RESEARCH COMMUNICATION

Inhibition of Heinz Body Induction in an *inVitro* Model and Total Antioxidant Activity of Medicinal Thai Plants

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Abstract

Herbs have been used for medicinal purposes for centuries and known to possess antioxidant properties that may help to reduce the risk of chronic diseases, cardiovascular disease, and cancer. We screen aqueous extracts from 20 medicinal plants in Thailand that were believed to possess anti-tumor activity, help immune-stimulating property and maintain blood stasis. The antioxidant activities were investigated in two bioassays. Firstly, we demonstrated inhibition of Heinz bodies induction caused by oxidants under in vitro condition. The percentages of Heinz body inhibition activity in plant extracts from Terminalia citrina, Cassia timoriensis, and Derris elliptica were the highest followed by Anamirta cocculus, and Oroxylum indicum respectively. In addition, we investigated total antioxidant activity in plant extracts by improved ABTS radical cation decolorization assay. The total antioxidant activity of the extract from Terminalia citrina was also the highest activity followed by Ficus pubigera, Derris elliptica, Anamirta cocculus, Caesalpinia sappan, and Oroxylum indicum respectively. Our results suggest medicinal Thai plants as valuable sources of antioxidants, which may have a potential anti-carcinogenic activity.

Key Words: Antioxidant activity - Heinz body inhibition - Thai plants - Terminalia citrina

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Introduction

Plants have played a crucial role in maintaining human health and improving the quality of human life for thousands of years. Also there are valuable components of seasonings, beverages, cosmetics, dyes, and medicines. The World Health Organization has estimated that 80% of the earth's inhabitants relied on traditional medicine for their primary health care needs, and most of this therapy involved the use of plant extracts or their active components (Bruneton, 1995). In Thailand, herbs have been used as food and medicine for centuries. There were believed to possess hypolipidemic, anti-tumor, or immune-stimulating properties that may be useful adjuncts in helping reduce the risk of cardiovascular disease and cancer. Self-prescribed herbal preparations are widely used today for a host of common ailments and conditions, such as anxiety, arthritis, cold, cough, constipation, fever, headaches, infection, insomnia, intestinal disorder, premenstrual syndrome, stress, ulcer, and weakness (Craig, 1999).

It is generally accepted that oxidative stress is involved in the tumor promotion stage because organic peroxides or radical-generating agents, such as benzoylperoxide are tumor promoters in mouse skin, while conversely, some radical scavengers can counteract them (Perchellet et al., 1995). Cancer is one of the most common causes of death in Thailand. Recently, resistance to anticancer drugs has been observed. Many chemical substances derived from herbs are known to be effective chemo preventive and anti-tumoral agents in a number of experimental models of carcinogenesis. Some Thai vegetables have been shown to induce a chemoprotective action (Jiwajinda et al., 2002; Murakami et al., 1995). The popular, green tea has been reported to provide protective effects against gastrointestinal cancer (Borrelli et al., 2004). There is increasing evidence for an association of a high consumption of fruits and vegetables and risk reducing of oral cancer (La Vecchia et al., 1997 and Morse et al., 2000) and prostate carcinoma (Thompson et al., 1997).

Nowadays, many researchers have found that the chemical substances in herbs contained a variety of phytosterols, triterpenes, flavonoids, saponins, and carotenoids, which have been accepted to be cancer chemoprotective (Steinmetz et al., 1991). Flavonoids have

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extensive biological properties that promote human health and help to reduce the risk of disease. Flavonoids extend the activity of vitamin C, act as antioxidants, protect LDL cholesterol from oxidation, inhibit platelet aggregation, and act as anti-inflammatory and anti-tumor agents (Manach et al., 1996; Smith et al., 1994; Cook et al., 1996). A variety of phenolic compounds, in addition to the flavonoids, are found in fruits, vegetables, and many herbs. Phenolics influence the quality and stability of foods by acting as flavorants, colorants, and antioxidants. Phenolic compounds, such as caffeic, ellagic, and ferulic acids, sesamol, and vanillin, also exhibit anti-carcinogenic activity and inhibit atherosclerosis as well (Decker et al., 1995).

