

Anti-carcinogenic activity of *Gynura procumbens* Extract through Cytochrome P450 and Glutathione S-transferase

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Abstract: This study aim to examine the molecular activity of cytochrome P450 and enzymatic GST μ (glutathione S-transferase μ) in Sprague-Dawley rat liver after induction of *G. procumbens* extract. Sprague Dawley female rats aged 40 days were divided into 5 groups, group I was the positive control which treated with 7,12-Dimethylbenz(a)anthracene (DMBA) 20 mg / kg bw; group II and III were given ethanol extract of *G. procumbens* 300 mg / kg bw and 750 mg / kg bw DMBA plus 20 mg / kg bw; group IV and V is a negative control which treated with the extract of 300 and 750 mg / kg bw without DMBA administration. In the third week of treatment, rat hepatic cytochrome P4501A1 activity was measured using the substrate 7-ethoxyresorufin-O-deethylase (EROD), while GST μ reacted with 1-Chloro-2,4-dinitrobenzene measured by spectrophotometry λ 340 nm. The results showed significant decrement of cytochrome P450 activity ($p < 0.05$) and the significant increasing of GST μ activity ($p < 0.05$) in rat which given *G. procumbens* extract at concentration 300 and 750 mg / kg bw. In conclusion, the ethanol extract of *G. procumbens* leaf acts as a blocking agent in carcinogenesis initiation phase which inhibits further progression towards malignancy.

Keywords: *Gynura procumbens*, Cytochrome P450, GST μ activity.

Introduction

World Health Organization reported that in every 11 minutes 1 patient died because of cancer and in every 3 minutes new cancer cases happened¹. According to Brauch, breast cancer incidence rates increased by 1-2% every year and cause of woman death, especially in industrialized countries such as in Germany one of three of women died at the age less than 60 years because of breast cancer. Based on world statistics, one of eight women has a risk for breast cancer¹.

Risk of breast cancer is modulated by mutation of some proteins such as BRCA1, BRCA2, ATM, CHEK2, PTEN, and TP53². At the protein level of mutation (missence mutation at residue 194), p53 will lose the ability to bind with its response elements in DNA^{3,4}. Treatments that have been used are surgery, radiotherapy, and chemotherapeutic drugs⁵. However, these treatments have some bad effects. Surgery will cause of re-multiplication surgery, radiotherapy resulted in other tissues damage the administration of anticancer chemotherapy has less selective pharmacological effects, and some types of cancer resistance⁶. Treatment of breast cancer in particular plant extracts such as ethanol extract of *Gynura procumbens* leaves which done by Sugiyanto et al. contain flavonoids and has been proved has the ability to inhibit lung tumors growth in mice induced by benzo (a) Pirene. Tasminatun states treatment of *G. procumbens* ethanolic leaf extract which given 3 times a week with dose of 250 mg / kg and 750 mg / kg after breast cancer initiation using DMBA induction on rat has a tendency to reduce the incidence of tumors by 10-20%^{7,8}.

7,12-Dimethylbenz(a)anthracene (DMBA) is a liver microsomal enzyme inductors, it is the most potent of the group polycyclic aromatic hydrocarbon (PAH) that can be metabolized to toxic compounds⁶. According

to Zhai et al., DMBA is involved in activation of cytochrome P450 enzyme (CYP) isoforms, CYP1A1, peroxidase into reactive intermediates that can damage DNA. The body reaction in maintaining the reactive intermediates is by increasing the enzyme glutathione S-transferase (GST) level as a reactive compound conjugates^{9,10}. The increasing of GST enzyme, will induce detoxification of carcinogen compounds which inhibit the next stages of cancer development. The important research studies is the role analysis of medicinal plant activity in carcinogenesis mechanism⁵. The mechanism is mediated by some factors in the body that involves regulation of enzymatic activity by cytochrome P450 (CYP1A1) and Glutathion-s-transferase (GST)⁹. The purpose of this research is to examine the molecular activity of cytochrome P450 and GST μ enzymatic (glutathione S-transferase μ) of rat liver after induction of *Gynura procumbens* extract in Sprague-Dawley strain rat.

