ORIGINAL ARTICLE

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Rec. Nat. Prod. 13:3 (2019) 189-198

records of natural products

Cytotoxic Activity-Guided Isolation Studies on *Fumana procumbens* (Dunal) Gren. & Godr.

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(Received July 19, 2018; Revised September 04, 2018; Accepted September 11, 2018)

Abstract: Among the plant secondary metabolites much attention has been given to naturally occurring phenolic compounds as attractive candidates for cancer treatment and prevention. *Fumana procumbens* (Dunal) Gren. & Godr (Cistaceae) is a perennial herb, distributed in inner and southwest Anatolia, Turkey. *F. procumbens* was shown to be rich in phenolic constituents especially flavonoids and biflavonoids which are known for their antioxidant and anticarcinogenic effects. Thus, this study aims to investigate the anticancer activity of *F. procumbens* extracts, fractions and pure compounds by MTT test against HeLa, MCF-7, and A549 as well as BEAS-2B cell lines for determining selectivity. Bioactivity-guided phytochemical investigation on *Fumana procumbens* (Dunal) Gren. & Godr. led to the isolation and identification of three biflavonoids: genkwanol A, dihydrodaphnodorin B and stelleranol. The structures were elucidated by extensive 1D- and 2D-NMR spectroscopic analysis in combination with MS experiments. This is the first report on the isolation of genkwanol A and stelleranol from the genus *Fumana* and from the family Cistaceae. As a result, no individual cytotoxic activity was obtained for biflavonoid compounds. However, the former fractions FrC and FrD as well as MeOH and *n*-BuOH extracts exerted moderate and significant cytotoxic activity which suggested a possible synergistic effect.

Keywords: Anticancer activity; Cistaceae; *Fumana procumbens*; cell viability; cytotoxicity; MTT test.

1. Introduction

Cancer is one of the main health problems which is characterized by uncontrolled growth and spread of abnormal cells. According to mortality data obtained from the National Center for Health Statistics (NCHS) cancer is the second leading cause of death after heart disease in both men and women in USA [1]. The World Health Organization (WHO) predicts about 15 million new incidents of cancer by 2020 [2]. Several types of cancer can be treated with chemotherapy, radiation therapy, hormonal therapy or surgery. Plant extracts and their pure compounds have been considered as a main source of potential chemotherapeutic treatments [3]. Indeed, medicinal plants and plant derived compounds have therapeutic and economical value in terms of drug discovery and development. Over

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The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products © May-June 2019 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.98.18.07.331

50% of drugs commercially used for anticancer therapy are derived from plants, such as polyphenols, brassinosteroids and taxols [4]. More to the point, a number of adverse effects may occur during chemotherapy, hence, natural therapies in cancer prevention and treatment may reduce side effects as more tolerable options.

Recently, phenolic compounds have attracted the attention of scientists owing to their diverse bioactivity potential. Phenolic compounds promote health benefits by reducing the incidence of degenerative diseases including cancer [5]. Flavonoids are from the polyphenolic compounds and they are physiologically active agents in plants and becoming of high interest for their health benefits [6].

One of the phenolic rich plant family, Cistaceae, comprises about 8 genera and 180 species of mainly Mediterranean plants. The species of this family have been used in traditional medicine as antimicrobial, antiulcerogenic, antidiarrheal, antirheumatic and vasodilator agents [7]. According to previous reports, Cistaceae family plants including *Cistus ladanifer* L., *Cistus populifolius* L. [8], *Cistus incanus* L., *Cistus monspeliensis* L.[9] and *Helianthemum lippii* (L.) Dum.Cours.[10] were demonstrated to have cytotoxic activity against several cancer cells. Another important but a small genus of Cistaceae is *Fumana* Dunal (Spach) genus, mainly distributed in the inner and coastal parts of Mediterranean countries. It is represented about 20 species on worldwide and with 10 species in the flora of Turkey [11, 12]. Few phytochemical data on the essential oil composition of *Fumana thymifolia* (L.) Verlot and methylated flavonol glycosides of *Fumana montana* Pomel. were reported. [13, 14]. In a recent study some flavonoid and biflavonoid compounds were isolated from the *F. procumbens* growing wild in Turkey [15]. Both flavonoid and biflavonoid type compounds have been shown to act as cytotoxic agents against different cancer cell lines as well as possess antigenotoxic activities [16, 17]. Therefore, this study aims to investigate the anticancer activity of *F. procumbens* with regard to its taxonomic relatedness with previously reported anticancer active species namely, *Cistus* and *Helianthemum*, and as a potential source of flavonoid and biflavonoid compounds. In the present study, according to activity-guided fractionation and isolation assay procedure, *in vitro* cytotoxic effect of the extracts, fractions and pure compounds of *F. procumbens* have been assessed by using MTT test, against HeLa, MCF-7, and A549 as well as BEAS-2B cell lines for determining selectivity.

