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Original article

Auraptene Promotes THP-1 Polarization to M1 Macrophages and Improves M1 Function

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Abstract

Background and objectives: Macrophages play an important role in tumor growth (M2 macrophage) or suppression (M1 macrophage). Auraptene, a prenyloxycoumarin compound extracted from *Citrus* plants, has anti-cancer and anti-inflammatory properties. The purpose of this study was to look into the effect of auraptene on macrophage polarization and the tumor microenvironment when a human monocyte cell line (THP-1) was co-cultured with human colorectal adenocarcinoma (HT-29). **Methods**: The toxicity of auraptene on THP-1 and HT-29 cells was determined by the MTT method. Using flow cytometry, the effect of auraptene on macrophage polarization was studied through THP-1 as a macrophage source. The effect of auraptene on the macrophage population was also studied in THP-1 co-cultured with HT-29. Furthermore, macrophage function was assessed by measuring IL-10 and IL-12 concentrations using the ELISA method, nitric oxide (NO) concentrations using the Griess method, and HT-29 apoptosis by flow cytometry. **Results**: The M1/M2 ratio of THP-1 exposed to auraptene increased significantly in both naive THP-1 and THP-1 co-cultured with HT-29. Auraptene significantly reduced tumor-protective IL-10 secretion in M1 ($p=0.0032$) and M2 ($p=0.0011$). Auraptene increased anti-tumor IL-12 in M2 significantly ($p=0.0011$). It increased M1 NO production ($p=0.0236$) while decreasing M2 NO production ($p=0.0001$). Auraptene also increased HT-29 apoptosis in M0 and M1 co-cultures ($p<0.0001$). **Conclusion:** Auraptene altered the release profiles and macrophage types to enhance the suppression of HT-29 cells.

Keywords: colonic neoplasms; coumarins; macrophage activation; tumor microenvironment

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Introduction

Cancer is regarded as one of the most common causes of death. It is estimated that approximately 13 million cancer-related deaths will occur by 2030 [1]. While colorectal cancer is considered the third most common and the fourth most deadly cancer globally [2], its resistance to treatment makes its cure much difficult [3]. On the other hand, tumor-associated macrophages (TAMs) play a pivotal role in modulating different types of cancer. A study showed that the increased infiltration of M1 macrophages in patients with colorectal cancer increases the chance of survival [4]. Macrophages are generally categorized as M1 or M2 types, and

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TAMs have been proved to express M2-like phenotypes [5].

Tumor-associated macrophages (TAMs) are heterogeneous tumor microenvironment (TME) cell populations that can form up to 50% of some solid neoplasms [6]. M1 macrophages express a series of pro-inflammatory cytokines, chemokines, and effector molecules such as IL-12, IL-23, TNF-α, iNOS, and MHCI/II. In contrast, M2 macrophages express a wide array of anti-inflammatory molecules, such as IL-10, TGF-β, and arginase1. In most tumors, infiltrated M2 phenotype macrophages provide an immunosuppressive microenvironment for tumor growth [7]. Macrophage cells are differentiated into precursor cells via specific factors [8,9]. M1 macrophages, which possess tumoricidal properties, are activated by cytokines such as IL-6, LPS, IL-12, IL-23, and IFN-γ and display CD80, CD86, TLR-2, TLR-4, iNOS, and MHC-II markers on their surface. On the other hand, M2 macrophages that play a significant role in the cancer growth are activated by IL-10, IL-13, IL-14, IL-4, and TGF-β, displaying CD163, CD206, and CD209 markers on their surface [10,11].

Auraptene, a natural coumarin compound abundant in citrus plants [12], has several important pharmacological properties, including anti-cancer, anti-inflammatory, immunomodulatory, metabolic control [12-14], and anti-parasite [15] properties. Studies have shown that auraptene prevents the growth of cancer cells in the G1 cell cycle and decreases the number of cancer cells in the s-phase [16,17]. Some of auraptene's anti-cancer mechanisms include the reduction of H_2O_2 production and superoxide anion (O_2^-) generation [18], phosphorylation of P53 [16], suppression of cyclin D_1 [16,19], lowering the regulation of myeloid cell leukemia type 1 gene [20], suppression of mitochondrial respiration, the glycolytic pathway, vascular endothelial growth factor (VEGF), angiogenesis, hypoxia-inducible factor [21], decreasing inflammation and pro-inflammatory cytokines (nuclear factor kappa β, TNF-α, STAT3, IL-1β, IL-6, NF-E related factor 2) [14], over-expression of P21, lowering the regulation of $ALDH_1$ and CD44 [22], suppression of the activity of matrix metalloproteinase 2, 9 [13], and reduction of the level of proteins involved in cell cycle regulation and DNA replication [17].

Furthermore, previous research has shown that oral auraptene supplements have antitumor properties and a sufficient bioavailability in the target tissue. [14,19,23]. Umbelliprenin is another sesquiterpene coumarin compound similar to auraptene (Figure 1) with antitumor, immunomodulatory, and macrophage M1 promoter properties [24]. The reasons for selecting this prenoxycumarin is that it is widely available in nature, safe for oral use [25], and has many pharmacological properties [12].

As noted before, this in vitro study was undertaken to investigate the impact of auraptene on macrophage activation as a component of the tumor microenvironment along with its anti-cancer impact on HT-29 cells.

