

Full Length Research Paper

Antioxidant and toxicity activities of *Artocarpus lakoocha* Roxb. heartwood extract

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The antioxidant activity of *Artocarpus lakoocha* heartwood extract was investigated from ethanol extraction by 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) decolorization, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and H₂O₂ scavenging assay. Polyphenolic; total phenolic, flavonoids and tannins were measured. Anti-oxidative stress was studied in AAPH-oxidized blood and glutathione (GSH), and malondialdehyde (MDA) was evaluated. Results showed that antioxidant activities were 128.30 ± 0.13, 55.86 ± 0.01, and 463.49 ± 0.01 µmol Trolox/g extracted from ABTS, DPPH, and H₂O₂ scavenging methods. One gram of extract contained total phenol (325.63 ± 2.99 mg GE), flavonoids (521.98 ± 0.01 mg QE) and tannins (124.03 ± 0.46 g TE), including rutin and resocinol. In the blood system, a low concentration of extract inhibited MDA progression and improved GSH, which was in contrast to a high concentration with its toxicity effect.

Key words: *Artocarpus lakoocha*, antioxidant, polyphenolic, glutathione, malondialdehyde.

INTRODUCTION

At present, various medicinal plants and plant products are promoted and used for treating various illnesses. Many of the medicinal plants used have potentially therapeutic compounds, with antioxidant, anti-inflammation, antibacterial and cytotoxic activities (Hayet et al., 2009). Basic knowledge of free radicals relating to many diseases is generally thought (Jasprica et al., 2007) to be because of excessive production, and less antioxidant protection, which results in the onset of numerous diseases and accelerated aging (Goze et al., 2009). Antioxidants are composed of enzymatic and non-enzymatic compounds that inhibit the chain reaction in lipid protein or DNA molecules (Velioglu et al., 1998). Phenolic compounds, such as flavonoid, phenolic acids, or phenolic diterpenes, are potentially dominant substances that show antioxidative activity (Kiselova et al., 2006), which plays an important role in absorbing and neutralizing some free radicals, quenching singlet and

triplet oxygen or directly decomposing peroxides (Javanmardi et al., 2003). *Artocarpus lakoocha* Roxb. (Moraceae) is a tropical tree widely distributed in the regions of South and Southeast Asia, including Nepal, India, Sri Lanka, Myanmar, southern China, Vietnam, Thailand, Malaysia and Indonesia. In Thailand, *A. lakoocha* is called 'Ma-Haad', and the dried aqueous extract prepared from the heartwood of this plant is known as 'Puag-Haad', which has been traditionally used as an anti-helminthic (Charoenlarp et al., 1989; Maneechai et al., 2009).

In 2009, research of this plant detected oxyresveratrol after ethanolic extraction (Maneechai et al., 2009), which supported strong tyrosinase-inhibitory activity, and its potential use as a skin-whitening agent (Likhitwitayawuid et al., 2006; Tengamnuy et al., 2006). In addition, flavonoids have been found in other species of *Artocarpus*, and they possess strong antioxidation (Toshio et al., 2003), anti-inflammation (Wei et al., 2005), and antiplatelet aggregation (Lin et al., 1996). Unfortunately, there have been few studies of *A. lakoocha* from ethanolic extraction, especially antioxidant activity, or phenolic content, including its toxicity. Thus,

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the aim of this study was to screen and determine all flavonoids in ethanolic extract from *A. lakoocha*, including assay activity on GSH and MDA in human blood from AAPH oxidation.

MATERIALS AND METHODS

Chemicals and reagents

Quercetin, Rutin, Pyrogallol, Gallic acid, Catechin, Caffeic acid, Resorcinol, 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2-Thiobarbituric acid (TBA), (1,1-diphenyl-2-picrylhydrazyl) (DPPH), glutathione (GSH), Trolox, and Horadised peroxidase were purchased from Sigma Chemical Company (St. Louis, MO, USA). Methanol and acetronitrite (HPLC Grade) were obtained from LabScan (Thailand). Folin-Ciocalteu reagent, Folin-Denis reagent and H₂O₂ (30%) were purchased from Merck (Germany).

