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Original article

# Bioactivity-guided isolation of compounds with antiproliferative activity from *Teucrium chamaedrys* L. subsp. *sinuatum* (Celak.) Rech. f.

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**Summary** *Purpose:* To reach active compounds from aerial parts of *Teucrium chamaedrys* L. subsp. *sinuatum* (TCS) under the guidance of anticancer assay. *Methods:* The active compounds of *T. chamaedrys* were isolated using chromatographic techniques including column and preparative thin layer chromatography. Isolated compounds were characterized using 1D and 2D NMR techniques and high-resolution HPLC–TOF/MS. In the present study, we evaluated *in vitro* antiproliferative activities of isolated compounds from TCS using xCELLigence Real Time Cell Analysis system (RTCA). *Findings:* Three phenolic compounds, namely luteolin-7-*O*-glucoside (cinaroside, compound 1), 5,6,3'-trihydroxy-7,4' dimethoxyflavone (nuchensin, compound 2), and (E)-*p*-coumaroyl-*O*-β-D-glucoside (compound 3) were isolated for the first time from *Teucrium chamaedrys* subsp. *sinuatum* (TCS). The phenolic compounds have antiproliferative effect against the HeLa (Human uterine cancer), C6 (Rat brain tumor) and PC3 (human prostate carcinoma) cells. *Conclusions:* The results demonstrated that *T. chamaedrys* subsp. sinuatum and its active components may be suggested as a promising natural antiproliferative agent against HeLa, C6 and PC3 cells.

**Key words:** phytochemical content, antiproliferative activity, HeLa, C6, PC3, *Teucrium chamaedrys* L. subsp. *sinuatum*

## Introduction

*Teucrium* L. (Lamiaceae) is a cosmopolitan genus that differs from other related genera. It comprises more than 300 species and almost 50 of these are known in Europe and distributed mainly in the Mediterranean basin. It is a perennial herbaceous plant with half-ligneous and shrub-like low stem being up to 50 cm high. *T. chamaedrys* subsp. *sinuatum* has a branched stem with oval serrated leaves and tiny blooms on the branch-tops. The plant inhabits rocky limestone areas, dry mountain meadows and pastures, edges of the sparse oak and pine forests up to 2600 m above the sea level in Central Europe, in the Mediterranean region and western Asia (1). Some species has been used to support medicinal treatments of certain diseases for a long time (2-4). *Teucrium chamaedrys* was used in the treatment of diseases such as abscesses, gout, digestive and respiratory tract infections (5). Among all disease groups the most serious one is cancer, therefore, there is excessive scientific and commercial interest in the continuing discovery of new anti-cancer products from natural sources (6). In cancer chemotherapy, plant-derived drugs became considerably important products due to the generally higher activities, therefore, many new drugs have been applied to treatment/prevention of cancer derived from secondary metabolites of the plants (7,8). Some drugs used to treat cancer have been isolated from natural products

(9). In addition, many studies exhibited the individual polyphenols or crude extracts of plants have potential anti-cancer effects, either antiproliferation, cancer cell cycle arrest or apoptosis (10,11). Many plant-derived new drugs have been applied for the treatment or prevention of various diseases due to their attractive biological activities (12). The recent study on antiproliferative effect demonstrated that the isolated flavonoids and fatty acids were effective from 100 to 1000 μg/ml (13). The interest towards *Teucrium* species has increased due to an anticancer activity evidenced for plant extracts and isolated compounds of these plants (14). *Teucrium* is one of the richest sources of diterpenes and more than 220 diterpenes were determined and they demonstrated insect-repellent and medicinal activities (15-17).

Cell-based *in vitro* screening of potential anticancer compounds is one of the most essential processes in new drug development. Conventional end point cytotoxicity assays, such as 3-(4, 5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT) assay (18,19), fluorescence microscopy (20), and the lactate dehydrogenase (LDH) assay (21), play an important role in the screening of anticancer activities of compounds isolated from natural sources. Although useful and widespread, these conventional methods are labor-intensive and require a sequence of manipulation steps which may lead to deviation of the results (19). Moreover, they can hardly meet the demands for real-time and dynamic monitoring of drug-cell interaction process. Therefore, it is imperative to develop a new method which can overcome these drawbacks.

The xCELLigence RTCA system (ACEABIO, USA) is an impedance-based, label-free biosensor technology which has a demonstrated applicability to provide continuous monitoring of cellular status, including cell growth, morphological changes and cell death in a real-time manner (22-24). As the cells interact with the microelectrodes integrated onto the bottom of the standard E-plate, the instrument measures the corresponding changes of electrical impedance automatically when changes occur in the biological status of the cells. The cellular responses are monitored and expressed as cell index (CI) (25). Thus, a label-free, non-invasive, real-time, automated detection can be performed using the xCELLigence system. Furthermore, a number of studies have proved that the new system is applicable for a variety of cell-based assay applications, such as compound-mediated cytotoxicity (26), cell invasion and migration (23), quality control of cells (27), and cell proliferation and differentiation (28,29). In this study, we reported the evaluation of the antiproliferative activities of crude extracts of TCS, the isolation and characterization of flavonoids and flavonoid glycosides from polar extracts. To the best of our knowledge, there is no report on the isolation of flavonoids and flavonoid glycosides and their antiproliferative activities against HeLa, PC3 and C6 cell lines from TCS. Thus, the present research reports the first bioassay-guided isolation of these compounds from TCS with antiproliferative activities.

## Material and Methods

### *General experimental procedures*

NMR spectra were recorded on Bruker Avance III Spectrometer at 400 MHz. Mass spectra was run on Agilent LC-TOF/MS spectrometer. IR spectra were recorded on KBr plates on Jasco 430 FT/IR Spectrometers. UV spectra were recorded in MeOH on JASCO V 530. Silica gel 60 $F_{254}$ (Merck) was used for TLC analyses. Spots on TLC were visualized by UV irradiation (254 and 366 nm), and by staining with $Ce(SO_4)_2$ (1% in 10% aqueous $H_2SO_4$) followed by heating (105 °C, 5 min).

### *Plant material*

Aerial parts of TCS were collected at vegetative state at an altitude of 1550 m in July 2011 in Bingol, Turkey (N:39°10'3125' E:40°22'1648'). The plant was identified by one of the co-authors, Prof. Dr. L. Behçet, and a voucher specimen (No: 782) was deposited in the Herbarium of Biology Department, Bingol University, Turkey. The plant aerial parts were air-dried, protected from direct sunlight and powdered. The powder was kept in a closed container at 10 °C.

### *Extraction and isolation of constituents*

Part of the air-dried and powdered plant material of TCS (whole plant; 126 g out of 960 g) was first

extracted with MeOH:$CH_2Cl_2$ (1:1; 3x1.5 L). After filtration, the solvent was removed by rotary evaporation to give a crude extract (16.98 g). An aliquot of the crude extract (12 g) was subjected to open column chromatography by using silica gel (250 g), eluted initially with hexane followed by hexane:$CH_2Cl_2$ (HD1 and HD5); $CH_2Cl_2$:EtOAc (DE1 and DE5); EtOAc:MeOH (ME1 and ME7) along a linear gradient and finally with 100% MeOH. ME-1U and ME-2U and ME-1A and ME-2A were obtained from the ME-1 and ME-2 fractions after purification by preparative thin layer chromatography (P-TLC) with MeOH:$CH_2Cl_2$ (1:4) as the solvent system. ME-1U and ME-2U parts exhibited the highest anticancer activity against HeLa and C6 cells. To increase the amount of ME-1U and ME-2U parts, aerial parts of TCS (834 g) was selectively extracted with n-hexane (5x11 L), $CH_2Cl_2$ (5.5 x 9 L), EtOAc (5 x 8 L), and MeOH (5.5 x 12 L) to yield 10.47 g, 16.18 g, 40.09 g and 22.6 g of extracts, respectively. The active fraction EtOAc was further separated with silica gel and eluted with a gradient of MeOH:$CH_2Cl_2$ (0:10 to 10:0) to yield seventy two fractions. Compound **2** (33 mg) was obtained from the fraction 52. Another active fraction of MeOH part (22.6 g) was dissolved in water (500 ml) and extracted with $CH_2Cl_2$ (4 x 0.5 L) and n-BuOH (6 x 0.5 L) to yield 2.73 g, 5.70 g of extracts, respectively. The n-BuOH extract (5.7 g) was subjected to column chromatography by using silica gel (150 g), eluted with EtOAc:$CH_2Cl_2$ (0:10 to 10:0, gradient), MeOH:EtOAc and (0:10 to 10:0, gradient) Compound **1** (7 mg) was obtained from the fraction 16 and Compound **3** (22 mg) was obtained from the fraction 18.

