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A new method for evaluating drug efficacy at the gene expression level

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Abstract: **Background**: Many natural medicines exert therapeutic effects through multitarget regulation, and using methods that simply evaluate the effects of a chemical drug on several targets to study natural products may not comprehensively reflect their efficacy. **Objectives**: This research attempts to explore and establish a new method to evaluate drug efficacy at the gene expression level using natural hepatoprotective drugs. **Methods**: Forty SPF male SD rats were randomly divided into a control group, a liver injury model group, a positive drug group (reduced glutathione), a curcumin treatment group and a puerarin treatment group. Carbon tetrachloride was used to damage liver cells and establish a liver injury model. Gene microarray technology was used to detect gene expression in liver cells. **Results**: Drug treatments significantly reduced the number of differentially expressed genes in the liver compared with model group and efficacy evaluation of drugs were curcumin > positive control drug > puerarin. The liver function indices and pathological analysis also supported the above results. **Conclusion**: Our findings demonstrated that the efficacy of different drugs can be evaluated at the level of gene expression.

Keywords: Differentially expressed genes; Efficacy evaluation; Gene detection technology; Natural medicine

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INTRODUCTION

Unlike chemical drugs and biological agents, many natural medicines exert therapeutic effects through multitarget regulation. The discovery and transformation of chemical drugs are based mainly on single-molecule general or targeted screening, but using methods that simply evaluate the effects of a chemical drug on several targets to study natural products may not comprehensively reflect their efficacy. Therefore, it is valuable to explore and establish a pharmacodynamic evaluation method that can ignore specific components, targets or complex signal transduction network mechanisms *in vivo* and we assume the principle of this method is to compare the degree of deviation between the health and disease states of the target organ and to verify whether the tested drug can correct the deviation.

Organs are composed of cells and the state and function of cells are regulated by their genes; hence, the comprehensive response of a target organ to drug treatment could be revealed at the level of gene regulation via gene detection technology (Wang *et al.*, 2017). Experiments had confirmed that there were significant differences in gene expression between healthy and diseased states and even between cells in different areas of target organ under pathological conditions (Kuppe *et al.*, 2022). Transcriptome analysis of cells from normal heart muscle and infarct areas was performed via RNA sequencing technology and the results revealed significant differences in gene expression between diseased and healthy states (Liao *et al.*, 2019). Studies using transcriptome sequencing and differential expression analysis have further confirmed that the active components of some natural products can abrogate changes in the expression of related genes caused by pathogenic factors (Liang *et al.*, 2018, Pratomo *et al.*, 2023).

Differentially expressed genes (DEGs) are used in drug development and in the study of the mechanism of action of drugs, since identifying modified target genes allows us to identify the mechanisms by which drugs exert their effects. The purpose of DEG analysis is often to compare gene expression patterns in diseased individuals with those in apparently healthy controls and/or those receiving therapy. Alterations in gene expression may be interpreted as modifications of a particular pathway that is involved in the pathogenesis of the disease and, in turn, may be a therapeutic target (Gardón *et al.*, 2022). Moreover, effective drug therapy may also abrogate disease-induced changes in gene expression.

Although the active ingredients and targets of natural medicines are complex, they are effective in promoting the recovery of the target organ from a damaged state to a normal state. Therefore, we speculate that the degree of deviation could be determined by the difference in the number of DEGs between the damaged and healthy states of the target organ. The efficacy of drugs could be measured by their ability to correct deviation (that is, reduce the number of DEGs). In this study, an animal model of liver injury was selected to evaluate the efficacy of two natural medicines (curcumin and puerarin, fig.1)

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with hepatoprotective effects (Datta *et al.*, 2023, Hernández-Aquino *et al.*, 2020, Wan *et al.*, 2021) and differences in gene expression of the target organ liver among each group were compared and analyzed with the help of gene chip detection technology to explore and establish a new method for evaluating drug efficacy at the level of gene expression.

MATERIALS AND METHODS

Animals and treatments

Animals were provided by the Department of Experimental Animals of Tongji Medical College. Forty SPF male SD rats were randomly divided into a control group, model group, positive drug group (reduced glutathione), curcumin treatment group and puerarin treatment group, with 8 rats in each group. Except for those in the control group, the other rats were injected intraperitoneally with 1 ml/kg body weight carbon tetrachloride (diluted in olive oil) twice a week for 6 weeks to damage liver cells and establish a liver injury model. Beginning at week 7, the positive drug group and drug treatment groups were administered 100 mg/kg body weight reduced glutathione, curcumin or puerarin by gavage for 14 days. Beginning at week 9, blood and liver samples of all the animals were collected after death.

