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Short Communication
Identification of Anticancer Compounds in Gallnuts Through PCA-constructed Secondary Metabolite Map

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Abstract

Background and Objective: Gallnuts have been traditionally used as a source of drugs for the treatment of cancerous diseases in folk medicinal system around world. To reveal the anticancer phytochemical foundation of Gallnuts, the secondary metabolite maps were constructed to correlate the chemistry with the cytotoxicity of crude extracts on human cancer cells. **Materials and Methods:** The growth inhibition activities were determined by MTT assay of 8 solvent extracts of gallnuts Galla Chinensis and Galla Turcica in the human liver cancer cell line 7721. The activities were then mapped onto the secondary metabolite profile of crude extracts by principal components analysis (PCA) of HPLC-UV data. The top five PCA components of the map discriminated extract activity mainly based on the differential content of five compounds, which two of them were then tested individually using MMT assay on cancer cells *in vitro*. **Results:** PCA-constructed secondary metabolite mapping quickly identified functional compounds from crude gallnuts extracts. Galla Chinensis and Galla Turcica constituents gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose inhibited the growth of human liver cancer cell line growth with activities (IC_{50}: 36.44±8.75 and 55.59±14.96 μg mL^{-1}, respectively) comparable to that of cisplatin (IC_{50}: 35.12±1.74 μg mL^{-1}). **Conclusion:** Gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose are identified as the anticancer constituents of Gallnuts. It thus strongly underpins a fundamental understanding of anticancer activities of gallnuts medicine and supports their ongoing clinical uses in China. Moreover, this work may confirm the potential of HPLC-PCA analysis as a tool for quick identification of functional compounds of plant extracts.

Key words: Gallnut, secondary metabolite, anticancer, principal components analysis, gallic acid

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gallnuts, as the pathological excrescences of the insect attack and deposition of the eggs on the young branches or twigs of plants are a group of very special natural products characterized of the plant-insect symbiont[1]. Galla Chinensis and Galla Turcica, two typical gallnuts most frequently recorded in western and eastern cultures are used as the traditional medicines for various body disorders, such as painful hemorrhoids, inflammation, diarrhea, dysentery, toothache, dental caries and cancers[2-3]. Galla Chinensis, mainly distributed in areas of south China, Sumatra and Malaysia, is formed on the family Anacardiaceae leaves or petioles parasitized by the Chinese sumac aphid Baker (mainly *Melaphis chinensis* Bell)[4]. Galla Turcica, native to and widely distributed in the Mediterranean coast countries, is formed on the young branches or twigs of *Quercus infectoria* Olivier parasitized by the gall wasps, *Cynips gallae-tinctoriae* Olivier[5]. The medicine Galla Chinensis and Galla Turcica are both the dry and clean gallnuts after removal of the larvae.

As claimed in TCM encyclopedia, gallnuts could be used as an important constituent in many prescriptions for the treatment of cancer-related symptoms[6]. Though their practices are based mainly on traditional beliefs handed down from generation to generation, the scientific evidences have recently been reported in literature. Tong *et al.*[7] reported that the ether fraction of Galla Chinensis presented a dose-dependent inhibition to the human glioblastoma cell[7] U251 with IC_{50} at 12.76 μg mL^{-1}. The ethyl acetate extracts of Galla Chinensis exhibited the significant inhibitory activities to the epidermal growth factor receptor, which was a validated target for human malignancies, with IC_{50} values[8] at 4.34 μg mL^{-1}. However, the foundation of chemical materials underlying the anticancer activities of gallnuts was barely reported. To the best of our knowledge, 12 chemical components have been identified from gallnuts to the date including gallic acid, syringic acid, ellagic acid, methyl gallate, β-sitosterol, 1,2,3,4,6-penta-O-galloyl-β-D-glucose, amentoflavone, purpurogallin, 1,2,3,4,6-tetra-O-galloyl-β-D-glucose, hexamethyl ether, isocryptomerin and methyl betulate[6]. Among them, gallic acid, ellagic acid, methyl gallate, β-sitosterol, 1,2,3,4,6-penta-O-galloyl-β-D-glucose, amentoflavone and purpurogallin have been reported to present anticancer activities elsewhere[9-15]. Nevertheless, besides the primary and secondary metabolic processes of natural products, the complex processes of gallnuts formation could generate abundance of bioactive metabolites, which includes components derived from plants, insects and mutual stimulation. A systematic investigation into the anticancer phytochemical foundation of the plant-insect symbiont gallnuts still remains an ongoing challenge. Therefore a multivariate data analysis (MVA)-guided method was employed in this study to identify the functional components of gallnuts crude extract for the anticancer activities determined *in vitro*.

