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Baicalein Reduces Metastasis and Heightens Caspase-Induced Apoptosis in Human Colorectal Cancer Cells

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Abstract

Current colorectal cancer (CRC) treatments exhibit unwanted cytotoxicity against healthy proliferating cells. Hence, these therapeutics demand higher specificity upon drug delivery, a task that may be facilitated by the discovery of anticancer agents bearing critical mechanisms of action. Baicalein is a flavonoid with promising anticancer activity, among other pharmacological benefits, and has therefore been of high clinical interest. We tested baicalein *in vitro* for its effect on several CRC hallmarks, including the suppression of metastasis (the spread of cancer cells from their initial site), the ability to induce apoptosis (cell death), and the inhibition of proliferation (the growth of cells). The suppression of the metastasis of CRC cells was recorded via two studies: the cell migration assay and the *in silico* docking of baicalein with toll-like receptor 4 (TLR4) and matrix metalloproteinase-9 (MMP-9). Results from the cell migration assay showed that baicalein inhibited metastasis by up to 25.76% ($p < 0.01$) in a concentration-dependent manner. We then reinforced these results by docking baicalein with TLR4 (binding affinity: −8.4 kcal/mol) and docking baicalein with MMP-9 (binding affinity: −7.9 kcal/mol), classifying strong binding affinities as those less than −6.0 kcal/mol. The induction of cell death was measured using a caspase activity assay. Again, a docking study was done to reinforce the findings from the primary *in vitro* experiment, though this time between baicalein and caspase-3 (binding affinity: −7.1 kcal/mol). Despite mixed observations in concentration dependence, caspase activity, relative to control, reached a maximal increase of 88.6% ($p < 0.01$), and results from the MTT assay demonstrated a survival rate, relative to control, of as low as 59.64%. Considerations for future studies include the testing of baicalein *in vivo* and on more aberrative CRC cell lines.

Keywords

Baicalein, Metastasis, Apoptosis, Proliferation, TLR4, MMP-9

1. Introduction

Colorectal cancer is the second-most common cause of cancer mortality in the United States for men and women combined [1]. Upon diagnosis, 70% - 75% of patients survive beyond year one, 30% - 35% beyond year three, and below 20% beyond year five [2]. Nevertheless, due to the uncontrollable cytotoxicity of many present treatments and the possibility of intrinsic or acquired drug resistance in the targeted cancer cells, the desire for novel anticancer agents of reduced cytotoxicity and increased immunity to drug resistance persists.

Baicalein (5,6,7-trihydroxy-2-phenylchromen-4-one) is a type of flavonoid originating from the Chinese skullcap herb *Scutellaria baicalensis* Georgi. Previous studies performed with baicalein underscore its ability to induce apoptosis via the activation of caspase proteases, specifically caspases −3, −8, and −9, inhibiting metastasis, a multistep phenomenon beginning with cell escape and ending with outgrowth and obstructing LPS-TLR4 binding [3] [4] [5]. Lipopolysaccharide (LPS) exists on the outer membrane of Gram-negative bacteria and is responsible for the dangerous drop in blood pressure associated with septic shock [6]. In colorectal cancer, studies have shown that LPS promotes inflammation through the various inflammatory cell pathways associated with cancer progression, including tumor necrosis factor-α (TNF-α). The study done by Chen *et al.* (2021) [5] demonstrated that baicalein interrupts the formation of LPS by binding directly to TLR4 and, consequently, inhibits CRC growth and angiogenesis, thereby reducing the metastatic potential.

1.1. Flavonoids as Anticancer Agents

Flavonoids are a class of plant-derived phenolic chemicals that function as secondary metabolites [7]. One of the principal causes of carcinogenesis is inflammation. Flavonoids, desirably, possess anti-inflammatory functions. Many flavonoids, such as wogonin and curcumin, are involved in the induction of cell death, either via the inhibition of autophagy or the triggering of apoptosis [8]. Flavonoids are moreover involved in the inhibition of angiogenesis, the formation of new blood vessels, and the suppression of metastasis via matrix metalloproteinase-9 (MMP-9) and interleukin-8 (IL-8). Flavonoids have also been shown to exhibit synergistic properties in chemoresistant colorectal cancer cells [9].

