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Evaluation and Characterization of Antioxidant Activity of Selected Herbs and Spices

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Abstract

This study investigated the antioxidant activity of selected herbs and spices, their effects in reducing the level of Reactive Oxygen Species (ROS) as well as their influence on the enzymatic activity of Superoxide Dismutase (SOD). Oxygen Radical Absorbance Capacity (ORAC) value was measured to determine the free radical scavenging capacity of the plant extracts, while a fluorescent probe was used to evaluate the ROS scavenging ability. An SOD assay was used to find out if the plants' aqueous extracts can stimulate the production of the SOD. Plant extracts which were found to exhibit high antioxidant activity such as curry leaves, rooibos tea and fenugreek showed superior free radical scavenging ability in hyperglycaemia-induced oxidative stress cell-line model. Plant extracts which exhibit low antioxidant activity such as Indian malabar, red silk cotton, cowitch, holy fruit tree and bitter gourd showed insignificant free radical scavenging ability. In addition, increased SOD activity was found in curry leaves and rooibos tea. This implied that the plants can increase the enzymatic activity of the SOD enzyme in order to reduce free radical levels, except for fenugreek which appeared to depend solely on the extract's ROS scavenging properties. Plant extracts such as Indian malabar, cowitch, bitter gourd, red silk cotton and holy fruit tree were found to have increase SOD activity, which may be due to the cellular defence mechanism stimulating the synthesis of SOD which was destructed by the ROS present in the cells. Further evaluation and characterization of the plants' for scientific proof is required to establish these traditional medicinal plants as effective means of disease treatment.

Keywords: Oxygen radical absorbance capacity, Reactive oxygen species, Superoxide dismutase

1. Introduction

Oxygen is required to produce energy for metabolic reactions in all animals, plants and bacteria except in certain anaerobic and aero-tolerant unicellular organisms. On the other hand, the free radicals of oxygen are capable of causing oxidative stress by reacting with cellular components which degrade or inactivate essential molecules. Oxidative stress occurs due to decreased antioxidant levels or increased production of reactive oxygen species (ROS) [1]. It can be defined as the imbalance in the ratio of ROS to antioxidant defences which eventually results in cell injury. Some examples of ROS are superoxide, singlet oxygen, triplet oxygen, ozone and hydroxyl and peroxyl radicals. Generally, cells are able to control or prevent the side effects of oxidative stress by up-regulating the antioxidant defences. This in turn can protect the cells from further oxidative damage.

Oxidative stress has also been linked to cell injury via a range of by-products such as 8- hydroxydeoxyguanosine (8-OHdG), protein carbonyl and lipid peroxides. The injury can be reversible in the early stages but eventually leads to irreversible damage [1]. Nevertheless, ROS such as mitochondrial superoxide is necessary to sustain and prevent the damage via pathogens and toxins, although overproduction of superoxide is closely related to disease pathologies such as diabetes and cancer.

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Research has shown several plants to possess anti-diabetic properties [2]. Curry leaves (*Murraya koenigii,* CL) were found to exhibit hypoglycaemic effects with increased hepatic glycogen content because of increased glycogenesis and decreased glycogenolysis and gluconeogenesis [2]. Rooibos tea (*Aspalathus linearis*, RT) was shown to increase glucose uptake and insulin secretion and suppressed the rise in fasting blood glucose level from previous research [3]. Fenugreek (*Trigonella foenum*, FG) aqueous leaf extract is known to have demonstrated hypoglycaemic and anti-hyperglycaemic effect [4]. Indian Malabar (*Pterocarpus marsupium*, IM), red silk cotton tree (*Bombax ceiba*, RSC), cowitch (*Mucuna pruriens*, CW), holy fruit tree (*Aegle marmelose*, HF), black mustard seed (*Brassica juncea*, BMS) and bitter gourd (*Momordica charantia*, BG) were found to reduce blood glucose level and exhibit hypoglycaemic effects from previous studies conducted. However, further research and scientific proof is required to establish these traditional medicinal plants as effective means of disease treatment compared with its western counterparts [5].

The objective of this study was to determine the antioxidant activity of the selected herbs and spices, their effects in reducing the level of reactive oxygen species and their influence on the enzymatic activity of superoxide dismutase in hyperglycaemia-induced oxidative stress cell-line model.

