Original Article

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Protection of antioxidative damage on erythrocytes of Thai traditional recipe "Ya-hom"

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Abstract

Antioxidant properties of the three formulas of Thai traditional Ya-hom (formula 1, formula 2, and formula 3) were studied. Water and methanolic extracts of Ya-hom were prepared. The free radical scavenging activity of these extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, using vitamin C and Trolox as reference standards. An *in vitro* oxidative hemolysis model using sheep red blood cells was performed to study the anti-hemolytic activity of extracts on free radical-induced damage to biological membranes using the 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) method. DPPH assay indicates the most potent activity of the methanolic extract of formula 3 with an IC_{50} value of 30.37 + 0.30 µg/ml, whereas the IC_{50} values of other extracts being ranged from 61.97 + 0.66 to 689.48 + 68.60 µg/ml. AAPH hemolysis assay also confirmed the most potent activity of the methanolic extract of formula 3 (0.5 mg/ml) with a prolonged 50% hemolysis time from the control group (from 70 to 180 min), while 50% hemolysis time for the other extracts ranged from 105 to 155 min and that of 0.5 mg/ml Trolox was 160 min. The phytochemical screening showed the presence of phenolic compounds in both water and methanolic extracts of the three formulas. Tannins were found in both water and methanolic extracts of formula 3 and in methanolic extracts of formulas 1 and 2. Flavonoids were found only in methanolic extracts of formulas 1 and 3. The result confirmed antioxidant activity of Ya-hom extracts which may be due to the presence of phenolic compounds, tannins, and/or flavonoids.

Keyword: Ya-hom; antioxidant; anti-hemolytic; DPPH; AAPH

1. INTRODUCTION

Among traditional medicines currently used in Thailand, Ya-hom is one of the most interesting and compelling folk formulas, with uses that include treatment for symptoms of fainting, as a mild cardiac stimulant, and as a remedy for stomach discomfort. Ya-hom is listed as a "National Herbal Medicine Product" in Thailand[(1)]. The commercially available of traditional Ya-hom in Thailand is available in different formulas with varied formula compositions. The main ingredients of traditional Ya-hom formula comprise young flower bud of *Syzygium aromaticum*, rhizome of *Angelica dahurica* Benth, stamen of *Nelumbo nucifera* Gaertn, infected wood of *Dracaena loureiri* Gagnep, sandal wood (*Santalum album* L.), licorice (*Glycyrrhiza glabra* L), flower of *Mesua ferrea* L, *Cinnamomum loureirii* Nees bark, *Cinnamomum verum* J.S. Presl bark, rhizome of *Ligusticum sinense* Olive. cv. Chuanxiong and *Aquilaria crassna* leaf[(1)]. To date, many studies have investigated the efficacy of Ya-hom formula, not only in animals, but also in humans. In the cardiovascular system, Ya-hom increased blood pressure in both rats and humans[(2, 3)] via 2 possible mechanisms; specifically, increasing vascular smooth muscle contraction and atrial contraction[(4)]. In addition, Ya-hom inhibited gastric acid and pepsin secretion and potentiated gastric visible mucus secretion[(5)].

Reactive oxygen species (ROS) are a group of reactive molecules, radicals and ions that are produced as a natural byproduct of the normal metabolism of oxygen. However, their

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levels can be increased dramatically during times of environmental stress. The potential targets for excess ROS in cells are membrane lipids, DNA, and proteins. ROS increases the chances of developing many human diseases and conditions, including cancer, neurodegenerative disease, heart disease, diabetes, inflammation, and skin aging[6-9]. In recent years, antioxidants have been subjected to many epidemiological studies that related their use to a reduction in the incidence of oxidative damage-related diseases. Each herbal ingredient in Ya-hom (*Syzygium aromaticum*, stamen of *Nelumbo nucifera*, *Glycyrrhiza glabra*, flowers of *Mesua ferrea*, *Cinnamomum verum* and *Aquilaria crassna* leaf) showed the antioxidant activity[10-17]. It has been revealed that Ya-hom contains a wide variety of natural antioxidants. However, only a few studies have demonstrated the antioxidant properties of the Ya-hom formula[17, 18]. Furthermore, it has been clinically proven that Ya-hom is safe with a median lethal dose (LD_{50}) of more than 5 g/kg body weight by oral administration and intraperitoneal injection in rats and mice[19].

The aim of this study was to use 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) hemolysis methods to investigate the antioxidant activity of the three most popular formulas of Ya-hom currently available in Thailand. The presence of phenolic compounds, tannins, and flavonoids in these three formulas was also investigated.

