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Stronger antioxidant and hepatoprotective components generated from Rubiaceae *Hedyotis diffusa* with biotinidase in the field of new biological energy technology

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Biological enzymes, as innovative biological technology in the field of new energy, were used in preparing the total flavonoids of Rubiaceae, *Hedyotis diffusa* in this study. The differences of the flavonoid compositions, contents, antioxidant and hepatoprotective activities on HL-02 cells damaged by H_2O_2 were compared between the new biotinidase energy and traditional technology.What's more, the antioxidant activity screening was investigated by H_2O_2-damaged HL-02 cells. The protection rates of total flavonoids and active components of *Hedyotis diffusa* on H_2O_2-damaged HL-02 cells were determined by MTT method, and the contents of ALT, AST, LDH, and ALP in cell culture medium were measured to investigate the liver protection of total flavonoids of *Hedyotis diffusa*. The results showed that the contents of isoflavones extracted by the new biological enzymatic energy technology were significantly higher than those by traditional reflux extraction, ultrasonic method and ultrasound-assisted enzymatic extraction method (p<0.05). The biological enzyme as new biological energy source could obtain new components with stronger antioxidant and hepatoprotective activities in *Hedyotis diffusa*, which was beneficial to the development and utilization of biological technology in the field of new energy.

Keywords: New biotinidase, total flavonoids, *Hedyotis diffusa*, biological energy source technology, antioxidant and hepatoprotective activties

INTRODUCTION

Hedyotis Diffusa is an annual herb, which attributed to the Rubiaceae and mainly distributed in the south of China and some Asian countries, such as Indonesia, Japan, Malaysia, Nepal, Thailand [1] and so on. Flavonoids are one kind of the main components of *Hedyotis diffusa* that exert various pharmacological activities including antioxidant, hepatoprotective and anti-tumor and so on [2-4]. The manufacture technologies of plant flavonoids are mainly divided into traditional preparation technology and modern extraction technology based on the energy pattern used for industrial applicability and environmental protection [5].

In this research, biological enzymes were used in preparing the total flavonoids of Rubiaceae, *Hedyotis diffusa*. The principle of the enzymatic technology in field of biological new energy is using the special biological enzymes to better release and extract biologically active substances by destroying their cell walls. It could also break down impurities such as protein, starch and pectin, and improve the productive rate of target biological activity components. Enzymatic production was used in combination with solvent extraction for the first time in this study. It has the advantages of low cost, high productive rate, strong specificity and reliability, simple operating equipment and environmental protection, which was suitable for industrial production by using new biological enzymes technology [6]. Compare the total flavonoids and active components of *Hedyotis diffusa* prepared by enzymatic energy with those prepared by the traditional productive technologies (reflux, ultrasonic method and ultrasound-assisted enzymatic extraction), there were differences in contents and pharmacological antioxidant and hepatoprotective activities remain to be further investigated.

Immediately following, 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2-H-tetrazolium bromide (MTT) was used to detect cell viability for screening active ingredients with antioxidant and liver-protecting activities [7]. This new biological enzymes energy technology would be instructive for future investigation on stronger antioxidant and hepatoprotective components generated from natural safe and non-toxic plant thereon which natural drugs would be established.

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EXPERIMENTS

Materials and Reagents

Hedyotis diffusa was collected from Jiangxi and certified by Professor Tianai Gao from Shanxi Provincial Food and Drug Inspection Institute. Biological cellulase: 30 units/mg (Zhejiang Yinuo Biotechnology Co., Ltd., food grade). Macroporous adsorbent resin AB-8 was purchased from Chengdu Grecia Chemical Technology Co., Ltd. HL-02 cells, RPMI-1640 medium, fetal bovine serum, 0.25% trypsin were purchased from Wuhan Dr. Biotech Co., Ltd. Dimethyl sulfoxide (DMSO), hydrogen peroxide, penicillin and streptomycin, MTT were obtained from Beijing Solibao Technology Co., Ltd. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) assay kits were purchased from Nanjing Jiancheng Biotechnology Research Institute. The experimental water was distilled water, and other reagents used were chromatographically grade.

Apparatus Total Flavonoids Preparetion

The total flavonoids of *Hedyotis diffusa* were extracted by biological enzymatic technology(A), reflux technology (B), ultrasonic technology (C) and ultrasound-assisted technology (D). Before extraction, *Hedyotis diffusa* were dried, grinded and sieved to get the solid powder (60 mesh). The solid powder would be ultrasonically treated with petroleum ether (1g:20mL) for 30minutes for degreasing, and dried and cooled. The extractions were as follow, all the processes were optimized. In enzymatic technology, 0.5% biological cellulase was added to per 1g of degreased sample and extracted at 55℃ for 1.5h in 30mL 50% ethanol solution with pH 5.0. The ultrasound-assisted method was performed in an ultrasonic cleaner according to the above process. Both reflux and ultrasonic processes used 50% ethanol as the extraction solvent, and the material-liquid ratio was 1:30. Then extracted for 1.5h under reflux at 80℃ or ultrasonic conditions, respectively. The four extracting technologies were purified by macroporous adsorption resin AB-8, and finally the total pure flavonoid extracts of *Hedyotis diffusa* were obtained.

