Chemical Composition, Antimicrobial, Antioxidant and Cytotoxic Activities and of Essential Oil from *Actinidia Arguta*

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Abstract

Chemical composition, antimicrobial, antioxidant, and cytotoxic properties of essential oil from *Actinidia arguta* (AEO) were evaluated. Gas chromatography-mass spectrometry analysis identified 56 chemical compounds, with the most abundant being Squalene (23.08%), γ -sitosterol (8.10%), and β -Tocopherol (7.01%). Whereas the AEO had significant antimicrobial activity against *Staphylococcus aureus* and *Saccharomyces cerevisiae*, it showed mild efficacy against *Bacillus subtilis* and *Microsporum canis*. On the contrary, the Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, were not susceptible to the AEO pressure. On the other hand, the AEO exhibited strong antioxidant activity against DPPH, β -carotene, and hydroxyl radicals, having an IC₅₀ values of 117.60, 73.60 and 35.15 μ g/mL, respectively. Additionally, compared to the PC-3 or HT-29 cell lines, the A549 cells were more susceptible to the AEO (IC₅₀ ; 6.067 mg/mL). Besides, the confocal laser scanning microscopy imaging showed that 16 mg/mL of the AEO induced apoptosis in the A549 cell lines. Our data indicate that the AEO might be useful in the food and pharmaceutical industry.

Introduction

There is a growing interest in the exploration of naturally-occurring bioactive compounds for industrial use. This development has been necessitated by the fact that there is increased resistance to a wide spectrum of commercial antibiotics (Fair and Tor 2014) as well as the toxicity associated with the synthetic antioxidants (Augustyniak et al. 2010; Ksouda et al. 2019). Essential oils are aromatic, subtle and volatile, found in various parts of the plant such as flowers, fruits, buds, leaves, bark, seeds, roots and wood (Sharma and Kumar 2015; Bączek et al. 2018; Saeed et al. 2018) (Cortes-Camargo et al. 2019; de Souza et al. 2019). They are secondary metabolites and play important biological roles in the plants, such as protection against microorganisms, insects or viruses (Singh et al. 2013; Houicher et al. 2018; Ksouda et al. 2019). Previous studies have demonstrated that essential oils have promising antimicrobial, antioxidant, antitumor or insecticidal activities (Soeur et al. 2011; Cabral et al. 2012; Bayala et al. 2014; Lesgards et al. 2014; Zoubiri et al. 2014; Thomas et al. 2017; Ali et al. 2020). Besides, the oils have been widely used in cosmetics, food, medicine, pharmaceutical and agricultural industries (Snoussi et al. 2018; Wang et al. 2018).

Actinidia arguta (Sieb. Et Zucc.) Planch. ex Miq. var. are small grape-sized fruits with edible green or red-colored skin, belonging to the *Actinidia* genus. It originated and widely cultivated in northern China. The *A. arguta* has a delicious taste and immense health benefits. It bears fruits rich in vitamins, polysaccharides, phenolics, flavones, alkaloids, as well as other essential minerals (Zhu et al. 2019). *A. arguta* is one of the richest sources of lutein (up to 0.93 mg/100 g FW) and vitamin C (up to 430 mg/100g fresh weight FW), *myo*-inositol (up to 982 mg/100g FW), and is considered as the most nutritious fruits (Latocha 2017). The rich nutritional value has prompted researchers to interrogate its anti-microbiology, antioxidant, anti-tumor or anti-inflammatory potentials (Latocha et al. 2013; An et al. 2016; Leontowicz et al. 2016).

Whereas some studies have reported the chemical composition and antimicrobial activity associated with the AEO (Matich et al. 2003), data on the antioxidant or antitumor activities of the AEO remain scant. Our study embarked on determining the chemical composition of the AEO as well as evaluation of its antimicrobial, antioxidant and cytotoxic properties. Our findings have set the basis for the use of the AEO in food, pharmaceutical or cosmetic industries.

Materials And Methods

Plant material

The *A. arguta* fruits were collected from the experimental farm of the Shenyang Agricultural University in September 2019.

