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Detoxification of 1,2-dihydroxy- 4-allylbenzene, a major phenolic compound in *Piper betle,* through glucuronidation using S9 protein of rat liver (*Sprague Dawley*)

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ABSTRACT

1,2-dihydroxy- 4-allylbenzene (DHAB) is a major phenolic compound in *Piper betle* leaf and also a metabolite of safrole metabolism. Epidemiologic studies showed people who have betel quid chewing habit are related to the incidence of oral submucous fibrosis and/or tumor formation. The aims of this research was to study the detoxification through the glucuronidation of DHAB using S9 protein of male rat liver (*Sprague Dawley)* and a co-factor of uridin 5′-diphosphoglucuronide acid (UDPGA). The results showed that glucuronidation of DHAB using S9 protein of rat liver resulted two isomers of glucuronide metabolites due to the availibility of two active hydroxyl groups that one of them can bind glucuronide but not two of hydroxyl groups at the same time.

Keywords: 1,2-dihydroxy- 4-allylbenzene, safrole, glucuronidation, S9 protein rat liver, *Piper betle*

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INTRODUCTION

1,2-dihydroxy-4-allylbenzene (DHAB) is a major phenolic compound in *Piper betle* leaf which is used for betel quid chewing for some Asian or Chinese and some other traditional medicines (Chung *et al*., 2008). It is also a major metabolite of safrole metabolism in rat and human in vitro experiment (Martati *et al*., 2011; Martati *et al*., 2012). The inflorescence of *Piper betle* used for betel quid chewing contains 9.74 mg/g wet weight of DHAB (Hwang *et al*., 1992). Chewing betel quid shows strong correlation to the incidence of oral cancer, leukoplakia and oral submucous fibrosis (IARC, 1985; Chang *et al*., 2001). Oral cancer cases in betel quid chewers are linked with alcohol drinker and tobacco smoker but It showed that betel quid chewing alone associated with oral cancer which was statistically significant (Ko *et al*., 1995). Safrole-DNA adducts were found in specimen of oral squamous cancer taken from Taiwanese patients who were tobacco betel quid chewers (Chang *et al*., 2001). The safrole-DNA adduct is formed through cytochrome P450 mediated formation of 1'-hydroxysafrole which are then sulfonated resulting stable safrole-DNA adduct in vivo and in vitro experiments (Miller and Miller, 1981). Moreover, in vitro experiment with Chinese hamster ovary cells showed DHAB-induced oxidative DNA damage resulted 8-OH-dG (8-hydroxydeoxy-guanosine) formation which was responsible for the chromosome aberration, micronucleus formation and cytotoxicity (Lee-Chen *et al*., 1996).

Some studies showed that DHAB has bioactivity properties for both toxicological or health benefits aspects. DHAB has antioxidant, antinitrosation, antimutagenic and anticarcinogenic properties against various mutagens and carcinogens, inhibits the benzopyrene-DNA interactions activated by mouse and rat liver S9 protein (Amonkar *et al*., 1989). In contrast, DHAB may induce mutations in the Ames test, glutathione (GSH) depletion, reactive oxygen species (ROS) production, cell cycle arrest at higher concentration (Chang *et al*., 2002).

Another source of DHAB is from metabolism of safrole. Figure 1 shows metabolism of safrole results 1′-hydroxysafrole, 3′-hydroxysafrole, 2′,3′-dihydroxysafrole dan 1,2-dihydroxy-4-allylbenzene (Martati *et al*., 2011; Martati *et al*., 2012). Safrole is a bioactive compound in some spices such as nutmeg, mace, cinnamon, star anise, ginger and black pepper (Periasamy *et al* ., 2016 ; Rocha *et al*., 2016) and a major compound in sassafras oil (*Sasafras albidum*). Intake of some food supplements containing those spices raise a health of concern (Al-Malahmeh *et al*., 2017; Berg *et al*., 2011). Pure safrole as flavoring agent has already banned by FDA (Food Drug Association) since December 1960 and by EFSA (European Food Safety Authority) food since 2008. Those prohibitions were taken because safrole is a genotoxic and carcinogenic compound in rodents assay. In rodents, safrole is metabolized in the liver through phase I metabolism mediated by cytochrome P450. Based on validated physiologically-based kinetic model of safrole metabolism for rat and human, the formation of DHAB was predicted 74.5 and 38.0% of the dose, respectively (Martati *et al*., 2011; Martati *et al*., 2012). The major detoxification pathway of 1'-hydroxysafrole resulted different pathway for rat and human which are glucuronidation and oxidation, respectively. DHAB undergoes conjugation reaction which could be glucuronidation, sulfation, and glutathione conjugations (Nakagawa *et al*., 2009). Therefore, it is necessary to study the detoxification of DHAB via glucuronidation in vitro. The aims of this research was to study the detoxification of glucuronidation of DHAB using S9 protein of rat liver and co-factor of uridin 5′-diphosphoglucuronide acid (UDPGA).