1.2. Induction of Apoptosis

Most cells are programmed to undergo cell death in a regulatory process called apoptosis. Due to its significance in tumor suppression, even the slightest dysfunction in apoptosis may lead to cancer [10]. Cells become cancerous in a two-

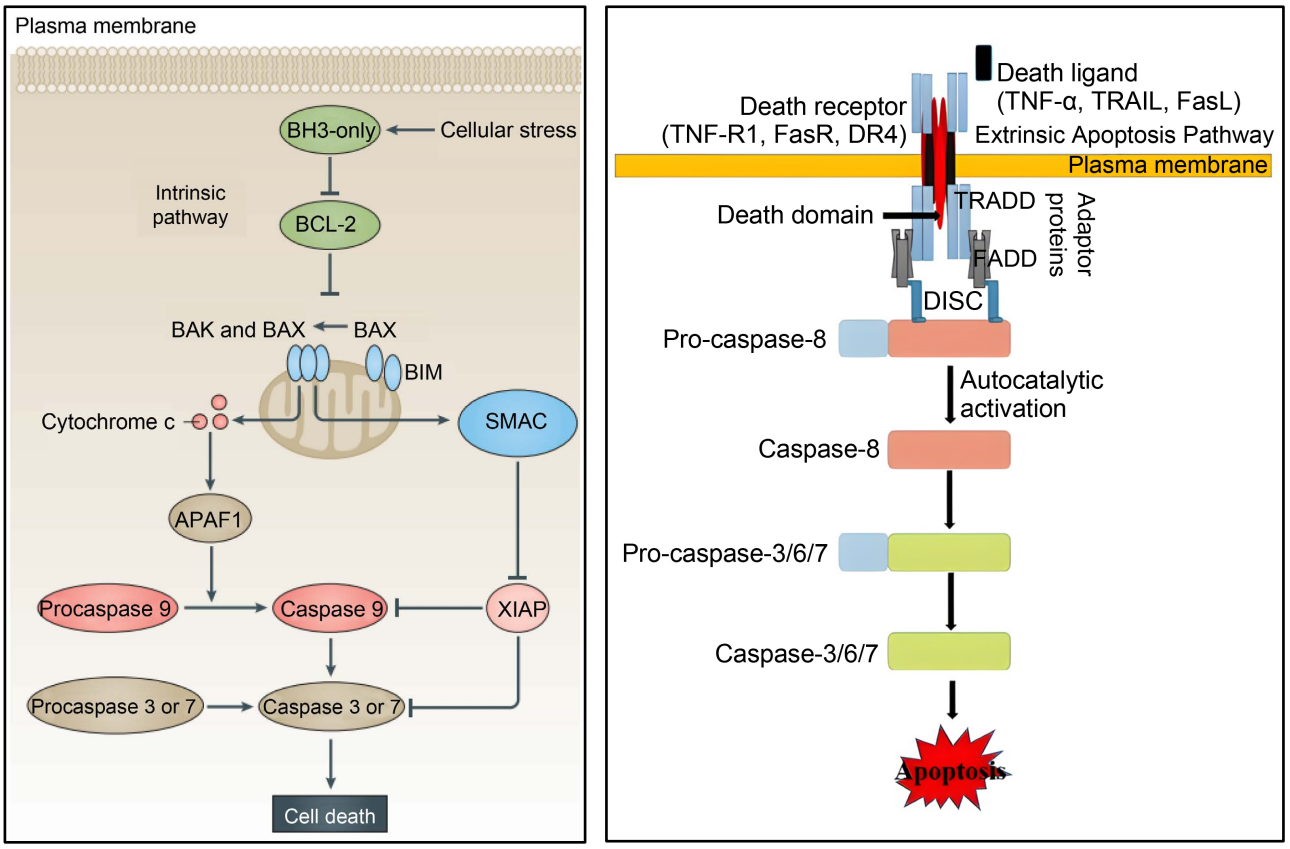
step process, wherein the absence of one will not lead to cancer. The first step involves hyperproliferation (rapid cell division) and relies on the mutation of proto-oncogenes. Such mutations allow cells to bypass various cell divisions, proliferations, and checkpoints. The second step, in turn, involves the deregulation of apoptosis (programmed cell death) and depends on the inactivation of the tumor suppressor genes. Tumor suppressor genes are unique in that, by the two-hit hypothesis, both alleles of a tumor suppressor gene must be affected for successful inactivation [11]. However, once cancerous, cells acquire innate resistance to programmed cell death in a process known as apoptosis evasion [12]. Consequently, one of the most sought-after characteristics of flavonoids, such as baicalein, is their ability to *induce* apoptosis through signaling cascades [13].

Signaling cascades that result in apoptosis occur in two ways: intrinsically (mitochondrial-driven) and extrinsically (via death receptors). For intrinsic signaling pathways, proteins belonging to the Bcl-2 superfamily are of the utmost importance to apoptosis since they either inhibit cell death (anti-apoptotic) or induce it (pro-apoptotic) [14]. Extrinsically, a death ligand, typically belonging to the tumor necrosis factor (TNF) receptor gene superfamily, binds to a death receptor and, in turn, (via the intermediary adaptor proteins) induces the autocatalysis of an initiator caspase (prefix pro-), which then prompts its active counterpart to cleave for activated caspase-3 (executioner caspase), thus achieving apoptosis (**Figure 1(b)**). One famous death ligand is Tumor necrosis factor-α (TNF-α), which is produced during the activation of macrophages, T-lymphocytes, and natural killer (NK) cells [15].

