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Inhibitory Effect of Selected Malaysian Herbal Plants on Glutathione S-transferase Activity

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Abstract: Many compounds from plants have been found to play an active role in inhibition and induction of GST activity. This study aims to evaluate the capability of five commonly used medicinal plants in Malaysia to inhibit the glutathione S-transferases (GST) activity *in vitro*. The ability of the plant extracts to inhibit GST activity was examined on rat liver cytosolic fraction and was analyzed using ultraviolet (UV) absorbance at 340 nm. When 1-chloro-2,4-dinitrobenzene (CDNB) was used as a substrate, tannic acid exerted inhibition with IC_{50} value of 6.18 μg mL^{-1}. The methanol extracts of *Orthosiphon stamineus* and *Cinnamomum iners* demonstrated the highest inhibitory activity against GST activity showing IC_{50} values of 35.20±8.72 and 35.55±3.84 μg mL^{-1}. It is noted that, at 250 μg mL^{-1} *C. iners* exhibited 1.3 more inhibition activity than tannic acid. The different parts of *Croton argyratum* plant were also evaluated on the GST inhibitory potential. Comparing the inhibition abilities of each part of Croton argyratum in decreasing order are leaves>roots> stems with IC_{50} values of 40.42, 57.88 and 143.80 μg mL^{-1}, respectively. The data may suggest potential use of *Orthosiphon stamineus* and *Cinnamomum iners* as herbal medicines with GST inhibitory effect.

Key words: Glutathione S-transferases inhibition, *Croton argyratum, Curcuma xanthorrhiza*, *Eurycoma longifolia, Orthosiphon stamineus*, *Cinnamomum iners*

INTRODUCTION

Herbal medicines which are utilized traditionally to cure illness may interact with human cytochrome P450s (Hanapi *et al*., 2010; Sharif, 2003) and with the drug metabolizing enzyme such as glutathione S-transferases (GST's) (Azizi *et al*., 2010; Coruh *et al*., 2007; Zhang and Wong, 1997). Indeed, focus on the interaction of the herbal compound with drug metabolizing enzymes has received increased attention. The CYP (phase I) and GST (phase II) catalytic enzyme play crucial role in drug metabolism pathway in mammals. Both enzymes determine the pharmacological and toxicological properties of the ingested drugs.

GST is a phase II enzyme that accounts for multifunctional role in the cell defense system against electrophilic compounds (Ploemen *et al*., 1994). GSTs also exhibit antioxidant properties due to their selenium-independent glutathione peroxidase activity (Widersten and Mannervik, 1995). GSTs are known to be associated with the cell's resistance to chemotherapy (Burg and Mulder, 2002), antibiotics (Arca *et al*., 1988) and insecticides (Fournier *et al*., 1992). Therefore, researchers are continuously investigating the evaluations of GST inhibitory potential.

Resistance of tumor cell to electrophilic xenobiotics such as alkylating agent after cancer chemotherapy treatment has been linked with the over-expression of certain GST isoenzyme (Burg and Mulder, 2002). GST is involved in the detoxification and metabolism of various anticancer drugs such as chlorambucil (Hayes and Pulford, 1995). Consequently, the effort to detoxify the drug during continuous chemotherapy treatment leads to the increase in the GST level (Tew, 1994). Thus, the plant exhibiting inhibitory potential on the GST activity will be crucial in increasing the efficiency of cancer chemotherapeutics agent (Athar *et al*., 2007). Moreover, there have been wide applications of the natural occurring GST inhibitors on treating the Alzhiemer's disease (Lovell *et al*., 1998; Sultana and Butterfield, 2004) and Parkinson (Kiyohara *et al*., 2010; Menegon *et al*., 1998) diseases.

The present study focuses in evaluating the rat liver glutathione S-transferases (GSTs) inhibitory potential by five local plants namely *Croton argyratum, Curcuma xanthorrhiza*, *Eurycoma longifolia*, Orthosiphon stamineus and *Cinnamomum iners* (Table 1).

