



Co-Chemotherapeutic Effect of n-Hexane Fraction of Binahong (*Anredera cordifolia* [Tenore] Steen.) on WiDr Colon Cancer Cell Line

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Abstract

BACKGROUND: The incidence of colon cancer ranks the third most common cancer case in Indonesia, with 12.8 cases per 100.000 community. Problems that arise from cancer treatment with chemotherapy are severe side effects. Thus, we develop cancer therapy using a natural material, namely, binahong leaves (*Anredera cordifolia*).

AIM: This study aims to investigate the co-chemotherapy activity of the n-hexane fraction of binahong (NFB) on the WiDr human colon cancer cells line.

METHODS: The sample was prepared by maceration using ethanol 70% and fractionation using n-hexane. Phytochemical screening was conducted with the thin-layer chromatography method. The antioxidant test was carried out using 1,1-diphenyl-2-picrylhydrazil assay, while the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay was designed for cytotoxic tests on WiDr colon cancer cells to identify the single effect and its combination with 5-fluorouracil as chemotherapy agents. Further, *in silico* test, molecular docking was used to determine the 4', 6, 7-trihydroxyaurone as an active compound on NFB against inhibitors of κ B kinases (IKK) and COX-2 proteins.

RESULTS: Phytochemical screening showed that NFB contained a flavonoid compound. NFB had a weak antioxidant activity with the inhibition concentration (IC_{50}) value of 2851 μ g/mL, while NFB had a relatively strong cytotoxic activity on WiDr colon cells with the IC_{50} value of 191 μ g/mL, and had the synergist activity in combination with 5-fluorouracil as a chemotherapy agent. The molecular docking showed the best interaction of 4', 6, 7-trihydroxyaurone against IKK, and COX-2 proteins with docking scores of -9.0 kcal/mol and -8.1 kcal/mol, respectively.

CONCLUSION: NFB had the potential to inhibit cell growth and had a synergistic effect in combination with 5-fluorouracil on WiDr colon cancer cells line.

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Introduction

The incidence of colon cancer continues to increase annually. According to GLOBOCAN (2018) data, colon cancer is the fourth of the world's most cancer incidents. In Indonesia, there are 12.8 incidence rates per 100.000, age standardized with 9.5% mortality from all of the cases [1]. Cancer treatments generally use medical treatments such as chemotherapy, radiation, and a combination of the two. However, they usually can cause several side effects such as pain, nausea, vomiting, and hair loss. Besides, the chemotherapy agents destroy cancer cells and attack the normal cells [2].

Binahong (*Anredera cordifolia* (Tenore) Steen.) is one of Indonesia's natural products that can be potentially developed for cancer treatment. In the past, people used binahong as traditional medicine for healing the wounds [3]. Numerous studies have been conducted to reveal the biological activity of binahong. The active compounds of binahong have an effect as an antioxidant, antibacterial, anti-inflammatory, and analgesic. Those active compounds are flavonoids,

glycosides, terpenoids, alkaloids, and saponins [4]. Based on the previous study by Rahardian (2018), binahong (*A. cordifolia* (Tenore) Steen.) had a vigorous cytotoxic activity with half-maximal inhibition concentration (IC_{50}) of 85.52 μ g/mL on HeLa cervical cancer cells [5]. However, its cytotoxic activity on another cancer cell, like WiDr human colon cancer, has not yet been tested. Furthermore, its combination with 5-fluorouracil as a common drug on colon cancer treatment has not yet been identified.

This study investigated the chemopreventive activity of the n-hexane fraction of binahong (NFB) on WiDr human colon cancer cells. It started from phytochemical screening conducted with thin-layer chromatography (TLC), antioxidant test using 1,1-diphenyl-2-picrylhydrazil (DPPH), single and combination cytotoxic test on WiDr cells designed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and *in silico* test using molecular docking by comparing the docking score of 4',6,7-trihydroxyaurone as the active compound in binahong leaves with 5-fluorouracil against COX-2 and inhibitors of κ B kinases (IKK) protein.

Methods

Extraction and fractionation

One kilogram of dried binahong leaves was extracted using ethanol 70% and using the maceration method for 5 days. The remaceration was then conducted for 2 days. The extract was fractionated using n-hexane. The evaporation of binahong fraction used a rotary evaporator on 60°C, and the result was a thick NFB.

