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Propolis as an Adjuvant for Colon Cancer Chemotherapy: Exploring its Potential on Apoptosis, Cell Cycle, and PI3K Expression

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Abstract

Propolis as co-chemotherapy is believed to reduce the severity of chemotherapy's adverse effects. The combination of propolis and chemotherapy is expected to induce apoptosis, arrest the cell cycle, and downregulate the expression of Phosphatidylinositol-3 kinase (PI3K), thereby reducing the proliferation of colon cancer cells (WiDr). The WiDr cells were obtained from the parasitology lab of the medical school at Universitas Gadjah Mada in Yogyakarta, Indonesia. Propolis is collected by beekeepers in Kabanjahe, North Sumatra. Cytotoxicity of the WiDr cell line was tested using the Microculture Tetrazolium Technique (MTT) assay. The therapy group was divided into seven subgroups. The experiment involved treating WiDr cells with different substances: K for control (normal), F for 20 µg/mL 5-FU, O for 5 µg/mL oxaliplatin, P for 7.5 µg/mL propolis, PF for a combination of 7.5 µg/mL propolis and 20 µg/mL 5-FU, PO for a combination of 7.5 µg/mL propolis and 5 µg/mL oxaliplatin, and FO for a combination of 5 µg/mL oxaliplatin and 20 µg/mL 5-fu. Flow cytometry was employed to investigate apoptosis, cell cycle, and PI3K profiles. The combination of propolis with either 5-FU or oxaliplatin enhanced both early and late apoptosis. Additionally, inhibition of the cell cycle at the G0-G1 and S phases was observed when propolis was combined with either 5-FU or oxaliplatin. PI3K expression was inhibited by propolis in combination with 5-fu or oxaliplatin. Propolis, used as co-chemotherapy, displayed anticancer efficacy through inducing apoptosis, boosting cell cycle, and suppressing PI3K expression.

Keywords: Apoptosis, Cell Cycle, PI3K, Propolis, WiDr.

Introduction

Propolis is a natural source of antioxidants and antitumor agents that can reduce the proliferation of cancer cells in prostate, cervical, breast, liver, and colon cancer cell line[1]. It is hypothesized that

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propolis' ability to promote apoptosis via both intrinsic and extrinsic routes will have a role in restraining cancer cells[2]. The effect of a single administration of propolis shows an inhibitory effect on cancer cells in the Widr cell line[3]. 5-flourouracil (5-FU) and oxaliplatin has been the standard drug for the management of colon cancer for a long time. Single 5-FU resistance with apoptosis below 20 percent is associated with the Wnt pathway[4]. Examination of Lgr5 expression on a single administration of 5-FU showed chemoresistance of 66.7%[5]. The combination of 5-FU and oxaliplatin showed an increase in the prediction of resistance by 85.7% of 71 patients by examining four markers, namely α-SMA, p-AKT, p-ERK, and surviving[6]. It is hoped that by combining propolis with chemotherapy, the unpleasant effects of the latter can be mitigated.

The anti-cancer effects of pharmaceuticals and natural substances are often evaluated by testing their impact on apoptosis and the cell cycle. It is hypothesized that propolis' ability to trigger apoptosis via both intrinsic and extrinsic routes would play a role in its anti-cancer effects[7]. Cell cycle inhibition of oxaliplatin was evident in the G2-M phase and S phase[8]. The combination of propolis with chemotherapeutic drugs certainly requires proof of cell cycle inhibition, which is key in cancer management.

Signal Phosphatidyl Inositol 3 Kinase (PI3k) is a mechanism that has long been studied in the biomolecular processes of cancer. This kinase pathway will be activated in cases of wound healing and treatment of several cases of skin diseases involving keratinocyte cells[9]. The mechanism of propolis improves skin damage caused by UV rays through direct targeting of the PI3K pathway[10]. The PI3K pathway is clearly highly expressed in colorectal cancer cases, so it is possible to develop targeted therapy via this pathway. PI3K through Akt (protein kinase B) expression plays an significance role in the growth of colorectal cancer, including metastases that occur[11]. PI3K expression analysis from combination propolis with 5-FU and combination propolis with oxaliplatin on WiDr colon cancer cells is one of the objectives of this research. The aim of this research is to compare the effectiveness of propolis alone with propolis combined with 5-FU or oxaliplatin in killing WiDr colon cancer cells.