2. Materials and Methods

2.1. Chemistry

2.1.1. General

NMR (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR) were measured on a Bruker AM 400 spectrometer and MS spectra on a LC/MS-MS Shimadzu 8040 instrument. Silicagel 60 (Merck, 70-230 mesh) was used for open column chromatography. Sephadex LH-20 (SP LH-20) (General Electric's Healthcare) was used for Gel Permeation Chromatography. LiChroprep RP-18 (Merck, 40-63 μm) was used for Flash Chromatography (Buchi). TLC analyses were carried out on pre-coated Kieselgel 60 F_{254} aluminum plates (Merck). Compounds were detected by UV fluorescence and spraying 30% H_2SO_4 in EtOH, followed by heating at 100 °C for 1-2 min.

2.1.2. Plant Material

Aerial parts of *F. procumbens* (Dunal) Gren. & Godr. were collected near the highway from Antalya 45 km to Isparta at about 1200 m altitude in June 2010. Plant species was identified by Dr. Mehmet Yavuz Paksoy (Munzur University, Tunceli, Turkey). The voucher specimen was deposited at the Herbarium of Hacettepe University, Faculty of Pharmacy (HUEF-10084).

2.1.3. Extraction & Isolation

Dried and powdered whole parts of *F. procumbens* (750 g) were extracted with 80% methanol (MeOH) (500 mL x 4) at 37°C. After the solvent evaporation (yield 4.6%), the crude MeOH extract (35 g) was partitioned between H_2O and *n*-butanol (*n*-BuOH). *n*-BuOH extract (16 g) was subjected to column chromatography on SP LH-20 (5 x 75 cm) and eluted with MeOH. Four main fractions (Fr. 1-4) [FrA: 4 g, FrB: 4.4 g, FrC: 4.9 g, FrD: 3.8 g] were obtained.

FrD (3.8 g) was subjected to SP LH-20 and eluted with MeOH to obtain three sub fractions. FrD1 (3 g) was subjected to Sigel column chromatography; eluted with a gradient cy-hexane:ethyl acetate:MeOH (10:10:1→10:10:5) solvent system yielding nine sub fractions.

Further purification of FrD1a (500 mg) was carried out on RP C18 column (1.5 cm x 15 cm) and eluted with H_2O:Acetonitrile (80:20→50:50) by using MPLC system coupled with a fraction collector (4-5 mbar, 10 mL/min) to give compound **3** (24 mg). FrD1c (197 mg) was subjected to SP LH-20 (2.5 x 50 cm) and eluted with MeOH to obtain compound **2** (7 mg). Further purification of FrD1d (400 mg) was performed on RP C18 column (1.5 cm x 15 cm) and eluted with H_2O:Acetonitrile (90:10→70:30) by using the same MPLC system used previously to give compound **1** (44 mg).

