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Antigenotoxicity Activity of Papaya (*Carica papaya* L.) Leaf Ethanolic Extract on *Swiss* Mice Induced Cyclophosphamide through Mammalian *In Vivo* Micronucleus Test

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Abstract

Cyclophosphamide (CPA) is an effective chemotherapeutic agent, but has side effect, causing DNA damage (genotoxic). Papaya leaf (*Carica papaya* L.) is known has flavonoid compound, quercetin. Quercetin is known has DNA protecting effect (antigenotoxic effect) by metabolism modulation. Thus, the aim of this research is to investigate the antigenotoxic effect of ethanolic extract of papaya (*Carica papaya* L.) leaf (EEPL) on CPA induced mice. The antigenotoxic effect was evaluated by mammalian *in vivo* micronucleus test. EEPL was orally administered as single treatment at dose 1000 mg/kgBW and in combination with CPA 50 mg/kgBW at dose 250 mg/kgBW; 500 mg/kgBW; and 1000 mg/kgBW. Molecular docking using PLANTS on CYP3A4 was performed to explore the antigenotoxic effect mechanism. The three different combination dose of EEPL with CPA significantly (p<0.05) decreased the amount of micronucleated polychromatic erytrhocyte (MNPCE)/1000 polychromatic erythrocyte (PCE) and also increased % PCE/(PCE+normochromatic erythrocyte (NCE), compared with single dose of CPA. Nevertheless, the antigenotoxic effect wasn't significant compared with each combination dose. The docking score result showed quercetin (-82,41) has more potent interaction to CYP3A4 than cyclophosphamide (-70,16) and both of them has similar active site at amino acid residue Ile 369 and Thr 309. The results obtained indicated that EEPL at dose 250 mg/KgBB is the optimal dose as antigenotoxic agent by interaction between quercetin with CYP3A4 based on molecular docking.

Keywords: antigenotoxic, *Carica papaya* L., MNPCE, *in vivo*

INTRODUCTION

Genotoxic effect is an effect of DNA damage that caused by chemical and physical agent that can modify nucleotide base and sugar phosphate backbone from DNA (Kastan, *et al*., 2004). One of the genotoxic agents is cyclophosphamide. This genotoxic effect caused by the activation of cyclophosphamide into its toxic metabolite, that is *phosphoramide mustard* (PM) (Shukla, *et al*., 2001).

One of the natural materials that has potency as antigenotoxic agent is Papaya leaves (*Carica papaya* L.). The methanolic extract of Papaya leaves contain quercetin 0.04 mg/g of dry leaves (Canini, *et al*., 2007). That have antigenotoxic effect by modulation of metabolism (Srinivas, *et al*., 2013). Thus, this study will explore the potency of ethanolic extract of Papaya leaves (EEPL) as the antignotoxic agent. Development of this study is mammalian *in vivo* micronucleus test to see the antigenotoxic potency of EEPL and molecular docking to observed the interaction between quercetin and CYP3A4 enzyme in inhibiting the formation of toxic metabolite of cyclophosphamide. The data resulted can be used to develop papaya leaves as one of the antigenotoxic agents to treat of genotoxic effect.

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This study is aimed to develop of EEPL as the potencial antigenotoxic agent that useful for repairing DNA damage that caused by side effect of chemotherapy.

MATERIAL AND METHODS

Collection and Plant Identification

The material of this study is papaya leaves from Grogol village, Salatiga, Central Java, and determined at Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada. About 400 g powders of papaya leaves was extracted by maceration method using 4 litres of ethanol 70%. Then that was filtered and taken the filtrate. The filtrate was evaporated by rotary evaporator until resulted the dry extract. After that tested thin layer chromatography (TLC) toward the content of quercetin. The stationary which is used is silica gel 60 plate F254. Eluent or mobile phase consist of toluene:acetic acid: formic acid (7:2:1).

In vivo Test with Mammalian *In Vivo* Micronucleus Test

Female Swiss mice strain aged 6-7 weeks with body weight 22.5-27.5 g as many as 42 tails were divided into 7 groups (Table 1). All treatment groups were sampling peripheral blood through vein of tails at the day-7 of treatment, 6 hours after giving CPA. The blood specimens were made peripheral blood smear preparation on the microscope slides, then methanol was fixed for 15 minutes and after that painted with Giemsa 10% in PBS pH 6.8 with incubation time 1 hour. The preparation was cleaned using aquadest. After the preparation dried, preparation was closed by the cover slip using Entellen and observed the MNPCE, PCE, and NCE under microscope with 1000x magnification.

