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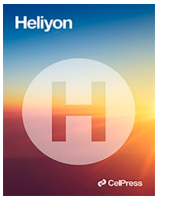

Identification of functional biomarkers of *Peganum harmala* and *Hypericum perforatum* using PCA-constructed secondary metabolite maps

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ABSTRACT

Peganum harmala L. (*P. harmala*), also known as Espand, Harmel, or Syrian rue, and *Hypericum perforatum* L. (*H. perforatum*), commonly known as St. John's wort, are two of the widely cultivated industrial crops and used worldwide in antihepatoma-related products. However, their main functional substances are still not clear, thus impeding the efficacy evaluations and quality controls of relative products around the world. In this work, the anti-hepatoma biomarkers of *P. harmala* and *H. perforatum* were clarified through the development of principal components analysis (PCA)-HPLC secondary metabolite mapping models. The chemical fingerprints of plant extracts were profiled by HPLC and then mapped to produce the secondary metabolite models using PCA. The models correlated the chemical information with the anti-hepatoma activities of plant extracts, thus indicating the functional inhibitors of *P. harmala* and *H. perforatum* against hepatoma cells. The activities of the identified compounds were validated by cytotoxic and apoptotic assays. The major inhibitors of *P. harmala* and *H. perforatum* against human hepatoma were determined to be harmine and quercetin, respectively. The IC_{50} values and the induced apoptotic rate of harmine on HepG2 cells were 20.7 ± 2.8 μM and 46.7 ± 3.5 %, respectively. The IC_{50} values and the induced apoptotic rate of quercetin on HepG2 cells were 49.5 ± 6.6 μM and 38.7 ± 2.6 %, respectively. In conclusion, the results significantly expanded the understanding of the biochemical foundations of *P. harmala* and *H. perforatum*, thus evidently supporting their current applications around the world. Moreover, harmine and quercetin could be used as biomarkers to evaluate the efficacy and quality of related products of industrial crops in therapeutic and health-improving applications.

1. Introduction

Empirical herbal applications using industrial crops are important supplementary or alternative remedies against various diseases in many regions of the world. Clearly, an indispensable step to evaluating such integrated applications and developing crop-based

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products is to clarify the functional biomarkers of the plant extracts or formulations that are relevant to their pharmacological applications. However, the complex chemical profiles of natural products and the corresponding large amounts of data produced in spectral analysis always preclude the efficiency and accuracy of the identification of certain compounds that contribute to functional effects. As a technique commonly used for multivariate data analysis, principal components analysis (PCA) has recently been applied to relate bioactivity to chemical ingredients in crude extracts of industrial crops [1,2]. Compared to conventional bioassay-guided isolation, the PCA-HPLC secondary metabolite mapping could significantly reduce the consumption of time, energy, and materials during the identification process, thus providing a competitive approach for fast, systematic screening of functional biomarkers in the products of industrial crops.

Peganum harmala L. (*P. harmala*) and *Hypericum perforatum* L. (*H. perforatum*) are two of the industrial crops widely used in the anti-hepatoma formula of traditional Chinese medicine [3,4]. Various parts of *P. harmala* have been traditionally used as herbal medicines to treat cancer, jaundice, colic, lumbago, coughs, rheumatism, hypertension, diabetes, and asthma around the world for centuries [5]. The anticancer mechanisms of *P. harmala* and its β-carboline alkaloids were composed of the complicated network regulation for the signaling associated with the autophagic and apoptotic death of cancer cells [6]. *H. perforatum*, commonly known as St. John's wort, presents comparable antidepressant activities to many prescribed drugs, such as fluoxetine, imipramine, paroxetine, or sertraline, thus being widely used in medicinal products for mild to moderate depression [7]. Besides, its anticancer effects have recently been validated in various *in vitro* and *in vivo* models. *H. perforatum* could enhance DNA repair from oxidative and alkylating damage [8], and induce apoptosis through prodeath autophagy [9].

Taken together, as two common herbal ingredients and important industrial crops, the functional biomarkers of *P. harmala* and *H. perforatum* for the traditional anti-hepatoma remedy have not been systematically identified yet, thus remaining an ongoing challenge. The objective of this study was to develop PCA-guided HPLC secondary metabolite mapping models to clarify the inhibitors of *P. harmala* and *H. perforatum* against human hepatoma cells. The results will reveal the phytochemical foundation of the anti-hepatoma applications of *P. harmala* and *H. perforatum*, and support the traditional uses, efficacy, and quality evaluation of the relative products in therapeutic and health-improving applications around the world. Moreover, the study will validate the feasibility of PCA maps as a competitive approach for functional biomarker identification of industrial crop products.