*Quantitative amount of isolated compounds*

Quantitative amounts of isolated compounds in TCS, ME1U, ME2U, ME1A and ME2A were determined by using Agilent Technologies 1260 Infinity HPLC System coupled with Agilent Technologies 6210 LC-TOF/MS detector (Agilent Zorbax SB-C18 column (3.5 μm, 4.6 x 100 mm)). Mobile phase A and B were water containing 0.1% formic acid and acetonitrile, respectively. The flow rate was 0.8 ml $min^{-1}$ and column temperature was 35 °C. Injection volume was 5 μL. Solvent program was as follow: 0-1 min 10% B; 1-12 min 40% B; 12-14 min 90% B; 14-17 min 90% B; 17-18 min 10% B; 18-25 min 10% B. Analyses were triplicated (30).

*Real-Time cell proliferation assays*

Antiproliferative tests were performed against HeLa, C6 and PC3 cells by using xCELLigence Real Time Cell Analyzer (RTCA) in an incubator (5% $CO_2$ and humidity 95%, 37 °C). Dulbecco's Modified Eagle Medium-High Glucose (DMEM) with 10% fetal bovine serum and 2% penicillin-streptomycin was used as cell culture medium during assessments. Firstly, 50 μL of medium was added to each well and the plate was left in the hood for 15 min and in the incubator for 15 min to let both the E-plate's golden electrode well bottoms and medium reach a thermal equilibrium. Then, the E-plate 96 was inserted into the RTCA SP station in the incubator and a background measurement was performed. After the E Plate 96 was ejected from the station, 100 μL cell suspensions were added to each well to obtain a 2.5 x $10^4$ cell/well concentration in each well except three wells. These wells were left without cell to check if there would be an increase in cell index (CI) originated from the medium. 100 μL medium was added to these wells instead of cell suspension. After leaving the E-plate in the hood for 30 min, it was inserted into the RTCA station and the second step measurement was initiated for 80 min. The cells adapted to the bottom of the wells and entered into a growth and division phase. After this step, the E-plate was ejected from the station. The solutions of samples in dimethyl sulfoxide (DMSO) (final concentration of DMSO was less than 1% in each well) and medium were added to the wells to obtain final concentrations of 10, 50 and 100 μg/mL. The final volume of the wells was completed to 200 μL with the medium. Then the E-plate was inserted into the station and the main measurement period was initiated for 48 hours. All the measurements were done in 10 min intervals and triplicated.

The real time cell analyzer (RTCA) system measures the electrical impedance across interdigitated microelectrodes integrated on the bottom of 96-well tissue culture plates (E-plates) (31). The impedance measured is affected by the biological status of the cells

interacting with the well surface. Cells that interact with the electrode modify the local ionic environment and lead to an increase in the impedance measured within the tissue culture well. Thus, the electrode impedance can be used to monitor cell number, viability, morphology, and adhesion degree (32). The impedance is displayed as the cell index (CI) value, which is a dimensionless parameter calculated by

$$\mathrm{CI} = \max_{i=1,\ldots,N}\left[\frac{R_{cell}(f_i)}{R_b(f_i)} - 1\right]$$

where $R_{cell}(f_i)$ is frequency-dependent electrode impedance at any time, $R_b(f_i)$ is background impedance measured at the initial time without cells, and N is number of the frequency points at which the impedance is measured.

## Results and discussions

### *Quantitative analysis of isolated compounds with HPLC-TOF/MS*

For the quantitative analysis of the isolated compounds, their solutions were prepared at different concentrations (25-250 μg/L). Calibration curve was created for each compound with a correlation coefficient ($R^2$) of 0.9983 for compound **1** and of 0.9908 for compound **2**. In the portions of ME1U, ME2U, ME1A and ME2A, the distribution of compound **1** was found as 9.39 ± 0.64, 4.36 ± 0.70, 2.43 ± 0.51 and 0.83 ± 0.16 mg/kg and of compound **2** as 0±0, 6.47 ± 0.67, 26.28 ± 1.02 and 23.62 ± 1.069 mg/kg, respectively. On the other hand the quantitative distribution of compounds **1** and **2** was determined as 285.9 ± 9.71 and 834.2 ± 7.937 mg/kg dried plant, respectively (Figure 1).

### *The chemical structure elucidation of compounds*

Compound **1** was isolated as solid with yellow color. Molecular formula of compound **1** was found to be $C_{21}H_{20}O_{11}$ using spectroscopic instruments. Corresponding to the formula, molecular ion peak was identified by HPLC-TOF/MS as 447.0948 $[M-H]^-$ (m/z) (Figure 2) and $^{13}C$ NMR and $^1H$ NMR chemical shift values were given in the Table 1. In FT-IR spectral data for the compound **1** the carbonyl group and OH absorption peaks were observed at 1654 and 3303 $cm^{-1}$ respectively. The melting point of the compound was found to be 205 °C. According to $^{13}C$ NMR spectrum of the compound; twenty one carbon signals were detected. The carbonyl carbon of the aromatic ring has a resonance at $\delta_C$= 182.3 ppm (C-4). Chemical shift value at $\delta_H$= 7.42 ppm H-2' (d, 2.0 Hz) proton has been observed to interact with $\delta_H$= 7.46 ppm H-6'(dd, 2.0 and 8.4 Hz) protons and H-1" anomeric proton were determined to interact with H-2' proton to be resonance in $\delta_H$= 3.55 ppm from COSY spectrum of the compound. Proton-carbon (C-H) pairings were determined by HETCOR spectra, C-H interactions were as follows;

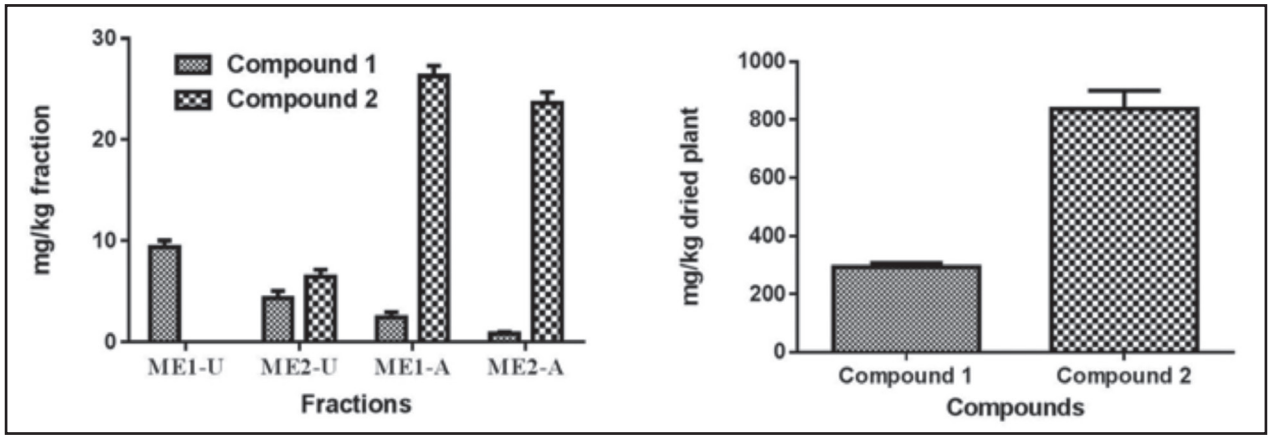


**Figure 1**. The amounts of the isolated compounds in fractions and dried plant (ME-1U and ME-2U and ME-1A and ME-2A were obtained from the ME-1 and ME-2 fractions after purification by preparative thin layer chromatography (P-TLC) with MeOH:$CH_2Cl_2$ (1:4) with the gradient solvent system