Body weight and liver index analyses

Body weight was recorded every weekend. The rats were sacrificed at the beginning of week 9, livers were exteriorized and weighed. The liver index (liver wet weight vs. body weight, mg/g) was calculated.

Plasma ALT/AST levels assay

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the plasma were analyzed via test kits (Nanjing Jiancheng Bioengineering Institute, China). The concentration of ALT/AST was determined by comparing the optical density values of the samples with the standard curve.

Histological examination

Each rat liver was processed to generate paraffin-embedded sections and then the sections were subjected to Masson's trichrome staining and evaluated under a light microscope. The percent area of collagen deposition in each section was calculated via Image-Pro Plus 6.0 software, the area of collagen was obtained by using software to accurately identify the color of collagen (blue area).

Liver tissue gene expression detection

Gene expression in liver tissue was detected via an Agilent rat microarray. RNA was extracted from liver tissue with the use of the mirVana RNA Isolation Kit (Applied Biosystem p/n AM1556) and the A260/A280 ratio of the RNA solution is a measure of RNA purity that typically ranges from 1.8 to 2.1. If the purity of total RNA is not high, it will affect the labeling efficiency of the probe and the results of chip hybridization, so the total RNA purification Kit (QIAGEN RNeasy Mini Kit) was used for purification.

After RNA extraction, quality inspection and purification, the liver tissues of each group were labeled, hybridized and scanned (using an Agilent Whole Genome Rat Microarray, 45018 total probes were included in the microarray). Key steps included: One-step synthesis of first and second strand cDNA, synthesis of Aautp-labeled cRNA, purification of cRNA, calculation of fluorescent molecule concentration and incorporation rate, Fluorescent labeling of cRNA samples, cRNA samples were fragmented and hybridized to microarrays (4×44K microarrays), washed and scanned in Agilent scanner at a resolution of 5μm. The scanner automatically scanned once with 100% and 10%PMT and the two results were automatically merged by Agilent software.

After the chip results were scanned with an Agilent scanner G2565BA, the feature extraction software from Agilent was used to read the data. The screening criteria for DEGs were as follows: log2 logarithmic transformation, T test $p< 0.05$ and threshold values of (-0.8, 0.8). The criteria of $p<0.05$ and 2-fold differential gene expression were used to determine significant DEGs.

Statistical analysis

The experimental results were analyzed via analysis of variance. The results are expressed as the mean ± standard deviation (SD). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Body weight and liver index

During the experiment, the weight of the rats in the control group increased normally, whereas the rats in the liver injury model groups exhibited weight impaired, which was alleviated after the mice were treated with the liver-protective drugs (fig. 2A). The liver index of the model group was higher than that of the control group, but the swelling of the liver decreased in the drug treatment groups (fig. 2B).

Plasma ALT/AST levels

Plasma ALT/AST levels are diagnostic indices of hepatocyte injury. As shown in fig. 3, ALT/AST levels in rats with CCl_4-induced liver injury were significantly increased and these levels were significantly reduced by drug treatment. The therapeutic effect of curcumin was stronger than that of the positive control drug, while the efficacy of puerarin was weaker.

Histological changes in the liver

Masson's trichrome staining revealed that the liver structure of the control group rats was normal and that there were few collagen fibers in the tissue.

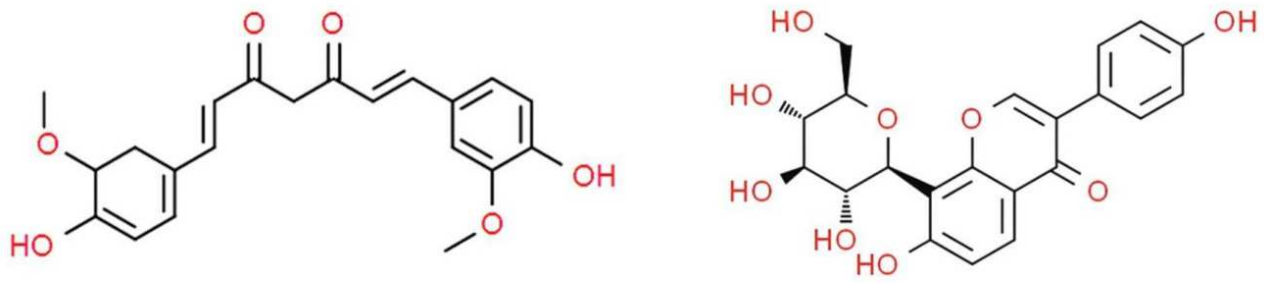

Fig. 1: Molecular structures of Curcumin and Puerarin.