Plant materials and extraction

Dry plant powder of *A. lakoocha* heartwood was obtained from Abhaibhubejhr Hospital, Prachinburi, Thailand. The heart wood of *Artocarpus lakoocha* has brown color and hard with termite resistant with a weight approximately 620 - 650 kg/cu.m³. Branch of *A. lakoocha* with leaves alternate and fruits in axillary position. Powders sample of *A. lakoocha* heartwood under microscope is similar to the previous studies (Palanuvej et al., 2007; Sambhandharaksa et al., 1962).

One hundred grams of dry plant material were extracted with 1,000 ml of 80% ethanol by stirring overnight at 4°C (cold room) and centrifuging at 3,000 rpm for 10 min. The supernatant was filtered through Whatman filter paper number 1 and evaporated using a rotating evaporator (60°C) followed by lyophilization to dryness and stored at -20°C until use.

Phytochemical screening assay

Screening of the chemical composition was analyzed by High Performance Liquid Chromatography (HPLC) (Kusirisin et al., 2009). Standard solution (rutin, pyrogallol, gallic acid, resorcinol, quercetin, catechin and caffeic acid) and extract at 20 mg/ml were dissolved in methanol and analyzed with an HPLC system condition (Agilent 1100 Series diode-array detector). All specific retention times of standards were eluted under a mobile phase (water: 0.4% acetic acid: methanol: acetronitrile, 70:20:5:5/ v:v:v:v) running within an ODS Hypersil (250 × 4 mm, 5 µm) column (oven at 25°C) and identified at 250 nm by 0.7 ml/min of flow rate.

Determination of total phenolic content

The total phenolic compound in plant extract was determined by Folin-Ciocalteu reagent (Javanmardi et al., 2003). The phenolic compound was oxidized to phenolates by the reagent at alkaline pH in a saturated solution of sodium carbonate, resulting in a blue molybdenum-tungsten complex that was detected at 764 nm. Fifty µl of plant extract were mixed with 2.5 ml of Folin-Ciocalteu's reagent diluted at 1/10, and 2.0 ml of sodium carbonate (7.5%, w/v). Then, the sample was incubated at 45°C for 15 min until the blue color was developed. The total phenolic content was calculated by comparing to standard gallic acid and expressed as milligrams of gallic acid equivalents to one gram of extract (mg GAE/g extract).

Determination of flavonoid content

The total flavonoid content was determined by the aluminum chloride colorimetric method (Joubert et al., 2008). The plant extract (0.5 ml of 1:10 g/ml) in methanol was mixed separately with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of (1 mol/l) potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The standard curve was set up by preparing various concentrations of quercetin in methanol. The result was expressed as quercetin equivalent (mg QE/g extract).

Determination of total tannin content

The total tannin content in the lyophilized plant extract was determined by modification of a previous method (Polshettiwar et al., 2007). The sample (0.1 ml) was mixed with 0.5 ml of Folin-Denis reagent followed by 1 ml of Na₂CO₃ (0.5% w/v) solution and distilled water (up to 5 ml). The absorbance was measured at 755 nm within 30 min of the reaction against the reagent blank. Total tannins in extract were expressed as equivalent to tannic acid (g TE/g extract).

ABTS decolorization assay

The ABTS radical scavenging activity was analyzed according to the method of Re and coworker (Re et al., 1999). ABTS was dissolved in distilled water to a concentration of 7 mmol/l. ABTS radical cation (ABTS^{•+}) was produced by reacting ABTS stock solution with 2.45 mmol/l of potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 - 16 h before use. Oxidation of the ABTS occurred immediately, but the absorbance was not maximal and stable until after more than 6 hours incubation. The ABTS^{•+} solution was diluted with dH₂O to give absorbance of between 0.68 and 0.72 at 734 nm. Stock solution of extract (20 mg/ml) in distilled water or 80% ethanol was diluted before introduction into the assay. The final concentration of extract was 1.0 - 0.125 mg/ml, with the final volume being 1 ml. All determinations were carried out at least three times on the standard and sample. The change in absorbance was recorded. The percentage of ABTS absorbance at 734 nm was calculated using the following formula:

$$\frac{(\text{Initial absorbance before adding extract} - \text{Final absorbance after adding extract}) \times 100}{\text{Initial absorbance before adding extract}}$$

Initial absorbance before adding extract

The initial reduction rate of A734 nm, with a 3, 20 s to 1 min lag and Factor = 1, was monitored and recorded. The data represented the ΔA/min. The antioxidant content in the plant extract was calculated and compared with the standard antioxidant, Trolox, at 1 mg.