Genkwanol A (***1***): Yellow powder; Negative ESI/MS *m/z*: 541 $[M-H]^-$; ^{1}H NMR (400 MHz, (DMSO-d_6) δ 10.56 (s, 2H, Ph-OH), 9.96 (s, 1H, Ph-OH), 9.57 (s, 1H, Ph-OH), 9.29 (s, 1H, Ph-OH), 7.04 (d, *J*=8.3 Hz, 2H, H-10"/14"), 6.80 (d, *J*=8.3 Hz, 2H, H-2'/6'), 6.68 (d, *J*=8.3 Hz, 2H, H-11"/13"), 6.52 (d, *J*=8.4 Hz, 2H, H-3'/5'), 6.10 (s, 1H, H-6), 5.76 (s, 1H, H-6"), 5.63 (s, 1H, H-8"), 5.48 (s, 1H, H-2"), 5.14 (d, *J*=4.6 Hz, 1H, OH), 4.62 (d, *J*=6.8 Hz, 1H, H-2), 3.78–3.63 (m, 1H, H-3), 2.58 (dd, *J*=16.1, 4.4 Hz, 1H, H-4α), 2.40 (dd, *J*=16.0, 7.3 Hz, 1H, H-4β). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 193.29 (C-4"), 172.65 (C-7), 168.32 (C-8α"), 161.29 (C-5), 159.44 (C-5"), 158.01 (C-4'), 157.64 (C-7"), 156.69 (C-12"), 151.64 (C-8α), 129.47 (C-1'), 129.29 (C-10"/14"), 127.29 (C-2'/6'), 123.63 (C-9"), 115.19 (C-11"/13"), 114.81 (C-3'/5'), 102.98 (C-4α"), 102.63 (C-4α), 101.59 (C-8), 96.46 (C-6"), 94.39 (C-3"), 91.29 (C-2"), 89.90 (C-6), 89.53 (C-8"), 80.13 (C-2), 66.48 (C-3), 27.78 (C-4).

Stelleranol (***3***): Yellow powder; Negative ESI/MS *m/z*: 557 $[M-H]^-$; ^{1}H NMR (400 MHz, CD_3OD) δ 7.14 (d, *J*=8.6 Hz, 2H, H-2'"/H-6'"), 6.79 (d, *J*=8.6 Hz, 2H, H-3'"/5'"), 6.67 (d, *J*=8.6 Hz, 2H, H-3'/5'), 6.59 (d, *J*=8.7 Hz, 2H, H-2'/6'), 6.14 (d, *J*=2.1 Hz, 1H, H-6"*), 6.11 (d, *J*=2.1 Hz, 1H, H-8"*), 5.98 (s, 1H, H-2"), 5.71 (s, 1H, H-6), 4.92 (overlapped with the solvent signal, H-2), 4.11 (brs, 1H, H-3), 2.66 (d, *J*=17.4 Hz, 1H, H-4α), 2.52 (dd, *J*=17.0, 3.7 Hz, 1H, H-4β). ^{13}C NMR (100 MHz, CD_3OD) δ 190.73 (C-4"), 189.01 (C-5), 169.82 (C-7), 168.34 (C-7"), 160.82 (C-8"α), 159.37 (C-8α), 158.27 (C-5"), 156.55 (C-4', C-4'"), 129.34 (C-2'"/C-6'"), 128.04 (C-1'), 127.14 (C-2'/C-6'), 122.46 (C-1'"), 114.47 (C-3'"/C-5'"), 114.32 (C-3'/C-5'), 108.49 (C-4α), 100.35 (C-6), 99.88 (C-4"α), 96.90 (C-6"*), 96.47 (C-8"*), 90.97 (C-2"), 85.20 (C-8), 80.47 (C-2), 80.28 (C-3"), 64.31 (C-3), 26.29 (C-4). *interchangeable

2.2. Cytotoxic Activity

2.2.1. Cell Lines and Cell Culture

Human breast cancer cell line (MCF-7), human cervical cancer cell line (HeLa), adenocarcinomic human alveolar basal epithelial cell line (A549), and human bronchial epithelial cell line (BEAS-2B) were used for cytotoxicity test of crude extracts, fractions and its pure compounds. HeLa kindly provided by Prof. Dr. Mustafa Ark (Faculty of Pharmacy, Gazi University) and A549, MCF-7 and BEAS-2B kindly provided by Prof. Dr. Engin Ulukaya (Faculty of Medicine, Istinye University). Cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heated-inactivated fetal bovine serum, 1% L-glutamine and 1% penicillin–streptomycin. BEAS-2B cells were cultured in RPMI supplemented with 20% heated-inactivated fetal bovine serum, 1% L-glutamine and 1% penicillin–streptomycin. Cells were maintained at 37°C and in a 5% CO_2 atmosphere with 95% humidity. When cells reach to confluency, they were detached with trypsin-EDTA (0.05%).