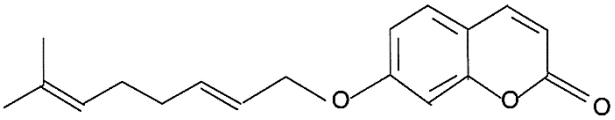

Figure 1. Chemical structure of auraptene

Materials and Methods

Ethical considerations

The ethics committee approval code in Shahid Beheshti University of Medical Sciences is IR.SBMU.RETECH.REC.1399.1206, which was approved on 8 April 2020**.**

Chemicals

Phorbol-12-myristate-13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder, Lipopolysaccharides from Escherichia coli (LPS), Roswell Park Memorial Institute medium (RPMI-1640), fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin, trypsin-EDTA, sulfanilamide 1% (w/v), N-(1-Naphthyl) ethylenediamine dihydrochloride (NED), standard nitrite solution ($NaNO_2$) were provided from Sigma-Aldrich (USA), recombinant human IL-4 (Cat # 200-04) , recombinant human IL-13 (Cat # 200-13), and recombinant human IFN-γ (Cat # 300-02) were bought from PeproTech (USA), and DMSO was obtained from Merck (Germany). FITC mouse anti-human CD68 (Cat # 562117), PE mouse anti-human CD80 (Cat # 557227), PE mouse anti-human CD163 (Cat # 560933) and the FITC Annexin V Apoptosis Detection Kit I (Cat # 556547), were purchased from BD Pharmingen (USA), human IL-10 (Cat # 3430-1H-6) and human IL-12 (p70) (Cat

#3455-1H-6) ELISA kits were bought from Mabtech Co. (Sweden).

Plant material

Auraptene, a prenyloxycoumarin, was purchased from Golexir pars Co. (Mashhad-Iran) in 2017. It has a molecular formula of $C_{19}H_{22}O_3$ and its molecular weight is 298.4 g/mol. Auraptene has been isolated from citrus family [26].

Cell lines

The human monocyte cell line (THP-1) and human colon cancer cell line (HT-29) were purchased from Pasteur Institute, Tehran, Iran.

Auraptene preparation

Two mg of auraptene was dissolved in 33.5 µL DMSO to make 200 mM auraptene as a stock solution, followed by a serial dilution. DMSO is toxic for THP-1 cells [27] and most human cancer cells [28]. In fact, DMSO's non-toxic concentration must be less than 0.25% *v/v* [29]; should its concentration surpasses such amount, it changes the permeability of the plasma membrane due to its interaction with this part's lipid [30].

Cell culture

THP-1 and HT-29 cell lines were cultured in Roswell Park Memorial Institute medium (RPMI-1640) and supplemented by 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, which were incubated at 37 °C, 95% humidity, and 5% CO2 conditions [31,32].

Cytotoxicity assay (MTT assay)

On the first day, THP-1 and HT-29 cell lines were seeded in triplicate at 5000 cells/well density in a 96-well plate, each containing 200 µL RPMI-1640. On the second day, the medium was refreshed, and the cells were treated with auraptene (12.5, 25, 50, 100, and 200 µM) for twenty-four hours. DMSO was the relevant control (concentration less than 0.25% v/v). The auraptene's cytotoxic effect was examined through the MTT method. Ten µL MTT reagent (5 mg/mL) was added to each well for four hours at 37 °C to achieve a final concentration of 0.5 mg/mL, and the supernatants were replaced by 100 µL DMSO. To solubilize formazan crystal, the plates were shaken gently at 37 °C for twenty minutes in the dark. Finally, the dye produced by formazan crystals was measured at 540 nm and 630 nm by utilizing an ELISA reader (BioTek). IC_{50} and IC_5 were determined via Graphpad Prism software [33].

Macrophage polarization and flow cytometry identification

In order to differentiate THP-1 monocytes into M0 macrophages, 3×10^4 (THP-1) cells were seeded in a 24-well plate. On the next day, 130 nM phorbol 12-myristate 13-acetate (PMA) was added for twenty-four hours, and incubated under 37 °C, 5% CO2, and 95% humidity. Subsequently, the old medium was removed, and a fresh PMA free medium was added for twenty-four hours. For the polarization of M0 to M1, 20 ng/mL IFN-γ and 10 pg/mL LPS were added for twenty-four hours. Moreover, to activate M0 to M2, 20 ng/mL IL-4 and 20 ng/mL IL-13 were added to the culture medium and incubated for twenty-four hours [34]. Also, to identify M0, M1, and M2 through flow cytometry, CD68, CD80, and CD163 markers were used, respectively [35]. The cells were separated using trypsin/EDTA and centrifuged for five minutes at 900 rpm. For each 50 µL sample, 5 µL FITC mouse anti-human CD68, PE mouse anti-human CD80, and PE mouse anti-human CD163 was added (single) and stored at 4 °C and wrapped in aluminum foil for half an hour. Then, flow cytometry was performed using a FACS Canto (CytoFLEX; Beckman Coulter, CA, USA), and the results were analyzed via Flowjo analysis software (Tree Star, Inc., USA) [36].

Cytokine assay (ELISA)

The amount of IL-10 and IL-12 secretion from 3×10^4 THP-1 cells under the effect of auraptene was measured by ELISA kits. The test was done according to the manufacturer's protocols, and the optical density was recorded at 450 nm by an ELISA reader. The ELISA test was conducted in duplicate for each sample.

Nitric oxide (NO) assay

Following the method of Griess test, 3×10^4 THP-1 cells were treated by auraptene for twenty-four hours and the supernatant of samples was centrifuged at 10,000 x g for 15 min at 4 °C. Then, 100 µL of cell culture supernatants and serial dilutions of standard nitrite solutions were combined by 50 µL sulfanilamide 1% (w/v) for 5-10 minutes at room temperature in triplicate in a 96-well, followed by adding 50 µL N-1-

naphthyl ethylene dihydrochloride (NED) 0.1% for 5-10 minutes in dark place. Absorbance was measured at 540 nm using the ELISA reader. The standard curve of nitrite was plotted and the curve was used to calculate the nitrite concentration of samples [37].

