Antioxidants activity and cholesterol regulation effect of *Caralluma flava N.E.Br* extract in HepG2 cells

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Herbal medicine has a long-standing history in many locations in Yemen and continue to provide useful and applicable tools for treating ailments. The objectives of the present study are to investigate antioxidant activity and cholesterol regulatory effect of *Caralluma flava N.E.Br* methanolic extract (CFM) through chemical and molecular approaches. CFM afforded the yield of 18 % (w/w) through methanolic extraction and contained considerable amount of phenolic and flavonoid compounds (9.22 ± 1.4 mg/g) and 3.35 ± 0.16 mg/g respectively). Antioxidant activity assays revealed that CFM are effective antioxidant with 76.8% DPPH scavenging activity bleaching inhibitory activity. Result from ß-carotene–linoleic acid assay shows that CFM efficiently retarded the oxidation of linoleic acid in the reaction system compared to control. CFM regulate the expression of cholesterol metabolism genes in HepG2 cells in a dose-dependent manner. The low-density lipoprotein receptor (LDLR) mRNA level was significantly up-regulated by 1.3, 2.4 and 3.2 fold compared to control cells. The mRNA level of 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCOA) was significantly suppressed by 12, 30 and 50% in a dose dependent manner compared to the control cells treated with 20, 50 and 100 µg/ml of CFM respectively. Apolipoprotein A-1 (Apo A-1) gene was up-regulated by 1.7 and 2.2 fold and apolipoprotein B100 (Apo B100) gene was suppressed by 11% and 24% in HepG2 cells treated with 50 and 100 µg/ml of CFM respectively, compared to control cells. In conclusion, CFM is a potential cardioprotector through enhancement of cholesterol regulation and exertion of antioxidant activity.

Keywords: *Caralluma flava* N.E.Br, *cholesterol regulation genes, Hepg2 cells.*

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1. Introduction

Herbal medicine has a long-standing history in many locations in Yemen and continue to provide useful and applicable tools for treating ailments. Genus of *Caralluma* has been known as a potential source for phytochemicals with medicinal usage also many species of the genus *Caralluma* are edible and used as the traditional medicine in many countries. *Caralluma flava* (CF) belongs to family Apocynaceae is a leafless, plant which grows wildly in many regions of Yemen. People in Yemen are consuming the CF plant freshly and used traditionally to treat of rheumatism, diabetes, leprosy, gastric ulcer and its juice as drops for ear inflammation (Dubaie *et al*., 2005). Based on the previous literature collection, few species of genus *Caralluma* are explored for pharmacological activities up to date. However, many other species (such as *Caralluma flava*, *Caralluma negevensis*, *Caralluma pauciflora*, and *Caralluma wissmannii*) have to be further explored (Malladi *et al*., 2018). Our Previous studies revealed that CF possesses multiple health-beneficial effects including anti-diabetic effect (Al-Naqeb *et al*., 2017), and antiulcer activity (Al-Naqeb *et al*., 2017).

There is a big interest in antioxidants due to their potentially positive effect against many diseases like cancer and coronary heart diseases. It has been reported that polyphenols protected the cardiovascular system, not only from oxidative stress but other damage because they possess other physiological effects, such as blood pressure reduction and inflammation decreasing action (Prahalathan *et al.*, 2012). Also, other antioxidant phytochemicals such as crocin, lycopene, carotenoid and allicin also show protective activities for cardiovascular system (Thushara *et al*., 2013).

The human HepG2 cell line has been used as a model for cholesterol regulation studies since they perform several of the normal biochemical functions specific to liver cells (Marc *et al*., 2004). The Stimulation of low-density lipoprotein receptor (LDLR) gene expression and the suppression of 3-Hydroxy-3-Methylglutaryl-CoA reductase (HMGCOA) gene are key mechanisms that control elevated plasma low-density lipoprotein cholesterol (LDLC) (Brown and Goldstein, 1997). Up-regulation of ApoA-1 gene and down-regulation of ApoB100 mRNA levels are also the key mechanism for cholesterol regulation.

Previous studies revealed that many species of the genus *Caralluma* possess multiple anti-hyperlipidemic effect (Ashfaq *et al*., 2017). To the best of our knowledge, there was no study has reported the reduction of cholesterol effect of *Caralluma flava* neither *in vitro* nor *in vivo*. Hence the aim of this study was to investigate the antioxidants activity of *Caralluma flava* methanolic extract CFM through different methods and the regulatory effect of CFM on cholesterol genes including LDLR, HMGCOA Apo B100 and Apo A1 in HepG2 cells.

