Original Research Paper

Inhibitory Effect of Crude Walnut Leaf Polysaccharides on Oxidative Damage of Proteins and Proliferation of SGC-7901 Cells

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Abstract: In this paper, we studied the inhibitory effect of crude Walnut Leaf Polysaccharides (WLP) on free radical-induced oxidative damage of proteins and anti-proliferation activity on human Gastric Cancer cells (SGC-7901). The oxidative degradation of Bovine Serum Albumin (BSA), mouse brain proteins (MBP) and Mouse Liver Proteins (MLP) induced by hydroxyl radical and alcoxyl radical was analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). In addition, MTT method was used to analyze the effect of WLP on cell viabilities of SGC-7901 cells. The results indicated that WLP significantly inhibited the oxidative damage of BSA induced by hydroxyl radical within the concentration range of 0.5~20 mg/mL. Similar protective effects were found in hydroxyl radical-induced oxidative damage on mouse tissue proteins. WLP inhibited the oxidative damage of three protein models induced by alcoxyl radical in a dose-dependent manner. Furthermore, WLP inhibited the growth of SGC-7901 cells in time- and concentration-dependent manner. In conclusion, WLP show significant inhibitory effect on protein oxidative damage induced by hydroxyl radical and alcoxyl radical as well as anti-proliferation capacity on SGC-7901 cells.

Keywords: Walnut Leaf, Polysaccharide, Protein, Oxidative Damage, SGC-7901 Cells

Introduction

Free radicals in the body are produced during normal metabolic processes. However, the excessive accumulation of free radicals will damage the body's protein, DNA and other biological macromolecules, which will lead to diseases such as cancer and diabetes, etc. (Gao *et al*., 2015; Lai *et al*., 2010). In recent years, free radicals-induced oxidative damage of proteins has become a hot topic in human nutrition and health (El-Azem *et al*., 2019).

Oxidative stress are mainly produced in mitochondria of human cells and related to progressive dysfunction of mitochondria which occurred in cancer (Pecorini *et al*., 2020). Thus, decrease ROS levels or oxidative stress in human cells can reduce the risk of cancer. With the deep understanding of oxidative stress, antioxidant substances have attracted the attention of many researchers. Although synthetic antioxidants have been proved to be effective in the treatment of many diseases, long-term and/or overdose consumption of some synthetic antioxidants such as Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT) may increase the risks of cancer (Seedevi *et al*., 2017; Xie *et al*., 2016). Studies have shown that foods with natural antioxidant ingredients can reduce the degree of oxidative damage in the body and decrease the risk of many diseases (Cerqueira *et al*., 2009; Chang *et al*., 2010; He *et al*., 2011; Palanisamy *et al*., 2017; Liu *et al*., 2018). Moreover, chemotherapy and surgery are the mainstream methods of cancer treatment, but some chemotherapy drugs have many limitations, such as limited efficacy of adverse reactions and high failure rate (Xie *et al*., 2013). Therefore, it is of great significance to extract and study natural active substances with antioxidant and anticancer activities from natural resources.

Several antioxidants and anticancer substances are found in nature. Among them, polysaccharides, an important source of functional food and drugs, are widely found in plants, animals and microbes etc. and possess the