2. Material and methods

2.1. Sample preparation

The samples, identified as *Peganum harmala* L. and *Hypericum perforatum* L. by Professor Pu Liu (Henan Engineering Technology Research Center for Funiu Mountain Wild Medicinal Source), were purchased from Tongren Drug Store, Luoyang. 50 g of voucher specimen each were deposited in our lab (access numbers: JG2022-021 and JG2022-022). The herbal materials were completely dried before the experiments. The dry herbs were minced and then extracted with 25 %, 45 %, 70 %, or 100 % ethanol (10 g/100 mL) to produce residues for freeze-drying. Meanwhile, materials were also extracted with methanol (200 g/2000 mL) twice. The produced residues were dried and then extracted with dichloromethane, ethyl acetate, and butanol to produce extracts for the following tests. Harmine (≥99 % purity) and quercetin (≥99 % purity) were purchased from Solarbio (Beijing, China).

2.2. Cytotoxicity assay

Briefly, 200 μL human hepatoma HepG2 cells with concentration at 1.5×10^4 cells/mL were cultured at the 96-well plate. Four hours later, a series of concentrations of *P. harmala* and *H. perforatum* extracts, harmine, quercetin and cisplatin (positive control) were added in 10 μL aliquots. The 0.01 % DMSO and sole solvent, which did not show any cytotoxicity in this study, were used as the negative control samples. After 24 h treatment, the cytotoxicity was evaluated using MTT-medicated spectrophotography. A Multiskan Spectrophotometer was employed to measure the optical density (O.D.) at 590 nm.

$$\text{Cytotoxicity } (\%) = ((\text{control O.D.} - \text{sample O.D.}) / \text{control O.D}) \times 100\%$$

The IC_{50} values of inhibitory effects of sample extracts were calculated as reported previously [2].

2.3. HPLC analysis

HPLC chromatograms were produced using a Waters HPLC coupled with a Thermo C18 column and the 2489 UV/Vis detector. For *P. harmala* extracts and harmine, the initial conditions of gradient elution were 10 % of eluent B (methanol) with a linear gradient of 50 % from 0 to 10 min, 70 % from 10 to 20 min, 80 % from 20 to 30 min, 85 % from 30 to 40 min, and 95 % from 40 to 45 min. This proportion maintained for 3 min and returned to the initial condition at 53 min 0.1 % phosphoric acid was used as eluent A. The UV detector was set at 270 nm. For *H. perforatum* extracts and quercetin, the HPLC conditions were selected as follows: equivalence elution with the ratio of 0.1 % phosphoric acid (eluent A) and acetonitrile (eluent B) at 84 : 16, and running time at 60 min. The UV detector was set at 360 nm. For both of herbs, the extracts samples were 5 mg/mL. The calibration curves of harmine and quercetin were obtained by preparing stock solutions (2000 μg/mL) in methanol. Subsequent dilutions were made to give 1000, 100, 10, 1, and 0.1 μg/mL. The linearity of the calibration curves was determined ($R^2 > 0.996$). The flow rate was 1 mL/min. Column temperature was 25 °C. The triplicated injections were made for each samples and the injection volume was 20 μL.

2.4. Multivariate data analysis

SIMCA-P 16.0 MVA software (Umetrics, Sweden) was used to perform the principal component analysis. HPLC chromatograms of *P. harmala* extracts at 270 nm comprised 3182 discrete regions acquired by data acquisition every second from 0.00 to 53.00 min. HPLC chromatograms of *H. perforatum* extracts at 360 nm comprised 3600 discrete regions acquired by data acquisition every second from 0.00 to 60.00 min. The resulting data was formatted and then processed by SIMCA-P 16.0. Six principal components were employed for PCA analysis to produce score and loading plots.

