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Cell Death Mechanisms in Cancer

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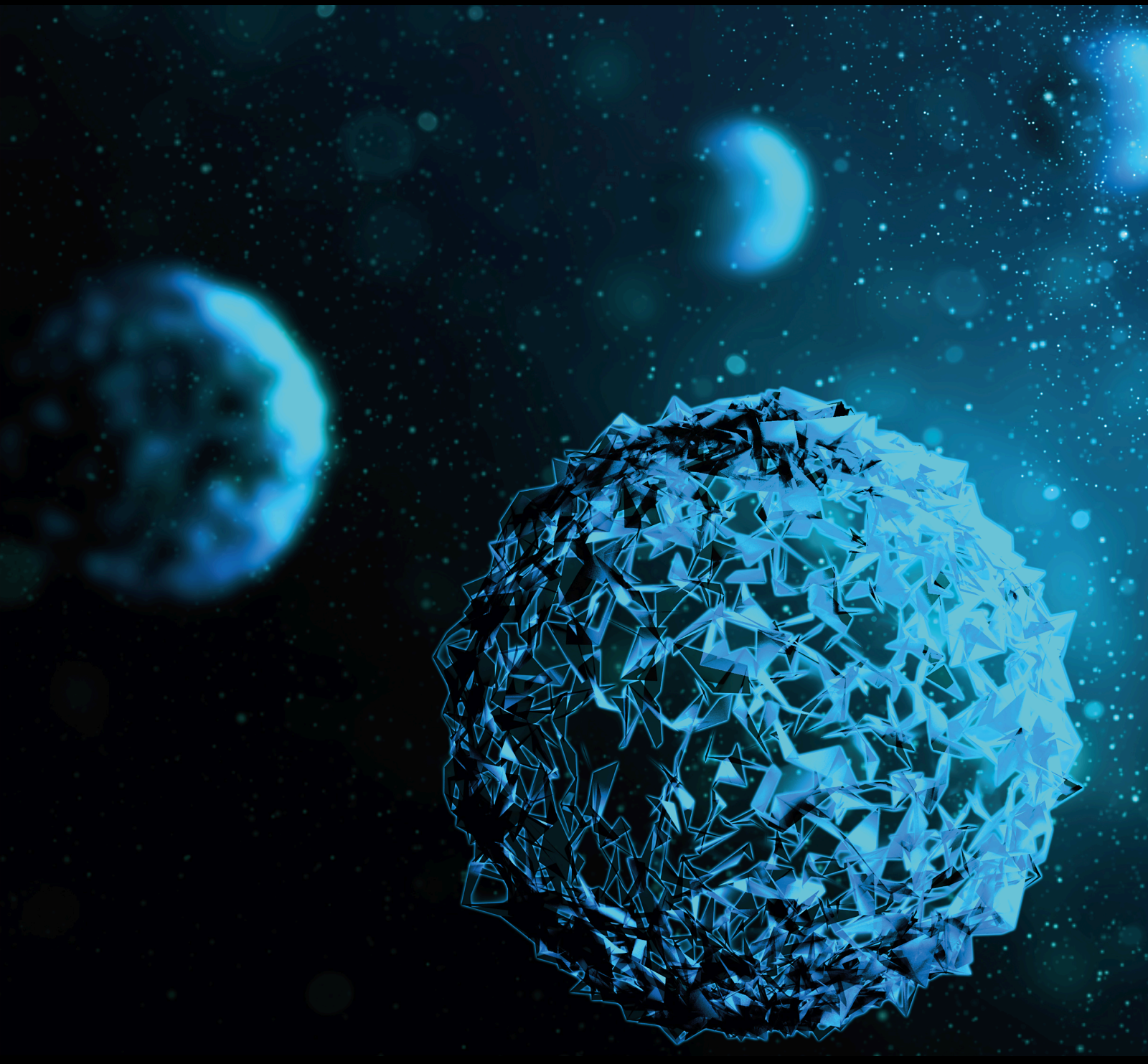

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Hindawi
BioMed Research International
Volume 2022, Article ID 4053074, 11 pages
https://doi.org/10.1155/2022/4053074

Research Article

Study on the Anticancer Activity of Prodigiosin from Variants of *Serratia Marcescens* QBN VTCC 910026

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Received 29 November 2021; Revised 8 February 2022; Accepted 12 March 2022; Published 23 April 2022

Academic Editor: Antonio Palumbo Jr

Prodigiosin (Pg), a secondary metabolism produced by numerous bacterial species, is known as anticancer, antibacterial, antifungal, immunosuppressant, antioxidant, antimalarial properties. Pg has been tested for antitumor activity in many different cancer cell lines but studies in LU-1, KB cell lines, and tumor-bearing mice are still limited. In this study, *Serratia marcescens* QBN VTCC 910026 strain (GenBank: KX674054.1) was mutated using Ethyl Methanesulfonate (EMS) to increase the production of Pg. One strain known as EMS 5 was capable of increasing prodigiosin biosynthetic yield by 52% when compared to the wild-type strain. Red bacterial pigmented colonies containing Pg were collected from solid media, lysed with acetone, purified with toluene: ethyl acetate at a ratio of 9: 1 (v/v), and then used to evaluate the potential anticancer activity. The purity of Pg was confirmed using a high-performance liquid chromatography (HPLC) method which indicated a 98% rate. Pg chemical formula which was determined using ^{1}H-NMR and ^{13}C-NMR spectroscopy, confirmed as prodigiosin (Pg). Human breast cancer cell lines MCF-7, oropharyngeal cancer KB, and particularly lung cancer LU-1 in vitro were used to test the anticancer activity of purified Pg compound. It showed a strong inhibitory ability in all the cancer cell lines. Furthermore, the isolated Pg had capable of inhibiting tumor growth, the tumor volume decreased by 36.82%, after 28 days. The results indicated that the bacterial prodigiosin from variants *Serratia marcescens* QBN VTCC 910026 strain is an encouraging fragment suitable for therapeutic applications.

1. Introduction

Prodigiosin (Pg) is a natural secondary metabolite that is biosynthesized by Gram-negative and Gram-positive bacteria such as *S. marcescens*, *V. psychroerythrus* [1], *V. ruber* sp. *nov*, *V. gazogenes* ATCC 29988 T and some other species such as *Streptomyces coelicolor* [2], *Pseudoalteromonas* sp. 1020R [3], *Janthinobacterium lividum* [4], *Zooshikella rubidus* S1-1 [5]. Prodigiosin is a tripyrrole rings pigment forming a pyrryldipyrrylmethane skeleton (two of the rings are directly linked to each other and the third is attached by way of a methane bridge) synthesized by *S. marcescens* with the molecular formula is $C_{20}H_{25}N_3O$ and a molecular weight of 323.44 Da. It is sensitive to light and insoluble in water. It is moderately soluble in alcohol and ether, and soluble in chloroform, methanol, acetonitrile and DMSO Three compounds, namely prodigiosin, undecyl prodigiosin and cycloprodigiosin hydrochloride, form the prodigiosin group and have biological activities for immunosuppression and apotosis induction of cancer cells. Cytotoxic effect of them requires the C-6 methoxy substituent. The A-pyrrole ring plays an important role both in co-nuclease activity and cytotoxicity of prodigiosin [6]. Pg and Obata x (OBX), two members of their prodiginine, small molecules active against cancer clinical trials are studying

[7]. Pg has been receiving much attention from researchers due to its immunosuppressive and anticancer properties on many drug-resistant cancer cell lines such as MDR1, BCRP, or MRP2 [8], or K562 human chronic myelogenous leukemia cells [9]. In addition, Pg has antibacterial and antifungal activities [10] and has little effect on normal cells. Pg has been tested for antitumor activity in more than 60 different cancer cell lines and the average IC_{50} value was 2.1 μg/mL. The anti-cancer potential of Pg was assumed to be due to this active substance causing apoptosis in cancer cells. Pg was found to be present in small granules near the cell nucleus, in the nucleus [11], cytoplasm [12], and the mitochondrial membrane [13], Pg has been shown to induce apoptosis in cells by four pathways: DNA damage, cell pH changes, cell cycle changes, and signal transduction interference. Pg intercalates into the DNA sequence (prefers AT sequence) in the small groove position, along with the presence of Cu^{2+} will promote oxidation leading to DNA damage and leading to cell death [14]. The bacterial prodigiosin induces apoptosis in JEG3 cells in vitro. and significantly inhibited the growth of JEG3 and PC3 cells in vivo experiment. The expressions level of protein makers such as P53 and Bax/Bcl-2 in JEG3 and PC3 were significantly higher than in untreated groups [14]. In this paper, we generated the variants of *Serratia marcescens* strain which was isolated from soil in Viet Nam. After purification and identification of Pg this compound was tested the anticancer activities in vitro and in vivo. Our results indicated that the Pg from variants of *S. marcescens* isolated from solid in Viet Nam possessed strong activities against cancer cells lines, tumor with high promising as an anticancer drug in future.

2. Materials and Methods

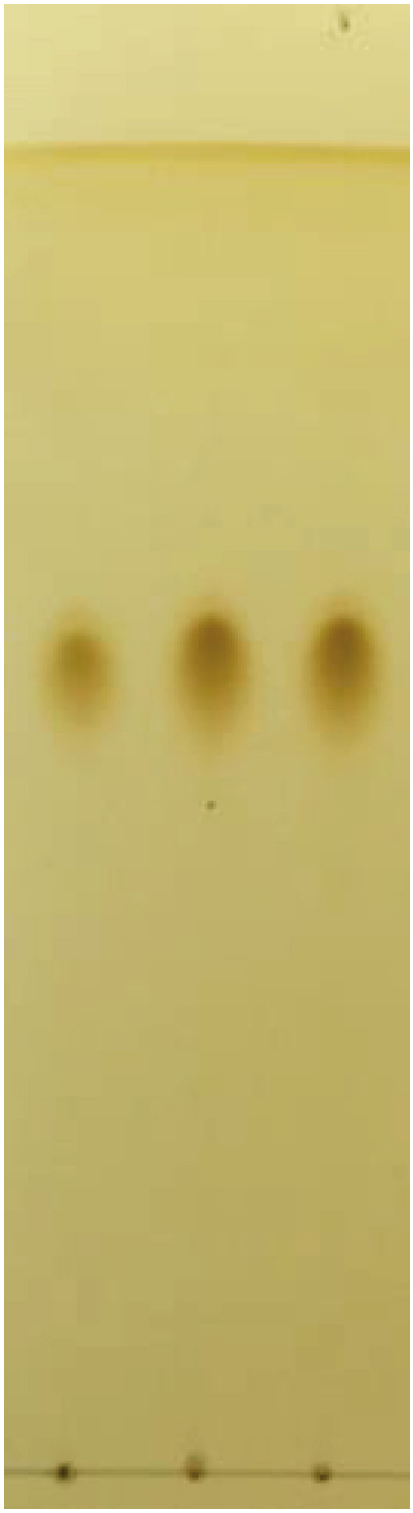
2.1. Chemical Reagents. Chemical, column chromatography Silica gel and solvents (methanol, ethyl acetate, chloroform, acetone, toluene) used in this study were from Merck (Darmstadt, Germany) or other suppliers (China). Thin-layer chromatography was performed on silica gel plates with 0.25 mm thick silica gel 60 F254 (Merck). Standard prodigiosin was provided by Sigma Aldrich (St. Louis, MO, USA). The strain of *Serratia marcescens* strain QBN VTCC 910026 (GenBank: KX674054.1) was isolated and identified by the members of the Enzyme Biotechnology laboratory, Institute of Biotechnology (Vietnam Academy of Science and Technology - VAST) in previous studies.

2.2. S. Marcescens QBN VTCC 910026 Culture. S. marcescens stored in glycerol 25% at -84°C was transferred into a cooler tray at -20°C. The bacteria were then streaked onto a LB agar dish for activation, followed by incubating at 28°C for 24 h until red colonies appeared. Next, a separated colony was isolated for shake culture in LB at 200 rpm, 28°C for 16 h. After that, *S. marcescens* was spread onto petri dishes of peanut medium using a glass spreader. The dishes were then incubated for 3 days until red pigment can be visibly seen. For Pg production, variant *S. marcescens* was grown on 2% peanut seed powder and 2% agar, then incubated on the tray (20 x 30 cm) at 28°C for 48 h. Cells grown on the surface was collected for Pg extraction and purification.

2.3. EMS Mutagenesis. S. marcescens strain QBN VTCC-910026 was subjected to chemical mutagenesis using ethyl methyl sulfonate (EMS) [15, 16]. Mutagenesis was achieved by inoculating LB medium with fresh culture of the selected isolate of *S. marcescens* and incubated at 28°C for 24 h. Then cell supernatants were centrifuged at 4000 rpm in 20 min at 4°C to obtain the pellets. These pellets were mixed with EMS at serial concentrations of 400, 800, 1600, 3200, or 6400 μg/mL overnight at 37°C. After incubating, the pellets were obtained by centrifugation at 12500 rpm for 10 min at 4°C. Washing these pellets with 0.2% Tween 20 from 4 to 6 times. Washed pellets were dissolved into 200 μL 0.2% Tween 20.50 μL of cell supernatants were used to spread evenly over the surface of the agar in each dish. Cultured dishes were inoculated at 28°C for 24 h.

2.4. Cell Lines and Cell Cultures. Metastatic Lewis lung carcinoma (LLC) cell line was used to induce cancer in mice (provided by Dr. Jeanette Maier, University of Milan, Italy). Fibroblast, HepG2, H460, LU-1, KB, and MCF-7 is supplied from American Type Culture Collection (ATCC) and grown at 37°C in the humidified atmosphere with 5% CO_2. RPMI (Gibco, USA) supplementing with 10% fetal bovine serum (FBS) and 1% antibiotics was used to culture cancer cell lines. For prodigiosin treatment, cells were cultured until the confluency reached 80% and washed with PBS before carrying out the experiments.

2.5. Extraction, Purification and Identification of Prodigiosin. 10 g of the wet cell was collected from a peanut-agar medium. The ethyl acetate: acetone (1: 1) solvent was selected to extract the red pigment from the cell of *S. marcescens* EMS 5 with the ratio of 1: 20 (w/v). The mixture was kept on a shaker at 200 rpm for 3 h at 28°C, and then centrifuged at 4000 rpm at 4°C for 15 min. After the sample was extracted 6 times, 300 mg of dry sediment was collected for purification with column chromatography (CC). In this study, the crude extract was mixed with SiO_2 at a ratio of 1: 1 (w/w), then was put on a column pre-immersed with absolute methanol. The extract in the column was eluted by toluene: ethyl acetate solvent system at a ratio of 9: 1 (v/v). Each segment passed through the column was collected with a volume of 50 ml, then checked for the presence of prodigiosin by thin layer chromatography. The identified segments were collected in groups to continue passing through the column or to dry for further studies. Thin layer chromatography was performed on a silica gel plate with 0.25 mm thick silica gel 60 F254. The solvent system was n-hexane: ethyl acetate (1: 1) as the mobile phase. The compounds were visible by iodine staining. Prodigiosin purity was determined by HPLC. 10 μl of sample was applied to the system. The purification experimental design was determined by LC/MS 1100 Agilent Ion sources ESL, column ODS C18, 3.0 x 150 mm, 3.5 μm, mobile phase MeOH: H_2O (80: 20; v/v). NMR 1D and 2D spectra were measure by Bruker AV 500 MHz in $CDCl_3$ solvent. ^{1}H NMR and ^{13}C NMR were determined at 500 MHz and 125 MHz, respectively. Tetramethylsilane (TMS) was used as the internal standard. The signals were obtained as singlet (s), doublet (d),



(a)

Figure 1: Continued.

