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# Protective effect of Insect tea primary leaf (*Malus sieboldii* (Regal) Rehd.) extract on H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in human embryonic kidney 293T cells

Jing Zhang<sup>1,2†</sup>, Hong Wang<sup>3†</sup>, Sha Yi<sup>1</sup>, Zemei Guo<sup>1</sup>, Yue Huang<sup>1</sup>, Weifeng Li<sup>4</sup>, Xin Zhao<sup>2\*</sup> and Huazhi Liu<sup>5\*</sup>

## Abstract

In this study, Insect tea primary leaf (*Malus sieboldii* (Regal) Rehd.) was used as the research object to investigate the protective effect of Insect tea primary extract (ITPLE) on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced oxidative damage in human embryonic kidney 293T cells (HEK 293T cells) and the mechanism of action of the main active components. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to determine the toxicity of ITPLE to HEK 293T cells in vitro as well as its protective effect against (H<sub>2</sub>O<sub>2</sub>)-induced oxidative damage in HEK 293T cells. In addition, various assay kits were used to measure oxidation-related indicators in HEK 293T cells, and quantitative polymerase chain reaction (qPCR) analysis was used to determine the mRNA expression levels of oxidation-related genes in HEK 293T cells. High performance liquid chromatography (HPLC) analysis was used to characterize active components in ITPLE. The experimental results revealed that the ITPLE had no toxic effect on cells in the range of 0–200 µg/mL, and, in this range, exhibited a concentration-dependent protective effect against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in HEK 293T cells. It was also found that the ITPLE can reduce the malondialdehyde (MDA) level and increase the levels of superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px), and catalase (CAT) in oxidative damage HEK 293T cells. The qPCR analysis results also showed that the ITPLE upregulated the mRNA expression levels of SOD, CAT, GSH and GSH-Px in HEK 293T cells damaged by H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. The HPLC analysis identified 7 bioactive components in the ITPLE, including neochlorogenic acid, cryptochlorogenic acid, rutin, kaempferin, isochlorogenic acid B, isochlorogenic acid A and hesperidin. This study reveals that ITPLE is rich in active compounds and has good antioxidant effect in vitro, thus it has the potential to be developed into a traditional Chinese medicine and functional drinks.

**Keywords:** Insect tea, Oxidative damage, Human embryonic kidney 293T cells, mRNA, H<sub>2</sub>O<sub>2</sub>

## Introduction

Insect tea is a traditional health drink in southern China. The production process of Insect tea is very special, in particular, the Insect tea primary leaves are fed to insect larvae, and then the excreta of the larvae are processed into tea like drinks [1]. Besides the influence of insects, the species of raw primary leaves (material leaves) also play an important role in the quality and function of Insect tea. The plant leaves used to make Insect tea mainly include Kuding tea (*Ligustrum robustum*

\*Correspondence: zhaoxin@cque.edu.cn; liuhuazhi8818@163.com

<sup>†</sup>Jing Zhang and Hong Wang contributed equally to this work

<sup>2</sup>Chongqing Collaborative Innovation Center for Functional Food, Chongqing Engineering Research Center of Functional Food, Chongqing Engineering Laboratory for Research and Development of Functional Food, Chongqing University of Education, Chongqing 400067, China

<sup>5</sup>First Affiliated Hospital of Gannan Medical University, Ganzhou 341000, Jiangxi, China

Full list of author information is available at the end of the article

(Roxb.) Bl.), Liubao tea (*Camellia sinensis*) and toringo (*Malus sieboldii* (Regal) Rehd.), etc. [2]. The fruit and leaf of toringo are both used in traditional Chinese medicine to help digestion and reduce inflammation [2]. It has been shown that the leaves of toringo contains abundant amino acids, polysaccharides, flavonoids and saponins [3]. However, the toringo leaves used for preparing Insect tea need to go through certain processing procedures. The leaves are first steamed, dried, and then added to rice soup for natural fermentation and ripening [4]. Afterwards, the composition and bioactivity of the leaves of toringo will change, but the research on the composition and function of the processed leaves is still lacking.

