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Berberine Inhibited Growth and Migration of Human Colon Cancer Cell Lines by Increasing Phosphatase and Tensin and Inhibiting Aquaporins 1, 3 and 5 Expressions

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Abstract: Introduction: Berberine is a natural isoquinoline alkaloid with anti-cancer properties. Nevertheless, the underlying mechanism of its action in human colorectal cancer (CRC) has not been thoroughly elucidated. We investigated the anti-cancer effect of berberine on HT-29, SW-480 and HCT-116 human CRC cell lines. **Methods:** Cell proliferation, migration and invasion were studied by MTT assay, wound healing, transwell chambers and flow cytometry. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunostaining were used to evaluate the expression of aquaporins (AQPs) 1, 3 and 5 in colon cancer cell lines before and after treatment with berberine (10, 30 and 100 μM). RT-qPCR and Western blotting were used to further explore the PI3K/AKT signaling pathway and the molecular mechanisms underlying berberine-induced inhibition of cell proliferation. **Results:** We demonstrated that treatment of these CRC cell lines with berberine inhibited cell proliferation, migration and invasion through induction of apoptosis and necrosis. HT-29, SW-480 and HCT-116 stained positively for AQP 1, 3 and 5, and berberine treatment down-regulated the expression of all three types of AQPs. Berberine also modulated PI3K/AKT pathway activity through up-regulating PTEN and down-regulating PI3K, AKT and p-AKT expression as well as suppressing its downstream targets, mTOR and p-mTOR at the protein level. **Discussion/Conclusions:** These findings indicate that berberine inhibited growth, migration and invasion of these colon cancer cell lines via down-regulation of AQP 1, 3 and 5 expressions, up-regulating PTEN which inhibited the PI3K/AKT pathway at the gene and protein levels, and that AQP 1, 3 and 5 expression level can be used as prognostic biomarkers for colon cancer metastasis.

Keywords: aquaporins; berberine; colorectal cancer; PI3K/AKT; PTEN

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1. Introduction

Water is an essential component of all living cells, and maintaining water balance is crucial for many biological functions. The mechanism by which water fluxes through cell membranes was debatable until the discovery and characterization of aquaporins (water channels). Aquaporins (AQPs) are expressed broadly throughout the animal and plant kingdom as well as in lower organisms. AQPs play a variety of functions in mammals, including those related to fluid homeostasis, glandular secretions, blood barriers function, immunity and inflammation, cell migration, angiogenesis, signal transduction and sensation [1].

Colorectal cancer (CRC) is one of the most common malignant tumors, ranking third in terms of recognition (6.1%) and second in terms of mortality (9.2%) [2]. Several studies revealed a positive correlation between AQPs and cancer invasion and metastasis due to their involvement in cell motility. For instance, a strong correlation was discovered between the expression of AQPs 1, 3 and 5 and lymph node metastasis in colon cancer patients [3]. Additionally, it was demonstrated that blocking AQP1 in colon cancer cells (HT-29) reduced migration and angiogenesis [4], and the silencing of AQP5 with shRNA decreased the migration and invasion of HCT-116 and SW-480 colon cancer cell lines [5]. These data suggested that AQPs are potential indicators and therapeutic targets for colon tumor metastasis and prognosis [6].

On the other hand, chemical drugs used in chemotherapy for the treatment of various kinds of cancer cause unpleasant side effects and are not very effective. Therefore, researchers are looking for compounds with greater efficacy and fewer side effects, with a preference for those derived from natural resources, such as plants, algae, and fungi.

Berberine (Supplementary Figure S1), an isoquinoline alkaloid isolated from traditional medicinal herbs, showed many bioactivities, including antioxidant, anti-inflammatory, cholesterol-lowering, anti-diabetic, anti-obesity, and antimicrobial activities [7–9]. More importantly, berberine was found to have an anti-tumor activity against many cancer types, including colorectal cancer [8], and the results showed that berberine could be a promising anti-tumor agent. Furthermore, berberine was demonstrated to induce G1/G0 phase cell cycle halt by suppressing cyclin D1 and boosting p27 and p21 in colon cancer HCT-116 and HT29 cells [10]. These authors also found that berberine decreased β-catenin, a negative regulator of fat mass and obesity-associated protein (FTO), suggesting a role in regulating stemness and the malignant behavior of colon cancer stem cells.

Berberine was also found to bind a unique region in the nuclear receptor retinoid X receptor α (RXRα), and this binding promotes RXRα interaction with nuclear β-catenin, thus leading to its degradation and to inhibition of tumor proliferation [11]. Moreover, berberine was found to inhibit the colon cancer cell viability by regulating β-catenin signaling in a concentration- and time-dependent manner through the translocation of β-catenin from the nucleus to the cytoplasm [12].

Additionally, berberine suppressed colorectal cancer cell growth by boosting calcium mobilization and the metabolism of fat-soluble vitamins. Moreover, berberine could impair mitochondrial function by inhibiting mitochondrial proteins (i.e., MRPL52), which are essential for protein synthesis in the mitochondria that are involved in many aspects of tumor progression through their mediation of aerobic energy conversion [13]. Furthermore, berberine caused apoptosis in colon cancer HCT-116 and HT29 cells [10] and inhibited the migration of SW480 cells by down-regulating the expression of glucose-regulated protein 78 (GRP78) and up-regulating the expression of cytokeratin [14]. High expression of GRP78 was found in patients with pathologic grade CRC, and when overexpressed, it was found to relocate from ER to the cell membrane in malignant cells where it promoted tumor growth and migration [15,16].

In HCT-116 cell line, berberine suppressed cell viability, caused cell apoptosis, activated caspase-3 activity and down-regulated miR-21 expression while enhancing the expression of integrin β4 (ITGβ4) and programmed cell death 4 (PDCD4) proteins [17]. This up-regulation of ITBβ4 and PDCD4 as well as the anti-cancer effects of berberine on cell viability, apoptosis rate and caspase-3 activity were abolished by overexpression of miR-21, suggesting the involvement of these three genes in mediating the action of berberine.

The precise mechanism by which berberine exerts its anti-tumor effects is still debatable [11,12], and many signaling pathways, such as β-catenin [10–12], AMPK [14] and PI3K-AKT [18], among other mechanisms [13,19], have been implicated. On the other hand, there is ample evidence from the literature on the involvement of AQPs in the migration and metastasis of CRC cell lines [3–5] and on their role as potential therapeutic targets for colon tumor metastasis and prognosis [6]. In this study, we investigated the expression levels of AQP1, AQP3 and AQP5 in HT-29, SW-480 and HCT-116 human colon cancer cell

lines to explore whether berberine down-regulates their expression and subsequently the migration and metastasis of these cell lines. Furthermore, since PI3K/AKT pathway mediates the action of growth factors through growth factor receptors, receptor tyrosine kinases or G-protein coupled receptors, subsequently promoting tumor growth and development, we explored whether berberine has an effect on the expression of the components of this pathway, including phosphatase and tensin (PTEN) and PI3K, AKT and mTOR.

2. Results

2.1. MTT Assay

Cell proliferation was inhibited following treatment with berberine in a concentration-dependent manner (Figure 1). There were no significant differences between the cell lines. The half maximal inhibitory concentration (IC50) of berberine on HT-29, SW-480 and HCT-116 cell lines were 34.6 ± 0.27, 44.3 ± 0.35 and 32.1 ± 0.43 µM, respectively.

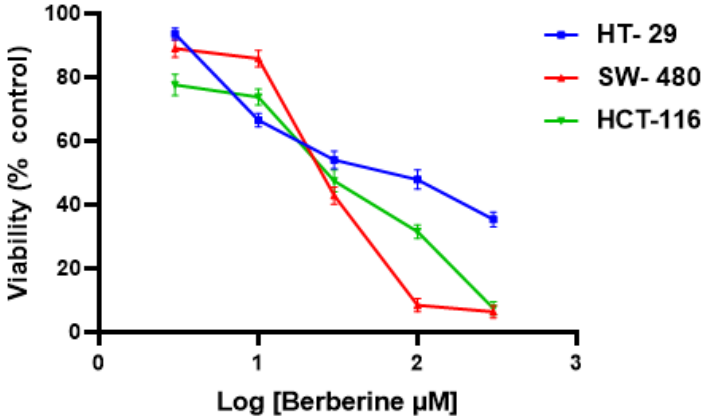

Figure 1. Inhibitory effect of berberine on cell viability. Viability was determined by MTT assay after 72 h of incubation. Data are the means ± SD of three independent tests and analyzed by one-way ANOVA (F = 40.1727; p = 0.1291). The values are expressed as percent of viability relative to the untreated cells that were considered as having 100% viability.

