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Mushroom Extract Protects against Hydrogen Peroxide-Induced Toxicity in Hepatic and Neuronal Human Cultured Cells

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Abstract: Hydrogen peroxide is an oxidative stress agent that is associated with depletion of intracellular glutathione and inhibition of antioxidant enzymes in different cell lines. Consumption of antioxidant-rich foods reduces cellular oxidative stress and its related health problems. This study aimed to assess the antioxidant properties of mushroom, *Agaricus bisporus* cultivar extract, against hydrogen peroxide induced oxidative stress in cultured human hepatic (HepG2) and neuronal (SH-SY5Y) cells. In this study, hydrogen peroxide caused significant oxidative stress in HepG2 and SH-SY5Y cells as demonstrated by glutathione depletion, impairment of total antioxidant capacity and inhibition of antioxidant enzymes (glutathione peroxidase, catalase and superoxide dismutase). *Agaricusbisporous* extract ameliorated the observed hydrogen peroxide-induced oxidative cellular insult as indicated by restoring the activity of glutathione and the assayed antioxidant enzymes to control levels. The results suggest that mushroom extract as antioxidant properties and protects against the oxidative stress induced by hydrogen peroxide-in cultured human hepatic and neuronal cells.

Key words: Mushroom cultivar extract, oxidative stress, human cell lines

INTRODUCTION

Fruits and vegetables are rich in tocopherols, flavonoids, phenolic acids, alkaloids, chlorophyll derivatives, or carotenoids that possess high antioxidant capacity and have significant health benefits (Gulcin, 2012; Hu, 2011). Dietary antioxidants have demonstrated potential for quenching free radicals generated as a result of various environmental insults, in addition to their role in primary prevention of human diseases (Esfahani *et al.*, 2011; Baldrick *et al.*, 2011).

Many investigations have been carried out to identify functional foods containing biologically active substances with potential in combating free radical generation. Recently, edible mushroom extracts were used as functional foods as a consequence of their therapeutic effects and antioxidant properties (Wasser and Weis, 1999; Ozen *et al.*, 2011; Ozturk *et al.*, 2011; Pal *et al.*, 2010).

There is little information regarding the antioxidant properties of the mushroom cultivar, *Agaricusbisporous*, extract (MCE) therefore this study was conducted to evaluate the protective effect of MCE against hydrogen peroxide (H₂O₂)-induced oxidative stress in cultured human hepatic (HepG2) and neuronal (SH-SY5Y) cells.

Results of this study will shed light on the importance of MCE as an antioxidant agent and its biological activity.

MATERIALS AND METHODS

Chemicals and supplies: All chemicals and supplies, unless otherwise specified, were purchased from Sigma Chemical Co (St Louis, MI, USA).

MCE preparation: Samples of mushroom (*Agaricus bisporus*) cultivar were bought from Gulf Mushroom ProductsCo, Muscat, Sultanate of Oman and transported to the Food Science and Nutrition laboratory on the day of harvesting. Mushroom samples were freeze dried in a freeze dryer for 3 days at -40°C Dried samples were ground to a fine powder using a mechanical grinder. Subsequently, the freeze dried powdered sample (5 g) was mixed with 100 mL of methanol, using a magnetic stirrer and incubated at room temperature for 24 h. The extracts were then filtered and centrifuged at 6000 g for 30 min at 4°C using a Sanyo, Harrier MSE centrifuge, 18/80 Rotor No. AO9R8326. After centrifugation, the supernatant was collected and concentrated under reduced pressure at 40°C for 3-4 h using a rotary evaporator to obtain the

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mushroom crude extract, which was stored in dark glass bottles at -40°C until used.