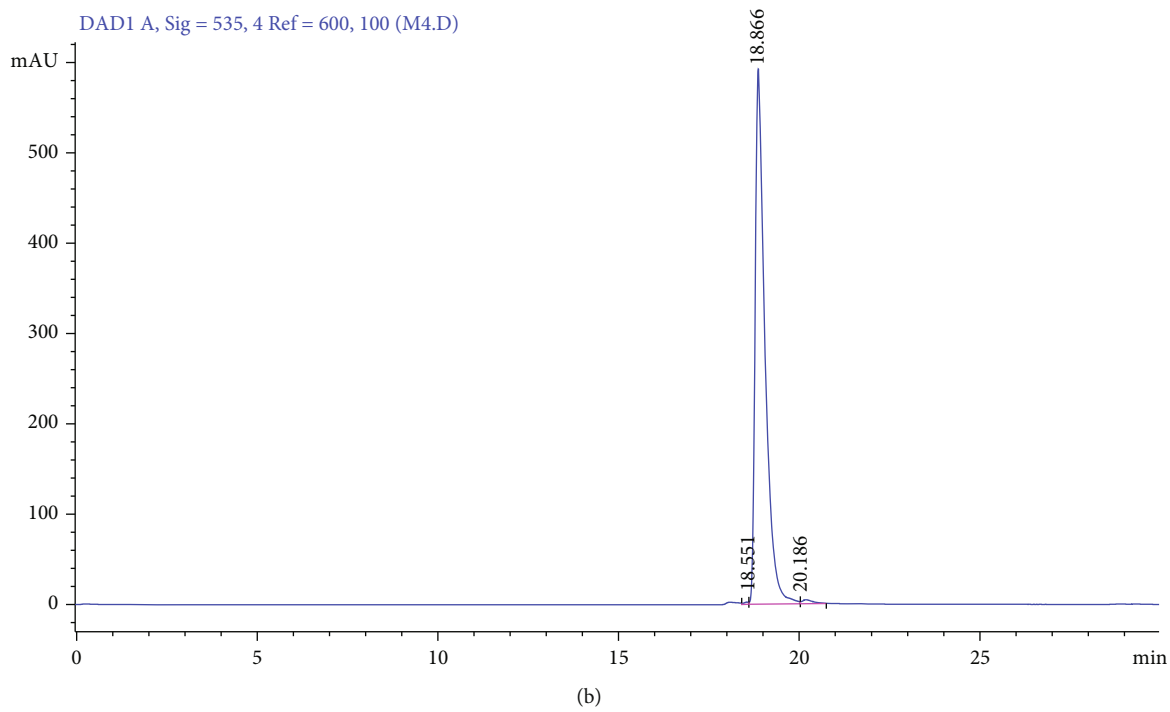


Figure 1: Purification of Prodigiosin. (a) TLC chromatography of the purified prodigiosin passing through the silica-gel column; lane 1: standard Pg, lane 2, 3: purified Pg. (b) HPLC of purified prodigiosin.

double doublet (dd), triplet (t), quintet (quint), and multiplet (m). MS spectra was measured by LC-MS Agilent 1100 (USA). The culture broth was analyzed by HPLC mobile phase in MeOH: H_2O =20: 80 for 2 min, MeOH: H_2O =20 – 100/80 – 0 for 17 min, MeOH: H_2O =100: 0 for 8 min, MeOH: H_2O =20: 80 for 5 min. The quantity of Pg in the culture broth was determined on HPLC based on standard Pg. Standard prodigiosin (Sigma) was diluted at various concentrations. Then, use a spectrophotometer to measure the absorbance at 535 nm.

2.6. Proliferation Assay. Concentration dependent effects on cell proliferation were measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Cancer cells after culture were transferred to 96 wells plate with a density of 2 x 10^4 cells/well and grown for 24 h at 37°C. Then, the culture media of cancer cells were replaced by a fresh one supplementing with different concentration of prodigiosin of (0.5; 1; 2; 4; 6; 8 and 10 μg/mL) for another 24 h. The control was used without adding prodigiosin. At the end of treatment, 5 mg/mL of MTT reagent was added to each well and incubated for 3 h. The MTT containing supernatant solution was removed and 100 μL of DMSO was added to each well to dissolve violet formazan crystal. The proliferation assay was measured at OD_{595}. Based on MTT results, we calculated the inhibitory concentration by using different concentrations of prodigiosin and the control without prodigiosin. The software TableCurve2Dv4 was used to determine IC_{50} value. Cell viability rate $= (\Delta_{\text{sample}} - \Delta_{\text{media}}/\Delta_{\text{control}} - \Delta_{\text{media}}) \times 100(\%)$

Δ_{sample} : Absorbance value of cancer cells treated with prodigiosin measured at OD_{595}.

$\Delta_{control}$: Absorbance value of cancer cells without treated with prodigiosin measured at OD_{595}.

Δ_{media} : Absorbance value of media measured at OD_{595}.

2.7. Tumor Suppression Capacity of Pg Using Tumorized Mice Model. Tumor induction of mice with the LLC cell line.

LLC cells were grown in DMEM medium supplemented with 10% bovine serum and 1% antibiotics at 37°C and 5% CO_2. Cells were harvested and injected into the thighs of mice at a concentration of 2x10^6 cells/mice (which is the tumor-causing concentration for mice reaching 100%). After 5 days of injecting LLC cells, if there is a tumor, then mice were used for next experiment. 18 healthy BALB/c mice, aged 10-12 weeks old, injected with LLC cells above were weighed, measured for tumor size at the injection site of LLC cells, and then divided to 3 groups (6 mice/group) [17]. After 5 days of tumor induction, mice were randomly divided into three groups which were (i) pathological control group receiving water with a volume of 0.3 mL/mouse; (ii) reference group receiving capecitabine at 200 mg/kg body weight (bw.) orally; (iii) sample treatment group receiving of Pg at 1 mg/kg bw. Tumor suppressive capacity of tested samples were compared with the pathological control group. The experimental mice were weighed and measured primary tumor sizes at the injection site every 7 days. The tumor volumes were calculated using the formula of previous studies [14, 15] as:$V = a \times (b^2/2)$

(V: tumor volume; a: length of the tumor; b: diameter of tumor)

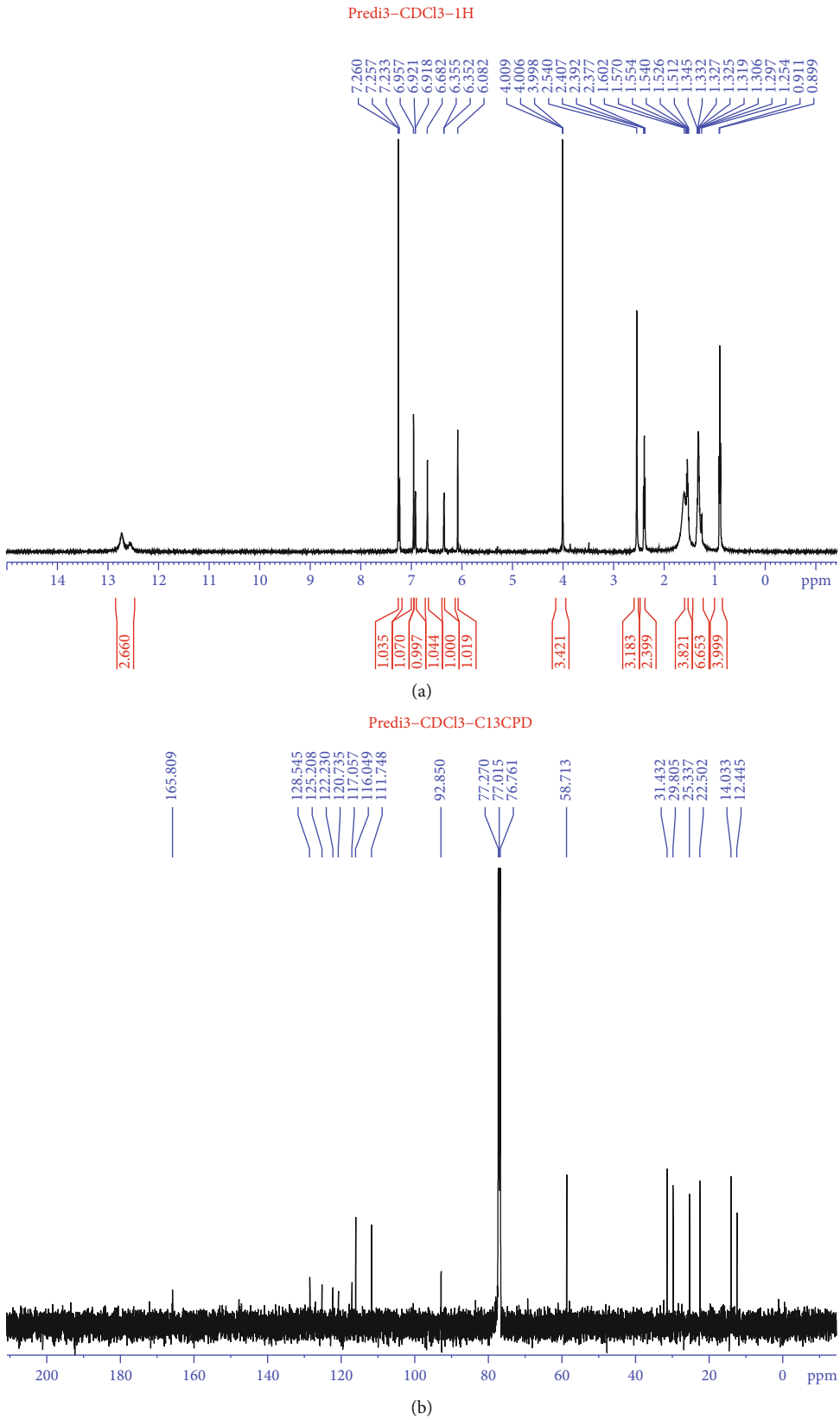


Figure 2: Spectra of purified compound. (a). ^{1}H NMR proton spectrum. (b). ^{13}C NMR spectrum of active prodigiosin purified from *S. marcescens* EMS 5.

3. Results

3.1. Mutagenesis on Serratia Marcescens QBN VTCC 910026. Ethyl methanesulfonate (EMS) was used as a mutagenic agent to produce a hyper producing strain in chemical mutagenesis experiments. *S. marcescens* strain QBN VTCC-910026 (GenBank: KX674054.1) was treated by EMS (Ethyl methane sulfonate) at different concentrations to generate variants lines. Some of variants produced Pg more than the wild type. However, most differences were not significant meaningful in statistic. The only one strain that produce Pg more than the wild type strain 52% (858 mg/L) was *S. marcescens* EMS 5 (Supplement 1). The prodigiosin productivity about 1.52 fold for the mutant *S. marcescens* EMS 5 compared with productivity of the wild type.

3.2. Purification and Identification of Prodigiosin from S. Marcescens EMS 5. An effective solvent system for Pg extraction from the culture of *S. marcescens* M10 was determine [18]. In this study, we have used the solvent ethyl acetate: acetone (1 : 1) to extract the prodigiosin from *S. marcescens* EMS 5. The cell-free extract was loaded on the silica gel column. 9 fractions were collected and checked on a TLC chromatography plate. The fractions 6–9 restrained a visible band corresponding to the standard prodigiosin as control (Supplement 2). The prodigiosin-contained fractions were collected and purified by passing the new silica gel column a second time. Two fractions were collected, and one single band of the putative compound was shown (Figure 1(a)). The HPLC result indicated that the purified compound harbored 1 single peak of pure prodigiosin reached 98% (Figure 1(b)).

The compound was isolated in the form of purple-red powder, melting temperature of 151-152°C. The spectral data indicated a peak of m/z $[M+H]^{+324.1}$ (m/z) corresponding to a molecular formula $C_{20}H_{25}N_3O$ (Supplement 3).

The ^{1}H NMR spectral data of purified compound from (Figure 2(a)) *S. mercescens* EMS 5 harboring broad singlet signals of 2 hydrogen atoms bind a nitrogen atom at 12.57 ppm (^{1}H) and 12.70 ppm, which corresponding to H-1and H-1. In the region of aromatic hydrogen and olefin, there are 6 resonant signals corresponding to 6 protons. Among of them, there are 3 singlet signals at δ_H 6.96, 6.68, and 6.08 ppm. The other 3 signals correspond to 3 protons belonging to the same spin system. The COSY ^{1}H-^{1}H spectra (Supplement 4) indicated the proton belonging to A ring: δ_H 7.23 (H-5), 6.92 (H-3) and 6.35 (H-4). The interaction constants of these protons were small enough (1.5 Hz), characterizing a heterozygous 5th ring, such as a pyrrole one.

In the high field, there are signals of an OMe group (s, 4.00), a Me group at δ_H 2.5 (s), a group of Me terminal carbon (t, 0.90), 2 groups of CH_2 (t, 2.39 and 1.54), and 2 groups of CH_2 in the form of overlay multiple at δ_H 1.32. The COSY spectra allow the identification of a spin system of an n-pentyl group.

Besides, there was an interaction of methyl group appeared on the COSY spectra (δ_H 2.54) and a CH_2 group (δ_H 2.39) with proton δ_H 6.68. This allowed us to define

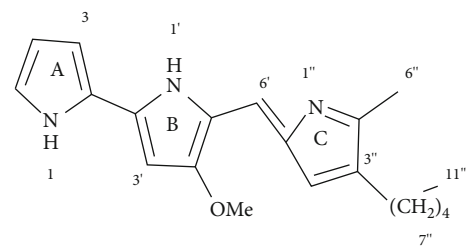


Figure 3: Chemical structure of purified prodigiosin.

H-4" (δ_H 6.68) and CH_3-6", CH_2-7" which belonging to the C ring.

The ^{13}C NMR (Figure 2(b)) and HSQC (Supplement 5) showed resonance signals of 20 carbon including 13 carbon sp^2 (6 CH and 7 quaternary carbons) and 1 methoxy group (δ_C 58.7), 2 methyl groups and 4 CH_2 groups. The HSQC spectra allowed the determination of the corresponding hydrogen-bound carbon. Specifically, in the A ring: 6.92/117.1 (H/C-3), 6.35/111.7 (H/C-4), 7,23/125,9 (H/C-5), OMe groups in the B ring at 6.08/92.9 (H/C-3'), 4.00/58.7 (H/C-7'), a CH olefin group at 6.96/117.1 (H/C-6'). In the C ring there is a CH group at 6.68/128.4 (H/C-4"), a methyl group 2.54/12.4 (H/C-6"), and corresponding groups of pentyl circuits.

The HMBC spectra (Supplement 6) determined the quaternary C (C-2, 120.7) of an A ring from the interaction of H_4-C_2, H_5-C_2, quaternary C of B ring (C2', 146.4; C-4', 165.8; C-5', 120.7) by the interaction of H-3'-C-2', C-5', H-6'-C-4', quaternary of C ring (C-2", 145.3; C-3", 125.2; C-5", 128.5) by the interaction of H-4"-C-2" and C-3", H-6"-C-2" and C-4". The proton interactions at 6.96 with C-4' and C'4" defined H-6'.

A methyl group of H-6" and a methylene group of H-7" had an interaction in HMBC with C-2"and C-4". From the above MS and NMR spectra, we can conclude the purified compound was Pg (Figure 3).

3.3. Inhibitory Activity of Pg against Cancer Cell Lines. With IC_{50} values on fibroblast normal cell lines more than 20 μg/ml (equivalent to 61.84 μM), purified Pg from *S. marcescens* EMS 5 had no effect on normal cells. Therefore, it's very potential to become medicine for cancer treatment.

