



## Research Article

# Antioxidant, Antiproliferative and Apoptotic Effects of Secondary Metabolites of Halotolerant *Aspergillus terreus* on Colon Adenocarcinoma Caco-2 Cells

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## Abstract

**Objective:** This study aimed to investigate the antioxidant and anticancer effects of the secondary metabolites of halotolerant *Aspergillus terreus*-2 (hAt-2) on colon adenocarcinoma (Caco-2) cells. The hAt-2 was isolated from Lake Tuz in Turkey. **Materials and Methods:** Ethyl acetate extract was prepared from the isolate of hAt-2 culture medium. The free radical scavenging activity and total phenolic content were evaluated using 2,2-diphenyl-1-picrylhydrazyl and Folin-Ciocalteu assays. The phenolic acids of the extract were evaluated using the high-performance liquid chromatography method. The cytotoxic effect of the extract was evaluated using 4-[3-(4-iodo-phenyl)-2-(4-nitrophenyl)-2H-5 tetrazolio]-1,3-benzene disulfonate assay. Cell proliferation was evaluated using the real-time cell analysis system (RTCA-DP). **Results:** The major phenolic acid in the hAt-2 extract was protocatechuic acid. The hAt-2 extract exhibited significant antiproliferative and apoptotic effects on Caco-2 cells in a time and concentration-dependent manner. The IC<sub>50</sub> value of the extract was detected as 1859 µg mL<sup>-1</sup> at 72 h using the RTCA-DP system. **Conclusion:** The present study suggested that the hAt-2 extract has antioxidant, antiproliferative and apoptotic properties to act as an anti-cancer agent on Caco-2 cells.

**Key words:** Anticancer, apoptotic, *Aspergillus terreus*, Caco-2, secondary metabolite

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Cancer is a complex multifactorial disease of the cells and a major public health concern in the world. Cancer development and progression are dependent on the cellular accumulation of free radicals that may be toxic to the surviving cells<sup>1-3</sup>. Colorectal cancer is among the top-ranking cancers in terms of diagnostic frequency and has the highest mortality rates<sup>4,5</sup>. Drug discovery against cancer, especially from the natural products is ventured throughout the world<sup>6</sup>. Natural products are of particular interest as chemopreventive agents because of their potentially low toxicity profiles and effectiveness<sup>7,8</sup>. Individuals use natural products for a variety of reasons, including preventing cancer and its recurrence<sup>8</sup>. New chemotherapeutic drugs are needed, in particular, to attenuate the varied harmful side effects of currently commonly used chemotherapeutics<sup>9-11</sup>.

Natural products are the potential source of new anticancer agents. Despite extensive progress in chemotherapy in the last decade alternative or adjuvant therapeutic agents still need to be discovered and developed. In the realm of drug discovery, natural products provide a vast pool for screening new antitumoral agents including candidate compounds to overcome drug resistance. Also, low-toxicity anticancer agents from fungi have become one of the research topics in the current pharmacy field<sup>12,13</sup>. Natural products may contribute to human survival. Hence, the interest in the field of natural antioxidants obtained from microfungi has increased. Natural products have been used in the pharmaceutical industry, especially in the treatment of cancer for the past 15 years<sup>14</sup>.

A comparison with other genera shows that *Aspergillus* species have been extraordinarily productive with regard to exometabolites (secondary metabolites)<sup>13</sup>. This study was undertaken to analyze the antioxidant, cytotoxic, antiproliferative and apoptotic effects of ethyl acetate extract of *Aspergillus terreus* (hAt-2), which was isolated from Tuz Lake (located in Turkey), on colon adenocarcinoma Caco-2 cells.

## MATERIALS AND METHODS

**Microorganism culture conditions and preparation of ethyl acetate extract:** The hAt-2 was isolated from different parts of Lake Tuz, which is the second biggest lake in Turkey. It was grown on the slant of malt extract agar and maintained at 25°C. Spore suspension of hAt-2 was prepared in sterile water and used for inoculation. The antioxidant medium was

prepared according to the modified formula of Malpure<sup>14</sup> and consisted of 3% sucrose, 0.1% yeast extract, 0.1% polypeptone, 0.3% (NH<sub>4</sub>)SO<sub>4</sub>, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05% KCl and 0.001% FeSO<sub>4</sub>·7H<sub>2</sub>O. Spore suspension (5 mL, 10<sup>7</sup> spores mL<sup>-1</sup>) of hAt-2 was inoculated into a 1000 mL Hilton flask containing 300 mL of the medium. The flasks were incubated at 25°C. Ethyl acetate extract was prepared from the isolate of hAt-2 culture medium, which included secondary metabolites.