Materials and Methods

Animal model for this research was Sprague Dawley female rats aged 40 days and weighing 25 ± 60 g which divided into 5 groups. Each group located in one cage, fed, and ad libitum drinking. Experimental animals acclimatized in experimental room and cage. Materials which needed for GST activity test are 2,4-dinitro-1-Chloro benzene (DCNB), 0.1 M phosphate buffers pH 6.5, the cytosolic fraction (protein content specific), GSH 50 mM (solution in distilled water). the materials of cytochrome P450 activity test are covers, β -naphthoflavone, corn oil, substrate 7-ethoxyresorufin-O-deethylase (EROD), 200 μ l microsomal protein (2 mg / ml), 0.05 M Tris-HCl buffer (pH 7.5), 100 μ l BSA (10 mg / ml in Tris buffer), 20 M MgCl₂ 25, 40 μ l solution of cofactor (NADP + 0.3282 g and 1.417 g of glucose 6-phosphate in 40 ml KCl), 2.5 units of glucose 6-phosphate dehydrogenase, 10 μ l substrate (1 mg in 10 ml of methanol ethoxyresorufin), DMSO (dimethyl sulfoxide) and methanol.

The equipments which used in this study are a set of surgical instruments, sterile disposable syringe, microsprit injector, ointment pots, glassware, cages, sterile tube, flask, pipette, electrical gram scales, pH meters TOA HM-60S, ultra centrifugator (Hitachi SCP 85H), electrical balance (Shimadzu, type LS-6DT), vortex, micropipette, and spectrophotometry λ 340 nm.

DMBA solution that treated to rats was prepared by dissolving 50 mg of 7,12-Dimethylbenz (a)anthracene / DMBA (Sigma Chem Co.) in 20.0 ml of corn oil-and resulted in concentration 2.5 mg / ml. DMBA treatment dose of 200mg/kg bw is calculated depends on rats body mass which increased every DMBA administration time

$$\text{Administration volume of DMBA} = \frac{\text{Body Weight Of Rat}}{1000} \times \frac{\text{Dose}}{\text{Concentration DMBA}}$$

G. procumbens leaves was dried and grounded into powder, then 500 grams of powder was extracted by maceration with 96% ethanol, 1.5 L. After 3 x 24 hours extract was filtered using rotary evaporator to obtain a thick extract. Total of 750 mg *G. procumbens* ethanolic extract were suspended in 0.5% CMC Na 25 ml to obtain the 30 mg / ml concentration. The *G. procumbens* ethanol extract treatment doses 300 mg / kg bw and 750 mg / kg bw were calculated depends on rats body mass which increased every administration time.

$$\text{Volume administration} = \frac{\text{Body Weight Of Rat}}{1000} \times \frac{\text{Dose}}{\text{Concentration Extract}}$$

Rats were divided into 5 groups: group I were as positive control with criteria of rat aged 40-53 days without treatment and the age of 54-61 days which given DMBA 20 mg / kg bw; Group II and III are rats aged 40-53 days which given ethanol extract of *G. procumbens* 300 mg / kg bw and 750 mg / kg bw and the age of 54-61 days given the ethanol extract of *G. procumbens* DMBA plus 20 mg / kg bw; group IV and V were negative controls, with criteria rats aged 40-61 days which given ethanol extract of *G. procumbens* 300 and 750 mg / kg bw without DMBA administration.

Measurement of Cytochrome P4501A1 Activity

At the age of 61 days, rats were anesthetized then dissected. Liver was homogenized with cold blender then centrifuged at 10,000 g for 20 min 40°C. Supernatant were resuspended at 100,000 g 40°C for 90 minutes. Pellet which resulted is a microsomal fraction contained cytochrome P450. For the measurement 200 μ l of

microsomal protein (2 mg / ml) mixed with 640 μ l 0.05M Tris-HCl buffer (pH 7.5), 100 μ l BSA (10 mg / ml in Tris buffer), 20 μ l 25 M $MgCl_2$, 40 μ l cofactor solution (0.3282 g 1.417 g NADP + and glucose 6-phosphate in 40 ml KCl), 2.5 units of glucose 6-phosphate dehydrogenase, 10 μ l substrate (1 mg of 7-ethoxyresorufin-O-deethylase / EROD in 10 ml of methanol), and 10 μ l 4'BF various concentrations in DMSO (α -naphthoflavone as a positive control and DMSO as a negative control). After incubation at 37°C for 2 min, the reaction was terminated by 2 ml of methanol administration. Samples were then centrifuged for 20 min at 2,000 g. Supernatant fraction was measured with a spectrophotometer (excitation wavelength 530 nm and emission 586 nm). Percentage barriers observed as

$$\frac{1 - (A \text{ sample} - \text{blank})}{(A - \text{blank DMSO})} \times 100$$

Measurement of GST activity

Rat liver samples homogenized in 0.25 M sucrose (pH 7.4) and cold centrifuged at 9000 g for 20 min. supernatant the centrifuged at 100,000 g for 1 h to obtain the cytosolic fraction. total of 15 mL cytosolic fraction was treated with 15 mL 50 mM GSH, 705 μ l R / 0.1 M phosphate buffers pH 6.5 and 15 mL 1-Chloro 2,4 dinitro benzene (CDNB) 50 mM, then measured at λ 345 nm from minute 0 to minute 3 using a spectrophotometer with a simple kinetic method.