The use of MVA to relate pharmacological activity to chemical constituents in crude extracts of natural products by metabolic profiling, or mapping has been developed and reported in recent years[16,17]. Compared to traditional bioassay-guided fractionation, MVA-guided method is characterized as minimum consuming on time, raw materials and solvents, thus providing an advanced technique for identification of bioactive agents of complex medicinal products.

To underpin a fundamental understanding of anticancer activities of gallnuts medicine and support their ongoing clinical uses in China, this study constructed HPLC secondary metabolite maps to clarify the anticancer phytochemical foundation of Galla Chinensis and Galla Turcica on human liver cancer cell line.

MATERIALS AND METHODS

The work was performed during March, 2018 to December, 2018 in natural products research laboratory, School of Chemical Engineering and Pharmaceutics, Henan University of Science and Technology.

Preparation of extract and standards: Galla Chinensis and Galla Turcica were purchased from Hao Yi Sheng Chinese herbal medicine store, Jinghua Street, Luoyang. Their identity kindly confirmed as Galla Chinensis and Galla Turcica by Associate Professor Xinsheng Wang at School of Chemical Engineering and Pharmaceutics, Henan University of Science and Technology. The voucher specimens have been deposited in natural products research laboratory, School of Chemical Engineering and Pharmaceutics (access number: JGG2018-033/034).

Galla Chinensis and Galla Turcica were minced. Portions of minced materials (10 g) were each minced with 100 mL of different solvents: 25, 70 or 100% EtOH and extracted by shaking for 24 h at room temperature. The solvents were completely removed at 35 under reduced pressure and the residue was freeze-dried overnight. Minced materials (200 g) was also extracted twice with 500 mL methanol and agitated at room temperature for 24 h. The extracts were filtered, solvent was completely removed at 35 under reduced pressure and the residue was lyophilized overnight. The dry

residues were then redissolved in 100 mL 10% methanol and sequentially extracted twice with 200 mL of hexane, dichloromethane, ethyl acetate and butanol. The remaining water phase was retained. Gallic acid (99% purity) and 1, 2, 3, 4, 6-O-pentagalloyl glucose (99% purity) were purchased from Solarbio (Beijing, China). The standards were dissolved in either 100% MeOH or a 0.01% dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) in PBS solution.

Cancer cell culture: The human hepatoma cell line 7721 was kindly provided by School of Medicine, Henan University of Science and Technology. Cells were grown in DMEM medium (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum, 100 g mL^{-1} penicillin and 100 g mL^{-1} streptomycin in a 5% carbon dioxide humidified incubator at 37 . Experiments were performed when cells were approximately 80% confluent.

Growth inhibition as measured by MTT assay: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Solarbio, Beijing, China) assay was performed as previously reported[17]. Briefly, 200 µL cell suspensions were seeded at a concentration of 1.5×10^4 cells mL^{-1} in a 96-well plate. After overnight incubation, Galla Chinensis and Galla Turcica extracts (50 µg mL^{-1}), Gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose (100-3.125 µg mL^{-1}) were added in 10 µL aliquots. The negative control was 10 µL of 0.01% DMSO in PBS' solution. Each concentration was tested three times in the same plate. Plates were run at least in triplicate. After 72 h incubation, 20 µL MTT solution was added and incubated at 37 for 4 h. The optical density (OD) was measured at 590 nm using a Multiskan spectrophotometer. The growth inhibition was determined using:

$$\text{Growth inhibition (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}}\times 100$$