In this study, 20 traditional Thai herbs believed to possess anti-tumor activity, have immune-stimulating properties and maintain blood stasis were examined for antioxidant activity, with reference to inhibition of Heinz body formation and hemoglobin precipitation caused by oxidants, as well as total antioxidant activity by improved ABTS radical cation decolorization assay. Antioxidant screening models in vitro would provide important preliminary data to help select plant extracts with potential anti-neoplastic properties for the future study.

Materials and Methods

Sample Preparation

Twenty dried medicinal plants (Table 1) were purchased from Traditional Thai Pharmacy in Bangkok. Each sample was prepared by boiling in water for 10 minutes with a ratio of herb to water at 1:20 w/v. The mixture was then shaken intermittently. After boiling, the mixture was cooled at room temperature, centrifuged at 2,500 rpm for 15 minutes. The supernatant of each extract was filtered through Whatman no.2 filter paper and then immediately analysis. Samples, not yet investigated, were stored at -20˚C until analyzed.

Table 1. Medicinal Thai Plants Studied. Their Families, Common /Thai Names, Part used and Therapeutic Applications

Scientific name	Family	Common name/ Thai name	Part used	Therapeutic applications
Adhatoda vasica Nees	Acanthaceae	Sa-nead	Leaf	Bronchitis, asthma, cough, local bleeding due to peptic ulcer, piles
Anamirta cocculus Wight & Arn.	Menispermaceae	Fish berry	Creeping stem	Blood stasis, fever, stimulate for central nervous system
Caesalpinia sappan L	Fabaceae	Sappan	Stem	Menstrual disorder, tonic, anti-tumor, blood stasis, cough
Cassia timoriensis DC	Leguminosae	Kee lek leard	Core	Menstrual disorder, tonic, anti-tumor, blood stasis
Croton sublyratus Kurz.	Euphorbiaceae	Croton	Core	Diarrhea, tonic for stomach, inflammation, blood stasis, menstrual disorder
Dalbergia cochinchinensis Pierre	Leguminosae	Siamese Rosewood/ Payung	Core	Anti-tumor, blood stasis
Derris elliptica Benth	Fabaceae	Hang lai Daeng	Creeping stem	Anti-tumor, blood stasis
Eurycoma longifolia Jack	Simaroubaceae	Pla lai peauk	Root	Tonic, malaria, aphrodisiac
Ficus pubigera Wall.	Moraceae	Mar grateub rong	Stem	Tonic for men, headache
Holarrhena pubescens Wall. ex Don.	Apocynaceae	Kurchi/ Mok luang	Bark	Diarrhea, inflammation
Mimusops elengi L	Sapotaceae	Bullet Wood	Root	Tonic for heart and lung, blood stasis, hepatitis
Morinda coreia Ham.	Rubiaceae	Yor Par	Core	Menstrual disorder, tonic for stomach , blood stasis
Oroxylum indicum L	Bignoniceae	Pae ka	Seed	Cough, anti-tumor, diarrhea, tonic
Plerocarpus indicus Willd.	Fabaceae	Padauk	Stem	Blood stasis
Plumbago indica L	Plumbaginaceae	Rose-colored Leadwort	Root	Menstrual disorder, tonic, hemorrhoids, appetizer
Plumbago zeylanica L	Plumbaginaceae	White-colored Leadwort	Root	Menstrual disorder, tonic, hemorrhoids, appetizer, antimicrobial, anti-tumor, reduce cholesterol
Randia siamensis Craib	Rubiaceae	Kud kauw	Seed	Menstrual disorder
Strychnos nux-vomica L	Strychnaceae	Nux-vomica Tree/ Snake Wood	Leaf	Tonic for heart, blood stasis, appetizer
Terminalia citrina Roxb. ex Fleming.	Combretaceae	Samor dee ngu	Seed	Blood stasis, antimicrobial
Zanthoxylum limonella Alston	Rutaceae	Makaen	Seed	Menstrual disorder, tonic for heart and stomach , blood stasis

Chemicals

Acetylphenylhydrazine (BDH Chemicals, England), crystal violet powder (Merck, Germany), KH2PO4 (Merck, Germany), Na2HPO4 (Merck, Germany), Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) (Aldrich chemical, USA), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid diamonium salt (ABTS) (Sigma-Aldrich, USA), potassium persulfate (Sigma-Aldrich, USA). All chemicals were of analytical grade and available locally.

