






In vitro cytotoxicity evaluation and phytochemical analysis of *Ajuga reptans* L. extracts

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ABSTRACT: The aim of this study was to evaluate phytochemical composition of *Ajuga reptans* L. (Lamiaceae) aerial parts (separately flower and leaf) methanol, aqueous-methanolic extracts, and their cytotoxic activities. The phytochemical analysis was performed high-performance liquid chromatography (HPLC). Caffeic acid, *p*-coumaric, gallic, chlorogenic, ferulic acids, kaempferol, rutin, quercetin, quercetin-3-O-galactoside, and quercitrin were used as reference substances by HPLC in all samples. The major compounds in the extract were found as ferulic acid, caffeic acid, rutin, and quercetin-3-O-galactoside. Cytotoxicity was investigated using methyl thiazole tetrazolium (MTT) assay. Cytotoxic evaluation of the extracts against cancer (MCF7, PC3, and A549) and healthy human embryonic kidney cell line (HEK293) cell lines by MTT. Compared to other cells, the methanol extract of *A. reptans* demonstrated high selectivity against PC3 cells (IC₅₀: 95 ± 0.99 µg/mL) and selectivity index was four times higher than reference drug colchicine. (IC₅₀: 95 ± 0.99 µg/mL, SI: 6.10). *A. reptans* demonstrated antiproliferative potential against prostate and lung cancer cells. Therefore, additional investigations are needed to study the mechanism of the cytotoxicity for *A. reptans*.

KEYWORDS: *Ajuga*; Cytotoxicity; Lamiaceae; HPLC.

1. INTRODUCTION

Ajuga genus (Lamiaceae) are perennial or annual herbaceous flowering plants, growing worldwide. Some species belonging to *Ajuga* genus are used in traditional medicine of different countries [1]. Several species of this genus have medicinal value but lack of information about therapeutic potential. *Ajuga reptans* L. (bugle) one of the important species of *Ajuga* genus used in the traditional medicine of many countries especially the eastern part of Europe [2]. *A. reptans* known in traditional medicine for its anti-inflammatory, wound healing and hepatoprotective properties [3,4]. The extracts obtained from *A. reptans* are used due to the contented iridoids (anti-inflammatory and wound healing) as antidiarrhoeic, antileucoreic, hepatoprotecting, and vulnerar [2,5]. Some active compounds, such as reptansterone [6], 28-epi-sengosterone [6], ajureptoside [7], ajugatansins A-D [8] and ajugavensin A-B [9] were identified in previous studies. Previous studies also reported that *A. reptans* flowers has rich source of anthocyanins and phenolic acids [10]. The use of natural products as anti-cancer agents has a long history that began with ethno-medicine and through the years. Nowadays, several drugs used in chemotherapy were isolated from plant species or derived from a natural compound [11]. In some studies, the extracts from different species of *Ajuga* genus were reported to have anticancer activity. Particularly, methanol and aqueous-methanolic extracts of genus aerial parts inhibit human breast carcinoma (MCF7), liver cancer (HepG2), human lung carcinoma (A549), hepatocellular carcinoma (LM3), human colon carcinoma cells (HT29) and human breast cancer (MDA-MB) cell lines [12]. In another study, the polar extracts of *A. bracteosa* exhibited significant activity against the human leukemia cell line (TPH-1), although nonpolar extracts were more active against the MCF7 cell line [13]. The aim of this

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present study was investigation of cytotoxic activity and phytoconstituents of methanol and aqueous-methanolic extracts obtained from aerial parts of *A. reptans*, collected in Sakarya (Türkiye), which was not previously reported. Furthermore, we evaluated the cytotoxic effects of methanol extracts and aqueous-methanolic extract of *A. reptans* both flower and leave part on MCF7, PC3, A549 cell lines and observed that difference in cytotoxic activity of extracts were not correlated with their similarities in composition of phenolic acids and flavonoids. Based on five phenolic acid standards and four flavonoid standards, we determined the chemical composition by HPLC analysis.

2. RESULTS

Qualitative chromatographic analysis by HPLC

Analyzing the *A. reptans* samples by HPLC (Table 1) on the basis of retention times as well as UV spectra of standard compounds, two phenolic acids (caffeic and ferulic acids), one flavonoid aglycone (rutin) and one flavonoid glycoside (quercetin-3-O-galactoside) were unequivocally identified (Figure 1 and Figure 2). The chemical composition and biological properties of different *Ajuga* species extracts differed significantly. Even within the same species, the chemical composition of plants can differ.

