http://dx.doi.org/10.4314/ajtcam.v11i4.21

COMBINATIVE EFFECTS OF THANH HAO MIET GIAP THANG (SWEET WORMWOOD AND TORTOISE SHELL DECOCTION) INGREDIENTS ON ANTIOXIDATIVE ACTIVITY *IN VITRO*

My Nuong Nguyen, Thuy Vy Nguyen, Thuy Duong Ho-Huynh*

Department of Genetics, Faculty of Biology, University of Science, Ho Chi Minh city, Vietnam
*** E-mail:** hhtduong@hcmus.edu.vn

Abstract

Background: Traditional formulae usually exhibit therapeutic effects through the combinations of different ingredients. The purpose of this study was to investigate *in vitro* anti-oxidative activity of Thanh Hao Miet Giap Thang (THMGT) (Sweet Wormwood and Tortoise Shell Decoction) formula and the interactions of its ingredients leading to the overall anti-oxidative effect.
Materials and Methods: We prepared 31 combinations containing two to four of the five ingredients including Herba *Artemisia apiacea* L (HbA), Carapax Trionycis *(Tryonix sinensis)* (CT), Rhizoma Anemarrhenae *(Anemarrhena asphodeloides)* (RzA), Radix Rehmanniae *(Rehmannia glutinosa* Libosch) (RdR), Moutan Cortex (*Paeonia suffruticosa*) (MC). These combinations were tested for anti-oxidative activity using DCFH-DA and DPPH assays on Hep G2 cells. We also analyzed changes in expression of genes involved in antioxidant defense system including Nuclear Factor Erythroid-Derived 2-Like 2 (*NFE2L2*), catalase (*CAT*), heme oxygenase-1 (*HO-1)*, glutathione peroxidase (*GPx*), cytoplasmic superoxide dismutase (*SOD1*), mitochondrial superoxide dismutase (*SOD2*).
Results: The complete formula and all combinations containing Moutan Cortex showed high antioxidant activity in both radical solution-based chemical assay and cellular-based assay. On the contrary, Carapax Trionycis displayed inhibitory effect on the overall antioxidant activity when present in a combination, an effect clearly emphasized in cellular-based assay. Hep G2 cells treated with the formula showed increased gene expression of *HO-1* and *SOD2* while expression of *CAT, SOD1, GPx* was unchanged.
Conclusion: Our results suggested that THMGT had anti-oxidative activity essentially through intrinsic reducing capacities and the overall activity of the formula resulted from enhancing and inhibiting interactions of ingredients.

Key words: Thanh Hao Miet Giap Thang, Sweet Wormwood and Tortoise Shell Decoction, antioxidant, traditional formula

Abbreviations: THMGT, Thanh Hao Miet Giap Thang; HbA, Herba *Artemisia apiacea;* CT, Carapax Tryonicis; RzA, Rhizoma Anemarrhenae; MC, Moutan Cortex; RdR, Radix Rehmanniae; ROS, Reactive oxygen species; NFE2L2, Nuclear Factor Erythroid-Derived 2-Like 2; CAT, catalase; GPx, glutathione peroxidase; SOD1, cytoplasmic superoxide dismutase; SOD2, mitochondrial superoxide dismutase; HO-1, heme oxygenase-1.

Introduction

Reactive oxygen species (ROS) are the products of oxygen-consuming metabolic processes in normal cells. ROS participate actively in cell signaling and have critical role in proper functioning of cells throughout the body (D'Autréaux and Toledano, 2007). Nevertheless, an excess of ROS can cause various diseases including degenerative disorders and cancers (Alfadda and Sallam, 2012). Therefore, the use of natural compounds having anti-oxidative properties is an attractive approach to counteract the harmful effect of excessive intracellular ROS (Brewer, 2011). Various vegetables and medicinal herbs showed anti-oxidative activity (Chen et al., 2004). Plant extracts rich in polyphenols and flavonoids have strong reducing power and free radicals scavenging activity (Brewer, 2011). Other compounds exert their activity through activation of endogenous anti-oxidative enzymes such as glutathione peroxidase (GPx) or heme oxygenase-1 (HO-1). Herbal formulae are also recognized for antioxidant activities (Ko et al., 1995; Wang, 2012)