**Cell culture:** The Caco-2 cells were cultured in RPMI 1640 containing 2 mM L-glutamine, 1.5 g L<sup>-1</sup> NaHCO<sub>3</sub> (sodium bicarbonate), 1% penicillin/streptomycin and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were grown to confluence in the growth medium. They were harvested at confluence with 0.05% trypsin-0.02% EDTA, plated in tissue culture dishes and grown in a fresh medium. A stock solution of the extract was dissolved in 0.1% dimethyl sulfoxide (DMSO) solution.

**Assessment of free radical scavenging activity using the 2,2-diphenyl-1-picrylhydrazyl method:** Scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals is a basic and easy method for assessing antioxidant activity<sup>15</sup>. The DPPH spectroscopic method has been used to exhibit the antioxidant capacity of the phenolic compounds<sup>16</sup>. This method is based on the measurement of free radical scavenging activity of the extract using DPPH radicals. The extract had a stronger radical scavenging effect owing to its higher phenolic content<sup>17</sup>. The reaction mixture was left at ambient temperature for 30 min in the darkness. The absorbance of the mixture solution was then measured spectrophotometrically at 517 nm. Each experiment was repeated three times and the average of the readings was taken. Butylhydroxytoluene (BHT) was used as the reference compound.

Different concentrations (9.6×10<sup>-4</sup>, 1.8×10<sup>-3</sup> and 3.6×10<sup>-3</sup> mg mL<sup>-1</sup>) of the extract were applied on Caco-2 cells and the percentage inhibition values were found to be 60.5±1.2, 79.2±1.36 and 80.1±2.0, respectively. The BHT was also used as a standard antioxidant with the same concentrations as those of the extract. The percentage inhibition values of the extract were found to be 42±1.03, 49±0.91 and 56±1.1, respectively. The results obtained for the extract were compared with those obtained for BHT. Experiments were performed in triplicate and the results were presented as average values with Standard Deviation (SD).

**Determination of the total phenolic content using the**

**Folin-Ciocalteu method:** Total phenolic content was determined using the Folin-Ciocalteu colorimetric method from the absorbance at 750 nm as described by Folin and Ciocalteu<sup>18</sup>. Gallic acid was used as a reference compound for determining the phenolic content of the compound<sup>19</sup>. This method gives a general measurement of the phenolic content of the metabolites.

**High-performance liquid chromatography analysis:**

The phenolic acid composition of the hAt-2 secondary metabolites was evaluated using reverse-phase column with gradient elution system in high-performance liquid chromatography (HPLC) (Shimadzu LC10AVP, Japan) from well-defined peaks<sup>20</sup>. The UV spectra of phenolic compounds were taken from reverse-phase analytic column (ODS3-C<sub>18</sub> (100, 4.6 and i.d., 3 mm) (Inertsil, Waldbronn, Germany) and transferred to a photo diode array detector and the analysis of phenolic compounds in the extract was performed using pre-prepared standards of phenolic acids. The HPLC analysis was performed according to the method proposed by Ozturk *et al.*<sup>20</sup> with some modifications. The HPLC gradient consisted of eluent A (methanol:water:formic acid 10:88:2) and eluent B (methanol:water:formic acid 90:8:2) with a flow rate of 1000  $\mu\text{L min}^{-1}$ : 0 min 15% B, 15 min 20% B, 5 min 35% B, 5 min 46% B and 6 min 100% B. The injection volume was 20  $\mu\text{L}$ . The detection was performed at 280 nm using a photo diode array detector SPD-M10Avp photodiode array (Shimadzu, Kyoto, Japan). Gallic acid (GA), protocatechuic acid (pro-CA), para-hydroxybenzoic acid, vanillic acid, caffeic acid (CA), chlorogenic acid (CHA), syringic acid (SA), para-coumaric acid (p-COU), Ferulic Acid (FA), ortho-coumaric acid (o-COU) and trans-cinnamic acid (tr-CIN) (Sigma-Aldrich, ABD) were used as phenolic acid standards in the HPLC assay. The retention time of phenolic acid peaks was procured from the standard phenolic acid mixture and hAt-2 ethyl acetate extract in 280 nm chromatograms. The UV spectra were compared and the phenolic acid contents of the extract were determined.

**WST-1 cytotoxicity test:** The viability of cells was measured using the 4-[3-(4-iodo-phenyl)-2-(4-nitrophenyl)-2H-5 tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Roche, Germany). The test is based on the cleavage of the tetrazolium salt WST-1 in formazan by mitochondrial dehydrogenases in viable cells. The formazan dye was quantified using a scanning multiwell spectrophotometer by measuring the absorbance of the dye at 420 nm. The Caco-2 cells were inoculated into

96-well culture plates at a density of  $5 \times 10^3$  cells well<sup>-1</sup>. After 24 h, they were treated with different concentrations (50, 100, 150, 200, 300 and 400  $\mu\text{g mL}^{-1}$ ) of the extract for 24 and 48 h. After incubation, cell proliferation reagent WST-1 (10  $\mu\text{L well}^{-1}$ ) was added and the absorbance was measured after 3 h. The absorbance of the samples was measured using an enzyme-linked immunosorbent assay reader (wavelength 420 nm). The measured absorbance was directly correlated to the number of viable cells. Cell viability rates were expressed as the percentage of the controls<sup>21,22</sup>.