2. MATERIALS AND METHODS

2.1 Materials

Ya-hom formula 1 (flowers of *Mesua ferrea*, stamen of *Nelumbo nucifera*, wood of *Dracaena loureiri* Gagnep, rhizomes of *Angelica dahurica* Benth, *Aquilaria crassna* leaves), formula 2 (*Syzygium aromaticum*, *Glycyrrhiza glabra*, wood of *Dracaena loureiri* Gagnep, *Cinnamomum verum* bark), and formula 3 (*Syzygium aromaticum*, flowers of *Mesua ferrea*, *Cinnamomum verum* bark, stamen of *Nelumbo nucifera*, *Aquilaria crassna* leaves) were obtained from a drug store in Bangkok, Thailand and used as received.

2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (vitamin C), and Trolox were purchased from Fluka (Switzerland). 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and phosphate buffered saline were purchased from Sigma-Aldrich (USA). Sheep red blood cells were obtained from the National Laboratory Animal Center, Mahidol University (NLAC-MU), Salaya Campus, Nakornpathom, Thailand.

2.2 Extraction

One hundred grams of each formula of Ya-hom was macerated with 1000 ml of 80% methanol or hot water (100 ^{o}C) for 24 hours and filtered to obtain liquid methanolic or water extracts. After filtering, the extracts were evaporated using a rotary evaporator and lyophilized to obtain dry crude extracts.

2.3 Phytochemical screening

Each extract was screened for the presence of flavonoids by Shinoda test and ammonia test; for phenolic compounds by 1% ferric chloride solution; and for tannins by gelatin solution and gelatin/salt solution[20-22].

2.4 Determination of the free radical scavenging activity by the DPPH assay[23]

The antioxidant activity of each extract was determined by assessing free radical scavenging ability in reaction to a stable DPPH free radical. Vitamin C or Trolox were used as the reference standards. The extract or the stock solutions of the standards (300 µl each) was mixed with 600 µl of 0.2 mM DPPH in absolute methanol. After incubation at 37^{o}C for 30 min, the absorbance of each solution was measured at 515 nm using a spectrophotometer (Pharmacia LKB Novaspec II, England). Concentration which provides 50% inhibition (IC_{50}) was determined. Triplicate samples of each were measured and averaged.

2.5 Determination of the anti-hemolytic activity by the AAPH assay

For hemolysis assay, 100 µl of 20% packed sheep red blood cells (RBC) was mixed

with 100 µl of 0.5 mg/ml extract in phosphate buffered saline (PBS), pH 7.4 and 200 µl of 200 mM AAPH. Every 30 min, during incubation at 37°C for a total of 3 hours, 4 µl of 0.15 M NaCl was added to each reaction mixture and centrifuged. The absorbance of the supernatant was measured spectrophotometrically at 540 nm[24, 25]. The anti-hemolytic effect of each extract was determined by the time for 50% hemolysis and was compared with that of PBS treated group as control and that of 0.5 mg/ml Trolox in PBS as reference standard. Every experiment was done in triplicate.

For hemolysis assay, 100 µl of 20% packed sheep red blood cells (RBC) was mixed with 100 µl of 0.5 mg/ml of extract in phosphate buffered saline (PBS) at pH 7.4 and then 200 µl of 200 mM AAPH was added and incubated at 37°C for 3 hours. Every 30 min during the incubation period, 4 µl of 0.15 M NaCl was added to each sample of the reaction mixture taken, and then centrifuged. The supernatant was separated for measuring the absorbance by spectrophotometric analysis at 540 nm[24, 25]. The anti-hemolytic effect of each sample was determined by calculating the time required for 50% hemolysis and was compared with that of PBS treated group as the negative control and that of 0.5 mg/ml Trolox in PBS as the positive control

3. RESULTS AND DISCUSSION

Phytochemical screening revealed the presence of phenolic compounds, tannins, and flavonoids, as shown in Table 1**.** The results showed that phenolic compounds are found in both methanolic and water extracts of all formulas, while tannins are found in both water and methanolic extracts of formula 3 and in methanolic extracts of formulas 1 and 2. Flavonoids were found only in methanolic extracts of formulas 1 and 3.

