Original Article

Effects of Ethanol and Aqueous Extracts of *Terminalia chebula, Cyperus rotundus, Tinospora crispa* and The Combined Remedy on Anti-oxidant Activities and Capacities

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Abstract

Introduction: Plant antioxidants have an important role in the prevention of many degenerative diseases. A Thai herbal remedy in Worayokasarn scripture consists of three plants, *Terminalia chebula* fruits (TC), *Cyperus rotundus* rhizomes (CR) and *Tinospora crispa* vines (TCL) has been used for longevity. Previous studies showed that polyphenol enriched extract of TC, CR methanol extract and TCL ethanol extract exerted free radical scavenging activity.

Objectives: To investigate ethanolic and aqueous extracts of the remedy and its plant ingredients on anti-oxidant activities and capacities.

Methods: The remedy and its plant ingredients were extracted by maceration (95% ethanol) and decoction techniques. All extracts were evaluated for anti-oxidant activities by DPPH and ABTS radical scavenging and FRAP assays. The total phenolic content (TPC) and total flavonoid content (TFC) were investigated.

Results: The remedy ethanol extract (RME) and aqueous extract (RMW) exerted DPPH scavenging activity (EC_{50} of 11.00 and 53.49 µg/mL, respectively), ABTS scavenging activity (EC_{50} = 41.66 and 34.06 µg/mL, respectively) and FRAP values (220.78 and 228.84 mg Fe^{2+}/g of extract, respectively). The TPC of RME and RMW were 48.16 and 64.95 mg GAE/g of extract, respectively, while the TFC (116.56 and 134.06 mg QE/g of extract, respectively). Moreover, TCW showed very strong DPPH (9.43 µg/mL) and ABTS (11.40 µg/mL) activities and the highest TPC (142.15 mg GAE/g of extract). TCE and CRE had the highest FRAP value (467.14 mg Fe^{2+}/g of extract) and TFC (182.30 mg QE/g of extract), respectively.

Conclusions: The remedy and the three herbal extracts had antioxidant activities and capacities. The results supported that the remedy should be selected to use for reducing and preventing oxidation.

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Introduction

Reactive oxygen species (ROS) are radical molecules containing the oxygen atom with an unpaired electron outside the atomic orbital named as an oxygen free radical.[1,2] In the cellular respiratory process, mitochondria utilizes about 98% of the inhaled oxygen and about 1 - 2% of total daily oxygen consumption become ROS which is one of the main causes of mitochondrial injury.[3,4] Large numbers of medicinal plants have been investigated for their anti-oxidant activity. Several studies on herbal plants have indicated the presence of phytochemicals such as phenolics, flavonoids, tannins, and proanthocyanidins as anti-oxidant.[5] The antioxidant contents of medicinal plants may contribute to protection from aging or degenerative disease. Thus, the inhibition of ROS may decrease the risk factors of degenerative diseases.

One remedy of Worayokhasarn scripture, a Thai traditional medicinal scripture, is used to promote long life. The remedy consists of three medicinal plants including *Terminalia chebula* Retz. (TC) fruits, *Cyperus rotundus* L. (CR) rhizomes, and *Tinospora crispa* L. (TCL) vines. Previous studies showed that polyphenol-rich extract of *T. chebula* fruits exerted DPPH radical scavenging activity with an IC_{50} value of 14 µg/mL and 96% ethanolic extract of *T. crispa* showed EC_{50} of 2.65 mg/mL.[6,7] The increase of methanol extract concentrations of *C. rotundus* increased the %inhibition of oxidation as determined by the DPPH method.[8] However, very little is known about the actions by which this remedy mediates its therapeutic effects. Thus, the remedy may use to for anti-oxidant purpose including the treatment and prevention of age-related or degenerative diseases.

