THE EFFECT OF ROSELLE (Hibiscus Sabdariffa L.) CALYX ETHANOLIC EXTRACT ON GST ACTIVITY AND DECREASING OF ALT AND AST LEVEL ON 7,12 DIMETHYL BENZ(A) ANTRASEN (DMBA) INDUCED RATS

By Zainur Rahman Hakim

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Zainur Rahman Hakim, Nurkhasanah, Laela Hayu Nurani

Postgraduate Program of Natural Drugs and Cosmetics, Pharmacy Faculty, Ahmad Dahlan University, Prof. Dr. Soepomo, S.H. St, Janturan, Warungboto, Umbulharjo,Yogyakarta, Postal Code 55164
Correspondence:
zainuralmubarok7@gmail.com
085643462477

ABSTRACT

Background : Oxidants or free radicals caused all abnormalities in this human body and to make liver cells damaged. Various natural products have been observing for drugs or compounds as antioxidants as Roselle (Hibiscus sabdariffa L).
Objective: The aim of this study was to determine ethanolic extract of roselle protect
Method: Roselle was extracted by maceration 60 %ethanol with a ratio of dry calyx and solvents are (1:3) stirer for 24 hours , then is filtered and concentrated by Rotary evaporator at 60°C . Maserate was transferred into a porcelain cup and evaporated on water bath at 60°C . Condensed extract is then stored in a sealed container and be identified .Forty-five Male Sprague Dawley rats age of 4 weeks divided into 9 groups: group I are baseline, group II and III was DMBA control group at day 7 and 35 (Single dose 75 mg/kgbw) . Group IV , V and VI are roselle extract treatment groups with variations each dose group of 10 mg / kg , 50 mg/kg and 100 mg/kgbw on day 7 induced DMBA 75 mg/kgbw. Group VII , VIII and IX treated with roselle extract in dose variation of each group 10 mg/kgbw, 50 mg/kgbw and 100 mg/kgbw. On day 35 induced DMBA 75 mg/kg BB . After that on day 7 and 35 after DMBA single dose, they were fasted (total 5 test animals each group) for 16 hours, then collect blood from eye (*orbital plexus*) and observed the SGOT and SGPT activity . After that, the animal were sacrified and hepar organ were observe GST gene expression by RT-PCR method. The data were analyzed using Kruska-wallis, Mann-Whitney, ANOVA, Least Significant Difference (LSD). Content of Polyfenol and Flavonoids use gallic acid and quercetin standart.
Outcome Measured : -
Result&conclusion: Results showed the increasing of ALT level (32.63±0.72) and AST level (27.09±1.51) at DMBA group compared to baseline ($p<0.05$). Significant differences occurred ALT and AST activity to DMBA control compared ($p<0.05$), decreasing of ALT and AST activity in a group of 10, 50, 100mg/kg/day compared to DMBA control group, a significant decrease of AST activity 18.45±0.92 ($p<0.05$) in the dose of 100 mg/kg/day

Keywords : Antioxidants , Roselle , glutathione peroxidase (GPx) , SGPT SGOT activity, GST activity, SD rats , DMBA

INTRODUCTION

Exposure to oxidants and free radicals due to higher levels of pollution in the environment cause various diseases that sometimes rarely encountered before such as skin cancer (Bicker and Athar, 2006), pulmonary disorders (Chen *et al*, 2008), abnormalities in brain nervous system (Pohanka, 2013), initiated cancer (Halliwell,2007). Recent studies proved evidence that disorder due to exposure to chemical oxidant compounds (Ciencewicki *et al*, 2008). DMBA (7,12-Dimethyl Benz(a) Anthracene) is polycyclic aromatic hydrocarbons compounds (PAHs), known as mutagenic, teratogenic, carcinogenic, cytotoxic and immunosupresif agents (Budi and Widyarini, 2010). It was *immunosuppressor* and a powerful organ-specific laboratory and widely used as carcinogen (Miyata *et* al,2001). This compound is a prooxidant and if its enters to human body will be metabolized and changed to Radical Oxigen Spices (ROS) epoxide dihidrodiol and radical cations (Hamid *et al*, 2009). These compounds also increasing liver damages in elevating levels of ALT, AST and ALP in rats (Radix, 2011).