activities of anti-oxidation, immunomodulation, bacteriostasis, anti-virus, antihypertensive and anticancer (Mzoughi *et al.*, 2018; Pan *et al.*, 2018; Thambiraj *et al.*, 2018; Hu *et al.*, 2017; Li *et al.*, 2017a; 2017b; Li and Shan, 2014; Cai *et al.*, 2016; Romdhane *et al.*, 2017; Teng *et al.*, 2013). Walnut tree is a deciduous tree native to southeast Europe (You *et al.*, 2013). Walnut fruit is one of the most common nuts in our daily life and is also considered as a natural functional food with high economic value because of its rich nutrition (Jeddou *et al.*, 2016; Almeida *et al.*, 2008). As we all know, the utilization of walnut is mostly in the fruit part, but the development of walnut leaves with strong functional activity is very few, which causes a great waste of resources. If the active ingredients in walnut leaves can be extracted and utilized, the economic benefits of walnut planting will be greatly improved. In traditional medicine, walnut leaf is mainly used as an astringent and preservative and has the effect of anti-diarrhea, anti-inflammatory, anti-ulcer and promoting bile flow (Tapia *et al.*, 2013). Previous studied reported that walnut leaf extract possessed the potential of anti-oxidation, anti-diabete and antihypertensive effects (Forino *et al.*, 2016; Pereira *et al.*, 2007). In addition, our previous studies found that WLP have antioxidant activities and anticancer effect on HepG2 cells (Yang *et al.*, 2019a; 2019b). However, the antioxidant activities of WLP from the perspective of protein oxidative damage and the anticancer effect of WLP on gastric cancer cell SGC-7901are not reported.

In order to fully reveal antioxidant and anticancer activities of WLP, in this study, WLP were extracted from walnut leaves using cellulase-assisted extraction method and the oxidative degradation of BSA, MBP and MLP was induced by Cu^{2+}/H_2O_2 and AAPH to assess the inhibited effect of WLP on oxidative damage of proteins. Moreover, the survival rate of SGC-7901 cells was detected by MTT colorimetry to evaluate the inhibited activity of WLP on the proliferation of gastric cancer cells.

Materials and Methods

Materials

Walnut leaves were collected from Zibo city, Shangdong Province in July 2018. WLP was extracted using cellulase assisted method as described in our previous study (Yang *et al.*, 2019b). SD rats were purchased from Shandong University Laboratory Animal Center. The treatment of SD rats during the experiment was in accordance with the relevant regulations of animal management methods. SD rats were fed with basal feed and drank water freely at 20-22°C with relative humidity of 45%-50%. BSA was obtained from Sigma (NY, USA). Human gastric cancer

cells (HL-7702) were obtained from Cell Bank of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium, Fetal Bovine Serum (FBS) and penicillin-Streptomycin (PS) were obtained from Gibco (NY, USA). Thiazolyl blue tetrazolium bromide (MTT) was obtained from Saiguo Biotechnology Co., Ltd (Guangzhou, China). Other reagents were analytical pure. The experimental water was ultra pure water.

Methods

Cellulase-Assisted Extraction of WLP

The walnut leaves were dried naturally and then crushed and sifted, then the powder of walnut leaves and cellulase were weighed accurately in proportion and added into a certain amount of distilled water. After soaking them fully, they were immersed in a shaking tank (Yiheng, China) for 2 h, centrifuged at 4000 r/min for 10 min and the supernatant was collected. Sevage reagent (chloroform: n-butanol = 4:1) was added to the polysaccharide solution for deproteinization and the polysaccharide and sevage reagent were mixed in a 1:1 ratio for 30 min. The deproteinized polysaccharide solution was taken and rotated to an appropriate volume under pressure at 50°C. Precooled anhydrous ethanol was added and placed overnight at 4°C. WLP was obtained after precipitation was collected and dried (Zhu *et al.*, 2014; Yang *et al.*, 2017; Yin *et al.*, 2018).

Preparation of Histone Protein

The brain and liver tissues of SD rats were rinsed with cold normal saline and some tissues of certain quality were cut out and put into Ripa and PMSF solution. The ice bath homogenate was centrifuged at 4°C (8000 r/min, 15 min). The supernatant was tissue protein. Histoprotein concentration was determined by BCA method.

Inhibition of WLP on Alkoxy Radicals-Induced Protein Oxidative Damage

BSA solutions or tissue protein solutions with a final concentration of 0.6 mg/mL were mixed with different concentrations of WLP and bathed in water at 37°C for 30 min. Then AAPH was added to the experimental group and the same volume of Phosphate Buffer (PBS, pH7.4) was used to replace AAPH in the control group. The samples were further treated in a water bath at 37°C. SDS-PAGE electrophoresis was used to analysis the protein samples after 4 h.