Curcuma xanthorrhiza is a traditional herb which originated from the ginger family (Zingiberaceae). This plant is used for various purposes from treatment of liver

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Table 1: Plants and their phytochemical content

Plant	Phytochemical content
Curcuma xanthorrhiza	Sesquiterpenoid, reducing sugars, saponins, anthraquinones, flavonoids, terpenoids and cardiac glycosides (Devaraj *et al*., 2010)
Croton argyratum	Diterpene, junceic acid, styryldehydropyrone and goniothalamin (Norizan *et al*., 2007)
Cinnamomum iners	Cardiac glycoside, flavonoid, polyphenol, saponin, sugar, tannin and terpenoid (Mustaffa *et al*., 2010)
Eurycoma longifolia	Quassinoids (Ang *et al*., 2002), beta-carboline alkaloid (Kuo *et al*., 2003)
Orthosiphon stamineus	Flavonoid, caffeic acid derivatives, and phenolic compounds (Malterud *et al*., 1989; Olah *et al*., 2003; Tezuka *et al*., 2000)

related disease to heart and joint disorders. Xanthorrizol is identified as the major constituent in the essential oil of this plant (Cheah *et al*., 2009; Devaraj *et al*., 2010).

Croton argyratum or known as "merkolan" by the locals is commonly grown in Borneo, Malaysia (Yusoff *et al*., 2010). Study noted that the leaves, stems (Horgen *et al*., 2001) and root (Norizan *et al*., 2007) possess medical properties . Both are used by the locals to stop purging and diarrhea

Literature search on *Cinnamomum iners* revealed that the bitterness of this plant is exploited by the locals to relieve fever, to treat digestive system related problem and to cure appetite related illness (Pengelly, 2004). Phutdhawong *et al*. (2007) reported on the antioxidant activity of *C. iners*. Furthermore this plant exhibits analgesic activity mediated peripheraly and proved to be non-toxic for consumption (Mustaffa *et al*., 2010).

Tongkat Ali or scientifically known as *Eurycoma longifolia* is a popular sought after herbal remedy for vast illness treatments and as additional supplements. Originating from the family of Simaroubaceae, some parts of the plants have known to possess antimalarial activity and plasmodicidal activities (Chan *et al*., 2004; Noor-Rain *et al*., 2007; Wernsdorfer *et al*., 2009). It is also commonly prescribed in traditional medicine for sexual insufficiency (Wahab *et al*., 2010).

Orthosiphon stamineus Benth. has gained its popularity as one of the most popular traditional folk medicines because of its anti-fungal and anti-diuretic properties (Olah *et al*., 2003). It is also served as beverages for treatment of kidney, urinary tract diseases, gout (Wright *et al*., 2007) and diabetes mellitus (Awale *et al*., 2003a, b).

The high medicinal values of the mentioned plants have generated our interest to investigate their inhibition on GST activity that might be beneficial for treating GST related disease.

MATERIALS AND METHODS

Chemicals: 1-chloro-2, 4-dinitrobenzene (CDNB), reduced gluthathione (GSH), cupric sulphate ($CuSO_4$), Folin-Ciocalteau's phenol reagent, sodium potassium tartarate and sodium carbonate (Na_2CO_3) were obtained from Sigma Chemicals Company (USA). Tannic acid that was used as the positive control was purchased from HmbG Chemical (German). Propane-1, 2-diol and Tween-80 were purchased from Fischer Scientific (UK). The powder of dipotassium hydrogen phosphate was obtained from Riedel-de Haen (Germany). Potassium chloride (KCl) was acquired from BDH Chemical (UK). All the reagents and organic solvents used throughout this project were of analytical grade. This project was conducted from December 2009 to July 2010 at Centre for Drug Research, Universiti Sains Malaysia, Malaysia.

Plant materials: the laboratory of Centre prepared The *Curcuma xanthorrhiza, Cinnamomum iners* and *Croton argyratum* extracts for Drug Research, Universiti Sains Malaysia (USM). *Eurycoma longifolia* and *Orthosiphon stamineus* extracts were obtained from Prof. Chan Kit Lam and Prof. Zahri Ismail respectively from the School of Pharmaceutical Sciences, Universiti Sains Malaysia. The purity of the extracts was verified by comparing the physical and spectroscopic data.

