Research Article

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In vitro and *in silico* investigations of the pro-apoptotic activity of *Opuntia ficus-indica* cladode extracts against K562 cells

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Abstract

Objectives: Cladodes of *Opuntia ficus-indica* (OFI) are thought to be an excellent source of bioactive compounds, making them an aspirant for the manufacture of health-promoting compounds. This study aims at exploring the pro-apoptotic effects of spiny and thornless OFI cladode extracts on the human immortalized myelogenous leukemia cell line (K562).
Methods: The ethanol extraction method was used for preparing cladode extract. Cytotoxicity was evaluated by MTT assays. Membrane permeability/damage was detected by annexin V-binding assays, and mitochondrial damage/alteration was detected by mitochondrial membrane potential measurements. The protein expression quantities of Bax and Bcl-2 were assessed by western blotting. The pro-apoptotic potentials of the main spiny and thornless OFI extract constituents were investigated structurally and mechanistically using protein–ligand docking and interaction profiling.
Results: Spiny OFI extract displayed a stronger cytotoxic effect than thornless OFI extract on K562 cells. In silico findings agreed with the pro-apoptotic action observed *in vitro*.
Conclusions: Finally, our findings imply that OFI extracts cause apoptosis in K562 cells in order to have anti-cancer effects.

Keywords: *Opuntia ficus-indica*; cancer; extract; apoptosis; *in silico*

Introduction

Cancer is a major public health issue and the world's second leading cause of death. In 2040, there are expected to be 16.3 million cancer-related deaths and 27.5 million newly diagnosed cancer cases [1]. Despite numerous therapeutic advances in elucidating carcinogenesis processes, the need to identify alternative approaches and complementary therapies for cancer prevention is great. Especially, the literature reports that medical plants are useful and available as chemoprotective agents against cancers worldwide. These medical plants are interesting as chemopreventive agents because of their potentially low side effects and toxicity profiles [2]. Furthermore, the anti-cancer effects of medicinal plants include antioxidant activity, suppression of angiogenesis in tumors, promotion of apoptosis, arrest of the cell cycle, and inhibition of proliferation of cells [2]. Regarding medicinal plants possessing anticancer qualities, *Opuntia ficus-indica* (L.) Miller is well-known for its advantageous biological characteristics [3].

O. ficus-indica (OFI) is a species of cactus in the genus *Opuntia* and family Cactaceae. Its primary growing regions are the Mediterranean region, Africa, the southwest region of the United States, and northern Mexico. Regionally, different part of OFI (fruit, roots and cladodes) are used in cosmetic industry and food as a vegetable or fruit for human consumption [4, 5]. In Cyprus, spiny (wild) and thornless (cultivated) form of OFI grow. The cladodes of OFI exhibit nutritional benefits attributed to their antioxidant activities and further functional properties of their compounds [6–8]. OFI cladodes, in particular, contain 6.7–11.73 % protein and amino acids such as alanine, isoleucine, and asparagine [9]. Several phenols and flavonoids including gallic acid, 3,4-dihydroxybenzoic, coumaric, 4-hydroxybenzoic, salicylic acid, ferulic acid, isoquercetin, nicotiflorin, isorhamnetin-3-O-glucoside, narcissin and rutin are found in cladodes of OFI [4]. These compounds give disease prevention and health promotion properties of cladode.

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Several experiments showed that cladodes of OFI showed hypocholesterolemic, anti-inflammatory, hypoglycemic, and neuroprotective effects [10–14].

In keeping with previous studies on chemical composition and biological activities, there are no reports addressing the specific effects of spiny and thornless ethanol extract of the OFI cladode with respect to cell viability, cytotoxic and pro-apoptotic effects in a human chronic myeloid leukemia cell line (K562). In this study, we aimed to examine spiny and thornless OFI cladode extract pro-apoptotic effects on relevant molecular mechanisms and proteins such as annexin-V, Bcl-2, and Bax in K562 cells.

Materials and methods

Preparation of cladode extracts

Spiny and thornless cladodes were collected Trikomo regions in Northern Cyprus in July. Collected cladode were washed with water, air-dried, and then they were peeled by hand. Extracts of thorny and thornless cladodes were obtained following the previously mentioned procedure [15]. The cladodes' thorns were removed, allowed to dry outside at 25 °C, and then sliced and crushed by a blender. Using the Soxhlet apparatus, 10 g of cladodes powdered flour was extracted with EtOH over the course of 3 h. EtOH was evaporated until dry using a rotary evaporator. The cladode extracts were freeze-dried for 36 h to get pure-dried extracts. Extract powders were dissolved in dimethyl sulfoxide (DMSO; 10 mg/mL) prior to use, and then they were promptly placed into the culture medium at the prescribed concentrations (using <0.05 % DMSO final concentration).