2. Materials and Methods

Herbs and spices were procured from the local wet market. Eagle's DMED, glucose and antibiotics were purchased from GIBCO, USA. Fetal Bovine Serum (FBS) was purchased from Hyclone. Human Umbilical Vein Endothelial Cells (HUVECs) and cervical cancer (HeLa) cells were purchased from ATCC (USA). Tissue culture treated T75 flasks, 48-well plates, 6-well plates were purchased from CellStar. Superoxide dismutase assay kit was purchased from Cayman chemicals (Ann Arbor, MI, USA). CM-H_2DCFDA was purchased from Invitrogen (molecular probe). KH_2PO_4 and K_2HPO_4 were obtained from MERCK, Singapore. Trolox was purchased from ACROS ORGANICS. AAPH was purchased from CalbioChem (USA). Fluorescein was purchased from Aldrich (USA). All other reagents were purchased from Sigma Chemicals, Singapore unless otherwise stated.

2.1 Preparation of Plant Extracts

Powdered plant (5g) was refluxed with 50ml of water for 2 hours. Followed by filtering the plant extract and repeat the refluxation in 50ml of water for another 2 hours. For fresh weight plants, 50g of plant was refluxed with 50ml of water. The plant extracts collected were combined and frozen overnight before transferring into freezer-dryer to be freeze–dry into powder form. Freeze-dry plant extracts were stored at -80°C.

2.2 Experimental Design of Cell Culture Model

HUVECs were cultured in 2% gelatine-coated 60mm 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. HeLa cells were cultured in tissue culture treated T75 flasks and grown in DMEM high glucose media supplemented with 10% FBS and 1% antibiotics. 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-EDTA. HUVECs were seeded at equal density (12500 cells per well) in gelatine coated 48-well plates in its respective media. HeLa cell were seeded at equal density (12500 cells per well) in 48-well plates and (125000 cells per well) in 6-well plates in its respective media. Both cells were exposed to the experimental condition for 4 days. On the day 4, the cells were treated with aqueous plant extracts in its respective concentrations. Three treatment groups were formed: Control cells in low glucose media (5.56mM), control cells in high glucose media (35mM) and cells treated with various plant concentrations in high glucose media (35mM).

2.3 Oxygen Radical Absorbance Capacity (ORAC) Assay

Fluorescein stock solution was made using 75mM potassium phosphate buffer (pH 7.4) and kept at –4°C for several months. Fresh fluorescein working solution was made daily by further diluting the stock solution in 75mM potassium phosphate buffer. Trolox standard

was prepared by dissolving 0.250g of Trolox in 50ml of 75mM potassium phosphate buffer to give a 0.02M stock solution. The stock solution was diluted with the same phosphate buffer to 200, 100, 50, 25 and 12.5 µM working solution. Twenty microliters each of sample, potassium phosphate buffer and Trolox standards were added into the wells according to the sample layout. Fluorescein working solution (160.0µl) was then added into the wells. AAPH solution was prepared last by diluting 0.110g of AAPH in 5.0ml of 75mM potassium phosphate buffer (pH 7.4). AAPH solution (20.0µl) was added last into the well via the automatic dispenser in the plate reader. Thus, the total volume for each well is 200µl and the fluorescence intensity was measure every 2 minutes for 2 hours by Tecan i-control multi well reader using Infinite 2000 software.

2.4 Detection of Intracellular ROS

To detect the intracellular production of ROS, the fluorescent dye CM-H_2DCFDA was used. HUVECs and HeLa cells were treated with different extract concentrations for a predetermined period. Cells were exposed to 20uM Carboxy-H_2DCFDA in phosphate buffered saline (PBS) for 45 minutes and washed twice in PBS. The intensity of viable cells was analyzed by Tecan i-control multi well reader using Infinite 2000 software.

2.5 SOD Assay

HeLa cells were treated with various aqueous plant extracts concentrations for a predetermined period. The cells were scraped off from the well and transferred into a clean microcentrifuge tube. The samples were sonicated and centrifuged to remove the cell pellets and the cell lysates were extracted. The supernatant of the samples were transferred into a new microcentrifuge tube. The SOD assay was performed according to the protocol provided in the assay kit by Cayman Chemicals. The results were analyzed by Tecan i-control multi well reader using Infinite 2000 software.

2.6 Statistical Analysis

Statistical analysis was carried out using SPSS 10.0 for Windows. Results are expressed as the mean ± S.E.M of 6 independent experiments. *P* values > 0.05 were considered to be significant.

3. Results

ORAC assay was used for the primary identification of plant extracts which exhibit high antioxidant activity. According to (Fig. 1), CL demonstrated superior radical scavenging ability, followed by RT and FG. IM, RSC and CW have the mid-range ORAC value. The least ORAC values were observed in HF, BMS and BG. After the ORAC values were measured, the plants were used to treat the diabetes induced cells to find out if plants with a high ORAC also exhibited high free radical scavenging properties *in vivo*.

