Supplementary information

Chemopreventive Effect of Arachidin-Rich Extract from Peanut Hairy Root Cultures on Inflammation-Induced Cholangiocarcinogenesis in a Cytokine-Treated Biliary Cell Model

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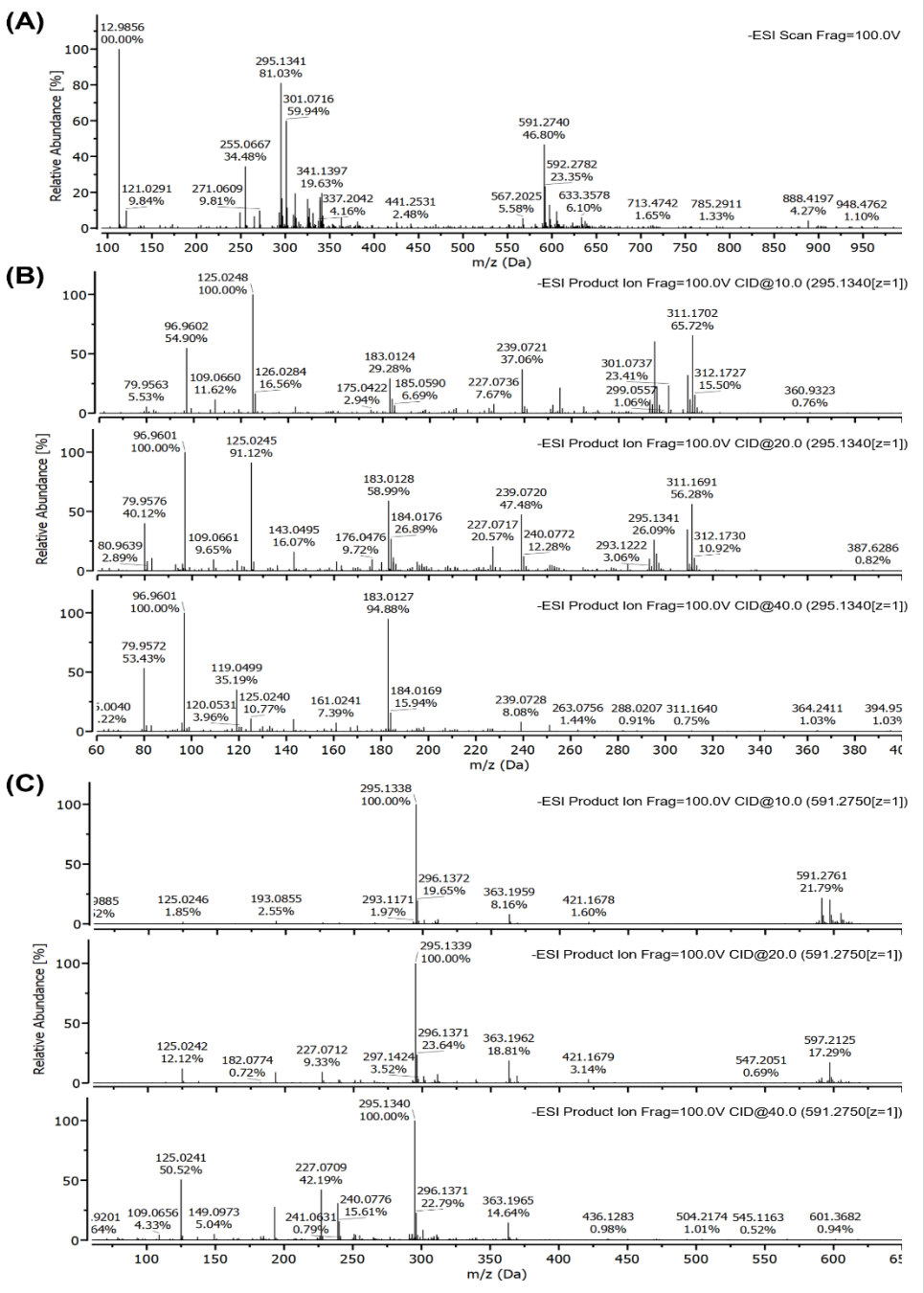
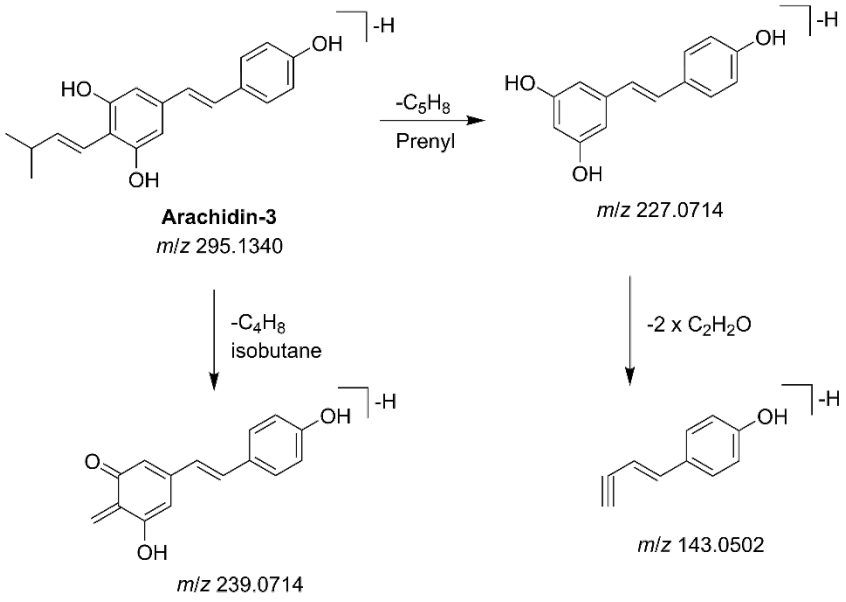
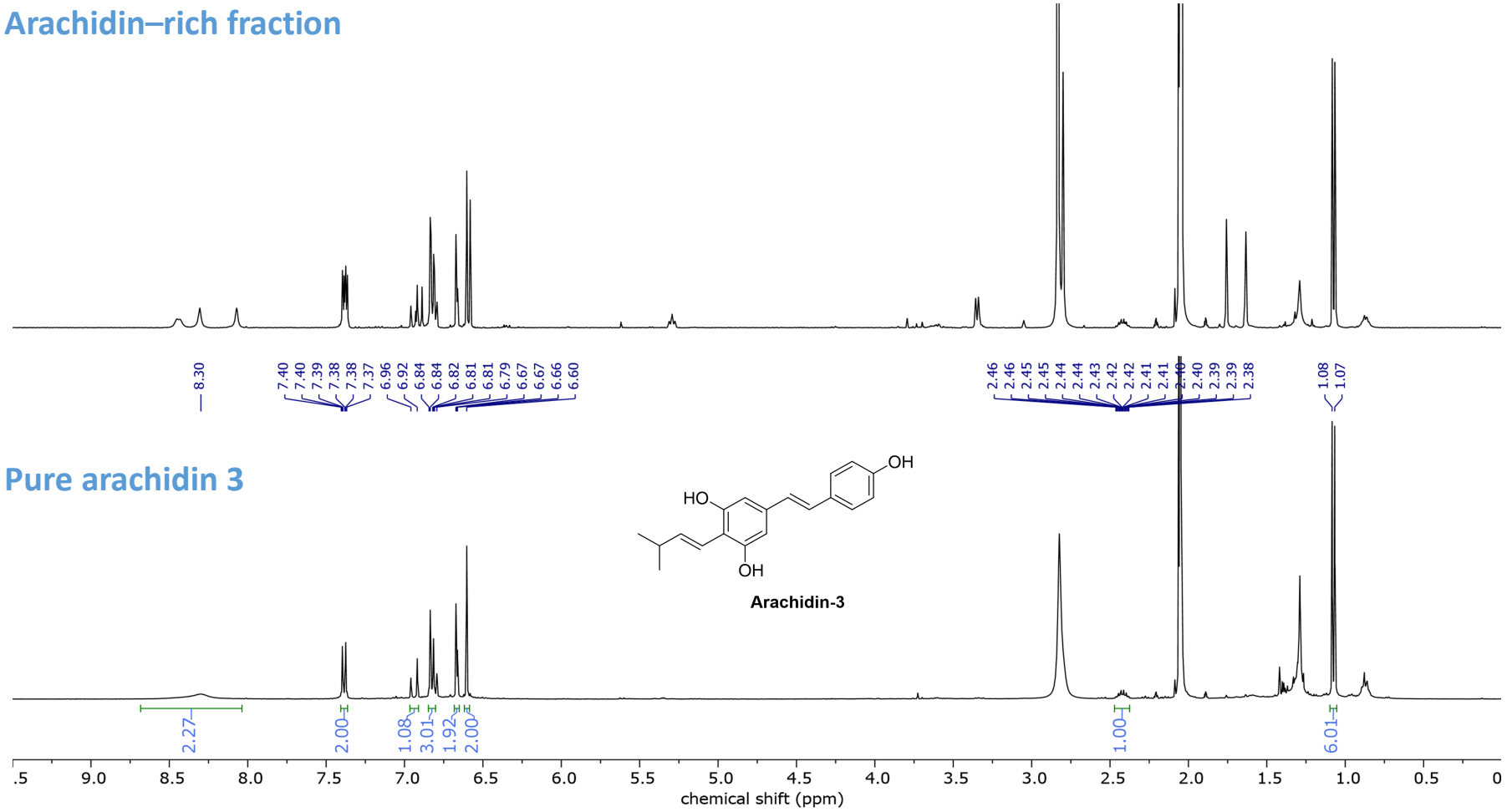


