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Original research paper

Upregulation of p53 by tannic acid treatment suppresses the proliferation of human colorectal carcinoma

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The present study's objective is to clarify the molecular mechanisms of tannic acid effects on the viability of human colorectal carcinoma (CRC). Tannic acid is stable for up to 48 h and is localized in both cytoplasm and nucleus. It dose-dependently inhibited the viability of CRC cell lines; SW-620 and HT-29 with IC_{50} values of 7.2 ± 0.8 and 37.6 ± 1.4 μmol L^{-1}. Besides, metastatic, invasive, and colony formation properties of CRC cells were significantly inhibited following the tannic acid treatment ($p < 0.001$). Tannic acid has been found to modulate enzyme, protein, and gene expressions of NQO1 in different levels and the upregulation of protein/gene expressions of p53 ($p < 0.001$), which leads the cells to trigger apoptosis. In conclusion, the present *in vitro* study may supply a significant background for *in vivo* studies in which the molecular mechanisms of antioxidant and chemopreventive activities of tannic acid will completely clarify.

Keywords: tannic acid, colorectal carcinoma, wound healing, cell viability, NQO1, p53

After cardiovascular diseases, cancer is the second most common cause of death, affecting millions worldwide (1). The uncontrolled cell cycle progression and the loss of apoptotic mechanisms are induced by activating oncogenes or deactivating tumor suppressor genes. Continually developing cancer cells have the potential to be metastatic. Therefore, early and effective treatments are essential in cancer treatment. Colorectal carcinoma (CRC), with its high morbidity and mortality rates, causes significant problems (2). A progressive change in genetic code during the development and progression of CRC results in tumor transformation from the normal colonic mucosa (3). Increased CRC incidence is related to malnutrition, smoking, intestinal inflammatory problems, polyps, genetic factors, and aging (4). Many attempts, including surgery, chemotherapy, and radiotherapy, were developed against CRC's proliferation and invasion (5–7). Although the use of chemically derived drugs, such as cisplatin-based therapy, prevents DNA repair mechanisms and induces apoptosis in cancer cells, it potentially causes severe side effects (8, 9).

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The protective effects of secondary plant metabolites on human health are well known (10). Phenolic compounds with aromatic rings containing hydroxyl groups are various plant-derived substances with biological activities ranging from antioxidant and anticancer properties (11, 12). One of the polyphenolic compounds is tannic acid (TA), produced from the secondary metabolism of plants. It can be obtained from various biological samples including grapes, grass, blackberries, and dates (13–15). TA has a central core composed of glucose esterified with gallic acid (Fig. 1d). TA's aromatic hydroxyl groups effectively form strong complexes with proteins and other macromolecules under particular environmental conditions. Antimicrobial, antifungal, antiviral, and cytotoxic properties of TA were demonstrated in many studies (16-19). TA's possesses inhibitory potential due to the regulatory effect of many enzyme activities (20, 21). One of these enzymes is NAD(P)H: quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2), a FAD-containing, homodimer protein (22). Two electron reducting properties of NQO1 make it critical against many procarcinogens and protect cells from oxidative stress and reactive oxygen species (ROS) (23).

The development of various cancer types is associated with a decrease in NQO1 activity by polymorphism (24, 25). Three protective strategies have been identified due to elevated NQO1 expression: one-step quinone detoxification, maintenance of endogenous antioxidants, and regulation of p53 stability (26). An important transcription factor, p53,

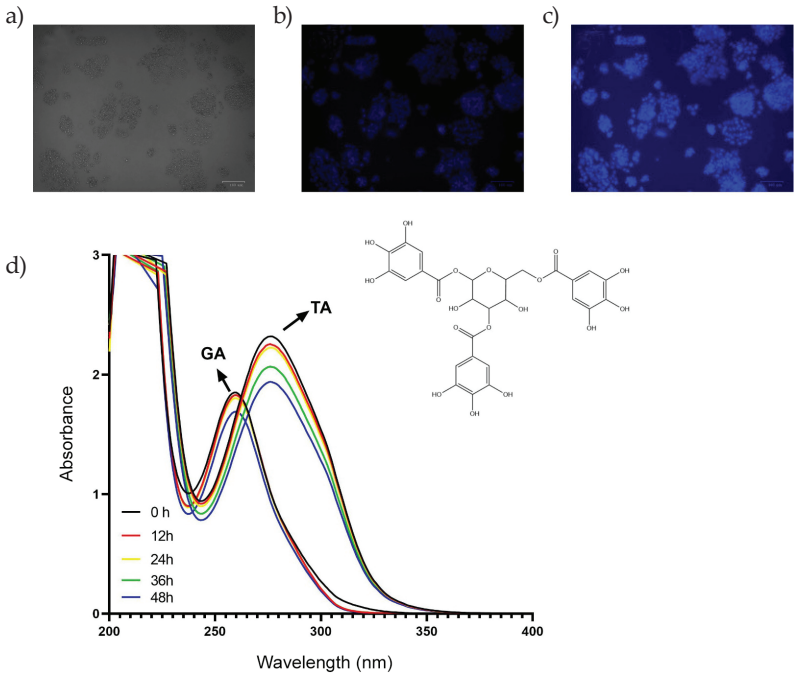

Fig. 1. Bio-imaging, structure, and stability of TA. SW-620 cells were treated with 5 μmol L^{-1} of TA and incubated for 45 min. The scale is 100 μm. a) brightfield; b) fluorescence; c) merge of the images; d) UV spectra and structures of TA and GA. λ_{ex} = 350 nm.

has a crucial role in the suppression of carcinogenesis, which induces either growth arrest or apoptosis (27). The upregulation of NQO1 inhibits proteasomal degradation of p53, p73 and p33 and maintains the stability of these enzymes (28). The increased level of NQO1 has been linked to the low level of CRC (29).