Microbial strains

The AEO were tested against six microorganisms, including two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) as well as two fungal strains (*Saccharomyces cerevisiae* and *Microsporum canis*). The susceptibility of the bacteria and the fungi to the essential oils were carried out using the disk diffusion method. All the strains were obtained from the Agricultural Culture Collection of China.

Human cell lines

Human colon cancer cell line (HT-29), human prostatic cancer cell line (PC-3), and human lung adenocarcinoma epithelial cell line (A549) were obtained from the College of Basic Medicine of China Medical University (Shenyang, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. The cells were grown at 37 °C in 95% humidified air and 5% CO₂.

Extraction of essential oil

The fruits were grinded into a homogenate and subjected to solvent extraction by N-hexane (Ghahramanloo et al. 2017). The obtained AEO was stored at 4°C in sealed glass vials. Percentage AEO yield was quantified as follows:

$$\text{AEO yield (\%)} = [\text{mass of AEO obtained (g)} / \text{mass of fresh fruit (g)}] \times 100$$

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The chemical composition of the essential oil was analyzed by GC/MS using Agilent 5973 EI mass selective detector coupled with Agilent GC6890, equipped with a HP-5MS fused capillary column (5% phenyl Methyl Silox) (30m×0.25mm, 0.25µm film thickness). Helium (99.999%) was used as a carrier gas with a flow rate of 1.0 mL/min. The initial temperature was programmed at 40°C, then increased 3°C/min

up to 80°C, then by 5°C/min up to 280°C. The temperature was maintained at 280°C for 20min, just as the injector and detector temperatures. The quadruple mass spectrometer was scanned over a range of 35-500amu at 1 scan per second, with a temperature of 150°C, ionizing voltage of 70 eV, and an ionic source temperature of 230°C. 2.0 µL of the AEO was injected with a split ratio of 10:1. Individual components of the AEO were identified on the basis of their retention indices (RI), and the compared with reference data using the Wiley7n.l library.

Antimicrobial activity assay

Agar diffusion method

The effect of the AEO on the bacteria was determined according to Marjana(Radunz et al. 2019), with few modifications. The bacterial cells were cultured in liquid Luria-Bertani media overnight at 37°C, while the fungal strains were cultured in Sabouraud dextrose broth at 28°C for 48h. The microbial suspensions were diluted to 10⁸ CFU/mL, while the fungal cells were diluted to 10⁶ CFU/mL. The microbial suspension (150 µL) were evenly spread on solid media. Thereafter, sterile 6mm diameter filter disks were placed on the media seeded with the microorganisms (3 disks per plate) and then AEO was dropped onto each paper disk (40 µL per disk). The treated plates were first kept at 4°C for 1h, then incubated at 37°C for 24h (bacteria), or at 28°C for 48h (fungi) (Lu et al. 2007). The antimicrobial activity was evaluated by measuring the diameter of growth inhibition zone surrounding the disks. All tests were performed in triplicates.

MIC and MBC/MFC

The AEO was dissolved in 1% (v/v) DMSO and then diluted to different concentrations (0.78-12.5 mg/mL). Minimum inhibitory concentration (MIC) value was measured following a protocol described by (Zhao et al. 2018), with slight modifications. Briefly, 10 µL from each of the incubated suspensions were transferred into the corresponding media and incubated at 37°C for 24h (bacteria) or at 28°C for 48h (fungi). The minimum concentration that inhibited the growth of the microorganisms was recorded as MBC (minimum bactericidal concentration) or MFC (minimum fungicidal concentration) (Ksouda et al. 2019). The experiments were done in duplicates.

Antioxidant activity

The antioxidant activity of the AEO was tested using the following spectrophotometric methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radical scavenging assays as described by (Lu et al. 2018), as well as the β-carotene bleaching test by Wang et al., 2008, with minor modifications. The AEO samples were tested at concentrations of 12.5 to 800 µg/mL and in triplicates. Butylated hydroxytoluene (BHT) was used as the positive control. IC₅₀ values were defined by linear regression analysis and depicted as means ± SD of the triplicates.

