Journal of Food and Drug Analysis

Volume 26 | Issue 3 Article 22

Mulberry leaf extract inhibit hepatocellular carcinoma cell proliferation via depressing IL-6 and TNF-α derived from adipocyte

Follow this and additional works at: https://www.jfda-online.com/journal

 Part of the Food Science Commons, Medicinal Chemistry and Pharmaceutics Commons, Pharmacology Commons, and the Toxicology Commons

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 4.0 License.

Recommended Citation
Chang, C.-H.; Chang, Y.-T.; Tseng, T.-H.; and Wang, C.-J. (2018) "Mulberry leaf extract inhibit hepatocellular carcinoma cell proliferation via depressing IL-6 and TNF-α derived from adipocyte," *Journal of Food and Drug Analysis*: Vol. 26 : Iss. 3 , Article 22.
Available at: https://doi.org/10.1016/j.jfda.2017.12.007

This Original Article is brought to you for free and open access by Journal of Food and Drug Analysis. It has been accepted for inclusion in Journal of Food and Drug Analysis by an authorized editor of Journal of Food and Drug Analysis.

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfda-online.com

Original Article

Mulberry leaf extract inhibit hepatocellular carcinoma cell proliferation via depressing IL-6 and TNF-α derived from adipocyte

Chun-Hua Chang [a], Yu-Tzu Chang [a], Tsui-Hwa Tseng [c,d,**], Chau-Jong Wang [a,b,*]

[a] Institute of Biochemistry, Microbiology and Immunology, Chung Shan Medical University, Taichung, Taiwan
[b] Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan
[c] Department of Medical Applied Chemistry, Chung Shan Medical University, Taichung, Taiwan
[d] Department of Medical Education, Chung Shan Medical University Hospital, Taichung, Taiwan

ARTICLE INFO

Article history:
Received 25 September 2017
Received in revised form
13 December 2017
Accepted 18 December 2017
Available online 1 February 2018

Keywords:
Hepatocellular carcinoma (HCC)
Obesity
Proinflammatory cytokines
Mulberry leaf extract (MLE)
Proliferation

ABSTRACT

Epidemiological studies have revealed that obesity and being overweight are associated with increased cancer risk. Adipose tissue is regarded as an endocrine organ that secretes proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which are related to the progression of hepatocellular carcinoma (HCC). In this study, adipocytes from 3T3-L1 cells were induced and stained with Oil Red O, which revealed marked intracellular lipid accumulation. Adding 15% conditioned medium (CM) from adipogenic -differentiated 3T3-L1 cells, which contained adipocyte-derived factors, to a culture medium of HepG2 cells was discovered to promote cell proliferation by a factor of up to 1.3 compared with the control. Mulberry leaf extract (MLE), with major components including chlorogenic acid and neochlorogenic acid, was revealed to inhibit CM-promoted HepG2 cell proliferation. The inhibitory effect of MLE on the proliferation of the signal network was evaluated. Expression of the CM-activated IκB/NFκB, STAT3, and Akt/mTOR pathways were reduced when MLE was administered. Although adipocyte-derived factors are complex, administrating anti-TNF-α and anti-IL-6 revealed that MLE blocks signal activation promoted by TNF-α and IL-6. Taken together, these results demonstrated that MLE targets the proliferation signal pathway of the inflammatory response of adipocytes in HCC and could be to prevent obesity-mediated liver cancer.

Copyright © 2018, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

* *Corresponding author*. Institute of Biochemistry, Microbiology and Immunology, Chung Shan Medical University, Taichung, Taiwan. Fax: +866 4 23248167
** *Corresponding author*. Chung Shan Medical University, Number 110, Section 1, Jiankuo North Road, Taichung 402, Taiwan.
E-mail addresses: tht@csmu.edu.tw (T.-H. Tseng), wcj@csmu.edu.tw (C.-J. Wang).

https://doi.org/10.1016/j.jfda.2017.12.007

1021-9498/Copyright © 2018, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Hepatocellular carcinoma (HCC) is a highly fatal disease. Common risk factors for the development of HCC include viral infections caused by the hepatitis B or hepatitis C viruses, excess alcohol intake, and some dietary carcinogens, aflatoxin in particular. In addition, growing evidence shows that obesity increases the risk of developing HCC [1,2]. Several pathophysiological mechanisms linking obesity to cancer have been suggested as related to the systemic and local effects of dysregulated adiposity [3]. Obese adipose tissue mainly releases proinflammatory cytokines consist of TNF-α, IL-6, leptin, resistin, angiotensin II, and plasminogen activator inhibitor 1 to induce inflammatory response. mRNA expression studies indicates that adipocytes can produce TNF-α, IL-1β and IL-6 [4–6]. TNF-α and IL-6 activate transcription factors NF-κB and STAT3, respectively, which can enhance cell growth and prevent apoptosis [7]. Additionally, adipose tissue functions as a key endocrine organ, releasing multiple bioactive substances that may be involved in a complex network of survival signal pathways such as PI3K/Akt/mTOR, which is believed to be critical during HCC development [8]. Therefore, cytokine blockers or natural diets that block signal pathways activated by adipocyte-derived factors may decrease the risk of developing obesity-promoted HCC.

