Research Article

Brazilian Green Propolis Inhibits Ox-LDL-Stimulated Oxidative Stress in Human Umbilical Vein Endothelial Cells Partly through PI3K/Akt/mTOR-Mediated Nrf2/HO-1 Pathway

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Propolis has been widely used as a dietary supplement for its health benefits, including cardiovascular protective effects. The aim of this study was to investigate the cytoprotective effects of Brazilian green propolis (BP) against oxidized low-density lipoprotein (Ox-LDL) induced human umbilical vein endothelial cells (HUVECs) damage. Our results suggested that treatment with BP rescued Ox-LDL-stimulated HUVECs cell viability losses, which might be associated with its inhibitive effects on the cell apoptosis and autophagy. We also noticed that BP restored Ox-LDL-stimulated HUVECs oxidative stress, by induced antioxidant gene expressions, including Hemeoxygenase-1 and its upstream mediator, Nrf2, which were mediated by the activation of the phosphorylation of PI3K/Akt/mTOR. Pretreatment with wortmannin, PI3K/AKT inhibitor, abolished BP induced Nrf2 nuclear translocation and HO-1 level. Our results demonstrated that BP protected HUVECs against oxidative damage partly via PI3K/Akt/mTOR-mediated Nrf/HO-1 pathway, which might be applied into preventing Ox-LDL mediated cardiovascular diseases.

1. Introduction

Atherosclerosis is a complex chronic inflammatory and metabolic disease, which is a consequence of oxidative stress, where homeostasis between endogenous antioxidants and reactive oxygen species is disrupted leading to lipid and protein oxidation in the vascular wall. Accumulation of lipoproteins in the vessel wall provides the initial trigger for vascular inflammation, causing endothelial dysfunction and monocyte recruitment [1]. Oxidized low-density lipoprotein (Ox-LDL) is a crucial factor in triggering the development of atherosclerosis [2]. It not only directly targets vascular endothelial cells (VECs) to induce endothelium injury or dysfunction, but can activate monocytes and macrophages by binding to scavenger receptors leading to the formation of plaque and secretion of proinflammatory cytokines [3, 4]. A mounting evidence indicated that endothelial cells dysfunctions may be harmful to the plaque vulnerability [5, 6]. Thus, protecting the endothelial cells against damage or death has been considered a novel target for the atherosclerotic treatment.

Previous reports suggested that oxidative stress plays an important role in Ox-LDL induced cell damage [7]. The imbalance of redox status of a cell can cause damage to cellular components or induce cell necrosis or apoptosis, and suppressing oxidative stress has the potential to be useful in cardiovascular diseases treatment [8]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is known as key modulator by upregulating of a number of detoxifying antioxidant enzymes, including heme-oxgenase-1 (HO-1), quinone oxidoreductase (NQO-1), glutathione S-transferase (GST), NAD(P)H, and γ-glutamyl cysteine synthetase catalytic subunit (GCLC) [9, 10]. Nrf2 is distributed mainly in the cytoplasm and its translocation to the nucleus is inhibited by a Kelch-like ECH-associated protein 1 (Keap1) under normal physiological conditions. Confirmation of Keap1 changes through interacting
with other inducers results in the release of Nrf2, which is
then free to translocate into the nucleus to regulate basal
antioxidant-response elements (ARE) expression including
HO-1[11]. Nrf2/HO-1 axis provides a theoretical basis for the
therapeutic effects against various oxidative stress-relevant
diseases, through counteracting oxidative stress injury to
resist inflammation, oxidation, mitochondrial damage, apopt-
osis, pyroptosis, and autophagy [12]. Additionally, vari-
os signaling cascades including mitogen-activated protein
kinase (MAPK), phosphatidylinositol 3-kinase (PI3K/Akt),
protein kinase C (PKC), c-jun N-terminal kinase (JNK),
and extracellular signal-regulated kinase (ERK) participate in
Nrf2 activation [13], etc.