2 Materials and Methods

2.1 Plant Collection.

Caralluma flava plant was collected from Rada, Albyda and from Bani Matr, Sana`a Yemen in 2014. The plant was identified and authenticated by a plant taxonomist at Department of Botany, Faculty of Agriculture, Sana`a University, Yemen. The plant was dried in oven at 45ºC and protected from the light. The dried plant was stored at 4ºC and protected from light prior use.

2.2 Chemicals

All the chemicals used in this study were analytical grade follows: Gallic acid (Sigma-Aldrich, Madrid, Spain); sodium carbonate (Na_2CO_3), methanol (99.8%) and Folin-Ciocalteu reagent (BDH Laboratory Supplies, Poole, England); 2.2-diphenyl-1-picrylhydraxyl (DPPH) (98.9%), L (+)-ascorbic acid, linoleic acid and catechin wrer from Sigma Chemical Co. St. Louis, USA; sulfoxide (DMSO) (Fisher Scientific, Loughborough, Leicestershire, UK) as well as aluminium trichloride ($AlCl_3$) (Merck, Darmstadt, Germany). For gene expression study, Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), sodium bicarbonate and phosphate buffered saline (PBS), were purchased from Sigma-Aldrich Co. (Sigma-Aldrich). RiboPure^{TM} 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 and supplied by Al-Shamel (Valencia, CA) while the primer and probes were supplied by Sigma-

Aldrich (St. Louis, MO) and synthesized by Integrated DNA Technologies, Inc (San Diego, CA).

2.3 *Caralluma Flava* Methanolic Extract Preparation

Dried *Caralluma flava* plant was finely ground using an electrical blinder (Waring Blender, Tokyo, Japan) and ground sample was extracted with methanol. For methanol extraction, powder plant was subjected to extraction with methanol at 1:5 (w/v) ratio for 48 h on stirrer and dark conditions. The mixture then was filtered using filter paper. The combined filtrate was concentrated by rotary evaporation at 40^{o}C. The extract was transferred into glass dark bottles and then stored at 4°C for subsequent analyses. (The *Caralluma flava* methanolic extract (CFM) showed yield of 18%).

2.4 Total Phenolic Content

Total phenolic content of CFM was determined using Folin–Ciocalteu assay. Briefly, 1 mg of CFM was dissolved in 10 ml of methanol. Then, 0.1 ml of these solutions was mixed with 2.5 ml of 10-fold diluted Folin-Ciocalteau reagent, and 2.0 ml of 7.5% sodium carbonate (Na_2CO_3). After incubation at 40°C for 30 min, the absorbance of the reaction mixtures were measured at 760 nm by using a spectrophotometer (Amersham 2100 Pro, UV-vis spectrophotometer, UK). Gallic acid was used as a standard and TPC of CFM was expressed in milligram gallic acid equivalent per 100 g dry weight sample (mg/g).

2.5 Total Flavonoid Content

Total flavonoid content (TFC) of CFM was estimated by the Aluminium chloride ($AlCl_{3)}$ method, adapted from Quettier-Deleu *et al*. (2000). In brief, 1 mg of CFM was dissolved in 10 ml DMSO for stock solution. Then, 500 µl from the CFM stock solution was mixed with 500 µl of 2% $AlCl_3$ and the mixture was left for 10 min. After that, the absorbance of the sample was measured at 435 nm using a spectrophotometer (Amersham 2100Pro, UV-vis spectrophotometer, UK). The TFC of CFM was expressed in mg rutin equivalent 100 g dry weight sample (mg/g).

2.6 DPPH Scavenging Activity

In brief, 50 µl of CFM at the concentration of 100 µg/ ml were added to 195 µl of 1,1-diphenyl-2- picrylhydrazyl (DPPH) DPPH methanolic solution (0.1 mM) in a 96-well plate. Then, the mixture was swirled thoroughly and left in darkness. The absorbance of the mixture

after 2, 10, 20, 30, 40, 50 and 60 min of reaction was respectively measured by a microplate reader (Opsys MR, Thermo Labsystems, Franklin, MA, USA) at 540 nm. Ascorbic acid and catechin were used as standards in this test at the same concentration (100 µg/ml) as the CFM.

2.7 The ß-carotene–Linoleic Acid Assay

Another method of determination the antioxidant activity of the CFM was evaluated using the ß-carotene–linoleic acid assay following the method of Amarowicz *et al*., 1993). In brief, a solution of b-carotene was prepared by dissolving 2 mg of b-carotene in 10 ml of chloroform. Two milliliters of this solution were pipetted into a 100 ml roundbottom 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 (Amersham 2100Pro, UV-vis spectrophotometer, UK). Sub-sequent absorbance readings were recorded over two hours period at 20 min intervals by keeping the samples in water bath at 50°C. Blank samples devoid of ß-carotene carotene, were prepared for background subtraction.