Treatment of THP-1 cells with auraptene

The amount of 3×10^4 THP-1 cells/well was seeded in triplicate in 24-well plate for twenty-four hours. Then, the old media was removed, and a new complete culture media containing 50 µM of auraptene was added to each well for twenty-four hours. THP-1 was polarized to M1 and M2 macrophages, and M1/M0 (CD80/CD68), M2/M0 (CD163/CD68), and M1/M2 (CD80/CD163) ratios were measured by flow cytometry. Finally, IL-10, IL-12 and Nitric oxide (NO) of the supernatant were measured.

Co-culturing HT-29 and different subtypes of macrophages

On the first day, 10^4 THP-1 cells were seeded in triplicate into each transwell insert (0.4 µm pore size Corning, Cat # 3450). On the second day, 130 nM PMA was added to transwell insert for twenty-four hours to differentiate into the M0. Then, the medium was refreshed (PMA free) for twenty-four hours. For polarization of M1, 20 ng/mL IFN-γ and 10 pg/mL LPS were added, and for M2 polarization, 20 ng/mL IL-4 and 20 ng/mL IL-13 were used for twenty-four hours. To set up the co-culture, 2×10^5 HT-29 cells were seeded in another plate, twenty-four hours prior to adding mature M0, M1, and M2 transwell inserts on the HT-29 plate. Finally, transwell insert media were changed and inserted to HT-29 plates without any direct contact (Figure 2) [38].

The effect of auraptene on co-culturing THP-1 and HT-29

Auraptene (50 µM) was added to two parts of the M0 and HT-29 co-culturing process. In this regard, first, it was added to PMA, and the co-culturing process continued. Then, the second part of auraptene was added to the transwell insert when it was placed on the HT-29 plate, and the co-culture was studied. It should be noted that auraptene was used only at one point in the co-culturing process.

The control group did not receive auraptene [24]. In each co-cultured group, apoptosis and necrosis of HT-29 cells were measured, and M1/M2 was calculated for THP-1 cells. As macrophage polarization-related factors which transform monocytes into M1 or M2 macrophages are removed from the environment after twenty-four hours, auraptene was added to the environment for merely twenty-four hours (Figure 3).

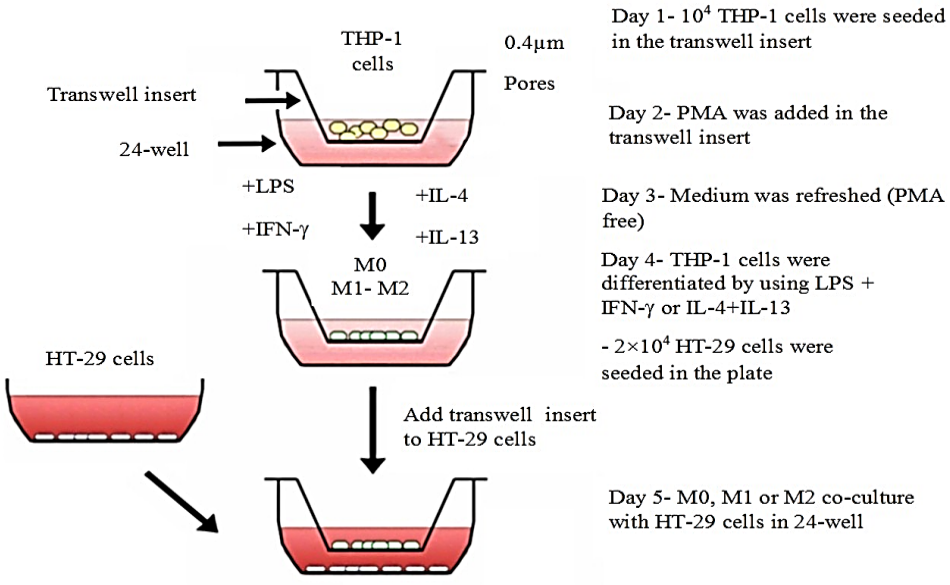

Figure 2. Co-culture of THP-1 with HT-29. THP-1 cells were seeded into the transwell inserts, where they were activated by PMA; addition of LPS and IFN-γ induced M1 macrophage, IL-4 and IL-13 induced M2 macrophage