Inhibition of WLP on Hydroxyl Radicals-Induced Protein Oxidative Damage

The method has been slightly modified by reference to the previous literature (Yuan *et al.*, 2013). WLP and protein samples were incubated the same way as above.

H_2O_2 (25 mmol/L) and $CuSO_4$ (0.1 mmol/L) solutions were added to the experimental groups and the PBS with the same volume (pH7.4) was used to replace H_2O_2 and $CuSO_4$ in the control group. After mixed, the samples were bathed in water at 37°C for 90 min, then the degree of protein oxidation was determined by SDS-PAGE.

Detection of Protein Oxidative Damage

The pre-treated samples were added with SDS loading buffer and treated at 95°C for 5 min. Then, equivalent samples were taken for 10% SDS-PAGE gel electrophoresis (Thermo, USA). After 70 min, the samples were stained with 0.1% coomassie bright blue R-250 and then the samples were decolorized with the decolorization solution. Finally, images were taken under the GelDoc XRS gel imaging system (Bio-Rad, USA) and the image band gray scale was analyzed by Image J software (Xiang *et al*., 2013).

Cell Culture and Activity Measurement of SGC-7901

SGC-7901 cells were cultured in a complete RPMI-1640 medium containing 10% FBS and 1% PS and the incubator conditions were set at 37°C and CO_2 concentration of 5%. The activity of SGC-7901 cells was determined by MTT colorimetry (Hu *et al*., 2017). The cultured cells were inoculated into 96-well plates at a density of 5×10^4/mL for 24 h, then treated with different doses of WLP for 12 h and 24 h, respectively. Then 20 μL of MTT solution was added to each well and incubated at 37°C for 4 h. After the unreacted dye was removed, 100 μL of DMSO was added to each well. Finally, the OD value was measured using a microplate reader (Bio-Rad, USA) at 570 nm. The survival rate of SGC-7901 cells was calculated according to the following formula:

$$The\ cell\ viability(\%) = 100 \times \frac{Absorbance\ of\ experimental\ group}{Absorbance\ of\ blank\ control\ group}$$

Statistical Analysis

The statistically analysis of the results were executed by Origin 8.0 and Excel 2019.All data were indicated as the means ± Standard Deviation (SD) and evaluated using Analysis of Variance (ANOVA). For all comparisons, a significant difference was judged to be statistically significant if $P<0.05$.

Results and Discussion

Inhibitory Effect of WLP on Oxidative Damage of BSA

AAPH could produce alkoxy radical after heat treatment. It can be seen from Fig. 1 that AAPH induced significant oxidative damage on BSA and the WLP in the concentration range of 0.1-2.5 mg/ml had an obvious inhibitory effect on the oxidative damage of BSA induced by alkoxy radicals ($P<0.01$) in dose-dependent manner.

Cu^{2+} catalyzes H_2O_2 to produce hydroxyl radicals. As can be seen from Fig. 2, the grayscale of the bands after hydroxyl radical treatment became lighter significantly ($P<0.01$), indicating that hydroxyl radical caused oxidative damage of BSA. Compared with the Cu^{2+}/H_2O_2-induced sample, the gray levels of BSA electrophoresis bands were significantly deepened after pretreatment with different concentrations of WLP ($P<0.01$), which indicated that WLP could inhibit the oxidative damage of BSA and the higher the concentration was, the more significant the protective effect was. When the concentration of WLP was 20 mg/mL, its protective effect on BSA was as high as 94.86%. These results indicated that WLP could significantly inhibit alcoxyl radical and hydroxyl radical induced BSA oxidative damage.