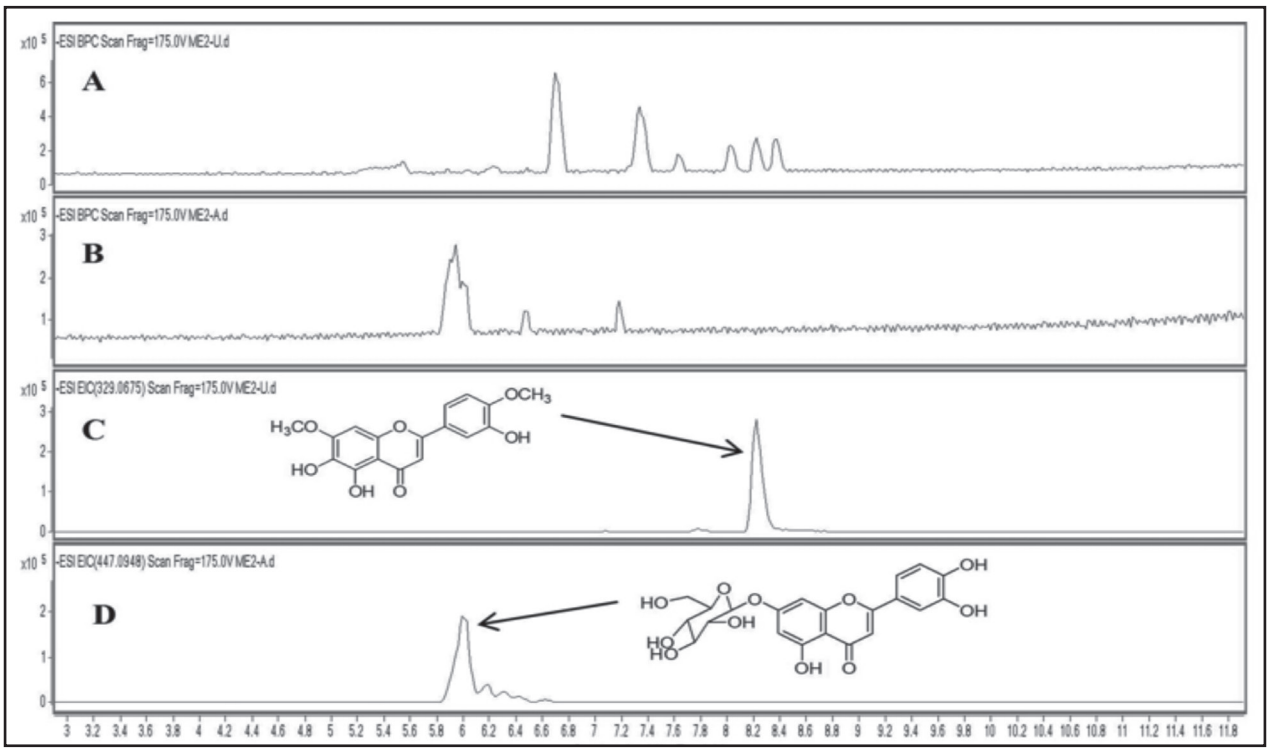


**Figure 2**. Active sub-fraction (A) and inactive sub-fraction (B), the more active compound (Compound **2**) (C), less active compound (Compound **1**) (D)

C-3 ($\delta_C$ = 103.6 ppm), C-6 ($\delta_C$ = 99.9 ppm) and C-8 ($\delta_C$ = 95.1 ppm) carbons correlates with the H-3 ($\delta_H$ = 6.76 ppm), H-6 ($\delta_H$ = 6.45 ppm) and H-8 ($\delta_H$ = 6.79 ppm) protons respectively. For ring B C-2'(δC=113.9 ppm) C-6' ($\delta_C$ = 119.6 ppm) and C-3'($\delta_C$ = 146.2) ppm carbons have been found to interact with H-2' ($\delta_H$ = 7.42 ppm) H-6' ($\delta_H$ = 7.46 ppm) and H-3' ($\delta_H$ = 6.92 ppm) protons. So the chemical structure of compound **1** was elucidated as a luteolin-7-O-glucoside (Figure 3). The obtained spectral data are consistent with the data existing in the literature (33).

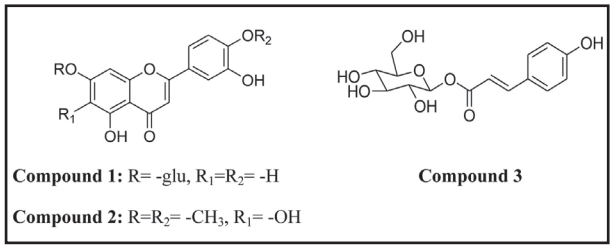


**Figure 3**. Isolated compounds from (*T. chamaedrys*) subsp. (*sinuatum*)

The physical appearance of the compound **2** was pale white powder. In the spectrum of FT-IR were determined bending and stretching vibration of functional groups represents ν = 3412 $cm^{-1}$ (OH bending), 2941 $cm^{-1}$ and 2830 $cm^{-1}$ (aliphatic C-H stretching), 1648 $cm^{-1}$ (C=O stretching), 1049 $cm^{-1}$ and 1011 $cm^{-1}$ (C-O-C symmetric stretching), 837 and 758 $cm^{-1}$ (substitute aromatic ring out of plane C-H bendings). From HPLC-TOF/MS spectrum was found to give a molecular ion peak in the fifteenth minute (-)-ESI MS: m/z 329.0675 $[M-H]^-$. In the $^1$H-NMR spectrum proton signals were compatible with the flavone skeleton that contains ring B (sinnamoil), which resonates at $\delta_H$ = 7.47 ppm (d, $J$ = 6.88 Hz) H-6' protons and at $\delta_H$ = 6.93 ppm H-2' proton, H-8 proton on ring A (benzoil) at $\delta_H$ = 6.88 ppm singlet, H-3 proton on ring C at $\delta_H$ = 6.75 singlet. Analyzing the spectrums of $^{13}$C-NMR were found to contain seventeen C atoms, ten of these quaternary C, five of methine C, two of methyl C, which substituted on C-4' and C-7 carbons, $\delta_C$ = 56.8 ppm and $\delta_C$ = 60.4 ppm respectively. In HETCOR spectrum of Compound **2** that shows C-H

interactions, H-3 ($\delta_H$ = 6.75 ppm) and H-8 ($\delta_H$ = 6.88 ppm) protons have been found to correlate with C-3 ($\delta_C$ = 103.1 ppm) and C-8 ($\delta_C$ = 91.9), respectively. Showing interactions of carbon-proton distance up to four bonds in HMBC spectrum ring B substituted on C-2 carbon was found that entry to the interaction distance with H-2', H-6' and H-3. The chemical structure is thus concluded as the compound of 5,6,3'-trihydroxy-7,4'-dimethoxyflavone (Figure 3). It is fully compatible with literature values (34).

Compound **3** was obtained as a white hulking powder resembling shredded coconut as a physical appearance. Spectrum of HPLC-TOF/MS of compound **3** showed a molecular ion peak at m/z 325.1471 $[M-H]^-$, which is resulting from molecule formula of $C_{15}H_{18}O_8$. The melting point of the compound was found to be 232 °C. Functional groups in the IR spectrum showed absorption bands for hydroxyl (3472 $cm^{-1}$) and benzene (1492 and 1552 $cm^{-1}$). The $^1$H-NMR spectrum of the compound **3** on the aromatic ring protons were observed at $\delta_H$ = 7.08 d ($J$ = 8.6) and $\delta_H$ = 7.52 d ($J$ = 8.6) on account of H-2/H-6 and H-3/H-5, respectively. The chemical shifts of the anomeric proton was determined at 5.53 ($J$ = 7.4) ppm in doublet form. $^{13}$C-NMR spec-