The IC_{50} value of Pg for HepG2 cancer cells, which was 8.75 μg/ml, equivalent to 27 μM, showed that purified Pg from *S. marcescens* EMS 5 strain did not have cytotoxic activity and had a weak inhibitory effect on the growth of HepG2 cancer cells (IC_{50}>4 μg/mL). Prodigiosin was resistant to H460 cells at the concentrations from 6 μg/mL to 10 μg/mL. The IC_{50} value of purified Pg reached 7.7 μg/mL, equivalent to 23 μM. The purified Pg was strongly resistant to MCF-7 cells at a concentration of 4-10 μg/mL. At the concentration of 2 μg/mL, purified Pg inhibited more than 50% of cells (IC_{50}<2 μg/mL). For SK-LU-1 cells, IC_{50} was 1.5 μg/mL, equivalent to 4.6 μM. Thus, purified Pg had cytotoxic activity and could inhibit the growth or killing of SK-LU-1 cancer cells. In KB carcinoma cells, at a concentration of 4 μg/mL, all cells shrank, and changed shape and some cells

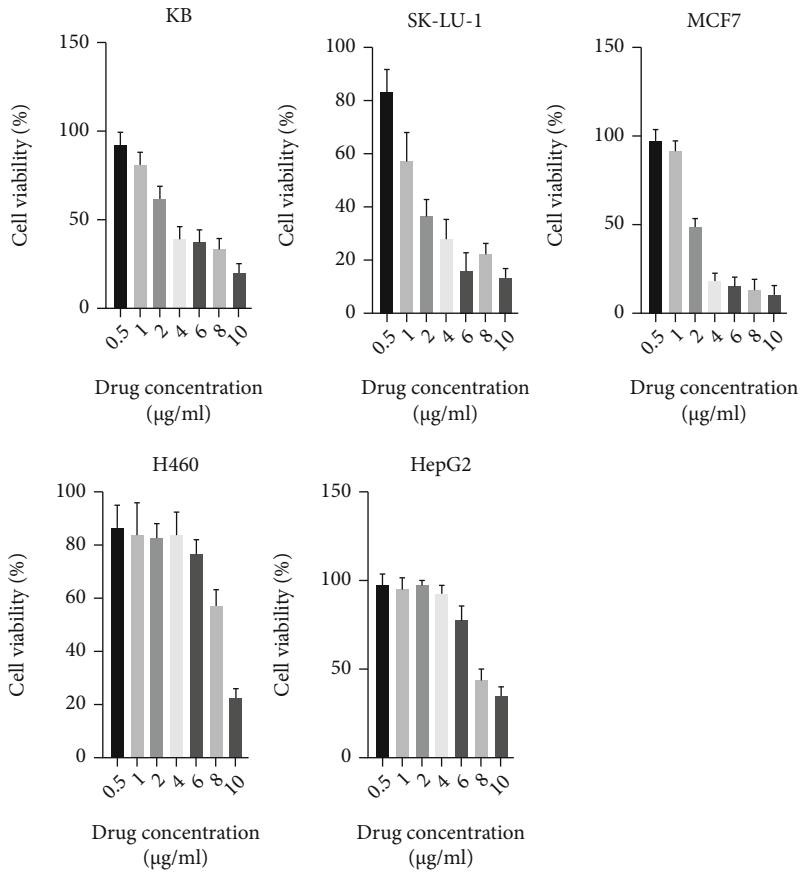


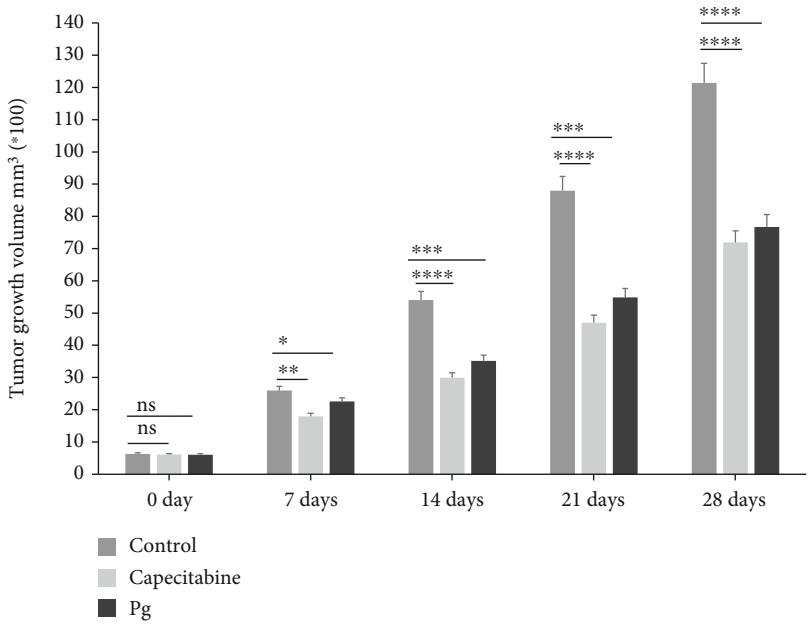
Figure 4: Inhibitory concentration of Prodigiosin (0.5; 1; 2; 4; 6; 8 and 10 μg/ml) on different cancer cell line KB, SK-LU-1, MCF7, H460 and HEPG2.

died. The cell viability treated with Pg at 4 μg/mL reached 40%. However, at the concentration of 10 μg/mL, this number accounted for 20% (Figure 4).

3.4. Tumor Growth in Experimental Groups. Tumor growth is a crucial indicator to assess the antitumor effect of the reagent. The results on the tumor in the thigh of mice are shown in Figure 5. The results (Figure 5 a) indicated that tumor volumes of the group administrated with Pg at the dose of 1 mg/kg/day, one time per 2 days: the 7th reduced compared to the control group. However, no statistical difference was found ($p > 0.05$). The same result was observed when comparing these two groups at the 14th, 21st and 28th day, but the difference was statistically significant ($p < 0.05$). The tumor volume of mice in the reference group orally treated with capecitabine at the dose of 200 mg/kg/day decreased at all measured points on the 7th, 14th, 21st, and 28th. The statistical difference of the tumor volume in this group was found ($p < 0.05$). The tumor volume of mice in the reference group that used capecitabine at the dose of 200 mg/kg/day remarkably decreased compared to the control group on the 7th, 14th, 21st, and 28th. The statistical difference in this group was found ($p < 0.05$). Again, as shown in Figure 5, the average tumor weight of all mice administered with Pg decreased to 7.23 g/mouse, compared with 13.31 g/mouse of the control group.

4. Discussions

The red pigment Pg is of great interest to many scientists and research because of its great potential in medicine. Pg has immunosuppressive, anticancer, antibacterial, antifungal, and antioxidant activity [14, 19]. This study reported that variants of *Serratia marcescens* QBN VTCC 910026 strain produce much more Pg production of 858 mg/L compared to HDZK-BYSB107 (0.656 g/L), *Zooshikella rubidus* S1-1 (0.048 g/L) and *Hahella chejuensis* KCTC 2396 (0.028 g/L) [5, 14, 20]. The Pg in *S. marcescens* were packed inside 2 membranes of peptidoglycan and lipopolysaccharide [11] so a solvent mixture of EtOAc and HCl 1% to extract the intracellular substances was used [12]. However, this solvent was not suitable to extract Pg from *S. marcescens* EMS 5. Consequently. a solvent system of ethyl acetate: acetone at a ratio of 1 : 1 (v/v) were used to disturb bacterial cell membrane and extracted Pg from *S. marcescens* EMS 5. The suitable solvent system was run on TLC chromatography was chloroform: ethyl acetate at the ratio of 1: 1 to separate compounds in crude pigment. This result is similar to Park's announcement in 2012 on choosing an appropriate solvent to extract Pg from cells of strain *H. chejuensis* M3349 [21]. Previously, Pg was pre-extracted in the chloroform phase and eluted by toluene and ethyl acetate, the purified compound was obtained by HPLC reached 14.3 g/L. One



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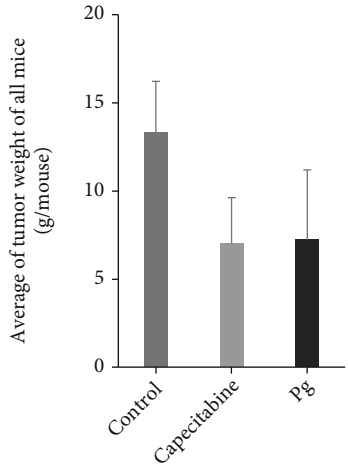

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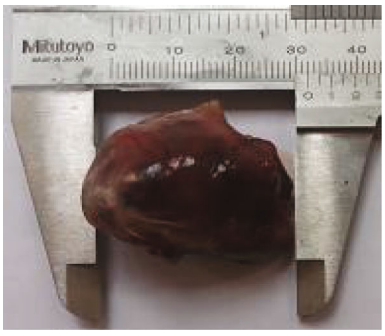

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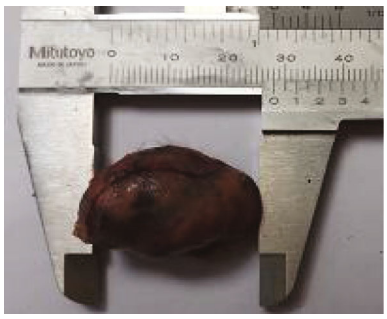

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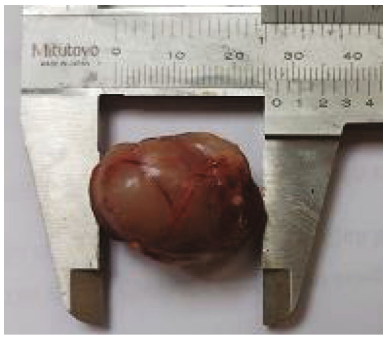

(e)

Figure 5: The effect of Pg on tumor in the thigh of mice before and after treatment. (a). The effect of Pg on tumor growth of mice after 28 days. Control: Healthy mice. Capecitabine: Mice were orally administrated with capecitabine at the dose of 200 mg/kg/day. PG: Mice were received with Pg at the dose of 2 mg/kg/day. Significance was determined using an unpaired two tailed t test: $****p < 0.0001$, $***p < 0.001$, $**p < 0.005$, $*p < 0.05$. Ns: Non-significant. Each value is expressed as mean ± SD. n = 10 per group. The average of tumor weight of all mice. (b). Tumor from mice without drug treatment. (c). Tumor was from mice orally administrated with capecitabine at the dose of 200 mg/kg. (d). Tumor was from mice injecting with Pg at the dose of 1 mg/kg (e).

advantage point of this study is that the culture of *S. marcescens* was carried out in an internal adsorbent bioreactor built by the research group and the supplement of minerals has boosted the Pg production [22]. Accordingly, fermentation and purification required more steps than our study. Among of the reasons why our Pg production was significantly low because the simple medium and long incubation time took major points for this limitations. In 2012, the Pg production of Park's research team was reduced by half after 2 days of incubation. Besides, Pg production was also affected by other inhibitors in the batch culture [21]. The molecular weight of Pg identified by LC-MS gave a signal peak at 324.1 m/z (Supplement. 2) which is almost similar to Pg from the study of Lin's research team (323.9 m/z) in 2019 [23]. The structure of prodigiosin was established by high-field ^{1}H-NMR, ^{13}C-NMR spectroscopy. The structure and molecular mass results were similar to prodigiosin from strain HDZK-BYSB107 [14] and prodigiosin from *Serratia* sp. KH-95 [22]. According to the American Cancer Society. The IC_{50} of reagent is below 20 μg/mL (crude extract or chemical fraction) or $IC_{50} \leq 4$ μg/mL (pure active substance) is considered as an anticancer agent. Thus, the IC_{50}>20 μg/mL (61.84 μM) from *S. marcescens* EMS 5 did not exhibit cytotoxic activity against fibroblast cells isolated from BALB/c mice. Pg has been tested on 60 different cancer cell lines [24], but some cell lines such as LU-1, KB had not been studied. After investigating some HepG2, KB, MCF-7, LU-1, H460 cancer cell lines, we found that purified Pg had a strong inhibitory ability on cancer cell lines: MCF-7 human breast, LU-1 lung cancer, KB carcinoma cells in vitro. The results of our study are also consistent with studies on testing the activity of prodigiosin on cells, which is to change cell shape, cell rupture, and death. The IC_{50} value of the KB line of Pg from *S. marcescens* EMS 5 was equivalent to the activity of Pg from *Labeo rohita* on some other lines. Specifically, Hela was 4.3 μM. HepG2 was 5.2 μM, and KB was 4.8 μM [25]. This value was not much higher than the IC_{50} value of Pg from *S. marcescens* on some other cancer cell lines such as cervical carcinoma (Hela-229) (IC_{50} achieved at 0.7 nM). The antitumor activity of prodigiosin compounds has been reported very limited in a few previous studies [14, 18]. The results (Figure 5(a)) showed that our purified Pg significantly reduced tumor volume compared to the control. In our previous research, Pg from *S. marcescens* M10 was injected into the mice abdominal cavity and inhibited 31.18% tumor growth. However, at this dose some mice died on days 19th and 22nd. Therefore, in this study, we injected Pg in the mice muscle with lower dose of Pg from *S. marcescens* EMS 5 to 1 mg/kg body weight. The results showed that the inhibitory activity of Pg is not significant difference from our previous study on days 7th and 14th. However, on days 21st and 28th the inhibitory activity of Pg from *S. marcescens* EMS 5 was significant inhibit tumor growth (inhibited 36.82% tumor growth after 28 days). The anticancer activity of antitumor compound was reported in other publications. The active ingredient TAT2 extracted from *A. tonkinensis* leaves have been shown to significantly inhibit tumor growth at 100 and 200 mg/kg body weight [26]. Prodigiosin inhibited Wnt/β-catenin signaling by targeting various sites in the pathway, including lipoprotein-receptor-related protein (LRP) 6, disheveled (DVL), and glycogen synthetase kinase-3β (GSK3β). The application of this compound hindered tumor development and decreased the production of phosphorylated LPR6, phosphorylate and unphosphorylated DVL2, Ser9 phosphorylated GSK3β, active β-catenin, and cyclin D1 [27]. In a combined therapy, prodigiosin/PU-H71 elevated caspases 3; 8; and 9 while decreasing mTOR expression. At the same time HSP90α, EFGR, VEGF were downregulated in the expression level. Thus, the compound possessed a high potential for the treatment of TNBC [28]. In an animal model, prodigiosin induced an antitumor activity on human carcinoma (JEG3) and prostate cancer cell line (PC3). The bacterial compound downregulated XIAP, cIAP-1 and IAP-2 [14].

5. Conclusions

The red pigment from variants of *Serratia marcescens* QBN VTCC 910026 was extracted and purified from the solid fermentation media by toluene: ethyl acetate solvent system with the ratio of 9: 1 (v/v). The purity of pigment confirmed by the high-performance liquid chromatography (HPLC) method presented as 98%. The red pigment was confirmed as prodigiosin using ^{1}H-NMR, ^{13}C-NMR spectroscopy. The purified prodigiosin has a strong inhibitory ability on human breast cancer cell lines MCF-7, lung cancer LU-1, oropharyngeal cancer KB in vitro. The prodigiosin from variants of *Serratia marcescens* QBN VTCC 910026 was shown to be capable to decreased the tumor volume by 36.82% compared to the control after 28 days. Thus, we concluded that the prodigiosin of variants of *Serratia marcescens* QBN VTCC 910026 could be deliberated as an anticancer drug in the future.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of Interest

The author(s) declare(s) that they have no conflicts of interest.

Acknowledgments

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.02-2018.332, "Renovated for higher-yield prodigiosin production from *Serratia marcescens* in Vietnam and identification of the molecular-biological mechanism by which prodigiosin works to cells".

Supplementary Materials

Supplement 1: Prodigiosin concentration; 1: Wild type; 2-36: variants of *S. marcescens*. Supplement 2: TLC chromatography of the supernatant of S. marcescens passing through the first column. S: Cell-free extract of prodigiosin by acetone.

