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Original Article

Hypocholesterolemic mechanism of phenolics-enriched extract from *Moringa oleifera* leaves in HepG2 cell lines

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Abstract

Previous studies have demonstrated the hypolipidemic activity of *Moringa oleifera* (MO) leaves via lowering serum levels of cholesterol, but the mechanism of action is unknown. In this study, we demonstrated the hypocholesterolemic mechanism of a phenolics-enriched extract of *Moringa oleifera* leaf (PMO) in HepG2 cells. When compared to the control treatment, PMO significantly decreased total intracellular cholesterol, inhibited the activity of HMG CoA reductase in a dose-dependent manner and enhanced LDL receptor binding activity. Moreover, PMO also significantly increased the genetic expressions of HMG CoA reductase and LDL receptor.

Keywords: *Moringa oleifera,* LDL receptor, hypolipidemic, HepG2 cell, HMG CoA reductase, phenolics

1. Introduction

Several epidemiological studies have shown that hypercholesterolemia is one of the risk factors for atherosclerosis and cardiovascular disease (CVD) (Yang, 2005). The liver is a major organ involved in cholesterol metabolism. Inhibition of the rate limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) in endogenous cholesterol biosynthesis and up-regulation of the low density lipoprotein (LDL) receptor are the most effective ways for lowering blood cholesterol and reducing cardiovascular event rates (Brown and Goldstein, 1997).

HMG CoA reductase inhibitors (Statins) are the drugs of choice for lowering blood cholesterol in people with or at high risk of cardiovascular disease. Although the drugs can decrease rates of mortality and morbidity, they also cause adverse side effects such as myopathy or liver dysfunction (Thompson *et al*., 2003). Therefore, studies of naturally occurring compounds as the regulators of cholesterol metabolism are of interest. Some natural compounds found in the human diet such as the plant flavonoid, quercetin, have positive effects on cholesterol metabolism both *in vitro* and *in vivo* (Moon *et al*., 2012; Jung *et al*., 2013), and catechins in green tea have been reported to lower CVD risk (Zheng *et al*., 2011). Several phenolic compounds in green tea,including catechin, epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) have shown remarkable effects on the clearance rate of cholesterol due to an increase in the expression of the LDL receptor (Brusill and Roach, 2006).

Moringa oleifera (MO), locally known as drumstick tree or horseradish tree, belongs to the Moringaceae family. It is a quick growing tree and widely distributed in tropical areas. The whole parts of MO have been used as traditional medicines because of their wide spectrum of biological activities, including antibacterial, anti- fungal, antiviral, anti-inflammatory, and antioxidant effects (Guevara *et al*., 1996; Fahey, 2005; Signh *et al*., 2009). Due to its hypolipidemic effects both in animal models and clinical trials (Ghasi *et al*., 2000; Chumark *et al*., 2008; Nambiar *et al*., 2010), the products

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from the dried leaves of MO was widely marketed as a functional food. Although MO has been reported to decrease lipid absorption (Jain *et al*., 2010) and inhibit cholesterol biosynthesis (Chumark *et al*., 2008), the exact mechanism of action has not yet been established. The present study aimed to investigate the mechanism underlying the hypocholesterolemic effect of *Moringa oliefera* leaf extract in human hepatoblastoma (HepG2) cells by determination of the activity, and mRNA expression of HMG CoA reductase enzyme and the LDL receptor.

2. Materials and Methods

2.1 Chemicals

Methanol (HPLC grade), isopropanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and formalin, (Fisher Scientific Co Ltd., United Kingdom); acetonitrile (HPLC grade), (Labscan, Bkk, Thailand); molecular biology agarose (Bio-Rad, Spain); 1kb DNA ladder, blue/orange 6X loading dye (Promega, U.S.A.); primer β-actin, 3-hydroxy-3-methylglutraryl coenzyme A (HMG CoA) reductase, and low density lipoprotein (LDL) receptor (Eurofins MWG Operon, Germany); omiscript reverse transcription Kit, TopTaq MasterMix kit (QIAGEN, Germany); novel juice (GeneDirex); RNA extraction kit (GE Healthcare, UK); Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and 1,1'-dioctadecyl-3,3,3',3'-tetramethylidocarbocyanin percholorate (Dil-LDL), (Invitrogen, UK); tryphan blue, neutral red, epigallocatechin-gallate (EGCG), and an HMG CoA reductase assay kit (Sigma, USA); cholesterol enzymatic kit (Randox, UK) and other analytical grade chemicals were used.