Inhibition of Heinz Body Induction

Buffer solution consisting of 1.3 parts of M/15 KH2PO4 (9.1 g of KH2PO4 dissolved in 1 L deionized water) was mixed with 8.7 parts of M/15 Na2HPO4 (9.5 g of Na2HPO4 dissolved in 1 L deionized water). Two hundred mg of glucose were added to 100 mL buffer solution.

Acetylphenylhydrazine solution was prepared by dissolving 100 mg of acetylphenylhydrazine in 100 mL buffer solution.

Crystal violet solution prepared by dissolving 2 g of crystal violet powder in 100 mL of 0.73% NaCl at room temperature. The solution was shaken for 5 min, and then filtered through Whatman No.2 filter paper. Working solution was prepared by adding equal volume of 0.73% NaCl to the filtered solution.

Two milliliters of extract (original dilution of 1:20) was mixed with 0.1 mL blood and incubated for 2 hours, and then 2 mL acetylphenylhydrazine was added. The mixture was incubated for another 2 hours, and then Heinz bodies were counted. If the test was positive, the extract was diluted in 2 and 4 fold and then was tested again. In the test system, each herb was tested in triplicate.

A positive control system was used, following the method described by Reinhart (1986), Sangkitikomol (2001), and Soogarun (2005). Two milliliters of acetylphenylhydrazine was added to 0.1 mL of packed red blood cells, and the mixture was incubated at 37˚C for 2 hours. Heinz bodies were counted under a microscope (1000x). A negative control was prepared by adding 2 mL of buffer solution into 0.1 mL of packed red blood cells. The negative control was incubated at 37˚C for 2 hours, and then Heinz body was observed.

We performed the counter stain of Heinz body by transferring solutions from each test and mixed it with crystal violet solution at equal volume. The mixture was left undisturbed at room temperature for 5 minutes. A thin smear was performed and the Heinz body was observed in at least 1000 red blood cells under a microscope (1000x).

Total Antioxidant Activity Assay

Total antioxidant activity was measured by using radical cation decolorization assay (Miller et al., 1993; Re et al., 1999). This assay based on the inhibition by antioxidants of the absorbance of the free radical cation from ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid diamonium salt). ABTS was incubated with potassium persulfate in order to produce the free radical cation ($ABTS^{o+}$). This had a relatively stable blue-green color, which was measured at 734 nm. Antioxidant compounds will suppress the absorbance of ABTSo+ to an extent on a time scale dependent on the antioxidant capacity in plasma. This assay was calibrated using Trolox (a water-soluble vitamin E analoque) as standard.

In brief, ABTS was dissolved in deionized water to make a 7 mM concentration solution. $ABTS^{o+}$ was produced by mixing ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and the mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. For our study, the $ABTS^{o+}$ solution was diluted with PBS, pH 7.4, to an absorbance of 0.70 ($\pm$0.02) at 734 nm. After addition of 1.0 ml of diluted $ABTS^{o+}$ (A734nm = 0.700 $\pm$ 0.200) to 10 µl of each plasma or Trolox standards (final concentration 0-15 µM) in PBS the absorbance reading was taken at 30 oC exactly 6 minutes after initial mixing. PBS blank were run in each assay. All determinations were carried out at least in triplicate. The percentage of absorbance inhibition at 734 nm was calculated and plotted as a function of antioxidants of Trolox for the standard reference data.

Results

The percentages of Heinz body formation in 20 medicinal plant extracts are shown in Table 2. The extracts from Anamirta cocculus, Cassia timoriensis, Derris elliptica, Oroxylum indicum, Plumbago indica and Terminalia citrina could completely inhibit the Heinz body formation at the dilution of 1:20. Furthermore, some extracts from Cassia timoriensis, Derris elliptica, and Terminalia citrina could

Table 2. Inhibition of Heinz Body Formation by Herbal Extracts

Family/spp.	% of Heinz body formation in different extract dilution*		
	1:20	1:40	1:80
Adhatoda vasica	100	100	100
Anamirta cocculus	0	3	9
Caesalpinia sappan	37	39	43
Cassia timoriensis	0	0	0
Croton sublyratus	100	100	100
Dalbergia cochinchinensis	79	95	100
Derris elliptica	0	0	0
Eurycoma longifolia	100	100	100
Ficus pubigera	100	100	100
Holarrhena pubescens	100	100	100
Mimusops elengi	85	98	100
Morinda coreia	85	91	100
Oroxylum indicum	0	39	87
Plerocarpus indicus	71	100	100
Plumbago indica	0	30	81
Plumbago zeylanica	11	32	32
Randia siamensis	98	98	100
Strychnos nux-vomica	49	61	79
Terminalia citrina	0	0	0
Zanthoxylum limonella	100	100	100