**Statistical analysis:** The WST1 data were evaluated as mean  $\pm$  standard error of mean of three independent experiments (n = 8). The results were analyzed using one-way analysis of variance and Tukey test. The  $p < 0.05$  was considered significant (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

**Determination of cell proliferation using real-time cell**

**analysis system:** The Real Time Cell Analysis (RTCA) method is a powerful and reliable tool in the discovery of new potential drugs because it facilitates technical optimization and quality control and has the possibility of monitoring in real time cell responses to compounds at an early stage of drug development in the pharmaceutical industry<sup>23</sup>.

The system measures electrical impedance across interdigitated microelectrodes incorporated at the bottom of tissue culture E-plates. The impedance measurement, which is indicated as Cell Index (CI) value, provides quantitative information about the condition of the cells, involving cell number, viability and morphology<sup>24-26</sup>. Cell viability is related to mechanisms involved in a variety of biological processes including cancer<sup>27</sup>. The background of the E-plates was measured in 100  $\mu\text{L}$  of the medium in the RTCA-DP station. Then, 100  $\mu\text{L}$  of a Caco-2 was added (10,000 cells well<sup>-1</sup>). E-plates were placed into the real-time cell analyzer and impedance was measured every hour. Approximately 24 h after seeding, when the cells were in the log phase of growth, they were treated with 100  $\mu\text{L}$  of the medium containing different concentrations of ethyl acetate extract (50, 100, 150, 200, 300 and 400  $\mu\text{g mL}^{-1}$ ) and impedance monitoring was continued for another 72 h. Impedance was expressed as CI values. Concentration-response curves at 72 h were generated to determine IC<sub>50</sub> values during incubation. A medium containing a final concentration of 0.1% DMSO served as the control. Also, fumagillin (100  $\mu\text{M}$ ) was used as a positive control. The electrical impedance was analyzed using

the RTCA-connected software of the RTCA-DP system as a dimensionless parameter called CI and also  $IC_{50}$  value was determined.

**Apoptosis detection by staining with annexin V-fluorescein isothiocyanate and propidium iodide:** Programmed cell death (apoptosis) is a normal physiological cycle in a healthy organism for destroying cells that are no longer needed. The apoptotic cycle includes translocation of membrane phosphatidylserine (PS) from the inner part of the plasma membrane to the membrane surface. The reaction allows the differentiation of early apoptotic cells, late apoptotic cells, necrotic cells and alive cells<sup>28-30</sup>.

The Annexin V-FITC Apoptosis Detection Kit (Cat. No. 556547; BD Biosciences Pharmingen, USA) was used to detect apoptosis, following the manufacturer's instructions. Briefly, Caco-2 cells ( $5 \times 10^5$  well<sup>-1</sup>) were seeded in six-well plates and treated with different concentrations of hAt-2 extract (25, 50 and 100  $\mu\text{g mL}^{-1}$ ) for 24 h. After the treatment, the cell suspensions were centrifuged at 1500 rpm for 5 min and the pellets were washed twice with 1 mL of cold phosphate-buffered saline. The cells were resuspended in 100 mL of binding buffer and stained with 5 mL of annexin V-FITC solution and 5 mL of Propidium Iodide (PI) solution for 20 min at room temperature in the dark. Then, the samples were diluted with 400 mL of binding buffer, processed for data acquisition and analyzed on a Becton-Dickinson FACSAria flow cytometer using FACSDiva Version 6.1.1 Software, USA. At least 10,000 cells were analyzed per sample. The fraction of cell populations in different quadrants was analyzed using quadrant statistics. The X and Y axes indicated the fluorescence of annexin V (green) and PI (red), respectively. Quadrant settings were optimized by comparing with the control group (nontreatment). It was possible to detect and quantitatively compare the percentages of gated populations in four of the delineated regions. Four distinct phenotypes were distinguishable: Viable (Annexin V/PI) lower left quadrant (Q3), early apoptotic (Annexin V/PI) lower right quadrant (Q4), late apoptotic (Annexin V/PI) upper right quadrant (Q2) and necrotic/damaged cells (Annexin V/PI) upper left quadrant (Q1)<sup>21,31</sup>.

