Influence of Extraction Method on Antioxidant and Nitric Oxide-Stimulating Activity of Herbal Mixtures in Human Endothelial Cells

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ABSTRACT

Nitric oxide (NO) plays an important role as main vasodilator. Aging and oxidative stress are the risk factors on reduction of NO bioavailability. These lead to endothelial dysfunction which is an early marker for atherosclerosis. In this study, three herbal mixtures from *Moringa oleifera, Butea superba* and *Kaempferia parviflora* were designed, namely CF-1, CF-2 and CF-3, and extracted by maceration and reflux extraction method. The biological activities were used as parameters for comparison of extraction techniques. These extracts were determined antioxidant activity by using DPPH radical scavenging assay and TNF-α-induced intracellular ROS generation in human endothelial cells. NO-producing activity of the herbal mixtures were also investigated in endothelial cells. The DPPH radical scavenging activity of herbal mixtures was significantly greater for maceration in comparison to reflux extraction. Antioxidant and NO-producing activity in endothelial cells of extracts prepared by maceration was similar to that of refluxing. Thus the maceration might be the suitable extraction technique for bringing out the potential of herbal mixture on improvement of endothelial function and preliminary preventing atherosclerosis.

Keywords: Antioxidant activity, Nitric oxide, Maceration, Reflux, Herbal mixtures.

INTRODUCTION

Erectile dysfunction (ED) is a critical health problem for million men across the world (Solomon et al., 2013). The incidence of ED on Thai men increases with age (Kongkanand et al., 2011). One of sharing risk factors of ED patients is atherosclerosis that correlated with impairment of nitric oxide (NO) bioavailability (Gandaglia et al., 2014). NO generated from endothelial nitric oxide synthase (eNOS) is a hallmark of endothelial function (Hedlund et al., 2000). Decreasing NO bioavailability contributes to endothelial dysfunction which is initial step in the development of atherosclerosis (Toda, 2012). Besides decreasing of NO bioavailability, oxidative stress is also involved in pathogenic atherosclerosis and cardiovascular diseases by directly reduces NO bioavailability (Cai & Harrison, 2000). Thus, increasing NO bioavailability and attenuating oxidative stress might reduce the incidence of atherosclerosis.

Over several years, using herbal supplements as an alternative medicine has become more popular. These bring about investigation of efficacy of herbal mixture as supplement. Thus we look for natural products from Thai herbal plants. Tubers of *Butea superba* Roxb. (Fabaceae family) was traditionally used for rejuvenation and treating ED (Tocharus et al., 2006). It has been reported to exert antioxidant, antibacterial, estrogenic and aphrodisiac activities (Vijayan et al., 2016). Next is *Moringa oleifera* belonging to the Moringaceae family. Leaves of this plant are reported as a rich source of β-carotene, protein, vitamin C, calcium, potassium, natural antioxidants, anti-pyretic, anticancer and antimicrobial agents (Anwar et al., 2007). The last is *Kaempferia parviflora* (Zingiberaceae family). Its rhizome has been used for treatment of ED, enhances male sexual function (Kotta et al., 2013), and possesses antioxidant and anti-inflammatory activity (Matsushita et al., 2015). Moreover, the investigation of medicinal plants has been begun with the extraction process, which is a crucial step in the screening process of the bioactive molecule from plant extracts (Azwanida, 2015). The different extraction methods affect the biological activities of plant extracts (Mutalib, 2014; Dhanani et al., 2017). Therefore, in the present study, three herbal mixtures of *B. superba*, *M. oleifera* and *K. parviflora* were designed and comparatively investigated their antioxidant and NO-increasing activity in endothelial cells. Also, the optimized condition in terms of two extraction techniques that effect on the quality of herbal mixture was investigated.

METHODOLOGY

Materials

Fetal bovine serum (FBS), penicillin-streptomycin and Dulbecco's modified Eagle's medium (DMEM) were bought from Gibco-Invitrogen (Grans Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), resveratrol, sodium nitrite, N-(1-naphthyl)-ethylene diamine dihydrochloride, tumor necrosis factor-α (TNF-α) and 2',7′-dichlorofluorescin diacetate (H_2DCFDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gallic acid and phosphoric acid were purchased from Merck (Kenilworth, NJ, USA). Sulfanilamide was obtained from Panreac (Barcelona, Spain).

Preparation of plant extracts

Leaves of *M. oleifera* were obtained from Chonburi Province. Rhizomes of *K. parviflora* and tubers of *B. superba* were perchased from local herb market at Pectchabun and Pathumthani Province, respectively. The plant samples were washed with tap water, chopped to small pieces, dried at 50°C and ground to powder. Herbal mixture was done by three forms. The first mixture was *M. oleifera* leaves and *K. parviflora* rhizomes (CF-1). The second, powder of *M. oleifera* leaves mixed with *B. superba* tubers (CF-2). And last is *K. parviflora* rhizomes which mixed with *B. superba* tubers (CF-3). These three mixtures were prepared by 1:1 ratio of plant powder. Then they were formulated together by two different extraction methods, maceration and reflux extraction method.