Thanh Hao Miet Giap Thang (THMGT) (Sweet Wormwood and Tortoise Shell decoction, Qing Hao Bie Jia Tang in Chinese) is an ancient Traditional Chinese Medicine formula, occasionally used in cancer supportive care in Vietnam. The formula includes five ingredients - Herba *Artemisia apiacea* L (HbA), Carapax Trionycis *(Tryonix sinensis)* (CT), Rhizoma Anemarrhenae *(Anemarrhena asphodeloides)* (RzA), Radix Rehmanniae *(Rehmannia glutinosa* Libosch) (RdR), Moutan Cortex (*Paeonia suffruticosa*) (MC). Antioxidant activity was reported for compounds isolated from Artemisia species (*A. annua, A. apiacea*) (Kim et al., 2003; Ferreira et al., 2010), MC (Matsuda et al., 2001; Chen et al., 2004; Yang et al., 2011; Furuya et al., 2012), RdR (Chen et al., 2004; Baek et al., 2012) and RzA (Chen et al., 2004; Chae et al., 2011). In multi-component traditional formulae, therapeutic effect is usually the results of complex interactions among components. In this study, we examined antioxidant activity of THMGT formula compared to the same activity of separate ingredients and different combinations of these ingredients in order to determine their possible interactions leading to antioxidant effect of the whole formula. Expression of some endogenous antioxidant genes was also analyzed to identify possible mechanism of action of the formula.

Materials and Methods

Preparation of THMGT and its ingredients

THMGT is composed of five ingredients in the following dosage: HbA (12 g), CT (12 g), RzA (20 g), RdR (16 g), MC (12 g). The decoction was routinely produced for clinical use in the Traditional Medicine Hospital HCMC. For research use, quantity of ingredients equivalent to ten times dosage was soaked in water for 30 minutes, boiled for 3 hours to obtain 720 ml aqueous extract and lyophilized to obtain dried powder. Combinations were prepared by mixing aqueous extracts of two, three, four ingredients at the same ratio as in the whole formula and subsequently lyophilized. Powders were prepared and kindly supplied by the Traditional Medicine Hospital HCMC. Before use, powders were re-dissolved in distilled water and 0.2 µm-filtered sterilized.

http://dx.doi.org/10.4314/ajtcam.v11i4.21

Cell lines and culture

Hep G2 cells (HB-8065) were purchased from The American Type Culture Collection (Manassas, Rockville). Cells were grown at 37°C and 5% CO_2, as monolayer cultures, in Eagle's Minimal Essential Medium supplemented with 10% FBS, 2 mM L-glutamine, 20 mM HEPES, 0.025 µg/ml amphotericine B, 100 IU/ml penicillin G, and 100 µg/ml streptomycin.

DPPH assay

The DPPH radical scavenging activity of extracts was determined as previously described (Herald et al., 2012). DPPH radical scavenging activity of samples was expressed as the scavenging percentage of free radicals after subtracting corresponding blanks and was determined using the following equation: (%) inhibition = 1 – [ODt/ODc] x 100 (%), where ODt and ODc are absorbance of test sample and control sample, respectively.

Intracellular ROS scavenging assay

The DCFH-DA method was used to determine the level of intracellular ROS (Cathcart et al., 1983) . Briefly, Hep G2 cells were seeded at 6 x 10^4 cells/well in 96-well plates. 24 hrs after seeding, cells were dark-incubated with DCFH-DA at concentration of 40 µM for 30 min and subsequently treated with extracts or quercetin as positive control. After an hour-treatment, extract-containing solution was removed and 600 µM AAPH was added. Fluorescent signals were collected every 10 min for one hour (excitation 485 nm, emission 538 nm). After subtracting the corresponding blanks and also the values measured at 0 min, intracellular ROS scavenging activity (%) was calculated as follows: (1-[Ft/Fc] x 100), where Ft and Fc are fluorescence values intensity of test samples and control sample, respectively.

Real-time RT-PCR

Total RNA was isolated from Hep G2 cells treated with different extract powders using Illustra RNAspin mini kit (GE Healthcare). Reverse transcription was performed using iScript reverse transcription supermix (Bio-rad) according to the manufacturer's instruction. Amplification of cDNA by real-time PCR was performed in 40 µl containing specific primers (Table 1), 1 µg cDNA, EvaGreen and master mix. PCR conditions were as follows: 95^0C for 10 min, 40 cycles of 95^oC at 30s, 59.5^oC at 30s, and 72oC at 30s. The relative changes in gene expression were analyzed by the ddCt 2(-Delta Delta C(T)) method with β-actin as internal control (Livak and Schmittgen, 2001).