Table 1. Retention time of standart phenolic compounds in *A. reptans* extracts (min), compounds detected in extracts (+) not detected (-).

| No | Phenolic compounds | Rt (min) | Detected |
|----|---------------------------|----------|----------|
| 1. | Quercetin-3-O-galactoside | 4.62 | + |
| 2. | Caffeic acid | 5.40 | + |
| 3. | Rutin | 8.57 | + |
| 4. | Ferulic acid | 10.41 | + |
| 5. | Gallic acid | 3.57 | - |
| 6. | Chlorogenic acid | 3.39 | - |
| 7. | Kaempferol | 22.23 | - |
| 8. | Quercitrin | 3.45 | - |

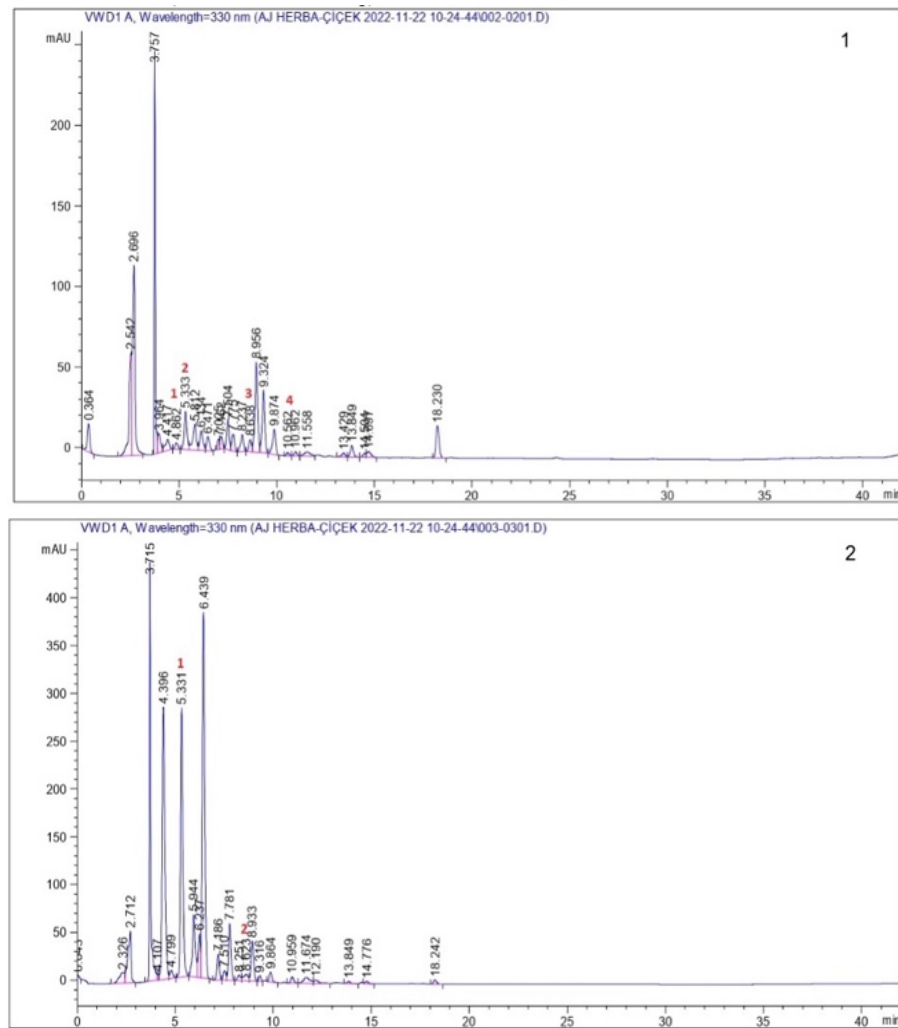


Figure 1: (1) HPLC chromatogram of AR flower meoh extract (1 (quercetin-3-galaktoside) R.T.: 4.86; 2 (caffeic acid) R.T.:5.33; 3 (rutin) R.T.:8.63; 4 (ferulic acid) R.T.:10.56). (2) HPLC chromatogram of AR flower meoh-water (1 (caffeic acid) R.T.:5.33; 2 (rutin) R.T.:8.62).