Phytochemical Screening using TLC

Phytochemical screening of NFB was performed to determine the presence of flavonoids. The TLC plate used silica GF₂₅₄ while the mobile phase was chloroform. The plate was dried in the oven at 110°C for 30 min and stored in a dry place. Samples were prepared and applied with 1–10 µl volumes to the origins of a silica GF₂₅₄ 1 cm above its bottom with a capillary tube's help. After the application of the sample on the plate, they were kept in TLC chambers. The mobile phase was allowed to move through the adsorbent phase up of the plate. The identification of the plate was carried out under 254 and 366 nm UV lights.

Antioxidant test using DPPH method

The stock solution was prepared by dissolving 15.8 mg of DPPH with 25 mL methanol, while the NFB was 50 mg on 50 mL methanol. The range concentration of NFB was 100, 200, 300, 400, and 500 µg/mL. This antioxidant test standard was quercetin with range concentration: 1, 2, 3, 4, and 5 µg/mL. Every tube contained 2 mL of NFB, 2 mL DPPH solution, and 6 mL methanol for every concentration. The reduction of absorbance was measured after 30 min. The concentration of NFB is required to reduce absorbance by 50% of the IC₅₀. The smaller the IC₅₀ value was, the greater the antioxidant activity of the NFB would be.

MTT cytotoxic assay test

The WiDr human colon cancer cells were seeded until confluent, and then, 5×10³ cells were plated into a 96-well microplate and incubated at 37°C for 48 h. The medium was removed and rinsed by phosphate-buffered saline. The stock solution of NFB and 5-fluorouracil was prepared with dimethyl sulfoxide (DMSO). The range of NFB was 15.625, 31.25, 62.5, 125, 250, and 500 µg/mL while the range concentration of 5-fluorouracil was 12.5, 25, 50, 100, and 200 µg/mL. The treated cells were incubated in the same condition as the previous step. The medium was then eliminated and added by MTT reagent 5 mg/mL in each well. The reaction between MTT and succinate hydrogenase of cells led to the formation of formazan crystals in 4–5 h.

After 5 h of incubation, 100 µL of sodium dodecyl sulfate (SDS) was added to each well to dissolve the formazan crystals. After that, the microplate was incubated for 24 h. Finally, the formazan complex was measured by an ELISA reader at 595 nm visible wavelength.

MTT cytotoxic assay test in combination

The cytotoxic effects of NFB and 5-fluorouracil on WiDr cells were tested using MTT reagent. The combination was designed to investigate the encouragement of one extract when the dose of the other extract remained fixed. The concentration range for the combination was IC₅₀ of extract and IC₅₀ of drugs that have already been obtained on previous cytotoxic tests. Both NFB and 5-fluorouracil concentrations varied in combination with IC₅₀ values of 1/2, 1/4, 1/8, and 1/16. This assay was based on the metabolic reduction of MTT to lead to the formation of formazan crystal. 5×10³ cells were plated into a 96-well microplate and incubated at 37°C for 48 h. Each well plate was seeded with 10 µL of 5×10³ cells and added with different doses of NFB and 5-fluorouracil that was diluted with DMSO. After 24 h, the medium was then eliminated and added by MTT 5 mg/mL in each well. The reaction between MTT and succinate hydrogenase of cells led to the formation of formazan crystals in 4–5 h. After 5 h of incubation, 100 µL of SDS was added to each well to dissolve the formazan crystals. After that, the 96-well microplate was incubated for 24 h. Finally, the formazan complex was measured by an ELISA reader at 595 nm visible wavelength.

In silico test using molecular docking

Computational studies in a virtual identification using molecular docking were usually used to screen natural product's database as bioactive compounds. First, IKK protein structure (PDB ID: 2GNG) and COX-2 protein structure (PDB ID: 6COX) were downloaded from a website "www.rcsb.org." The Autodock Vina application was used to select the Grid Box submenu and adjust the docking area of proteins and ligands. The area was related to the root mean square deviation (RMSD) value at each conformation. RMSD value of <2Å was the selected value. The visualization process is then carried out to determine the position of the bond between the protein and the ligand in 3D using DS Visualizer.