Cytotoxicity assay

Determination of IC₅₀

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Hamdi et al. 2018) was used to evaluate the cytotoxicity of the AEO. Briefly, 1×10^6 cells were seeded in 96 well plates for 24 h. The cells were treated with different concentrations of the AEO samples (1-32 mg/mL) for 48 h. Untreated cells were used as the negative control. Up to 0.5 mg/mL of MTT was added into the cells and incubated for 4 h. Thereafter, the medium was replaced by 100 μ L of DMSO to dissolve formazan crystals. Absorbance was detected on a StateFax-3200 microplate reader (AEARNES, CA, USA) using a wavelength of 570 nm and a reference wavelength of 630 nm. The IC₅₀ was calculated by a liner regression analysis with 95% confidence limits. The inhibition of cell proliferation was approximated using the following formula:

$$\text{Cell growth inhibition ratio (\%)} = (1 - A_t / A_c) \times 100\%$$

where A_t is absorbance of the test sample, A_c is the absorbance of negative control.

Confocal laser scanning microscopy (CLSM) assay

A total of 1×10^5 A549 cells were seeded in a 6 well plate for 24 h (37°C, 5% CO₂). 16 mg/mL of the AEO samples was added and incubated for 48 h. Untreated cells were used as the negative control. The medium was removed, washed twice in cold PBS, and then the cells were digested with 0.25% trypsin. The cells were recovered by centrifugation at 3000 $\times$ g, washed twice in cold PBS, followed by final centrifugation at 3000 $\times$ g for 5min. The density of the cells was adjusted to 1×10^3 /mL with cold PBS. 1mL of acridine orange (1 mg/mL) was added into the cells and then incubated at room temperature and in darkness for 5 min. A laser confocal microscope was used to image the DNA morphology of the cells.

Statistical analysis

Each of the experiments was performed in triplicate. The mean value was calculated, and the experimental results were expressed as the mean $\pm$ standard deviations (SD). Besides, one-way ANOVA in SPSS Statistics 22.0 software (IBM, USA) was used to analyze the significant differences between the data sets. Significance was set at $p < 0.05$.

Results

Chemical composition of the essential oil

The extraction yielded 1.04% (m/m) of the AEO with a yellowish to orange color. The AEO composition, along with the retention time are listed in Table 1. GC-MS experiment identified 92.09% (52 constituents) of the total composition (Fig. 1). The major compounds detected in the AEO were: Squalene (23.08%), γ -sitosterol (8.10%), β -tocopherol (7.01%), Stigmast-7-en-3-ol (5.67%), and 2-Hexenal (2.59%). Overall, the AEO comprises of sterols (26.73%), triterpenes (26.35%), alkanes (23.42%), phenols (7%), alcohols (4.23%), aldehydes (4.11%) and esters (3.89%).

Antimicrobial activity analysis

The in vitro antimicrobial activity of the AEO against Gram-positive, Gram-negative bacteria as well as fungal organisms was assessed. As shown in Table 2, the AEO exerted significant activity against *S. aureus* and *S. cerevisiae* (Inhibition zone; $19.5 \text{ mm} \pm 0.54$ and $20.5 \text{ mm} \pm 0.48$, respectively) but mild activity against *B. subtilis* ($17.2 \text{ mm} \pm 0.35$) and *M. canis* ($16.8 \text{ mm} \pm 0.57$). However, the *E. coli* and *P. aeruginosa* did not show any susceptibility to the AEO pressure (Inhibition zone; $8.5 \text{ mm} \pm 0.12$ and $10 \text{ mm} \pm 0.21$, respectively). Also, our study has shown that the AEO exhibited bactericidal effect against *B. subtilis* and *E. coli* (MBC/MIC < 4) as well as two fungicidal effect against *S. cerevisiae* and *M. canis* (MFC/MIC < 4). On the other hand, it exhibited a bacteriostatic effect against *S. aureus* and *P. aeruginosa* (MBC/MIC > 4).

