

Research Article

The Synergistic Effect of Combination of Pentagamavunone-1 with Diosmin, Galangin, and Piperine in WiDr Colon Cancer Cells: *In vitro* and Target Protein Prediction

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ABSTRACT

Pentagamavunone-1 (PGV-1) is a curcumin analog with a prominent anti-cancer potency *in vitro* and *in vivo* for several cancer types, including colon cancer. Combining PGV-1 with natural compounds such as diosmin, galangin, and piperine can enhance its effectiveness due to their promising chemoprevention properties. We aimed to evaluate the effectiveness of combining PGV-1 with diosmin, galangin, or piperine for colon cancer by using *in vitro* and bioinformatic approaches to predict their target proteins. WiDr cells were used as a model for colon adenocarcinoma (COAD). The cell viability under a single or combination treatment of PGV-1 and diosmin, galangin, or piperine was evaluated using direct counting by the trypan blue exclusion test. SwissTargetProtein, UALCAN, and OncoLnc were utilized to predict target proteins of the compounds in COAD, the expression level of target proteins in COAD, and the survival rate of patients with overexpressed target proteins, respectively. The IC₅₀ values for PGV-1, diosmin, galangin, and piperine were 2.8×10^{-2} µg/mL, 81 µg/mL, 7 µg/mL, and 172 µg/mL, respectively. All the tested natural compounds showed synergistic effects when combined with PGV-1 at low concentrations. Eleven proteins that were overexpressed in COAD were identified as potential targets. Overlapped predicted targets of PGV-1 and galangin or piperine were CDK1, MET, and TOP2A. The high expression of another set of predicted target proteins, SCD, CA9, and SQLE, led to lower survival rates in COAD patients. We concluded that combinations of PGV-1 with natural compounds can synergistically enhance its anti-cancer activity for colon cancer.

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INTRODUCTION

Colon cancer is the most newly diagnosed cancer cases globally in 2020 after breast, lung, prostate, and nonmelanoma of skin (Sung et al. 2021). Despite having the highest level of a 5-year survival among the other 18 cancers or cancer groups in Southeast Asia, the survival rates widely vary among countries (Allemani et al. 2018). On the other hand, the

evolving lifestyle including diet may contribute to higher incidence of colon and rectal cancer in young adults (Stoffel & Murphy 2021). Colorectal cancer is in the top four causes of cancer-related death worldwide (Argiles et al. 2020). In the United States alone, colon cancer ranks as the third largest mortality in cancer after breast cancer and lung and bronchial cancer during 2014–2018 (Siegel et al. 2021). The adjuvant treatment by using chemotherapy agents such as 5-fluorouracil (5-FU) and oxaliplatin is involved in the treatment. However, the risk including contraindications, liver and renal dysfunction, and heart failure, have to be monitored and assessed constantly (Argiles et al. 2020). Thus, the advancement in colon cancer treatment as well as the development of highly potent chemotherapy with low side effects is important.

A curcumin analog Pentagamavunon-1 (PGV-1) (Figure 1) has been developed initially to support chemotherapy as a combination agent (co-chemotherapy) for anti-cancer. PGV-1 sensitizes doxorubicin-resistant breast cancer cells (Meiyanto et al. 2014) and synergistically increases the effectiveness of 5-FU in colon cancer cells (Meiyanto et al. 2018). Eventually, PGV-1 itself has been proven as a potent anti-cancer candidate with low side effects and minimal relapse for leukemia, breast adenocarcinoma, cervical cancer, uterine cancer, and pancreatic cancer (Lestari et al. 2019). *In vitro* and *in vivo* assays show that PGV-1 suppresses tumour cell growth and migration via various mechanisms, e.g. metabolic and enzymatic regulation, cell cycle arrest and senescence, and signal transduction, in leukemia (Lestari et al. 2019), breast cancer (Meiyanto et al. 2019; Wulandari et al. 2020; Meiyanto et al. 2021), and colon cancer (Wulandari et al. 2021). Indeed, PGV-1 is a promising anti-cancer candidate.