Inhibitory Effect of WLP on Oxidative Damage of MBP

Protein plays a key role in the growth and functional development of brain cells. Under the condition of oxidative stress, the balance between strong oxidants and antioxidants in the body is destroyed and a large number of free radicals are generated to induce oxidative damage of tissue proteins such as brain proteins, leading to the occurrence of brain diseases (Butterfield *et al*., 2001).

As can be seen from Fig. 3, 50 mmol/L of AAPH caused significant oxidative degradation of MBP. WLP at 0.5 mg/mL had no significant inhibitory effect on the oxidative damage of MBP induced by alkoxy radicals. However, the oxidative degradation degree of MBP was significantly reduced after pretreatment with 2, 3.5 and 5 mg/mL of WLP. The above results indicated that WLP with concentrations of 2-5 mg/mL had significant protective effects on alkoxy radical induced MBP oxidation.

As shown in Fig. 4, the degree of MBP oxidation induced by hydroxyl radical increased significantly compared with the blank group ($P<0.01$). After the pretreatment of 0.01-0.1 mg/mL WLP, the gray level of hydroxyl free-radical-induced MBP bands increased significantly and the higher the concentration of WLP, the more obvious the protective effect on MBP ($P<0.01$). When the concentration of WLP was 0.1 mg/mL, the protection rate was up to 99.40%. The above results indicated that WLP inhibited hydroxyl radical induced MBP oxidative damage.
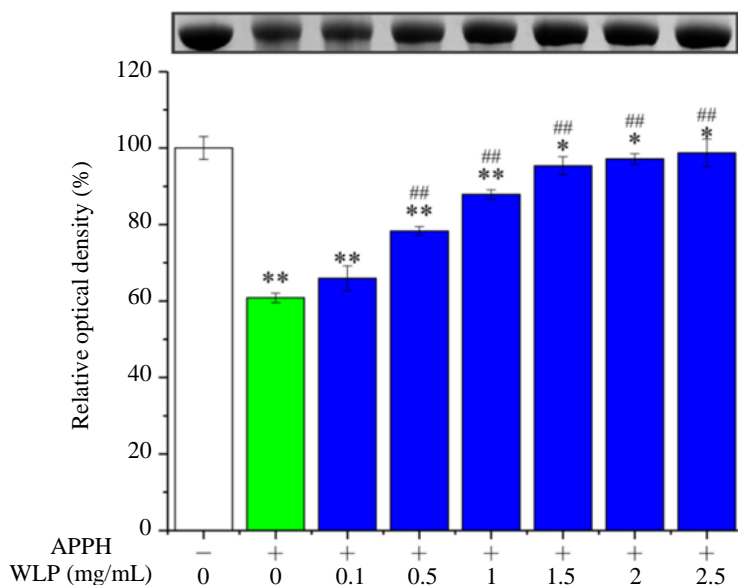
Fig. 1: Inhibitory effect of WLP on alcoxyl radical-induced oxidative damage of BSA. -. inexistent; +. existent. Compared with the blank group, *. Difference was significant (*P*<0.05), **. Difference was extremely significant (*P*<0.01); compared with the induction group, **#.**Difference was significant (*P*<0.05), **##.**Difference was extremely significant (*P*<0.01). Similarly hereinafter
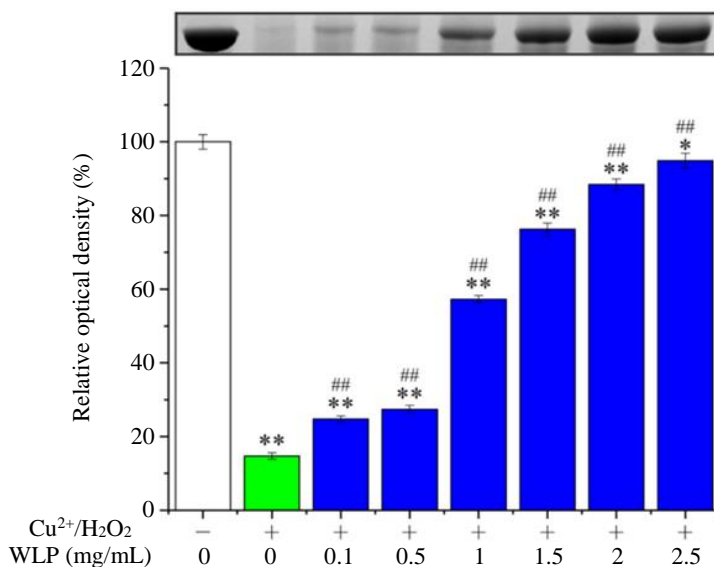

