

Full Length Research Paper

***Monechma ciliatum* methanolic extract regulates low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase genes expression in HepG2 cells**

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***Monechma ciliatum* methanolic extract (MCME) obtained from *Monechma ciliatum* seedcake showed high total phenolic compounds with high antioxidant activity. The regulatory effects of MCME at 10, 20 and 50 µg/ml on low density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) were investigated in human HepG2 cell line using quantitative real-time polymerase chain reaction. LDLR mRNA level was increased significantly by 1.4, 2.6 and 4.3 fold in MCME treated cells at 10, 20 and 50, respectively, compared to untreated cells. Whereas, HMGCR mRNA level was decreased significantly by 38, 63 and 80% in MCME treated cells at 10, 20 and 50, respectively, compared to untreated cells. The effect of MCME was concentration dependent, and different doses showed significant differences in regulation of both LDLR and HMGCR genes. The present study showed that MCME effectively regulated the expression of LDLR and HMGCR genes influencing the cholesterol metabolism in HepG2 cells.**

Key words: Antioxidant activity, β -carotene-linoleic acid assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH), gene expression, low density lipoprotein receptor, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *Monechma ciliatum*.

INTRODUCTION

Monechma ciliatum (also known as black mahlab in Sudan), a member of family Acanthaceae, is an annual glabrous herb, 30 - 65 cm high. The seeds are used as flavoring material in different food products e.g. Kisra (traditional fermented sorghum flour sheets) and bread. The seeds are also used as an effective laxative, and contain a fixed oil which emits a sweet and pleasant odor.

It is further used in traditional Sudanese fragrances, lotion and other cosmetics used for wedding preparation and childbirth (Sharief, 2001). *M. ciliatum* was used in remedy of general body pain, liver, cold, diarrhea and sterility in women and its leaves methanolic extract showed oxytocic property *in vivo* and *in vitro* (Ayoub and Babiker, 1981; Uguru, et al., 1995). Previous studies clearly indicate that methanolic extract of *M. ciliatum* has high phenolic compounds with high antioxidant activity (Mariod et al., 2010a,b). There is an increasing interest in polyphenols due to their potentially positive effect against certain diseases, namely: mainly some forms of cancer and coronary heart diseases, and they have been

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reported to have *in vitro* antioxidant activities, even higher than the common antioxidants, vitamin C and E (Rice-Evans et al., 1997). Salleh et al. (2002) examined polyphenol-rich extracts of 12 edible plants for their potential to inhibit low density lipoprotein (LDL) oxidation and the modulation (up- or down-regulate) of low density lipoprotein receptor (LDLR) activity in HepG2 cells, and they found that most plant extracts studied demonstrated a positive association between antioxidant activity and the ability to up-regulate LDL receptor.

The human HepG2 cell line is considered a good model for studying the regulation of hepatic low density lipoprotein cholesterol (LDLC) catabolism, cholesterol metabolism, lipid synthesis and lipoprotein synthesis since they perform several of the normal biochemical functions specific to liver cells (Marc et al., 2004). LDLR is known to primarily regulate exogenous cholesterol by removing more than 70% of the human LDLC from the blood circulation (Sebelyl et al., 2003). Cholesterol synthesis is controlled by the regulation of genes encoding cholesterol synthetic enzymes, especially HMGCR which is the rate-limiting enzyme in this process (Brown and Goldstein, 1986). The up-regulation of LDLR and the down-regulation of HMGCR are key mechanisms that control elevated plasma LDLC (Brown and Goldstein, 1997). Inhibitors of HMGCR, also known as statins, are commonly used in the treatment of hypercholesterolemia, an important risk factor for coronary heart disease and stroke (Gould et al., 2007). Our group previous study (Al-Nageeb and Ismail, 2009) clearly showed that thymoquinone rich fraction extracted from *Nigella sativa* seeds using a supercritical fluid extraction technique and pure commercial thymoquinone are effective in regulating Apo A-1 and Apo B100 genes that influence cholesterol metabolism in HepG2 cells.

In this study, we investigated the effects of MCME on the regulation of genes encoding the LDLR and HMGCR genes in HepG2 cells. In the first part of this study, the cytotoxic effect of MCME with respect to the growth of HepG2 cells was determined using the 3-(4,5-dimethylthiazole-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay in order to select the appropriate doses that resulted in cell viability that was greater than 70% in order to perform gene expression experiments. In the second part of this study, MCME activity on the regulation of LDLR and HMGCR genes was investigated using real-time polymerase chain reaction (PCR) with a TaqMan assay.