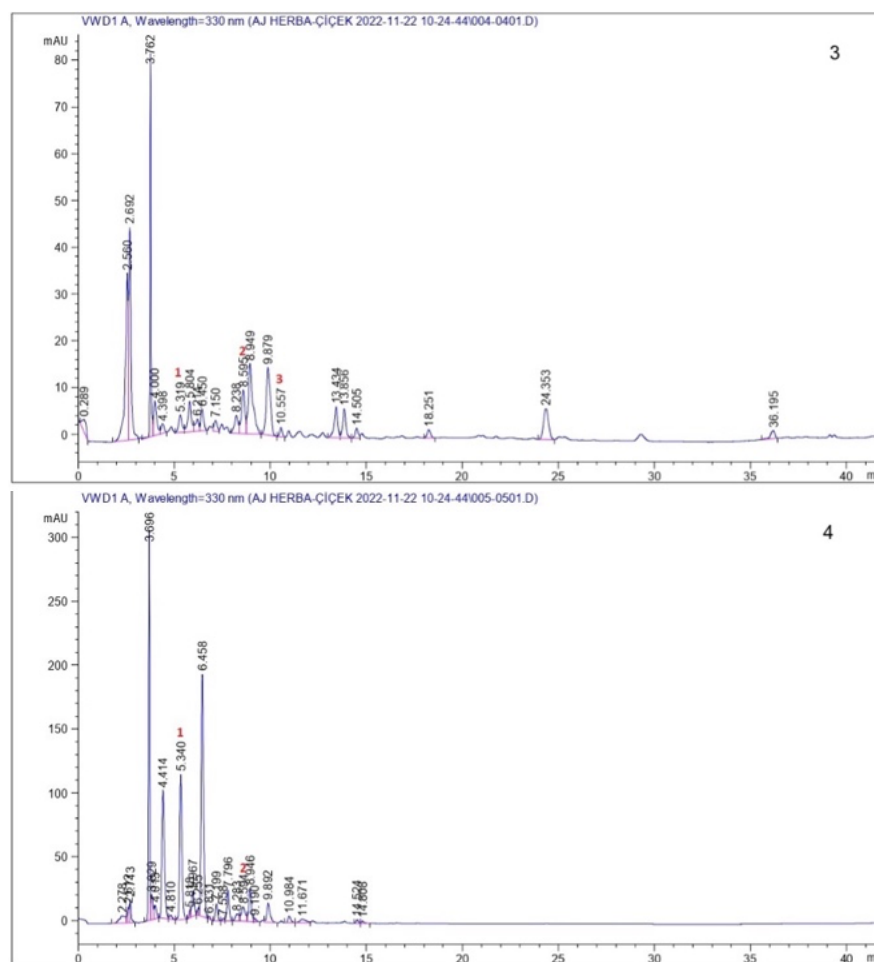


Figure 2: (3) HPLC chromatogram of AR aerial part meoh (1 (caffeic acid) R.T.:5.31; 2 (rutin) R.T.:8.59; 3 (ferulic acid) R.T.:10.55). (4) HPLC chromatogram of AR aerial part meoh-water (1 (caffeic acid) R.T.:5.34; 2 (rutin) R.T.:8.59)

From this aspect, our *A. reptans* flower extract showed similarity and differences to analyzed samples from Romania. In previously analyzed extracts, cinnamic acid derivatives, two flavonoid aglycones and one flavonoid glycoside were observed [4]. Compared to other study that reported the chemical composition of *A. reptans*, there is a variation in the chemical composition. The compounds of the flower of *A. reptans* in Romania were caffeic acid, *p*-coumaric acid, ferulic acid, luteolin, apigenin, and quercitrin. On the other hand, the best of our knowledge, this is the first report on polyphenols of *A. reptans* flower methanol and aqueous-methanolic extract.

Cell viability

In vitro assessment of cytotoxic activity of the methanol and aqueous-methanolic extracts different part of *A. reptans* against human cancer cell lines (A549, MCF7 and PC3) and normal embryonic kidney cell line (HEK293) was performed using the MTT assay. The IC₅₀ values of each extract of *A. reptans* against A549, MCF7, PC3 and HEK293 cell lines are presented in Table 2. Cytotoxic effects exhibited all extracts IC₅₀ values of 163 ± 2.01, 160 ± 1.29, 137 ± 1.02, and 95 ± 0.99 µg/mL against the PC3 cell line, respectively. The methanol extracts of flower significantly reduced the cell viability in the PC3 cell line (selectivity index (SI) = 6.10) while a significant decrease was observed in the MCF7 cell line only at 300 µg/mL compared to the control (SI = 1.91). MTT assay showed that the methanolic extract of *A. reptans* flower (AS1) show higher cytotoxic effects against PC3 cell lines compared to MCF7 and A549 cell lines and non-selective towards the HEK293 (Table 2). The aqueous-methanolic extract of *A. reptans* flower (AS2) also demonstrated cytotoxic effect against the A549 and PC3 cell lines with IC₅₀ values 133 ± 1.40 and 137 ± 1.02 µg/mL, respectively. Treatment of both methanol (AS3) and aqueous-methanolic (AS4) extracts of *A. reptans* aerial parts revealed cytotoxic activity towards PC3 cell line with IC₅₀ values 160 ± 1.29 and 163 ± 2.01 µg/mL, respectively. The results were compared to the