EXPERIMENTAL

Chemicals

Tannic acid (TA; T0200), phenylmethanesulfonylflouride (PMSF; P7626), bicinchoninic acid (D8284), bovine serum albumin (BSA; A7511), and DCPIP were obtained from Sigma-Aldrich (USA). NQO1 (Anti-NQO1 antibody (ab34173, 31 kDa), p53 (ab32389, 53 kDa), GAPDH (ab181602, 36 kDa), p53 (PAb 240, 53 kDa), and Goat Anti Rabbit IgG (ab216773) were purchased from Abcam (UK). Bax (Proteintech (50599-2), 21 kDa), Bcl-2 (Proteintech (12789-1), 26 kDa) were obtained from Proteintech (USA). Alamar blue was purchased from Invitrogen Life Technologies). Non-fat dry milk (170-6404) and tetramethyl ethylene diamine (TEMED; 161-0801) were from Bio-Rad Laboratories (USA). Primers were designed by Iontek (Turkey). Leibovitz (L-15), EMEM, L-glutamine were obtained from ATCC (American Type Culture Collection, USA). All purchased chemicals and solvents were of the analytical standard at the highest grade of purity available.

Biology

In vitro *bioimaging*. – The penetration and the localization of TA in SW-620 cells were detected *via* fluorescence microscopy technique. Cells were seeded into a glass-bottom dark 24-well plate and incubated overnight at 37 °C and 5 % CO_2. Cells were then washed with 10 mmol L^{-1} PBS and pretreated with DAPI (1/1000) for 30 min. The excess amount of DAPI was removed, and cells were incubated with serum-free growth medium containing 5 μmol L^{-1} of TA for 45 min. Following the incubation, the cells were washed twice with PBS to remove the excess TA, and fluorescence intensity was monitored with a fluorescence microscope (ZOE Bio-Rad, USA) at an excitation wavelength of 355 nm and an emission wavelength of 433 nm (30).

Stability studies of TA. – To determine TA stability in *in vitro* conditions, 40 μg^{-1} of tannic acid and its monomeric subunit gallic acid were dissolved in FBS free growth medium and change in the UV-spectra monitored by spectrophotometer (Shimadzu, Japan) at 37 °C up to 48 h.

Cell viability and proliferation studies. – Human colorectal carcinoma cell lines, SW620, and HT-29 colon cancer cells and human healthy epithelial cell line CCD-18Co were purchased from ATCC. SW620, HT-29, and CCD-18Co cells were grown in Leibovitz (L-15) and EMEM growth mediums, respectively, supplemented with 10 % FBS (fetal bovine serum) and 2 mM glutamine and were grown at 37 °C and 5 % CO_2. The growth media used for these cell lines were also used for the dilution of TA. 3×10^4 cells were transferred to 24-well plates and treated with various TA concentrations ranging from 0–200 μmol L^{-1} and were incubated 48 h. The proliferation and viability of colon cells were determined by

using Alamar blue (31). Cells were treated with Alamar blue (10 % of the well) for 3 h, and the cell suspension was transferred to a 96-well plate. The change in the color was measured spectrophotometrically by reading absorbance at 570 nm and 600 nm. The IC_{50} values were calculated using the sigmoidal plot of the cell viability.

Wound healing assay. – To determine the effects of TA on metastatic properties of human CRC cells, *in vitro* wound healing studies were performed. Before the wound healing studies, cells were seeded into 6-well plates and treated with TA t the equivalent concentration of IC_{50} values (7.2 μmol L^{-1} for SW-620 and 37.6 μmol L^{-1} for HT-29) for 48 h. Control groups were supplemented with the growth medium without TA. After 48 h, 5×10^4 of SW620 and HT-29 cells were seeded into 24-well plate using CytoSelect 24-well Wound Healing inserts. Following overnight incubation at 37 °C, and 5 % CO_2, the inserts were removed and the cells were washed with 10 mmol L^{-1} PBS to remove unattached cells. Reduction in the scratch areas was monitored by a Trinocular inverted microscope (VWR, USA). The cell movement was monitored for 24 h, and the images were taken with a digital camera embedded in the microscope. The wound healing percentage was calculated based on the reduction in the scratch area measured by Image J software.

