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A Comparative Study on the Antioxidant Activity of Commonly Used South Asian Herbs

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ABSTRACT

The antioxidant activities of curry leaves, fenugreek seeds, Indian malabar leaves, red silk cotton tree leaves, cowitch leaves, holyfruit tree leaves, and black mustard seeds were compared. Their effects on reactive oxygen species (ROS) and superoxide dismutase (SOD) activity were investigated. The Oxygen Radical Absorbance Capacity (ORAC) assay determined the antioxidant potential of the extracts, while the ROS scavenging ability was explored in hyperglycemia-induced human umbilical vein endothelial cells (HUVECs). The SOD assay determined if the extracts stimulated the enzyme activity in the HUVECs. Curry leaf and fenugreek extracts had high ORAC values and superior free radical scavenging abilities compared with the rest of the extracts. The curry leaf extract had also increased the SOD activity. Fenugreek extract had not increased the SOD activity of the HUVECs. Thus, the two herbs displayed two distinct pathways of action for scavenging of ROS.

Key words: Oxygen radical absorbance capacity, Reactive oxygen species, Superoxide dismutase

INTRODUCTION

South Asia is widely known for its copious consumption of spices. Due to its geographic overlap with the tropics, South Asia is gifted with a variety of plants which are found in bloom throughout the year. These spices are also known to contribute to the trademark flavor and fragrances of South Asian food which have become quite popular even in Western countries. The amounts of spices added to food items vary with regional and cultural practices in South Asian households, which in turn results in a difference in the taste for the same food item prepared in different locations. Due to the presence of compounds such as capsaicin, most South Asian spices are capable of stimulating the trigeminal nerve by adding a punch and pungency to food items. These sensations have led to the popularity of South Asian food as means of pleasure and satisfaction throughout the world.

South Asian spices are also known for their medicinal properties and are thus consumed or added to food products as herbs. Some of the common household spices/herbs have been hypothesized as being potent antioxidants which are able to scavenge Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), thereby reducing oxidative stress levels.[1] Due to this hypothesis, they are considered as effective remedies for oxidative stress–related diseases such as diabetes.[2] In brief, diabetic hyperglycemia is known to result in increased ROS and RNS production by (1) the upregulation of the polyol pathway, (2) increased protein kinase-C (PKC) activation, (3) advanced glycation end-products (AGE), and (4) the up-regulation of the hexosamine pathway.[3-5] Therefore, antioxidants have been considered as the most effective remedy against the long-term complications of diabetes which are initiated by these pathways.

A few studies conducted recently have demonstrated the effectiveness of some South Asian herbs/spices as remedies for diabetes.

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For instance, curry leaves (CL; *Murraya koenigii*) were observed to have reduced damage to retinal, hepatic, and renal tissues in hyperglycemic cell models.[6] Similarly, the aqueous extract of fenugreek seeds (FG; *Trigonella foenum*) had also shown positive effects in hyperglycemic cell models.[5,6] Despite this scientific evidence, some of the commonly used South Asian herbs/spices have not been explored for their hypothesized antioxidant potential and, thereby, their effect against ROS and RNS in hyperglycemic conditions. Although they have been incorporated in the diet of diabetics in South Asia for generations, the lack of scientific evidence hinders their recognition as effective means of combating the disease. Herbs/spices such as Indian malabar leaves (IM; *Pterocarpus marsupium*), red silk cotton tree leaves (RSC; *Bombax ceiba*), cowitch leaves (CW; *Mucuna pruriens*), holy fruit tree leaves (HF; *Aegle marmelos*), and black mustard seeds (BMS; *Brassica juncea*) are commonly used, but seem to lack the scientific evidence as to their effectiveness against diabetes-induced oxidative stress.[7,8] Even in the instance of CL and FG, research has failed to substantiate their potential as antioxidants and free radical scavengers.

In view of these gaps, the objective of this study was to qualitatively determine the antioxidant activity of CL, FG, IM, RSC, CW, HF, and BMS, their effects against ROS, and their influence on the enzymatic activity of superoxide dismutase (SOD) in a hyperglycemia-induced oxidative stress cell-line model. These herbs were selected because they are consumed more frequently than others regardless of the health condition of the consumers. Although these herbs have been hypothesized to have antioxidant and anti-diabetic effects, the overall effects and claims have not been fully elucidated qualitatively or quantitatively.