Fig. 2: Inhibitory effect of WLP on hydroxyl radical-induced oxidative damage of BSA

Inhibitory Effect of WLP on Oxidative Damage of MLP

The liver is an important organ involved in metabolism and plays a key role in protein synthesis. However, free radicals can easily induce protein damage in the liver and affect the health of the liver (Zhang *et al*., 2013).

It can be seen from Fig. 5 that WLP interfered with protein bands deepen, indicating that the concentration of WLP in the range of 0.5-2 mg/mL could effectively inhibit the MLP damage caused by alkoxy free radical and presented a concentration dependent relationship. When the concentration of WLP was up to 2 mg/mL, the protective effect of MLP on MLP oxidative damage induced by alkoxy radical reached 99%. The above

results showed that WLP prevented the oxidative attack of alkoxy radicals on MLP.

As can be seen from Fig. 6, the band of histone induced by hydroxyl radical became lighter (*P*<0.01), indicating that hydroxyl radical induced oxidative

degradation of MLP. WLP with a concentration range of 0.5-10 mg/mL inhibited hydroxyl radical-induced oxidative damage of MLP in a dose dependent manner. The results showed that WLP could inhibit the oxidative degradation of MLP induced by hydroxyl free radical.
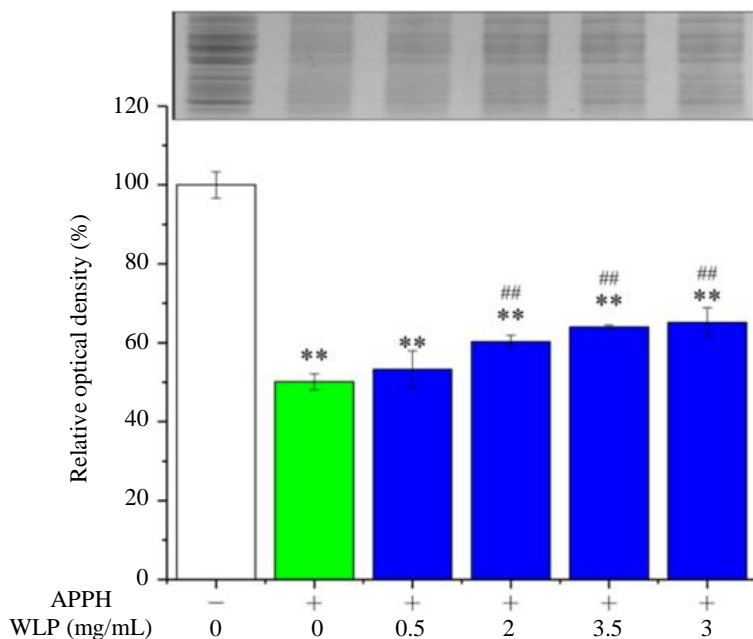