2.2.2. Exposures

National Cancer Institute (NCI) guidelines recommended that promising anticancer substances should have an IC_{50} value ≤ 20 μg/mL and ≤ 4 μg/mL for extract and pure compounds, respectively [18]. Besides, the NCI has performed *in vitro* anticancer drug screening program at l00 μg/mL for natural product extracts or fractions as highest concentration [19]. In this context, crude extract and fractions were tested at 0.4-100 μg/mL with half-log dilution, while pure compounds were tested at 0.6-20 μg/mL in 6 concentration points. Extracts, fractions, and pure compounds were dissolved in dimethylsulfoxide (DMSO) at 0.2% (v/v) final concentration in the cell culture since no toxic effects on cell growth were observed at this concentration.

2.2.3. Cytotoxicity Test

The cytotoxic activity of these materials was determined by MTT assay. Cell suspensions in phenol red free medium at 6×10^3 cells/well were seeded into 96-well tissue culture plates at 200 μL/well for 24 h incubation. Then, the supernatant was removed and the cells were treated at 200 μL/well with determined concentrations of the extract, fractions or pure compounds in phenol red free medium in triplicate. Untreated and DMSO (0.2%) treated cells were used as negative and vehicle controls, respectively while doxorubicin (final concentrations; 0.3, 0.56, 1, 1.8, and 3 μg/mL) was used as a positive control. Each experiment was performed in triplicate (at least n=6). The plates were incubated with test chemicals for 48 h. After the incubation period, the supernatant was removed and 200 μL of fresh medium and 50 μL MTT solution (1 g/L) were added to the each well. The cells were incubated for 3 h at 37°C. The resulting formazan crystals were dissolved in 160 μL of DMSO and finally, the absorbance at 570 nm was evaluated using a spectrophotometer (SpectraMax i3x; Molecular Devices, San Jose, CA, USA). The reading absorbance values were converted to the percentage of the control. The percent of cell viability in the vehicle control was assumed 100.

2.3. Statistical Analysis

Data were analyzed using GraphPad Prism software (GraphPad Prism 7.0 (demo version), GraphPad Software, Inc., CA, USA). The significance of differences in means between the cytotoxic effects of each extract, fraction and pure compound at different concentrations on cells were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. Nonlinear regression analysis (dose-response) was used to determine the IC_{50} of doxorubicin. A value $p \leq 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Structure Elucidation

The structure of isolated compounds (Figure 1) were elucidated by 1D- and 2D- NMR analyses and comparison with literature data; genkwanol A (**1**) [20, 21],dihydrodaphnodorin B (**2**) [15, 22, 23], stelleranol (**3**) [24],

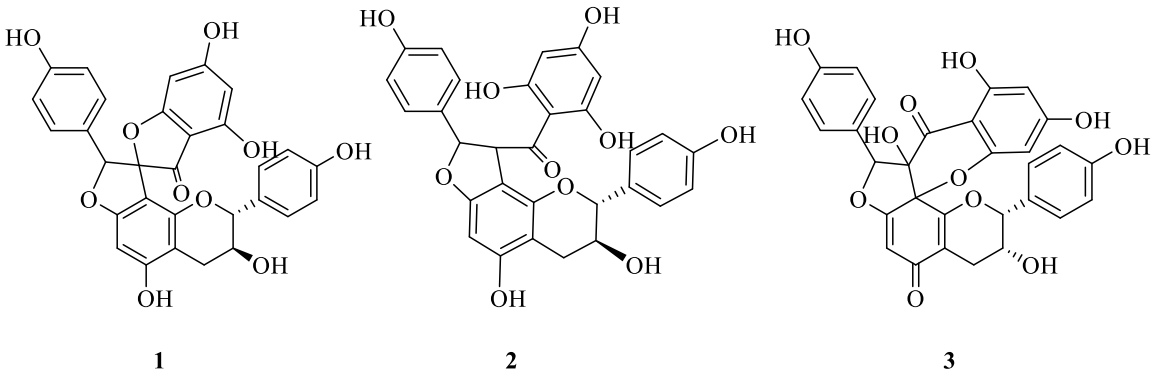

Figure 1. Structures of the isolated compounds (**1-3**)