Colony formation assay. – To observe the effects of TA on colony formation properties of Human CRC cells, a soft agar colony formation assay was performed. SW620 and HT-29 cells were seeded into a 24-well plate and treated with 7.2 μmol L^{-1} and 37.6 μmol L^{-1} of TA, respectively for 48 h at 37 °C and 5 % CO_2. The cells were harvested, counted with TC20 automated cell counter, and 5×10^3 of cells were transferred into a 6-well plate containing 1 % base agar as described in our previous study and incubated at 37 °C and 5 % CO_2 for 15 days (32). The colonies were then stained with 0.1 % toluidine blue and the number of colonies was counted and analyzed by Image J software.

Cell invasion assay. – To determine the effects of TA on cell invasive properties of SW-620 and HT-29 cells, the cells were seeded into a 24-well plate and treated with 7.2 and 37.6 μmol L^{-1} of TA, respectively for 48 h at 37 °C and 5 % CO_2, then harvested and transferred into the BioCoat Matrigel Invasion Chambers. According to the manufacturer's instructions, the invasive properties of cells were determined (BD Biosciences, USA).

Apoptosis assays. – Following the treatment of SW-620 and HT-29 cells with an equivalent concentration of IC_{50} values for 48 h, the cells were harvested by trypsin-EDTA, and the ratios of apoptotic and necrotic cells were calculated using Annexin V-FITC and 7-Aminoactinomycin D (7-AAD dye) (BD Biosciences), according to the manufacturer's instructions. Analyses of the cells were performed by The NovoCyte Flow Cytometry Systems (Acea, USA).

Protein lysis and Western blotting assay. – Following the cell treatment with TA (concentration equivalent of IC_{50} values for 48 h), the protein extraction from SW620 and HT-29 cells was performed using RIPA buffer as described manufactural protocols, and the BCA method was used to determine the protein concentrations by using Bovine serum albumin (BSA) as protein standard (33, 34). The whole-cell extracts were used for protein expression through SDS-PAGE, followed by Western blot. 15 μg of protein were separated on precast 7.5 % SDS-PAGE gels and transferred to PVDF membrane. After blocking with 5 % non-fat dry milk, primary antibodies (NQO1, p53, Bax, Bcl-2, and GAPDH) were added and incubated

overnight at 4 °C. Following TBST wash, the secondary antibody was added, and bands were visualized by the enhanced chemiluminescence (ECL) system. The densitometric analysis was performed with Image J software.

Determination of NQO1 activity. – NQO1 activity was measured using the 2,6 dichlorophenolindophenol (DCPIP) substrate, as described by Ernster *et al.* (22) using the spectrophotometric method and the conditions optimized by Karakurt and Adali (13). This method is based on the reduced absorption of the reduced form of DCPIP by NQO1 absorbing light at 600 nm (Fig. 5a).

cDNA synthesis and quantitative mRNA expression by real-time PCR. – Total RNA from SW-620 and HT-29 cells was extracted with QIAzol (Qiagen, USA), and the quality and quantity of the RNA were analyzed with 2100 Bioanalyser instrument (Agilent Technologies, USA). The samples that have RIN (RNA Integrity Number) higher than 7 were used for cDNA synthesis. The cDNA synthesis was performed by using iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.), and the cDNA synthesis conditions included priming for 5 min at 25 °C and reverse transcription for 20 min at 46 °C, and the reaction was stopped by incubation for 1 min at 95 °C. The samples were held at 4 °C until qRT-PCR studies. The alterations in the mRNA expression of NQO1 were determined with qRT-PCR using Bio-Rad CFX Connect (Bio-Rad Laboratories, Inc.). Primers were designed with the primer and controlled with Primer-Blast (NIH). The sequences of the forward (F) and reverse (R) primers were: Specific for NQO1 is F-5′-AAG GCA GTG CTT TCC ATC AC-3′, and R-5′-AGG CTG CTT GGA GCA AAA TA-3′. Specific for p53 is F-5′-CCT ATG GAA ACT ACT TCC TGA AAA C-3′, and R-5′-GTA GAT TAC CAC TGG AGT CTT CC-3′. Specific for Bax is F-5′-ATG GAC GGG TCC GGG GAG-3′, and R-5′-ATC CAG CCC AAC AGC CGC-3′. Specific for Bcl-2 is F-5′-AAG CCG GCG ACG ACT TCT-3′ and R-5′-GGT GCC GGT TCA GGT ACT CA-3′. GAPDH was used as a housekeeping gene for normalization. Specific for GAPDH is F-5′-GCC AAA AGG GTC ATC ATC TC-3′, and R-5′-TGA GTC CTT CCA CGA TAC CA-3′. 20 μL of the reaction mixture was initially denaturated at 95 °C for 3 min, then 35 cycles of following conditions; denaturation at 95 °C for 15 s, and annealing at 60 °C for 30 s. The mRNA expression of specific genes was calculated by the $2^{-\Delta\Delta Ct}$ method, which is extensively used, and reliable data analysis (35).

Statistical analysis

GraphPad Prism v8.0 was used for the calculations and statistical analysis of the obtained data. The results were expressed as mean ± standard deviation (SD) of three independent experiments. The obtained results were analyzed using Two-Way repeated measure analysis of variance (ANOVA) and unpaired *t*-test. Anderson-Darling test was used for assessing normality. The significance level was set to $p < 0.05$.

