Determination of total phenolics, flavonoid contents and antioxidant activity of different mBHT fractions: A polyherbal medicine

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Abstract: In this study, antioxidant activity, total phenolic and flavonoids content of four different fractions from the traditional Korean polyherbal medicine of Modified Bo-yang-Hwan-o-Tang (mBHT) was determined using spectrophotometric methods. Antioxidant activity of fractions was expressed as percentage of DPPH radicals inhibition and IC_{50} values (µg/ml). Values in percentage ranged from 48.35 to 77.43%. The reducing powers of all the extracts were comparable with that of positive control sample of Butylated hydroxyl tolune (BHT) and ascorbic acid which was found to be dose dependent. Total phenolic content ranged from 106.83±0.002 to 188.661±0.002mg/g, expressed as gallic acid equivalents. The total flavonoid contents varied from 28.44±0.001 to 105.25±0.001mg/g, expressed as quarcetin equivalents. Ethyl acetate fractions of mBHT showed the highest phenolic (188.66 mg GAE/g) and flavonoids (105.25 mg QAE/g) contents and strong antioxidant activity. Total phenolics and flavonoid content of all the mBHT fractions were found reasonably correlated with IC_{50} of DPPH (R^2=0.980 and 0.932, respectively). The high contents of phenolic compounds indicated that these compounds responsible for antioxidant activity. Therefore, ethyl acetate fractions of mBHT can be regarded as promising candidates for natural plant sources of antioxidants.

Keywords: mBHT, bioactive fraction, total phenolics content, total flavonoids content DPPH scavenging, reducing power.

INTRODUCTION

Free radicals are responsible for normal metabolic processes such as digestion and the conversion of food into energy. They are quite helpful for many of body's natural functions. However, the uncontrolled productions of free radicals are associated with the onset of a large number of diseases such as cancer, rheumatoid arthritis, cirrhosis, arteriosclerosis and degenerative processes associated with aging. Usually, the food system might produce highly reactive oxygen free radicals, which are capable of oxidizing bio-molecules, resulting in cell death and tissue damage (Kumar and Kuttan 2009). Therefore, antioxidants are being considered as free radical scavengers, which prevent and repair damage done by free radicals (Bruce *et al*., 1993).

A number of antioxidants from both natural and synthetic origin have been considered for use in the treatment of various human diseases (Cuzzocrea *et al*., 2001). Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT) are suspected to be highly carcinogenic and being harmful to immune system (Grice 1988 and Namiki 1990). In connection with this, natural antioxidants such as flavonoids, phenolics, tannins, curcumin and terpenoids are found in various plants (Prakesh *et al.,* 2007 and Arulpriya *et al*., 2010), which function as reducing agents, hydrogen donators, free radicals scavengers and singlet oxygen quenchers and therefore being called as cell saviors (Fattouch *et al*., 2007). External addition of antioxidants might overcome the effect of free radicals on the body, and in turn could prevent the occurrence of many diseases. The search for appropriate anti-inflammatory and antioxidant agents has recently focused on plants used in traditional medicines because of leads provided by natural products that may be better for treating oxidative stress related diseases (Sarker and Nahar, 2004).

Modified Bo-yang-Hwan-o-Tang (mBHT) also named as JP05, is a polyherbal medicine composed with twelve different herbs, and long been used as a prescription for stroke, senile and vascular dementia, ischemic brain, and heart damages (Jeong *et al*., 2008) Recently, mBHT has been reported its biological properties such as vasoprotection in brain endothelial cells (Son *et al*., 2010) anti-apoptosis in neuronal cells (Mahesh *et al*., 2011) and anti-cerebral ischemia in rats (Jung *et al*., 2011 and Choi *et al*., 2011) by our laboratory. Moreover, the inhibitory effect of methylene chloride fraction isolated from mBHT on microglia-mediated neuroinflammation was recently reported (Jung *et al*., 2012). However, their antioxidant activities with different solvent fractions were not studied. Several reports have also revealed that the majority of the antioxidant activity may be from biochemicals such as flavonoids, isoflavones, flavones, anthocyanins, catechins and other phenolics (Alothman *et al*., 2009 and Isabelle *et al*., 2010).