HPLC instrumentation: The chemical profiles of samples and standards were performed using a Waters series HPLC system comprising a Waters e2695 Separations Module with degasser, a variable wavelength 2489 UV/Vis detector and an autosampler. The UV detector was set at 210 and 270 nm and data was acquired using Empower 3 software. The column used was a Thermo C18, 5 µm (250×4.6 mm) maintained at ambient room temperature. Gradient elution of the samples and standard were performed using 0.01% formic acid (eluent A) and methanol (eluent B). The separation condition of HPLC was developed originally in this study. For Galla Chinensis, the gradient elution initial conditions were 10% of eluent B with linear gradient to 45% from 1-13 min, to 54% from 13-25 min, to 90% from 25-27 min, this proportion being maintained for 2 min. The column was then returned to the initial condition at 30 min. For Galla Turcica extracts, the gradient elution initial conditions were 10% of eluent B with linear gradient to 37% from 1-10 min, to 51% from 13-25 min, to 90% from 25-27 min, this proportion being maintained for 2 min. The column was then returned to the initial condition at 30 min. The flow rate was 1 mL min^{-1}. Dry residues of Galla Chinensis and Galla Turcica extracts were dissolved in methanol at 5 mg mL^{-1}. The sample injection volume was 10 µL: three injections were performed for each sample and standard.

Multivariate data analysis (MVA): Principal components analysis (PCA) was adopted and performed using SIMCA-P 14.1 MVA software (Umetrics, Sweden) as reported before[17]. HPLC chromatograms of both of Galla Chinensis and Galla Turcica extracts at 270 nm comprised 1801 discrete regions by data acquisition every second from 0.00-30.00 min. The resulting data were formatted as an ARW file and exported into Microsoft® Excel 2010. The total 1801 integral regions were retained for further analysis. The resulting data were exported into SIMCA-P 14.1 for analysis. The processing method for PCA was mean-centring without scaling and rotation. Five principal components were chosen for PCA analysis.

Statistical analysis: The data were shown as mean±standard deviation (SD). Statistical comparison among treatments was carried out using one-way analysis of variance (ANOVA). The statistical significances between control and sample groups were calculated by the Student's t-test. Data were taken as significant where $p<0.05$.

RESULTS

Growth inhibitory effects of Galla Chinensis and Galla Turcica extracts for human cancer cells *in vitro*: The cytotoxic effects of different gallnuts crude extracts including both of Galla Chinensis and Galla Turcica were determined on human hepatoma 7721 cancer cell line *in vitro* using MTT assay. As shown in Fig. 1, all of the gallnut crude extracts showed the cytotoxicity at the dose of 50 µg mL^{-1} and could prevent over 50% proliferation of 7721 cancer cells *in vitro*. For Galla Chinensis (Fig. 1a), 25% extract showed highest effects and water extract showed lowest effects. Similarly, the extracts of Galla Turcica also presented strong cytotoxicity to 7721 cancer cells *in vitro*. However, the most effective extract was 100% ethanol (Fig. 1b) and the least cytotoxic extract was water fraction.