Propolis, a resinous material that honeybees (Apis mel-
ilifera L.) collect from various plant resources, is used widely
as a health and functional food [14]. Abundant polyphenolic
constituents have been identified in propolis [15]. In recent
years phenolic compounds have been considered as intra-
cellular direct antioxidants, by scavenging oxidizing species,
and to exert an indirect effect, by inducing the upregulation of
the synthesis of endogenous antioxidant enzymes such as
superoxide dismutase, catalases, and peroxidases [16]. Our
recent studies have demonstrated that propolis has shown
excellent antioxidant activities, which might be attributed to
the activation of PI3K/Akt/mTOR, an important signaling
pathway in mediating cellular survival, proliferation, differen-
tiation, apoptosis, and metastasis [17]. However, the rela-
tionship between Nrf2/ HO-1 activation and PI3K/Akt/mTOR
signal pathway on protecting endothelial cells has not been
fully elucidated. The present study was designed to verify
the hypothesis that Brazilian green propolis could inhibit
apoptosis and autophagy induced by Ox-LDL in HUVECs by
PI3K/Akt/mTOR-mediated Nrf2/ HO-1 pathway.

2. Materials and Methods

2.1. Materials. Dulbecco’s modified Eagle's medium (DMEM)
and fetal bovine serum (FBS) were obtained from Gibco (USA).
Sulfurhodamine B (SRB), acridine orange (AO),
Hoechst 33258, 2′,7′-dichlorodihydrofluorescin (DCHF),
and JC-1 were from Sigma Co. (USA). Primary antibodies
against β-actin, PI3K, and p-PI3K were from Santa Cruz
Biotechnology (USA). Primary antibodies against caspase
3, PARP, LC3B, p70S6K, p-p70S6K, p-mTOR, mTOR, Akt,
p-Akt, Beclin 1, Atg7, and Nrf2 and secondary antibody
(horseradish peroxidase) were from Cell Signaling Technol-
ogy (USA). Primary antibodies against Bcl-2 and Bax were
from ABclonal biotech (USA). Primary antibody against p62
was from BD Transduction Laboratories (USA). Primary
antibody against HO-1 was from Abcam (USA). Primary
antibody Lamin B was from Beyotime (China). Secondary
antibody for immunofluorescence, donkey anti-rabbit IgG
Alexa Fluor-488, was purchased from Life Technologies
(USA). Wortmannin was obtained from Selleck (USA). Ox-
LDL was purchased from Beijing Xiesheng Biotechnology
(China). DPPH, ABTS, α-tocopherol (Vitamin E), gallic acid,
quercetin, and the standards used in HPLC analysis were
purchased from Sigma (St. Louis, MO, USA). All other
reagents were of ultrapure grade.

2.2. Preparation of Ethanol Extracted Brazilian Propolis. 
Brazilian green propolis collected in Minas Gerais State of
Brazil was used in present study, where Baccharis dracun-
culifolia DC was the main botanical source. The extraction
method was as follows. 95% (v/v) ethanol was used to
extract raw Brazilian green propolis in the dark for 24 h
with a constant stirring. The supernatant was filtered to
remove the residues. Then the solvent was evaporated in a
rotary evaporator under a reduced pressure at 50°C until
reaching a constant weight. The ethanol extracted Brazilian
green propolis (EEBP) was redissolved in ethanol when it
is used. The major chemical constituents were analyzed by
6510 Liquid chromatography-tandem quadrupole time of
flight mass spectrometry (HPLC-DAD/Q-TOF-MS). In brief,
Proshell 120SB-C18 column (2.1 mm×100 mm, 2.7 μm) was
used in the chromatographic experiments. The mobile phases
used were methanol/water in gradient elution mode (0–2
min,15%–30%; 10–25 min, 30%–90%; 25–30 min, 90%; 30–
31 min, 15%). The sample injection volume was 2 μL, and
the concentration of propolis was 10 mg/mL. The column
temperature was maintained at 30°C and the flow rate was
set to 0.2 mL/min. The UV detector was set at 254 nm.
The optimal MS conditions were set as follows: electrospray
ionization (ESI); flow rate and temperature were 11.0 L/min
and 350°C; nebulizer, 40 psi, respectively.

2.3. Determination of Total Phenolic Content (TPC). The TPC
was measured by the modified Folin-Ciocalteu method [18].
In brief, 100 μL EEBP (300, 500, and 700 μg/mL) was mixed
with 100 μL Folin-Ciocalteu reagent and kept for 5 min after
vortex in the dark. Then the sample was incubated with 300
μL 2% sodium carbonate at room temperature for 2 h. Finally,
200 μL solution was injected into 96-well plate and measured
at 765 nm. In the experiment, different concentrations of
gallic acid (0, 5, 10, 20, 40, 60, 80, and 100 μg/mL) were used
as a standard and the results were expressed as gallic acid
equivalent (GAE) per gram sample.