DMBA can caused cancer through DNA binding-radical formation (DNA adducts), DNA damage and production of chronic inflammation. Several establish studies proved that DMBA mediates molecular, biochemical, genetic, and organ histopathology changes in patients with cancer (Gimenez and Slaga, 1993; Miyata *et al*.,2001). The damage due to Radical Oxigen Spices (ROS) was actually can be prevented by the presence of a primary antioxidant enzyme activity consisting of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Mates *et al* , 1999). Asni *et al* (2009) reported that the formation of oxygen radicals due to hypoxia can be compensated by an increase in primary antioxidants such as GST and catalase. Gluthation transferase (GST) catalyze gluthation formation of radicals into compounds that are easier to excretion. However, if the amount of ROS is excessive so the ability of GST become limited (Meiyanto *et al*. 2007).Therefore, we need a prevention system by consuming foods or fruits containing antioxidants. One of the plants are reported to have potential benefits as antioxidants is Hibiscus sabdariffa L. (Ross, 2003). The active substance in roselle that functioned as antioxidants such as gossypetin, anthocyanin, hibiscine glucoside (Wiyarsi, 2011). Based on this background, we wanted to prove whether the roselle calyx ethanolic extract have antioxidant activity in rats.

Results of research conducted by Kelv (2012) concluded that Hibiscus sabdariffa calyx extract dose of 250 mg/kgbw lowered malondialdehyde which is the end product of lipid peroxidation (oxidative stress is arising due to the triggered by free radicals) of 28.0 % in rats fed cooking oil and roselle calyx extract dose of 500 mg/kgbw lowered malondialdehyde 50.2 %. Ariati(2012) on her research concluded that the water fraction Hibiscus sabdariffa calyx can lower total cholesterol, LDL, and can reduce levels of ALT. Research on roselle also performed by Wijayanti (2013), which is that the ethanol extract of Hibiscus sabdariffa calyx has the ability to lower blood sugar levels (100 mg/kgbw), cholesterol and triglyceride levels returned to normal conditions. Based on this background, we wanted to prove the effect ethanolic extract of Hibiscus sabdariffa calyx has antioxidant activity on sprague dawley rats induced DMBA by determine GST, SGPT and SGOT activity.

METHODS

Extraction and Qualitatif Analysis

Hibiscus sabdariffa calyx was collected from Malang, East Java, Indonesia and identified by botanist at Laboratory of Biology, Faculty of Biology, Ahmad Dahlan University. The calyx were than dried at 60°C and powdered. 1500 g of roselle calyx powder were macerated by 7.5 liters ethanol 70 % (1:5), stirred for 3 hours and allowed for 24 hours. The extract filtered and evaporated with a rotary vacuum evaporator at 60°C and 100rpm, then concentrated on water bath with a temperature of 60-70°C to obtain a viscous extract. Roselle calyx ethanolic extract (RCEE) were weighed and the yield was calculated (Anonymous, 2004) Qualitative TLC (thin layer chromatoghrapy) analysis by radix (2011) with silica gel_{254} using the mobile phase (toluene:acetone:formic acid) (6 : 6 : 1).

Determination of Total Flavonoid Content

Total flavonoid content was determined according to Chang *et al* (2002) using quercetine as reference standard. Both roselle and rutin (50 mg) were dissolved in 50 mL ethanol. Subsequently, the quercetine solution was diluted to provide a series of concentrations (0.010; 0.015; 0.020; 0.025 and 0.030 mg/mL). The sample solution (0.5 mL) was added with 1.5 mL methanol, 0.1 mL of 10% AlCl3, 0.1 mL natrium acetate 1M and 2.8 mL distilled water, and then incubated for 30 minutes. Furthermore, absorbance was measured in 415 nm wavelength, and distilled water with 10% $AlCl_3$ and natrium acetate 1M was used as a blank. Total flavonoid content was expressed in gram quercetine equivalent (QE) of each 50 grams roselle dry weight.