Antioxidant activity analysis

DPPH radical scavenging assay

DPPH radical scavenging test is most common method used to evaluate antioxidant activity of compounds (Ksouda et al. 2019; Zhou et al. 2020). Our data showed that the AEO exhibited strong and concentration-dependent scavenging activity on DPPH ($\text{IC}_{50} = 117.60 \text{ } \mu\text{g/mL}$) as shown in Fig. 2a. A concentration between $12.5\text{--}800 \text{ } \mu\text{g/mL}$ of the AEO reduced DPPH $\cdot$ by between $15.11\% \pm 1.08\%$ to $82.72\% \pm 2.52\%$. The AEO activity was comparable to the activity shown by the synthetic antioxidant agent BHT ($\text{IC}_{50} = 5.27 \text{ } \mu\text{g/mL}$).

β -carotene bleaching test

The β -carotene bleaching test was used to evaluate the potential of the AEO to inhibit formation of conjugated diene hydroperoxides from linoleic acid oxidation in the emulsion (Miraliakbari and Shahidi 2008). Compared to BHT ($99.26\% \pm 3.88\%$; $\text{IC}_{50} = 6.81 \text{ } \mu\text{g/mL}$), $800 \text{ } \mu\text{g/mL}$ of the AEO bleached β -carotene by $88.48\% \pm 2.80\%$, with an IC_{50} of $73.60 \text{ } \mu\text{g/mL}$, as shown in Fig. 2b. The remarkable β -carotene bleaching activity might be associated with the fact that nonpolar antioxidants exhibit high antioxidant activity in emulsions. The nonpolar antioxidants concentrate at the lipid/air surface of the emulsions (Ksouda et al. 2019).

Hydroxyl radical scavenging assay

As the strongest free radical in reactive oxygen species, hydroxyl ($\cdot\text{OH}$) can react rapidly with almost all biological macromolecules in cells (Radunz et al. 2019). Our data showed that the scavenging activity of the AEO on the hydroxyl was concentration dependent as shown in Fig. 2c. $800 \text{ } \mu\text{g/mL}$ of the oil resulted in the highest scavenging activity ($98.76\% \pm 2.42\%$) and the IC_{50} value was determined as $35.15 \text{ } \mu\text{g/mL}$. However, compared to the AEO, BHA exhibited higher hydroxyl radical scavenging ability, with the highest scavenging activity of $99.45 \pm 2.31\%$ $\mu\text{g/mL}$ and an IC_{50} value of $6.06 \text{ } \mu\text{g/mL}$.

Cytotoxicity activity analysis

Determination of IC₅₀

MTT assay was used to determine the cytotoxicity of 1-32 mg/mL of the AEO on HT-29, PC-3 or A549 cell lines, exposed for 48h. The AEO inhibited 78.63%, 60.42% or 57.31% proliferation of A549, HT-29 or PC-3 cells respectively as shown in Fig.4. The IC₅₀ values were 6.067 mg/mL, 11.905 mg/mL or 13.646 mg/mL for the A549, HT-29 or PC-3 cell lines respectively (Table 3), compared to the control group ($P<0.05$). This finding indicate that whereas the effect was cell-specific, the AEO had a significant inhibitory effect on tumor growth.

Confocal laser scanning microscopy (CLSM) assay

DNA damage of the A549 cells was studied by CLSM. Fluorescence staining by acridine orange showed that whereas the control cells had normal morphology (Fig. 3a), the A549 cells that were subjected to the AEO for 48 h (Fig. 3b) or 72 h (Fig. 3c) exhibited typical apoptotic characteristics. There was presence of dense yellow-green staining in the nucleus or cytoplasm, formation of cell membrane vesicles, lysed nuclei as well as apoptotic bodies. The CLSM results confirmed the ability of the AEO to induce apoptosis in A549 tumor cells, thus anti-tumor activity.

Discussion

The extraction yield and composition of essential oil are various according to extraction methods and reagents. In this study, we have reported for the first time the presence of γ -sitosterol, Stigmast-7-en-3-ol and β -Tocopherol in the AEO. On the contrary, whereas we identified 0.96% of ethyl butyrate in the essential oil, Yang et al., 2006 showed a high (86.89%) relative content of ethyl butyrate in volatile components of *A. arguta*. Besides, Xin et al. (G. Xin 2009) studied the aroma from *A. arguta* fruits and found that the olefin content accounted for 51.71% of the volatile oil, but the acid was not detected. These chemical differences may be due to variability in varieties, regional differences, maturity stages or extraction and detection methods.