Hydrogen peroxide scavenging assay

The scavenging activity of plant extract on H₂O₂ was modified from the protocol of Büyükbacı (2008). The hydrogen peroxide was activated by peroxidase, generating a hydroxyl radical that modulated the ABTS non radical to ABTS cation radical. The H₂O₂ (0.003%) was mixed with 0.1 ml of extract at different concentrations. Then, 0.8 ml of 0.1% ABTS and 0.1 ml of 10 U/ml peroxidase were added to 0.8 ml of 0.1 mmol/l phosphate buffer (pH 6.0). The solution was then incubated at 37°C for 15 min. Absorbency at 414 nm was measured. Data were represented as percentage of hydrogen peroxide scavenging compared to standard

Trolox.

DPPH radical scavenging assay

The radical scavenging activity of plant extracts against the DPPH radical was measured according to the method of Thitilerdecha (Thitilerdecha et al., 2010). Fifty μ l of plant extract were added to 2.95 ml of DPPH solution (6.4 mg of DPPH in 100 ml of absolute ethanol). The absorbance at 515 nm was measured after the solution had been allowed to stand in the dark at room temperature for 30 min. Ethanol was used as the blank solution and Trolox as a positive control. The scavenging activity of the DPPH radicals was calculated by plant extracts according to the following formula:

DPPH* scavenging activity (%) =

$$\frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100\%$$

Protective effect in the human blood system

The model for this study was modified from the original method (Ximenes et al., 2009). Heparinized blood was provided from healthy subjects. The character of the blood was as follows: hemoglobin 10-16 g/dl, hematocrits 40-50%, white blood cell 5,000 - 10,000 cells/cu.mm., MCV 80-95 fL, MCH 27 - 32 pg. and MCHC 32-36 g/dl. A two milliliter sample of blood either untreated or treated with 50 μ l of AAPH (100 mM) in the presence or absence of plant extract was incubated at 37°C for 2 h. After incubation, whole blood was centrifuged at 3,000 rpm for 5 min, and the total glutathione (GSH) level in erythrocyte and malondialdehyde (MDA) in plasma was detected by DTNB (Beutler et al., 1963) and TBARS (Roman et al., 2002) methods, respectively.

Total GSH determination

The method for detecting blood GSH was modified from the original Beutler protocol (Beutler et al., 1963). Whole blood (0.4 ml) was mixed with 1.0 ml of distilled water and 1.6 ml of precipitant solution. Supernatant of 0.2 ml was separated by filtering with Whatman filter paper no. 1. Clear supernatant of 0.5 ml was added to 0.5 ml of phosphate buffer (pH 7.4) and 0.5 ml of DTNB reagent. The absorbance was detected at 412 nm within 5 min. The total glutathione concentration was measured and compared to standard GSH.

Malondialdehyde (MDA) determination

The protocol followed the Roman protocol (Roman et al., 2002). Two hundred and fifty μ l of plasma were mixed with 0.75 ml of H₃PO₄ (0.44 mol/L), and stored at room temperature for 10 min. Then, 0.25 ml of TBA (w/v, 0.6%) was added, and the sample incubated in a water bath (90°C) for 30 min. A pink color developed after reaction was stopped with cool water. The MDA was measured by HPLC at 532 nm. The analytical HPLC system condition was performed in an Agilent 1100 Series diode-array detector high performance liquid chromatograph. The MDA was detected by diode array detector wavelengths at 532 nm and separated in a ODS Hypersil (250 \times 4 mm, 5 μ m) column. Isocratic elution was employed with a mobile phase consisting of 65% KH₂PO₄: 35% methanol, at 1 ml/min of flow rate. The MDA

concentration was compared with standard malondialdehyde (bis) acetate.

Statistical analysis

All the grouped data were statistically evaluated with SPSS 16.00 software. Data were expressed as mean \pm S.E. and analyzed statistically using One-way ANOVA. Differences were considered statistically significant at P < 0.05.