Animal: Fifteen Sprague Dawley rats weighing from 150 to 200 g provided by Animal House of Universiti Sains Malaysia were used in the study. The rats were housed in a temperature controlled room at 22±2°C under a 12 h light/ 12 h dark cycle for one week prior to sacrifice. The animals were fed with water and food *ad libitium.*

Preparation of rat cytosolic fraction: All the rats were anaesthetized with diethyl ether and followed by cervical dislocation. The liver was removed from each rat immediately after the sacrifice. The livers were washed with ice-cooled water and potassium phosphate buffer (pH 7.4). Furthermore, the samples were suspended in three volumes of phosphate buffer (pH 7.4) and were homogenized using Potter-Elvehjem homogenizer (Azizi *et al*., 2010). The homogenate were then centrifuged at 12500x g for 20 min. All subsequent steps were carried out at 4°C. The resulting supernatant was further centrifuged at 100,000x g for 60 min. The supernatants obtained were the cytosolic fraction. The total protein content of each cytosol sample was determined using Lowry method (Pomory, 2008). All preparations were stored at -80°C until used.

Determination of GST Activity on various plant extracts: *In vitro* GST activity was determined as described previously by Habig *et al*. (1974) with certain

modifications (Azizi *et al*., 2010). The experiments were conducted at room temperature. The mixture consisted of 0.1 M potassium phosphate buffer at pH 6.5, 30 mM of CDNB as the substrate, 30 mM GSH and GST enzymes (0.125 mg mL^{-1}). Rat liver cytosolic fractions were prepared and used as the GST enzyme source to determine GSH conjugation towards GST activity. The concentrations of plant extracts and tannic acid ranging from 0.01-1000 and 0.01-250 μg mL^{-1} were used. The concentration of all plant extracts ranged from 0.01-1000 μg mL^{-1} except for *Croton argyratum* leave extract which ranged from 0.01-250 μg mL^{-1}. The reason for not determining the activity above 250 μg mL^{-1} was because maximum absorbance was reached at concentration higher than that. Five replicates were used for each measurement. The enzyme activity was detected *in vitro* through the measurement of the conjugation activity with CDNB at 340 nm using Plate CHAMELEON™ (Hidex Oy, Findland) for 5 min. The specific GST activity for each plant extract was measured based on the formation of GSH conjugate with CDNB.

Statistical analysis: All experiments were carried out in 5 replicates and expressed as the Mean±SD. All the computation analysis was carried out using GraphPad Prism® 5 software.

RESULTS AND DISCUSSION

All the values were obtained graphically by non-linear regression analysis of the remaining enzyme activity (unit mg^{-1}) versus the logarithm of natural product concentration (μg mL^{-1}). Their inhibitory effects on GST activity were shown in Fig. 1 and 2. The IC_{50} values were obtained from extracts showing more than 50% inhibition (Appiah-Opong *et al*., 2007). Concentration of extracts for 50% inhibitory effect on GST activity was summarized in Table 2.

Of all the plant extracts tested result shows that *Croton argyratum, Orthosiphon stamineus and Cinnamomum iners* equally inhibit the GST activity relative to a known inhibitor, tannic acid. However, *Curcuma xanthorrihiza* and *Eurycoma longifolia* extracts showed negligible inhibition. Figure. 1 shows plant exhibiting strong inhibition with more than 50% inhibition on GST activity. Figure 2 represents weak inhibition of plant extracts towards GST activity. Inhibition activity of all the plant extracts on Fig. 1 coincides with the IC_{50} value from Table 2 with all the plant extracts expressed IC_{50} values except for *Curcuma xanthorrihiza* and *Eurycoma longifolia*. Table 2 summarizes the IC_{50} values of all the extracts from the inhibition result.