Methods

Chemicals and Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-Striazine (TPTZ), 2,2' Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Aluminium chloride 6-hydrate ($AlCl_3 \bullet 6H_2O$), Folin & Ciocalteu's reagent, Iron (lll) chloride hexahydrate ($FeCl_3 \bullet 6H_2O$), Potassium persulphate ($K_2O_8S_2$), Sodium acetate ($CH_3COONa \bullet 3H_2O$), Sodium carbonate (Na_2CO_3), Sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), 6-hydroxy-2, 5, 7, 8-tetramethylchroman -2-carboxylic acid (Trolox), butylated hydroxytoluene (BHT), gallic acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol was purchased from Merck, Germany. Glacial acetic acid from RCI Labscan, Thailand

Plant Materials

Fresh *T. chebula* fruits were purchased from Prachin Buri province, Thailand. Fresh *C. rotundus* rhizomes were purchased from Surin province, Thailand. Fresh *T. crispa* vines were purchased from Ubon Ratchathani province, Thailand. All plants were identified by a botanist at the Department of Thai Traditional and Alternative Medicine of Thai Traditional Medicine Research Institute. The voucher specimen numbers are shown in Table 1.

Plant Extractions

The remedy was mixed dried TC fruits, dried CR rhizomes and dried TCL vines in a 1:1:1 ratio following the Worayokasarn scripture. A 150 g fine powder of the remedy, the TC fruits, the CR rhizomes or the TCL vines was extracted by 95% ethanol maceration and decoction with water. Codes of the extract from the different solvents are shown in Table 1.

Table 1 Thai herbal plants in the remedy of the Worayokasarn scripture

Scientific name (Family)	Thai name	Part of Use	Voucher specimen number	Extraction	Code
Terminalia chebula Retz. (Combretaceae)	Sa-Mo-Thai	Fruit	TTM-c No. 1000665	Ethanol Maceration	TCE
				Water Decoction	TCW
Cyperus rotundus L. (Cyperaceae)	Haw-Mu	Rhizome	TTM-c No. 1000666	Ethanol Maceration	CRE
				Water Decoction	CRW
Tinospora crispa L. (Menispermaceae)	Bo-Ra-Phet	Vine	TTM-c No. 1000667	Ethanol Maceration	TCLE
				Water Decoction	TCLW
Remedy	-	-	-	Ethanol Maceration	RME
				Water Decoction	RMW

Measurement of Total Phenolic Contents

TPC was determined by the modified Folin-Ciocalteu method.[9] A 20 µL of the extract was added with 100 µL of Folin-Ciocalteu's reagent and 80 µL of Na_2CO_3, respectively. After incubation at room temperature for 30 minutes, absorbance values of the sample were measured at 765 nm by a microplate reader. All measurements were performed in triplicate. TPC was calculated by comparing the absorbance of sample with the calibration curve of gallic acid and the result was expressed as mg gallic acid equivalent per gram (mg GAE/g).

Measurement of Total Flavonoid Contents

TFC was determined according to the method of Zhishen, et.al. with slight modification.[10] A 500 µL of the extract (10-500 µg/mL concentrations) was added to 75 µL of 5% $NaNO_2$ and 150 µL of 10% $AlCl_3$, respectively. After incubation at room temperature for 5 min, the reaction mixture was treated with 500 µL of 1 M NaOH. The mixture was added to 275 µL water and incubated for 30 minutes at room temperature. The absorbance was measured at 510 nm by a microplate reader. TFC was calculated by comparing the absorbance of sample with the calibration curve of quercetin and the result was expressed as mg quercetin equivalent per gram (mg QE/g).

Determination of DPPH Radical Scavenging Activity

The antioxidant activity scavenging effect on DPPH radical was determined by a method of Yamasaki et al.[11] The extract was dissolved in absolute ethanol or distilled water (6.25 - 100 µg/mL concentrations with two-fold dilutions). 100 µL of extracts and then 100 µL of 6×10^{-5} M DPPH in absolute ethanol were transferred into a 96-well microplate and incubation for 30 min in the dark at room temperature. The absorbance was measured at 520 nm by a microplate reader. BHT was used as positive control. The experiment was tested in triplicate. The DPPH scavenging activity was represented as EC_{50} of by calculating the 50% effective concentration by using Prism program (GraphPad, USA).

