Palm Oil-Derived Phytosterol: Glutathione Antioxidant Status in Rats Exposed to Carbon Tetrachloride

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INTRODUCTION

Links between oxidative stress and adverse health effects have been suggested for several groups of diseases, including cardiovascular, respiratory, gastrointestinal and neurological (Marx 1987; Nur Azlina et al., 2009, Nur Azlina et al., 2013; Lee et al., 2013). It is well known that these changes are mediated by free radical damage to lipids, proteins, and DNA. Protection from damage occurs through the action of multiple antioxidants either by endogenously produced or provided through dietary intake (Lobo et al., 2010). Uncontrolled production of reactive oxygen species contributes to the pathogenesis of diseases such as cancer and cardiovascular disorders (Vivancos and Moreno, 2005; Azadeh and Mohammad 2011). There were reports which associate lipid peroxidation with coronary or peripheral arterial disease (Lee et al., 2013). Lipid peroxidation is a process in which lipids are oxidized to form radicals and therefore capable of causing extensive tissue. Lipid peroxidation may be the mechanism through which several cardiovascular risk factors may promote cardiovascular disease (Rumly et al., 2004). It is generally believed that oxidative stress may contribute to carcinogenesis by oxidative DNA modification and that antioxidants might prevent human from cancer. A growing body of evidence has demonstrated that lipid peroxidation may actually inhibit most cancer cells (Zanetti et al., 2003; Gago-Dominguez et al., 2005). Therefore, reducing lipid peroxidation is important in prevention of cardiovascular diseases and cancer. Studies have shown this may be achieved through the antioxidant properties of phytosterols. Phytosterols or plant sterols are a group of steroid alcohols, phytochemicals naturally occurring in plants.

ABSTRACT

The aim of this study was to assess the antioxidant effect of phytosterol from palm oil by studying its ability to improve antioxidant status of rats induced with oxidative stress by carbon tetrachloride (CCl4). The rats were divided into four groups of normal control (NC), carbon tetrachloride (CCl4), phytosterol (P) and phytosterol plus carbon tetrachloride (P+CCl4). The P and P+CCl4 groups received weekly phytosterol pre-treatment via subcutaneous injections at 140 mg/kg rat weight for 5 weeks while the NC and CCl4 groups only received olive oil (vehicle). Carbon tetrachloride at the dose determined by a preliminary study was given as single oral dose to induce lipid peroxidation in the CCl4 and P+CCl4 groups. After 24 hours, the rats were sacrificed and the heart, liver, kidney and lung were isolated for the determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) levels. Carbon tetrachloride caused significant reduction in the GSH:GSSG ratio in all major organs. Phytosterol pre-treatment as in the P+CCl4 group significantly increased the GSH:GSSG ratio in major organs. The present findings indicate that phytosterols keep tissue glutathione concentration in normal levels which may indicate improving antioxidant status in major organs of the rats treated with carbon tetrachloride.
They have chemical structure which are similar to cholesterol (Weihrauch and Gardner 1978, Weingartner et al., 2009) and exist in several forms in plants (Law, 2000; Katan et al., 2003; Abumweis et al., 2007) including β-sitosterol, campesterol, stigmasterol and cycloartenol (Ostlund 2002). Phytosterols are natural components found in the human diet. They are found in corn, wheat and rice. Phytosterol intake varies according to the type of diet, European take about 150-400 mg/day (Morton et al.,1995; Susan et al., 2010) while vegetarian Japanese have higher intake (Nair et al., 1984; Seki et al., 2003).

Phytosterols are well known for their ability to lower plasma cholesterol level (Klingberg et al., 2008) by interfering with the absorption of cholesterol from the gastrointestinal system (Jones 1999; Hayes et al., 2004; Jia et al., 2007). Phytosterols have also been shown to possess anticancer properties against certain types of cancer (Choi et al., 2007; Mendilaharsu et al., 1998; De Stefani et al., 2000; McCann et al., 2003; Ju et al. 2004). These studies imply that phytosterols maybe useful in prevention of both cardiovascular disease and cancer.