RESULTS AND DISCUSSION

Dynamic cellular localization of TA in human CRC cells was monitored with fluorescence microscopy technique, and it was found that TA may penetrate from the outer cell membrane and localize both in cytoplasm and nucleus (Fig. 1b). TA was found to be a

stable compound in growth mediums of human CRC cells (Fig. 1d). Herein, UV spectra of 40 μg mL^{-1} of TA, and its monomeric subunit gallic acid (GA) were monitored up to 48 h at 37 °C with UV-spectrophotometer and no spectrum shift was observed during the experiment. The results suggested that TA can remain relatively stable (10 % degradation) in neutral pH (7.4) at 37 °C up to 48 h. The studies proved that especially alkaline conditions lead to TA's inactivation, and it can be stable at pH 6, 7, and 8 (36). Under alkaline condition (pH > 10), TA is converted to GA that is esterified to TA's glucose moiety. Analyses of TA and GA UV spectrums proved that both compounds have different maximum absorbance. TA's maximum absorbance is at 280 nm, whereas GA's maximum is at 260 nm. Therefore, the conversion of TA to GA can be detected by measuring the absorbance changes at 280 nm and 260 nm (37).

The cytotoxic potency of TA was determined by Alamar Blue assay. Treatment of human CRC cell lines, SW-620 (metastatic) and HT-29 (invasive), with TA leads to suppression of cell growth in a dose-dependent manner (Fig. 2a and 2b). On the other hand, when healthy colon epithelial cells (CCD-18Co) were treated with TA, the cell viability of CCD-18Co was slightly reduced (IC_{50} > 200 μmol L^{-1}) (Fig. 2c). Following the TA treatment of SW-620 and HT-29 cells, the calculated IC_{50} values were 7.2 ± 0.8 and 37.6 ± 1.4 μmol L^{-1}, respectively (Fig. 2c). The viability of SW-620 and HT-29 cells was almost completely reduced after 15 and 100 μmol L^{-1}, respectively. The inhibitory potential of TA on human

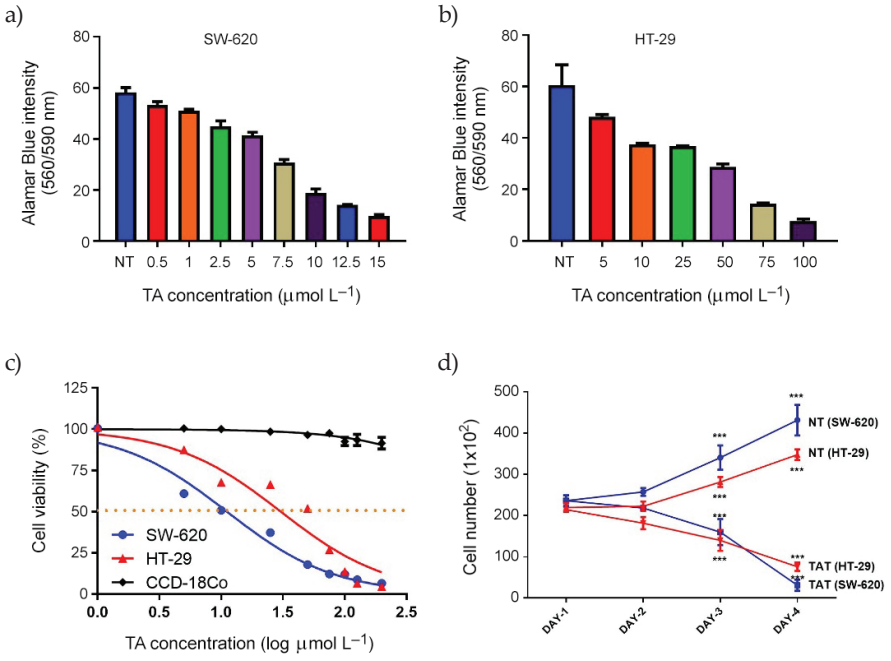

Fig. 2. Cytotoxic effects of TA on SW-620 and HT-29 cells. a) and b) TA dose-dependently inhibits the viability of SW-620 and HT-29 cells; c) determination of IC_{50} values of TA on human CRC cells, CCD-18Co, was used as a healthy colon epithelial cell; d) growth curve SW-620 and HT-29 cells. Data are displayed as mean ± SD. ***, $p < 0.0001$ for TA treated group (TAT), and non-treated group (NT).

CRC cells was observed at 24 h and increased time-dependently (Fig. 2d). Alteration of gene and protein expressions varies from cell to cell and the compound used for treatment. It may have taken 24 h for changes in gene and protein expressions to appear after treating cells with tannic acid. As indicated in Fig. 2d, TA's inhibitory effect on cell proliferation is cytotoxic, not cytostatic.