MATERIALS AND METHODS

Materials

Materials: S9 protein from liver of rat (*Sprague-Dawley)* was obtained from BD Gentest (Worburn, USA). Uridine 5′-diphosphoglucuronide acid (UDPGA) was purchased from Sigma-Aldrich. Acetonitrile chromatography grade was obtained from Biosolve. Dimethyl sulfoxide (DMSO)

was purchased from Acros Organic (New Jersey, USA). Tris (hydroxymethyl) aminomethane and alamethicin (from *Trichoderma viride*) were obtained from Roche Diagnostics Germany.

Glucuronidation of DHAB

Reaction of glucuronidation of DHAB was performed as previously described for 1'-hydroxy alkenylbenzenes (Punt *et al*., 2008;Al-Subeihi *et al*., 2011; Martati *et al*., 2011). The incubation mixtures were consisted of 10 mM UDPGA, 0.2 mg protein S9/mL, 0.2 M Tris-HCl (pH 7.4) and 10 mM $MgCl_2$ (Table I) Pre-incubation with 0.025 mg/mL alamethicin for 15 min on ice was carried out to maximize glucuronidation enzyme activity (Fisher *et al*., 2000). After that, the mixture was incubated in a water bath at 37°C for 1 min. Then, 1 uL substrate of DHAB from a 200 time concentration stock solution in DMSO was added. Incubation was performed for 10 min. The reaction was stopped by adding 50 µL of cold acetonitrile. Sample was centrifuged at 16,000 g for 5 min. Aliquots was stored at -20°C prior HPLC analysis. Blank was made as the same for sample but without UDPGA added. Each treatment was repeated three times.

Table I. Composition materials for glucuronidation of DHAB

Materials	Blank (µL)	Sample (µL)
0.2 M Tris-HCl pH 7.4 and 10 mM $MgCl_2$	196	146
10 mM UDPGA	0	50
S9 protein of rat liver	2	2
0.025 mg/mL alamethicin	1	1
DHAB 50 µM	1	1

HPLC Analysis

Quantification of metabolites of glucuronidation reaction was performed on a HPLC equipped with photodiode array detector and column Alltima C18 5 µm, 150 x 4.6 mm (Grace Alltech, Breda, The Netherlands). The gradient elutions were nanopure water containing 0.1% (v/v) acetic acid and acetonitrile. The flow rate was set at 1 mL/min. Gradient was programmed from 10% to 30% acetonitrile in 30 min. After which, the acetonitrile was increased to 100% in 2 min and kept for 2 min. After that, the acetonitrile was decreased to 10% in 2 min and kept for 10 min. Chromatograms were compared between samples and blanks. The peaks appeared in the sample but not in blank were metabolite of glucuronides of DHAB. The absorbance of the peak was measured from 200 to 400 nm to obtain the UV spectra and confirm the identity of the peak.

RESULTS AND DISCUSSION

Glucuronidation is a glucuronic acid transfer from UDPGA to a large variety of aglycones (endo- and xenobiotics) catalyzed by UGTs (uridine diphosphate glucuronosyltransferases), a class of membrane bound enzymes of endoplasmic reticulum (Fisher *et al*., 2000). In metabolism of some alkenylbenzenes group, glucuronidaion is a major detoxification pathway (Punt *et al*., 2008; Al-Subeihi *et al*., 2011; Martati *et al*., 2011; van den Berg *et al*., 2012). The addition of alamethicin in the incubation of glucuronidation reaction was performed to insert it in to the membranes and forming pores, therefore, it removes the latency of UGT activity (Fisher *et al*., 2000). Glucuronidation of DHAB with the cofactor of UDPGA resulted two metabolites (peak at time retention of 25.690 min dan 26.394 min) as shown in Figure 2A and Figure 2B. A blank (without co-factor of UDPGA) shows no metabolite formed. This showed that glucuronidation of DHAB resulted two isomer of glucuronides metabolites. The UV spectra of the peaks shows that the two glucuronide metabolites have different UV spectra (Figure 2 (1) and Figure 2 (2)). Figure 2 (3) shows UV spectra of a substrate of DHAB. The peak area of the DHAB glucuronide appeared at 25.690 min and at 26.394 are 74.962 ±

9.765 and 200.563 ± 27.687, respectively. It shows that DHAB glucuronide peak appeared at 26.394 min is bigger than peak at 25.690 min. The concentration of each DHAB glucuronide was not quantified because un-available of the commercial standard compound of DHAB glucuronides.