Cell culture, treatment and cytotoxicity assay

K-562 cell line (Cat No: CCL-243, ATCC) was cultured in RPMI-1640 (Cat No: FG1215, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % FBS (FBS; Cat No: FBS-11B Capricorn Scientific), 1 % L-glutamine (Cat No: K 0283, Merck millipore) and 1 % penicillin–streptomycin (Cat no: A 2212, Pan Biotech), and maintained in a humidified atmosphere of 5 % carbon dioxide at 37 °C. By using the MTT assay, the inhibitory effect of OFI extracts on cell proliferation was ascertained. In basic terms, the cells were propagated in 96-well plates. After 24 h of culture, cells were subjected to various doses of OFI (spiny and thornless extracts: 0, 10, 25, 50, 75, and 100 g/mL, respectively) for 24 and 48 h. Following treatment, each well received 10 µL of MTT (Cat No: 11465007001, Merck), and the plate was incubated for a further 4 h at 37 °C. The absorbance of DMSO-solubilized violet formazan crystals, which was directly proportional to cell viability, was measured at 570 nm with a hybrid multi-mode microplate reader (Synergy H1; Bio Tek Instruments, Inc., Winooski, VT, USA). A plot of percent cell viability vs. *OFI* extracts (spiny and thornless) concentration was graphed (n=3), and the concentration that gave half-maximal inhibition of the cell growth (IC_{50}) was determined graphically.

Determination of apoptosis

Apoptosis of K-562 cells upon OFI extracts treatment was determined by using both Tali apoptosis assay and MMP assay. In Tali apoptosis assay, cells were initially stained with 5 µL of annexin V Alexa Fluor 488 (Tali™ Apoptosis Kit - Annexin V Alexa Fluor™ 488 & Propidium Iodide Cat. No: A10788, Invitrogen) in darkness for 20 min. Following centrifugation, they were then stained with 1 µL of propidium iodide (PI) in darkness for 1 min. The fluorescence of both dyes was measured simultaneously by using a benchtop cell analyzer (Tali Image-Based Cytometer; Invitrogen, Carlsbad, CA, USA). Green fluorescence and red fluorescence (with or without green fluorescence) were used to differentiate apoptotic cells (annexin V-positive/PI negative) from dead cells (annexin V-positive/PI positive or annexin V-negative/PI positive), respectively. In MMP assay, cells were stained with the JC-1 probe according to the instructions provided with the test kit. The alternating fluorescence emission color of the dye was quantitated with a hybrid multi-mode microplate reader endowed with appropriate filters (Synergy H1; Bio Tek Instruments, Inc., Winooski, VT, USA). Green fluorescence and red fluorescence were used to differentiate apoptotic cells with poor mitochondrial integrity (low $\Delta\Psi m$) from healthy cells with good mitochondrial integrity (high $\Delta\Psi m$), respectively.

Western blotting

The effect of OFI extract on the expression of apoptotic proteins [Bax (1:200; Cat no: sc-20067, Santa Cruz Biotechnology), Bcl-2 (1:200; Cat no: sc-7382, Santa Cruz Biotechnology), and β-actin (1:200; Cat no: sc-130657, Santa Cruz Biotechnology) in K562 cells was assessed using the western blot method. K562 cells were subjected to varying concentrations of OFI extracts on a medium with 1 % serum. To prepared the whole cell extract, cells (5.0×10^5 cells/mL) were lysed with 1× radioimmune-precipitation assay (RIPA) buffer containing protease inhibitor cocktail (Cat No: 11873580001; Sigma Aldrich, St. Louis, MO, USA), The amount of protein in the obtained K562 cells lysates was determined using the Lowry method

and 50 g of protein were loaded into 12 % SDS-PAGE (for 90 min) and then blotted onto the membranes (Schleicher and Schuell, 0.45 m, German). All membrane was blocked with 1 % BSA including 0.05 % Tween 20 (for 1 h, at +22 °C), before incubating primary antibody for 14 h. Densitometric studies were performed using the Bio-Rad Molecular Analyst software after a 1 h incubation period with the secondary antibody on membranes treated with the primary antibody (free edition, www.totallab.com).