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Hence, in the present study, to identify major biological active fraction of mBHT, we investigated the antioxidant activity of four different fractions of mBHT such as butanol, ethylacetate, methylene chloride and water in tube tests using the determination of total phenolics, flavonoids contents and antioxidant assay.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, quercetin, butylated hydroxytoluene (BHT), Folin-Ciocalteu's phenol reagent and $AlCl_3$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium hexacyano ferrate, methanool, ethanol (HPLC grade) obtained from Merck (Darmstadt, Germany). Sodium bicarbonate was purchased from DC Chemical Co., Ltd (Daegu, Republic of Korea). Ferric chloride was purchased from Duksan pure chemicals Co Ltd., (Gyeonggido, Republic of Korea). Water was obtained from water distillation plants in our laboratory. UV spectra UV-Visible spectra measurements were done using ASYA-HITECH, UVM-340 (GimbH, Austria).

Sample collection

mBHT was extracted freshly in the year of 2010 along with water according to described as previously (Son *et al*., 2010; Mahesh *et al*., 2011; Jung *et al*., 2011; Choi *et al*., 2011; Jung *et al*., 2012). Next, the dried water extract of mBHT (300 mg, yield of 30%) was fractionated with different solvents such as butanol, ethylacetate, methylene chloride and water. All fractions were separately concentrated using rotary evaporator (Buchi, USA) under reduced pressure at 40°C. The voucher specimens for mBHT (08001C) and each fraction (08001C-BU, -EA, -MC and -W) have been deposited at the Herbarium of the Korean Medicine R&D Center Dongguk University. The concentrated extracts were ground using a pulverizer. The final yield of butanol, ethylacetate, methylene chloride and water fractions were found to be 11.0%, 1.5%, 3.5% and 64% respectively and was stored at 4ºC until use. For stock solutions, 10mg/ml of each sample was dissolved with methanol. Finally each sample was used to explore their phenolics and flavonoids contents and the antioxidant activity.

Determination of total phenolics content

The content of phenolics in each sample was determined using spectrophotometric method (Singletion *et al*., 1999) with minor modifications. Methanolic solution of each sample in the concentration of 1mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5ml of each methanol sample, 2.5ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5ml 7.5% $NaHCO_3$. Blank was concomitantly prepared**,** containing 0.5ml methanol, 2.5ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45^{o}C for 45 min. The absorbance was determined using spectrophotometer at λ max =765nm (ASYA-HITECH, UVM-340). The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in the total phenolic contents were expressed as a gallic acid equivalence (mg of GAE/g of sample).

Determination of total flavonoids content

The content of flavonoids in each sample was determined using spectrophotometric method (Quettier *et al*., 2000) with minor midification. The sample contained 1ml of methanol solution of the extract in the concentration of 1 mg/ml and 1ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The samples were incubated for 1 hr at room temperature (RT). The absorbance was determined using spectrophotometer at λmax =415nm. The same procedure was repeated for the standard solution of quercetin and the calibration graph was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/mL) on the calibration line and then, the content of flavonoids in each sample was expressed as a term of quarcetin equivalent (mg of QAE /g of sample).

DPPH radical scavenging assay

The ability of each sample to scavenge DPPH free radicals was assessed by the standard method (Tekao *et al*., 1994) with a little modification (Kumarasamy *et al*., 2007). The stock solution of samples was prepared in methanol to achieve the final concentration of 1mg/mL, and diluted at different concentrations of 50, 125, 250, 500, 750, and 1000µg/mL. Diluted solutions (1 ml each) were mixed with 1mL of methanol solution of DPPH at concentration of 1mg/mL. After 30 min incubation in darkness at RT, the absorbance was recorded at 517 nm. The commercial known antioxidant, butylated hydroxytoluene (BHT) and ascorbic acid were used as positive control. DPPH solution in the absence of each sample was used as a control and the 80% methanol was used as a blank. The percentage of DPPH radical scavenging was calculated using the following equation: DPPH scavenging effect (%) = ((A0- A1)/ A0) x 100 Where A0 was the absorbance of the positive control and A1 was the absorbance in the presence of the test sample. The actual decrease in absorption induced by the test was compared with the positive controls. The IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH radicals.

Reducing power assay

Total reducing power was determined as described method, previously (Yildirim *et al*., 2001). Various concentrations of each sample (250-1000µg/mL) were separately mixed with 1mL of 0.2M sodium phosphate buffer (pH=6.6) and 1mL of 1% potassium ferric cyanide, followed by incubation at 50ºC for 20min. 1mL of 10% TCA was added to the mixture, which was then centrifuged at 3000 rpm for 1 min. Finally 2mL of the supernatant solution were mixed with equal volume of distilled water. Absorbance was measured at 700 nm after the addition of 0.5 ml of 1% $FeCl_3$. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid and BHT were used as standard compounds.