Chemical composition of MCE: Samples were analyzed for chemical composition (moisture, protein, fat, carbohydrates and ash) using the Association of Official Analytical Chemists (AOAC) procedures (Horwitz and Latimer, 2005). The moisture content was determined by heating 1 g of sample in a thermostatically controlled oven to a constant weight at 105°C ; the crude protein content ($N \times 4.38$) of the samples was estimated by the macro Kjeldahl method (Horwitz and Latimer, 2005). The crude fat was determined by extracting a known weight of powdered mushroom extract sample with petroleum ether, using Soxhlet apparatus; the ash content was determined by incineration at 550°C for 24 h.

Total carbohydrates were calculated by the difference:

$$\text{Total carbohydrates} = 100 - (\text{g moisture} + \text{g protein} + \text{g fat} + \text{g ash})$$

Total polyphenol content of MCE: Total phenolic content was measured by the Folin-Ciocalteu assay of Singleton and Rossi with slight modifications (Singleton and Jr. Rossi, 1965). In brief, 10 μL of mushroom extract was placed in a test tube and 3 mL of distilled water was added to it. Then, 250 μL Folin-Ciocalteu reagents were added and the mixture was vortexed. After a short, 5 min, incubation period 750 μL of 1.9 mmol L^{-1} sodium carbonate was added. Then, 990 μL of water was added to make a total volume of 5 mL. The final reaction mixture was incubated for 2 hrs at room temperature. The absorbance at 765 nm was measured and compared with Gallic Acid standards. The phenolic content of the mushroom crude extract was expressed as mg Gallic Acid Equivalents.

Total flavonoids content of MCE: Flavonoids were determined according to the aluminum chloride colorimetric assay of Kim *et al.* (2003). Distilled water (4 mL) was added to 1 mL of mushroom extract; then, 5% sodium nitrite solution (0.3 mL) was added, followed by 10% aluminum chloride solution (0.3 mL). The mixtures were incubated at ambient temperature for 5 min and then 2 mL of 1 mmol L^{-1} sodium hydroxide were added. Immediately, the volume of reaction mixture was made to 10 mL with distilled water. The mixture was thoroughly vortexed and the absorbance of the pink color developed was determined at 510 nm. A calibration curve was prepared with Catech in and the results were expressed as mg Catechin Equivalents.

Cell culture : Cultured human hepatic (HepG2) hepatic and neuronal (SH-SY5Y) cells were purchased from the American Type Culture Collection (ATCC; Manassas,

VA, USA) and were frozen in liquid nitrogen before culturing. Cells were grown as monolayer in 10-cm petri dishes that contained 10 mL of DMEM (for HepG2 cells) or α -MEM (for SH-SY5Y cells), supplemented with 1% PSF and 10% FBS. Cultured cells were placed in an incubator chamber containing humidified 95% air and 5% CO_2 at 37°C . For passage, confluent cells were detached with 1.0 mL trypsin-EDTA solution. They were then re-suspended in 12 mL of cell culture medium and seeded at 100,000 cells per well in six-well culture plates, then incubated for 24 h before conducting the experiment.

The experimental design of this study was to incubate HepG2 and SH-SY5Y cells, for 60 min with either different doses of H_2O_2 (an oxidative stress-inducing agent) or 1 mmol/L L-buthione-[S,R]-sulfoximine (BSO), a selective inhibitor of glutathione *in-vivo* synthesis (Griffith *et al.*, 1979), in the presence or absence of MCE. After treatment, the cells were washed with modified Krebs's solution, scraped, pelleted and re-suspended in 1 mL of 100 mmol L^{-1} phosphate buffer, pH 7.4. Cell membranes were disrupted by sonication on ice and the cell lysates centrifuged at 4°C , at 10,000 g, for 20 min. Supernatants were separated from cell debris and used for subsequent biochemical assay measurements. All measurements were normalized to the protein content of the cell lysates.