Molecular docking

The solution NMR structures of (i) human Bcl-2 in complex with an acylsulfonamide-based inhibitor (PDB ID: 2O21) [16], (ii) human Bcl-x_L in complex with the same dual Bcl-2/Bcl-x_L inhibitor (PDB ID: 2O1Y) [16] and (iii) human Mcl-1 in complex with the selective inhibitor topotoclax (PDB ID: 6OVC) [17] were retrieved from RCSB Protein Data Bank [18]. The 3D conformers of narcissin (compound ID: 5481663) and nicotiflorin (compound ID: 5318767) were downloaded from PubChem [19]. The ligands were docked in moderate-precision mode onto the proteins, which were prepared via the Protoss optimization routine [20, 21], using the JAMDA fully automated protein–ligand docking tool [22–24] available at https://proteins.plus/. The sphere within a radius of 6.5 Å from the native (co-crystallised) inhibitor was used to define the conformational search space. Based on the computed JAMDA score and additional structural parameters listed for known flavonoids, the optimal docking solution was chosen [25]. Favorable non-covalent interactions between the anti-apoptotic proteins and the flavonol glycosides were estimated by using Discovery Studio v16.1.0 (Dassault Systèmes BIOVIA Corp., USA).

Statistical analysis

GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. All data were reported as mean±SD One-way analysis of variance (ANOVA) was employed to compare multiple groups followed by Bonferroni's multiple comparison test to discover the differences between multiple groups. Significant values of $p<0.05$ were considered.

Results

Effects of OFI extracts on K562 cells viability

To evaluate the anti-cancer activity of spiny and thornless ethanol extracts of the OFI cladode with various concentrations (10–100 µg/mL) were screened on K562 myeloid cancer cell lines for 24 and 48 h using the MTT assay. The spiny OFI cladode extract exhibited potent antiproliferative activities with IC_{50} values of 44.67 µg/mL for 48 h on K562 cells (Figure 1A and B). Furthermore, the IC_{50} value of thornless ethanol extracts of the OFI cladode after 48 h was 44.11 µg/mL in the K562 cells (Figure 1A and B).

Apoptosis is characterized by a reduction in the ΔΨm level of the mitochondrial membrane and malfunction [26]. K562 cells were exposed to varying concentrations of thornless and spiny ethanol extracts of the OFI cladode (10–100 µg/mL) for 48 h before being incubated with JC-1 dye to ascertain the role played by the mitochondria in the induction of apoptosis by OFI. Based on our results, as indicated in Figure 2A, both spiny and thornless ethanol extracts of the OFI cladode caused loss of ΔΨm level in a dose-dependent manner in K562 cells.

Furthermore, it was determined that cell proliferation is suppressed and viability is reduced in the treatment of K562 cells with OFI cladode extract obtained spiny and thornless for 48 h. Inhibition of K562 cell proliferation was determined with a decrease of about 22 and 11 %, especially after the addition of 100 µg/mL spiny and thornless OFI cladode extracts, due to increased extracts concentrations according to the cell count results using TALI image-based cytometer after trypan blue staining.

After 48 h of treatment with four different spiny OFI extracts, the highest significant variation in inhibition was 45 % at 50 g/mL. Consistent with ~30 % reduction in cell proliferation, when annexin V/PI labelling was performed, it was discovered that 40 % of the total cell population was apoptotic at the IC_{50} concentration (44.67 g/mL) and 45 % at 50 g/mL. The results of the JC-1 assay revealed that viability decreased by 35 % upon the addition of spiny OFI cladode extract at 50 µg/mL concentrations. The cytotoxic effect of thornless OFI cladode extract showed the inhibition of 20 % in the K562 cells after 48 h (Figure 2B). Overall, it may be possible to say that spiny OFI cladode extract is more sensitive to the cytotoxic effect of K562 cells than thornless OFI cladode extract.

Western blotting results

We investigated whether anti-apoptotic Bcl-2 and proapoptotic Bax antibodies were associated with the antiproliferative activity of spiny and thornless OFI cladode extract using western blot. Our cell results showed that the spiny and thornless OFI cladode extract significantly inhibits the growth of K562 cells. We found that spiny OFI extract (30 and 50 µg/mL) in K562 cells exhibited increased efficacy on the Bax/Bcl-2 ratio due to decreasing effect on the level of Bcl-