Experimental

Twenty-five male Sprague dawley rats weighing 30-70 g were obtained from Departement of Animal Development and Research, Gajah Mada University, adapted for one week in a well-ventilated room, good humidity, quite light (12 hours of light and 12 hours dark). The maintenance done according to animal handling guideline in this departement. The rats than divided into five groups: Baseline, negative control, and dose variation RCEE groups at 10, 50, 100 day mg/kgbw/day for 7 days and at day 8^{th} all groups (exp.baseline) induced by single dose 75 mg/kgbw of DMBA intragastric. 7 days after DMBA induction, all groupswere fasted for 16 hours and collected blood from eye (*orbital plexus*) to observed the SGOT and SGPT activity. The animal were sacrified and liver organ were observed GST activity.

Sample Preparation

Blood samples were centrifuged at 4°C and 10,000 rpm. The serumes (top layer) then pipetted into the container for observing ALT and AST activity. Liver were chilled with liquid phosphate buffer pH 7.5 become to homogenates, than centrifuged at 4°C and 10,000 rpm and the serumes were collected and centrifuge 105,000 rpm, the final serume containing cytosolic GST for enzyme activity assay.

Determination of ALT and AST

All determination of ALT and AST activity was followed to Diasys® procedure kit. Determination of ALT used mixture reagent (RI), consist of 140 mmol/L of TRIS pH 7.15, L - alanine 700 mmol/L and LDH (laktate dehydrogenase) ≥ 2300 U/L; (RII) consist of 2-oksoglutarate 85 mmol/L and NADH 1 mmol/L. Sample solution contains a mixture of RI and RII with a ratio of 4 : 1. A total of 600 mL of reagent kits SGPT reacted with 60 mL of

sample, vortex and incubated at room temperature for 1 minute and read sample absorbance using a spectrophotometer at 334 nm wavelength. The results were then calculated the average is then multiplied by a correction factor (971) at a temperature of 25°C/30°C. Determination of AST used mixture reagent (R1) 80 mmol/L of TRIS pH 7.8, L-aspartate 240 mmol/L, MDH (malate dehydrogenase) ≥ 600 U/L and LDH (laktate dehydrogenase) ≥ 600 U/L, then made a mixture of reagents SGOT (R2) 2- oksoglutarate 12 mmol/L and NADH 0.18 mmol/L. Sample solution contains a mixture of R1 and R2 with a ratio of 4 : 1. A total of 1000 µL of reagent kits SGOT reacted with 100 µL of sample, vortex and incubated at room temperature for 1 and read absorbance using a spectrophotometer at 334 nm wavelenght. Results then read back after 1.2 and 3 minutes. The results were then calculated the average and multiplied by a correction factor (971) at a temperature of 25°C / 30°C.

Gluthation S Transferase (GST) activity test

Determination of cytosolic protein concentration (products) before GST enzym using the Biuret reagen with Bovine serume albumin (BSA) 1 % as a comparison standart. The absorbance both of BSA and samples were measured at a wavelength of 546 nm with incubation time 20 minutes. Absorbance of samples plotted to BSA standard curve to obtain the protein content (Yulius, 2001)