2.8 Measurement of Cells Viability

In order to avoid a cytotoxic effect of CFM on HepG2 cell proliferation, the cytotoxicity study was carried out using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) proliferation assay. HepG2 cells were treated with different concentrations of CFM in order to select the appropriate doses that resulted in proliferation rate of HepG2 cells greater than 70% to perform the gene expression experiments. HepG2 cells were plated in 96 well plates at a density of 1×10^5 cells per well, incubated overnight at 37°C in a humidified atmosphere including 5% CO_2. CFM was dissolved in DMSO and DMSO concentration was controlled to be not more than 0.1%. Then, the extract further diluted in Dulbecco's minimum essential medium (DMEM) to obtain a range of concentrations between 0.5 and 200 µg/ml. The cells were exposed to the extract for 72 hours. After this incubation period, MTS (20 µl) was added into each well and

the plates were then incubated for an additional four hours. Absorbance was recorded at 490 nm by microplate reader (Opsys MR, Thermo Labsystems, Franklin, MA, USA).

Cells viability was calculated by using this formula:

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

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

2.9 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 1×10^5 cells/well. Twenty-four hours before the start of experiment, the medium was changed to DMEM supplemented with 10% human lipoprotein deficient serum (HLPDS). Then, the cells were divided into 4 groups, the first was treated with CFM at dose of 20 µg/ml, the second group was treated with CFM at dose of 50 µg/ml, the third group was treated with CFM at dose of 100 µg/ml and the fourth group was not treated and served as a control group.

2.10 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 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.

2.11 Quantitative real-time PCR

TaqMan Primers and probes specific for LDLR, HMGCR and beta-actin were designed from of 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. GenBank accession number code for Apo A-1 is NM_000039, Apo B100 is M15421 and for beta actin gene is EF095209.

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 mix, 2 µL of

Gene	Sequence	Size (bp)
LDLR F	CACGGTGGAGATAGTGACAATG	
LDLR R	GGGCTTCTTCTCATTTCCTCTG	73
LDLR P	TCACCAAGCTCTGGGCGACGTTGCT	
HMG-COAR F	GGGACCAACCTACTACCTC	
HMG-COAR R	GGTCGAAGATCAATTTACAA	210
HMG_COAR P	TCACCAAGCTCTGGGCGACGTTGCT	
Beta actin F	CCAACTGGGACGACATGGAG	
Beta-actin R	TCAAACATGATCTGGGTCATCTTC	144
Beta actin P	AATCTGGCACCACACCTTCTACAATGAGC	
Apo B100 F	CCATACAGTGAGCCAGCCTTG	
Apo B100 R	ATGCCATCCTTCTGAGTTCAGAG	119
Apo B100 P	CCTTCCGAGCCCTGGTGCCAGC	
Apo A-1 F	AACCTAAAGCTCCTTGACAACTG	
Apo A-1 R	CTCCTCCAGATCCTTGCTCATC	149
Apo A-1 P	CAGCGTGACCTCCACCTTCAGCAAGC	

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). The sequence of the primers and probes are presented in Table 1.

Table 1. TaqMan specific primers and probes used in this study

Note: F = forward, R = reverse, P = probe and bp = base pair

Real-time PCR amplification of cDNA was performed for 40 cycles. After an initial incubation for 15 min at 95ºC the PCR cycle consisted of a denaturation period for 15 seconds at 94ºC and an annealing period for 60 sec at 50ºC. Analysis of the gene expression data was performed using a $\Delta\Delta C_T$ method of relative quantification, according to a previous reported by Kenneth and Thomas (2001). RotorGene analysis software (version 6.0) was used to analyze all of the results from the PCR assays.

2.12 Statistical Analysis

Each experiment was repeated thee times and the data are reported as the mean ± SD. Statistical significance was determined using one-way ANOVA or two-way ANOVA post hoc test: Tukey using GraphPad Prism 7 software.

3. Results

3.1 Yield, TPC and TFC

The yield of CFM through methanolic-aqueous extraction was 18 ± 0.61 % (w/w). On the other hand, TPC and TFC of CFM were 9.22±1.4 mg/g and 3.35 ±0.16 mg/g, respectively.

3.2 DPPH Scavenging Activity

Figure 1 shows DPPH scavenging activity of CFM, ascorbic acid as standard control. Result from this assay clearly showed that CFM exhibited high antiradical activity towards DPPH radical, although the scavenging activity was significantly lower than the pure antioxidant standard, ascorbic acid and catchine ($p<0.0001$). Right after 10 minutes of the reaction, CFM (100 µg/ml) scavenged more than 50% of the total radicals in the reaction system. Subsequently, the scavenging activity of CFM was gradually increased to 67% at the end of the experiment.