Reactive oxygen species (ROS) produced by aerobic metabolism in humans, mainly include superoxide anion radical ( $O_2^{\cdot-}$ ), peroxide anion ( $O_2^{2-}$ ), hydroxyl radical ( $\cdot OH$ ), hydroxyl ion ( $OH^-$ ), hydrogen peroxide ( $H_2O_2$ ), etc. ROS can cause oxidative stress (OS) in human cells. OS is an imbalance between oxidation and antioxidation in vivo, which tends to oxidize, resulting in inflammatory infiltration of neutrophils, increase of protease secretion and production of a large number of oxidation intermediates. OS is a negative effect produced by free radicals in the cells, and is considered to be an important factor leading to aging and diseases [5]. OS can damage cell structures and affect function in human cells, resulting in molecular degradation, apoptosis, inflammation and ultimately leading to various types of chronic diseases, such as cardiovascular disease, neurodegenerative diseases, kidney disease and cancer [6, 7]. The development of these diseases is closely related to the oxidative changes of key physiological molecules in the body, including the regulation of proteins, lipids, carbohydrates, nucleic acids, gene expression and inflammatory response [8].

In this study, the components of the processed leaves of toringo (Insect tea primary leaf) extract (ITPLE) were analyzed. In addition, since the available evidence indicates that  $H_2O_2$  is the main form of ROS in vivo, which can generate  $\cdot OH$  through Fenton reaction and intracellular  $Fe^{2+}$  and then cause chain reaction,  $H_2O_2$  is used in this study as an inducer of OS. Also, an in vitro human embryonic kidney 293T (HEK 293T) cell injury model was established and used to investigate the inhibitory effect of ITPLE on OS-induced cell injury, and to preliminarily analyze its potential active components, so as to provide certain reference basis for the protective effect of Insect tea primary leaf on human chronic diseases.

## Materials and methods

### Extraction of insect tea primary leaf

After the Insect tea primary leaf was freeze-dried, 100 g of the Insect tea primary leaf was taken out and crushed, and extracted in 2 L of 80% aqueous methanol solution

(v/v). Then, the filtrate of the extract was evaporated and the evaporated drying extract (ITPLE) was used for subsequent cellular assays.

### Cell culture

After thawing, HEK 293T cells (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) were resuspended in high glucose Eagle's Minimum Essential Medium (EMEM) medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin–streptomycin double antibody solution, and cultured in a Heracell 150i  $CO_2$  incubator (Thermo Fisher Scientific Inc.) with a saturated humid environment at 37 °C and 5%  $CO_2$ .

### Assay of ITPLE toxicity on HEK 293T cells

HEK 293T cells were suspended in EMEM medium at a concentration of  $1 \times 10^4$  cells/mL. Then, 200  $\mu L$  of this cell suspension was seeded into a 96-well cell culture plate and cultured for 24 h at 37 °C. After the cells adhered to the plate, the original culture medium was suctioned off, discarded, and replaced with fresh medium containing ITPLE in the range of 0–300  $\mu g/mL$ , and adherent cells were further cultured for 48 h. Afterwards, the medium was suctioned off, discarded, and replaced with medium containing the MTT reagent (5 mg/mL) and cells were cultured for 4 h. Subsequently, the medium was discarded, and 200  $\mu L$  of dimethyl sulfoxide (DMSO) was added and reacted in the dark for 30 min. Eventually, the optical density (OD) value was measured at 494 nm on an Evolution™ 350 UV–Vis spectrophotometer (Thermo Fisher Scientific Inc.), and the cell proliferation rate was determined [9].

### Effect of ITPLE on $H_2O_2$ -induced oxidative damage in HEK 293T cells

After the cells were attached to the plate as described in ITPLE toxicity determination section, the medium was suctioned off, discarded, and replaced with fresh medium containing 0.3 mmol/L  $H_2O_2$  and cells were cultured for 4 h to induce oxidative damage in HEK 293T cells. Subsequently, the culture medium was discarded, and fresh medium containing ITPLE as described in sub-Sect. “Assay of ITPLE toxicity on HEK 293T cells”. Eventually, after treatment with ITPLE, the absorbance value was measured and the cell proliferation rate was determined [9].

### Determination of MDA, SOD, GSH, GSH-Px and CAT in HEK 293T cells subjected to oxidative damage

As described in ITPLE toxicity determination section, after treating the cells with ITPLE for 48 h, cells in the culture plate were washed with phosphate-buffered saline

(PBS). Then cells were detached from the plate by adding 200  $\mu$ L of trypsin solution per well, resuspended in complete medium and centrifuged at 4000 rpm for 15 min. Afterwards, the supernatant was discarded, and the process was repeated once. Then, the 800  $\mu$ L lysate (solarbio, Beijing, China) was added to the cell, and after full lysis, the lysate cells were centrifuged for 5 min (8000 rpm), and the supernatant was taken out. The levels of MDA, SOD, GSH, GSH-Px and CAT in the cell homogenate were determined according to the instructions of the corresponding kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