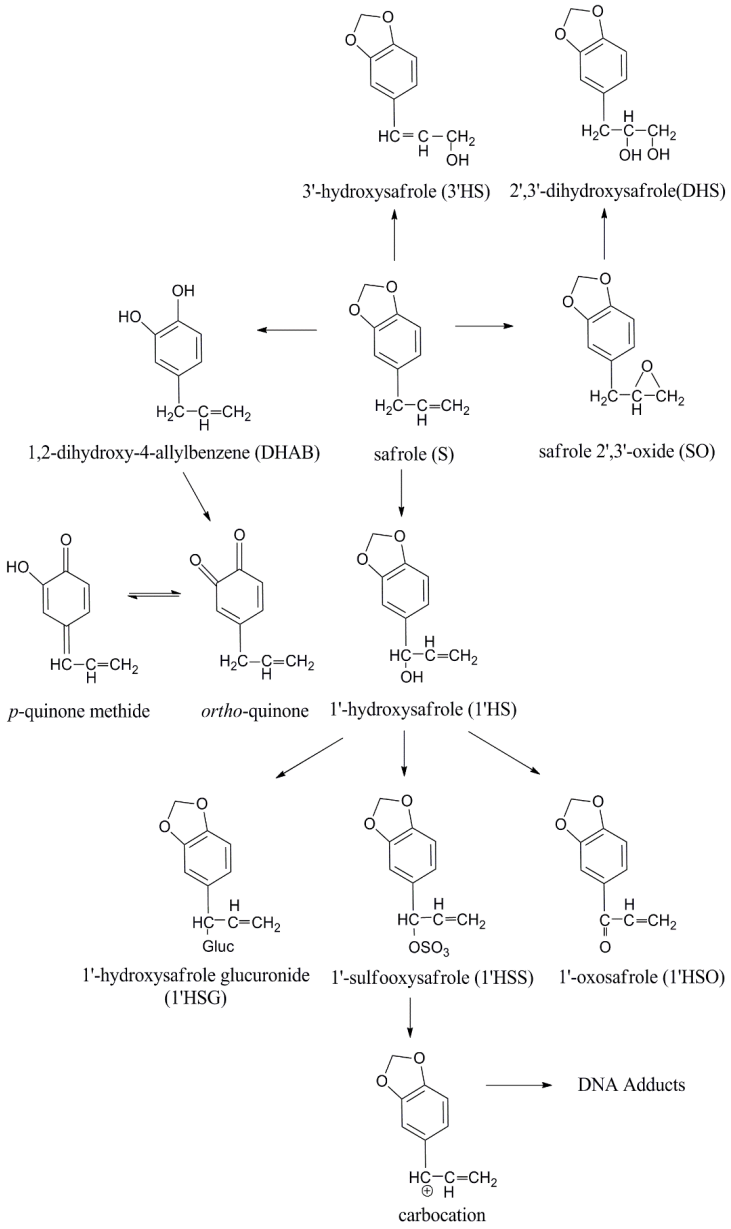

Figure 1. Metabolisme of safrole (Martati *et al*., 2011)

Experiment of Nakagawa *et al*., (2009) resulted that hydroxyl groups of DHAB are mostly converted to sulfat and/or glucuronides conjugates in the hepatocytes rat Male F344/Jcl exposed to DHAB (Nakagawa *et al*., 2009). It was found only one glucuronide of DHAB, although, there was one unknown peak next to the DHAB glucuronide showed in the chromatogram that was possibly another DHAB glucuronide (Nakagawa *et al*., 2009). The two hydroxyl groups in DHAB provide functional groups for conjugates but only single hydroxyl binds glucuronic acid (Figure 3). This is supported by (Nakagawa *et al*., 2009) that the diglucuronides of DHAB were not present as trace metabolites in

incubation DHAB with rat hepatocyte suspensions. Glucuronidation plays an important role in detoxifying or inactivating some endogenous compounds and xenobiotics (Fisher *et al*., 2000). The glucuronide metabolites are readily excreted in the urine. In rat hepatocytes, DHAB showed more toxic in cells than the metabolites of glucuronides, sulfate dan GSH conjugates showing that those phase II reactions play important role in detoxification of DHAB (Nakagawa *et al*., 2009). In vitro mutagenicity test using *Salmonella typhimurium* TA97, TA98, TA100 and TA102 showed that exposure of cells with DHAB increased the number of those bacterial revertants indicated that DHAB were mutagenic compounds (Lee-Chen *et al*., 1996). In addition, exposure the cells with DHAB in the presence of S9 rat liver decreased the number of revertants. This indicated that metabolites of phase II reactions have less mutagenicity than those of DHAB (Lee-Chen *et al*., 1996).