The apoptotic pathways that flavonoids disrupt are mitochondria-driven (intrinsic). Kim *et al.* (2012) [16] demonstrated that the apoptotic properties of baicalein in CRC cells stem from the increased expression of the pro-apoptotic Bax protein and the decreased expression of the anti-apoptotic Bcl-2 protein. As shown in **Figure 1**, the Bax and Bcl-2 proteins exist strictly in the intrinsic pathway. Kim *et al.* used HT-29 cells, which bear a close resemblance in origin to the cells used in our present study.

1.3. Inhibition of TLR4

Pattern recognition receptors (PRRs) are proteins tasked with detecting *specific* molecular structures known as pathogen-associated molecular patterns (PAMPs) [18]. Toll-like receptors (TLRs), a constituent of PRRs, are among the most investigated PRRs due to their unique signaling methods. Toll-like receptor 4 (TLR4) is a protein capable of recognizing lipopolysaccharide (LPS). Trouble starts when LPS, also known as endotoxin, binds to TLR4. Mechanistically, the binding of LPS to TLR4 triggers the dimerization of TLR4 and consequently recruits downstream signaling, leading to gene expression connected to inflammation. By blocking the LPS-TLR4 signaling pathway, Baicalein inhibits tumor growth, adhesiveness, and metastatic capability [19]. In addition to LPS-induced TLR4 signaling, the role of baicalein may also be connected to the TLR4/HIF-1α/VEGF



(a) (b)

Figure 1. (a) (left) Summary of the intrinsic (mitochondrial-driven) apoptotic pathway. Here, the ultimate step is caspase-9's activation of procaspase-3 and procaspase-7. The penultimate step, wherein caspase-9 is activated, traces back to Cytochrome c (cyt. c) and a protease (APAF1). This figure is taken from Ashkenazi *et al.*, 2017 [15]. (b) (right) Summary of the extrinsic apoptotic pathway. Unlike its counterpart, the extrinsic pathway begins with extracellular initiation, usually via a receptor, like the one shown above. This figure is taken from Kashyap *et al.*, 2021 [17].

signaling pathway. In the study by Chen *et al.*, 2021 [5], baicalein is shown to inhibit CRC growth and metastasis via TLR4/HIF-1*α*/VEGF axis in vivo, supporting the translation of baicalein into novel TLR4-targeting therapeutics for CRC treatment.

1.4. Reduction of Metastasis

Metastasis is the disruption of the primary site of cancer, and, in terms of cancer staging, it is a characteristic of the final and most aggressive stage. This cancer hallmark has been deemed the primary cause of death in patients with colorectal cancer (CRC) [20]. In breast cancer, baicalein has been observed to suppress the proliferation, migration, and invasion of breast cancer cells in a time- and dose-dependent manner [21]. Previous studies indicate that baicalein can inhibit CRC cells' proliferation and colony formation ability [3]. The study by Chai *et al.* (2017) is one of the few metastatic-focused projects focusing on baicalein and CRC. The authors demonstrate that following treatment with baicalein, one saw decreased matrix metalloproteinase expression and a potential synergistic role of

combining baicalein with an extracellular regulated kinase (ERK) inhibitor to reduce MMP expression further. This phenomenon of anti-metastasis due to downregulated MMP expression is not particularly novel. Chandrashekar *et al.* (2012) [22] also explored the inhibition of metastasis due to matrix metalloproteinase MMP-2/-9 reduction. However, the brunt of metastatic research has been spent on other cancers, such as hepatocellular carcinoma (HCC), breast cancer, and ovarian cancer. In this study, the cell migration assay will assess the restrictive potential of baicalein.

1.5. Aim

Studies have demonstrated the potential of baicalein in downregulating the expression of TLR4 and MMP-9. However, one limitation is that most studies on baicalein have been preclinical, meaning that the effects on humans have yet to be elucidated [23]. The present study utilizes baicalein's inhibitory and apoptotic abilities to test its impact on COLO320DM, a type of adenocarcinomatous (gland-developing) CRC. Metastatic inhibition, if found at high rates, can likely be attributed to baicalein's inhibitory ability on TLR4 and MMP-9 [24]. Change in caspase activity, if found to occur at regular intervals and significant rates, may likely be attributed to its ability to activate caspase-induced mechanisms of apoptosis. MTT, if found to regulate cell survival at regular intervals and with decent success, may likely be attributed to both metastatic inhibition and caspase activity, given that cytotoxicity is broadly defined as the degree of toxicity imposed on a cell, thus leading to that cell's impairment or death. We investigate all three anticancer properties of this drug in human colorectal cancer cell lines in hopes of adding to the existing arsenal of anticancer treatment for colorectal cancer.