**Table1**. $^1$H and $^{13}$C NMR data for compounds **1-3** (in DMSO-$d_6$; $^{13}$C: 100 MHz; $^1$H: 400 MHz)

| | **Compound 1** | | **Compound 2** | | **Compound 3** | | |
|---|---|---|---|---|---|---|---|
| C/H | $\delta_C$ | $\delta_H$ (Hz) | $\delta_C$ | $\delta_H$ (Hz) | C/H | $\delta_C$ | $\delta_H$ (Hz) |
| $C_2$ | 164.9 | | 164.7 | | $C_1$ | 130.3 | |
| $C_3$ | 103.6 | 6.76 s | 103.1 | 6.75 s | $C_2$ | 130.3 | 7.52 d (*J*=8.6) |
| $C_4$ | 182.3 | | 182.6 | | $C_3$ | 116.8 | 7.08 d (*J*=8.6) |
| $C_5$ | 161.5 | | 146.2 | | $C_4$ | 159.0 | |
| $C_6$ | 99.9 | 6.45 s (*J*=1.9) | 129.1. | | $C_5$ | 116.8 | 7.08 d (*J* =8.6) |
| $C_7$ | 163.4 | | 153.0 | | $C_6$ | 130.3 | 7.52 d (*J*=8.6) |
| $C_8$ | 95.1 | 6.79 s (*J*=1.9) | 91.9 | 6.88 s | $C_\alpha$ | 127.9 | 6.32 d (*J*= 15.6) |
| $C_9$ | 157.4 | | 150.3 | | $C_\alpha$ | 144.4 | 7.75 d (*J*= 15.6) |
| $C_{10}$ | 105.7 | | 105.0 | | C=O | 166.5 | |
| $C_{1'}$ | 121.8 | | 121.9 | | *Glucose* | | |
| $C_2$' | 113.9 | 7.42 d (*J*=2.0) | 113.9 | 6.93 | $C_{1'}$-H | 99.4 | 5.53 d (*J*=7.4) |
| $C_{3'}$ | 146.2 | | 150.3 | | $C_{2'}$-H | 73.4 | 5.05 |
| $C_{4'}$ | 150.4 | | 152.5 | | $C_{3'}$-H | 74.0 | 4.22 |
| $C_{5'}$ | 116.4 | 6.91 d (*J*=8.4) | 113.9 | 6.76 d (*J*=6.9) | $C_{4'}$-H | 71.4 | 4.11 |
| $C_{6'}$ | 119.6 | 7.46 dd (*J*=8.4 *J*= 2.0) | 119.5 | 7.47 d (*J*=6.9) | $C_{5'}$-H | 77.1 | 4.28 dd (*J*=7.1/2.3) |
| -$OCH_3$ | | | 60.4 | | $C_{6'}$-H | 64.7 | 4.31 dd (*J*=11.8/7.1) |
| -$OCH_3$ | | | 56.8 | | | | 4.11 dd (*J*=11.8/2.3) |
| 4'-OH | | 10.41 s | | | | | |
| 3'-OH | | 12.98 s | | 12.95 s | | | |
| 5-OH | | 9.47 s | | 9.45 s | | | |
| 6-OH | | | | 10.02 s | | | |
| *Glucose* | | | | | | | |
| 1" | 100.2 | 5.07 d (*J*=7.6) | | | | | |
| 2" | 76.1 | 3.55 | | | | | |
| 3" | 69.4 | 3.27 | | | | | |
| 4" | 71.1 | 3.91 | | | | | |
| 5" | 76.2 | 3.46 | | | | | |
| 6" | 61.0 | 3.73 d ( *J*=8.1) | | | | | |
| | | 3.45 d ( *J*=8.1) | | | | | |

trum showed the resonance of fifteen carbon atoms. Of these, the carbonyl, α and β carbons detected at $\delta_C$= 166.5 ppm, $\delta_C$ = 127.9 ppm and $\delta_C$ = 144.4 ppm respectively. The glucose moiety carbons were resonanced in the range of $\delta_C$= 64.7 to $\delta_C$= 99.4 ppm. The glucose moiety substituted on carboxyl group was determined from HMBC where carbonyl carbons (C=O; $\delta_C$= 166.5 ppm) interacts with anomeric protons at 5.53 ppm ($J$ = 7.4). Based on these findings the compound **3** was identified as (E)-p-Coumaroyl-*O*-β-D-glucoside (Figure 3). The spectral data (MS, NMR, IR) were compared with the value included in the previous literature the results were found to be compatible (35).

*Real-Time Cell Proliferation Assays*

To monitor the real-time kinetic responses and dynamic growth of cancer cells against the added samples (crude extract, sub-fractions and isolated compounds), the cells were allowed to enter log growth phase by continuously monitoring with real-time cell analysis system (xCELLigence). These phases are formed at different times in each experiment: For the crude extract one hour later, for sub-fractions twenty-five hour later, for isolated compounds fourtyhour later. For this process HeLa and C6 cells were seeded onto a 96 well E-plate and monitored in real-time until composed stage of the log growth phase, at which point samples were added at different concentrations. After interaction of the cells with samples, changes in cell numbers were recorded instantly as Cell Index (CI). RTCA (xCELLigence, SP, ACEABIO, USA) with microelectronic impedance sensors were developed to measure living cell characteristics such as cell growth, adhesion, differentiation, migration, cytotoxicity, receptor tyrosine kinase activation (36), G protein-coupled receptor–ligand interactions (37), host–pathogen interactions (38) and β-cell injury (39). Proliferation assays conducted with HeLa and C6 cancer cell lines at different concentrations (250 and 500 **µg/mL**) of the crude extract of *T. chamaedrys* L. subsp. *sinuatum* have demonstrated significant antiproliferative activity in compared with control and 5-fluorouracil (5-FU) that is anticancer agent (Figure 4A). Growth graphs are obtained by Real-Time Cell Analyzer (xCELLigence System) specially for each cell (HeLa and C6) while the rapid proliferation observed in HeLa cells, which shows that cell adhesion was immediately, this was a little slowly in C6 cells ($p$ < 0.05).

Non-polar sub-fractions (HD1 and HD5) exhibited considerable antiproliferative effect as compared with the control. Sub-fractions HD5 showed the highest anticancer activity against the HeLa cell line at both concentrations while HD3 and HD4 against C6 cells at the concentration of 250 µg/mL and 500 µg/mL respectively. This case showed us that increase

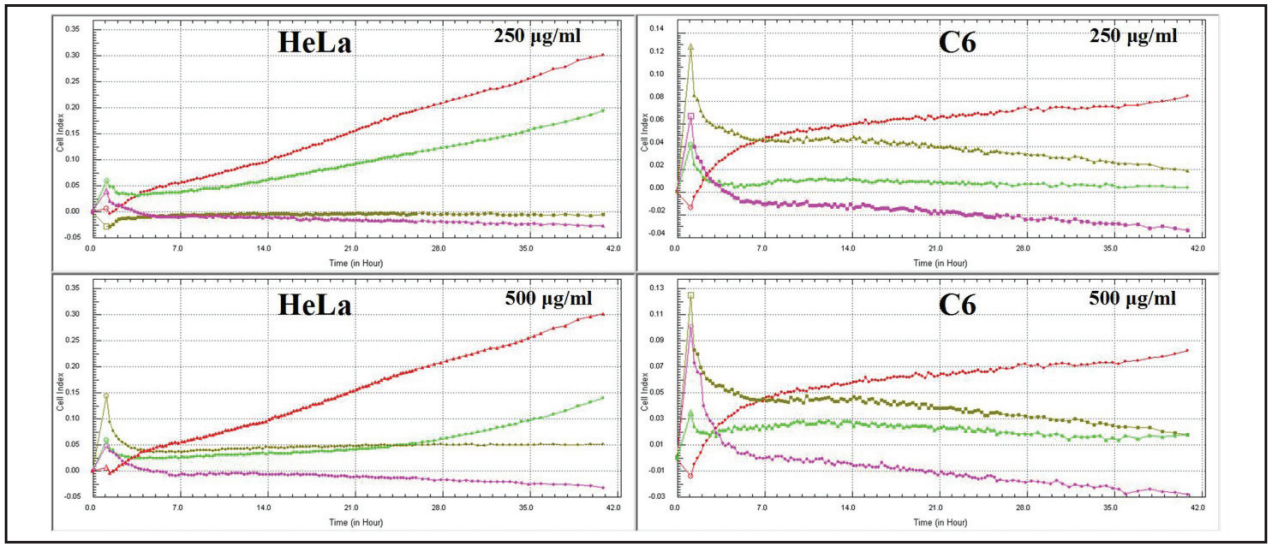


**Figure 4a**.The proliferation assays of the crude extracts of *T. chamaedrys* subsp. *sinuatum* [■TCS extract ■DMSO ■5-FU ■Ctrl]