2.2 Preparation of phenolics-enriched extract from *Moringa oleifera* leaf

Fresh leaves of *Moringa oleifera* were collected in July, 2013 from Khon Kaen Province, Thailand and the voucher specimen (HB186/56) of plant was kept at the Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University. The samples were dried and pulverized with an electric grinder to obtain a free flowing powder. One kilogram of dried leaf powder was extracted with seven liters of 70% ethanol in a stainless steel pot for three days, then filtered through cotton and filter paper Whatman® No. 1. The extract was concentrated using a vacuum evaporator at 50°C and then freeze-dried in a lyophilizer. The dried phenolics-enriched extract of *Moringa oleifera* leaves (PMO) was kept at -20°C until further used.

2.3 High performance liquid chromatography (HPLC) analysis of PMO

Six commercial available phenolic compounds (caffeic acid, p-coumaric acid, rutin, isoquercetin, quercitrin, and quercetin) (Figure 1) were used as reference standards for PMO standardization. The HPLC analysis was performed on an Agilent Technologies 1260 machine using an Agilent hypersil ODS column (5 µm, 4.6 x 250 mm) at 30°C. The gradient mobile phase consisted of solution A (acetonitrile:water: phosphoric acid = 80:19.8:0.2) and solution B (0.2% phosphoric acid) at a flow rate of 1.0 ml/min. The gradient solvent elution was 100% solution B initially, and the gradient program was as follows: 0-5 min: isocratic at 0% solution A; 5-10 min: gradient to 5% solution A; 10-15 min: isocratic at 5% solution A; 15-20 min: gradient to 10% solution A; 20-30 min: isocratic at 10% solution A; 30-35 min: gradient to 15% solution A; 35-45 min, isocratic at 15% solution A; 45-50 min: gradient to 20% solution A; 50-55 min: isocratic at 20% solution A; 55-80 min: gradient to 50% solution A. The UV detection wavelength was set at 210 nm and the injection sample volume was 20 µl.

2.4 HMG CoA reductase activity assay

Based on the oxidation of NADPH, the HMG CoA reductase activity was evaluated by a kit assay following manufacturer's instruction. The reaction was started by the addition of HMG CoA reductase to the assay mixture containing buffer, NADPH, HMG CoA, and test samples; then the absorbance at 340 nm was continuously determined for 20 min. The initial velocity of each sample was measured as the decreasing rate of absorbance, and the rate of reaction in the

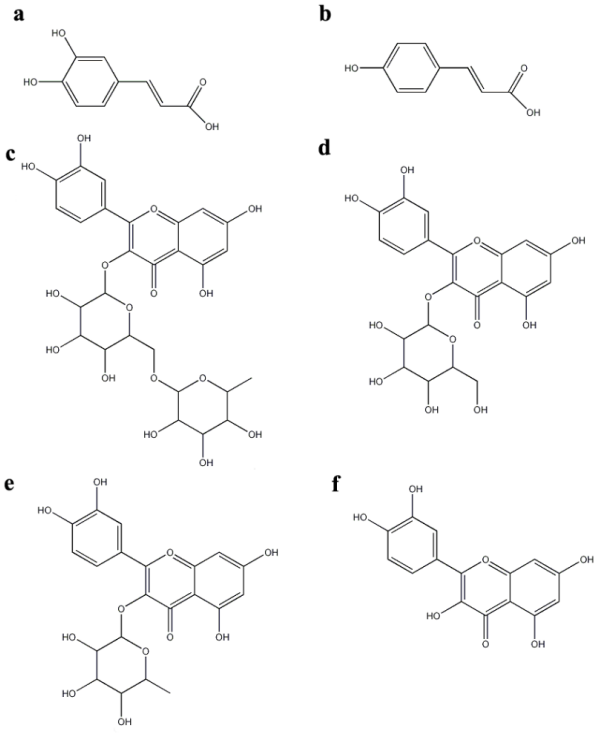

Figure 1. Chemical structures of caffeic acid (a), *p*-coumaric acid (b), rutin or quercetin 3-rutinoside (c), isoquercetin or quercetin 3-glucoside (d), quercitrin or quercetin rhamnoside (e), and quercetin (f).

units of ΔAbs_{340}/min was then calculated for the enzyme specific activity.