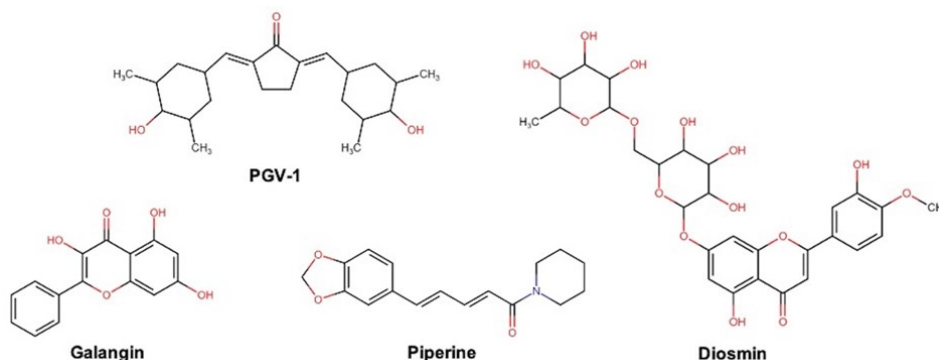


Figure 1. Chemical structures of PGV-1, diosmin, galangin, and piperine (Musyayyadah et al. 2021; Hasbiyani et al. 2021; Endah et al. 2021).

Considering its high cytotoxicity, PGV-1 can be combined with less cytotoxic compounds to lower the PGV-1's dosage but maintain its effectiveness toward cancer cells. Numerous compounds from natural sources have been reported to have cytotoxic activities and are conceivable as combination therapy for colorectal cancer (Rejhova et al. 2018). Recently, combination of PGV-1 with diosmin, galangin, or piperine (Figure 1) presented promising anti-cancer synergisms in triple negative breast cancer cells (Musyayyadah et al. 2021; Hasbiyani et al. 2021; Endah et al. 2021). Diosmin is a methoxy flavonoid that can be found abundantly in *Citrus* sp. (Chen et al. 2020; Utomo et al. 2020) with various pharmacological activities including anti-cancer activity (Zheng et al. 2020) while maintaining its safety (Russo et al. 2018). Another flavonoid that also possesses anti-cancer activities to various cancers is galangin from the rhizome of *Alpinia galanga* (Huang et al. 2020). Additionally, alkaloid piperine found in the most widely used spices, black pepper or other Piper species, is also a promising cancer chemoprevention agent

based on its activity on several cancer cell lines (Manayi et al. 2018). Reinforcing the mitotic failure as proven *in vitro*, the bioinformatic analysis confirms that PGV-1 and those above natural compounds target cell cycle protein regulators (Musyayyadah et al. 2021; Hasbiyani et al. 2021; Endah et al. 2021).

Following the combination for breast cancer cells, we further evaluate the PGV-1 combination with diosmin, galangin, or piperine in colon cancer cells. Those three compounds were chosen because of their safety and well-known health beneficial effects. In this study we assessed the synergistic effect of PGV-1 by diosmin, galangin, or piperine in colon cancer cells by a cell viability assay. Thus, their combination with PGV-1 would provide an alternative co-chemotherapy treatment. Further evaluation on PGV-1 and the natural compounds' target proteins that are overexpressed in colon cancer and their effects on patients' survival rate was performed by bioinformatic analysis.

MATERIALS AND METHODS

Materials

PGV-1 with 95% purity was obtained from Cancer Chemoprevention Research Centre (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada (UGM). Diosmin (catalog number #Y0000094), galangin (#282200) and piperine (#P49007) were purchased from Sigma Aldrich (USA). The tested compound was firstly dissolved in dimethyl sulfoxide (DMSO, Sigma) as the stock solution with a concentration of 100,000 μM . The serial dilution was then prepared by diluting the stock in the complete medium with a maximum DMSO concentration of less than 1% in the final concentration. The solution was always prepared freshly before the cell treatment.

