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Dynamic monitoring of the cytotoxic effects of *Cousinia iconica* extracts on A549 and RL95-2 cells using the xCELLigence RTCA system

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Asteraceae family plants have potential anticancer activity. In this study, the cytotoxic effects of *C. iconica* were investigated using different tumor cell lines: Non-small lung cancer (A549) and human endometrium carcinoma cell line (RL95-2). The cytotoxicity of the extracts was determined with MTT assay and the xCELLigence Real-Time Cell Analyser (RTCA). As a result, the ethyl acetate sub-extract and methanol extract of *C. iconica* were found cytotoxic to RL95-2 cell line with 271 and 51 µg/mL IC_{50} values, respectively. According to RTCA results, the IC_{50} values of ethyl acetate sub-extract were 44.82, 60.89, and 55.22 µg/mL and methanol extract 265.52, 250.48, and 246.65 µg/mL, for 24, 48, and 72-h exposure, respectively. RL95-2 cells are more sensitive to ethyl acetate sub-extract than methanol extract. This study is the known first study to show the cytotoxic effects of *C. iconica* extracts on both A549 and RL95-2 cell lines.

Keywords: Asteraceae, Cytotoxicity, RL95-2, xCELLigence

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Cancer after cardiovascular diseases is the second leading cause of death in the world. According to data from the World Health Organization, more than 8 million people worldwide die from cancer every year[1]. Cancer is a disease that occurs as a result of the uncontrolled proliferation of cells in organs or tissues of the body. Despite differences in formation and type, common features of cancers include invasion and metastasis, stimulation of cell division, genomic instability, resistance to cell death and induction of angiogenesis[2]. Nowadays, the use of many synthetic drugs is limited due to their possible side effects. In this respect, natural products are preferred[3].

The Asteraceae family is the largest family of flowering plants with approximately 1,100 genera and 25,000 species. The family includes various plants such as food, poisonous plants and medicine[4]. *Cousinia* Cass. is the most widespread genera of the Asteraceae family, represented by 600-700 species. In Turkey, this genus is described as 38 species in 6 sections and 26 of them are endemic. *Cousinia iconica* Hub-Mor. is one of these endemic species distributed in open areas, scrublands, and stony slopes[5].

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According to the literature, *Cousinia* species have been investigated on phytochemical constituents[6-8], and biological activities, such as antimicrobial[9], cytotoxic[10-12] and antioxidant and enzyme inhibition activities[12,13] properties until now. But there are not cytotoxic activity studies on *C. iconica* species in literature. Therefore, it was aimed to investigate the cytotoxic effect of methanol extract and sub-extracts of *C. iconica* against human non-small lung (A549) and endometrium carcinoma (RL95-2) cell lines by MTT and Real-Time Cell Analyzer (xCELLigence) method.

Material and Methods

Plant material

The aerial parts of plant material was collected in July 2017 from Konya. The voucher no. of the plant was KNYA 11.040. The herbarium sample was stored at the Herbarium of Selcuk University.

Extract preparation

The flowering plant material were air-dried (500 g). Then powdered and extracted. The extraction method was maceration (600 mL methanol x 3 for 24 h) at room temperature. After extraction the combined macerates were filtered. The evaporated extract (CIM)

was dispersed with distilled water, and then subsequently partitioned with different polarity of solvents. The *n*-hexane sub-extract (CIH), ethyl acetate sub-extract (CIE), *n*-butanol sub-extract (CIB) and water sub-extract (CIW) were obtained.

Materials

Dimethyl sulfoxide (DMSO 0.1%) was used for the stock solutions of the extracts and the reference compound cisplatin. DMSO (Cat No: A3672), Dulbecco's modified eagle's medium with F-12 nutrient mixture (DMEM: F-12) (Cat No: 01-170-1A), penicillin/streptomycin solution (Cat No: 03-031-1C). Fetal bovine serum (FBS) (Cat No: S0115), insulin (Cat No: I9278) and trypsin-EDTA (T3924). E-plate (05 232 368 001).

Cell culture

Human lung (A549) and endometrial cancer cell lines (RL95-2) were cultured in F12 Kaignhn's medium and DMEM: F-12 (supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin solution, and 0.005 mg/mL insulin), respectively. The cells were incubated at 37°C in a humidified, 5% CO_2 atmosphere. Trypsin (0.25% EDTA) was used to remove them from the flasks for subculture. Stock solutions of the extracts were prepared in dimethyl sulfoxide (DMSO) and the final DMSO concentration in the medium was less than 0.1%.