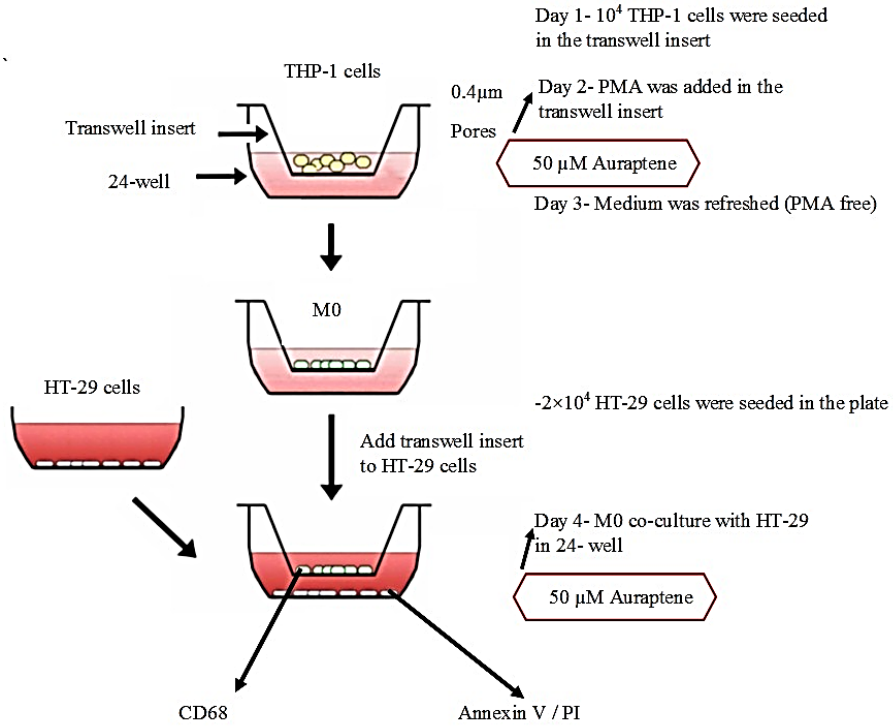

Figure 3. Co-culturing of M0 with HT-29. Auraptene was added to the PMA (day 2) or to the co-culture (day 4)

To study the effect of 50 µM of auraptene on co-culturing M1 and HT-29, auraptene was added in three points alone: first, it was added with PMA, and the co-culturing process continued. Second, 50 µM of auraptene was added to IFN-γ and LPS, and co-culturing was carried out. Third, 50 µM of auraptene was added to the transwell insert when it was placed on the HT-29 plate. As for the control group, auraptene was not added (Figure 4) [24].

To evaluate the effect of 50 µM of auraptene on co-culturing M2 and HT-29, auraptene was added in three stages alone: first, it was added to PMA, and the co-culturing was done. Second, 50 µM of auraptene was added to IL-4, IL-13 and the co-culturing process continued. Third, 50 µM of auraptene was inserted into the transwell insert when it was placed on the HT-29 plate. As for the control group, auraptene was not added in any stages (Figure 5) [24].

Assaying apoptosis and necrosis via flow cytometry

To study the apoptosis and necrosis of HT-29 cells, Annexin V-FITC/PI double staining was carried out Moreover, 10^5 HT-29 cells were harvested using trypsin/EDTA that contained 0.02% EDTA, 0.05% trypsin in PBS, followed by the transformation of HT-29 cells to tubes and centrifuge. Then, the cells were washed three times with 1X cold PBS, and the supernatant was carefully removed. The cells were re-suspended in the 1X binding buffer to 100 µL. Five µL Annexin V/PI staining solution was added to the tubes which were then gently swirled and stored in a dark place for twenty minutes. Next, 400 µL 1X binding buffer was added to each tube and mixed. Finally, the cells were analyzed via flow cytometry (Beckman Coulter Epics XL.MCL) [39].

Statistical analysis.

The data were analyzed, and the comparisons between groups' means were made using parametric or non-parametric tests via GraphPad Prism software (GraphPad Software, USA). P values <0.05 were considered to be significant.

Results and Discussion

After twenty-four hours, the IC_{50} values for auraptene in HT29 and THP-1 were found to be 42.14 µM (95% CI: 34.35 to 51.69) and 81.81 µM (95% CI: 69.81 to 95.87), respectively (Figure 6). The IC_5 of auraptene on THP-1 cells was approximately 50 µM, so this concentration was not toxic to THP-1 cells, it was chosen for further investigation.

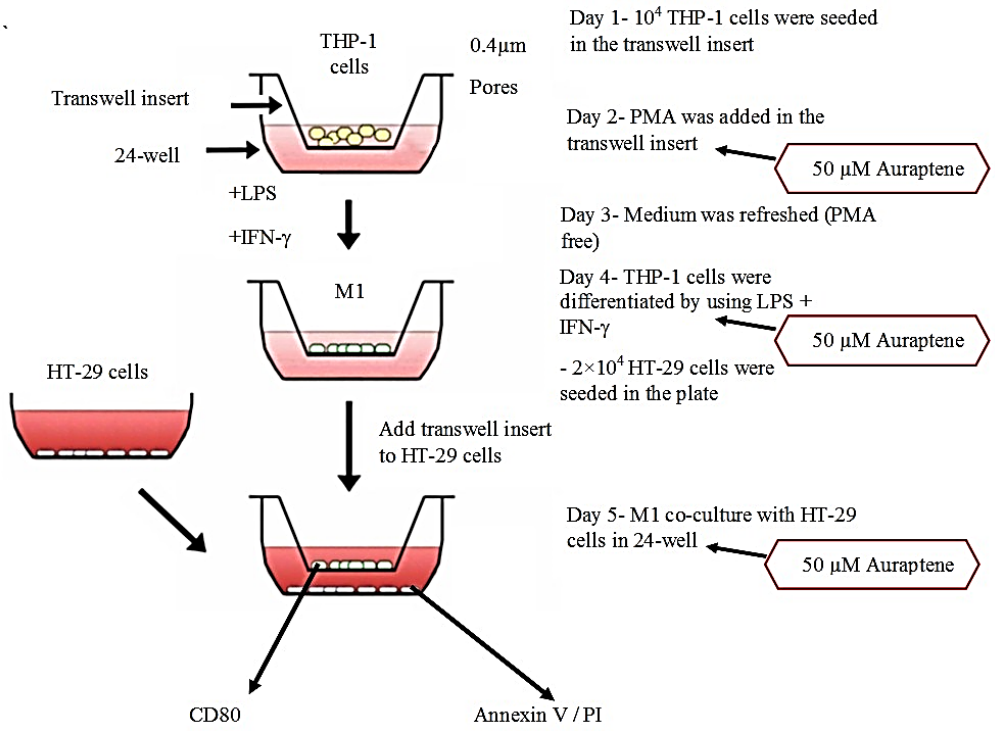

Figure 4. In co-culturing M1 and HT-29; auraptene was added to PMA, IFN-γ + LPS, and the co-culturing stages.