Results

This research began with the extraction process on the simplicia powder of binahong leaves (*A. cordifolia* (Tenore) Steen.) using the maceration method with 70% ethanol solvent ratio 1:10 (1 kg powder:10 l 70%

ethanol). A total of 1 kg of binahong leaf powder were put into a closed vessel and then immersed for 5 days in 70% ethanol solvent. The obtained maceration was then filtered using a flannel cloth and followed by a remaceration process for 2 days with the same process. The maceration results showed a total liquid extract of 4.5 L. Furthermore, the fractionation process was carried out from the ethanol extract partitioned liquid-liquid with n-hexane as a solvent with a ratio of 1:1. Based on the fractionation process of the binahong leaf of n-hexane extract, a fraction of 2 L was obtained. The NFB leaves were then concentrated by evaporating using a rotary evaporator at 60°C. Thus, the dense fraction of binahong leaves was found as much as 3.4 g with a yield percentage of 3.43%.

The TLC method was selected as a qualitative test for chemical compounds in NFB using the silica gel GF254 as stationary phase and the chloroform as mobile phase. The motion phase was first saturated in the chamber. Furthermore, NFB as a sample was spotted using a capillary tube on the GF254 silica gel plate measuring 10 cm × 5 cm and put into the chamber, waiting to reach the elution limit. After the limit was reached, the stationary phase was then dried, and the spots were observed under UV rays of 254 nm and 366 nm, while for the color reaction, TLC plates were evaporated using ammonia. Based on the qualitative test of flavonoids using the TLC method, it was suspected that NFB contained flavonoid class compounds in spots number 4 and 5 [Figure 1]. Based on observations under UV light 254, brown spots observed under UV light 366 showed a purple glow. It is in line with Anderson and Markham's (2006) statement that flavonoid compounds would show blackish-brown spots under 254 nm UV, while under 366 nm UV, the spots would show luminescence. The spots appeared both at UV 254 nm and UV 366 nm observations [6]. The spot number 4 and 5 resulted in Rf = 0.85 and 0.925, which showed that NFB contained flavonoids [Figure 1].

In NFB, the antioxidant activity was analyzed using the DPPH method. The positive control used in this study's antioxidant test was quercetin. Based on Fowler and Koffas research, binahong leaves contained flavonoid compounds in the flavonol group quercetin [7]. The antioxidant test obtained an IC₅₀ value of NFB and quercetin (positive control), which was 2851 and 6.87 µg/mL, respectively [Figures 2 and 3].

The MTT assay was selected as a method in the cytotoxic test. It was carried out by reducing the yellow salt of tetrazolium MTT through the reductase system; thus, the cytotoxic activity of FHDB in cells could be determined. Colon cancer WiDr was compared with chemotherapy agent 5-fluorouracil. The absorption of data was read using an ELISA reader at a wavelength of 595 nm. At the lowest concentration of 31.25 µg/mL, the NFB could kill WiDr cells by 29.91%, while at the highest concentration of 500 µg/mL, NFB could kill WiDr cells by 100%. The parameter used in the analysis of the

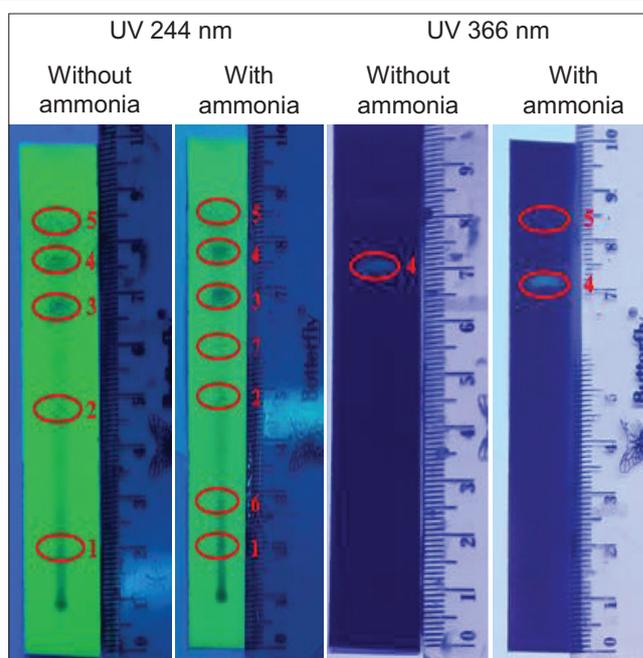


Figure 1: Flavonoid of n-hexane fraction of binahong (NFB) observations in number 4 and 5 with an Rf = 0.85 and 0.925, showing that NFB contained flavonoids compounds

cytotoxic test was the IC₅₀ value. In the cell percentage graph, the IC₅₀ value was 191 µg/mL [Figure 4]. When it was compared with chemotherapy agent 5-fluorouracil at the lowest concentration of 12.5 µg/mL, it could kill WiDr cells by 30.96%, and at the highest concentration of 200 µg/mL, it could kill WiDr cells by 42.47%. The calculations were then carried out, and the IC₅₀ of a 5-fluorouracil value obtained was 311 µg/mL [Figure 5].