2.5. Apoptosis assay

The HepG2 cell suspension (1.5×10^4 cells/mL, 800 μL/well) were loaded into the 24-well plate with cover slides in the bottom. Four hours later, quercetin and harmine were added at a concentration of 50 μM. 0.01 % DMSO and camptothecin were used as the negative and positive controls, respectively. After 12 h of treatment, the apoptosis was then detected using an Annexin-V-FLUOS staining kit (Roche Applied Science, Germany).

2.6. Statistical analysis

The data were shown as mean ± standard deviation. Statistical comparison among treatments was carried out using one-way

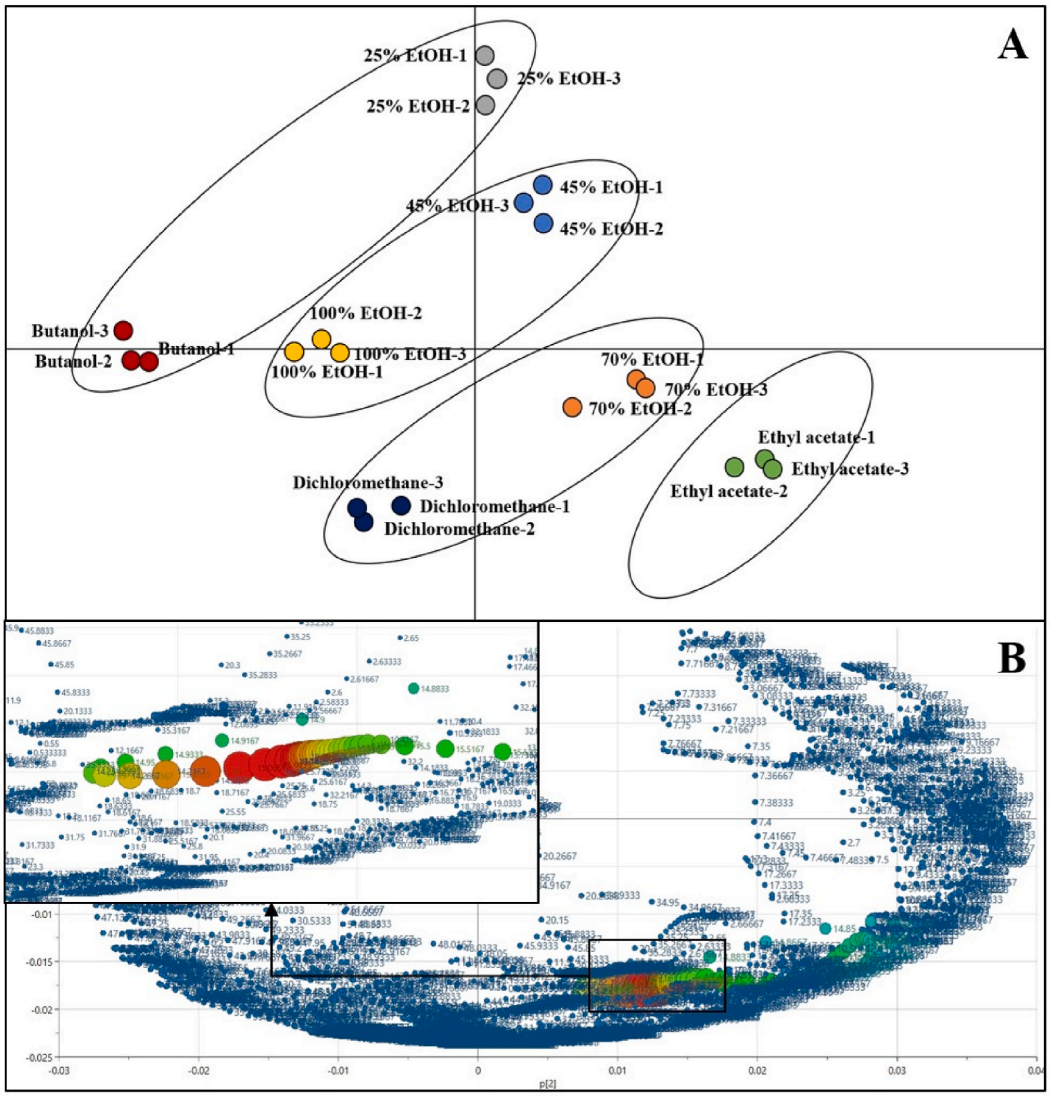

Fig. 1. PCA scores plot (A) and loading plot (B) for *P. harmala* extracts.

analysis of variance. The statistical significances between control and sample groups were calculated by the Student's t-test. Data were taken as significant where $p < 0.05$.