#### qPCR analysis of mRNA expression levels in HEK 293T cells subjected to oxidative damage

Total RNA was isolated from HEK 293T cells treated as described in sub-Sect. “[Determination of MDA, SOD, GSH, GSH-Px and CAT in HEK 293T cells subjected to oxidative damage](#)”, using Trizol reagent (Thermo Fisher Scientific Inc.). Briefly, 1  $\mu$ L of oligo (dT) 18 primer (500 ng/ $\mu$ L) and 1.0  $\mu$ L of total RNA (1.0  $\mu$ g/ $\mu$ L) were added to 10.0  $\mu$ L of nuclease free water and heated at 65 °C for 5 min. Then, a reagent mixture containing 4.0  $\mu$ L of 5 $\times$  reaction buffer, 1.0  $\mu$ L of ribolock RNase inhibitor (20 U), 2.0  $\mu$ L of 10 mM dNTP mix and 1.0  $\mu$ L of 200 U/ $\mu$ L reversible reverse transcriptase (Thermo Fisher Scientific Inc.) was added to the total RNA system, and the cDNA was synthesized at 42 °C for 60 min and 700 °C for 5 min. Then, 1.0  $\mu$ L cDNA, 1.0  $\mu$ L each (10  $\mu$ m/ $\mu$ L) of forward and reverse primers (Thermo Fisher Scientific), 10.0  $\mu$ L premix and 7.0  $\mu$ L sterile double-distilled water were mixed for cDNA amplification. The amplification conditions were as follows: denaturation at 95 °C for 3 min, annealing at 60 °C for 30 s, extension at 95 °C for 1 min, for 40 cycles, performed on an ABI StepOne Plus qPCR system (Thermo Fisher Scientific Inc.). The mRNA expression of SOD, CAT, GSH and GSH Px were detected by qPCR analysis. The primer sequences are shown in Table 1. The cDNA of each gene was amplified three times in parallel, and the average CT value was used. The housekeeping gene Actin was used as an internal reference and the related gene was calculated according to  $2^{-\Delta\Delta CT}$  method [10].

#### Determination of the composition of ITPLE by HPLC analysis

A 10 mg/mL stock solution of ITPLE was prepared in DMSO t, and then diluted with an aqueous 50% methanol solution to obtain a liquid sample with a final concentration of 2 mg/mL. The diluted ITPLE solution was tested (injection volume: 10  $\mu$ L) on the Ultimate 3000 high performance liquid chromatography

**Table 1 Sequences of the primers used for the HEK 293T cells**

Gene name	Sequence
SOD	Forward: 5'-AGATGGTGTGGCCGATGTGT-3' Reverse: 5'-TCCAGCGTTTCTGTCTTTGTA-3'
CAT	Forward: 5'-TGTTGCTGGAGAATCGGGTTC-3' Reverse: 5'-TCCAGTTACCATCTTCTGTGTA-3'
GSH	Forward: 5'-TACGGCTCACCCAATGCTC-3' Reverse: 5'-CTATGGCAGCTGGTCAAATA-3'
GSH-Px	Forward: 5'-GTCGGTGTATGCCTTCTCGG-3' Reverse: 5'-CTGCAGCTCGTTCATCTGGG-3'
Actin	Forward: 5'-TCAAGAAGGTGGTAAGCAGG-3' Reverse: 5'-AGCGTCAAAGGTGGAGGAGTG-3'

**Table 2 Gradient elution condition in this study**

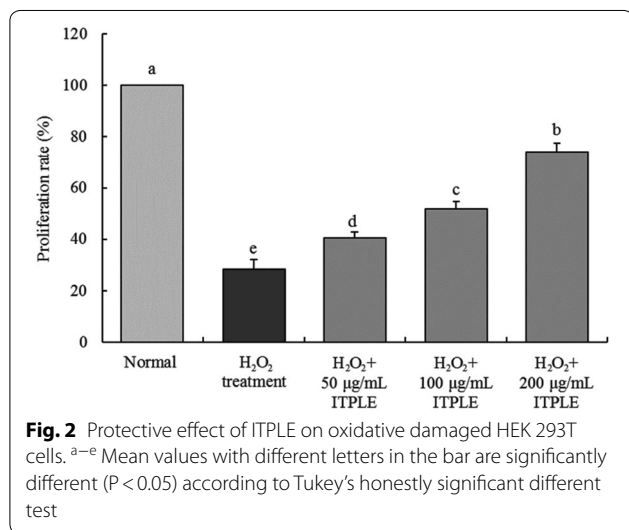
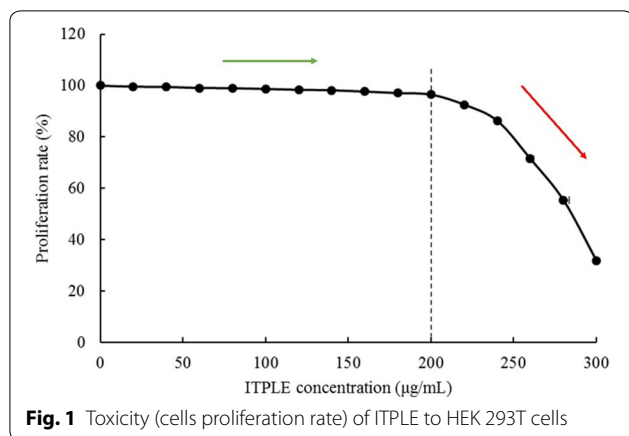
Time (min)	0.5% acetic acid water: acetonitrile
0	88:12
15	80:20
35	60:40
45	30:70
50	0:100
55	0:100