Accumulated evidence suggests that a high intake of plant foods is associated with a lower risk of developing a chronic disease and cancer. Plant polyphenols, which have manifold biological roles, have received substantial attention [9–12]. They generally present in fruits, vegetables, and tea. Mulberry leaves (*Morus alba* L.), which are commonly used to feed silkworms, are known to be rich in polyphenols such as quercetin and caffeic acid [13] and are used as a traditional medicine to treat several metabolic diseases including dyslipidemia, diabetes, fatty liver disease, and hypertension [14–16]. Previous research has revealed that mulberry leaf extract (MLE) can effectively inhibit the proliferation and migration of vascular smooth muscle cells, improve vascular endothelial function, and reduce atheroma burden [17,18]. Ann et al. discovered that mulberry leaves have beneficial effects on obesity-related fatty liver disease through their regulation of hepatic lipid metabolism, fibrosis, and the antioxidant defense system [19]. Because of the etiological link between obesity and liver cancer, plant polyphenols—with their antioxidant and anti-inflammatory properties—have drawn increasing interest for their possible role in chemoprevention. However, little is known about the effect of plant polyphenols on the progression of adipocyte-induced HCC progression. Hence, the present study investigated the inhibitory effect of MLE on adipocyte-derived factor-induced HepG2 cell proliferation and attempted to verify the molecular mechanism involved.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F-12), minimum essential media, fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). In addition, 3-(4,5-dimethylthiazol-zyl)-2,5- diphenyltetrazolium bromide (MTT), antimouse IgG peroxidase conjugate, antirabbit IgG peroxidase conjugate antibodies, and β-actin antibodies were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-p38, NF-κB (p65), STAT3, IκB, mTOR, and GSK3β antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antiphosphorylated p38 (Thr 180/Tyr 182), phosphorylated STAT3 (Ser 727), phosphorylated mTOR (Ser 2448), phosphorylated IκB (Ser 32/36), and phosphorylated GSK3β (Ser21/9) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). IL-6 and TNF-α antibodies were purchased from Abcam Biotechnology (Abcam, Cambridge, MA, USA).

2.2. Preparation of MLE

Mulberry leaves were collected in Dadu Township, central Taiwan. Fresh mulberry leaves (200 g) were harvested and immediately dried at 50 °C. The dried leaves were then heated at 90 °C in 3 L of deionized water for 1 h. After filtration, the residue was removed and the aqueous extract was lyophilized (−80 °C, 12 h) to obtain MLE. The dried powder was stored at −80 °C and MLE was filtrated using a 0.22-μm filter prior to use in the following experiments.

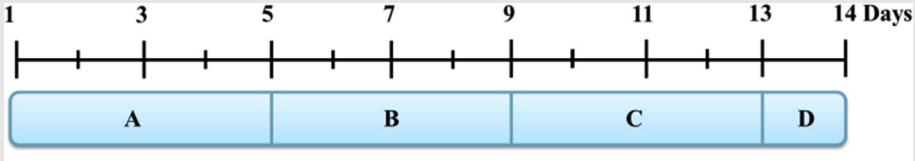
2.3. Cell culture

Murine 3T3-L1 fibroblast cells (BCRC 60159) and HepG2 cells (BCRC 60025) were purchased from Bioresource Collection and Research Center. The 3T3-L1 cells were grown in DMEM (Invitrogen Inc.) supplemented with 10% FBS, 4 mM L-glutamine, and 1% penicillin-streptomycin. HepG2 cells are a human hepatoblastoma cell line. Cells were maintained in a minimum essential medium (Invitrogen Inc.) supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate (Invitrogen Inc.), and then incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.4. Adipocyte formation assay and preparation of CM

The 3T3-L1 cells (preadipocytes) were seeded at a density of 10^6/mL in six-well plates and grown to confluence. Cell differentiation into adipocytes was induced using DMEM supplemented with 10% FBS, 4.5 μM rosiglitazone (RSG), 1 μg/mL insulin, 0.25 μM dexamethasone (DEX), and 0.5 mM isobutylmethylxanthine (IBMX) for 4 days, with the medium replaced every 2 days. On day 5, the medium was replaced with a medium containing 10% FBS supplemented with 1 μg/mL insulin for 4 additional days, with the medium again replaced every 2 days. On day 9, the medium was replaced with a medium containing 10% FBS for 4 days, with the medium replaced every 2 days. At this point, preadipocytes were induced to become adipocytes and lipid droplets became clearly visible. On day 13, the medium was replaced with a serum-free medium. The observed induction of adipogenic differentiation from preadipocytes is summarized in Table 1.

Table 1 – Induction of adipogenic differentiation from preadipocytes (3T3-L1). Adipose differentiation of 3T3-L1 preadipocytes was induced as described in Materials and Methods.



	DMEM (high glucose 4.5 g/L)		RSG (4.5 μM)	DEX (0.25μM)	IBMX (0.5 mM)	Insulin (1 μg/ ml)
	10% FBS	Non serum				
A	+	-	+	+	+	+
B	+	-	-	-	-	+
C	+	-	-	-	-	-
D	-	+	-	-	-	-

The medium was collected as a conditioned medium (CM) and stored at −80 °C until use.

2.5. Oil Red O staining

Adipocyte differentiation was detected by staining lipids with Oil Red O (Sigma–Aldrich). Oil Red O stock solution (5 mg/mL) was prepared in isopropanol and stored at 4 °C prior to use. Fresh Oil Red O working solutions were prepared by mixing the stock solution with distilled water (6:4), followed by incubation for 10 min and filtration through a 0.22-μm filter. Cells were washed twice with phosphate buffered saline (PBS) and fixed with 4% formaldehyde for 1 h at room temperature. Subsequently, the fixative solution was removed and the cells were washed twice with PBS. The Oil Red O working solution was added and covered the cells for 30 min. The cells were then washed with distilled water, dried, and examined using an inverted optical microscope.

2.6. Cell viability

The HepG2 cells were seeded at a density of 5×10^4/mL in 24-well plates and treated with CMs of various concentrations (0%–25%) for 24 or 48 h. After incubation, their cytotoxicity was determined using an MTT assay. In brief, the medium was changed and MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. After washing with PBS, 1 mL isopropanol was added to the purple blue formazan, and the absorbance was measured spectrophotometrically at 563 nm.

2.7. WST assay

Cell proliferation was measured using the WST-1 cell proliferation reagent kit (Roche Applied Science, Mannheim, Germany). HepG2 cells were plated in 24-well plates at a density of 5×10^4 cells per well. After treatment with 15% CM and MLEs of various concentrations, 10 μL/well of WST-1 was added to the medium, followed by incubation at 37 °C in a humidified atmosphere of 5% CO_2 for 0.5 h. Cellular viability was determined by measuring the cells' absorbance using an ELISA reader at 440 nm.