Methods

Cell Culture

WiDr cells representing colon adenocarcinoma (COAD) (Chen et al. 1987) as the most common colon cancer were a collection of CCRC, Faculty of Pharmacy, Universitas Gadjah Mada (Wulandari et al. 2021). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI) medium (Gibco, USA) complemented with 10% fetal bovine serum (FBS) (Gibco, USA) and a final concentration of 50-100 IU/mL penicillin and 50-100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco, USA). The cells were incubated with 5% CO_2 in the 37 °C incubator.

Cell Viability Assay with Direct Counting Method

WiDr cells (1.5×10^4 /well) were distributed into 24-well plates. After reaching about 80% confluency after overnight incubation, the cells were treated with a serial concentration of PGV-1 or natural compounds. Cells were incubated for 24 h and then assayed using the trypan blue exclusion test as modified from previous method (Strober 2015). One part of the cell suspension was mixed with one part of 0.4% trypan blue, incubated for 3 min at room temperature, and the viable cells as unstained cells were counted on the haemocytometer under a microscope. Each concentration was done in triplicate with three times counting for each well. The inhibitory concentration of 50% (IC_{50}) value was calculated by a regression analysis on log concentration versus percentage of cell viability (Musyayyadah et al. 2021). The IC_{50} values were then used as a guide for the combination assay. The combination index (CI) was calculated as described previously (Musyayyadah et al. 2021) by normalizing the percentage of cell viability with respect to the IC_{50} value.

Target Protein Prediction of PGV-1 and Natural Compounds in Colon Cancer

SwissTargetProtein (<http://www.swisstargetprediction.ch>) was used to identify the target proteins (Daina et al. 2019) for PGV-1, diosmin, galangin, and piperine. The UALCAN database (<http://ualcan.path.uab.edu>) was used to access the TCGA dataset for proteins that are overexpressed in colon cancer. To determine overlapping protein targets of the compounds and the overexpressed protein, diagram Venn was utilized using InteractiVenn (<http://www.interactivenn.net>) (Heberle et al. 2015). A MacBook Air with 1,8 GHz Intel Core i5 and 8 GB RAM was used for this analysis.

Expression of Predicted Target Proteins in COAD

UALCAN provides access to the TCGA dataset for the expression of predicted protein targets in normal cells and COAD (Chandrashekar et al. 2017). The collected data from the TCGA dataset was then processed using GraphPad Prism V9. A Hewlett-Packard laptop with AMD Ryzen 3 processor and 4 GB RAM was used for this analysis.

Survival Rate of COAD Patients with Overexpressed Target Proteins

The TCGA dataset for survival rate was accessed using OncoLnc (<http://www.oncolnc.org>) (Anaya 2016). The upper and lower percentiles were both 50:50 to create the optimized result based on the number of samples of COAD patients. All the collected information was prepared using GraphPad Prism V9. The same computer specification as the section above was used for this analysis.

Statistical Analysis

Statistical significance was assayed using GraphPad Prism V9. The parametric test used Student's *t*-test to determine the significance between groups with a 95% confidence level ($p < 0.05$). The normality data was confirmed using the One-Sample Kolmogorov-Smirnov test (Musyayyadah et al. 2021).

RESULTS AND DISCUSSION

Cytotoxicity of PGV-1, Diosmin, Galangin, and Piperine in WiDr Cells

Cytotoxic effect of PGV-1, diosmin, galangin and piperine on WiDr cells was carried out by the direct counting method. Administration of PGV-1 (0.025-2 μ M) or diosmin, galangin, and piperine (10-1,000 μ M) for 24 h showed that all compounds had a dose-dependent cytotoxic activity in WiDr cells (Figure 2). As predicted, PGV-1 showed a remarkable cytotoxic activity with an IC_{50} of 0.08 μ M (Figure 2a). Among the tested natural compounds galangin exhibited the strongest cytotoxicity at IC_{50} of 26 μ M, followed by diosmin and piperine, 133 μ M and 603 μ M, indicating moderate cytotoxicity and not cytotoxic, respectively (Figure 2b, Table 1), based on cytotoxicity classification by World Health Organization (WHO) (Niyibizi et al. 2020).