## RESULTS

### **Determination of free radical scavenging activity (DPPH):**

The total phenolic content and free radical scavenging activity of the extract were determined in this study. The total phenolic content was expressed as gallic acid equivalents

(mg GAE  $\text{g}^{-1}$  extract). The percentage inhibition values of the extract were  $60.5 \pm 1.2$ ,  $79.2 \pm 1.36$  and  $80.1 \pm 2.0\%$  (w/w) and the total phenolic content was found to be  $78 \pm 1.32$  mg of GAE  $\text{g}^{-1}$  of extract (dry weight).

The free radical scavenging potential of the extracts compared with that of BHT was also tested by the DPPH method. Percentage inhibition value is a parameter widely used to measure the free radical scavenging activity. That is, a higher percentage inhibition value corresponds to a higher antioxidant activity<sup>15</sup>. These data indicated that the extract had the same activity as that of BHT. The hAt-2 extract showed the highest DPPH radical scavenging ability ( $EC_{50} = 47.30 \pm 0.95$  and  $51.36 \pm 0.98 \mu\text{g mL}^{-1}$ ,  $p > 0.05$  vs BHT), which was similar to that of the reference compound BHT ( $EC_{50} = 51.08 \pm 0.67 \mu\text{g mL}^{-1}$ ). It was observed that extract percentage inhibition values were  $60.5 \pm 1.2$ ,  $79.2 \pm 1.36$  and  $80.1 \pm 2.0$  compared with BHT ( $87.11 \pm 2.06$ ). The results of DPPH radical scavenging activity testing indicated that the hAt-2 extract had similar antioxidant activities. The highest free radical scavenging activity of the extract was  $80.1 \pm 2\%$  at a concentration of  $3.6 \times 10^{-3}$  mg  $\text{mL}^{-1}$ .

### **Determination of the total phenolic content (Folin-Ciocalteu method):**

The Folin-Ciocalteu method was used to determine the Total Polyphenolic (TP) content in the extract of hAt-2 using the UV/Vis spectrophotometric method. The TP is known to be responsible for the antioxidant activity<sup>19</sup>. The total phenolic content of the extract was determined using Folin-Ciocalteu assay. Phenolic complexes have redox properties and act as antioxidants<sup>32</sup>. Their free radical scavenging ability is facilitated via their hydroxyl groups<sup>33</sup>.

**HPLC result:** Most of the phenolic acids have absorption maxima in the ultraviolet absorption spectra at a wavelength of 280 nm. They were identified by matching their retention times (peak normalization) and ultraviolet spectra with those of authentic standards, using the HPLC-photodiode array detection system. All of the phenolic acids were resolved entirely from each other (Fig. 1, 2). The phenolic acid amounts in extract are given in Table 1. The HPLC results showed that pro-CA dominated in the extract. Also, pro-CA, GA, FA, CA, CHA, SA, p-COU, tr-CIN and o-COU acids were detected in the extract of hAt-2.

All these results demonstrated that the free radical scavenging activity of ethyl acetate extract of hAt-2 was found in parallel with the total phenolic content. The inclusion of a high amount of pro-CA ( $64.34$  mg  $\text{g}^{-1}$ ) from the family of phenolic acids in the extract verified significant antioxidant activity.