Materials and Methods

Procedure

There were seven subgroups within the therapy group. K= control (normal), F = WiDr cells treated with 20 µg/mL 5-FU, O = WiDr cells treated with 5 µg/mL oxaliplatin, P = WiDr cells treated with 7.5 µg/mL propolis, PF = WiDr cells treated with a combination of 7.5 µg/mL propolis and 20 µg/mL 5-FU, PO = WiDr cells treated with a combination of 7.5 µg/mL propolis and 5 µg/mL oxaliplatin, FO = WiDr cells treated with combination of 5 µg/mL oxaliplatin and 20 µg/mL 5-FU. Flow cytometry was used to examine cellular apoptosis, cell cycle progression, and PI3K profiles.

MaduEfi's Propolis Sample and Preparation of Total Extract

The beekeepers at madu efi's bee farm in Kabanjahe, North Sumatera, Indonesia, provided the sample of propolis. Plants such as pine merkusii, callandraspp, and casuarina spp were used in the rearing of Trigona bees in the Kabanjahe habitat. The propolis was fractioned with ethanol after being mixed with hexane and filtered three times. Evaporation took place after collecting the filtrate. The ethanol extract of propolis is expected to have a high content of phenolics and flavonoids[12].

Cytotoxicity Assay

The WiDr cells came from the Universitas Gadjah Mada Medical School's Parasitology Laboratory in Yogyakarta. 96 well plates were used for the WiDr culture. After that, fresh medium was added, and the test solutions of varying concentrations were mixed with the co-solvent DMSO (Sigma) before being placed in an incubator at 37 degrees Celsius and 5% carbon dioxide[13, 14].

In this assay, WiDr cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco) and 0.5% fungizone (Gibco) in flasks in a humidified atmosphere (5% CO2) at 37°C. The inoculum was spread at 1 x 104 cells/mL with an optimum volume of 0.1 mL per well. After 24 hours of incubation, the medium was discarded and treated with propolis, 5-FU and oxaliplatin. After 24 hours incubation, the cells were incubated with 0.5 mg/mL MTT for 4 hours at 37°C. Viable cells react

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with MTT to form purple formazan crystals. Purple formazan crystals. After 4 hours, 10% SDS (Sigma) in 0.01N HCl (Merck) was added as a stopper to dissolve the formazan crystals. The cells were incubated for 24 hours at room temperature and protected from light. After incubation, the cells were vortexed and the absorbance was measured using a microplate reader at using a microplate reader at λ 595 nm. The absorbance data from each well was converted to the percentage of viable cells[15, 16].

Apoptosis and Cell Cycle Expression Analysis

WiDr cells (1 x10^6Cells/well) were placed in 6 wells and then incubated for 24 hours. After that, the cells were exposed to the test solution, and then incubated for 24 hours. According to the best absorbance of the two drug combinations, the best test solution was found with a propolis concentration of 7.5 µg/mL, oxaliplatin of 5 µg/mL and 5-FU of 20 µg/m. In cell cycle analysis we used PI kit (BioLegend) added to sediment and resuspended and incubated at 37oC for 30 min. In the apoptosis test, Annexin V and propidium iodide were added, while in the cell cycle test, propidium iodide was added[17].

PI3K Expression Analysis

WiDr cells were plated in a six-well plate and incubated for 24 hours. The cells were then treated with propolis, 5FU and oxaliplatin according to the grouping and incubated for 24 hours. The floating and adherent cells were collected in a conical tube using 0.025% trypsin. Cold PBS and centrifuged at 2500 rpm for 5 minutes. The supernatant was separated while the precipitate was collected. The sediment cells were fixed with 70% ethanol and allowed to stand for 2 hours at 20 C. PI3K FITC was incorporated and incubated at 37ºC for 10 minutes[14]. Samples were analysed using a FAC flow cytometer.

