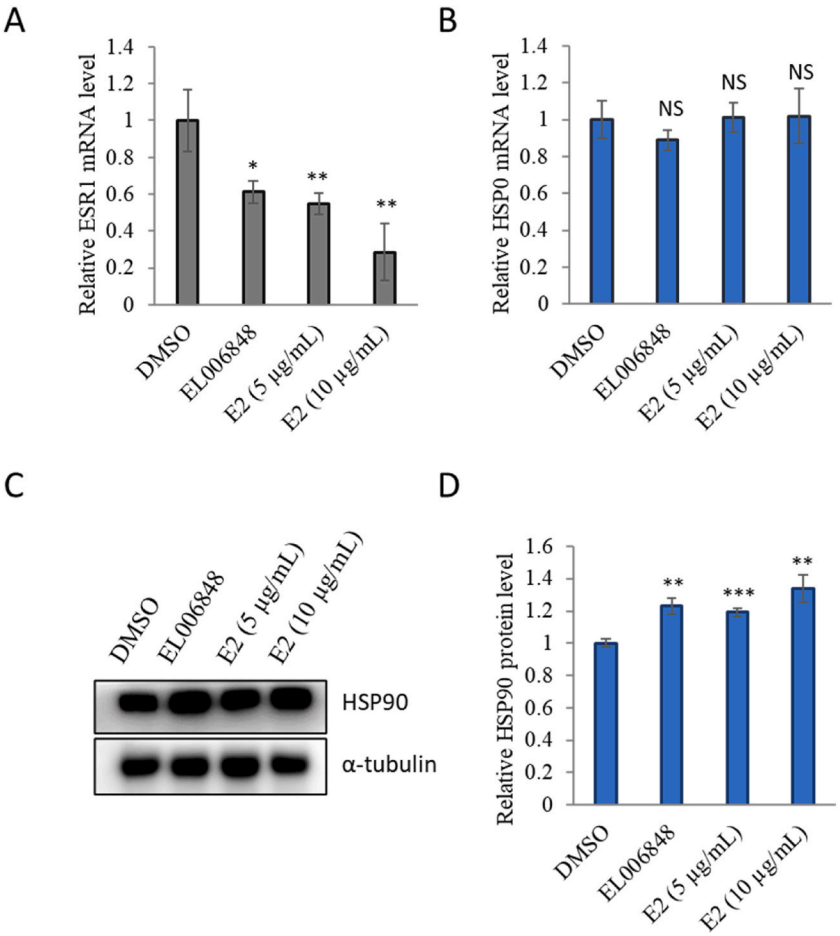


Fig. 6. E2 modulates expression of ESR1 and HSP90. H1975 cells were treated with the crude extract (10 μg/mL) and two concentrations of E2 (5 and 10 μg/mL) for 48 h, followed by qRT-PCR or Western blotting. (A) ESR1 mRNA expression levels. (B) HSP90 mRNA expression levels. (C, D) HSP90 protein levels. Data are presented as the mean ± SD, $n = 3$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS, no significant compared with the DMSO-treated group.