Supplementary Figure S1. Negative electrospray ionization (ESI-MS/MS) spectra of arachidin-3-rich fraction. (A) Full scan mass spectrum showing the deprotonated molecular ion $[M-H]^-$ at *m*/*z* 295.1341, corresponding to the molecular formula $C_{19}H_{19}O_3$ of arachidin-3 and the dimer ion $[2M-H]^-$ at *m*/*z* 591.2740. (B) Product ion (MS/MS) spectra of the precursor ion *m*/*z* 295.1340

obtained at collision-induced dissociation (CID) energies of 10, 20, and 40 eV. Increasing collision energy shifts the abundance from the precursor to characteristic fragment ions at *m*/*z* 183.01, 125.02, and 96.96. (C) Product ion spectra of the dimer precursor ion *m*/*z* 591.2750 at CID energies of 10, 20, and 40 eV. At lower energy (CID 10 eV), the dimer predominantly dissociates into the monomeric ion (*m*/*z* 295.13), while higher energies induce further fragmentation consistent with the pattern observed in (B).

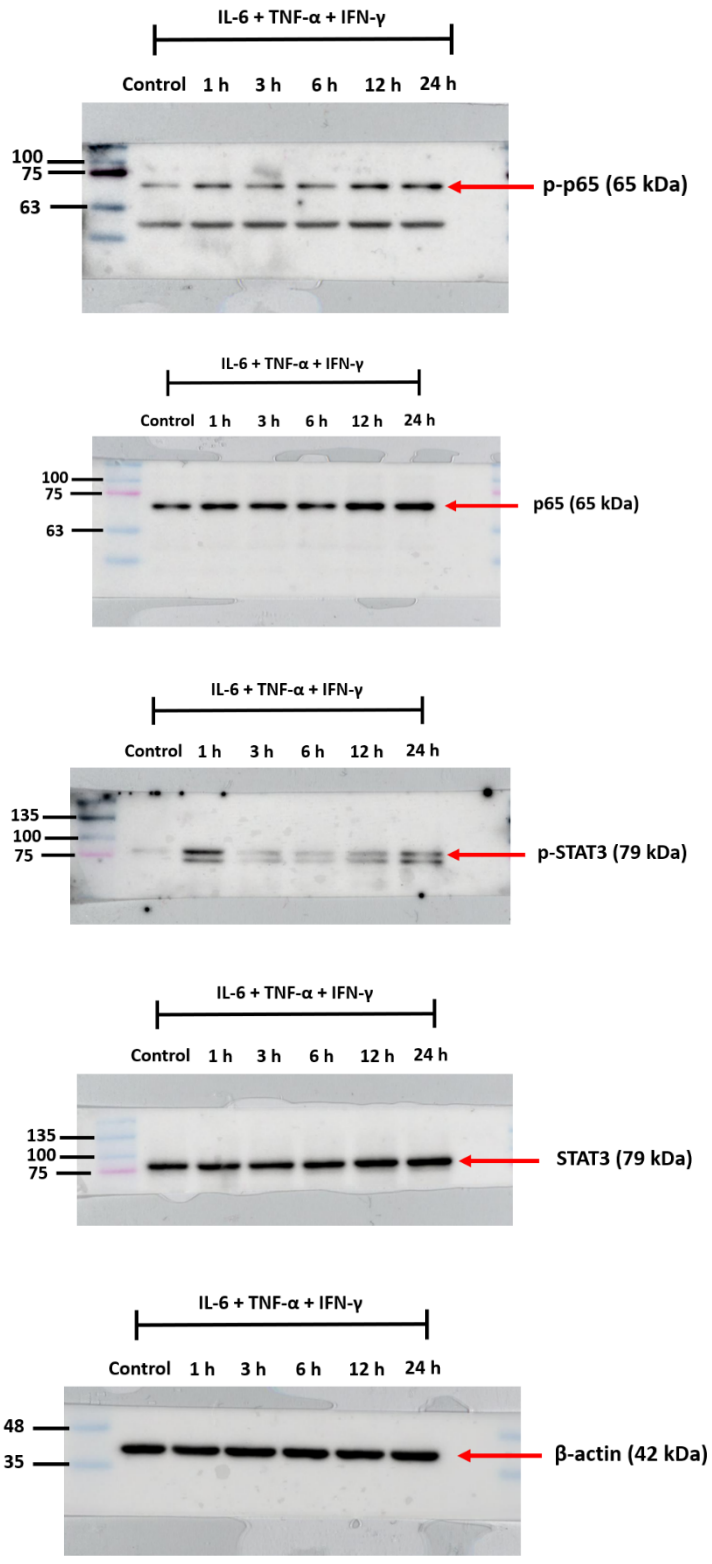


Supplementary Figure S2. Proposed mass spectrometry fragmentation pathway of arachidin-3 in negative electrospray ionization mode (ESI-MS/MS). The deprotonated precursor ion $[M-H]^-$ at *m*/*z* 295.1340 undergoes fragmentation *via* two distinct pathways. The primary pathway involves the neutral loss of a prenyl moiety to yield the resveratrol core ion at *m*/*z* 227.0714. This intermediate subsequently undergoes sequential fragmentation, losing two ketene units to form the product ion at *m*/*z* 143.0502. A competitive pathway involves the neutral loss of isobutene from the prenyl side chain to generate the fragment ion at *m*/*z* 239.0714.