2.8. BrdU incorporation assay

The BrdU Cell Proliferation ELISA colorimetric assay kit (Roche Applied Science, IN, USA) was used to quantitate cell proliferation according to the manufacturer's protocol. HepG2 cells were seeded at a density of 10^4 in 96-well plates and incubated overnight. The cells were then treated with adipocyte-CM and combined with MLE in various concentrations (1–4 mg/mL) for 48 h. BrdU solution (10 μM) was added to each well and left for 24 h at 37 °C. Cellular proliferation was determined by measuring the cells' absorbance using an ELISA reader at 450 nm.

2.9. Nuclear extraction

Nuclear and cytoplasmic fractionations were performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Thermo Scientific). Briefly, 2×10^6 HepG2 cells were induced with an adipocyte-CM and treated with MLE of different concentrations (1–4 mg/mL) for 48 h. After centrifugation (500 g for 5 min), the supernatant was removed. Then, 200 μL of CER I buffer was added to the cell pellets, which were then vigorously shaken for 15 s. The cell mixtures were incubated on ice for 10 min, and then added to 11 μL of CER II buffer. The cell mixtures were again incubated on ice for 1 min and shaken vigorously for 5 s. The cell suspension was subsequently centrifuged at 16,000 g for 5 min. The supernatants (cytoplasmic extract) were kept on ice. The cell pellets were resuspended using 100 μL of nuclear extraction reagent buffer (NERbuffer) on ice for 40 min with 15 s of vigorous shaking every 10 min. Following centrifugation at the maximum speed

of 16,000 g for 10 min, the supernatants (nuclear extracts) thus obtained was transferred to a new Eppendorf tube and stored on ice or at −80 °C until use.

2.10. Western blot analysis

2×10^6 cells per well were seeded in 10-cm dishes in the presence of 0.5–4.0 mg/mL MLE and 15% CM. Total protein extracts were prepared in radioimmunoprecipitation assay buffer (RIPA buffer) (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% NP-40) containing protease inhibitors. Protein concentration was measured using a Bradford protein assay kit (Bio-Rad, USA). Cell lysate with an equal amount of protein (50 μL) was loaded onto 8%–10% SDS-PAGE and transferred onto a nitrocellulose membrane (PALL, USA). The membranes were blocked in 5% milk in Tris-buffered saline (TBS) plus 0.5% Tween for 1 h at room temperature and then incubated with specific primary antibodies at 4 °C overnight, after which the secondary antibody was conjugated to horseradish peroxidase for 1 h. The immunocomplex was revealed using enhanced chemiluminescence with a chemiluminescence detection kit (Millipore, USA) and was exposed using a Fuji-Film LAS-4000 mini (Tokyo, Japan). Protein quantity was determined using FujiFilm Multi Gauge 2.2.

2.11. Statistical analysis

Data reported are means ± standard deviations of three independent experiments analyzed using analysis of variance. Significant differences were defined as $P < 0.05$.

3. Results and discussion

3.1. Induction of adipogenic differentiation from preadipocyte 3T3-L1

Mulberry leaves have recently been discovered to have a beneficial effect on obesity-induced hepatic lipogenesis and fibrosis [19]. However, the effect of MLE on obesity-mediated hepatic cancer cell proliferation remains unknown. To address this deficiency in the literature, the effect of MLE on adipocyte-mediated hepatic cancer cell proliferation and its signaling pathway was investigated in this study. Cancer-associated adipocytes were previously proposed to interact reciprocally with cancer cells and affect cancer progression [20]. To clarify the chemopreventive potential of MLE regarding obesity-associated HCC *in vitro*, adipocyte from 3T3-L1 preadipocytes was prepared according to the process presented in Table 1. Postconfluent 3T3-L1 preadipocytes were discovered to have a fibroblast-like shape (Fig. 1, left) when treated with differentiation medium containing a mixture of RSG, DEX, IBMX, and insulin. On day 13, round adipocytes were observed after adipogenic differentiation (Fig. 1, middle). In addition, cells with intracellular lipid accumulation were markedly stained by Oil Red O, suggesting that 3T3-L1 preadipocytes differentiate into mature adipocytes (Fig. 1, right).

3.2. Promoting cell proliferation of HepG2 using CM from adipocytes

Fig. 2A illustrated the effect of CM from adipocytes on the viability of HepG2 cells. Cell viability was highest when treatment with 15% CM for 24 h and 48 h was performed; therefore, 15% CM was used in the subsequent experiments.

3.3. Inhibiting CM-induced proliferation in HepG2 cells using MLE and its components

Previous studies have reported that natural polyphenols have anticancer potential [21], so whether MLE inhibits CM-induced HepG2 proliferation was investigated. Treatment with CM for 24 or 48 h was demonstrated to significantly promote HepG2 proliferation, whereas MLE treatment decreased this effect at both treatment durations (Fig. 2B). When treatment with MLE was administered at 2 and 4 mg/mL, the absorbance was lower than that of the control group, implying that MLE not only inhibited CM-promoted HepG2 proliferation but also blocked the endogenous proliferation effect of HepG2 cells. In our previous report, the composition of MLE were identified using high-performance liquid chromatography and liquid chromatography–mass spectrometry. Eight phenolic compounds were identified: neochlorogenic acid (35.5%), cryptochlorogenic (31.7%), chlorogenic (23.8%), rutin (9.2%), isoquercitrin (5.6%), astragalin acid (5.3%), nicotiflorin (3.5%), and protocatechuic acid (1.3%) [22] The effect of major phenolic acids on CM-induced proliferation was also investigated. CGA and nCGA were discovered to significantly inhibit CM-promoted proliferation and endogenous-mediated proliferation in HepG2 cells (Fig. 2C).