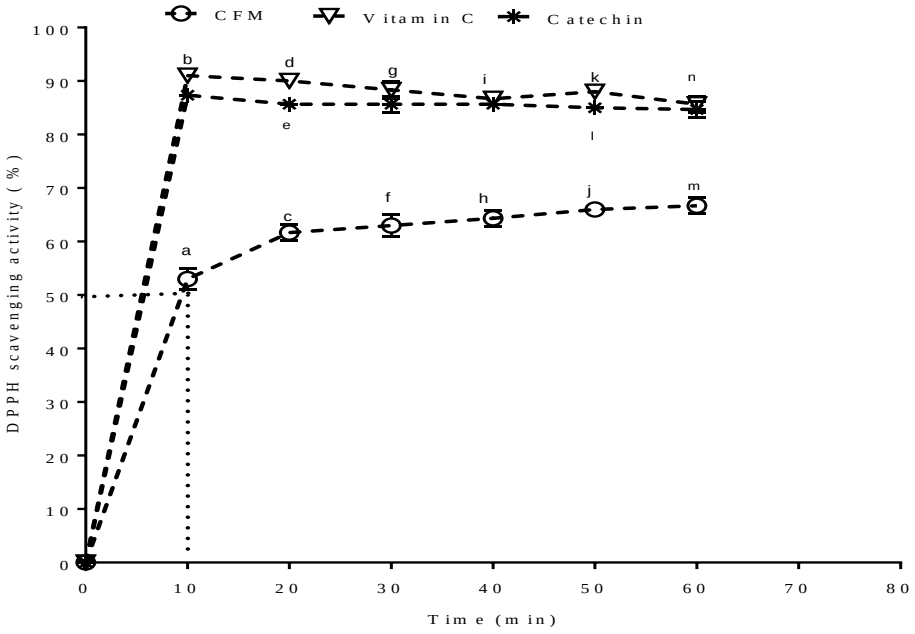

Figure (1). DPPH scavenging activity of *Caralluma flava* methanolic extract. Catechin and vitamin C were used as standard antioxidants. Data represent the mean of three samples ± STD. Within each time point, different characters statistically significant different among

the extract and standards p<0.0001. Analysis was carried out using Two-way ANOVA using GraphPad Prism 7.

3.3 Antioxidant Activity Determination

Figure 2 shows beta-carotene bleaching inhibitory activity of CFM and butylated hydroxyanisole (BHA) at 50°C. Result from ß -carotene–linoleic acid assay shows that BHA and CFM efficiently retarded the oxidation of linoleic acid in the reaction system compared to control, CFM was better in their effect on reducing the oxidation of ß -carotene than control but it was less in antioxidant activity than BHA and its degradation rate of ß -carotene clearly depends on its antioxidant activity.

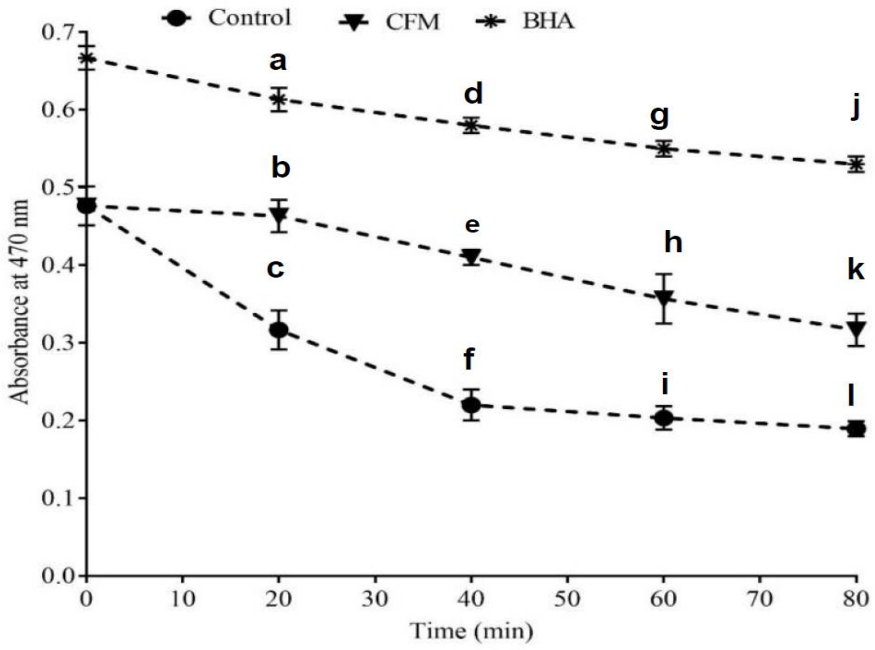

Figure (2). Effect of Caralluma flava methanolic extract on oxidation of ß-carotene/linoleic acid at 50°C. BHA: butylated hydroxyanisole. Data represent the mean of three samples ± STD. Within each time point, different characters statistically significant different among extract, control and standard p<0.0001. Analysis was carried out using Two-way ANOVA using GraphPad Prism 7.