MATERIALS AND METHODS

Sample material, standards and reagents

All solvents used were of HPLC grade, including methanol, ethyl acetate, hexane, chloroform, butylated hydroxyanisole (BHA), β -carotene, linoleic acid and Folin-Ciocalteu reagent as well as polyoxyethylene sorbitan monopalmitate (Tween 40), which were purchased from Fisher Scientific (Fisher Scientific Co Ltd., Ottawa,

ON). Gallic acid was obtained from Fluka Chemie AG (Buchi, Flawil, Switzerland). Dulbecco's minimum essential medium (DMEM), FBS, penicillin, streptomycin, trypsin, human lipoprotein deficient serum (HLPDS) were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Co., St. Louis, MO). MTT, sodium bicarbonate and PBS, were also purchased from Sigma-Aldrich Co. (Sigma-Aldrich Co., St. Louis, MO). RiboPure™ RNA isolation kit was purchased from Ambion (Ambion, Austin, TX). First Strand cDNA Synthesis Kit was purchased from Ferments (MBI Ferments Inc, Vilnius, Lithuania). The Quantict Probe Real-Time PCR master mix was purchased from Qiagen (Valencia, CA) while the oligo (dT) primer and probes were supplied by Sigma-Aldrich (St. Louis, MO) and synthesized by Integrated DNA Technologies, Inc (San Diego, CA).

M. ciliatum seeds were obtained from a local store at Khartoum North, Sudan. The seeds were cleaned under running tap water for 10 min, rinsed twice with distilled water and air-dried in oven at 40°C for overnight. The seeds were grinded to powder using an electric grinder (National, Model MX-915, Kadoma, Osaka, Japan) for 10 min and then passed through a 35 mm (42 mesh) sieve. The cakes were obtained after oils were extracted from the ground seeds by extraction with *n*-hexane (bp 50 - 60°C) using Soxhlet apparatus for 6 h following the AOCS (1993) method Aa 4 - 38 and the obtained seedcakes were used to extract phenolic compounds.

Extraction of phenolic compounds

Twenty grams of the dried ground seedcakes from *M. ciliatum* were extracted with 80% methanol by sonication (Hwasin Technology, Seoul, Korea) to obtain *M. ciliatum* methanolic extract (MCME) with solid to solvent ratio of 1:10 (w/v) at room temperature for 1 h, the obtained extract was homogenized at 13000 rpm for 15 min followed by sonication (Hwasin Technology, Seoul, Korea) at constant temperature of 30°C for 1 h. The MCME was filtered through filter paper Whatman no 1. Then solvent was removed by using rotary evaporator (Buchi, Flawil, Switzerland). The yield of the extract was measured before kept at -80°C for further analysis.

Determination of TPC in MCME

The total phenolic content (TPC) of MCME was determined using the Folin-Ciocalteu reagent (Zhou and Yu, 2006). The reaction mixture contained 1 ml of MCME and each individual fraction, 0.5 ml of the Folin-Ciocalteu reagent, 3 ml of 20% sodium carbonate and 10 ml of distilled water. After 2 h of reaction at ambient temperature, the absorbance at 765 nm was measured using spectrophotometer (Shimazu, Co., Ltd., Kyoto, Japan) and used to calculate the phenolic contents, using gallic acid as a standard. The total phenolic contents were then expressed as gallic acid equivalents (GAE) in mg/g dry sample.

Antioxidant activity (AOA) measurement

The antioxidant activity (AOA) of CMME was addressed using the following methods.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test

The antioxidant activity of the extract, on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Gordon et al. (2001). A methanolic solution (100 μ l) of the extract was placed in a cuvette and 0.5 ml of a methanolic solution of DPPH (50 mg DPPH/100 ml MeOH) was added. After 30 min,

Table 1. Regulation of LDLR gene by MCME in HepG2 cells incubated with 10% HLPDS.