1–9: Fraction 1 to fraction 9 of cell-free extract of prodigiosin when passing through the silica gel column. Supplement 3: MS spectrum of prodigiosin. Supplement 4 COSY spectrum of prodigiosin. Supplement 5: HSQC spectrum of prodigiosin. Supplement 6: HMBC spectrum of prodigiosin. *(Supplementary Materials)*

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Hindawi
BioMed Research International
Volume 2022, Article ID 3254420, 12 pages
https://doi.org/10.1155/2022/3254420

Review Article

Molecular Mechanisms of Coffee on Prostate Cancer Prevention

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Received 24 August 2021; Accepted 9 April 2022; Published 22 April 2022

Academic Editor: Ming Yi

Prostate cancer (PCa) is one of the most common types of cancer among men, and coffee is associated with a reduced risk of developing PCa. Therefore, we aim to review possible coffee molecular mechanisms that contribute to PCa prevention. Coffee has an important antioxidant capacity that reduces oxidative stress, leading to a reduced mutation in cells. Beyond direct antioxidant activity, coffee stimulates phase II enzymatic activity, which is related to the detoxification of reactive metabolites. The anti-inflammatory effects of coffee reduce tissue damage related to PCa development. Coffee induces autophagy, regulates the NF-κB pathway, and reduces the expression of iNOS and inflammatory mediators, such as TNF-α, IL-6, IL-8, and CRP. Also, coffee modulates transcriptional factors and pathways. It has been shown that coffee increases testosterone and reduces sex hormone-binding globulin, estrogen, and prostate-specific antigen. Coffee also enhances insulin resistance and glucose metabolism. All these effects may contribute to protection against PCa development.

1. Introduction

The prostate is a gland localized between men's bladder and penis and surrounds the urethra. It secretes prostate fluid that protects sperm. Prostate cancer (PCa) develops when mutated semen-secreting prostate cells start proliferating uncontrollably. If PCa is not treated, it may metastasize, usually to lymph nodes, to hip bone, and then to other organs. Ordinarily, it is not diagnosed in the early stages because it is initially asymptomatic [1]. PCa is the second most common type of cancer among males, accounting for 14.1% of all cancers in men (WHO 2020).

Cancer, in general, develops due to successive mutations in genes, which alter cell morphology and physiology [2]. Oxidative stress leads to inflammation through redox pathways, increasing inflammatory marker circulation [3, 4]. The inflammatory response causes tissue injury and DNA damage [3, 5]. This disorder affects cell cycle and cell division, apoptosis signaling, and DNA repair mechanisms, leading to cancer [2].

Besides nonmodifiable risk factors, such as age, family history, and ethnicity, PCa development can also be influenced by diet and environmental factors, through epigenetics, which involves changes of gene transcription without any alteration in the nucleotide sequence [1]. Consumption of high content of natural phytochemicals from plants is associated with health benefits [4].

Coffee can be a major source of biochemical compounds [6] that can contribute to protection against PCa. In 2020, coffee consumption worldwide was 167.2 million bags (60 kg/bag) ((ICO) 2021). The main varieties produced are Arabica (*Coffea arabica*, 58.5%) and Robusta (*Coffea canephora*, 41.5%) ((ICO) 2021). Coffee has shown *in vitro* antiproliferative effects against PCa cell lines [7–11].

There are some meta-analyses with epidemiological data exploring the association of coffee intake and PCa risk

[12–17]. However, no reviews so far have explored how coffee can have those beneficial effects on PCa. Therefore, the aim of this review is to detail molecular mechanisms that can be involved in the capacity of coffee and its bioactive compounds to prevent and treat PCa.

2. Methods

In this review, we searched at Web of Science, Scopus, and PubMed databases. The search terms included "Coffee" OR "*Coffea arabica*" OR "*Coffea canephora*" OR "chlorogenic acids" OR "CGA" OR "caffeine" on the title or abstract. Those were combined with specific terms of each possible effect with the Boolean operator "AND" for each search. For the antioxidant, the terms were "anti-oxidant" OR "oxidative stress" OR "Reactive oxygen species". For the anti-inflammatory, the terms were "anti-inflammatory" OR "inflammation" OR "cytokines". Other terms were "mutation", OR "DNA damage" OR "transcriptional factors" OR "signaling pathways" OR "testosterone" OR "steroids hormones" OR "insulin resistance".

Papers retrieved were filtered for original articles, published in English in the last 10 years. The association between coffee intake and prostate cancer risk has sometimes produced inconsistent results. To circumvent, summarize, and assess the quality of current evidence on the subject, observational studies on molecular mechanisms on existing findings were considered. Only papers produced in high impact factor journals were considered and used in this research. It was also observed the heterogeneity, evidence of small-study effects, and excess significance bias. Table 1 summarizes the relevant findings from 38 articles included in the present review.

3. Bioactive Compounds in Coffee

Coffee contains many well-known bioactive compounds that can be related to anticancer effects. Caffeine is a trimethylxanthine and is the major bioactive compound in coffee [18]. Virtually, all caffeine is absorbed and its main effect is the stimulation in the central nervous system and adenosine receptor inhibition [18, 19]. It has been observed that caffeine increases cancer cell death and protects against mutagenicity [20].

Chlorogenic acids (CGAs) are a group of polyphenols formed between transcinnamic and quinic acid [19, 21]. CGAs prevent free radical damage and modulate inflammation, regulation of glucose, and lipid metabolism [20]. Trigonelline is generated from the nicotinic acid and regulates key enzymes in glucose and lipid metabolism, preventing cell invasion and inhibiting cancer cell proliferation [18]. Diterpenes are the main lipidic fraction in coffee, primarily cafestol and kahweol, and have anticarcinogenic, antioxidant, and anti-inflammatory activity [18, 20]. Maillard reaction forms melanoidins during roasting, which has antioxidant, anti-inflammatory, and antimicrobial effects [18].

However, the chemical composition of coffee can vary depending on various factors. The first factor is the variety. Robusta coffee has more chlorogenic acids and caffeine than Arabica [22]. During coffee farming, the soil, altitude, sun exposure, rain, and temperature can affect coffee composition [23]. During processing, roasting, grinding, and brewing also affect the final composition [10].

4. Antioxidant Activity Induced by Coffee

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced endogenously by mitochondrial respiration and exogenously by exposure to oxidizing agents [24]. Oxidative stress results from cellular production of oxidant molecules surpassing the capacity of antioxidants to overcome these damages and may lead to PCa development [25]. Oxidative stress causes the oxidation of crucial biomolecules, causing DNA damage and oxidizing key enzymes involved in gene expression [24].

Coffee presents high antioxidant capacity *in vitro* [10, 26, 27]. Coffee's antioxidant compounds include caffeine, phenolic compounds (mostly CGAs), trigonelline, diterpenes (cafestol and kahweol), and melanoidins [20, 26]. A mechanism for coffee's antioxidant activity is described in Figure 1.

Some studies have reported a significant increase in total plasma antioxidant capacity following coffee consumption, even after a single serving (200 or 400 mL). These effects of a single dose were lost in long term, but in recurrent consumption of medium roast coffee (150 mL/day), plasma antioxidant activity was increased by up to 26% [25].

Coffee significantly increased antioxidant response element (ARE) activation, which could induce the expression of genes related to the cellular antioxidant system [28, 29]. ARE proteins are a part of the complex antioxidant system that protects cells from oxidative damage by neutralizing free radicals and oxidizing agents [30]. ARE-related genes are in the cell defense promoter regions, which include phase II detoxifying enzymes and enzymes involved in antioxidant defense [29, 31].

Coffee consumption increases the intracellular activity of phase I (cytochrome P450) and II enzymes [6], such as glutathione reductase (GR), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione S-transferases (GST), superoxide dismutase (SOD), and catalase (CAT) ([32], Valadão [29, 30, 33–36]). This increase in GSH has been attributed to polyphenols [34].

It has been hypothesized that compounds with antioxidant properties generally increase the messenger RNA (mRNA) expression of antioxidant-related enzymes. But the expression of those enzymes can be downregulated in some antioxidant-treated cells because the compounds may have directly ameliorated the prevailing oxidative stress [34].

A low-molecular-weight coffee fraction supplementation in rats was able to reduce noncoding microRNA-124-3p and increase the expression of mRNA involved in GPx coding, raising the expression of this enzyme, probably due to caffeine [37]. Noncoding microRNA is an epigenetic factor, can act as tumor suppressors or oncogenes, and may be downregulated or upregulated in PCa. They are short regulatory RNA molecules that cannot be translated into amino acids and may disturb the mRNA purpose. This might

Table 1: Effects of coffee or coffee compound *in vitro*, on cell, animal, or epidemiological models.

Model	Coffee sample/compound	Outcomes	Reference
In vitro	Green and roasted *Coffea arabica* and *C. robusta*	(-) Hydroxyl (OH°) radicals DNA damage	[43]
In vitro	Coffee phenolics and caffeine metabolites	(-) DNA single-strand breaks	[60]
LNCaP, LNCaP-SF, PC-3, and DU145 cells	Kahweol, cafestol, caffeine, caffeic acid, CGA, and trigonelline	(-) Proliferation and migration of PCa cells (-) AR, CCR2, and CCR5	[85]
Xenograft study of SCID mice		(-) PCa growth	
PC-3 cells PC-3 xenografts in athymic nude mice	Dark roasted *Coffea arabica*	(-) NF-κB activity and DNA-binding (+) Apoptosis (+) Modulation of gene expression	[8]
MC3T3-E1 cells	CGA	(-) H_2O_2 damage (+) HO-1 and Nrf2 (+) PI3K/Akt	[33]
B16F10 cells	*SCG Coffea arabica*	(-) PI3K/Akt and MAPK	[56]
AML-12 cells	*Coffea arabica* light, medium, city, and French roasts	(+) GSH (+) mRNA related to GSH	[34]
RAW 264.7 cells		(-) TNF-α and IL6	
RAW 267.4 cells	SCG	(-) NO production	[26]
CCD-18Co cells	Phenolic compounds from green coffee	(-) ROS production	[40]
HepG2 and KYSE70 cells	Decaffeinated commercial coffee	(+) UGT (-) BaP-induced damage (-) ROS production (+) AhR and Nrf2	[38]
RINm5F and 3T3-L1 cells	CGA	(+) Insulin secretion (+) PPAR-y and GLUT4	(Sanchez et al. 2017)
EA.hy926 cells	Green and light roast *Coffea arabica*	(+) Redox status (+) GSH	[30]
Caco-2 cells	Turkish, filter, and instant coffee	(+) AhR and Nrf2 (+) CYP1A1 expression	[66]
HT29 cells	Blend of green and roasted *Coffea arabica*	(+) Nrf2 transcription and translocation (+) ARE and GST	[29]
HT29 cells	*Coffea arabica*	(+) Nrf2 translocation	[62]
HeLa cells	SCG extracts	(-) ROS level (-) Induced DNA strand breaks	[41]
U-937 cells	SCG	(-) TNF-α, IL-6, and IL-10	[48]
SH-SY5Y	Kahweol	(-) ROS and RNS (+) Regulation of PI3K and MAPK pathways	(de [44])
HCT116	Caffeic acid	(+) Regulation of PI3K/Akt	[93]
MCF-7, MDA-MB-231, T47D, and Tam-R cells	Caffeine and caffeic acid	(-) ER abundance (-) IGF1R and pAkt	[63]
C57BL/6 mice	*Coffea arabica* light, medium, city, and French roasts	(-) Liver necrosis (-) IL-6 (-) TNF-α (+) NF-κB pathway	[28]
C57BL/6 mice	Low-molecular-weight from regular and decaffeinated *Coffea canephora*	(-) MicroRNA-124-3p (+) mRNA related to GPX Decaffeinated coffee had no effect	[37]
C57BL/6 mice	Regular and decaffeinated coffee	(+) Autophagic flux (+) Lipidation of LC3B (-) p62/SQSTM1 (-) mTORC1 (+) Deacetylation of cellular proteins	[52]
HtgUGT1A mice	Caffeic acid		[39]

Table 1: Continued.

Model	Coffee sample/compound	Outcomes	Reference
		(+) UGT (-) ROS	
Sprague Dawley rats	*Coffea arabica* oil	(-) PSA	(Cueto et al. 2016)
Him-OFA rats	Regular and decaffeinated *Coffea arabica*	(-) Hepatic foci frequency (-) Aflatoxin DNA damage (+) UGT	[59]
Wistar rats	A medium roast of *Coffea arabica*	(+) SOD, CAT, and GPx	[36]
Wistar rats	Green *Coffea arabica*	(-) Lipid peroxidation (+) GSH, SOD, CAT, and GR	[35]
Wistar rats	Green *Coffea canephora* and caffeine	(-) Carrageenan-induced paw oedema	(Pergolizzi et al. 2018)
Wistar rats	Green *Coffea arabica*	(-) IL-1β, TNF-α, and Nos2 (-) Interstitial inflammation index	[47]
Humanized SHBG transgenic mice	Caffeine	(+) Hepatic SHBG production (-) Akt phosphorylation	[77]
Cross-sectional clinical trial	Caffeinated beverages	Coffee consumption positively associated with SHBG concentration	[78]
Prospective clinical trial	Caffeinated and decaffeinated coffee	(-) CRP, IL-6, and TNFR-2 (+) Adiponectin and SHBG (-) Estrone (+) Testosterone (-) C-peptide and IGFBP-3	[50]
Prospective clinical trial	Coffee intake	(-) Urinary 8-OHdG (-) Ferritin	[61]
Prospective clinical trial	Coffee intake	(+) AhR (+) CYP1A1/A2	[65]
Intervention clinical trial	Green and roasted *Coffea arabica* blend	(-) Spontaneous DNA strand breaks	([57]; 2014; 2011)
Intervention clinical trial	*Coffea arabica*	(-) DNA strand breaks (+) Nrf2 signaling	[31]
Intervention clinical trial	Caffeinated and decaffeinated coffee	(+) Insulin sensitivity (+) GLP-1 and GIP	[89]

Legend: (-) = reduction/inhibition; (+) = increase/activation/improvement; CGA = chlorogenic acid; SCG = spent coffee ground.

influence RNA silencing and gene expression at posttranscriptional and translational levels [1].

Coffee has also been shown to inhibit oxidative stress through UDP-glucuronosyltransferases (UGT) activation, which catalyze the detoxification of reactive metabolites [38]; similar results were observed for caffeic acid [39]. Coffee reduced ROS production/concentration [38, 40, 41].

Antioxidant properties observed in coffee are mainly attributed to CGAs, which have one to two aromatic rings linked to hydroxyl groups and donate hydrogen atoms, reducing free radicals [24, 40, 42, 43]. Their oxidation products, phenoxyl radicals, are promptly stabilized by resonance stabilization. CGAs react with different sources of free radicals at a varied pace; their relative efficiency is species-specific [24].

Isolated kahweol protected mitochondria from redox stress and prevented the formation of ROS and RNS (De Oliveira, De Souza, and Fürstenau [44]).

5. Anti-Inflammation Effects

Inflammation is a physiological reaction to tissue damage induced by exogenous or endogenous agents. Exogen factors include pathogens, allergens, foreign bodies, and pernicious substances. Endogenous causes originate from cell signaling due to injured or malfunctioning tissues [24].

Inflammation may be related to PCa development because inflammatory cells are often present in the prostate microenvironment of adult men and are related to PCa precursor lesions, called proliferative inflammatory atrophy, which is abundant in cells that may be predisposed to genomic mutations, and inflammatory stress can provoke epigenetic changes, concomitant with the rupture of the epithelial barrier [45].