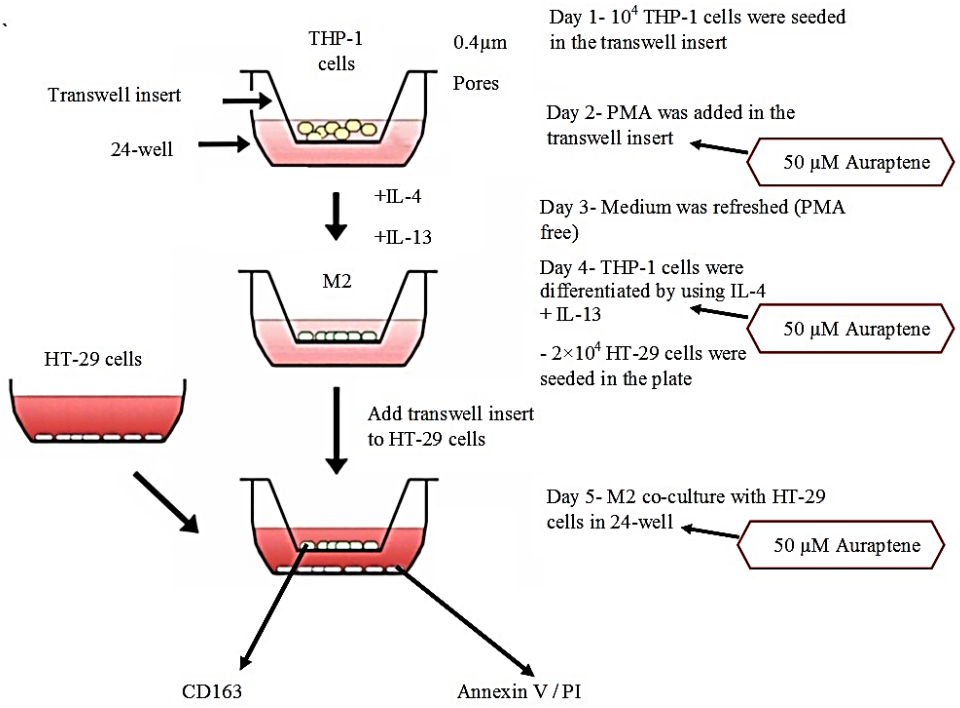

Figure 5. Co-culture of M2 and HT-29; auraptene was added to PMA, IL-4 + IL-13, or the co-culture.

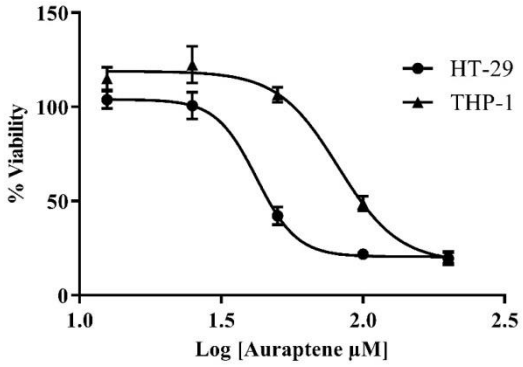

Figure 6. Cytotoxicity of twenty-four hours THP-1 (triangle) and HT-29 (circle) exposure to different concentrations of auraptene (AUR) measured via MTT assay; Each point represents the mean ± SD of triple assays.

In this study, auraptene toxicity in HT-29 cells was found to be concentration-dependent. While in another study, the toxicity of auraptene on MCF7 cells was dose-dependent (toxicity started at <75 µM [40].

Another research showed that auraptene dramatically reduced cell viability in the U87 GBM cell line in an increasing-concentration manner after twenty-four hours [41]. It should be noted that auraptene is a natural coumarin that has been proved to improve the outcome of current chemoradiotherapy treatments for colon cancer (HT-29) cells [22].The results indicated that auraptene significantly increased the M1/M0 (from 2.115±0.075 to 6.148±1.743, p=0.0018), M1/M2 (from 1.99±0.455 to 2.65±0.336, p=0.0284), and M2/M0 (from 1.106±0.251 to 2.342±0.730 p=0.0093) ratio in culture media (Figure 7). Auraptene increased induction of polarization of THP-1 into M1, and M2.

Another coumarin, i.e., umbelliprenin , increased M1/M0 and M1/M2 but decreased M2/M0, promoting the M1 pathway [24]. As found by another study, the CD206 expression in M2 macrophages in colon cancer tissues was found to be 1.8 times greater than that of the normal tissues [42]. Doxycycline suppressed M2 macrophage polarization in human and mouse bone marrow in a dose-dependent manner. [43].

The results of the present study showed that auraptene significantly increased the M1/M2 ratio in co-cultured + auraptene (4.973±0.9315) compared to M0 control co-culture (2.647±0.7008) (p=0.0066).

In PMA + auraptene group (12.27±4.901) (p=0.0199) and LPS + IFN-γ + auraptene group (12.40±2.082) (p=0.0185), M1/M2 fraction was significantly enhanced compared to M1 control co-culture (4.406±0.561). Moreover, auraptene had no effect on M1/M2 ratio in co-cultured M2 and HT-29 (Figure 8).