MATERIALS AND METHODS

The herbs/spices were procured from the Little India Ayurvedic Medicinal Hall. The authenticity of the spices was affirmed by Miss. Lee Yian Hoon and samples were placed in the herbarium of Temasek Polytechnic which consisted of 296 other plant species. Eagle's DMEM (Dulbecco's Modified Eagle Medium) glucose, and antibiotics were purchased from Gibco–Invitrogen, Oregon, USA. Fetal Bovine Serum (FBS) was purchased from Hyclone (Oregon, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Virginia, USA). Tissue culture treated T75 flasks, 48-well plates, and 6-well plates were purchased from CellStar (Singapore). SOD assay kit was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). 5-(and-6)-chloromethyl -2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2D-CFDA) was purchased from Invitrogen (Molecular Probe, Oregon, USA). KH_2PO_4 and K_2HPO_4 were obtained from Merck, Singapore. Trolox was purchased from Acros Organics, Singapore. 2,2'-Azo-bis (2-amidinopropane) dihydrochloride (AAPH) was purchased from CalbioChem (Massachusetts, USA). Sodium fluorescein was purchased from Sigma-Aldrich (Michigan, USA). All other reagents were purchased from Sigma-Aldrich, Singapore, unless otherwise stated.

Preparation of aqueous spice extracts

Aqueous extracts of the leaves or seeds of CL, FG, IM, RSC, CW, HF, and BMS were prepared to mimic actual cooking durations and temperatures utilized in the preparation of South Asian food items as closely as possible. The leaves or seeds of the herbs of 50 g each were refluxed with 50 ml of water for 2 h at 60°C. The remnants were filtered and the extraction was repeated. The collected extracts were combined and freeze-dried overnight. The freeze-dried plant extracts were powdered and stored at − 80°C till the analyses were carried out. The extraction rate of each of the herbs was 25 mg/ml.

Oxygen radical absorbance capacity assay

The ORAC assay was carried out according to the method of Huang *et al*.[9] Sodium fluorescein stock solution (4.19×10^{-3} mM) was made using 75 mM potassium phosphate buffer (pH 7.4) and stored at − 4°C. Sodium fluorescein working solution was made daily by further diluting the stock solution in 75 mM potassium phosphate buffer. Trolox standard was prepared by dissolving 0.250 g of Trolox in 50 ml of 75 mM potassium phosphate buffer to give a 0.02 M stock solution. The stock solution was diluted with the same phosphate buffer to 200, 100, 50, 25, and 12.5 μM working solutions. Twenty microliters each of sample, potassium phosphate buffer, and Trolox standards was added into the wells of a 96-well microplate. Sodium fluorescein working solution (160 μl) was then added into the wells. AAPH solution was prepared last by diluting 0.110 g of AAPH in 5.0 ml of 75 mM potassium phosphate buffer (pH 7.4). AAPH solution (20.0 μl) was added last into the well via an automatic dispenser. Thus, the total volume for each well was 200 μl, and the fluorescence intensity was measured every 2 min for 2 h by Tecan Infinite® PRO reader with i-control™ software.

Experimental design of the hyperglycemic cell culture model

HUVECs were cultured in 2% gelatine-coated 60-mm Petri dishes and grown in DMEM low-glucose media supplemented with 20% FBS and 1% antibiotics. The Petri dishes were incubated at 37°C in 5% CO_2. The flasks were incubated at 37°C in 5% CO_2. Sub-culturing was carried out when the respective growth conditions were confluent by the use of trypsin-ethylenediaminetetra acetic acid (EDTA). HUVECs were seeded at equal density (12,500 cells per well) in gelatine-coated 48-well plates in their respective media. The cells were exposed to the experimental condition for 4 days. On day 4, the cells were treated with the plant extracts which were dissolved in water at concentrations of 0.75 and 1.0 mg/ml, which could be converted to the estimated typical amounts of consumption of the herbs when they are added to food. Two control groups of the cells were formed: (1) low-glucose (LG) media (5.56 mM) and (2) high-glucose (HG) media (35 mM). The cells treated with the various plant concentrations were exposed to HG media (35 mM) for 1 week. All treatments were carried out in triplicates.

Detection of intracellular ROS

The detection of intracellular ROS was carried out according to the method used by Brownlee.[10] CM-H_2DCFDA was used to detect the concentration of intracellular ROS. In brief, the cells were exposed to 20 μl of 20 μM CM-H_2DCFDA dis-

solved in phosphate-buffered saline (PBS) for 45 min. Excess CM-H_2DCFDA was removed by washing the cells twice using PBS. The intensity of viable cells was analyzed by Tecan Infinite PRO reader with i-control software.