Maceration method

The plant powders were packed in a fabric filter, soaked in 95% ethanol for seven days and swirled twice a day. Ratio of plant mixture per solvent was 1:10 (g per mL). The extracts were filtered using Whatman No.1 filter. After that the plant residues were re-extracted again once. Then, the extracts were concentrated using a rotary evaporator. At last the crude extracts were stored at -20 °C.

Reflux extraction method

Reflux extraction method was used the same ratio of plant mixture and solvent as maceration method. These mixtures were mixed in round bottom flask and reflux at 100 °C for 6 h. After that, the extracts were filtered using Whatman No.1 filter and concentrated using a rotary evaporator and kept at -20 °C.

DPPH radical scavenging activity

DPPH radical scavenging method was used to evaluate *in vitro* antioxidant activity and performed as described by Srisook et al. (2012). Concisely, 50 µL of sample solution (concentration of 1,000, 500, 250, 125 and 62.5 µg/mL) was mixed with 100 µl of 0.2 mM DPPH solution dissolved in methanol. The reaction was performed in 96-well microplate and incubated in the dark for 30 min. The absorbance was measured at 517 nm by microplate reader (Versamax, Molecular Devices, USA.). Gallic acid was used as positive control.

Cell culture

Human umbilical vein endothelial cells (EA.hy926 cell line) was obtained from American Type Culture Collection (ATCC). EA.hy926 cells were cultured in DMEM containing 10% (v/v) FBS, penicillin (100 Units/mL) and streptomycin (100 µg/mL) and incubated at 37 °C in CO_2 incubator (5% CO_2 (v/v) in air). Cells were subcultured by trypsinization.

Measurement of intracellular reactive oxygen species (ROS) production by cell-based DCF assay

Generation of intracellular ROS was detected by H_2DCFDA staining probe as the method of Bandar et al. (2013). Briefly, EA.hy926 cells were subcultured into 6-well plate with a number of 3×10^5 cells per well. Cells were pre-treated with serum-free DMEM containing the plant extracts at 100 µg/mL for 1 h. Then cells were treated with TNF-α (10 ng/mL) for 2 h. Next cells were rinsed with warm PBS buffer before incubation with 50 µM of H_2DCFDA for 30 min in the dark. After incubation, the cells were washed with PBS and scraped in PBS on ice. Fluorescent intensity was measured by fluorescence spectrophotometer (Cary Eclipse, Agilent, USA.). Oxidized-fluorescent DCF is excited at wavelength of 485 nm and emitted at 521 nm. Gallic acid was used as positive control.

Determination of NO production by Griess reaction

Nitrite is an oxidation product of NO radical. Cells were subcultured and seeded into 24-well plate with a number of 1×10^5 cells per well. Cells were treated

with the plant extracts at 100 µg/mL for 24 h. Amount of nitrite in the culture media was determined by Griess reaction (Srisook et al., 2012). Nitrite concentration was calculated from standard curve of sodium nitrite. Resveratrol was used as positive control.

Statistical analysis

The data is expressed as mean ± SD of at least three experiments. Statistical significance was tested by using one-way analysis of variance (ANOVA) of Minitab 16, follow by Tukey test for multiple comparison. A value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

In this study, three new herbal mixtures (CF-1, CF-2 and CF-3) were designed. The two extraction methods were used to evaluate the antioxidant and NO-stimulating activity of the herbal mixtures. Maceration and reflux extraction methods were performed at the same ratio of plant mixture and solvent (1:10) while extraction times were different. The result shown in Table 1, the extraction yields between maceration and reflux extraction of CF-1 and CF-3 mixture were obtained in almost the same amounts. This means the reflux extraction method, in this case, can reduce extraction time from fourteen days to one day. However extraction yield by maceration was higher than that by reflux method in case of the CF-2 mixture.

Antioxidant activity of the herbal mixtures was determined by two test systems, *in vitro* DPPH radical scavenging and cell-based DCF assay. The DPPH is a stable radical. DPPH assay is widely used method to screen free radical scavenging activity of plant antioxidants. It is based on a single electron transfer reaction (Apak et al., 2013). The herbal mixtures obtained by maceration extraction had the lower EC_{50} value, meaning that it demonstrated the superior antioxidant activity. Among extracts obtained from maceration method, CF-1 showed the greatest DPPH activity and subordinated with CF-2 and CF-3, respectively. In reflux, the activity of CF-1 and CF-2 was comparable, however, greater than CF-3 (Table 1). Gallic acid was used as a positive control with an EC_{50} value of 6.47±0.22 µg/mL. The higher DPPH radical scavenging activity was obtained from herbal mixtures prepared by maceration compared to refluxing. According to our results, heating treatment in the reflux method could decompose antioxidant compounds showing DPPH radical scavenging activity. This result concurs with the data from Sultana et al. (2009) which reported that samples obtained by the maceration method exhibited the higher DPPH radical scavenging activity than those extracted by the reflux method. However, the data from our study is disagreement with the results of other studies in which the plant material extracts prepared by the reflux method showed the higher DPPH radical scavenging activity than those extracted by the maceration method (Phongtongpasuk and Poadang, 2014; Stanojevic et al., 2016).

