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Investigation of antioxidant activity and effect on cell viability of pyrrolidine compounds in MCF-7 (breast cancer) and DLD-1 (colon cancer) cell lines

Abstract. In this study, it is aimed to investigate the effects of pyrrolidine compounds that are thought to be new drug candidates with effect on cell viability on cancer (MCF-7 and DLD-1) and normal (MCF-12A and CCD-18CO) cell lines. The effect on cell viability of the pyrrolidine compounds was determined for 24 hours at different concentrations (25-100 µM) on cancer (MCF-7 and DLD-1) and normal (MCF-12A and CCD-18CO) cell lines by comparing MTT and RTCA assays. The antioxidant activities of the pyrrolidine compounds were determined at different concentrations (25-200 mM) with DPPH, metal chelating and reducing methods. In addition, the calculation of the structural properties of the new pyrrolidine scaffolding with the DFT method was presented. It has been determined that pyrrolidine compounds reduce the number of MCF-7 cancer cells according to a negative control by half of and inhibit the DLD-1 cancer cells with the MTT method. IC_{50} value for MCF-7 cell was determined as 100 µM. Metal chelation and DPPH activities have close results. The IC_{50} value of DPPH activity of the compounds ranged from 108 to 142 µg/mL.
Key words: pyrrolidine, cell viability, antioxidant activity, DFT, ADME

Introduction

Despite the growing research interest and current medical treatments, Breast and Colon Cancer are amongst the leading causes of death, and mortality is still very high depending on the type of cancer [1; 2].

Radiation therapy is a very effective treatment method for cancer [3; 4]. Radiation therapy can lead to cell death. Therefore, cell viability is an essential and important parameter for the treatment of human cancer [5].

Cell viability is defined as the ability of cells to perform specific functions as well as to perform the mitotic potential of divisible cells [6].

Cell analysis is critical and indispensable for preclinical evaluation of agents considered to have anticancer properties. In a typical anticancer drug study, the IC^{50} value of the test compound is first determined by "50 % killing" (the concentration of the drug that provides 50 % inhibition of the cell) [7].

It is observed that the total success rates of cancer drug candidates are very low and 23% less likely to be successful in phase III of clinical studies in terms of their therapeutic properties [8].

Among the recommended ways that potentially reduce cancer progression, using naturally occurring agents or synthetic substances and reducing the risk of developing cancer by reducing oxidative stress, has attracted great attention [8]. Antioxidants protect against oncogenic transformation with anticancer drugs that produce free radicals and thus reduce the painful side effects of radiation and chemotherapy [9].

The i*n vitro* study about the cell viability effects of six fucoidans on nine different cell lines (HL-60, Raji, HeLa, ARPE-19, OMM-1, A-375, HCT-116, HepG2, HaCaT) has been published.1 MDA-MB-231, MCF-7, U-145, A2780 cell lines and investigated its effect on cell viability by MTS assays [10]. Resazurin-based cell viability assay was performed using the HCC38, MCF-7, MCF-10A and MDA-MB-436 cell lines.7 BALB / 3T3 and HepG2 cells were evaluated with three different cytotoxicity assays (MTT, NRU, and LDH) [11].

In our study, we present a new drug candidate from pyrrolidine compounds that will have effect on cell viability in MCF-7 and MCF-12A, DLD-1 and CCD-18CO cell lines. Using a domino Diels-Alder reaction for the hybrid synthesis approach, we synthesized pyrrolidine analog designed to examine its cytotoxic effect for MCF-7 (breast cancer) and DLD-1 (colon cancer) cell lines while enabling the exploration of their antioxidant activity.

This research, it was studied by comparing two different pyrrolidine compounds **I** and **II**. MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide) and RTCA (real-time cell analysis) assays were determined by comparing cancer (MCF-7 and DLD-1) and normal (MCF-12A and CCD-18CO) cell lines. The significance of differences between data sets in MTT assay was analyzed statistically by ANOVA with SPSS 20.0 program for cancer and normal cell lines. Besides, the antioxidant activities of the pyrrolidine compounds were determined (25-200 mM) at different concentrations by comparing two different compounds **I** and **II**.

In addition, according to both: molecular electrostatic potential, frontier molecular orbital analysis, dipole moment - which were computed DFT/B3LYP/method 6-31 G(d,p) basis set in the ground state- and Lipinski rules score, some pharmacokinetic predictions (via Swiss ADME free web tool) exhibited correlation (Figure 1).

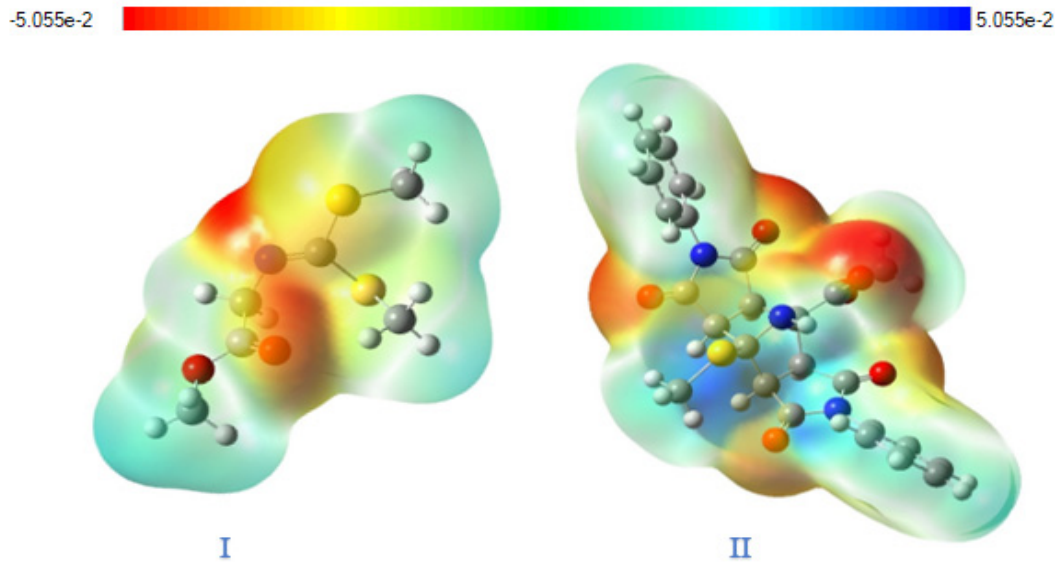


Figure 1 – Electrostatic potential mapped over the electron density of compound I and II