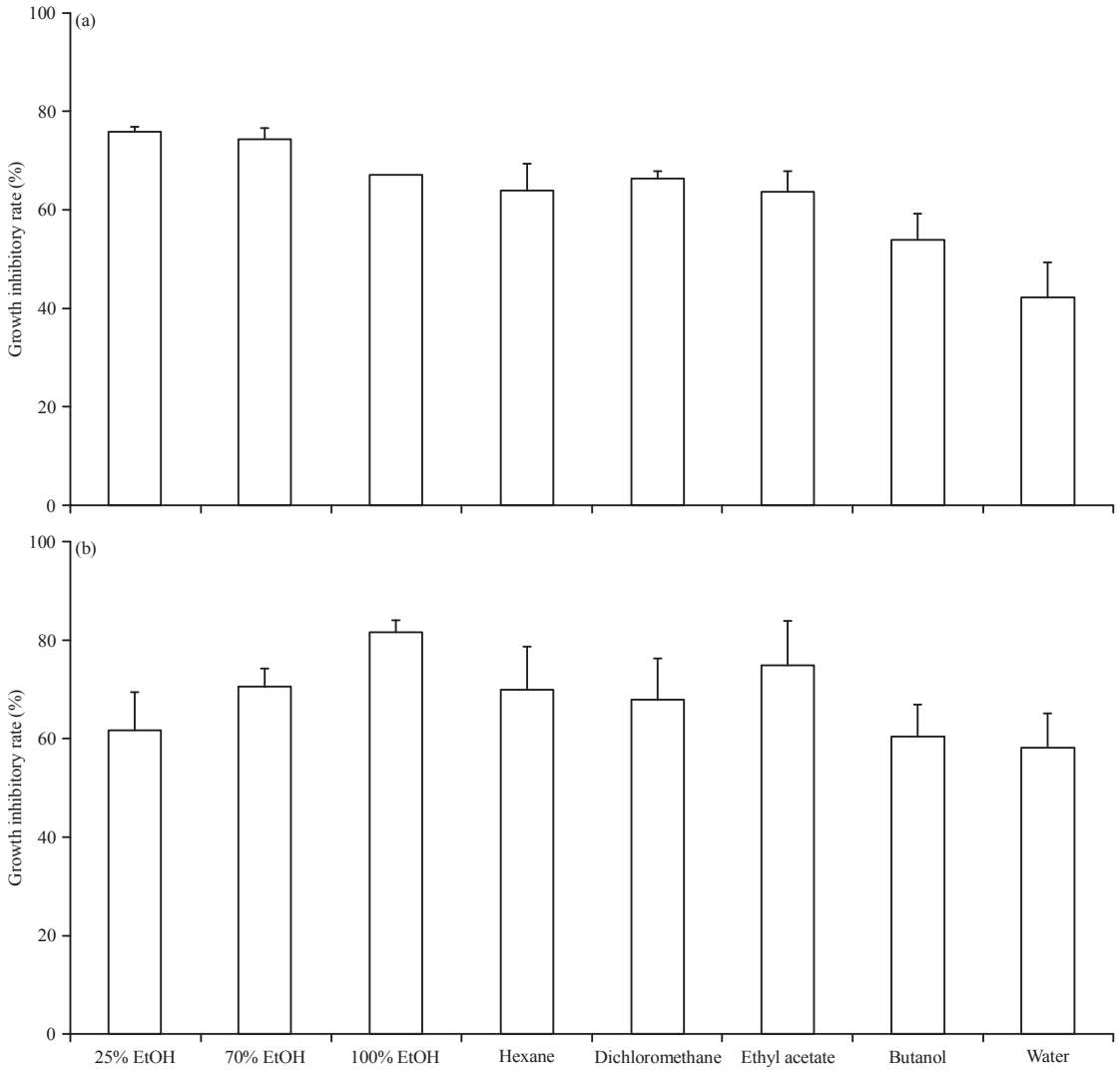

Fig. 1(a-b): Growth inhibition of crude extracts (50 μg mL^{-1}) of (a) Galla Chinensis and (b) Galla Turcica on human hepatoma 7721 cancer cell line

HPLC-PCA map: As shown in Fig. 2 and 3, each point represented one HPLC dataset for a given extract and each extract was tested in triplicates. On supervision, as illustrated by the ellipses superimposed in Fig. 2, all 24 samples of crude extracts of Galla Chinensis could be discriminated into three groups. This was consistent with the experimental value of each sample's inhibitory effects on human cancer cell lines *in vitro*. Crude extracts with inhibitory rates (<55%) were considered as poor inhibitors: water and butanol extracts. Those of 100% aqueous EtOH, hexane, dichloromethane and ethyl acetate showed similar but intermediate inhibition (63%<Inhibition rates<68%). The more potent inhibition (>74%) was located in the crude extracts from 25% EtOH and 70% EtOH. This map demonstrated good correlation between composition and the inhibition. Furthermore, as the variance was mainly due to the top 5 component (PC1-5, 97.3%), it could determine the contributing variables (HPLC peaks) using the PCA Contribution Scores Plot. Peaks with retention times of 5.25 (Gallic acid), 7.95 (Unknown), 11.37 (Unknown), 12.93 (Unknown) and 14.25 min (1, 2, 3, 4, 6-O-pentagalloyl glucose) contributed most to PC1-5, as confirmed by spiking samples with commercial standards. This indicated that the variance of growth inhibitory effect of Galla Chinensis extracts on liver cancer cell line 7721 depended on the concentration of these compounds. Moreover, according to the location of correlated peaks in the Biplot combining the score and loading plots, gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose, in particular, had the highest contribution to the most active 25% EtOH and 70% EtOH extracts.