2.5 Cell culture

HepG 2, human hepatoma cells were grown in 25 cm^2 polystyrene flasks containing DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% penicillin-streptomycin at 37°C in a humidified atmosphere at 5% CO_2. The culture medium was changed twice a week and the cells were subcultured once a week.

2.6 Measurement of intracellular cholesterol

HepG2 cells were seeded in a 100 mm cell culture disc. After reaching 80% confluence, the media was discarded and cells were pre-incubated in serum-free DMEM for 12 hrs, then treated with various concentrations of test sample in DMEM free serum and incubated at 37°C in a humidified atmosphere at 5% CO_2 for 24 hrs. A modification of the method was used to extract cholesterol (Mizoguchi *et al*., 2004). After washing with 10 mM phosphate buffer saline, pH 7.4 (PBS), the cells were extracted with a mixture of hexane-isopropanol (3:2) and dried using a vacuum evaporator at 50°C. The dried residue was suspended in 0.2 ml of isopropanol and assayed for cholesterol content using an enzymatic test kit according to the manufacturer's instructions. The remaining cellular protein was dissolved in 0.5 N NaOH for protein determination (Lowry *et al*., 1951).

2.7 LDL uptake assay

LDL-receptor activity of HepG2 cells was determined by measuring the uptake of fluorescent labeled Dil-LDL (Teupser *et al*., 1996). The cells were seeded overnight at a density of 10^6 cells/well in a 12-well tissue culture plate and pre-incubated in serum-free DMEM for 12 hrs, then treated with various concentrations of test sample for 24 hrs. After washing with PBS, the cells were incubated with a fluorescent dye (Dil-LDL) at a concentration of 6 μg/mL for 4 hrs, twice washed with PBS, then 0.5 ml of isopropanol was added to each well and the cultures were gently shaken on a TITRAMAX 1000 shaker (Heidolp, Germany) for 15 min. The isopropanol containing fluorescent dye was centrifuged at 500 g for 15 min and the fluorescent intensity in the supernatant was determined at 520 nm (excitation) and 578 nm (emission) wavelengths by a microplate reader (Beckman coulter DTX 990 multimode detector, USA). The remaining cellular protein was dissolved in 0.5 N NaOH for protein determination (Lowry *et al*., 1951).

2.8 Expression of HMG CoA reductase and LDL receptor genes

Semi-quantitative-reverse transcription-polymerase chain reaction (RT-PCR) was used to study the effect of PMO on the gene expression of HMG CoA reductase and LDL receptor. The overnight incubated cells (at a density of 10^6 cells/well), were pre-incubated with serum-free DMEM for 12 hrs; then treated with various concentration of PMO at 37°C in 5% CO_2 for 24 hrs. The cells were harvested and total RNA was extracted by RNA isolation kit according to the manufacturer's instructions. The purity of RNA was confirmed with the spectrophotometric absorbance ratio at 260/280 nm and RNA quantity was determined at the absorbance of 260 nm. Total RNA (40 ng) were subjected to RT-PCR using a Two Step RT-PCR kit and performing by thermo cycler (Biometra, Germany). Specific oligonucleotides were based on published sequences (Suarez *et al*., 2004). The product sizes were 471, 408, and 661 bp, and 26 , 29, and 30 cycles for HMG CoA reductase, LDL receptor and β-actin, respectively. Each cycle consisted of 90°C denaturation for 1 min, 60°C primer annealing for 1 min, and 72°C primer extension for 2 min. cDNA was strained (NOVEL JUICE) and analyzed by electrophoresis on 1.5% agarose gel. RT-PCR product density was determined by Gel Documentation and a system analysis machine (Gel Documentation InGenius L, Bio-rad Lab, Hercules, CA, USA). The data were expressed as the relative mRNA expression level with β-actin.