2.2. Flow Cytometry

To evaluate the apoptotic effects of berberine, colon cancer cell lines were treated for 24, 48 or 72 h with different concentrations of berberine, and flow cytometric analyses were performed by double staining with annexin-V FITC/PI. Exposure of HT-29 cells to berberine showed an increase in apoptosis (early and late) in a concentration-dependent manner. After 72 h of treatment, more cells moved into necrosis wherein almost 49% of the cells exhibited necrosis at 100 µM (Figure 2A). The effect of berberine on SW-480 cells was less noticeable, and the maximum necrosis after 72 h at 100 µM was about 31% (Figure 2B). HCT-116 cells were more sensitive to berberine at different concentrations and all time points tested with 60% necrosis observed after 72 h at 100 µM (Figure 2C). The data on annexin-V FITC/PI staining for the 3 cell lines at 24, 48 and 72 h are provided as Figure S2.

DNA binding dyes like toluidine blue were used to discriminate between normal cells and those that are undergoing apoptosis. HT-29 cells treated with 34.6 µM of berberine for 72 h displayed apoptotic features. Some cells appeared round with dense chromatin, ruptured plasma membrane, apoptotic bodies (Figure 3A), and fragmented nucleus (Figure 3B). In SW-480 cells, pronounced apoptotic changes, such as chromatin condensation (Figure 3C), nuclear fragmentation, cell swelling and formation of apoptotic bodies (Figure 3D), were observed after exposure to 44.3 µM of berberine for 72 h. HCT-116 displayed obvious apoptotic features, such as cell swelling, nucleus with condensed chromatin (Figure 3E), fragmented nucleus, vacuolated cytoplasm, ruptured plasma membrane, and apoptotic bodies (Figure 3F).

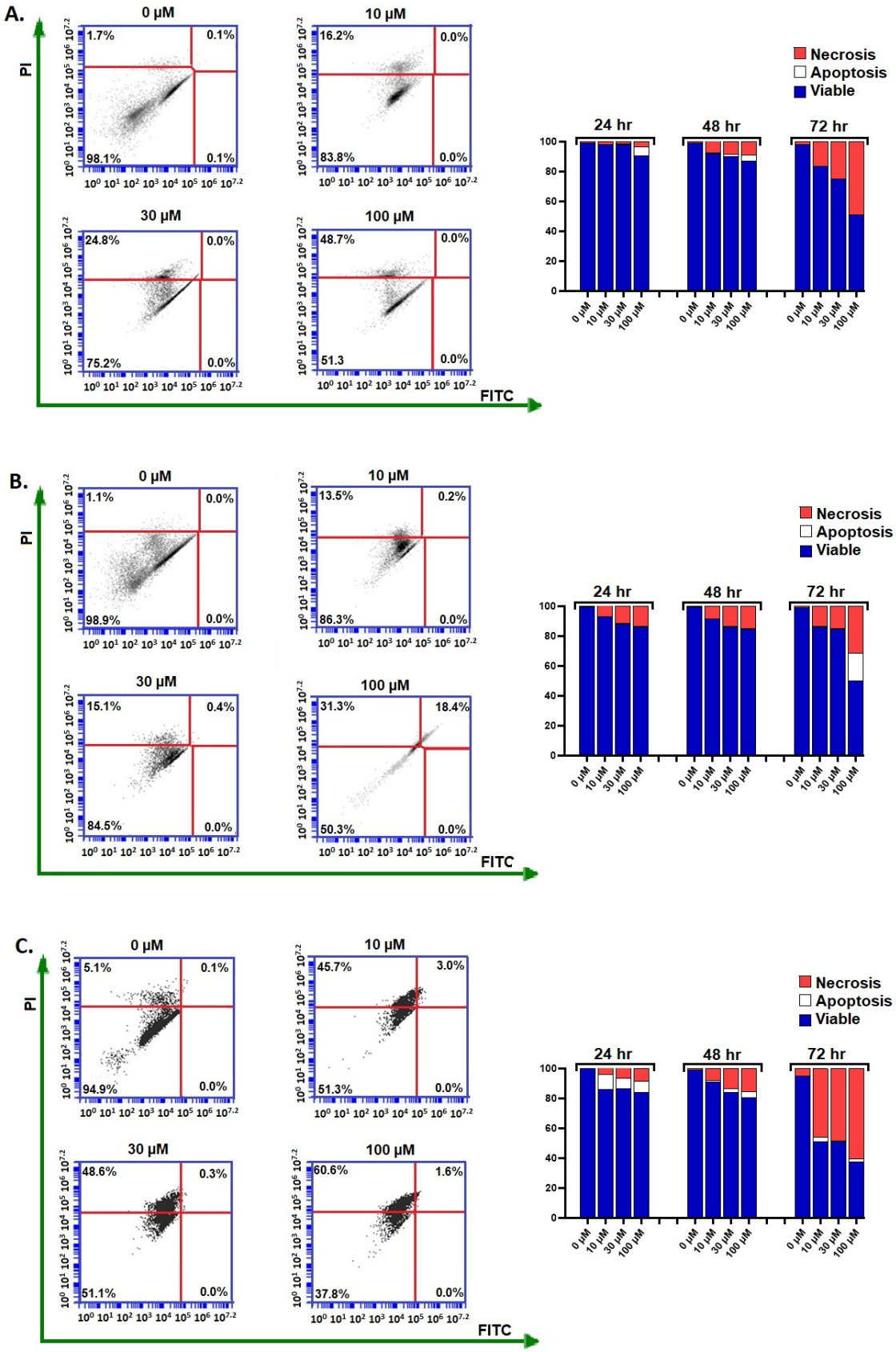

Figure 2. Representative flow cytometry plots using annexin V-FITC/PI staining for apoptosis and necrosis after 72 h (**left panels**). (**A**) HT-29, (**B**) SW-480 and (**C**) HCT-116 cells were treated with the

indicated concentrations of berberine. Cell count percentages for each cell type over 24, 48 or 72 h are shown (**right panels**). Flow cytometry plot analyses for the data in the right panel are provided in Figure S2.

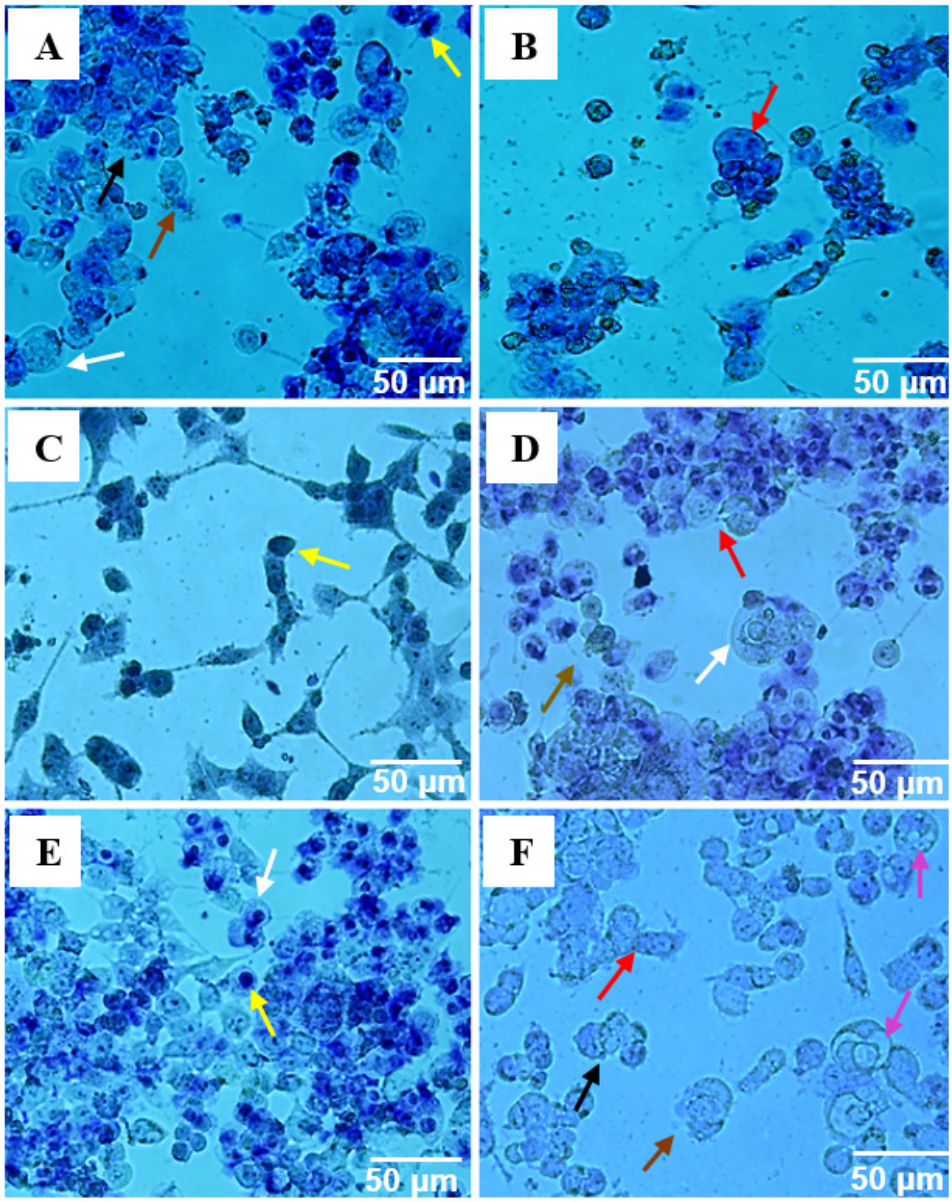

Figure 3. Berberine-induced apoptosis in HT-29 (**A**,**B**), SW-480 (**C**,**D**) and HCT-116 (**E**,**F**) cells. Chromatin condensation is indicated by yellow arrow; ruptured plasma membrane by black arrow; apoptotic bodies by brown arrow; cell swelling by white arrow; nuclear fragmentation by red arrow; and vacuolated cytoplasm by violet arrow. Magnification was 400× with scale bars of 50 µm.