Alteration in the SW-620 and HT-29 cell mobility was measured by wound healing assay (Fig. 3a), a standard *in vitro* technique for determining two-dimension cell migration

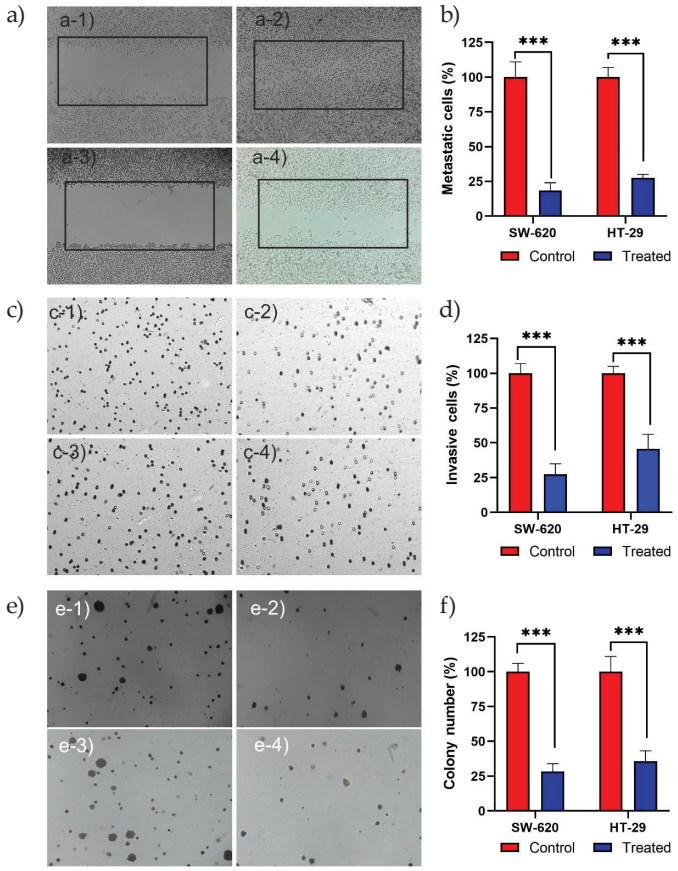

Fig. 3. Tannic acid modulates migration, invasion, and cellular anchorage-independent growth properties of CRC cells. Treated and non-treated (control) SW620 (a-1/a-2; c-1/c-2; e-1/e-2) and HT-29 (a-3/a4; c-3/c-4; e-3/e4) cells were subject to wound healing, matrigel invasion, and soft agar colony formation assays. a, c and e are the representative images, b, d, and f quantitate analysis of wound healing, invasion, and colony formation assays. The representative images of control and TA treated groups for wound healing, invasion and colony formation assay were shown in a-1(0 h)/a-2 (24 h), c-1(0 h)/c-2 (24 h), and e-2(0 h)/e-2(15 days) and a-3(0 h)/a-4 (24 h), c-3(0 h)/c-4 (24 h), and e-3(0 h)/e-4(15 days), respectively. Data are expressed as mean ± SD. Asterisks signify the level of significance: ***$p < 0.0001$ ($n = 6$ per group).

(38, 39). The cells were pretreated with TA for 48 h and transferred and subjected to cell scratching assay for 24 h. The number of migrated cells was significantly ($p < 0.001$) reduced in SW-620 (82 %) and HT-29 (73 %) cell lines when compared to non-treated cells (Fig. 3b). Mechanical forces, molecular interactions, and biochemical cascades are activated after gap formation (40, 41). The other important issue during wound healing assay is TA's concentration since overdose may cause apoptosis and necrosis. Besides, the TA's concentration should not exceed the IC_{50} value to detect only migrating cells, not to proliferated cells. To figure out the TA's effect on CRC cells' invasive potential, Matrigel invasion assay was performed (Fig. 3c). Cells were forced to invade through a two-chamber system separated by a cell-permeable membrane (42, 43). TA significantly inhibited the invasive potential of SW-620 and HT-29 cells by 73 % and 55 % ($p < 0.0001$) (Fig. 3d). To evaluate the cellular transformation and cells' ability to form colonies *in vitro*, Soft Agar Colony Formation Assay (Fig. 3e) was performed (44, 45). TA significantly inhibited colony formation potential of SW-620 and HT-29 cell as 72 % ($p < 0.0001$) and 65 % ($p = 0.0044$), respectively (Fig. 3f). A significant correlation was observed between the migration, invasion, and colony formation ratios of CRC cells ($p < 0.05$). These results indicate that TA reduces cell migration, invasion, and colony formation in CRC cells *in vitro* (Fig. 3). To elucidate TA's contribution to the apoptotic process of SW-620 and HT-29 cells, phosphatidylserine residues externalization on the outer plasma membrane of apoptotic cells was monitored with flow cytometry (Fig. 4a), and alteration in protein expression was measured by Western blot (Fig. 4c). TA significantly trigger the cancerous cells to apoptosis in a different manner. As shown in Fig. 4b, TA increased early rate apoptosis (13 %) and late apoptosis (6 %) in SW-620 cells. On the other hand, in HT-29 cells, TA treatment increased not only early (6 %) and late (12 %) apoptosis but also necrosis (3 %), which is a toxic process and energy-independent mode of death. The apoptotic pathway includes many genes and proteins, and cascades (46, 47). Cancerous cells have been developed many strategies such as up-regulation of Bcl-2 and down-regulation of Bax to prevent apoptosis. Following the TA treatment, a non-significant alteration was observed in Bcl-2 protein expressions neither SW-620 nor HT-29 cells. On the other hand, the Bax protein expression was significantly increased in SW-620 ($p < 0.0001$) and HT-29 ($p < 0.005$) cell following the TA treatment (Fig. 4d). SW-620 cells were obtained from lymph node where colon adenocarcinoma was metastasized. On the other hand, HT-29 cells are less metastatic. The mRNA and protein expression studies were found to be correlated with each other and flow cytometer analyses. TA treatment increased the proapoptotic Bax protein level by 3.05-fold and gene expression as 4.02 fold in SW-620 cells. Besides, they increased 1.86-fold and 2.0-fold, respectively, in HT-29 cells. The obtained results demonstrated that protein and mRNA expressions were correlated with each other.