Table 1: The primers used for Real-time RT-PCR

Gene	Sequences	Reference
NFE2L2	F: 5′-TTCAGCCAGCCCAGCACATC-3' R: 5′-CGTAGCCGAAGAAACCTCATTGTC-3′	(Garbin et al., 2009)
SOD1	F: 5'-AGGTCCTCACTTTAATCCTCTATCCA-3' R: 5'-ACCATCTTTGTCAGCAGTCACATT-3'	(Duarte et al., 2012)
SOD2	F: 5'-GGACACTTACAAATTGCTGCTTGT-3' R: 5'-AGTAAGCGTGCTCCCACACAT-3'	(Duarte et al., 2012)
CAT	F: 5'- ACTTTGAGGTCACACATGACATT -3' R: 5'- CTGAACCCGATTCTCCAGCA -3'	(Wang et al., 2012)
HO-1	F: 5'-CAGGAGCTGCTGACCCATGA-3' R: 5'-AGCAACTGTCGCCACCAGAA-3'	(Jian et al., 2011)
GPx	F: 5'-ACGATGTTGCCTGGAACTTT-3' R: 5'-TCGATGTCAATGGTCTGGAA-3'	(Walshe et al., 2007)
beta-actin	F: 5'- CCTGGCACCCAGCACAAT -3' R: 5'- GCCGATCCACACGGAGTACT -3'	(Mitsuhashi et al., 2008)

Each value represents mean ± SD, (n=3). Data denoted * ($p<0.05$) are significant increase compared to corresponded combinations without MC analysed by one-way ANOVA with Tukey's multiple comparison test. Data denoted $^+$ ($p<0.05$) are significant reduction compared to corresponded combinations without CT analysed by one-way ANOVA with Tukey's multiple comparison test. Numbering of combinations was used in the text"

Results

Antioxidant activities of THMGT and its ingredients on Hep G2 cells

Anti-oxidative activity of THMGT was determined by DPPH and DCFH-DA assays. The extracts of THMGT and its components were initially tested at 10% (v/v). Effective concentration for 50% scavenging (EC_{50}) of the extracts which had radical scavenging percentage greater than 50%, were determined (Shoemaker et al., 2005). In DPPH chemical assay, MC and HbA exhibited the highest free radical scavenging capacity followed by THMGT > RzA > RdR. CT had no effect (EC_{50} > 5.11 mg/ml). In DCFH-DA assay, MC expressed the highest antioxidant activity followed by HbA > RzA > THMGT. RdR (EC_{50} > 8.71 mg/ml) and CT (EC_{50} > 5.11 mg/ml) showed no antioxidative activity (Table 2).

Combinations of ingredients from THMGT exhibited antioxidant activity

To determine the role of each ingredient in THMGT antioxidant activity, a panel of twenty five combinations containing two to four ingredients was prepared and tested using DPPH and DCFH-DA assays on Hep G2 cells. THMGT was prepared at the concentration of EC_{50}. The concentration of each ingredient alone or mixed into combinations was similar to its concentration present in the whole formula at EC_{50}.

In DPPH assay, 15 combinations (2-16) containing MC have antioxidant activity exceeding 30 %, whereas all five ingredients (1, 17, 25, 29, 31) and 11 combinations (18-24, 26-28, and 30) free of MC expressed low or no antioxidant activity (< 30 %) (Table 3). Results obtained with DCFH-DA assay showed 13 combinations (2, 3, 5, 6, 8, 10, 12-16, 18, 22) having antioxidant activity >30 %. Eleven of these combinations contained MC.

http://dx.doi.org/10.4314/ajtcam.v11i4.21

All five ingredients and 13 other combinations; (4, 7, 9, 11, 19, 20, 21, 23, 24, 26-28, 30) exhibited low or no antioxidant activity (< 30 %). Interestingly, comparison between 9 pairs of combinations with/without CT (2/7, 3/9, 5/11, 6/12, 8/14, 13/16, 18/21, 27/28, 31/30) showed statistically significant higher antioxidant activity of combinations free of CT than those containing CT. Taken together, combinations containing MC and free of CT showed high antioxidant activity concordantly detected by both assays. Combinations containing MC + CT exhibited high antioxidant activity revealed by DPPH assay which showed low activity with DCFH-DA assay. In fact, the inhibitory effect of CT was mildly detected in chemical assay (DPPH) but greatly emphasized in cellular system (DCFH-DA). Combinations free of MC exhibited no or low antioxidant activity with both assays, except two combinations (18, 22) containing RzA, RdR and HbA that expressed moderate activity.

Table 2: Antioxidant activities of THMGT and its ingredients assessed by DPPH and DCFH-DA assays

Samples	EC_{50}(mg/ml)	
	DPPH	DCFH-DA
THMGT	0,17 ± 0,02[b]	4,08 ± 0,30[d]
HbA	0,04 ± 0,00[a]	1,32 ± 0,15[b]
RzA	0,34 ± 0,03[d]	2,99 ± 0,42[c]
RdR	1,26 ± 0,08[c]	>8,71(~10%)
MC	0,01 ± 0,01[a]	0,20 ± 0,01[a]
CT	> 5,11 (~10%)	> 5,11(~10%)

Each value represents mean ± SD (n = 3). Data denoted [a, b, c, d] ($p<0.05$) are significant compared to other ingredients analyzed by one-way ANOVA with Tukey's Multiple comparison test.