Table 1. Phytochemical screening of Ya-hom extracts

% Yield / Phytochemical screening	Formula 1		Formula 2		Formula 3	
	Methanolic extract	Water extract	Methanolic extract	Water extract	Methanolic Extract	Water extract
% Yield	6.27	4.58	4.65	3.95	13.71	6.22
Phenolic compounds	+	+	+	+	+	+
Tannins	+	-	+	-	+	+
Flavonoids	+	-	-	-	+	-

Regarding free radical scavenging activity, it was shown that methanolic extracts of all formulas showed higher potency than water extracts. This was most notably the case in formula 3, which demonstrated an IC_{50} of 30.37 ± 0.30 µg/ml, while the IC_{50} of vitamin C and Trolox, reference standard, were 17.47 ± 0.01 µg/ml and 22.75 ± 0.02 µg/ml, respectively (as shown in Table 2). The IC_{50} values of other extracts ranged from 61.97 ± 0.66 to 689.48 ± 68.60 µg/ml.

From the AAPH hemolysis method, for which data is shown in Figures 1 and 2 and Table 2, it was found that the methanolic extract of formula 3 possessed the most potent anti-hemolytic properties. This extract at a concentration of 0.5 mg/ml extended 50% hemolysis time from 70 minutes (control group or PBS treated group) to 180 minutes, while the 50% hemolysis time for Trolox (0.5 mg/ ml) was 160 minutes.

It was found that the methanolic extract of Ya-hom, especially formula 3, showed a strong antioxidant activity with the DPPH method, but its potency is less than that of vitamin C and Trolox, the comparative agents used in this study. However, the IC_{50} for methanolic extract of formula 3 was less than 50 µg/ml.

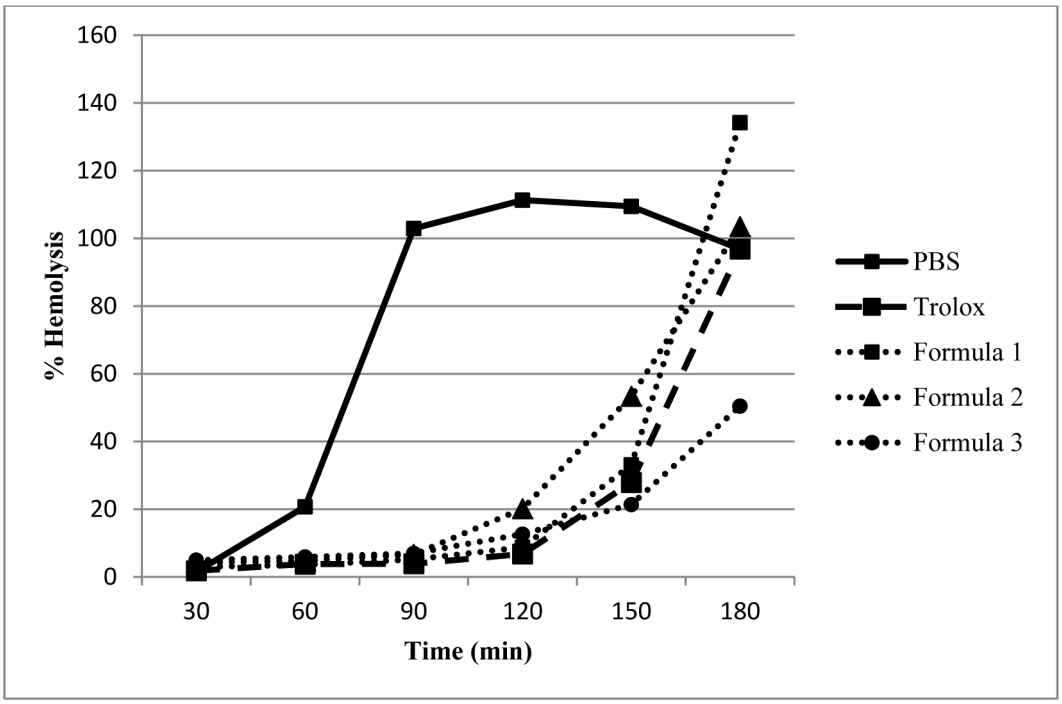

Figure 1. Effect of methanolic extract of Ya-hom formula on AAPH-induced hemolysis

