Activity of Noni Fruit (*Morinda citrifolia* L.) Ethanolic Extract on Class *mu* Glutation S-Transferase of Lung Rat

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**Abstract**

One of the main modalities of cancer treatment is chemotherapy, which uses chemicals that are generally electrophilic. These xenobiotic compounds sometimes do not produce effective response due to activity of glutathione S-transferase (GST) which inactivate the xenobiotics. Several natural phenolic compounds were reported to inhibit GST activity in vitro. Noni fruit (*Morinda citrifolia* L.) which contains flavonoids and other phenolic compounds such as scopoletin and morindon is proposed to interfere GST activity. This study aimed to analyze the effect of ethanolic extract of Noni fruit *in vivo* on GST activity in lung rat using 1,2-dichloro-4-nitrobenzene (DCNB). This substrate is a specific for class *mu* GST. First, rats were administered with ethanolic extract of Noni and dimethylbenz(α) anthracene (DMBA) for two weeks. The cytosolic fraction of lung was isolated then the GST activity was determined by simple kinetic program which was automatically calculated using spectrophotometer. The results showed that ethanolic extract of Noni in 1 and 5% (w/v) of concentration induced class *mu* GST activity, whereas 10% (w/v) of concentration inhibited class *mu* GST activity. After a treatment with DMBA, all tested concentrations of ethanolic extract of Noni inhibited class *mu* GST activity of lung rat significantly. These results indicated that Noni fruit extract can be further developed as a supportive agent of a chemotherapy drug.

**Keywords:** DMBA, GST, *Morinda citrifolia* L., spectrophotometer.

**INTRODUCTION**

Cancer is one of the leading causes of death in industrialized and developing countries with an estimation of 18.1 million of new cases and 9.6 million of cancer deaths (Bray, *et al*., 2018). Several methods of cancer therapy have been carried out intensively, such as surgery, chemotherapy, radiation, and hormonal therapy. Unfortunately, several chemotherapeutic agents are failure because of increasing of glutathione S-transferase (GST) activity. This enzyme accelerates the elimination process of electrophilic xenobiotic (Allocati, *et al*., 2018). For this reason, efforts are needed to reduce the GST activity in order to increase the efficacy of chemotherapeutic agents with electrophilic xenobiotics such as...
as cisplatin, 5-fluorouracil, and adriamycin.

Glutathione S-transferase is a superfamily enzyme which is naturally found in both eukaryotes and prokaryotes. According to their cellular localization, GSTs in eukaryotes are divided into three families, that are cytosolic, mitochondrial, and microsomal GST (Sheehan, et al., 2001; Oakley, 2011). This enzyme is found in several tissues such as liver, kidney, lung, and small intestine (Van Haften, et al., 2003). Based on the structure and function, GST is grouped into 12 classes including alpha, zeta, mu, phi, sigma, omega and theta (Antonella, et al., 2003; Oakley, 2011) depending on their amino acid sequence (Guneidy, et al., 2017). To analyze GST activity, a substrate that is specific to certain GST isoenzymes can be used. The 7-chloro-4-dinitrobenzo-2-oxa-1,3-diazol (CDNB) is a common substrate used for all classes of GST (Sheehan, et al., 2001).

GST has several important biological roles, including involve in the synthesis of leukotriens and prostaglandins, cell protection against toxic molecules and oxidative stress (Hayes, et al., 2005) and catalyzes conjugation of electrophilic xenobiotics (including many carcinogens) with glutathione (GSH) to make a hydrophilic molecules which easier to be eliminated in a metabolism process. This faster elimination of electrophilic xenobiotics eventually causes resistance to cytostatic drug (Allocati, et al., 2018).

Natural phenolic compounds such as polyphenols, flavonoids, and curcumin have been known as inhibitor compounds of GST activity in vitro (Sudibyo, 2000; Hamed, et al., 2014; Arinc and Yilmaz, 2014; Guneidy, et al., 2017). Other research reported that pentagamavunon-0 (a curcumin analogue) inhibited class mu GST activity but not of class phi of rat kidney (Sudibyo and Supardjan, 2002).