standard cytotoxic drug (Colchicine) (Table 2). On the other hand, cytotoxic activity of *Ajuga orientalis* L. extracts was tested by Oran et al. (2022) against a variety of human cancer cell lines, including, human colon adenocarcinoma cells (Caco2) and MCF7 [15]. The ethanolic extract of *A. orientalis* demonstrated high selectivity against MCF7 cells and low selectivity against Caco-2 cells whereas the aqueous extract demonstrated remarkable selectivity against both tested cancer cells. Furthermore, the cytotoxic effect of ethanolic extracts of *A. genevensis*, *A. chamaepitys*, and *A. laxmannii* against various cell lines, including murine colon carcinoma cell line (C26) and murine melanoma cell line (B16.F10). The antiproliferative potential of ethanolic extracts of *A. genevensis*, *A. chamaepitys*, and *A. laxmannii* on murine colon carcinoma cell line (C26) and murine melanoma cell line (B16.F10) was investigated [16]. The ethanolic extract of *A. laxmannii* exhibited the most potent cytotoxicity ($IC_{50} = 176.3$ and $236.8 \mu\text{g/mL}$) on C26 cells and B16.F10 cells, respectively. Moreover, compared to other cells lines, the methanolic extract of *A. reptans* flower demonstrated selectivity against PC3 cancer cells and lowest selectivity against MCF7 cells.

Table 2. IC_{50} values and standard deviation of extracts and standards on A549, MCF7, PC3, and HEK293 cells for 24 hours ($\mu\text{g/mL}$) and selective index (SI) of extracts. Methanolic extract of *A. reptans* flower (AS1), aqueous-methanolic extract of *A. reptans* flower (AS2), methanol (AS3) and aqueous-methanolic (AS4) extracts of *A. reptans* aerial parts.

| Extract | $IC_{50}(\mu\text{g/mL})$ | | | | Selectivity Index (SI) | | |
|------------|---------------------------|-----------------|------------------|------------------|---|--|---|
| | A549 | PC3 | MCF7 | HEK293 | $IC_{50}\text{HEK293}/IC_{50}\text{A549}$ | $IC_{50}\text{HEK293}/IC_{50}\text{PC3}$ | $IC_{50}\text{HEK293}/IC_{50}\text{MCF7}$ |
| AS1 | 112 ± 1.55 | 95 ± 0.99 | 303 ± 0.99 | 580 ± 2.55 | 5.17 | 6.10 | 1.91 |
| AS2 | 133 ± 1.40 | 137 ± 1.02 | 401 ± 2.11 | 660 ± 2.06 | 4.96 | 4.81 | 1.64 |
| AS3 | 303 ± 2.05 | 160 ± 1.29 | 339 ± 1.87 | 448 ± 1.99 | 1.47 | 2.80 | 1.32 |
| AS4 | 254 ± 1.99 | 163 ± 2.01 | 410 ± 2.59 | 703 ± 2.81 | 2.76 | 4.31 | 1.71 |
| Colchicine | 9.28 ± 1.01 | 0.98 ± 0.99 | 52.11 ± 0.85 | 24.53 ± 1.02 | 2.58 | 24.48 | 0.46 |