3.2 Cytotoxicity

The extracts, fractions and pure compounds of *F. procumbens* were studied with the aim of finding new drugs capable of cytotoxic effect against breast, cervical and lung cancers. Although standard chemotherapeutics are not totally specific to cancer cells, it is strongly expected that an ideal anticancer agent should discriminate cancerous cells from normal cells. Therefore, selective anticancer activity of the compounds should be scrutinized thoroughly during development process. In this context, in the present study, the cytotoxic effect of *F. procumbens* on cancer cells were compared with that of normal BEAS-2B cells. Inhibition effects of the extracts, fractions and compounds on cell viability were determined for different concentrations by using MTT assay. 50% cell viability inhibition was considered as clinically significant for cytotoxicity. Doxorubicin which is a potent anticancer agent was used as a positive control. When the cells were treated with doxorubicin against HeLa, MCF-7, A549, and BEAS-2B cells, the IC_{50} values were determined as 1.41 ± 0.35, 1.52 ± 0.57, 1.29 ± 0.27, and 0.09 ± 0.01 µg/mL, respectively (Figure 2). The results of cytotoxic activity of extracts, fractions and three pure compounds of *F. procumbens* against four human cell lines (HeLa, MCF-7, A549 and BEAS-2B) are shown in Figure 3 and Figure 4.

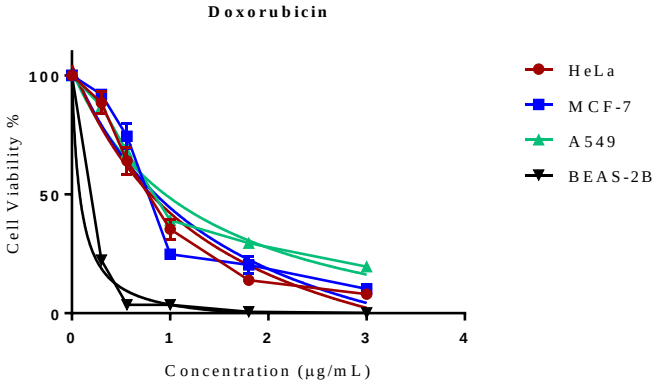

Figure 2. Effects of doxorubicin treatment in HeLa, MCF-7, A549, and BEAS-2B cells.

80% MeOH extract obtained from the plant was primarily assayed for cytotoxicity at 0.4, 1.2, 3.7, 11.1, 33.3, and 100 µg/mL concentrations in HeLa, MCF-7, and A549 cell lines. Clinically significant decline in the viability was only observed in HeLa cells at 100 µg/mL concentration which was resulted in 36.4% viability, compared to the vehicle control ($p<0.0001$). In parallel, MCF-7 cells treated with 80% MeOH extract displayed statistically but not clinically significant inhibition (86.3%) in cell viability at the highest dose ($p=0.0380$). Although 80% MeOH extract showed 75-80% cell

viability in A549 cells at all concentrations ($p<0.0001$), these declines were not dose dependent. On the other hand, 80% MeOH extract treated BEAS-2B cells showed higher cell viability (72%) compared to HeLa cells, therefore, we can conclude that 80% MeOH extract has a reasonable selectivity for cancer cells.