STATISTICAL ANALYSIS

All experiments were repeated at least three times, and the results were presented as the mean ± S.D. Statistical significance was analyzed with one-way analysis of variance (ANOVA), pair-wise and multiple-comparison testing between groups, as well as the Turkey test using Graph Pad Prism 5.0 software (Graph Pad software, Inc., CA, USA. p value less than 0.05 was considered to be statistically significant.

RESULTS

Effect of mBHT fractions on total phenolics and flavonoids contents

Table 1 shows the yield and the total phenolics and flavonoids contents of mBHT fractions with butanol, ethyl acetate, methylene chloride and water. The water fraction had the highest yield amongst the four fractions.

The total phenolic content of mBHT fractions was determined by Folin-Ciocalteu method (Singletion *et al*., 1999), which is known as a gallic acid equivalent. Among the mBHT fractions, ethyl acetate fraction was significantly showed the highest [(188.66±0.006) mg/g)] amount of phenolic contents followed by the butanol fraction (151.92±0.003) mg/g], the methylene chloride fraction [(124.72±0.05) mg/g], and the water fraction [(106.83±0.002) mg/g].

We next measured the total flavonoid contents of mBHT fractions by aluminium chloride method with quercetin as a standard (Quettier *et al*., 2000). Among the fractions, the ethyl acetate fraction of mBHT was showed the highest [(105.25±0.003) mg/g)] amount of flavonoids contents followed by the butanol fraction [(45.55±0.001) mg/g], the methylene chloride fraction [(43.25±0.004) mg/g], and the water fraction [(28.44±0.001) mg/g]. The contents of total phenolics (188.661-106.83 mg GAE/g DW) and flavonoids (105.25-28.44mg quercetin equivalent/g DW) of all the mBHT fractions were found reasonably correlated with IC_{50} of DPPH (R^2=0.980 and 0.932, respectively). Furthermore, it was clearly noted that, the ethyl acetate extract of mBHT was given higher values of total phenolics (188.66 mg GAE/g) and flavonoids (105.25mg QAE/g) compared those of the other fractions (fig. 1a, b).

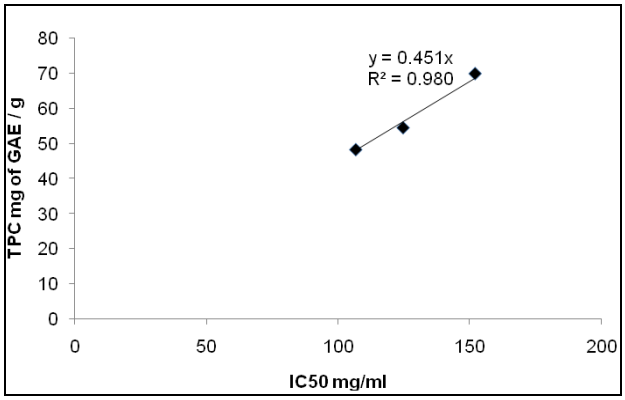

Fig. 1a: Linear correlation between DPPH IC_{50} and TPC of mBHT fractions

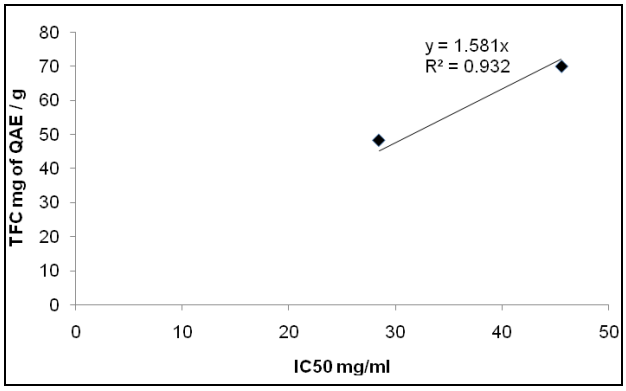

Fig 1b: Linear correlation between DPPH IC_{50} and TFC of mBHT fractions

Effect of mBHT fractions on DPPH radicals scavenging activity

To investigate the free radical scavenging activity of fractions, we measured DPPH radicals scavenging activity (Tekao *et al*., 1994; Kumarasamy *et al*., 2007). As shown in fig. 2, the scavenging activity of DPPH was significantly increased in the fractions with butanol, ethylacetate, methylene chloride and water in a dose-dependent manner. Among the fractions, the ethyl acetate fraction was exhibited a maximum DPPH radicals scavenging activity (73.99±0.36) %, followed by butanol (69.96±0.36) %, methylene chloride (54.57±0.55) % and water (48.35±0.21) %, at 1mg/ml respectively. Whereas positive control samples of BHT (Butylated hydroxyl tolune) and ascorbic acid was found to be DPPH scavenging activity with 74.87±0.21% and 77.43±0.2%, at 1mg/ml, respectively. The IC50 value of each fraction with ethyl acetate, butanol, methylene chloride, and water and BHT and ascorbic acids were 0.675, 0.714, 0.916, 1.034, 0.667 and 0.645 mg/mL, respectively.

