

RESEARCH ARTICLE

Insights into the antineoplastic mechanism of *Chelidonium majus* via systems pharmacology approach

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Background: The antineoplastic activity of *Chelidonium majus* has been reported, but its mechanism of action (MoA) is unsuspected. The emerging theory of systems pharmacology may be a useful approach to analyze the complicated MoA of this multi-ingredient traditional Chinese medicine (TCM).

Methods: We collected the ingredients and related compound-target interactions of *C. majus* from several databases. The bSDTNBI (balanced substructure-drug-target network-based inference) method was applied to predict each ingredient's targets. Pathway enrichment analysis was subsequently conducted to illustrate the potential MoA, and prognostic genes were identified to predict the certain types of cancers that *C. majus* might be beneficial in treatment. Bioassays and literature survey were used to validate the *in silico* results.

Results: Systems pharmacology analysis demonstrated that *C. majus* exerted experimental or putative interactions with 18 cancer-associated pathways, and might specifically act on 13 types of cancers. Chelidonine, sanguinarine, chelerythrine, berberine, and coptisine, which are the predominant components of *C. majus*, may suppress the cancer genes by regulating cell cycle, inducing cell apoptosis and inhibiting proliferation.

Conclusions: The antineoplastic MoA of *C. majus* was investigated by systems pharmacology approach. *C. majus* exhibited promising pharmacological effect against cancer, and may consequently be useful material in further drug development. The alkaloids are the key components in *C. majus* that exhibit anticancer activity.

Keywords: systems pharmacology; mechanism of action; traditional Chinese medicine; *Chelidonium majus*

Author summary: Tremendous and persistent efforts have been made to seek novel antineoplastic drugs in the everlasting fight against cancers. In this work, we integrated several systems pharmacology approaches to uncover the MoA of *C. majus*' antineoplastic activity. We have found that the alkaloids in *C. majus* may be the significant components for its anticarcinogenic effect. Our research unlocked the antineoplastic mechanism of this medicinal herb that was employed as an analgesic in TCM. The polypharmacological character of *C. majus* can be utilized for further drug research and development.

INTRODUCTION

Traditional Chinese medicine (TCM), mainly consisting of medicinal herbs, has been widely utilized both pharmaceutically and clinically in China and other countries. At present, TCM still takes a place in eastern

countries. Although TCM is clinically effective for medical treatment [1], its mechanism of action (MoA) is rather ambiguous. One hypothesis is that TCM acts in a sophisticated way, with multiple components affecting multiple targets simultaneously, so the individual study on one specific target is usually insufficient [2]. Therefore,

the therapeutic mechanism of TCM needs to be investigated in a more holistic way.

Chelidonium majus is a medicinal herb belonging to the Papaveraceae family that distributes in Europe, Asia and North America. While *C. majus* is utilized as an analgesic in TCM formula, the polypharmacological effects of *C. majus* have also been discovered. It was reported to exert anti-infectious, anti-inflammatory and antineoplastic effects [3,4]. The anticancer effect of *C. majus* was observed by a plenty of studies, yet the molecular MoA of *C. majus* remains unclear.

As the development of systems biology and network pharmacology, systems pharmacology [5,6] has emerged to be a novel methodology for us to understand the complex mechanism of multi-component TCM. Systems pharmacology treats the human body as a closely integrating and dynamically changing system, which coincides with the TCM theory. Therefore, systems pharmacology appears to be a powerful approach in understanding the MoA of multi-component TCM.

Recently, we developed a series of network-based methods to predict drug-target interactions, namely network-based inference (NBI) [7], substructure-drug-target network-based inference (SDTNBI) [8] and balanced SDTNBI (bSDTNBI) [9]. These methods do not rely on the three-dimensional structures of targets, and hence show great advantages in target prediction. The SDTNBI and bSDTNBI methods can be used to predict potential targets for both old drugs and new chemical entities, with substructures to bridge the gap between new compounds and old drugs. Our previous studies have demonstrated that both SDTNBI and bSDTNBI methods are powerful tools to uncover the pharmacological and

toxicological mechanisms of TCM with complex compositions [10,11], and bSDTNBI outperformed SDTNBI.