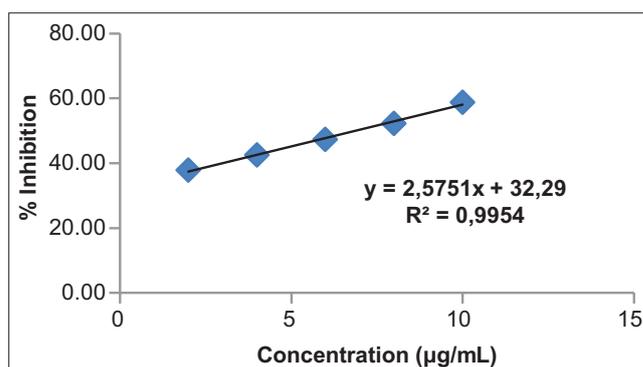


Figure 2: Inhibition graphic of quercetin

A combination test on NFB with chemotherapy agent 5-fluorouracil was performed using the MTT assay to determine the potency of the two samples when they were combined and tested against WiDr cancer cells. Ideally, when a chemotherapy agent was combined with samples from natural ingredients, it would have a synergistic effect against cancer cells and reduce chemotherapy agents' side effects; thus, it could provide optimal benefits [8]. Combination tests were carried out after obtaining an IC₅₀ value of cytotoxic tests carried out previously. They were used as the basis for the preparation of concentration series. The CI value obtained from the combination test was

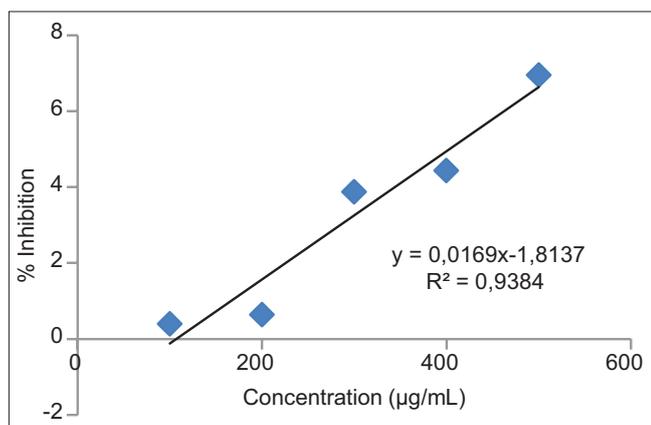


Figure 3: Inhibition graphic of n-hexane fraction of binahong

between 0.80–28.92 [Table 1]. In other words, the more significant the dose of FHDB combination was, the smaller the dose of chemotherapy agent 5-fluorouracil would be, and the more synergistic the effect would be.

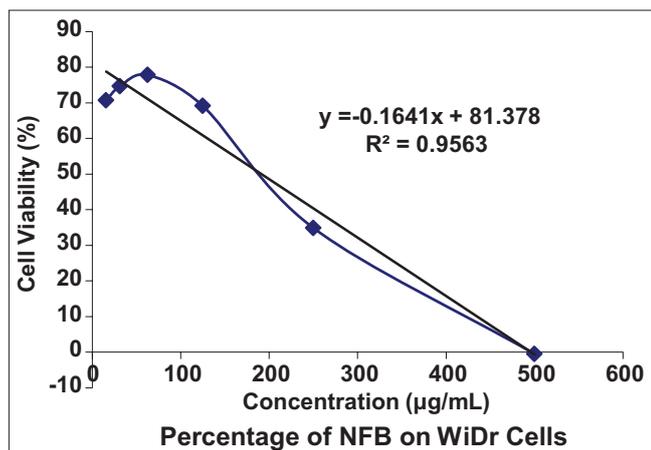


Figure 4: Graphic percentage of n-hexane fraction of binahong on WiDr cells

Based on the combination of NFB and 5-fluorouracil, CI calculation 0.80 was obtained. CI value ranged from 0.7 to 0.9, and it indicated a mild synergistic activity, going up to medium [Figures 6 and 7].