Effect of mBHT fractions on reduction capacity

In this study, we monitored Fe^{2+} by measuring the formation of Perl's Prussian blue with absorbance at 700 nm. The presence of reductants (antioxidants) in the sample would result in reduction of the Fe^{3+} ferric cyanide complex to the ferrous form (Yildirim *et al*., 2001). Therefore, the strengthening compounds of reducing power have a stronger peroxide reducing ability. table 2 shows the reductive capabilities of different concentrations of ethyl acetate, butanol, methylene chloride and water extracts of mBHT, standard BHT and ascorbic acid. It was found that the reducing power of mBHT fractions was increased in a concentration dependent manner. Among four fractions, the ethyl acetate fraction was showed the highest reducing ability (absorbance 2.382 at 1000µg/mL). The activity was higher than that of BHT (absorbance 2.117 at 1000µg/ml), and was slightly relevant to ascorbic acid (absorbance 2.413 at 1000 µg/mL).

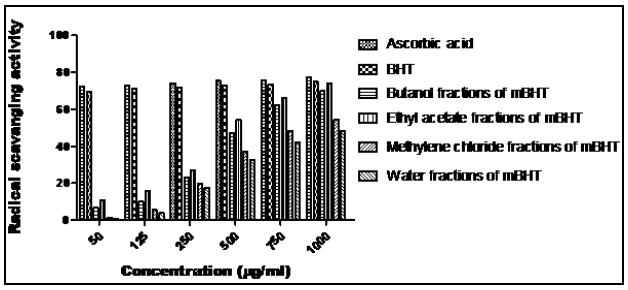

Fig. 2: Free radical scavanging activity of mBHT fractions. The free radicals scavenging activity was measured in the fractions by DPPH assay.

DISCUSSION

Recently, plant antioxidant has developed during the recent days, might be due to the appearance of tremendous side effects of certain commercially available antioxidants. In medicinal plant point of view, a plenty of different types of potential bioactive compounds with antioxidant activity that play a significant role in terminating the generation of free radicals.

Modified Bo-yang-Hwan-o-Tang (mBHT) also known as JP05, is a traditional polyherbal medicine composed of twelve different herbs being used as a prescription for stroke, senile and vascular dementia, ischemic brain and heart damages (Jeong *et al*., 2008). In our laboratory, the biological activity of mBHT has been reported well as vasoprotection in brain endothelial cells (Son *et al*., 2010), anti-apoptosis in neuronal cells (Mahesh *et al*., 2011) and anti-cerebral ischemia in rats (Jung *et al*., 2011 and Choi *et al*., 2011). Recently, the inhibitory effect of methylene chloride fraction isolated from mBHT on microglia-mediated neuroinflammation was also reported (Jung *et al*., 2012). Though, their antioxidant activities with different solvent fractions were not studied. Hence, in this study, we evaluate the antioxidant activity of different solvent fractions of mBHT such as butanol, ethylacetate, methylene chloride and water in tube tests using the determination of total phenolics, flavonoids contents and DPPH, reducing power assays.

In our reports, it has been reported that the yield of extractable antioxidant compounds was highest in ethyl acetate fraction of mBHT in comparison with butanol, methylene chloride and water. In the present study, the relative antioxidant ability of mBHT, a polyherbal medicine and its fractions with butanol, ethylacetate, methylene chloride and water was investigated by DPPH radicals scavenging assay and reducing power assay.

DPPH assay is a free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reducing capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. High reduction of DPPH is related to the high scavenging activity performed by particular sample (Lachumy *et al*., 2010). IC_{50} was calculated as amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by 50%. The lower the IC_{50} value, the higher the antioxidant activity. Ethyl acetate fractions of mBHT showed the lowest IC_{50} value with highest antioxidant activity. In our study, mBHT and its four fractions were able to scavenge DPPH radicals in order as follows: the ethyl acetate fraction > the butanol fraction > the methylene chloride fraction > the water fraction.

