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Oral administration of neem (*Azadirachta indica* A. Juss) leaf extract increases Cyclin D1 expression in hepatocyte regeneration in acetaminophen-induced hepatotoxic wistar rats

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ABSTRACT

Background: Acetaminophen is widely used. Inappropriate dose acetaminophen administration can induce hepatotoxicity which characterized by hemorrhage and necrosis. Necrosis may be followed by regeneration of viable hepatocytes outside the necrotic area which depends on antioxidant, proliferation mediator, and cell cycle activator including cyclin D1. Neem (*Azadirachta indica* A. Juss) leaf is a strong antioxidant which has an abundance flavonoids. This study aims to prove oral administration of neem leaf extract increases cyclin D1 expression in hepatocyte regeneration in acetaminophen-induced hepatotoxic Wistar rats.

Methods: The experimental study was a post-test only control group design using 24 hepatotoxic Wistar rats induced by 315 mg acetaminophen/200g rat body weight (BW) which is equal to 250 mg acetaminophen/kg human BW. Liver toxicity was determined by measuring serum SGPT level. The experimental animals divided into

four groups: P0, P1, P2, and P3. All groups got a standard therapy of N-acetylcysteine. P1, P2, and P3 groups were given 50 mg/200 g BW, 100 mg/200 g BW and 200 mg/200 g BW neem leaf extract respectively, twice a day for seven days. The animals were subsequently terminated, and cyclin D1 expression was evaluated by immunohistochemistry.

Result: The P0, P1, P2 and P3 groups showed 12.67% (n=6; SD=1.033), 17.5% (n=6; SD=1.225), 31.33% (n=6; SD=1.506) and 42.00% (n=6; SD=2.828) cyclin D1 expression respectively. One way ANOVA test revealed D1 expression was significantly different between groups ($p = 0.000$). Post hoc multiple comparisons analysis revealed D1 expression between all groups were significantly different ($p = 0.000$).

Conclusion: Oral administration of neem leaf extract increases cyclin D1 expression in hepatocyte regeneration in acetaminophen-induced hepatotoxic Wistar rats, which increases along with the dosage.

Keywords: acetaminophen-induced, hepatotoxic, liver regeneration, neem leaf extract, cyclin D1

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INTRODUCTION

Acetaminophen is widely used and often used without medical supervision. Using this drug with inappropriate dose can induce hepatotoxicity. Acetaminophen toxicity was the main cause of acute liver failure, and it contributes up to 46% of all cases in Europe and United States, and the incidence tends to increase every day.¹⁻⁶ N-acetylcysteine (NAC) is still used as a standard therapy for acetaminophen-induced hepatotoxicity. However, Young et al. (2009) revealed that long-term NAC administration inhibits liver regeneration.⁷

Liver toxicity is characterized by hepatocyte hemorrhage and necrosis. These changes will be followed by regeneration of viable hepatocyte outside of the necrotic area. Liver regeneration process depends on antioxidant, proliferation mediators, and cell cycle activator including cyclin D1.¹⁻²

Quiescent cells such as hepatocyte, after stimulated by mitogenic signals (cyclin-dependent kinase

4 (CDK4), cyclin-dependent kinase 6 (CDK6), and cyclin D), will enter G1 phase and subsequently starts the cell cycle. The decision of continuing cell cycle depends on cellular RAS induction, and it will increase cyclin D1 level at G2 phase. Cyclin D1 is suggested as an active button and a key regulator of continuous cell cycle progression. Cyclin D1 induction is the most reliable biologic marker of cell cycle progression.⁷⁻⁹

Neem, known as *mimba* in Indonesia, has a high potential as an antioxidant due to its abundance flavonoids, such as quercetin, gallic acid, (+)gallocatechin, (-)epicatechin, (+)catechin and epigallocatechin. It was proved that some herbs, including neem leaf, have hepatoprotective effect.³⁻⁵ Hence this study was conducted to prove that oral administration of neem leaf extract increases cyclin D1 expression in hepatocyte regeneration in acetaminophen-induced hepatotoxic Wistar rats.

METHODS

Chemicals

Acetaminophen drops for infants (Indofarma pharmaceutical Ltd.), *glutamic pyruvic transaminase* (SGPT) BR opt. reagent (Glory Diagnostics Ltd.), N-acetylcysteine effervescent tablets (Zambon Ltd.), 96% ethanol food grade (Bratacochem Ltd.), paraffin wax (Leica Ltd.), 10% neutrally buffered formalin (Leica Ltd.), and rabbit anti-cyclin D1 antibody (Lab Vision Corp.) were used in this study.