RESULTS AND DISCUSSION

Chemical profiles and phenolic compounds

Previous screening study of *A. lakoocha* heartwood was done by thin layer chromatography, and showed the oxy-resveratrol mainly active compound and also had bezoic acid by capillary zone electrophoresis (Palanuvej et al., 2007). But in a 70% ethanolic-extract system can separated many flavonoid or phenolic compounds (Marston et al., 2006) thus, this study interested in some active compound such as catechine, flavonoid or isoflavone. In this study, found the chemical profiling by HPLC in the ethanol extract of *A. lakoocha* heartwood revealed the presence of a chemical entity with respect to specific retention times (RT) (Figure 2) when compared to the chromatogram of standards (Figure 1). The chemical compounds in *A. lakoocha* extract at 20 mg/ml showed rutin (4.61 min), pyrogallol (7.22 min), gallic acid (8.39 min), resorcinol (10.33 min), quercetin (14.29 min), catechin (31.32 min) and caffeic acid (36.96 min). Due to using a low volume sample injection of only 6 μ l from a stock 20 mg/ml, two dominant peaks of rutin and resorcinol could be calculated, and the concentrations compared to the standard solution of 653.44 and 4.78 mg/g extract.

Polyphenolic compounds such as phenols, flavonoids and also tannins are very important plant constituents. A previous report showed that total phenolics correlated to antioxidant activity ($R^2 = 0.79$) (Akinmoladun et al., 2010), and their effects reduced superoxide radicals and lipid peroxidation. This study found that *A. lakoocha* extract contained different totals of polyphenolic compounds (Table 1). One gram of dry weight *A. lakoocha* extract contained a total amount of phenols, flavonoids and tannins as follows: 325.63 mg GE, 521.98 mg QE and 124.03 g TE, respectively. This result was similar to previous studies on other species of the genus, *Artocapus*, which found flavonoids (Toshio et al., 2003; Wei et al., 2005). Furthermore, in oxyresveratrol (phenolic compound), resveratrol traces of some flavonoids and resorcinol were searched for in the heartwood of this plant (Maneechai et al., 2009; Poopyruchpong et al., 1978). These compounds found in this plant also possessed strong antioxidant, anti-inflammatory and neuroprotective properties (Maneechai et al., 2009).

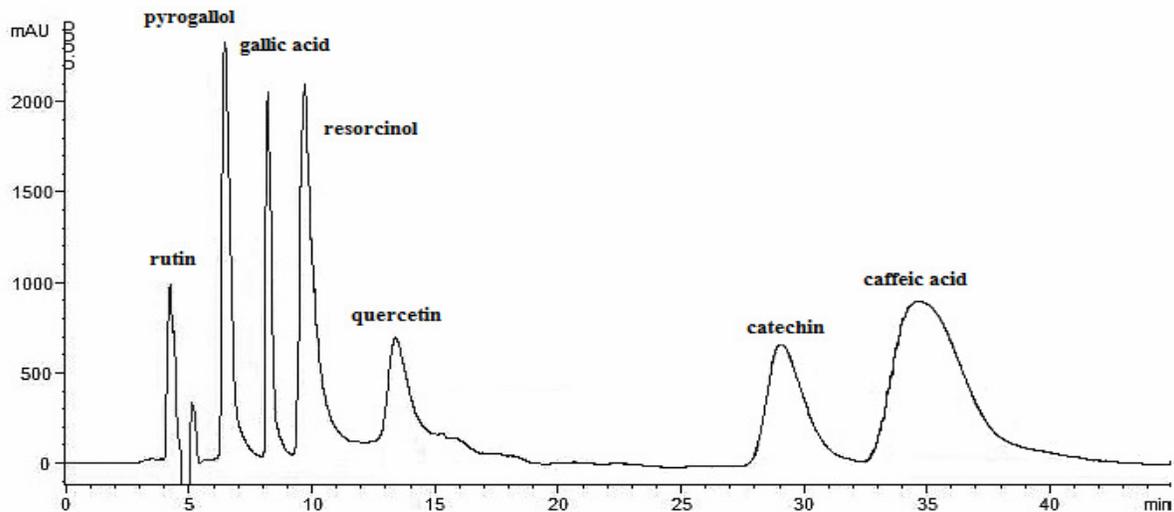


Figure 1. Chromatogram of all standards at 20 mg/mL; rutin (4.55 min), pyrogallol (6.96 min), gallic acid (8.7 min), resorcinol (10.23 min), quercetin (13.61 min), catechin (29.08 min), and caffeic acid (34.93 min).