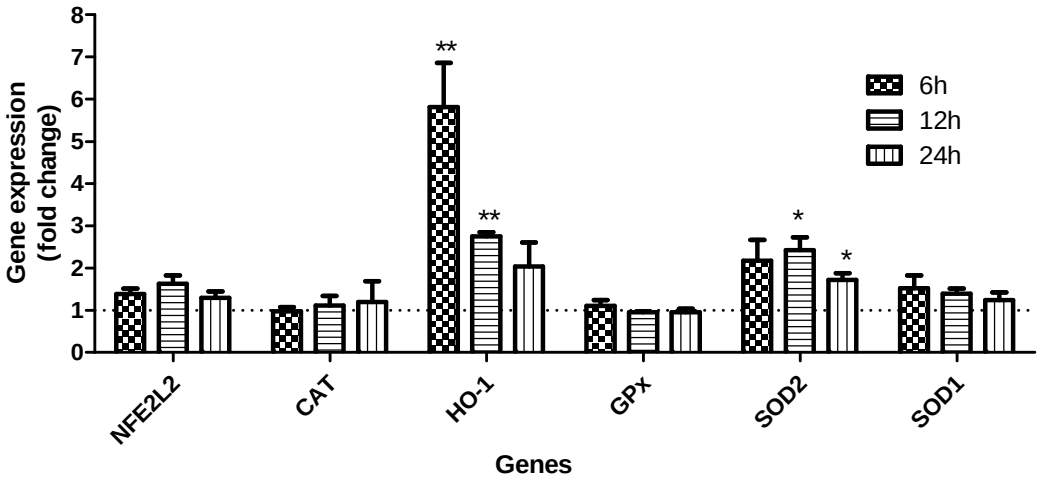

Figure 1: Hep G2 cells were treated with 4 mg/ml THMGT or H2O for 6, 12 and 24 h, then total RNA **was** isolated. Gene expression of NFE2L2, CAT, HO-1, GPx, SOD1 and SOD2 **was** determined by real-time RT-PCR. Data **were** presented as mean values of fold change for antioxidant genes mRNA levels in THMGT-treated Hep G2 cells relative to those of control cells. Both control and treatment values were normalized to the internal control gene beta-actin. Data **were** an average of three independent experiments. Error bars (SD) **were** shown. The statistical differences between the treatment and control were analyzed by two-tailed paired Student's t-tests (*$p<0.05$; **$p<0.01$).

THMGT induced *HO-1* and *SOD2* expression in Hep G2 cells.

We analyzed changes in expression of some genes involved in antioxidant defense system including *NFE2L2), CAT), HO-1, GPx, SOD1, SOD2*. Hep G2 cells treated with THMGT showed significant increase of *HO-1* gene expression beginning at 6 hours after induction, while *SOD2* increase mRNA level at 12 and 24 hours after treatment (Figure 1.). Nrf2 and other endogenous antioxidant enzymes such as *CAT, GPx,* and *SOD1* showed no significant changes in mRNA levels.

Discussion

THMGT and some of the ingredients showed antioxidant activity at various levels. High antioxidant activity of MC observed in this study was in agreement with previous reports (Matsuda et al., 2001; Chen et al., 2004; Rho et al., 2005; Yang et al., 2011; Furuya et al., 2012) . HbA, RzA and RdR are also recognized for their antioxidant activity (Kim et al., 2003; Chen et al., 2004; Chae et al., 2011; Baek et al., 2012). We obtained concordant results with both assays DPPH and DCFH-DA for MC, HbA and CT. In DCFH-DA assay, THMGT exhibited lower capacity than RzA compared to results obtained with DPPH assay. DCFH-DA assay not only reflects the chemical free radical scavenging capacity of antioxidants, it

http://dx.doi.org/10.4314/ajtcam.v11i4.21
also takes into account the uptake and metabolism of compounds within the cell (Badarinath et al., 2010; Liu and Finley, 2005). Thus, difference in antioxidant activity of THMGT observed between the two assays could be a result of low permeability and/or complex interactions among metabolized compounds of THMGT inside the cell that negatively affects the overall intracellular antioxidant effect, as previously reported with other natural compounds (Palafox-Carlos et al., 2012; Wang, 2012).

Table 3: Effects of THMGT on expression of several antioxidant genes.