Apart from DPPH scavenging activity, antioxidant activity was also determined by cell-based DCF assay using H_2DCFDA, which is hydrolyzed by intracellular esterases to form $DCFH_2$. It is oxidized by Fenton reaction-generated ROS and forms the highly fluorescent dichlorofluorescein (DCF) (Cohn et al., 2008).

ROS in endothelial cells mainly generated by NADPH oxidase (Frey et al., 2009), enzyme catalyzed the production of superoxide which rapidly reacts with NO to form peroxynitrite leading to decrease in NO bioavailability cells. Therefore, the herbal mixtures were evaluated their effect on the level of ROS in endothelial cells. Intracellular ROS generation was obviously increased upon TNF-α exposure for 2 h. ROS generation in cells pre-treated with all extracts was significantly decreased compared to TNF-α-stimulation (Figure 1). Change between two extraction methods did not affect ROS scavenging activity of CF-1 and CF-2. However, CF-3 prepared by refluxing showed significant greater ROS scavenging activity than that of maceration method. This might be attributed to an extraction under the reflux condition resulting in the change of components with scavenging effect of ROS in cells treated with TNF-α. The obtained data indicated that scavenging activity of plant extract is depended on specific phytochemical constituents, which can influence their antioxidant effect (Sittisart, & Chitsomboon, 2014).

Table 1. Extraction yield and DPPH radical scavenging activity (EC_{50}) of herbal mixtures

Herbal mixture	%Yield		EC_{50} (µg/mL)	
	Maceration	**Reflux**	**Maceration**	**Reflux**
CF-1	12.80	11.59	561.59 ± 25.04^{Aa}	902.85 ± 0.05^{Ab}
CF-2	12.37	8.83	653.64 ± 10.47^{Ba}	800.88 ± 0.06^{Ab}
CF-3	8.33	7.73	711.51 ± 17.97^{Ca}	1020.00 ± 0.03^{Bb}
Gallic acid	-		6.47±0.22	

* A, B and C shows significantly differences among herbal mixtures prepared with the same extraction condition.
* a and b: shows significantly differences between the herbal mixtures prepared with the different extraction condition.

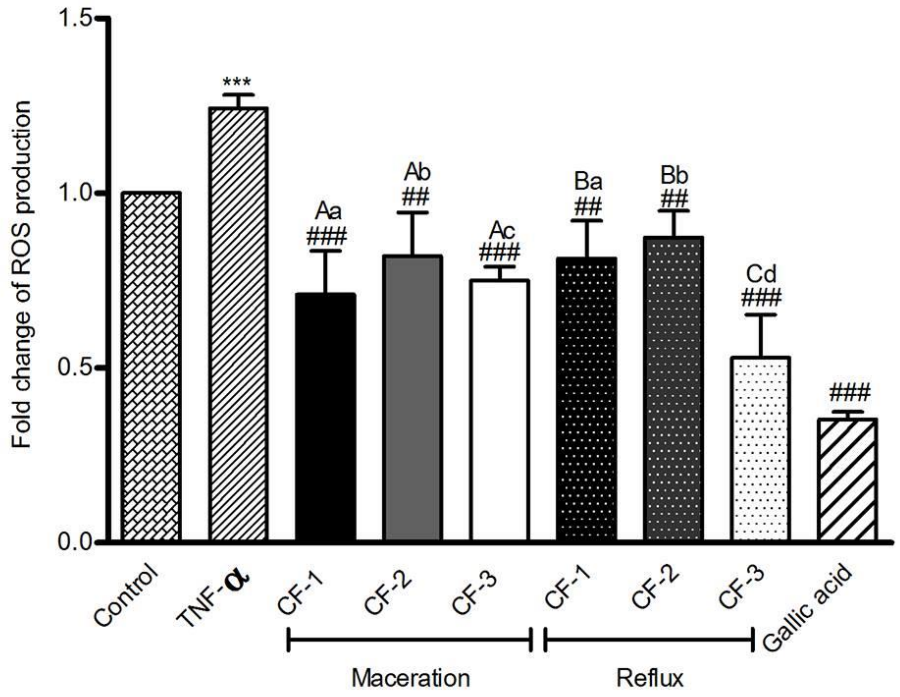

Figure 1 Inhibition effect of herbal mixture on TNF-α-induced intracellular ROS production. Cells were pre-treated with extracts at 100 µg/mL or 250 µM gallic acid, and followed by stimulation with TNF-α for 2 h. The fluorescence intensity of DCF-stained cells was quantified; the result was expressed as mean ± S.D. of three independent trials. Data are presented as fold change of ROS production of control cells. ***$P < 0.001$; when compared with control. $^{###}P < 0.001$, $^{##}P < 0.01$; when compared with TNF-α. A, B and C shows significantly differences among herbal mixtures prepared with the same extraction condition. a, b, c and d: shows significantly differences between the herbal mixtures prepared with the different extraction condition.