This study proved that while treating THP-1 cells with auraptene in co-culturing with HT-29 significantly enhanced M1/M2 ratio in differentiating THP-1 from M0 and M1, it did not affect the M2 polarization pathway.

We found in another study that umbelliprenin significantly increased M1/M2 ratio in umbelliprenin +PMA when M0 was co-cultured with the human gastric adenocarcinoma cell line (AGS). It was also suggested that when M1 was co-cultured with the AGS in umbelliprenin + PMA, the M1/M2 ratio was increased, and contrary to this study's findings, umbelliprenin increased M1/M2 in+PMA and IL4 + IL-13 group when M2 was co-cultured with the AGS [24].

We used the approximate IC_{50} concentration of auraptene on HT-29 cells in this study, so we expected to see an increase in apoptosis. Auraptene 50 µM, which was slightly higher than the IC_{50}, caused a small significant increase in early apoptosis in HT-29 co-cultured with M0 (PMA + auraptene group) (Figure 9A). In M1 co-cultured with HT-29, auraptene increased early apoptosis of HT-29 in the PMA +auraptene and LPS + IFN-γ +auraptene groups, but had little effect in the co-culture+auraptene group despite its direct presence in the culture media. As a result, even though auraptene was not present in the media, it increased the function of M1 macrophages to induce HT-29 apoptosis (Figure 9B). Late apoptosis and necrosis of HT-29 cells were greatly suppressed in M2- HT-29 co-culture, indicating that TAM macrophage has a stronger effect in protecting HT-29 cells (Figure 9C).

In our previous research findings, IC_5 of umbelliprenin did not affect apoptosis and necrosis of AGS cells [24]. Another study has found that *Ferula Szowitsiana* root-extracted auraptene increases caspase-3 and caspase-8 in MCF-7 cells [44]. However, the current study showed that early and late apoptosis of HT-29 cells that were co-cultured with THP-1 cells in the M1 polarization pathway (at the PMA + auraptene and LPS + IFN-γ + auraptene) considerably increased. Moreover, it was found that auraptene+co-culture in M2 co-culture

dramatically reduced the HT-29 cells' apoptosis, while auraptene+co-culture in M1 co-culture was not considerably affected in this regard.

The results showed that auraptene reduced the concentration of IL-10 in M1 culture media compared to the control group (p=0.0032). Moreover, while auraptene significantly reduced the concentration of IL-10 in M2 culture media (p=0.0011) (Figure 10), it increased the concentration of IL-12 in M2 culture media (p=0.0011) and had no effect in M1 culture media (Figure 10).

In a study comprising 48 patients with colorectal cancer and 27 volunteers, Stanilov et al.(2010) found that the serum IL-10 concentration in colorectal cancer patients was significantly greater than that of the volunteers [45]. Another study suggested that the levels of IL-10 in 146 patients with colorectal cancer had increased. Furthermore, it was claimed that lower IL-10 concentrations were associated with a reduced likelihood of cancer recurrence and a higher chance of survival [46]. In splenocyte cells, another similar coumarin compound (umbelliprenin) increased IL-10 [47]. In a rat model of rheumatoid arthritis generated by complete Freund's adjuvant (CFA), auraptene and umbelliprenin had no significant influence on IL-10 levels compared to the control group (p>0.05) [48]. While another study found that auraptene significantly raised the IL-10 gene expression index at all three doses (10, 30, and 90 µM) [49] and that auraptene significantly reduced the amounts of IL-10 in M1 and M2 cultured cells, which is favorable for tumor suppression.

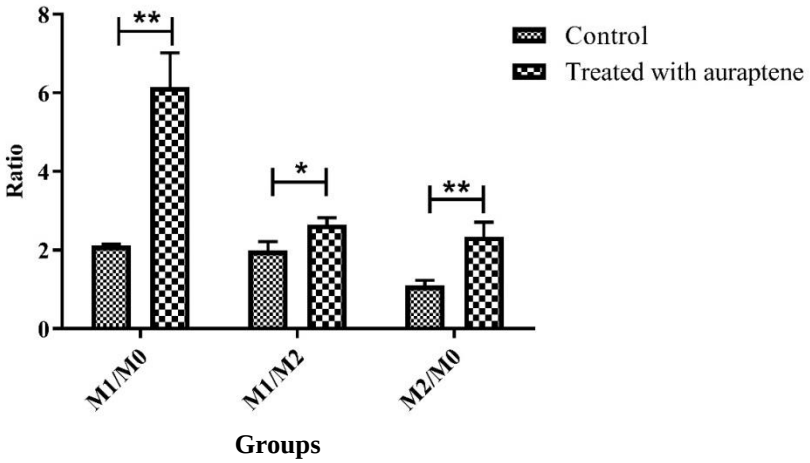

Figure 7. The effect of auraptene on ratio of intact THP-1 cells' polarization; the figure shows the M1/M0, M1/M2, and M2/M0 ratio in treated and untreated macrophages. Each bar represents the mean ± SD of the three independent experiments. *: p<0.05, **: p<0.01