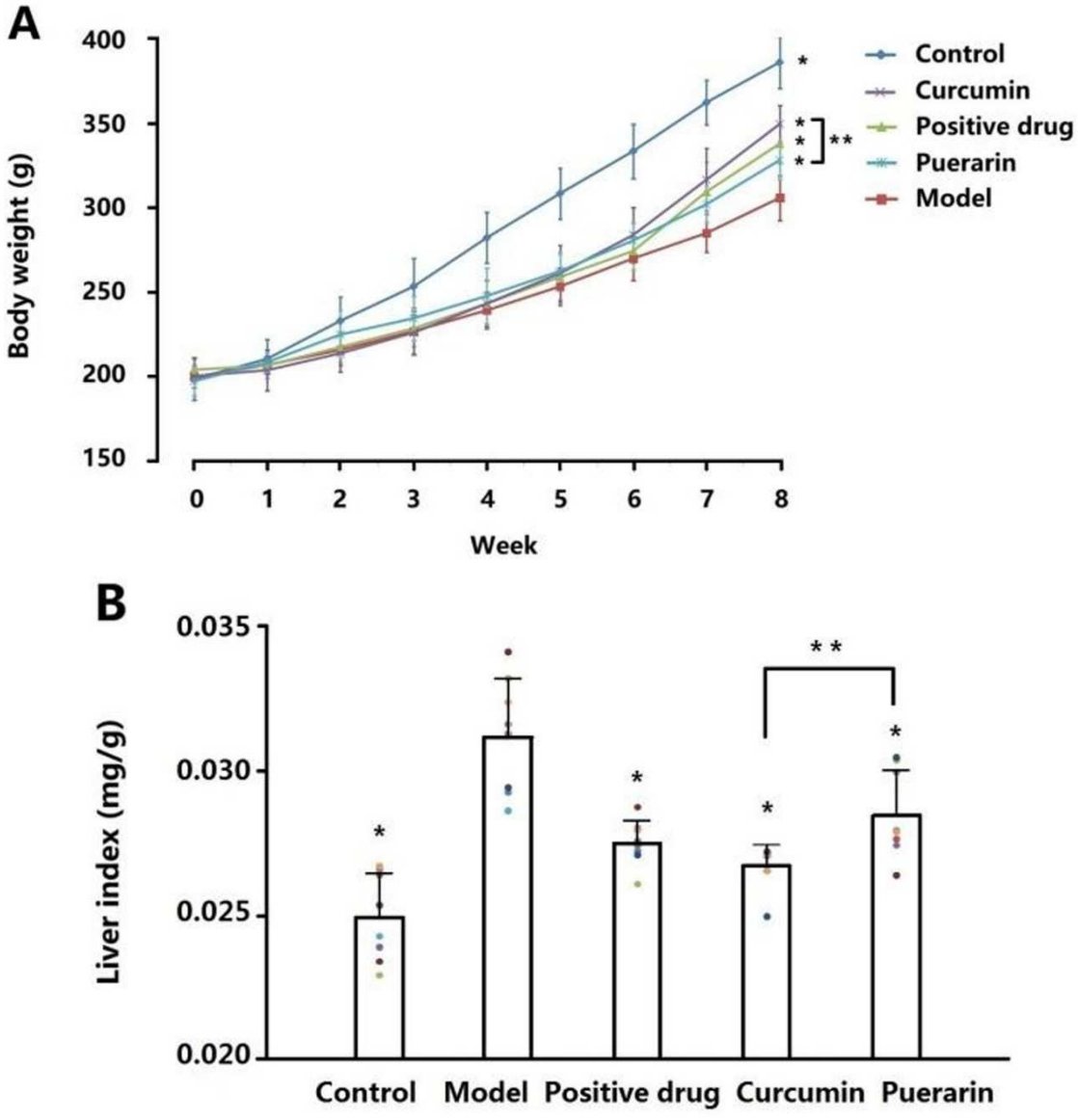

Fig. 2: Body weight (A) and liver index (B) of rats ($\overline{X}$±SD), the number of animals n=8, *p<0.05 compared with the control group, **p<0.05 compared with the Puerarin group.