Materials and methods

*Synthesis of compound **II**.* Methyl-2-((bis(methylthio)methylene)amino)acetate (212.61 mg, 1.11 mmol) and N-phenylmaleimide (824.29 mg, 4.76 mmol) were added to a round bottom flask which was subsequently evacuated and purged with N_2 before the addition of anhydrous toluene (15 mL). The reaction was allowed to stir at a reflux of 72 hours. The reaction mixture was allowed to cool down and was removed by evaporation and the crude product was purified by column chromatography (hexane:ethylacetate (1:1)) to give a colorless solid. Yield = 189.23 mg (38%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7,67 (s, 1H), 7.47-7.42 (m, 7H), 7.36-7.28 (m, 3H), 3.94 (s, 3H), 3.67 (d, J = 6.8 Hz, 2H), 3.30 (d, J = 6.8 Hz, 2H), 2.49 (s, 3H), ^{13}C NMR (101 MHz, $CDCl_3$) δ_C 173.68, 172.10, 171.42, 131.91, 128.95, 128.67, 127.05, 69.54, 60.74, 52.18, 49.62, 42.27, 13.19 ppm; HRMS(EIC): Exact Mass 491.12; $[M+H]^+$ ion 492.1502, $[M-H]^+$ ion 490.1452 m/z. anal. calculated for $C_{25}H_{21}N_3O_6S$: C, 61.09; H, 4.31; N, 8.55 Found: C, 60.86; H, 4.25; N, 8.46; Melting point: 209-207 °C.

Estimation of antioxidant activity. Main stocks of the compounds were prepared to be 1 mg / mL in DMSO. Compounds' doses in five different concentrations (25-50-100-150-200 µg / mL) were prepared. The antioxidant activity of the compounds was determined by free radical scavenging (DPPH), metal chelation and reduction activities.

DPPH (Free radical scavenging) activity assay. DPPH activity were made according to Brand-Williams et al., 1995 [12]. Compound activities was compared with standard antioxidant compounds Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and Trolox (25-200 µg / mL). The absorbance of the compounds was measured at 517 nm. Free radical scavenging effect (%) = [(A0 –

A1)/ A0] x 100 (A0 = control absorbance and A1 = sample solution absorbance).

Metal chelating (Fe^{+2} ions chelating) activity assay. The metal chelating activity of the compounds were made according to Decker and Welch, 1990 [13]. Compound activities were compared with standard antioxidant compounds EDTA, butylated hydroxytoluene (BHT) and Trolox (25-200 µg / mL). The absorbance of the compounds was measured at 562 nm. Metal chelating activity (%) = [(A0 - A1) / A0] x 100. (A0 = control absorbance and A1 = sample solution absorbance).

Reducing activity assay. Measurements of the reducing activity of the compounds were made according to Oyaizu, 1986 [14]. The absorbance of the compounds was measured at 700 nm. Compound activities were compared with standard antioxidant compounds EDTA, Gallic and Trolox.

Assessment of cell viability activity

Cell Lines. In vitro studies were used MCF-7 (ATCC® HTB22™) (breast adenocarcinoma), MCF-12A (ATCC® CRL-10782™) (normal breast epithelium), DLD-1 (colon adenocarcinoma) (ATCC® CCL221™) and CCD-18CO (normal colon epithelium) (ATCC® CRL-1459™) normal cell lines.

Cell culture. Cancer (MCF-7 and DLD-1) and normal (MCF-12A and CCD-18CO) cell lines were cultured using 25 g / 100 mL sodium bicarbonate, 10% fetal bovine serum (FBS) DMEM (Dulbecco's Modified Eagle Medium), EMEM (Eagle's Minimum Essential Medium) and RPMI-1640 (Roswell Park Memorial Institute) mediums containing 1 % penicillin/streptomycin in 25cm^2 or 75cm^2 flask, 5% CO_2 and it was produced by incubation for 24 hours. Stock solutions were prepared with dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany) and controls were performed with EMEM, RPMI 1640 and DMEM mediums with 0.1% DMSO concentration without compounds [15].

MTT assay. The MTT (5 mg/ mL) method, a colorimetric assay, was used to investigate cell viability activities of compounds in cell lines. MCF-7 and MCF-12A, DLD-1 and CCD-18CO (1x10^4 cells / mL) cells, and compounds (1.56-3.12-6.25-12.5-25-100 µM) were incubated for 24 hours in 96-well plates (Costar, USA). After incubation, mediums were aspirated and 100 µL MTT was added to each well. Plates were incubated for two hours at 37 °C. The viability activity of the compounds was calculated by measuring OD (optical density) with a microplate reader (Thermo Scientific, USA) at 570 nm. The activities of the compounds were compared with the anticancer drug curcumin (200 µM) and mitomycin (200 µM) positive controls [16].

Statistics. The significance of differences between data sets was analyzed statistically by ANOVA with SPSS 20.0 program for MCF-7, DLD-1, MCF-12A and CCD-18CO. The conclusions were indicated as ID50 ± SE (standard error of the mean) for cell lines.

iCELLigence system real-time cell analyzer assay. iCELLigence ™ real-time cell analyzer (RTCA) is used for real-time monitoring of cancer cell proliferation, viability and cytotoxicity and has 16-well microtiter plates with a gold microelectrode biosensor array. MCF-7 and DLD-1, MCF-12A and CCD-18CO cell lines were loaded into DMEM, EMEM and RPMI media containing 10% FBS at 1 x 10^4 cells/ 400 µL on E-plates and incubated at 37 °C with 5% CO_2. After waiting for the cells to adhere to the E-plates for 24 hours, the synthesized compounds were added to the wells and effects of compounds on cell proliferation were monitored in real time for up to 48 hours [17].