2.9 Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean+S.D. One-Way ANOVA and multiple comparisons were used to test for significant differences ($P<0.05$) using SPSS version 19.0.

3. Results

3.1 Phenolic content of PMO

By comparing the retention times of HPLC chromatograms and spiking with each standard compound, at least six phenolic compounds were determined to be present in PMO, which were 17.63±0.13 mg/g of caffeic acid (RT=38.09 min), 28.6±1.13 mg/g of p-coumaric acid (RT=47.95 min), 11.47± 1.07 mg/g of rutin or quercetin 3-rutinoside (RT=62.14 min), 80.23±0.78 mg/g of isoquercetin or quercetin 3-glucoside (RT=63.17 min), 62.48±0.65 mg/g of quercitrin or quercetin 3-rhamnoside (RT=67.98 min), and 1.88±0.15 mg/g of quercetin (RT=75.30 min), and several unidentified peaks (Figure 2). The content summation of these six phenolics was 202.3 mg/g (20.23%), indicating the phenolics-enriched extracted of MO (PMO). Among these phenolic compounds, isoquercetin and quercitrin were the major constituents.

3.2 PMO decreased intracellular cholesterol

As shown in Figure 3a, PMO (at 100-400 μg/ml concentrations) significantly decreased intracellular cholesterol in HepG2 cells in a similar fashion to standard EGCG.

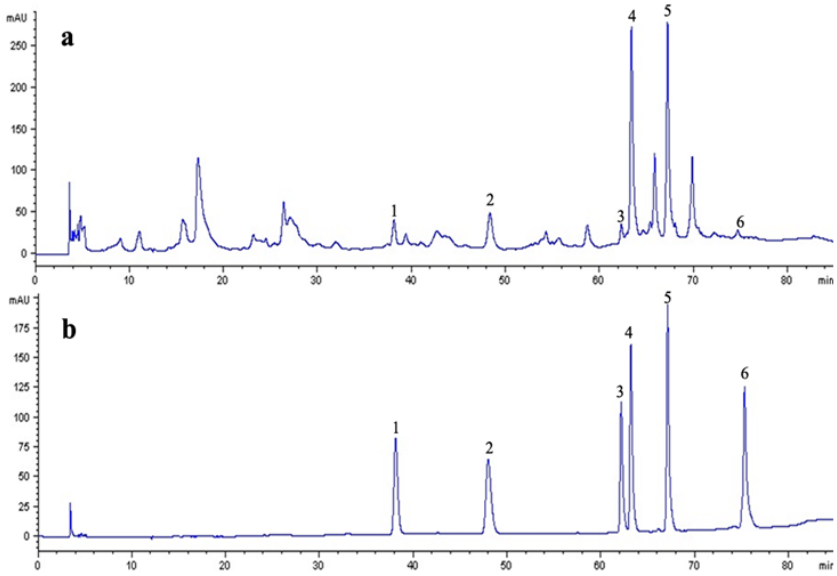

Figure 2. HPLC chromatograms of PMO (a) and six standard phenolic compounds (b), 1 = caffeic acid, 2 = *p*-coumaric acid, 3 = rutin, 4 = isoquercetin, 5 = quercitrin, 6 = quercetin.