Mengkudu (Morinda citrifolia L.) which is known as Noni has been used by Polynesians as a traditional drug for more than 2000 years (Wang, et al., 2002). This herb is reported to have extensive therapeutic effects as antibacterial, antiviral, antitumor, analgesic, hypotensive agent, anti-inflammatory and immune system enhancement (Wang, et al., 2002; West, et al., 2018). Several active compounds have been identified from Noni fruit including scopoletin (a phenolic coumarin), anthraquinone, flavonoids, carotenoids, rubiadine, terpenoids, noniosides, morindon, and beta carotene (Almeida, et al., 2019). Ethanolic extract of Noni at 26.6 and 133.1 mg/kg body weight administered orally to experimental rat was reported able to increase activity of class mu GST in liver (Atiah, 2006) and kidney organ (Rosaningsetyas, 2006). This study aimed to analyze the effect of ethanolic extract of Noni fruit in vivo on GST activity in lung rat induced by DMBA using 1,2-dichloro-4-nitrobenzene (DCNB) substrate which is a specific substrate for class mu GST.

MATERIALS AND METHODS

Materials

Matured Noni fruit was taken at the Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. The Noni fruit was yellowish green in color. Male rats (Wistar strain), 2 months of age, 175-210 g of weight were from Sanggar Kegiatan Belajar, Bantul, Yogyakarta, Indonesia. Bovine serum albumin (BSA), 7,12-dimethyl benz(α)anthracene (DMBA), and 1,2-dichloro-4-nitrobenzene (DCNB) were purchased from Sigma (St. Louis, Missouri, USA). Glutathione (GSH), ethanol, sodium carbonate, sodium nitrite, aluminium chloride, sodium hydroxide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, copper sulphate pentahydrate, Folin-Ciocalteu reagent and potassium sodium tartrate were purchased from Merck (Darmstadt, Germany). Corn oil was purchased from PT. Nutrifood Indonesia, Bogor, Indonesia.

Methods

Preparation of Noni extract

A number of 5 kg of fresh Noni fruit were
blended and macerated with 70% ethanol in a closed vessel for 3 days while stirring occasionally. The obtained filtrate was evaporated using vacuum rotary evaporator (Heidolph VV 2000) at 60°C, then dried by freeze drying (Free Zone®). A number 45 g of dried extract were collected. The determination of phenolic compounds in dried extract of Noni was performed with Folin-Ciocalteu reagent.

Maximum Wavelength and Operating Time Measurement for Protein Determination

The Lowry method used in protein determination is a simple and specific method with high reproducibility and still shows good sensitivity at low concentration up to 5 µg protein. From this measurement, obtained operating time was 45-55 min and λ max 749 nm.

Treatment of Experimental Rat

A number of 50 male rats were randomly divided into 10 groups which consist of 5 rats each group. Each group was placed in one cage. Feed and drinking water were replaced every day, while the husks were replaced every two days. The cages were placed in room with 25°C of temperature and lighting (approximately 15 Watt lamps) on for 8 h and off for 16 h. For adaptation purpose, rats were kept in the cages for one week. For the treatment of each group, it can be seen at Figure 1. Since the dried extract of Noni was completely dissolved in water, we did not use a suspending agent such carboxy methyl cellulose for this purpose.

Preparation of Pulmonary Cytosolic Fraction

After the treatments were completed, rats were fasted for 24 h before being sacrificed by cervical dislocation and their lungs were taken for the preparation of the cytosolic fraction containing GST by multilevel centrifugation method (Hitachi SCP 85 H) according to Lundgren, et al. (1987) with a slight modification, that are time and variation of ultracentrifugation speed. Determination of protein content was carried out by spectrophotometry using Lowry method and BSA comparison standard at maximum wave length.

The organ was taken after the rats had been fasted 24 h (only given by tap water) to minimize the nutritional content of food in the blood which might affect the GST activity. Lung was homogenized by a cold blender (4°C) at 440 rpm in phosphate buffer (pH 7.5) to avoid extreme changes which disrupt the its biological activity.
Specimen were centrifuged at 10,000 x g for 30 min at 4°C to precipitate the collapse of the cells, cell nuclei, and mitochondria. The supernatant was collected and recentrifuged at 105,000 x g for 90 min to precipitate microsomes. The supernatant from this step was cytosolic fraction containing unrefined GST.