It is well-known that NO is a major vasodilator by activating soluble guanylyl cyclase (Hedlund et al., 2000). eNOS-catalyzed NO diffuses to smooth muscle cells and causes relaxation of smooth muscle resulting in vasodilation (Forstermann and Sessa, 2012). Several lines of evidence indicate that NO bioavailability is decreased by increasing ROS (Schulz, et al., 2011; Pierini and Bryan, 2015). Thus enhancing NO bioavailability can improve vascular function. The synthesis of eNOS-catalyzed NO can be modulated either by increasing eNOS activity or by increasing eNOS expression (Forstermann and Sessa, 2012). In the present study, all herbal mixtures, except CF-1 prepared by reflux, significantly increased NO production in endothelial cells (Figure 2). Resveratrol, a positive control, was significantly increased NO production which was in accordant with previous reports (Liu et al., 2017; Srisook et

al., 2018). There was no significant difference in capability between the herbal mixtures CF-2 and CF-3 prepared by two extraction methods. The results obtained in the present study suggest that all herbal mixtures might improve endothelial dysfunction by enhancing NO bioavailability via suppression of oxidative stress as well as inducing NO production, and be considered as a candidate for development as dietary supplement for improving endothelial function and preventing atherosclerosis.

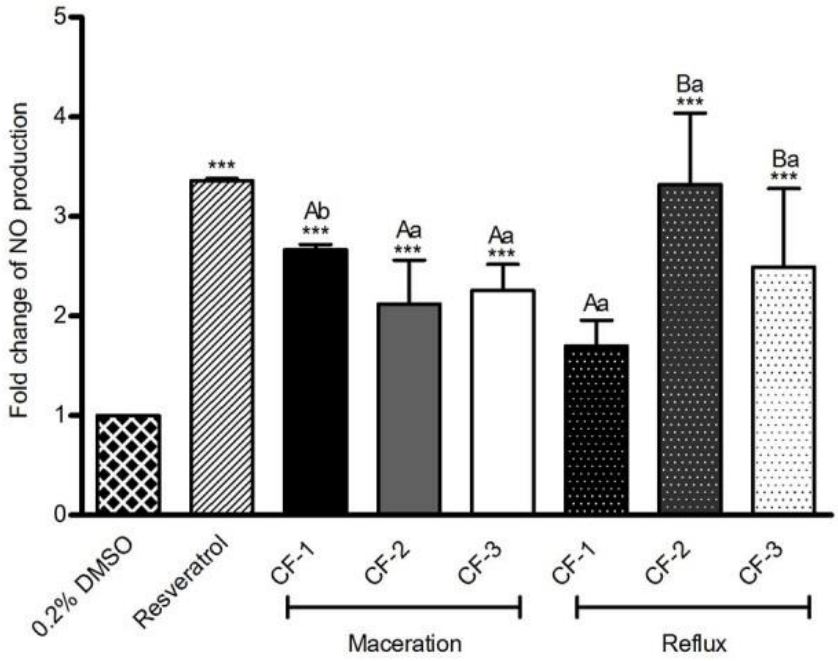

Figure 2 Effect of herbal mixture on NO production in endothelial cells. Cells were treated with extracts at 100 µg/mL or 100 µM resveratrol for 24 h. NO production was investigated by Griess reaction. The result was expressed as mean ± S.D. of three independent trials. Data are presented as fold change of NO production of 0.2% DMSO-treated cells. ***P < 0.001; when compared with 0.2% DMSO. A and B show significantly differences among herbal mixtures prepared with the same extraction condition. a and b show significantly differences between the herbal mixtures prepared with the different extraction condition.

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CONCLUSIONS

In this study we compared two extraction methods for antioxidant and NO-stimulating activity of herbal mixtures prepared from *B. superba*, *M. oleifera* and *K. parviflora*. The results indicate that the different extraction methods had an effect on biological properties of the herbal mixtures. It is concluded that the maceration extraction method exhibits better biological activities than that of refluxing, while both extraction methods give similar extraction yields. Moreover, all herbal mixtures prepared by maceration showed a similar extent of antioxidant and NO-producing activity.

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