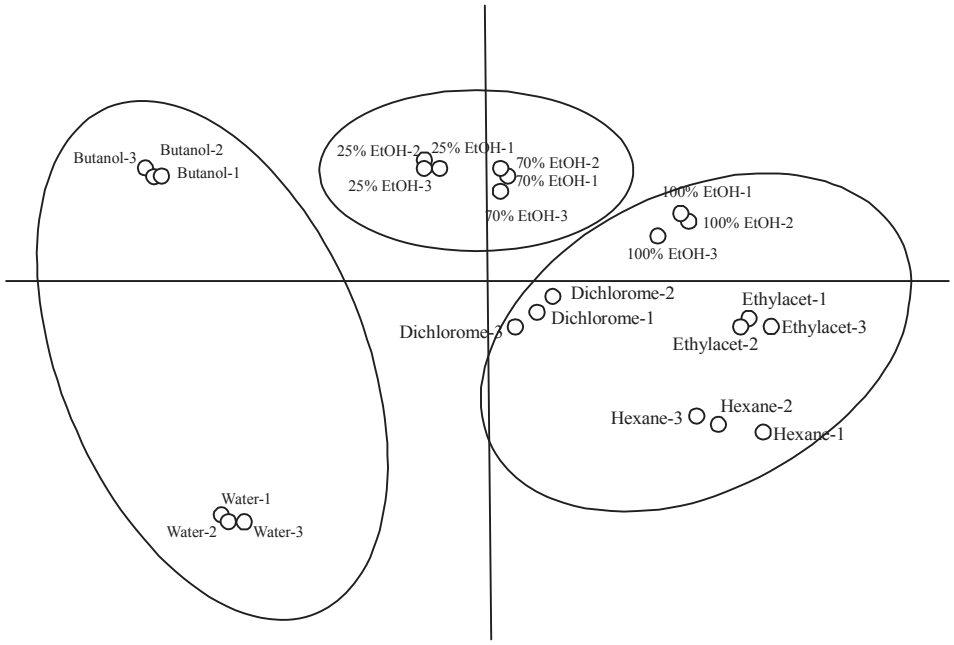

Fig. 2: PCA scores plot for Galla Chinensis crude extracts. Each point on the plot represents one HPLC dataset of an extract and points with the same name indicate replicates (n = 3)