(HPLC) system (Thermo Fisher Scientific) after passing a 0.22  $\mu$ m organic filter membrane from Standard products (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China). The chromatographic conditions were as follows: Hypersep C18 column (4.6 mm  $\times$  150 mm, 5  $\mu$ m); mobile phase A, 0.5% acetic acid water; mobile phase B, acetonitrile; flow rate, 0.5 mL/min; column temperature: 25 °C; detection wavelength, 280 nm; gradient elution conditions were as shown in Table 2.

## Results

#### Toxicity of ITPLE to HEK 293T cells

As shown in Fig. 1, after ITPLE treatment, the proliferation of HEK 293T cells was not inhibited in the concentration range of 0–200  $\mu$ g/mL, with cells basically maintaining normal proliferation. However, when the concentration exceeded 200  $\mu$ g/mL, the cell proliferation was inhibited. Thus, these results indicate that ITPLE has no toxic effect on HEK 293T cells in the concentration range of 0–200  $\mu$ g/mL. Accordingly, the concentrations of 50, 100 and 200  $\mu$ g/mL were selected for subsequent experiments.



### Protective effect of ITPLE against oxidative damaged in HEK 293T cells

As shown in Fig. 2, compared with normal HEK 293T cells, the proliferation rate (28.3%) of HEK 293T cells subjected to  $H_2O_2$ -induced oxidative damage decreased significantly ( $P < 0.05$ ). Compared with the HEK 293T cells subjected to oxidative damage without ITPLE treatment, the proliferation rate of HEK 293T cells similarly subjected to oxidative damage, but treated with 50 (51.8%) or 100 (40.6%) or 200  $\mu\text{g/mL}$  (73.9%) of the ITPLE increased significantly ( $P < 0.05$ ), and the increased proliferation was positively correlated with the concentration of action.

### SOD, GSH, GSH-Px, CAT and MDA levels in HEK 293T cells subjected to oxidative damaged and treated with the ITPLE

The results shown in Fig. 3 reveal that the levels of SOD, GSH, GSH Px and CAT in normal HEK 293T cells were

the highest, while the level of MDA was the lowest. The ITPLE significantly ( $P < 0.05$ ) inhibited in a concentration-dependent manner the  $H_2O_2$ -induced changes in the levels of SOD, GSH, GSH Px, CAT and MDA in HEK 293T. Clearly, the results showed that the ITPLE can regulate the oxidation related indicators in HEK 293T cells, thus attenuating the oxidative damage induced by  $H_2O_2$ .

### SOD, GSH, GSH-Px and CAT mRNA expression levels in HEK 293T cells subjected to oxidative damaged and treated with the ITPLE

The results of the qPCR analysis (Fig. 4) revealed that the mRNA expression levels of SOD, CAT, GSH and GSH-Px decreased significantly ( $P < 0.05$ ) in HEK 293T cells subjected to  $H_2O_2$ -induced oxidative injury. The mRNA expression levels of SOD, CAT, GSH and GSH-Px increased significantly ( $P < 0.05$ ) in HEK 293T cells subjected to oxidative damage.