3.4 Cell Viability Assay

The growth of HepG2 cells in the presence of CFM at different concentration (50 to 350 µg/ml) was examined. Under the experimental conditions, CFM exhibited growth inhibitory effects on HepG2 cells over a period of 72 hours. As shown in Figure 3, the IC_{50} of CFM for HepG2 cells was approximately 200 µg/ml.

3.5 *In vitro* Gene Expression Study

3.5.1 *In vitro* Gene Expression Study of LDLR

The effect of CFM at 20, 50 and 100 µg/ml on the regulation of LDLR gene is shown in Figure 4. When cells were incubated with HLPDS and treated with CFM at different doses, LDLR mRNA level was significantly increased by 1.3, 2.4 and 3.2 folds respectively, in a dose dependent manner when compared to the untreated cells. Different characters statistically significant different from the negative control. Analysis was carried out using One-way ANOVA using GraphPad Prism.

3.5.2 Gene Expression Study of HMGCR

The effect of CFM (20, 50 and 100 µg/ml) on the regulation of HMGCR gene is shown in Figure 5. When cells were incubated with HLPDS and treated with CFM at different doses, HMGCR mRNA level was significantly decreased by 12, 30 and 50% in a dose dependent manner compared to the control untreated cells. (The percentage of suppression was calculated as follows: % suppression = ($2^{-\Delta\Delta CT}$ control cells) - ($2^{-\Delta\Delta CT}$ treated cells) x 100)

3.5.3 Regulation of Apo B100 gene

Figure 6 shows the effect of CFM on the suppression level of Apo B100 mRNA. The mRNA level was suppressed by CFM and the suppression level was increased by increasing the dose applied. Apo B100 was significantly suppressed by 11% and 24% in HepG2 cells treated in 50 and 100 µg/ml of CFM respectively compared to control cells. There was no significant different ($p \leq 0.05$) in the suppression level in cells treated with 20 µg/ml of CFM compared to control cells.

4. Discussion

Antioxidants play important role in the prevention of free radical formation and helps in reducing various disorders such as cancer and cardiovascular disease. In the present study, we performed experiments aimed at determining the antioxidants activity of CFM and investigate the effect of the CFM on the regulation of the key genes involved in cholesterol metabolism *in vitro* using human HepG2 cells.

Phenolic compounds such as phenolic acids and flavonoids are major dietary antioxidants derived from plants (Balasundram *et al*., 2006). The total flavonoid content in this study

(9.22±1.4 mg/g) of CFM reported in the present study is slightly lower than that founded by Karthishwaran *et al*. (2017), (TPC = 10.14 mg/g. dw) that may due to the different extraction solvent and procedure. However, CFM reported in the present study was in agreement to the CFM reported by our previous study (Al-Naqeb, 2017), However, TFC (3.35 ±0.16 mg/g) of CFM in the present study slightly lower to the finding (TFC = 4.13 mg/ g) of Karthishwaran *et al*. (2017) due to the different extraction procedure. Since the CFM in the present study is originated from a Yemen to the one used by Karthishwaran *et al*. (2017), this suggests that the TPC variation in CFM is probably due to the environmental factors such as climates, sun exposure and soil composition that alters the phenolic metabolism of CF plant. The active ingredient in this plant are pregnane glycosides and triterpenes (Marwah *et al*., 2007).

DPPH scavenging activity assay evaluates the hydrogen-donating ability of the chain-breaking antioxidants, the antioxidants that capable to donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation (Lugasi *et al*., 1998). Result from DPPH scavenging activity assay clearly showed that CFM exhibited high antiradical activity (67%) towards DPPH radical, despite of its low concentration (100 µg/ml) in the reaction system. Besides serving as an effective radical scavenger, the result from DPPH assay also reveals the efficacy of CFM as an antioxidant. CFM spontaneously scavenged more than 50% of the total radicals within the first ten minutes of the reaction, suggesting that CFM is capable to scavenge fast-degrading free radicals in an efficient and short time manner.