Group	Average C _T of LDLR	Average C _T of beta actin	ΔC _T	ΔΔC _T	2 ^{-ΔΔC_T} (Fold)
Control	22.74 ± 1.34	19.55 ± 1.56	3.19	0	1
10 μg/ml MCME	22.63 ± 1.52	19.91 ± 0.97	2.72	-0.47	1.4
20 μg/ml MCME	20.22 ± 0.78	18.39 ± 0.89	1.83	-1.36	2.6
50 μg/ml MCME	19.59 ± 0.56	18.52 ± 0.94	1.07	-2.12	4.3

Results are expressed as means ± SD (n = 3). ΔC_T = ΔC_T LDLR- ΔC_T of β-actin, ΔΔC_T = ΔC_T treated cells - ΔC_T control.

absorbance at 515 nm was determined using a spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan), and IC₅₀, was reported which is the concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time period. All determinations were performed in triplicate.

The β-carotene–linoleic acid assay

The antioxidant activity (AOA) of the MCME was evaluated using the β-carotene–linoleic acid assay following the method of Amarowicz et al. (1993). In brief, a solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two milliliters of this solution were pipetted into a 100 ml round-bottom flask. After chloroform was removed under vacuum, using a rotary evaporator at 40°C, 40 mg of purified linoleic acid, 400 mg of Tween-40 as an emulsifier, and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into a series of tubes containing 200 μl of the extract (200 ppm in methanol). The total volume of the system was adjusted to 5 ml with methanol. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm with a Shimadzu spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). Sub-sequent absorbance readings were recorded over a 2 h period at 20 min intervals by keeping the samples in a water bath at 50°C. Blank samples devoid of β-carotene, were prepared for background subtraction

Measurement of cell viability

In order to avoid a cytotoxic effect of MCME on HepG2 cell proliferation, the cytotoxicity study was carried out using the MTT proliferation assay. HepG2 cells were treated with different concentrations of MCME in order to select the appropriate doses that resulted in cell proliferation greater than 70% to perform the gene expression experiments.

HepG2 cells were plated in 96 well plates at a density of 1 × 10⁵ cells per well, incubated overnight at 37°C in a humidified atmosphere including 5% CO₂. MCME was dissolved in DMSO and DMSO concentration was controlled not to be more than 0.1%. Then, the extract was further diluted in Dulbecco's minimum essential medium (DMEM) to obtain a range of concentrations between 12.5 and 100 μg/ml. The cells were exposed to the extract for 72 h. After this incubation period, 20 μl MTT (5 mg/ml) were added into each well and the plates were then incubated for an additional 4 h. Absorbance was recorded at 490 nm by microplate reader (Opsys MR, Thermo LabSystems, Franklin, MA, USA). Cells viability was calculated by using this formula:

$$\% \text{ Viability} = \frac{\text{Absorbance samples} - \text{Absorbance blank}}{\text{Absorbance control} - \text{Absorbance blank}} \times 100$$

The dose-response curve was plotted and the concentration which gave 50% of cell growth (IC₅₀) was calculated.

Gene expression study

HepG2 cells were seeded in a 6 well plate in DMEM, supplemented with 10% fetal bovine serum (FBS) at a density of 1 × 10⁵ cells/well. Twenty-four hours before the start of experiment, the medium was changed to DMEM supplemented with 10% HLPDS, then, the cells were divided into 2 groups. Each group of the cells was further divided into 4 groups, the first of which was treated with MCME at dose of 10 μg/ml, the second group was treated with MCME at dose of 20 μg/ml, the third group was treated with MCME at dose of 50 μg/ml and the fourth group was not treated and served as a control group.

RNA extraction and cDNA synthesis

The total RNA was isolated from HepG2 cells using the RiboPure RNA isolation kit according to the manufacturer's instructions. The purity of the extracted RNA was determined by measuring the ratio of the optical density at 260 and 280 nm using a spectrophotometer (BioRad, USA). The total RNA concentration was determined by measuring the absorbance at 260 nm. The integrity and size distribution of the total RNA was determined by using a 1.5 agarose gel. The 18S and 28S RNA bands were visualized under UV light using gel image instrumentation. RNA samples were reverse transcribed into first-strand cDNA using the First Strand cDNA Synthesis Kit #K1612 (Ferments), according to the manufacturer's instructions.