SOD assay

The SOD assay was performed according to the protocol provided in the assay kit by Cayman Chemicals. The absorbance of the cells was measured at 450 nm using the Tecan Infinite PRO reader with i-control software. The equation used for the calculation of the SOD activity is as follows:

$$\text{SOD(U/ml)} = \left(\frac{\text{Sample linear rate} - \text{y-intercept}}{\text{Slope}} \right) \times \left(\frac{0.23 \text{ ml}}{0.01 \text{ ml}} \right) \times \text{sample dilution}$$

Statistical analysis

Statistical analysis was carried out using SPSS 10.0 for Windows. Results are expressed as the mean ± SEM of six independent experiments. $P > 0.05$ were considered to be significant.

RESULTS

The ORAC assay was able to differentiate the herbal extracts which had a superior antioxidant activity. According to Figure 1, CL had the highest ORAC value, followed by FG. IM, RSC, and CW had mid-range ORAC values. The least ORAC values were observed in HF and BMS.

Under high glucose conditions, cells are known to produce more intracellular ROS as compared to cells incubated under low glucose conditions. Thus, after treatment with the fluorescent dye, the HUVECs incubated under high glucose conditions fluoresced more than the cells incubated in low glucose conditions as shown in the confocal microscopy images in Figure 2. This further confirms the finding that high glucose conditions are able to increase the oxidative stress via ROS production. On a qualitative and comparative note with regards to the confocal images, the cells treated with CL and FG had less fluorescence intensities than the HG control cells, which indicate reduced presence of free radicals. Along the same lines, BMS – which had the least ORAC value among all herbal extracts – displayed higher fluorescence intensity than the LG control as well as the CL- and FG-treated cells. However, its intensity was still less than the HG control cells, indicating its ability to quench the presence of free radicals, although not to the same extent as CL and FG. The images displayed a clear correlation with the rest of the results where CL and FG had the highest antioxidant and radical scavenging activities and BMS had the least in both parameters.

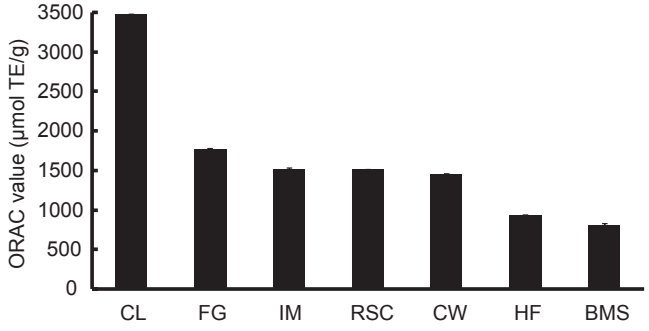

Figure 1. ORAC values of the aqueous plant extracts

CL and FG at 1.0 mg/ml showed the highest decrease in the intracellular ROS level close to the LG control cells, as seen in Figure 3. Overall, CL and FG at both concentrations had the highest reduction among all the herbal extracts. These observations support the findings from ORAC assay that the plants with the highest antioxidant capacity have the most effective free radical scavenging properties. Overall, CL, FG, IM, RSC, and CW displayed statistically significant reductions of the fluorescence intensity levels, compared with the HG control ($P < 0.05$). The trend in the reduction was comparable to the ORAC values of the herbs. HF and BMS did not show a statistically significant reduction of the fluorescence intensity as compared with the HG control. All herbs had the highest reduction in the fluorescence intensity at 1.0 mg/ml instead of 0.75 mg/ml.

As shown in Figure 4, CL concentration at 1.0 mg/ml displayed the highest SOD activity, followed by its concentration at 0.75 mg/ml. None of the other herb concentrations demonstrated the stimulation of SOD activity. This observation reveals that CL is able to scavenge free radicals by two pathways: (1) by scavenging the radicals through the bioactive compounds present in the extract itself and (2) by stimulating the activity of SOD. In addition, by the inactivity of SOD as displayed by the cells treated with FG, it could be concluded that the ROS scavenging ability of this herb is due to the antioxidant behavior of the bioactive compounds present in the spice's extract itself.