Measuring Instruments

A Becton Dickinson (BD) FACS Canto II from Gajah mada university, Yogyakarta, Indonesia was used on apoptosis, cell cycle, and PI3K expression. Analysis Cell cycle according to the role of DNA (Sub G0-G1, G0-G1, S, and G2/M) was calculated using ModFit Lt. 3.0 s[15]. Collected cells in the M2 area compare with control cell will be marker of expression from PI3K.

Statistical Analysis

For statistical analysis, we employed SPSS, version 24.0 (SPSS Inc., Chicago, Illinois). Parametric testing (Anova one way) was used for variables with a normal distribution ($p > 0.05$), while the *Kruskal-Wallis* test was used for those with an aberrant distribution (p 0.05).

Results and Discussion

Effect on Apoptosis and Cell Cycle

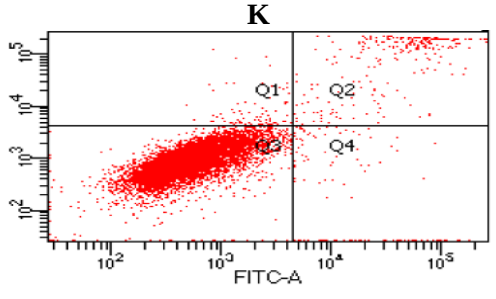
Table 1, presents quantitative data on apoptosis induction, and Fig. 1 displays the flow cytometry results. The combination of propolis with these two ingredients (5-FU or oxaliplatin) showed good results in improving early and late apoptosis. The apoptosis value is determined from the Q4 area which indicates an early apoptotic state. The highest apoptosis value in was shown by a combination of 5-FU and propolis (11, 80 %) compared to the control (0, 83 %).

Table 2. displays the effect of propolis on cell cycle modulation, while Fig. 2 shows the results of flow cytometry. A higher percentage than the control indicates that the cells experience an obstacle in the preparation of DNA material to be synthesized, thereby inhibiting the cell cycle process. Propolis combinations that showed cell cycle inhibition were found in the 5-FU combination as well as the combination and oxaliplatin in G0-G1 and S. In the sub-G0-G1 phase, the increase only occurred in the combination of propolis and 5-FU (0.73%), while in the G2-M phase, the increase occurred in the combination of propolis and oxaliplatin (49.13%).

Table 1. Apoptosis values of WiDr cells after treatment combination PF and FO.

Group	Apoptosis
K	0,83
F	11,33
O	11,07
P	1,73
PF	11,80

K = control sel ; F = 5-FU; O = oxsaliplatin; P = propolis; PF = propolis + 5-FU; FO = 5-FU + oxaliplatin.



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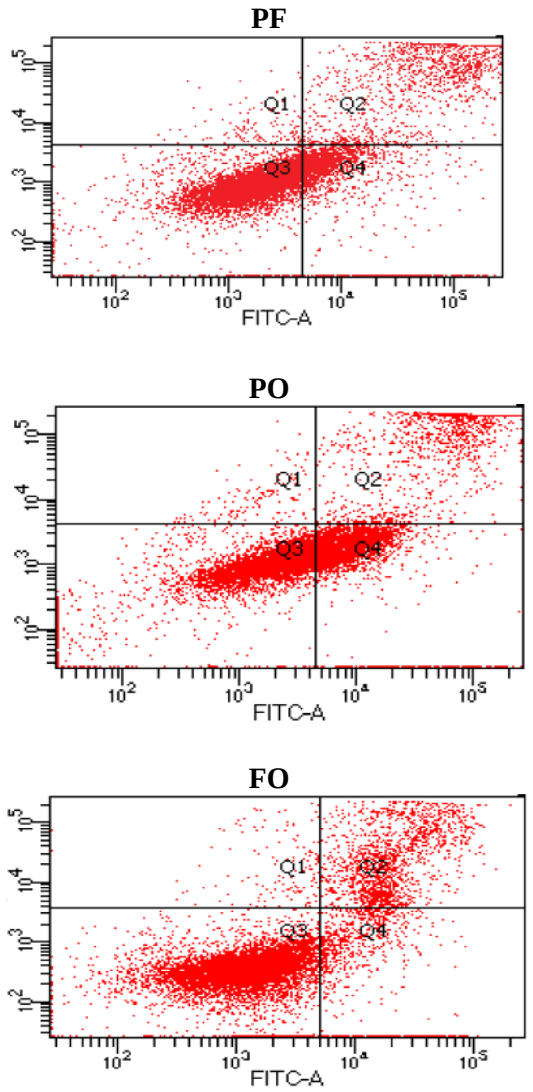