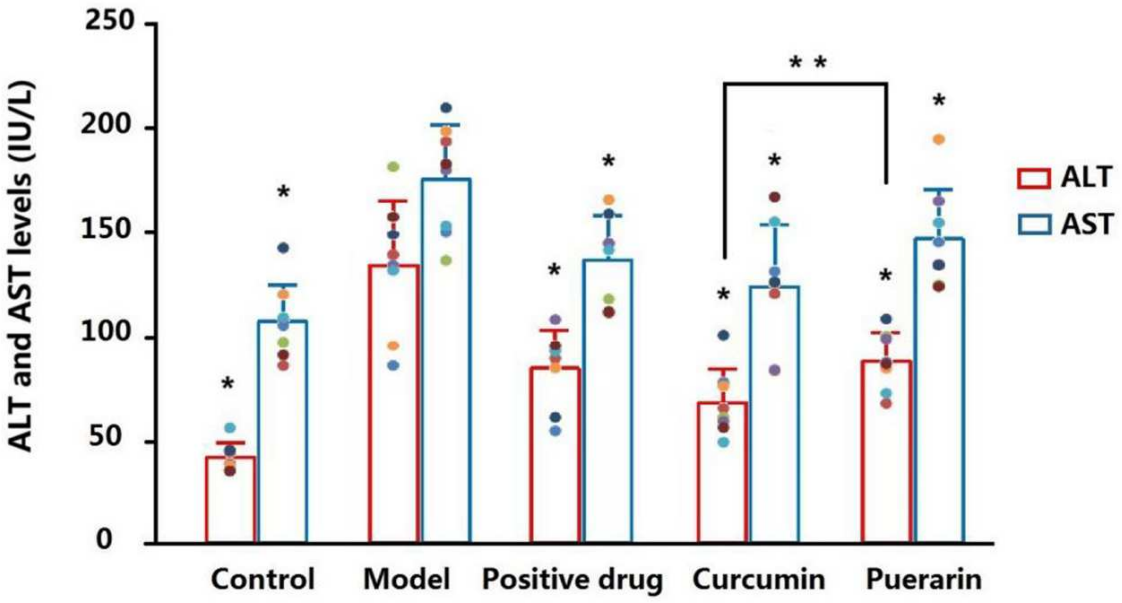

Fig. 3: Plasma ALT/AST levels of rats ($\bar{X}$±SD), the number of animals n=8, *p<0.05 compared with the control group, **p<0.05 compared with the Puerarin group.

Compared with those of the control group, the pathological sections of the liver injury group presented obvious necrosis of hepatocytes, which formed a large number of fibers (fig. 4A) and the percentage of collagen fibers was significantly greater in the liver injury group than in the control group. In the drug treatment groups, the positive control drugs curcumin and puerarin alleviated liver tissue injury, reduced the area of collagen fibers and promoted the recovery of tissue to a normal state. The therapeutic effect of curcumin was stronger than that of the positive control drug, while the efficacy of puerarin was weaker (fig. 4B). The area percentage of collagen fibers in the liver injury model group was 2.54±0.75%, while it decreased to 1.19±0.24% and 1.81±0.35% in the curcumin and puerarin treatment groups respectively.

Table 1: Numbers of differentially expressed genes (DEGs) between groups.

Groups	Number of DEGs
Model vs. Control	1358±135
Postive drug vs. Control	761±44*
Curcumin vs. Control	324±33* #
Puerarin vs. Control	1050±127*

Data were assumed as mean±SD, the number of animals n=8, *p<0.05 compared with the model group, #p<0.05 compared with the Puerarin group.

Results of the gene microarray test

The experimental results (table. 1) revealed that, compared with that in the normal control group, the gene expression of target organs in the model group was significantly different and positive drug treatment significantly reduced the number of DEGs. The tested drugs curcumin and puerarin also had obvious therapeutic effects, which reduced the number of DEGs in the liver. The efficacy evaluation results were curcumin > positive control drug > puerarin (fig.5). Pathological sections of liver tissue and ALT/AST levels from each group also supported the above conclusions.