Tabel I. Test Animal Treatment

Group	Treatment						
	Day-1	Day -2	Day -3	Day -4	Day -5	Day -6	Day -7
I. Negative control	-	-	-	-	-	-	-
II. Control of EEDP dose 1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB	EEDP doses 1000 mg/ Kg BB	EEDP doses 1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB	EEDP dose1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB
III. Control Cyclophosphamide (CPA) dose 50 mg/ Kg BB	-	-	-	-	-	CPA dose 50 mg/ Kg BB	CPA dose 50 mg/ Kg BB
IV. Control of solvent CMC-Na	CMC-Na	CMC-Na	CMC-Na	CMC-Na	CMC-Na	CMC-Na + CPA dose 50 mg/ Kg BB	CMC-Na + CPA dose 50 mg/ Kg BB
V. EEDP dose 250 mg/ kg BB + CPA dose 50 mg/ Kg BB	EEDP dose 250 mg/ Kg BB	EEDP dose 250 mg/ Kg BB	EEDP dose 250 mg/ Kg BB	EEDP dose 250 mg/ Kg BB	EEDP dose 250 mg/ Kg BB	EEDP dose 250 mg/ Kg BB + CPA dose 50 mg/ Kg BB	EEDP dose 250 mg/ Kg BB + CPA dose 50 mg/ Kg BB
VI. EEDP dose 500 mg/ kg BB + CPA dose 50 mg/ Kg BB	EEDP dose 500 mg/ Kg BB	EEDP dose 500 mg/ Kg BB	EEDP dose 500 mg/ Kg BB	EEDP dose 500 mg/ Kg BB	EEDP dose 500 mg/ Kg BB	EEDP dose 500 mg/ Kg BB + CPA dose 50 mg/ Kg BB	EEDP dose 500 mg/ Kg BB + CPA dose 50 mg/ Kg BB
VII. EEDP dose 1000 mg/ kg BB + CPA dose 50 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB + CPA dose 50 mg/ Kg BB	EEDP dose 1000 mg/ Kg BB + CPA dose 50 mg/ Kg BB

Molecular Docking

Molecular docking was done as final confirmation to compare the nterctoin of content coumpound of EEDP, quercetin with cyclophosphamide made by using Marvin Sketch software. While the set of protein complex structure was obtained from Protein Data Bank (PDB) that downloaded from site http://ww.pdb.org/home/home.do.

Data Analysis

Counted the amount of MNPCE/100 PCE and % PCE/(PCE+NCE). MNPCE is young red blood cells which experienced genotoxicity, signed as violet cells which have micronucleus like spot. PCE is young red blood cells which have no experienced as genotoxicity, signed as violet cells but have no micronucleus like spot. NCE is mature red blood cells, but have no cell coloured violet. In genotoxic compound, amount of MNPCE/1000 PCE is high but amount of %PCE/(PCE+NCE) is low.

Results that obtained of this study were analysed with post hoc tukey test with trust level 95%. The result of molecular docking was docking score between quercetin-CYP3A4 and cyclophosphamide- CYP3A4. If docking score quercetin-CYP3A4 is lower than cyclophosphamide- CYP3A4, it show that quercetin which probably contained in the EEDP will be potential antigenotoxic agent.

RESULTS AND DISCUSSION

Qualitative Test Results of Active Ingredients Compounds

Test thin layer chromatography (TLC) aims to confirm the presence of compounds *quercetin* in EEDP. The system used is silica gel G stationary phase and the 60 F254 mobile phase mixture of toluene: ethyl acetate: formic acid (7:2:1 v / v / v). TLC profile EEDP in seen in Fig. 1.

The result of TLC observed by visible light (a), UV 254 nm (b), and UV 366 nm (c) shows EEDP and quercetin have the same spot hRf at 25. The same value of hRf shows the types of compounds that have the same relative polarity. This shows great possibilities EEDP containing quercetin compounds.

Mammalian In Vivo Test micronucleus

The Principle of *mammalian in vivo micronucleus test* is the formation of micronuclei in the process of hematopoiesis, which is one indicator of DNA mutations caused by exposure of genotoxic compounds. Observations by microscope shows cells with MNPCE, PCE and NCE on (Fig. 2). After that, count the number of MNPCE/1000 PCE as genotoxic parameters and % PCE/PCE + NCE as a parameter of toxicity. This study uses manually cell counter because of easier, cheaper, and has the same accuracy with an automated method (Criswell, *et al*., 1998).