Under high glucose (HG) conditions, cells are known to produce more intracellular ROS as compared to cells incubated under low glucose (LG) conditions. Thus, after treatment with the fluorescent dye, the HeLa cells incubated under HG condition fluoresced brighter than the cells incubated in LG condition as shown in (Fig. 2). This further confirms the finding that high

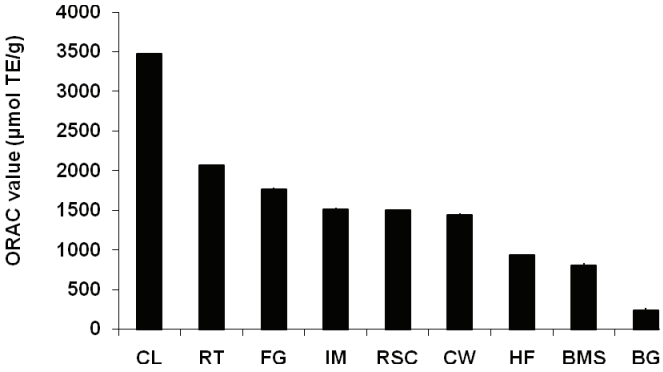

Fig. 1. ORAC values of various aqueous plant extracts - (CL) curry leaves, (RT) rooibos tea, (FG) fenugreek, (IM) Indian malabar, (RSC) red silk cotton tree, (CW) cowitch, (HF) holy fruit tree, (BMS) black mustard seed and (BG) bittergourd.

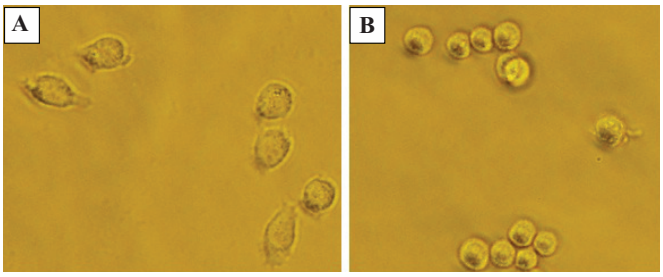

Fig. 2. A representative image of HeLa cell incubated in (A) low glucose (LG) media and (B) high glucose (HG) media for 5 days after treatment with the fluorescent dye.

glucose/hyperglycaemia can increase oxidative stress via ROS production.

Both HeLa cells and HUVECs were used to determine the selected plants' free radical scavenging properties *in vivo*. HeLa cells treated with BMS aqueous extract showed no significant reduction in the ROS fluorescence intensity as seen in (Fig. 3A). On the other hand, HeLa cells treated with IM, HF, RCS, CW and BG aqueous extracts showed a decrease in the fluorescence intensity at 0.75 to 1.00mg/ml (Fig. 3A–B). Moreover, HeLa cells treated with RT aqueous extract demonstrated a gradual decrease in the fluorescence intensity at a low treatment concentration of 0.05 to 0.25mg/ml (Fig. 4A–B). In addition, HeLa cells treated with CL and FG aqueous extracts exhibited a reduction in fluorescence intensity at 0.50 to 1.00mg/ml (Figure 4B).

After the cells were treated with the respective aqueous extracts, FG-treated HeLa cells were found to exhibit signs of apoptosis (Fig. 5–6). The cells treated