Molecular properties prediction via DFT and Swiss ADME. Theoretical studies including geometry optimization, total energy, dipole moment were carried out using DFT/ B3LYP methods at the 6-31G(d,p) basis set and TD-DFT were performed to assign the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). The results are exhibited with Gaussian 09W software package and Gauss-View 5.0 graphical interface programs [18; 19].

Lipinski rules scores, lipophillicity (log Po/W) bioavailability score (BAS), gastrointestinal absorption (GI), skin permeation (log Kp), topological polar surface area (TSPA) prediction are illustrated in Table 1 and Figure 2 [20; 21].

Table 1 – *In sillico* ADME properties of compound **I** and **II**

Compounds	H-bond acceptors/ donors	TPSA	GI absorbtion	BAS	Log *Kp*	Log *S* (ESOL)	Log $P_{O/W}$
I	3:0	89.26 Å^2	High	0.55	-6.20 cm/s	-1.84	2.43
II	7:1	138.39 Å^2	High	0.55	-8.69 cm/s	-3.35	2.64

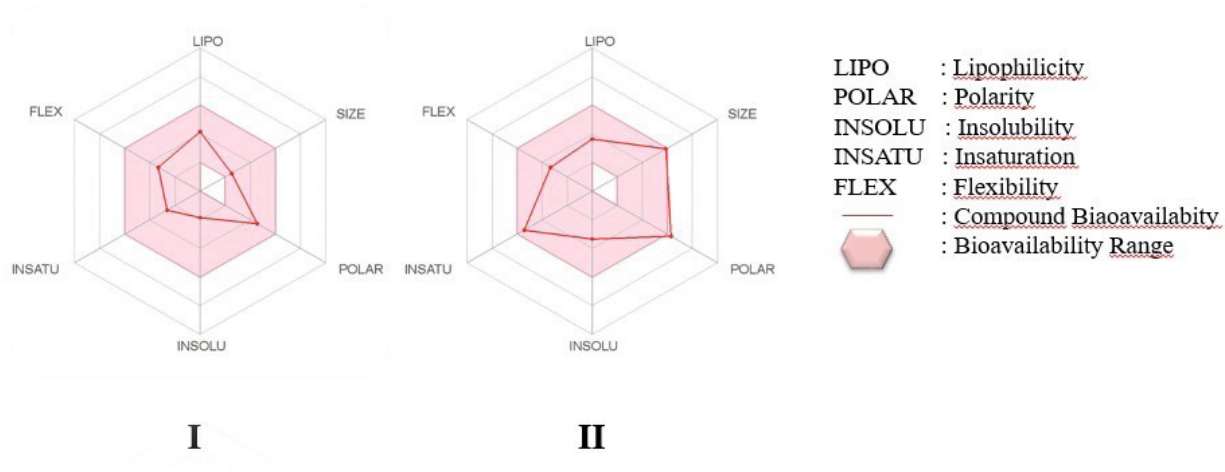
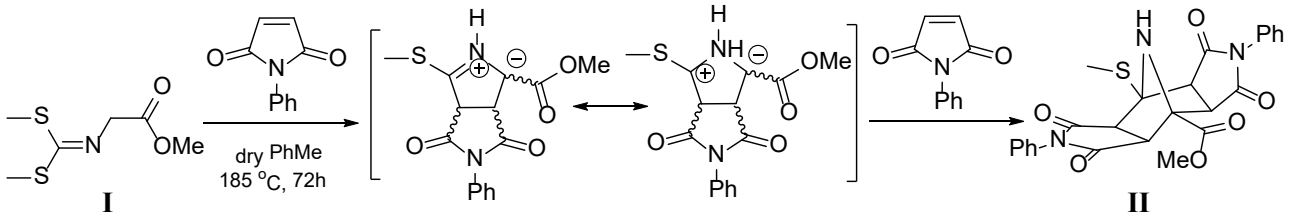


Figure 2 – Compound **I** and **II** anlayzed using Swiss ADME software

Results and discussion

Domino Diels-Alder approach has been employed for the synthesis of pyrrolidine derivative (**II**) from **I** and outlined in Scheme 1. In our previous work, the pyrrolidine-isoindole hybrid compound was synthesized and its exact structure was determined by single-crystal Xray analysis [22].



Scheme 1 – Synthetic route for **II**

In this work, the structure of the compound **II** was confirmed by 1H-13C NMR, HRMS and its purity was checked by elemental analysis. Compound **II** was prepared in one pot; methyl 2-((bis(methylthio) methylene)amino)acetate (I) reacted with N-phenylmaleimide. The 1H NMR spectrum of **II** showed peaks at 2.49 ppm, and 3.94 ppm attributed to the S-CH3 and O-CH3 protons respectively, which was indicated in literature [23; 24].

Antioxidant activity. As a result of the metal chealating activity and DPPH Activity: **II** › **I**, the reducing activity was found at a very low value compared to standard synthetic antioxidant compounds. These two compounds showed moderate activity **II** › **I**. These values approximately correlated with IC_{50} value of MTT and RTCA assays made for cell viability. Antioxidant activity IC_{50} values range from 108 to 142 (Figure 3-5).

Cell viability activity. In our study, MTT and RTCA analyses were performed to investigate whether the pyrrolidine compounds have cell viability in cell lines. Similar effects were observed in both MTT and RTCA assays. The two compounds were evaluated against four cell lines. For the effects on cell viability of the compounds, cancer cell lines (MCF-7 and DLD-1) and normal cell lines (MCF-12A and CCD-18CO) were used in vitro. For this purpose, cells were incubated with compounds (**I-II**) in multiple concentrations (25, 50, 100 µM) (the best doses obtained in MTT assays) for 24 hours at 37 °C. Cell effects on cell viability optical density (OD) values were calculated at 570 nm. The effects of compounds on in vitro proliferation in both breast and colon cancer were based on optical density (OD) values: **II** > **I**.