3. DISCUSSION

Flavonoids and phytosteroids are found in large quantities in *A. reptans*. It also contains anthocyanins, phenolic acids, and other secondary metabolites. Many reports were published on the chemical composition of *A. reptans*, mentioning the evidence of numerous bioactive compounds, among them terpenoid, sterols, phenolic acids and flavonoids. However, there are few references in the literature on the phenolic profile of this species. The chemical composition and biological properties of different *Ajuga* species extracts differed significantly. Even within the same species, the chemical composition of plants can differ. These variations are associated with several major factors that may have impact on the yield, composition, solvent, and tissues of plant organs [14]. Toiu et al. (2017) showed that three flavonoids (quercitrin, apigenin and naringenin) and three phenolic acids (caffeic, *p*-coumaric and ferulic acids) appeared to predominate in the ethanolic extract of *A. reptans* [4]. However, Ghita et al. (2011) reported the presence of caffeic acid, chlorogenic acid, apigenin, and luteolin in the extract of the aerial part of *A. reptans* methanolic extract [2]. These results are in accordance with our findings. The study for anti-cancer agents from natural sources was successful worldwide; active extracts and compounds were investigated and nowadays used to treat cancer [14]. Traditional medicine knowledge is helpful to lead the search for plants with potential cytotoxic activity. The present study was undertaken to evaluate the cytotoxic activity of *A. reptans*, the traditional medicines, that are used in the treatment of cancer and cancer-related illnesses in the country. Ethnopharmacological data has been one of the common ways for the discovery of biologically active constituents from plants extracts. The advantage of the ethnopharmacological knowledge is that the literature may already allow for rationalization with respect to the therapeutic potential of a reputed use. On the basis of reported traditional uses, this plant *A. reptans* is selected for the study of the potential anti-cancer activity. In this study, methanol and aqueous-methanolic extract of *A. reptans* was used for the investigation of cytotoxic activity. Extracts of *A. reptans* were investigated using a MTT assay on three human cancer cell lines, MCF7, A549, PC-3 and normal cell line HEK293. A mitochondrial enzyme in living cells, succinate-dehydrogenase cleaves the tetrazolium ring and converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan formed is directly comparable to the number of viable cells, according to Mosmann [17].

Plants contain many phytochemical compounds, including phenolic acid and flavonoid compounds. Because of their antioxidative and possibly anticarcinogenic properties, the effect of these phytochemicals is currently of great interest. In addition to their metal-chelating abilities, phenols and flavonoids act as free radical scavengers and reducing agents [18]. Rutin, as a glycoside of quercetin (flavanol) has several beneficial

pharmacological properties including anticancer, antiproliferative, anti-carcinogenic, and anti-oxidative stress effects [19]. There are *in vitro* studies that evaluate the effect of rutin on the proliferation of cancer cell lines, such as the breast, colon, prostate, and lung [20]. Rutin caused growth inhibition in human glioblastoma cell lines (U251) through induction of apoptosis and regulation of expression of the pro- and antiapoptotic genes (Bcl-2, Cas-3, Bax, and TP53). Rutin also decreased mitochondrial membrane potential. It has shown *in vitro* antiangiogenic properties on SW480 (human colon adenocarcinoma cell line) through cell cycle arrest at G1 phase and regulation of microRNAs (miRNAs), long noncoding RNAs (lncRNAs), messenger RNAs (mRNAs), and transcription factors (TFs) [21–24]. The chemopreventive activities of rutin also have been confirmed in animal models in several research papers [19].

Phenolic acids are a subclass of plant phenolics, divided into benzoic and cinnamic acids, that are associated with potent anticancer abilities in various *in vitro* and *in vivo* studies [25]. Caffeic acid (3,4-dihydroxycinnamic acid, CA), had a potent inhibitory effect on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion and TPA-induced formation of DNA of mouse skin and inhibitory effect on the synthesis of DNA, RNA and protein in cultured HeLa cells [26]. CA derivatives most potent anticancer activity showed on colon-HCT 116, breast-MCF-7 and lung-NCI H460 cells [27]. Several studies showed that ferulic acid cytotoxic effects in the colorectal cancer Caco-2 cell line by elongating S/G2 phase and reducing G1 phase. It also showed cytotoxicity on prostate cancer PC-3 and LNCaP cell lines, inhibited cell proliferation, invasion and induced apoptosis at 300 μ M and 500 μ M, respectively [28].

Studies showed that cytotoxic effect of *Ajuга* genus not only correlated with phenolic compounds, since there was no obvious correlation between phenolic compound content and cytotoxic activity. For that reason, additional qualitative and quantitative analyses of *A. reptans* extracts are needed to explain the cytotoxic activity.

4. CONCLUSION

In the present study, our results demonstrated that the methanolic and methanol-water extract of *A. orientalis* flowers and aerial parts has similar phenolic and flavonoid contents. The extracts of the mentioned plant showed moderate *in vitro* cytotoxic activity. The aerial parts (separately flower and leaves) of *A. reptans* were examined to determine their cytotoxic activity against, MCF7, PC3, and A549. All extracts were evaluated for their cytotoxic activities using the MTT assay. Statistical significance was determined in comparison to the reference drug colchicine and the selectivity index was determined. Compared to other extracts, methanolic extract of *A. reptans* flower was the most potent on PC3 cell line and selectivity index was four times better than colchicine. Further biological and phytochemical investigations are recommended to evaluate their potential as cytotoxic therapeutics.