The Combination Effect of PGV-1 and Diosmin/Galangin/Piperine in WiDr Cells

Based on the IC_{50} values, a combination cytotoxicity test was executed to determine the effect of the natural compounds in increasing PGV-1's effect. One-eighth, quarter, and half of IC_{50} of natural compounds was combined with $\frac{1}{2}$ IC_{50} or IC_{50} of PGV-1. The same direct counting method as the single cytotoxicity assay was employed and the CI was calculated for

Table 1. The IC₅₀ values of PGV-1, diosmin, galangin, and piperine on WiDr cells.

Compound	IC ₅₀
PGV-1	0.08 μM (2.8×10 ⁻² μg/mL)
Diosmin	133 μM (81 μg/mL)
Galangin	26 μM (7 μg/mL)
Piperine	603 μM (172 μg/mL)

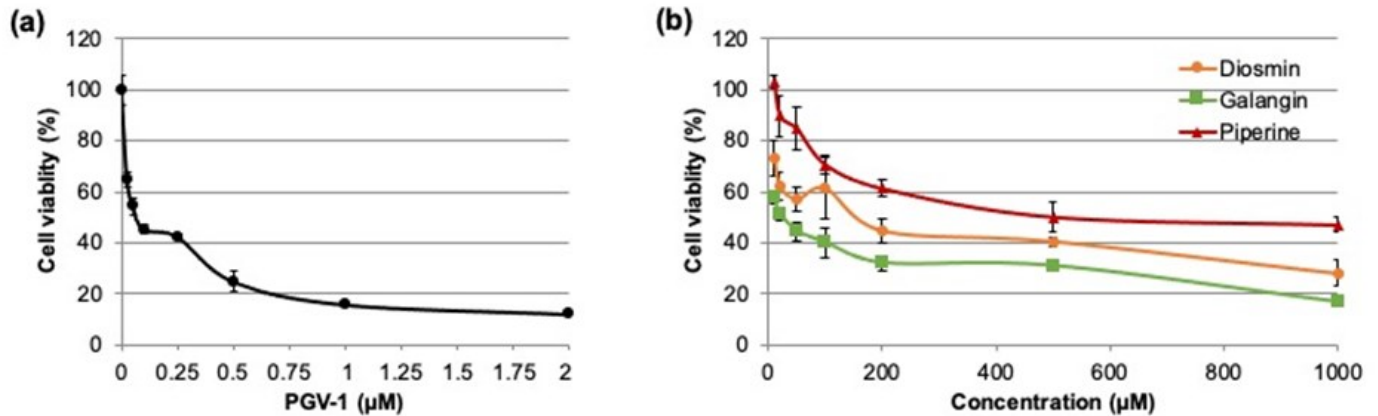


Figure 2. Cytotoxicity of PGV-1 and natural compounds on WiDr cells. Cytotoxicity of PGV-1 (a); diosmin, galangin, and piperine (b). The cell viability was assayed by direct counting and the percentage is presented as average ± SE (*n*=3). The IC₅₀ value was calculated by a regression analysis on log concentration versus percentage of cell viability.

each concentration combination point by normalizing the percentage of cell viability to the respective IC₅₀. CI value of less than 0.9 indicates a synergistic effect (Ikawati & Septisetyani 2018). Even though piperine was not toxic in WiDr cells, almost all the combinations with PGV-1 showed synergistic effects (Figure 3c, Table 2). The combination of PGV-1 and piperine at low concentration resulted in a CI value of 0.2 demonstrating a strong synergy (Table 2). Likewise, all the tested concentrations of galangin gave synergy when combined with low concentration of PGV-1 (Figure 3b, Table 2). Diosmin, on the other hand, showed synergistic effects in all the tested combinations (CI 0.3-0.9) (Figure 3a, Table 2). Taken together, the three tested natural compounds have the ability to inhibit WiDr cell proliferation in combination with PGV-1.