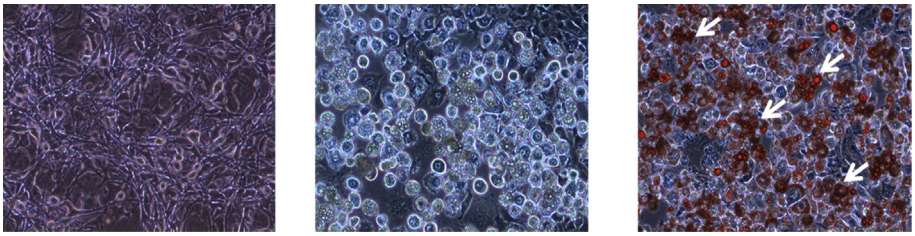


Fig. 1 – The differentiation of adipocytes was evaluated using Oil Red O staining. Mouse preadipocytes, 3T3-L1 cell line showed a fibroblast-like shape (left panel). Preadipocytes were induced into adipogenic differentiation by differentiation-induced media (middle panel). Adipose differentiation of 3T3-L1 preadipocytes evaluated by Oil-red-O staining of lipid droplets (right panel). The adipogenic-differentiated cell showed positive staining for Oil-red-O. Red-colored expression indicated lipid droplets in differentiated adipocyte (arrowhead).

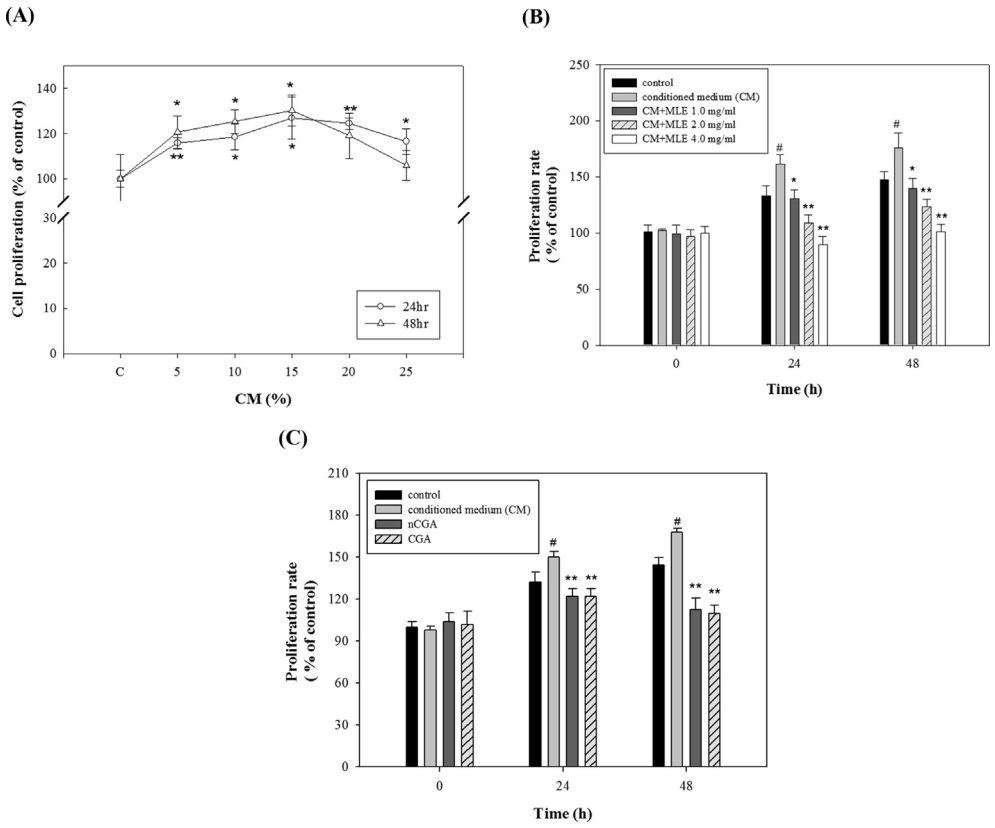


Fig. 2 – Inhibition of adipocyte-conditioned medium (CM)-induced cell proliferation by MLE in HepG2 cells. (A) 5×10^4 HepG2 cells were treated with CM of different concentrations (0%–25%) in serum-free culture medium for 24 and 48 h. Cell viability was analyzed using the MTT assay as described in the text (B) 5×10^4 HepG2 cells per well were treated with adipocyte-conditioned medium alone and in combination with MLE of various concentrations (1, 2 and 4 mg/mL) for 24 and 48 h (C) 5×10^4 HepG2 cells per well were induced with adipocyte-conditioned medium and treated with nCGA (0.1 mg/mL) and CGA (0.1 mg/mL) for 24 and 48 h. Cell proliferation was evaluated using the WST assay. The result represents an average of four independent experiments ±SD. The result represents an average of three independent experiments ±SD. $^{\#}p < 0.05$ compared with the control.$^{*}p < 0.05$, $^{**}p < 0.005$ versus CM group.

3.4. *Deactivation of CM-induced NF-κB, STAT3, and mTOR using MLE*

Adipocytes have been proposed to induce a low-grade inflammatory response that in turn increases TNF-α and IL-6 expression. One previous study reported that TNF-α and IL-6 can stimulate the proliferation and progression of HCC [20]. TNF-α and IL-6 activate NF-κB and STAT3, respectively. The effect of MLE on the phosphorylation of IκB and p38 MAPK, which are upstream of NF-κB (Fig. 3A), was evaluated. The results revealed that CM increased the expression of p-IκB but not that of p-p38 and enhanced the nuclear translocation of NF-κB p65. MLE treatment decreased the expression of p-IκB and nuclear translocation of NF-κB p65. In addition, CM increased the expression of pSTAT3, which was reversed by the MLE treatment (Fig. 3B). Therefore, MLE inhibited the NF-κB and STAT3 signaling pathways, which are involved in HCC proliferation. Adipocyte-derived factors are complex and may activate other signaling networks such as that of mTOR, which is frequently upregulated in HCC and is associated with cell proliferation. The effect of CM on the mTOR pathway was thus examined. CM was revealed to increase the phosphorylation of Akt and mTOR, which was reduced by the administration of MLE (Fig. 3C). These results demonstrated that MLE can block the PI3K/Akt/mTOR signaling pathway.