2.3. Wound Healing Assay

A wound healing experiment was performed to confirm the inhibitory effect of berberine on cell migration in HT-29, SW-480 and HCT-116 cells. As shown in Figures 4 and S3, treatment of HT-29 with berberine showed an obvious reduction in wound closure compared to the untreated cells (Figure 4A). For SW-480 cells, the highest wound closure reached 38% at 10 µM after 72 h. (Figure 4B). At high concentration (100 µM), cells displayed flimsy migration abilities compared to the lower concentrations, and wound closure was only 10%. HCT-116 cell migration was generally slow at all concentrations, being 25% at 10 µM and 7% at 100 µM (Figure 4C).

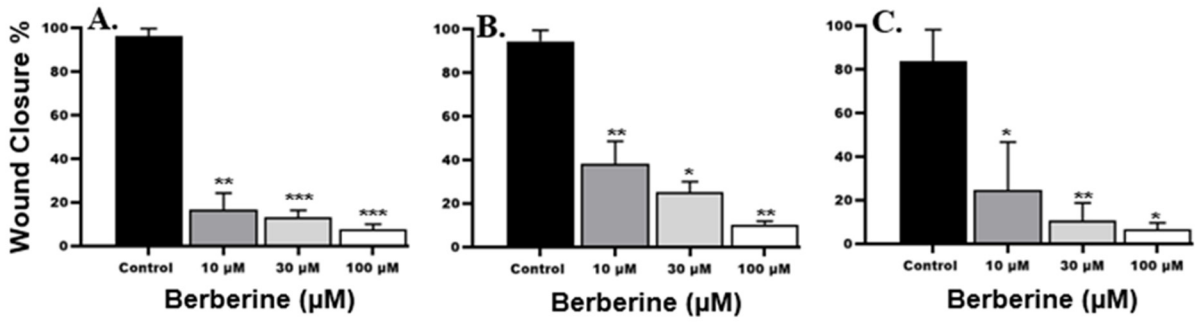

Figure 4. Effect of berberine on wound healing of HT-29 (**A**), SW-480 (**B**) and HCT-116 (**C**). Wound-healing assay after treatment with (0 µM (control), 10, 30 or 100 µM) for 72 h was analyzed by one-way ANOVA for control and treated group followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.002$ and *** $p < 0.0001$.

2.4. Transwell Migration and Invasion Assay

Figure 5A shows that HT-29 and HCT-116 cell lines migration was significantly decreased in the presence of berberine compared to the controls. Berberine also reduced the migration ability of SW-480 cells. Using Matrigel chamber assay, we found that berberine decreased the invasion capacity of tested colon cancer cell lines (HT-29, SW-480 and HCT-116) in a manner similar to that observed with the migration assay (Figure 5B).

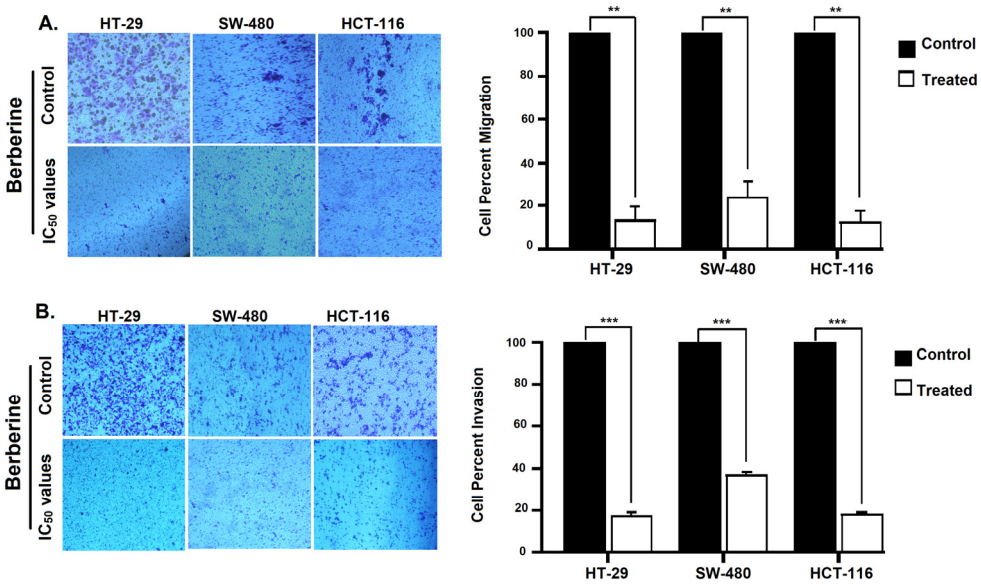

Figure 5. Effect of berberine on HT-29, SW-480 and HCT-116 cell migration (**A**) and invasion (**B**). Migration of colon cancer cell lines was assayed by transwell chamber assay whereas invasion was assayed by Matrigel. Analysis was done by two-way ANOVA for control and treated group followed by Sidak's multiple comparisons test. Magnification was 20X. ** $p < 0.01$ and *** $p < 0.001$.

2.5. Immunofluorescence Staining of AQP 1, 3 and 5

2.5.1. HT-29

Immunofluorescence staining was used to determine whether the expression of AQP1, AQP3 and AQP5 changed in HT-29 cells after treatment with berberine. HT-29 cells were positive for AQP1, AQP3 and AQP5. In particular, strong staining for AQP1 was evident in the cell membrane of HT-29 (Figure 6A). In contrast, both AQP3 and AQP5 were positively stained but with lower intensity than AQP1 (Figure 6B,C). Upon treatment with berberine (34.6 µM) for 72 h, AQP1, AQP3 and AQP5 staining was still detectable, and comparison of fluorescence intensity showed that treated cells had significantly lower fluorescence for AQP1 than their untreated control.

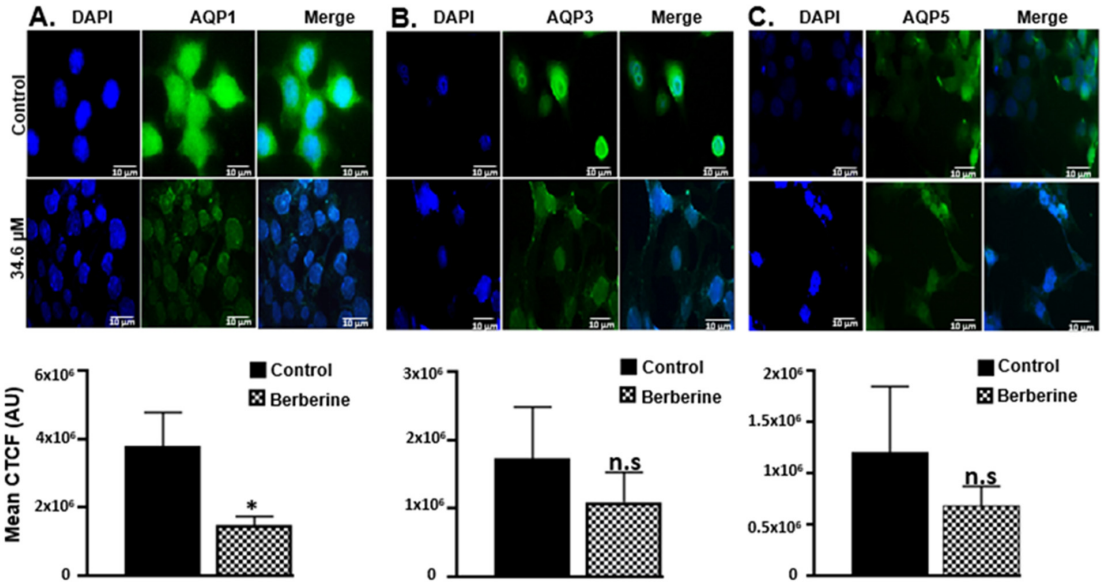

Figure 6. Berberine decreased the staining intensity of AQP 1, 3 and 5 on HT-29 cells. Immunofluorescence images (**upper panels**) and quantification (**lower panel**) in HT-29 cells of AQP1 (**A**), AQP3 (**B**) and AQP5 (**C**). Cells were incubated with 0 µM of berberine (control) or with the IC50 of berberine (34.6 µM) for 72 h. The mean corrected total cell fluorescence for AQP1, AQP3 and AQP5 was calculated using Image J software (http://rsb.info.nih.gov/ij/(NIH), accessed on 23 December 2022). Labeled AQPs' positive staining is identified by green fluorescence and the nuclei with blue (DAPI). Statistical analysis was done by paired *t*-test. Magnification was 63× with scale bars of 10 µm. * $p < 0.05$. n.s, not significant.