Determination of GST activity used rat liver cytosolic fraction following conditions incubation mixture (Habig et al. ,1974)with less modification: into 1 ml cuvette put 960 mL of 0.1 M phosphate buffer pH 6.5, 17.5 mL cytosolic fraction (containing GST) rat liver, 20 mL of GSH (Gluthation) 50 mM (solution in distilled water), and 20 mL of CDNB (1-chloro-2,4-dinitrobenzene) 50 mM (dissolved in ethanol). GS-DNB conjugate product was measured at a wavelength of 340 nm from minute 0 to 3 minutes using a simple kinetic program. Measurement results are in the form of data rate (rate = Δ absorbance/min)

$$V = \frac{Rate}{\Delta\varepsilon_{GS-DNB} \cdot d \cdot c}$$

Notes

$\Delta\varepsilon_{GS-DNB}$: Extinction coefficient ($9{,}6\ mM^{-1}cm^{-1}$)

d : Cuvette width (cm)

c : Protein concentration (mg/ml)

V :velocity of conjugate formation

Statistical Analysis

The data were presented as mean ± the standard error of the mean (SEM). That value represents potencial of the protected effect of RCEE to liver. All data were analyzed statistically using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P-values less than 0.05 were considered significant.

RESULTS AND ANALYSIS

Extraction and Qualitatif Analysis

The result extraction of 1500 g rosella dry calyx with 70% ethanol yielded 615,45 g viscous ethanolic extract (41,03%). It means that each 100 g dry weight of RCEE contained total flavonoid equivalent to quercetine of 41,03 g. Qualitative Analysis was done to observe chromatography profile on TLC (Thin Layer Chromatography) plate and demonstrated by RF (Retention Factor) value. RF value showed the quercetine

(flavonoid) content on the extract. The result showed a weak spot on TLC as high as quersetin standart and RF value was 0,87. Quercetine was used as comparison standart because most of plants have this compound.

Total Flavonoids Contents

In this study, total flavonoid contentwas determined using a modified method basedon the procedure of Chang *et al.* (2002) using quercetine as a reference standard. Principally, the procedure is related tothe formation of flavonoid and $AlCl_3$ complex that produces a yellow coloured solution. Theabsorbance is measured spectrophotometrically at maximum wavelength of 415 nm.The total flavonoid content was equivalent to quercetine milligram per gram dry material of the extract.The absorbances of a series concentrations of quercetine were plotted to their concentration to yield a linearcalibration curve of quercetine (y = 0,04263x-0,14195) with coefficient of correlation (r) value of 0,99159.

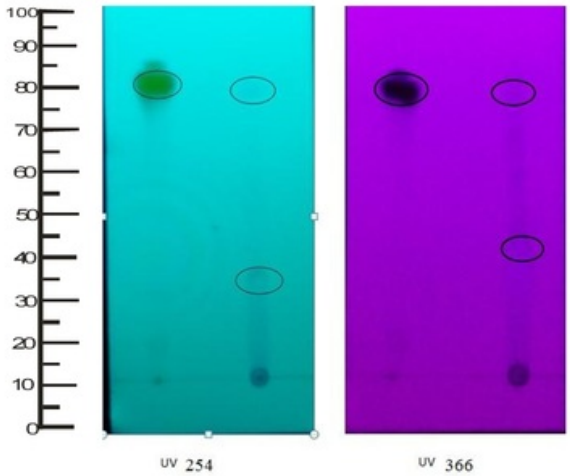

Figure 1.TLC analysis

Table 1. Total Flavonoids

Sample Replication	Abs	X (µg/mL)	Flavonoid (/50 g RCEE)	$\dot{X} \pm SD$
1	0,336	11,219	0,37	0,363±0,012
2	0,310	10,612	0,35	
3	0,331	11,105	0,37	