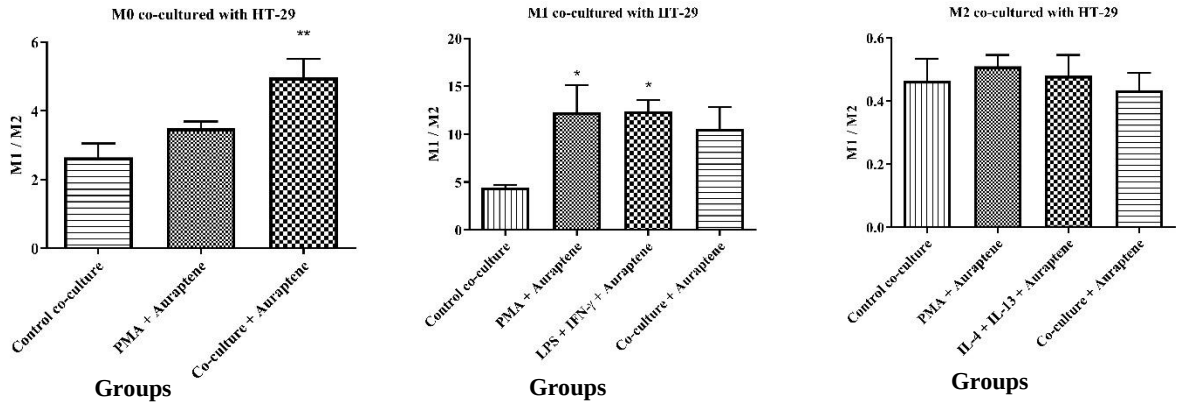

Figure 8. Effect of auraptene on M1/M2 ratio in different co-culturing stages; the ratio was determined by flow cytometry in M0, M1, and M2 polarization processes. Each bar represents the mean ± SD of the three independent experiments. *: p<0.05; **: p<0.01

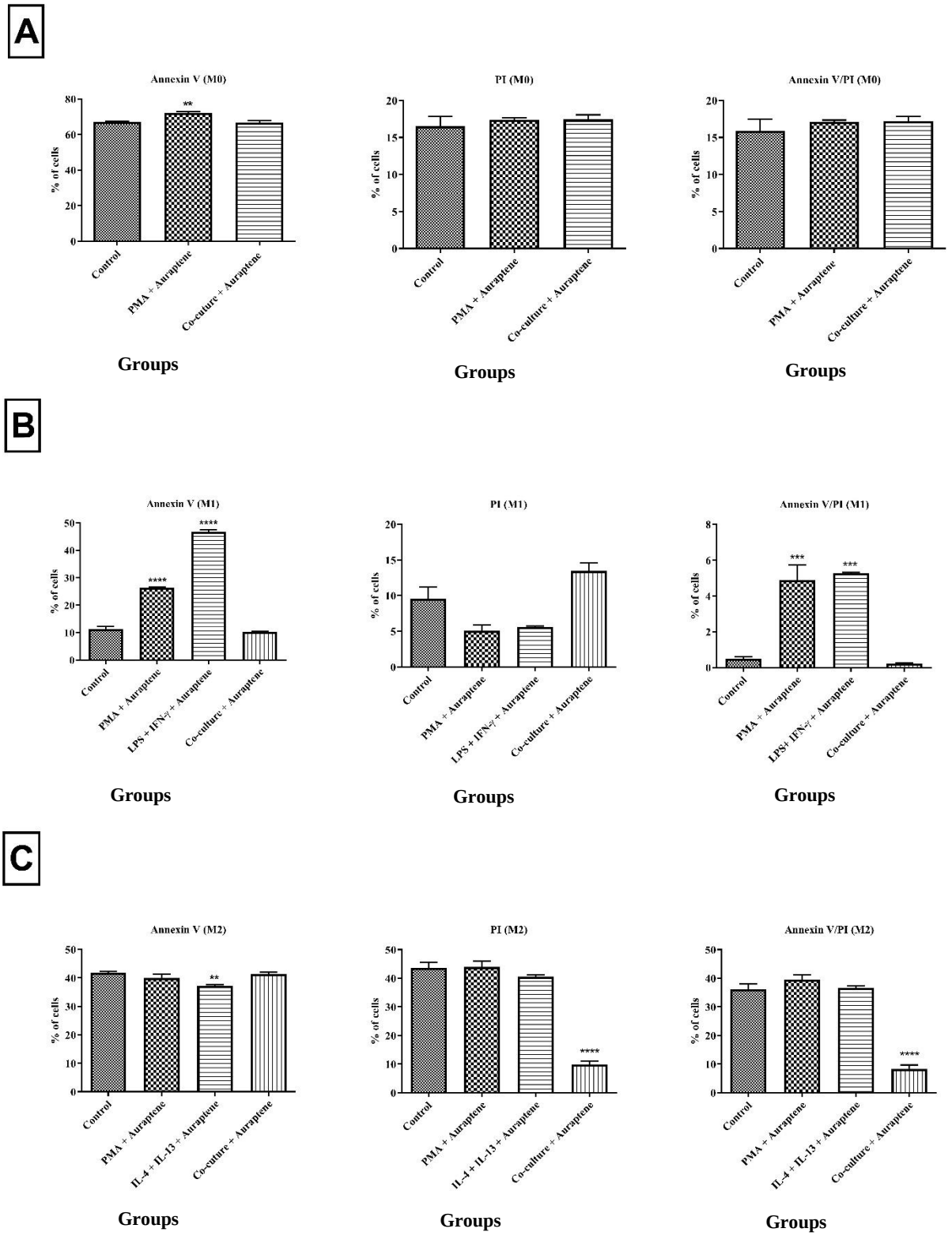

Figure 9. Assaying flow cytometry analysis of HT-29 apoptosis in different co-culturing stages. Annexin V/ PI assay of HT-29 cells co-cultured with auraptene-treated (50 µM) (A) M0, (B) M1, and (C) M2 cells. Each bar represents the mean ± SD of the three independent experiments. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$

IL-12 has been previously shown to have anti-cancer effects in colorectal cancer studies [50]. In our findings, auraptene significantly increased the concentration of IL-12 in M2 culture media.