It has been demonstrated that Bax and Bcl-2 expressions are regulated by tumor suppressor gene; p53 (48). *TP53* gene (expresses p53 protein) activates DNA repair proteins and induces apoptosis is mutant in more than 50 % of human cancer (49–51). Western blot and qRT-PCR studies showed that after TA treatment, protein and mRNA expressions of P53 in SW-620 cells were significantly ($p < 0.001$) elevated as 1.54-fold and 1.78-fold, respectively (Fig. 5d and 5e). This alteration might explain the increased levels of Bax gene expression since Bax is involved in p53-mediated apoptosis (52). To elucidate the reason for p53, the NQO1 enzyme, protein, and mRNA expression were investigated. NQO1 is responsible for detoxification of several natural and synthetic compounds by two-electron reduction and the stability of tumor suppressor proteins p53 (53–55).

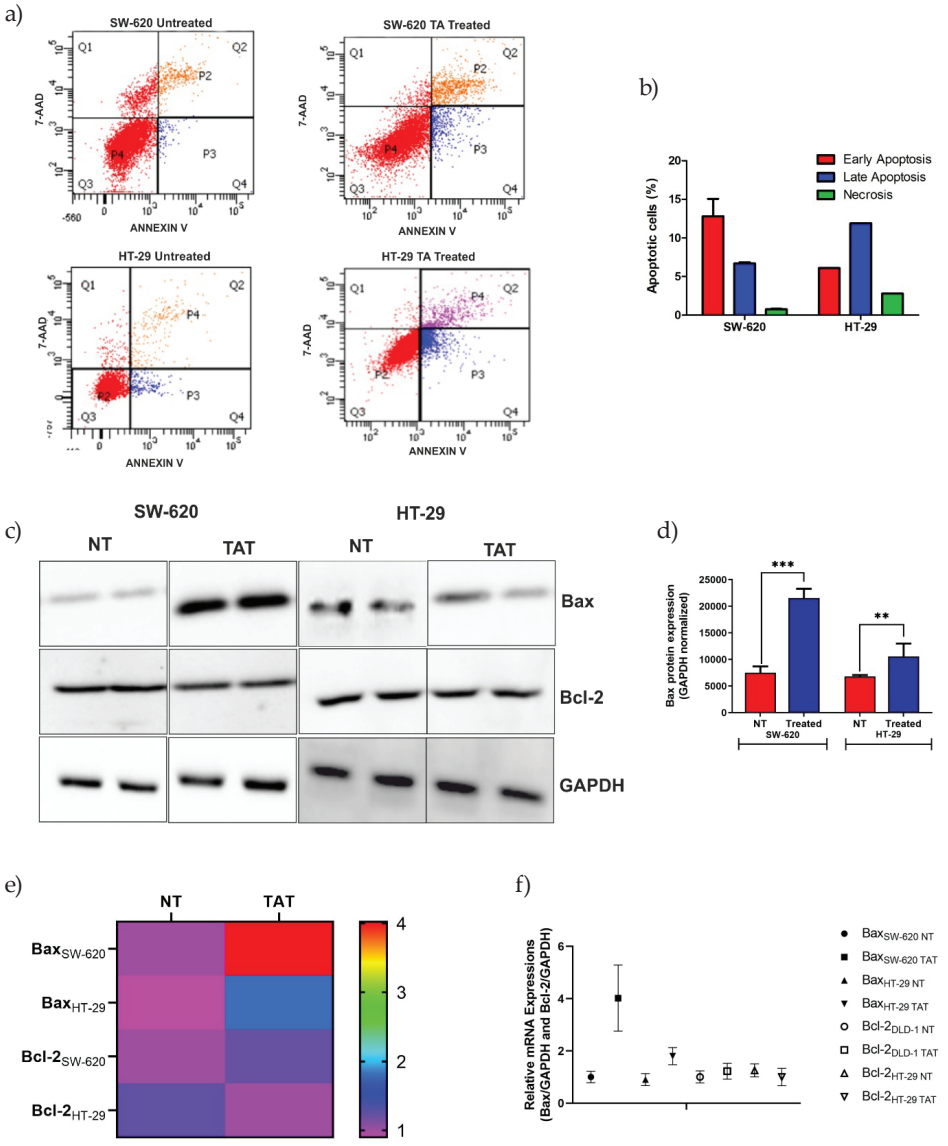

Fig. 4. Effects of TA on apoptosis of SW-620 and HT-29 cells. The cells were treated with an equivalent concentration of IC_{50} values of TA for 24 h. a) representative charts of apoptosis after Annexin V-FITC /7AAD