3.5. *Effect of anti-IL-6 and anti-TNF-α on CM-induced signal activation*

To determine whether CM promotes signal activation through the TNF-α and IL-6 mediated signaling pathways, the phosphorylation of p-STAT, p-IκB, p-Akt, and p-mTOR was performed using the administration of anti-IL-6 and anti-TNF-α. The results indicated that anti-IL-6 blocked the CM-activated STAT pathway whereas anti-TNF-α blocked the CM-activated IκB/NF-κB pathway (Fig. 4A and B). Furthermore, administration of anti-IL-6 and anti-TNF-α also blocked the CM-activated Akt/mTOR signaling pathway (Fig. 4C). Finally, treatment with MLE, anti-IL-6, or anti-TNF-α were all demonstrated using BrdU incorporation analysis to inhibit CM-induced HepG2 proliferation (Fig. 5). Thus, MLE blocks signal activations promoted by TNF-α and IL-6, resulting in the antiproliferation of HepG2 cells.

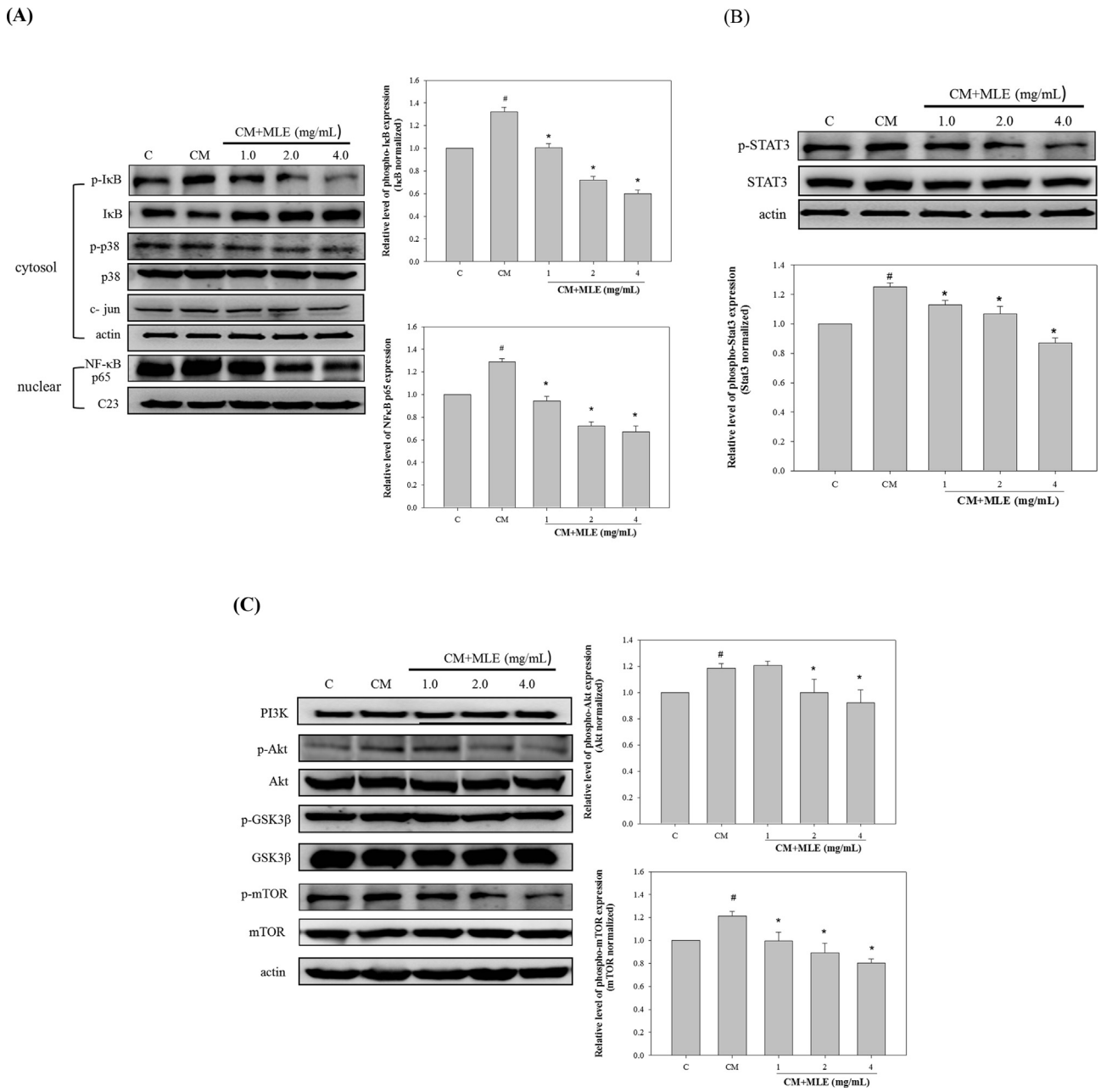


Fig. 3 – Effects of adipocyte-conditioned medium (CM) induced cell proliferation by MLE on protein levels of TNF-α/IL-6 signaling-related proteins in HepG2 cells. HepG2 cells were induced with adipocyte-conditioned medium and treated with MLE of various concentrations (1–4 mg/mL) for 48 h. Whole cell extracts (50 μg/lane) were separated on 8–10% SDS PAGE followed by Western blot analyses. Each target protein band was detected with respective antibody. β-actin and C23 were used as the cytosolic and nuclear protein loading control, respectively. Quantification of phosph-IκB and NFκB(A), phosph-Stat3/Stat3(B) and phosph-Akt/Akt and phosph-mTOR/mTOR(C) protein level. The graph represents mean values of three independent experiments, and error bars represent means ± SD of the experiments. $^{\#}p < 0.05$ compared with the control.$^{*}p < 0.05$ versus CM group. C, control; CM, adipocyte-conditioned medium.