2.5.2. SW-480

SW-480 cells also showed positive staining for AQP1, AQP3 and AQP5. A more pronounced fluorescence intensity was recorded for AQP3 in untreated SW-480 cells compared to AQP1 and AQP5 (Figure 7B). The pattern of AQP1 distribution was similar to AQP5 in untreated cells (Figure 7A,C). Berberine (44.3 µM) inhibited the expression of all three types of AQPs in SW-480 cells.

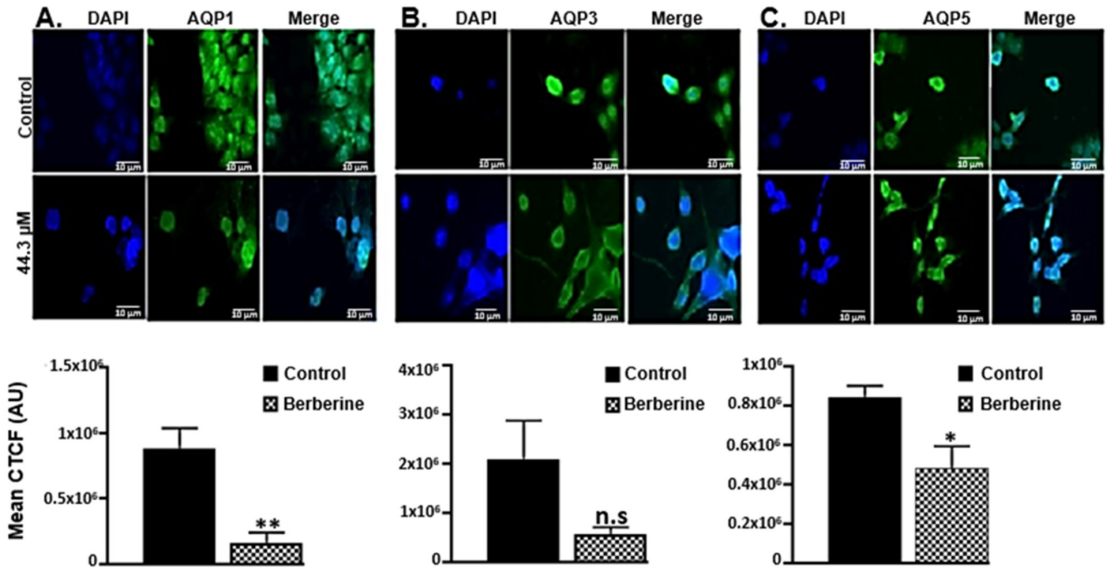

Figure 7. Berberine decreased the staining intensity of AQP 1, 3 and 5 on SW-480 cells. Immunofluorescence images (**upper panels**) and quantification (**lower panel**) of AQP1 (**A**), AQP3 (**B**) and AQP5 (**C**)

are shown. Cells were incubated with 0 μM of berberine (control) or with the IC50 of berberine (44.3 μM) for 72 h. The mean corrected total cell fluorescence for AQP1, AQP3 and AQP5 was calculated using Image J software (http://rsb.info.nih.gov/ij/(NIH), accessed on 23 December 2022). Labeled AQPs' positive staining is identified by green fluorescence and the nuclei with blue (DAPI). Analysis was done by paired *t*-test. Magnification was 63× with scale bars of 10 μm. * $p < 0.05$, ** $p < 0.01$. n.s, not significant.

2.5.3. HCT-116

The positive immunofluorescence of AQP1, 3 and 5 demonstrated the presence of AQPs in HCT-116. AQP5 exhibited slightly stronger fluorescence intensity in HCT-116 plasma membrane compared to weaker intensity for AQP1 and AQP3 (Figure 8). Decreased fluorescence intensity for AQPs was observed after treatment with berberine (32.1 μM) for 72 h.

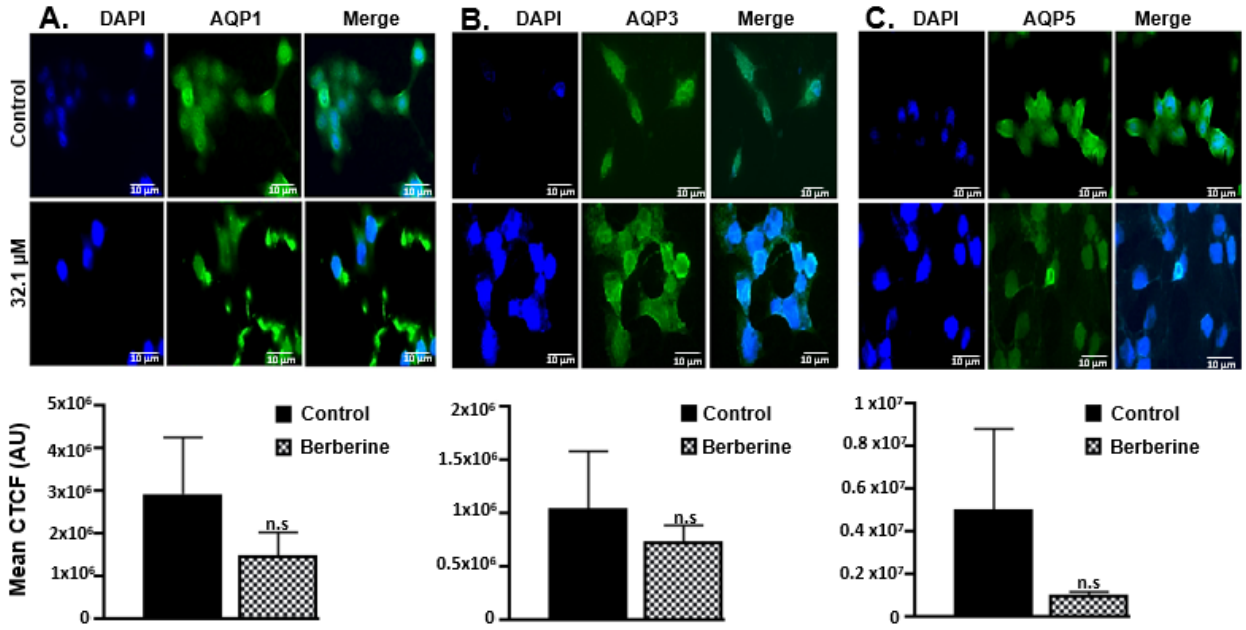

Figure 8. Berberine decreased the staining intensity of AQP 1, 3 and 5 on HCT-116 cells. Immunofluorescence images (**upper panels**) and quantification (**lower panel**) of AQP1 (**A**), AQP3 (**B**) and AQP5 (**C**) are shown. Cells were incubated with 0 μM of berberine (control) or with the IC50 of berberine (32.1 μM) for 72 h. The mean corrected total cell fluorescence for AQP1, AQP3 and AQP5 was calculated using Image J software (http://rsb.info.nih.gov/ij/(NIH), accessed on 23 December 2022). Labeled AQPs' positive staining is identified by green fluorescence and the nuclei with blue (DAPI). Analysis was done by paired *t*-test. Magnification was 63× with scale bars of 10 μm. n.s, not significant.

2.6. RT-qPCR

2.6.1. PI3K and PTEN Gene Expression

Figure 9A shows PI3K expression in HT-29 cells in the absence and the presence of berberine. The level of PI3K mRNA decreased after 24 and 48 h of treatment in response to all tested concentrations of berberine. A marked down-regulation in the level of PI3K mRNA was observed after 72 h of exposure at 100 μM in HT-29.

Berberine also reduced PI3K gene expression in SW-480 by about 2 folds (Figure 9B). Likewise, the downregulation in PI3K gene in HCT-116 was observed at all tested concentrations and at different time points. However, a significant down-regulation of gene expression (about 13 folds) was observed when HCT-116 cells were treated with berberine at 100 μM after 72 h of incubation (Figure 9C).