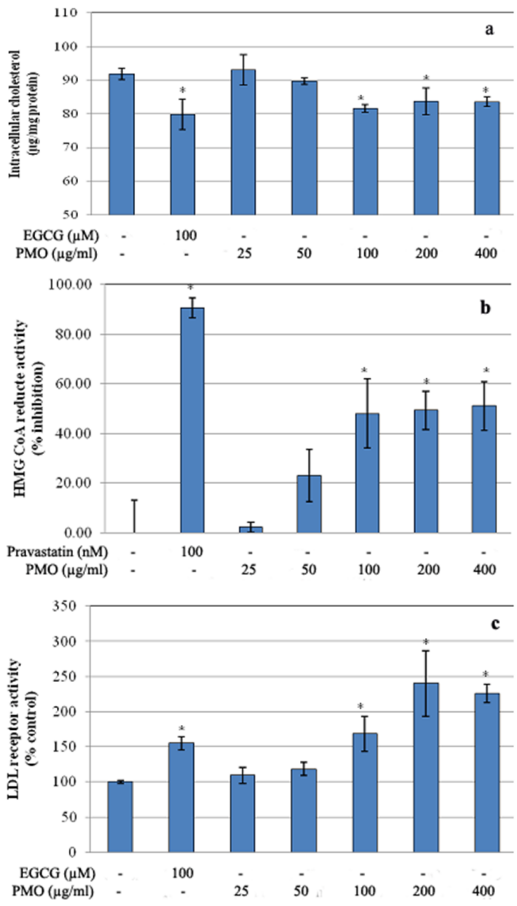

Figure 3. Effect of PMO on intracellular cholesterol content (a), HMG CoA reductase (b), and LDL receptor binding activity (c). Values are expressed as mean±SD, (n=3); * Significant differences from control at p <0.05.

3.3 PMO inhibited HMG CoA reductase activity

PMO significantly inhibited HMG CoA reductase activity in a dose-dependent manner. The inhibitions were 23.08±10.47, 48.10±14.02, 49.43±7.70, and 51.18±9.80 % at 50, 100, 200, and 400 µg/ml of PMO, respectively (Figure 3b).

3.4 PMO increased LDL receptor binding activity

PMO increased the LDL receptor binding activity. The amount of LDL uptake by HepG2 cells was initially significantly greater than that of control with the addition of 100 µg/ml of PMO, and attained a plateau onward from 200-400 µg/ml of PMO (Figure 3c). It is interesting to observe that PMO at doses of 100-400 µg/ml increased the activity of LDLR greater than the positive control EGCG at 100 ìM.

3.5 Effect of PMO on the expression of cholesterol metabolism-related genes

PMO significantly increased the genetic expression of both LDL receptor and HMG CoA reductase (Figure 4). PMO (25-400 µg/ml) had a significantly higher effect on the expression of LDL receptor gene than EGCG (100 µM). Similarly, PMO at 100-400 µg/ml concentrations significantly enhanced the expression of HMG CoA reductase gene at a level comparable to that of EGCG (100 µM).

4. Discussion

The aim of the present study was to elucidate the underlying mechanism of the hypocholesterolemic effect of PMO. Since the liver plays a central role ensuring the

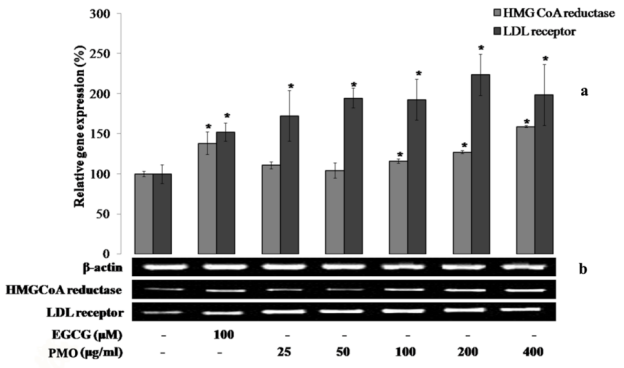

Figure 4. Effect of PMO on mRNA expression of LDL receptor and HMG CoA reductase genes (a) and their electrophoresis bands (b). Results are expressed as mean±SD (n=3), * Significant differences from control at $p < 0.05$.

systemic homeostasis of glucose and lipid metabolism via the ability to metabolise cholesterol and triglycerides, as well as the synthesis of lipoproteins (Wu *et al*., 1984; Javitt, 1990), HepG2 cells were used as a liver model.

The findings that PMO decreased intracellular cholesterol and HMG CoA reductase activity in HepG2 cells were in agreement with a previous report that PMO decreased the cholesterol accumulation in rat liver (Gashi *et al*., 2000). The direct inhibitory effect of PMO on HMG CoA reductase activity can deplete intracellular cholesterol, similar to the previous report that statins can inhibit HMG CoA reductase activity and decrase intracellular cholesterol in HepG2 cells (Scharnagle *et al*., 2001). EGCG was used as a positive control in this study, because it was earlier reported to block HMG CoA reductase activity in liver microsomes (Cuccioloni *et al*., 2011), and decrease intracellular cholesterol in HepG2 cells (Bursill *et al*., 2001).