**Determination of GST Activity**

The determination of GST activity was performed with DCNB as the substrate (Habig, et al., 1974). Incubation mixture consisted of 0.1 M phosphate buffer pH 7.5; cytosol fraction; 50 mM GSH and 50 mM DCNB. As the blank, 0.1 M phosphate buffer pH 7.5 was used. The GS-CNB conjugate product was measured at λ 345 nm with simple kinetic methods using a spectrophotometer (Genesys-5 Milton Roy). The measurement results are a rate of reaction (Δ absorption per minute). Protein concentration in the cytosol fraction was determined by Lowry methods (Lowry, et al., 1951) using bovine serum albumine (BSA) to make a protein standard curve.

**Calculation of Percent (%) Inhibition or Induction**

From the absorption measurement of GS-CNB conjugate product, the value of Δ absorption per minute (rate) was obtained.  

\[ V = \frac{\text{GST activity}}{\text{Rate/ΔεGS-CNB, cuvette thickness/protein concentration in the mixture}} \]

\[ ΔεGS-CNB = 8.5 \text{ mM}^{-1}\text{cm}^{-1} \]

\[ \text{Percent inhibition or induction} = \frac{[V\text{ control} - V\text{ treatment}]}{V\text{ control}} \times 100\% \]

**Data Analysis**

Since the obtained data of GST activity calculation were parametric, the Anova test was carried out by SPSS for Windows program to see whether there were differences between control and treatment of administration of Noni fruit extract and DMBA in GST activity.

**RESULTS**

**Ethanolic Extract of Noni Contained Phenolic Compounds**

Determination of phenolic compounds in dried extract of Noni was positive because it formed a blue color with the Folin-Ciocalteu reagent. This method used sodium carbonate because reaction of phenolic substances with the Folin-Ciocalteu reagent occurs in an alkaline atmosphere (Prior, et al., 2005).

**Tabel 1. GST activity of each treatment measured by spectrophotometry.**

<table>
<thead>
<tr>
<th>Group of Rat</th>
<th>Protein Content ±SD (mg/mL)</th>
<th>GST Activity ±SD (nmol/min/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.0895±5.29 x 10⁻⁴</td>
<td>345.08±24.65</td>
</tr>
<tr>
<td>II</td>
<td>0.0515±1.72 x 10⁻⁴</td>
<td>402.58±23.46</td>
</tr>
<tr>
<td>III</td>
<td>0.0664±9.02 x 10⁻⁴</td>
<td>413.85±18.28</td>
</tr>
<tr>
<td>IV</td>
<td>0.0711±3.06 x 10⁻⁴</td>
<td>310.52±21.95</td>
</tr>
<tr>
<td>V</td>
<td>0.0866±1.01 x 10⁻³</td>
<td>331.11±31.19</td>
</tr>
<tr>
<td>VI</td>
<td>0.0511±5.51 x 10⁻⁴</td>
<td>319.48±38.62</td>
</tr>
<tr>
<td>VII</td>
<td>0.0929±2.78 x 10⁻³</td>
<td>561.83±67.20</td>
</tr>
<tr>
<td>VIII</td>
<td>0.1008±3.93 x 10⁻³</td>
<td>301.99±18.30</td>
</tr>
<tr>
<td>IX</td>
<td>0.1101±2.84 x 10⁻³</td>
<td>198.54±15.60</td>
</tr>
<tr>
<td>X</td>
<td>0.0776±1.00 x 10⁻³</td>
<td>272.87±15.157</td>
</tr>
</tbody>
</table>

Note:

**Group 1**, rats without treatment and were only given by food and drink for 14 days. **Groups 2, 3, and 4**, rats were given by ethanolic extract of Noni orally with level of 1, 5, and 10%, respectively, once a day for 14 days.

**Group 5**, rats were treated by corn oil as DMBA solvent control, intragastrically, 3 times a week for 2 weeks. **Group 6,** rats were treated by DMBA in corn oil, intraperitonially at a dose of 20 mg/kg body weight, 3 times a week for 1 week.