2.4. Total Flavonoids Contents (TFC). The TFC was deter-
mined by the modified method [18]. 150 μL EEBP (300, 500,
and 700 μg/mL) was mixed with 10 μL aluminum nitrate (100
g/L) and 10 μL potassium acetate (9.8 g/L). The absorbance
was measured at 415 nm after being incubated at room
temperature for 1 h. In the experiment, concentrations of
quercetin (4–80 μg/mL) were used as a standard to determine
TFC in EEBP. The results were expressed as milligrams
quercetin equivalent per gram sample.

2.5. DPPH Radical-Scavenging Test. DPPH radical-scavenging
activity of EEBP was tested by the modified method [18, 
19]. Different concentrations of EEBP (70–280 μg/mL) were
prepared for the reaction. The reaction mixtures in the 96-
well plates consisted of the sample (50 μL) and DPPH radicals
(50 μL, 0.5 mg/mL) dissolved in ethanol and kept in the dark
for 30 min. The absorbance was measured at 517 nm against
a blank. All determinations were performed in triplicate.
The scavenging activity of the samples was expressed as the IC₅₀
value, the concentration required to scavenge 50% of DPPH
radicals.
2.7. **Cell Culture.** HUVECs were generous gift from Atherosclerosis Research Institute of Taishan Medical University of China. HUVECs were cultured in high-glucose DMEM medium supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin. Cellswere grown and maintained in a humidified incubator at 37°C and 5% CO₂. All studies were performed using 80-90% confluent cells before treatment. To induce vascular endothelial damage, HUVECs were treated with designed concentrations of Ox-LDL (20, 40, and 80 µg/mL).

2.8. **Cell Viability Assay.** Cells were seeded in 96-well plates. After treatment with EEBP (1.25, 2.5, and 5 µg/mL) combined with Ox-LDL (40 µg/mL) for 3, 6, and 12 h, cell viability was measured by SRB assay. Briefly, 100 µL 10% trichloroacetic acid was used to precipitate cells at 4°C for 1 h. After 5 washes in deionized water, the cells were stained with 50 µL of 0.4% (W/V) SRB solution at room temperature for 20 min and then washed five times with 1% acetic acid. 100 µL of 10 mM Tris base was used to dissolve the bound dye. The optical densities were measured at the wavelength of 492 nm using microplate spectrophotometer.

The viability (%) was expressed as (OD of treated group/OD of Ox-LDL group) ×100%. The viability of the Ox-LDL group was set at 100%.

2.9. **Acridine Orange (AO) and Hoechst 33258 Staining.** AO and Hoechst 33258 were used to test the morphological changes of nuclei and apoptosis, respectively. At 6 h, cells were stained with AO (5 µg/mL) at room temperature for 5 min or stained with Hoechst 33258 (10 µg/mL) for 15 min. After that, cells were observed under a laser scanning confocal microscope (Olympus FV1200, Japan) or a TE2000S fluorescence microscope (Nikon, Japan), respectively.

2.10. **Real-Time PCR Analysis.** After treatment with EEBP (1.25, 2.5, and 5 µg/mL) combined with Ox-LDL (40 µg/mL) for 6 h, total RNA from HUVECs was extracted by RNA extraction kit (Carry Helix, China) in accordance with the manufacturer's protocol. The cDNA was synthesized from RNA with PrimeScript RT Kit (TaKaRa, Dalian, China) in accordance with the manufacturer's protocol. Quantitative PCR was performed with SYBR Premix EX Taq (TaKaRa, Dalian, China) using a Real-time PCR Detection System (Agilent StrataGene Mx3000, USA). The expression of the housekeeping gene β-actin was used to normalize the expression levels, and the results were expressed as 2^ΔΔCt. The primer pairs were the following: HO-1 (sense, 5'-TCTTGGCCTGGCGTTCTTACC-3'; antisense, 5'-GGATGTGCTTTTCCGTTGGGG-3'), Nrf2 (sense, 5'-CAACACTTCCAGGTTGCGCCC-3'; antisense, 5'-AGTACGTAACGCTAGCCGAA-3'), β-actin (sense, 5'-GCGTTTCCGAAAGTTGCC-3'; antisense, 5'-CGCGGGATATCATCATCATTCCAT-3').