Oxidative stress biochemical assays: The following parameters were measured in the cell lysates using assay kits and according to the manufactures' instructions: glutathione (GSH) concentration with glutathione assay kit (K251, Biovision, Mountain View, CA, USA), Total Antioxidant Capacity (TAC) using Randox assay kit (Randox Laboratories Crumlin, UK), glutathione peroxidase (GPx) assay kit (Oxis International, Inc., Foster City, CA, USA), catalase (CAT) assay kit (Cayman Chemical Co., Ann Arbor, MI, USA), superoxide dismutase (SOD) assay kit (Cell Technology Inc., Mountain view, CA, USA).

Protein measurement: The protein content of cell lysates was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard and protein contents were expressed as mg mL^{-1} of sample.

Cells viability assay: MTS [-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], in the presence of phenazine methosulfate (PMS), is chemically reduced into formazan product, which is soluble in tissue culture medium and its color absorbance can be measured at 490-500 nm (Malich *et al.*, 1997). A colorimetric method using an MTS assay kit (Cell Titer 96[®] Aqueous Non-Radioactive Cell Proliferation Assay; Promega Corporation, Madison, WI, USA) was

carried out using 96-well microtitreplates and the production of formazan was followed at 490 nm using a standard 96-well plate reader. The intensity of the color produced was proportional to the number of living HepG2 or SH-SY5Y cells.

Statistical analysis: Statistical analysis was performed using GraphPad Prism (version 5.03; GraphPad Software Inc., San Diego, CA, USA). The results are expressed as Means±Standard Error of Means (SEM). Comparisons of groups were performed using one-way analysis of variance, followed by Tukey's test. The Student's unpaired t-test was used for pair wise comparisons. The values at the level of $p < 0.05$ were considered to be significantly different.

RESULTS

Table 1 represents the chemical analysis of Mushroom Cultivar Extract (MCE). Data reveals that MCE is a good source of total polyphenols content (234.53 ± 3.54 Gallic Acid Equivalents mg g^{-1}), flavonoids content (0.76 ± 0.05 Catechin Equivalents $0.76 \pm 0.05 \text{ mg g}^{-1}$). The chemical composition shows also that MCE has high contents of carbohydrates and crude proteins with average values of 59.92 ± 0.45 and $34.63 \pm 0.31 \text{ mg g}^{-1}$ respectively. The moisture, crude fat and total ash contents of MCE were 14.35 ± 0.43 , 2.94 ± 0.28 and $9.51 \pm 0.87 \text{ mg g}^{-1}$, respectively.

Effect of H_2O_2 and MCE on cells viability and GSH depletion: H_2O_2 decreased HepG2 and SH-SY5Y cells viability in a dose-dependent manner. Figure 1a shows that the cells viability decreased gradually and reached a plateau of cells death at a concentration of H_2O_2 as high as 0.1%. Treatment of HepG2 and SH-SY5Y cells with H_2O_2 caused a dose-dependent decrease in GSH concentration, where the intracellular GSH concentration depleted gradually as the H_2O_2 concentration increased and reached a plateau of depletion at 0.1% H_2O_2 (Fig. 1b).

Table 1: Chemical analysis of Mushroom cultivar extract

| Parameters | Values |
|---|-------------------|
| Moisture content (%)* | 14.35 ± 0.43 |
| Crude Fat (%)* | 2.94 ± 0.28 |
| Crude Protein (%)* | 34.63 ± 0.31 |
| Total ash content (%)* | 9.51 ± 0.87 |
| Carbohydrate (%) | 52.92 ± 0.45 |
| Polyphenol content/Gallic Acid Equivalents (mg g^{-1}) | 234.53 ± 3.54 |
| Flavonoids content/Catechin Equivalents (mg g^{-1}) | 0.76 ± 0.05 |

*Chemical Composition/100g of sample. All the measurements were taken in triplicate and the mean values were calculated. Data expressed as Means±SEM