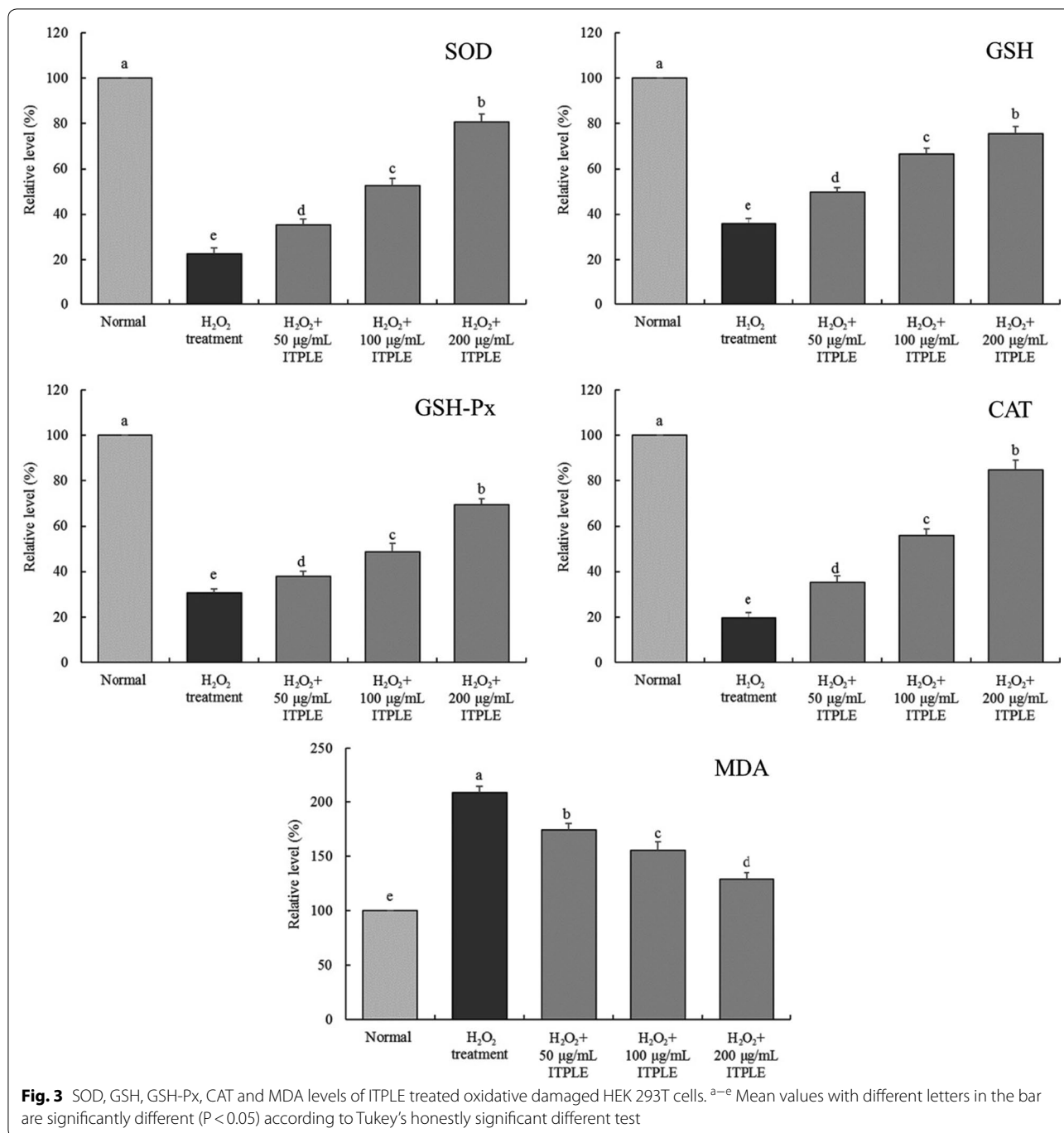
### Constituents of ITPLE

As shown in Fig. 5, the results of the HPLC analysis revealed that the ITPLE was composed of neochlorogenic acid, cryptochlorogenic acid, rutin, kaempferin, quercetin, isochlorogenic acid A and isochlorogenic acid B. In particular, the content of kaempferin and isochlorogenic acid B were higher than that of the other chemical components.

### Discussion

Oxidative stress can cause lipid peroxidation of polyunsaturated fatty acids in biofilm, and then cell oxidative damage, resulting in a series of diseases [11]. In order to avoid the damage caused by oxidative stress, human beings can resist oxidative stress through their own antioxidant system and intake of exogenous antioxidants. In particular, the regulation enzyme system including SOD, CAT, GSH-Px and so on plays a role in resisting oxidative stress [12]. It has been proved that many plants have the effect of improving the damage caused by oxidative stress, and have been used in practical application [13–15]. However, many application cases are based on the inheritance of experience and traditional medicine, and lack of in-depth study on the active components of plants and their mechanism of action. The purpose of this study was to analyze the components of Insect tea primary leaf (*Malus sieboldii* (Regal) Rehd.) that had been used in traditional Chinese medicine, and to studied the role of inhibiting oxidative stress, and to accumulated theoretical basis for the application of this plant in other diseases in the future.