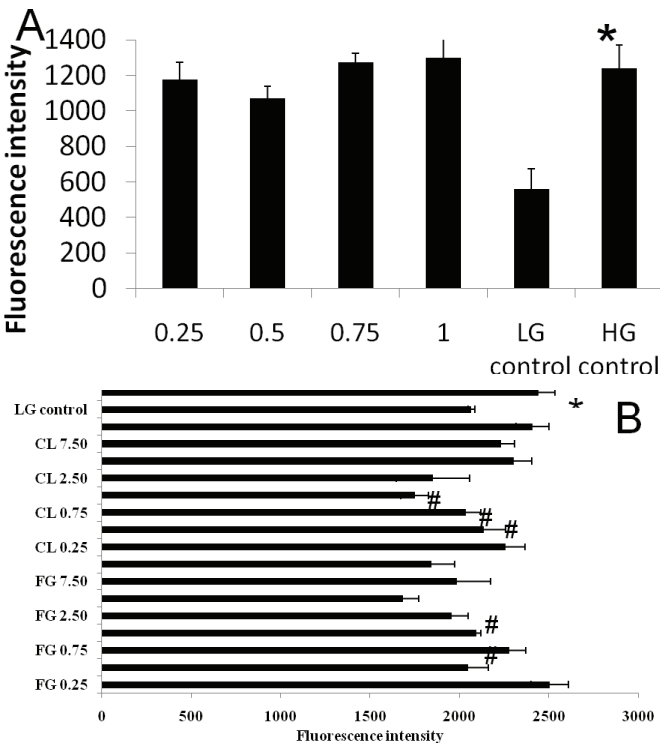

Fig. 3. Fluorescence intensity of the HeLa cells (A) incubated in 30mM glucose after treatment with the rooibos tea extracts (mg/ml) and the low glucose control of 5.6mM without treatment (B) 30mM glucose without treatment and 30mM glucose treated with the CL and FG extracts. *p < 0.05 versus low glucose (LG) control; #p < 0.05 versus high glucose (HG) control

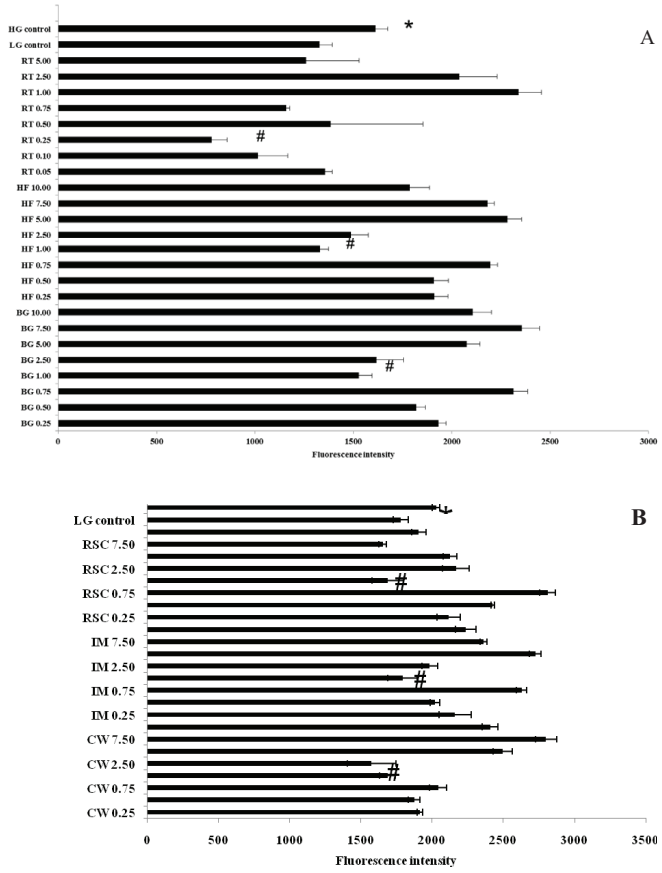

Fig. 4. Fluorescence intensity of the HeLa cells (A) incubated in 30mM glucose after treatment with the RT, HF and BG extracts (mg/ml) and the low glucose control of 5.6mM without treatment (B) incubated in 30mM glucose after treatment with the rooibos tea extracts (mg/ml) (B) 30mM glucose without treatment and 30mM glucose treated with the RSC, IM and CW extracts and the low glucose control with 5.6mM without treatment. *p < 0.05 versus low glucose (LG) control; #p < 0.05 versus high glucose (HG) control

with ≥2.5mg/ml FG aqueous extract appeared to be circular, highly vacuolated and detached from the base of the culture plate. This finding suggests that FG might also have some anti-cancer properties.

The same experiment using the optimal aqueous extracts concentration was conducted for the HUVECs. CL 0.75, FG 0.50 and RT 0.25 aqueous extracts showed a decrease in the intracellular ROS level close to the LG control cell as seen in (Fig. 7). This supports the findings from ORAC assay that the plants with a higher antioxidant capacity have better free radical scavenging properties.