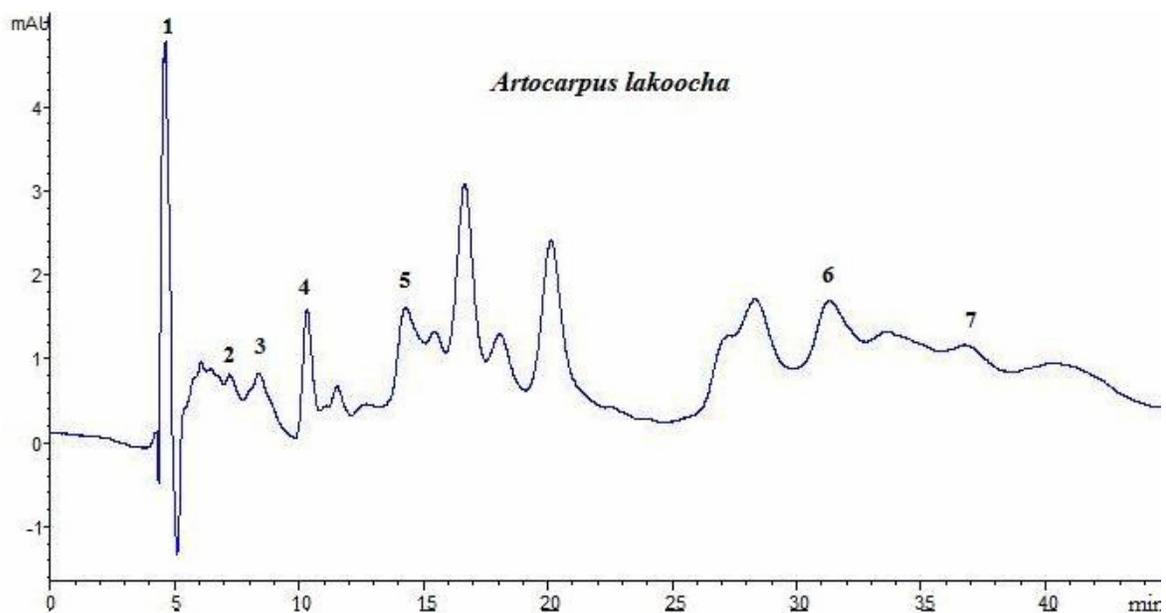


Figure 2. Chromatogram of chemical profiles from *A. lakoocha* heartwood extract, ethanolic extract at 20 mg/ml, was analyzed by HPLC at a wavelength of 250 nm. The chromatogram shows rutin (1), pyrogallol (2), gallic acid (3), resorcinol (4), quercetin (5) catechin (6) and caffeic acid (7).

Table 1. Polyphenolic compound contents of *A. lakoocha* heartwood extract.

1 g of <i>Artocarpus lakoocha</i>	Content
Phenols (mg GE)	325.63 ± 2.99
Flavonoids (mg QE)	521.98 ± 0.01
Tannins (g TE)	124.03 ± 0.46

Values express mean ± SE (n=3), GE = gallic acid equivalent, QE = quercetin equivalent, and TE = tannin equivalent.

Antioxidant activities

The *A. lakoocha* extract comprised antioxidant activity, as shown in Table 2. *A. lakoocha* is one of many plants that contain many phenolics (flavonoids and phenolic acids), generally known as rather strong antioxidants (Jasprica et al., 2007). Several methods have been proposed to measure free radical scavenging capacity (RSC), regardless of the individual compounds that contribute to the

Table 2. Antioxidant activity comparisons of *A. lakoocha* heartwood extract.

1 g of <i>Artocarpus lakoocha</i>	Activity ($\mu\text{mol Trolox}$)
ABTS	128.30 \pm 0.13
DPPH	55.86 \pm 0.01
H ₂ O ₂ scavenging	463.49 \pm 0.01

Values express mean \pm SE (n = 3).

total free radical scavenging capacity of a plant product. For the evaluation of plant activity, easy, rapid and reliable methods can be very useful, such as measuring the disappearance of color stable free radicals such as the 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS⁺), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical and hydrogen peroxide (H₂O₂) assay (Jasprica et al., 2007; Re et al., 1999).