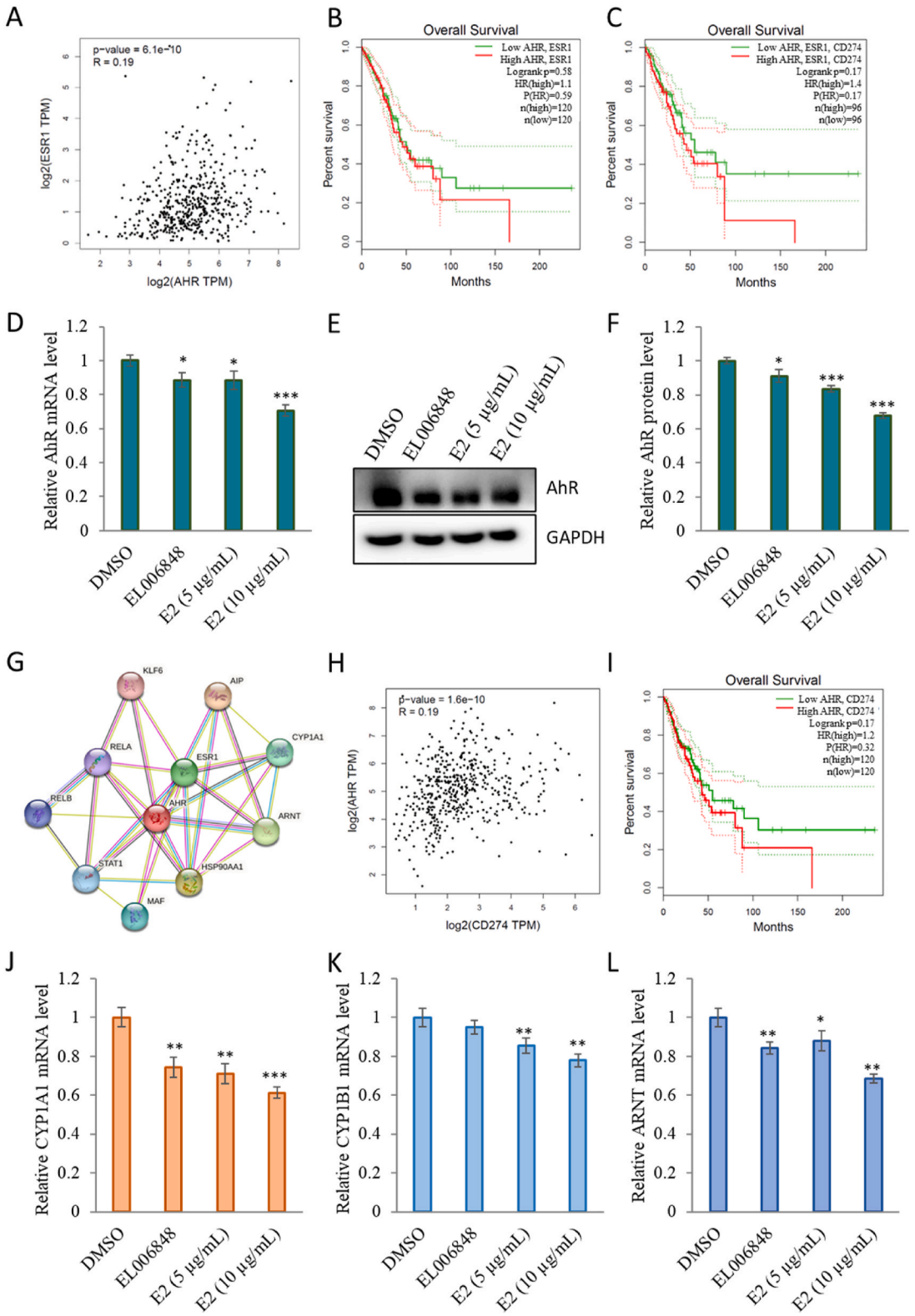


Fig. 7. Expression of PD-L1 is regulated via AhR and ESR1. (A) The GEPIA web tool was used to analyze the relationship between expression of ESR1 and that of AhR mRNA in lung cancer samples. (B, C) Overall survival of LUAD patients with cancers showing expression of ESR1-AhR or ESR1-AhR-CD274 according to the GEPIA database. (D–F) AhR mRNA and protein levels were downregulated by the crude extract and both concentrations of E2 (5 and 10 μg/mL) after exposure for 48 h. Data are presented as the mean ± SD, $n = 3$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$,

compared with the DMSO-treated group. (G) AhR network analysis by STRING. (H) The GEPIA web tool was used to analyze the relationship between expression of AhR and that of CD274 mRNA in lung cancer samples. (I) Overall survival curve of LUAD patients expressing AhR-CD274 according to the GEPIA database. (J–L) Expressions of CYP1A1, CYP1B1, and ARNT mRNA were suppressed by the crude extract and both concentrations of E2 (5 and 10 μg/mL) after treatment for 48 h. Data are presented as the mean ± SD, $n = 3$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared with the DMSO-treated group.

scoring transcriptional regulator that was downregulated, we examined expression of ESR1 mRNA by qRT-PCR after exposure to the crude extract and E2 (at 5 and 10 μg/mL) in H1975 cell line (Fig. 6A). HSP90 is a chaperone protein that helps to stabilize and fold many proteins within cells; indeed, HSP90 works with oncogenic proteins in cancer cells [39,40]. Indeed, overexpression of ESR1 tumor tissues are highly dependent on HSP90 [41], also a complex of HSP90 and AhR [42]. A previous report shows that the HSP90 inhibitors 17-AAG and geldanamycin increase protein level of HSP90 in A549 cells [39]. Our own results showed that the crude extract and E2 upregulated expression of HSP90 mRNA and protein (Fig. 6B–D). Taken together, the data suggest that E2 regulates expression of several genes related to ESR1.