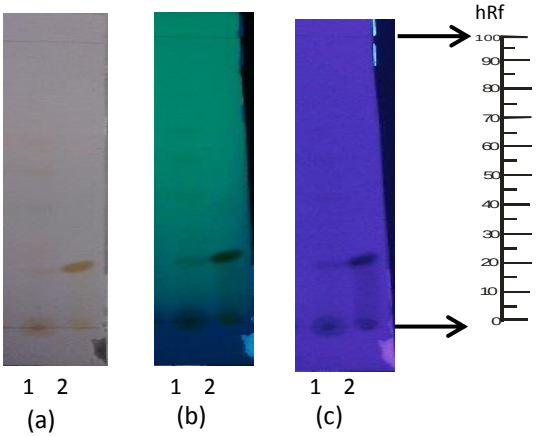

Figure 1. Profile of thin layer chromatography ethanolic extract of papaya (EEDP). Elution is done with the stationary phase silica gel 60 F254 and mobile phase mixture of toluene: ethyl acetate: formic acid (7: 2: 1). EEDP (1) and quercetin (2) have the same spot on the HRF 25.

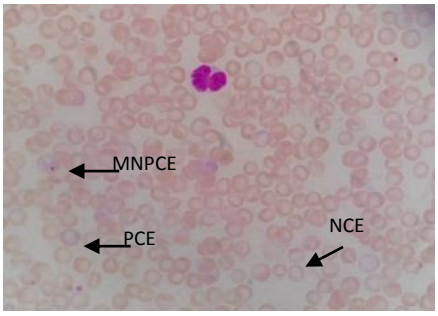

Figure 2. The appearance blood smear test animals. Peripheral blood smears with Giemsa staining was observed through a light microscope with a magnification of 1000x lens (with oil immersion). Black arrow (⟶) shows an example of each cell MNPCE, PCE, or NCE.

Treatment EEDP with combination dose 250; 500; 1000 mg/KgBW with SIF 50mg/KgBW can reduce the number of MNPCE/1000 PCE significantly (p<0.05) compared to treatment SIF, but the decline did not differ significantly between treatment dose (Fig. 3a). This shows character antigenotoxic of EEDP with optimal dose at 250 mg/KgBW of 2.06 times (Table II). In addition, the combination treatment was significantly (p <0.05) were able to increase the % PCE/(PCE + NCE) compared to treatment SIF, but the decline did not differ significantly between treatment dose (Fig. 3b). This shows EEDP not toxic at doses of tests with optimal doses at 250 mg / KgBW by 1.82 times (Table 2).

Overall, the study is same with the previous studies showing that some flavonoids have been tested *in vitro* and *in vivo* has antigenotoxic effect (Utesch, *et al*., 2008). The decline Genotoxicity of SIF by EEDP likely influenced by interactions of CYP3A4 with quercetin thus reducing the formation of metabolites genotoksiknya. It also like the theory that prevention of mutagenesis can be done through a reduction metabolic activity of mutagenic compounds (Ruddon, 2007).

Table 2.. Changes in the number MNPCE / 1000 PCE and PCE / (PCE + NCE) in test animals

The treatment group	MNPCE/ 1000 PCE	The ratio of the number MNPCE	PCE/ (NCE+PCE)	Ratio %PCE
Control	15±6.93	-	4.89±0.71	-
CMC-Na	15±2.65	-	4.23±0.78	-
EEDP 1000 mg/KgBW	9.67±2.52	-	6±1.35	-
SIF 50 mg/KgBW	65.33±10.02*	-	2.59±0.05*	-
SIF 50 mg/KgBW + EEDP 250 mg/KgBW	31.67±4.04**	2.06	4.73±0.43**	1.83
SIF 50 mg/KgBW + EEDP 500 mg/KgBW	29±2.65**	2.25	5.14±0.45**	1.98
SIF 50 mg/KgBW + EEDP 1000 mg/KgBW	23.33±3.06**	2.80	5.69±0.53**	2.19

the amount shown mean ± SD of three mice
the ratio is comparation between combination effect and single cyclophosphamide effect
*** *p*<0.05 compared with controls based on *post hoc Tukey test***
**** *p*<0.05 compared with cyclophosphamide treatment based on *post hoc Tukey test***

Kelompok perlakuan	1	2	3	4	5	6	7
CMC-Na	-	+	-	+	+	+	+
SIF 50 mg/KgBB	-	-	+	-	+	+	+
EEDP 250 mg/KgBB	-	-	-	-	+	-	-
EEDP 500 mg/KgBB	-	-	-	-	-	+	-
EEDP 1000 mg/KgBB	-	+	-	-	-	-	+