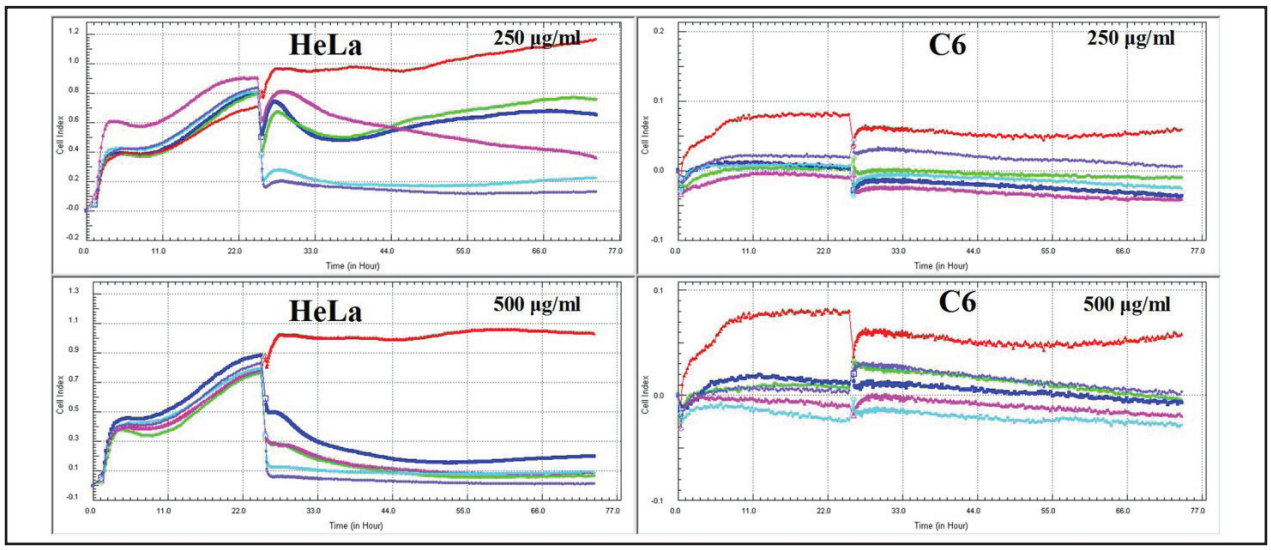


**Figure 4b**. The proliferation assays of HD1 and HD5 sub-fractions of the crude extracts of T. chamaedrys subsp. sinuatum [■HD1■HD2 ■HD3 ■HD4 ■HD5 ■Ctrl]

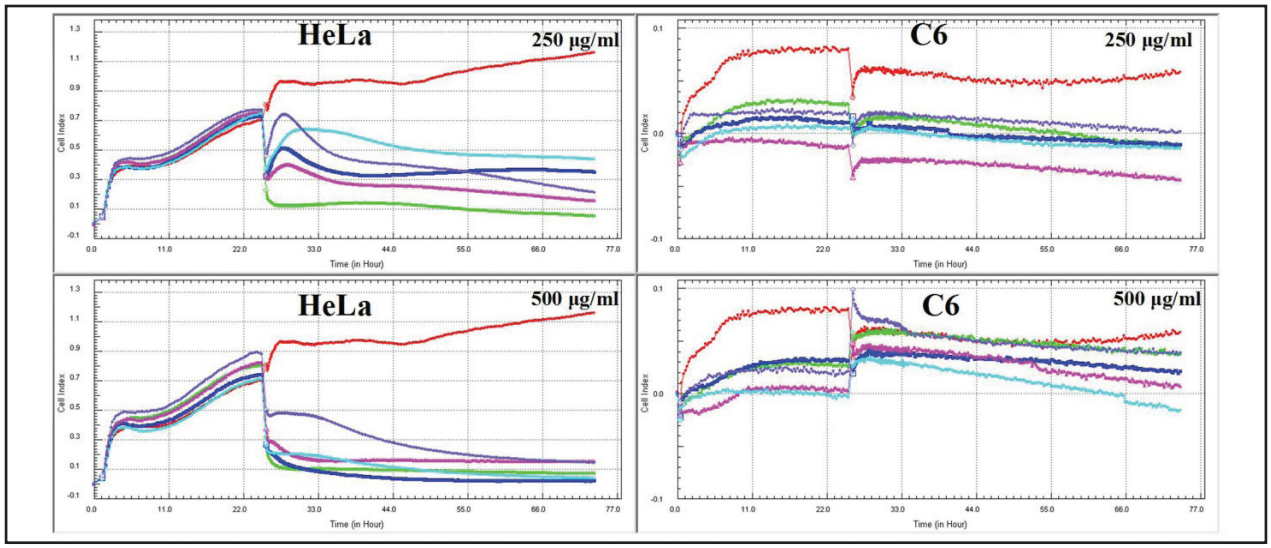


**Figure 4c**. The proliferation assays of DE1 and DE5 sub-fractions of the crude extracts of T. chamaedrys subsp. sinuatum [■DE1■DE2 ■DE3 ■DE4 ■DE5 ■Ctrl]

antiproliferative activity in parallel to the increase in polarity gradient of hexane: dichloromethane. This was proved as the sub-fractions HD1 and HD2 showed relatively weak activity when compared with sub-fractions HD3, HD4 and HD5 at concentration of 250 µg/mL against HeLa cells (Figure 4B).

While the sub-fractions DE1, DE2, DE3 and DE4 showed strong antiproliferative activity in mid-polarity gradient DE5 showed weak activity partially against both cell lines (Figure 4C). Increase or decrease in the cell index value of cell index is related to adhesion of the cells in each well. Higher CI values means greater cell adhesion.

Conversely, as cells die, they reduce adhesion, thus reduction in the Cell Index levels are seen. The addition of ME7 from polar sub-fractions showed a sharp decrease in the value of CI in particular against HeLa cells at a concentration of 250 µg/mL compared to the

other sub-fractions (Figure 4D). These results showed that usual of the polar sub-fractions were further evidence of cytotoxic effects, according to the non-polar sub-fractions ($p < 0.05$). All graphs showing the number of cells were obtained from the xCELLigence software as real-time changing time intervals.

The portions of ME1U, ME2U, ME1A and ME2A that were obtained by preparative thin layer chromatography showed quite different anticancer activities ($p < 0.05$). As shown in Figure 4E, portions of ME1U and ME2U showed a sharp decrease in the cell index against the HeLa cell line.

Dose-dependent antiproliferative effects of the isolated compounds were examined at three different concentrations (10, 50 and 100 µg/mL). Compound **2** showed the highest anti-proliferative activity in all concentrations against both HeLa and C6 cell lines with the exception of that C6 cell line at the concentration of 10 µM at which Compound **1** showed the highest anti-proliferative activity ($p < 0.05$). Other isolated

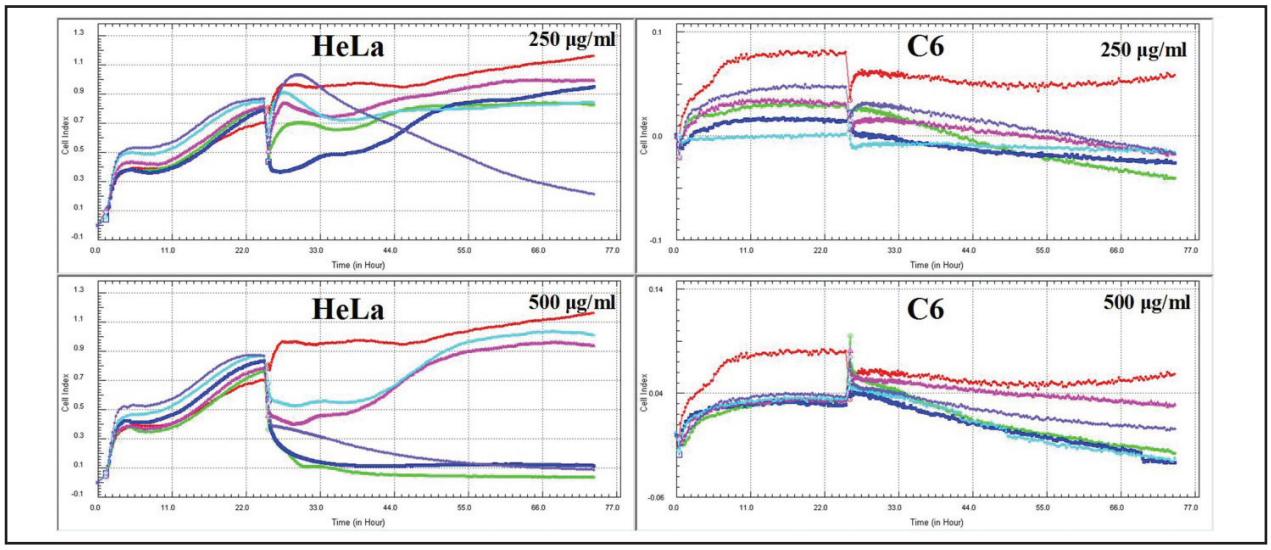


**Figure 4d**. The proliferation assays of ME3 and ME7 sub-fractions of the crude extracts of T. chamaedrys subsp. sinuatum [■ME3■ME4 ■ME5 ■ME6 ■ME7 ■Ctrl]