Unlike DPPH assay, ß-carotene–linoleic acid assay uses a biologically relevant matrix, linoleic acid as an initiator to the oxidation process (Ferreria *et al*., 2006). The presence of antioxidants in the extract will minimize the oxidation of ß-carotene carotene by hydroperoxides. Hydroperoxides formed in this system will be decomposed by the antioxidants from the extracts. Thus, the degradation rate of ß-carotene - depends on the antioxidant activity of the extracts. Most studies showed that there was no correlation between TPC and BCB (Mariod *et al*., 2006).

Reduction of serum cholesterol of some *Caralluma* genus has been reported. Serum cholesterol level was significantly reduced in non-diabetic rats treated with *Caralluma russeliana* stem extract compared with control groups (A Zari and Al-Thebaiti, 2018). Cholesterol-decreasing activity of *Caralluma radscendens* aqueous extract was also reported

(Sakore *et. al.*, 2012). The best of our knowledge there was no study has been carried out on the cholesterol regulation genes of *Caralluma genus* in human hepG2 cells. The human HepG2 cells line are considered as a good model for studying the regulation of hepatic low-density lipoprotein cholesterol (LDLC) catabolism, cholesterol metabolism, lipid synthesis and lipoprotein synthesis, since they are representative to perform several of the normal biochemical functions specific to liver cells (Marc *et al*., 2004). These cells have been shown to express the major enzymes required for metabolism of the intra and extracellular cholesterol (Sebelyl *et al*., 2003).

The cells viability assays were performed in order to demonstrate the cytotoxic effects of CFM on HepG2 cell growth. Result indicates a significant decrease (50%) in HepG2 viability at 220 µg/m of CFM after a 72 hours incubation period. The effect of CFM on viability and proliferation of HepG2 cells lines has not been reported so far. In this study, the different doses of CFM at 20, 50 and 100 µg/ml below the IC_{50} values obtained by MTS assay did not alter the proliferation rate of HepG2 and were thus selected to perform the *in vitro* gene expression experiments with cells that had a proliferation rate of greater than 70%.

Through our gene expression study, CFM had enhanced the regulation of LDLR and suppression of HMGCR genes expression ($p=0.0001$). Interestingly, CFM treated HepG2 cells exhibited a significant up-regulated LDLR gene expression and down-regulated HMGCR gene expression in a dose-dependent manner. This elucidates that CFM is capable to provide an optimal cholesterol regulation/ lowering effects through enhancement of LDLC uptake from the plasma and suppression of cholesterol biosynthesis in the liver, simultaneously. Plant extracts with optional antioxidants activity have been reported to regulate the genes involved in cholesterol metabolism in HepG2 cells. LDLR expression was stimulated by the ethanolic extract of Corylus avellana L. extract and the effect was suggested due to the antioxidant activity of the extract (Benassia *et al*., 2019). Methanol extract of Cajanus Cajan L. leaves up-regulation of the LDLR as well as down-regulation of HMGCOA in in HepG2 cells (Chen *et al*., 2019). Monechma ciliatum methanolic extract, effectively regulated the expression of LDLR and HMGCR genes influencing the cholesterol metabolism in HepG2 cells and the activity was suggested due to the antioxidant activity (Mariod *et al*., 2010). Vanillin rich antioxidant fraction showed potential antioxidant activity and regulated genes involved in cholesterol metabolism including LDLR and HMGCR in

dose-dependent manner in HepG2 cells (Al-Naqeb et al., 2010). Thymoquinone rich antioxidants fraction extracted from Nigella sativa seeds using a supercritical fluid extraction technique was effective in regulating of LDR and HMGCOA genes in vitro and in vivo (Al-Naqeep et al., 2009).

Previous studies using HepG2 cells had shown that the rate of cholesterol synthesis and the availability of cellular cholesteryl esters regulated Apo B100 gene expression (Oyen *et al*., 1999). Up-regulation of ApoA-1 gene and down-regulation of ApoB100 mRNA levels by CFM treatments suggest that CFM could regulate cholesterol by different mechanisms that involved the important apolipoprotein genes, including Apo A-1 and Apo B100.

We observed that lower equivalent concentrations of CFM showed higher activity in up-regulation of cholesterol regulation genes in HepG2 cells. That suggests the effect of CFM on regulation of tested genes demonstrates that CFM contains a group of natural compounds and has unique properties in regulation of key genes involved in cholesterol regulation. Experiments to isolate and identify these compounds are recommended to be carried out. Our study suggests that CFM is a potential nutraceutical for the prevention of cardiovascular diseases due to its dual cardio-protective mechanisms, namely enhancement of cholesterol regulation and exertion of antioxidant activity.