Figure 1. Images showed apoptosis analysis using flow cytometry and stained using Annexin V on WiDr cells K = control cells; PF = Cells treated with propolis at 7,5 µg/mL. Moreover, 5-FU at 20 µg/mL; PO Cells treated with propolis at 7,5 µg/mL and oxaliplatin at 5 µg/mL; FO Cells treated with 5-FU at 20 µg/mL and oxaliplatin at 5 µg/mL.

Table 2. Cell cycle process of WiDr cells after combination PF, PO, and FO.

Group	Sub G_0-G_1	G_0-G_1	S	G_2-M
K	0,17	55,03	6,90	10,57
F	0,63	75,40	9,00	7,63
O	0,47	13,30	18,57	58,57
P	0,23	64,03	8,20	13,87
PF	0,73	77,57	8,60	6,37
FO	0,07	68,70	12,77	11,83

K = control sel ; F = 5-FU; O = oxsaliplatin; P = propolis; PF = propolis + 5-FU; FO = 5-FU + oxaliplatin.

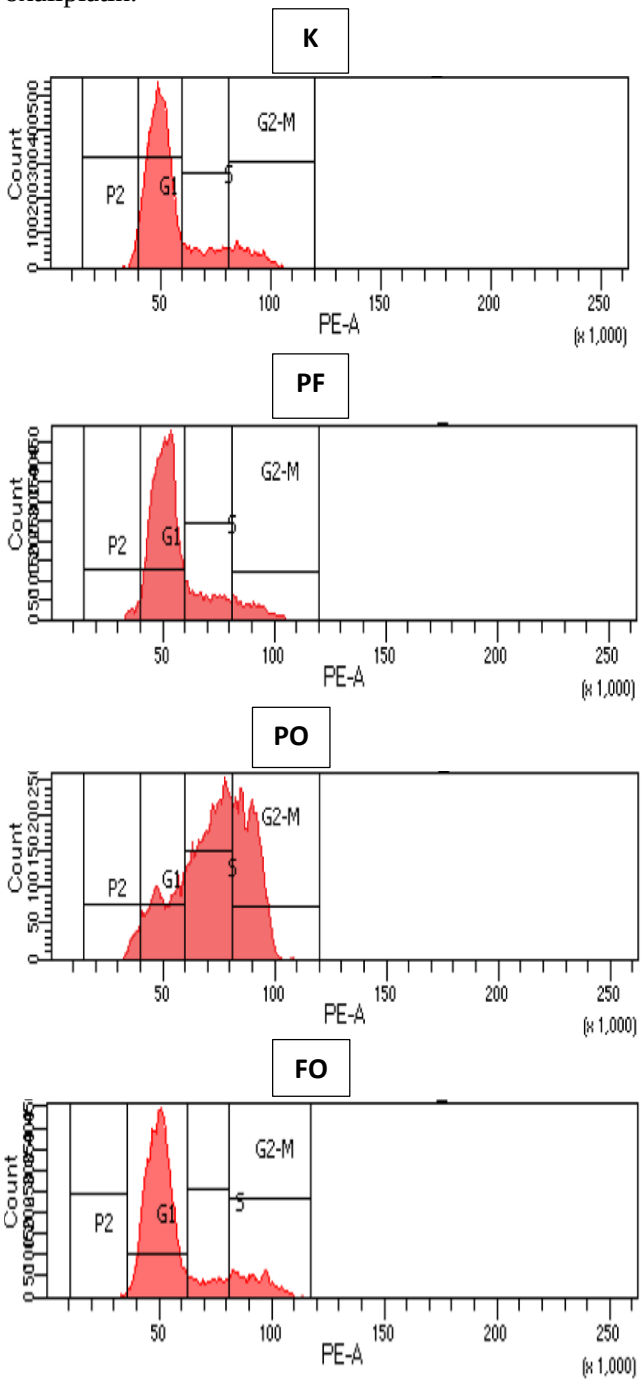

Figure 2. Images showed cell cycle analysis using flow cytometry on WiDr cells (a) control cells, (b) Cells treated with propolis at 7,5 µg/mL. and 5-FU at 20 µg/mL (c) Cells treated with propolis at 7,5 µg/mL and oxaliplatin at 5 µg/mL (d) Cells treated with 5-FU at 20 µg/mL and oxaliplatin at 5 µg/mL.

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Results from Table 3. Kruskal wallis analysis results show variables that are significantly different ($p<0.05$) between each group are in the Apoptosis variable and the Cell Cycle variable.

Table 3. Analysis of Apoptosis and Cell Cycle from Combination 5-FU, Oxaliplatin and Propolis.

Variable	Group	Mean Rank	Sig
Apoptosis	K	2,00	
	F	18,33	
	O	9,33	
	P	5,00	0,005*
	PF	14,33	
	PO	10,00	
	FO	18,00	
Cell cycle	K	2,00	
	F	10,50	
	O	19,83	
	P	5,00	0,003*
	PF	8,50	
	PO	17,17	
	FO	14,00	

K = control sel ; F = 5-FU; O = oxsaliplatin; P = propolis; PF = propolis + 5-FU; PO = propolis + oxaliplatin; FO = 5-FU + oxaliplatin.