Research on the development of natural antibacterial, antifungal agents has attracted much attention (Fair and Tor 2014). As different solvent extracts possess different concentration and extent of bioactive principles, their antibacterial activity is also variates (Rajput et al. 2021). In this study, AEO had significant antimicrobial activity against *Staphylococcus aureus* and *Saccharomyces cerevisiae*, mild efficacy against *Bacillus subtilis* and *Microsporum canis*. However, the Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, were not susceptible to the AEO pressure. This phenomenon suggests that the volatile oil might be acting on the peptidoglycan layer in the cell wall of Gram-positive bacteria. Also, the antimicrobial activity was mainly attributed to squalene, which was not only in higher amounts in the extracted oil but also has been proved to have strong antibacterial activity (Popa et al. 2015). Our data demonstrates that the AEO possesses antimicrobial activity, thus, might be useful in the pharmaceutical and food industry. Antioxidant assay showed AEO exhibited high antioxidant activities probably due to

the diverse constituents that might be working in synergy. The compounds such as squalene, γ -sitosterol and β -tocopherol have been reported to have antioxidant activities (Hidayathulla et al. 2018; Shimizu et al. 2019; Weber et al. 2020). Antioxidant activity assays in this research indicating AEO might be used in various pharmaceutical and food industries.

Apoptosis plays an important role in tumorigenesis, and might inform damaged cells by apoptosis, mediating the "suicide" death of tumor cells (Al-Sheddi et al. 2019; Huang et al. 2020). In addition, previous literature reports that some plant essential oils can activate additional pro-apoptotic pathways specific for cancer cells (Abu-Darwish et al. 2016; Zhao et al. 2017; Vasilijevic et al. 2018; Laghezza Masci et al. 2020; Li et al. 2020). The composition of plant essential oil varies from plant to plant, thus, their diverse anti-tumor mechanisms. We, for the first time, show that AEO could significantly inhibit the proliferation and induce apoptosis in A549 cells, indicating that AEO can be used in the development of natural anti-tumor drugs. However, more studies are needed to reveal the molecular mechanisms defining the effect of the AEO on cancer cells.

Conclusions

The essential oil extracted from *A. arguta* mainly comprised of squalene, γ -sitosterol and β -Tocopherol. The AEO exhibited potential antimicrobial, antioxidant and cytotoxic activities, which might be a function of synergy among the compounds. Besides, the activity might be regulated by other secondary components which play a significant role in defining the aroma, density, texture, color, cellular penetration, lipophobia, and hydrophilicity of the AEO (Emami et al. 2016). Therefore, the AEO harbors huge potential that can be used in the phyto-pharmaceutical and food industry.

Abbreviations

AEO, *A. arguta* essential oil; GC-MS, Gas chromatography-mass spectrometry; CLSM, Confocal laser scanning microscopy; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal bovine serum; RI, retention indices; MIC, Minimum inhibitory concentration; MBC, Minimum bactericidal concentration; MFC, Minimum fungicidal concentration; DPPH, 1,1-diphenyl-2-picrylhydrazyl; BHT, Butylated hydroxytoluene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Declarations

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Author contributions: H.W. and Y.Y. conceived and designed the experiments; H.Q. and S.T. performed the experiments; Z.W. and Y.Y. analyzed the experiment data; H.W. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Tables

Tabel 1 Compositions of the essential oil from fresh fruits of *Actinidia arguta*