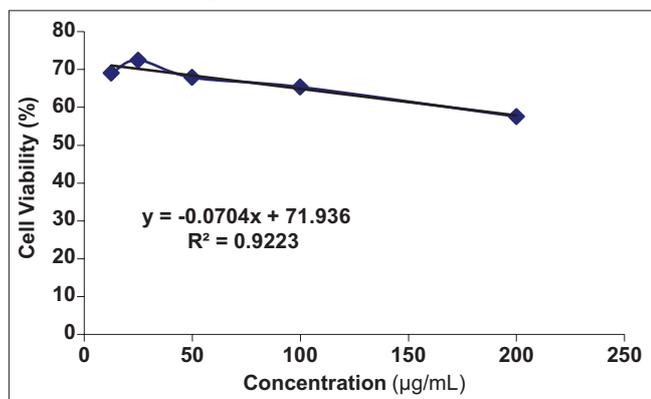


Figure 5: Graphic percentage of 5-fluorouracil on WiDr cells

In this study, the *in silico* test used the molecular docking method carried out using the Autodock Vina application and DS Visualizer to visualize the results

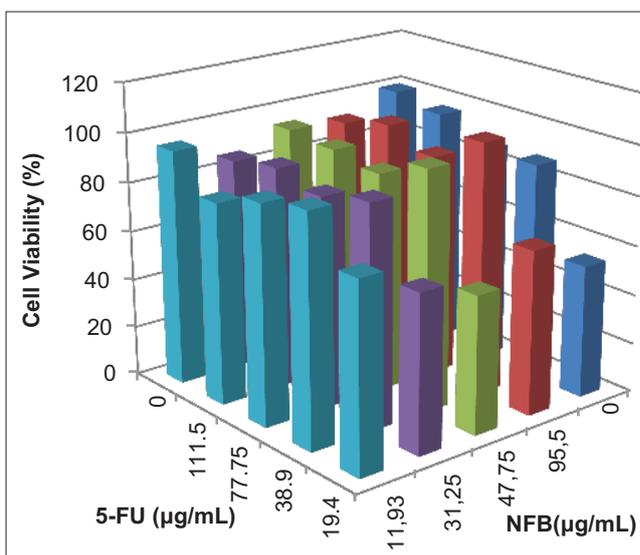


Figure 6: Viability of graphic of combination between n-hexane fraction of binahong and 5-fluorouracil on WiDr cells

in two dimensions and three dimensions. YASARA was also used to function and measure the RMSD value of the test compound. The *in silico* test using molecular docking showed that the lowest docking score of 4',

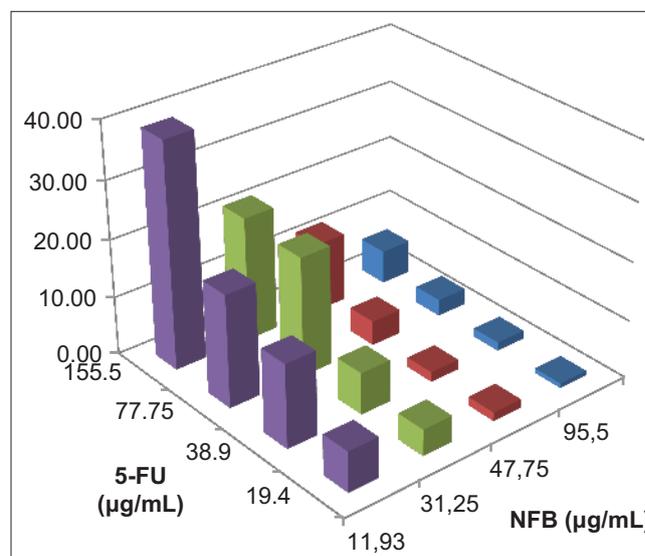


Figure 7: The score of combination index

6, 7-trihydroxyaurone compound was obtained in IKK protein with docking score -9.0 kcal/mol and an RMSD value of 0.184 Å when it was compared to the chemotherapy agent of 5-FU [Figure 8]. Similarly, the compound of 4', 6, 7-trihydroxyauron was targeted at COX-2 protein with a docking score of -8.1 kcal/mol with an RMSD value of 1.659 Å [Figure 9].