Neem leaf extraction

The fresh neem leaves were collected in the morning from a garden within 98 m above the sea level at Kediri Regency of East Java and then dried at room temperature, protected from direct sunlight until it had a crisp texture and could be processed into 100 mesh gradient powder. The leaf extract was made using maceration procedure according to Anief (2015) by using 96% ethanol food grade as solvent at room temperature.¹⁰

Experimental Animal

Experimental subjects were male Wistar strain rats (*Rattus norvegicus*), four months of age, with average body weight of 200 grams. The hepatotoxicity was induced by a single dose of 315 mg acetaminophen/200 gram body weight (BW) of rats (equal to 250 mg/kg BW for human).¹¹ The Wistar rats chosen were male because they had stronger physical. A 4 months old rat has similarities with a 12 years old teenager human.¹²

At the beginning of the study, 28 Wistar rats were given a single dose of 315 mg/200 g BW acetaminophen (equal to 250 mg/kg BW for human) through an orogastric tube. Hepatotoxicity was determined in 36 hours after acetaminophen administration, by standard examination of SGPT serum level using spectrophotometry method, at 340 nm. Rats with SGPT level > 889 U/L (more than 20x normal level were considered as hepatotoxic rats).⁶ Twenty-four rats with the highest SGPT level were selected as

experimental subjects. These 24 hepatotoxic rats were randomly divided into four groups. The groups were P0, P1, P2, and P3 which consisted of 6 rats each. The P0 only got 28 mg/200 g BW NAC as initial dose and continued with 42 mg/200 g BW twice a day through an orogastric tube. The other three groups also got NAC with the same dose as group P0. The P1 group was given 50 mg/200 g BW neem leaf extract, twice a day. The P2 group was given 100 mg/200 g BW neem leaf extract, twice a day. The P3 group was given 200 mg/200 g BW neem leaf extract (as maximum dose) twice a day.³ The rats were terminated after seven days of the experiment.

Cyclin D1 immunohistochemistry and interpretation

Livers of the rats were taken, processed and stained with cyclin D1 immunohistochemistry. Cyclin D1 immunostaining was performed by using streptavidin-biotin peroxidase method with monoclonal antibody anti-cyclin D1 SP4 clone, diluted 1:100, and by using antigen retrieval with 700-watt microwave heating in citrate buffer solution (pH 6.0) for 20 minutes.¹³

Cyclin D1 expression was evaluated with binocular microscope Olympus CX31. Hepatocyte with brown nuclear staining with moderate to strong intensity was considered cyclin D1 positive. The cyclin D1 expression counting was done by defining quantitative score based on the number of hepatocytes with positive cyclin D1 among 100 cells under 400 times magnification at the "hot spot" area, around necrotic areas. The score was presented in percentage.¹⁴⁻¹⁸

Statistical analysis

Data normality was tested by using Shapiro-Wilks test. Data homogeneity was tested by using Levene's test. The effect of neem leaf extract to cyclin D1 level in all groups was analyzed using analysis of variance (one-way ANOVA) on the confidence level of $p=0.05$. The different levels of cyclin D1 expression between each group tested with post hoc multiple comparisons test on the confidence level of $p=0.05$.

RESULTS

The SGPT level of 28 experimental rats after acetaminophen administration range from 1484 U/L to 1683 U/L. All rats were considered as rats with acute liver injury (acute hepatotoxic). Rats with the 24 highest SGPT level were selected as experimental subjects. All experimental rats had a homogeneous SGPT level with p value=0.200 (>0.05).

The phytochemistry testing reports of neem leaves extract, based on testing report No. 033a/UN.14.24/UPTLA/2017 by Analytic Laboratory Udayana University on 19th to 20th March 2107 are shown in Table 1.

Table 1 Phytochemistry testing reports of neem leaf extract

No.	Parameters	Units	Methods	Results
1.	Tannin	mg/100 g TAE wb	Spectrophotometry	4481.98
2.	Flavonoid	mg/100 g QE wb	Spectrophotometry	3457.30
3.	Total Phenolic	mg/100 g GAE wb	Spectrophotometry	3906.25
4.	Antioxidant Capacity	mg/L GAEAC wb	Spectrophotometry	10302.72
5.	IC 50%	mg/mL wb	Spectrophotometry	1.05
6.	Betha Carotene	mg/100 g wb	Spectrophotometry	16817.13