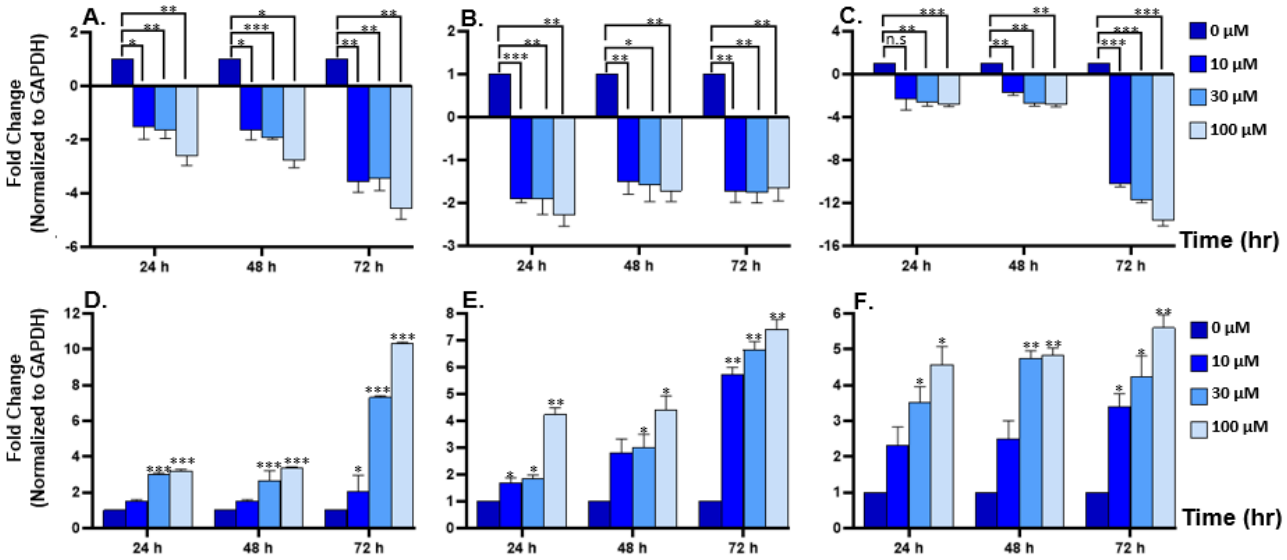

Figure 9. Effect of berberine on PI3K (**upper panel**) and PTEN (**lower panel**) mRNA expression levels in HT-29 (**A**,**D**), SW-480 (**B**,**E**) and HCT-116 (**C**,**F**) cells. Cells were incubated with berberine (0 µM, 10 µM, 30 µM, 100 µM) for 24, 48 and 72 h. Expression levels were normalized against GAPDH gene expression as a housekeeping gene. Values are presented as fold change. Data are expressed as mean ± SD of two independent experiments and analyzed by two-way ANOVA for control and treated groups followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, n.s, not significant, compared to the control.

Treatment of all three cell lines with berberine caused an increase in PTEN expression at all time points of incubation, but the effects were more obvious after 48 and 72 h., especially with 30 µM and 100 µM. The fold increase in PTEN expression after 72 h of incubation at 100 µM was ranging between 6–10 folds for the 3 cell lines (Figure 9D–F).

2.6.2. AQP1, AQP3 and AQP5 Gene Expression

After 24 h of incubation with berberine, HT-29 cells exhibited insignificant increase (<2 folds) in AQP1 mRNA (Figure 10A). After 48 and 72 h, there was a significant down-regulation of AQP1 mRNA at all concentrations (5–8 folds). Likewise, SW-480 and HCT 116 cells incubated for 24, 48 and 72 h, with berberine showing decreased AQP1 gene expression (2–16 folds) at all concentrations used (Figure 10B,C).

HT-29 cells incubated for 24, 48 and 72 h with berberine exhibiting a significant down-regulation (2–7 folds) in AQP3 gene expression at all tested concentrations (Figure 10D). In SW-480 and in HCT 116 cells, AQP3 gene expression was down-regulated (2–9 and 1–10 folds, respectively) in the presence of different concentrations of berberine (Figure 10E,F).

Upon incubation of HT-29 cells with different concentrations of berberine, AQP5 gene expression was down-regulated (2–9 folds) at all incubation periods. Of notice was an insignificant up-regulation observed at 10 µM after 24 h of incubation (Figure 10G). SW-480 and HCT-116 cells responded to berberine treatment by a significant down-regulation (2–27 folds) (Figure 10H,I).

2.7. Western Blot

Figure 11 demonstrates that the expression of PTEN was significantly increased in the three cell lines in a concentration-dependent manner. Figure 11A also shows that berberine slightly increased the total level of AKT and mTOR in HT29 cells but decreased p-AKT at the100 µM level and p-mTOR substantially for all concentrations.

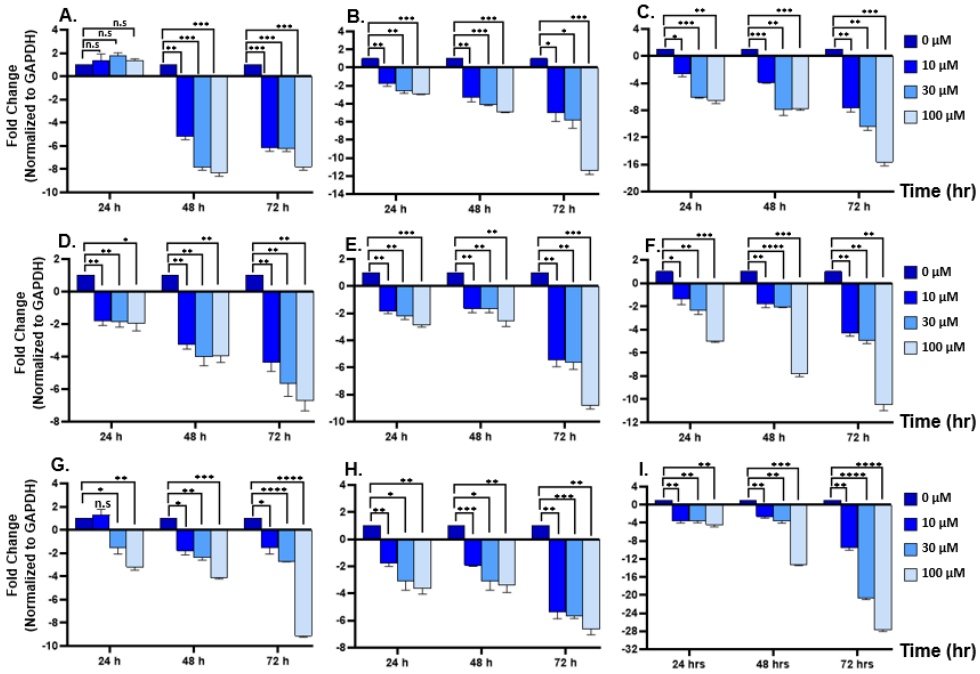

Figure 10. Effect of berberine on AQP1, AQP3 and AQP5 mRNA expression level in HT-29 (**A**,**D**,**G**), SW-480 (**B**,**E**,**H**) and HCT-116 (**C**,**F**,**I**) cells. Cells were incubated with berberine (0 μM, 10 μM, 30 μM, 100 μM) for 24, 48 and 72 h. Expression levels were normalized against GAPDH gene expression. Data are expressed as mean ± SD of two independent experiments and analyzed by two-way ANOVA for control and treated groups followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, n.s, not significant, when compared to the untreated control.

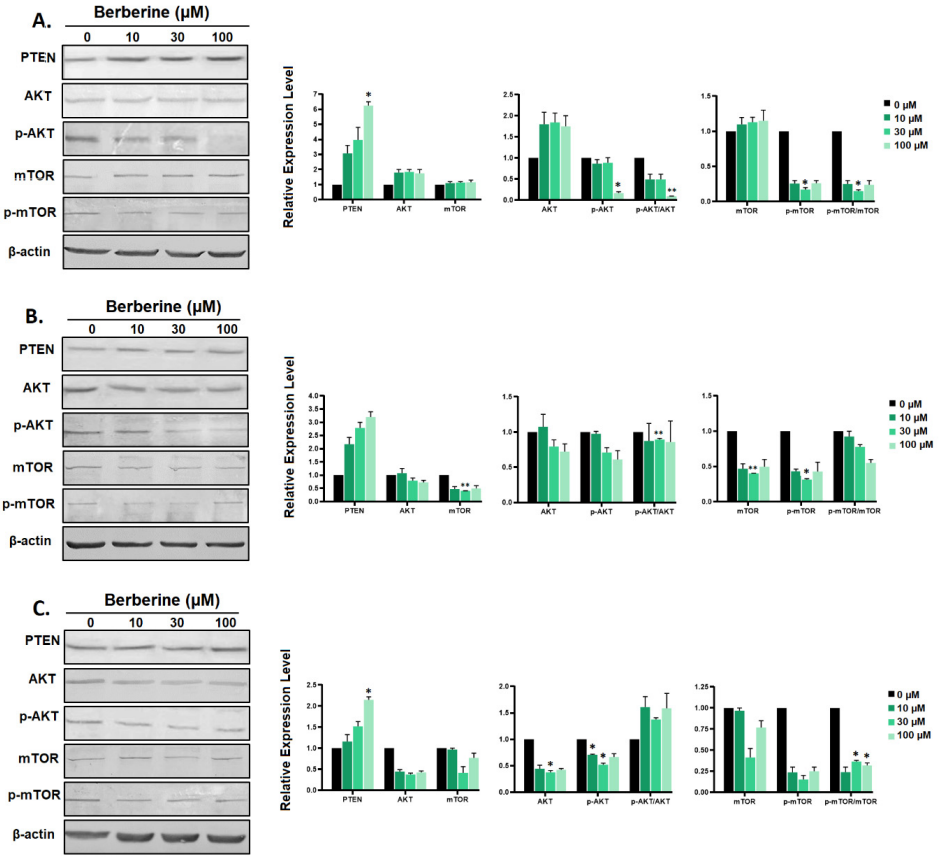

Figure 11. Effect of berberine on the expression of PTEN, AKT, p-AKT, mTOR and p-mTOR in CRC

cell lines as demonstrated by Western blot. (**A**) HT-29, (**B**) SW-480, (**C**) HCT-116. Cells were incubated with berberine (0 μM, 10 μM,30 μM,100 μM) for 24, 48 and 72 h. The relative expression of protein bands was normalized to β-actin. Data are expressed as mean ± SD of two independent experiments and analyzed by two-way ANOVA for control and treated groups followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$ when compared to the untreated control.