stains with flow cytometry analyses; b) analyses and ratios of apoptosis of SW-620 and HT-29 cells; c) representative Western blot bands of Bax and Bcl-2 proteins in SW-620 and HT-29 cell; d) analyses of Bax protein expression in SW-620 and HT-29 cells; e) heat map analyses of mRNA expression of Bax and Bcl-2 genes in SW-620 and HT-29 cells; f) analyses of Bax and Bcl-2 gene expression. The results are expressed as mean ± SD of three different experiments ($n = 6$). $^{**}p < 0.001$ and $^{***}p < 0.0001$).

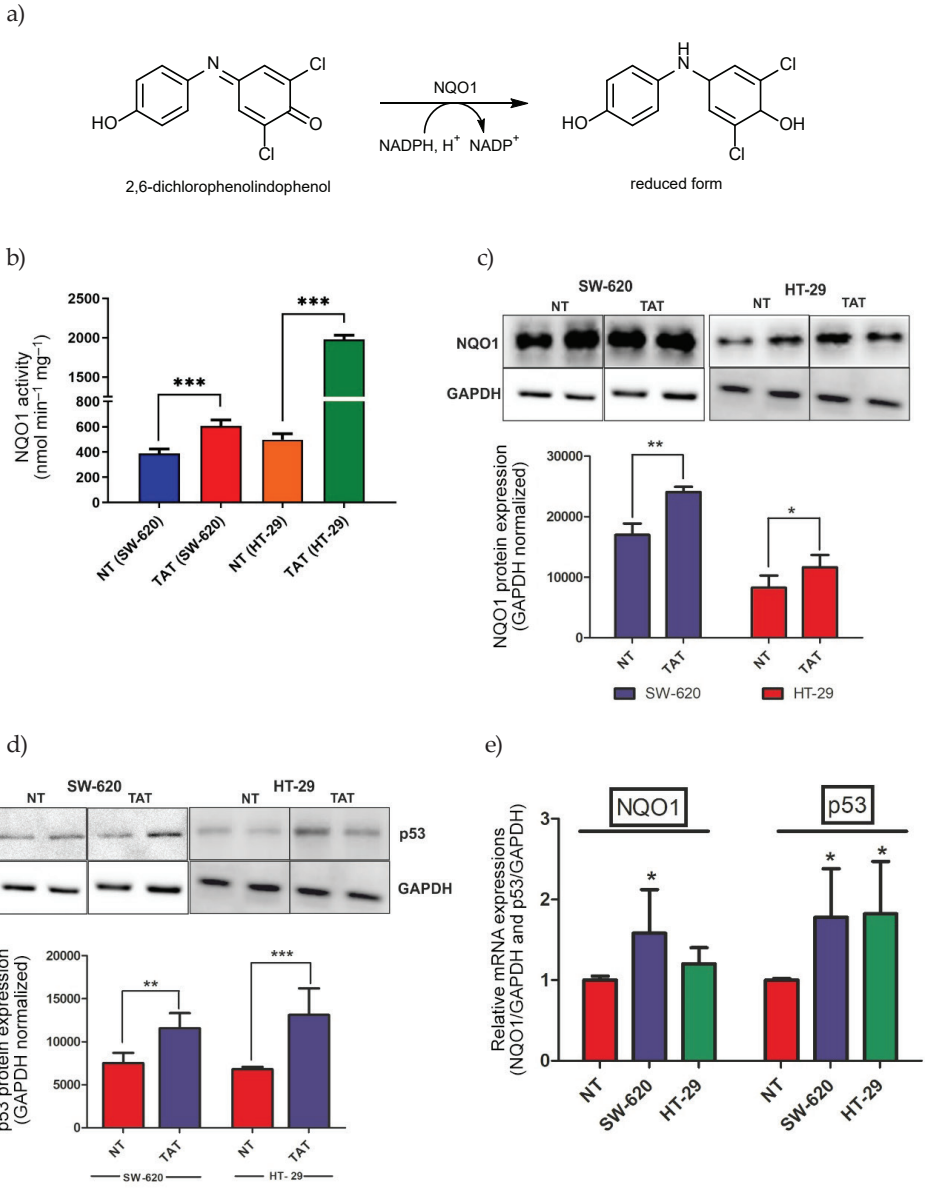

Fig. 5. Effects of TA on the expression of NQO1 and p53. a) Reduction of DCPIP by NQO1; b) *in vitro* effects of TA on NQO1 enzyme activity of SW-620 and HT-29 cells; c) and d) Effects of TA on NQO1 and p53 protein expressions of SW-620 and HT-29. Representative immunoblot and band density analysis of protein expression of the non-treated and TA treated groups; e) mRNA relative expression profiles of *NQO1* and *TP53* genes. The results are expressed as mean ± SD of three different experiments ($n = 6$). *$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$.