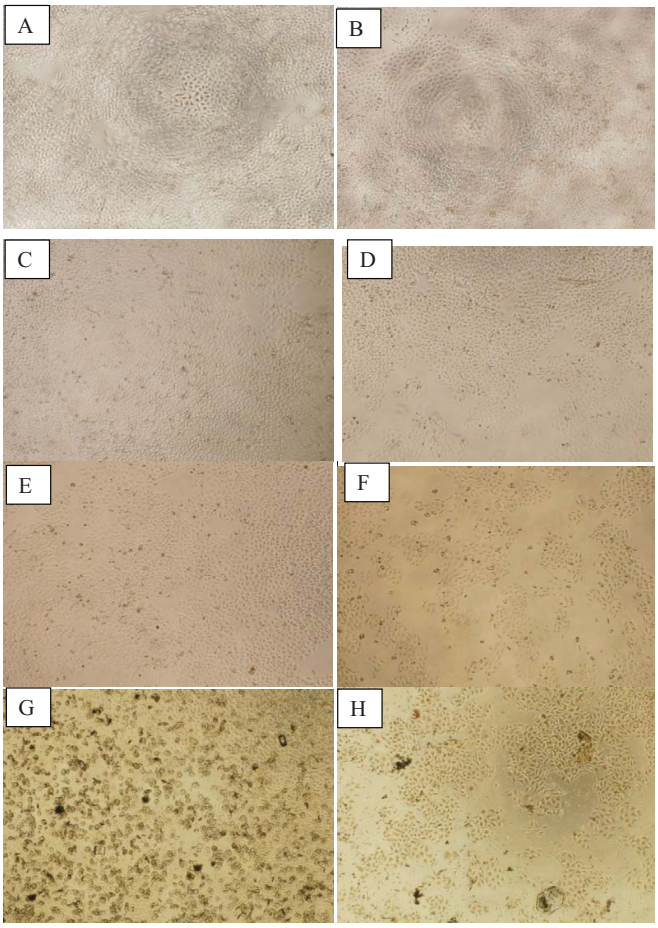

Fig. 5. A representative 40X magnification reverse microscope images of HeLa cells in 48-well plates. Cells incubated in (A) 5.6mM glucose with treatment, (B) 30mM glucose without treatment, (C) 30mM glucose after treatment with 0.25mg/ml of FG, (D) 30mM glucose after treatment with 0.50mg/ml of FG, (E) 30mM glucose after treatment with 1.00mg/ml of FG, (F) 30mM glucose after treatment with 2.50mg/ml of FG, (G) 30mM glucose after treatment with 5.00mg/ml of FG, (H) 30mM glucose after treatment with 10.00 mg/ml of FG.

The optimal aqueous extract concentrations for each plant were also used to treat the HeLa cells for the SOD assay. 0.75 mg/ml HF and CL, 1.00mg/ml RSC, BG, CW and IM and 0.25mg/ml RT aqueous extracts were found to increase the SOD enzyme activity in the cell as shown in (Fig. 8). These show that the plants can stimulate the cells to produce more SOD enzymes to cope with the increase production of intracellular ROS in particular superoxide.

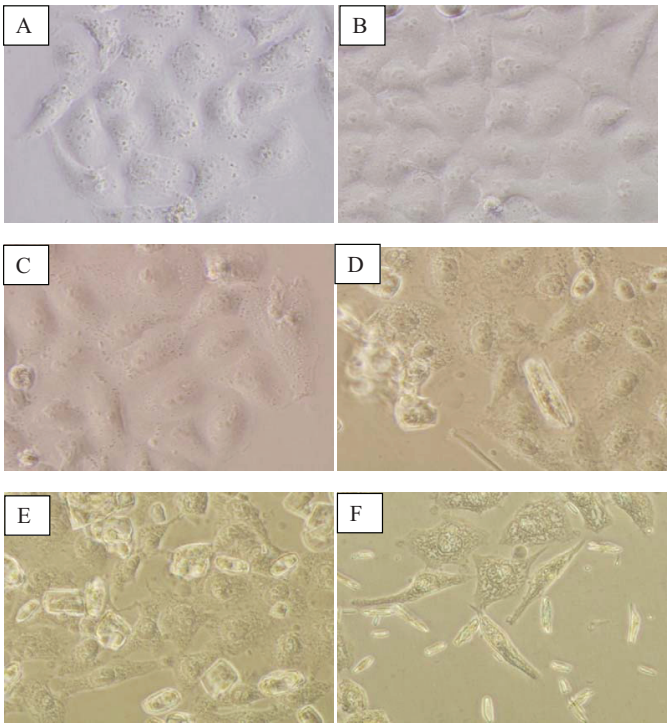

Fig. 6. A representative 400X magnification reverse microscope images of HeLa cells in 48-well plates. Cells incubated in (A) 5.6mM glucose with treatment, (B) 30mM glucose without treatment, (C) 30mM glucose after treatment with 1.00mg/ml of FG, (D) 30mM glucose after treatment with 2.50mg/ml of FG, (E) 30mM glucose after treatment with 5.00mg/ml of FG, (F) 30mM glucose after treatment with 10.00mg/ml of FG.