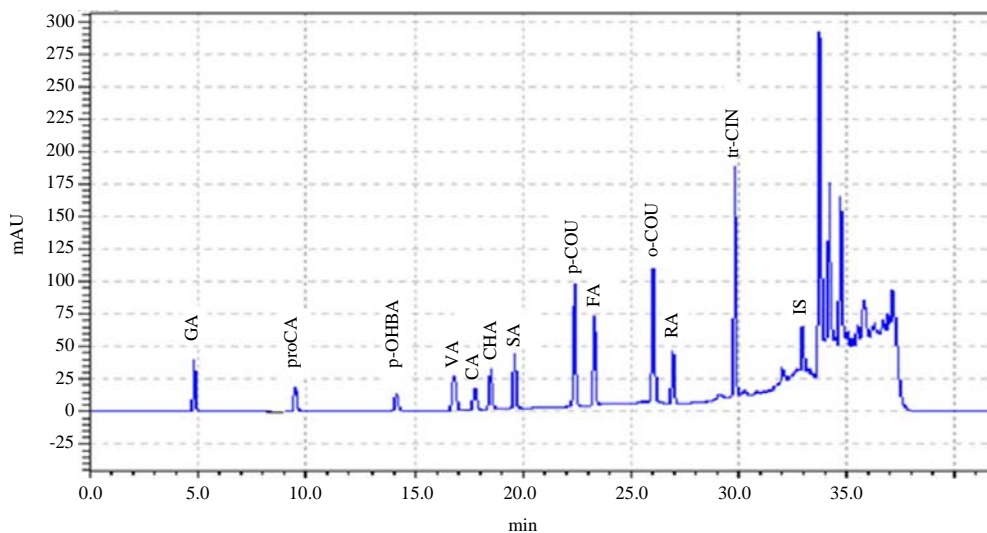


Fig. 1: HPLC chromatogram of standard phenolic acid content

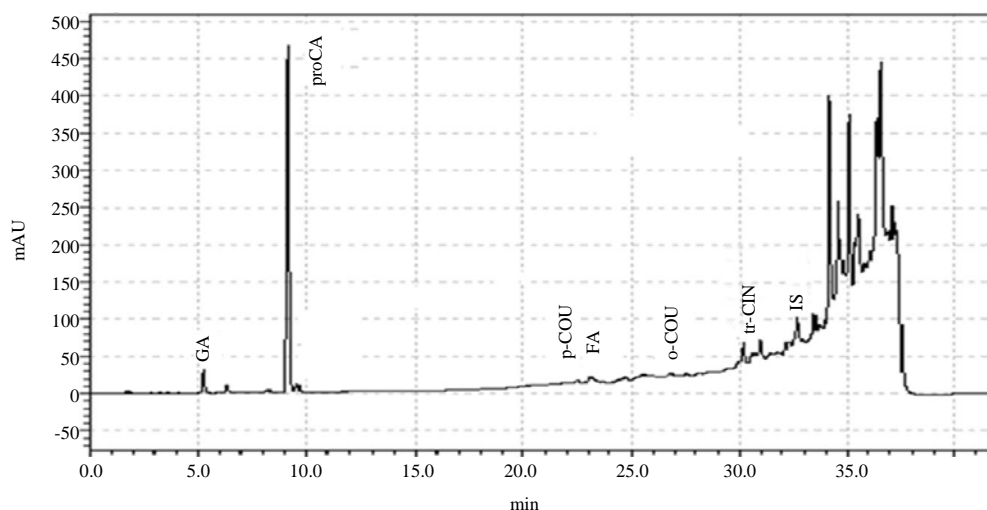


Fig. 2: HPLC chromatogram of certain phenolic acids in hAt-2 extract

Table 1: Phenolic acid amounts ( $\text{mg g}^{-1}$ ) of hAt-2 secondary metabolites by HPLC

Phenolic acid amounts	hAt-2 ( $\text{mg g}^{-1}$ )
Gallic acid (GA)	1.49
Protocatechuic acid (Pro-CA)	64.34
Para-hydroxybenzoic acid (p-OHBA)	-
Vanillic acid (VA)	-
Caffeic acid (CA)	5.83
Chlorogenic acid (CHA)	3.43
Syringic acid (SA)	3.13
Para-coumaric acid (p-COU)	2.40
Ferulic acid (FA)	8.21
Orto-coumaric acid (o-COU)	2.13
Trans-cinnamic acid (tr-CIN)	1.35

Table 2: Cell inhibition percentages of hAt-2 extract on Caco-2 cells using WST-1 method

hAt-2 (h)	WST-1 method					
	50	100	150	200	300	400
	( $\mu\text{g mL}^{-1}$ )					
24	22.31	32.43	37.23	62.72	67.69	69.25
48	7.80	8.35	20.93	56.81	65.44	69.89

**Cytotoxic effects of hAt-2 on Caco-2 cells:** The WST1 assay results indicated that hAt-2 secondary metabolites inhibited Caco-2 cell proliferation, especially at a concentration of  $200 \mu\text{g mL}^{-1}$  and above. Cell proliferation values were less than 50% at concentrations of 200, 300 and  $400 \mu\text{g mL}^{-1}$  after 24 h and 150, 200, 300 and  $400 \mu\text{g mL}^{-1}$  after 48 h. Cell proliferation values of the extract on Caco-2 cells are shown in Fig. 3. Table 2 shows calculated percentages of cell inhibition of the extract on Caco-2 cells.

**Assessment of the effects of hAt-2 secondary metabolites on Caco-2 cell**

**Proliferation using RTCA-DP:** The aim of this method was to assess the effect of hAt-2 extract on intestinal epithelium Caco-2 cells using a real-time cell electric impedance sensing system (RTCA-DP). Effects of hAt-2 extract on cell

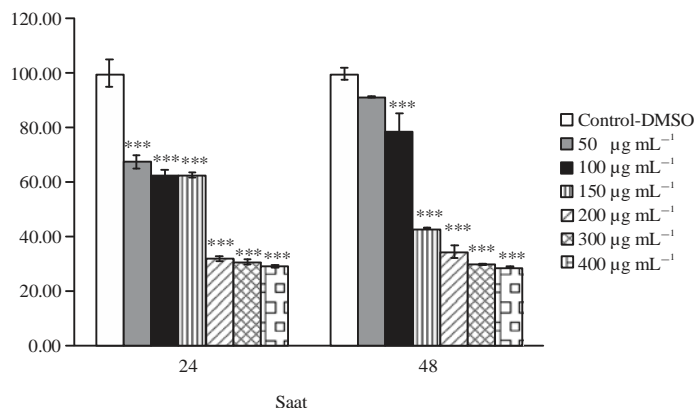


Fig. 3: Cytotoxicity analysis of hAt-2 extract on Caco-2 cells. Cell proliferation (%) was presented as Mean  $\pm$  Standard Deviation (SD) from three independent experiments (WST-1 method; Control: 0.1% DMSO, n = 8, p<0.001\*\*\*)