3. Results and discussion

The chromatographic analysis of industrial crop extracts from different solvents often produces similar chemical profiles and large amounts of data, thus impeding visual and manual identification to target certain compounds that have particular biological effects (Figs S1 and S2). In this work, the non-parametric HPLC-PCA model was developed to correlate the secondary metabolites of industrial crops with the growth inhibitory activity of crude extracts on hepatoma cells, which successfully mapped the different bioactivity groups and then determined the main grouping contributors (functional biomarkers).

3.1. PCA-HPLC secondary metabolite mapping of P. harmala

As shown in Fig. 1A, the score plot of PCA presented the secondary metabolite profiles of *P. harmala* extracts in 3D space. Generally, samples with a similar chemical profile were grouped closely, while distinct samples were dispersed. Based on the supervised diversity, a total of 21 samples could be discriminated into four groups (Fig. 1A), which was consistent with the anti-hepatoma effects of each sample (Table 1). Extracts with IC_{50} values over 396 μg/mL (25 % ethanol and butanol extracts) showed relatively weak inhibitory effects. 45 % and 100 % ethanol extracts displayed relatively low-intermediate inhibition ($211 < IC_{50} < 264$ μg/mL), and 70 % ethanol and dichloromethane extracts demonstrated relatively high-intermediate inhibition ($136 < IC_{50} < 176$ μg/mL). The relatively strong inhibition ($IC_{50} < 96$ μg/mL) was exhibited by the ether acetate extract. As the variance produced in this PCA was mainly due to the first six principal components (97.2 %), the contributing variables (signals of HPLC chromatograph) were thus determined using the PCA loading plot (Fig. 1B). The peak (large red spots) with a retention time of 15.122 min (harmine) contributed most to the location of the most potent extracts for inhibition of cancer cells. By comparing the HPLC data of samples with that of the commercial standards, the 15.122 min peak was identified as harmine (Figs S1 and S3). As shown in Table 1, the concentration of harmine in the seven *P. harmala* extracts were determined using the standard calibration curve. In general, the stronger inhibitory activity of extracts was correlated with the higher concentration of harmine. Therefore, the variance of the anti-hepatoma effects of *P. harmala* extracts could be proposed to mainly depend on the concentration of harmine. However, as the magnitude of inhibitory effects was not 100 % coherent for harmine concentrations, this could not fully explain the differential inhibitory activity of different employed solvent extracts. The results of Table 1 suggested that other compounds additionally contributed to the antihepatoma effects of *P. harmala* extracts, which was consistent to the existence of many signals (orange, yellow and green spots) around harmine spots in the contribution plots (Fig. 1B).

3.2. PCA-HPLC secondary metabolite mapping of H. perforatum

Regarding the *H. perforatum* extracts, 21 samples were manually grouped into three clusters by the ellipses illustrated in Fig. 2A. This grouping, resulting from the chemical composition, is consistent with that grouped using the cytotoxic effects of *H. perforatum* extracts against hepatoma cells *in vitro* (Table 2). The 25 and 45 % ethanol extracts with IC_{50} values over 365 μg/mL were considered as relatively weak inhibitors. Those of 70 and 100 % ethonal, ethyl acetate, and butanol, with IC_{50} values between 221 and 286 μg/mL, showed relatively intermediate inhibition. The crude extract of dichloromethane, with IC_{50} at 166.7 μg/mL, has the strongest cytotoxicity. This map demonstrated the importance of the link between the chemical composition and the anti-hepatoma activity of *H. perforatum*. In addition, the first six principal components determined 98.3 % of the variance of this PCA map. The loading plot was also employed to analyze the contributing variables. As revealed in Fig. 2B, variables (large red spots), indicating the corelated HPLC peak with retention times of 35.35 min, contributed most to the location of the most potent extracts for inhibition of cancer cells. By comparing with the commercial standards, the 35.35 min peak was identified as quercetin (Figs S2 and S4). Moreover, the concentrations of quercetin in the extracts were positively correlated with the inhibitory activity of the extracts (Table 2), further indicating its main functional role in the anti-hepatoma effects of *H. perforatum*. Similarly, the magnitude of inhibitory effects was non-coherent for quercetin concentrations in ethyl acetate and dichloromethane extract solvent systems (Table 2). It could also be explained by the activity contribution of the minor functional compounds indicated by orange, yellow and green spots in the contribution plot (Fig. 2B).