Determination of ABTS Radical Scavenging Activity

ABTS method was modified from a previous method.[13] ABTS reagent was prepared with 7.7 mg/mL $ABTS^{\bullet+}$ in MQ water and 1.2 mg/mL Potassium persulphate ($K_2O_8S_2$) in MQ

water in a 1:1 ratio. A 20 µL sample solution (various concentrations at 6.25-100 µg/mL) and 180 µL of the ABTS reagent were mixed and incubated for 6 minutes at room temperature. Trolox and BHT were used as the positive control. The absorbances were measured at 734 nm using a microplate reader. The antioxidant activity was presented as IC_{50} of ABTS activity.

Determination of Ferric Reducing Antioxidant Power (FRAP) Activity

FRAP assay was slightly modified from Benzie and coworkers.[12] A 20 µL of the extract (2.5 - 100 µg/mL concentrations), Trolox (5 - 300 µg/mL concentrations), or Ferrous sulfate (5 to 800 µg/mL concentrations) solution was added to 180 µL of FRAP reagent (300 mM Acetate buffer, 10 mM TPTZ and 20 mM $FeCl_3 \bullet 6H_2O$ in 10:1:1 ratio) and then incubated for 8 minutes at room temperature. The absorbances were read at 593 nm by a microplate reader. The antioxidant activity was demonstrated as IC_{50} of FRAP activity.

Statistical Analysis

All experiments were carried out by triplicate. The data were performed as mean ± standard error of the mean (SEM). The data were analyzed by ANOVA and post-hoc analysis with least significant difference. A *P-value* of less than 0.05 was considered statistical significance.

Results

The remedy in this study contained dried fruits of *T. chebula,* dried rhizomes of *C. rotundus* and dried vines of *T. crispa*. The antioxidant activities of the extracts were investigated by DPPH and ABTS radical scavenging and FRAP assays. The content of phenolics and flavonoids were measured.

Our results found that RME and RMW exerted antioxidant activities by DPPH assay with EC_{50} values of 11.00 ± 0.50 and 53.49 ± 14.00 µg/mL, respectively. Moreover, the RME significantly inhibited DPPH scavenging activity better than the positive control, BHT (17.64 ± 1.49 µg/mL) but the RMW did not inhibit oxidation better than BHT (Figure 1). EC_{50} DPPH values of TCE, TCW, CRE, CRW, TCLE, and TCLW were 9.43 ± 1.74, 10.47 ± 0.97, 40.23 ± 0.20, > 100, 70.80 ± 0.81, and > 100 µg/mL, respectively. The results found that TCE showed the highest effective antioxidant by using DPPH assay as shown in Figure 1. In addition, the TCE and TCW significantly inhibited DPPH activity better than BHT.

For ABTS scavenging activity, RME, RMW, TCE, TCW, CRE, CRW, TCLE, and TCLW exerted anti-oxidant activity with EC_{50} value of 41.66 ± 1.20, 34.06 ± 1.13, 21.14 ± 1.37, 11.40 ± 0.84, > 100, > 100, > 100 and, > 100 µg/mL, respectively less than the positive control, BHT (14.79 ± 1.72 µg/mL) and Trolox (9.40 ± 1.86 µg/mL) as shown in Figure 2. Our results showed that RME and RMW inhibited ABTS activity. Moreover, TCW showed the highest ABTS•+ scavenging activity and did not differ BHT and Trolox (Figure 2).

The extracts had FRAP values were 54.29 ± 0.23 to 382.56 ± 4.52 mg Fe^{2+}/g of extract and TEAC values were 54.58 ± 0.22 to 375.39 ± 4.41 mg Trolox/g of extract. As shown on figure 3, TCW showed the highest effective antioxidant by determining FRAP assay (FRAP value was 467.17 ± 29.07 mg Fe^{2+}/g of extract and TEAC value was 458.04 ± 28.41 mg Trolox/g of extract) shown in Figure 3. Furthermore, TCE and TCW had FRAP and TEAC values higher than RME and RMW but CRE, CRW, TCLE, and TCLW had the values less than the RME and RMW.