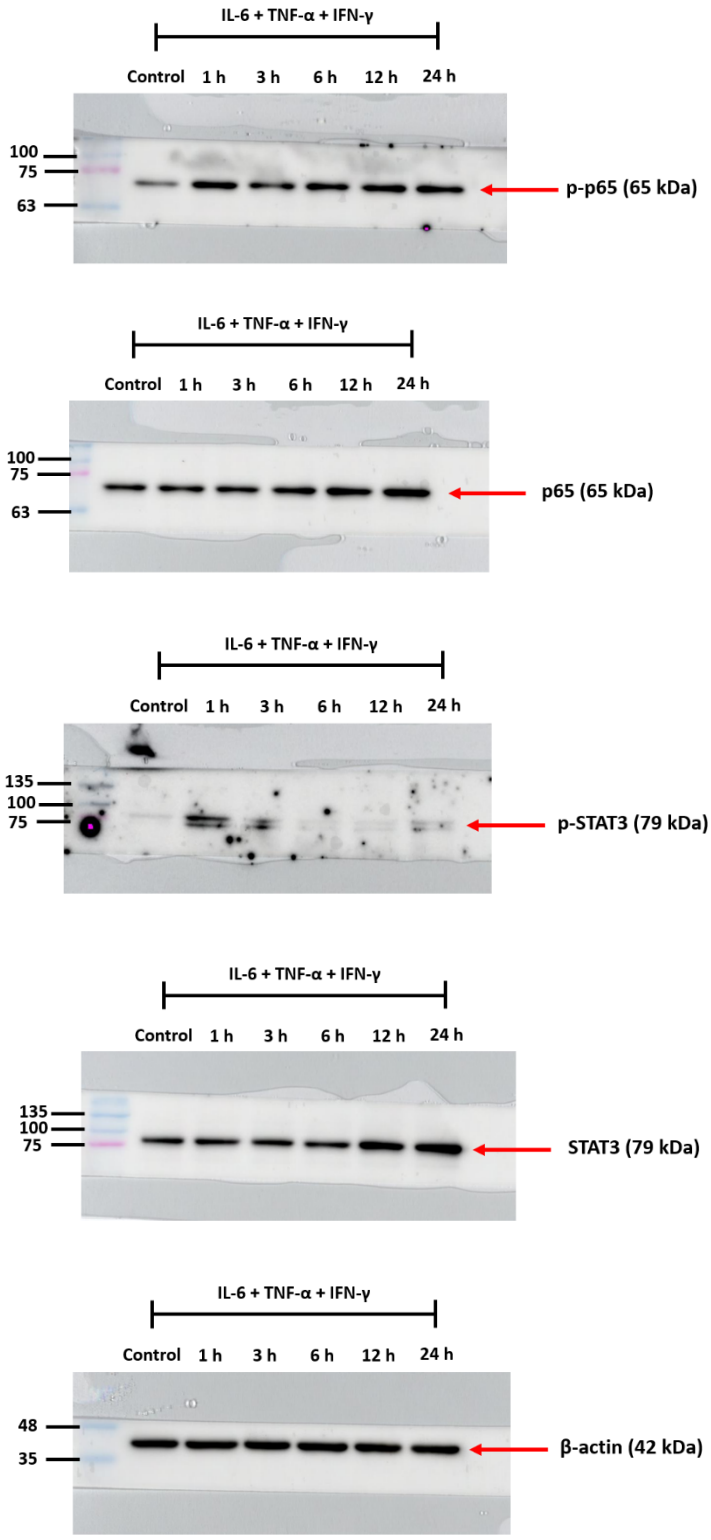


Supplementary Figure S3. Comparison of the ^{1}H NMR spectra (400 MHz, acetone-d_6) of an arachidin–rich fraction and pure arachidin-3.

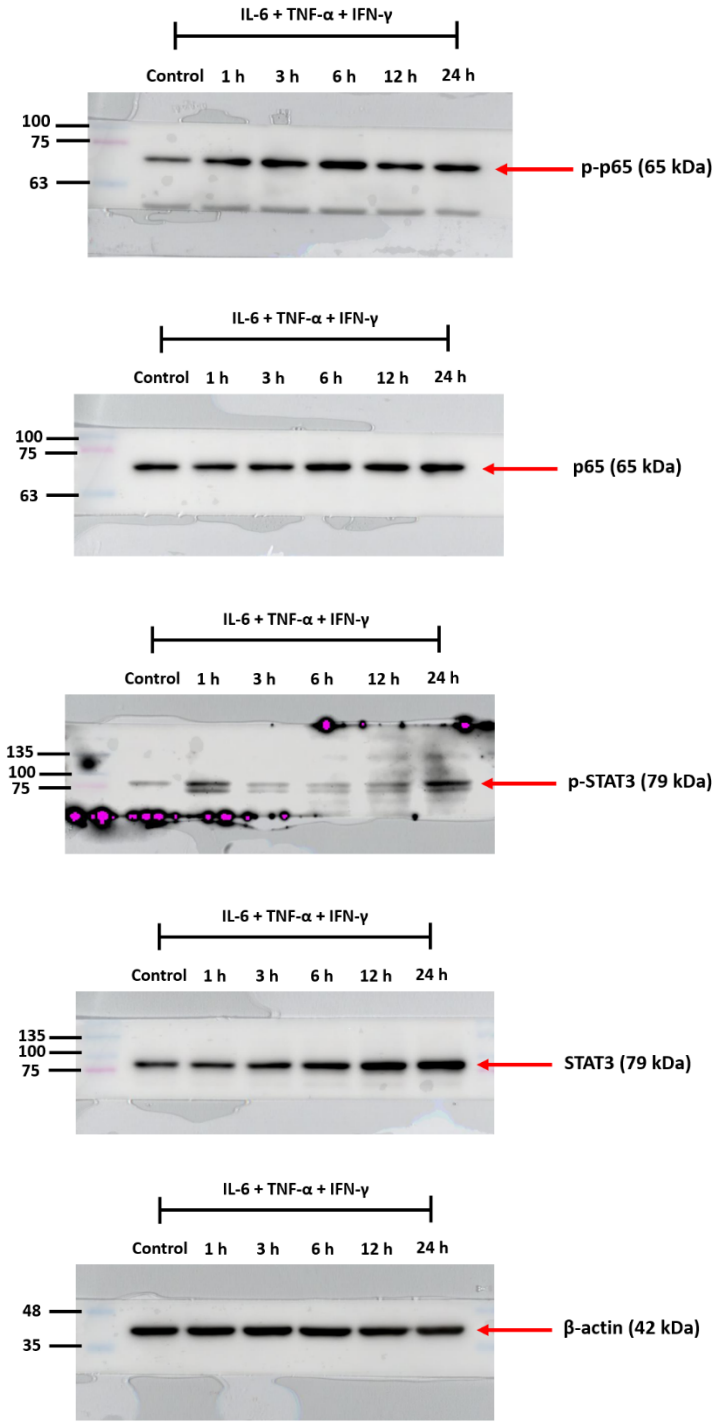
N1


IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
100
75
63
p-p65 (65 kDa)
IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
100
75
63
p65 (65 kDa)
IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
135
100
75
p-STAT3 (79 kDa)
IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
135
100
75
STAT3 (79 kDa)
IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
48
35
β-actin (42 kDa)