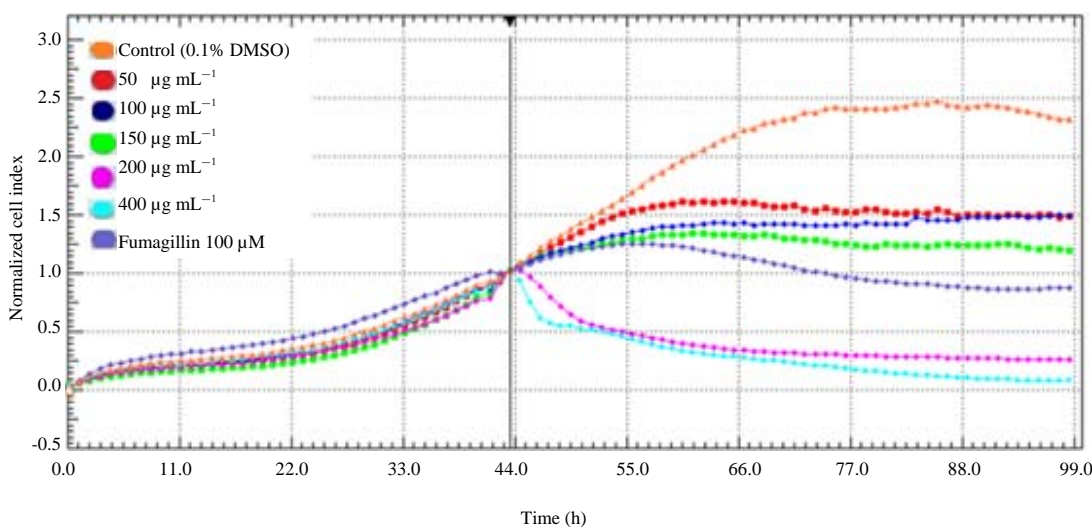


Fig. 4: Real-time cell monitoring of hAt-2 secondary metabolite on Caco-2 by using RTCA-DP system

Table 3: Flow cytometry cell percentages of hAt-2 extract on Caco-2 cells

hAt-2 secondary metabolite	Alive cells (Q3%)	Necrotic cells (Q1%)	Early apoptotic cells (Q2%)	Late apoptotic cells (Q4%)
25 ( $\mu\text{g mL}^{-1}$ )	14.2	8.0	60.5	17.3
50 ( $\mu\text{g mL}^{-1}$ )	9.4	6.3	68.9	15.4

proliferation were evaluated after the exposure of Caco-2 cells to concentrations of 50, 100, 150, 200 and 400  $\mu\text{g mL}^{-1}$ . The hAt-2 extract decreased the CI value of Caco-2 cells in a time and concentration-dependent manner (Fig. 4). According to CI values,  $\text{IC}_{50}$  was calculated as 185.9  $\mu\text{g mL}^{-1}$  at the end of 72 h using RTCA system analysis software (Fig. 5).

**Apoptosis detection using flow cytometry:** Different concentrations (25, 50 and 100  $\mu\text{g mL}^{-1}$ ) of the extract were analyzed by flow cytometry to determine the most effective

concentration to cause apoptosis. All concentrations of the extract demonstrated the remarkable apoptotic effect on Caco-2 cells at the end of 24 h (Fig. 6, Table 3). The highest apoptotic value was determined as 84.3% (early+late apoptotic cell percentage) at a concentration of 50  $\mu\text{g mL}^{-1}$ . An apoptotic rate of less than 10% indicated that three different concentrations of the extract caused the death of Caco-2 cells via apoptosis. The present findings demonstrated that 50  $\mu\text{g mL}^{-1}$  of the hAt-2 secondary metabolite-induced death of maximum Caco-2 cells via apoptosis (Table 3).