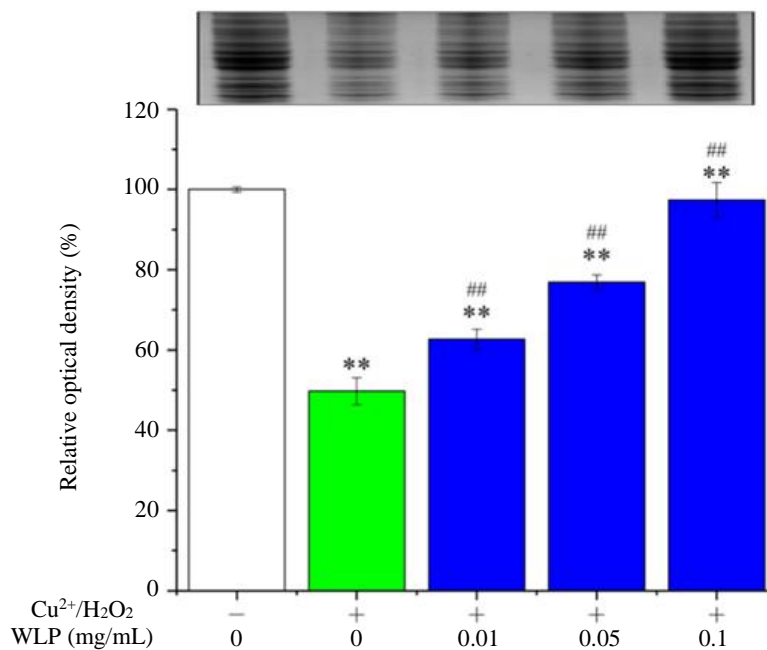**Fig. 3:** Inhibitory effect of WLP on alcoxyl radical-induced oxidative damage of MBP

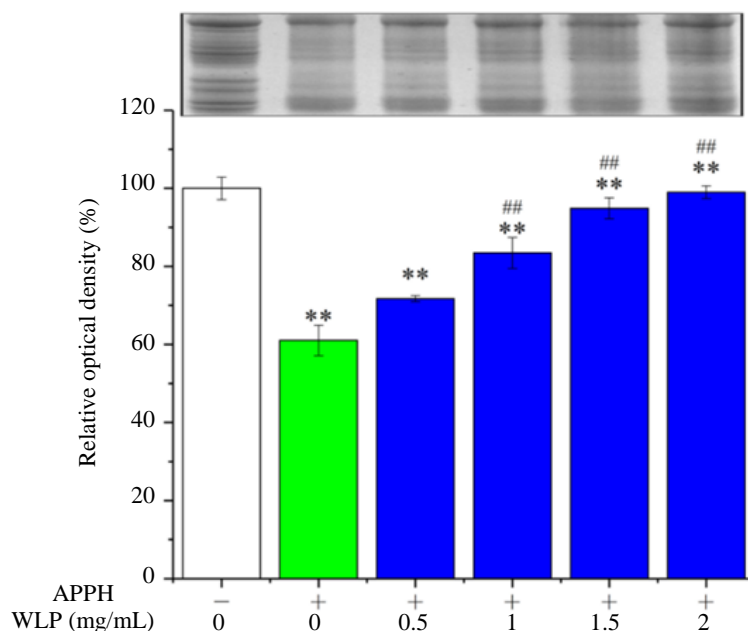**Fig. 4:** Inhibiting effect of WLP on hydroxyl radical-induced oxidative damage of MBP

Fig. 5: Inhibitory effect of WLP on alcoxyl radical-induced oxidative damage of MLP
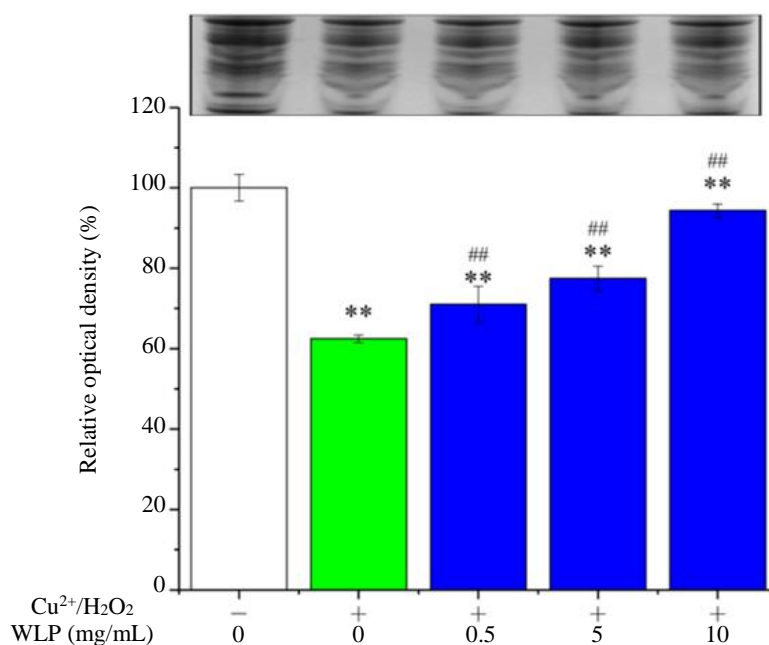

