Original Article

The Effect of Hydro-alcoholic Extract of *Rheum Turkestanicum* Roots against Oxidative Stress in Endothelial Cells

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Abstract

Introduction: Cardiovascular disorders (CVD) are a common cause of mortality worldwide. Oxidative stress is thought to be a major factor leading to CVD. Anti-oxidants such as medicinal plants may have a role in the mitigation of vascular problems through free radicals scavenging. In this study, we evaluated the protective effects of *Rheum turkestanicum* against hydrogen peroxide (H_2O_2)-induced toxicity in endothelial cells (BAE-1). **Methods:** To evaluate the protective effect of *R. turkestanicum* against H_2O_2 toxicity, four groups comprised of control group (the cells without any treatment), H_2O_2 group (the cells incubated with H_2O_2 (200 μM)), and treatment groups (the cells treated with *R. turkestanicum* (12200 μg/ml) alone or 24h before exposure to H_2O_2). Quercetin (30.23 μg/ml) was used as a bioactive ingredient of the extract. Then the cell viability, reactive oxygen species, lipid peroxidation, and apoptosis were evaluated. **Results:** H_2O_2 exposure reduced cell viability to 13.6 ± 1.6%, enhanced ROS generation to 1445 ± 80.7%, lipid peroxidation (LPO, 290 ± 13% of control), and apoptotic cells ($P < 0.001$). In contrast, compared with H_2O_2 group, *R. turkestanicum* and quercetin significantly restored the cell viability to 80.3 ± 1.6 and 87.2 ± 2.1%, ROS formation to 186 ± 10 and 129 ± 1%, as well as LPO to 130.7 ± 7.7 and 116 ± 2.5 of control, respectively ($P < 0.001$). Therefore, the extract reduced H_2O_2-induced toxicity in BAE-1 cells by scavenging of free radicals. **Conclusion:** Our findings demonstrated that the extract might reduce toxicity of endothelial cells by attenuation of oxidative stress, which can be related to the presence of active ingredients including quercetin.

Keywords: *Apoptosis, endothelial cells, oxidative stress, quercetin, Rheum turkestanicum*

Introduction

Endothelial cells are involved in vascular hemostasis, angiogenesis, inflammation responses, and vascular contraction.[1,2] Studies have shown that dysfunction of endothelial cells contributes to the pathogenesis of cardiovascular diseases (CVD).[2] Most of the risk factors associated with CVD disturb the cell function and processes like apoptosis.[3] Oxidative stress has been suggested as the common characteristic of risk factors of CVD including hypertension, aging, obesity, unhealthy diet, and low-physical activity.[3]

Oxidative stress is induced by production of excessive amount of ROS and exhausted antioxidant defense systems. ROS cause DNA injury and mitochondrial dysfunction which consequently results in apoptotic cell death.[4]

Endothelial dysfunction during atherosclerosis is associated with the release of arachidonic acid and inflammatory cytokines, including tumor necrosis factor-α (TNF-α), Interleukin-1 (IL-1) and IL-6, and induces apoptosis of endothelial and vascular smooth muscle cells. Inflammation can induce oxidative stress, which in turn can inflammation.[3,5]

Among different types of ROS, hydrogen peroxide (H_2O_2) is common, which is used extensively in *in vitro* studies to induce endothelial cell injuries.[6] Although the production of free radicals plays a role in the pathogenesis of different disorders, however, medicinal herbs with anti-oxidant properties such as polyphenols, beta-carotene, and tocopherols may reduce H_2O_2-induced toxicity in endothelial cells.[3] Recent studies have reported *Crocus sativus*,[7] *Phyllanthus emblica*,[8] *Melissa officinalis*,[9] and pomegranate seed oil diminished H_2O_2 toxicity through scavenging of free radicals.[10] *R. turkestanicum* belongs to *Polygonaceae* family and grows in north-east and central Asia particularly