Table 2. The combination index values.

Compound (μM)	Combination index	
	PGV-1 (μM)	
	0.04	0.08
Diosmin		
17	0.5*	0.8*
33	0.5*	0.5*
67	0.6*	0.5*
Galangin		
3	0.7*	1.7
6	0.5*	1.0
13	0.5*	0.9
Piperine		
75	0.2*	0.6*
150	0.4*	0.8*
300	0.7*	1.3

Remark: asterisk indicates synergistic combination.

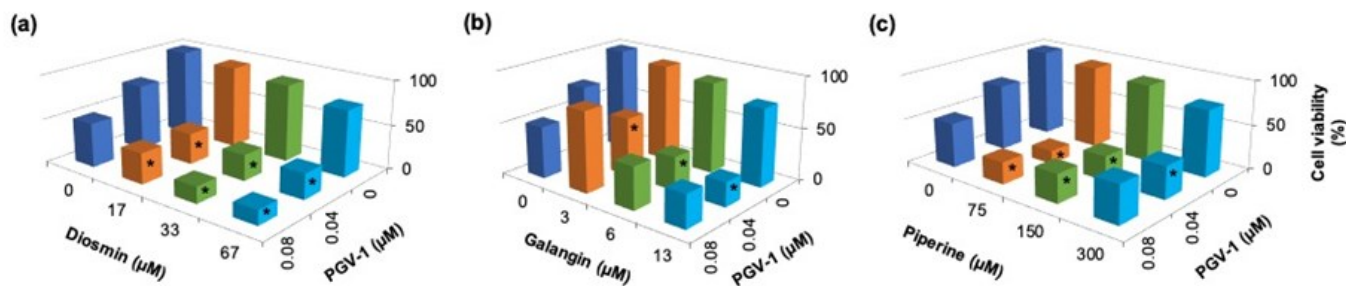


Figure 3. The effect of combination of PGV-1 and diosmin (a), galangin (b), or piperine (c) on WiDr cells. Cells were treated with PGV-1 at $\frac{1}{2}$ or IC_{50} and natural compounds at $\frac{1}{8}$, $\frac{1}{4}$, or $\frac{1}{2}$ IC_{50} in triplicate. The combination index was calculated as previously described by normalizing the percentage of cell viability to the respective IC_{50} value. Asterisk indicates synergistic combination.

Predicted Target Proteins of PGV1 and Diosmin/Galangin/Piperine in Colon Cancer

Predicted target proteins of PGV-1, diosmin, galangin and piperine were explored using the SwissTargetPrediction database ensuing in 100 protein targets from each compound (Figure 4a-c). To find out whether the target proteins of each compound are overexpressed in colon cancer, we searched for overexpressed proteins in the TCGA database using UALCAN, resulting in 300 overexpressed proteins in COAD (Figure 4a-c). To discover the overlap between the PGV-1 and/or each natural compound target protein with the overexpressed protein in COAD, we processed them by using a Venn diagram. Of the 100 predicted compound target proteins, the following proteins are overlapping with overexpressed proteins in COAD: PGV-1: SCD, TOP2A, CDK1, and MET (Figure 4a); diosmin: MMP7, CA9, MMP1, SQLE, and AURKA (Figure 4a); galangin: CA9, MMP3, NEK2, MET, CDK1, and TOP2A (Figure 4b); piperine: AURKA and CDK1 (Figure 4c). A summary of overlapping proteins (11 proteins) can be seen in Table 3.