Combinations		Antioxidant activity (%)	
		DPPH assay	DCFH-DA assay
1	MC	24.63 ± 3.86	14.49 ± 3.04
2	MC-RzA	45.89 ± 7.89*	36.20 ± 2.74*
3	MC-HbA	45.06 ± 6.11*	54.53 ± 3.17*
4	MC-CT	31.06 ± 4.27^{+}	7.69 ± 0.67^{+}
5	MC-RdR	33.95 ± 4.01	41.26 ± 1.45*
6	MC-RzA-HbA	52.00 ± 4.94*	52.99 ± 3.99*
7	MC-RzA-CT	46.41 ± 6.15*	14.51 ± 1.80*$^{+}$
8	MC-RzA-RdR	46.78 ± 10.54*	45.69 ± 8.43*
9	MC-HbA-CT	37.08 ± 7.67*	19.35 ± 3.52^{+}
10	MC-HbA-RdR	47.43 ± 2.39*	50.94 ± 4.66*
11	MC-CT-RdR	35.05 ± 2.25*	13.45 ± 3.26^{+}
12	MC-RzA-HbA-CT	45.92 ± 11.16*	38.93 ± 1.76*$^{+}$
13	MC-RzA-HbA-RdR	57.33 ± 13.45*	62.20 ± 2.85*
14	MC-RzA-CT-RdR	45.15 ± 7.10*	31.07 ± 1.14*$^{+}$
15	MC-HbA-CT-RdR	43.61 ± 4.12*	38.15 ± 4.48*
16	THMGT	46.77 ± 1.49	39.67 ± 6.06*$^{+}$
17	RzA	7.73 ± 2.97	2.67 ± 3.95
18	RzA-HbA	23.69 ± 2.75	30.88 ± 0.37
19	RzA-CT	6.63 ± 3.68	-0.47 ± 2.08
20	RzA-RdR	5.25 ± 6.70	-15.12 ± 2.65
21	RzA-HbA-CT	20.88 ± 2.24	-0.06 ± 4.23^{+}
22	RzA-HbA-RdR	29.84 ± 7.20	33.75 ± 2.39
23	RzA-CT-RdR	12.36 ± 3.49	14.05 ± 5.64^{+}
24	RzA-HbA-CT-RdR	21.44 ± 4.54	24.39 ± 4.32
25	HbA	17.61 ± 4.68	16.98 ± 7.41
26	HbA-CT	12.87 ± 2.24	14.07 ± 3.66
27	HbA-RdR	15.34 ± 2.67	26.35 ± 2.96
28	HbA-CT-RdR	13.26 ± 2.20	11.38 ±7.03^{+}
29	CT	-4.98 ± 6.71	-7.06 ± 4.03
30	CT-RdR	-0.44 ± 4.25	4.69 ± 4.28^{+}
31	RdR	4.03 ± 4.21	19.58 ± 5.52

In a multi-component formula, curative effects result from interactions among different components (Rong et al., 2008; Wang et al., 2014). We generated a panel of all possible combinations of ingredients from THMGT and tested their anti-oxidative capacity in order to determine possible combinative effects of components on the overall activity of the whole formula. A good correlation was observed for results obtained by DPPH and DCFH-DA assays. Combinations containing MC exhibited high radical scavenging capacity whereas those free of MC showed no or low antioxidant activity in both assays. The high activity observed cannot be totally attributed to MC since MC alone exhibited moderate antioxidant activity. Thus, combinative effects among MC and the other ingredients could lead to enhanced activity, as previously described for other herb combinations (Prakash et al., 2009; Ferreira et al., 2010; Palafox-Carlos et al., 2012). Concordant high antioxidant activity observed in both chemical and cell-based assays suggested free radicals scavenging as the main mechanism of action for the anti-oxidative capacity of some combinations containing MC. On the other hand, differences in antioxidant activity of other combinations were observed between chemical and cell-based assay, especially in

http://dx.doi.org/10.4314/ajtcam.v11i4.21
combinations containing CT. In fact, the addition of CT into a combination exerted a significant inhibitory effect clearly emphasized in cell based assay. These results suggested interactions between cellular metabolized compounds of CT with the other components that decreased the intrinsic antioxidant activity of the combination and/or negatively regulated antioxidant enzymes. Antagonism between molecules present in combinations of natural compounds were previously reported (Palafox-Carlos et al., 2012).