2. Methodology

2.1. Materials

Baicalein (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich®, St. Louis, MO, USA) and serially diluted to the desired concentrations (×1000, ×100, ×10) with Minimum Essential Medium (MEM). The ×1 concentration was 0.013 M, or 0.35% (w/v). COLO320DM (CCL-220) (ATCC, Manassas, VA, USA), a cell line of human Caucasian colon adenocarcinoma origin, was cultured in RPMI 1640 + 10% h.i. FBS at 37˚C. Both baicalein and COLO320DM were stored at −20˚C Pipettes (Thermo Fisher Scientific Finnpipette™, Waltham, MA, USA), Fisher Healthcare™ PROTOCOL™ Hema 3™ Fixative and Solutions I/II (for staining), and tissue culture plates (Sigma-Aldrich®, St. Louis, MO, USA) were used accordingly for the three assays (cell migration, caspase, and MTT). The caspase and MTT assays required the use of a microplate reader (Model 1680; Bio-Rad Laboratories Inc., Hercules, CA, USA) and Microplate Manager 4.0 software (Bio-Rad Laboratories Inc., Hercules, CA, USA) upon analysis.

2.2. Dilutions

Four microcentrifuge tubes were used for the four concentrations of baicalein, abbreviated as "BAC." Tubes contained 900 μL of MEM, aside from the stock solution, BAC ×1. To start, 1000 μL of DMSO was added into a tube containing 3.5 mg of baicalein to allow for the dilution of BAC ×1. After that, 100 μL of BAC ×1 was transferred into BAC ×10. Transferring 100 μL of the higher concentration to the next highest concentration, thus forming a 1-to-9 ratio of baicalein to MEM, was known as a serial dilution. Serial dilutions followed until we reached BAC ×1000, a 1/1000th dilution of the stock solution. Aliquots were all homogenized using a vortex mixer (Fisher Scientific, Hampton, NH, USA) before transfer. For example, before transferring 100 μL of BAC ×10 into BAC ×100, BAC ×10 would be homogenized for a few seconds to maximize dissolution.

2.3. Cell Migration Assay Protocol

This assay aimed to determine baicalein's ability to inhibit COLO320DM metastasis. A 6-well plate was used to conduct this study. BAC ×100, BAC ×10, and BAC ×1 each received one well, whereas BAC ×1000 received two. Wells were then filled with 10 μL of their respective concentrations. The sixth well was used for control and thus only contained the COLO320DM cell culture. Once the concentrations were in, the plate was gently swirled to ensure relative homogeneity within the wells. The following step was essential to migration analysis. Three horizontal scratches (per cell) were made using a sterile pipette tip to simulate the wounds in the given migration. The scratches were meant to be as straight (important, affected measurement), visible, and consistent (distance between each scratch) as possible.

Afterward, wells were treated with MEM before being placed in the oven overnight. The following day, MEM was drained from all 6 wells so that the pipette was angled vertically to narrowly touch the base of the well's side. This adding-removing process was replicated for the next three steps. We next added Fisher Healthcare™ PROTOCOL™ Hema 3™ Fixative stain (1000 μL) into each of our 6 wells; 1 minute later, we completely removed the fixative from all 6 wells. Then, we added Fisher Healthcare™ PROTOCOL™ Hema 3™ staining (1000 μL) to each of our 6 wells, and following a few minutes wait, removed the staining liquid accordingly: this staining process was done to ensure the adhesion of the CRC cells to the well. To finish, we added distilled water (1000 μL) to each well and, after 1 min, drained all 6 wells identically to before.

With each procedure, wells were double-checked to ensure no liquid or bubbles were withstanding. The CRC cells were then analyzed and photographed cyclically using the processing program ImageJ, whereby for each pipette marking that had been made initially, and three photographs were taken across that one line. This process was repeated three times for each cell, with respect to the three markings, meaning that we had 54 images overall.

Images were then uploaded to Microsoft Paint 3D for analysis. Using the Paint 3D "select" feature, lines (6 per wound) were drawn vertically from one side of the wound to the other at our discretion. Accounting for variability within measurements for each wound, we measured multiple times (6 times), then averaged those values for a scratch average. Our concentration average would hence be the average of the 9 scratch averages, leaving us in total with 324 measurements (px.), 54 scratch averages (px.), and ultimately, 6 concentration averages (px.).

2.4. Caspase Activity Assay Protocol

This assay aimed to determine the baicalein's ability to induce apoptosis in COLO320DM cells. Apoptosis, as mentioned before, is the act of programmed cell death. As cancer cells find ways to evade apoptosis, the body's conventional methods of controlled death fail. However, baicalein addresses this aberration by triggering apoptosis by upregulating caspase proteases, including caspase-3 and caspase-9, in human colorectal cancer cells such as HCT-116 (Wang *et al.*, 2015).