3.5. AhR acts in concert with ESR1, and the fatty acid component E2 downregulates the AhR pathway

The GEPIA web analysis tool showed that expression of ESR1 correlates with that of AhR in lung cancer (Fig. 7A). Therefore, we investigated the effects of co-overexpression and co-underexpression of ESR1-AhR and ESR1-AHR-CD274 (PD-L1) on overall survival mediated by GEPIA. High expression of both ESR1 and AhR in lung cancer is associated with poor survival (Fig. 7B). Concomitant high expression of the ESR1-AhR-CD274 genes also results in poor survival (Fig. 7C). The endolichenic fungus crude extract and its fatty acid component E2 significantly downregulated expression of AhR mRNA and protein (Fig. 7D–F). Next, we extracted the AhR linkage gene map using the STRING web tool to further characterize the AhR-mediated effects of E2 (Fig. 6G). Expression of AhR correlates with that of PD-L1 in lung cancer; high expression of both is associated with lower survival, independent of ESR1 (Fig. 7H and I). The crude extract and the fatty acid-containing E2 fraction suppressed AhR-regulated expression of CYP1A1, CYP1B1, and ARNT mRNA (Fig. 7J–L). These results suggest that the ESR1 and AhR pathways are downregulated by the crude extract and E2, and that downregulation of PD-L1 is mediated by ESR1 and AhR.

3.6. Inhibitory effects of E2 on NF-κB and STAT3

Finally, we performed a reporter assay, qRT-PCR, and Western blot analyses to determine whether the effects of the crude extract and E2 on AhR non-canonical pathways, and their inflammatory effects, were due to inhibition of STAT3 and NF-κB. E2 significantly downregulated STAT reporter activity and the mRNA and protein levels of STAT3. The crude extract downregulated STAT-luc activity, but failed to downregulate its mRNA and protein significantly (Fig. 8A–B, E-F). However, the crude extract downregulated NF-kB-luc activity at a concentration of 10 μg/mL, although it failed to inhibit NF-kB mRNA expression and protein level. E2 downregulated NF-kB-luc activity, and NF-kB mRNA and protein levels, significantly at 10 μg/mL concentrations (Fig. 8C–D, E, G). These results show that E2 fatty acids inhibit inflammation-related markers, as well as the non-canonical AhR pathway, STAT3, and NF-kB (Fig. 8C–E, G).

4. Discussion

Inhibition of immune checkpoints provides a promising way to prevent cancer progression [43]. PD-L1 expressed by cancer cells causes cancer progression by binding to programmed death-1 (PD-1), thereby facilitating immune escape. Therefore, blocking PD-1/PD-L1 blockade is an important target [44]. The mechanisms underlying modulation of immune checkpoints are unclear. Recently, AP-1, IRF-1, MYC, STAT3, NF-kB, and AhR were identified as regulators of PD-L1 [11,45].

The findings of the current study are as follows: (1) endolichenic fungus extract and its fatty acid component-containing subfractions downregulated expression of PD-L1 mRNA and total protein levels at concentrations that were non-toxic in H1975 cell line. The crude extract and subfractions E1 and E2 suppressed expression of PD-L1 surface protein (induced by AhR ligand (BaP)); (2) the crude extract and fraction E2 suppressed multiple immune checkpoint molecules (HVEM, VISTA, 4-1BBL, Galectin-9, and VTCN1); (3) exposure to E2 led to downregulation of ESR1, FOXG1, GATA3, HNF4A, MYF5, NFATC2, NFATC4, and POU2AF1, and upregulation of ATF3, ATF4, EGR1, CREBBP, NFATC3, TBP, SP1, and SP2; (4) the crude extract and E2 suppressed the canonical AhR pathway; (5) E2 suppressed expression of STAT3 and NF-kB, which are associated with inflammation, and function in the non-canonical AhR pathway; and (6) E2 contains mainly palmitic acid, stearic acid, and oleic acid. Collectively, the data suggest the therapeutic potential of the fatty acid components contained within endolichenic fungi, EL006848 (Fig. 9).