N2


IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
100
75
63
p-p65 (65 kDa)
IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
100
75
63
p65 (65 kDa)
IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
135
100
75
p-STAT3 (79 kDa)
IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
135
100
75
STAT3 (79 kDa)
IL-6 + TNF-α + IFN-γ
Control 1 h 3 h 6 h 12 h 24 h
48
35
β-actin (42 kDa)

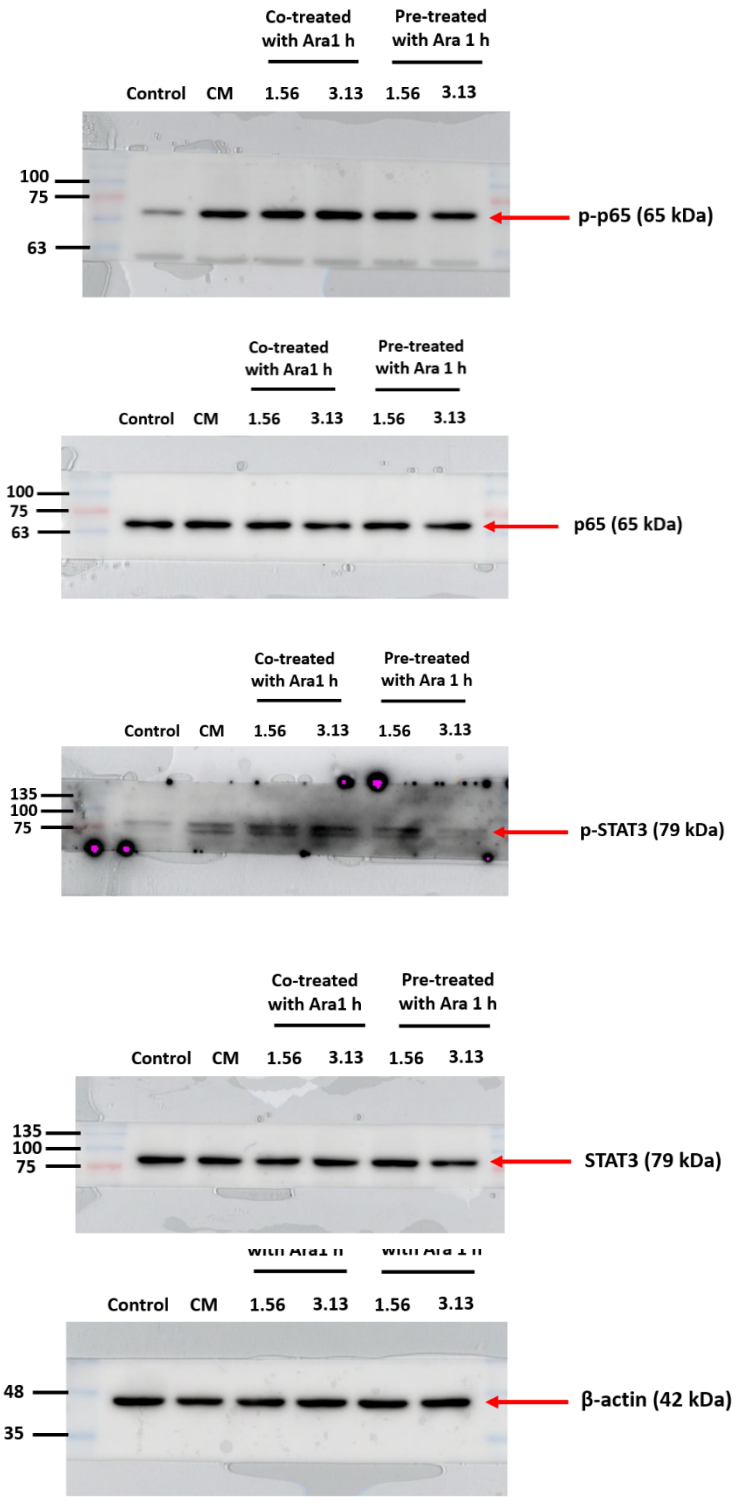
N3



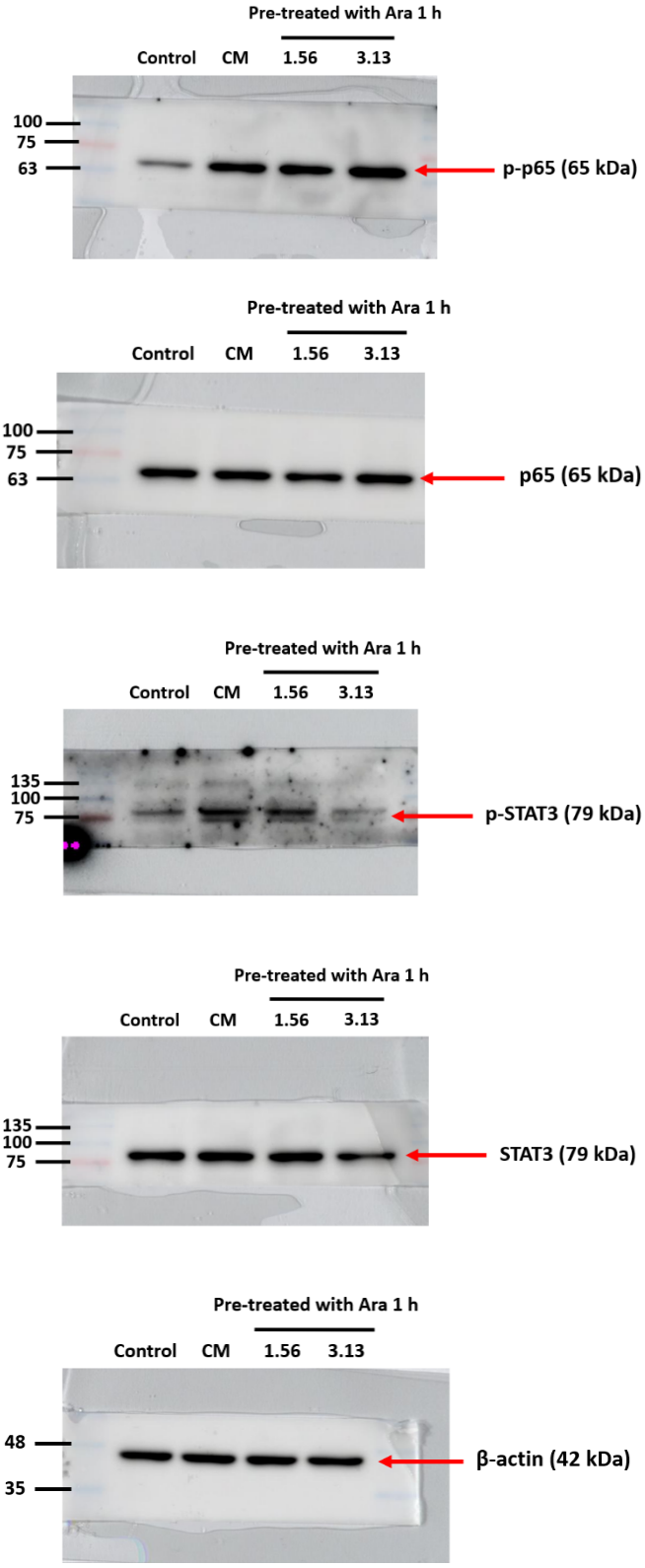
Supplementary Figure S4. *Full-length, unprocessed western blot images showing the effects of the cytokine mixture on the activation of p-p65 and p-STAT3 in MMNK-1 cells.* The blot images represent the expression levels of each protein analyzed by western blotting using ECL detection

reagents. Blot replicates N1, N2, and N3 are shown, with replicate N3 corresponding to the representative results presented in Figure 2A.