Beneficial health effects of an array of herbal preparation have been ascribed to the ability of plant extracts to scavenge free radicals, and in such a way as to prevent the development of various diseases (e.g. diabetes, rheumatoid arthritis, Alzheimer's) (Jasprica et al., 2007). Owing to the complex nature of phytochemicals, measuring total antioxidative efficiency using spectrophotometric stable free radical (ABTS⁺, DPPH⁺ and H₂O₂ scavenging assays) is an easy way to evaluate the antioxidant activity of different extracts (Jasprica et al., 2007). In this work, ABTS radical cation, DPPH cation radical and H₂O₂ scavenging were used with a spectrophotometer to establish the kinetics of antioxidative behavior of the most commonly present phenolics in *A. lakoocha*. In three methods of antioxidant assays has different hypothesis. H₂O₂ scavenging is prepared in water soluble containing the ABTS and peroxidase enzyme, thus this can be confirmed in case of active compound in *A. lakoocha* extract are water-soluble ability as well as in the ABTS decolorization method (van Meeteren et al., 2004). In ABTS decolorization is a system from potassium persulfate and ABTS thus, any active compound in *A. lakoocha* extract must be act with ABTS radical directly whereas in H₂O₂ scavenging test, active compound can act to H₂O₂ or ABTS or enzyme. Whereas in DPPH method, all reagents was prepared in ethanol solvent, thus some active compound in *A. lakoocha* extract from ethanol must be active and have high activity when compared to ABTS and H₂O₂ system (Moon et al., 2009). The results showed that the antioxidant activity in one gram of extract was 128.30 \pm 0.13, 55.86 \pm 0.01 and 463.49 \pm 0.01 $\mu\text{mol Trolox}$ from ABTS, DPPH, and H₂O₂ scavenging methods, respectively (Table 2).

The human erythrocyte model that co-incubates between whole blood and 100 mM AAPH was studied. AAPH is an organic radical after incubation at room temperature (Ximenes et al., 2009). After incubation at 37°C for 6 h, at the low concentration of 10 $\mu\text{g/ml}$, the

extract inhibited lipid peroxidation but did not protect glutathione from AAPH oxidation. Whereas, at higher concentrations of 50 and 100 $\mu\text{g/ml}$, the GSH was more oxidized (Figure 3), and activation of the lipid peroxidation (Figure 4) was higher than in the red cell control. Results and many repeats showed that the levels of GSH did not improve after being treated with extracts in a series of concentrations, which was the same result as in lipid peroxidation (Figures 3 and 4). The results could be explained from possible high dose of flavonoid in *A. lakoocha*. Although there are the benefits of antioxidant and phytochemical action in plants, which contain phenols, flavonoid, or tannin that scavenge some radicals, the oxidation process of membrane proteins or lipid compartments is inhibited by their acting as chain-breaking, peroxy-radical scavengers (Jasprica et al., 2007; Pan et al., 2007, and Rudnicki et al., 2007). An interesting result of this study presented a higher condition of lipid peroxidation in whole blood by AAPH. In results of co-incubation with *A. lakoocha* extract at varying concentrations, MDA from lipid peroxidation was inhibited after using a significantly ($p < 0.001$) low concentration (10 $\mu\text{g/ml}$ extract). It is important to emphasize that those flavonoids, phenols or tannins are able to scavenge free radicals.

However, at high concentrations of extract (50 and 100 $\mu\text{g/ml}$), results showed that the MDA level increased when compared with the red cell control group, which was treated with AAPH. From the results of this study, it was found that *A. lakoocha* extract contained a high content level of phenols, flavonoids and tannins. Previous evidence showed that the flavonoids and tannins were phenolic compounds that naturally occurred in plants and inhibited lipid peroxidation, free radical scavengers, active oxygen and chelate iron ions, and inactivated lipoxygenase. The tannin used was beneficial to health and acted as a chemo-preventive agent against carcinogens or mutagens and free radical scavengers, and stopped damaging reactions (Sudha and Gnanamani, 2008). However, serious attention has to be given to unspent tannins, because they even act as pro-oxidants when there is a redox active metal (Khan et al., 2000; Labieniec et al., 2003). Tannin exhibited its strong pro-oxidant action in the presence of Fe (II) and also stronger antioxidant effect by its ability to quench hydroxyl radical and singlet oxygen-mediated DNA bases. In a previous *in vitro* study, flavonoids such as quercetin and myricetin were shown to decrease glutathione in the nuclear of isolated rat liver. A previous study (Choi et al., 2003) confirmed that chronic administration of quercetin (a member of the flavones family) significantly decreased glutathione concentration and glutathione reductase activity in rats. It has been well known that dysfunction of glutathione metabolism (glutathione concentration or the enzymes involved in the glutathione pathway) causes oxidative damage. Lipid peroxidation is a well known example of oxidative damage that affects cell membranes, lipoproteins, and other