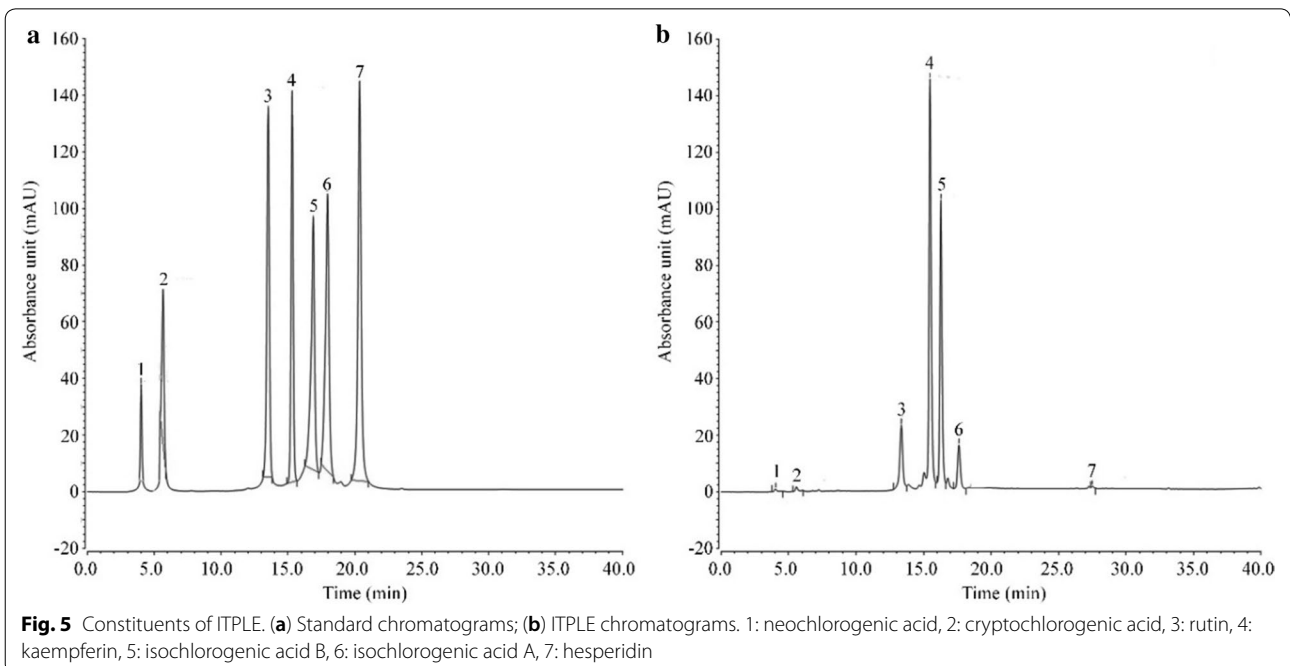
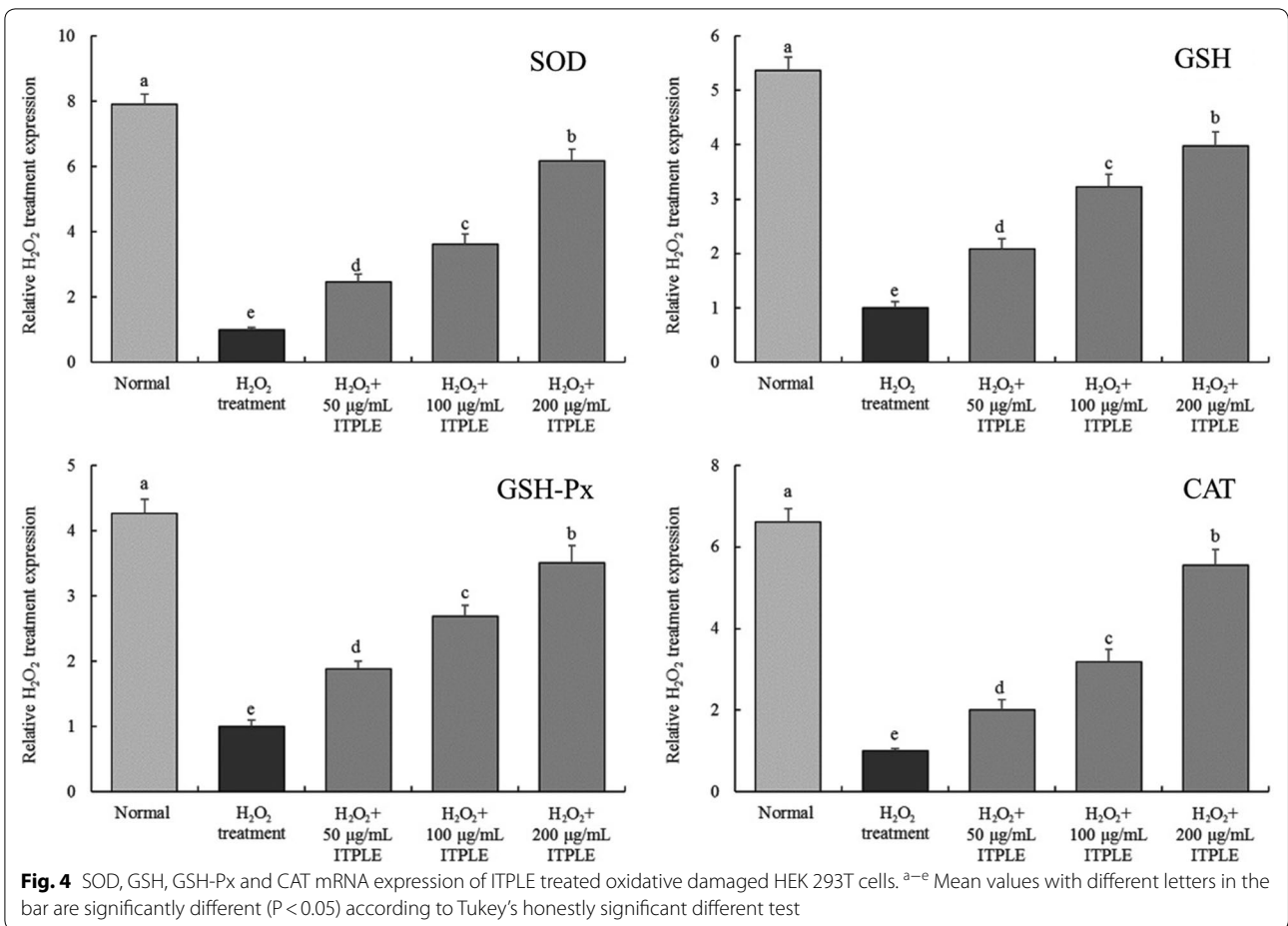
MDA is the final metabolite of membrane lipid peroxidation in vivo, which can reflect the degree of tissue peroxidation. MDA released from cell membrane can