DISCUSSION

To verify whether the efficacy of these natural products in treating liver injury can be compared at the gene expression level, we used carbon tetrachloride to establish a mouse model of liver injury and detected the number of DEGs in liver tissue between normal and damaged conditions. The results revealed that the liver function of the model rats with liver injury was significantly impaired and the number of DEGs between the model group and the control group was 1358±135. Compared with the control treatment, positive drug treatment significantly abrogated abnormal gene expression in the target organ liver and the number of DEGs was reduced to 761±44. Curcumin and puerarin also alleviated liver damage, reducing the number of DEGs to 324±33 and 1050±127, respectively. The above experimental results showed that, liver gene expression in the model group was significantly different compared with the normal control group and the positive control drug could significantly reduce the difference caused by cell injury. According to GO Biological Process analysis, the hepatoprotective functions of curcumin and puerarin were mainly reflected in DNA-dependent transcription regulation, transcription from RNA polymerase II promoter, cell proliferation and differentiation, etc. Molecular network analysis was mainly reflected in MAPK signaling pathway, cell cycle and pathways in cancer.

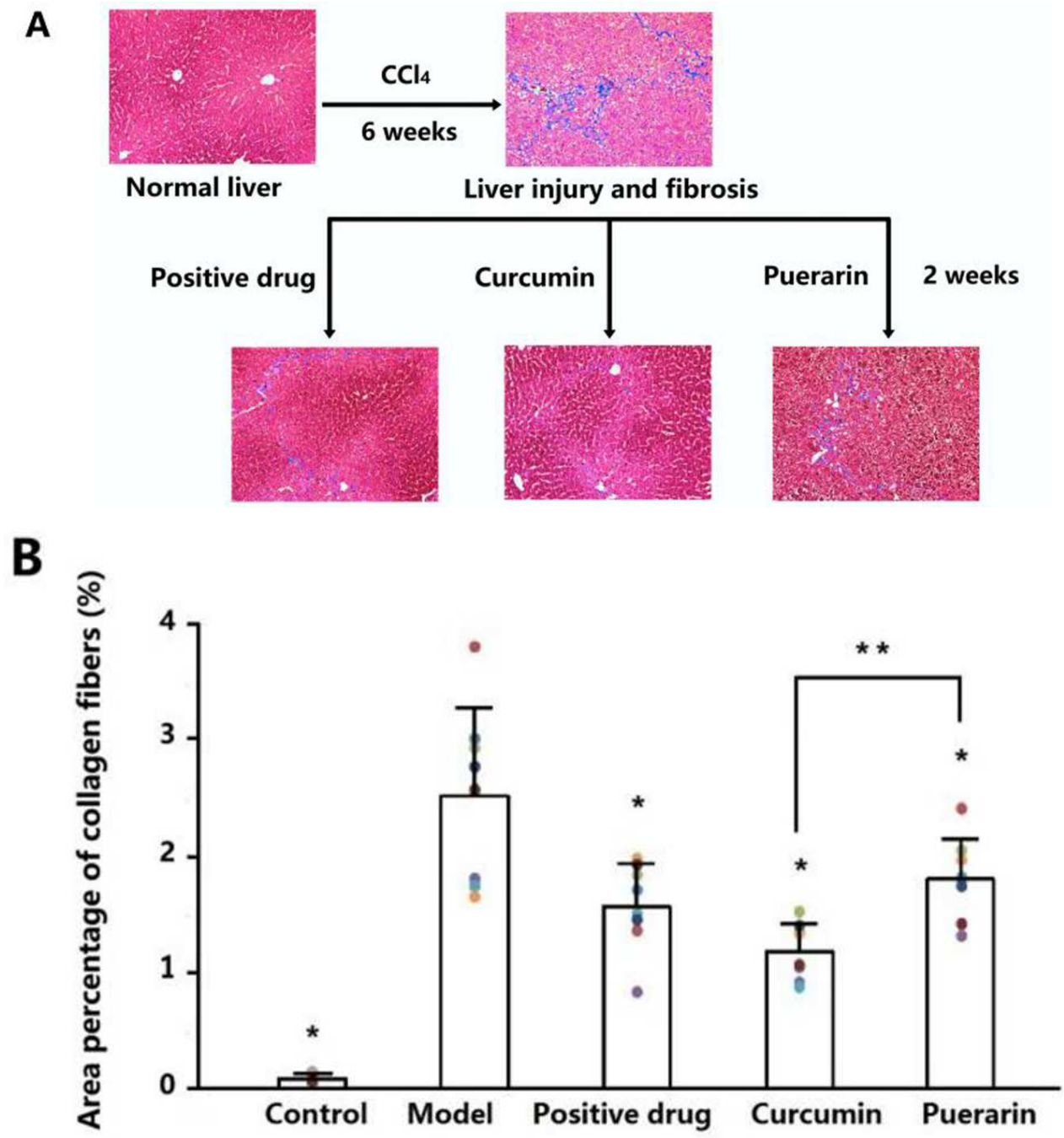

Fig. 4: Liver sections (A) and fibrotic areas (B) of the rats ($\overline{X}$±SD), the number of animals n=8, *p<0.05 compared with the control group, **p<0.05 compared with the Puerarin group.

Although the tested drugs curcumin (a polyphenol found from Turmeric and its mechanisms of alleviating liver injury include downregulating Smad pathways, blocking NF-κB proinflammatory cytokine production and decreasing the number of liver remodeling cells) and puerarin (a flavonoid derived from Pueraria lobata, which treatment decreases oxidative stress and inflammation response and contribute to prevent injury factors induced lipid metabolism disorder) had different mechanisms, they showed the same obvious therapeutic effects, which could reduce the number of DEGs in the liver. The efficacy evaluation results were curcumin > positive drug > puerarin, the histopathological staining results and the ALT/AST levels of each group also supported above conclusions.

Previous studies revealed differences between the pathological and normal states of target organs at the level of gene expression, as well as regional differences related to disease pathology (Zhao, 2023).