Quantitative real-time PCR

TaqMan Primers and probes specific for LDLR, HMGCR and β-actin were designed from human (Homo sapiens) adapted from the NCBI (National Center for Biotechnology Information) GenBank Database (www.ncbi.nlm.nih.gov), synthesized by Integrated DNA Technologies, Inc (San Diego, CA) and supplied by Sigma Aldrich. GenBank accession number code for LDLR is BC014514, for HMGCR is BC033692 and for beta actin gene is EF095209 (Table 1). Real-time PCR was performed using the Quantict probe real time PCR master mix according to the manufacturer's instructions. The real-time quantitative PCR for each sample was performed in triplicate. Briefly, a reaction (25 μl) containing 12.5 μl of master mixed with 2 μl of 400 nM of each forward and reverse primer, 1 μl of the probe (200 nM) and 1 μl of the template cDNA (10 ng/μl).

Real-time PCR amplification of cDNA was performed for 40 cycles. After an initial incubation for 15 min at 95°C the PCR cycle consisted of a denaturation period for 15 s at 94°C and an annealing period for 60 s at 50°C. Analysis of the gene expression data was performed using a ΔΔC_T method of relative quantification, according to a previous report by Kenneth et al. (2001). RotorGene analysis software (version 6.0) was used to analyze all the results from the PCR assays.

Statistical analysis

Each experiment was repeated three times and the data are

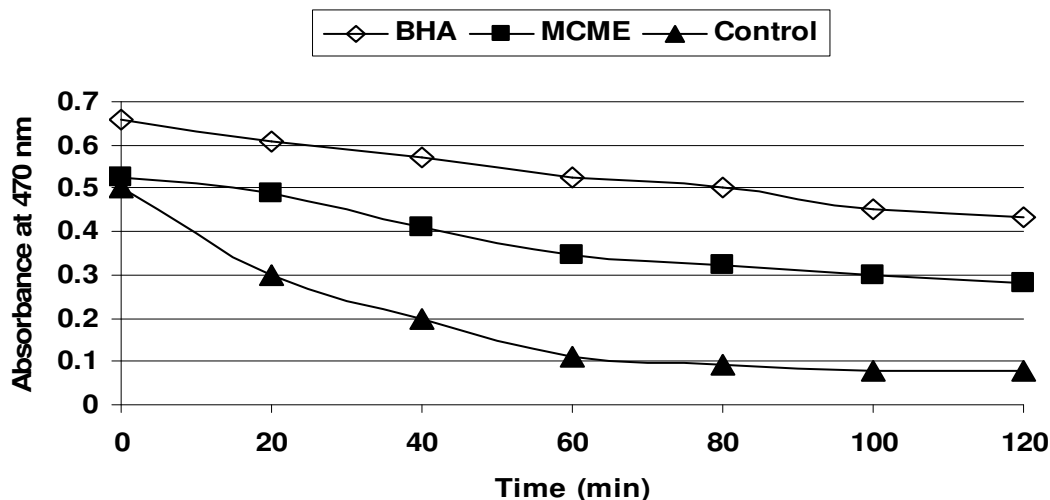


Figure 1. Effect of MCME on oxidation of β -carotene/linoleic acid at 50°C. BHA: butylated hydroxyanisole, MCME: *Monechma ciliatum* methanolic extract.

reported as the mean \pm SD that was analyzed using the SPSS window program version 14.0. A one-way ANOVA was used to compare the results from different treatments and control cells. The statistical significance was indicated by P values <0.05 .

RESULTS AND DISCUSSION

Total phenolic and antioxidant activity

The content of the total phenolic compounds of MCME was found to be 17.1 ± 0.04 (mg GAE/g extract). This finding was in agreement with the results of our previous study (Mariod et al., 2009). The DPPH radical scavenging capacity assay was used to examine the antioxidant activity of MCME. The extract was assayed over a range of dilutions to establish the concentration of the extract required to scavenge 50% of the DPPH radical present in the assay medium, referred to as the IC_{50} and defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Lower IC_{50} value reflects better DPPH radical scavenging activity (Molyneux, 2004). Under the assay conditions employed here, the IC_{50} of ascorbic acid as standard was $0.017 \mu\text{g/ml}$, and the MCME possessed lower DPPH radical scavenging activity with the lowest IC_{50} value of $0.450 \pm 0.1 \mu\text{g/ml}$. In the BCB assay, the oxidation of linoleic acid generates peroxy free radicals is due to the abstraction of hydrogen atom from diallylic methylene groups of linoleic acid (Kumaran and Karunakaran, 2006). The free radical then oxidizes the highly unsaturated β -carotene. The presence of antioxidants in the extract will minimize the oxidation of β -carotene by hydroperoxides. Hydroperoxides formed in this system will be decomposed by the antioxidants from the extracts. Thus, the degradation rate of β -carotene depends on the antioxidant activity of the extracts. Most studies showed that there was no correlation between TPC and BCB

(Amarowicz et al., 1993; Matthaüs, 2002; Mariod et al., 2006).