In conclusion, CFM exhibits high antioxidant and cholesterol lowering activities in the present study through chemical and molecular assays. Our results clearly show that CFM regulate LDLR, HMGCOA, Apo A-1 and Apo B100 genes in vitro at transcription level. Further animal study is needed to determine whether CFM can reduce plasma cholesterol levels by the observed mechanism in HepG2 cells study.

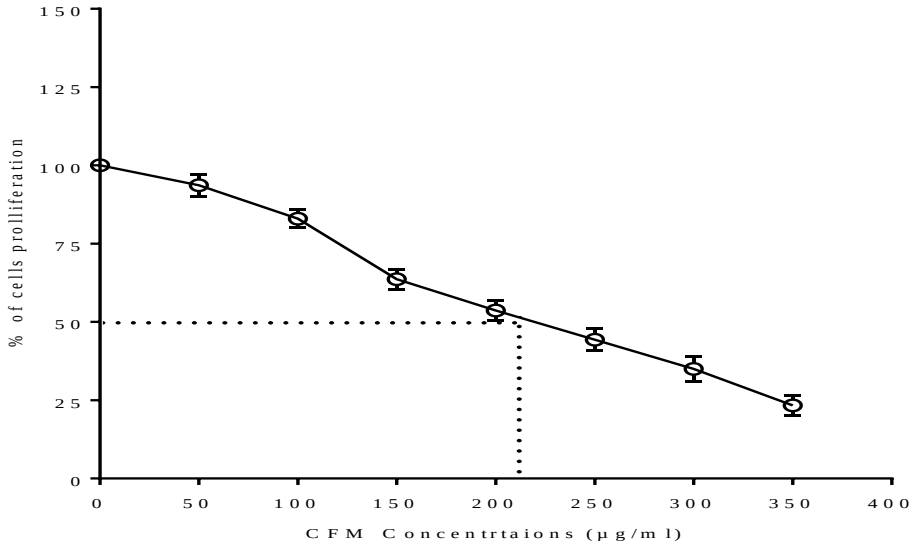

Figure (3). Cytotoxic effect of *Caralluma flava* methanolic extract against HepG2 cells. IC_{50} values (200 µg/ml) obtained after 72 hours using MTS assay. Each value presents the average of 6 replicates ± SD.

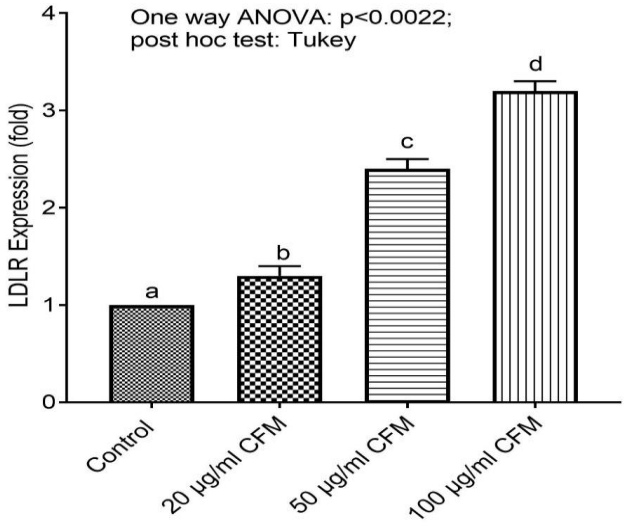

Figure (4). Effect of *Caralluma flava* methanolic extract treatments on LDLR mRNA levels in HepG2 cells. Data represent the mean of three samples ± STD. Different characters statistically significant different from the negative control. Analysis was carried out using One-way ANOVA using GraphPad Prism 7.