Molecular studies proved that NQO1 enzyme activity significantly elevated in many solid tumors, which suggests its crucial importance in cancer therapy (56). NQO1 indirectly inhibits the deacetylation of histone H3 lysine 9 which inhibits Bax transcription and decreased apoptosis (57). Besides, overexpression of NQO1 decreased Bax mRNA expression and stimulated Bax and Caspase-3 expressions in hepatocellular carcinoma (58). TA treatment of SW-620 and HT-29 cells significantly ($p < 0.0001$) increased NQO1 enzyme activity as 1.56-fold and 3.97-fold, respectively (Fig. 5b). Treatment with TA also increased NQO1 protein expression in SW-620 and HT-29 cells 1.5-fold ($p < 0.005$) and 1.4-fold ($p < 0.05$), respectively (Fig. 5c). When NQO1 mRNA expression was investigated in those cells, the correlation between protein and mRNA expressions of NQO1 was observed in SW-620 (1.58-fold, $p < 0.05$). On the other hand, no significant correlation was observed between protein and gene expressions as NQO1 mRNA change in HT-29 cells did not alter significantly in HT-29 cells (1.2-fold, $p = 0.072$) (Fig. 5e). Tannic acid treatment increased very similarly in NQO1 mRNA, protein, and enzyme activity as 1.56-fold, 1.5-fold, and 1.58-fold, respectively. It was quite different in HT-29 cells since no alteration was observed at the mRNA level, while protein and enzyme activities were increased as 1.4-fold and 3.97 fold, respectively. Studies have demonstrated that sometimes there is a poor correlation between mRNA and enzyme activity results (59, 60). The alteration of enzyme activity might be due to the alteration in protein expression and post-translational modification, such as the addition of functional groups or proteolytic cleavage of regulatory subunits (61).

Increased NQO1 protein expression and enzyme activity may be due to the transcriptional modulation of the NQO1 gene; however, it may be a posttranscriptional modification in HT-29 cells. NQO1 protein expression was also found significantly ($p = 0.0002$) higher in SW-620 cells than HT-29 cells (2.05-fold). SW-620 cells are aggressive colorectal carcinoma cells, whereas HT-29 cells are much more adherent. Therefore, SW-620 cells have developed aggressive defense systems against chemotherapeutic agents. A single nucleotide polymorphism (SNP) in NQO1, in which cysteine is converted to threonine at the position 609 (C609T), is associated with low NQO1 activity in human CRC (62). There is a direct correlation between NQO1 activity and tumorigenicity (24, 63). TA treatment increased the mRNA and protein expressions of NQO1, and an increased level of NQO1 has been demonstrated to enhance p53 protein stability. p53 has a crucial role in the Arf/p53/p21 and DDR pathway so that it can regulate growth arrest and apoptosis. The activity of p53 is highly connected to its structure. NQO1 directly binds to p53 whose regulation is controlled by the E3 ubiquitin ligase MDM2 and protects it from 20S proteasomes degradation. Decreased p53 activity, due to the presence of arginine instead of proline at codon 72, has been linked to low chemotherapy-induced apoptosis (64). *In vitro* studies are the first choice methods for explaining molecular mechanisms due to many samples, rapid results, and animal ethics. However, *in vitro* results are not entirely reliable based on the complex communication between cells and tissues and the inability to create the microenvironment *in vivo* conditions such as the appropriate dose. The expression of hundreds of genes is up or down-regulated when a primary hepatocyte cell is isolated from its original environment. Besides, the mimic of xenobiotic metabolism is too complex for *in vitro* conditions. There is no interaction between different cells, and it is difficult to understand the long term consequences of agents. Therefore, in further studies, TA's *in vitro* effects on human CRC cells must be validated with *in vivo* experiments.

CONCLUSIONS

The present study concluded that TA has the potential for cancer chemoprevention. It significantly inhibited the viability of human CRC and is stable up to 48 h at 37 °C. Active phenolic groups of TA can bind to proteins and nucleic acids, which elevated activity and stability of them. The treatment of TA promotes the activation of the NQO1 enzyme that enhances the stability of p53 proteins. p53 protein is a crucial protein in cell arrest and apoptosis; therefore, increased stability (activity) of p53 promotes apoptosis and causes inhibition of human CRC cells. A decreased level of p53, either mutation or deletion, is an effective strategy of cancerous cells against apoptosis, making p53 an ideal target during anti-cancer drug design. Herein, efficient, reliable, and efficiently handled alternative compounds gain vital importance. The modulatory effect of TA on gene and protein expressions makes it a valuable chemo protectant against human CRC. TA either directly or indirectly induces apoptosis and inhibits the viability of CRC cells. This *in vitro* study may supply an essential background for *in vivo* studies in which the molecular mechanisms of antioxidant and chemopreventive activities of TA will be clarified entirely.

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