4. Discussion

Epidemiological studies have demonstrated that obesity and being overweight are associated with increased risk of cancers such as HCC. In obese patients, lipid accumulation in the liver increases the demand on the endoplasmic reticulum, thus provoking oxidative stress, causing the production of reactive oxygen species, and activating inflammatory pathways. Oxidative stress can induce DNA damage, which leads to genomic instability. The enhanced production of proinflammatory cytokines such as TNF-α and IL-6, which can lead to hepatic inflammation, promotes abnormalities in liver cells [23]. Researchers have reported a number of biologically active

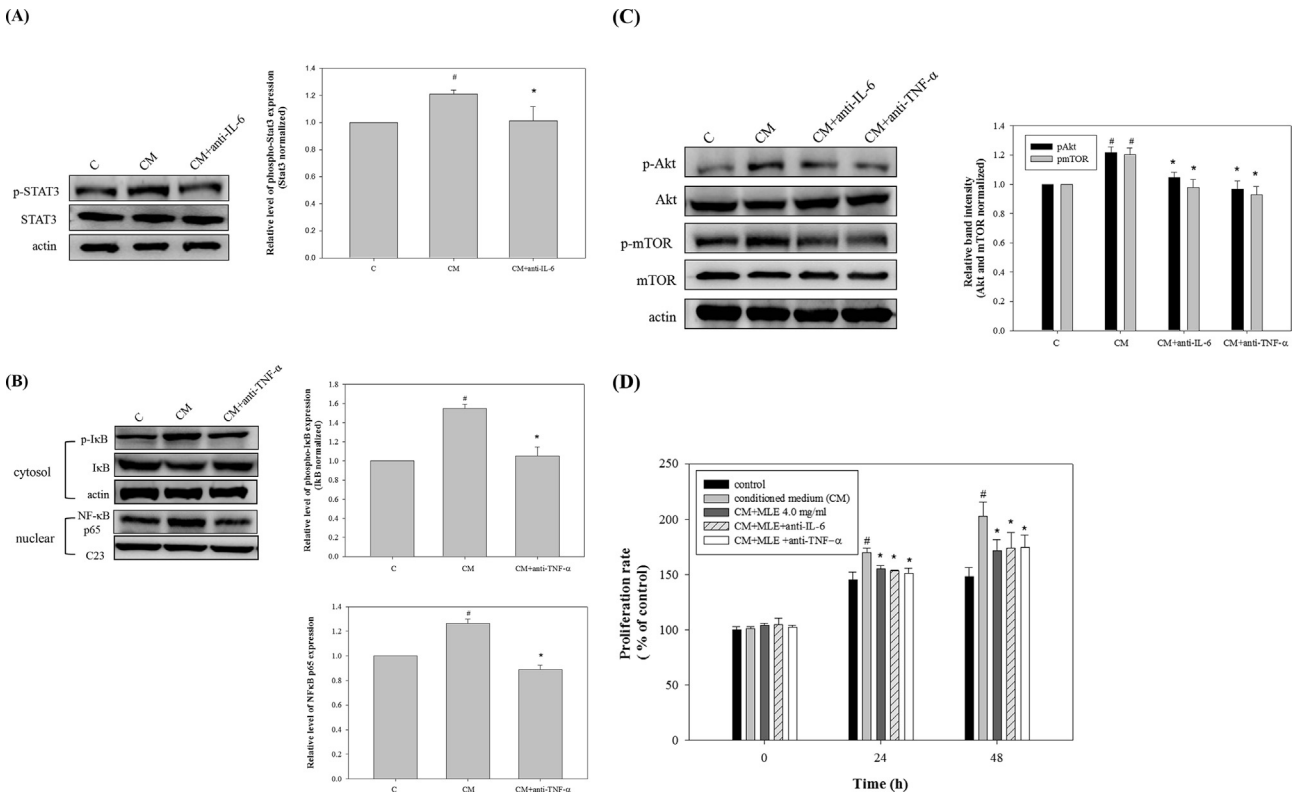


Fig. 4 – Inhibition of adipocyte-conditioned medium (CM)-induced signal activation by anti-IL-6 and anti-TNF-α in HepG2 cells (A–C) HepG2 cells were induced with adipocyte-conditioned medium and treated with anti-IL-6 (10 μg/mL) or anti-TNF-α (1 ng/mL) for 48 h. Whole cell extracts (50 μg/lane) were separated on 10% SDS PAGE followed by Western blot analyses. Each target protein band was detected with respective antibody. β-actin was used as an internal control. Quantification of the pSTAT3, NF-κB p65, pIκB, pAkt and pmTOR protein level were put on right site. (D) HepG2 cells were treated with or without adipocyte-conditioned medium and the HepG2 cells were presence MLE 4 mg/ml with anti-IL-6 or anti-TNF-α for 0, 24 and 48hours. Proliferation was measured by the WST assay. All the experiments were performed thrice in triplicates. $^{\#}p < 0.05$ versus control group and * $p < 0.05$ versus CM group.

compounds in MLE that have effective anti-inflammatory, antioxidant, and hepatoprotective activities [24,25]. This study discovered that administration of a medium from cultured adipocytes increased the proliferation of HepG2 (Fig. 2), which was associated with activating the IL-6 and TNF-α signaling pathways (Fig. 4). The results also demonstrated that MLE treatment decreased CM-promoted HepG2 proliferation by blocking proliferation signaling pathways including the STAT3, IκB/NFκB, and Akt/mTOR pathways.