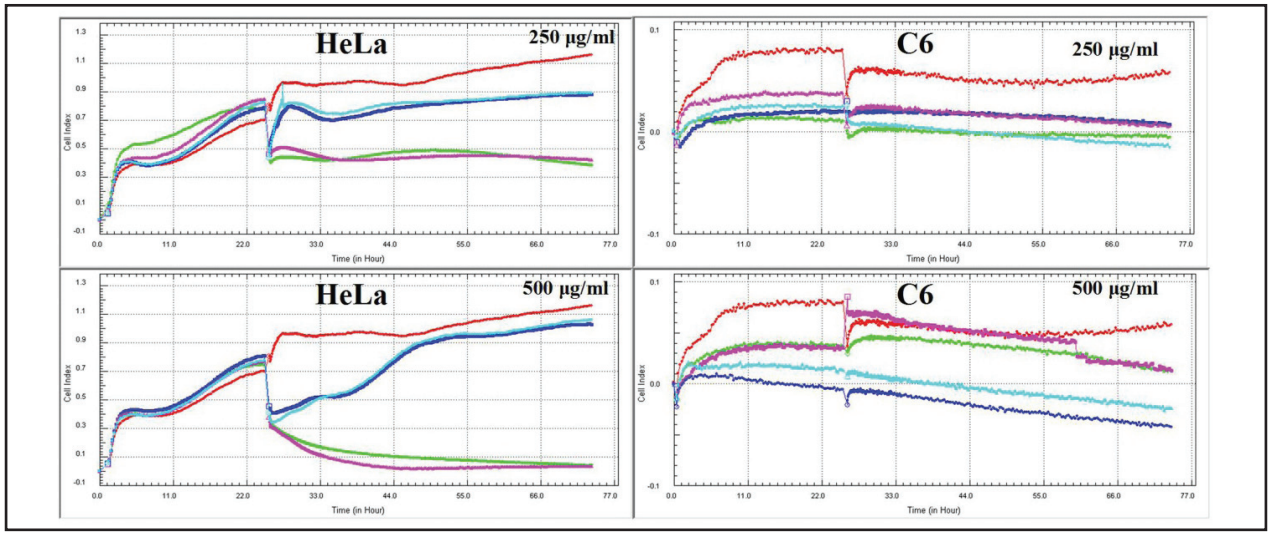


**Figure 4e**. The proliferation assays of ME1U, ME1A, ME2U and ME2A sub-fractions of the crude extracts of T. chamaedrys subsp. sinuatum [■ME1U ■ME1A ■ME2U ■ME2A ■Ctrl]

compounds also exhibited significant anti-proliferative activity when compared to control and 5-FU (Figure 4F). One of the privileges of the xCELLigence System is that adhesion and proliferation data in the same wells can be monitored in real time. For example, as shown in Figure 4F, process that is seen in the first four hour period can be named as adhesion, while the process in the next succeeding period as recovered data of cell proliferation. As demonstrated by the previous cytotoxicity assays, information about the status of the cell morphology can be obtained after an expose to a toxic substance (40,41). But the instant information of cells, such as proliferation, death, adhesion and morphology is obtained by xCELLigence system; this characteristic makes this system superior to single-point assays (42). The features mentioned above make the xCELLigence system an appropriate evaluation method for testing the response of cells to toxic substances (43,44).

The effects of TCS extract and isolated compounds 1 and 3 against the growth of PC3 cells did not exhibit cytotoxicity at neither of the tested concentrations; however, the cytotoxicity increased with increasing concentration from 10 to 50 and 100 µg/mL for compound **2**. Results showed excellent agreement with the activity of HeLa and C6 cells against those isolated compounds ($p < 0.05$). The activity may be due to the methoxy groups rather than glucoside bonded to flavone. The methoxy-substituted flavone exhibited the highest activity; glycosylation seems to be ineffective on the antiproliferative activity as seen in compounds 1 and 3 (Figure 4G). The most active compound was the 5,6,3'-trihydroxy-7,4'-dimethoxyflavone (compound **2**), followed by its 3-glycoside derivative luteolin-7-*O*-glucoside (compound **1**), and (E)-p-coumaroyl-O-β-D-glucoside (compound **3**). In general, the most active flavones are methoxy-substituted ones; glycosylation seems to be ineffective on the biological activity (45).

In our continuous endeavor to enhance the activity studies on natural products (46,47), we will continue with a great curiosity to explore antiproliferative activities of the medicinal plants especially the ones that are specific to the eastern region of Turkey, natural products

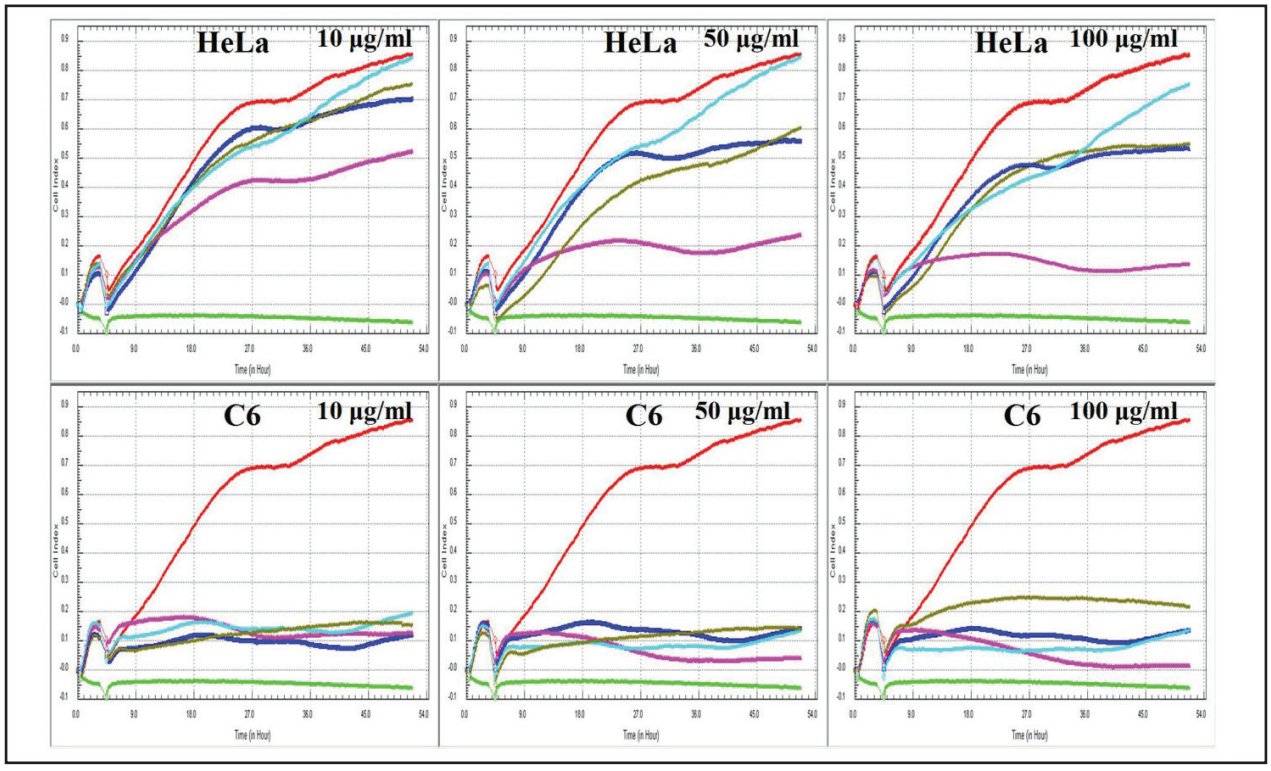


**Figure 4f**. Anti-proliferative effects of isolated compounds on HeLa and C6 cells. Proliferation of the cells monitored by xCELLigence 54-hour in a period of time [■Cmpd 1 ■Cmpd 2 ■Cmpd 3 ■5-FU ■Ctrl ■Medium]