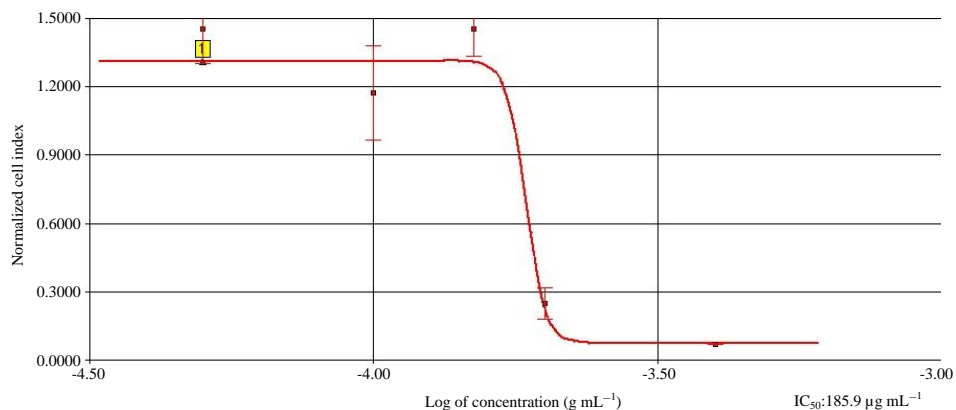


Fig. 5: Effect of hAt-2 extract extract during 72 h exposure on the viability of cells were determined based on the concentration-response curves of the cell index and determined  $IC_{50}$  rates by the RTCA DP system on the viability of Caco-2 line ( $IC_{50}$ :  $185.9 \mu\text{g mL}^{-1}$ )

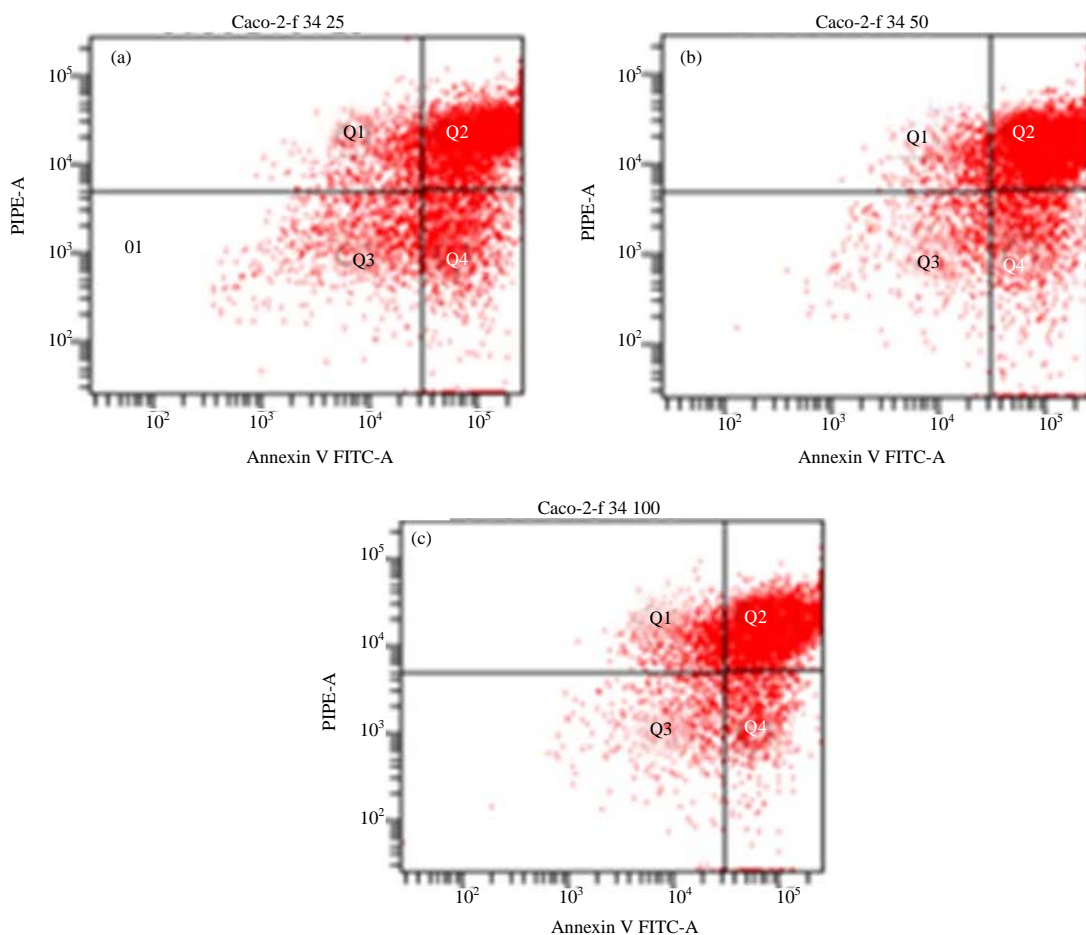


Fig. 6(a-c): Typical quadrant analysis of annexin V-FITC/propidium iodide flow cytometry of Caco-2 cells treated with hAt-2 extract. Caco-2 cells were cultured for 24 h in medium with the extract at concentrations of (a) Control,  $25 \mu\text{g mL}^{-1}$ , (b)  $50 \mu\text{g mL}^{-1}$  and (c)  $100 \mu\text{g mL}^{-1}$ . At least 10,000 cells were analyzed per sample and quadrant analysis was performed. The proportion (%) of cell number is shown in each quadrant. Q1: Necrotic cells, Q2: Late apoptotic cells, Q3: Viable cells and Q4: Early apoptotic cells