In SW-480, the lowest concentration of berberine (10 μM) did not affect AKT protein expression, but the higher concentrations decreased AKT and mTOR while p-AKT was insignificantly affected and p-mTOR was decreased (Figure 11B). In HCT-116, AKT protein was reduced by about 50%; mTOR was insignificantly reduced; p-AKT was 40–50% reduced; and p-mTOR was reduced by 4 folds (Figure 11C).

3. Materials and Methods

3.1. Cell Lines Culturing

The cell lines used were the human colorectal cancer cell lines HT-29, SW-480 and HCT-116 (Sigma-Aldrich). Cells were cultured in a high glucose (4.5 g/L) medium, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin mixture. During the experiment, cells were kept in 25-cm^2 T-flasks (Corning, NY, USA) under the standard conditions of 37 °C and 5% CO_2.

3.2. MTT Assay

HT-29, SW-480 and HCT-116 cancer cell lines were treated with berberine in concentrations of 3, 10, 30, 100 and 300 μM for 72 h. After incubation at 37 °C in a humidified incubator, the old media was removed and replaced by RPMI-1640 media with 5% FBS. Fifteen μL of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT reagent (5 mg/mL in phosphate buffered saline) was added to each well and incubated for 3 h at 37 °C. The medium was then aspirated carefully, and the formazan crystals contained in the cells were solubilized with 100 μL solubilization solution/stop mix for 24 h. After shaking for 1 min, the absorbance was read on a microplate reader (BioTek elx808, Agilent, Technologies, Inc., Santa Clara, CA, USA) at the wavelength of 570 nm [14]. The effect of berberine on growth inhibition was calculated as viability percentage using the formula:

$$\text{Viability } \% = \frac{[\text{Mean optical density of the sample}]}{[\text{Mean optical density of control}]} \times 100$$

The IC50 value of each cancer cell line was calculated from three independent determinations using Graph Pad prism software, version 8.

3.3. Apoptosis Assay

3.3.1. Flow Cytometry

The colorectal cancer cell lines (HT-29, SW-480 and HCT-116) were cultured in T25 culture flasks at seeding density of 1 × 106/flask. Cells were allowed to grow at 37 °C, 5% CO_2 and 95% relative humidity until 80–85% confluence. Cells were exposed to three concentrations of berberine (10, 30 and 100 μM) for 24, 48 and 72 h, incubated under standard conditions of 37 °C, 5% CO_2 and 95% relative humidity and stained according to the manufacturer's instructions (Promega, Minneapolis, MN 55413, USA). In brief, cells were gently resuspended in cold binding buffer (1X) and incubated for 15 min in Annexin V incubation reagent at room temperature in the dark. The quantification was recorded using BD Accuri™ C6 Plus Flow Cytometer (BD Medical Devices Company, Franklin Lakes, NJ, USA). For each sample, 10,000–20,000 events were collected. The results were displayed in the form of dot plots divided into four quadrants. Viable cells are both annexin V-FITC and propidium iodide negative (AN−/PI−); early apoptotic cells are annexin V-FITC positive and propidium iodide negative (AN+/PI−); late apoptotic cells, which are both annexin V-FITC and propidium iodide positive (AN+/PI+), and necrotic cells or completely

ruptured cells are annexin V-FITC negative and propidium iodide positive (AN−/PI+). The used excitation wavelength was 488 nm, and annexin V-FITC fluorescence was detected in FL1 (emission was 533/30 band pass filter) while propidium iodide was detected in FL2 (emission was 585/40 band pass filter). The controls used were untreated double-stained cells [20].

3.3.2. Toluidine Blue Staining

To evaluate the morphological changes in cells exposed to berberine, cells were counted using a hemocytometer (Neubauer chamber) in the presence of trypan blue (Sigma-Aldrich, Budapest, Hungary). Cells/well (2×10^5) were cultured in 6-well plates and were allowed to grow to a confluence of 80–85%. HT-29, SW-480 and HCT-116 cells were treated with previously determined IC50 concentrations of berberine for each cell type (34.6 µM, 44.3 µM and 32.1 µM, respectively) and incubated at 37 °C for 72 h. The old media was removed, and cell lines were washed twice with PBS (1 mL/well) for 5 min, followed by 1% toluidine blue (Sigma-Aldrich) [21]. The morphology of cells was examined, and images were captured using a ZEISS Axio Vert.A1 microscope equipped with Leica Flexacam1 camera.

3.4. Wound Healing Assay

The wound healing method was used to detect cell motility. HT-29, HCT-116 and SW-480 cells were seeded (1.6×10^5 cells/well) in 6-well plates and allowed to attach to the surface until 85–90% confluence. Under standard conditions of 37 °C temperature and 5% CO_2 and after 24 h, the confluent monolayers were scratched using a 200 µL sterile pipette tip. Cells were supplied with FBS-free DMEM media, and the various concentrations of berberine (10, 30 and 100 µM) were added. Cell migration toward the wound area was photographed and measured using a ZEISS Axio Vert.A1 microscope equipped with Leica Flexacam1 camera at 10X (Boeco, Hamburg, Germany). The distances between the edges were taken at 0, 24, 48 and 72 h at the same position of the wound. Wound closure (%) was quantified using the percentage change in the normalized measurement of area divided by the original open area as follows: Wound Closure % = $\frac{A(0)-A(t)}{A(0)} \times 100$, where $A(0)$ is the area at time zero (0) and $A(t)$ is the area after incubation time (t) [22].

3.5. Migration Assay

Migration properties of HT-29, SW-480 and HCT-116 were assayed using transwell chambers containing filters with a pore size of 8.0 µm (Corning Inc., Cambridge, MA, USA). Cells (1 × 105) were added into the upper chamber and incubated until 85–90% confluence at 37 °C. The medium in the upper chamber was aspirated and replaced with serum-free media 24 h. after seeding. In the lower chamber, a medium containing 10% FBS was used as a chemoattractant and a positive control. Cells were allowed to migrate for 72 h in the absence or presence of berberine (the IC50 for each cell type), and the migrating cells were then fixed with 4% paraformaldehyde for 10 min and permeabilized using 100% methanol for 2 min. Cells at the transwell chamber filter were stained with 2% crystal violet (C3886, Sigma). Then images of migrating cells were captured for five randomly selected fields using a ZEISS Axio Vert.A1 microscope equipped with Leica Flexacam1 camera at 20X, and cells were counted and expressed as percent of the control [23].

3.6. Invasion Assay

The invasion ability of cells assessed using the transwell chambers containing filters with a pore size of 8.0 µm (Corning Inc., Cambridge, MA, USA) were precoated with thawed and liquefied Matrigel. Matrigel (30–40 µL) was added to a 12-well transwell insert and solidified in a 37 °C incubator for 15–30 min to form a thin gel layer. Cells (1×10^5) were added to the upper chamber and incubated until 85–90% confluence at 37 °C. The medium in the upper chamber was aspirated and replaced with serum-free media 24 h after seeding. In the lower chamber, a medium containing 10% FBS was used as a chemoattractant and a positive control. Cells were allowed to migrate for 72 h in the

absence or the presence of berberine (the IC50 for each cell type). The cells that penetrated through the Matrigel to the lower surface of the filter (invaders) were then fixed with 4% paraformaldehyde for 10 min and permeabilized using 100% methanol for 2 min. Cells at the lower surface of the transwell chamber filter were stained with 2% crystal violet (C3886, Sigma). Then images of migrating cells were captured for five randomly selected fields using a ZEISS Axio Vert.A1 microscope equipped with Leica Flexacam1 camera at 20X, and the number of cells was counted and expressed as a percent compared to the control [24].

3.7. Staining Protocol for Aquaporins (Immunofluorescence)

Colon cancer cell lines at a density of 1×10^4 cells/well were plated in 6-well plates containing clean coverslips and were incubated for 24 h. HT-29, SW-480 and HCT-116 cells were then incubated for 72 h with berberine 34.6 μM, 44.3 μM and 32.1 μM, respectively. After treatment, the cells on the coverslips were rinsed with PBS twice and then fixed with 4% paraformaldehyde at room temperature for 10 min. After 3 times of washing with PBS for 5 min each, cells were permeabilized with PBS containing 0.3% Triton-X100 for 5 min and blocked with 5% normal goat serum (NGS) for 30 min. For immunofluorescence labeling, the cells on the coverslips were incubated overnight with the primary AQP antibodies (1:50) (AQP1 IHC antibody, AQP3 IHC antibody and AQP5 IHC antibody) at 4 °C. Cells were washed 4 times in PBS for 5 min, blocked again in PBS with 5% NGS and then incubated with goat anti-rabbit IgG (H + L) secondary antibody, FITC (1:200) (VK307556; Invitrogen, Waltham, MA, USA) in the dark for 1 h. Cell lines were washed in PBS twice for 5 min and were incubated with DAPI (300 nM), a fluorescent nuclear label, for 5 min. Cells were then washed two more times with PBS and mounted on slides. Images were acquired using Olympus CKX41 inverted phase contrast fluorescence microscope with Optika Pro5 camera (Microscope Central, Tustin, CA, USA). Fluorescence density was quantified, and the mean corrected total cell fluorescence was calculated using Image J V1.8.0 software (software 1.48q, Rayne Rasband, National Institutes of Health, USA; http://rsb.info.nih.gov/ij/(NIH), accessed on 25 December 2022) [25].