In this study, we investigated the anticancer MoA of *C. majus* with systems pharmacology approach, together with bioassays and literature survey. We firstly collected the chemical components and known targets of *C. majus* from several databases. Then, putative targets were predicted for each ingredient via bSDTNBI method. Afterwards, pathway enrichment analysis was performed on the basis of experimental and putative compound-target interactions (CTIs). Network analysis was subsequently conducted to unravel the potential mechanism of *C. majus*, and genetic prognostic factors were analyzed to determine the certain cancer types on which *C. majus* might be effective. The key components from *C. majus* that exert potent anticancer activity were also identified.

RESULTS

The whole workflow of this study was illustrated in Figure 1, including three major steps: data collection and analysis, network construction and analysis, experimental validation.

Collection and analysis of *C. majus*' ingredients

Totally 44 chemical components of *C. majus* were obtained from three TCM databases, including TCM Database@Taiwan [12], TCMSP [13] and TCMID [14]. Duplicated molecules were removed. The logP value and molecule weight of each compound were then calculated to build the chemical space (Figure 2). The compounds

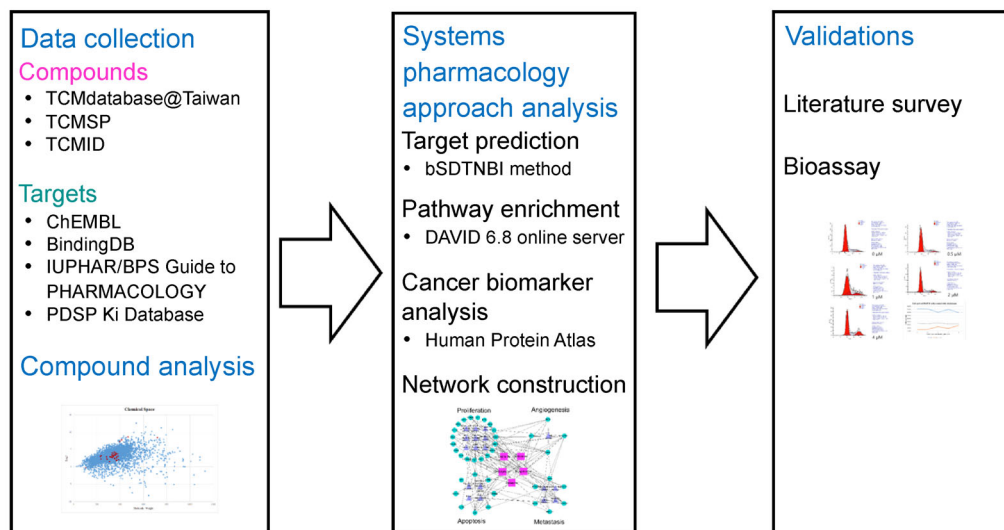


Figure 1. The schematic diagram of the workflow used in the study.