Cell Culture

HL-02 cells were cultured in RPMI-1640 added with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a constant temperature incubator with 5% CO_2 at 37℃. According to cell growth, subculture was carried out for 2 days to ensure that all cells used for the experiment were in a logarithmic growth phase. The normal control group cultured with RPMI-1640. The model group cultured with 200 μmol/L H_2O_2. The administration groups treated with 125, 62.5, 31.25 μg/mL total flavonoids prepared by different technologies and compounds including vitexin, isovitexin, naringin, genistein, apigenin and chrysin (16, 8, 4 μmol/L, respectively). Bifendate (12 μmol/L) was given as positive control group. The survival rates of HL-02 cells were determined by MTT method.

Screening for Liver Protective Activity

The model of H_2O_2-induced HL-02 injury was used to investigate the protective effects of total flavonoids on liver damage. H_2O_2 at different concentrations (50, 100, 200, 400, 600, 800 μmol/L) were applied to HL-02 cells for 4h. The absorbance (OD) value was measured by MTT method and the cell survival rates were calculated to determine the appropriate modelling concentration of H_2O_2. The administration groups and the positive control group were treated with the samples as in "3" for 12 h in advance. 125 μmol/L bifendate was used as a positive control. After treatment according to the above method, the OD was measured and the protection rates for HL-02 cells were caculated: Protection rate (%) = ($OD_{\text{administration group}}$-$OD_{\text{model group}}$) / ($OD_{\text{control group}}$-$OD_{\text{model group}}$)× 100%.

Optimization of cellulase-assisted enzymatic extraction conditionsDetermination of ALT, AST, LDH and ALP in Supernatant

The *in vitro* activities of hepatocellular leakage ALT, AST, LDH and ALP were assayed as markers and indices of hepatotoxicity of HL-O2 cells. HL-02 cells in logarithmic growth phase were digested with trypsin and seeded in 12-well plates, cultured for 24h. After collected, the contents of AST, ALT, LDH and ALP in the cell culture media were measured according to the instructions of the kits. The activities were expressed as the percentages of the activities over the corresponding control groups.

Data Analysis

T-test analysis was used to compare the differences between different groups. When $p < 0.05$, there was a statistical difference. All experiments were repeated at least 3 times in parallel.

RESULTS AND DISCUSSIONS

Concentration Selection of H_2O_2 Modeling

The survival rates of H_2O_2 at concentrations (50~800μmol/L) on HL-02 cells were shown in Fig.1. The results showed that the survival rate of HL-02 cells decreased with the increase of H_2O_2 concentration. When the concentration of H_2O_2 was 200μmol/L, the cell survival rate was about 60%, which was significantly lower than that of the control group ($p < 0.05$). So, the concentration 200μmol/L of H_2O_2 was selected for hepatocyte damage modeling.

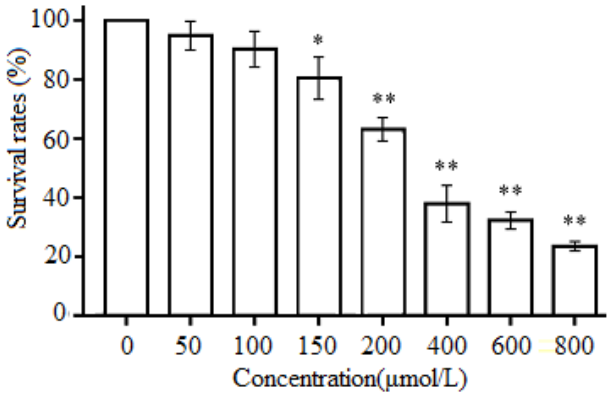

Fig.1. Cell survival rate of HL-02 cells by different concentrations of H_2O_2. * (p<0.05), ** (p<0.01) indicated that there was a significant difference from the model group; ## (p<0.01) indicatesd that there was a significant difference from the control group

Screening for Liver Protecive Activities

Furthermore, as shown in Fig.2, the highest protection rates of biological enzymatic technology (sample A) were obtained among four extraction technologies,respectively. It was up to 59.30 ± 0.81% and even more higher than the positive control bifendate at 125μg/mL. The hepatoprotective activity of total flavonoids prepared by enzymatic technology was also significantly higher than those of total flavonoids prepared by other method at the same concentration (p<0.05).