* % Heinz body formation was observed in at least 1000 red blood cells under a microscope (1000x).

completely inhibit the Heinz body formation up to the dilution of 1:80. Caesalpinia sappan and Strychnos nux-vomica could partially inhibit (at < 50%) the Heinz body formation in the dilution of 1:20. On the other hand, Adhatoda vasica, Croton sublyratus, Eurycoma longifolia, Ficus pubigera, Holarrhena pubescens, and Zanthoxylum limonella could not inhibit the Heinz body formation at any dilutions.

The total antioxidant activity of all medicinal plant extracts are shown in Table 3. We found that the extract from Terminalia citrina was the highest activity followed by Ficus pubigera, Derris elliptica, Anamirta cocculus, Caesalpinia sappan, Oroxylum indicum, Holarrhena pubescens, Plumbago indica, Plerocarpus indicus, Cassia timoriensis, Zanthoxylum limonella, Plumbago zeylanica, and Strychnos nux-vomica, Randia siamensis, Adhatoda vasica, Dalbergia cochinchinensis, Mimusops elengi, Eurycoma longifolia, respectively. Nevertheless, total antioxidant activity from the extract of Morinda coreia extract was the lowest.

We found that plant extract from Terminalia citrina was the highest not only in the total antioxidant activity but also in the Heinz body inhibition activity

The percentages of Heinz body inhibition of some medicinal plants are shown using different extract concentrations, since acetylphenylhydrazine induction prooxidant activity. The results in Figure 1 are shown that some medicinal plant extracts from Plumbago indica, and Oroxylum indicum act proportional increase to inhibit Heinz body formation at high concentration.

Table 3. The Percentage of Heinz Body Inhibition Activity at the 1:20 of Extract Dilution Comparing to the Total Antioxidant Activity of All Medicinal Plant Extracts

Family/spp.	Antioxidant status	
	% Heinz body inhibition activity*	Total Antioxidant Activity (mM/g)**
Adhatoda vasica	0	1.206
Anamirta cocculus	100	9.825
Caesalpinia sappan	63	9.531
Cassia timoriensis	100	5.422
Croton sublyratus	0	0.556
Dalbergia cochinchinensis	21	1.206
Derris elliptica	100	18.397
Eurycoma longifolia	0	0.834
Ficus pubigera	0	18.669
Holarrhena pubescens	0	8.122
Mimusops elengi	15	1.028
Morinda coreia	15	0.466
Oroxylum indicum	100	8.884
Plerocarpus indicus	29	5.931
Plumbago indica	100	4.013
Plumbago zeylanica	89	1.222
Randia siamensis	2	1.493
Strychnos nux-vomica	51	3.200
Terminalia citrina	100	33.740
Zanthoxylum limonella	0	5.059

* % Heinz body inhibition activity = 100 - % Heinz body formation at the 1:20 of extract dilution