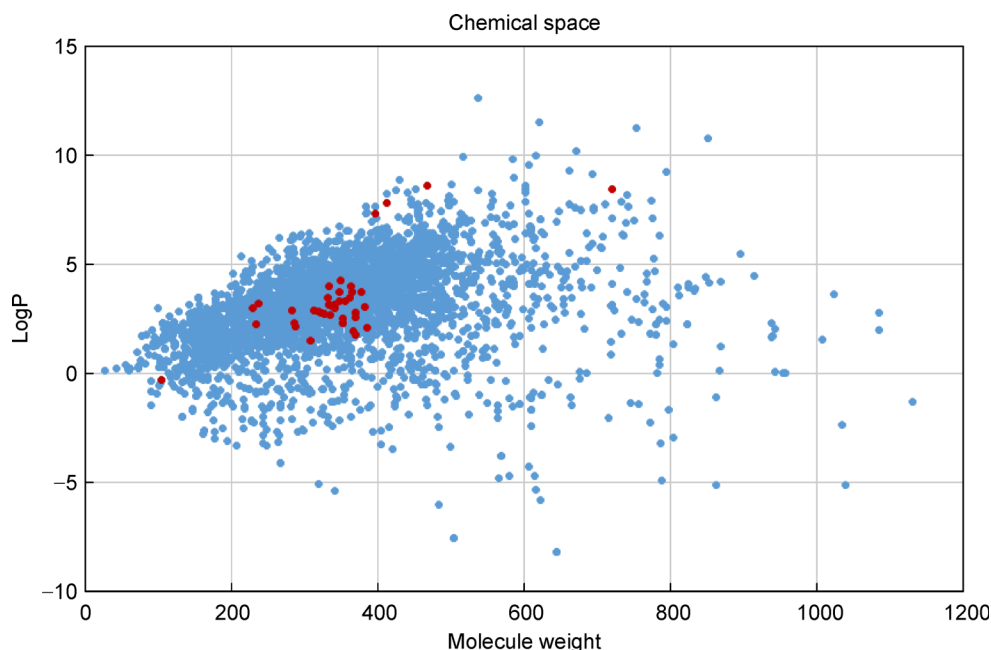


Figure 2. Chemical space analysis between ingredients of *C. majus* (red dots) and the compounds from the bSDTNBI model (blue dots).

from *C. majus* exhibit high superposition with the chemical space of the compounds from the bSDTNBI model we built before [11], except four compounds with logP value higher than 7, which are lupeol acetate, chelidimerine, ergosterol and spinasterol. Most of the ingredients have logP values greater than zero, which implies their not-so-good water solubility. The results showed that all of *C. majus*' ingredients fitted well in the bSDTNBI model, which consists of 1495 herbal ingredients and 2385 drugs.

Target retrieval and prediction

We collected 201 known CTI pairs between 15 ingredients and 111 targets from four databases, including ChEMBL [15], BindingDB [16], IUPHAR/BPS Guide to PHARMACOLOGY [17], and PDSP Ki Database [18]. Afterwards, we predicted top 20 putative targets for each 44 ingredient using our bSDTNBI method. Duplicated CTIs were removed. In all, we obtained 1025 CTIs between 44 ingredients and 173 targets.

Pathway enrichment analysis

The 173 known and putative targets of 44 *C. majus*' ingredients were then uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 online server [19,20] for pathway enrichment analysis. These pathways are associated with

chemical metabolism, nervous system, cancer, infection, and inflammation, which are consistent with the fact that *C. majus* has been clinically used as an analgesic in TCM [21]. Meanwhile, *C. majus* exerts significant association with the cancer-related pathways, which indicates that *C. majus* might exert promising antineoplastic activity.

Besides, *C. majus*' ingredients interact with 37 targets enriched in 18 crucial cancer-associated pathways. 22 of them were known targets and 13 ones were putative targets. The 18 cancer-associated pathways included cell cycle pathway, apoptosis pathway, PI3K-Akt signaling pathway, NF- κ B signaling pathway, *etc.* The pathways were highly correlated with cell division, cell proliferation, apoptosis, metastasis and angiogenesis [22–26]. These pathways were perceived as the potential mechanisms of *C. majus*' anticancer activity. A network was constructed to illustrate the MoA of *C. majus*' anticancer activity (Figure 3). From Figure 3, we can see that several components are vastly discovered, such as sanguinarine, luteolin, berberine, and chelerythrine. There are 38 known CTIs and 118 putative CTIs, and 44 ingredients have all interacted with the 37 cancer-associated targets.

Cancer biomarker analysis

Although the ingredients from *C. majus* can modulate several signal pathways that are highly associated with cancers, *C. majus* might exert therapeutic activities against several certain types of cancers. We therefore