Table 2: IC_{50} values (μg mL^{-1}) for rat liver cytosolic GSTs for various plant extracts

Plants	Parts	IC_{50} value (μg mL^{-1})
	Leaves	40.42±6.63
Croton argyratum ethanolic extracts	Roots	57.88±9.65
	Stems	143.80±33.35
Curcuma xanthoriza aqueous extracts	Rhizome	ND
Orthosiphon stamineus methanolic extracts	Leaves	35.55±3.84
Eurycoma longifolia aqueous extracts	Roots	ND
Cinnamomum iners methanolic extracts	Leaves	35.20±8.72
Tannic Acid (Positive Control)	Powder	6.18±1.03

All values are Mean±SD for n = 5 determination. ND (not determined) due to percentage of inhibition being less than 50%

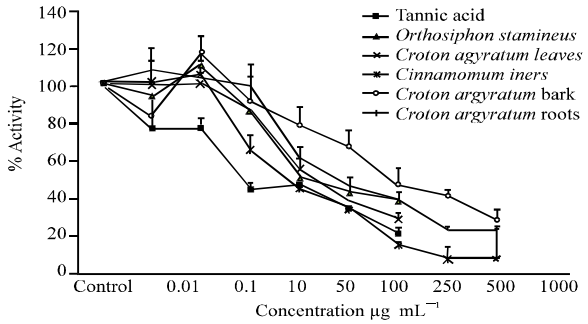

Fig. 1: The inhibitory effect on rat cytosolic GST activity with increasing concentration of varied plant extracts. Assays were performed in 5 replicate for each sample

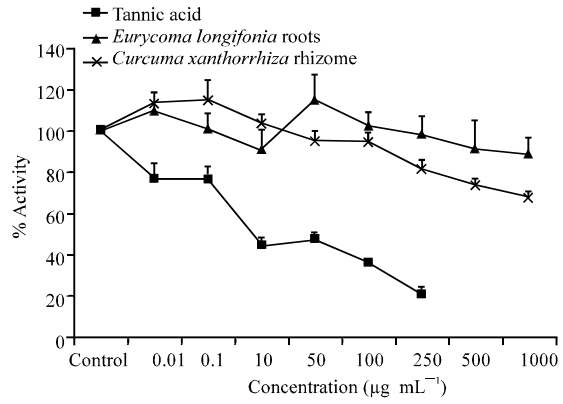

Fig. 2: Effect on rat cytosolic GST activity with increasing concentration of *Curcuma xanthorrhiza* and *Eurycoma longifolia*. Assays were performed in 5 replicate for each sample

Based on Fig. 1, at 50 μg mL^{-1}, *C. iners* showed 1.04 times higher inhibition activity relative to tannic acid. At still higher concentration range of 50 to 250 μg mL^{-1}, *Cinnamomum iners* extract exhibited much higher inhibitory potential relative to tannic acid. At the high concentration of 250 μg mL^{-1}, *C. iners* is 1.30 times more

than tannic acid in inhibitory activity. Meanwhile, *Croton argyratum* bark had showed the lowest inhibition activity at the same concentration range. Figure 2 demonstrated the comparable inhibition activity between *Eurycoma longifolia* root and *Curcuma xanthorrhiza* rhizome. However, both of them are weaker inhibitor compared to tannic acid. To date, there are no published reports in *in vitro* GST inhibitions by all the selected plants used in this experiment.

Tannic acid was used as the positive control and showed an IC_{50} value of 6.18±1.03 μM. CDNB is a common substrate used in GSTs activity assays. Jemth and Mannervik (1997), however, reported that certain GST isoenzymes demonstrate very low activity towards CDNB. Tannic acid which is a hydrolysable type of tannin (Robbers *et al*., 1996) has been commonly used as a positive control due to the non competitive inhibition towards CDNB and competitive inhibition on GSH characteristics (Zhang and Das, 1994). The author also proved that tannic acid is a potent inhibitor amongst other polyphenol tested. Tannin is a type of polyphenol that is involved in the inhibition or induction of some enzymes (Chung *et al*., 1998). Zhang and Wong (1997) revealed that plant polyphenols are responsible for the inhibition of GST activity of cancer cells.