No.	Compound	Retention Time	Relative content (%) ^a
1	Ethyl butyrate	4.614	0.96
2	Hexanal	4.820	1.52
3	2-Hexenal	6.321	2.59
4	Hexanol	6.872	1.56
5	Nonane	7.865	0.12
6	Benzeneethanol	16.813	0.08
7	Benzoic acid ethyl ester	25.270	0.68
8	Hexedecane	30.698	0.13
9	Heptadecane	32.968	0.21
10	Octadecane	35.116	0.23
11	α -Terpineol	35.582	0.21
12	Neophytadiene	35.949	0.20
13	Cyclotetradecane	36.825	0.12
14	Nonadecane	37.162	0.21
15	Eicosane	39.122	0.24
16	Isopropyl palmitato	39.608	0.18
17	1-Octadecene	40.757	0.24
18	Heneicosane	40.987	0.59
19	Cyclohexane decyl	42.621	0.62
20	Docosane	42.782	0.93
21	Hexadecanamide	42.621	0.66
22	1-Naphthalenamine-N-phonyl	43.535	0.56
23	Tricosane	44.502	1.42
24	9-Octadecenamide	45.586	1.47
25	Octadecanamide	45.992	0.61
26	Tetracosane	46.153	1.81
27	Linoleic acid butyl ester	47.312	1.17
28	Pentacosane	47.745	2.36

29	Hexacosane	49.273	2.28
30	Tetracosane	50.191	0.37
31	Heptacosane	50.346	0.16
32	Heptacosane	50.747	2.06
33	Eicosane	51.639	0.49
34	Hexacosane	51.794	0.45
35	Octacosane	52.189	2.08
36	Schizandrin	52.494	0.27
37	Squalene	52.670	23.08
38	Octadecane	53.183	0.44
39	Nonacosane	53.557	2.27
40	Triacotane	55.058	1.27
41	β -Tocopherol	56.116	7.01
42	Hentriacotane	56.837	1.19
43	Hexacosanol	56.944	1.59
44	Cholest-5-en-3-ol	57.371	1.37
45	Docosane	58.953	0.78
46	Campesterol	59.759	0.72
47	Stigmasta-5,22-dien-3-ol	60.545	1.16
48	tritriacotane	61.544	0.59
49	1-Eicosanol	61.800	0.79
50	γ -sitosterol	62.153	8.10
51	β -Amyrin	63.131	1.55
52	Stigmast-7-en-3-ol	63.809	5.67
53	α -Amyrin	64.391	1.72
54	9,19-cyclolanostan-3-ol,24-methylene	66.357	1.61
55	9,19-cyclolanostan-3-ol,acetate	67.954	1.08
56	A-Friedooleanan-3-one	69.007	0.26
Total %			92.09

% of identified compounds	7.91
Number of identified compounds	56

Table 2 Evaluation of antibacterial activity of *Actinidia arguta* essential oil.

Microorganisms		Diameter of the inhibition zone (mm±SD)		Essential oil	
		Antibiotics	Essential oil	MIC(mg/mL)	MBC/MFC(mg/mL)
Gram-positive		Gentamycin			MBC
	<i>B. subtilis</i>	23.5±0.23	17.2±0.35	1.56	3.125
	<i>S. aureus</i>	25.3±0.41	19.5±0.54	0.78	3.125
Gram-negative	<i>E. coli</i>	16.8±0.37	8.5±0.12	6.25	12.5
	<i>P. aeruginosa</i>	18.5±0.39	10±0.21	1.56	6.25
		Amphotericin			MFC
Fungi	<i>S. cerevisiae</i>	24.3±0.52	20.5±0.48	1.56	3.125
	<i>M. canis</i>	21.6±0.58	16.8±0.57	3.125	6.25

Table 3 Cytotoxic effect of AEO

Cell lines	IC ₅₀ (mg/mL)
HT-29 cells	11.905
PC-3 cells	13.646
A549 cells	6.067

Figures

Figure 1

GC-MS chromatogram of *Actinidia arguta* essential oil

Figure 2

Antioxidant activity of AEO: DPPH radical scavenging activity (a), inhibition of β -carotene bleaching assay (b) and hydroxyl radical scavenging activity (c).

Figure 3

Cytotoxic effect of AEO against A549, PC-3, HT-29 cells at different concentrations.

Figure 4

Assessment of ultra-structural morphology of A549 cells by CLSM. Magnification: 200× a: Untreated cells; b: A549 cells treated with AEO for 48h; c: A549 cells treated with AEO for 72 h.

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