**Kruskal Wallis test, p<0,05 significantly diffeent*

Table 4. shows that there were significant differences ($p<0.05$) in apoptosis and cell cycle between the groups, as determined by Kruskal Wallis post hoc analysis.

Table 4. Analyses Post hoc apoptosis and cell cycle from each group combination propolis, 5-FU and oxaliplatin.

	Group	Apoptosis	Cell cycle
		P	***p***
K	F	0,046*	0,050*
	O	0,046*	0,050*
	P	0,043*	0,037*
	PF	0,046*	0,046*
	PO	0,046*	0,050*
	FO	0,043*	0,050*
F	O	0,050*	0,050*
	P	0,046*	0,037*
	PF	0,275	0,178
	PO	0,050*	0,050*
	FO	0,507	0,050*
O	P	0,046*	0,037*
	PF	0,050*	0,046*
	PO	0,827	0,077
	FO	0,046*	0,050*
P	PF	0,046*	0,034*
	PO	0,046*	0,037*
	FO	0,043*	0,037*
PF	PO	0,127	0,046*
	FO	0,046*	0,046*
PO	FO	0,046*	0,050*

K = control sel ; F = 5-FU; O = oxaliplatin; P = propolis; PF = propolis + 5-FU; PO = propolis + oxaliplatin; FO = 5-FU + oxaliplatin.
**p<0,05, Post hoc Kruskal Wallis*

Propolis combined with 5-fluorouracil and oxaliplatin has been shown to be effective in reducing cancer cell growth by boosting apoptosis and inhibiting the cell cycle. Propolis contains useful phenolics (caffeic acid and vanillin) that have a role in increasing apoptosis and decreasing the cell cycle[18, 19]. The ability of propolis to trigger apoptosis is associated with the caspase pathway, especially caspase 3, which stimulates apoptosis in cancer cells[20, 21]. Researchers should continue studying cyclin D1, caspase 3, caspase 7, and caspase 9 to learn more about propolis's anti-cancer effects.
Propolis contains several flavonoids that have been shown to suppress cancer growth. These flavonoids include quercetin, quercetin-3β-D-glucoside, and luteolin. Propolis contains luteolin, which blocks G0-G1 of the cell cycle and triggers apoptosis[22, 23]. Combination medications containing natural components like propolis have the potential to combat 5-FU resistance[24, 25].