The effect of MCME on oxidation of β -carotene/linoleic acid at 50°C is shown in Figure 1. It was clear that the presence of antioxidants in the MCME reduced the oxidation of β -carotene by hydroperoxides from this extract. There were significant differences ($P < 0.05$) between the extract, control and BHA effect as shown in Figure 1. MCME was better in their effect on reducing the oxidation of β -carotene than control but it was less in antioxidant activity than BHA and its degradation rate of β -carotene clearly depends on its antioxidant activity.

Cell viability assay

The cell viability assays were performed in order to demonstrate the cytotoxic effects of MCME. In order to avoid a strong reduction of cell viability, the cytotoxicity study was carried out using MTT assay by treating HepG2 cells with different concentrations of MCME. The growth of the HepG2 cells in the presence of various concentrations of MCME (12.5 - $100 \mu\text{g/ml}$) was examined. Under the experimental conditions, MCME exhibited growth inhibitory effects on HepG2 cells over a 72 h period. The IC_{50} for HepG2 cells was approximately $83 \mu\text{g/ml}$ as shown in Figure 2.

Regulation of LDLR gene by MCME

From the amplification plot for LDLR gene (raw data not shown), the β -actin gene had C_T values ranging from 18 - 19 cycles and LDLR gene had C_T values between 19 - 23 cycles. The effects of MCME at 10, 20 and $50 \mu\text{g/ml}$ on the regulation of LDLR gene are shown in Table 1. Triplicate results were summarized in Figure 3. When

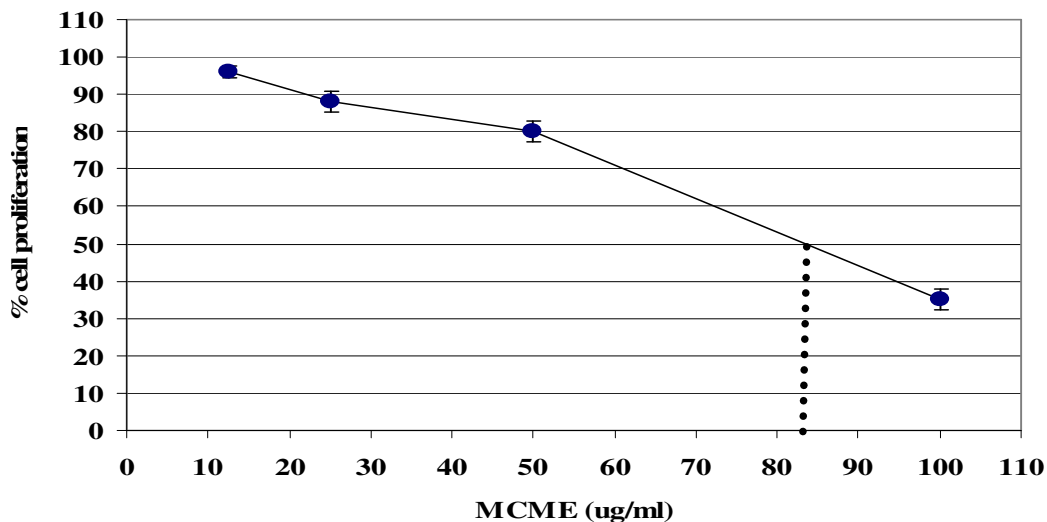


Figure 2. Cytotoxic effect of MCME against HepG2 cells. IC₅₀ values (70 µg/ml) obtained after 72 h using MTT assay. HepG2 cells were seeded into 96-well plate and incubated overnight and then cells were exposed to medium containing different concentrations (12.5 - 100 µg/ml) of MCME for 72 h. Each value presents the average of 6 replicates ± SD.