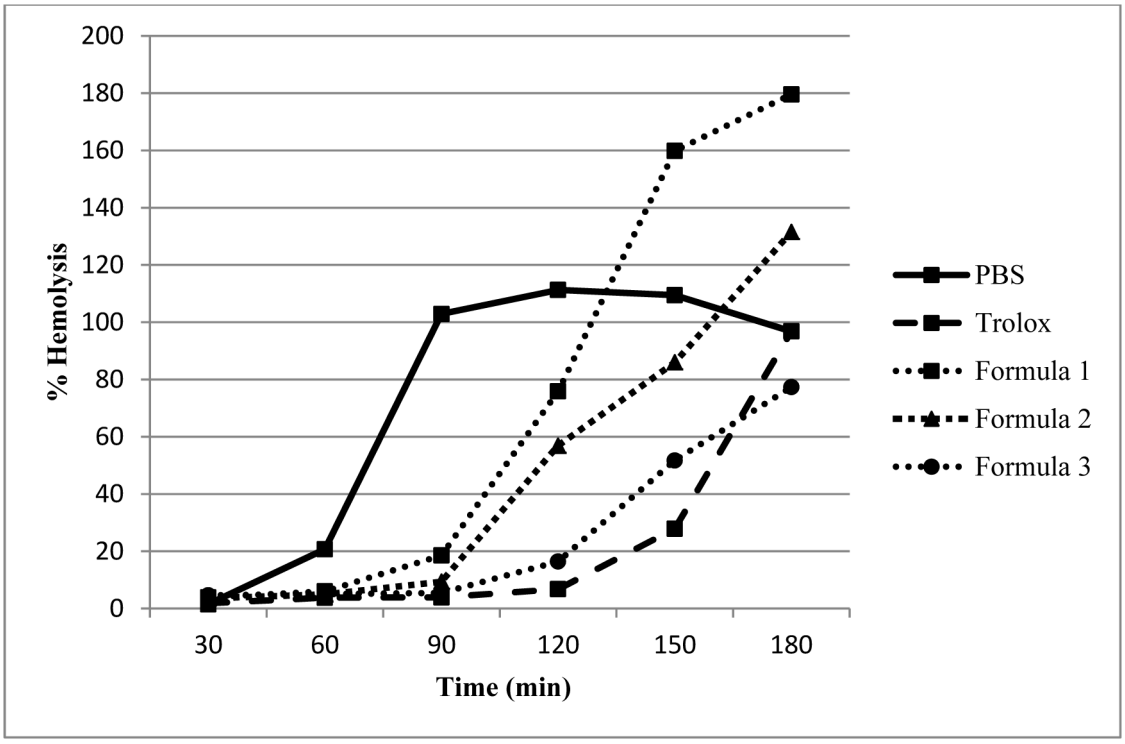

Figure 2. Effect of water extract of Ya-hom formula on AAPH-induced hemolysis

Table 2. IC_{50} (μg/ml) and time (min) for 50% hemolysis of Ya-hom extracts

	IC50 (μg/ml) by DPPH method	Time (min) for 50% hemolysis by AAPH method
Vitamin c	17.47 ± 0.01	
Trolox	22.75 ± 0.02	160
Formula 1 : Methanolic extract	61.97 ± 0.66	155
: Water extract	689.48 ± 68.60	105
Formula 2 : Methanolic extract	121.37 ± 0.08	145
: Water extract	488.23 ±9.36	115
Formula 3 : Methanolic extract	30.37 ± 0.30	180
: Water extract	137.15 ± 7.18	148

Based on Cervantes-Cervantes[26], it may conclude that the methanolic extract of Ya-hom formula 3 exhibited high antioxidant activity, while that of Ya-hom formulas 1 and 2 showed moderate and mild antioxidant activity, respectively. From the AAPH hemolysis method, it was found that the methanolic extract of formula 3 possessed the most potent antioxidant property. It has been suggested that the ability of Ya-hom formula to extend AAPH-induced RBC hemolytic time is due to its contribution to the protection of the erythrocyte membrane, which is rich in polyunsaturated fatty acids from peroxidation[27]. Oxidative damage to erythrocyte membranes (lipid and protein peroxidation) may be implicated in hemolysis associated with some hemoglobinopathies, oxidative drugs, transition metal excess, radiation, and deficiencies in some erythrocyte antioxidant systems[28]. AAPH, a water-soluble free radical generator, was used to imitate the *in vivo* condition of oxidative stress. Peroxyl radicals are generated by thermal decomposition of an azo compound in the presence of oxygen and they cause lipid peroxidation of red blood cell membranes, resulting in cell lysis. The protective effect of the Ya-hom formula against red blood cell lysis induced by AAPH may be due to its antioxidant activity via lipid peroxyl scavenging. This phenomenon may strongly explain its protective effect against free radicals and also its stabilizing effect on the red blood cell membrane.

4. CONCLUSION

It was concluded that the methanolic extracts of Ya-hom exhibited the significantly higher free radical scavenging activity and reduced AAPH-induced erythrocyte hemolysis. Phenolic compounds, tannins, and flavonoids were also found in methanolic extracts in all of the investigated Ya-hom formulas. It emphasizes the effect of these compounds in the Ya-hom which were thought to be responsible for the observed antioxidant activity. Therefore, the findings of this study confirm the defensive antioxidant activity of Thai traditional Ya-hom formula.

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