Coffee compounds can decrease chronic inflammation and, therefore, protect against DNA degradation, consequently decreasing the risk of disease [46]. The

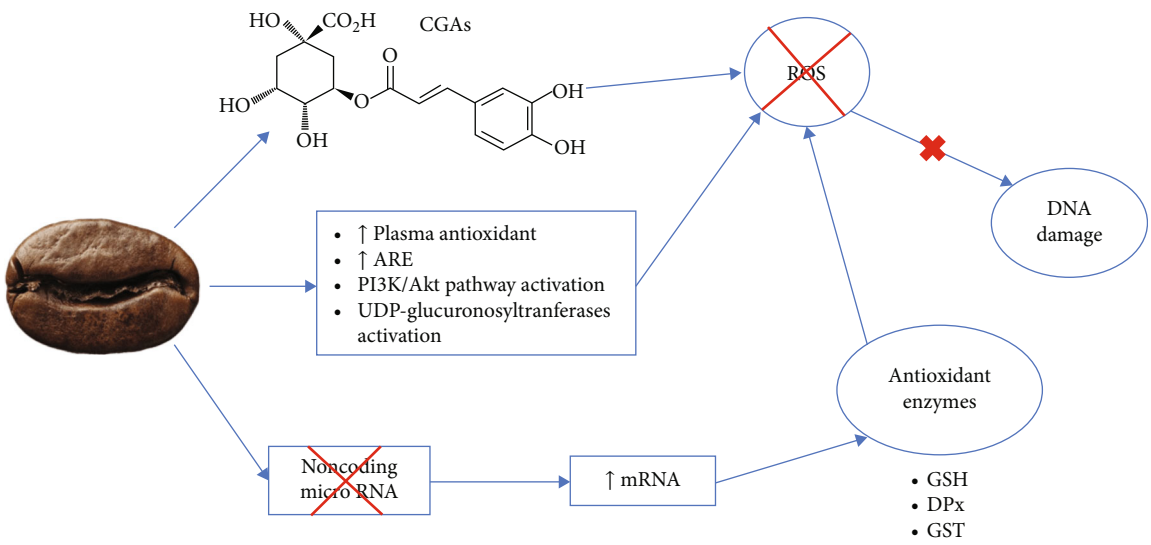

Figure 1: Mechanism of coffee's antioxidant activity.

inflammation process is characterized by the raised production of proinflammatory cytokines, such as C-reactive protein (CRP), interleukins (IL), and tumor necrosis factors (TNF) [34].

A proposed mechanism for coffee's anti-inflammatory effects is described in Figure 2. In animal models, green coffee intake reduced inflammatory markers, such as TNF-α [47, 48], IL-6, IL-10 [48], IL-1β, and Nos2 and reduced interstitial inflammatory index [47]. It has been observed in clinical trials that coffee intake over several weeks had a prevailing anti-inflammatory action evaluated by serum markers [49]. In addition to blood inflammatory markers, topical treatment of coffee and caffeine on mice's paws displayed a considerable inhibition of the carrageenan-induced oedema development [27].

It has been observed that coffee can inhibit TNF-α-induced NF-κB activity and DNA-binding in PCa cells. Coffee also regulated the expression of inflammatory and cancer-related genes probably through the NF-κB signaling pathway. Coffee downregulated genes related to invasion (MMP9) and inflammation (NF-κB2, CD40, EDN1, and ICAM1) and upregulated genes related to the antioxidant system, such as NFE2L2, HMOX1, NQO1, and GCLC [8].

The TNF-α and IL-6 expressions were decreased by lightly roasted coffee extract, and the levels raised as roasting levels were exacerbated [34]. Inducible nitric oxide synthase (iNOS) expression declined, which produces proinflammatory mediators, such as NO [26, 34]. Coffee exhibited an inverse relation with CRP, IL-6, and TNFR2, with similar results both in caffeinated and decaffeinated coffee [50].

Autophagy selectively degrades cellular components to minimize cell injury [3]. Autophagy is related to inflammation and immunity regulation and has tumor-suppressive properties [51]. It has been observed that caffeinated and decaffeinated coffee can induce autophagy. Chronic and acute administration of coffee raised the autophagic flux in different tissues and reduced the content of the autophagic substrate sequestosome-1 (p62/SQSTM1). This occurs through lipidation of microtubule-associated protein 1 light chain 3 β (LC3B-I), rising the electrophoretic mobility in sodium dodecyl sulfate PAGE (SDS-PAGE), and converting to LC3B-II. Coffee also caused inhibition of the mammalian target of rapamycin complex 1 (mTORC1) enzymatic activity, probably related to deacetylation of cellular proteins [52]. Caffeine is known to induce hepatic autophagy, being hepatoprotective ([53, 54], Salomone, Galvano, and Li Volti [55]).

Indeed, caffeine is the main compound with overall anti-inflammatory activity in coffee and can reduce TNF-α and IL-6 production in a dose-dependent way. Caffeine can modulate nuclear factor-κB (NF-κB) activation [28], which provokes the expression of inflammatory genes, including iNOS, cyclooxygenase-2 (COX2), and cytokines [34].

Nonetheless, 26 anti-inflammatory metabolites have been identified in coffee [48]. CGAs decrease the generation of inflammatory mediators by inhibition of protein tyrosine phosphatase 1B (PTP1B), minimizing proinflammatory cytokine genes expression and regulating NF-κB activation [56]. As a result, COX is suppressed, causing a reduction of IL-6 and IL-8 and TNF-α release [28, 34]. Great CGA concentration represses IL-1B mRNA, provoking considerably less cell adhesion and inflammation [46].

Kahweol lessens COX2 and monocyte chemoattractant protein-1 (MCP-1) quantities, meaning it could be antiangiogenic. Kahweol also decreases iNOS in rats' carrageenan paw oedema. During roasting, trigonelline is fractionated in nicotinic acid, which is a promising anti-inflammatory agent, as it diminishes MCP-1 and enhances adiponectin in adipocytes infused with TNF-α [46].

6. Protection against DNA Damage

Coffee's antioxidant and anti-inflammatory effects result in protection against DNA damage. A proposed mechanism for coffee protection against DNA damage is described in Figure 3. It has been observed a reduction in spontaneous

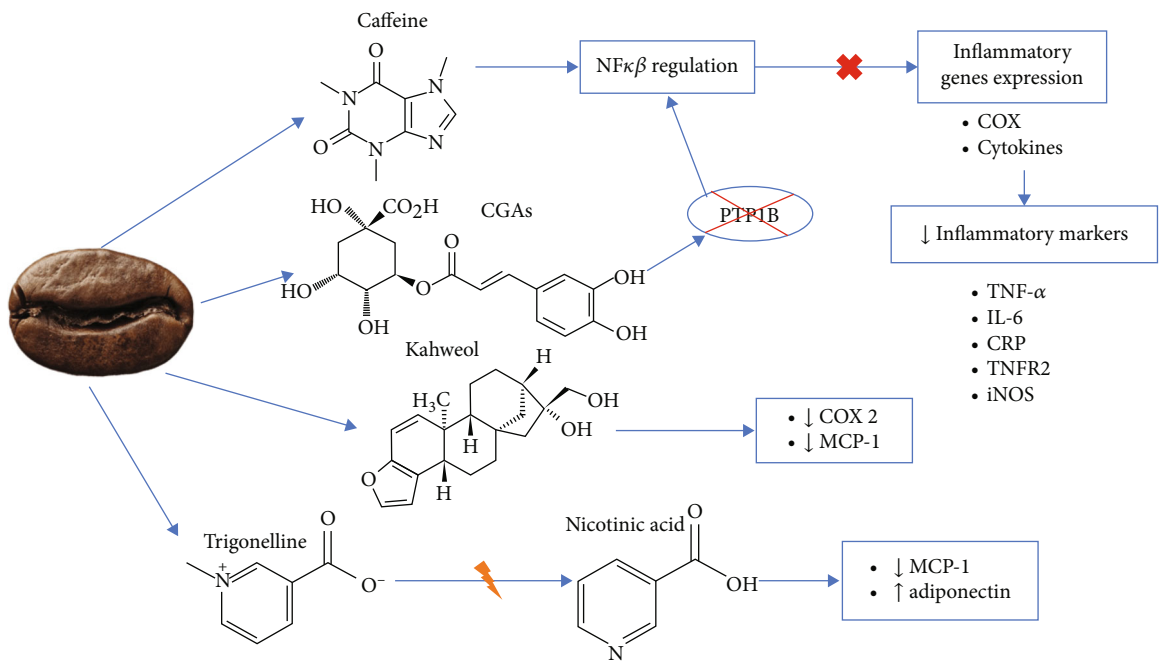

Figure 2: Mechanism of action of coffee for anti-inflammatory activity.

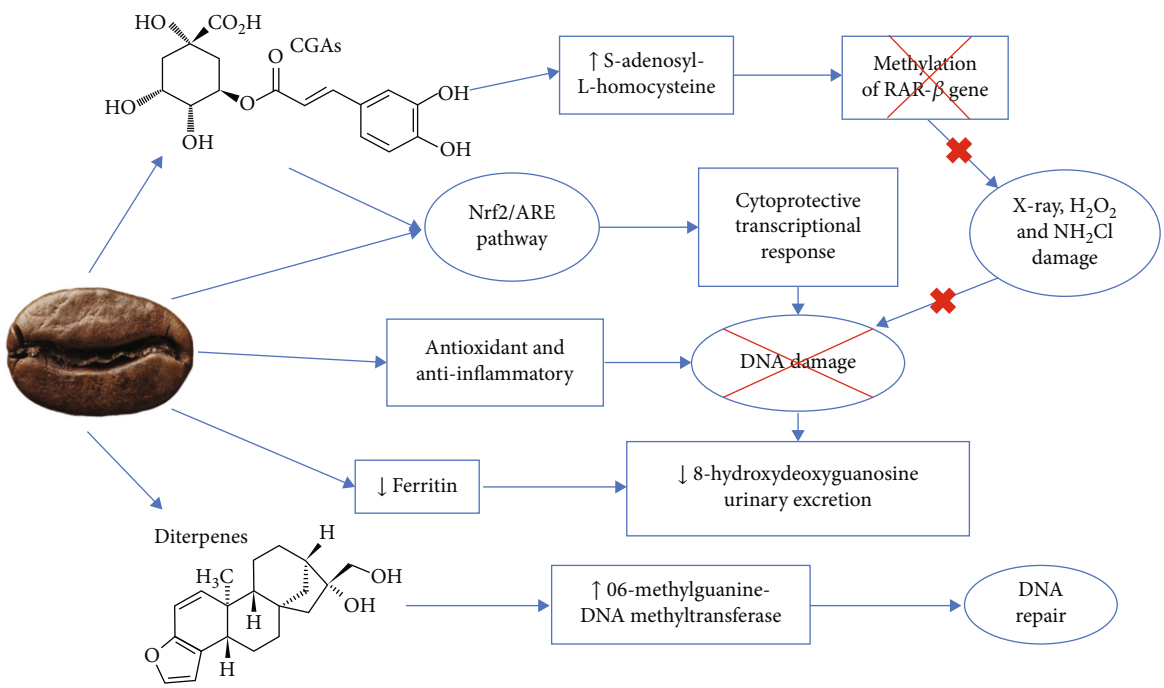

Figure 3: Mechanism of action of coffee for protection against DNA damage.

DNA strand breaks after only 2 hours of coffee consumption, with further decrease when more coffee was consumed [57]. Similar results were observed in 4 weeks [32, 58] and 8 weeks [31] of coffee intake.

These observations imply a defensive impact of coffee on DNA integrity. It has been observed that coffee reduced oxidative DNA damage induced by hydroxyl (OH°) radicals [43], H_2O_2 [33, 41], Ro photosensitizer [41], and benzo[α]-pyrene (BaP) [38]. Additionally, coffee presents strong chemopreventive properties against DNA damage caused by aflatoxin, probably due to induction of GST [59].

Isolated compounds found in coffee, such as CGA and caffeine metabolites, reduced DNA single-strand breaks caused by ROS [60]. CGA isomers displayed a protective action against X-ray, H_2O_2, and NH_2Cl-induced DNA plasmid chromosome breaks [24].

Urinary 8-hydroxydeoxyguanosine excretion tended to reduce with coffee intake, which is a biomarker of systemic

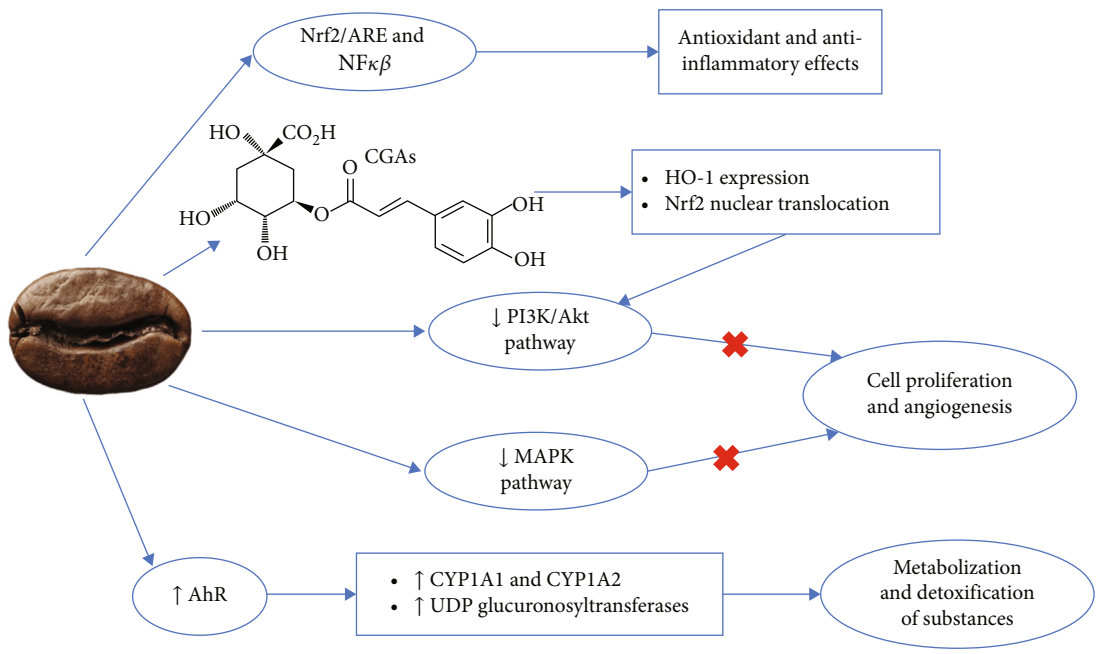

Figure 4: Mechanism of action of coffee for modulation of transcriptional factors.

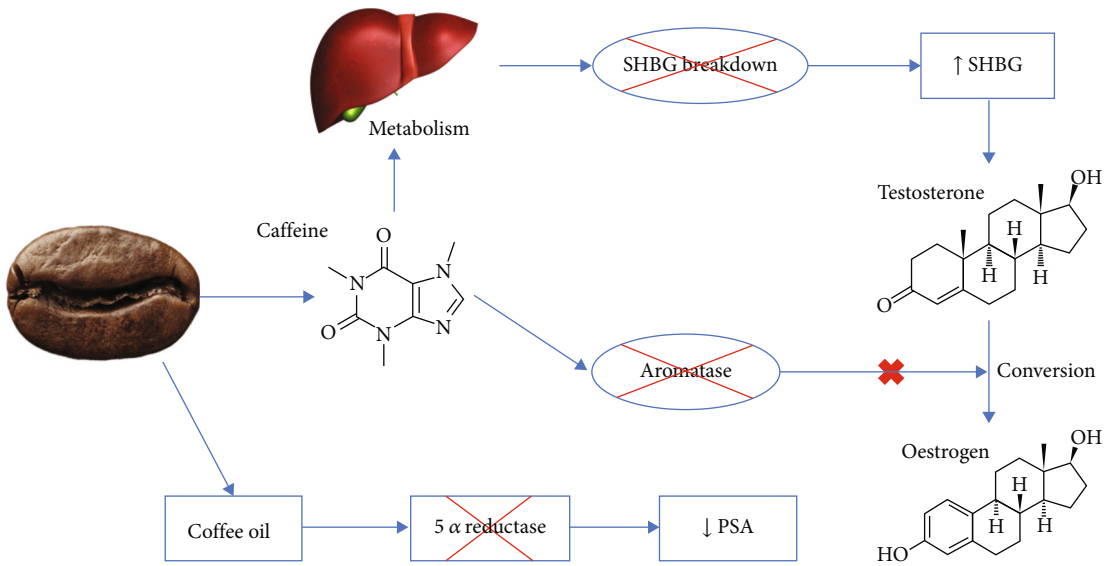

Figure 5: Mechanism of action of coffee for controlling steroid metabolism.

oxidative DNA damage and repair. This result was associated with lower serum ferritin and, indeed, coffee has iron-chelating properties [61]. Coffee and its diterpene components kahweol and cafestol raised the expression of DNA repair protein O6-methylguanine-DNA methyltransferase and some phase II enzymes [57].