Phytosterols were found to exert antioxidant effects on the oxidation of methyl linoleate in solution. They also suppressed the oxidation and consumption of α-tocopherol in β-linoleoyl-γ-palmityl phosphatidylcholine (PLPC) liposomal membranes (Yoshida and Niki, 2003). This study showed that phytosterols can act as antioxidants and free-radical scavengers. In another study by Vivancos and Moreno (2005), it was reported that phytosterol increased the activities of antioxidant enzymes, superoxide dismutase and glutathione peroxidase in cultured macrophage cells with oxidative stress induced by phorbol 12-myristate 13-acetate. This finding provided another mechanism whereby phytosterols were able to protect cells from oxidative stress damage by increasing the activities of antioxidant enzymes.

There are limited published reports on the effects of phytosterol from palm oil on endogenous antioxidant levels. Therefore we have conducted a study on the effects of pre-treatment with phytosterol on the level of reduced and oxidized glutathione levels in plasma and organs of rats exposed to carbon tetrachloride. This is important to justify the role of phytosterols in their ability to control oxidative stress and prevention of its related diseases.

MATERIAL AND METHODS

A preliminary study was conducted to determine the suitable dose of carbon tetrachloride which can induce lipid peroxidation in various organs of the rat model. Carbon tetrachloride is an old animal model; it was discovered that the dose of carbon tetrachloride that produced significant lipid peroxidation in the liver was 0.5 ml/kg (Hismiogullari et al., 2014).

After the dose selection, 24 male Sprague-Dawley rats weighing between 175 to 200 grams were obtained from the UKM Animal House. The rats were housed in plastic cages at room temperature (29±3°C) and daily dark/light cycle. They were fed standard food pellets (Gold Coin, Malaysia) and distilled water ad libitum. They were allowed to adjust to the new environment for a week before the study was started. The study was approved by the Universiti Kebangsaan Malaysia, Animal Ethic Committee.

The rats were randomly divided into 4 groups of normal control (NC), Carbon tetrachloride (CCl)$_4$, Phytosterol (P) and Phytosterol + Carbon tetrachloride (P+CCl$_4$). The latter two groups were pre-treated with subcutaneous injection of phytosterol (MPOB, Malaysia) at the dose of 140 mg/kg, once a week for five weeks (modified from Yoshida and Niki 2003). These phytosterols were extracted from palm oil and composed of 60% β-sitosterol and 40% stigmasterol and campesterol. The NC and CCl$_4$ groups only received 2 ml/kg olive oil (vehicle) (Oomerbhoy Ltd, Mumbai) subcutaneously once a week for the same duration. Rats in the CCl$_4$ and P + CCl$_4$ groups were then given a single dose of 0.5 ml/kg carbon tetrachloride (BDH Chemicals, England) diluted in olive oil via oral gavage to induce lipid peroxidation. Rats in the NC and P groups only received equivalent amounts of olive oil (vehicle). After 24 hours, the rats were then sacrificed humanely and the liver, heart, kidneys and lungs were dissected out. The organs total glutathione and oxidized glutathione levels were measured according to the method of Griffith (1979). Reduced glutathione (GSH) is considered to be one of the most important scavengers of reactive oxygen species (ROS), and its ratio with oxidised glutathione (GSSG) may be used as a marker of oxidative stress (Zitka et al., 2012).

The results are expressed as mean ± SEM. The statistical significance of the data was determined using one-way analysis of variance (ANOVA) and post hoc Tukey test. The level of significance was taken as p <0.05.

RESULTS

Reduced glutathione (GSH) is the major endogenous antioxidant in life organism. The results are expressed by the ratio of GSH to the oxidized form of glutathione (GSSG). Exposure to CCl$_4$ resulted in a significant reduction of hepatic glutathione level by 38.8% (P=0.027) compared to the control (NC) group as shown in Figure 1.