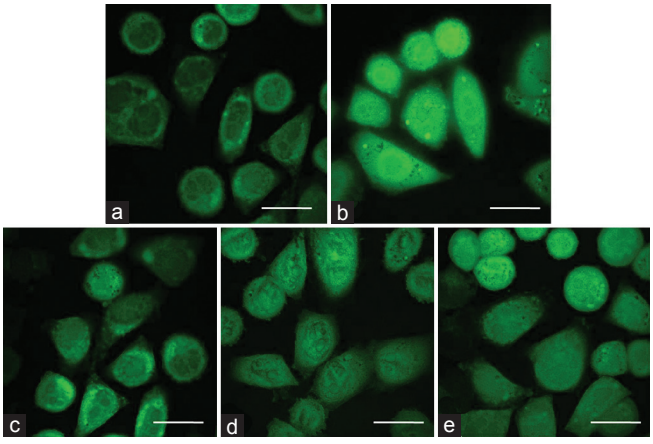

Figure 2. Representative confocal microscopy images of the HUVECs incubated in (a) low-glucose (LG) media and (b) high-glucose (HG) media for 4 days, as well as cells treated for 1 week with (c) HG + CL (1.0 mg/ml) (d) HG + FG (1.0 mg/ml), and (e) HG + BMS (1.0 mg/ml). Scale bars indicate 100 µm. All cells were treated with 20 µl of 20 µM CM-H_2DCFDA. The fluorescence intensity of the LG control cells was comparatively less than that of HG control cells. The cells treated with CL and FG had less fluorescence intensities than the HG control cells, which indicate reduced presence of free radicals. In comparison, BMS, which had the least ORAC value, displayed higher fluorescence intensity than the LG control and CL- and FG-treated cells. However, its intensity was still less than that of HG control cells. The images displayed a correlation with the rest of the results where CL and FG had the highest antioxidant and radical scavenging activities and BMS had the least in both parameters

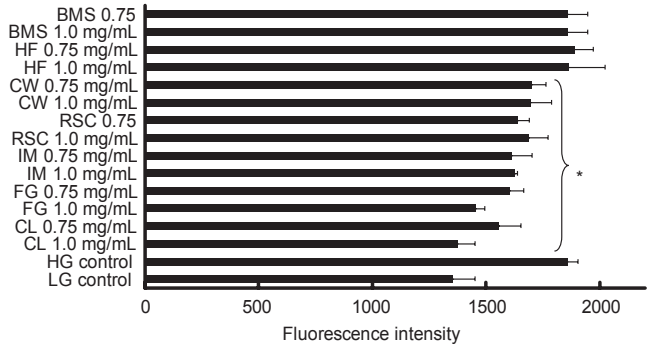

Figure 3. Fluorescence intensity of the HUVECs incubated in (i) 35 mM glucose after treatment with the respective aqueous plant extracts (mg/ml) for 1 week, (ii) 35 mM glucose without treatment, and (iii) 5.6 mM glucose without treatment. *$P < 0.05$ versus low glucose (LG) control; #$P < 0.05$ versus high glucose (HG) control

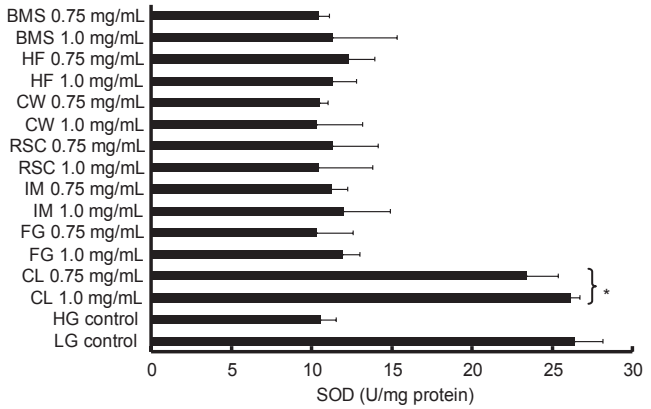

Figure 4. SOD activity of the HUVECs incubated in (i) 35 mM glucose after treatment with the respective aqueous plant extracts at various concentrations (mg/ml) for 1 week, (ii) 35 mM glucose without treatment, and (iii) 5.6 mM glucose without treatment. *$P < 0.05$ versus low-glucose (LG) control; #$P < 0.05$ versus high-glucose (HG) control

DISCUSSION

The ORAC assay quantifies the peroxyl radical scavenging capacity by measuring the ability of potential antioxidants to inhibit the fluorescein oxidation by peroxyl radicals.[11] Thus, this assay was used as an initial screening to estimate the radical scavenging ability of the spices. However, such chemical assays are unable to characterize the behavior of potential antioxidants under actual physiological conditions. Therefore, it has been recommended by many researchers to utilize at least two different types of assays for the investigation of antioxidant activities of samples.[12-14] Also, the ORAC assay itself could be an unjust means of screening for antioxidant activity since it focuses only on the scavenging of the peroxyl radical. Therefore, in order to complement the ORAC results, the antioxidant activity of the extracts and their effectiveness against intracellular ROS was determined with HUVECs using CM-H_2DCFDA.