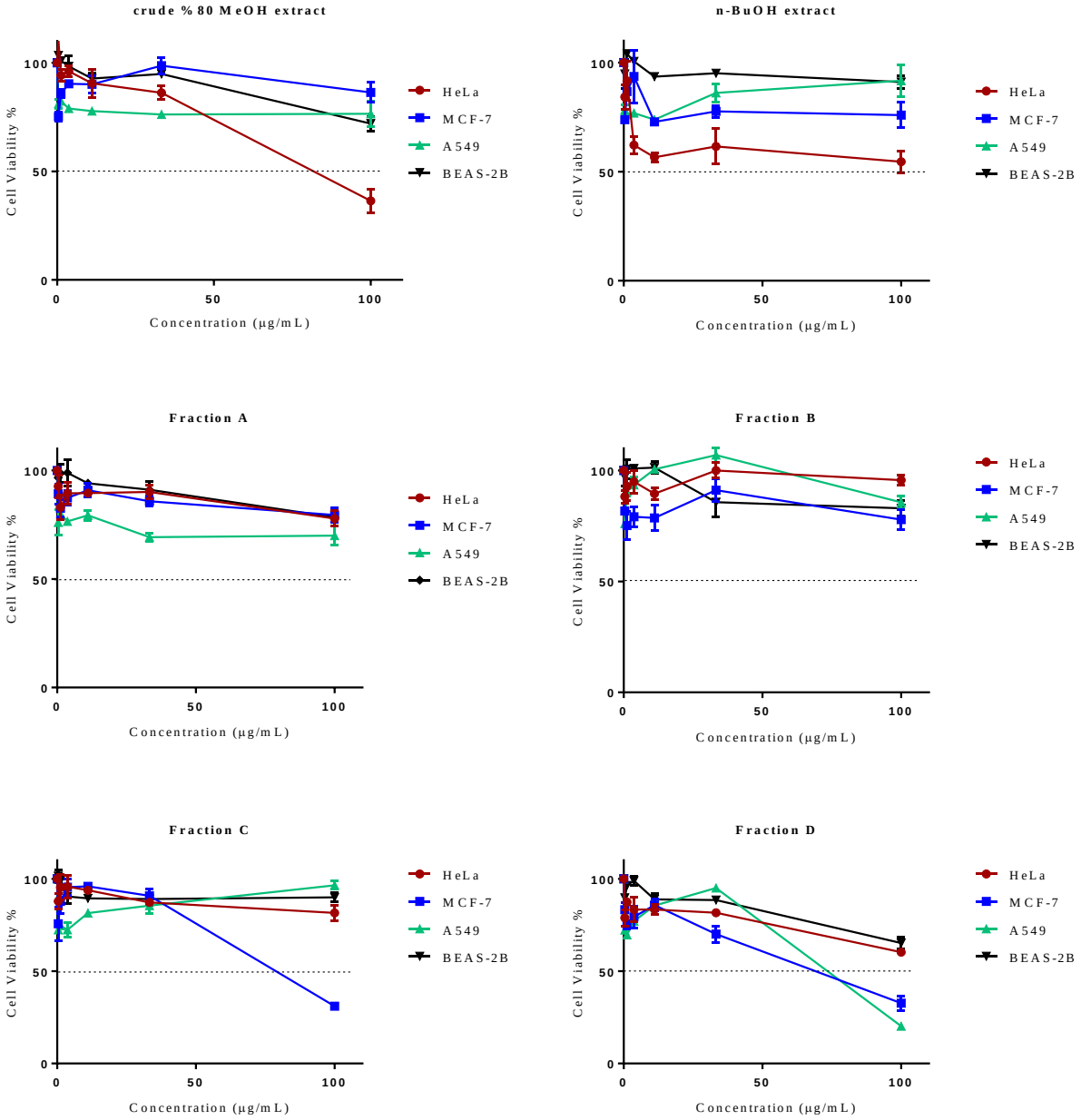

Figure 3. Cell viabilities (%) after 48 h exposure to extract and fractions of *F. procumbens* (The plot represents values of mean cell viability % ± SEM)

n-BuOH extract was assessed for cytotoxic activity at the same doses with 80%-MeOH extract and statistically significant decrease in cell viability was observed in HeLa and MCF-7 cell lines. The cell viability was between 54.6-62.2% at ≥ 3.7 µg/mL concentrations in HeLa cells. This decline was remarkable but not significant in terms of clinical threshold value. On the other hand, the cell viability started to decrease at ≥ 11 µg/mL concentrations ranging between 72.8% and 77.6% in MCF-7 cells. Although statistically significant decrease in cell viability up to 75% were observed for A549 cells exposed to the *n*-BuOH extract at lower doses, the response was not significant at the highest dose.

Although exposure to FrA caused a slight viability inhibition at the highest dose (69.9-79.4%) in all cancer cells, only A549 cells responded to all concentrations of FrA without dose dependent manner. After treatment with FrB, there was no clinically significant cytotoxic effect on HeLa cells while statistically significant loss in cell viability was detected at the highest dose in MCF-7 and A549 cells (77.9% and 85.7% respectively). According to the results of the present study, neither FrA nor FrB demonstrated promising cytotoxic effect.