Expression of Predicted Target Proteins in COAD and the Survival Analysis

To differentiate the expression of the above target proteins in normal cells and cancer cells, we further explored data from the TCGA database. To facilitate access to TCGA, third party websites such as UALCAN were used. We confirmed that the eleven target proteins are significantly overexpressed in COAD when compared to normal cells, all with p values lower than 1×10^{-11} (Figure 5a). Each target protein was then examined for its impact on the patients' survival. From those eleven proteins, we then analysed the effect of high and low expression of the protein in COAD patients. While overexpression 8 of 11 proteins did not negatively

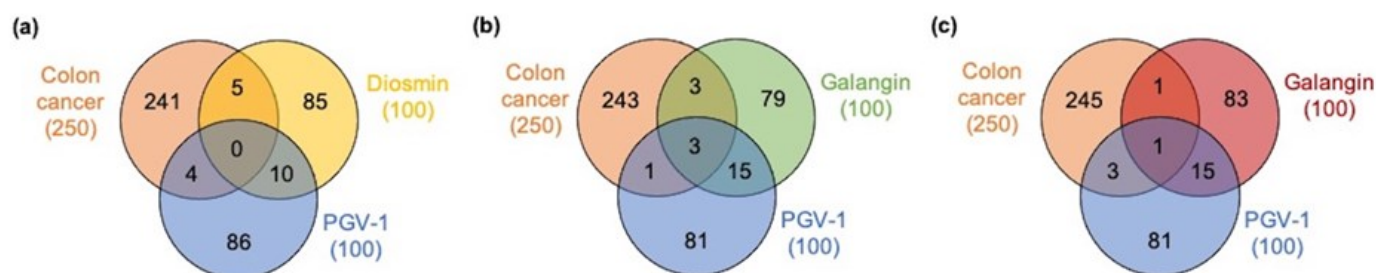


Figure 4. Intercept between overexpressed proteins in colon cancer and target proteins of PGV-1 and diosmin (a), galangin (b), and piperine (c). Predicted target proteins of the compounds was acquired from SwissTargetPrediction. Overexpressed proteins in colon adenocarcinoma were acquired from UALCAN. Venn diagram was created by InteractiVenn.

Table 3. Overlapped target proteins.

Target	Common name	Compound
Cyclin-dependent kinase 1	CDK1*	PGV-1 and galangin/piperine
Hepatocyte growth factor receptor	MET*	PGV-1 and galangin
DNA topoisomerase II alpha	TOP2A*	PGV-1 and galangin
Acyl-CoA desaturase	SCD	PGV-1
Carbonic anhydrase IX	CA9	diosmin, galangin
Serine/threonine-protein kinase Aurora-A	AURKA	diosmin, piperine
Matrix metalloproteinase 7	MMP7	diosmin
Matrix metalloproteinase 3	MMP3	galangin
Matrix metalloproteinase 1	MMP1	diosmin
Squalene monooxygenase	SQLE	diosmin
Serine/threonine-protein kinase NEK2	NEK2	galangin

Remark: asterisk indicates indicates overlapped target protein of PGV-1 and the natural compound.

affect patients' survival rate (data not shown), overexpression of SCD ($p = 0.726$), CA9 ($p = 0.678$), and SQLE ($p = 0.462$) have shown to decrease the survival rate in COAD patients (Figure 5b).

Discussion

We confirmed the high potency of PGV-1 against WiDr colon cancer cells. In this study, we obtained the IC_{50} value of 0.08 μM , which is lower than those previously reported, 18 μM (Meiyanto et al. 2018) and 12 μM (Wulandari et al. 2021). Possibly because of the difference of cell viability assays that were used. In this study we employed a direct counting method rather than an enzymatic colorimetric assay (Aslanturk 2017; Lestari et al. 2019). Nevertheless, the tested natural compounds displayed a similar cytotoxicity profile in WiDr cells as described formerly in 4T1 triple negative breast cancer cells, from the highest to the lowest one is galangin, diosmin, and piperine at the IC_{50} of 120 μM (Hasbiyani et al. 2021), 389 μM (Musyayyadah et al. 2021), and 800 μM (Endah et al. 2021), respectively. Accordingly, the most potent among the tested natural compounds is galangin, followed by diosmin and piperine (Figure 2c). Based on the classification by WHO (Niyibizi et al. 2020), galangin and diosmin are cytotoxic ($IC_{50} < 90 \mu\text{g}/\text{mL}$), while piperine is not ($IC_{50} > 90 \mu\text{g}/\text{mL}$). Taken together, galangin is the most promising chemoprevention agent among the three tested natural compounds.