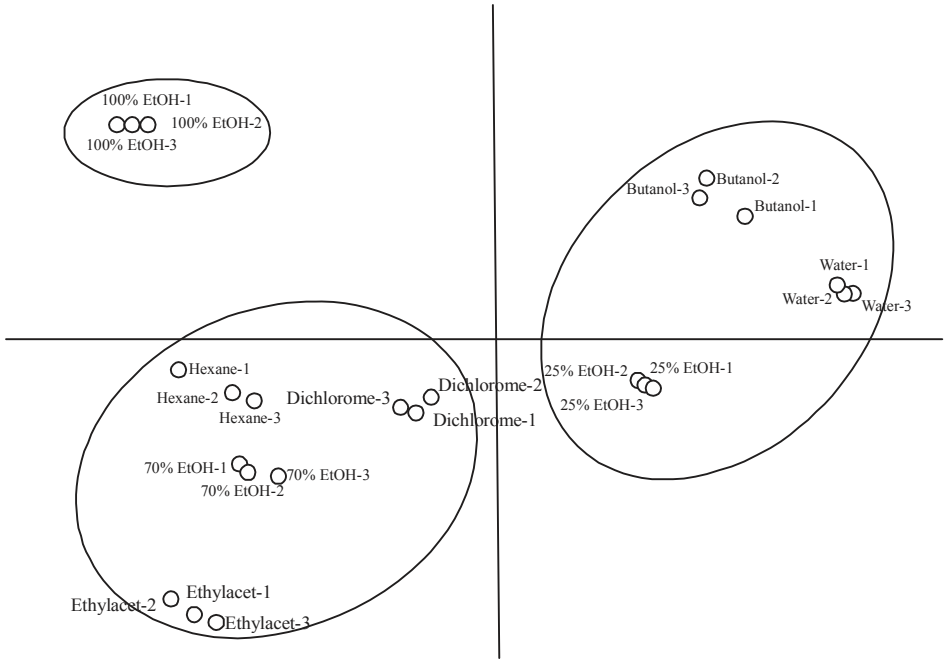

Fig. 3: PCA scores plot for Galla Turcica crude extracts. Each point on the plot represents one HPLC dataset of an extract and points with the same name indicate replicates (n = 3)

Similarly, as illustrated by the ellipses superimposed in Fig. 3, all 24 samples of crude extracts of Galla Turcica could be discriminated into three groups as well. This was also consistent with the each sample's inhibitory effects on human liver cancer cells. The more potent inhibition (>82%) was located in the crude extracts from absolute EtOH. Crude extracts of 70% aqueous EtOH, dichloromethane and ethyl acetate showed intermediate inhibition (68%<Inhibitory rate

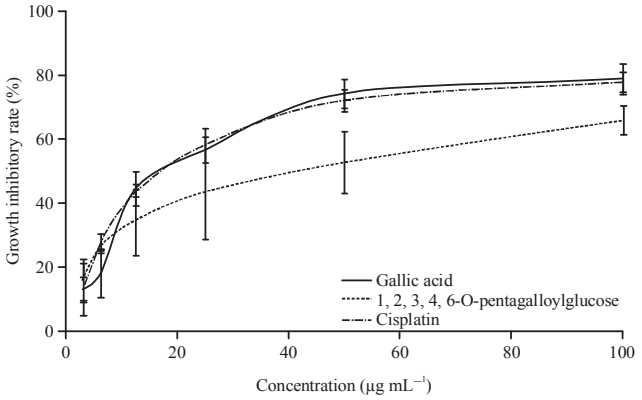

Fig. 4: Growth inhibition of gallic acid, 1, 2, 3, 4, 6-O-pentagalloyl glucose and Cisplatin (positive control) on human hepatoma 7721 cancer cell line

<76%). Those with inhibitory rates (<63%) were considered as poor inhibitors: 25 aqueous EtOH, butanol and water. This map also demonstrated good correlation between composition and the inhibition. The variance was mainly due to the top 5 principal component (PC1-5, 93%). 5.26 (Gallic acid), 9.37 (Unknown), 9.63 (Unknown), 14.4 (1, 2, 3, 4, 6-O-pentagalloyl glucose) and 16.27 min (Unknown) contribute most to PC1-5, as confirmed by spiking samples with commercial standards. This indicated that the variance of growth inhibitory effect of Galla Turcica extracts depended on the concentration of these compounds. Moreover, according to the location of correlated peaks in the bioplots, gallic acid contributed greatly to the activity of 100% EtOH, while 1, 2, 3, 4, 6-O-pentagalloyl glucose, in particular had the important contribution to the 70% EtOH, dichloromethane and ethyl acetate extracts.

Validation of Gallnuts inhibitors of human cancer cells: Based on the analysis above, gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose were identified as major anticancer compounds within both of Galla Chinensis and Galla Turcica. To further validate this conclusion, the cytotoxicity of Gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose were tested on 7721 cells *in vitro* using MTT assay. As shown in Fig. 4, both of the compounds could inhibit the growth of hepatoma 7721 cancer cells in a dose-dependent manner. The gallic acid presented a similar cytotoxic effect as the positive control cisplatin in the doses tested (100-3.125 μg mL^{-1}). The effect of 1, 2, 3, 4, 6-O-pentagalloyl glucose was a little lower than that of gallic acid. The IC_{50} values for gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose were found to be 36.44±8.75 and 55.59±14.96 μg mL^{-1}, respectively. These are comparable to that of the antineoplastic cisplatin (IC_{50}: 35.12±1.74 μg mL^{-1}).