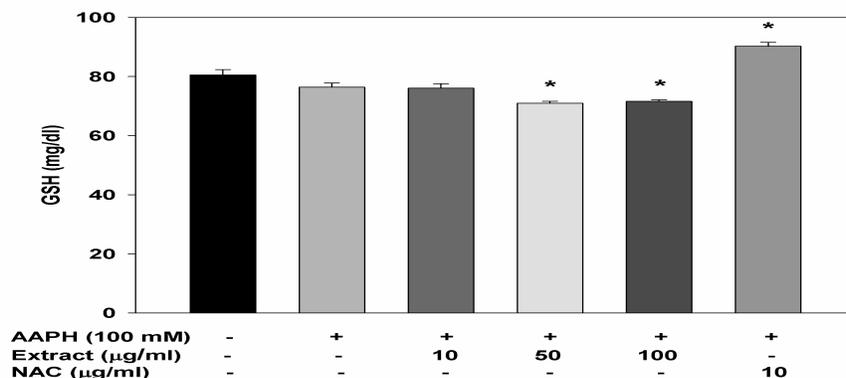


Figure 3. Effect of *Artocarpus lakoocha* extract on GSH in AAPH-oxidized whole blood. Whole blood was co-treated with 10, 50 and 100 µg/ml of extract in the presence of 100 mM AAPH at 37°C for 6 h. Erythrocyte GSH was detected by DTNB reagent. The positive control of antioxidant was compared by using standard N-acetylcysteine (NAC) at 10 µg/ml. Values are given as mean ± SE (n = 3). *Statistical significance was at $p < 0.01$ and compared with the red cell control.

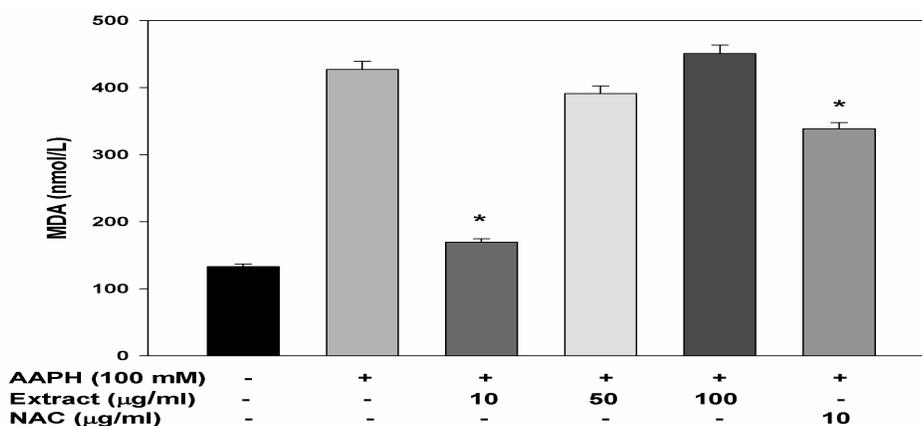


Figure 4. Effect of *Artocarpus lakoocha* extract on lipid peroxidation in red blood cells. Whole blood was treated with 10, 50 and 100 µg/ml of extract in the presence of 100 mM AAPH at 37°C for 6 h. The plasma was separated for determining the malondialdehyde concentration by TBARS assay. The positive control of antioxidant was compared by using standard N-acetylcysteine (NAC) at 10 µg/ml. Values are given as mean ± SE (n=3). *Statistical significance was at $p < 0.001$ and compared with red cells treated with AAPH.

lipid-containing structures (Choi et al., 2003). Moreover, there are no reports on the toxicity or its pro-oxidant activity of *A. lakoocha* extract previously, but in our study found the toxicity of this plant that need more studies and confirmed in the future.

Conclusion

A. lakoocha refers to antihelminthic, antiherpetic and skin-whitening agents in clinical terms. In this study, we found that *A. lakoocha* extract from ethanolic extraction contained total and important antioxidants and

polyphenolic compounds, especially tannins and flavonoids. However, the high concentration of extract might be toxic. Thus, modification of ethanolic extract from *Artocarpus lakoocha* should be carefully used and studied more in the future.

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