![Fig. 1: Mean GSH: GSSG in liver. Different letters indicate significant differences between group at p<0.05. Data is expressed as mean ± SEM.](image)

Rats treated with phytosterols showed no significant different in the hepatic glutathione level compared to the non-exposed control. The finding suggests that phytosterols can restore...
Similar result was observed in renal tissue. Phytosterols pre-treatment caused renal GSH:GSSG ratio elevation in rats induced with carbon tetrachloride compared to the CCl₄ group (Figure 2).

In the heart, there was also a significant reduction in GSH:GSSG ratio for the CCl₄ group compared to the normal control group (Figure 3).

Phytosterols pre-treatment maintained the cardiac GSH:GSSG ratio induced by carbon tetrachloride towards the normal value. In the lungs, a similar observation was shown, where there was a significant reduction of the GSH: GSSG ratio in the CCl₄ group (Figure 4).

These ratios however, were maintained to the normal control value after CCl₄ exposure in rats pre-treated with phytosterols. The mean values for the reduced and oxidized glutathione for all groups are shown in Table 1.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control GSH (nmol/mg)</th>
<th>Control GSSG (nmol/mg)</th>
<th>CCl₄+Phytosterol GSH (nmol/mg)</th>
<th>CCl₄+Phytosterol GSSG (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.6434</td>
<td>1.1433</td>
<td>0.7390</td>
<td>0.8290</td>
</tr>
<tr>
<td>Lung</td>
<td>0.4000</td>
<td>0.1182</td>
<td>0.3438</td>
<td>0.3606</td>
</tr>
<tr>
<td>Heart</td>
<td>0.1828</td>
<td>0.2240</td>
<td>0.1456</td>
<td>0.1599</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.0873</td>
<td>0.0150</td>
<td>0.0721</td>
<td>0.0741</td>
</tr>
</tbody>
</table>

DISCUSSION
Humans are continuously exposed to different chemicals from food, air and soil (Hasegawa 1995). Some of these chemicals induced free-radical-mediated lipid peroxidation leading to disruption of biomembranes and dysfunction of cells and tissues (Ayala et al., 2014, Cho et al., 2003). Carbon tetrachloride is an extensively used xenobiotic to induce lipid peroxidation and toxicity (Rudnicki et al., 2007). In our study, carbon tetrachloride has reduced the endogenous antioxidant levels as shown by the reduction in the GSH:GSSG ratio in all organ indicating an increased in oxidative stress levels in the rats. The failure of the endogenous antioxidant defence system was attributed to the carbon tetrachloride-induced generation of free radicals. We observed a significant depletion of glutathione content in all major organs following exposure to this stressor.

Reduced glutathione (GSH) acts as a non-enzymatic antioxidant that reduces H₂O₂, hydroperoxides (ROOH) and xenobiotic toxicity (Kadiska et al., 2000). Reduced glutathione is readily oxidized to glutathione disulfide (GSSG) by any of the selenium-containing GPX isozymes, as well as the reaction with ROOH or xenobiotic compounds that may subsequently cause the reduction of GSH level. The GSSG is either rapidly reduced by glutathione reductase and NADPH or utilized in the protein folding process in the endoplasmic reticulum. There, GSSG is recycled by protein disulfide isomerase to form GSH. Because of these recycling mechanisms, GSH is an extremely efficient intracellular buffer for oxidative stress (Cantinet et al., 2007). Mechanistic studies on CCl₄-induced toxicity reveal that GSH conjugation plays a critical role in the elimination of toxic metabolites, which are the major cause of liver pathology (Lee et al., 2008). In the present study, the liver, heart, kidneys and lungs content of GSH was significantly decreased in carbon tetrachloride-intoxicated rats compared with control rats, suggesting a role of GSH in CCl₄-induced toxicities. The
mechanism of multiple organ protection by palm-derived phytosterols against CCl₄ toxicity might be due to restoration of the GSH level. Pre-treatment with phytosterol extract increased the levels of GSH which may possibly be due to enhancing of GSH synthesizing enzyme activities such as c-glutamylcysteine synthetase (c-GCS) and GSH synthetase, suggesting that these phytosterols could preserve the carbon tetrachloride-induced depletion of GSH in major organ. These however need further investigation. Similarly, Oyedemi and Afolayan (2011) had showed that aqueous leaves extract of Leonotis leonurus extract effectively increased the percentage inhibition of GSH. While Kamisah et al., (2011) had shown palm-derived vitamin E had the ability to reduce oxidative stress in rat’s stomach.