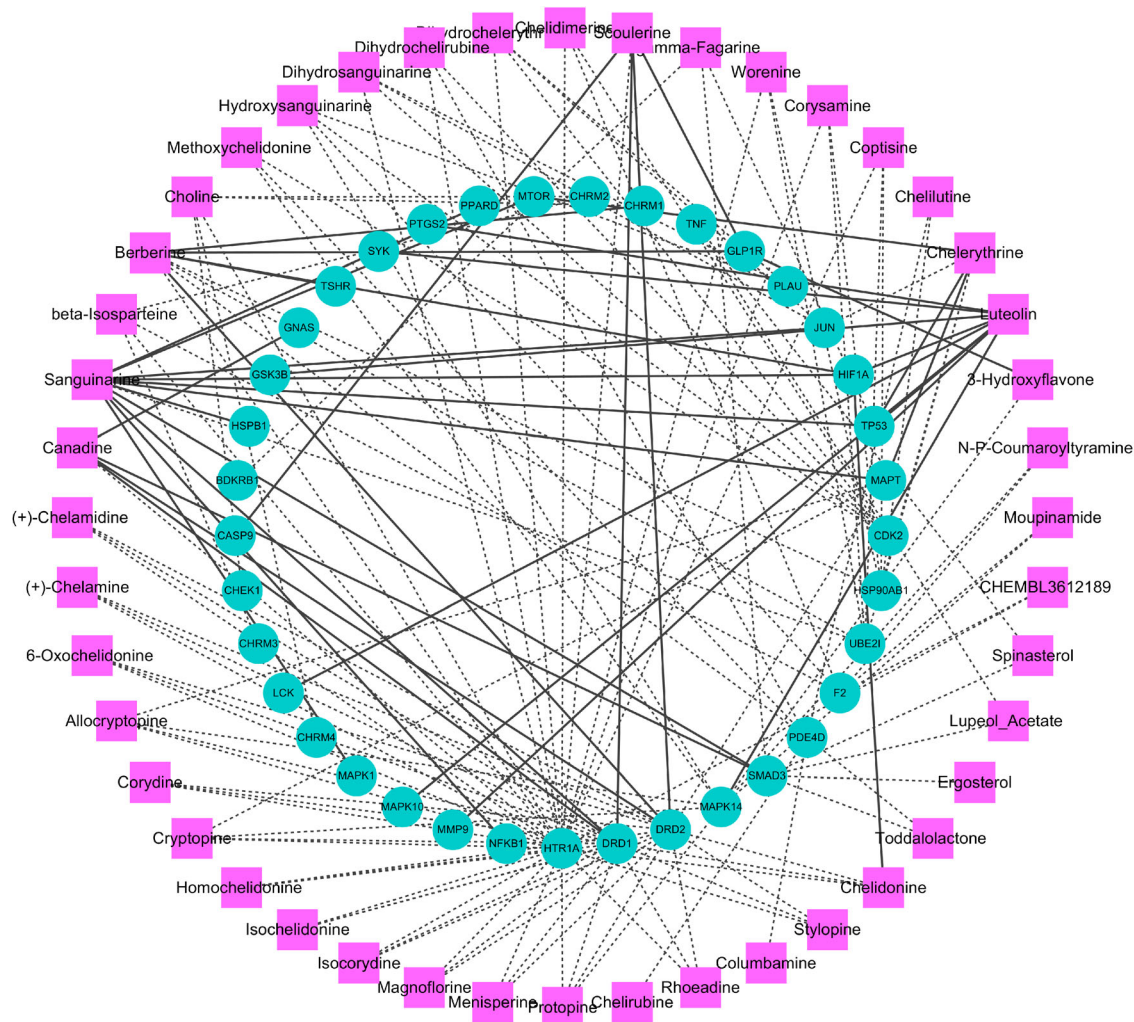


Figure 3. The network of *C. majus*' ingredients with their known and putative targets. The ingredients were denoted by magenta square, and targets were denoted by cyan circle. Known CTIs were represented in solid lines, while putative CTIs were represented in dotted lines. This figure, together with Figure 4, was prepared via Cytoscape 3.6.0.

investigated the known and putative targets whose expression levels are significantly associated with the prognosis of cancer patients, which were referred as the genetic prognostic factors. The genetic prognostic factors were obtained from the Human Protein Atlas [27]. The known and putative targets were mapped into the genetic prognostic factors. Among the 173 known and putative targets of *C. majus*, 22 targets were identified as the genetic prognostic factors of 13 types of cancers, including renal cancer, liver cancer, endometrial cancer, pancreatic cancer, head and neck cancer, urothelial cancer, ovarian cancer, colorectal cancer, thyroid cancer, prostate cancer, melanoma, lung cancer, and breast cancer (Table 1). Theoretically, *C. majus* might exert positive therapeutic activities against these 13 specific types of cancers.