Cell survival signaling plays a critical role in the pathogenesis of cancer. Most studies on IL-6 signaling in hepatic epithelia have focused on proliferation and the characterization of the pathways involved [26]. Numerous different pathways are known to be activated by IL-6, such as the STAT3, p38/MAPK, and PI3K/AKT pathways. The CM was revealed herein to activate the STAT3 and Akt/mTOR pathways, but this activation was reduced by the administration of MLE to HepG2 cells (Fig. 3). TNF-α, crucial to cancer-related inflammation, can activate p38/MAPK and I kappa B kinase (IKK) [27] IKK activation is associated with the activation of NFκB, a transcription factor. The role of NFκB in cancer cells appears to involve the regulation of cell proliferation, control of apoptosis, and stimulation of invasion/metastasis. The present study additionally revealed that Akt/mTOR activation in HepG2 is also mediated by TNF-α. The CM-activated IKK and NFκB, but this activation was reduced by the administration of MLE to HepG2 cells (Fig. 3). Adipose tissue is considered to be not simply a reservoir of stored energy but also an active secretory organ that releases inflammatory cytokines, adipokines, and growth factors, leading to an increased risk of HCC development [28]. Whether these signal activations are regulated by other components released by adipocytes merits further investigation. The carcinogenesis of HCC is a multifactor and complex process involving chromosomal aberrations, epigenetic alterations, and the activation of complex signaling pathways. A previous study reported that mTOR is frequently upregulated in HCC and that growth factors such as insulin-like growth factor II are secreted from HepG2 [29]. HepG2 may activate endogenous proliferation signaling pathways in a serum-free culture (control). Comparing Figs. 3 and 4 reveals that MLE inhibited not only CM-activated proliferation signaling but also endogenous proliferation signaling, thus demonstrating its anti-HCC role of targeting proliferation signaling pathways.

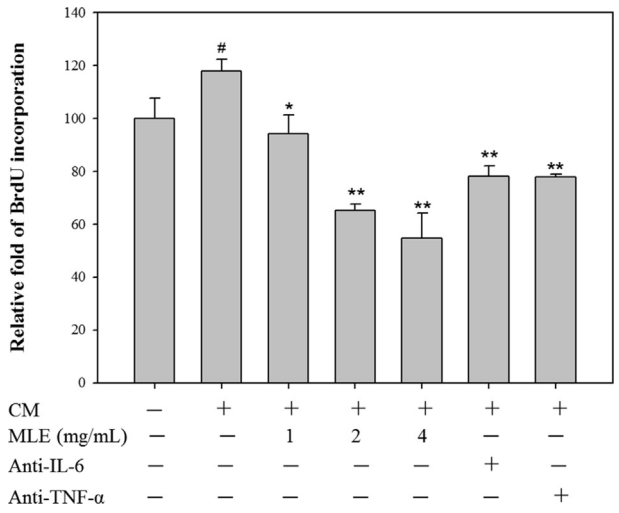


Fig. 5 – Effects of adipocyte-conditioned medium (CM) induced cell proliferation by MLE, anti-IL-6 and anti-TNF-α in HepG2 cells. 10^4 HepG2 cells per well were induced with adipocyte-conditioned medium and treated with different concentration MLE (1, 2 and 4 mg/mL), anti-IL-6 (10 μg/mL), and anti-TNF-α (1 ng/mL) for 48 h. Cell proliferation was analyzed by BrdU incorporation assays. The values represents an average of three independent experiments ±SD. $^{*}p < 0.05$, $^{**}p < 0.005$ versus CM group. $^{\#}p < 0.05$ compared with the control.

In recent years, the biological properties of plant polyphenols with chemopreventive potential have attracted increasing interest. CGA and its isomer nCGA have been associated with anticarcinogenic, anti-inflammatory, and antioxidant activities and have been demonstrated to reduce the risk of cardiovascular disease and type 2 diabetes [22,30]. CGA and nCGA can suppress breast cancer cell viability and growth while having no effect on MCF-10A normal breast epithelial cells. In Caco-2 human colon cancer, both CGA and nCGA also showed similar effects [22,31]. In our previous report, 0.5% of MLE exhibited a significant effect to decrease the obesity induced NAFLD, inflammation and oxidative stress. The polyphenol composition analysis revealed that chlorogenic and its isomers account for approximately 0.47 mg when mice were fed with 10 g diet containing 0.5% MLE. We speculated that administration 2.35 mg/day of chlorogenic and its isomers could prevent NAFLD in human (500 g diet/day). Thus, we hypothesized that chlorogenic and its isomers should be effective in preventing obesity-induced liver cancer at similar concentrations CGA was also shown to suppress carbon tetrachloride-induced NF-κB activation and decrease the levels of TNF-α, IL-6, and IL-1β in rat serum [32]. The composition of MLE except for nCGA and CGA includes other polyphenols. Studies showed that isoquercitrin suppress colon cancer proliferation and inflammation [34]. Rutin, a polyphenolic bioflavonoid, has anti-tumor effect on lung cancer cells. When cells were treated with rutin caused a significant reduction in lipid peroxidation and LDH activity; restored antioxidant enzyme activity and modulated the expression of inflammatory [35]. Additional studies revealed that astragalin acid inhibited TNF-α induced NF-κB activity and suppressed tumor growth and induced cancer cell apoptosis in vivo [36]. Obesity, an abnormal or excessive fat accumulation in adipose tissue, is considered a chronic inflammatory disease and increases the risk of developing HCC. Chronic overexpression of inflammatory mediators in cell microenvironments enhances tumor promotion and progression. This paper is the first report on polyphenol-containing MLE inhibiting adipocyte-derived factor-enhanced hepatoma cell proliferation through the blocking of inflammatory mediator-activated signaling pathways.

In conclusion, the results presented herein demonstrated that MLE targets the proliferation signal pathways of the inflammatory response of adipocytes in HCC and has potential for chemoprevention in obesity-mediated liver cancer.

Conflicts of interest

The authors do not have any possible conflicts of interest.

Acknowledgements

This work was supported by Ministry of Science and Technology Grant (MOST 104-2632-B-040-002), Taiwan.