** Total Antioxidant Activity was reported in mM trolox equivalent in 1 g of herb (dry weight)

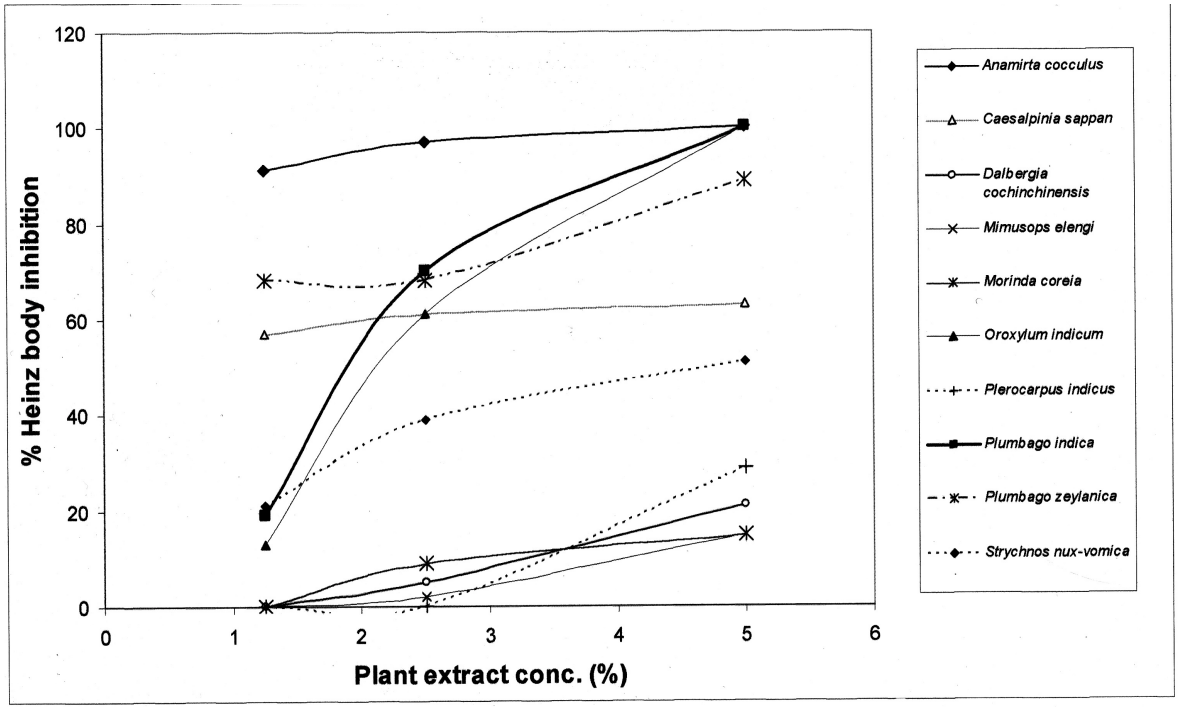

Figure 1. The Percentages of Heinz Body Inhibition of Some Medicinal Plants as a Function of Their Extract Formations (% of undiluted extract). Some herbal extracts act proportional increase to inhibit Heinz body formation at high concentration

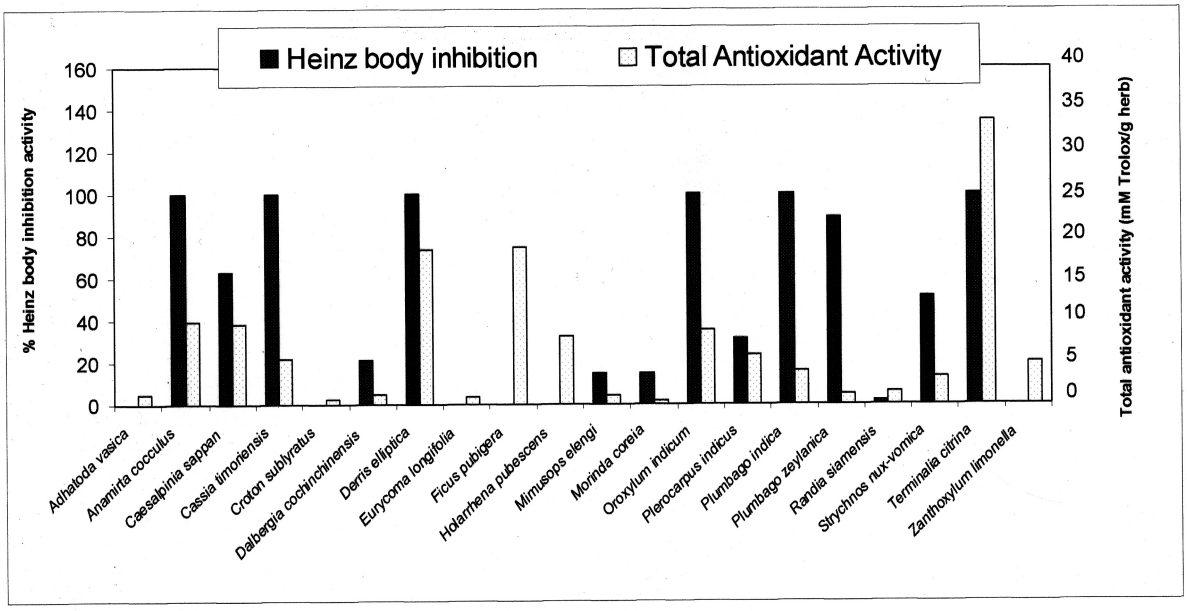

Figure 2. The Percentages of Heinz Body Inhibition Activity in the Plant Extract (at the dilution of 1:20) Versus Total Antioxidant Activity

Figure 2 presents relation between the percentages of Heinz body inhibition activity of the plant extract at the dilution 1:20 and the total antioxidant activity.