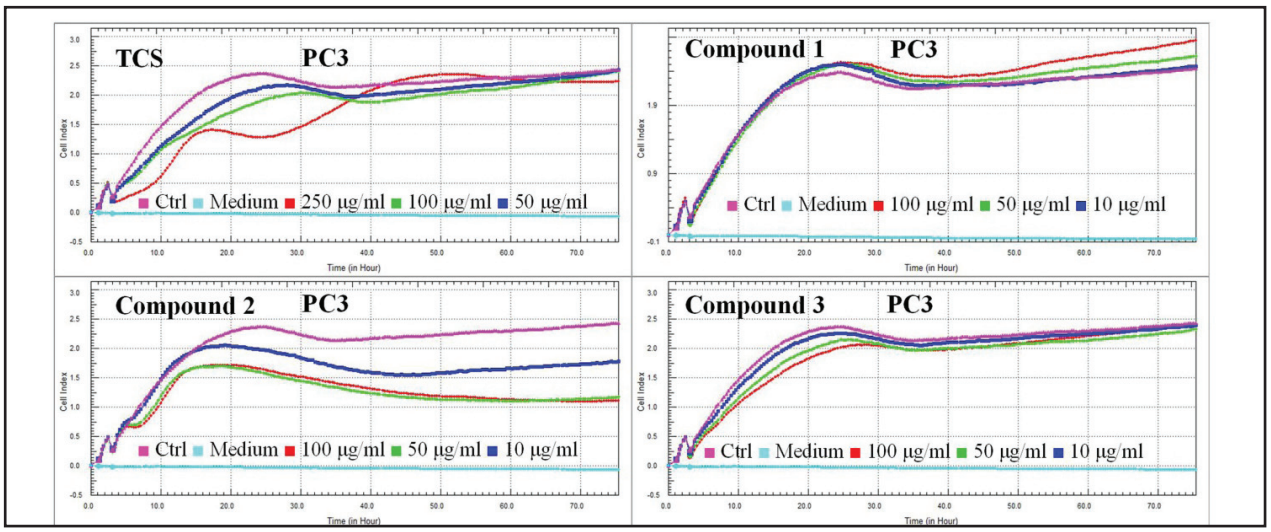


**Figure 4f**. Anti-proliferative effects of isolated compounds on HeLa and C6 cells. Proliferation of the cells monitored by xCELLigence 54-hour in a period of time [Cmpd **1** Cmpd **2** Cmpd **3** 5-FU Ctrl Medium]

that we isolated from them and not imparted to literature with new technologies.

Stankovic and his research group reported that extracts from different *Teucrium* species caused cytotoxicity and induced apoptosis on the selected malignant cell lines: HCT-116, HeLa, Fem-x, K562 and MDA-MB-361 cells (14,49). For these purposes, it is important to evaluate the antiproliferative effects of *T. chamaedrys* subsp. *sinuatum* and its individual bioactive compounds on HeLa and C6 cancer cells in vitro. We also choose later to work PC-3 prostate cancer cells which are very invasive, poorly differentiated and have a mesenchymal phenotype in order to better evaluate anticancer activity of pure compounds (49,50). Therefore, this cell line is an excellent choice to study several anti-cancer features of a candidate compound. Flavonoids have several anti-cancer activities including carcinogen inactivation, induction of apoptosis, antioxidation, and reversal of the drug resistance (51). Our results suggest the important function of *T. chamaedrys* subsp. *sinuatum* in cancer therapy, subfraction and pure bioactive compounds from this species may prevent proliferation of selected tumor cells.

## Conclusion

The bio-guided fractionation of *T. chamaedrys* subsp. *sinuatum* resulted in isolation of bioactive compounds, namely luteolin-7-*O*-glucoside, 5,6,3'-trihydroxy-7,4'-dimethoxyflavone and (E)-*p*-Coumaroyl-*O*-β-D-glucoside were isolated for the first time from TCS. The cytotoxic effects of the sub-fractions and pure compounds, obtained by bio-guided fractionation of TCS, against HeLa, C6 and PC3 cells were determined using the impedance-based xCELLigence system. Among all the tested compounds 5,6,3'-trihydroxy-7,4'-dimethoxyflavone was highly effective at all concentrations compared to other compounds and 5-FU. This study provided some useful references for the further research on *T. chamaedrys* L. subsp. *sinuatum* and the screening of leading anticancer compounds from traditional medicines.

## Conflict of interest

The authors confirm that this article content has no conflict of interest.

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## References

1. Ekim T. Teucrium L. In: Davis PH. editor. Flora of Turkey and east Aegean islands. Edinburgh University Press. 1982; 7: 53-75.
2. Ulubelen A, Topcu G, Sonmez U. Chemical and biological evaluation of genus Teucrium. St. Nat. Prod. Chem. 2000; 13: 591–648.
3. Menichini F, Conforti F, Rigano D, Formisano C, Piozzi F, & Senatore F. Phytochemical composition, anti-inflammatory and antitumour activities of four Teucrium essential oils from Greece. Food Chem. 2009; 115: 679-686.
4. Hasani-Ranjbar S, Nayebi N, Larijani B, & Abdollahi M. A systematic review of the efficacy and safety of herbal medicines used in the treatment of obesity. Int. J. Pharm. 2010; 6: 315–325.
5. Bruno M, Rosselli S, Maggio A, Piozzi FL, Scaglioni NA, Arnold NE, Simmonds MSJ. Neoclerodanes from Teucrium. orientale. Chem. Pharm Bull. 2004; 52: 1497-1500.
6. Erenler R, Telci I, Ulutas M, Demirtas I, Gul F, Elmastas M, & Kayir O. Chemical Constituents, Quantitative Analysis and Antioxidant Activities of Echinacea purpurea (L.) Moench and Echinacea pallida (Nutt.) Nutt. J. Food Biochem. 2015; 39: 622-630.
7. Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981-2002. J. Nat. Prod. 2003; 66: 1022–1037.
8. Balunas MJ, Kinghorn AD. Drug discovery from medicinal plants. Life Sci. 2005; 78: 431–441.
9. Gordaliza M. Natural products as leads to anticancer drugs. Clin. Tr. Oncol. 2007; 9: 767–776.
10. Pierini R, Gee JM, Belshaw NJ, & Johnson IT. Flavonoids and intestinal cancers. Brit. J. Nutr. 2008; 99: ES53–ES59.
11. Loa J, Chow P, & Zhang K. Studies of structure–activity relationship on plant polyphenol-induced suppression of human liver cancer cells. Cancer Chemoth. Pharm. 2009; 63: 1007–1016.
12. Böhm H, Boeing H, Hempel J, Raab B, & Kroke A, Flavonols, flavones and anthocyanins as native antioxidants of food and their possible role in the prevention of chronic diseases. Eur. J. Nutr. 1998; 37: 147–163.
13. Demirtas I, Gecibesler IH, Yaglioglu AS, Antiproliferative activities of isolated flavone glycosides and fatty acids from Stachys byzantine. Phytochem. Lett. 2013; 6: 209–214.
14. Stankovic MS, Curcic MG, Zizic JB, Topuzovic, MD, Solujic SR, Markovic SD, Teucrium plant species as natural sources of novel anticancer compounds: antiproliferative, proapoptotic and antioxidant properties. Int. J. Mol. Sci. 2011; 12: 4190–4205.
15. Coll J, & Tandrón YA. Isolation and structure elucidation of three neoclerodane diterpenes from Teucrium fruticans L. (Labiatae). Phytochem. 2005; 66: 2298-2303.
16. Piozzi F, Bruno M, Rosselli S, & Maggio A. Advances on the chemistry of furano-diterpenoids from Teucrium genus. Heterocycles. 2005; 65: 1221-1234.
17. Stankovic MS, Topuzovic M, Solujic S, Mihailovic V. Antioxidant activity and concentration of phenols and flavonoids in the whole plant and plant parts of Teucrium chamaedrys L. var. glanduliferum Haussk. J. Med. Plant Res. 2010; 4: 2092–2098.
18. Twentyman P, and Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br. J. Cancer. 1987; 56: 279-285.
19. Fotakis G, and Timbrell JA. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol. Lett. 2006; 160: 171-177.
20. Borenfreund E, and Puerner JA. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 1985; 24: 119-24.
21. Weyermann J, Lochmann D, and Zimmer A. A practical note on the use of cytotoxicity assays. Int. J. Pharm. 2005; 288: 369-376.
22. Boyd JM, Huang L, Xie L, Moe B, Gabos S, and Li XF. A cell-microelectronic sensing technique for profiling cytotoxicity of chemicals. Anal. Chim. Acta. 2008; 615: 80-87.
23. Bird C, and Kirstein S. Real-time, label-free monitoring of cellular invasion and migration with the xCELLigence system. Nat. Methods. 2009; 6: 5-6.
24. Urcan E, Haertel U, Styllou M, Hickel R, Scherthan H, and Reichl FX. Real-time xCELLigence impedance analysis of the cytotoxicity of dental composite components on human gingival fibroblasts. Dent. Mater. 2010; 26: 51-58.
25. Zhang LL, Li-Na MA, Dan YAN, Zhang CE, Dan GAO, Xiong Y, & Xiao-He XIAO. Dynamic monitoring of the cytotoxic effects of protoberberine alkaloids from Rhizoma Coptidis on HepG2 cells using the xCELLigence system. Chinese J. Nat. Med. 2014; 12: 428-435.
26. Otero-González L, Sierra-Alvarez R, Boitano S, and Field JA. Application and validation of an impedance-based real time cell analyzer to measure the toxicity of nanoparticles impacting human bronchial epithelial cells. Environ. Sci. Technol. 2012; 46: 10271-10278.
27. Irelan JT, Wu MJ, Morgan J, Ke N, Xi B, Wang X, Xu X, and Abassi YA, Rapid and quantitative assessment of cell quality, identity, and functionality for cell-based assays using real-time cellular analysis. J. Biomol. Screen. 2011; 16: 313-322.
28. Steinbach SK, El-Mounayri O, Dacosta RS, Frontini MJ, Nong Z, Maeda A, Pickering JG, Miller FD, and Husain M. Directed differentiation of skin-derived precursors into functional vascular smooth muscle cells. Arterioscler Thromb. Vasc. Biol. 2011; 31: 2938-2948.