Previous studies show that fatty acids suppress cancer progression [46,47]. The extract obtained from the fungus strain used in the present study contains oleic, palmitic, and stearic acids. A previously study showed that oleic acid suppresses interferon gamma-induced expression of PD-L1 by human lung carcinoma cells [43]. Another reported that oleic acid regulates inflammation, as well as various immunomodulators, through Th2/GATA-3 and interleukin-17/RORγt [48]. Inhibition of PD-L1, EGFR, and tyrosine kinases by palmitic acid has been reported [49]. Although fatty acids are commonly used in nano-emulsions, we showed that a derivative of palmitic acid improved T cell immune responses by inhibiting PD-L1 [50]. A previous study also showed that stearic acid, another major fatty acid, forms a nanocomplex with ibrutinib, leading to immunotherapeutic effects [51]. The results of our study are consistent with these, moreover we also show the potential anticancer effects of multiple immune checkpoints with a fatty acid-rich

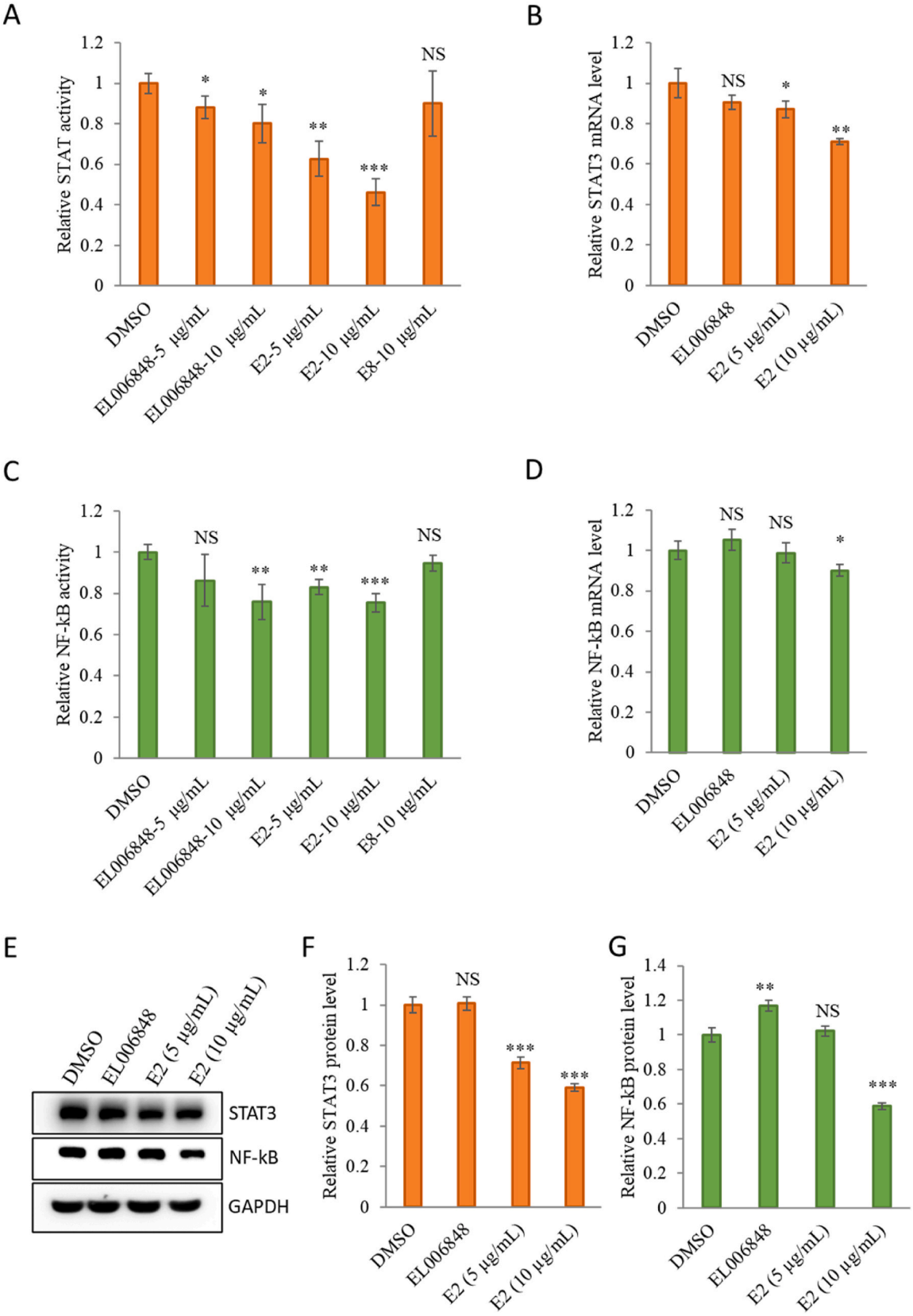


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Fig. 8. Inhibitory effects of E2 on STAT3 and NF-κB. (A and C) HEK293T cells were transfected with NF-κB or STAT3 luciferase reporter constructs and then treated with crude extract or E2 (5 and 10 μg/mL) for 24 h. E8 was used as the control. Luciferase activity was determined in a Dual-Luciferase reporter assay system and normalized against Renilla luciferase activity. (B and D) qRT-PCR analysis of STAT3 and NF-kB mRNA expression. H1975 cells were treated for 48 h with crude extract or E2 (5 and 10 μg/mL). (E–G) Immunoblot analyses (with the indicated antibodies) of H1975 cells treated for 48 h with crude extract or E2 (5 and 10 μg/mL). GAPDH was used as a loading control. Data are presented as the mean ± SD, $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS, no significant compared with the DMSO-treated group.