Overall, the HUVECs treated with the plant extracts showed a reduction in the fluorescence intensity as compared with the HG control group. This is because the fluorescence intensity of the probe is affected by the amount of mitochondrial ROS present in the cells. The concentration of ROS is directly proportionate to the fluorescence intensity. Thus, although not quantified, the least fluorescence intensity was shown in the cells treated with CL at 1.0 mg/ml.

ROS scavenging properties of the plants could be further characterized by measuring the SOD activity of the cells. An increase in SOD activity indicates a higher expression of the enzyme. SOD assists in the conversion of intracellular superoxide to H_2O_2. Antioxidant compounds which do not display radical scavenging ability using chemical assays such as ORAC have been known to display the stimulation of SOD expression, thereby reducing the concentration of ROS.[15-18] Given this aspect, it was deemed vital to explore the stimulation of SOD expression of all the herbal extracts, especially those with a low ORAC value. Nevertheless, IM, CW, HF, RSC, and BMS did not show any significant ability to stimulate the expression of SOD. Since the ORAC values and the fluorescence intensities of the extract-treated cells of these herbs were comparable, it could be concluded that these herbs also engage in scavenging free radicals by the antioxidant compounds present in their extracts themselves. To determine whether these herbs may show considerable radical scavenging activities at higher concentrations than those investigated in this study, further investigations are required. However, since the concentrations of 0.75 mg/ml and 1.0 mg/ml translate to daily consumption amounts of the herbs, whether increased amounts would result in toxicity or adverse effects on the flavor of food itself is to be determined by further research.

Although the results from this study could be considered preliminary, they are of importance since the root cause of diseases such as diabetes and cardiovascular disease is known to be oxidative stress caused by ROS.[19] Since many of the current treatments for these diseases have been known to be ineffective on a long-term basis, the phytochemicals and phytomedicines have been touted as the more reliable and efficient method for disease prevention and treatment.[20] Plants which have been used in traditional medicinal practices have been tested throughout generations for their efficacies and safety, thus making them a more plausible means of obtaining good health and wellness. Despite the current lack of scientific background to most of the claims of traditional origin, plant-based medicines are slowly, but surely gaining popularity over the Western counterparts, with more research being conducted of the nature of this study.[21] Most of the traditional herbal medicines do not display their efficacy overnight. However, the herbs have reduced side effects and the disease condition is considered on a holistic basis rather than in isolation during treatment, which in turn leads to reduced side effects than Western medicines.[22]

CONCLUSIONS

In conclusion, CL and FG were found to exhibit higher antioxidant potential due to their varied abilities to reduce the increased ROS levels. Although IM, CW, HF, RSC, and BMS did not display any antioxidant potential, the herbs should not be dismissed based on their performance in these assays alone. Despite ROS-induced oxidative stress being the root cause of diabetes and its complications, the histories of the herbs spanning over many centuries as

effective alone are strong reasons to continue their applications for therapeutic purposes. One also has to bear in mind that these spices are used in combinations for culinary or medicinal purposes. Therefore, the full extent of their abilities in disease treatment is yet to be elucidated.

In this aspect, CL and FG could be considered as potent radical scavengers on their own. Although their actual performance in physiological systems requires further exploration, they have presented their capabilities as antioxidants through the preliminary screening of this study itself. The efficacy of these two herbs will be further characterized *in vivo* to better understand their mechanisms of action and to determine whether they are able to behave holistically by reducing the biochemical pathways leading to diabetic complications.

Identification of the bioactive compounds present in these two herbs will also be of interest and a future direction from the current study. Although phenolic compounds present in CL and FG have been claimed as the responsible parties for their medicinal properties, this hypothesis requires further confirmation and has to be substantiated with characterization of the extract.[23-26]

A combination of herbs and its efficacy will also be explored in future studies. Since CL and fenugreek displayed two different pathways of action, a concoction consisting of the two herbs as well as the optimum percentages to be added will be further investigated in future studies. This will also shed light upon other possible combinations of herbs of similar nature, which could result in enhancement of the antioxidant and anti-diabetic properties.

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