The cell viability decreased to 31.1% after exposure to the highest dose of FrC on MCF-7 cells indicating a clinically significant cytotoxic effect ($p<0.0001$) and remarkable selectivity compared to 90% cell viability in BEAS-2B. In other cancer cells, exposure to FrC resulted in around 80% cell viability at which did not provide any significant anticancer activity.

Among fractions, the most promising cytotoxic effect was detected for FrD. The results indicated that FrD significantly inhibited the growth of MCF-7 cells at 100 µg/mL concentration with 32.7% cell viability. Also FrD exhibited the strongest cytotoxic activity against A549 cells with 20.2% cell viability. On the other hand, cell viability was decreased significantly to approximately 80% at lower doses and it decreased to 60% at the highest dose compared to vehicle control ($p<0.0001$) in HeLa cells. Even though FrD caused significant decrease in cell viability (65.2%) at the highest dose in BEAS-2B cells, it was clearly observed that FrD showed higher selectivity to cancer cells.

Considering the bioactivity results, FrD was selected for further purification to determine the active principles responsible for the cytotoxic activity. So various conventional chromatographic methods were used for isolation of the major compounds. Three biflavonoids: genkwanol A, dihydrodaphnodorin B and stelleranol were isolated and their structures were elucidated by extensive 1D- and 2D-NMR spectroscopic analysis in combination with MS experiments.

According to the published articles, genkwanol A was previously isolated from several plant species from the family Thymelaeaceae, namely *Thymelaea microphylla* Coss. & Durieu [25], *Wikstroemia indica* (L.) C.A. Mey. [26], *Stellera chamaejasme* L. [27] and *Daphne genkwa* Siebold & Zucc. [28]. Besides *F. procumbens*, dihydrodaphnodorin B was isolated from *S. chamaejasme* [27], *Daphne odora* Thunb. [22] and *D. genkwa* [29] and stelleranol was isolated from *S. chamaejasme* [30], *Diploniorpha sikokiana* (Franchet & Savatier) Honda [31], *T. microphylla* [25], *W. indica* [32], *D. genkwa* [29] and *D. holosericea* (Diels) Hamaya [33].

Biflavonoids are dimeric types of flavonoids which are linked by a C-C or C-O-C bond, displaying several biological activities, such as antibacterial, antifungal, antioxidant, antiviral, analgesic, anti-inflammatory and anticancer [34]. In order to understand the contribution of the isolated biflavonoids to the cytotoxic activity, we investigated their cytotoxic activity on the same cell lines at concentration ranges of 0.6-20 µg/mL for duration of 48 h. Although some concentrations of genkwanol A, dihydrodaphnodorin B and stelleranol decreased the cell viability to a statistically significant lower levels compared to the vehicle control, none of them was clinically important.

In previous literatures, genkwanol A was demonstrated to have moderate activity against microtubule polymerization and HIV-1, as well as display antifungal effect [26]. Although Yan et al. (2014) revealed the phytotoxic effect of genkwanol A and dihydrodaphnodorin B against *Arabidopsis thaliana* (L.) Heynh. seedlings (IC_{50}: 74.8 µg/mL and 27.3 µg/mL, respectively), no data on cytotoxic activity of genkwanol A has been reported so far [27, 35]. Sun et al. showed antibacterial activity of dihydrodaphnodorin B against *Staphylococcus aureus* [29]. Previously stelleranol was shown to have a potent anti-respiratory syncytial virus activity [24].

In our study, genkwanol A negatively affected the viability only in A549 cells at the highest concentration. However, the viability level was only reached to a value of 83.2% ($p<0.0001$). No clinically significant cytotoxic effect was determined for dihydrodaphnodorin B which showed at least 90% cell viability on cancer or noncancer cell lines. The cell viability after exposure to stelleranol began to decrease at lower doses in A549 cells however, this decline has only reached to 80% similar to genkwanol A. The other investigated cell lines showed only 10% inhibition on cell viability.