The setup for this assay was identical to that of the cell migration assay: one well for control, two wells for BAC ×1000, and one well each for the remaining concentrations—BAC ×100, ×10, and ×1. Each well contained 10 μL of its designated concentration, aside from Control, which was left untreated. Cells were then placed into the incubator overnight at 5% CO_2 and 37˚C. Following incubation, cells were prepared for analysis, beginning with draining. Wells were fully drained using a sterile pipette into tubes of five. Tubes were then centrifuged to foster the formation of cell pellets. Afterward, 500 μL of fixation buffer was added to each tube. Caspase assay buffer (50 μL), followed by caspase lysis buffer (45 μL), and, ultimately, the appropriate sample (5 μL) were then added to each well. Caspase substrate (5 μL) was then added to assess the reaction. Cells were read at 15-minute intervals, starting at $t = 0$ and ending at $t = 60$, using a microtiter plate reader (415 nm). The percent change in caspase activity was then calculated as the sample average minus that of the control, then divided by the control.

2.5. MTT Assay Protocol

This assay aimed to determine baicalein's effect on COLO320DM proliferation. A 96-well tissue culture plate was used. Of the 12 rows ("row" = 1 × 8 wells), 4 were used for baicalein and another for control. The eight wells per row (e.g., "Row 1" for "BAC ×1000", "Row 2" for "BAC ×100", and so on) contained 5.0 μL. The culture plate was then placed in the oven (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37˚C with 5% CO_2 for 24 h. Following the 24 h, 10 μL of MTT reagent was added into each of the 40 wells, and a second incubation was prepared under the same conditions, except shortened to 3 h, as the first. This second incubation was done to enable the conversion of the MTT reagent into purple formazan. Cells were then read using a microtiter reader at

595 nm, and the resulting data was logged by Microplate Manager 4.0 software.

3. Results

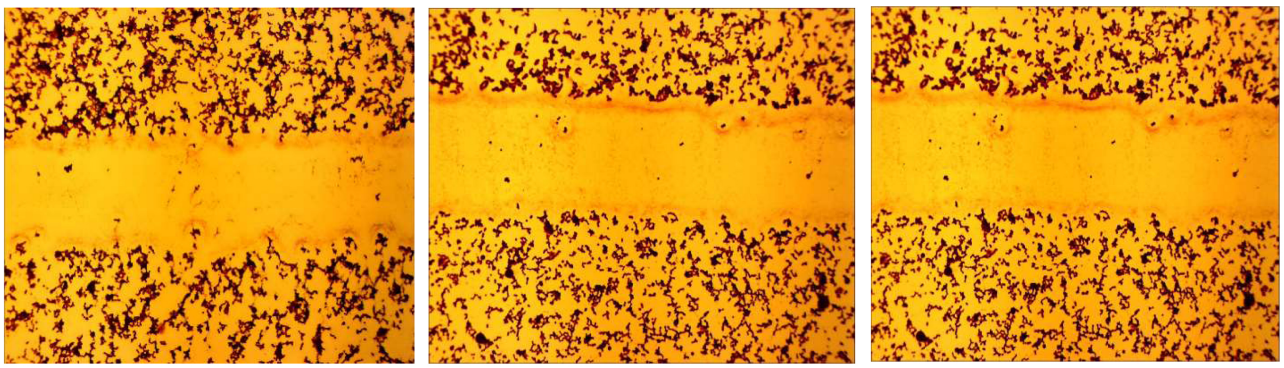
3.1. Baicalein Suppresses Metastasis

The results from the images were assessed for their average wound width (distance from front to front). Changes in wound width are detected by comparison to the control well. Metastasis was therefore analyzed indirectly and explained as the migratory feasibility, in that wider gaps meant greater inhibition, and vice versa. Wounds were measured in pixels (px.) using Microsoft Paint 3D, though ultimately represented as percentages relative to Control. Although the inconsistency posed by BAC ×1000 (2), wound width and concentration exhibited a strong positive relationship, indicating baicalein's ability to inhibit metastasis. (**Figure 2**)