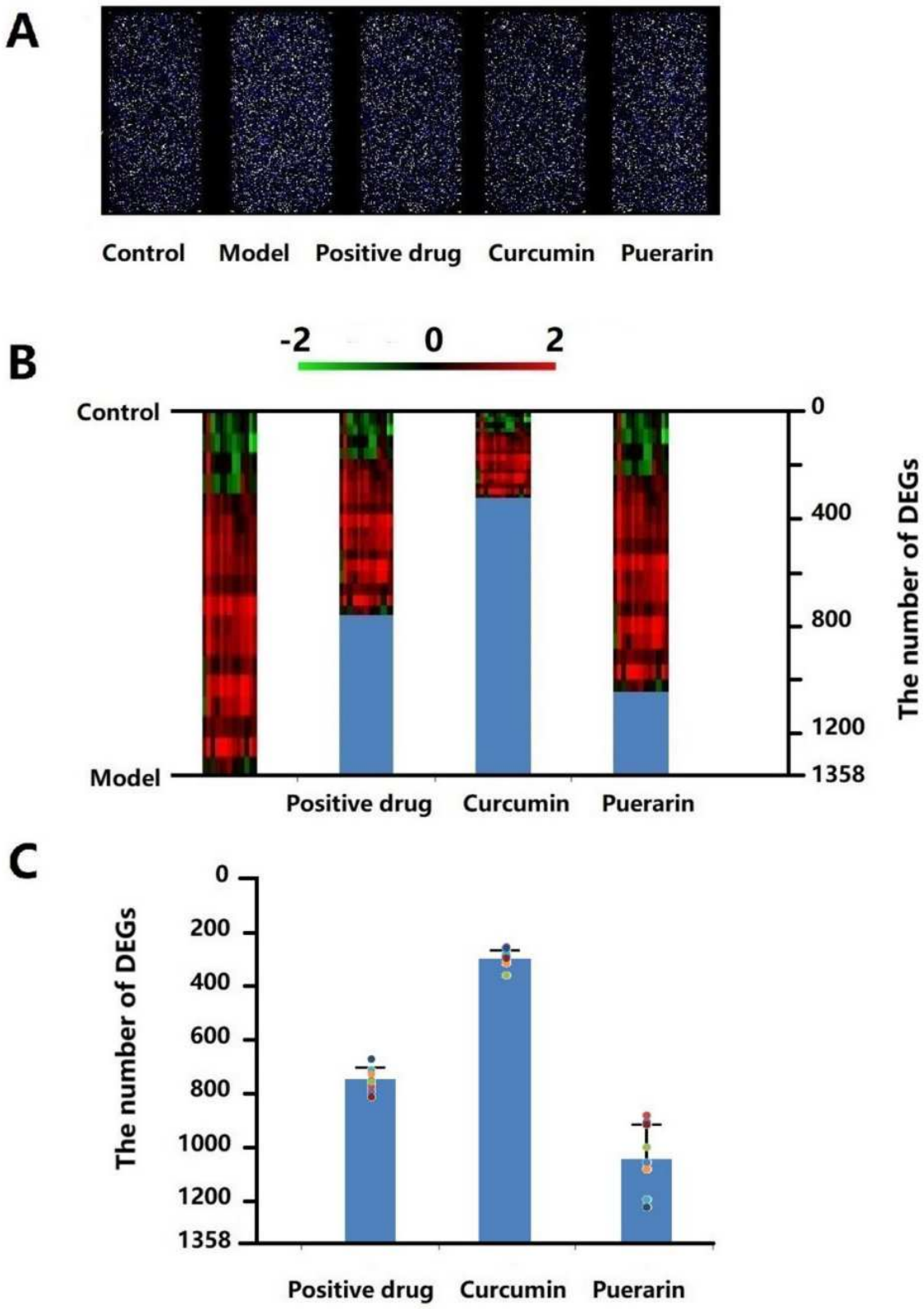

Fig. 5: The results of the gene microarray scan (A), number of differentially expressed genes(DEGs) (B) and efficacy evaluation of drugs (C) ($\overline{X}$ ±SD), the number of animals n=8. Analysis of variance showed significant difference between groups ($p<0.05$).

Some natural product active substances act on target organs after entering the body to produce indirect long-term conditioning effects, but the immediate direct effect may not be obvious and its long-term systemic efficacy may be more advantageous if it is evaluated at the genetic level (Newman *et al.*, 2020). Natural products, especially those derived from traditional herbs, can significantly regulate a wide range of biological activities and modulate human gene expression through multiple mechanisms. Many biological properties of natural products have been discovered, but how they achieve efficacy is unknown because the clear mechanisms by which natural products regulate gene expression have received little attention (Chen *et al.*, 2022), hindering further drug development.

For some drugs with complex active ingredients and targets, if the goal is to promote target organ recovery from a diseased state to a normal state (Bai *et al.*, 2024, Cheng *et al.*, 2023, Su *et al.*, 2019), then the degree of injury could be measured by the number of DEGs between the disease state and the healthy state of the target organ and the efficacy of the drug could be measured by its ability to reduce the degree of deviation, that is, reduce the number of DEGs.

Evaluating drug efficacy from the level of gene expression ignores the specific components, target sites or complex signal transduction network mechanism in the body and focuses on the target organ as a whole (Cao *et al.*, 2024, Yang *et al.