Determination of hepatotoxicity and antioxidant pattern

Once hepatocytes are damaged, ALT, AST, LDH and ALP will be released out of cells into the culture medium. So, they are frequently used as biomarkers of hepatotoxicity. When liver damage occurs, a large amount of those would be released and the contents were significantly increased. Herein, the activities of ALT, AST, LDH and ALP in cellular supernatant were tested in order to further investigate the protective effects of total flavonoids of *Hedyotis diffusa* prepared by different methods on HL-02 cell injury induced by H_2O_2. They were significantly elevated in model groups compared with the control groups (p< 0.01) (shown in Fig.3-6).

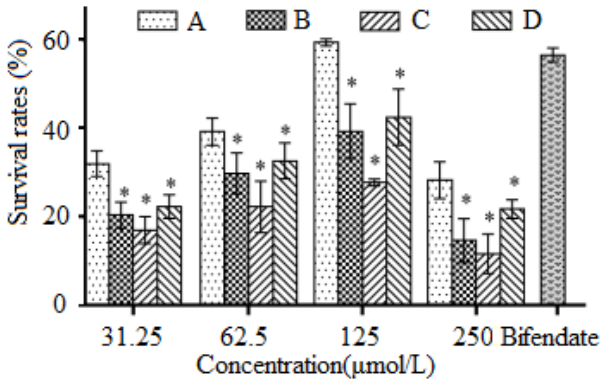

Fig.2. Protective activities of total flavonoids prepared by four different technologies. A, biological enzymatic technology; B, reflux technology; C, ultrasonic technology; D, ultrasound-assisted technology. * (p<0.05), ** (p<0.01) indicated that there was a significant difference from the model group; ## (p<0.01) indicatesd that there was a significant difference from the control group.

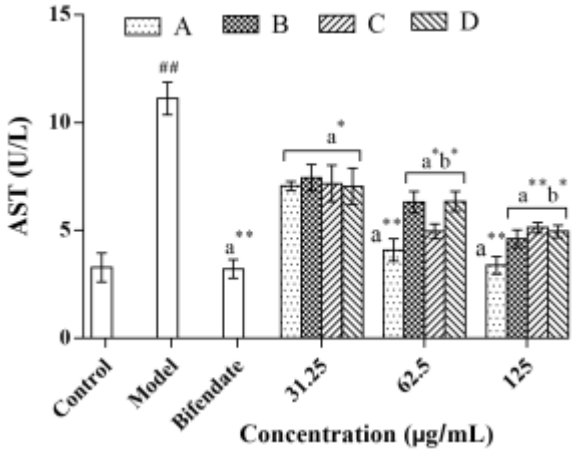

Fig.3. Detections of AST for hepatotoxicity and antioxidant pattern in H_2O_2-induced hepatotoxic HL-O2 cells for 24h. Bifendate (5mg/mL) was as positive control. A, biological enzymatic technology; B, reflux technology; C, ultrasonic technology; D, ultrasound-assisted technology. ##(p<0.01) indicated there was a significant difference compared with control group; a* (p<0.05), a** (p<0.01) indicated that there was a significant difference compared with model group; b* (p<0.05) indicated there was a significant difference compared with sample A (biological enzymatic technology)

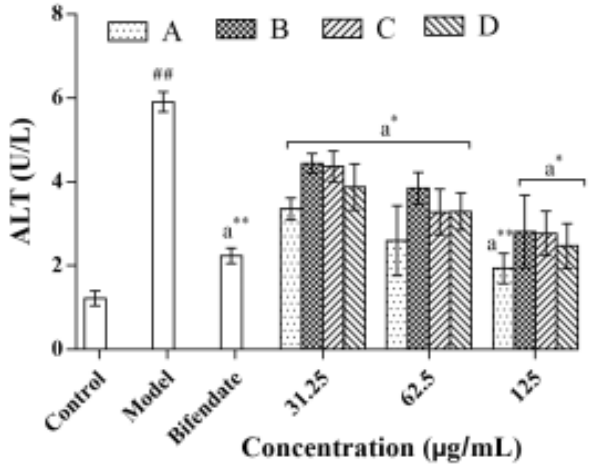

Fig.4. Detections of ALT for hepatotoxicity and antioxidant pattern in H_2O_2-induced hepatotoxic HL-O2 cells for 24h. Bifendate (5mg/mL) was as positive control. A, biological enzymatic technology; B, reflux technology; C, ultrasonic technology; D, ultrasound-assisted technology. $^{\#\#}(p<0.01)$ indicated there was a significant difference compared with control group; a* ($p<0.05$), a** ($p<0.01$) indicated that there was a significant difference compared with model group; b* ($p<0.05$) indicated there was a significant difference compared with sample A (biological enzymatic technology)