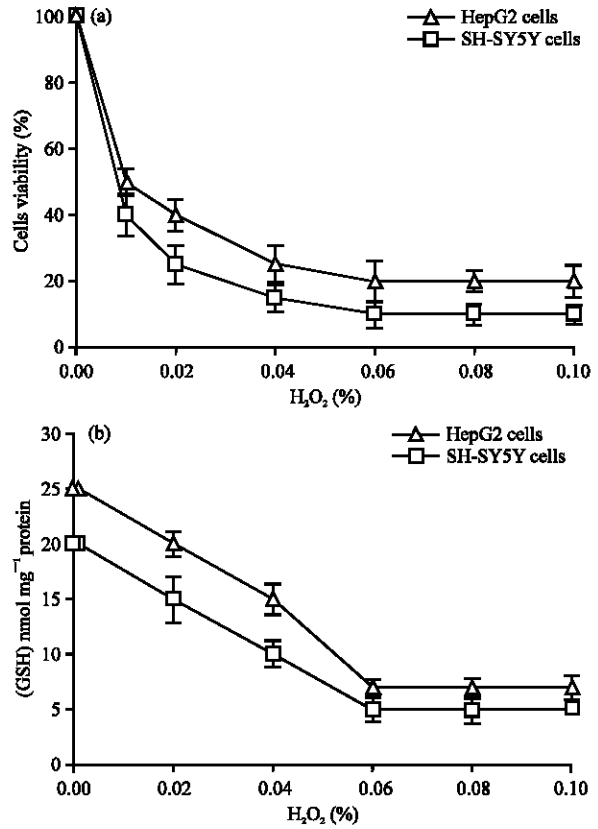


Fig. 1(a-b): Dose-dependent decrease of hydrogen peroxide (H_2O_2) on hepatic (HepG2) and neuronal (SH-SY5Y) cells viability (a) and on glutathione level (b). HepG2 and SH-SY5Y cells were treated for 60 min with different doses of and then subjected to MTS assay and GSH measurement. H_2O_2 decreased cells viability and induced GSH depletion in a dose-dependent manner and reached a plateau of inhibition at a concentration as high as 0.1%. Cell viability is expressed as percent of control. Results are the Means±SEM of six measurements

As presented in Fig. 2, in a dose-dependent manner, MCE ameliorated the 0.1% H_2O_2 -induced cytotoxicity in hepatic and neuronal cells and significantly increased cell viability and its protective effect reached a plateau at a concentration of $100 \mu\text{g mL}^{-1}$ (Fig. 2). In addition $100 \mu\text{g mL}^{-1}$ MCE completely (100%) counteracted the GSH depletion in the 0.1% H_2O_2 -treated cells. Therefore, for all subsequent experiments, $100 \mu\text{g mL}^{-1}$ MCE was applied to explore its antioxidant properties.

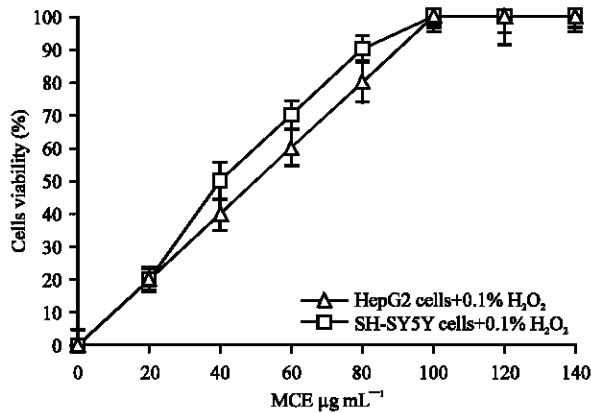


Fig. 2: Dose-dependent effect of Mushroom Cultivar Extract (MCE) against 0.1% H₂O₂-induced hepato- and neuro-cytotoxicity. HepG2 and SH-SY5Y cells were treated for 60 min with 0.1% H₂O₂ in the presence of different concentrations of MCE (20, 40, 60, 80, 100, 120 and 140 µg mL⁻¹) and then subjected to a MTS assay. MCE, in a dose-dependent manner, ameliorated the 0.1% H₂O₂-induced cells death and significantly restored 100% of cells viability plateau at a concentration of 100 µg mL⁻¹. Cells viability are expressed as percent of control, cells treated with 0 µg mL⁻¹ MCE and 0% H₂O₂. Results are the Means±SEM of six measurements