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of Iran. Based on the recent pharmacological studies, *R. turkestanicum*has been used as an anticancer, antidiabetic, and antihypertensive.[11] Recent studies have revealed a protective effect of this plant against toxic agents, such as doxorubicin,[12] cisplatin,[13] gentamicin,[14] mercuric chloride,[15] and hexachlorobutadien.[16] Antioxidant activity of some plants of *Rheum* genus has been attributed to the presence of bioactive components that scavenge free radicals (IC_{50} value in DPPH assay: 2.8-11.8 μM).[17] *R. turkestanicum* counter acted hexachloro butadien and mercuric chloride-induced nephrotoxicity through its antioxidant properties indicated by attenuated lipid peroxidation and increased total thiol content. The antioxidant activity of the plant was also involved in the neuroprotective and anti-apoptotic effects that have been linked to the suppression of ROS generation and lipid peroxidation.[18]

Most of the pharmacological effects may be attributable to the active compounds such as polyphenolic and flavonoid. These bioactive components have found to possess antioxidant and free radicals scavenging potential.[19] Quercetin as a bioactive ingredient is found in *Rheum* species including *R. turkestanicum*.[20] In this investigation, we evaluated the protective effects of *R. turkestanicum* and quercetin against H_2O_2-induced toxicity in bovine aortic endothelium (BAE-1) cells.

Materials and Methods

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), propidium iodide (PI), H_2O_2, Triton X-100, thiobarbituric acid (TBA), sodium citrate and quercetin were provided from Sigma. Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin ×100 were obtained from Gibco. Dimethyl sulfoxide (DMSO) was purchased from Merck. BAE-1 cell line was prepared from the Pasteur Institute (Tehran, Iran).

Preparation of extract

R. turkestanicum roots were collected from the Kalat region (Khorasan Razavi, Iran). This plant was identified by M.R. Joharchi, Ferdowsi University of Mashhad Herbarium (voucher specimen No. 21377). The roots were dried and crushed into a powder by electric micronizer. The soxhlet extract was prepared by 70% ethanol, then dried and kept at −20°C until downstream processes.

Cell culture

BAE-1 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/ml penicillin and streptomycin at 37°C in a humidified atmosphere (90%) containing 5% CO_2.

Assessment of cell viability

The cell viability was evaluated by MTT assay. The cells were seeded, pre-treated with extract (12200 μg/ml) and quercetin (30.23 μg/ml) for 24 h. After 24 h of incubation, the cells were exposed to H_2O_2 at a dose of 200 μM for 30 min (the concentration was chosen based on the previous study).[12,21] Then, the MTT solution was added to each well and incubated for 3h, DMSO was used to dissolve the Formazan precipitate. The absorbance of samples was determined at 600 nm on an ELISA reader.[18]

Measurement of reactive oxygen species

DCFH-DA method was used to determine the intracellular ROS level. The cells were pretreated with the extract (12200 μg/ml) and quercetin (30.23 μg/ml) for 24 h, then H_2O_2 (200 μM) was added to cells for 30 min.[12] After 30 min exposure to H_2O_2, the cells were treated with DCFH-DA and incubated for 30 min. Fluorescence strength was measured by a microplate reader at 504 nm for excitation and 524 nm for emission.[22]

Lipid peroxidation assay

ROS can destroy membrane lipids and produce a variety of breakdown products including alcohols, ketones, aldehydes, and others. Malondialdehyde (MDA) is a main product of lipid peroxidation (LPO). MDA reacts with thiobarbituric acid (TBA) to generate fluorescence adduct. LPO was measured by TBA reactive substance (TBARS) assay. The amount of LPO was estimated by TBARS fluorescence intensity.[23] After the incubation, the cells were scraped and centrifuged at 13,000 ×*g* for 30 min at 4°C.[23] Then, 400 μl of trichloroacetic acid (15%) and 800μl of TBA (0.7%) were added to 500 μl of cells. The mixture was vortexed and then heated for 40 min in a boiling water bath. Subsequently, 200 μl of the sample was transferred to 96-well plate, and the fluorescence intensity was read with excitation and emission of 480 and 530 nm, respectively. The experiment was carried out in triplicate.