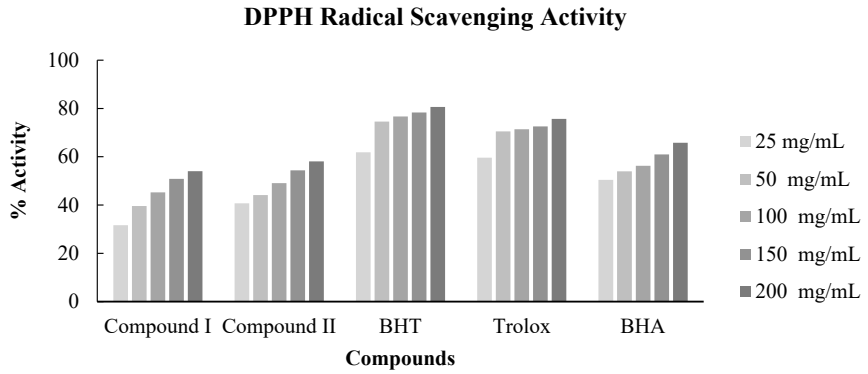


Figure 3 – DPPH activities of studied compounds and BHT, Trolox and BHA as standards

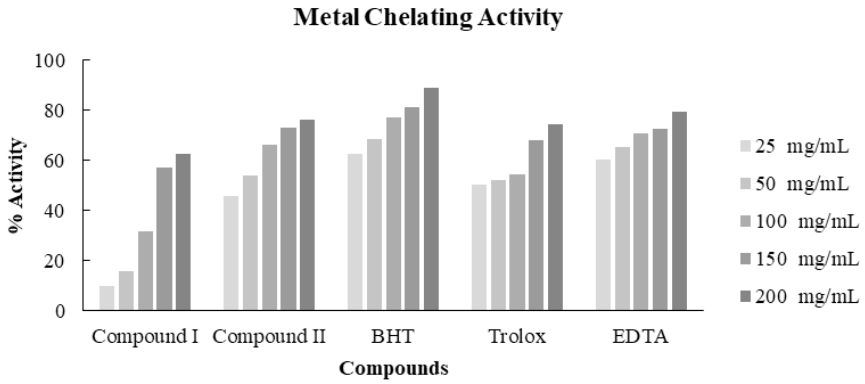


Figure 4 – Metal chelating activities of studied compounds compared to BHT, Trolox and EDTA as standards

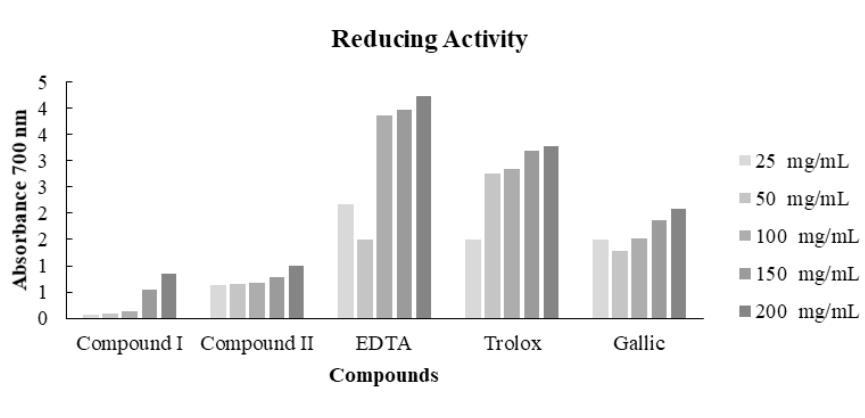


Figure 5 – Reducing activities of studied compounds and EDTA, Trolox and Gallic as standards

Jiao et al., 2018 [10] studies showed that Sorafenib suppresses cancer cell viability and tumor growth by MTS experiments on four cell lines. 7studies obtained data with reproducible results using Resazurin based cell viability experiments using 4 different cell lines. 11studies reported a decrease in cell viability after incubation of BALB / 3T3 and HepG2 cells with iron chloride or molybdenum trioxide with three different cytotoxicity experiments.

We compared antiproliferative and cytotoxicity activities of pyrrolidine compounds with two different cell viability assays. The results of the two

different methods showed similar properties for the MCF-7 cell line. Both MTT and RTCA assays gave the most effective result for compound **II** in the MCF-7 cell line and IC_{50} value: 100 µM. Compound **II** has cytotoxic activity for the MCF-7 cell line. MTT and RTCA assays showed different effects in DLD-1 cancer cell line. The effect of DLD-1 cell on cell viability in the MTT assay gave the most effective result for compound **II** (100 µM) and has an antiproliferative effect. In the RTCA assay, the effect of compound **II** on the cell viability of DLD-1 cell is almost nonexistent (Figure 6-9).