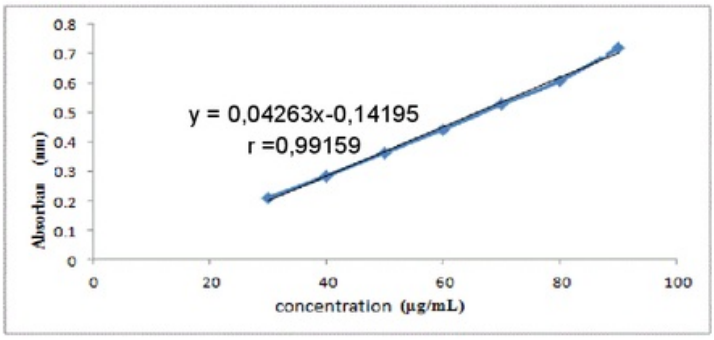

Figure 2. Flavonoid Standard Curve

The ortho hydroxy group of the quercetin binds the Al^{3+}. The color changes (color loss) of RCEE from red-purple to dark blue after the addition of $AlCl_3$ was due to the presence of hydroxyl groups on antosian compound that binds to metal Al^{3+}.

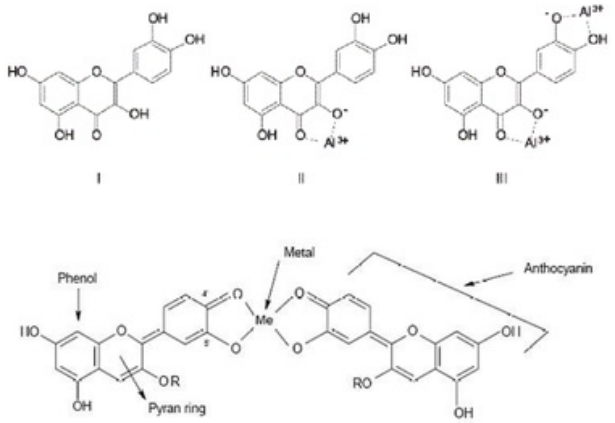

Figure 3. Quercetine and Anthocyanin Metal Complexes

Determination ALT and AST Activity

ALT and AST determination of the activity carried out on seven days after administration of DMBA induction to see how much liver damage caused. Detailed results are presented in the following table.

Table 2. ALT and AST Activity

Kelompok	SGPT	SGOT
Baseline	23,60±0,55	18,64±0,65
DMBA	32,63±0,72	27,09±1,51
Rosela 10 mg/kgBW	31,19±0,83	24,28±0,69
Rosela 50 mg/kgBW	25,37±0,61	20,15±0,63
Rosela 100 mg/kgBW	23,67±0,73	20,03±0,83

Result data above showed a significant improvement that DMBA incrased ALT and AST levels. ALT (Alanine transaminase) and AST (Aspartate transaminase) is a transaminase enzyme that is normally located in the liver and other organs. ALT enzymes in the body catalyzed changed reversibly of L-alanine and α- ketoglutarate into Pyruvate and L-Glutamate, whereas AST catalyzed L-Aspartate catalyze change + α - ketoglutarate into oxaloacetate and L - Glutamate be reversibly . Normally these changes (becomes Piruvate and Oksaloasetate) is intended to produce energy in the metabolic processes such as glycolysis . But if there is damage to cellular proteins severe then the process is not reversible . So that this enzyme will continue and increase in blood serum.