Determination of apoptosis

PI-staining was used to identify the apoptotic cells. A sub-G1 peak, indicative of DNA fragmentation, is observed after cell incubation in a hypotonic phosphate-citrate buffer, containing a DNA-binding dye (such as PI). In the histogram, DNA-free apoptotic cells absorb less stain, which can be observed on the left side of the peak. On the basis of the described protocol, the cells were treated after seeding in a 24-well plate. Then, adherent and floating cells were harvested, and incubation was performed at 4°C in darkness overnight, using a hypotonic buffer (750 μl; 50 μg/ml of PI in 0.1% triton X-100 and 0.1% sodium citrate). Finally, a FACS can system (Becton Dickinson) was used to perform flow cytometry, yielding a total of 10^4 events.[12]

Statistical analysis

All obtained data were expressed as mean ± SEM from three independent experiments. Graph Pad Prism version 6 was used to statistically analyze the data. The statistical analysis was performed using Graph Pad Prism version 6. Statistical evaluation was done using one-way analysis of variance, followed by Tukey *post hoc* test. The minimum level of significance was $P < 0.05$.

Results

Effect of hydro-alcoholic extract of *R. turkestanicm* on cell viability

The toxicity effect of *R. turkestanicum* was evaluated on BAE-1cells at different doses (12200µg/ml) by MTT assay. Results indicated that the extract had no toxic effect on cell viability [Figure 1].

Hydro-alcoholic extract of *R. turkestanicm* attenuated cell death following H_2O_2 toxicity

The results revealed H_2O_2 increased cell death at a dose of 200 µM ($P < 0.001$) compared with the control group, while quercetin and different concentrations of extract (25200 µg/ml) reduced cell death and had a protective effect against H_2O_2-induced toxicity on BAE-1 cells ($P < 0.001$) [Figure 2]. In the cells treated with 100 and 200, the viability significantly increased, comparedwith that in the groups treated with 12, 25, 50µM ($P < 0.001$). The cell viability was significantly elevated in the 12 and 100 µM treated groups versus 25 and 200 µM group, respectively ($P < 0.01$, $P < 0.001$). Although, there were significant differences between the extract (12-200 µM) and control group ($P < 0.001$), the viability increased concentration-dependently in the cells treated with the extract. Similarly, significant differences were observed between the 12 and 100 µM treated groups and quercetin ($P < 0.001$).

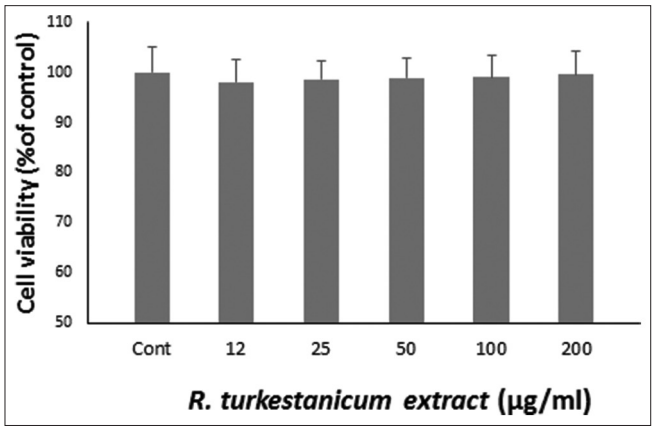

Figure 1: Effect of hydro-alcoholic extract of *R. turkestanicm*on BAE-1 cell viability. The cells were pretreated with different concentrations of *R. turkestanicm* for 24 h followed by 30 min incubation with H_2O_2. The cell viability was determined via the MTT assay. The data were expressed as percentage viability of control. The results are mean ± SEM. The test was done in triplicate ($n = 3$)

Hydro-alcoholic extract of *R. turkestanicm* reduced ROS

Our findings showed that H_2O_2 elevated the level of ROS in the cells ($P < 0.001$). Quercetin and the extract significantly reduced ROS generation in comparison with H_2O_2 group, in a dose-dependently manner [$P < 0.001$, Figure 3].Compared to the control group, the extract concentrations < 100 µM (50, 25, and 12µM) exhibited remarkable differences ($P < 0.001$).Intracellular ROS significantly decreased in the cells treated with 100 and 200 µM of the extract, compared with that in the groups treated with 12, 25, and 50µM of the extract ($P < 0.001$). Therefore, the inhibiting effect of the extract on intracellular ROS was concentration-dependent. ROS content was significantly reduced in the 12 µM treated groups versus 25 µM of the extract ($P < 0.01$), respectively. Similarly, significant differences were observed between the groups treated with the extract at 12100 µM and quercetin ($P < 0.001$).