DISCUSSION

In this study, HPLC-PCA secondary metabolite maps revealed that gallic acid and 1, 2, 3, 4, 6-O- pentagalloyl glucose acted as two of the functional anticancer compounds within gallnuts medicines Galla Chinensis and Galla Turcica.

Traditionally, bioassay-guided fractionation was most popular method used in the determination of functional compounds within herbal medicines. However, the interminable processes and large consumables lead to very low efficiency in most of cases during isolation. Nevertheless, the bioactive compounds could be easily lost as there's no global supervision during the fractionation[17]. Multivariate data analysis has thus been employed for the metabolic profiling of herbal secondary metabolites in many recent studies[18-19]. This work further demonstrated the potential of HPLC-PCA analysis as a tool for identification of functional compounds of plant extracts quickly and efficiently.

The anticancer activities of gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose were confirmed in the *in vitro* validation tests and those effects were comparable to that of the antineoplastic cisplatin. Gallic acid has been previously reported to inhibit carcinogenesis in animal models and *in vitro* cancerous cell lines[20-22]. The anticancer mechanisms of gallic acid identified in various cells include modulation of apoptosis-related proteins, activation of ATM kinase, cyclooxygenase inhibition, GSH depletion, ribonucleotide reductase inhibition, ADAMs inhibition, vascular endothelial growth factor inhibition, UDP-glucose dehydrogenase inhibition and NF-κB inhibition[23-27]. 1,2,3,4,6-tetra-O-galloyl-β-D-glucose was used to report to present significant cytotoxic effects on human MDA-MB-231 breast cancer cells with IC_{50} value as low[14] as 1.2 μM. This impressive effect could be achieved through targeting on the overexpression of lactic acid dehydrogenase-A and metabolism genes of MDA-MB-231 cancer cells[14]. Though the anticancer function reported in the literature above, the anticancer roles of gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose in gallnuts Galla Chinensis and Galla Turcica were herein reported for the first time.

These finding in the work partly provided the scientific evidence to support that gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose were the major anticancer constituents of Galla Chinensis and Galla Turcica. However, their anticancer effects need to be validated individually in a broad of *in vitro* and *in vivo* cancerous models. The underlying anticancer mechanisms should be elucidated in future studies. More important, more phytochemical compounds of gallnuts should be purified, especially the

unknown ones identified in this study. It was aiming to obtain powerful anticancer agents or lead compounds from gallnut medicines.

CONCLUSION

PCA-constructed secondary metabolite mapping quickly identified functional compounds from crude gallnuts extracts. Gallnuts (Galla Chinensis and Galla Turcica) constituents gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose were inhibitors of human liver cancer cell line growth with activity comparable to that of cisplatin (IC_{50}: 35.12±1.74 μg mL^{-1}). The further pharmacological investigation and clinical trials were expected to expand the knowledge of the wild traditional medicinal resource gallnuts, aiming to finally transform it to the valuable authorized anticancer drugs.

SIGNIFICANCE STATEMENT

Overall, the study performed herein evident that gallnuts contained a number of anticancer phytochemical components. Gallic acid and 1, 2, 3, 4, 6-O-pentagalloyl glucose were firstly confirmed to be the direct anticancer constituents of Galla Chinensis and Galla Turcica. It thus strongly underpinned a fundamental understanding of anticancer activities of gallnuts medicine and supports their ongoing clinical uses in China. Moreover, this work demonstrated the potential of HPLC-PCA analysis as a tool for identification of functional compounds of plant extracts quickly and efficiently. This approach was an important first step to build the scientific foundation of herbal medicines used in folk medicine systems around world.

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