The significant increase in genetic expression of HMG CoA reductase and LDL receptor genes as well as the significant stimulation of the LDL receptor binding activity, suggest a vital role of PMO in the regulation of cholesterol metabolism. In the cholesterol feedback regulation to control the level of intracellular cholesterol and lipid metabolism, a group of key lipogenic transcription factors, called sterol regulatory element binding proteins (SREBPs), affect several genes related to lipid metabolism such as HMG CoA reductase and LDL receptor genes (Wang *et al*., 1993; Vallett *et al*., 1996). In general, the SREBPs are synthesized as inactive proteins. When the intracellular cholesterol is depleted, the SREBPs are translocated from ER to Golgi bodies, which are activated by two proteinase enzymes, S1P and S2P. Then active SREBPs are translocated to the nucleus and promote the transcription of SREBPs target genes such as HMG CoA reductase and LDL receptor gene (Bengoechea-Alonso and Ericsson, 2007). Interestingly, the findings that PMO enhanced the expression of LDL receptor and HMG-CoA reductase mRNA, and increased LDL receptor activity are similar to those of a previous report on the effect of HMG CoA reductasae inhibitor statin (Scharnagl *et al*., 2001). Surprisingly, PMO increased the expression of the HMG CoA reductase mRNA, but the intracellular cholesterol was still depleted. This phenomenon may suggest competitive inhibition against the substrate HMG CoA of PMO, as previously observed for mevastatin (Endo *et al*., 1976). Moreover, PMO may modulate the phosphorylation of HMG CoA reductase to the inactive form, as observed for garlic extract (Liu and Yeh, 2002). Therefore, PMO may regulate cholesterol metabolism via the cholesterol feedback regulation. However, the mechanism of PMO on the HMG CoA reductase activity, SREBP expression and activity should be further investigated. Taken together, our results provide the underlying mechanism to support the previous finding that MO extracts decreased blood cholesterol in animals (Ghasi *et al*., 2000; Chumark *et al*., 2008). Furthermore, our results suggest that PMO affected cholesterol levels by blocking cholesterol synthesis, thereby reducing the liver cholesterol and increasing the expression and production of LDL receptors.

A number of phenolic compounds were reported to have hypocholesterolemic effect. For example, quercetin at 75 μM concentration significantly induced LDL gene expression, accompanied by an increase in nuclear SREBPs (Moon *et al*., 2012). Rutin was shown to inhibit oleic acid induced lipid accumulation via inhibition of transcription of HMG CoA reductase in HepG2 cells (Wu *et al*., 2011). Moreover, rutin and quercitin in buckwheat protein were reported to have cholesterol-lowering activity (Fabjan *et al*., 2003; Tomotake *et al*., 2007). A diet containing 0.2% of caffeic acid significantly reduced both HMG CoA reductase activity and total cholesterol in high-cholesterol diet induced rats (Yeh *et al*., 2009), isoquercetin significantly decreased total cholesterol and triglyceride in diabetic mice and high-cholesterol diet induced rabbits (Kamada *et al*., 2005; Zhang *et al*., 2011) and *p*-coumaric acid also significantly decreased total cholesterol and triglyceride in the isoproterenol induced myocardial infarcted rats (Roy and Stanely Mainzen Prince, 2013). With the findings that PMO contained considerable amount of caffeic acd, *p*-coumaric acid, rutin, isoquercetin, quercitrin and quercetin, therefore, these six phenolic compounds may confer the hypolipidemic effect.

5. Conclusions

Our data demonstrate a vital role of PMO in LDL-cholesterol metabolism as an inducer of LDL receptor expression and binding activity by blocking HMG CoA reductase activity. These findings suggest the underlying mechanism of hypocholesterolemic action of *Moringa oleifera*, which occurred via the inhibition of HMG CoA reductase activity, enhancement of LDL receptor binding activity and up-regulation of LDL receptor gene expression.

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