4. Discussion

ORAC assay was used to quantify the plants peroxyl radical scavenging capacity by measuring the antioxidants ability to inhibit the fluorescein oxidation by peroxyl radicals [6]. Thus, the plants' radical scavenging ability can be identified using this method. The selected plants' ORAC result showed that CL > RT > FG have the highest radical scavenging ability. However, *in vitro* chemistry based assays do not hold much value when it comes to bioactivity of the antioxidants and it is a recommended practise to use at least two different types of assays for the investigation of antioxidant activities of samples [7–8]. Therefore, detection of intracellular ROS assays was also conducted on both the HeLa cells and HUVECs using the fluorescence maker, CM-H_2DCFDA.

HeLa cells were used for the initial rapid screening of the intracellular ROS assay. This is to identify the optimal

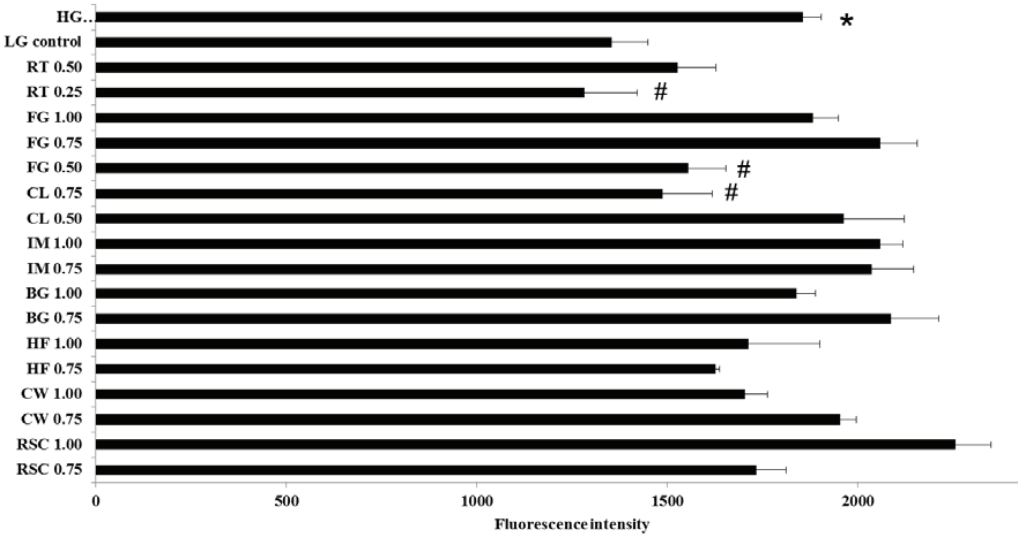

Fig. 7. Fluorescence intensity of HUVECs incubated in (i) 30mM glucose after treatment with respective aqueous plant extracts (mg/ml), (ii) 30mM glucose without treatment and (iii) 5.6mM glucose without treatment. *p<0.05 versus low glucose (LG) control; #p<0.05 versus high glucose (HG) control