Coffee constituents, such as CGAs, trigonelline, and kahweol, are regulators of the Nrf2/ARE signaling pathways ([57, 62], De Oliveira, De Souza, and Fürstenau 2020). It has been observed raised levels of Nrf2-dependent enzymes after consumption of coffees rich in CGA. Likewise, an inverse correlation between Nrf2 transcription and DNA strand breaks has been observed after coffee consumption [58].

Both Nrf2 and phosphorylated Nrf2 (pNrf2) are involved in the induction of Nrf2/ARE-dependent gene transcription. Alterations in Nrf2 translocation mediate the ARE-linked cytoprotective transcriptional response rather than a change in total Nrf2 concentration. There is evidence of pNrf2 translocation to the nucleus and an increase in pNrf2 with the decrease in Nrf2 due to coffee consumption [29, 31, 62].

7. Modulation of Transcriptional Factors

Coffee may prevent PCa development through modulation of transcriptional factors, which mechanism is shown in Figure 4. Some of those factors have already been discussed,

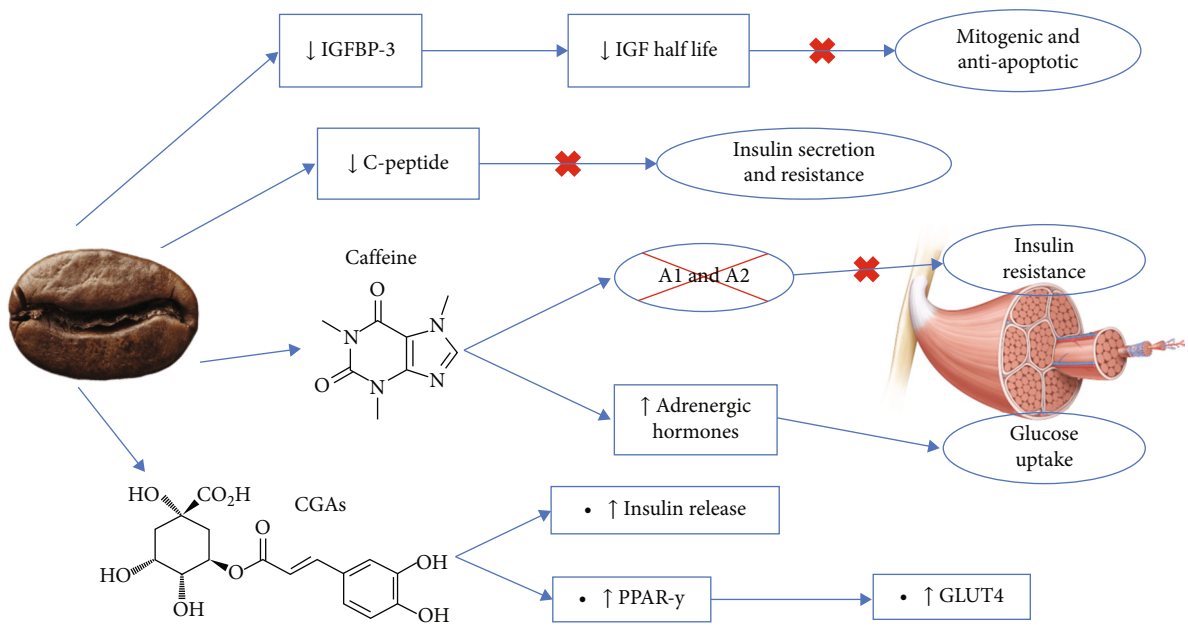

Figure 6: Coffee's mechanism for enhancing insulin resistance.

such as NF-κB and Nrf2/ARE pathways. Coffee may also affect phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt), activator protein 1 (AP-1), aryl hydrocarbon receptor (AhR), and mitogen-activated protein kinase (MAPK) pathways.

Coffee can downregulate PI3K/Akt signaling pathway [56], as well as its compounds, like caffeine [63] caffeic acid, and kahweol (De Oliveira, De Souza, and Fürstenau [44]). This pathway is protooncogenic and is responsible for metabolism, cell cycle, survival, and angiogenesis. It is frequently engaged in heme-oxygenase 1 (HO-1) expression and transcription of several kinds of cells. CGA promotes HO-1 expression and Nrf2 nuclear translocation, which might be related to PI3K/Akt signaling pathway [33].

The MAPK group comprises extracellular responsive kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. MAPK signaling pathways are regulated by coffee [56] and kahweol (De Oliveira, De Souza, and Fürstenau [44]). There is an association between the restriction of cell growth and the lesser activation/phosphorylation of MAPKs, indicating a capacity to decrease cancer cell proliferation [64].

AhR has an adjusting role in the manifestation of CYP1A1 and CYP1A2 genes, which are involved in the metabolization of several substances [65]. Coffee activates AhR pathways genes, inducing UGT, which is related to detoxification and clearance of reactive metabolites [38, 66].

There is evidence that CGA secures against cancer caused by external factors. Its defensive actions can be associated with the omission of NF-κB, AP-1, and MAPK activation concerning ROS effects [64]. Specific effects of CGAs in transcriptional pathways leading to cancer growth suppression are reviewed elsewhere [21].

8. Controlling Steroid Metabolism

Historically, it was believed that androgens were involved in PCa development. However, more recent studies found no relation between testosterone levels and/or testosterone therapy and increased risk of PCa [67–73]. Furthermore, androgen deprivation therapy (ADT) is effective against most types of PCa, but its effect is probably related to androgen receptor (AR) expression, which is a well-established component of PCa [74–76].

Therefore, the relationship between androgenic hormones and PCa development is not completely understood. But coffee is involved in steroid metabolism, and a proposed mechanism is described in Figure 5.

Caffeinated coffee is associated with higher concentrations of total testosterone. This is probably related to sex hormone-binding globulin (SHBG), which is a sex hormone transport protein and functions as a regulator of their activity. Decreased SHBG may be linked to an elevated risk of PCa [50]. Coffee and caffeine are linked to greater SHBG concentration [50, 77, 78]. Caffeine and SHBG are primarily metabolized by the liver, so one possible explanation is that caffeine intake might cause SHBG rise by impacting its catabolism [50]. Another possibility is that caffeine increases hepatic SHBG production by upregulation adiponectin synthesis and decreasing Akt phosphorylation [77].

Besides that, studies have shown that coffee and caffeine can affect estrogen metabolism by inhibiting aromatase, the prime enzyme responsible for the transformation of androgen to estrogen [50, 79]. Serum estrogen and estrogen receptors (ER) are associated with PCa development, and the molecular mechanisms involved are reviewed elsewhere [80, 81]. Coffee intake is related to reduced levels of estrogen

[50], possibly related to the presence of compounds with estrogenic activity in coffee [82]. Caffeine and caffeic acid reduced the ER expression [63].

Coffee oil has significantly decreased the prostate-specific antigen (PSA) compared to the control *in vivo* (Cueto et al. [83]). PSA is a specific PCa marker and used for diagnosis, and its concentration is usually remarkably high in PCa [84]. Therefore, a reduction in PSA levels suggests that coffee oil has defensive actions on the inflammatory status and against prostate hypertrophy. It has been hypothesized that the coffee oil effect was due to 5 alpha-reductase enzyme inhibition (Cueto et al. [83]).

AR has been associated with increased proliferation and altered migratory potential in PCa cells [74]. Coffee diterpenes (kahweol and cafestol) decreased the nuclear AR in AR-positive PCa cells, inhibiting their signals and inducing apoptosis. Coffee diterpenes also caused a reduction in CCR2 and CCR5, without raising their ligands (CCL2 and CCL5) [85].

9. Enhancing Insulin Resistance

Fasting serum insulin and insulin resistance are associated with PCa development. Insulin could act as a growth factor and probably raise the androgen entry in prostatic cells by reducing the effect of SHBG. Insulin resistance could raise insulin-like growth factor (IGF), which has mitogenic and antiapoptotic properties, which promote cell proliferation [86]. High circulating insulin also produces proinflammatory responses [4].

Besides its antioxidant and anti-inflammatory activities, coffee exerts specific effects that improve glucose and insulin status and is well recognized for preventing and treating type 2 diabetes mellitus (T2DM) [87, 88]. A summary of the mechanism of coffee on glucose metabolism is expressed in Figure 6. These effects are observed both in caffeinated and decaffeinated coffee; however, they might be more significant in decaffeinated coffee ([89, 90], Kumar [91]).

It has been observed that caffeinated coffee consumption reduces IGF-binding protein-3 (IGFBP-3), which extends IGF half-life. C-peptide also decreased due to coffee intake, which is a marker for insulin secretions, indicating lower insulin production and resistance [50]. Moreover, caffeic acid and caffeine reduced IGF-1 receptor expression [63].

It has been observed that incretins raised, and blood glucose reduced after coffee intake. Incretins, such as GLP-1 and GIP, are related to glucose reduction. Additionally, coffee has norharman β-carboline, which inhibits α-glucosidase activity, which lessens carbohydrate absorption, reducing postprandial glucose levels [89].

Caffeine acts as an antagonist of the A1 and A2 adenosine receptors, which in the skeletal muscle are related to insulin resistance [18, 19, 49]. Caffeine has a synergic effect with adrenergic hormones, which increases glucose intake by tissues. Most of the population quickly metabolizes caffeine; thus, it has acute effects. There is evidence that habitual caffeine intake leads to tolerance to these effects [90].

CGAs are probably responsible for the long-term effects. It has been hypothesized that CGA stimulates insulin release, being a secretagogue. CGA increased the expression of peroxisome proliferator-activated receptor-y (PPAR-y), which is essential in insulin sensitivity. Glucose transporter type 4 (GLUT4) also increased due to CGA, and its expression is stimulated by PPAR-y [92].

10. Conclusion

Coffee may reduce the risk of developing PCa through many molecular mechanisms. The ones are antioxidant and anti-inflammatory activities, protection against DNA damage, modulation of transcriptional factors, regulation through microRNA, enhancing steroid metabolism, and enhancing insulin resistance. However, studies vary on serving portions and it is not possible to determine an ideal coffee intake. Therefore, more molecular studies are fundamental to confirm such effects, determine intake recommendations, and assure safety. Coffee may impact PCa through other factors, but more studies are necessary to expand the knowledge on this area and to verify the real extension of the association of coffee consumption and its effects on PCa development.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

The manuscript was presented as a Master's thesis: Montenegro, Júlia. "Compostos bioativos, atividade antioxidante e atividade antiproliferativa em linhagens celulares de câncer de próstata de extratos de café obtidos por extração assistida por micro-ondas (MAE)." Master's thesis, 2020. (http://www.unirio.br/ccbs/nutricao/ppgan_pt/dissertacoes-e-teses/dissertacoes-e-teses-defendidas/2020/2020/compostos-bioativos-atividade-antioxidante-e-atividade-antiproliferativa-em-linhagens-celulares-de-cancer-de-prostata-de-extratos-de-cafe-obtidos-por-extracao-assistida-por-micro-ondas-mae/view).

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

This research was funded by the FAPERJ grant numbers 202.073/2016 and 210.145/2016, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Grant 001, and Universidade Federal do Estado do Rio de Janeiro (UNIRIO).

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Hindawi
BioMed Research International
Volume 2021, Article ID 3420168, 10 pages
https://doi.org/10.1155/2021/3420168

Review Article

Differences of Key Proteins between Apoptosis and Necroptosis

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Received 23 August 2021; Revised 8 November 2021; Accepted 13 November 2021; Published 12 December 2021

Academic Editor: Antonio Palumbo Jr

Many different types of programmed cell death (PCD) have been identified, including apoptosis and necroptosis. Apoptosis is a type of cell death that is controlled by various genes. It is in charge of eliminating aberrant cells such as cancer cells, replenishing normal cells, and molding the body as it develops. Necroptosis is a type of programmed cell death that combines necrosis and apoptosis. In other words, it takes on a necrotic appearance, although cells die in a controlled manner. Various investigations of these two pathways have revealed that caspase-8, receptor-interacting serine/threonine-protein kinase 1 (RIPK1), and RIPK3 are crucial proteins in charge of the switching between these two pathways, resulting in the activation or inhibition of necroptosis. In this review, we have summarized the key proteins between apoptosis and necroptosis.

1. Introduction

Cell death has two types: unpredictable cell death and programmed cell death. Programmed cell death (PCD) refers to cell death that occurs throughout development and is not accidental [1]. PCD results in either lytic or nonlytic morphology depending on the signaling pathways involved [2].

Apoptosis, autophagy, necroptosis, pyroptosis, and other PCDs have comparable forms and kinds. Eukaryotic cells commit suicide by apoptosis, which is a highly conserved mechanism [3]. Autophagy is an intracellular evolutionarily conserved catabolic degradation process [4]. Necroptosis is a necrotic programmed cell death that has a high immunogenicity and interacts with autophagy and apoptosis [5]. Pyroptosis is an activation of inflammatory caspases that causes a lytic kind of inflammatory cell death [6].

Many studies have been conducted regarding PCD routes in recent years. The purpose of this review is to summarize the primary mechanism involved in apoptosis and necroptosis and among PCD and analyze proteins involved in each pathway. And in conclusion, another purpose is to understand the relationship between these pathways which will be helpful in the cancer disease research.

2. Apoptosis

Apoptosis causes a cell to stop growing and dividing. It is a process that causes cell's contents to flow into its surrounding environment [7]. Apoptosis is characterized by membrane blebbing, nuclear chromatin condensation, cell shrinkage, DNA breakdown into nucleosomal units, and the formation of apoptotic bodies [8]. Apoptosis can be triggered by either extracellular death receptors (such as tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), and Fas cell surface death receptor (FAS)) or intracellular stimuli (such as nutrient deprivation, irreparable genetic damage, severe osmotic stress, and hypoxia) [1].

The extrinsic pathway (Table 1) includes connections mediated by transmembrane death receptors, TNF receptor, and extrinsic pathway and possesses genes of the superfamily [1]. Binding between death receptors and ligands will initiate this pathway, resulting in the development of a "death-

Table 1: Apoptosis mechanism in cancer disease.