Fig. 6: Inhibitory effect of WLP on hydroxyl radical-induced oxidative damage of MLP

Inhibitory Effect of WLP on SGC-7901 Cells

The inhibitory effect of WLP on cell viability of SGC-7901 cells was shown in Fig. 7. WLP displayed obvious inhibitory activity on human gastric cancer cell SGC-7901. When the amount of additive was 9 mg/mL, the antiproliferation activity of polysaccharide increased and the cell survival rate decreased significantly. Compared with 12 h, the activity of SGC-7901 cells cultured with WLP for 24 h was weaker. The results showed that the growth of SGC-7901 cells could be inhibited by WLP in a concentration and time-dependent manner.
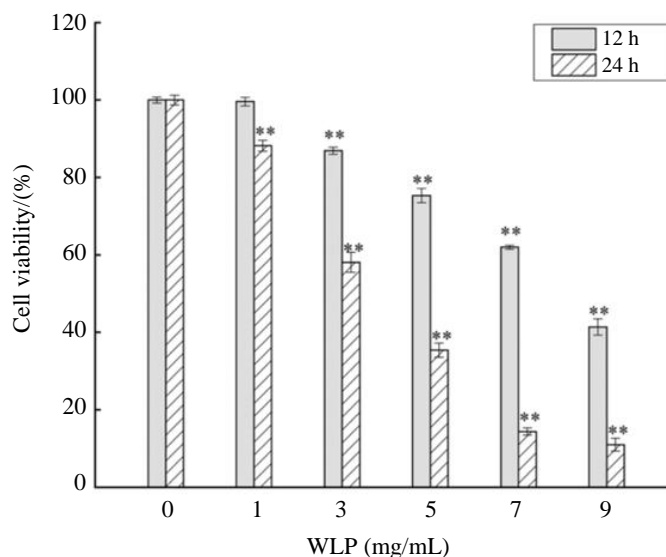
Fig. 7: Inhibitory effect of WLP on cell viability of SGC-7901 cells

Conclusion

In this paper, the antioxidant activity of WLP was investigated from the perspective of biomacromolecule. Our results proved that WLP significantly inhibited the oxidative damage of proteins (BSA, MBP and MLP) in hydroxyl radical and alkoxy radical induced systems. Moreover, this work preliminarily confirmed that WLP could reduce the cell viability of human gastric cancer SGC-7901 cells in concentration- and time-dependent manners. This study provides a basis for evaluating the antioxidant and anticancer activities of WLP and give ideas for the comprehensive utilization of agricultural waste walnut leaves.

Acknowledgement

Funding Information

Author's Contributions

Shuqing Yang: Participated in the whole experiment process and also contributed to the interpretation of the results and manuscript preparation.

ZhikeXie, Shuyan Yu and Ming He: Participated in part of the experimental design.

Qing Liu: Ameliorated the manuscript.

Haifang Xiao: Contributed to the study design, the interpretation of the results and manuscript preparation.

Yuanda Song: Contributed to the guidance of experimental design and ameliorated the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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