29. Dreesen O, Chojnowski A, Ong PF, Zhao TY, Common JE, Lunny D, Lane EB, Lee SJ, Vardy LA, and Stewart CL. Lamin B1 fluctuations have differential effects on cellular proliferation and senescence. J. Cell Biol. 2013; 200: 605-617.
30. Hossain MB, Barry-Ryan C, Martin-Diana AB, and Brunton NP. Optimisation of accelerated solvent extraction of antioxidant compounds from rosemary (Rosmarinus officinalis L.), marjoram (Origanum majorana L.) and oregano (Origanum vulgare L.) using response surface methodology. Food Chem. 2011; 126: 339-346.
31. Atienza JM, Yu N, Kirstein SL, Xi B, Wang X, Xu X, and Abassi YA, Dynamic and label-free cell-based assays using the real-time cell electronic sensing system. Assay Drug Dev. Technol. 2006; 4: 597-607.
32. Xing JZ, Zhu L, Gabos S, Xie L, Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity. Toxicol In Vitro. 2006; 20: 995-1004.
33. Lin YP, Chen TY, Tseng HW, Lee MH, Chen ST. Neural cell protective compounds isolated from Phoenix hanceana var. formosana. Phytochem. 2009; 70: 1173–1181.
34. Nagao T, Abe F, Kinjo J, and Okabe H. Antiproliferative Constituents in Plants 10. Flavones from the Leaves of Lantana montevidensis BRIQ. and Consideration of Structure–Activity Relationship. Biol .Pharm. Bull. 2002; 25: 875-879.
35. Katsuragi H, Shimoda K, Yamamoto R, Ishihara K, and Hamada H. Glycosylation of Capsaicin Derivatives and Phenylpropanoid Derivatives Using Cultured Plant Cells. Biochem. Ins. 2011; 4: 1–12.
36. Atienza JM, Yu N, Wang X, Xu X, Abassi Y. Label-free and real-time cellbased kinase assay for screening selective and potent receptor tyrosine kinase inhibitors using microelectronic sensor array. J. Biomol. Screen. 2006; 11: 634–643.
37. Abassi YA, Jackson JA, Zhu J, O'connell J, Wang X, Xu X. Label-free, real time monitoring of IgE-mediated mast cell activation on microelectronic cell sensor arrays. J. Immunol. Methods. 2004; 292: 195–205.
38. Mou X, Wan S, Li Y, Zhang S, Sun M, Liu F, et al. Phenotypic pattern-based assay for dynamically monitoring host cellular responses to Salmonella infections. PLoS One. 2011; 6: 1-12.
39. Guan N, Gao W, He M, Zheng M, Xu X, Wang, X, Wang MW. Dynamic monitoring of β-cell injury with impedance and rescue by glucagons-like peptide-1. Anal. Biochem. 2012; 423: 61–69.
40. Willingham MC, Cytochemical methods for the detection of apoptosis. J. Histochem. Cytochem. 1999; 47: 1101-1109.
41. Barrett KL, Willingham JM, Garvin AJ, Willingham MC. Advances in cytochemical methods for the detection of apoptosis. J. Histochem. Cytochem. 2001; 49: 821-832.
42. Kirstein SL, Atienza, J.M., Xi, B., Zhu, J., Yu, N., Wang, X., et al., Live cell quality control and utility of real-time cell electronic sensing for assay development. Assay Drug Dev. Technol. 2006; 4: 545-553.
43. Xing JZ, Zhu L, Jackson JA, Gabos S, Sun XJ, Wang XB, and Xu X, Dynamic monitoring of cytotoxicity on microelectronic sensors. Chem. Res. Toxicol. 2005; 18: 154-161.
44. Zhu J, Wang X, Xu X, Abassi YA. Dynamic and label-free monitoring of natural killer cell cytotoxic activity using electronic cell sensor arrays. J. Immunol. Methods. 2006; 309: 25-33.
45. Walle T. Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass?. Semin. Cancer. Biol. 2007; 17: 354–362.
46. Demirtas I, Sahin A, Ayhan B, Tekin S, and Telci I, Antiproliferative Effects of the Methanolic Extracts of Sideritis libanotica Labill. subsp. Linearis. Rec. Nat. Prod. 2009; 3: 104-109.
47. Gecibesler IH, Demirtas I, & Koçak A, Based on the Quantity, Temperature and Time, The Examination of Variability in Odorous Components of Tanacetum abrotanifolium from Turkey: An Exclusive Gradient Work. J. Essent. Oil Bear. Pl. 2015; 18: 840-843.
48. Stankovic MS, Mitrovic TL, Matic IZ, Topuzovic MD, and Stamenkovic SM, New values of Teucrium species: In vitro study of cytotoxic activities of secondary metabolites. Not. Bot. Horti. Agrobot. Cluj. Napoca. 2015; 43: 41-46.
49. Kandouz M, Alachkar A, Zhang L, Dekhil H, Chehn AF, Yasmeen A, and Al Moustafa AE. Teucrium polium plant extract inhibits cell invasion and motility of human prostate cancer cells via the restoration of the E-cadherin/catenin complex. J. Ethnopharmacol. 2010; 129: 410-415.
50. Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang CZ, and Huang J. PC3 is a cell line characteristic of prostatic small cell carcinoma. Prostate. 2011; 71: 1668-1679.
51. Chahar MK, Sharma N, Dobhal MP, and Joshi YC. Flavonoids: A versatile source of anticancer drugs. Pharmacogn. Rev. 2011; 5: 1-12.

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