Discussion

We investigated the antioxidant activity of Thai herbs, extracted by boiling in the water in order to mimic the preparation of traditional medicine in Thailand. The medicinal plant extracts were diluted in various dilutions for inhibition of Heinz body induction using in vitro model. Regarding Fenton reaction, hemoglobin in erythrocytes was oxidized by hydrogen peroxides and a ferrous ion catalyst (Halliwell et al., 1984, Halliwell et al., 1985, Puppo et al., 1988). The peroxide is broken down into a hydroxide ion and a hydroxyl free radical. The hydroxyl free radical is the primary oxidizing species which oxidize and break apart organic molecules. In our assay, phenylhydrazine (Miller et al., 1970; Beutler, 1969; Beutler et al., 1955), the free radical intermediate product, oxidized polyunsaturated fatty acids in cell membranes of erythrocytes. As a result of the denaturation of hemoglobins in erythrocytes, the formation of intracellular precipitates known as Heinz bodies occurs. Flavonoids and nonflavonoids, commonly found in herbs, react as an antioxidant by inhibiting the oxygen radical formation through the enhanced oxidation of Fe2+ ion as the prooxidant. Both substances effectively inhibited the formation of thiobarbituric acid-reactive substances, a marker of lipid peroxidation of microsomes from rat liver. (Yoshino et al., 1998) Thus, administration of diluted plant extracts with phenylhydrazine, resulting in the denaturation of hemoglobins in erythrocytes in vitro model was demonstrated. In our case, assessment of Heinz bodies is a useful gauge in evaluating susceptibility of red blood cells to the oxidant stress.

The medicinal plants that shown high significant activity in two bioassay systems are Terminalia citrina, Derris elliptica, Cassia timoriensis, Anamirta cocculus, and Oroxylum indicum. However, Ficus pubigera had significantly high total antioxidant activity but had not the ability to suppress Heinz body formation.

In chemical analysis of Terminalia citrina, Burapadaja and Bunchoo (1995) was isolated the active compounds from the extract of Terminalia citrina. They have reported five known tannins identified as corilagin, punicalagin, 1,3,6-tri-O-galloyl-beta-D-glucopyranose, chebulagic acid, and 1,2,3,4,6-penta-O-galloyl-beta-D-glucopyranose by comparison of their physical and spectral data with those of authentic samples. (Burapadaja and Bunchoo, 1995) The antioxidant properties of polyphenols may change as a consequence of their oxidation state. Polyphenols with an intermediate oxidation state can exhibit higher radical scavenging activity than nonoxidized polyphenols. (Gil et al.,2000, Kaur C et al., 2001) The higher antioxidant activity of the partially oxidized polyphenols could be attributed to their increased ability to donate a hydrogen atom from the aromatic hydroxyl group to a free radical and/or to the capacity of their aromatic structure to support unpaired electrons through delocalization around the p-electron system. (Kikugava et al., 1990) Our data suggest that the biological effects exhibited by Terminalia citrina, under these experimental conditions, could be related to a principle effect of the tannin compounds evidenced in the extract. In particular, it has been reported that these natural compounds show scavenging activity against free radicals (Yokozawa et al., 1997; Yokozawa et al., 1998; Nakagawa and Yokozawa, 2002). Polyphenol tannic acid inhibits the hydroxide ion formation from Fenton reaction by

complexing ferrous ions (Lopes et al., 1999). Thus, we have not seen Heinz body formation in some herbs containing high dose of tannins.

The present results propose that the extract of Terminalia citrina has antioxidant activity, which indicates its effectiveness in protection form diseases caused by overproduction of free radicals. Further studies would be required to evaluate in vivo assay.

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