V = Product conjugate GS-CNB

V = (speed of reaction / $\Delta\epsilon$ GS-CNB) thick cuvette / protein levels in the incubation mixture

$\Delta\epsilon$ GS-DNB = 9.6 mM-1cm-1

Data were analyzed using one-way ANOVA with Tukey post-hoc test at 95% confidence level to compare GST μ and CYP450 activity between treatment groups. Statistical tests carried out with SPSS® software for Windows.

Results

Data of cytochrome P4501A1 activity which reacted with EROD and GST μ reacted with 1-Chloro 2,4 dinitro benzene listed in Table 1.

Table 1 Data cytochrome P450 activity and GST activity

Group	P450 Activity	GST Activity
DMBA 20 mg/kg.bw	1,2768 ^a \pm 0,13	5,1244 ^a \pm 1,43
Gynura p 300 mg/kg.bw + DMBA 20 mg/kg.bw	0,7298 ^b \pm 0,28	8,1390 ^b \pm 1,18
Gynura p 750 mg/kg.bw + DMBA 20 mg/kg.bw	0,9212 ^{ab} \pm 0,34	7,1408 ^{ab} \pm 1,03
Gynura p 300 mg/kg.bw	0,6024 ^b \pm 0,24	5,6250 ^a \pm 2,15
Gynura p 750 mg/kg.bw	0,8452 ^b \pm 0.36	8,1718 ^b \pm 1,59

The analysis results of the data normality test showed that the activity of cytochrome P450 is normally distributed ($p > 0.05$). One-way analysis of variance test showed the effect of *G. procumbens* extract administration were significantly increase the activity of cytochrome P450 ($p > 0.05$) on rat liver which initiated with DMBA 20 mg / kg bw. Further test showed the highest value of cytochrome P450 activity is in the treatment group I (0.13 ± 1.2768 A), but it was not significantly different with treatment group III (0.34 ± 0.9212 ab).

The normality test data of Kolmogorov Smirnov showed that data was normally distributed ($p > 0.05$). One-way analysis of variance test showed that ethanol extract of *G. procumbens* has significant affect to GST μ activity. Further tests showed that treatment group V showed the highest level of treatment effect and significantly different with group I and IV, but it is not significantly different from groups II and III.

Discussion

G. procumbens extract has effect on rat hepatic microsomal cytochrome P450 fraction activity which initiated with DMBA 20 mg / kg bw. The highest level of cytochrome P450 activity was in the treatment group I (0.13 ± 1.2768 A) which given 20 mg of DMBA / kg body weight at the age of 54-61 days. Material which used in this study such as 7,12-Dimethylbenz(a)anthracene (DMBA), PhIP (2-amino-1-methyl-6-phenylimidazo (4-5-b) pyridine) and NMU (nitrosomethylurea) were known as carcinogen that can induce breast cancer^{11,12,13,14}. DMBA is one of PAHs (polycyclic aromatic hydrocarbons) compound. This reactive compound will easily bind covalently to tissue macromolecules including DNA¹⁵. PAH metabolism in mammals conducted by the activity of CYP gene that encodes cytochrome P450 enzyme, the enzyme is regulated by Aromatic Hydrocarbon Receptor (AHR), and cytochrome P450 metabolic activity which responsible against PAHs and aromatic amines¹⁵.

DMBA activation mechanism has been described by Melendez-Colon et al. that involves cytochrome P-450 enzyme and peroxidase or become reactive intermediate that can damage DNA and the formation of the radical dihydrodiol epoxide cation¹⁶. Dihydrodiol epoxide ecosyclic will bind to amino group of purine base of DNA then adducts covalently into a stable form, while the radical cation would bind purine N7 or C8 which becomes unstable and cause depurination Dihydrodiol epoxide is responsible for tumors initiation by forming radical cation¹⁶.