Table 1: The combination index score of combination between n-hexane fraction of binahong and 5-fluorouracil on WiDr cells

Concentration of n-hexane fraction of binahong (µg/mL)	Concentration of 5-fluorouracil (µg/mL)			
	155.5	77.75	38.9	19.4
95.5	4.32	2.86	1.48	0.80
47.75	8.86	4.21	1.68	1.48
31.25	15.90	20.54	7.20	4.52
11.93	28.92	19.41	14.44	6.99

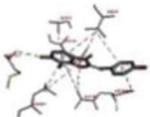
Active compounds	3D structure	Root mean square deviation (Å)	Docking score (kcal/mol)	Conformation
5-fluorouracil		1.552	-5.3	2
4',6,7-trihydroxyaurone		0.184	-9.0	2

Figure 8: 3D structure and docking score of n-hexane fraction of binahong on IKK protein

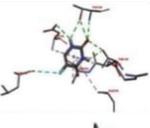
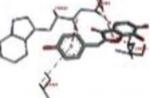
Active compounds	3D structure	Root mean square deviation (Å)	Docking Score (kcal/mol)	Conformation
5-fluorouracil		1.524	-7.6	6
4',6,7-trihydroxyaurone		1.659	-8.1	6

Figure 9: 3D structure and docking score of n-hexane fraction of binahong on COX-2 protein

Discussion

The potential of NFB as co-chemotherapy on WiDr colon cancer cells has not yet been evaluated. In this study, WiDr cells represented a mammalian cell line derived from a 78-year-old woman's colon. WiDr cells were derived from the other colon cancer cell, namely, HT-29 [9]. Free radical scavenging effects of NFB at different concentrations were measured with quercetin as a positive control compound using the DPPH method. The result demonstrated that NFB had a weak activity with an IC_{50} value of 2851 $\mu\text{g/mL}$ compared to quercetin as a positive control with an IC_{50} value of 6.87 $\mu\text{g/mL}$. It was due to NFB not containing enough flavonoids and that there was not enough potency in the DPPH free radical reduction process.

Further on this research, the cytotoxic activity was observed to determine the potency of NFB in inhibiting WiDr colon cancer cell proliferation. The cytotoxic activity was observed using the MTT assay. The results showed that NFB was quite strong to inhibit WiDr cell growth with an IC_{50} value of 191 $\mu\text{g/mL}$ while the IC_{50} value of 5-fluorouracil was 311 $\mu\text{g/mL}$. The next step was investigating NFB's effect in combination with 5-fluorouracil, a standard chemotherapeutic agent on WiDr colon cancer cells. The combination of 1/2 IC_{50} , 1/4 IC_{50} , 1/8 IC_{50} , and 1/16 IC_{50} , either NFB or 5-fluorouracil, showed a synergistic effect in inhibiting WiDr cell growth, which scored 0.8 ($CI < 1$). It indicated that the compounds of NFB had the potential to be developed as co-chemotherapeutic agents on WiDr colon cancer cells.

On *in silico* test using molecular docking, the 4', 6, 7-trihydroxyaurone compound was obtained

in IKK protein with a docking score of -9.0 kcal/mol (RMSD value of 0.184 Å), when it was compared to the agent 5-FU chemotherapy. Similarly, compound 4',6,7-trihydroxyaurone was targeted at COX-2 protein with a docking score of -8.1 kcal/mol (RMSD value of 1.659 Å). The nuclear factor-kappa B (NF- κ B) signaling pathway was a key regulator of inflammation and correlated with carcinogenesis [10]. In an inactive state, NF- κ B dimers were bound to IKK. The NF- κ B signaling pathway was initiated by cytokines, such as interleukin-1 and tumor necrosis factor, which stimulated the IKK complex. The three NF- κ B essential modulators (IKK α , IKK β , and IKK γ or NEMO) were involved in the classical pathway. In colon cancer, IKKB induced the activation of NF- κ B in intestinal epithelial cells and its corresponding inflammation appeared to have an essential role in tumor formation [11]. On the other hand, COX-2 proteins were overexpressed in about 80% of colon cancers and 40% of colon adenomas relative to normal mucosa [12]. This overexpression might give prognostic information in human colon cancers and other epithelial cancers. Therefore, blocking the IKK and COX-2 signaling pathway with molecular docking could represent the potential 4', 6, 7-trihydroxyaurone compound on NFB.

Conclusion

The NFB had the potential to inhibit cell growth and had a synergistic effect in combination with 5-fluorouracil on WiDr colon cancer cells line based on *in vitro* and *in silico* assay.

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