## DISCUSSION

Natural products are the main source of anticancer agents<sup>12</sup>. Especially *Aspergillus* has a large number of species-specific exometabolites called secondary metabolites. *Aspergillus* is one of the most chemically active of all fungi, producing a wide variety of secondary metabolites (exometabolites)<sup>13,34</sup>. The chemodiversity of *Aspergillus* species is extremely high and hence many bioactive components may be extracted from *Aspergillus* in the future. This potential of *Aspergillus* may contribute to the discovery of new drug alternatives and also encourage experimental studies on chronic illnesses such as cancer. Hence, more detailed molecular approaches are needed to explore fully this potential<sup>13</sup>.

Specific dietary supplements can be as effective as chemopreventive agents<sup>13</sup>. Cancer is considered to be a serious public health concern in both developed and developing countries, of which colon cancer is the fourth most commonly diagnosed cancer in the world. In this regard, natural product extracts continue to be the most promising source of new drugs for cancer. As fungi are known as one of the most productive groups for useful natural products, the present study attempted to detect the anticancer compounds in two isolated soil fungi<sup>35</sup>.

The classical and molecular identification of hAt-2 was performed in this study. The hAt-2 secondary metabolites displayed time-dependent inhibition of Caco-2 cell growth and proliferation. The growth inhibition values were correlated with the flow cytometric analyses and cytotoxicity of the secondary metabolites. Therefore, it may be concluded that hAt-2 secondary metabolites possess potent antiproliferative and apoptotic properties against Caco-2 cells. The HPLC results of the extract showed that pro-CA dominated in the extract. Also, different amounts of pro-CA, GA, FA, CA, CHA, SA, p-COU, tr-CIN and o-COU acids were detected.

The studies on the phenolic content of halotolerant or halophilic fungi are fairly seldom. In parallel to the present HPLC result, another study of the same group reported that the fermentation liquid of *Penicillium* sp., had GA and pro-CA. The HPLC results of the extract showed that pro-CA dominated in the extract. Besides pro-CA, different amounts of GA, FA, CA, CHA, SA, p-COU, tr-CIN and o-COU acids were also detected. The present study focused on the potential of secondary metabolites from halotolerant fungi. The free radical scavenging activity of secondary metabolites was determined on the basis of the elimination of DPPH radicals and the total phenolic content was determined by the

Folin-Ciocalteu reaction. The antioxidant activity of the secondary metabolites of hAt-2 was positively correlated with the total phenolic content of the extract. Antioxidants obtained from natural sources have attracted immense attention because of their free radical scavenging ability<sup>36</sup>. Free radicals are involved in the development of a number of chronic disorders such as cancer, neurodegeneration and inflammation<sup>37,38</sup>. The presence of antioxidants such as phenolics, flavonoids and tannins may provide protection against a number of deadly diseases<sup>35</sup>.

Fungi are a major source of biologically active secondary metabolites, which play a major role in the treatment of many human and animal diseases<sup>34,39,40</sup>. A secondary metabolite is a chemical compound produced by a number of fungal species. A fungal secondary metabolite is a chemical compound produced by a limited number of species in a genus, an order or even a phylum, which has a high differentiation power<sup>34</sup>. *Aspergillus* contain species that produce a large number of species-specific exometabolites with a high degree of chemoconsistency. The chemodiversity of *Aspergillus* is extremely high and hence many more bioactive compounds can be extracted from it in the future. Both ecological and genetic/molecular approaches are needed to fully explore this treasure of natural products<sup>13</sup>. In brief, metabolites including new compounds were isolated and identified from the fermentation broth of hAt-2 under 10% salinity conditions in this study. High salt stress affected the quantity and profile of secondary metabolites. The new metabolite demonstrated antioxidant, antiproliferative and apoptotic activities under high-salt-stress conditions. The typical metabolites of hAt-2 showed antiproliferative effects with low cytotoxicity.

## CONCLUSION

In conclusion, significant activities were revealed in hAt-2 secondary metabolites. This novel study described the potent antiproliferative and apoptotic effects of hAt-2 extract on the growth of human colon cancer cells. The low toxicity of hAt-2 secondary metabolites makes them attractive for *in vivo* studies on colon cancer prevention and treatment. The present results also demonstrated that hAt-2 secondary metabolites might serve as the source of polyphenols confirmed by their antioxidant activities and contribute to the discovery of new drug alternatives.

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