Nrf2 antioxidant response pathway is considered as the first line of cellular defense against harmful effects of oxidative stress (Jeong et al., 2005; Almazari et al., 2012). *NFE2L2* expression is induced by natural chemo-preventive compounds (Jeong et al., 2005; Almazari et al., 2012). Induction of NFE2L2 gene expression and activation of Nrf2 protein induce expression of antioxidant enzymes including CAT, SOD, GPx, HO-1 (Zhu et al., 2005). In this study, when expression level of several genes involved in antioxidant response system was determined, no gene expression change was observed in Hep G2 cells treated with THMGT for *NFE2L2*, *CAT*, *SOD1*, and *GPx; HO-1* expression, and to a lesser extent, *SOD2* increased under THMGT treatment. Since butanol extract from *Artemisia apiacea* has been shown to induce rat *SOD*, *CAT* and *GPx* (Kim et al., 2003); the absence of gene induction in this study could result from water low solubility of substances such as artemisinin (Wright et al., 2010). Natural compounds and herbal formula can induce *HO-1* expression (Motterlini et al., 2000; Rong et al., 2008) . *HO-1* expression is induced through Nrf2 binding to ARE (antioxidant response element) of *HO-1* gene promoter region (Jeong et al., 2005; Liu et al., 2007; Almazari et al., 2012). In this study, *HO-1* gene expression was upregulated four to six fold by THMGT without any detectable induction of *NFE2L2* gene. These results suggested that: (1) THMGT exerted its effects through Nrf2 protein activation or stabilization rather than transcriptional induction as reported for carnosol that increased nuclear Nrf2 protein levels (Martin et al., 2004), (2) there was a non-linear correlation between *NFE2L2* gene induction and the induction of Nrf2-targeted antioxidant genes by THMGT, as previously recorded (Jeong et al., 2007), (3) THMGT activated the PI3K survival signaling pathway leading to the activation of HO-1 protein (Martin et al., 2004; Almazari et al., 2012). Therefore, the increased gene expression of *HO-1* under THMGT treatment could be a tentative response of tumor cells to cytotoxic effect of the formula and was not involved in anti-oxidative defense system. *SOD2*, a mitochondrial superoxide dismutase, is essential for life and is highly regulated at multiple levels (Borrelli et al., 2014). Some natural compounds and their derivatives upregulate gene expression of *SOD2* (Priego et al., 2008). Since cancer cells produced more superoxide anions, the concentration of ROS, H_2O_2 in particular, is higher than in normal cells. Results from this study suggested that an increase of *SOD2* expression, together with an unchanged expression of *CAT* and *GPx*, leading to accumulation of hydrogen peroxide. Thus, anti-proliferation activity of THMGT (data not shown) could be induced by increasing intracellular H_2O_2 concentration (Priego et al., 2008).

This study reported that THMGT exerted anti-oxidative activity mainly through free radical scavenging capacity. The overall effect of THMGT resulted from enhancing interactions among MC and other ingredients and inhibitory effect of CT when combined with other ingredients.

Acknowledgment

This work was supported by grant from Vietnam National University of Ho Chi Minh City (B2011-18-08TD).