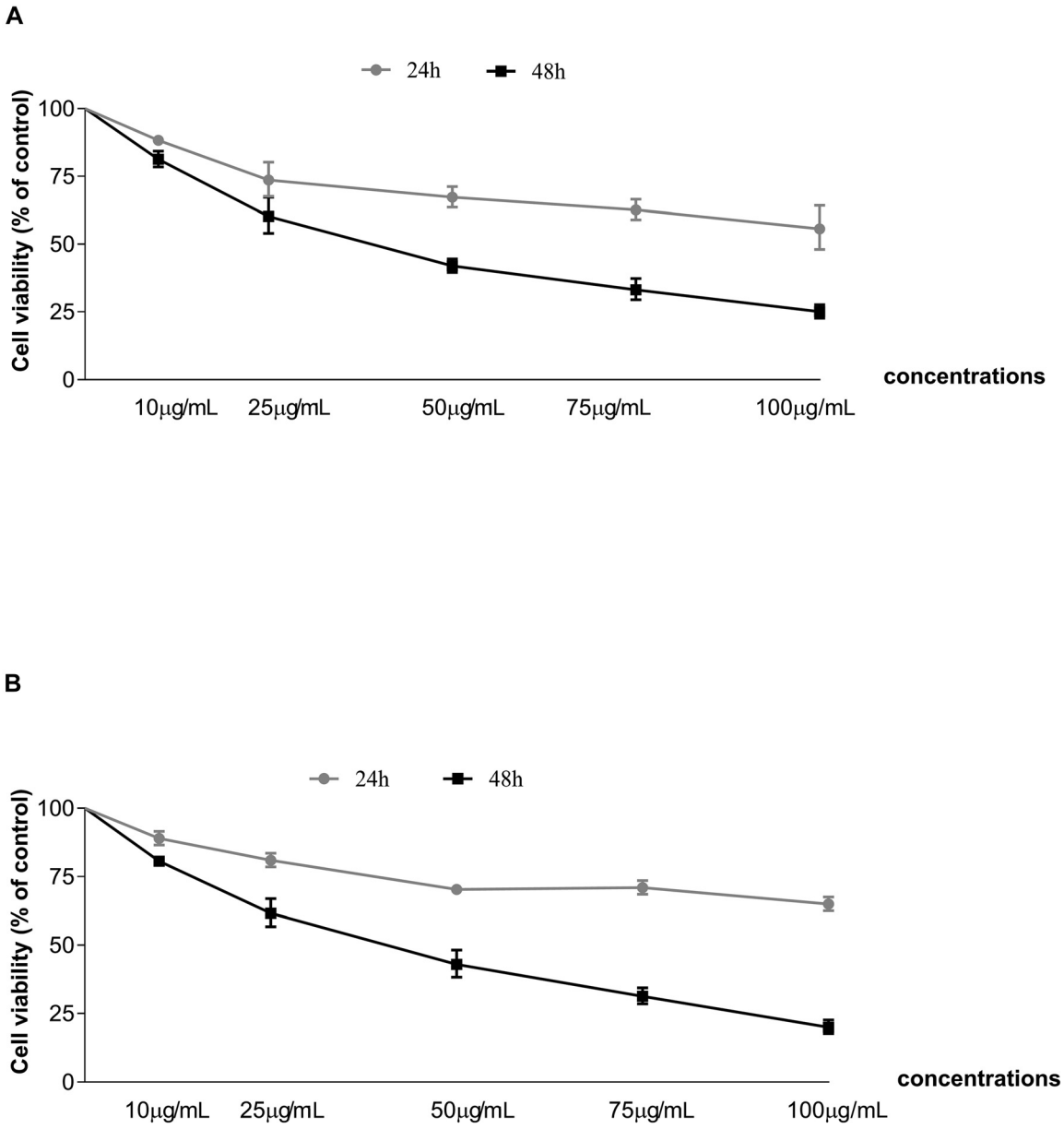

Figure 1: Effect of cell growth inhibition by different (A) spiny and (B) thornless OFI extracts. K562 cells were treated with each OFI extract (10, 25, 50, 75, and 100 μg/mL) for 24 and 48 h. The cell proliferation inhibition was measured using the MTT assay. Values are expressed as mean±SD of independent experiments.

2 expression and increasing effect on the level of Bax expression at 48 h ($p<0.001$) when compared control group (Figure 3A–C). The Bax/Bcl-2 ratio increased 1.23-fold ($p<0.001$) at 30 μg/mL. Also, we found that thornless OFI extract (30 and 50 μg/mL) was not found to have any effect on the Bcl-2/Bax ratio at 48 h in K562 cells (Figure 3B and C). It was determined that thornless OFI extract in K562 cells exhibited at 48 h increased efficacy on the level of Bcl-2 expression ($p<0.05$), but not a significant increasing effect on the level of Bax expression (Figure 3C).

Protein–ligand docking and interaction profiling results

The results of our computational studies revealed that both the isorhamnetin glycoside narcissin (i.e. isorhamnetin 3-*O*-rutinoside) (Figure 4A) and the kaempferol glycoside nicotiflorin (i.e. kaempferol 3-O-rutinoside) (Figure 4B) could fill in the vacant space in the inhibitor-binding groove of human Bcl-2. The flavonol groups of the ligands appeared to form conventional hydrogen-bonding interactions with Gly145 and