Table 1
The IC_{50} values of inhibitory effects of *P. harmala* extracts on human hepatoma cells HepG2 and the harmine concentration.

Extracts	IC_{50} (μg/mL)	Harmine concentration (μg/mL)	Effects
25 % ethanol	465.3 ± 21.5	50.8 ± 0.1	Relative weak
Butanol	396.3 ± 10.6	21.7 ± 0.0	
45 % ethanol	264.7 ± 23.3	72.8 ± 0.1	Relative low-intermediate
100 % ethanol	211.3 ± 15.3	14.8 ± 0.2	
70 % ethanol	176.0 ± 6.0	112.1 ± 3.0	Relative high-intermediate
Dichloromethane	136.3 ± 18.6	1163.7 ± 4.4	
Ethyl acetate	96.0 ± 14.9	1434.2 ± 0.9	Relative strong

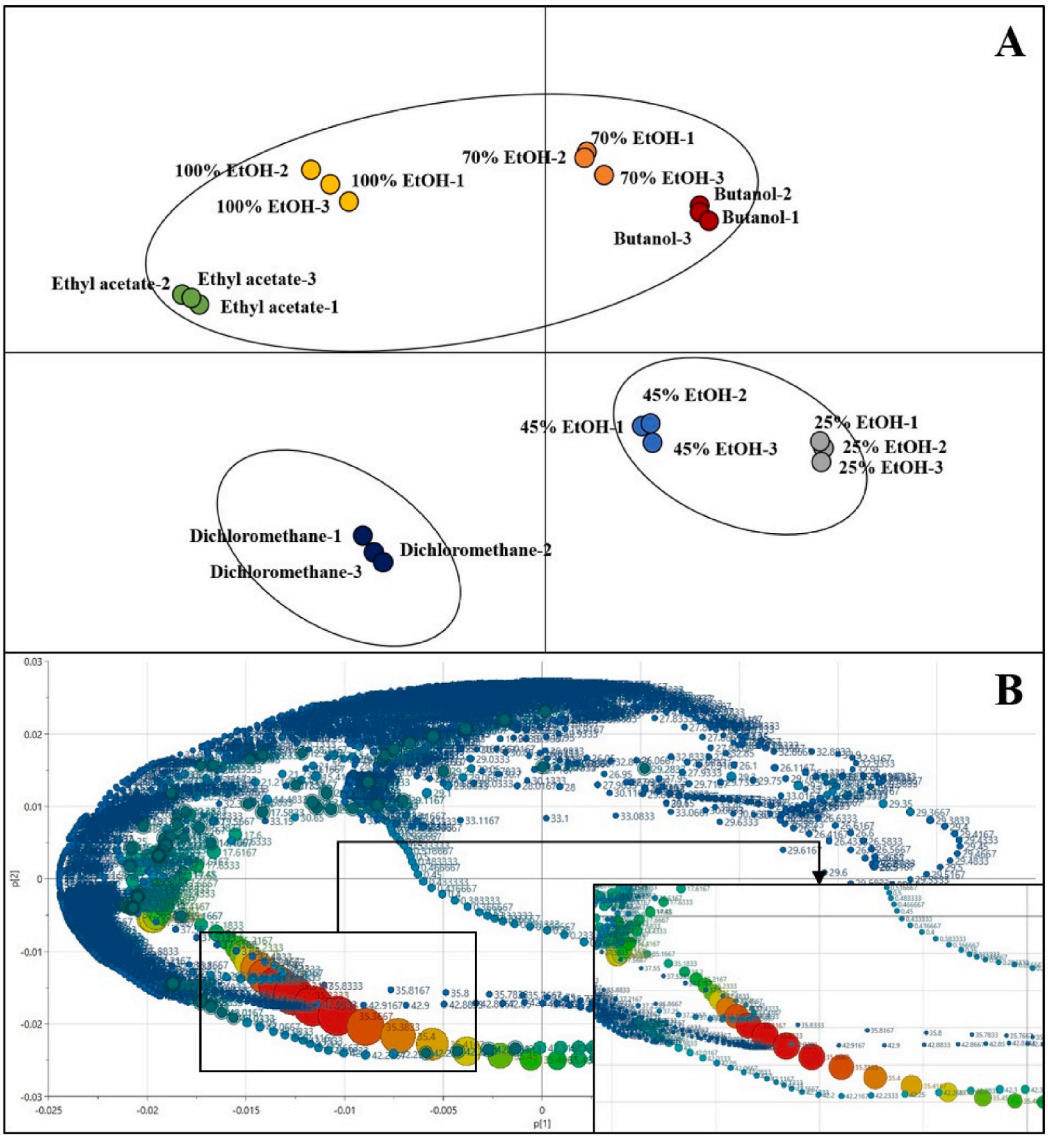

Fig. 2. PCA scores plot (A) and loading plot (B) for *H. perforatum* extracts.

Table 2
The IC_{50} values of inhibitory effects of *H. perforatum* extracts on human hepatoma cells HepG2 and the quercetin concentration.

Extracts	IC_{50} (μg/mL)	Quercetin concentration (μg/mL)	Inhibitory effects
25 % ethanol	365.3 ± 26.7	1.58 ± 0.0	Relative weak
45 % ethanol	451.2 ± 27.0	1.66 ± 0.1	
Butanol	286.0 ± 18.7	3.66 ± 0.0	Relative intermediate
70 % ethanol	265.0 ± 20.5	3.67 ± 0.1	
100 % ethanol	243.8 ± 10.7	15.14 ± 0.1	
Ethyl acetate	221.6 ± 15.3	29.48 ± 1.3	
Dichloromethane	166.6 ± 18.3	18.5 ± 0.8	Relative strong

3.3. Validation of herbal inhibitors of human hepatoma cells

The grouping contributors, harmine and quercetin, extracted from complex multivariate chemical datasets were predicted to be the main functional biomarkers contributing to the anti-hepatoma activities of *P. harmala* and *H. perforatum*, respectively. To further validate the prediction above, harmine and quercetin were evaluated for growth inhibition in HepG2 cells. As shown in Fig. 3A, both