Table 2: Protective effect mushroom cultivar extract against hydrogen peroxide-induced impairment of oxidative stress markers in HepG2 hepatic cells

| Parameter | Control | MCE | H ₂ O ₂ | H ₂ O ₂ +MCE |
|----------------------------------|-----------|-----------|-------------------------------|------------------------------------|
| CAT (µmol/min mg ⁻¹) | 85.47±3.6 | 82.96±2.4 | 18.46±1.4 ^a | 81.12±4.6 |
| GPX (µmol/min mg ⁻¹) | 2.14±0.5 | 2.31±0.8 | 0.68±0.2 ^b | 2.51±0.7 |
| SOD (µmol/min mg ⁻¹) | 41.21±4.8 | 42.11±5.3 | 9.15±1.4 ^c | 41.41±6.6 |
| TAC (nmol mg ⁻¹) | 96.12±7.2 | 93.94±5.6 | 21.68±3.5 ^d | 94.94±7.3 |
| GSH (nmol mg ⁻¹) | 20.27±1.2 | 19.86±1.6 | 5.36±0.5 ^e | 20.17±1.1 |

H₂O₂: Hydrogen peroxide; MCE: Mushroom cultivar extract; CAT: Catalase; GPX: Glutathione peroxidase; SOD: Superoxide dismutase; TAC: Total antioxidant capacity; GSH, glutathione, HepG2 cells were incubated for 60 min with 0.1% H₂O₂ in the presence or absence of 100 µg mL⁻¹ MCE. Data expressed as Means±SEM of six measurements, MCE was compared with control group and there was no statistical significant difference, p>0.05, H₂O₂, MCE and H₂O₂ + MCE groups were compared with control group. Data with (a, b, c, d and e) superscripts are significantly lower than control group, p<0.05. Whereas, data with no superscripts are not statistically significant different

Effect of H₂O₂ and MCE on oxidative stress markers of hepatic and neuronal cells: The data presented in Table 2 and 3 illustrates that treatment of HepG2 or SH-SY5Y cells with 0.1% H₂O₂ caused inhibition of the measured antioxidant enzymes (CAT, GPx and SOD) and induced GSH depletion and TAC impairment to a level that is significantly lower than the controls groups

Table 3: Protective effect of mushroom cultivar extract against hydrogen peroxide-induced impairment of oxidative stress markers in SH-SY5Y neuronal cells

| Parameter | Control | MCE | H ₂ O ₂ | H ₂ O ₂ +MCE |
|----------------------------------|------------|------------|-------------------------------|------------------------------------|
| CAT (µmol/min mg ⁻¹) | 94.63±4.7 | 93.78±3.5 | 20.36±2.3 ^a | 93.96±5.1 |
| GPX (µmol/min mg ⁻¹) | 3.51±0.9 | 3.49±0.7 | 0.79±0.3 ^b | 3.64±0.6 |
| SOD (µmol/min mg ⁻¹) | 50.36±7.1 | 51.62±6.7 | 10.71±4.9 ^c | 50.78±7.3 |
| TAC (nmol mg ⁻¹) | 104.32±9.4 | 103.45±8.3 | 25.93±9.7 ^d | 104.13±8.7 |
| GSH (nmol mg ⁻¹) | 25.19±2.5 | 25.23±2.1 | 7.18±1.5 ^e | 24.75±1.9 |