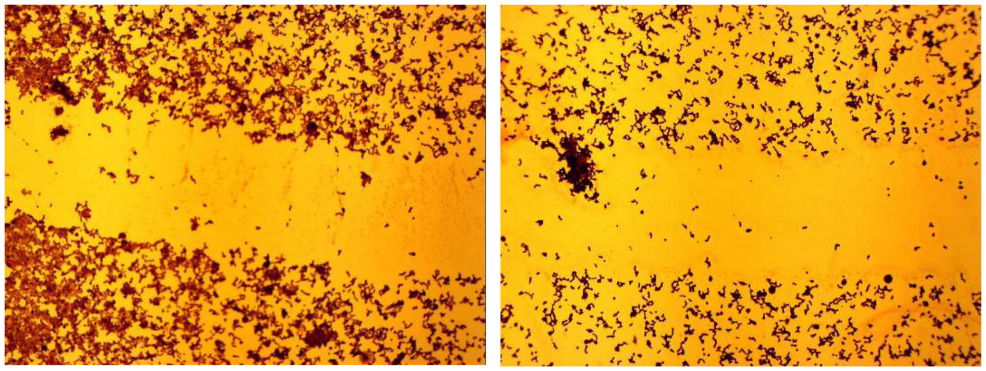
The chart below summarizes cell migration data. Concentrations were measured in percentages relative to the control and recorded as significant (**Figure 3**).

3.2. Molecular Docking Elicits High Binding Affinities

Molecular docking is an *in silico* technique for predicting binding characteristics between a ligand(s) and a macromolecule [25]. We docked the ligand baicalein with the macromolecules toll-like receptor 4 (TLR4), matrix metalloproteinase-9



(a) Control wound. (b) BAC ×1000 wound. (c) BAC ×100 wound.



(d) BAC ×10 wound. (e) BAC ×1 wound.

Figure 2. (a) Control wound. (b) BAC ×1000 wound. (c) BAC ×100 wound. (d) BAC ×10 wound. (e) BAC ×1 wound.

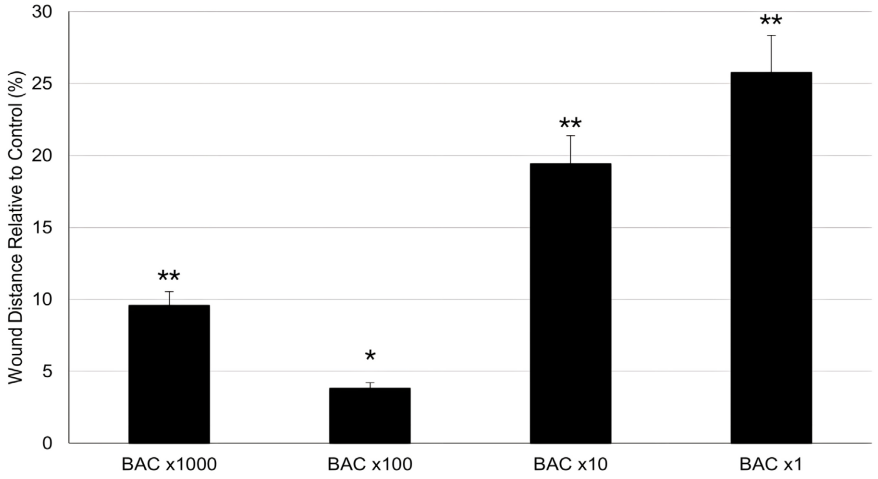


Figure 3. Baicalein on cell migration ("*" = $p < 0.05$; "**" = $p < 0.01$).

(MMP-9), and caspase-3. The structures of baicalein ($C_{15}H_{10}O_5$) and TLR4 (PDB ID: 2Z63), MMP-9 (PDB ID: 2OW2), and caspase-3 (PDB ID: 1RE1) were retrieved from the public databases PubChem and the Protein Data Bank (PDB), respectively (**Figure 4**).

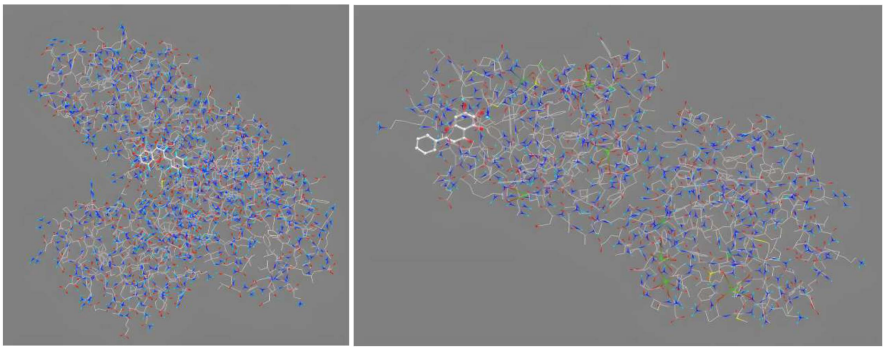
3.3. Baicalein Induces Apoptosis

Baicalein induces apoptosis in a concentration-dependent manner. The highest concentration of baicalein, labeled as BAC ×1, amounted to an increase in caspase activity of 88.6%. Incidentally, we omitted the lowest concentration of baicalein from the chart due to its unusually high change in caspase activity (80.7%). This irregularity was not explicitly known, and the rest three concentrations were all found to be statistically non-significant with $p > 0.05$. However, the highest concentration of baicalein demonstrated a p-value that was extremely close to 0.05 ($p = 0.058$) (**Figure 5**).