compounds exhibited dose-dependent cytotoxicity against these hepatoma cells *in vitro*. The IC_{50} values of harmine and quercetin were found to be 20.7 ± 2.8 μM and 49.5 ± 6.6 μM, whereas those of cisplatin (the positive control) were 23.5 ± 6.2 μM. Moreover, a proportion of HepG2 cells exposed to harmine and quercetin treatment showed an early apoptosis phenotype (Fig. 3B&C) with the apoptotic rate at 46.7 ± 3.5 % and 38.7 ± 2.6 %, respectively.

The effect of harmine against hepatoma cells reported in this work is superior to those reported for many other types of cancer cells, such as cervical cancer cells [10], colon cancer [11], gastric cancer [12], and lung cancer [13]. Harmine has been found to induce the autophagic death of cancer cells through the enhanced phosphorylation of adenosine monophosphate-activated protein kinase. Moreover, it reduced the expression of both p-Akt/Akt and p-mTOR/mTOR to induce the apoptotic and autophagic death of cancer cells [11,12]. Additionally, its effects on the malignant cell also involved rescued cell-cell adhesion, loss of anchorage-independent growth, reorganization of the actin cytoskeleton, and inhibition of cell motility [14]. Meanwhile, harmine demonstrated anti-invasive and anti-metastatic effects by regulating RECK signaling and pro-metastatic factors such as AKT, ERK, MMP-9, and VEGFs [11]. In addition to the effects reported above, this work first indicated the anticancer action of harmine against hepatoma cells, thus confirming its main biomarker role for the anticancer activities of *P. harmala*. However, it was noteworthy that, harmine, with its higher lipophilicity and a larger volume of distribution, could distribute to the peripheral compartment of animal models *in vivo* [15]. It was then metabolized and further converted to other hydroxylated products, which might lead to central neurological symptoms, including systemic muscle tremor, convulsion and even opisthotonos, and acute cardiovascular toxicity, including increases of the