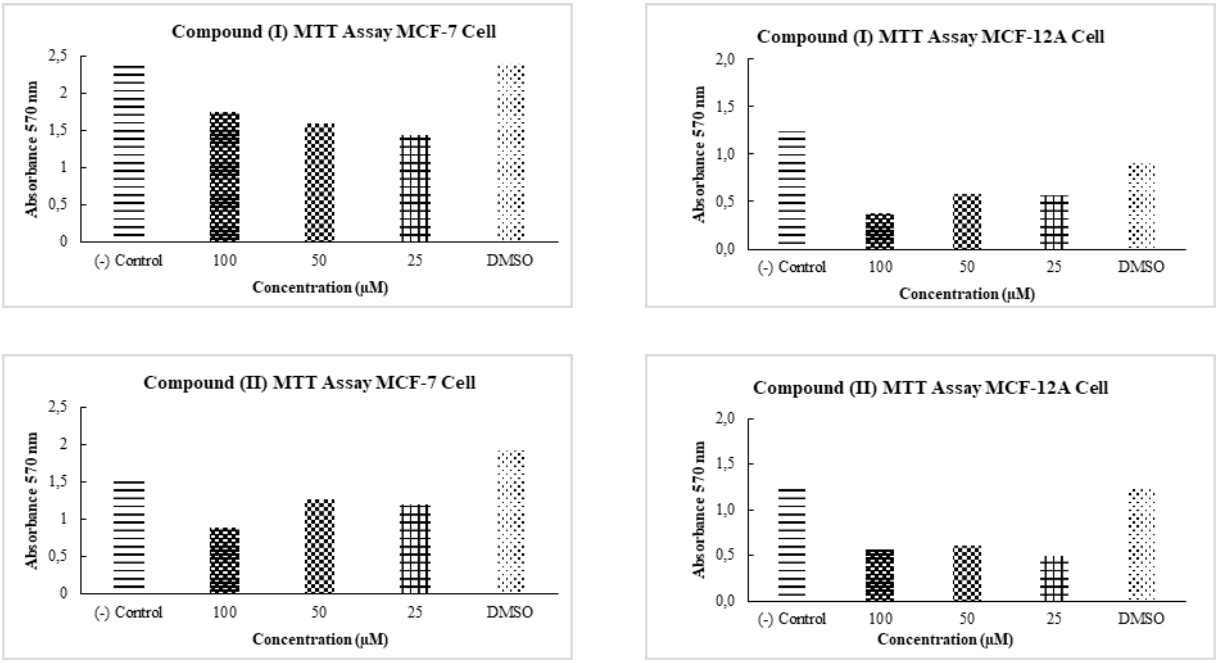


Figure 6 – MTT assay of increasing concentrations of compounds on MCF-7 and MCF-12A cells

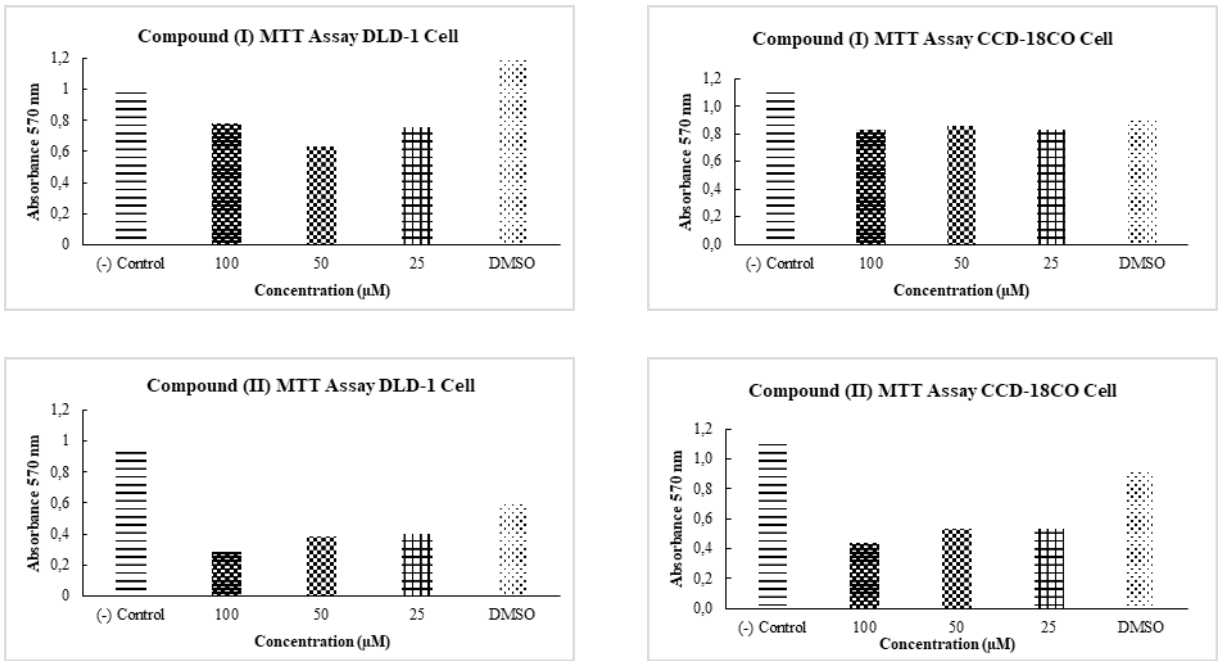


Figure 7 – MTT assay of increasing concentrations of compounds on DLD-1 and CCD-18CO cells