Hydro-alcoholic extract of *R. turkestanicm* attenuated lipid peroxidation

H_2O_2 increased MDA as lipid peroxidation index ($P < 0.001$). Pretreatment of cells with different doses of extract and quercetin attenuated lipid peroxidation and the level of MDA ($P < 0.001$) [Figure 4]. In the cells treated with the extract at 100 and 200µM, MDA level significantly decreased, compared with that in the groups treated with 12, 25µM of the extract ($P < 0.001$). Also, the differences between the cells treated with 50 µM and the cells treated with 12, 25, and 200 µM of the extract were significant ($P < 0.001$).Although, there were significant differences between the extract at different concentration (12100µM) and control group, the MDA level concentration-dependently decreased in the cells

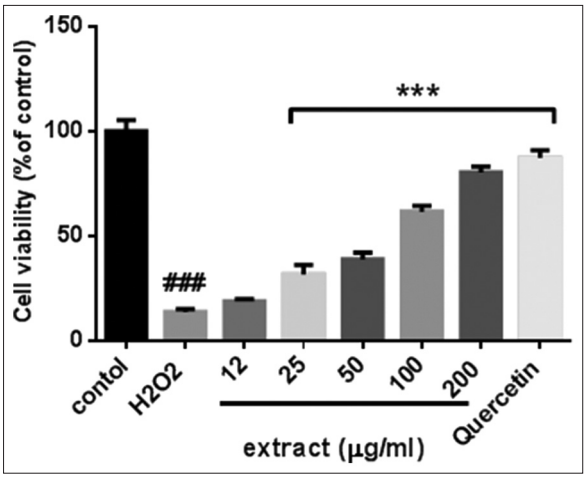

Figure 2: The protective effects of hydro-alcoholic extract of *R. turkestanicm* against H_2O_2-induced BAE-1 cells toxicity. The cells were pre-treated with different concentrations of *R.turkestanicm* for 24 h followed by 30 min incubation with H_2O_2, and the cell viability was quantified by MTT assay. The data were expressed as percentage viability of control. The results are mean ± SEM. The test was done in triplicate ($n = 3$). (*$P < 0.001$ compared with H_2O_2, ###$P < 0.001$ compared with control)**

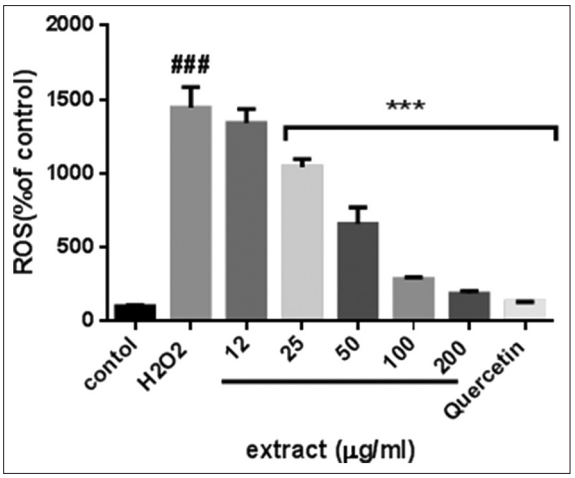

Figure 3: Effects of hydro-alcoholic extract of *R. turkestanicm* on ROS production in BAE-1 cells. The cells were pre-treated with different concentrations of *R.turkestanicm* and quercetin for 24 h followed by 30 min incubation with H_2O_2. The intracellular ROS was estimated via fluorescence intensity. The results were expressed as percentage of fluorescence intensity of control. The results are mean ± SEM. The test was done in triplicate (*n* = 3). (**P* < 0.001 compared to H_2O_2, ###*P* < 0.001 compared with control)**