Cytotoxic combination assay is a convenient approach to measure the potency of a combination in cancer cells (Avand et al. 2018). In correspondence with the cytotoxicity, the combination assay revealed that galangin shows a solid synergistic effect with low concentration of PGV-1 (Figure 3, Table 2). Each concentration of diosmin also gives synergistic effects in colon cancer cells, similar to the results in breast cancer cells (Musyayyadah et al. 2021). On the contrary to the finding in 4T1 cells (Endah et al. 2021), piperine shows synergism with PGV-1 in WiDr cells, especially at the low concentration of PGV-1. Based on these data, all the three tested natural compounds are promising to be combined with PGV-1.

It is interesting that galangin, diosmin, and piperine act similarly in terms of potentiating of PGV-1 in colon cancer cells despite the different cytotoxic potency. A follow up bioinformatic analysis uncovered that diosmin, galangin, and piperine target the same and different overexpressed proteins in COAD as PGV-1 (Figure 4). Those different protein targets may contribute in resulting a synergistic effect when combined with PGV-1. Most of the overexpressed proteins in COAD that are targets of PGV-1 have roles as catalytic enzymes in degrading extracellular

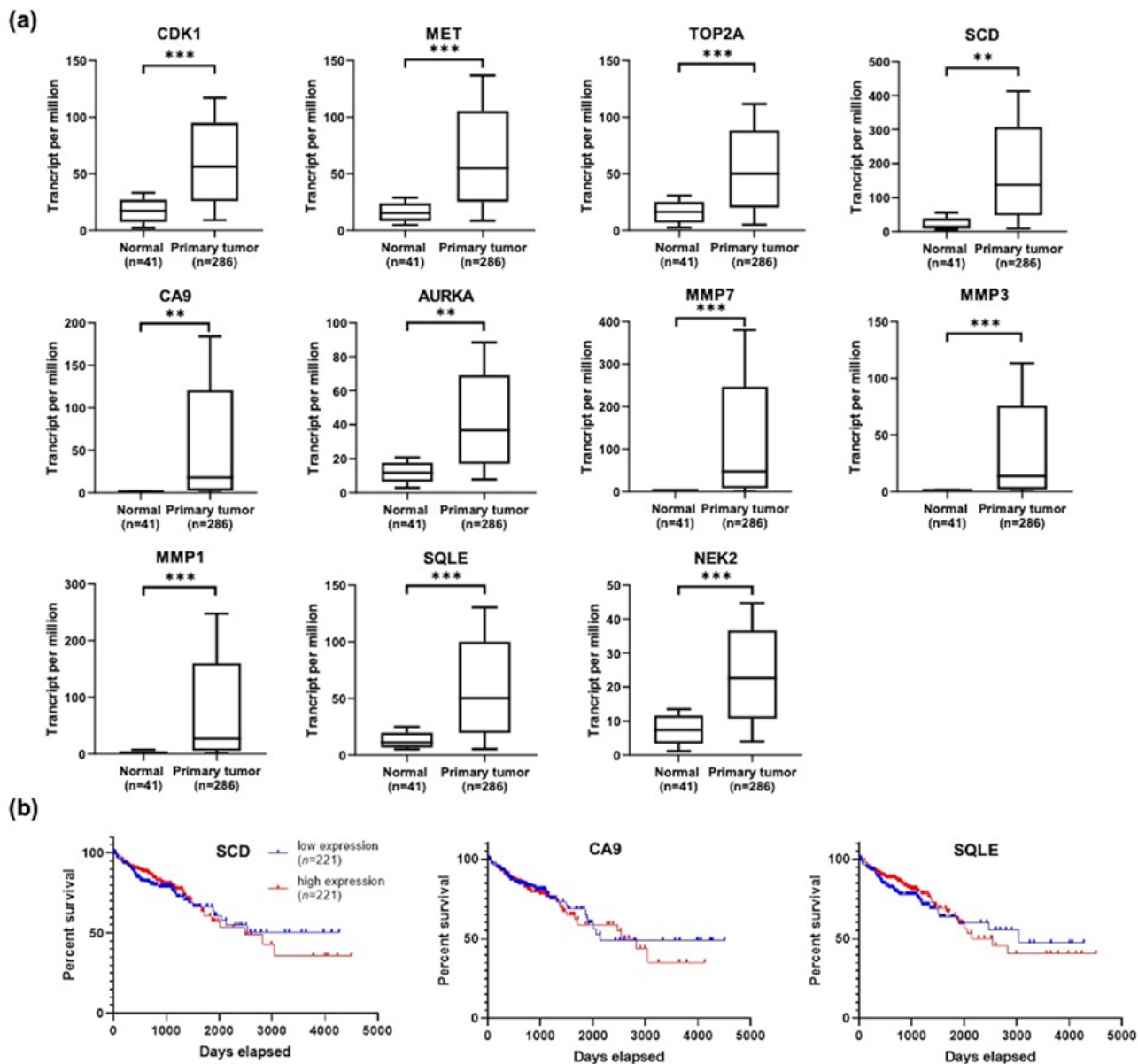


Figure 5. Expression of target proteins in colon cancer (a) and the survival analysis (b). Expression of predicted protein targets was obtained from the TCGA database of UALCAN. Asterisks indicate significant differences between normal and colon adenocarcinoma (**, $p < 0.01$; ***, $p < 0.001$). The percent of survival was analysed by the TCGA dataset accessed via OncoLnc.