PI3K Expression

Treatment with propolis showed an accumulation of cells in the M2 area on the expression of PI3K with the flow cytometry method. The result from PF treatment shows PI3K (7,07 %). PO treatment shows PI3K (7,07 %). FO treatment shows PI3K (1,33%). The accumulation cells compared to control cells show the inhibition of PI3K to WiDr cells. Even when compared to the combination of two chemotherapeutic medicines, 5-FU and oxaliplatin, propolis and 5-FU or propolis and oxaliplatin inhibited PI3K expression.
Table 5. shows the PI3K expression of all groups. The best inhibitory expression was seen in the increase of PI3K value compared to the control group, which was seen in the combination group of propolis with 5-Fu.

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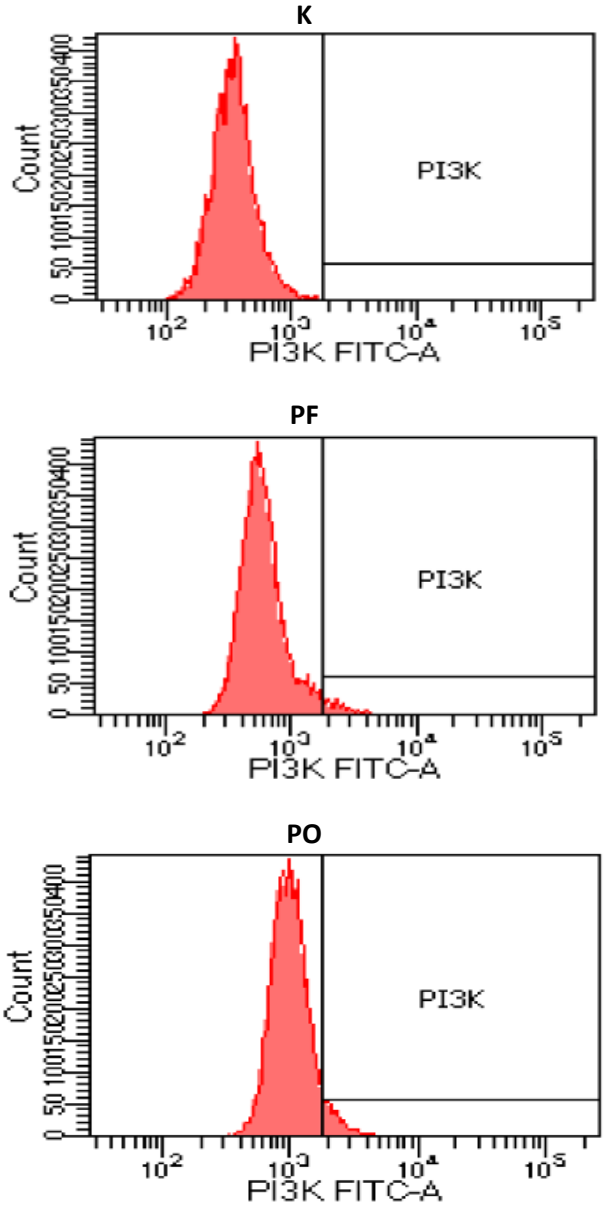
Table 5. PI3K expression of WiDr cells after combination PF and FO.

Group	PI3K
F	2,97 ± 0,63
O	6,27 ± 0,11
P	5,27 ± 0,89
PF	7,07 ± 4,53
FO	1,33 ± 0,05

F = 5-FU; **O** = oxsaliplatin; **P** = propolis; **PF** = propolis + 5-FU; **FO** = 5-FU + oxaliplatin.