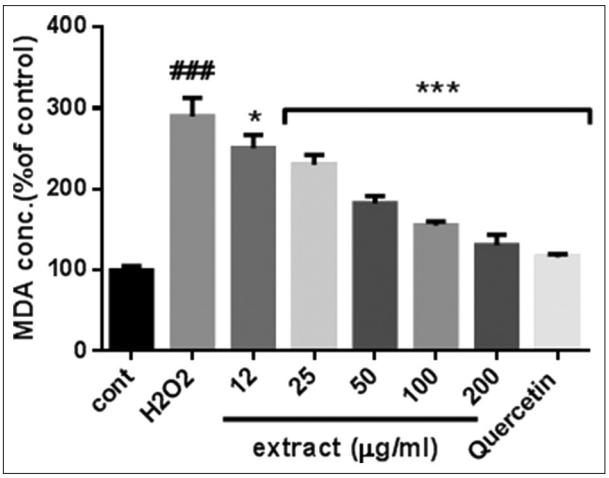

Figure 4: Effects of hydro-alcoholic extract of *R. turkestanicm*on lipid peroxidation in BAE-1 cells. The cells were pre-treated with different concentrations of *R. turkestanicm* and quercetin for 24 h followed by 30 min incubation with H_2O_2. The results are mean ± SEM. The MDA level was determined via TBARS fluorescence intensity. The results were expressed as percentage of fluorescence intensity of control. The test was done in triplicate (*n* = 3). (P* < 0.05, ****P* < 0.001 compared to H_2O_2, ###*P* < 0.001 compared with control)**

treated with the extract. Similarly, significant differences were observed between the cells treated with the extract at 12100 µM and quercetin ($P < 0.001$).

Hydro-alcoholic extract of *R. turkestanicm* reduced apoptotic cells

As shown in Figure 5, H_2O_2elevated apoptotic cells ($P < 0.001$) while quercetin and extract counteracted apoptotic cell following H_2O_2-induced apoptosis.

Discussion

In this research, we evaluated the protective effects of *R.turkestanicum* hydro-alcoholic extract against H_2O_2-induced toxicity in BAE-1 cells for the first time. H_2O_2elevated cell death, apoptotic cells, ROS generation, and MDA at a dose of 200µM. Pretreatment of cells with the extract reduced cell death, apoptotic cells, and oxidative stress via attenuation of lipid peroxidation and ROS production, dose-dependently. Quercetin as an active ingredient which is found in *R. turkestanicum*[20] restored cell viability and decreased oxidative stress following H_2O_2-induced oxidative stress at a dose of 30.23 µg/ml. *R. turkestanicum* reduced doxorubicin-induced toxicity in cardiomyocytes (H9C2 cells),[12] glutamate toxicity in PC12 cells.[18] The protective effects of this herb against toxic agents such as gentamicin,[14] mercuric chloride,[15] hexachlorbutadien,[16] and cisplatin[13] have been also demonstrated. Another study showed that *R.turkestanicum* prevented cardiac injury following STZ-induced diabetic in rats via attenuation of lactate dehydrogenase and creatine phosphokinase.[19] These studies have revealed the protective effects of *R.turkestanicum* may be related to the presence of active ingredients that scavenge free radicals and attenuate oxidative stress.[20] Bhushan *et al*. (2007) reported that *R. turkestanicum* induced apoptosis through ROS generation in human leukemic cells, HL60 and NB4. ROS formation dose-dependently has been elevated in the cells treated with *R. turkestanicum*. Therefore, *R. turkestanicum* and its components may act as both pro-oxidant and antioxidant, depending on the redox state of the biological environment.[24] Rheum species is composed of different ingredients including anthrones, anthraquinones, quercetin, resveratrol, anthocyanins, acylglucosides, stilbenes, organic acids, and vitamins.[20] The presence of anthraquinone derivatives including emodin, aloe-emodin, rhein, chrysophanol, physcion, and danthron as the main biologically active constituents of *Rheum* genus including *R. turkestanicum* have been identified.[25,26] Anti-inflammatory and anti-apoptotic effects of emodin have reduced myocardial infarction in rat heart via elevation of antioxidant capacity.[27] Moreover, it suppresses the expression of Toll-like receptor 4 and p38 mitogen activating protein kinase following viral myocarditis.[28] Quercetin counteracted cardiac inflammation after ischemia-reperfusion by suppressing the activity of signal transducer and activator of transcription 3 (STAT3).[29] Also, quercetin declined infarct size via activating the PI3K/Akt signaling pathway and modulating the expression of Bcl-2 and Bax proteins.[30] Additionally, it reduced doxorubicin-induced cardiotoxicity in H9C2 cells and mice by the upregulation of Bmi-1 expression and suppression of oxidative stress.[31] On the basis of the *in vitro* and *in vivo* studies, resveratrol revealed cardio-protective properties against doxorubicin toxicity through enhancing the antioxidant enzymes activity and attenuation of pro-apoptotic proteins such as