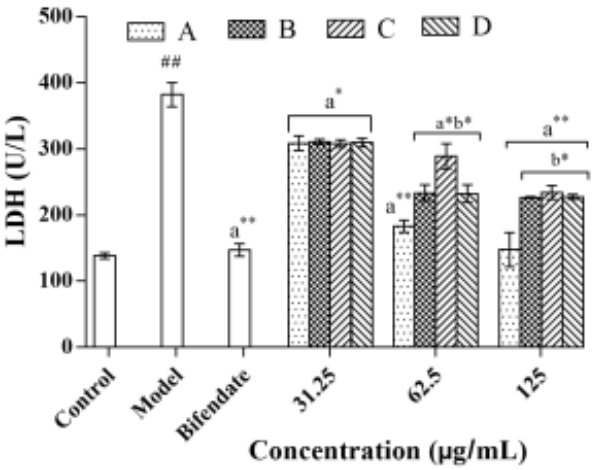

Fig.5. Detections of LDH for hepatotoxicity and antioxidant pattern in H_2O_2-induced hepatotoxic HL-O2 cells for 24 h. Bifendate (5mg/mL) was as positive control. A, biological enzymatic technology; B, reflux technology; C, ultrasonic technology; D, ultrasound-assisted technology. $^{\#\#}(p<0.01)$ indicated there was a significant difference compared with control group; a* ($p<0.05$), a** ($p<0.01$) indicated that there was a significant difference compared with model group; b* ($p<0.05$) indicated there was a significant difference compared with sample A (biological enzymatic technology)

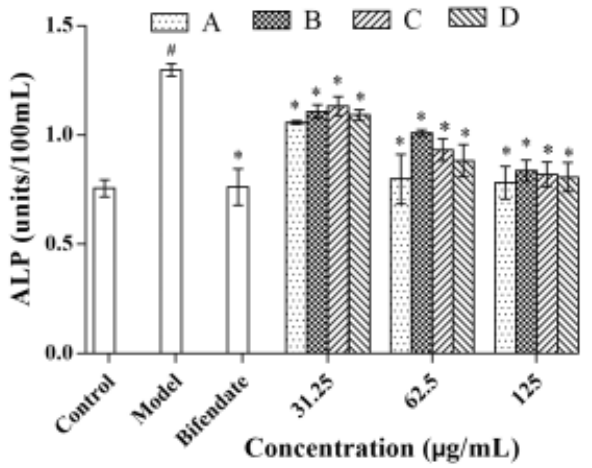

Fig.6. Detections of ALP for hepatotoxicity and antioxidant pattern in H_2O_2-induced hepatotoxic HL-O2 cells for 24h. Bifendate (5mg/mL) was as positive control. A, biological enzymatic technology; B, reflux technology; C, ultrasonic technology; D, ultrasound-assisted technology. $^{\#\#}(p<0.01)$ indicated there was a significant difference compared with control group; a* ($p<0.05$), a** ($p<0.01$) indicated that there was a significant difference compared with model group; b* ($p<0.05$) indicated there was a significant difference compared with sample A (biological enzymatic technology)

While, treatment of total flavonoids in cell supernatant at 31.25, 62.5, 125 μg/mL significantly reduced ALT, AST, LDH and ALP contents, which revealed a remarkable improvement in a dose-dependent manner compared with the model groups and positive control bifendate (125 μg/mL), as shown in Fig.3-6. At 62.5, 125 μg/mL, the effect of total flavonoids prepared by biological enzymatic technology (sample A) on reduce the content of AST and LDH were significantly higher than those of other three method at same concentrations ($p<0.05$).

CONCLUSIONS

Biological enzymes, as innovative biological technology in the field of new energy, had been widely used in various fields, including food raw material development, wastewater treatment and plant extraction applications. What's more, the effects of total flavonoids prepared by enzymatic method on reducing the contents of AST and LDH and protecting the H_2O_2-induced liver damage were significantly higher than those of other three method ($p<0.05$). In conclusion, the innovative biological enzymatic technology could improve the contents of active ingredients (about 3.5 times or more) with hepatoprotective effect. The total

flavonoids prepared by biological enzymatic technology had more effective on antioxidant and hepatoprotective activities. The biological enzymes as new biological energy source could obtain new components with stronger antioxidant and hepatoprotective activities in *Hedyotis diffusa*, which was beneficial to the development and utilization of biological technology in the field of new energy.

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ABBREVIATIONS

MTT - 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
ALT - alanine aminotransferase
AST - aspartate aminotransferase
LDH - lactate dehydrogenase
ALP – alkaline phosphatase
DMSO - Dimethyl sulfoxide
OD - absorbance

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