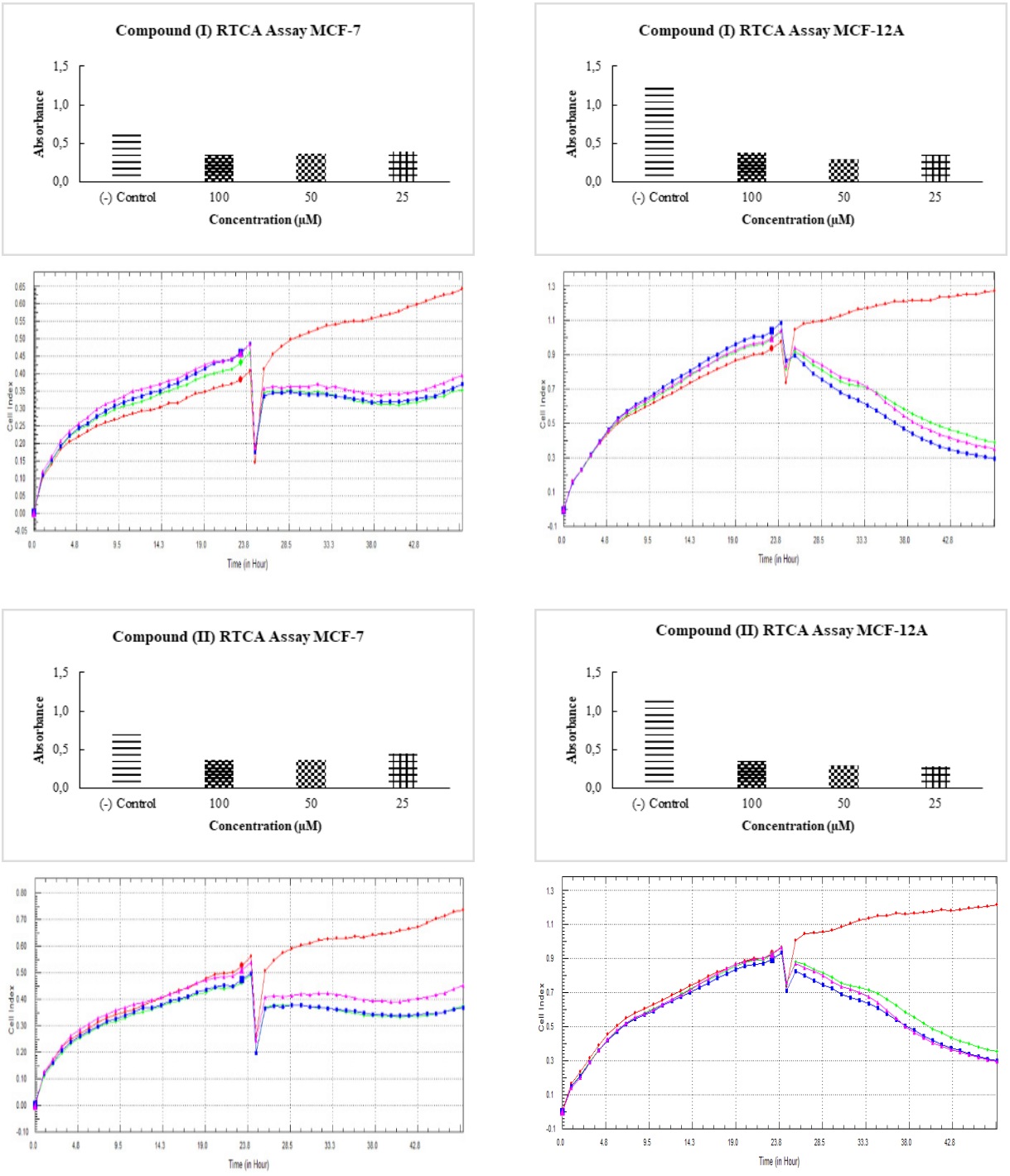


Figure 8 – Real-time cell analysis of increasing concentrations of compounds on MCF-7 and MCF-12A cells. (Red; Negative control, Green; 100 µM, Blue; 50 µM, Pink; 25 µM)

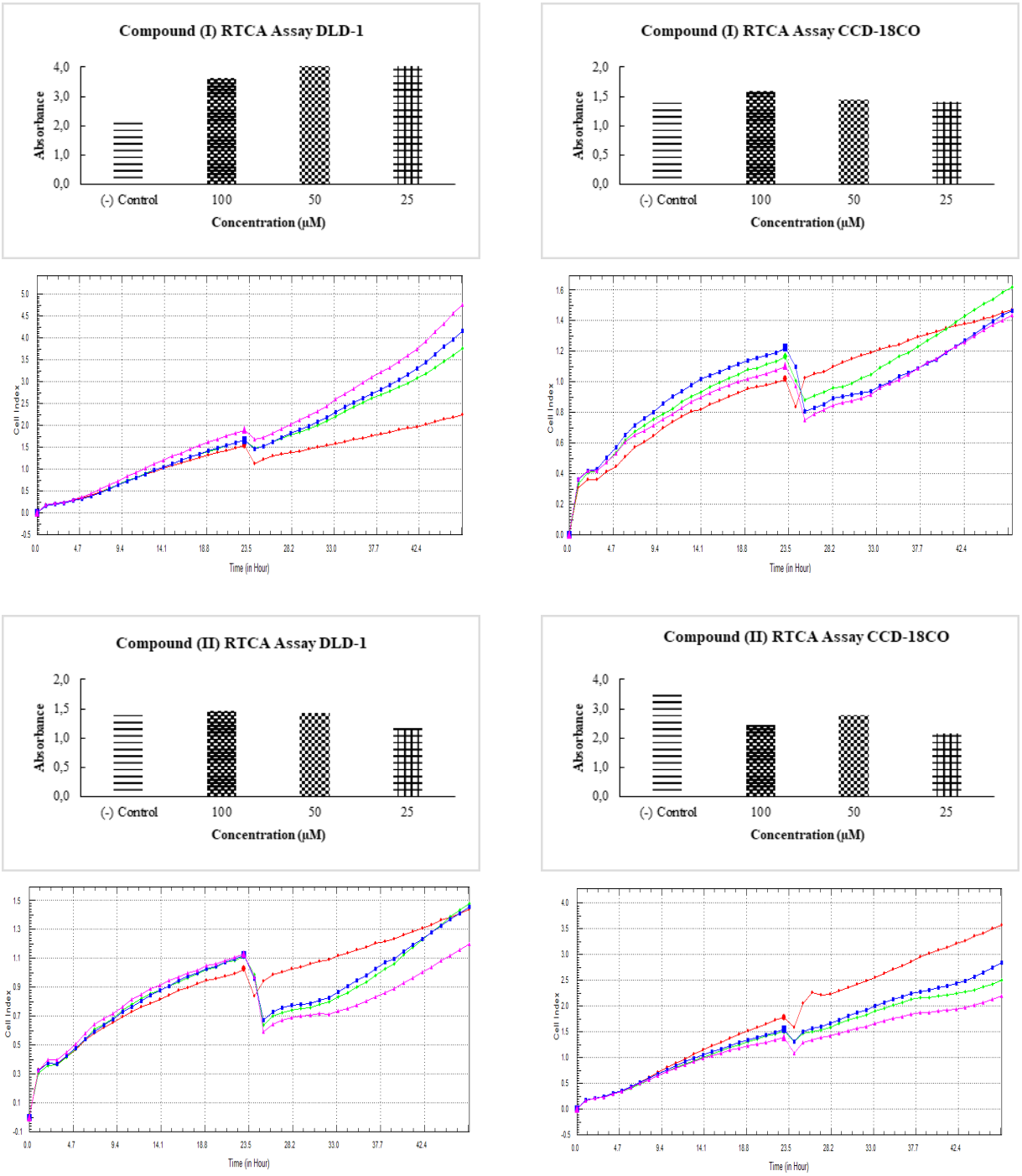


Figure 9 – Real-time cell analysis of increasing concentrations of compounds on DLD-1 and CCD-18CO cells. (Red; Negative control, Green; 100 µM, Blue; 50 µM, Pink; 25 µM)

Compound **I** and **II** concentrations were compared across four cell lines. According to the results of ANOVA analysis (SPSS 20.0), there was a significant difference between them. Post hoc analyses were also conducted to understand the relationships between the groups. Therefore, the homogeneity of the variance test result was determined, and different tests were used depending on these results. Games-Howell and Tukey tests were used for multiple comparisons of compound **I** and compound **II** in cancer cell (MCF-7 and DLD-1) and normal cell (MCF-12A and CCD-18CO) ($p<0.005$) (Table 2 and 3, Figure 10-13). The acquired data was evaluated using SPSS 20.0 analysis and defined as IC_{50} values.