react with protein and nucleic acid, cause cross-linking polymerization, inhibit protein synthesis, damage cell membrane structure and function, change its permeability, and affect normal physiological activities of the body [16, 17]. Therefore, the level of MDA can be used to monitor the degree of lipid peroxidation and indirectly reflect the degree of cell and body damage. Both human endogenous antioxidant enzymes of CAT, SOD, GSH-Px and

non enzyme GSH can effectively improve oxidative stress injury [18]. SOD can catalyze superoxide anion radical disproportionation to produce oxygen and hydrogen peroxide, which plays an important role in the balance of oxidation and oxidation resistance, and is closely related to the occurrence and development of many diseases [19]. The biological function of catalase is to promote the decomposition of hydrogen peroxide in cells, so that





it will not further produce toxic free radicals of hydrogen and oxygen, so as to protect the function of antioxidant enzyme system, which is also of great significance for human growth and metabolism [20]. GSH is characterized by active sulfhydryl (-SH), which is the most important functional group. It can participate in various important biochemical reactions of the body, protect the sulfhydryl group of important enzyme protein from oxidation and inactivation, and ensure energy metabolism and cell utilization. Meanwhile, it can directly reduce the free radicals to acidic substances by the combination of sulfhydryl and free radicals in the body, so as to accelerate the excretion of free radicals and fight against the damage of free radicals to important organs [21]. GSH-Px can reduce toxic peroxides to nontoxic hydroxyl compounds, and promote the decomposition of  $H_2O_2$ , so as to protect the structure and function of cell membrane from the interference and damage of oxides [22]. SOD can convert the excess  $O_2^-$  of the body into  $H_2O_2$ , which is then converted into  $H_2O$  by CAT and GSH-Px [22]. CAT has a high affinity for  $H_2O_2$  and can reduce the toxic  $H_2O_2$  to  $H_2O$  [23]. In addition, GSH can react directly with ROS, reduce it, and act as the substrate of GSH-Px, catalyze GSH to GSSG, reduce alkane hydroperoxides to hydroxyl compounds, and promote the decomposition of  $H_2O_2$  [24]. The cooperation of CAT, SOD, GSH-Px and GSH can play the role of resisting oxidative stress in the body and protect the body together [25]. In this study, through the detection of kits and mRNA analysis, it was observed that the ITPLE could increase the SOD, CAT, GSH, GSH-Px levels and reduce the MDA level of HEK 293T cells, thus reducing the oxidative damage caused by  $H_2O_2$ .