*, 2020). This method uses the healthy state as a reference to comparatively study the comprehensive drug response of the target organ in the disease state and verify whether the drug achieves the effect of promoting recovery and treating diseases by reducing the number of DEGs. Therefore, it is suitable for the evaluation of the efficacy of natural products, especially drugs made from a mixture of multiple ingredients.

CONCLUSION

Curcumin and puerarin alleviated liver damage caused by carbon tetrachloride in SD rats and efficacy evaluation of drugs were curcumin > positive control drug > puerarin. The results of gene chip detection showed drug treatments significantly reduced the number of DEGs in the liver compared with model group. The efficacy of drugs were consistent with their ability to reduce the number of DEGs, demonstrating that the efficacy of different drugs can evaluated at the level of gene expression.

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Authors' contributions
Mosi Chen and Qi Li performed the experiments, participated in data collection, and drafted the manuscript. Genlin Chen, Li Dai and Libing Tian conducted statistical analysis, and contributed to the study design. Shuai Shao and Dehong Yu contributed to data collection and interpretation. Chenglan Su revised the manuscript. All authors critically reviewed and approved the final version of the manuscript.

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Data availability statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval
All animal experiments were approved by the Ethics Committee of Laboratory Animals of Tongji Medical College of Huazhong University of Science and Technology and the approval number is 20230306129. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest
All authors have read and approved the article and declare no conflict of interest.

Supplementary data
https://www.pjps.pk/uploads/2026/04/SUP1776929904.pdf

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