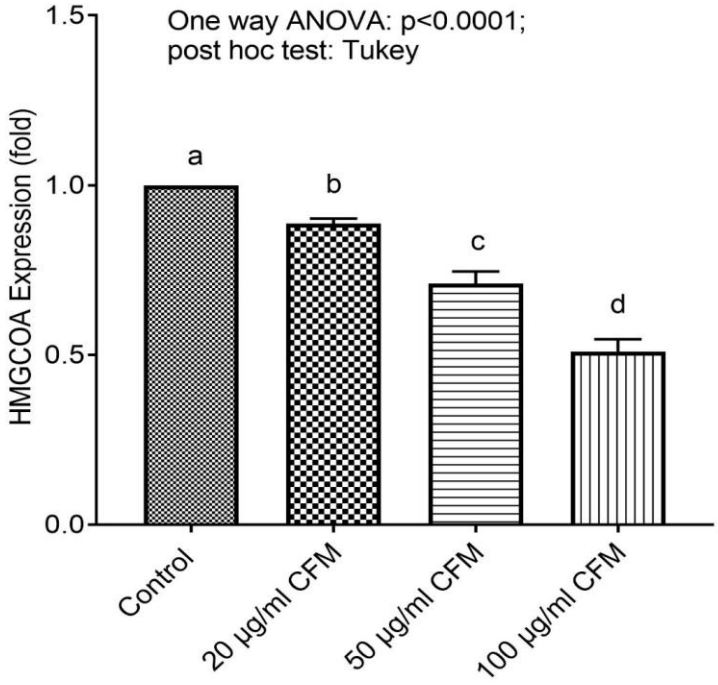

Figure (5). Effect of *Caralluma flava* methanolic extract treatments on HMGCOA mRNA levels in HepG2 cells. Data represent the mean of three sample s± STD. Different characters statistically significant different from the negative control. Analysis was carried out using One-way ANOVA using GraphPad Prism.

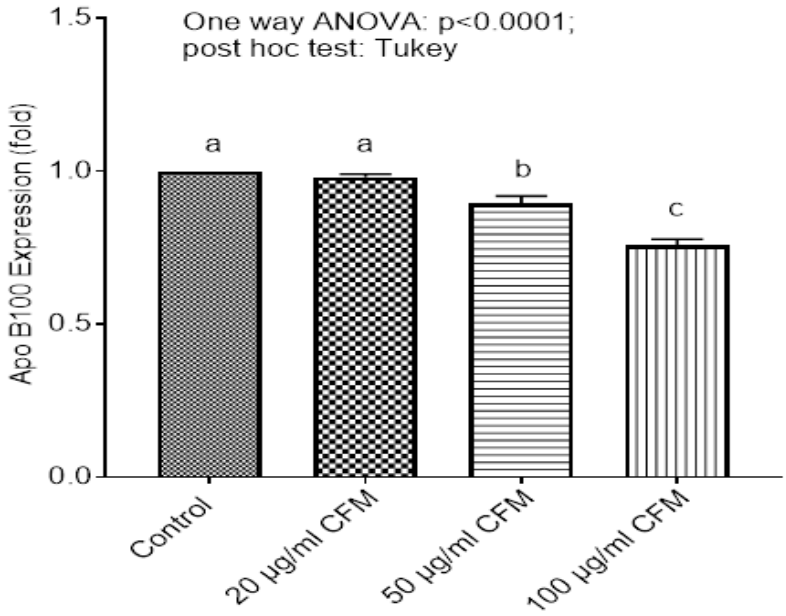

Figure (6). Effect of *Caralluma flava* methanolic extract treatments on Apo B100 mRNA levels in HepG2 cells. Data represent the mean of three samples ± STD. Different characters statistically significant different from the negative control. Analysis was carried out using One-way ANOVA using GraphPad Prism.

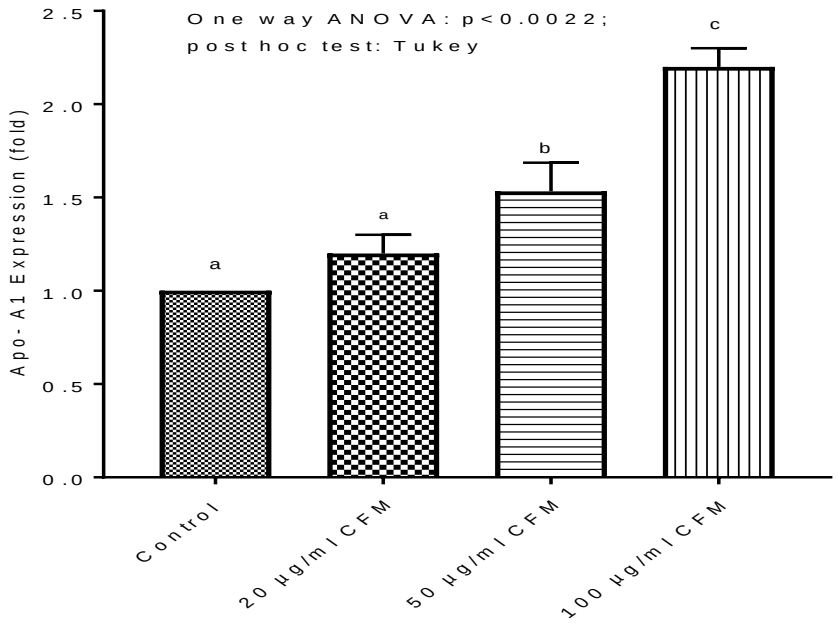

Figure (7). Effect of *Caralluma flava* methanolic extract treatments on Apo A1 mRNA levels in HepG2 cells. Data represent the mean of three samples ± STD. Different characters statistically significant different from the negative control. Analysis was carried out using One-way ANOVA using GraphPad Prism.

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