**Group 7,** rats were treated by DMBA in corn oil, intraperitonially at a dose of 20 mg/kg body weight, 3 times a week followed by ethanolic extract of Noni fruit orally at level of 1, 5, and 10%, respectively, in distilled water, once a day for 7 days.
Measurement of GST Activity

The measurement of conjugate product was carried out at λ 345 nm, which is the maximum wavelength of GS-CNB complex. The measurement was also linear within 3 minutes with changes in absorption less than 0.05 per minute. Therefore, measurements of the conjugate product were performed from minute 0 to 3. The results of determination of GST activity showed that treatment with ethanolic extract of Noni at 1 and 5% of dose (group II and III) and DMBA for 2 weeks (group VII) increased the GST activity. Interestingly, the GST activity decreased after DMBA treatment which followed by extract etanolic of Noni (group VIII-X), as shown in Table 1.

Ethanolic Extract of Noni Inhibited GST Activity After Treatment with DMBA

A compound has the ability to inhibit GST activity if its GST activity value is smaller than the control, whereas it has an inducing activity when the GST activity value due to the treatment is greater than control. The results of the calculation of the percentage values are listed in Table 2 and 3.

**Table 2. The induction percentage of GST activity compared to control.**

<table>
<thead>
<tr>
<th>Group of Rat</th>
<th>GST Activity ±SD (nmol.min⁻¹/mg protein)</th>
<th>Inhibition Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without treatment</td>
<td>345.08±24.65</td>
<td>-</td>
</tr>
<tr>
<td>Corn oil 2 weeks</td>
<td>331.11±31.19</td>
<td>-</td>
</tr>
<tr>
<td>Extract 1%</td>
<td>402.54±23.46</td>
<td>16.66 **</td>
</tr>
<tr>
<td>Extract 5%</td>
<td>413.85±18.28</td>
<td>19.93 **</td>
</tr>
<tr>
<td>DMBA 2 weeks</td>
<td>561.83±67.20</td>
<td>69.79 **</td>
</tr>
</tbody>
</table>

**Table 3. The inhibition percentage of GST activity compared to control.**

<table>
<thead>
<tr>
<th>Group of Rat</th>
<th>GST Activity ±SD (nmol.min⁻¹/mg protein)</th>
<th>Inhibition Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without treatment</td>
<td>345.08±24.65</td>
<td>-</td>
</tr>
<tr>
<td>DMBA 2 weeks</td>
<td>561.83±67.20</td>
<td>-</td>
</tr>
<tr>
<td>Extract 10%</td>
<td>310.52±21.95</td>
<td>10.01 **</td>
</tr>
<tr>
<td>DMBA 2 weeks + extract 1%</td>
<td>301.99±18.30</td>
<td>46.25 **</td>
</tr>
<tr>
<td>DMBA 2 weeks + extract 5%</td>
<td>198.54±15.60</td>
<td>64.66 **</td>
</tr>
<tr>
<td>DMBA 2 weeks + extract 10%</td>
<td>272.87±15.07</td>
<td>59.44 **</td>
</tr>
</tbody>
</table>

a) = GST activity compared to control (without treatment)
b) = GST activity compared to DMBA 2 weeks
* = statistically significant (p=0.05)
Inhibition percentage was counted by the mean data

**DISCUSSION**

This study aimed to analyze the effect of ethanolic extract of Noni fruit on GST activity in lung rat. Ethanol was chosen in the extraction process of the active compounds because it is a universal solvent which can dilute many kind of chemical compounds. Alcoholic solvent such as ethanol 70-80% is the best choice for extracting flavonoids (Robinson, 1995), as contained in Noni fruit. Extraction vessels were placed in the dark place and wrapped in a black chamber in order to reduce the influence of sunlight because the presence of sunlight can induce the damage of chemical contents especially flavonoids.

Because there was no purification of obtained GST, enzyme activity was measured in micromole product yielded per minute per milligram protein in the final incubation medium. In other word, determination of GST activity was carried out to find out how much enzyme that catalyzed the conjugation reaction of GSH and DCNB measured by spectrophotometry (Habig, et al., 1974). Phosphate buffer was used as reaction medium because it did not inhibit GST activity (Clark, et al., 1991). The pH value used was 7.5 because optimum GSH conjugation reaction with DCNB was in that pH value (Habig, et al., 1974). Other research also showed that the conjugation reaction of GSH and DCNB was optimum at pH 7.5 to 9.0 (Booth, et al., 1961). Sudibyo (1997) also reported that 0.1 M phosphate buffer at pH 7.5 was the optimum reaction condition of GST from rat liver to its substrate.