In-vitro cytotoxic activity assay

To determine the cytotoxic effect of extracts on the A549 and RL95-2 cell line, MTT (3-(4,5-dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide) assay was performed. Extracts were diluted over a wide range of concentrations. 20000 cells per well were seeded into 96 well plates. After overnight incubation, the medium was removed from the plate, and cells were treated with extracts that were dissolved in a fresh medium. Cells were incubated for 24 h in an incubator supplied with 5% CO_2. After treatment, extracts were removed from the plate. Firstly 90 µL fresh medium and 10 µL of 5 mg/mL MTT stock solution were added to each well. The plate was incubated in a plate shaker for 5 min. Final concentration of MTT solution was 0.5 mg/mL. Then, cells were incubated for 4 h in a 5% CO_2 incubator. Formazan crystals were observed on the plate. MTT solution was removed from the plate and formazan was dissolved using DMSO solution. Absorbance was measured at 570 nm wavelength using a microplate reader. Viability results were calculated after dividing each group's absorbance by the control group's absorbance[14].

Real-time cell analysis (RTCA)

The xCELLigence Real Time Cell Analyser system (ACEA Biosciences, San Diego, CA) is a commercial system designed to allow for continuous real-time monitoring of cellular viability in vitro in a non-invasive, label-free manner. According to MTT assay results, novel concentrations were determined to assess the effects of the extracts on cell viability of CIE (30-750 µg/mL) and CIM (30-750 µg/mL). In order to measure the cytotoxic response of RL95-2 cells in real-time, cells were seeded on gold microelectrodes embedded at the bottom of 96 well E-plate at a density of $2x10^4$ cells/well[15]. The impedance was recorded at 15 min intervals. The extracts were added to the culture at their growth phase. All incubations were performed for 96 h. The effects of the extracts were assessed by the RTCA-SP system software using the sigmoidal dose-response curves. IC_{50} values were defined at 72 h.

Statistical analysis

GraphPad Prism Software Version 8.2.1 (La Jolla, CA, USA) was used for statistical analysis. Data are expressed as the mean ± SD. Comparisons between the experimental groups were performed using one-way analysis of variance followed by post-hoc Dunnett's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of the extracts on the cell viability

As a result, among the extracts, CIH, CIE, and CIM sub-extracts were more cytotoxic to RL95-2 with 355±0.05, 51±0.03, and 271±0.03 µg/mL IC_{50} values, respectively. It was shown that other extracts did not decrease cell viability and the IC_{50} values were not calculated. Besides that, the cytotoxic effect of all extracts was determined against the A549 cell line. According to the results, only the CIH sub-extract was found more cytotoxic than the others (IC_{50}=498±0.05 µg/mL) (Table 1).

By RTCA, the extracts decreases the viability of the cells

To monitor and validate the accuracy of the MTT assay in evaluating the cytotoxic effects of CIE and CIM, the cell viability was assessed using the

xCELLigence system (xCELLigence, Roche/ACEA Biosciences, CA). Continuous CI alterations resulting from changes in cell number, morphology, and adhesion on the microelectrodes were measured by the RTCA SP instrument for approximately 96 h.

Table 1 — The 24 h cytotoxicity results of *C. iconica* extracts and sub-extracts by MTT assay

Extracts	24 h IC_{50} values (µg/mL)	
	A549	RL95-2
CIM	*In*	271.47±0.03*
CIH	498.02±0.05	355.47±0.05*
CIE	*In*	51.78±0.03*
CIB	*In*	*In*
CIW	*In*	*In*
Cisplatin	24.45±0.07 µM	20.05±0.02 µM

Cells were treated with various concentrations of CIM, CIH, CIE, CIB, CIW, and cisplatin for 24 h. The absorbance was determined after 4 h-incubation of the cells with MTT. Cell viability is presented as the mean ± SD at least three independent experiments. *** p<0.001 compared with the untreated control. *In*, inactive; CIM: *C. iconica* methanol extract, CIH: *C. iconica n*-hexane sub-extract, CIE: *C. iconica* ethyl acetate sub-extract, CIB: *C. iconica n*-butanol extract, CIW: *C. iconica* water sub-extract.

The concentration-response curves and the viability of treated cells at the indicated time points are exhibited in Figure 1 and Figure 2 for CIE and CIM, respectively. Control groups without compound treatment indicated normal cell growth in electronic microwells.

A correlation was noted between results obtained from the xCELLigence system and from the MTT assay for CIE on RL95-2. According to the results, it was observed that CIE and CIM have an antiproliferative effect at the concentrations of 30, 60, 125, and 250 µg/mL and the cell viability decreased at all concentrations in a time-dependent manner (Fig. 1).