2.11. **Immunofluorescence Assay.** Immunofluorescence assay was performed as previous method [21]. In brief, cells were fixed in 4% paraformaldehyde (w/v) at room temperature for 15 min and then blocked with 5% donkey serum (v/v) for 20 min. After adding the primary antibody-LC3B (1:100) and second antibody (1:200) (FITC-IgG), a laser scanning confocal microscope (Olympus FV1200, Japan) was used for fluorescence detection. Analysis used the Image-Pro Plus software (USA). Images are representative of three independent experiments.

2.12. **Western Blotting Analysis.** Western blotting analysis was performed as previous method [21]. After treatment with EEBP (1.25, 2.5, and 5 µg/mL) combined with Ox-LDL (40 µg/mL) for 6 h, cells were washed twice with ice-cold PBS. Total cell lysates and nuclear extracts were prepared by cell lysis buffer and a nuclear extraction kit (Thermo Scientific, USA), respectively. Protein concentration was measured by the Bradford method. Equal amounts of protein were separated by 12-15% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. 5% skim milk was used to block the membranes at room temperature for 1 h. After that, the membranes were probed with primary antibodies (1:1000) at 4°C for 16 h and incubated with second antibodies (1:3000-5000) at 37°C for 1 h after three washes in TBST. The bands were visualized using an enhanced chemiluminescent detection kit (Thermo Electron Corp., USA).

2.13. **Intracellular Reactive Oxygen Species (ROS) Analysis.** To determine ROS production, cells were washed with basal DMEM medium after treatment and then stained with 0.5 mL DCFH at 37°C for 30 min in the dark. After three washes with basal DMEM medium, the fluorescence was monitored with a confocal laser scanning microscope (Olympus FV1200, Japan). The photographs were representative of three independent experiments. Results were shown as the relative fluorescence intensity ratio compared with Ox-LDL group.

2.14. **Mitochondrial Membrane Potential Measurement.** JC-1 probe was used to test mitochondrial membrane potential. In brief, JC-1 probe was added to cells after EEBP treatment at 37°C for 15 min in the incubator, and cells were washed three times with basal DMEM medium. Then the fluorescence...
and the flavonoid content was 51.9%. The phenolic concentrations ranged from 49 to 100 mg GAE/g. Green propolis from the state of São Paulo have reported that was 83.20 ± 4.78 mg GAE/g. Some studies with ethanoic extracts of EEBP (1.25, 2.5, and 5 μg/mL) could dramatically augment the mRNA expression of Nrf2 and its downstream signal molecular –HO-1 at 6 h (Figure 1(b)). AO and Hoechst 33258 staining also indicated that EEBP treatment obviously inhibited nuclear concentration and fragment caused by Ox-LDL (Figures 1(c) and 1(d)).

To further confirm EEBP inhibited apoptosis in Ox-LDL-stimulated HUVECs, the hallmarks of apoptosis, caspase 9, Bax, Bcl-2, caspase 3, and PARP levels were measured by western blotting analysis. After EEBP treatment, the antiapoptosis protein Bcl-2 level significantly improved, and the level of proapoptosis protein Bax evidently decreased. Furthermore, the cleaved caspase 9, caspase 3, and PARP levels were significantly suppressed after EEBP treatment (Figure 2).

2.15. Statistical Analysis. Data are from at least three independent experiments and expressed as means ± S.E.M. Statistical analysis involved the paired Student t test and ANOVA with SPSS Ins (PASW Statistics 18). Differences were considered statistically significant at P < 0.05.

3. Results

3.1. Components Identified in EEBP. A total of 7 compounds were identified and quantified in EEBP based on HPLC-DAD/Q-TOF-MS analysis (Table 1). As shown in Table 1, p-coumaric acid (11.72 mg/g) and artemepillin C (39.80 mg/g) were the dominant ingredients in EEBP.

3.2. Total Phenolic Contents (TPC), Total Flavonoid Contents (TFC), and Free Radical-Scavenging Activities of EEBP. The contents of TPC and TFC in EEBP were shown in Table 2. The content of TPC was 111.40 ± 4.78 mg GAE/g, and TFC was 83.20 ± 4.54 mg/g. Some studies with ethanoic extracts of green propolis from the state of São Paulo have reported that the phenolic concentrations range from 49 to 100 mg GAE/g, and the flavonoid content was of 51.9 ± 2.4 mg/g [22].