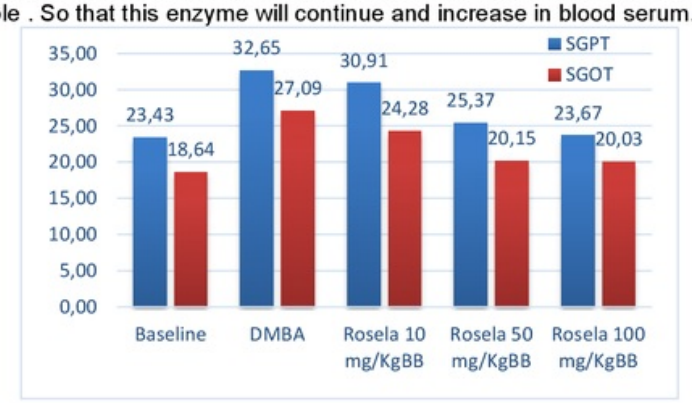

Figure 4. Decreasing ALT and AST activity profile

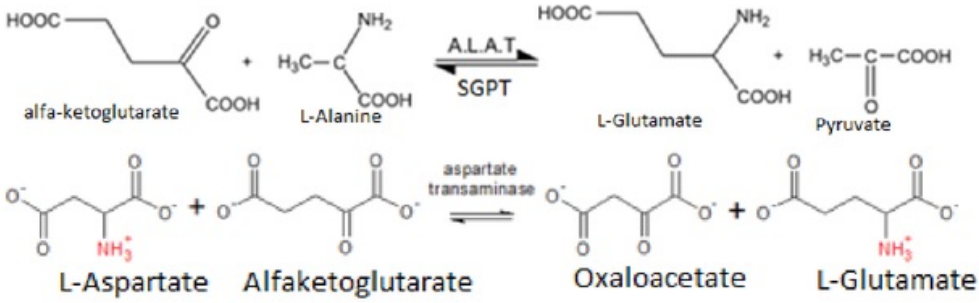

Figure 5. Transaminase Reaction

Determination GST Activity

Determinating of protein content of rat cytosol fraction before specific activity of GST aims to determine how much protein per unit volume in the cytosolic fraction rats liver. Protein content data is then used to calculate specific GST activity cytosolic

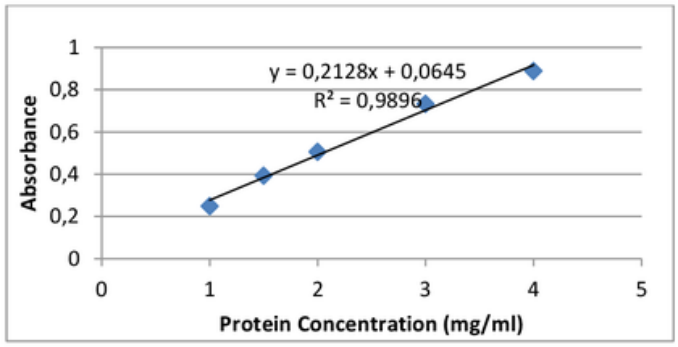

Figure 6. The standard curve of BSA

In this study, absorbance of BSA standard plotted to yeild a linear calibration curve with coefficient of callibration (0.9896). this curve was used to obtain protein content (c). Protein data is then calculated by the formula enzyme activity as described earlier.

Table 3. The protein content

Groups	Protein Content (mg/mL)
Baseline	1,703
DMBA	0,519
10 mg/kgbw	2,292
50 mg/kgbw	2,538
100 mg/kgbw	1,001

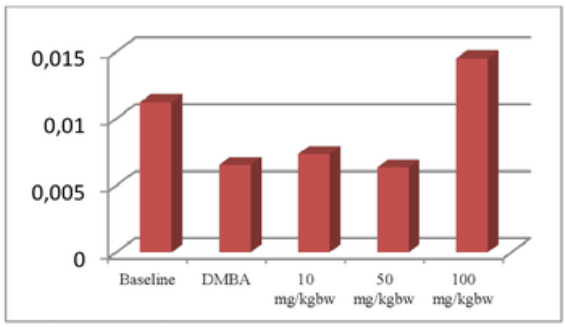

Figure 7. The GST activity of rosella extract treated rats.

From the results of the determination of the protein content of rat liver cytosol fraction showed presence decrease in protein levels on DMBA groups compared to the control. Increased levels of protein in a dose groups I, II , and III compared to the control group becausetheir inhibition of protein biosynthesis. DMBA, a member of the polycyclic aromatic hydrocarbons, is present in the environment as a product of incomplete combustion of complex hydrocarbons. Being an indirect carcinogen, DMBA requires metabolic activation to become a carcinogen. DMBA is metabolized by cytochrome

