3. MATERIALS AND METHODS

3.1 Chemical and Reagents

1,1 diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT) and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co.,St. Louis,U.S.A. The standard 1,1,3,3 tetramethoxypropane (TMP) was obtained from S.V. Medico, Thailand. Trichloroacetic acid was obtained from E. Merch (Darmstadt, Germany). Reduced glutathione and 5,5′ dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Fluka. Paracetamol was obtained from NIDA Pharma. Inc., Thailand (Lot 032239)

3.2 Plant Preparation

P. speciosa were purchased from a local market in Hat Yai, Songkhla, Thailand, and kept frozen. The seeds were separated from the empty pods and cleaned.

To determine antioxidant activity, the seeds were blended in a blender. One gram of the seeds were homogenized with 20 ml of distilled water, or homogenized after boiling for 5 min, with 20 ml of distilled water. The homogenate were centrifuged at 30,000 rpm for 20 minutes at temperature of 5 °C. The supernatant are stored at 4 °C until being tested.

For the treatment in rats, the fresh or boiled (5 min boiled in 100 °C water) seeds were prepared by blending with distilled water in a blender to make a concentration of 0.5 g/ml.

3.3 Animals

Male Wistar rats weighing 200-250 g were obtained from Division of Animal House, Faculty of Science, Prince of Songkhla University, Hat Yai Campus. Animals were housed in controlled rooms, temperature 25 °C, with a

12 hour light/dark cycle and had free access to standard laboratory food and water ad libitum. Rat were fasted for 12 hours before starting the experiment, but had free access to water.

3.4 Experimental Design

In vivo experiment: Rats were divided into six groups. Group 1 served as control and received 0.9% saline at a single dose of 1.5 ml/kg intraperitoneally, group 2 was given *P. speciosa* at a single dose of 6 g/kg orally, group 3 was given *P. speciosa* 6 g/kg orally daily for 7 days, group 4 was treated with paracetamol at a single dose of 500 mg/kg intraperitoneally, group 5 was pretreated with *P. speciosa* seeds 6 g/kg orally 1 hour before giving a single dose of paracetamol, 500 mg/kg intraperitoneally, and group 6 was pretreated with *P. speciosa* seeds 6 g/kg orally for 7 days before administration of a single dose of paracetamol, 500 mg/kg intraperitoneally.

3.4.1 Serum collection

Blood samples were collected from the retro-orbitol plexus under light anesthesia with diethylether at 12 hours after paracetamol injection. Blood samples were centrifuged at 3,000 rpm for 30 minutes and serum was separated and stored at 4 °C until assayed.

3.4.2 Tissue preparation for lipid peroxidation and reduced glutathione determination

Rats were killed by cervical dislocation at 3, 12 and 24 hours after paracetamol injection. Livers were excised immediately washed in ice-cold 0.9% NaCl, dissected to remove connective tissues and weighed. The samples were kept on ice and rapidly processed.

3.5 Method

3.5.1 Measurement of hepatocellular enzymes in serum

Serum aspartate aminotransaminase (AST), alanine aminotransferase

(ALT), and alkaline phosphatase (ALP) were determined using a standard clinical chemistry analyzer (Vitalab Flexor E) on the same day. The results of AST, ALT and ALP activity were expressed in international units (IU) per liter.

3.5.2 Assay of reduced glutathione

Principles

5,5/ dithio-bis (2-nitrobenzoic acid), DTNB, is a disulfide compound which can be reduced by sulfhydryl group of reduced glutathione (GSH) and formed yellow color. The yellow color is measured by spectrophotometer at 412 nm.

Procedure

Reduced glutathione was measured by colorimetric method according to Anderson, (1985). 0.5 g of liver tissue was homogenized in 2.5 ml 5% sulfosalicylic acid. After centrifugation at 3,300 rpm for 15 min, 25 µl of supernatant was mixed with 710 µl buffer (143 mM sodium phosphate plus 6.3 mM EDTA, pH 7.5) and 175 µl of distilled water. Then 100 µl of DTNB solution was added into the mixture solution. The absorbance of colour developed was detected at 412 nm. The reference cuvette contained 0.5% sulfusalicylic acid 200 µl, buffer 710 µl and DTNB 100 µl. GSH levels was quantity from a standard curve plotted with different concentration of reduced glutathione (appendix-8) and values were presented as µmole/g liver.