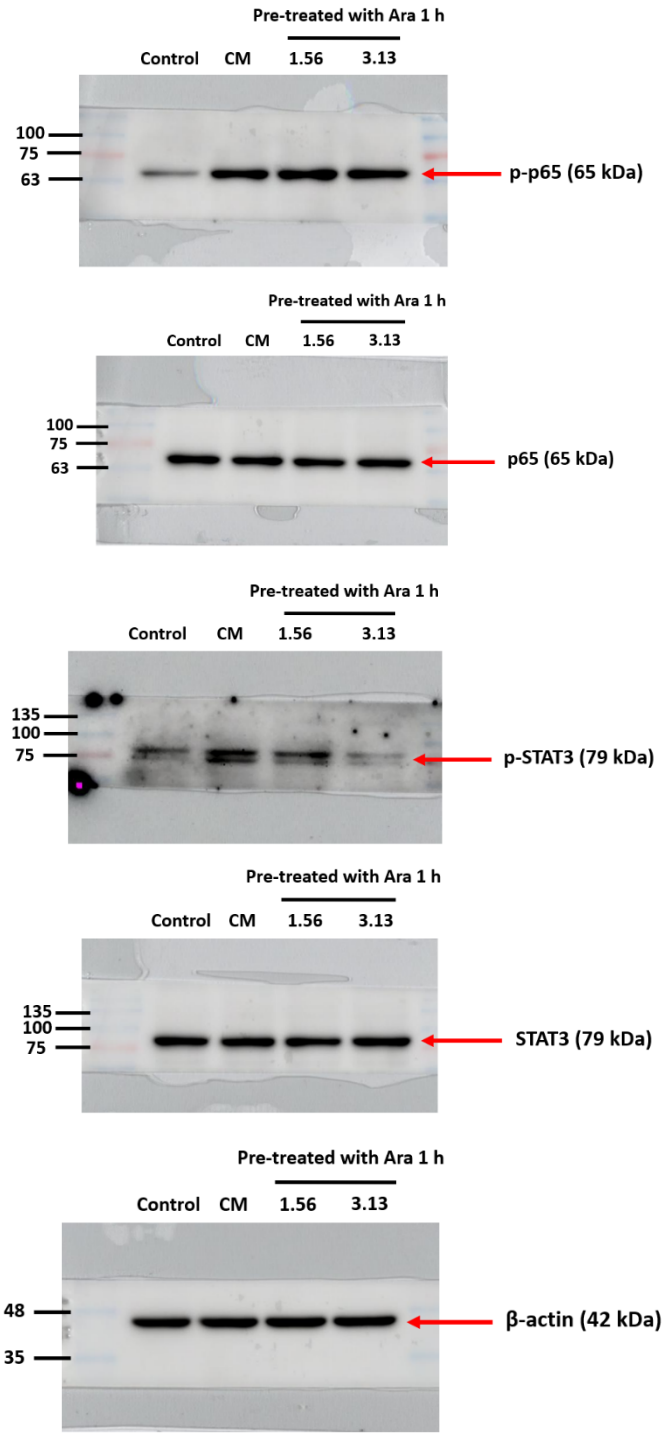
N1



N2

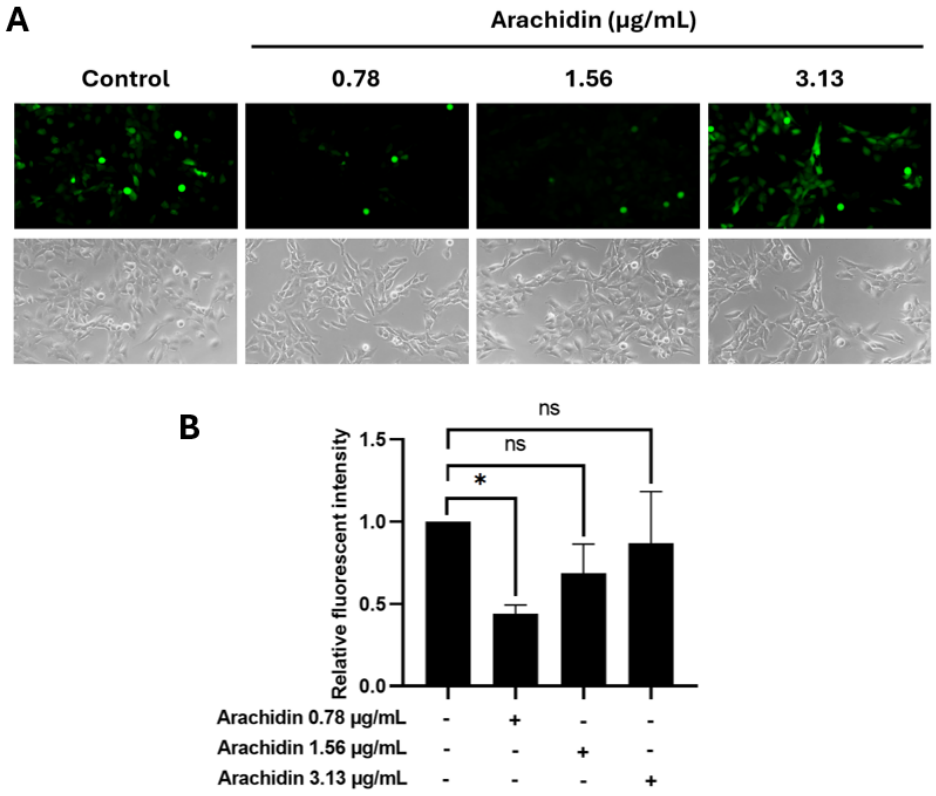

Pre-treated with Ara 1 h
Control
CM
1.56
3.13
100
75
63
p-p65 (65 kDa)
Pre-treated with Ara 1 h
Control
CM
1.56
3.13
100
75
63
p65 (65 kDa)
Pre-treated with Ara 1 h
Control
CM
1.56
3.13
135
100
75
p-STAT3 (79 kDa)
Pre-treated with Ara 1 h
Control
CM
1.56
3.13
135
100
75
STAT3 (79 kDa)
Pre-treated with Ara 1 h