And the DPPH free racial scavenging activity (IC_{50}) of EEBP was 179.11 ± 4.09, and ABTS' (IC_{50}) was 72.10 ± 0.40, which was lower than Chinese propolis [23].

was monitored with a confocal laser scanning microscope (Olympus FV1200, Japan). Results were shown by ratio of red to green fluorescence as compared with the Ox-LDL group.

3.4. EEBP Suppressed Autophagy in Ox-LDL-Stimulated HUVECs. Besides EEBP suppressed apoptosis, we also wondered about the effect of EEBP on autophagy. As shown in Figure 3(a), when challenged with EEBP (1.25, 2.5, and 5 μg/mL), cells stained with anti-LC3 antibody showed a significantly decreased punctuated pattern compared with Ox-LDL group (Figure 3(a)). Western blotting results also demonstrated that EEBP treatment obviously inhibited LC3 I to LC3 II shift. The levels of p62, the hallmark of autophagic flux, were obviously enhanced after EEBP treatment. Moreover, Beclin 1 and Atg7 levels which also are involved in autophagy also decreased during the process (Figures 3(b) and 3(c)).

3.5. EEBP Ameliorated Oxidative Stress in Ox-LDL-Stimulated HUVECs. The Ox-LDL led to oxidative stress-induced HUVECs damage, which was regarded as an important step in the process of atherosclerosis. To find the protective mechanism of EEBP on Ox-LDL-stimulated HUVECs, we firstly tested the antioxidant genes levels of Nrf2 and HO-1. Nrf2 is a key regulator in the cellular adaptive response to oxidative stress [24]. The activation of Nrf2 is involved in the protection of cells against oxidative stress. In Ox-LDL-stimulated HUVECs, we found that EEBP (2.5 and 5 μg/mL) could dramatically augment the mRAN expression of Nrf2 and its downstream signal molecular –HO-1 at 6 h.

Table 1: HPLC-DAD/Q-TOF-MS analysis on EEBP.

<table>
<thead>
<tr>
<th>compounds</th>
<th>Retention time</th>
<th>[M+H]⁺</th>
<th>EEBP (content mg/g)</th>
<th>regression equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>kaempferol</td>
<td>28.4</td>
<td>287.055</td>
<td>1.05</td>
<td>y = 94864x + 129824</td>
</tr>
<tr>
<td>caffeic acid</td>
<td>17.2</td>
<td>181.0495</td>
<td>1.51</td>
<td>y = 32392x + 22456</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>20.22</td>
<td>165.0546</td>
<td>11.72</td>
<td>y = 45243x + 73332</td>
</tr>
<tr>
<td>purenebio</td>
<td>9.8</td>
<td>155.0339</td>
<td>0.34</td>
<td>y = 23525x + 22623</td>
</tr>
<tr>
<td>artemepillin C</td>
<td>34.7</td>
<td>301.1798</td>
<td>39.80</td>
<td>y = 109286x - 69009</td>
</tr>
<tr>
<td>naringenin</td>
<td>27.0</td>
<td>273.0612</td>
<td>0.04</td>
<td>y = 137541x + 229226</td>
</tr>
<tr>
<td>ferulic acid</td>
<td>21.4</td>
<td>195.0652</td>
<td>0.14</td>
<td>y = 126715x + 159085</td>
</tr>
</tbody>
</table>

Table 2: The contents of TPC and TFC in EEBP and the free radical scavenging activities of EEBP.

<table>
<thead>
<tr>
<th>sample</th>
<th>TPC (mg GAE/g)</th>
<th>TFC(mg/g)</th>
<th>DPPH(IC_{50})</th>
<th>ABTS' (IC_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEBP</td>
<td>111.40±4.78</td>
<td>83.20 ± 4.54</td>
<td>179.11 ± 4.09</td>
<td>72.10 ± 0.40</td>
</tr>
</tbody>
</table>
Figure 1: Effects of ethanol extracted Brazilian green propolis (EEBP) on cell viability in Ox-LDL treated HUVECs. (a), Effects of Ox-LDL (20, 40, and 80 μg/mL) on cell viability in Ox-LDL induced HUVECs at 6 h. (b), Effects of EEBP (1.25, 2.5, and 5 μg/mL) on cell viability in Ox-LDL induced HUVECs for 3, 6, and 12 h. Cell viability was tested by SRB assay and illustrated in column figures. (* P < 0.05, ** P < 0.01 vs Ox-LDL, n=3). Data are means ± S.E.M. (c), AO staining showed treatment with EEBP depressed nuclear condensation and fragmentation in Ox-LDL induced HUVECs (200×). (d), Hoechst 33258 staining suggested that treatment with EEBP inhibited apoptosis in Ox-LDL induced HUVECs (200×).