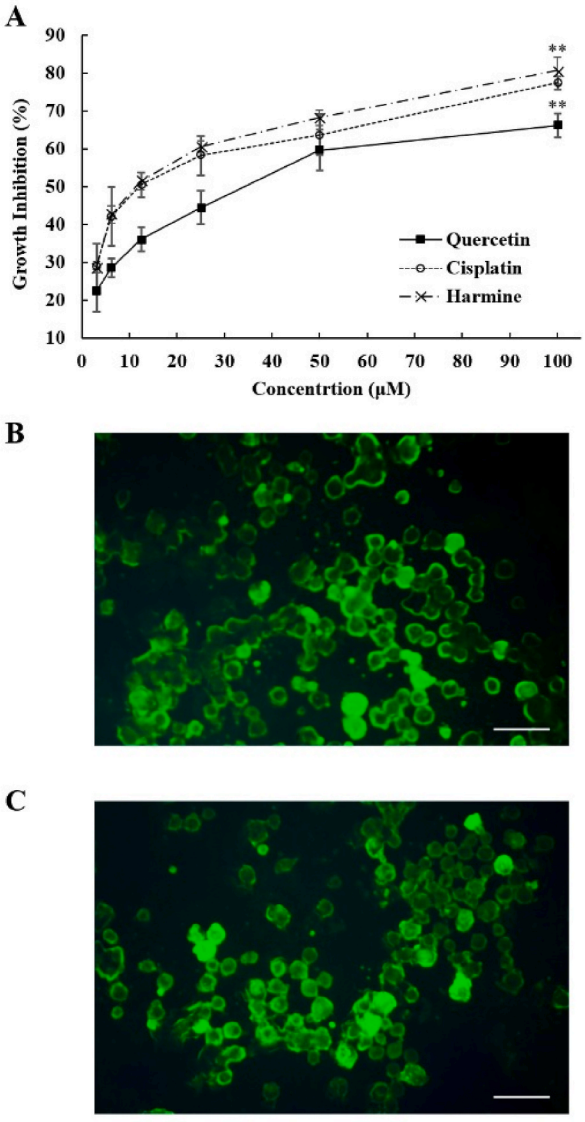

Fig. 3. The growth inhibition (A) and apoptosis (B&C) induced by harmine and quercetin on human hepatoma cells HepG2 *in vitro*. **$p < 0.05$. Scale bar, 50 μm.

myocardial enzymes lactate dehydrogenase, creatine kinase, and creatine kinase-MB, decreases in the blood pressure and heart rate [15,16]. For the mice models, the half lethal dose of harmine was 26.9 mg/kg [16].

Previous evidence-based research has reported that quercetin has anticancer actions on malignant cells of colon cancer, breast cancer, ovarian cancer, and lymphoma to induce cell cycle arrest, apoptosis and autophagy, tumor angiogenesis, and metastasis inhibition [17]. These anticancer actions are exerted mainly through the modulation of the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin, Wnt/β-catenin, and mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 pathways [18]. Moreover, the *in vivo* animal experiments indicated quercetin had no genetic damage, kidney or liver toxicity [19]. The clinical trials have shown no toxicity or side effects of quercetin-containing supplements on the population, thus demonstrating the safety of quercetin application [20]. Meanwhile, the International Cancer Institute confirmed that quercetin was not a carcinogen in 1999 and FDA demonstrated that quercetin was safe to use under expected conditions in 2010 [21]. The result of this work, together with the previous literature reports, indicated the critical role of quercetin as the functional biomarker for *H. perforatum*-based remedies.

4. Conclusions

Using the PCA-HPLC secondary metabolites mapping approach, harmine and quercetin were determined to be the major anti-hepatoma constituent of *P. harmala* and *H. perforatum*, respectively. This study verified the effectiveness of PCA-HPLC secondary metabolite mapping as a tool quickly identify the biomarkers in the complicated chemical composition of crude extracts of industrial crops. Moreover, the results significantly expanded the understanding of the biochemical foundations of *P. harmala* and *H. perforatum*, and evidentially supported the current supplementary applications. Most importantly, harmine and quercetin could be used as the major biomarkers to evaluate the efficacy and quality of the related therapeutic formulations and healthy-improving products around the world.

Data availability statement

The data associated with our study have not been deposited in a publicly available repository. Data will be made available on request.

CRediT authorship contribution statement

Jiayu Gao: Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Xinyi Yang:** Visualization, Validation, Investigation, Formal analysis, Data curation. **Ying Liang:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Formal analysis. **Dongyi Hu:** Software, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23565.

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