The cell viability decreased all of the concentration and time-dependent manner for CIM. However, the CI values close to each other for 30, 60, and 125

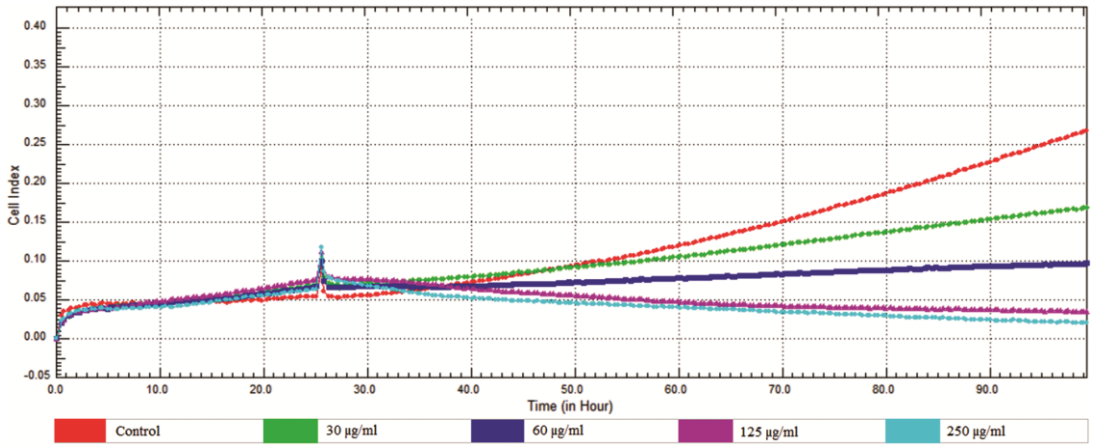

Fig. 1 — Dose and time-dependent cytotoxic effect and alteration of CI of CIE determined by xCELLigence system. Cells were treated with various concentrations of CIE for 72 h. The CI was calculated from four repeated measurements. CIE: The ethyl acetate sub-extract of *C. iconica*

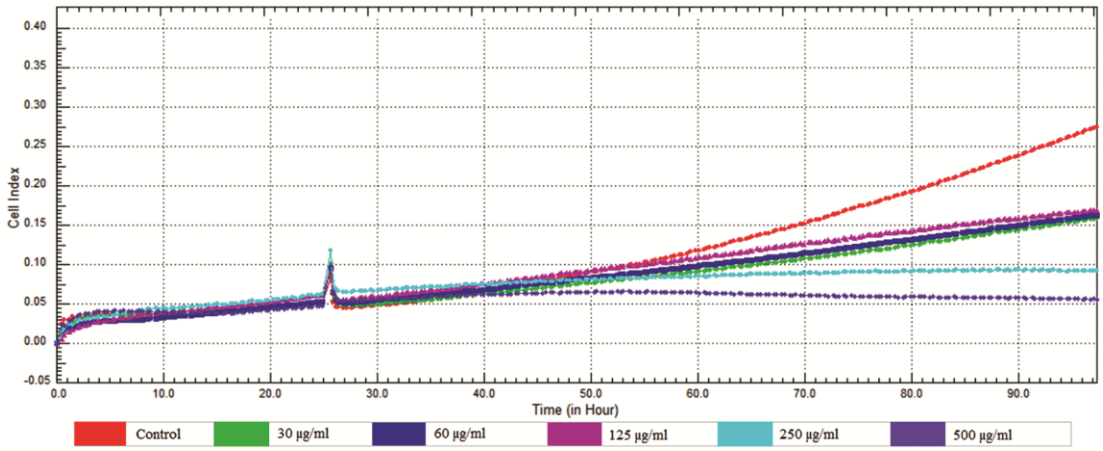

Fig. 2 — Dose and time-dependent cytotoxic effect and alteration of CI of CIM determined by xCELLigence system. Cells were treated with various concentrations of CIM for 72 h. The CI was calculated from four repeated measurements. CIM: The methanol extract of *C. iconica*

Table 2 — IC_{50} values of RL95-2 cells for 24, 48 and 72 h[1].

Extracts	24 h IC_{50} values (µg/mL)	48 h IC_{50} values (µg/mL)	72 h IC_{50} values (µg/mL)
CIE	44.82	60.89	55.22
CIM	265.52	250.48	246.65

[1]The IC_{50} of CIE and CIM were obtained based on the dose-response curves of CI during 72 h exposure and calculated from repeated experiments (n = 4) with the real-time xCELLigence system.

µg/mL at 72 h (Fig. 2). As shown in Figure 1 and Figure 2, the concentration-response curves of the given time points corresponded well with the same dose of the MTT for CIE.