H₂O₂: Hydrogen peroxide; MCE: Mushroom cultivar extract; CAT: Catalase; GPX: Glutathione peroxidase; SOD: Superoxide dismutase; TAC: Total antioxidant capacity; GSH, Glutathione, SH-SY5Y neuronal cells were incubated for 60 min with 0.1% H₂O₂ in the presence or absence of 100 µg mL⁻¹ MCE. Data expressed as means±SEM of six measurements, MCE was compared with control group and there was no statistical significant difference, p>0.05, H₂O₂, MCE and H₂O₂+MCE groups were compared with control group. Data with (a, b, c, d and e) superscripts are significantly lower than control group, p<0.05. Whereas, data with no superscripts are not statistically significant different

(p<0.05). In addition, concomitant treatment of cells with 100 µg mL MCE protected cells against the 0.1% H₂O₂-induced hepato and neuro- toxicities and ameliorated the inhibited antioxidant enzymes and restored GSH and TAC to their basal level.

Effect of BSO and MCE on cells viability and GSH depletion: BSO at a concentration of 1 mmol L⁻¹ significantly (p<0.05) decreased cell viability and induced a depletion of the intracellular GSH concentration, whereas the concomitant treatment of cells with 100 µg mL⁻¹ MCE restored cell viability up to 100% and reversed the BSO-induced GSH depletion (Fig. 3a-d).

DISCUSSION

Overproduction of free radicals is considered as one of the etiological factors contributing to aging and the development of non-communicable diseases. GSH is biosynthesized within cells and it primarily provides cells with a reducing milieu that maintains the thiol groups of intracellular proteins and keeps antioxidants such as ascorbate and α-tocopherol in an active state (Deneke, 2000; DiMascio *et al.*, 1991). GSH is the major intracellular antioxidant which can undergo oxidation to the disulfide (GSSG), oxidized form, when scavenging reactive oxygen species and a reduced GSH/GSSG ratio is an indication of oxidative stress.

H₂O₂ is an oxidizing agent that generates free radicals and causes GSH-depletion which is associated with a reduction in the activity of antioxidant enzymes including CAT, GPx and SOD (Guizani *et al.*, 2011; Ha *et al.*, 2010; Tarozzi *et al.*, 2009). The SOD enzyme is highly efficient at dismutating O₂^{•-} to H₂O₂; CAT catalyzes removal of H₂O₂ to stable O₂ and GPx removes H₂O₂ by