References

1. Alfadda, A. A., and Sallam, R.M. (2012). Reactive oxygen species in health and disease. J. Biomed. Biotechnol., 2012.
2. Almazari, I., Park, J.-M., Park, S.-A., Suh, J.-Y., Na, H.-K., Cha, Y.-N., and Surh, Y.-J. (2012). Guggulsterone induces heme oxygenase-1 expression through activation of Nrf2 in human mammary epithelial cells: PTEN as a putative target. Carcinogen, 33: 368–376.
3. Badarinath, A. V., Rao, K.M., Chetty, C.M.S., Ramkanth, S., Rajan, T.V.S., and Gnanaprakash, K. (2010). A Review on *In-vitro* Antioxidant Methods : Comparisions, Correlations and Considerations. Int. J. Pharmatech Res., 2: 1276–1285.
4. Baek, G.-H., Jang, Y.-S., Jeong, S.-I., Cha, J., Joo, M., Shin, S.-W., Ha, K.-T., and Jeong, H.-S. (2012). Rehmannia glutinosa suppresses inflammatory responses elicited by advanced glycation end products. Inflammation, 35: 1232–1241.
5. Borrelli, A., Schiattarella, A., Bonelli, P., Tuccillo, F.M., Buonaguro, F.M., and Mancini, A. (2014). The Functional Role of MnSOD as a Biomarker of Human Diseases and Therapeutic Potential of a New Isoform of a Human Recombinant MnSOD. Biomed Res. Int., 2014: 476789.
6. Brewer, M.S. (2011). Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. Compr. Rev. Food Sci. Food Saf., 10: 221–247.
7. Cathcart, R., Schwiers, E., and Ames, B.N. (1983). Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Anal. Biochem., 134: 111–116.
8. Chae, S., Piao, M.J., Kang, K.A., Zhang, R., Kim, K.C., Youn, U.J., Nam, K.-W., Lee, J.H., and Hyun, J.W. (2011). Inhibition of Matrix Metalloproteinase-1 Induced by Oxidative Stress in Human Keratinocytes by Mangiferin Isolated from Anemarrhena asphodeloides. Biosci. Biotechnol. Biochem., 75: 2321–2325.
9. Chen, I., Chang, H., Yang, H., and Chen, G. (2004). Evaluation of Total Antioxidant Activity of Several Popular Vegetables and Chinese Herbs : A Fast Approach with ABTS / H 2 O 2 / HRP System in Microplates. J. Food Drug Anal., 12: 29–33.
10. D'Autréaux, B., and Toledano, M.B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat. Rev. Mol. Cell Biol., 8: 813–824.
11. Duarte, P.M., Napimoga, M.H., Fagnani, E.C., Santos, V.R., Bastos, M.F., Ribeiro, F. V, Araújo, V.C., and Demasi, A.P.D. (2012). The expression of antioxidant enzymes in the gingivae of type 2 diabetics with chronic periodontitis. Arch. Oral Biol., 57: 161–168.
12. Ferreira, J.F.S., Luthria, D.L., Sasaki, T., and Heyerick, A. (2010). Flavonoids from Artemisia annua L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. Mol. 15: 3135–3170.
13. Furuya, R., Hu, H., Zhang, Z., and Shigemori, H. (2012). Suffruyabiosides A and B, two new monoterpene diglycosides from moutan cortex. Mol. 17: 4915–4923.
14. Garbin, U., Fratta Pasini, A., Stranieri, C., Cominacini, M., Pasini, A., Manfro, S., Lugoboni, F., Mozzini, C., Guidi, G., Faccini, G., et al. (2009). Cigarette smoking blocks the protective expression of Nrf2/ARE pathway in peripheral mononuclear cells of young heavy smokers favouring inflammation. PLoS One, 4: e8225.
15. Herald, T.J., Gadgil, P., and Tilley, M. (2012). High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. J. Sci. Food Agric., 92: 2326–2331.