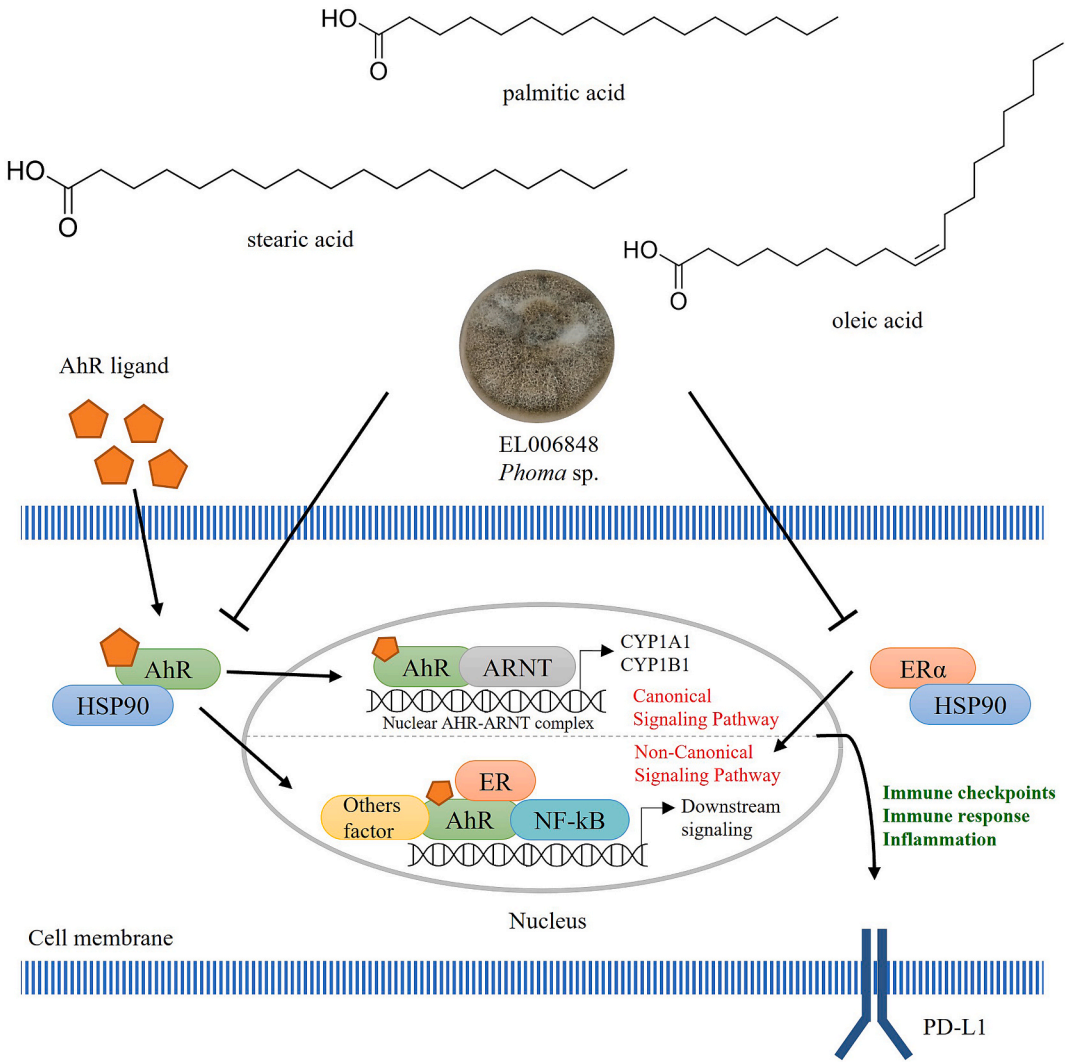

Fig. 9. Schematic overview of the immune checkpoint and transcriptional regulators inhibited by fatty acid component from EL006848, *Phoma* sp. Endolichenic fungi produce mainly oleic, palmitic, and stearic acids. The crude extract and its subfraction (E2) downregulate transcriptional regulators ESR1, AhR, ARNT, STAT3, and NF-kB. Downregulation of these transcription factors in the cell nucleus is thought to be responsible for downregulation of immune checkpoint molecules. Fatty acid components in the crude extract and subfractions inhibit expression of PD-L1.

fraction.

The estrogen receptor 1 (ESR1) and its genetic variations are associated with increased cancer risk and metastasis [52]. PD-L1 expression in cancer cell lines is negatively regulated by the estrogen receptor [53]. Here, we show that E2 downregulated both ESR1 and PD-L1 levels in the lung cancer cell line H1975. Along with ESR1, E2 downregulated many transcriptional regulators associated with ESR1. Furthermore, the extract and E2 affected the levels of the HSP90 protein, which is part of the ESR1 gene network. HSP90 regulates many mechanisms, including immunomodulation in cancer cells, and works together with other cellular proteins [54].

The AhR resides in the cytoplasm, where it forms a complex with HSP90, AIP and p23. Binding of AhR ligands such as BaP and

TCCD results in nuclear translocation of AhR, where it forms a complex with the nuclear translocator, thereby regulating expression of CYP1A1 and CYP1B1 [55–57]. In addition, AhR regulates cellular processes by binding to NF-kB and other transcriptional factors in the nucleus [58,59]. To further characterize the effect of E2 on non-canonical pathways associated with AhR, we also examined NF-kB and STAT3, both of which are responsible for driving inflammation. AhR regulates immune-related pathways together with NF-kB or STAT3 [60,61]. Our results show that both the crude extract and fatty acid component E2 suppressed expression of the AhR and AhR-related genes.