3.8. Reverse Transcription—Quantitative PCR (RT-qPCR)

The colorectal cancer cell lines (HT-29, SW-480 and HCT-116) were cultured in T25 culture flasks at seeding density of 1×10^6/flask. Cells were allowed to grow at 37 °C, 5% CO_2 and 95% relative humidity to a confluence of 80–85% and then exposed to three different concentrations of berberine (10, 30 and 100 μM) and incubated for 24, 48 and 72 h. [26] Total RNA was extracted using SV Total RNA Isolation System kit (Promega, Minneapolis, MN 55413, USA) [27]. The first strand of cDNA was synthesized using Go-ScriptTM Reverse Transcriptase System (Promega, USA) [28]. BIO-RAD PTC-200 Thermal Cycler PCR 96 well (Bio-Rad, Hercules, CA, USA) was used and the thermal conditions for cDNA synthesis were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Finally, microcentrifuge tubes were incubated at 4 °C for 24 h. [29]. Lyophilized primer pairs (forward and reverse primers) for the selected genes (PI3K, PTEN, AQP1, AQP3 and AQP5), in addition to the housekeeping gene (GAPDH), were purchased from integrated DNA technologies (IDT), USA. The sequences of primer pairs for all genes were selected according to the literature (Table 1). Analyses of mRNA expression levels of the tested genes were assessed using Sso Advanced Universal SYBR® Green Supermix (BIO-RAD, USA). PCR amplification was performed in RT-qPCR CFX96 real time PCR machine (Bio-Rad, USA), using the cycling conditions as indicated above. Fold change was calculated using the $2-\Delta\Delta Ct$ method [30].

3.9. Western Blot Analyses

The effect of berberine on the PTEN/AKT/mTOR pathway in HT-29, SW-480 and HCT-116 cells was evaluated by Western blotting to examine the underlying mechanism of berberine-mediated inhibition of cell proliferation and metastasis as described by Cipak Gasparovic et al. [35]. Colon cancer cell lines (HT-29, SW-480 and HCT118) were seeded into

T25 culture flasks at a density of 1×10^6 cells/flask and cultured to 85–90% confluence and then incubated with berberine (0, 10, 30 and 100 µM) under standard conditions of 37 °C, 5% CO_2 and 95% relative humidity for 72 h. Subsequently, total protein was extracted from cells using RIPA lysis buffer system (SC-24948A; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) supplemented with phosphatase inhibitor cocktail IV (ab201115; Abcam, Cambridge, UK). The total protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., San Diego, CA, USA, and 20 µg of protein samples per lane were separated via 8% SDS-PAGE and transferred onto PVDF membranes, which were blocked overnight with 3% bovine serum albumin (BSA) in tris-buffered saline (TBS) at room temperature. Membranes were subsequently incubated overnight with the following primary antibodies at 4 °C: PTEN (AF847, R&D System; 1:1000), p-AKT-S473 (AF887, R&D System; 1:1000), AKT (MAB2055, R&D System; 1:1000), p-mTOR-S2448 (ab109268, Abcam; 1:1000), mTOR (AHO1232, Thermo Fisher Scientific; 1:1000) and beta actin (2118S; Cell Signaling Technology, Inc.; Danvers, MA, USA; 1:1000). Following the primary antibody incubation, the membranes were washed using TBS and Tween® 20 and incubated at room temperature for 1 hr with HRP-conjugated goat anti-rabbit secondary antibody (AS014, ABclonal; 1:1000). Protein bands were visualized using a SuperSignal™ West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific, Inc., USA) on a gel imaging system (GE Healthcare, Buckinghamshire, UK), and the band density was quantified by densitometric analysis using Image J V1.8.0 software (Software 1.48q, Rayne Rasband, National Institutes of Health, USA; http://rsb.info.nih.gov/ij/(NIH), accessed 26 December 2022).

Table 1. List of primers sequences used for the genes PI3K, PTEN AQP1, AQP3, AQP5 and GAPDH.

Target Gene	Primer Sequence	Reference
PI3K	Forward: 5′ GAAGTTGCTCTACCCAGTGTCC 3′ Reverse: 5′ GATAGCCGTTCTTTCATTTGG 3′	[31]
PTEN	Forward: 5′ CAAGATGATGTTTGAAACTAT 3′ Reverse: 5′ CTTTAGCTGGCAGACCACAA 3′	[32]
AQP1	Forward: 5′ ATT TTCTGG GTG GGGCCATT 3′ Reverse: 5′ GGG CCAGACCCCTTC TAT TT 3′	[33]
AQP3	Forward: 5′ ACC CCT CTG GAC ACT TGG AT 3′ Reverse: 5′ GGGTTG TTGTAG GGGTCAA 3′	[33]
AQP5	Forward: 5′ TCC ATTGGCCTGTCTGTCAC 3′ Reverse: 5′ CTT TGATGATGGCCACACGC 3′	[34]
GAPDH	Forward: 5′ CATCACTGC CACCCAGAA GA 3′ Reverse: 5′ GTCAAAGGTGGAGGAGTGGG 3′	[34]

3.10. Statistical Analyses

Data obtained were statistically analyzed, and graphs were plotted using Graph Pad Prism (Version 8). All values are represented as the mean ± SD. Statistical comparison of wound healing experiments were performed by one-way ANOVA that was followed by Dunnett's multiple comparisons test. Migration and invasion were analyzed by two-way ANOVA that was followed by Sidak's multiple comparisons test. Immunofluorescence experiments were performed using paired *t*-test. Gene expression and Western blot experiments were analyzed by two-way ANOVA that was followed by Dunnett's multiple comparisons test. Significance was declared when $p < 0.05$.

4. Discussion

Colorectal cancer (CRC) remains a major cause of mortality worldwide. Currently, there are a variety of treatment options, including surgical treatment, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, among others. Despite advances in the diagnosis and treatment of CRC, the relative survival rate of patients is below 40% [14]. Invasion, metastasis, and resistance to drugs are the main characteristics of cancer that

compromise tumor therapy [36]. Therefore, it appears necessary to discover novel drugs that target CRC biomarkers in order to halt tumor survival and development.

Berberine has shown an anti-tumor role in a variety of solid tumors, such as prostate cancer [37], nasopharyngeal cancer [38] and colon cancer [39], where it can effectively inhibit malignant tumors of various tissue sources. Other workers showed that berberine has an anti-cancer function, which was attributed mainly to its prevention of the spread of cancer cells [40]. Only a few studies have discussed the mechanism of berberine impact on cell proliferation, migration, and apoptosis of CRC cells, despite the fact that numerous key proteins of several pathways are controlled in a variety of cancer cells treated with berberine [41,42]. In accordance with these studies, we evaluated the impact of berberine on cell growth, migration, invasion, apoptosis, and expression level of certain genes (PI3K, PTEN, AQP1, AQP3 and AQP5) in HT-29, SW-480 and HCT-116 colon cancer cell lines.

The MTT assay was used in this research to determine the cytotoxic effect of berberine and to calculate IC50 values. When calculated on a molar basis, the IC50 values for these cell lines were 58% and 59% lower than those found by Zhao and colleagues (2022) for HT-29 and SW-480 cells but comparable to those of HCT-116. According to these researchers, berberine inhibited these cell lines' abilities to proliferate in a concentration- and time-dependent way [43]. In support, Dai et al. (2019) demonstrated that berberine suppressed cell viability on HT-29 and HCT-116 cells over 12–72 h at concentrations varying from 10 M to 100 M [44] in both a concentration- and time-dependent way. Additionally, Li et al. (2021) found that berberine had a broad inhibitory effect on the viability of the HT-29, SW-480 and HCT-116 cell lines with IC50 values of 16.22 M, 61.5 M and 55.27 M, respectively [13].

Significant effort has been invested into discovering naturally occurring chemopreventive or chemotherapeutic reagents capable of inhibiting colon tumor growth [8]. Apoptosis is one of the main mechanisms to prevent the development of cancer [45]. In the flow cytometry experiment (Figure 2), berberine decreased cell population that were Annexin V negative and PI negative (viable cells) in a concentration- and time-dependent manner, but it increased the Annexin V positive and PI positive (apoptotic cells) cell population as well as the Annexin V negative and PI positive (necrotic cells) cell population. This is confirming the data from the MTT assay, and altogether they indicate that berberine induced tumor cell death through apoptosis and necrosis. Cell count values indicated that HCT-116 cells were more sensitive to berberine than HT-29 and SW-480 cells after 72 h of exposure. This is partly consistent with the findings of Gong and colleagues (2019), who showed that apoptosis was promoted by up to 28% when human colon cancer cell lines (SW-480 and HT-29) were treated with berberine at concentrations as low as 20 μM after 24 h [14]. Other researchers studying the apoptotic effect of berberine on HT-29 and HCT-116 cells also concluded that 40 μM increased apoptosis up to 11% after 48 h [44].