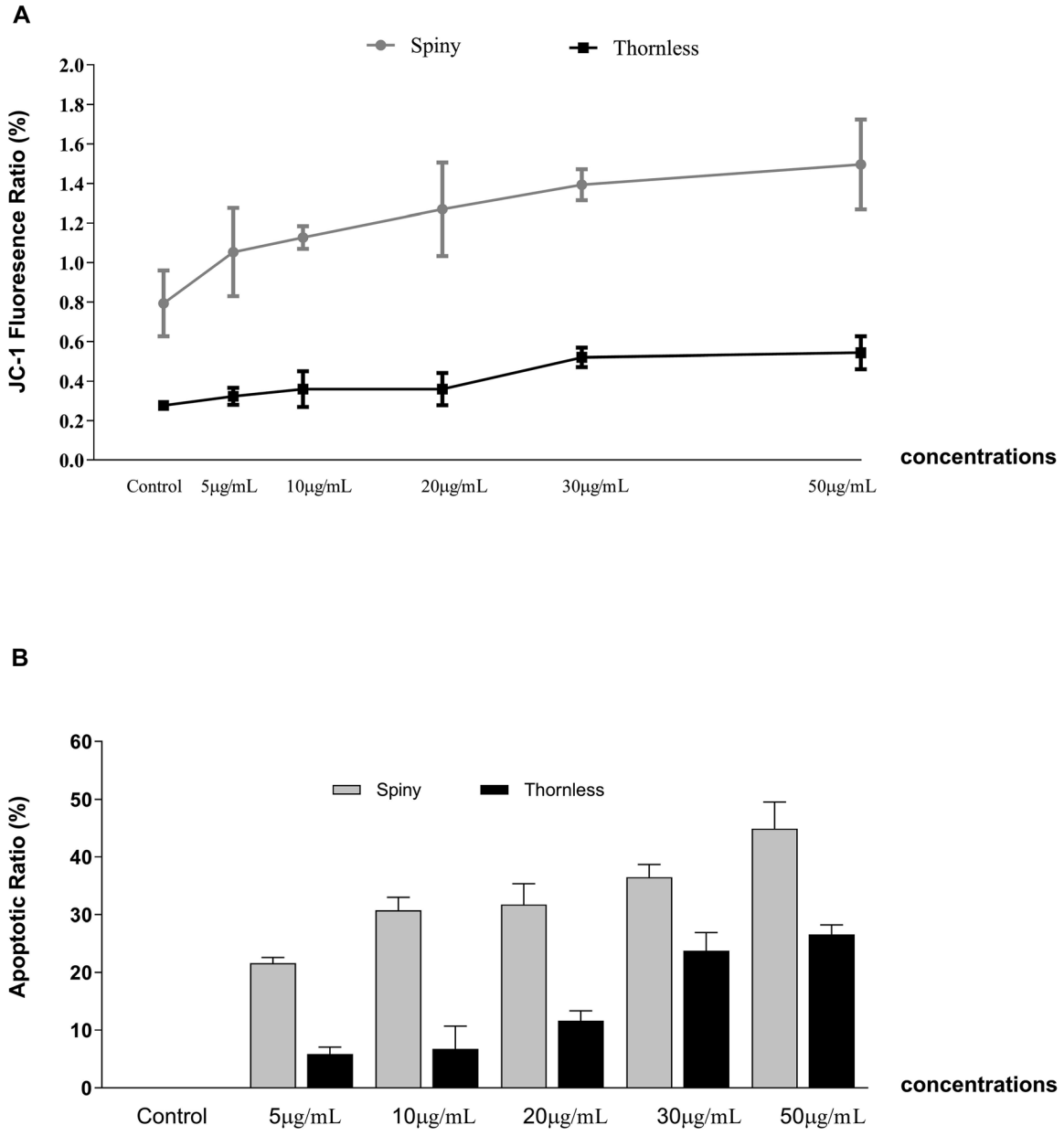

Figure 2: (A) Mitochondrial membrane potential was detected by using JC-1 dye, and (B) apoptotic cell ratios were evaluated using the Tali apoptosis kit in K562 cells (n=3). ***The data was significant when compared with control group (p=0.0008). ****The data was significant when compared with control group (p<0.0001).

Arg146 as well as a pi–pi stacking interaction with Phe104. Arg146 was in sufficiently close proximity to the A-rings of the ligands to also form a cation–pi interaction with them. Asp111, Glu136 and Ala149 could establish conventional hydrogen-bonding interactions with the glucose and rhamnose sugar groups of the ligands. With its additional methoxyl group at the C-3′ position, narcissin was also able to interact with Tyr108 through a weaker carbon hydrogen bond. A somewhat similar bonding pattern was observed for the predicted Bcl-x_L–narcissin (Figure 4C) and Bcl-x_L–nicotiflorin (Figure 4D) complexes. Ala93, Arg100 and Gly138 appeared to form conventional hydrogen bonds with the flavonol groups of the ligands, while Glu92 and Tyr195 appeared to form conventional hydrogen bonds with the disaccharide moieties of the molecules. Phe97, which corresponds to Phe104 in Bcl-2, could be involved in a pi–pi stacking interaction with the C-rings of the ligands. Considering flavonol glycosides in complex with Mcl-1 (Figure 4D and E), Met231 and Met250 were estimated to play a key role in stabilising the bound (docked) conformations of the molecules by establishing sulphur–pi interactions