In fact, the docking of baicalein with caspase-3 elicits a relatively strong binding affinity, thus supporting baicalein's successful activation of caspase-induced apoptosis.

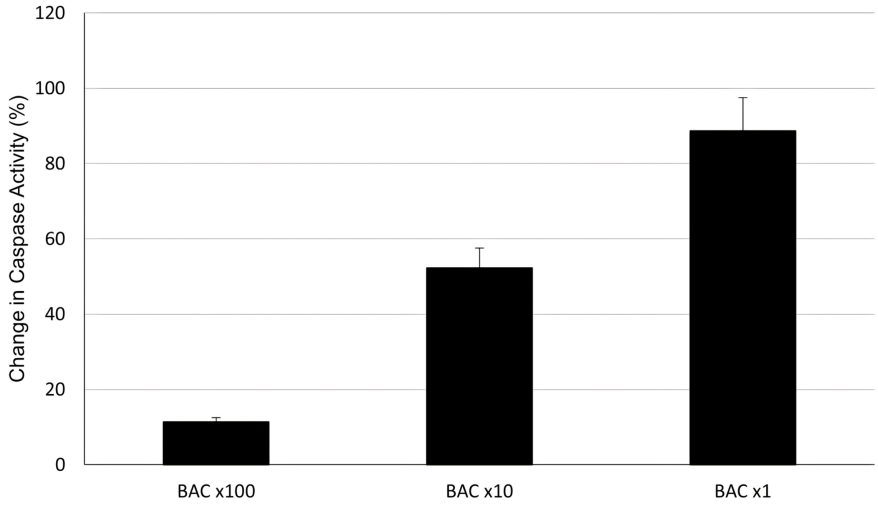
3.4. Baicalein Inhibits Proliferation

The MTT assay measured the survival of CRC cells when treated with baicalein relative to that of a control group of CRC cells. Results from this assay were unexpected in that the lowest concentration of baicalein performed the best, which went against the concentration dependence of the other two assays. We owed this irregularity to a color change in the higher concentrations of baicalein. The MTT assay, being colorimetric, is therefore unreliable if the chemical's color interferes with that of what the assay requires. We hypothesized that this color change stemmed from the oxidation of baicalein. However, this oxidation took over a day to transpire. The colors of baicalein had not changed when we conducted the caspase activity assay, the other colorimetric study (**Figure 6**).

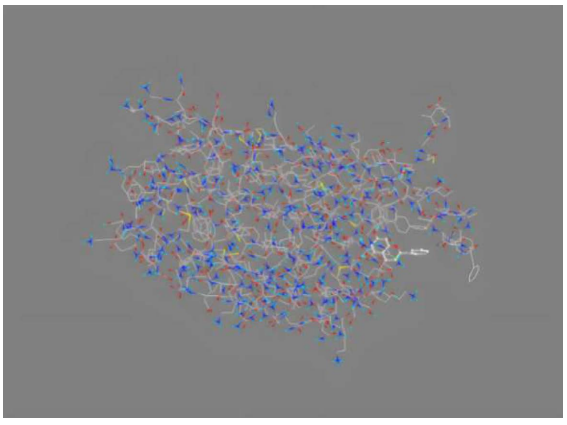


(a) (b)

Figure 4. (a) (left). Docking of baicalein (ligand, centerpiece) and TLR4 (macromolecule, overarching structure) using PyRxAutoDockVina (binding affinity: −8.4 kcal/mol). (b) (right). Docking of baicalein (ligand, far left) with MMP-9 (macromolecule, overarching structure) using PyRxAutoDockVina (binding affinity: −7.9 kcal/mol).



(a)



(b)

Figure 5. (a) Baicalein on caspase activity ("*" = $p < 0.05$; "**" = $p < 0.01$). "Change in Caspase Activity (%)" = $100 \times \left(\frac{C_{sample}}{C_{control}} \right)$. (b) Docking between baicalein and caspase-3 (PDB ID: 1RE1) reveals a binding affinity of −7.1 kcal/mol.