The anti-oxidant capacities were measured the TPC and the TFC values. TPC of all extracts were between 26.54 ± 0.97 to 142.15 ± 2.37 mg GAE/g of extract. In addition to, TCW showed the highest TPC value as shown in Figure 4. TFC of all extracts were between 62.02 ± 15.09 to 182.30 ± 11.24 mg QEU/g of extract. Moreover, CRE showed the highest antioxidant capacity by using TFC (Figure 5).

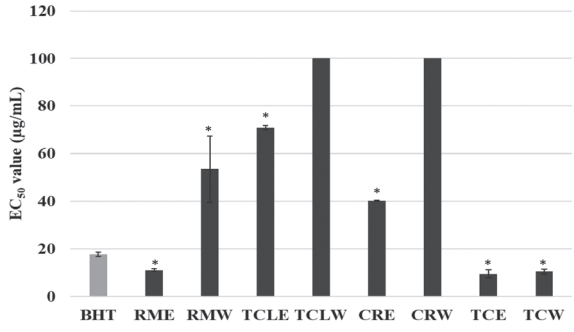


Figure 1 DPPH activities of the aqueous and the ethanol extracts of the remedy, TC, CR, and TCL when compared with BHT.
*P-value < .05 vs BHT.

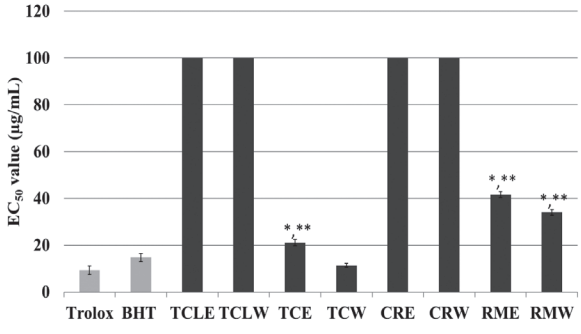
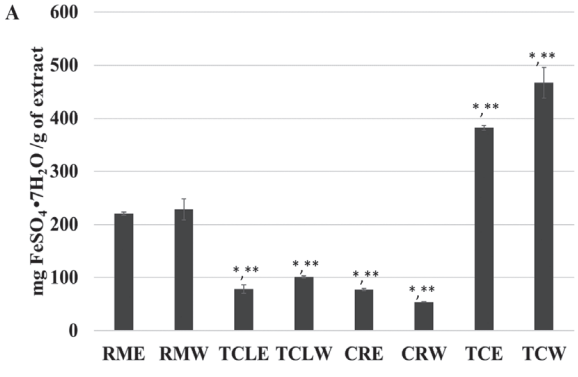


Figure 2 ABTS activities of the aqueous and the ethanol extracts of the remedy, TC, CR, and TCL when compared with Trolox and BHT.
* P-value < .05 vs BHT,
** P-value < .05 vs Trolox.



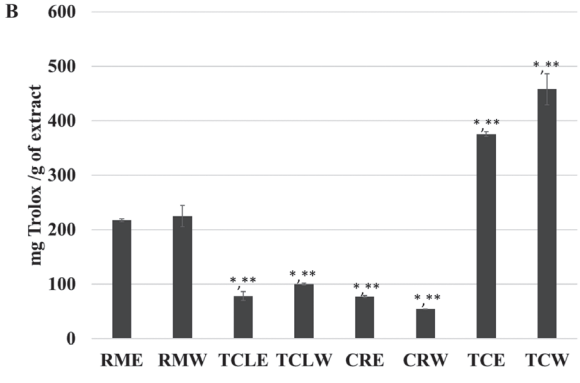


Figure 3 FRAP activities of the aqueous and the ethanol extracts of the remedy, TC, CR, and TCL when compared with $FeSO_4 \cdot 7H_2O$ (A) and Trolox (B).
* P-value < .05 vs RME,
** P-value < .05 vs RMW.