http://dx.doi.org/10.4314/ajtcam.v11i4.21
16. Jeong, J.-M., Choi, C.-H., Kang, S.-K., Lee, I.-H., Lee, J.-Y., and Jung, H. (2007). Antioxidant and chemosensitizing effects of flavonoids with hydroxy and/or methoxy groups and structure-activity relationship. J. Pharm. Pharm. Sci., 10: 537–546.
17. Jeong, W.-S., Keum, Y.-S., Chen, C., Jain, M.R., Shen, G., Kim, J.-H., Li, W., and Kong, A.-N.T. (2005). Differential expression and stability of endogenous nuclear factor E2-related factor 2 (Nrf2) by natural chemopreventive compounds in HepG2 human hepatoma cells. J. Biochem. Mol. Biol., 38: 167–176.
18. Jian, Z., Li, K., Liu, L., Zhang, Y., Zhou, Z., Li, C., and Gao, T. (2011). Heme oxygenase-1 protects human melanocytes from H2O2-induced oxidative stress via the Nrf2-ARE pathway. J. Invest. Dermatol., 131: 1420–1427.
19. Kim, K.S., Lee, S., Lee, Y.S., Jung, S.H., Park, Y., Shin, K.H., and Kim, B.-K. (2003). Anti-oxidant activities of the extracts from the herbs of Artemisia apiacea. J. Ethnopharmacol., 85: 69–72.
20. Ko, K.M., Yick, P.K., Poon, M.K.T., Chet, C.T., Ng, K.H., and Kong, Y.C. (1995). Schisandra Chinensis-derived antioxidant activities in “Sheng Mai San” a compound formulation,in vivo andin vitro. Phyther. Res., 9: 203–206.
21. Liu, R.H., and Finley, J. (2005). Potential cell culture models for antioxidant research. J. Agric. Food Chem., 53: 4311–4314.
22. Liu, X., Peyton, K.J., Ensenat, D., Wang, H., Hannink, M., Alam, J., and Durante, W. (2007). Nitric oxide stimulates heme oxygenase-1 gene transcription via the Nrf2/ARE complex to promote vascular smooth muscle cell survival. Cardiovasc. Res., 75: 381–389.
23. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25: 402–408.
24. Martin, D., Rojo, A.I., Salinas, M., Diaz, R., Gallardo, G., Alam, J., De Galarreta, C.M.R., and Cuadrado, A. (2004). Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J. Biol. Chem., 279: 8919–8929.
25. Matsuda, H., Ohta, T., Kawaguchi, A., and Yoshikawa, M. (2001). Bioactive Constituents of Chinese Natural Medicines. VI. Moutan Cortex. (2): Structrues and Radical Scavenging Effects of Suffruticosides A, B, C, D, and E, and E and Galloyl-oxypaeoniflorin. Chem. Pharm. Bull. (Tokyo)., 49: 69–72.
26. Mitsuhashi, M., Ogura, M., Endo, K., Obara, K., Izutsu, H., Targan, S.R., Maemura, M., Tachikawa, D., and Shinagawa, A. (2008). Ex vivo induction of mRNA in human whole blood as a new platform of drug and dietary supplement development. Pharm. Res., 25: 1116–1124.
27. Motterlini, R., Foresti, R., Bassi, R., and Green, C.J. (2000). Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. Free Radic. Biol. Med., 28: 1303–1312.
28. Palafox-Carlos, H., Gil-Chávez, J., Sotelo-Mundo, R.R., Namiesnik, J., Gorinstein, S., and González-Aguilar, G. a (2012). Antioxidant interactions between major phenolic compounds found in “Ataulfo” mango pulp: chlorogenic, gallic, protocatechuic and vanillic acids. Mol., 17: 12657–12664.
29. Prakash, O., Singh, G.N., Singh, R.M., Madan, S., and Mathur, S.C. (2009). Interactions of herbal extract combinations against free radical scavenging activity. Pharm. Biol., 47: 729–733.
30. Priego, S., Feddi, F., Ferrer, P., Mena, S., Benlloch, M., Ortega, A., Carretero, J., Obrador, E., Asensi, M., and Estrela, J.M. (2008). Natural polyphenols facilitate elimination of HT-29 colorectal cancer xenografts by chemoradiotherapy: a Bcl-2- and superoxide dismutase 2-dependent mechanism. Mol. Cancer Ther., 7: 3330–3342.
31. Rho, S., Chung, H.-S., Kang, M., Lee, E., Cho, C., Kim, H., Park, S., Kim, H.-Y., Hong, M., Shin, M., et al. (2005). Inhibition of production of reactive oxygen species and gene expression profile by treatment of ethanol extract of Moutan Cortex Radicis in oxidative stressed PC12 cells. Biol. Pharm. Bull., 28: 661–666.
32. Rong, J., Cheung, C.Y.-H., Lau, A.S.-Y., Shen, J., Tam, P.K.-H., and Cheng, Y.-C. (2008). Induction of heme oxygenase-1 by traditional Chinese medicine formulation ISF-1 and its ingredients as a cytoprotective mechanism against oxidative stress. Int. J. Mol. Med., 21: 405–411.
33. Shoemaker, M., Hamilton, B., Dairkee, S.H., Cohen, I., and Campbell, M.J. (2005). In vitro anticancer activity of twelve Chinese medicinal herbs. Phytother. Res., 19: 649–651.
34. Walshe, J., Serewko-Auret, M.M., Teakle, N., Cameron, S., Minto, K., Smith, L., Burcham, P.C., Russell, T., Strutton, G., Griffin, A., et al. (2007). Inactivation of glutathione peroxidase activity contributes to UV-induced squamous cell carcinoma formation. Cancer Res., 67: 4751–4758.
35. Wang, X.-H. (2012). Free radical scavenging activity of Chinese traditional four substances decoction (FSD) extracts. African J. Pharm. Pharmacol., 6: 3113–3117.
36. Wang, F., Zhao, S., Li, F., Zhang, B., Qu, Y., Sun, T., Luo, T., and Li, D. (2014). Investigation of Antioxidant Interactions between Radix Astragali and Cimicifuga foetida and Identification of Synergistic Antioxidant Compounds. PLoS One, 9: e87221.
37. Wang, Y., Wei, Y., Zhang, H., Shi, Y., Li, Y., and Li, R. (2012). Arsenic trioxide induces apoptosis of p53 null osteosarcoma MG63 cells through the inhibition of catalase. Med. Oncol., 29: 1328–1334.
38. Wright, C.W., Linley, P. a, Brun, R., Wittlin, S., and Hsu, E. (2010). Ancient Chinese methods are remarkably effective for the preparation of artemisinin-rich extracts of Qing Hao with potent antimalarial activity. Mol., 15: 804–812.
39. Yang, J., Kim, J., Sa, Y.J., Kim, M.O., Jeong, H.J., Yeon, C., and Kim, M.J. (2011). Antioxidant, antibacterial and a-glucosidase inhibitory activities of different extracts of Cortex Moutan. African J. Biotechnol., 10: 9438–9444.
40. Zhu, H., Itoh, K., Yamamoto, M., Zweier, J.L., and Li, Y. (2005). Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. FEBS Lett., 579: 3029–3036.