Protein	Up/ down	Description	Article related to apoptosis	References
(1) Extrinsic pathway				
FADD	Up	The key adaptor that transmits death signals via death receptors	Induction of apoptosis in HL-60 cells by luteolin necessitates FADD-caspase-8-mediated apoptosis	[12, 13]
FasL and Fas	Up	A critical death ligand and its receptor	Treatment with SCU, on the other hand, increases expression levels of Fas and Fas ligand (FasL) known to activate cleaved caspase-3, caspase-8, and polymeric adenosine diphosphate ribose (PARP) while decreasing the expression of death receptor 4 (DR4)	[12, 14]
TRAIL	Up	TNF family death ligand	Cancer cells are destroyed whereas primary esophageal cells are protected when primary esophageal cells are cultured in a mixed population with type I cancer cells and treated with TRAIL in the presence of a caspase-9 inhibitor	[12, 15]
DR4 and DR5	Up	Death receptors for TRAIL	Casticin enhances TRAIL-induced apoptosis by downregulating cell survival proteins and inducing DR5 via ROS	[12, 16]
(2) Intrinsic pathway				
Bcl-2	Down	Regulate cell behavior through programmed cell death	The estrogenic actions of certain flavonoids may be responsible for upregulation of the Bcl2 gene in apoptotic MCF7 cells after flavonoid therapy	[17, 18]
BH3-only proteins	Up	To exert their intrinsic proapoptotic activities, all BH3-only molecules require multidomain BH3 proteins (Bax and Bak)	Phenoxodiol induces melanoma cell apoptosis by inducing p53-dependent BH3 proteins (PUMA, Noxa, and Bad) and p53-independent Bim protein, resulting in Bax activation and downstream events	[19, 20]
Bcl-x_L	Down	Functions as apoptosis inhibitors	Fisetin, an HSF1 inhibitor, acts as a triple inhibitor, lowering expression levels of Bcl-2, Mcl-1, and Bcl-x L via downregulation of their chaperones, BAG3 and HSP70. As a result, fisetin might be beneficial in combating single agent-induced resistance	[17, 21]
BAX and BAK	Up	Results in the release of cytochrome c and activates caspases derived from mitochondria	Calycopterin treatment increases the Bax/Bcl2 ratio in HepG2 cancer cells, causing mitochondrial damage and subsequent cytochrome C release	[18, 19]
p53	Up	An important proapoptotic factor and tumor inhibitor	N101-2 treatment decreases expression levels of cyclin A and p-pRb while increasing expression levels of p53, p21, and p27	[19, 22]
(3) Caspase and caspase inhibitors				
Caspase-8	Up	Initiator caspase that promotes the activation of caspase-3	The ligand binding to the transmembrane death receptor initiates the extrinsic apoptotic pathway, which leads in caspase-8 activation	[18]
Caspase-10	Up	Activation of signal transduction cascade is initiated by a caspase initiator	Caspase-10 is cleaved in response to flavone treatment	[17, 23]
Caspase-3	Up	Caspase effector	Fisetin activates caspase-3 and caspase-7 in a dose-dependent way. Such caspase activation coincides with PARP cleavage	[21]
IAPs (XIAP, cIAP1/2)	Down	Inhibitors of apoptosis proteins	Survivin, an inhibitor of apoptosis (IAP) family member, showed a reduction in expression following DHM therapy, perhaps due to p53 activation	[24]

included signaling complex" (DISC) consisting of TNF receptor 1 (TNFR1), Fas-Fas-L, death receptor 3 (DR3), death receptor 4 (DR4), and tumor necrosis factor superfamily 10 (known as TRAIL/Apo2L) [7].

The extrinsic route has various death receptors and ligands. We will focus on Fas-L and TNF receptors as examples. FAS will form a trimer when it is mixed with Fas-L. As a result, the FAS death effector domain, which can be coupled to the adaptor protein FAS-associated death domain (FADD), is exposed. To generate an apoptosis-inducing signal complex, procaspase-8 and procaspase-10 can attach to this domain, cleaving these procaspases to activate caspase-8 and caspase-10 which then trigger caspase-3 and caspse-7 as effector caspases, thus breaking the target protein and causing apoptosis [7].

RIPK1 and the TNF receptor- (TNFR-) associated death domain (TRADD) are also adaptor proteins linked to the cytoplasmic portion of the TNA receptor to create apoptosis complex I when the TNF trimer binds to the TNF receptor trimer [7]. Complex I was the first complex found in the respiratory system's components with a mechanistic explanation for its involvement in inducing apoptosis [9]. In the absence of proteins (such as cIAP and FLIP) that suppress a natural cell death, complexes such as RIP1, FADD, and caspase-8 will assemble in the cytoplasm, resulting in apoptosis via caspase chain reaction (apoptosis complex II) [7, 9].

The intrinsic pathway (Table 1), also known as the mitochondrial apoptosis pathway, involves various stimuli that operate on a variety of cell targets. It is caused by intrinsic lethal stimuli such as hypoxia, endoplasmic reticulum (ER) stress, metabolic stress, and DNA damage. Substances secreted by the mitochondria can trigger this type of apoptosis linked to the extrinsic pathway [7]. In extrinsic pathway, caspase-8 and caspase-10 are capable of cleaving BH3-interacting domain death agonist (BID) to truncated BID (tBID). BAX and BCL2-antagonist/killer (BAK) are activated by tBID.

Inherent fatal stimuli can stimulate BH3-only proteins which in turn activates BAX and BAK. Activated BAX and BAK can form mitochondrial permeability transition (MPT) pores on mitochondrial outer walls, allowing cytochrome c to release into the cytoplasm due to mitochondrial outer membrane permeability (MOMP) induction and act as a signaling molecule (proapoptotic proteins, including Smac/Diablo, cytochrome c, and HrtA2/Omi) in the cytoplasm to facilitate the formation of apoptosome with adapter protein apoptotic protease activating factor 1 (APAF1) in the cytoplasm. When an apoptosome is produced, caspase-9 is triggered, followed by cascades that activate caspase-3 and caspase-7. Activated caspase-3 and caspase-7 will lead to the destruction of cellular components through apoptosis [1, 7].

In addition, ripoptosome formation has been linked to the apoptotic pathway [10]. Ripoptosome is a 2 MDa protein complex that includes caspase-8, FADD, several cFLIP isoforms, and RIPK1 as key components. It can be produced by a range of cellular circumstances such as DNA damage, genotoxic stress, and IAP depletion by medicine such as teniposide/etoposide and SMAC mimetics. Teniposide is a semisynthetic phospholotoxin derivative that has similar mechanisms of action, effects, and toxicity to etoposide. Furthermore, both medicines have the property of blocking DNA synthesis in the premitotic stage of cell division by inhibiting the topoisomerase II enzyme. And they are combined with anticancer treatments. Therefore, we think that it is good to mention the substance used in combination with anticancer drugs as an example.

Flavonoids have been used in several studies to cure cancer by triggering apoptosis. Scutellarein can activate the Fas-mediated extrinsic apoptotic pathway in Hep3B cells [14]. Apigenin promotes cell death in human breast cancer MDA MB-231 and MCF-7 cells, resulting in considerable toxicity and, most importantly, apoptosis [25]. GL-V9 also induces apoptosis of human breast cancer cell lines [26]. Isoliquiritigenin, a flavonoid, can similarly stop melanoma cells from proliferating and migrating by inhibiting miR-27a expression [1, 27].

3. Necroptosis

Necroptosis is a type of controlled cell death that has characteristics of both apoptosis and necrosis [28]. In other terms, it is an inflammatory-mediated cell death or programmed form of cell necrosis.

As previously stated, apoptosis involves a set of mechanisms that can result in cell death. The death of cells or tissues caused by pathogenic infection, cellular injury, or noxious stimuli refers to necrosis. In another sense, necrosis is an uncontrolled and unrestricted type of cell death [8]. Necrosis occurs quickly as a result of extreme physicochemical stress such as mechanical stress, heat, osmotic shock, cell freeze-thawing, and acidity [8, 29]. Loss of plasma membrane integrity, increased cell volume, organelle swelling, lack of internucleosomal DNA breakage, and cellular collapse are all symptoms of necrosis [8]. Overexpression of certain proinflammatory proteins, such as nuclear factor kappa B, promotes cell membrane rupture and leaking of cell contents into the surrounding regions, culminating in a cascade of inflammation and tissue damage [7].

Necroptosis (Table 2) is a kind of controlled cell death characterized by morphology of necrosis. It is dependent on RIPK3 and mixed lineage kinase domain-like proteins (MLKL) [30]. It is characterized morphologically by swelling of organelles, increased cell volume, cellular collapse, permeabilization of the plasma membrane, and release of cellular contents [8].

Necroptosis induction is the activation of serine/threonine kinase RIPK1 via a cascade of signaling pathways involving the tumor necrosis factor (TNF) receptor superfamily, T cell receptors, interferon receptors, Toll-like receptors (TLRs), cellular metabolic and genotoxic stresses, or various anticancer agents that induce necroptosis [1, 12, 30, 31]. Additionally, necrostatin-1 (Nec-1), a particular necroptosis inhibitor, inhibits necroptosis as demonstrated by decreased necrotic ultrastructural changes and lower expression levels of RIPK1, RIPK3, phosphorylated MLKL, PGAM5, DRP1, and cytoplasmic HMGB1 [32, 33].

In TNFR1 signaling, TNF activates TNFR1 and causes the recruitment of RIP1 kinase, TRADD, TRAF2, and cIAP1/2, producing a transient complex known as complex I [1, 34]. TNF stimulates RIPK1 and the cellular inhibitor

Table 2: Necroptosis mechanism in cancer disease.

Protein	Up/ down	Description	Article related to necroptosis	References
TNF	Up	Tumor necrosis factor	Fisetin significantly increases TNF and IK expression while decreasing pNF-, and pIK expression	[35]
RIPK1 and RIPK3	Up	Receptor-interacting protein kinase-1 and 3	In the presence of ZVAD, MCF-7 cells express substantially more RIPK1 and RIPK3 in response to Que than in the absence of ZVAD	[33]
TNFR1	Up	TNF's receptor following the formation of two TNFR complexes	Cell survival, apoptosis, or necroptosis can result from TNFR1 stimulation caused by damage, cellular stress, or infection	[8, 36]
			TNF-α, TNFR1, and necroptosis protein expression are all inhibited by PPO, indicating that PPO can protect neurons by preventing TNF-α-induced necroptosis	[37]
FADD	Up	Fas-associated protein with death domain (FADD). Activation of MLKL	Necroptosis is caused by caspase-8, FADD, and RIPK3 (complex IIa/b)	[36]
			FADD expression in HepG2 is reduced by fisetin treatment. FADD protects intestinal epithelial cells from RIP3-induced cell necrosis	[35]
MLKL	Up	MLKL (mixed lineage kinase like) after rapid plasma membrane rupture and inflammatory response via DAMP and cytokine release	M1 CM significantly increases expression levels of MLKL, RIPK3, and p-MLKL after quercetin treatment	[36, 38]

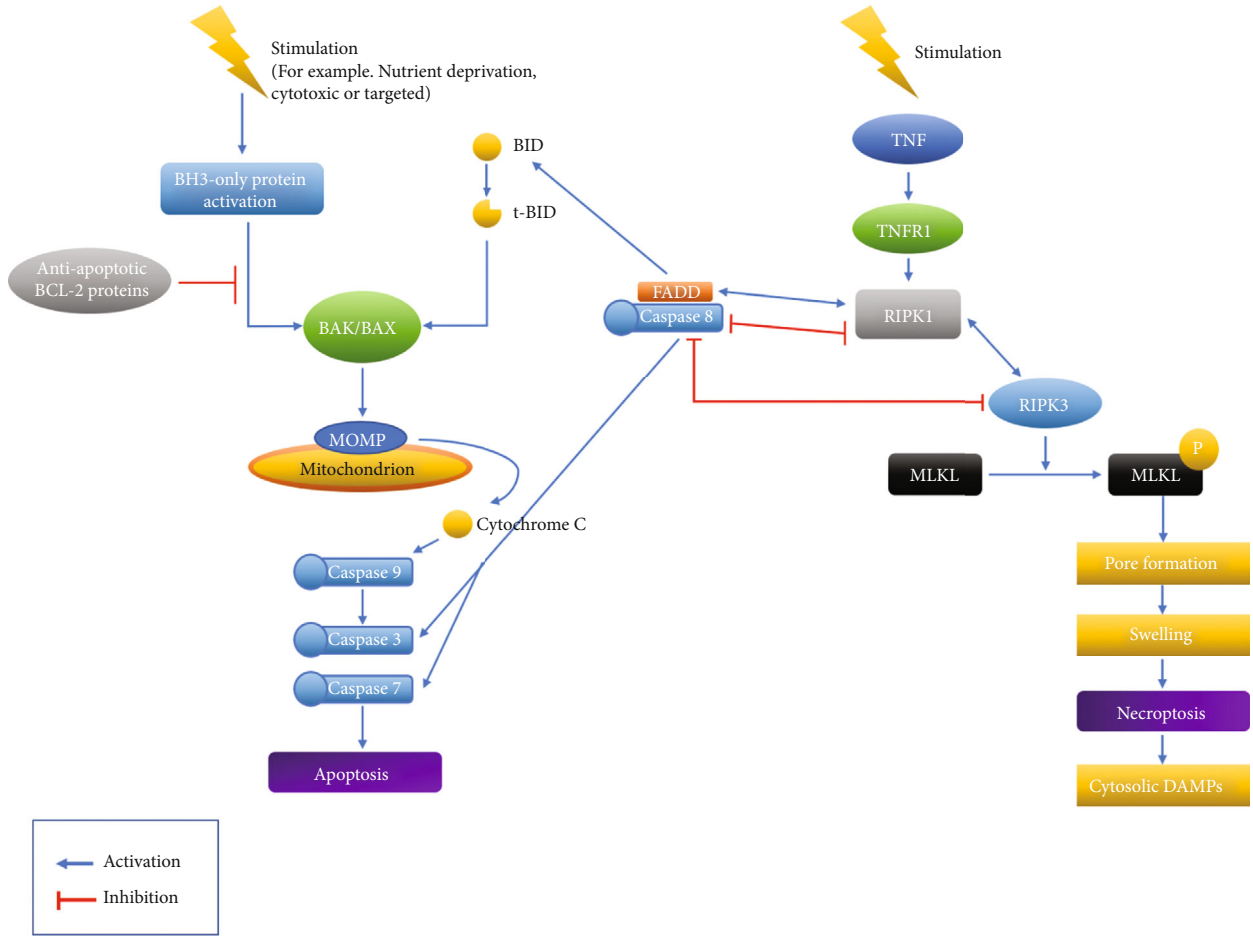

Figure 1: Apoptosis and necroptosis pathways. Among the pathways of apoptosis and necroptosis, activation and inhibition are explained with a focus on caspase-8.

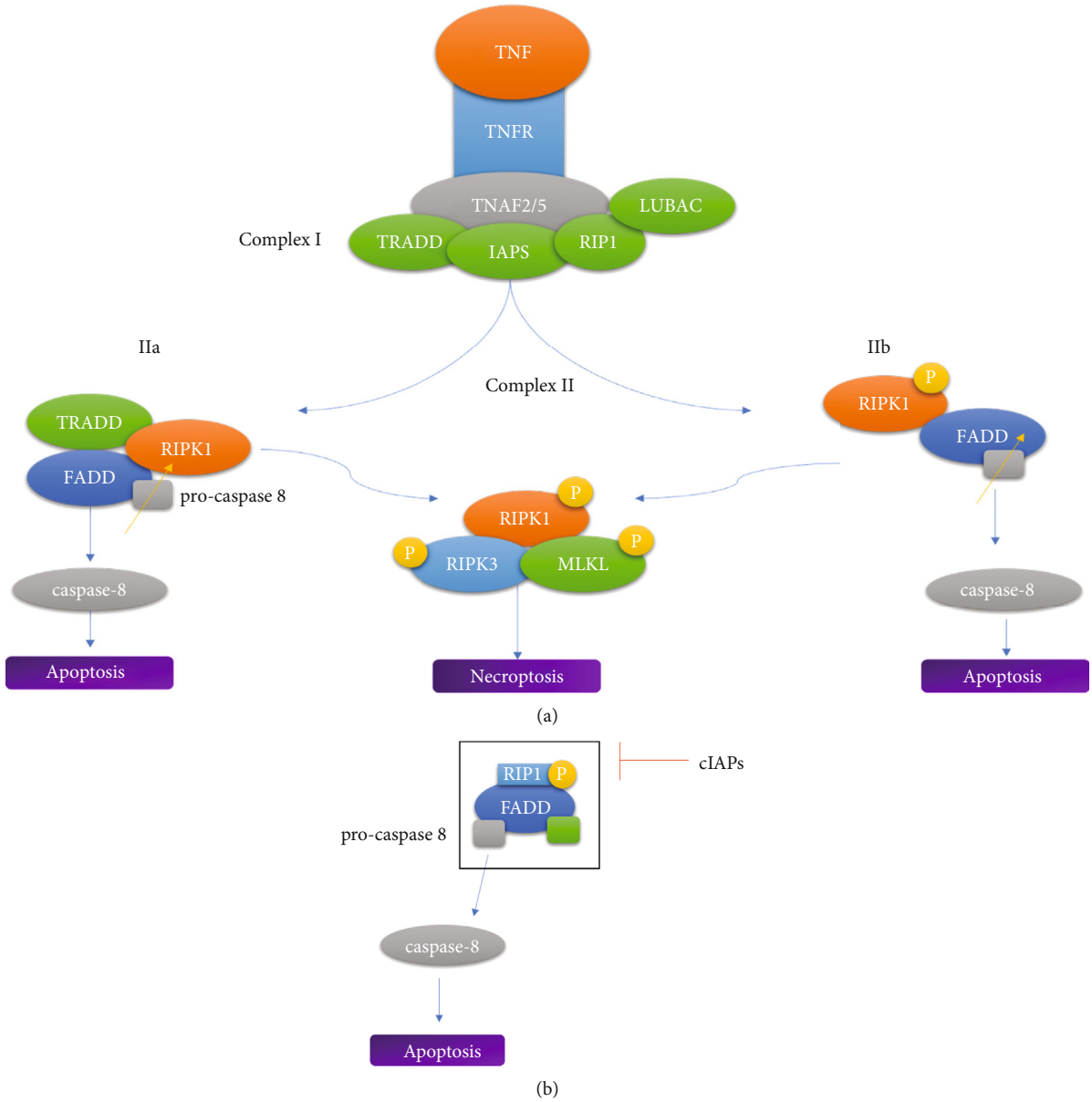

Figure 2: Necrosome and ripoptosome. (a) When complex I is activated by stimulation, complex II is formed and RIPK1 is dissociated. (b) When cIAPs block the ripoptosome, RIPK1 is isolated.