Control
CM
1.56
3.13
48
35
β-actin (42 kDa)

N3

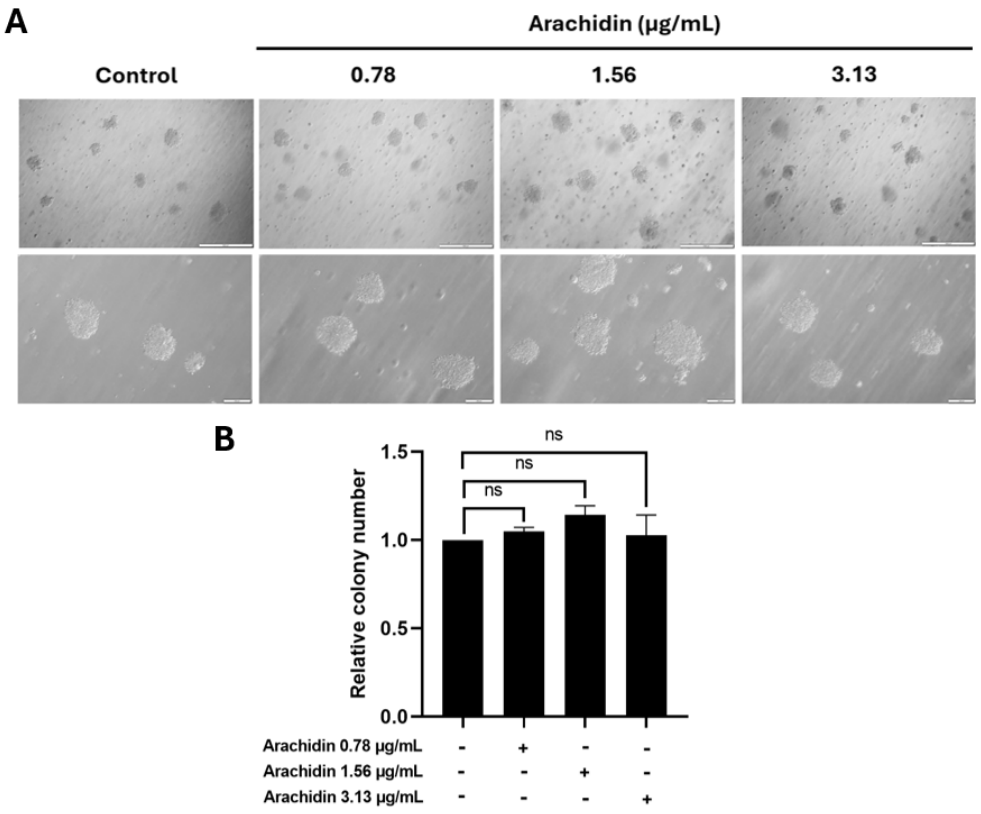


Supplementary Figure S5. *Full-length, unprocessed western blot images showing the effects of arachidin-rich extract on cytokine mixture-induced activation of p-p65 and p-STAT3 in MMNK-1 cells.* The blot images represent the expression levels of each protein analyzed by western blotting