3.6. EEBP Activated PI3K/Akt/mTOR Signaling Pathway. Furthermore, activation of Nrf2 requires regulation of protein kinases, including the MAPK cascade, PI3K/Akt, and PKC signaling pathways. The phosphorylation of PI3K activated Akt and then mTOR can integrate upstream activating signals through PI3K/Akt pathway and become phosphorylated form, which negatively regulates autophagy [25]. To further determine the antioxidant mechanism of EEBP depressing oxidative stress induced by Ox-LDL, we investigated the effects of EEBP treatment on PI3K/Akt/mTOR signaling pathway. As shown in Figure 5, cells challenged with EEBP evidently increased the phosphorylation levels of PI3K, Akt, and mTOR, and the phosphorylation level of p70S6K, the
direct downstream target of mTOR, was also significantly elevated.

3.7. EEBP Attenuated Oxidative Stress Was PI3K/AKT/mTOR Dependent. Mounting evidence has demonstrated that activation of Nrf2 may be accompanied by Nrf2 nuclear translocation and bind with the ARE sequences. As shown in Figure 6(a), EEBP treatment enhanced Nrf2 accumulation in the nucleus, but Nrf2 level in the nucleus was significantly depressed after wortmannin treatment, a PI3K/Akt inhibitor (Figure 6(a)). Furthermore, the protein expressions of p-Akt, p-p70S6K, and HO-1 were inhibited in cells challenged with wortmannin as well as EEBP treatment (Figures 6(b) and 6(c)), indicating that EEBP attenuated oxidative stress was PI3K/Akt/mTOR dependent.

4. Discussion

In this study, we showed the antioxidant activity of the Brazilian green propolis is the key effects for its protecting in vascular endothelial cells against Ox-LDL challenge. Importantly, we have shown that Brazilian green propolis promoted activation of PI3K/Akt/mTOR pathway, further enhancing the cellular antioxidant system. This increased antioxidant activity attenuated apoptosis and inhibited autophagy of HUVECs induced by Ox-LDL.

The chemical constituents of propolis including poplar propolis and green propolis vary greatly according to plant origins and geographical and climatic features of the collection site [26, 27]. Thus we tested the chemical constituents of this sample, and the predominating components in the sample used are p-coumaric acid and artepillin C, which can
be seen as the markers of green propolis [27]. Moreover, Brazilian green propolis is rich in antioxidant polyphenols, which exerts excellent antioxidant activity.

Oxidative stress is an imbalance between prooxidants and antioxidants, and it has been postulated to be closely associated with cardiovascular diseases, including atherosclerosis. Nrf2 nuclear translocation augments expression of antioxidant-response element HO-1 and NQO1. HO-1 is induced by a variety of conditions associated with oxidative stress. Ishikawa et al. (2001) demonstrated that mildly oxidized LDL markedly induced HO-1 in human aortic endothelial damage [28]. Accumulating evidence demonstrates that natural plant-derived pharmacological modulators upregulate Nrf2/ARE to prevent chronic diseases including cardiovascular disease [29]. Here, BP, one of the plant-derived natural products, shows excellent antioxidant activities through attenuating ROS, protecting mitochondrial membrane potential, activating the antioxidant gene expression of Nrf2 and HO-1. Thus, Brazilian green propolis could improve redox conditions against oxidative stress to protect endothelium cells and further to prevent cardiovascular diseases. Additionally, various signaling cascades including mitogen-activated protein kinase are involved in the activation of Nrf2/HO-1 [30]. The present study showed Brazilian green propolis inhibiting oxidative stress was dependent PI3K/AKT/mTOR activation. Other studies have suggested that poplar type propolis stimulated antioxidant genes expression via p38/Erk-Nrf2 pathway [30]. There are more than 600 kinds of chemical compositions identified in propolis from different plant sources. The chemical compositions of poplar propolis and Brazilian green propolis vary greatly. The chemical constituents of poplar type propolis are mainly flavonoids, phenolic acids, and esters. However, p-coumaric acid and artepillin C are the dominant ingredients in Brazilian green propolis. The difference in chemical constituents may lead to the difference in the antioxidant mechanism.