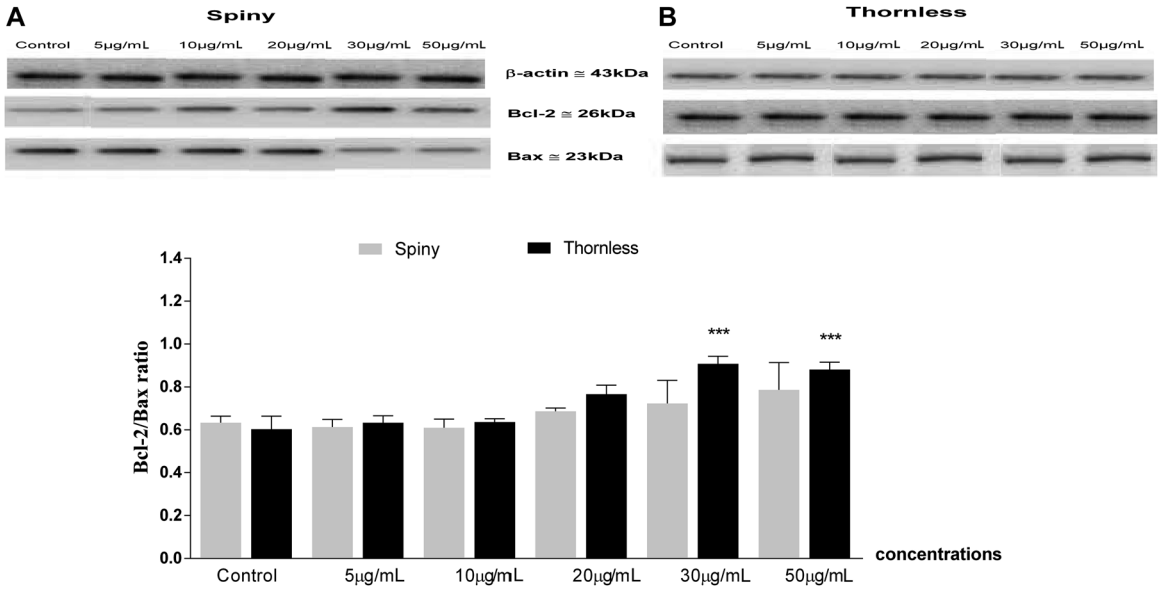

Figure 3: The expression levels Bax and Bcl-2 after spiny (A) and thornless (B) OFI extract application in K562 cells. (C) The expression levels of Bax/Bcl-2 ratio after spiny and thornless OFI extract application in K562 cells. Western blots were determined using the antibodies directed against Bax and Bcl-2. The immunoblots indicated the Bcl-2 (≅ 26 kDa), and Bax (≅ 23 kDa) levels (duplicated). β-actin (≅ 43 kDa) was used to normalize the amount of protein loaded in each lane. ***The data was significant when compared with control group ($p<0.001$).

with the flavonol groups of them. In addition, Val253 and Arg263 were able to establish conventional hydrogen-bonding interactions with the terminal sugar groups of the ligands.

Discussion

OFI is a plant that belongs to the *Cactaceae* family and is commonly found in arid areas of the world [27]. The spiny and thornless form of the OFI is found in Cyprus. In our study, the cytotoxic and pro-apoptotic effects were determined of both spiny and thornless OFI cladode extracts. This is the first study to focus on the anticancer effects of OFI cladode extracts on K562.

OFI cladodes contain of protein, polyphenols, flavonoids, lipids, vitamins, minerals, amino acids such as isoleucine, alanine, and asparagine are found [4]. The powerful antioxidant and anti-cancer properties of OFI cladodes may be related to their rich flavonoids and polyphenolic content. *In vitro* studies using active substances (such as oputinol, OFI fruit extract, indicaxantin) isolated from various OFI classes have shown that cell viability decreases in a dose- and time-dependent manner in KB oral carcinoma, colon, brain cancer and melanoma cell lines [28–30]. In addition, it was observed that the cell proliferation, antioxidant and pro-apoptotic properties of extracts of *O. ficus-indica* stems obtained using different extraction methods depend on the extraction method and cell type, as well as dose and time dependence [31].