TAE : tannic acid equivalent

QE : quercetine equivalent

GAE : gallic acid equivalent

GAEAC : gallic acid equivalent antioxidant capacity

IC 50% : inhibitory concentration against free radical DPPH 0.1 mM

wb : wet base

Table 2 Expression of cyclin D1 in the livers of the experimental rats

Code of Experimental Rats	Cyclin D1 Expression (%)
P0-1	14
P0-2	11
P0-3	13
P0-4	12
P0-5	13
P0-6	13
P1-1	17
P1-2	19
P1-3	17
P1-4	19
P1-5	17
P1-6	16
P2-1	30
P2-2	34
P2-3	32
P2-4	31
P2-5	30
P2-6	31
P3-1	40
P3-2	46
P3-3	42
P3-4	44
P3-5	42
P3-6	38

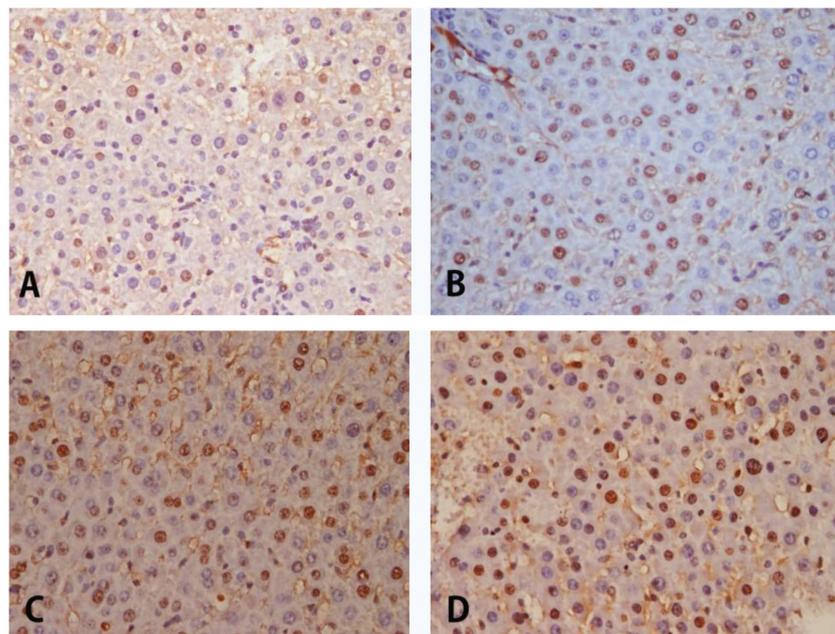


Figure 1 Cyclin D1 expression. Rat P0-1 with 14% positive cells (A), Rat P1-2 with 19% positive cells (B), Rat P2-6 with 31% positive cells (C), Rat P3-6 with 44% positive cells (D) (400x)

Cyclin D1 expression range was 11-46% (Table 2). Mean Cyclin D1 expression of P0, P1, P2 and P3 groups were 12.67% (SD= 1.033), 17.5% (SD= 1.225), 31.33% (SD= 1.506) and 42% (SD= 2.828) respectively. Microscopic finding of cyclin

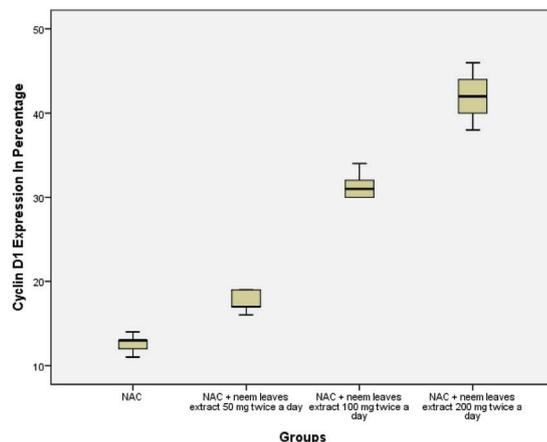


Figure 2 Descriptive analysis of cyclin D1 expression in all groups (minimum value, maximum value, range, means, and standard deviation)

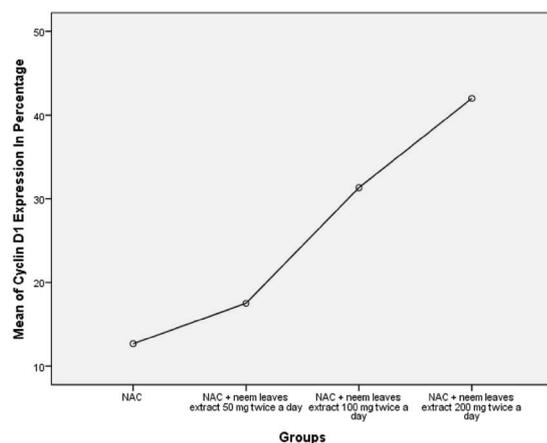


Figure 3 Plots of Mean Cyclin D1 Expression for All Groups

D1 immunohistochemistry staining is shown in Fig. 1. The descriptive analysis in each group is shown in Fig. 2.