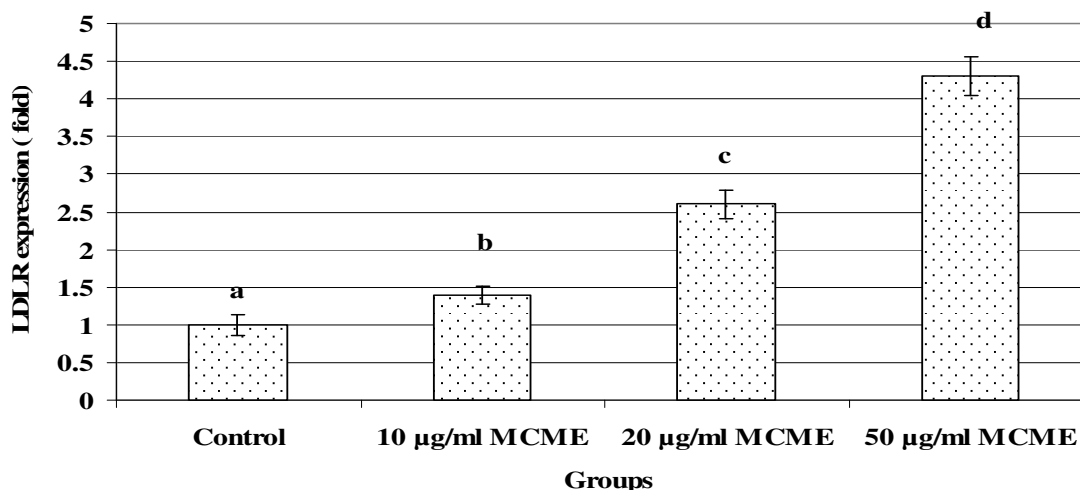


Figure 3. Effect of MCME treatments on LDLR mRNA levels in HepG2 cells. Level of LDLR mRNA measured by relative quantitative RT-PCR in HepG2 cells kept under control conditions or incubated with MCME at 10, 20 and 50 µg/ml for 24 h. LDLR mRNA levels were collected for beta actin. Data represent the mean of three samples ± SD. Different alphabets indicates significant difference from the control (HLPDS) at P < 0.05 level of significance (n = 3).

cells were incubated with HLPDS and treated with MCME at different doses, LDLR mRNA level was increased significantly by 1.4, 2.6 and 4.3 fold in MCME treated cells at 10, 20 and 50 respectively, compared to untreated cells. The effect of MCME was concentration dependent. Different doses showed significant difference in LDLR mRNA. LDLR activity was significantly up-regulated by MCME extracts (Figure 3). Although inhibition of LDL oxidation by a range of different plant sources has been widely reported (Salleh et al., 1998),

relatively few studies have focused on the effects of phytochemicals on LDLR activity (Lovati et al., 1992; Pal et al., 1999). The LDLR modulation effects have not been described previously for the MCME used in this study.

Regulation of HMGCR gene by MCME

The effects of MCME at 10, 20 and 50 µg/ml on the regulation of HMGCR gene are shown in Table 2.

Table 2. The effects of MCME at 10, 20 and 50 µg/ml on the regulation of HMGCR gene.

Group	Average C _T of HMGCR	Average C _T of beta actin	ΔC _T	ΔΔC _T	2 ^{-ΔΔCT} (Fold)
Control	20.74 ± 0.67	19.55 ± 1.56	1.19	0	1
10 µg/ml MCME	21.80 ± 0.75	19.91 ± 0.97	1.89	0.7	0.62
20 µg/ml MCME	22.03 ± 0.85	19.39 ± 0.89	2.46	1.45	0.37
50 µg/ml MCME	22.74 ± 0.84	19.52 ± 0.94	3.22	2.30	0.20

Results are expressed as means ± SD (n = 3). ΔC_T = ΔC_T HMGCR- ΔC_T of β-actin, ΔΔC_T = ΔC_T treated cells - ΔC_T control.