We showed that the fraction rich in fatty acids suppressed the level of PD-L1 dependent on and independent of BaP-induced AhR. PD-L1 can be constitutively expressed in many tumors, and its expression can be maintained and regulated by multiple factors [7]. In addition, the prognostic role of PD-L1 is unclear. It has been interpreted that this may be due to the differences between different lung cancer cohorts [62]. Presence of PD-L1 marker in resected tumor tissue or biopsy samples taken by appropriate methods is important for the strategy of treatment [63]. How it is regulated in other immune checkpoint markers, including PD-L1, is still not clearly elucidated. Therefore, we analyzed the effect of E2 on 84 transcription regulators to investigate the factors underlying suppression of other immune checkpoints, including PD-L1. A summary of all the targets used in the study eventually formed the gene network, E2 observed to affect the expression of AhR/ARNT and ESR1-related genes. Due to the existence of links between different regulators of immune checkpoints and cellular responses, further research is needed to investigate how fatty acid components regulate immune checkpoints through their mechanism of action.

5. Conclusion

We investigated the therapeutic effects of crude extracts and subfractions obtained from an endolichenic fungi containing oleic acid, palmitic acid, and stearic acid components. The results showed that these fatty acid components regulate multiple immune checkpoints, including PD-L1, as well as transcriptional regulators, in a lung cancer cell line. Surface protein expression of PD-L1 induced by the AhR ligand BaP was suppressed by E2, suggesting that this mechanism is regulated via the AhR/ARNT axis. ESR1, STAT3, and NF-kB, both of which are involved in the non-canonical AhR pathway, were downregulated by E2. Future studies should include comprehensive assessments of the potential benefits and harms of using fatty acids as a treatment for cancer. Studies should examine the utility of fatty acids as adjuvants to established treatments to prevent cancer development and immune escape.

Declarations

Author contribution statement

Mücahit Varlı: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Men Thi Ngo: Performed the experiments; Analyzed and interpreted the data.

Seong-Min Kim, İsa Taş, Rui Zhou, Chathurika D. B. Gamage, Sultan Pulat, So-Yeon Park: Performed the experiments; Analyzed and interpreted the data.

Nüzhet Cenk Sesal, Jae-Seoun Hur: Contributed materials..

Kyo Bin Kang, Hangun Kim: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.

5.1. Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19185.

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