Carbon tetrachlorides is known to cause injuries to the organs of the body (Reynolds et al., 1984) including liver (Nevin et al., 2005; Shim et al., 2010), kidneys (Ozturk et al., 2003), heart, lungs, gastrointestinal tract and central nervous system (ATSDR 2005). The primary targets for carbon tetrachloride toxicity are liver and kidneys (IPCS 1999). In the body, carbon tetrachloride is metabolised by cytochrome P-450 enzymes to reactive trichloromethyl radical. The radical is oxidized further, forming the even more reactive trichlomethyl peroxyl radicals (McGregor and Lang, 1996; IPCS 1999). Lipid peroxidation occurs via reactive metabolic intermediates of carbon tetrachloride, in particular the trichloromethylperoxyl radical (IPCS 1999).

Phytosterols taken into the body are incorporated into the cell membranes (Awad et al., 2004) and are highly concentrated in the lungs, adrenal cortex, intestinal epithelia and ovaries (Sanders et al., 2000). In our study, we found that phytosterol pre-treatment was able to prevent the reduction in the GSH:GSSG ratios in the liver, kidneys, heart and lungs. Based on these findings, pre-treatment with phytosterols maintained the endogenous antioxidant system thus showing its ability to reduce lipid peroxidation and protect the organs against damages by lipid peroxidation. This is consistent with the finding that pre-treatment with other antioxidants, such as vitamin E reduced the hepatoxic action of carbon tetrachloride (IPCS 1999). Other studies had shown, herbs with high antioxidant properties like Nigella sativa oil decreased lipid peroxidation and were capable of preventing the rise of lipoperoxidation due to the nephrotoxic effect of Amikacin (Iman and Mahmoud, 2011). While herbs containing antioxidant was able to protect the liver against carbon tetrachloride effect as demonstrated by the increase in non-protein sulhydryl level (Syed et al., 2008), reduced malondialdehyde level (Patrick et al., 2010; Bashandy and Alwasel 2011) and liver enzymes (Prakash et al., 2008; Mohammed et al., 2008).

In a study by Mora-Ranjeva et al.,(2006), phytosterol in the form of sitosterol and stigmasterol were incorporated in to human keratinocytes (SVK14 line) and exposed to ultraviolet. It was found that sitosterol induced significant decrease (~30%) in lipid peroxidation whereas stigmasterol markedly increased lipid peroxidation (+70%). This study had shown that the effects of phytosterol on lipid peroxidation also depended on the form of phytosterol. In our study, we used phytosterols derived from palm oil with β-sitosterol making up 60% of the component while campesterol and stigmasterol made up the rest. The high level of stigmasterol in palm-derived phytosterols could explain the reasons for the maintenance of the endogenous glutathione content by its ability to reduce lipid peroxidation (Ajayi and Malachi 2016).

The phytosterols dosage used in this study was equivalent to the intake of 10.5 grams per week in a 75-kg man. This is high considering that the daily intake of phytosterols in the human diet is about 200 to 300 mg/kg body weight (Susan et al., 2010, Ostlund 2002, Morton et al., 1995). With regard to its toxicity, administration of phytosterols in rats did not show any subchronic toxicity or teratogenic effects (Xiemei et al., 2015). In human study, daily intake of 9.0 grams of phytosterols for eight weeks did not show any adverse effects (Katan et al., 2003).

As a conclusion, the findings indicate therapeutic usefulness of phytosterols in reducing lipid peroxidation as shown by its ability to maintain endogenous antioxidant levels in the liver, kidneys, heart and lungs in CCl₄-induced oxidative stress condition. Thus, this agent may be useful as an agent to protect major organs against chemical-induced toxicity in-vivo.

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