5. MATERIALS AND METHODS

5.1. Chemicals

The standard chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and the HPLC-grade solvents were purchased from Merck. Methyl thiazole tetrazolium (MTT) reagent were purchased from Sigma-Aldrich (Germany).

5.2. Plant material and extraction

The plant of *Ajuга reptans* L. was identified and collected in May 2022 from Geyve district of Sakarya, Türkiye. The aerial parts of *A. reptans* was authenticated by Dr. Ayse Esra Karadag at Department of Pharmacognosy, Medipol University Faculty of Pharmacy, Istanbul, Türkiye. The Voucher specimen No. is IMEF 1268. The dried aerial parts of *A. reptans* (200 g) were both flower and aerial parts separately cut into small pieces, powdered, and then both extracted with methanol and methanol-water (4:1) mixture for 36 h. The viscous semi-solid extract was collected in a tared conical flask the solvent was removed by rota-evaporator and last traces of solvent being removed under vacuum *etuv*. The yield was found 10 g for flower methanol extract, 13 g for aerial parts methanol extract, 8 g for flower aqueous-methanolic extract, and 12 g for aerial parts aqueous-methanolic extract.

5.3. Qualitative chromatographic analysis with HPLC systems

For High Pressure Liquid Chromatography (HPLC) analysis 2 mg each extract of *A. reptans* was dissolved in 5 mL methanol and filtered by 0.22 µm membrane filters. HPLC analysis of standards and extracts was performed using Agilent 1200 series instrument. Flavonoids were separated on a C-18 reverse phase HPLC column (Agilent, 250 mm x 4.6 µm, particle size 5 µm) at 25°C. Eluent A was the water and eluent B was acetonitrile. Separation was performed in an isocratic step at %15 of B for 15 min followed by a linear gradient from 15% to 95% of B in 5 min, then to 100% of B in 2 min and 85% of B in 5 min with a flow rate at 1 mL/min [29]. Flavonoids were identified by comparing their retention times and corresponding UV-Vis absorption spectra with caffeic acid, *p*-coumaric, gallic, chlorogenic, ferulic acids, kaempferol, rutin, quercetin, quercetin-3-O-galactoside, and quercitrin standards.

5.4. Cell culture

Human breast cancer cell line MCF7, human prostate cancer cell line PC3, human lung cancer cell line A549, human embryonic kidney cell line HEK293 were grown in suitable medium [Dulbecco's modified Eagle's medium/high glucose (DMEM/High)], RPMI 1640 (with L-Glutamine with Phenol Red and without HEPES), Dulbecco's Modified Eagle's Medium" (DMEM/Gibco) supplemented with 10%(v/v) heat-inactivated fetal bovine serum, L-glutamine (2mM), antibiotic-antimycotics solution (100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B), at 37 °C in a humidified incubator containing 5% CO₂ [30]. The cells were passaged every 3 days.

5.5. MTT Assay

Cell viability was essentially determined using the MTT assay as described by [17]. The studied cells were situated at 5 ×10⁴ cells into each well of 96-well tissue culture plates (Nunc, Denmark) and incubated for 24 h. After this procedure, all the tested extracts and Colchicine (positive control) were dissolved in DMSO (0.5%) individually and added to culture wells at varying concentrations (1-1000 µg/mL). After 24 h of incubating period, 30 µL MTT solution (0.5 mg/mL in Phosphate Buffered Saline) was added to each well and the cells were incubated for 4 h. at 37°C. Purple formazan crystals were generated via the reduction product of the MTT agent by the mitochondrial dehydrogenase enzyme of intact cells. These crystals were dissolved in 150 µL DMSO and the absorbance was read by Spectramaxi3 (OD570 nm). The percentage of living cells was calculated based on the medium control. The extracts concentrations that reduced absorbance to 50% of control values were described as IC₅₀ values.

$$\text{Viability \%} = (\text{Absorbance}_{\text{extract}} / \text{Absorbance}_{\text{control}}) \times 100$$

$$\text{Selectivity Index} = \text{IC}_{50} \text{ Health cell line} / \text{IC}_{50} \text{ Cancer cell line}$$

5.6. Statistical analysis

All repeated experiments were conducted in triplicate. Statistical analysis and IC₅₀ values were determined by using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA; Version 8.4.3). The data were expressed as mean ± standard deviation (SD).

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