REFERENCES

[1] Petta S, Craxi A. Hepatocellular carcinoma and non-alcoholic fatty liver disease: from a clinical to a molecular association. Curr Pharmaceut Des 2010;16:741–52.
[2] Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. Hepatology 2010;51:1820–32.
[3] Hefetz-Sela S, Scherer PE. Adipocytes: impact on tumor growth and potential sites for therapeutic intervention. Pharmacol Ther 2013;138:197–210.
[4] Harvey AE, Lashinger LM, Hursting SD. The growing challenge of obesity and cancer: an inflammatory issue. Ann N Y Acad Sci 2011;1229:45–52.
[5] Coppack SW. Pro-inflammatory cytokines and adipose tissue. Proc Nutr Soc 2001;60:349–56.
[6] Makki K, Froguel P, Wolowczuk I. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. ISRN Inf 2013:2013.
[7] Grivennikov SI, Karin M. Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage. Ann Rheum Dis 2011;70(Suppl. 1):i104–8.
[8] Malley CO, Pidgeon GP. The mTOR pathway in obesity driven gastrointestinal cancers: potential targets and clinical trials. BBA Clin 2016;5:29–40.
[9] Russo M, Russo GL, Daglia M, Kasi PD, Ravi S, Nabavi SF, et al. Understanding genistein in cancer: the "good" and the "bad" effects: a review. Food Chem 2016;196:589–600.
[10] Rodríguez M, Estrela J, Ortega Á. Natural polyphenols and apoptosis induction in cancer therapy. J Carcinog Mutagen S 2013:6.
[11] Stagos D, Amoutzias GD, Matakos A, Spyrou A, Tsatsakis AM, Kouretas D. Chemoprevention of liver cancer by plant polyphenols. Food Chem Toxicol 2012;50:2155–70.

[12] Devi KP, Rajavel T, Nabavi SF, Setzer WN, Ahmadi A, Mansouri K, et al. Hesperidin: a promising anticancer agent from nature. Ind Crop Prod 2015;76:582–9.
[13] Chan KC, Yang MY, Lin MC, Lee YJ, Chang WC, Wang CJ. Mulberry leaf extract inhibits the development of atherosclerosis in cholesterol-fed rabbits and in cultured aortic vascular smooth muscle cells. J Agric Food Chem 2013;61:2780–8.
[14] Flores MB, Rocha GZ, Damas-Souza DM, Osorio-Costa F, Dias MM, Ropelle ER, et al. Obesity-induced increase in tumor necrosis factor-alpha leads to development of colon cancer in mice. Gastroenterology 2012;143:741–753 e1–4.
[15] Ou TT, Hsu MJ, Chan KC, Huang CN, Ho HH, Wang CJ. Mulberry extract inhibits oleic acid-induced lipid accumulation via reduction of lipogenesis and promotion of hepatic lipid clearance. J Sci Food Agric 2011;91:2740–8.
[16] Kobayashi Y, Miyazawa M, Kamei A, Abe K, Kojima T. Ameliorative effects of mulberry (Morus alba L.) leaves on hyperlipidemia in rats fed a high-fat diet: induction of fatty acid oxidation, inhibition of lipogenesis, and suppression of oxidative stress. Biosci Biotechnol Biochem 2010;74:2385–95.
[17] Chan KC, Ho HH, Peng CH, Lan KP, Lin MC, Chen HM, et al. Polyphenol-rich extract from mulberry leaf inhibits vascular smooth muscle cell proliferation involving upregulation of p53 and inhibition of cyclin-dependent kinase. J Agric Food Chem 2010;58:2536–42.
[18] Yang M-Y, Chang Y-C, Chan K-C, Lee Y-J, Wang C-J. Flavonoid-enriched extracts from Nelumbo nucifera leaves inhibits proliferation of breast cancer in vitro and in vivo. Eur J Integr Med 2011;3:e153–63.
[19] Ann JY, Eo H, Lim Y. Mulberry leaves (Morus alba L.) ameliorate obesity-induced hepatic lipogenesis, fibrosis, and oxidative stress in high-fat diet-fed mice. Genes Nutr 2015;10:46.
[20] Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. Cell 2010;140:197–208.
[21] Bae MH, Lee MJ, Bae SK, Lee OH, Lee YM, Park BC, et al. Insulin-like growth factor II (IGF-II) secreted from HepG2 human hepatocellular carcinoma cells shows angiogenic activity. Cancer Lett 1998;128:41–6.
[22] Noratto G, Porter W, Byrne D, Cisneros-Zevallos L. Identifying peach and plum polyphenols with chemopreventive potential against estrogen-independent breast cancer cells. J Agric Food Chem 2009;57:5219–26.
[23] Deng T, Lyon CJ, Bergin S, Caligiuri MA, Hsueh WA. Obesity, inflammation, and cancer. Annu Rev Pathol 2016;11:421–49.
[24] Ou TT, Kuo CY, Chyau CC, Lee HJ, Peng JS, Wang CJ. Improvement of lipopolysaccharide-induced hepatic injuries and inflammation with mulberry extracts. J Sci Food Agric 2013;93:1880–6.
[25] Tang C-C, Huang H-P, Lee Y-J, Tang Y-H, Wang C-J. Hepatoprotective effect of mulberry water extracts on ethanol-induced liver injury via anti-inflammation and inhibition of lipogenesis in C57BL/6J mice. Food Chem Toxicol 2013;62:786–96.
[26] Johnson C, Han Y, Hughart N, McCarra J, Alpini G, Meng F. Interleukin-6 and its receptor, key players in hepatobiliary inflammation and cancer. Transl Gastrointest Cancer 2012;1:58–70.
[27] Cawthorn WP, Sethi JK. TNF-alpha and adipocyte biology. FEBS Lett 2008;582:117–31.
[28] Duan XF, Tang P, Li Q, Yu ZT. Obesity, adipokines and hepatocellular carcinoma. Int J Cancer 2013;133:1776–83.
[29] Korkina LG, De Luca C, Kostyuk VA, Pastore S. Plant polyphenols and tumors: from mechanisms to therapies, prevention, and protection against toxicity of anti-cancer treatments. Curr Med Chem 2009;16:3943–65.
[30] Farah A, Monteiro M, Donangelo CM, Lafay S. Chlorogenic acids from green coffee extract are highly bioavailable in humans. J Nutr 2008;138:2309–15.
[31] Thurow T. Effect of chlorogenic acid and neochlorogenic acid on human colon cancer cells. 2012.
[32] Shi H, Dong L, Jiang J, Zhao J, Zhao G, Dang X, et al. Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway. Toxicology 2013;303:107–14.