Auraptene significantly increased the concentration of the NO in M1 culture media ($p=0.0236$) and significantly reduced NO concentration in M2 culture media ($p<0.0001$) compared to the control group (Figure 11).

The role of NO in colon cancer is controversial [51]. Although normal macrophages produce cytotoxicity by generating NO via the iNOS route with the substrate l-arginine, this pathway is inhibited in M2 macrophages where ornithine and polyamines are synthesized to promote tumor cell proliferation.

The generation of NO and iNOS is inhibited as soon as TAMs take on the M2-like phenotype [52]. NO promotes lymphangiogenesis and metastasis to lymph nodes, probably through VEGF-C participation, leading to overexpression of MMP-2 and MMP-9 and the downregulation of TIMP-2 and TIMP-3 [51]. Moreover, according to the current study's findings, auraptene significantly increased NO concentration in the M1 cell line culture medium while dramatically decreased NO concentration in M2 cell line culture media.

Conclusion

This study showed that auraptene had fewer cytotoxicity effects on THP-1 than on HT-29 cells and that it improved polarization of naive THP-1 to M1, promoted M1/M2 ratio in the process of co-culturing M0 and M1 with HT-29 cells, and increased apoptosis of HT-29. Also, it was found that auraptene affected NO, IL-10, and IL-12 in both M1 and M2 macrophages in favor of tumor suppression.

Acknowledgment

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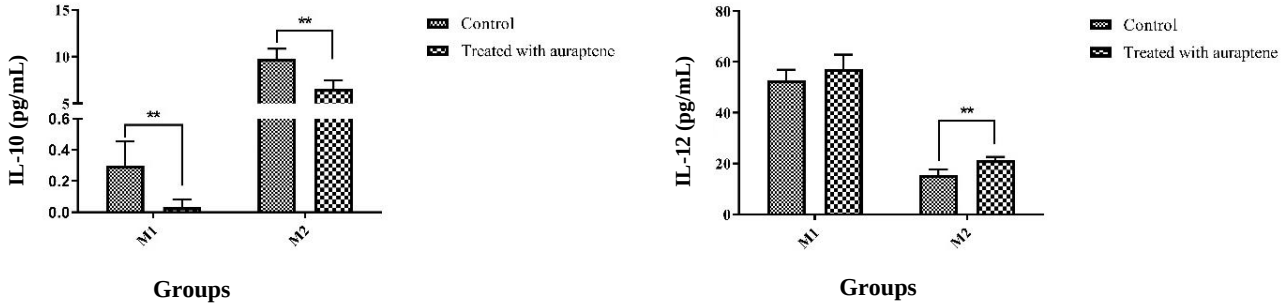

Figure 10. Effect of auraptene (50 μM) effect on IL-10 and IL-12 release profile using ELISA kit; Each bar represents the mean±SD of the three independent experiments; **: $p<0.01$

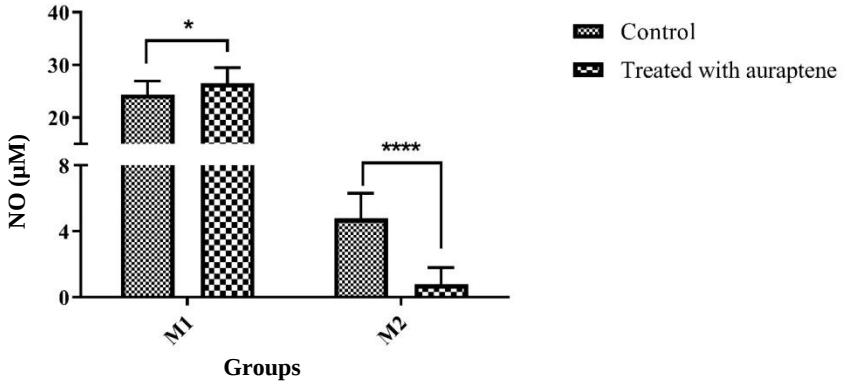

Figure 11. Nitric oxide assay in the supernatant of auraptene-treated (50 μM) M1 and M2 macrophages. Each bar represents the mean±SD of the three independent experiments; *: $p < 0.05$; ****: $p < 0.0001$

Author contributions

Seyed Ali Ziai and Afshin Jalali were involved in design of study; Afshin Jalali performed the acquisition of data; Afshin Jalali, Mostafa Haji Molla Hoseini, Mitra Rezaei and Seyed Ali Ziai contributed to analysis and interpretation of results; Seyed Ali Ziai and Afshin Jalali prepared the draft of the manuscript and revised it. All authors approved the final draft of the manuscript**.**

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper's content.

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Abbreviations

NO: nitric oxide; TAMs: tumor associated macrophages; TME: tumor microenvironment; VEGF: vascular endothelial growth factor; AUR: auraptene; PMA: phorbol 12-myristate 13-acetate; GBM: glioblastoma multiforme; M1: M1 macrophage; M2: M2 macrophage; M0: M0 macrophage; UMB: umbelliprenin; iNos: inducible nitric oxide synthase; VEGF-C: vascular endothelial growth factor-C; MMP-2: matrix metalloproteinase-2; MMP-9: Matrix metallopeptidase-9; TIMP2: tissue inhibitor of metalloproteinases 2; TIMP-3: tissue inhibitor of metalloproteinases-3; CFA: complete Freund's adjuvant; PBS: phosphate buffered saline; IL: interleukin; TNF-α: tumor necrosis factor-α; MHC: major histocompatibility complex; IFN-ɤ: Interferon gamma; TLR-4: Toll-like receptor -4; CD: cluster of differentiation