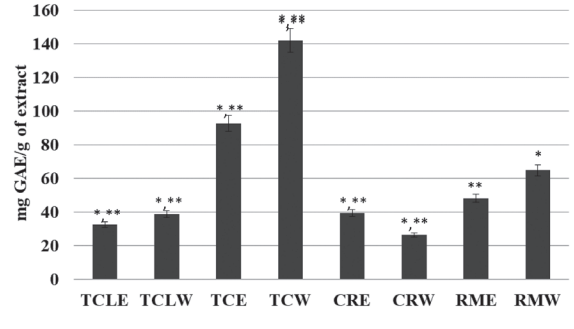


Figure 4 TPC of the aqueous and the ethanol extracts of the remedy, TC, CR, and TCL.
* P-value < .05 vs RME,
** P-value < .05 vs RMW.

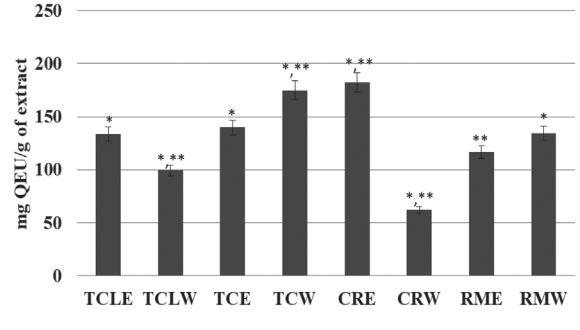


Figure 5 TFC of the aqueous and the ethanol extracts of the remedy, TC, CR, and TCL.
* P-value < .05 vs RME,
** P-value < .05 vs RMW.

Discussion

Oxidative stress can be explained as the imbalance between oxidants and antioxidants. Oxidative stress has been suggested to be the cause of aging and degenerative diseases. Antioxidants have already been found in plant materials and supplements. We first investigate the ethanolic and water extract of the remedy on antioxidant activities and capacities.

ABTS, FRAP, and DPPH are methods that measure the antioxidant activities. The results found that TCE and RME presented the most effective antioxidant by using DPPH assay. Previous studies showed that the polyphenol-rich extract of *T. chebula* fruits and 96% ethanolic extract of *T. crispa* exerted DPPH radical scavenging activity with EC_{50} of 14 µg/mL and 2.65 mg/mL, repectively.[6,7] For *C. rotundus,* methanolic extract inhibited of oxidation by determining the DPPH method in dose-dependence manner.[8] Furthermore, TCW showed the highest effective antioxidant by ABTS and FRAP determinations.

Phenolic and flavonoid compounds are the most common antioxidants in herbal plants. The antioxidant capacity was determined by TPC and TFC methods. TPC was inversely proportional to the EC_{50} value of the extract in the DPPH and ABTS assays, as evidence that an extract with high TPC was a better activity on scavenging the free radicals. Our results presented TCW and CRE had the highest antioxidant compound by measuring TFC and TPC, respectively. The results related to the previous studies which have reported that the extract with the highest TPC was also the best antioxidant activity. Several previous studies have reported the TPC and TFC of the plant utilized in this study. 70% aqueous ethanol extract of *T. chebula* had TPC value of 118.5 mg GAE/g of extract[14]. 70% ethanol and aqueous extracts of *C. rotundus* had TPC value of 57.383 ± 2.869 and 4.050 ± 0.202 µg GAE/mg extract, respectively and showed TFC value of 109.065 ± 5.453 and 26.822 µg QE/mg extract, respectively.[8] The aqueous *T. crispa* extract had TPC value of 213.16 ± 1.31 mg GAE/g dry stem weight and TFC value of 62.07 ± 39.76 mg QE/g dry stem weight.[15] Our results presented the ethanol and aqueous extracts of the remedy had TPC and TFC according with the previous studies.

In conclusions, the ethanol and aqueous extracts of the remedy had antioxidant activities and capacities. The remedy may be used for reducing oxidative stress relating with aging and degenerative stress. Moreover, the remedy could be used in a further study to investigated its anti-oxidant effect in an animal model and clinical trial.

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Conflict of Interest The authors have no conflict of interest to declare.

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