Table 2 – *In vitro* cell viability of cell lines by MTT assay after treating MCF-7 and MCF-12A cell lines with varying concentrations of studied compounds for 24 hours

Compounds	Concentration (μM)	Cell lines ID50 [μm] ± SE	
		MCF-7	MCF-12A
I	25	1,445±0,075	0,566±0,106
	50	1,598±0,127	0,579±0,139
	100	1,752±0,079	0,382±0,059
II	25	1,197±0,086	0,493±0,059
	50	1,257±0,067	0,608±0,097
	100	0,885±0,100	0,563±0.040

Table 3 – *In vitro* cell viability of cell lines investigated by MTT assay after treating DLD-1 and CCD-18CO cell lines with varying concentrations of compounds **I** and **II** for 24 hours

Cell lines	Concentration (μM)	Compounds ID50 [μm] ± SE	
		I	II
DLD-1	25	0,758±0,056	0,402±0,037
	50	0,630±0,037	0,383±0,086
	100	0,779±0,027	0,283±0,062
CCD-18CO	25	0,822±0,003	0,822±0,003
	50	0,718±0,001	0,718±0,001
	100	0,724±0,007	0,724±0,007

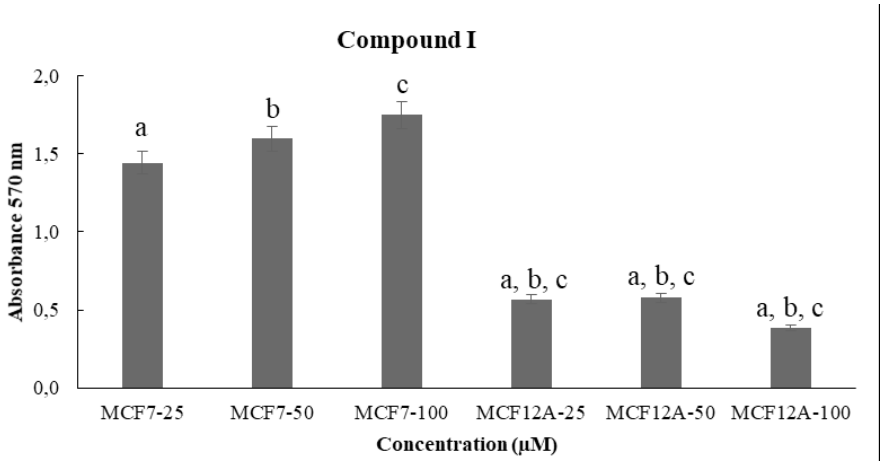


Figure 10 – Comparison between MCF-7 and MCF-12A cell lines in terms of cell viability.
Note: a, b, c indicate significant difference for compound **I**

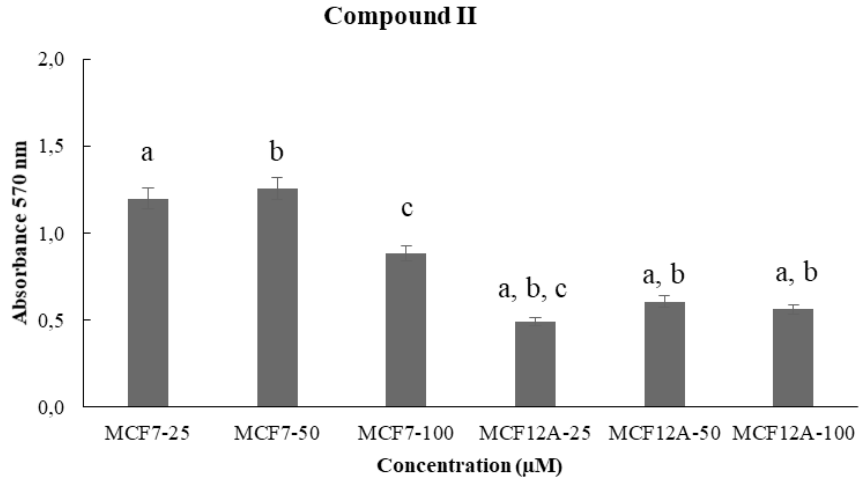


Figure 11 – Comparison between MCF-7 and MCF-12A cell lines in terms of cell viability.
Note: a, b, c indicate significant difference for compound **II**

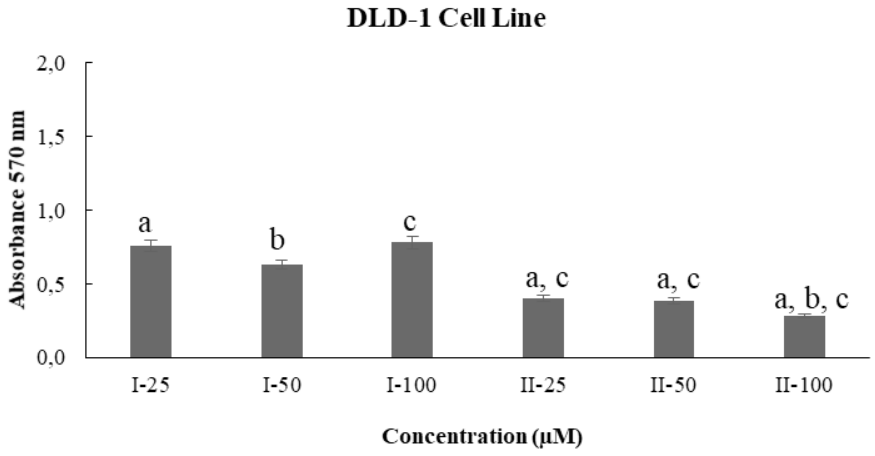


Figure 12 – Comparison between compound **I** and **II** in terms of cell viability.
Note: a, b, c indicate significant difference for DLD-1 cell line