Fig. 3 shows that the PI3K expression of the propolis and 5-FU combination (PF) is almost 7 times higher than the two combinations of oxaliplatin and 5-FU (FO) chemotherapy drugs, indicating that the PF group had the best PI3K inhibition.



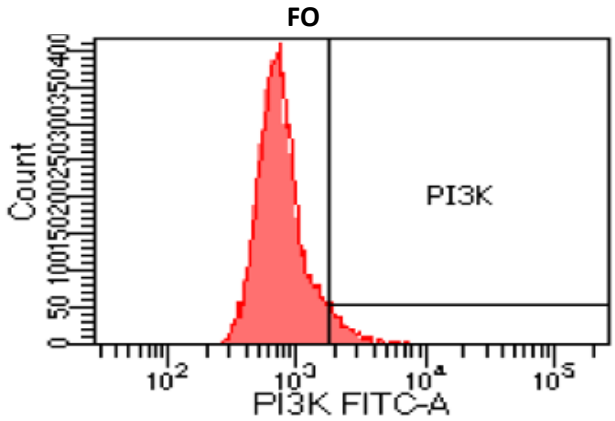


Figure 3. Images showed expression of PI3K using flow cytometry and on WiDr cells K = control cells; PF = Cells treated with propolis at 7,5 µg/mL and 5-FU at 20 µg/mL; PO = Cells treated with propolis at 7,5 µg/mL and oxaliplatin at 5 µg/mL; FO = Cells treated with 5-FU at 20 µg/mL and oxaliplatin at 5 µg/mL.

Table 6. shows that the ANOVA test revealed a significant difference of 0.004 ($p<0.05$) among the five treatment groups. The increased expression of PI3K suggests the inhibitory effect of the Propolis on the PI3K pathway.

Table 6. Analysis of PI3K expression from combination propolis, 5-FU dan Oxaliplatin on WiDr cell.

Group		Mean	F	Sig
PI3K	F	2,9667		
	O	6,2667		
	P	5,2667	5,688	0,004*
	PF	7,0667		
	FO	1,6000		

F = 5-FU; **O** = oxsaliplatin; **P** = propolis; **PF** = propolis + 5-FU; **FO** = 5-FU + oxaliplatin.
**Anova test p<0,05 significant differences.*

Table 7. shows that there are 3 groups with different significance, namely the O, P and PO groups. The best PI3K inhibition expression was shown in the combination group of propolis with oxaliplatin.

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Table 7. Results of post hoc Anova Analysis of PI3K.

	Test	Group		Mean Dif	Sig
PI3K	Games-Howell	Control	F	-1,36667	0,449
			O	-4,66667*	0,042
			P	-3,66667*	0,047
			PF	-5,46667	0,551
			PO	-5,46667*	0,010
			FO	0,26667	0,994

F = 5-FU; **O** = oxsaliplatin; **P** = propolis; **PF** = propolis + 5-FU; **PO** = propolis + oxaliplatin; **FO** = 5-FU + oxaliplatin.
*p<0,05, *post hoc Anova test*

Propolis is a natural substance with anticancer properties due to its ability to regulate PI3K expression. Propolis's ability to inhibit the PI3K pathway can be attributed to the presence of caffeic acid phenethyl ester in the substance[26]. The role of propolis here is related to the content of caffeic acid phenethyl ester, quercetin, and apigenin. This is in line with research on extracts from these three ingredients showing inhibition of PI3K activity in research on anti-skin agents due to ultraviolet[10, 27]. The use of metformin and calcitriol is reported to increase the tumor inhibitory effect of 5-FU so it is hoped that it will reduce 5-FU resistance through the PI3K/Akt/Mtor pathway. The PI3K pathway is expected to stimulate aerobic glycolysis[28]. Based on mean difference, PI3K expression from the combination group of propolis with oxaliplatin was most different from the control.