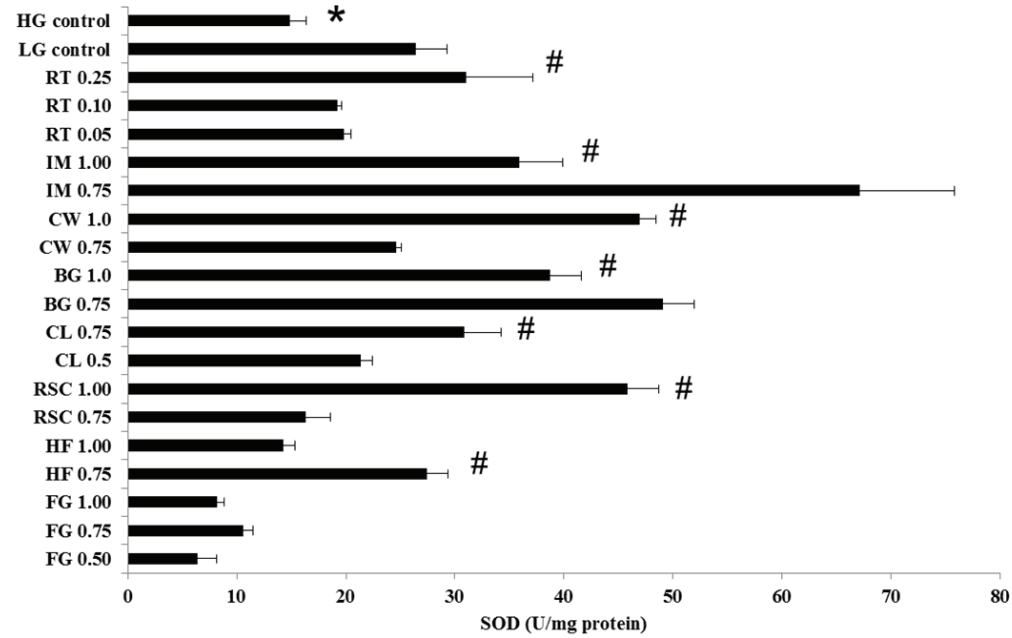

Fig. 8. SOD activity of HeLa cells incubated in (i) 30mM glucose after treatment with the respective aqueous plant extracts at various concentrations (mg/ml), (ii) 30mM glucose without treatment and (iii) 5.6mM glucose without treatment. *p<0.05 versus low glucose (LG) control; #p<0.05 versus high glucose (HG) control

aqueous plant extract concentrations (mg/ml) which were effective in reducing the ROS level in the cells and also to rule out plant extracts which were ineffective in reducing the increase intracellular ROS produced under high glucose conditions.

Plant extract-treated HeLa cells which showed a decreased in the fluorescence intensity indicated its ROS scavenging properties. This is because the fluorescence intensity is affected by the amount of mitochondrial ROS present in the cells. A higher amount of mitochondrial

ROS results in a greater degree of fluorescence intensity and vice versa. Optimal plant extract concentrations were identified for all the selected plants except BMS which showed no significant decreased in the fluorescence intensity in the entire treatment group. BMS has shown a low ORAC value and showed insignificant decreases of fluorescence intensity in the treatment group (Fig. 4A). This implies that the plant contains little or no ROS scavenging properties. Therefore, it was not used for any further evaluation and characterization of its antioxidant properties. For the rest of the selected plants, its optimal plant concentration varies from one another because different plants have different active compounds of various percentages which can alter different pharmacodynamic properties in the cells. Hence, the effective dosage of the plant extracts varies from one another.

Additionally, an interesting finding was observed for FG-treated HeLa cells. FG was found to exhibit apoptosis in cells treated with ≥2.5mg/ml aqueous extract. This observation was also identified in recent studies that FG was found to induce anti-cancer activity via apoptosis due to flavonoids present in the extract. These flavonoids were also shown to inhibit carcinogenesis in animal models and induce apoptosis in tumour cells [9–11]. It can differentially induce apoptosis in cancer cells and not in the normal counterparts. Moreover, any agents which can induce apoptosis in tumors have the potential to be used for anti-tumor therapy. Further investigations are underway to isolate and characterize FG active ingredient that contributes to its effects [12]. Thus, the decrease in the fluorescence intensity of ≥2.5mg/ml in FG extract treated HeLa cells might be due to the decrease in cell density in the wells. In order to prevent any error in analyzing and interpretation of the results, the optimal aqueous extracts treatment used for FG was selected between 0.50mg/ml to 1.00mg/ml. This is to ensure that the cells' density in each well remains constant.

HeLa cells were selected for rapid screening in this experiment because HeLa cells grow and proliferate very rapidly. This allows more experiments to be conducted with a wider screening of the extracts. Brownlee [12] also mentions that 'all cells – whether it be diseased or not, will respond the same way to both high and low glucose conditions'. This is because the increased glucose level in the cells will affect the ATP syntheses pathway in the same for every cell, which eventually results in the increase in mitochondrial ROS production [13]. In addition, the results obtained in this experiment (Fig. 3) also support the argument stated by Brownlee [12], as the HeLa cells incubated in HG condition exhibited higher fluorescence intensity than those incubated in LG condition.

In general, cancer cells produce a greater amount of ROS as compared to the normal cell lines even though the fluorescence marker will only bind specifically to the mitochondrial ROS presence in the cells [14–15]. There might still be some interference in the HeLa cells which will affect the fluorescence intensity. Hence, to ensure that the data obtained is accurate, reliable and reproducible, HUVECs were used to justify the findings obtained from the HeLa cells.

Both the HeLa cells' and HUVECs' intracellular ROS assay were performed using the same methods. However, for the HUVECs, it is treated with the optimal plant extracts found in the experiment conducted when using HeLa cells. This is to correlate the two results obtained from both HeLa cells' and HUVECs' and also to compare the trend - the decrease or increase in fluorescence intensity of the optimal aqueous extracts used in both cells. This is also to confirm the results and reduce any form of interference from affecting it.