The only study in the literature about the anti-cancer effects of OFI cladode extract against cancer cells was reported by Heikal et al. (2021). It has been demonstrated that the generated OFI cladode extracts have antiproliferative effects on MCF-7 breast cancer cells and PC3 prostate cancer cells both *in vitro* and *in vivo* [32]. Our study's findings concur with those of the earlier investigation. Our results showed that the effective doses of spiny and thornless OFI extract were similar in K562 cells.

Scientific reports published in recent years have brought attention to the existence of OFI fruit and cladode molecules, which may have important biological advantages for human health. Both fruit and cladode of OFI are known for their high content of flavonoids and polyphenols which are exhibited antioxidant and anti-inflammatory properties [33, 34]. Akacha et al. (2018) demonstrated that OFI cladode extract protected the small intestine from toxic damage caused by MTX in Wistar rats [35]. In the only study conducted with K562 cells, Sreekanth et al. (2007) showed that betanin isolated from OFI fruits caused dose- and time-dependent inhibition of cell proliferation (IC_{50}=40 µM) and induced apoptosis through the intrinsic pathway [36]. However, proapoptotic properties of cladode extract of spiny and thornless OFI have not been studied in K562 cells yet. In the current investigation, we assessed apoptotic ratio of both spiny and thornless cladode extracts of OFI in K562 cells. Spiny OFI cladode extract was more effective in apoptosis in K562 cells. In agreement with our study, in contrast to OFI