matrices and allowing cell migration or invasion, including MMP3, CDK1, AURKA, MMP1, MMP7, MET, SQLE, SCD, and CA9 (Panther Classification System, <http://www.pantherdb.org>). The distinguished effects of galangin are predicted to be caused due to its aim on proteins that are involved in the cell cycle modulation (CDK1 and TOP2A). Meanwhile, diosmin and piperine target proteins that are associated with metabolism (CA9, SQLE) and metastasis (MMP7 and MMP1). Previous studies stated that PGV-1 has a strong antimetastatic activity in colon cancer (Wulandari et al. 2021). Thus, it is worthwhile to further consider the combined effect on metastasis, for example by migration and invasion assay. Additionally, an in silico study by molecular docking can also predict the molecular mechanism of the compounds (Kusuma et al. 2022).

CONCLUSIONS

Among the three tested natural compounds, galangin possesses the highest cytotoxicity in WiDr cells with an IC_{50} of 26 μ M (7 μ g/mL). Nevertheless, based on the combination index, diosmin and piperine also give

synergistic effects, whereas galangin at the concentration of one per eight IC₅₀ exhibits a strongest synergism. Galangin and piperine share predicted protein targets with PGV-1 that are overexpressed in COAD, including CDK1, MET, and TOP2A. On the other hand, the natural compounds also predicted to target proteins that are overexpressed in COAD differently than PGV-1: diosmin targets CA9, AURKA, MMP7, MMP1, and SQLE; galangin targets CA9, MMP3, and NEK2; while piperine targets AUKA. The synergistic effect could also possibly be caused by those different protein targets.

AUTHOR CONTRIBUTION

MI, DDPP, EM designed the study. HM, YMP, UMZ, FW carried out the laboratory works. MI, HM, EM analyzed the data. MI, HM, UMZ wrote the manuscript. EM gave the final approval for the publication. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the research and the research funding.

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