of apoptosis protein 1 (cIAP1), which phosphorylates the inhibitor of the kappa B kinase (IKB) complex, resulting in the activation of nuclear factor kappa B (NF-κB) which leads to cell survival [35]. RIPK1 is modified by enzyme cylindromatosis (CYLD) in complex I, resulting in complex II which includes FADD, RIPK1, TRADD, and caspase-8 as key components. This step determines whether complex II leads to apoptosis or necroptosis. Inhibition of cIAP1 causes the formation of complex IIa which stimulates the caspase cascade and induces apoptosis [1]. RIPK1 and RIPK3 will form necrosome/complex IIb, a cytoplasmic necroptotic protein complex structure, if caspase-8 activity is suppressed [1, 33]. The necroptotic signal transduction pathway is triggered by complex IIb. Upon formation of complex IIb, oligomerization of mixed lineage kinase domain-like protein (MLKL) occurs, which is a downstream effector of necroptosis that initiates the necroptotic process [1, 35].

The development of numerous human diseases, including cancer, has been related to necroptosis [1]. Apigenin causes ROS-dependent necroptotic cell death through mitochondrial dysfunction due to ATP deficiency [39]. Prunetin (PRU) can induce necroptosis in a gastric cancer cell line [40]. Flavonoids can cause cancer cells that are resistant to apoptosis to undergo necroptosis [33].

4. Comparison of Proteins in Both Apoptosis and Necroptosis Pathways

Necroptosis is an immunologically silent inflammatory form of controlled necrotic cell death [41]. Controlled necrotic

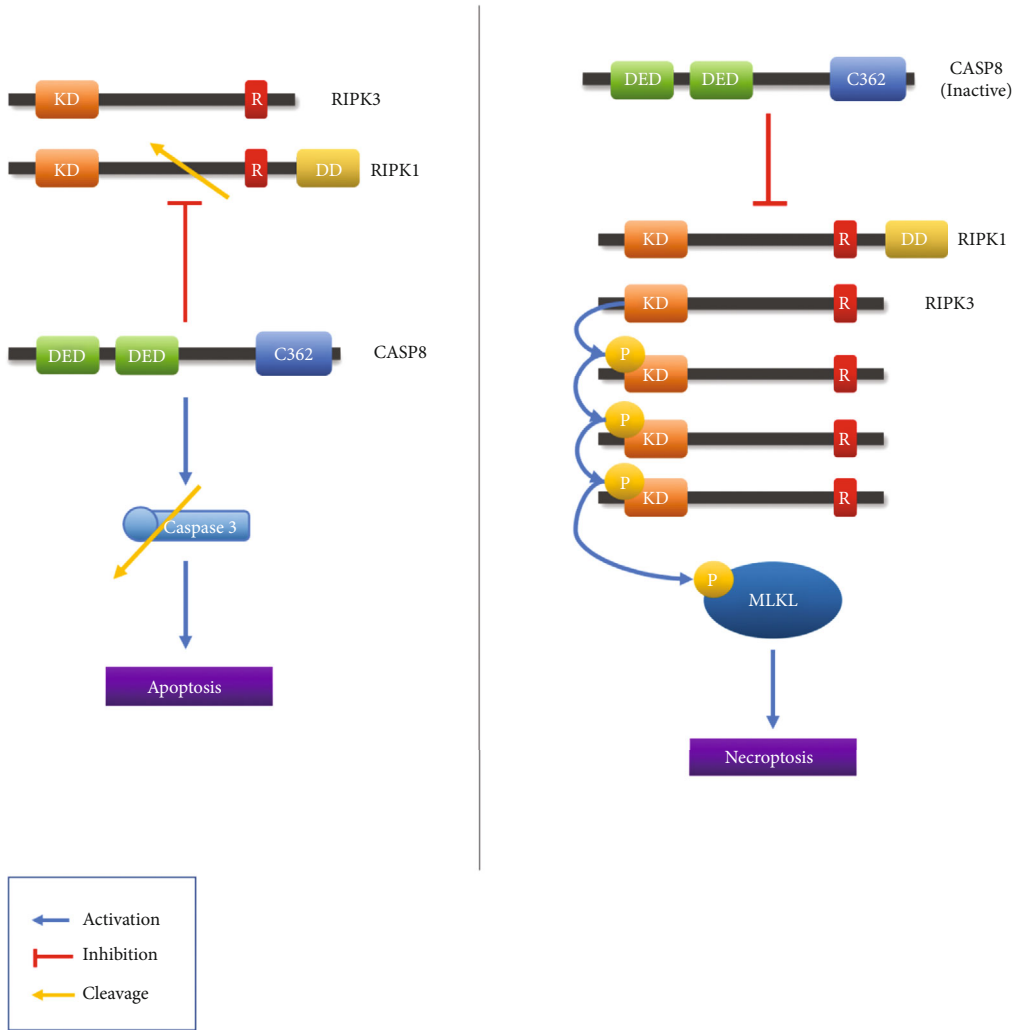

Figure 3: CASP8, RIPK1, and RIPK3 effect on apoptosis and necroptosis. Caspase-8 plays a role in apoptosis and necroptosis. In apoptosis, caspase-3 is cleaved to cause apoptosis, and in necroptosis, RIPK1 is inhibited to prevent necroptosis.

cell death has recently been discovered in several forms, all of which have morphological characteristics such as increased cellular volume, organelle swelling, and plasma membrane rupture. They have diverse triggers that involve various metabolic processes [41]. In terms of apoptosis and necroptosis, "caspase-8" is a protein that is associated with both apoptosis and necroptosis (Figure 1). Caspase-8 is an extrinsic apoptosis initiator caspase that suppresses necroptosis mediated by RIPK3 and MLKL [42]. Aside from its functions in apoptosis and necroptosis, new *in vitro* investigations have shown that caspase-8 is a scaffolding protein that can initiate cytokine synthesis independently of its enzymatic activity [42]. The scaffolding function of caspase-8 has also been implicated in activating inflammasome in macrophages triggered by double-stranded RNA (dsRNA) [42]. In addition, independent of cell death, the activity of enzyme caspase-8 is essential for NF-κB activation and cytokine secretion in response to activated antigen receptors, Fc receptors, or Toll-like receptors (TLRs) [42].

When comparing the relationship of two-concerning caspase-8 between apoptosis and necroptosis, RIPK1 and RIPK3 are also involved (Figure 1). Upstream pathways activate RIPK3 by interacting with three other proteins in the mammalian genome that have conserved RIPK homotypic interaction motifs through RIPK homotypic interaction motif-dependent protein-protein interactions: ZBP1/DAI, TRIF, and RIPK1 [41]. RIPK1 is the link between RIPK3 and death receptor signaling. TLR3 and TLR4 are activated by TRIF, which then activates RIPK3. ZBP1/DAI primarily activates RIPK3 in response to viruses [41].

By functioning as a signaling hub within the apoptotic and necroptotic cell death pathways, RIPK1 controls cell survival, cell death, and inflammation. It possesses activities that are both kinase dependent and kinase independent [41]. RIPK1 can form a complex with FADD and caspase-8, in line with a previously reported signaling axis that interacts with FADD and caspase-8 to increase cell death [43, 44]. The amino acid Asp325 of RIPK1 is required for avoiding aberrant cell death in response to TNF, indicating that caspase-8 cleavage of RIPK1 is a method of disassembling death-inducing complexes [44]. Furthermore, irrespective of its kinase activity, RIPK1 functions as a scaffold to inhibit

Table 3: Key proteins involved in both apoptosis and necroptosis.

Protein	Relation of apoptosis and necroptosis	References
RIPK1, RIPK3	In response to TNF, RIPK1 Asp325 is needed for reducing aberrant cell death	[44]
	Inhibiting caspase-8-mediated apoptosis as well as RIPK3-MLKL-dependent necroptosis fully prevents RIPK1-mediated embryonic mortality	[41]
Caspase-8	Caspase-8 is a molecular switch that controls apoptosis and necroptosis, as well as protecting tissues from injury	[42]
	Caspase-8 cleaves itself, other proteins, or both in order to prevent necroptosis	[44]

Table 4: The relationship between disease and key proteins.

Key proteins	Effect in disease	Related diseases	FDA-approved inhibitor (company)	References
Caspase-8	Mediator	Inflammation and disease in rodent malaria	None	[52]
RIPK1	Activation of RIPK1	Skin diseases, including melanoma, psoriasis, and systemic lupus erythematosus	Sunitinib (Pfizer), Pazopanib (GSK, Novartis)	[53, 54]
RIPK3	Mediator	Sepsis-associated organ injury and chronic lung diseases	Dabrafenib (GSK, Novartis)	[54, 55]

apoptosis and necroptosis in many tissues and this role is critical for avoiding inflammation and maintaining tissue homeostasis, according to several studies [41, 45, 46]. Inhibition of RIPK1 kinase activity by genetic and pharmacological means, on the other hand, has shown a critical function for RIPK1 kinase-dependent apoptosis and necroptosis in the development of inflammatory and degenerative diseases in a variety of tissues [41]. These studies have revealed that RIPK1 has opposing roles in preventing and promoting cell death, implying that its kinase-independent and kinase-dependent actions must be carefully balanced to maintain tissue homeostasis and to avoid cell death and illness [41].

Caspase-8 cleaves RIPK1 to prevent cell death and inflammation [41]. It is known that caspase-8 can inhibit necroptosis but can activate apoptosis [42]. RIPK1, RIPK3, cFLIP, and CYLD are among the proteins that caspase-8 can cleave to regulate necroptosis [41]. According to a research, Asp325 in RIPK1 is essential for avoiding abnormal cell death throughout development as well as TNF-induced cell death in a variety of cell types. Caspase-8 can cleave Asp325 of RIPK1 in cell-death signaling complexes, causing the complexes to disassemble and the death signal to be terminated [44].

RIPK1 dissociates from complex I and forms either complex IIa (Ripoptosome) or complex IIb when ubiquitin carboxyl-terminal hydrolase (CYLD) (a Lys63-deubiquitylating enzyme) or cIAPs block it (necrosome) (Figure 2). When deubiquitylated RIPK1 interacts with FADD-caspase-8-FLIPL via their DDs, complex IIa is produced [46]. When RIPK1 recruits RIPK3 via their mutual RIPK homotypic interaction motifs, the riptosome is complete (RHIM). RIPK1 and RIPK3 can blanket the ripoptosome and enhance caspase-8-mediated apoptosis by having a large number of caspase-8 cleavage at sites. When caspase-8 is present as a homodimer, it is fully digested, resulting in apoptosis. When caspase-8 forms heterodimers with FLIPL, its activity toward RIPK1 is unaffected but its activity toward other apoptotic substrates including pro-caspase-3 and BID is decreased [47, 48]. As a result, despite the disassembly of the ripoptosome, cells are unable to undergo apoptosis [11, 47, 48].

Caspase-8 prevents necroptosis by cleaving the proteins RIPK1 and RIPK3. Death receptors such as DNA-dependent activator of IFN (DAI) regulatory factors can produce necroptosis as an alternate type of programmed death when caspase-8 is blocked. Necroptosis, on the other hand, produces inflammation by secreting a high number of proinflammatory cytokines and molecular patterns associated with damage (DAMPs). Caspases-8 may change the balance between apoptosis and necroptosis, making it a critical regulator of the inflammatory tumor microenvironment [47–49].

5. Conclusion

In this review paper, caspase-8, RIPK1, and RIPK3 are identified as key proteins involved in both apoptosis and necroptosis (Figure 3, Table 3). Apoptosis is a kind of controlled cell death marked by cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA breakage [12]. Necroptosis is a type of controlled cell death that is not dependent on caspases and has features that are halfway between necrosis and apoptosis [50]. We thought that it is important to examine the relationship between the two pathways. Therefore, the key proteins between these two pathways are investigated.

Caspase-8 cleaves BID to tBID during apoptosis. It has the ability to activate BAX and BAK. As a result, it activates the pathways of apoptotic mechanism. The role of caspase-8 in necroptosis is becoming more complex than its role in apoptosis (Figure 1). In necroptosis, RIPK1 and RIPK3 can inhibit caspase-8. Furthermore, RIPK1 and RIPK3 are also crucial in the transition from apoptosis to necroptosis. Caspase-8, on the other hand, is a key factor in the transition from necroptosis to apoptosis [51]. Therefore, the three key proteins involved in both

apoptosis and necroptosis are intertwined to act cordially that initiates different cell death.

Overexpression of key proteins has the potential to cause illness (Table 4). These proteins are strongly linked to apoptosis and necroptosis. We think that by targeting these key proteins, therapeutic strategies for illnesses like cancer and others listed in Table 4 can be created.

The mechanisms underlying the relationship among caspase-8, RIPK1, and RIPK3 still remain to be clarified. Therefore, cell death studies targeting caspase-8, RIPK1, and RIPK3 among cell pathways have been recently conducted. We that think this review paper will provide the foundation basic knowledge for future research.

Abbreviations

PCD: Programmed cell death
RIPK1: Receptor-interacting serine/threonine protein kinase 1
TNF: Tumor necrosis factor
TRAIL: TNF-related apoptosis-inducing ligand
FAS: Fas cell surface death receptor
DISC: Death-included signaling complex
TNFR1: TNF receptor 1
DR3: Death receptor 3
FADD: FAS-associated death domain
TRADD: TNFR-associated death domain
ER: Endoplasmic reticulum
BID: BH3-interacting domain death agonist
tBID: Truncated BID
BAK: BCL-2-antagonist/killer
MOMP: Mitochondrial outer membrane permeability
APAF1: Apoptotic protease activating factor 1
FasL: Fas ligand
PARP: Polymeric adenosine diphosphate ribose
MLKL: Mixed lineage kinase domain-like proteins
TLRs: Toll-like receptors
Nec-1: Necrostatin-1
cIAP 1: Cellular inhibitor of apoptosis protein 1
IkB: Inhibitor of kappa B kinase
NF-κB: Nuclear factor kappa B
DAI: DNA-dependent activator of IFN
DAMPs: Damage-associated molecular pattern.

Conflicts of Interest

The authors clearly declare that they have no conflict of interest in this study.

Authors' Contributions

Min Yeong Park and Sang Eun Ha contributed equally to this work.

Acknowledgments

This study was supported by the National Research Foundation of Korea funded by Ministry of Science and ICT (grant no. 2020R1A2B5B01001807).

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