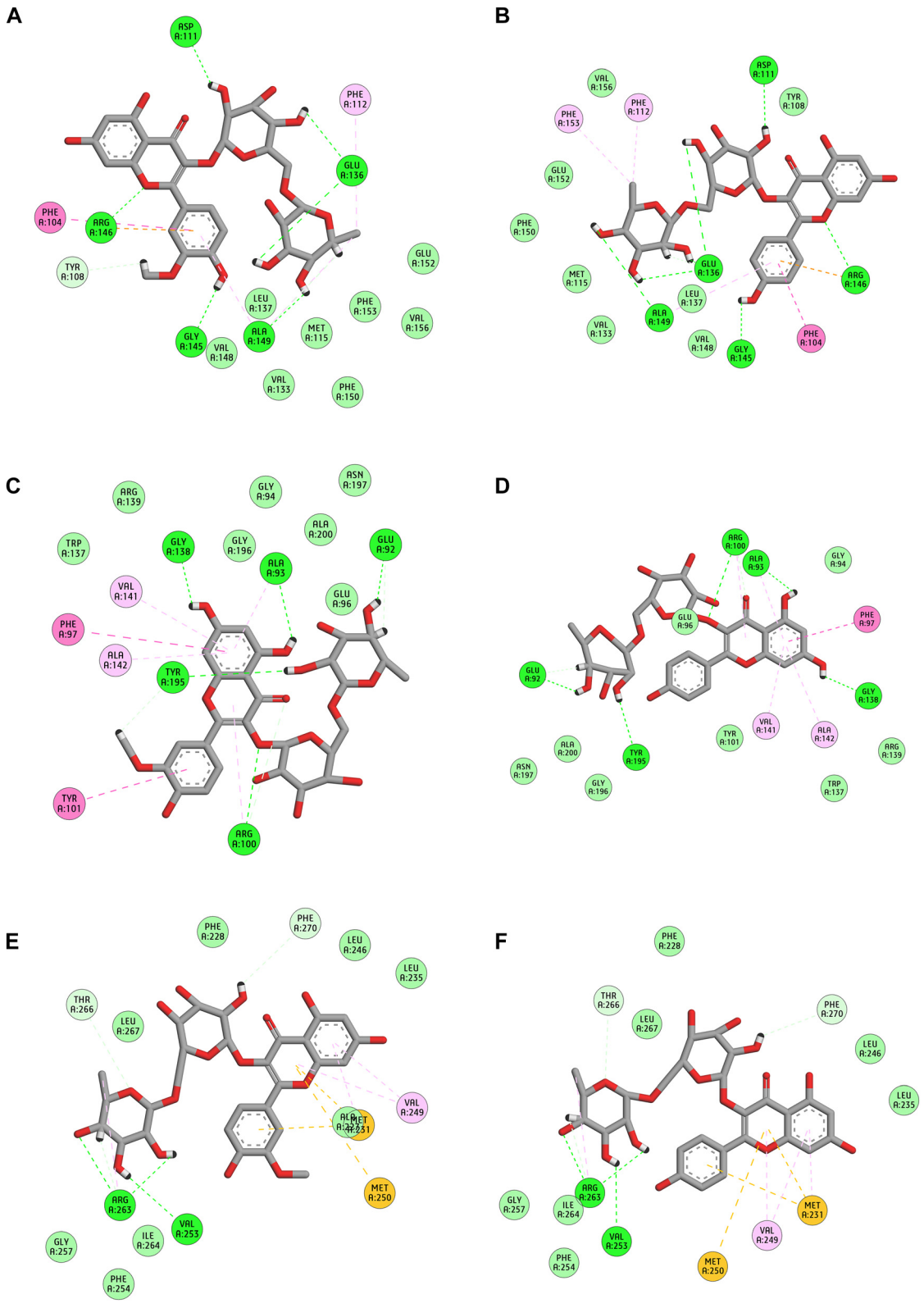

Figure 4: Favorable non-covalent interactions occurring between the predicted (A) Bcl-2–narcissin, (B) Bcl-2–nicotiflorin, (C) Bcl-xL–narcissin, (D) Bcl-xL–nicotiflorin, (E) Mcl-1–narcissin, and (F) Mcl-1–nicotiflorin complexes. Magenta dashed lines correspond to pi–pi stacking interactions, pink dashed lines correspond to hydrophobic interactions, lime green dashed lines correspond to conventional hydrogen bonds, light green dashed lines correspond to weak hydrogen bonds, bright orange dashed lines correspond to electrostatic interactions, and light orange dashed lines correspond to sulfur–pi interactions. The images (A)–(F) were rendered using Discovery Studio Visualizer, v16.1.0 (Dassault Systèmes BIOVIA Corp., San Diego, CA, USA).

cladodes without spines, Boutakiout et al. (2018) provided evidence that spiky cladodes have higher levels of antioxidant activity and phenolic compounds [37]. It can be concluded that the higher proapoptotic effects of spiny OFI extract may be related to higher phenolic content.

Bcl-2, mitochondrial membrane protein, inhibits apoptotic death in cells. It can bind Bax proteins and block proapoptotic cytochrome *c* protein released from mitochondria. Bcl-2/Bax (anti/pro-apoptotic) ratio is an important biomarker for showing the apoptosis capacity of agents in experimental studies [38]. It was previously documented that isorhamnetin diglycosides, which were extracted from an alkaline OFI extract, induced colon cancer cells (HT-29) to undergo apoptosis [39]. Serra et al. (2013) found that OFI fruit juices had antiproliferative effects on the human colorectal cancer cell line (HT29) [40]. In our study, the spiny OFI cladode extract exhibited a statistically lower Bcl-2/Bax ratio due to the high level of Bax expression and low level of Bcl-2 expression in K652 cells. Overall, the data in this present study demonstrated that the natural extracts obtained from spiny OFI cladodes were richer in antioxidants and proapoptotic compounds than thornless OFI cladodes extract in K562 cells.

Besides comparatively measuring Bcl-2 and Bax expression levels, we also tested in an *in silico* setting whether two dominant phenolic compounds found in OFI, namely narcissin and nicotiflorin, held the potential to be housed at the BH3-binding site the of the pro-survival proteins Mcl-1, Bcl-2and Bcl-xL from human. In cancerous cells, the anti-apoptotic Bcl-2 family of proteins (Mcl-1, Bcl-2 and Bcl-xL) sequesters the pore-forming effector proteins (such as Bak and Bax) and pro-apoptotic BH3-only proteins (such as Bid, Bad and Bim). This increases the survival of cancer cells, as well as to therapeutic resistance in many cancer types. Gossypol, a naturally occurring polyphenol obtained from the cottonseed plant, has been demonstrated to function as a BH3 mimetic and to obstruct the physical interactions between pro- and anti-apoptotic proteins, thereby promoting the death of cancer cells. As a BH3 mimetic, gossypol, a naturally occurring polyphenol sourced from the cottonseed plant (*Gossypium* spp.), has been demonstrated to promote cancer cell death by preventing direct physical interactions between pro- and anti-apoptotic proteins [41, 42]. Structurally similar flavonoid compounds, which mimic the Bim BH3 peptide, have also been designed and synthesized in an attempt to release bound pro-apoptotic proteins and prime cancer cells for death [43]. These studies have paved the way for further research on naturally occurring flavonoids with BH3 mimetic activity Pull-down assays, intrinsic fluorescence measurements, and NMR chemical shift perturbation investigations, for example, have shown that quercetin directly binds to the BH3-binding groove of Bcl-xL and Bcl-2 and induces apoptosis in cancer cells [44]. Our results imply that the two natural flavonol glycosides in question can be accommodated well in the BH3-binding groove of anti-apoptotic proteins and that they can establish favorable non-covalent interactions with the key residues that are involved in binding to BH3-only proteins or BH3-mimetic drugs.

It could be concluded that spiny and thornless OFI cladode extracts had proapoptotic effects in K562 cells. Moreover, spiny OFI cladode extract was more effective in apoptosis in K562 cells. Docking simulations also predicted their proapoptotic activities.

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Informed consent: Not applicable.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
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Data availability: Not applicable.

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