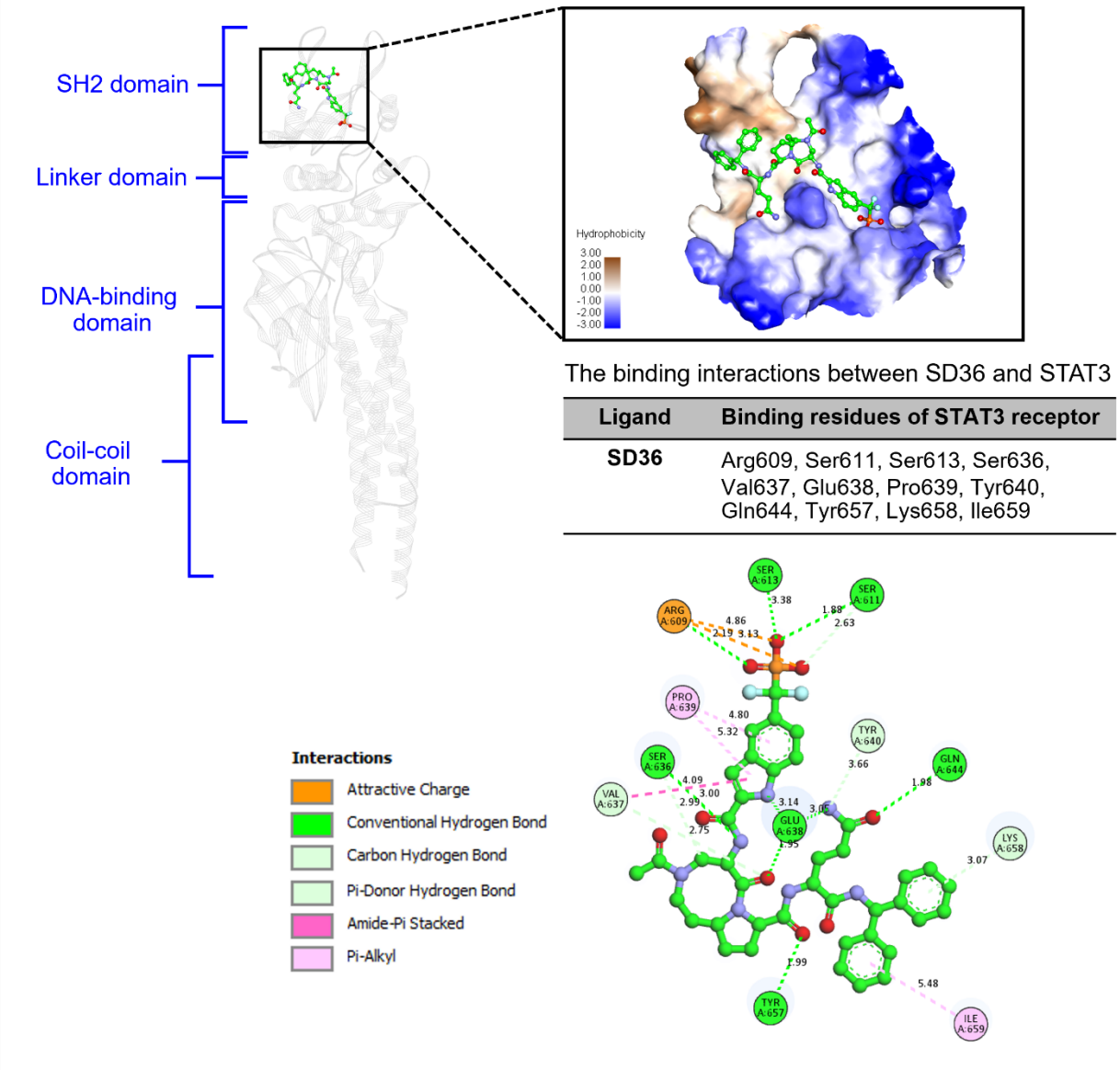
using ECL detection reagents. Blot replicates N1, N2, and N3 are shown, with replicate N3 corresponding to the representative results presented in Figure 3A. CM indicates cytokine mixture consisting of IL-6, TNF-α, and IFN-γ.



Supplementary Figure S6. *Effects of arachidin-rich extract on ROS production assessed by DCFH-DA assay.* (A) Representative fluorescence images of DCFH-DA staining. (B) Quantitative analysis of relative fluorescence intensity. Data are presented as mean ± SD from three independent experiments. *ns* = not significant; **p*< 0.05 indicates a significant difference compared to the control group.



Supplementary Figure S7. *Effects of arachidin-rich extract on tumorigenic potential assessed by soft agar colony formation assay.* (A) Representative images colony formation in soft agar. (B) Quantitative analysis of relative colony number. Data are presented as mean ± SD from three independent experiments. *ns* = not significant.



The binding interactions between SD36 and STAT3

Ligand	Binding residues of STAT3 receptor
SD36	Arg609, Ser611, Ser613, Ser636, Val637, Glu638, Pro639, Tyr640, Gln644, Tyr657, Lys658, Ile659

Supplementary Figure S8. *Predicted binding interactions of standard inhibitor (SD36) within the STAT3 SH2 domain.* Predicted two-dimensional (2D) and three-dimensional (3D) molecular docking representations illustrate the binding mode of SD36 within the SH2 binding pocket of STAT3.