It is known that DMBA that is included in polycyclic aromatic hydrocarbons (PAHs) will form a dihydrodiol epoxide in the presence of cyto-
chrome P-450 and microsomal hydrolase epoxide. This metabolite will bind with nitrogen atom number 7 (N7) or carbon atom number 8 (C8) of purine bases and forming a unstable products and trigger the formation of apurinic sites (AP) as a result of a spontaneous depurination process (Melendez-colon, et al., 1999; McCarty, et al., 2009). Epoxide compound which is easy to be an electrophilic form will induce the expression of GST, a phase II metabolism enzyme. The electrophilic compound will be conjugated with glutathione to be a hydrophilic metabolite and easy to eliminate (McCarty, et al., 2009). GST induction might be mediated by antioxidant response elements and its transcription factor Nrf2, which is involved in the induction of many phase II enzymes (Thimmulappa, et al., 2002).

As shown in Table 2, treatment with DMBA for 2 weeks increased GST activity 69.79%. GST plays a regulatory role in cellular signaling in the process of protein interactions with critical kinases involved in controlling stress response, apoptosis and proliferation (McIlwain, et al., 2006). That is why in cancer case, GST activity will increase and cancer treatment with some electrophilic drug will not be effective.

From Table 2, it can be seen that treatment of Noni extract at concentration 1 and 5% increased GST activity. Some phenolic compounds were reported to be involved in the induction cellular defence system including detoxifying and antioxidant enzyme systems (Huang, et al., 2010). Flavonoids also induce enzyme expression of phase II biotransformation such as GST and quinon reductase (Ren, et al., 2003). This induction effect of GST activity might be because of iridoid, a natural product found in Noni fruit. Iridoid was reported to induce GST activity at 100 µM (Mukanganyama, et al., 2011). It is interesting because iridoid was also reported to inhibit GST activity at low concentration but induce it at high concentration. Those opposite results indicate that there could be other molecular interactions occuring the substrates and the enzyme. Contrary to iridoid and some flavonoids which induce GST activity, other research reported that some kind of flavonoids such as quercetin and hesperidin inhibited GST activity competitive mechanism; naringenin and rutin by mixed-type mechanism (Arinc and Yilmaz, 2014; Karakurt, et al., 2015); while ellagic acic inhibited GST activity by noncompetitive mechanism (Karakurt, et al., 2015). It indicate that different chemical structure including phenolic content in Noni fruit, such as such as scopoletine, proxirionine, and flavonoids including morindon and rubiadin (Bangun and Sarwono, 2002) could have difference binding site at GST enzyme and produce difference effect as Arinc and Yilmaz (2004) and Karakurt, et al. (2015) mentioned.

In other results, as shown in Table 3, the administration of Noni fruit extract was able to inhibit GST activity after administration of DMBA significantly (p=0.05). Extract with 1, 5, and 10% content inhibited GST activity of 46.25; 64.66 and 59.44%, respectively. This is beneficial when administration of Noni is combined with chemotherapy agent such as adriamycin, cisplatin, 5-fluorouracil, and vincristine because the therapeutic effect of those agents will be optimum (Ali, et al., 2016). Wang, et al. (2002) reported that ethanolic extract of *Morinda citrifolia* L. were able to indirectly suppress the tumor growth by increasing the immune response by inhibiting the production of tumor necrosis factor-alpha (TNF-α), which is a promoter of endogenous tumors and interleukin-1, interleukin-12, and nitrate oxide (Brown, 2012).

**LIMITATION OF STUDY**

This study still needed many improvements in order to get adequate result and conclusion, including purification of the extract and determining the extract effect of Noni to several classes of GST.

**CONCLUSION**

Ethanolic extract of *Morinda citrifolia* L. at level 1 and 5% induce GST activity. It means in this concentration, with more intensive research, the
extract can be used as chemoprevention agent. In other hand, after treatment with DMBA, the extract inhibit GST activity which means it can be used as a promising supportive cancer therapy because the electrophilic drug such as busulfan and cisplatin are not eliminated too early.

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