3.5.3 Determination of lipid peroxidation by thiobarbituric acid reaction (TBAR)

Principles

The assay is based upon the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product of polyunsaturated fatty acid oxidation, to form pink-coloured TBA-MDA complex in acidic and boiling temperature condition. The TBA-MDA complex is measured by spectrophotometer at 532 nm. (Draper and Hadley, 1990).

Procedure

Lipid peroxidation of rat liver using thiobarbituric acid was measured by a modified method of Ohkawa *et al*., (1979) as follows : One gram of rat liver tissue was homogenized in 3 ml of 50 mM potassium phosphate buffer (pH 7.0) by using a Heidolph homogenizers with a Teflon pestle. To 0.3 ml of liver homogenate in test tube, 1.5 ml of 10% trichloroacetic acid solution and 1.5 ml of 0.67% thiobarbituric acid solution were added. The mixture was boiled in water bath at 95 °C for 60 min and then cooled with tap water at room temperature. After centrifugation at 3,000 rpm for 15 min, the absorbance of sample was measured at 532 nm. 1,1,3,3 tetramethoxypropane (TMP) was used as a standard of malondialdehyde (appedix-9).

3.5.4 1,1 Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

Principles

This assay is based on the measurement of the scavenging ability of antioxidant towards the stable radical, 1,1 diphenyl-2-picrylhydrazyl (DPPH). The free radical DPPH is reduced to the corresponding hydrazine when it reacts with hydrogen donors, which evaluates the absorbance at 520 nm.

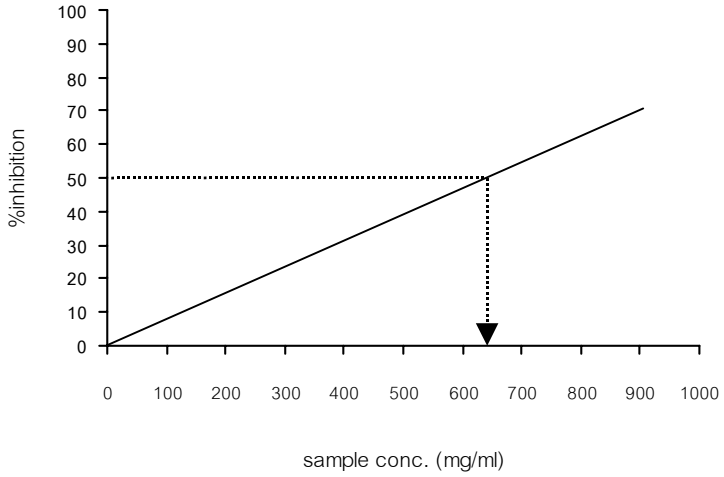
Procedure

DPPH scavenging activity was determined using a modified method of Hatano *et al*.,1989. A portion of *P. speciosa* sample was mixed with the same volume of 60 µM DPPH in 70% ethanol and allowed to stand at room temperature for 30 minutes. The absorbance was then measured at 520 nm. BHT, which is one of the well-known antioxidants, was used for a positive control. The assay was done in triplicate. The rates of absorbance for control and samples were calculated for %inhibition. The EC_{50} of each sample was obtained by plotting between %inhibition and sample concentration (µg/ml).

Calculation

$$\% \text{ inhibition} = \left(\frac{\text{O.D.control} - \text{O.D.sample}}{\text{O.D.control}} \right) \times 100$$

EC_{50} = efficient concentration of sample at 50% inhibition



When X = sample concentration at 50% inhibition

3.5.5 Histopathological study of liver

The livers of 3 rats per each group were fixed in 10% formalin and stained with hematoxylin and eosin dye. The histological sections were examined under a light microscope and the extent of necrosis was graded as followed : normal sections (0), minimal centrilubular necrosis (+1), extensive necrosis confined to centrilubular region (+2), necrosis extending from central zone to midzone or further to portal triad (+3).

3.6 Statistical analysis

Results are expressed as mean ± S.E.M. The biochemical parameters were statistically assessed by Kruskal-Wallis test followed by Mann-Whitney U test for differences between groups. In addition reduced glutathione and lipid peroxidation data were analyzed using one-way analysis of variance (ANOVA) with Scheffe' s for multiple comparison. Differences at a p-value of less than 0.05 were considered as the level of significance in all statistical tests.