The CI values of the treated cells decreased from 30 µg/mL in a concentration-dependent manner for CIE (Fig. 1). In contrast, the CI values of the control kept rising until the maximal value at about 72 h after the treatment. To compare CIE and CIM extracts with each other, their 50% inhibitory concentrations (IC_{50}) were calculated for 24, 48, and 72-h exposure. According to RTCA, the IC_{50} values of CIE were 44.82, 60.89, and 55.22 µg/mL, and for CIM 265.52, 250.48, and 246.65 µg/mL, respectively (Table 2). Real-time xCELLigence measurements show that RL95-2 cells are more sensitive to CIE than CIM (Figure 1 and Figure 2).

Discussion

Based on previous studies on cytotoxic activity of *Cousinia* species it was shown that there is significant activity against different cell lines. In a study, various *Cousinia* species were examined against fibrocarcinoma cell line and *C. verbasciflora* was found more active (IC_{50}=18.4 ± 0.59 µg/mL)[16]. In another study, desoxyjanerin and raserolit were isolated from *C. aitchisonii* dichloromethane extract and subjected to cytotoxic screening. As a result, these compounds were found more cytotoxic against breast cancer MCF-7 cell line with 4.5 µg/mL and 4.6 µg/mL, IC_{50} values, respectively[10]. Besides that, *C. shulabadensis* also evaluated and it was shown that it has an MMP inhibitory effect (IC_{50}=109 µg/mL)[16]. In another study four *Cousinia* species were investigated for cytotoxic activity against A549 and Colo205 cell lines. According to the results fraction-3 from *C. stenocephala* was found most active than others (IC_{50}=49.2±0.51 µg/mL)[12]. In another study, these four species were evaluated against HepG2 (human hepatocellular carcinoma) cell line. In a result, *C. davisiana* methanol extract (CD) and ethyl acetate sub-extract (CDE) showed more cytotoxic activity with 150 and 89 µg/mL IC_{50} values, respectively[17]. In a study, the cytotoxic activity of *C. ermenekensis* methanol extract was determined against A549, Colo205, HepG2, and Beas-2b cell lines. As a result, it was found more active against Colo 205 cell line (IC_{50}=69 µg/mL)[11]. In another study, six flavonoids and diterpene compound grindelic acid were isolated from *C. alata* and were evaluated for cytotoxic activity. As a results, among the compounds grindelic acid showed promising cytotoxic activity against the *Artemia salina nauplii* and antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella enteritidis*[18]. In a study, the *in-vitro* growth inhibitory effects of methanol extracts from *C. harazensis* and *C. calocephala* were investigated on A2780, T-47D, A549 and Hep-G2 cell lines. According to the results, *C. harazensis* was found most active on Hep-G2 with IC_{50} of 4.521 µg/mL[19]. According to the study, 7 sesquiterpene lactones, and 4 lignans were isolated from the aerial parts of *C. turkmenorum*. Among the isolated compounds, aguerin B showed the most cytotoxic activity against MCF7 cell lines with IC_{50} value of 18.9 µM[20].

Based on these reports the investigation of the cytotoxic effect of *C. iconica* extracts were carried out against A549 and RL95-2 cell lines by two methods. According to the results of xCELLigence RTCA and MTT methods, the CIE sub-extract was found to be more active for RL95-2 cells than the others. The IC_{50} value of the CIE has the closest IC_{50} value to the positive control cisplatin.

It was shown that these results support previous studies. To the best of our knowledge, the activity of the CIE sub-extract may be explained with the phytochemical composition of the sub-extract. According to our previous study on this species, the substances of the CIE sub-extract were qualified[13]. It is clear that, the vast majority of these compounds were known for their anticancer capacities such as apigenin[21], caffeic acid, chlorogenic acid[22], and betulinic acid[23]. So, we can consider that the cytotoxic effect of CIE on the RL95-2 cell line may be explained by the presence of these compounds.

Conclusion

This is the first research on the cytotoxic activity of *C. iconica*. Moreover, the limited studies on *Cousinia* species and identified compounds combined with activity evaluation will shed new light on the

advanced studies. Furthermore, this study suggests a detailed investigation on clarifying the mechanism of action.

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Conflicts of Interest

No conflict of interest is associated with this work.

Authors' Contributions

We declare that this work was done by the authors named in this article, and the authors will bear all liabilities about claims relating to the content of this article. OT and LP contributed to collecting plant samples and extract preparation. LP performed qualitative chemical profiling of the phytoconstituents. AKKA and ED carried out cytotoxic activity tests and data analysis. LP, AKKA, ED, and OT were designed the study and contributed to the critical reading of the manuscript. All authors read and approved the final manuscript.

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