The most inspiring aspect of the present study is that it explores the regulatory mechanism controlling vascular endothelial cells apoptosis and autophagy stimulated by Ox-LDL. Ox-LDL inhibits mTOR and induces apoptosis and autophagy in vascular endothelial cells [31]; Bcl/Bax-caspase9-caspase3 pathway maybe was involved in Ox-LDL-induced HUVECs apoptosis [32]. Many studies also have confirmed that propolis can modulate the expression of the Bcl-2 family genes, producing upregulation of the antiapoptotic Bcl-2 and downregulation of the proapoptotic Bax [33]. Here we found that Ox-LDL-induced HUVECs apoptosis could be inhibited by Brazilian green propolis, in which Bcl2/Bax-caspase9-caspase3 pathway maybe was involved.
Figure 4: Continued.
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FIGURE 4: EEBP ameliorated oxidative stress in Ox-LDL-stimulated HUVECs at 6 h. (a), mRNA levels of HO-1 and Nrf2. (b), Expression of HO-1 and Nrf2 of total cell lysate in Ox-LDL-stimulated HUVECs. (c), Expression of Nrf2 of nuclear lysate in Ox-LDL-stimulated HUVECs. (d), Expression of Nrf2 of cytoplasmic lysate in Ox-LDL-stimulated HUVECs. (e), Fluorescent micrographs of ROS obtained in Ox-LDL induced HUVECs at 6 h. (f), Quantification of relative quantity of ROS in Ox-LDL induced HUVECs at 6 h. Values represent the relative fluorescent intensity per cell determined by laser scanning confocal microscopy. (g), Fluorescent micrographs of mitochondrial membrane potential obtained in Ox-LDL induced HUVECs at 6 h. (h), Quantification of relative fluorescent intensity per cell determined by laser scanning confocal microscopy. Values are represented as ratio of red to green fluorescence (* P < 0.05, ** P < 0.01 vs Ox-LDL, n=3). Data are means ± S.E.M.

FIGURE 5: EEBP activated PI3K/Akt/mTOR signaling pathway. (a), Expression of Akt, p-Akt, PI3K, and p-PI3K after treatment with EEBP in Ox-LDL induced HUVECs at 6 h, respectively. (b), Quantification of relative expression quantity of p-Akt and p-PI3K in Ox-LDL induced HUVECs at 6 h. (c), Expression of mTOR, p-mTOR, p70S6K, and p-p70S6K after treatment with EEBP in Ox-LDL induced HUVECs at 6 h. (d), Quantification of relative expression quantity of p-mTOR and p-p70S6K in Ox-LDL induced HUVECs at 6 h (* P < 0.05, ** P < 0.01 vs Ox-LDL, n=3). Data are means ± S.E.M.

through increasing Bcl-2 and decreasing Bax levels and inhibited the activation of both caspases 9 and 3. On the other hand, autophagy is now known to become dysfunctional in atherosclerosis. Basal autophagy can protect plaque cells against oxidative stress, but excessively stimulated autophagy may lead to endothelium cells death to aggravate plaque rupture. We demonstrated here that the effects of Brazilian green propolis on autophagy might be associated with a mechanistic target of mTOR-dependent signaling pathway. In summary, propolis alleviated Ox-LDL-induced HUVECs damage by suppressing apoptosis and autophagy. The underlying mechanism may, at least in part, involve activating PI3K/Akt/mTOR and enhancing antioxidant gene HO-1 and Nrf2 expression. Our study provides a potential

Data Availability
The data used to support the findings of this study are included within the article.

Conflicts of Interest
The authors declare no conflicts of interest.

Authors’ Contributions
Wenwen Yuan and Huasong Chang contributed equally to this work.

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endothelial cells and restricts atherosclerosis in apolipoprotein E−/− mice,” *Scientific Reports*, vol. 4, article 5519, 2014.