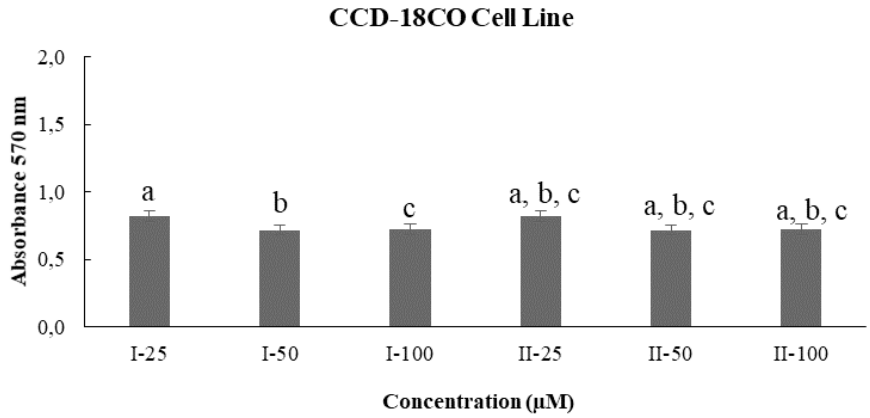


Figure 13 – Comparison between compound **I** and **II** in terms of cell viability.
Note: a, b, c indicate significant difference for CCD-18CO cell line

*The calculation results for **I** and **II**.* The geometries were optimized in the gas phase using Gaussian 09 package of programs [18]. The results obtained from the ab initio TD-DFT calculations with B3LYP /6,31G(d,p). The energy gap between HOMO and LUMO energy level calculated for the compound **I** and **II** were 5.432 eV, 5.074 eV respectively, as given in Figure 14, therefore compound **II** is more reactive than **I**.

The calculated dipole moment values showed compound **II** (3.46 Debye) at a higher value than compound **I** (2.1271 Debye). When dipole moments are compared, it is seen that compound **II** is at the highest value and also these results correlated with biological activity of both MTT and antioxidant results; which is similiar in literature. [25; 26].

MEP is to determine the reactive electrophilic and nucleophilic attack regions, as potential increases red, orange, yellow, green and blue respectively. It is seen that both compounds **II** and **I** ester and imide side red color which represents the negative charge.

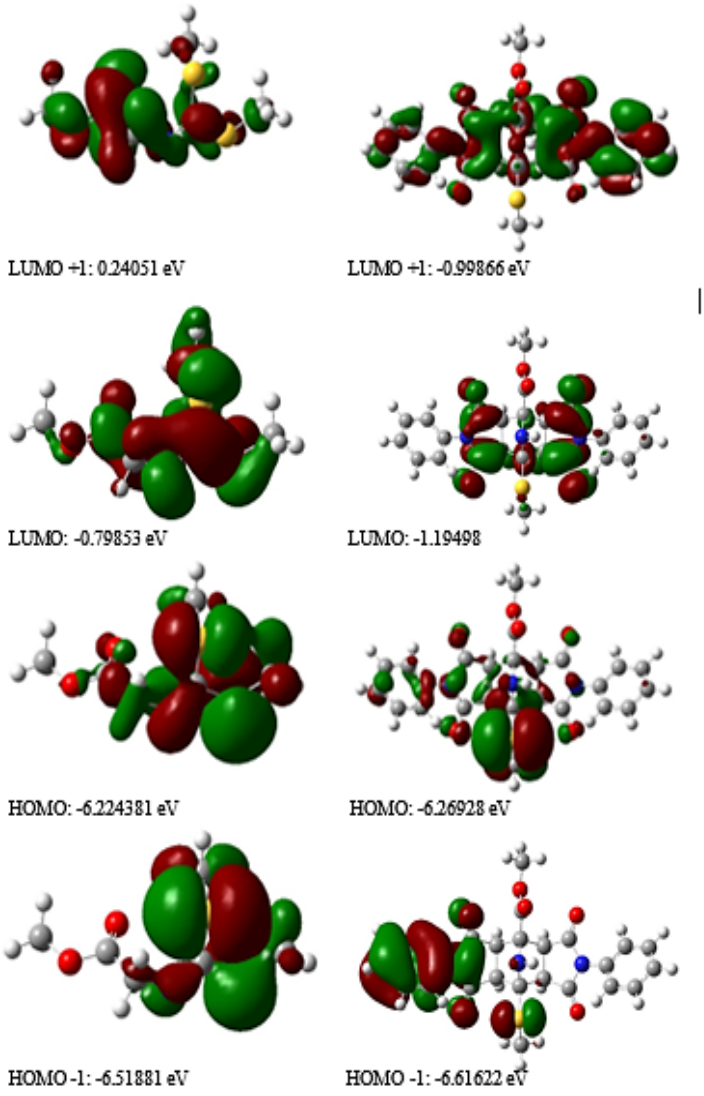


Figure 14 – Frontier molecular orbitals surfaces and energy levels

Represent the ADME properties of compound **I** and **II**. According to the Lipinski's rules: molecules should have five or fewer H-bond donors and ten or fewer H-bond acceptors.

Compound **I** has three H-bond acceptor and **II** has one H-bond donor and seven acceptor. Lipophilicity result in compound **II** is higher than compound **I**.

Conclusion

In brief, in our study, we determined the effect of pyrrolidine-derived compound on cell viability by comparing MTT and RTCA analysis on MCF-7, MCF-12A, DLD-1 and CCD-18CO cell lines. The significance of differences between data sets in MTT assay was analyzed statistically by ANOVA with SPSS 20.0 program for the four cell lines. Besides, the antioxidant activities of the pyrrolidine compounds were determined at different concentrations via free radical scavenging, metal chelating and reducing methods. Cell viability studies investigating pyrrolidine compounds of cancer (MCF-7 and DLD-1) and normal (MCF-12A and CCD-18CO) cell lines, have not been found in literature. This study is unique for both compounds and cell lines. Based on *in sillico* ADME studies and DFT calculations, we concluded that compounds **I** and **II** have biologically active. We think that these compounds give effective results in cell viability and that pyrrolidine compounds or new derivatives, that can be synthesized, will be useful for new drug candidates with in molecular research.

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