Neochlorogenic acid, cryptochlorogenic acid, isochlorogenic acid A and isochlorogenic acid B are chlorogenic acid compounds, all of which have good antioxidant effects [26]. The research also showed that they played the role of antibacterial, antiviral, increasing leukocyte, protecting liver and choleric, anti-tumor, lowering blood pressure, reducing blood lipid, clearing free radicals and stimulating central nervous system through their antioxidant abilities [27–31]. In addition, *in vitro* experiments also show that isochlorogenic acid B has a good scavenging effect on hydroxyl radicals, hydrogen peroxide is easy to produce hydroxyl radicals and cause oxidative damage [32], which suggests that isochlorogenic acid B should have a good inhibitory effect on hydrogen peroxide induced oxidative damage. Rutin, as a commonly used antioxidant, has a strong ability of scavenging free radicals. In pharmacology, it has the functions of anti-bacterial and anti-inflammatory, anti radiation, regulating the permeability and brittleness of capillary wall, preventing vascular rupture, hemostasis, etc. [33].

Kaempferoside is also an antioxidant. Preliminary studies have also shown that kaempferoside has been detected in a variety of traditional Chinese medicine ingredients. It can be inferred that kaempferoside has certain efficacy in treating inflammation and improving immunity [34]. It has been shown that the phenolic hydroxyl of kaempferoside can scavenge hydrogen peroxide, so as to play the role of kaempferoside in inhibiting the oxidative damage caused by peroxidation [35]. Quercetin is also used as an antioxidant in the food industry. At the same time, research shows that quercetin has good expectorant and antitussive effects [36]. These seven substances have good antioxidant effect, these ingredients are also detected in traditional Chinese medicine and functional food, they also have a variety of disease prevention and treatment effects [26–36]. In this study, the ITPLE contained these active substances, their own functions and synergistic effects constituted the inhibition of oxidative damaged HEK 293T cells, which might play the preventive or therapeutic effect on some diseases.

In conclusion, in this study, we evaluated the protective effect of the ITPLE against OS at the cellular level and preliminarily examined the relationship between the active compounds and their efficacy. The results of biochemical and molecular biological experiments showed that the ITPLE had excellent inhibitory effects on oxidative damage in HEK 293T cells. In particular, ITPLE could enhance the production of antioxidant enzymes and other antioxidant substances in HEK 293T cells induced by OS, thereby protecting cells from oxidative damage. Through the analysis of the composition of ITPLE, we found that it contains several antioxidant compounds, whose mutual synergistic effects have a cytoprotective effect. Therefore, it can be concluded that ITPLE showed excellent inhibitory effects against oxidative damage in HEK 293T cells. However, this study had certain limitations, for instance, this study was carried out in an *in vitro* cell model of oxidative injury, and further animal experiments are needed in the future. Additionally, the synergistic mechanism between the active components of ITPLE needs further study.

#### Authors' contributions

JZ and HW performed the majority of the experiments and wrote the manuscript; SY, ZG, YH and WL contributed to the data analysis; HL and XZ designed and supervised the study, and checked the final manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

Datasets used and/or analysed during the current study that are not included in the manuscript are available from the corresponding author on reasonable request.

**Competing interests**

All authors declare that there is no competing of interests.

**Author details**

<sup>1</sup> Environment and Quality Inspection College, Chongqing Chemical Industry Vocational College, Chongqing 401228, China. <sup>2</sup> Chongqing Collaborative Innovation Center for Functional Food, Chongqing Engineering Research Center of Functional Food, Chongqing Engineering Laboratory for Research and Development of Functional Food, Chongqing University of Education, Chongqing 400067, China. <sup>3</sup> Department of Cardiothoracic Surgery, First Affiliated Hospital of Chengdu Medical College, Chengdu 610500, China. <sup>4</sup> School of Life Science and Biotechnology, Yangtze Normal University, Chongqing, China. <sup>5</sup> First Affiliated Hospital of Gannan Medical University, Ganzhou 341000, Jiangxi, China.

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