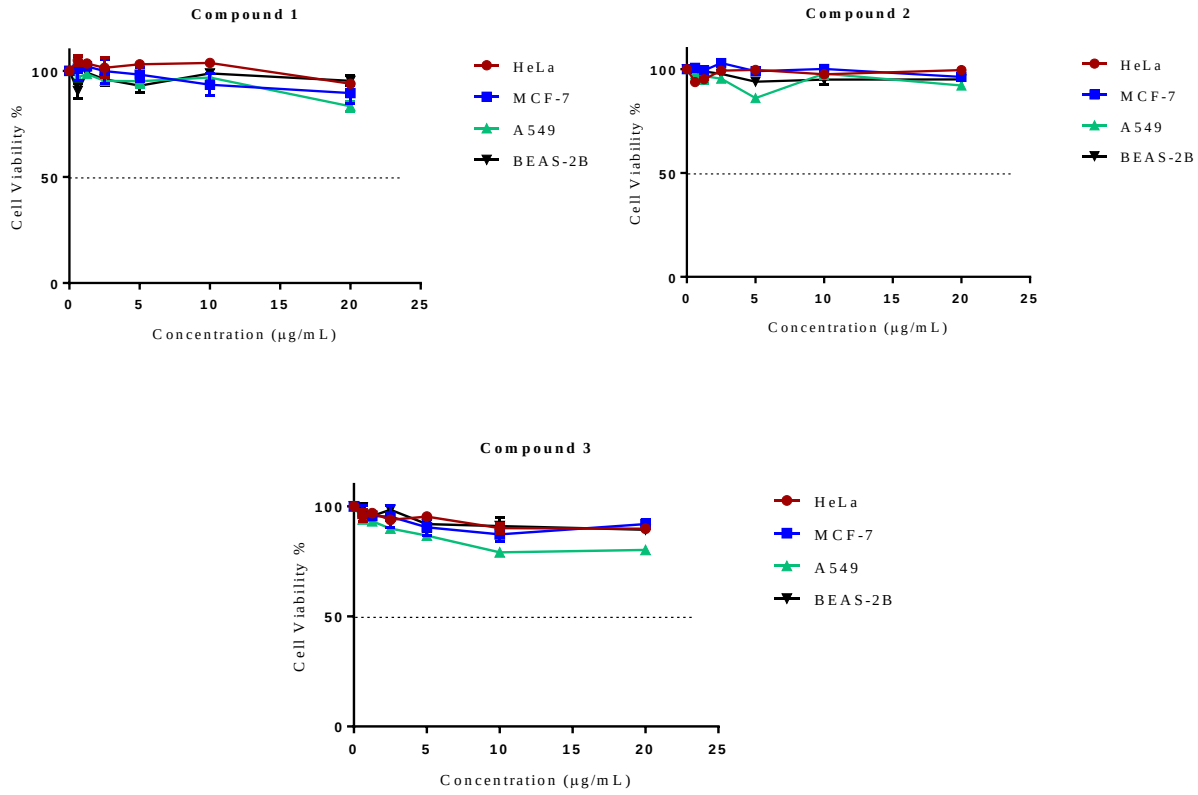

Figure 4. Cell viabilities (%) after 48h exposure to pure compounds of *F. procumbens* (The plot represents values of mean cell viability % ± SEM)

In conclusion, our findings suggest no individual cytotoxic activity for biflavonoid compounds considering recommendation of the National Cancer Institute (NCI) guidelines. However, the former fractions FrC and FrD as well as MeOH and *n*-BuOH extracts exerted moderate and significant cytotoxic activity which suggested a possible synergistic effect. Indeed, in previous reports, mixtures of herbal polyphenol compounds demonstrated better bioactivities when compared to the effect of individual compounds [36, 37]. This study is the first step towards enhancing our understanding of anticancer potential of the *F. procumbens* plant. In the future, by elucidating the toxicity mechanisms of MeOH and *n*-BuOH extract, FrC and FrD might be worthy in developing candidate anticancer agents. Therefore, our further studies will be in progress for investigating the activity of the mixtures of these bioflavonoids in different ratios in order to reveal a possible synergism among the aforementioned biflavonoid compounds.

Acknowledgments

This study was financially supported by Gazi University Scientific Researches Unit (Grant 02/2018-11).

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