P4501A1 in liver microsomes and by cytochrome P4501B1 in primary bone marrow stromal cells to form diol epoxides and other toxic ROS. The toxic metabolites of DMBA, including diol epoxides, are capable of binding to adenine residues of DNA causing chromosomal damage (Guerin, 1978). Accumulation of reactive oxygen species in cells damages the defence mechanisms of the body including the DNA structure and enzymatic balance triggering the process of cancer pathogenesis (Mates *et al.*, 2000). The reactive oxygen species (ROS) are continously produced within the cell, particularly during mitochondrial electron transport chain, and in peroxisomes (Cadenas and sies, 1968). Futhermore, foreign compounds such as toxins, drugs, and foods or exposure to environmental factors such as pollutants, heavy metals or ultraviolet radiations can cause ROS (Hanukoglu, 2006). Whereas excess of ROS induces oxidative modification of cellular macromolecules, which leads to aberrant cell functions causing menbrane and DNA damage, enzyme inactivation, and ultimately, cell death (Circu and Aw, 2010). Endogenous enzymatic and non-enzymatic antioxidant defenses, and exogenous antioxidants supplied by the diet play a povital role in such protective mechanisms. (Masella *et al.*, 2012)

The decreasing GST activity on DMBA groups showed the hepatotoxic event. On the RCEE group (100 mg/kgbw) showed the hepatoprotective effect by increasing GST activity. It means that roselle "accompanied" effect to decreasing oxidant in RCEE group.there was possible mechanism such as inhibing DMBA the Cyp 450 metabolism to epoxid or scavaging the radicals of ROS products. The GST activity from rat liver rat are presented in table. Based results of statistical analysis using one-way ANOVA, there were significant differences between DMBA and Baseline, control group compared three groups treatment at the level of 95 %.

Table 3. GST activity

Dosing Group	GST Activity
Baseline	0.011233[a]
DMBA	0.006533[b]
10 mg/kgBW	0.007333
50 mg/kgBW	0.006333
100 mg/kgBW	0.0145

The reaction between GSH and CDNB is nucleophilic aromatic substitution. The processinvolves intermediates compounds are known as Meisenheimer complex.

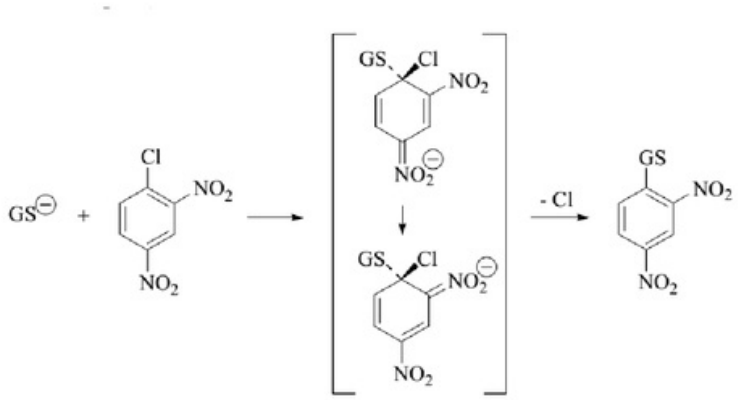

Figure 8. Meisenheimer complex (Josephy,1997)

Measurement of conjugates product in GSH with CDNB reaction was catalyzed by GST performed at a maximum wavelength 340 nm. The GS - DNB conjugate products started from minute 0 - 3 (Habig *et al.*,1974). from the results shown significant improvement in RCEE dose of 100 mg / kgbw . this indicates protection by GST activity by increasing the speed of GSH with CDNB bond. Other other study roselle has antioxidant and hepatoprotective activity in fish CCl4 induced significantly by lowerin mechanism of lactate dehydrogenase (LDH), glutamate oxalate transaminase (GOT), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH - Px) (Yin, 2011).

CONCLUSION

From the result and statistical analysis, we can conclute that RCEE (Roselle Calyx Ethanolic Extract) can functioned as antioxidant by incrasing GST activity and Decreasing ALT and AST activity mechanism.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest

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