Methanolic extracts of *Cinnamomum iners* and *Orthosiphon stamineus* are the most effective GST inhibitors on rat liver cytosolic GST with IC_{50} values of 35.20±8.72 and 35.55±3.84 μg mL^{-1} (Table 2), respectively. Judging on the low IC_{50} values of both extracts, the data might correlate them as potent inhibitors of GST *in vivo*. The inhibitory potential on GST activity can be associated with the concentration of polyphenols content (Das *et al*., 1984) and total flavonoid content (Ghazali and Waring, 1999). This property proposed that these chemicals may possess important pharmacological and toxicological effects (Middleton *et al*., 2000). Corresponding to this fact is the presence of flavonoid and polyphenolic compounds detected in both plants (Table 1) (Malterud *et al*., 1989; Mustaffa *et al*., 2010; Olah *et al*., 2003) that might have contributed to the strong inhibitory effect on GST activity.

Gringauz (1997) also reported that *Orthosiphon stamineus* (OS) contain high amount of tripenoids and flavonoids. Furthermore, the methanolic extract of OS is found to possess anti-tumor activity through enhancement of anti-proliferative effect on cancer cell (Sahib *et al*., 2009a). Sahib *et al*. (2009b) also reported on the *in vitro* antiangiogenic activity of the OS methanolic extract. Meanwhile the antioxidant activity of *Cinnamomum iners* is largely associated with its polyphenolic content (Pang *et al*., 2009). Their team also reported on the involvement of *Cinnamomum iners* in signaling pathway by inhibiting the proliferation of various cell lines. In agreement with this, Mustaffa *et al*. (2010) also reported on the high antioxidant capacity of the standardized leave methanolic extract of *Cinnamomum iners*. When the IC_{50} values of the plants were compared to the IC_{50} value obtained from tannic acid, all the plants show weaker inhibition than tannic acid.

Croton argyratum or *Croton argyratus* has yet to receive huge attention from scientists judging on the lack of literature on this plant. Noor-Rain *et al*. (2007) reported on antiplasmodial activities of this plant towards *P. falciparum*. In addition, the isolated compounds of *Croton argyratum* such as goniothalamin (Norizan *et al*., 2007) from the root exhibited anti-cancer properties against a range of human tumor and animal cell lines (Umar-Tsafe *et al*., 2004). Besides plant extracts, essential oils are also been screened for their potential usage. The lowest IC_{50} value (40.42±6.63 μg mL^{-1}) can be observed (Table 2) on leaf extract compare to other extracts of this plant indicating that the leaf is a better GST inhibitor. The stem of *Croton argyratum* showed 50% inhibition at concentration of 143.80±33.35 μg mL^{-1} (Table 2) representing the lowest inhibition on GST activity.

It was proposed that *E. longifolia* possesses high medicinal values due to the possession of wide range of quassinoids (Table 1) (Jiwajinda *et al*., 2002). However, the direct effect of quassinoids on GST activity has not been proved yet. Although methanol extract of *Curcuma xanthorrhiza* has been reported to possess cancer chemopreventive potential (Park *et al*., 2008), the author did not specify the exact compound that contributed to such effect. Alone, xanthorrizol exhibits inhibition on the tumor nodules in the lung tissue (Choi *et al*., 2004). Meanwhile, it is reported that combination of both xanthorrizol and curcumin (from the rhizome of *Curcuma longa*) inhibit the proliferation of cancer cells (Cheah *et al*., 2009). Present data showed no inhibition on GST activity in the root extract of *E. longifolia* and rhizome of *Cucurma xanthoriza* (Table 2).

The present results might substantiate the potential use of *Orthosiphon stamineus* and *Cinnamomum iners* extracts for drug discovery and development as adjuvant in chemotherapy. They can be subjected to further study on the drug development for cancer and other diseases which may cause an over expression of GSTs.

CONCLUSION

The GST inhibition abilities of 5 plant extracts have been compared relatively to tannic acid; extracts from *Cinnamomum iners* proved a more potent GST inhibitors compared to the other local plant extracts studied. Meanwhile, the stem of *Croton argyratum* is the least effective GST inhibitor. Following this encouraging

finding, further studies in the determination of the major constituents from *Cinnamomum iners* plant extract in contributing to the GST inhibitory effect are essential to fully understand its GST inhibition mechanism.

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