The second treatment group had low P450 activity, but GST activity is quite high compared to other treatments. P450 CYP1A1 isoform often associated with activation of DMBA in rodents¹⁷. *G. procumbens* ethanol extract administration will suppressed the activity of P450 in mice. *G. procumbens* ethanol extract contains flavonoids which leads to form flavones or flavonols¹⁸. This study is related to research of Zhang et al. that the flavonols, kaempferol, and quercetin could significantly inhibit CYP1A1 in cultured rat hepatocytes.

The results showed that the activity of CYP1A1 have some significant differences between the treatment groups. It is related to research which conducted by Zhai et al. that some flavonoid compounds have high potency and selectivity for inhibiting CYP1A isoenzymes. The main compound on the flavonoid extract could possibly inhibit the CYP1A1 isoenzyme before and during the initiation which has specific activity with DMBA metabolite. Specific activity of P450 isoenzymes CYP1A1 and CYP1A2 can be inhibited by flavonoids^{9,20}.

The results showed that the GST μ activity is highest in the group V which has been treated with *G. procumbens* extract 750 mg / kg bw for 8,1718b \pm 1.59. Glutathione S-transferase (GST) is known as a large group of enzymes that play a role in detoxification. Membrane-bound enzyme, microsomal GST and leukotrienes C4 synthetase are encoded by a single gene. Appelt and Reicks research was conducted to prove that the expression level of GST is an important factor in determining the sensitivity of cells against toxic. Chemopreventive agents act as blocking agents that affect the initiation phase of cancer, such as detoxification of carcinogenic compounds through the increasing of GST expression²². This is related to Appelt and Reicks research²¹ which showed high GST activity on the second week in rats with a high isofalvonoids diet. Some of flavonoids such as genistein, rutin, silymarin, catechins, and anthocyanins are able to increase the expression of GST^{21,22,23}.

G. procumbens extract contains flavonoids, unsaturated sterols, triterpenes, polyphenols, tannins, saponins, and essential oils^{24,25,26}. Class of terpenoid compounds also have been proven to stimulate detoxification through induction of GST. Such as carvone, cineol, famesol, geraniol, limonene, menthol, perillyl-alcohol, and α - pinene²⁷. Based on research conducted by Kensler and Crowell has been shown that monoterpenes have chemopreventive effects through the induction of GST in the cancer initiation phase. Diterpene compounds like cafestol and kahweol may increase the expression and activity of placental GST^{28,29,30}.

Treatment group V were issued phase II enzymes to metabolize more xenobiotic compounds, in this case DMBA, which will increase the expression of GST enzymes in liver. This is in line with the nature of DMBA as a bifunctional enzyme inducer that can induce phase I and phase II of metabolism through activation of aryl hydrocarbon receptor and Nrf2¹⁰. AHR translocation will occur after its transcription factor binds to the transporter ARNT nuclear chaperones. AHR-ARNT complex will binds and activates the XRE gene. XRE

activation will cause the expression of cytochrome p450 enzymes and transcription factor Nrf2. These enzymes will metabolize xenobiotic compounds and produce electrophilic metabolites. These metabolites will activate Nrf2-ARE pathway which will induce the enzymes of phase II metabolism, including GST enzymes¹⁹.

The treatment group extract 750 mg / kg DMBA + GST activity was not able to raise as much as treatment group extract 300 mg / kg + DMBA. This might be due to a dose 750 mg / kg were able to induce GST in high number, but it will led the extract and GST conjugated in a fairly short period of time. Many of the compounds that induce GST is a substrate for GST itself or the result of cytochrome P450 metabolism that can act as a GST substrate¹⁷.

Several in vivo studies prove that the ethanol extract of *G. procumbens* leaf was able to inhibit lung and stomach tumor growth in mice which induced by benzo (α) pyrene^{7,31}. However, research which conducted by Yen et al. showed that the concentration of flavonoids was also influence the level of GST activity in the same way as treatment group extract 750 mg / kg + DMBA. Quercetin at 200 mM concentration coulful significantly decreased GST activity in human lymphocytes. Naringenin at concentrations of 25-50 mM could increase the activity of GST at higher concentrations³².

Conclusions

G. procumbent 300 and 750 mg / kg bw significantly decreased the cytochrome P450 activity ($p < 0.05$), but significantly increased the GST μ activity ($p < 0.05$). Therefore the ethanol extract of *procumbens* leaf acts as a blocking agent in the initiation phase of carcinogenesis which inhibits further development towards malignancy.

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