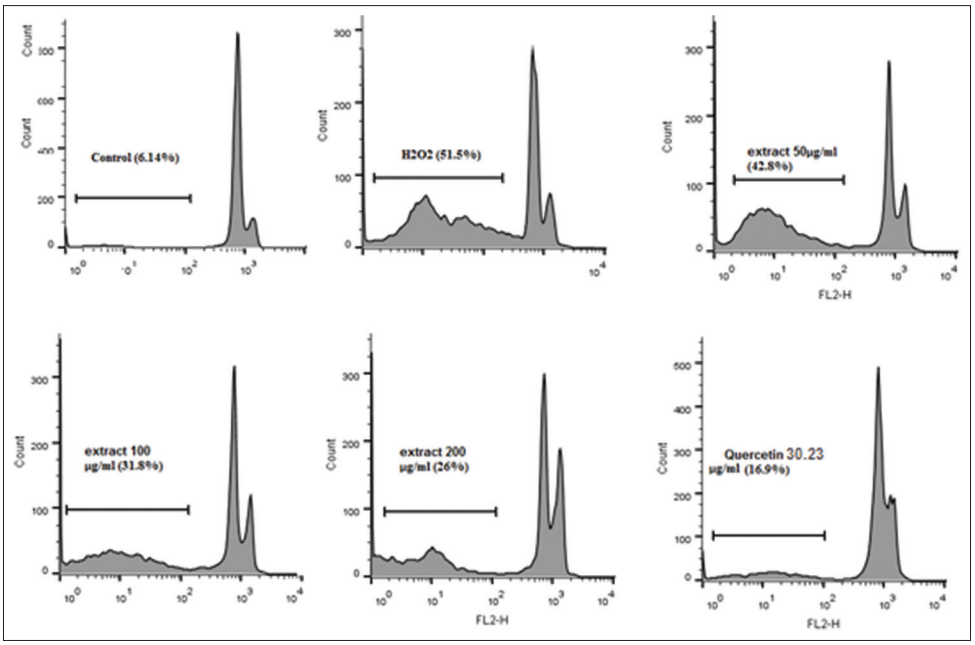

Figure 5: Effects of hydro-alcoholic extract of *R. turkestanicm* on BAE-1 apoptotic cells. The cells were pretreated with different concentrations of *R. turkestanicm* and quercetin for 24 h followed by 30 min with H_2O_2. They were stained with PI for flow cytometric analysis. The flow cytometry histograms representing the cells with reduced DNA content accumulated in the sub-G1 region

p53, Bax, and caspase3.[32-34] Resveratrol reduced ROS generation following palmitic acid-induced oxidative stress in human aortic endothelial cells.[35] Rhein decreased H_2O_2-induced toxicity in human umbilical vein endothelial cells via inhibition of ROS production and apoptosis.[21] According to these findings, the protective effect of extract against oxidative stress may be mediated via active ingredients.

Conclusion

Our findings showed the protective effect of *R. turkestanicum* and quercetin against H_2O_2-induced toxicity in endothelial cells. These beneficial properties are related to the presence of active ingredients, probably by suppression of free radicals, lipid peroxidation, and apoptotic cell death. The present study proposed that this extract can be a potential cardio-protective agent in the prevention of cardiovascular disease. However, more investigations are needed to warrant these findings.

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Authors' contribution

Azar Hosseini, Mohammad Soukhtanloo, BizhanMalaekeh-Nikouei designed the study. Azar Hosseini, Arezoo Rajabian, Sahar Sheikh conducted the experiments. Azar Hosseini and Arezoo Rajabian wrote and revised the manuscript.

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Conflicts of interest

There are no conflicts of interest.

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