The results showed 0.25mg/ml RT, 0.75mg/ml CL and 0.50mg/ml FG aqueous plant extracts treated HUVECs demonstrate large extent of ROS scavenging ability especially RT in decreasing the ROS presence in the cells, with the rest of the selected plants exhibiting no or insignificant ROS scavenging ability. This indicates that high ORAC level plant extracts also exhibit prominent ROS scavenging ability which can be further characterized to identify its mechanism used to reduce the increase production of ROS. Insignificant decrease in the fluorescence intensity is observed for IM, CW, HF, RSC and BG plant extracts treated HUVECs but not Hela cells. This might be due to the different mechanisms of action of the extract exhibited in the HeLa cells and HUVECs. The low ORAC values found in IM, CW, HF, RSC and BG extract might also indicate that the plant extracts have insignificant or no antioxidant ability at all.

ROS scavenging properties of the plants is further characterised by measuring the SOD activity of the cells. An increase in SOD activity indicates that the cells contain a larger amount of SOD enzyme expression. SOD

helps to convert intracellular superoxide to H_2O_2. For the SOD sample preparation, the HeLa cells were seeded in 6-well plates. This is because protein concentration and SOD activity of the cell lysates extracted using 48-well plates were too low. Therefore, 6-well plates were chosen as it can accommodate a greater amount of cells and increase the yield of protein harvested from each well.

An increased intracellular SOD activity was observed in RT, CL, HF, CW, RSC, IM and BG plant extracts. Only FG-treated cells showed no increase in the SOD activity. However, FG has an ORAC value higher than most of the selected plants and the treated cells exhibited a decreased in fluorescence intensity in both HeLa cells and HUVECs. As demonstrated by Gifford *et al.*, [16], this implies that FG might contain bioactive compounds which can mimic the action of SOD enzymes and directly scavenge the ROS presence in the cells.

On the other hand, CL and RT plant extracts have the highest ORAC values. The plants were identified to show a decreased in ROS levels in both HeLa cells and HUVECs and also exhibited an increased in SOD activity. This indicated that the bioactive compound in both plant extracts can scavenge the ROS presence in the cells. Furthermore, the increased SOD activities in the cell can also suggest that both plant extracts can stimulate the cells to increase the synthesis of the SOD to help and cope with the increased ROS production due to hyperglycaemia.

IM, CW, HF, RSC and BG did not show any significant ORAC values and ability to decrease the ROS level in the cells. Moreover, cells treated with these plants were found to have an increase in SOD activities which further confirmed the plants do not have any ROS scavenging properties. The increased SOD activity might be produced by the cellular defence mechanism to cope with the increased ROS production. However, the increase in the ROS in the cells would have denatured SOD protein structure, which in turn affects the function of the enzymes resulting in a slight decreased in ROS level in the cells. Thus, the plants do not exhibit any significant antioxidant ability to reduce the ROS level in the cells.

5. Conclusions

In conclusion, CL, RT and FG were found to exhibit higher antioxidant activity due to their ability to reduce the increased ROS levels in hyperglycaemia-induced oxidative stress. Furthermore, CL and RT were identified to increase the enzymatic activity of the SOD enzyme. This shows that CL and RT can cause [1] direct scavenging/catalysing of the mitochondrial ROS and [2] indirect breakdown of the ROS by stimulating the production of SOD enzymes to catalyse the mitochondrial ROS. FG can only scavenge the mitochondrial ROS directly and does not increase the synthesis of SOD.

IM, RSC, HF, CW and BG were seen to have insignificant antioxidant activity because the plants did not show any reduction in the ROS levels. Furthermore, the high SOD activity in these plant-treated cells suggests that the plant extracts' bioactive compound did not contained any antioxidant properties. This may be due to the cellular defence mechanism stimulating the synthesis of SOD which was destructed by the ROS present in the cells. Therefore, the plants do not exhibit any significant antioxidant ability to reduce the ROS level.

Further evaluation and characterization of the plants' antioxidant mechanism for is required to establish these traditional medicinal plants as a more effective means of disease treatment. This will help to form a connection between preliminary investigations in a cell-line model and *in vivo* animal studies. It also serves as a rapid and convenient method for the pharmacological and biochemical screening of bioactive molecules before testing in animal models of greater physiological complexity. For further characterization, the plant's bioactive compound can be identified by doing chemical analysis such as high performance liquid chromatography. The identified active compound can then be isolated and re-tested on cell-line models and animal models to confirm its effectiveness. If successful, the therapeutic index of the bioactive compound can be established in animal models to identify and prevent any toxicity and harmful effects from occurring before testing the drugs in a clinical trial to monitor the effectiveness of the drugs on human. The antioxidant properties of the drug can be used to treat diseases which are associated with increased oxidative stress such as diabetes mellitus to reduce the patients' risks of contracting diabetes complications and also the adverse side-effects.

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