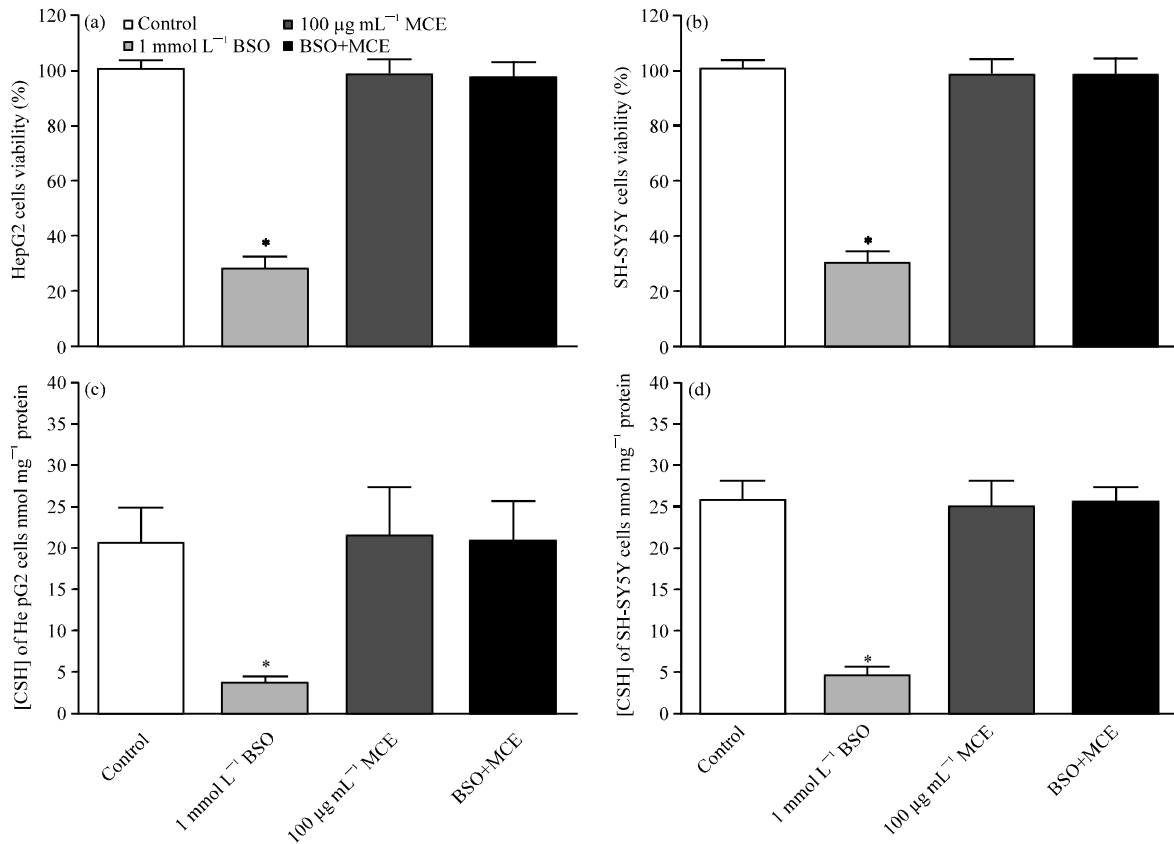


Fig. 3(a-d): Effects of BSO and Mushroom Cultivar Extract (MCE) on cells viability (a and b) and GSH levels (c and d) of HepG2 and SH-SY5Y cells, the x-axis represents the tested groups. Cells were treated for 60 minutes with 1 mmol L⁻¹ BSO in the presence or absence of 100 µg mL⁻¹ MCE and then subjected to a MTS assay for GSH assay measurements. Concomitant treatment of cells with 100 µg mL⁻¹ MCE restored the BSO-induced hepato- and neuro-cells death up to 100% and reversed the BSO-induced GSH depletion. Results are the Means±SEM of six measurements. *Significantly lower than the control group (p<0.05)

coupling its reduction to H₂O with oxidation of GSH into an inactive form, GSSG (Halliwell and Gutteridge, 2007).

In this study, depletion of hepato- and neuro-cellular GSH has been induced by acute treatment of cells with either H₂O₂ or BSO, a selective inhibitor of the γ-glutamylcysteine synthetase, which catalyzes the rate-limiting step of GSH *de-novo* synthesis (Griffith *et al.*, 1979). The results indicate that concomitant treatment of hepatic and neuronal cells with 100 µg mL⁻¹ MCE reversed the BSO and H₂O₂-induced GSH depletion. Based on the fact that in contrast to H₂O₂, BSO induces GSH depletion in a mechanism that does not involve oxidation or free radicals attack, a potential interpretation of the observed protective effects of MCE is that MCE targets both the GSH *de-novo* synthesis pathway and protects the cell membrane against oxidative damage generated by the oxidizing agent, H₂O₂.

CONCLUSION

Collectively, in the studied cell lines, MCE appears to be of significance in the cellular recovery of GSH and the subsequent improvement of the cellular redox milieu as evident by restoring the inhibited antioxidant enzymes and augmented TAC status. Research derived from this study has suggested that MCE acts as a potent antioxidant and improves the hepato- and neuro-cellular redox capacity in a mechanism that involves combating oxidative stress. This finding is an asset to establish a base line data concerning the antioxidant biological activities of MCE, a topic that has very limited information in the literature and further *in-vivo* studies are needed to clearly demonstrate the beneficial role of MCE against hepato- and neuro-toxicities.

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