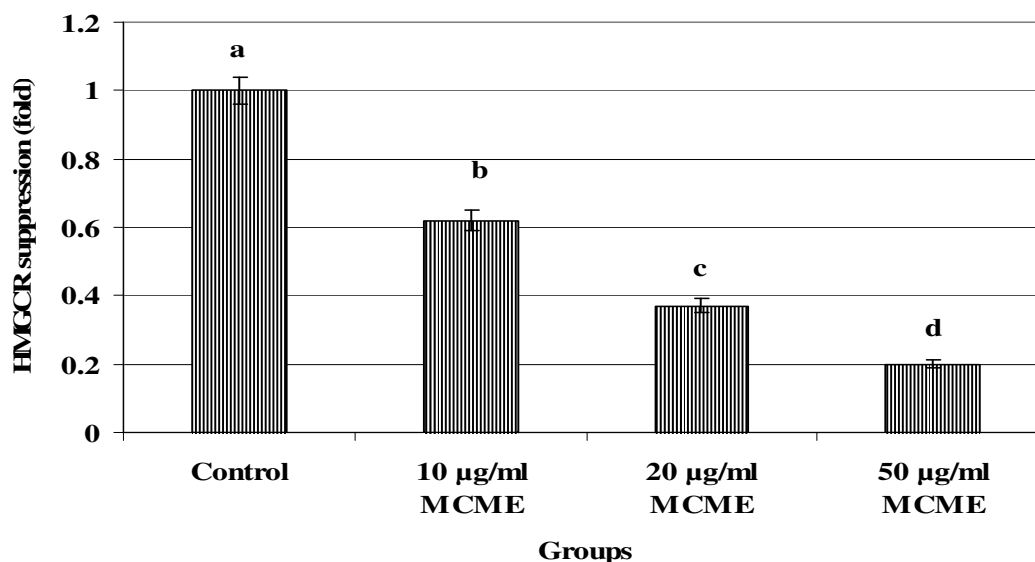


Figure 4. Effect of MCME treatments on HMGCR mRNA levels in HepG2 cells. Level of HMGCR mRNA measured by relative quantitative RT-PCR in HepG2 cells kept under control conditions or incubated with MCME at 10, 20 and 50 µg/ml for 24 h. HMGCR mRNA levels were collected for beta actin. Data represent the mean of three samples ± SD. Different alphabets indicates significant difference from the control (HLPDS) at P < 0.05 level of significant (n = 3).

Triplicate results were summarized in Figure 4. When cells were incubated with HLPDS and treated with MCME at different doses, HMGCR mRNA level was decreased significantly by 38, 63 and 80% in MCME treated cells at 10, 20 and 50, respectively, compared to untreated cells. The effect of MCME was concentration dependent. Different doses showed significant decrease in HMGCR mRNA. The percentage of suppression was calculated as follows:

$$\% \text{ Suppression} = \left[\frac{2^{-\Delta\Delta CT}}{\text{Control cells}} \right] - \left[\frac{2^{-\Delta\Delta CT}}{\text{Treated cells}} \right] \times 100$$

Regulation of HMGCR gene by MCME in HepG2 cells incubated with 10% HLPDS

In this study, three different doses of MCME at 10, 20 and 50 µg/ml below the IC₅₀ values obtained by MTT assay were selected. We identified MCME as new regulators of the LDLR and HMGCR genes. When the

cells were treated with MCME at different doses, MCME increased the expression level of the LDLR gene in HepG2 cells. Higher dose of MCME demonstrated a higher efficiency in increasing the LDLR expression when compared with untreated cells, however, at the same time suppressing the level of HMGCR. Interestingly, some hypocholesterolemic agents such as sterols cause a suppression of HMGCR and LDLR mRNA levels in HepG2 cells from the same line (Larsen et al., 1994). A series of benzamides have also been shown to up-regulate the LDLR activity without affecting cholesterol biosynthesis (Ashton et al., 1996). However, we have found that MCME shows the opposite effects on these two genes, lowering HMGCR levels and increasing the LDLR mRNA expression levels. The ability of MCME to suppress cholesterol synthesis while expressing LDLR activity suggests that this MCME may prove to be useful as a cholesterol lowering agent. Our results also indicated that this regulation was at the transcriptional level.

Although HepG2 cells are considered as good model to study the cholesterol metabolism, some of the changes

that accrue in HepG2 cells may not take place *in vivo*, thus there is a need to perform an animal study to validate and confirm the findings obtained in this *in vitro* study.

In conclusion, the up-regulation of the LDLR gene and the down-regulation of the HMGCR gene by MCME in HepG2 cells suggested that MCME regulated cholesterol metabolism through two primary mechanisms. First, the uptake of LDL cholesterol was mediated via the up-regulation of LDLR and secondly, cholesterol regulation was mediated by the suppression of the HMGCR gene that inhibited the synthesis of cholesterol by decreasing the activity of the HMGCR enzyme activity. Our results suggest that MCME can procure desirable health benefits in the prevention of cardiovascular disease.

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