(a)

Kelompok perlakuan	1	2	3	4	5	6	7
CMC-Na	-	+	-	+	+	+	+
SIF 50 mg/KgBB	-	-	+	-	+	+	+
EEDP 250 mg/KgBB	-	-	-	-	+	-	-
EEDP 500 mg/KgBB	-	-	-	-	-	+	-
EEDP 1000 mg/KgBB	-	+	-	-	-	-	+

(b)

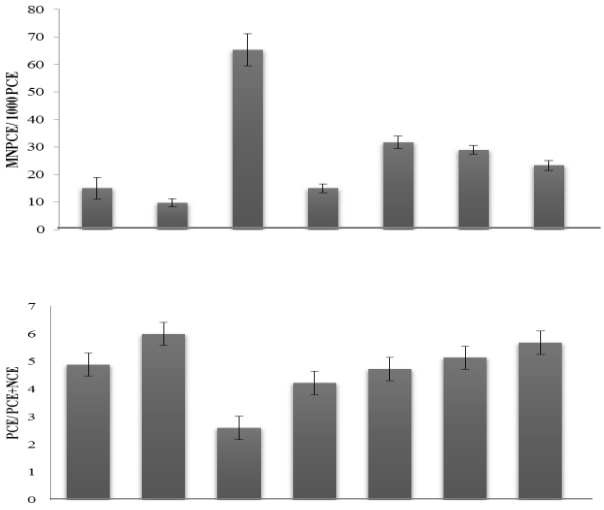

Figure 3. Effect EEDP treatment on the profile of peripheral blood smear test animals. Peripheral blood smear test animals were observed by light microscope with a magnification of 1000x, then calculated the number of MNPCE / 1000 PCE (a), and the% PCE/(PCE + NCE) (b). Significance between groups are indicated by numbers on the graph.

Molecular Docking

Results docking of CYP3A4 showed that *quercetin* docking score (-82.41) lower than cyclophosphamide (-70.16) (Table 3). This suggests that quercetin is a more potent when interact with CYP3A4 than cyclophosphamide. These results are supported by visualization of interaction shows the similarities between the bond sites of quercetin and cyclophosphamide on CYP3A4 at Thr Ile 369 and 309 amino acid residue through hydrophobic bonding (Fig. 4).

CONCLUSION

EEDP have antigenotoxic effects against cyclophosphamide and not toxic at the dose test with an optimal dose of 250 mg/KgBW. Antigenotoxic effect of EEDP was predicted by interactions of quercetin compound with CYP3A4 at Thr Ile 369 and 309 amino acid residue through hydrophobic bond. Thus, EEDP can inhibit the metabolism of cyclophosphamide into toxic metabolites, *i.e*, phosphoramide mustard.

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Table 3. Docking scores of quercetin and cyclophosphamide on CYP3A4

No.	Ligand name	Docking score	RMSD
1.	*Quercetin*	-82,41	3,00
2.	Cyclophosphamide	-70,16	

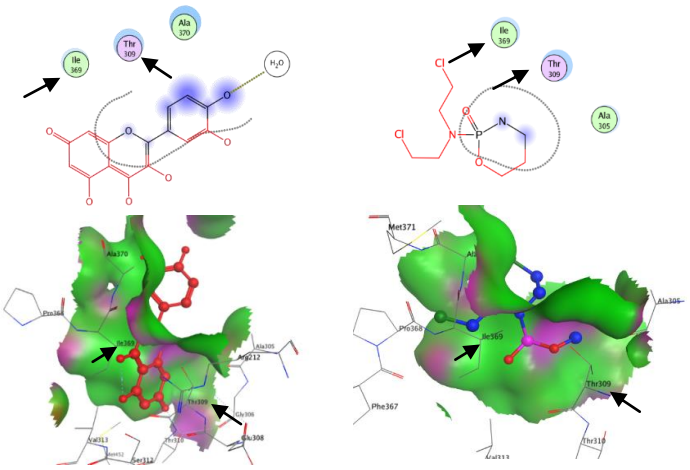

Figure 4. Interaction of cyclophosphamide and quercetin on CYP3A4. Visualization interactions on CYP3A4 showed quercetin compound (a) and cyclophosphamide (b) have the same connective site at Thr Ile 369 and 309 amino acid residue. The sign → showed the same amino acid residues at the site of connective.

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