The significance level (p) of normality shows a normal distribution (> 0.05) using Shapiro-Wilks test for P0, P1, P2, and P3 group which had value 0.473, 0.101, 0.212, and 0.960 respectively. The result of homogeneity of cyclin D1 expression using Levene's test for each group was homogeneous with a value of $p= 0.246 (> 0.05)$.

The one-way ANOVA test to compare mean of cyclin D1 expression after administration of neem leaf extract showed a significant difference of variance between the four groups of experimental rats with a significance level of 0.000 (<0.05). The graph of a plot of the mean cyclin D1 expression for all groups is shown in Fig. 3.

Post hoc multiple comparisons analysis showed a significant difference between P0 and P1, P2, and P3 groups with a significance level of 0.000 (<0.05). P1 group had a significant difference with P0, P2,

and P3 with a significance level of 0.000. P2 group had a significant difference with P0, P1, and P3 with a significance level of 0.000. P3 group had a significant difference with P0, P1, and P2 with a significance level of 0.000.

DISCUSSION

After seven days of oral administration of neem leaves extract (50 mg, 100 mg, and 200 mg), twice a day, through an orogastric tube, the hepatocyte cyclin D1 expression counts were significantly increased when compared to control group which only got NAC administration as standard therapy. The control P0 group which only got NAC had the lowest cyclin D1 expression. The P1 group which got both NAC and 50 mg neem leaves extract, twice a day, had cyclin D1 expression mean higher than the P0 group. The P2 group which got NAC and 100 mg neem leaves extract, twice a day, had cyclin D1 expression mean higher than P0 and P1 group. The P3 group which got NAC and 200 mg neem leaves extract, twice a day (which was the highest dose), had the highest cyclin D1 expression mean.

Significance level analysis of variance (ANOVA) had a value of $p = 0.000$ (<0.05), concluded that the cyclin D1 expression variance of treatment groups significantly different. This significant ANOVA test result needs advanced test using multiple comparisons *post hoc* test which compares mean between treatment groups to define the mean difference of cyclin D1 more detail. The result of multiple comparisons *post hoc* tests between groups showed that all the significance level of variance analysis had a value of 0.000 (<0.05). This means that the cyclin D1 variances between the four groups of treatment were significantly different.

Neem (*Azadirachta indica* A. Juss) leaf extract was used in this study based on phytochemistry testing contain very high tannin, flavonoid, total polyphenol, and beta-carotene. Antioxidant activity test also revealed that neem leaves extract had high antioxidant capacity.

Oral administration of neem leaves extract on hepatotoxic acetaminophen-induced rats could neutralize the effects of free radical *N-acetyl-p-benzoquinone* (NAPQI) that produced as a metabolite of acetaminophen in the liver. Quercetine, catechin, other flavonoids, and beta-carotene which are abundant in neem leaf extract have three principal basic activities as an antioxidant. Firstly, cell scavenging activity from H_2O_2 and O_2 that formed on the xanthine oxidase metabolism pathway. Secondly, by preventing lipid peroxidation of cell membrane lipid bilayer, induced by *radical oxygen species* (ROS). And thirdly, by increasing glutathione-S

transferase (glutathione reductase) enzyme activity, one of the most important enzymes for protecting cell membrane integrity on mammals, particularly hepatocyte and erythrocyte cell membrane.^{4,19-23}

Liver stem cells could found on the border of portal system and hepatocyte lobules, there were in zone 3 of liver parenchyma. These stem cells can differentiate into hepatocytes and bile duct cells.¹⁶ Antioxidant's protection from the free radical activity will support a good regeneration process because the stem cells outside the necrotic area, particularly on vena centralis area and the portal system, can multiply and differentiate, to become a hepatocyte, and replace the necrotic hepatocytes, if there is no interruption from the free radicals. The antioxidant activity or scavenging of free radicals will inhibit the ceasing process of stem cell activity through p38 MAPK-p16Ink4a-retinoblastoma(Rb) pathway, which can be induced by free radicals.²⁴

CONCLUSION

Oral administration of neem leaf extract increases cyclin D1 expression in hepatocyte regeneration in acetaminophen-induced hepatotoxic Wistar rats, which increases along with the dosage of the neem leaf extract.

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