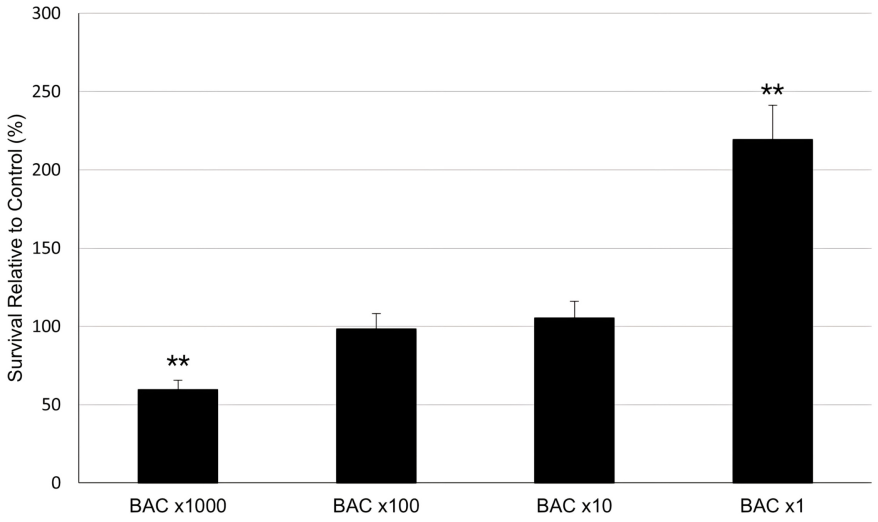


Figure 6. Baicalein's effects on cell survival ("*" = $p < 0.05$; "**" = $p < 0.01$). "Survival Relative to Control (%)" = $100 \times \left(\frac{A_{sample}}{A_{control}} \right)$.

4. Discussion

Our assay and docking results are largely in line with the literature review. We have concluded that baicalein's only observable side effect is that its higher concentrations might naturally change color, which would affect the results of colorimetric assays such as caspase activity and MTT. We hypothesized that this color change may have been due to the oxidation of the chemical. Cell migration results indicated that baicalein inhibited metastasis in a concentration-dependent manner that can be connected to the downregulation of MMP-9 and the inhibition of TLR4. It was later shown through molecular docking that baicalein could inhibit TLR4 and downregulate MMP-9. Baicalein's ability to limit LPS-to-TLR4 interaction could prevent inflammation, thereby reducing the chance of carcinogenesis. Additionally, since MMP-9 is implicated in cell metastasis, the inhibition of MMP-9 reinforced the results from the cell migration assay that baicalein was an effective inhibitor of metastasis. Caspase activity results were also concentration-dependent and demonstrated baicalein's strengths in setting off pro-apoptotic signaling pathways, leading to an increase in the induced cell death of colorectal cancer (CRC) cells. MTT results demonstrated the effects of baicalein on CRC proliferation. However, the results from the MTT assay were unusual in that the lowest concentration of baicalein fared best in inhibiting proliferation. Further testing should be done on more aberrative non-adenocarcinomatous CRC cell lines, such as CL40, HCT-116, and CR4. Previous literature indicates that baicalein's effect has been modeled consistently from cancer to cancer. Its caspase activity demonstrates that Baicalein's restrictive potential is similarly translatable to pathways such as MMP-2, PI3K/AKT, and NF-κB [26]. For instance, studies have illustrated baicalein's ability to synergistically inhibit the NF-κB signaling pathway [27].

Lastly, the author acknowledges that the present study bears several limitations. First, the results from the molecular docking of baicalein with toll-like receptor 4 (TLR4), matrix metalloproteinase-9 (MMP-9), and caspase-3, despite entailing a best-fit approach, are not comparable to those of an *in vivo* or *in vitro* study. Second, a flow cytometric study could have been done to quantify baicalein's effects on the inhibition of TLR4 and the downregulation of MMP-9, which would have been considerably more advantageous, and regardless helpful to have had, to the data acquired from molecular docking. Third, it was known that the cell migration assay did not wholly reflect the proper metabolic processes of CRC metastasis or, in that respect, the metastasis of any form of cancer. Overarchingly, we studied baicalein from an *in vitro*, rather than *in vivo*, standpoint. The *in vivo* implementation of baicalein may reveal new mechanisms of action and corroborate the results of *in vitro* experiments [28].

5. Conclusion

We have demonstrated that baicalein is an effective and concentration-dependent inhibitor of metastasis and inducer of apoptosis in colorectal cancer (CRC) cells. The inhibition of metastasis, one of the key anticancer characteristics of flavonoids, was directly measured using the cell migration assay and indirectly measured using *in silico* molecular docking. The ability to induce cell death, another notable anticancer trait of flavonoids, was indirectly measured using the caspase activity assay, which relied on the induced presence of caspase-3, a protein entailed in apoptosis. Paradoxically, the inhibition of proliferation, a quality of flavonoids that lacked in the extensiveness of the literature on metastasis and apoptosis, grew more prominent as the concentration of baicalein decreased. Altogether, this paper advances the use of flavonoids as individual or synergistic anticancer agents in the hallmarks of metastasis, apoptosis, and proliferation. The implementation of flavonoids into the current array of chemotherapeutics is a well-documented process that can extensively aid in resolving the issues of drug resistance and collateral cytotoxicity against healthy proliferating cells.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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