Buparlisib and apitolisib are examples of inhibitors of the PI3K/Akt/Mtor pathway that were developed together with other chemotherapy drugs such as paclitaxel for breast cancer cases that have entered phase III clinical trials[29]. Patients with metastatic head and neck squamous cell carcinoma benefit more from a combination of buparlisip plus paclitaxel than from paclitaxel alone[30]. Table 5 shows that when propolis was used in conjunction with 5-fluorouracil and oxaliplatin, PI3K expression was suppressed to a greater extent than when either drug was used alone.

Conclusion

The best apoptosis inhibition was shown by the combination of oxaliplatin and propolis. In cell cycle sub phases G0-G1 and G0-G1, the best combination was found when propolis was combined with 5-FU, while in G2-M phase and S phase, the best combination was found in the combination of propolis and oxaliplatin. The best inhibitory expression of PI3K was shown in the combination group of propolis with oxaliplatin.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee at Universitas Sumatera Utara.
- No animal studies are present in the manuscript.

Authors' Contribution Statement

S.E.M contributed to conception, design of the study and manuscript preparation. P.A.Z.H and I.P.N performed data acquisition and experimental laboratory work, A.L and P.C.E contributed to data analysis. R.E were involved in article drafting. All authors have approved the final version of the manuscript.

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البروبوليس كمساعد للعلاج الكيميائي لسرطان القولون: استكشاف إمكاناته في موت الخلايا المبرمج، ودورة الخلية، وتعبيرPI3K

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الخلاصة

يُعتقد أن البروبوليس كعلاج كيميائي مشترك يقلل من شدة الآثار الضارة للعلاج الكيميائي. من المتوقع أن يؤدي الجمع بين البروبوليس والعلاج الكيميائي إلى تحفيز موت الخلايا المبرمج، وإيقاف دورة الخلية، وتقليل تنظيم التعبير عن (PI3K 3-) وبالتالي تقليل تكاثر خلايا سرطان القولون WiDr. تم الحصول على خلايا WiDr من مختبر الطفيليات بكلية الطب بجامعة غادجاه مادا في يوجياكارتا بإندونيسيا. يتم جمع ا البروبوليس من قبل النحالين في كابانجاهي، شمال سومطرة. تم اختبار السمية الخلوية لخط خلايا WiDr باستخدام مقايسة تقنية Microculture Tetrazolium (MTT).

تم تقسيم مجموعة العلاج إلى سبع مجموعات فرعية. تضمنت التجربة معالجة خلايا WiDr بمواد مختلفة: K للمجموعة الضابطة، F لـ 20 ميكروغرام/مل FU-5، O لـ 5 ميكروغرام/مل أوكساليبلاتين، P لـ 7.5 ميكروغرام/مل بروبوليس ، PF لمزيج من 7.5 ميكروغرام / مل بروبوليس و 20 ميكروغرام / مل FU-5، PO لمزيج من 7.5 ميكروغرام / مل بروبوليس و 5 ميكروغرام / مل أوكساليبلاتين، و FO لمزيج من 5 ميكروغرام / مل أوكساليبلاتين و 20 ميكروغرام / مل FO-5. تم استخدام قياس التدفق الخلوي للتحقيق في موت الخلايا المبرمج، ودورة الخلية، وملامح PI3K. أدى الجمع بين البروبوليس مع FU-5 أو أوكساليبلاتين إلى تعزيز موت الخلايا المبرمج المبكر والمتأخر. بالإضافة إلى ذلك، لوحظ تثبيط دورة الخلية في المرحلتين G0-G1 وS عندما تم دمج البروبوليس مع FU-5 أو أوكساليبلاتين. تم تثبيط تعبير PI3K بواسطة البروبوليس بالاشتراك مع FU-5 أو أوكساليبلاتين. أظهر البروبوليس، المستخدم كعلاج كيميائي مشترك، فعالية مضادة للسرطان من خلال تحفيز موت الخلايا المبرمج، وتعزيز دورة الخلية، وقمع تعبير PI3K.

الكلمات المفتاحية: موت الخلايا المبرمج، دورة الخلية، PI3K، البروبوليس، WiDr.