To determine how berberine produces its anti-cancer impact, we used toluidine blue stain to monitor the morphology of colorectal cancer cell lines after 72 h of treatment with the IC50 values for each cell line. Cell images (Figure 3) revealed pertinent morphological changes. Cells separated from the bottom of the well and took on a rounded form. Furthermore, the cells exhibited blebbing membranes, compacted chromatin, and fragmented nuclei. These modifications are typical of apoptosis [46].

Cancer cell motility and metastasis are the main reasons for the failure of tumor chemotherapy [47]. We performed wound healing, migration, and invasion assays to visualize the possible effect of berberine on the migratory and invasive ability of cell lines. The results of wound healing assay indicated that berberine impaired cell motility in a concentration- and time-dependent manner. Transwell migration and invasion assays showed that treatment with the IC50 values of berberine for 72 h significantly inhibited migration and invasion capacity of HT-29, SW-480 and HCT-116. However, wound healing, migration and invasion assay results indicated that SW-480 cells were more aggressive than HT-29 and HCT-116 cells after 72 h of exposure. These observations are consistent with those of other researchers studying anti-metastatic effect of berberine on SW-480

and HCT-116 through transwell migration assay who concluded that berberine inhibited migration in a concentration-dependent manner after 24 h of exposure [48].

Colorectal cancer molecular assessment is in the spotlight because of its impact on prognosis and therapy prediction [49]. The PI3K/AKT pathway is implicated in cell survival and proliferation since molecular aberrations and/or overactivation of the PI3K/PTEN/AKT pathway are common in various types of cancer [50]. The PTEN protein is principally involved in the homeostatic maintenance of PI3K/AKT signaling initiated by EGFR activation (or activation of other tyrosine kinase receptors or G-protein-coupled receptors) [51]. It typically works as a tumor suppressor by directly opposing the PI3K function by dephosphorylating the lipid-signaling second messenger PI 3,4,5-triphosphate (PIP3), a lipid product of the PI-3-kinase (PI3K). Therefore, its purpose is to prevent the downstream signaling processes, such as AKT and the mammalian target of rapamycin (mTOR), from being activated [52].

On the other hand, PI3Ks catalyze cell cycle biochemical reactions, which are involved in cell growth and survival. Thus PTEN, which opposes the activity of PI3Ks, is implicated in the inhibition of cell cycle progression and induction of cell death [53].

To explore the mechanism of action of berberine, we used RT-qPCR and immunoblot assays to see if berberine affected PI3K and PTEN expression. RT-qPCR analysis revealed that PI3K gene expression was down-regulated by berberine at various doses and times of incubation. PI3K gene expression was down-regulated by 2 folds in SW-480 cells, while it was reduced by 4 and 13 fold in HT-29 and HCT-116, respectively (Figure 9A–C).

The expression of PTEN was substantially up-regulated after exposure to berberine in the three cell lines in a concentration-dependent manner, as demonstrated by RT-qPCR and immunoblot assay. PTEN controls the PI3K/AKT pathway by dephosphorylating the PI3K product PIP3, which prevents the pathway from promoting cell viability and tumor growth. Therefore, the increase in PTEN expression demonstrated at the level of RNA expression (Figure 9D–F) and at the protein level (Figure 11) was concomitant with a down-regulation of AKT, p-AKT, mTOR and p-mTOR in SW-480 and HCT-116 (Figure 11B,C). In HT-29, there was a small increase (<2 fold) in AKT protein, but p-AKT and p-mTOR were significantly down-regulated, especially at 100 μM (Figure 11A). The decreased levels of p-AKT and p-mTOR in these colon cancer cell lines validate the conclusion that the anti-tumor activity of berberine could be, at least in part, due to its action as an inhibitor of PI3K/AKT/mTOR pathway [53].

These observations are consistent with a previous study demonstrating that berberine treatment dose-dependently up-regulated PTEN expression, as well as inhibited the expression of PI3K, AKT and mTOR proteins in SW-480 cells [54]. A more recent preclinical study showed the capacity of berberine to potentiate therapeutic efficacy when acting synergistically with other natural compounds. Okuno and his coworker (2022) found the synergistic anti-tumorigenic effects of berberine when administrated with oligomeric proanthocyanidins in CRC cell lines, and the effects were mediated by enhancement of cellular apoptosis and inhibition of AKT and p-AKT significantly in CRC cells [18].

Several studies revealed a positive correlation between AQPs' expression and CRC carcinogenesis. For instance, Kang et al. (2008) found that AQP5 was overexpressed in colon cancer cell lines and colon cancer tissues [55], and Jiang (2009) showed that AQP1 was highly expressed in colon cancer [56]. Others found a strong relationship between AQPs 1, 3 and 5 expressions and lymph node metastasis in patients with colon cancer [3]. Consistent with these studies, we demonstrated the presence of AQP1, AQP3 and AQP5 in tested colon cancer cell lines by immunofluorescence and by data from RT-qPCR experiments, and we showed a decline in the immunofluorescence and the expression of the 3 AQP types.

To understand the underlying regulatory mechanisms, Li and his colleagues (2013) provided remarkable data showing that human epidermal growth factor (hEGF) can up-regulate the expression of AQP3 protein and the migration ability of the human colorectal carcinoma cell line HCT-116 in a dose- and time-dependent manner whereas an AQP3 inhibitor, CuSO4, blocked the enhanced migration ability of HCT-116 cells. Along the

same line, hEGF-induced overexpression of AQP3 was inhibited by the inhibitor LY294002 of PI3K/AKT, whereas the inhibitor U0126 of ERK had only a minor effect on the hEGF-induced AQP3 up-regulation [6]. In consistency with these findings, the present experiment demonstrated that berberine significantly downregulated the expression of AQPs in the 3 cell lines, suggesting a potential role for berberine in inhibiting the metastasis of these cell lines.

Our study confirms the capacity of berberine to disrupt PI3K/AKT/mTOR pathway through up-regulation of the tumor suppressor PTEN. PTEN antagonizes PI3K and, subsequently, the activation of the downstream AKT and mTOR at both RNA and translational level. Moreover, this study revealed that berberine showed anti-tumor activity, not only through PTEN/PI3K/AKT/mTOR pathway but also through the downregulation of AQP1, AQP3 and AQP5 expressions. Since AQPs have been shown to promote malignancies [6] by accelerating cell migration via extension of the leading edges (lamellipodia) of migrating cells to speed the rate of movement, the present study suggests that berberine could therapeutically target colon tumor metastasis. To our knowledge, this is the first study to examine the impact of berberine on the expression of aquaporins (AQPs) in colon cancer cell lines and their implication in the underlying molecular pathways that cause inhibition of cell proliferation. Moreover, the finding that berberine can up-regulate PTEN and down-regulate PI3K, AKT, mTOR and AQPs 1, 3 and 5 at the same time confirms the pleiotropic effect of berberine and sheds light on the possible crosstalk between AQPs and other molecular pathways involved, particularly the PI3K/AKT signaling pathway and its downstream targets, mTOR and p-mTOR.

5. Conclusions

The current study findings support a fundamental role of water channels in cell migration, a process that is central to a variety of biological phenomena, such as angiogenesis, wound healing, tumor spread and organ regeneration. Berberine was found to have a significant anti-CRC activity as MTT, and wound healing assays showed that it had a significant anti-CRC activity. Additionally, the integrative gene expression in our study showed that berberine had a remarkable inhibitory effect on AQP1, 3 and 5 gene expression, possibly regulating tumor metastasis. The study also revealed a novel mechanism for the anti-tumor activity of berberine through the induction of PTEN gene expression and subsequent suppression of the PI3K/AKT/mTOR signaling pathway, which regulates cells' proliferation. The study indicates a multi-target mechanism of action for berberine.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules28093823/s1, Figure S1: The chemical structure of berberine. Figure S2: Representative flow cytometry plots using annexin V-FITC/PI staining for apoptosis and necrosis after 24, 48 and 72 h. (left panels). (A) HT-29, (B) SW-480 and (C) HCT-116 cells were treated with the indicated concentrations of berberine. Cell count percentages for each cell type over 24, 48, or 72 h. are shown (right panels). Figure S3. Effect of berberine on HT-29 (A), SW-480 (B) and HCT-116 (C) cell migration. Migration of colon cancer cell lines was assayed by wound-healing assay and analyzed by one-way ANOVA followed by Dunnett's multiple comparison test for control and treated groups at 0, 24, 48, and 72 h. Figure S4. Original gels for ß-actin and PTEN for HT-29, SW-480, and HCT-116 cell lines. Western blots for PI3K, AKT, p-AKT, m-TOR, and p-mTOR are available upon request.

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