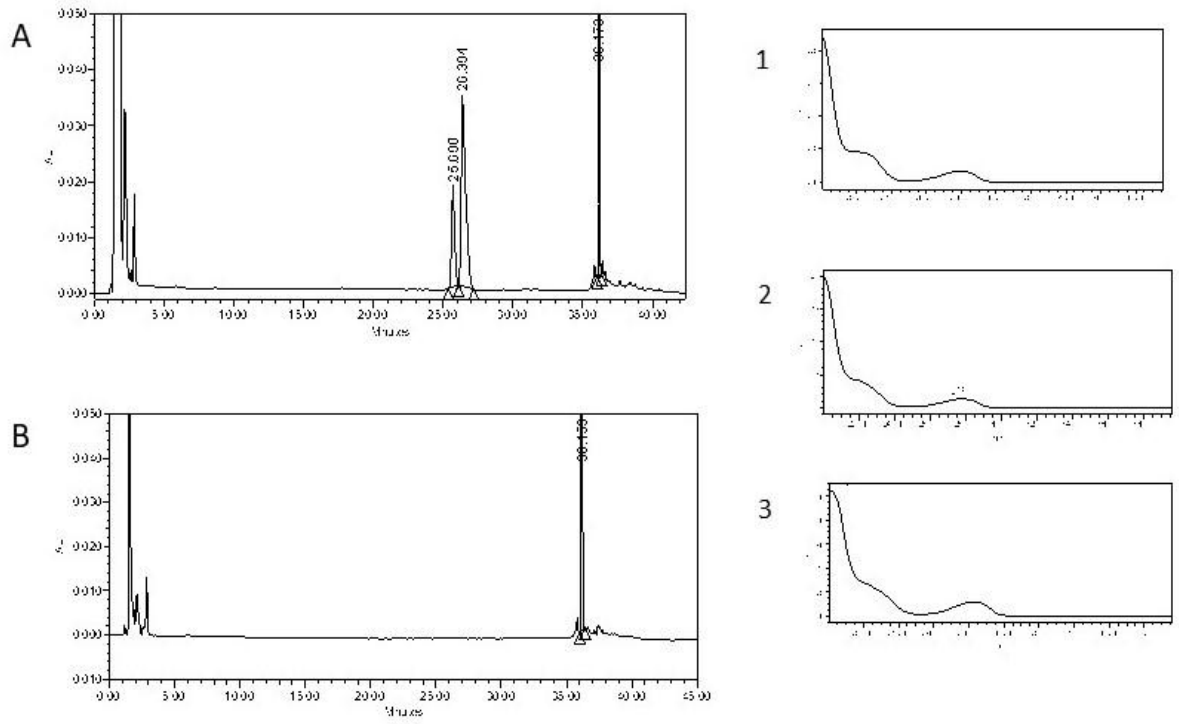

Figure 2. HPLC chromatogram of an incubation of DHAB (0.25 μM) with male rat liver S9 homogenates in the presence of UDPGA as a cofactor (A) and without UDPGA (B). UV Spectrum of DHAB glucuronide peak at 25.690 min (1); DHAB glucuronide peak at 26.394 min (2); DHAB peak at 36.173 min (3)

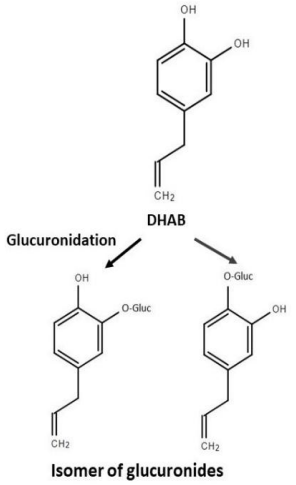

Figure 3. Proposed metabolites of glucuronidation of DHAB mediated rat liver S9 protein

CONCLUSION

Detoxificaton of DHAB through the glucuronidaton using rat liver S9 protein resulted two isomer metabolites of glucuronides due to the availibility of two active hydroxyl groups that one of them can bind glucuronide but not two of hydroxyl at the same time Those two glucuronides have un-equal concentration of the metabolite.

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