To determine the reduction capacity of mBHT fractions, we measured reductant, Fe^{3+} ferric cyanide complex to the ferrous form as a marker of antioxidant in each extracts by Perl's Prussian blue formation method (Yildirim *et al*., 2001). In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to $Fe^{2+}$by donating an electron. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. In this study, it appears that the ethyl acetate fraction of mBHT possess a strong hydrogen donating capabilities to act as antioxidant.

The total Phenolics present in plants extracts have received considerable attention because of their potential antioxidant activity. Phenolic compounds undergo a complex redox reaction with the phosphotungstic and phosphor-molybdic acids present in the Folin-Ciocalteau reagent (Singletion *et al*., 1999). Good correlation was found between phenolic contents of the different mBHT fractions (R^2 0.980) and their IC_{50} values. The result suggests that a very good plant antioxidant activity from the activity of phenolic compounds. Many studies have conclusively shown close relationship between total phenolic contents and antioxidative activity of the fruits, plants and vegetables (Kähkönen *et al*., 1999; Choi *et al*.,

Table 1: Extraction yield and the contents of total phenolics and flavonoids in four fractions isolated from mBHT

Fractions of mBHT	Extraction yield (% yield (w/w)	Total phenolic contents (mg of GAE/g)	Total flavonoid contents (mg of QAE/g)
Butanol	11.0%	188.66 ± 0.003^a	105.25 ± 0.001^a
Ethyl acetate	1.5%	151.92 ± 0.006^b	45.55 ± 0.001^b
Methylene chloride	3.5%	124.72 ± 0.005^c	43.25 ± 0.004^b
Water	64.0%	106.83 ± 0.002^d	28.44 ± 0.001^c

Values are mean (n=3) ± SD. Values with the different superscript letter are statistically different (p<0.05. GAE – gallic acid equivalents. QAE – quarcetin equivalents.

Table 2: Reducing power of different fractions isolated from mBHT

Con (ug/ml)	Absorbance at 700 nm					
	Ascorbic acid (Control)	BHT (Control)	Butanol	Ethyl acetate	Methylene chloride	Water
250	1.714 ±0.006^a	1.313±0.002^a	0.816±0.001^a	0.971±0.002^a	0.752±0.001^a	0.547±0.001^a
500	2.035 ±0.014^b	1.881±0.003^b	1.372±0.002^b	2.213±0.001^b	1.203±0.001^b	0.867±0.001^b
750	2.255±0.005^c	2.068±0.001^c	1.622±0.002^c	2.291±0.002^b	1.561±0.001^c	1.156±0.001^c
1000	2.413±0.002^d	2.117±0.002^d	1.955±0.003^d	2.382±0.003^c	1.736±0.001^d	1.237±0.001^d

Values are mean (n=3) ± SD. Values within a column followed by different letters are significantly different (P<0.05).

2011). Hence, our findings indicate that the antioxidant activity of the plant extracts might be exerted by the phenolic compounds in the plant.

Flavonoids and phenolics in vegetables, fruits, spices, and medicinal herbs might prevent cancer through antioxidant action and/ or the modulation of several protein functions (Sakakibara 2003). Interestingly, a good correlation was also found between flavonoids contents of the different solvent fractions of mBHT ($R^2$0.932) and their IC_{50} values. . The total flavonoid contents of mBHT fractions were ranged from 105.15mg/g to 28.44mg quercetin/g weight. It may be due to the variation of environmental conditions, which can modify the constituents inside of the plant. This depicts that flavonoids which are subgroups of phenolic compounds had also exerts an antioxidant activity. Therefore, the antioxidant activity of ethyl acetate fraction of mBHT might be exerted by both phenolic and flavonoids compounds.

CONCLUSSION

As we know, this is the first reports that envisage the antioxidant activities of the different solvent fractions of mBHT such as butanol, ethyl acetate, methylene chloride and water. In our reports, it has been reported that the yield of extractable antioxidant compounds was highest in ethyl acetate fraction of mBHT than the other fractions. The high contents of phenolic, flavonoids compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity indicated that ethyl acetate extract of mBHT contribute to the strong antioxidant activity. Hence, ethyl acetate fractions of mBHT could be a good source of antioxidant phenolics and might be an alternate to synthetic antioxidants. Further studies are materialized for the isolation and identification of individual phenolic compounds and also *in vivo* studies are needed for better understanding their mechanism of action as antioxidant.

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