Identification of key anticancer components in *C. majus*

C. majus mainly composes of several alkaloids including chelidone, chelerythrine, sanguinarine, coptisine, berberine and their derivatives. Among them, chelidone, sanguinarine, chelerythrine, berberine and coptisine are the dominant components [28]. Through our study, we found that alkaloids were the dominant antineoplastic components in *C. majus*. We have observed relevant mechanisms to explain the promising antineoplastic activity that alkaloids have showed. A tripartite network was constructed to illustrate the function of the five dominant components in the anticancer activity of *C. majus* (Figure 4). The 18 cancer-associated pathways can be classified into 4 clusters based on their function in

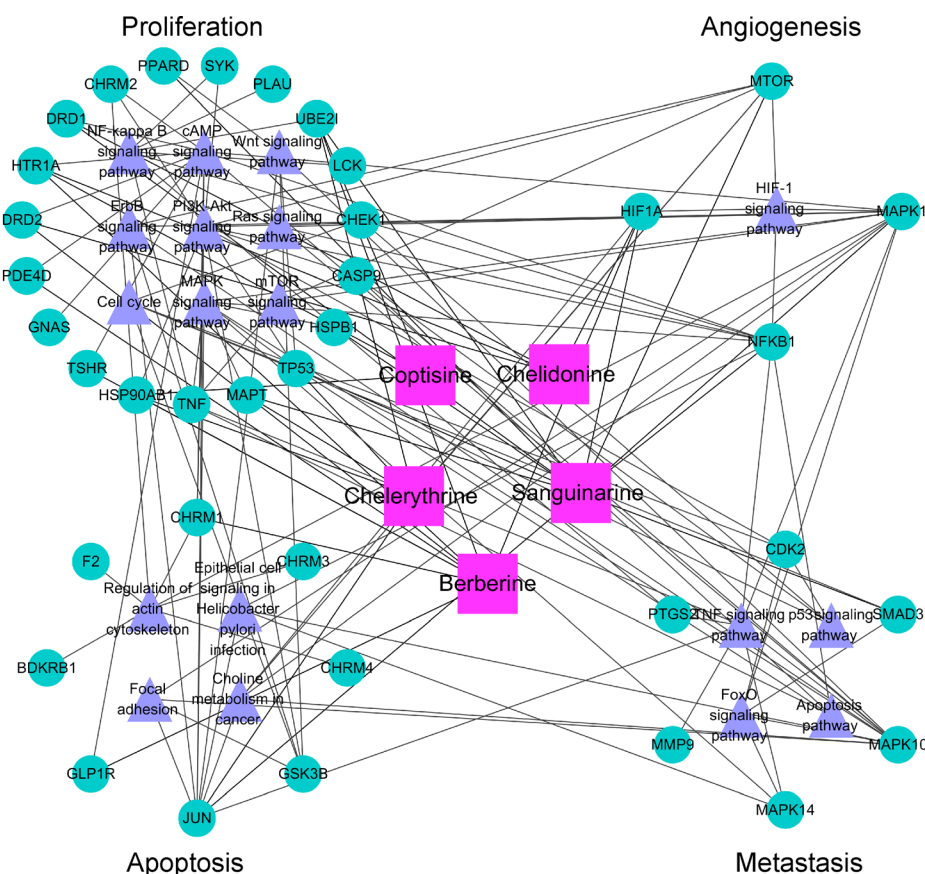


Figure 4. The tripartite network of 5 key anticancer ingredients, the targets, and cancer-associated pathways. These pathways were classified into four clusters based on their biological functions in cancer. The compounds were denoted by magenta square, the targets by cyan circle, the pathways by purple triangle.

Table 1 The relationships of cancer types and genetic prognostic factors

Cancer type	Genetic prognostic factor*
Renal cancer	CDK2, CHEK1, F2, GNAS, GSK3B, HSP90AB1, LCK, MAPK10, MMP9, MTOR, NFKB1, PDE4D, PLAU, PTGS2
Liver cancer	CDK2, CHEK1, GSK3B, MAPK1, MMP9, PPARD, UBE2I
Endometrial cancer	LCK, MMP9, PLAU, TP53
Pancreatic cancer	CHEK1, PLAU, SMAD3
Head and neck cancer	CDK2, LCK, PLAU
Colorectal cancer	CHEK1, HSPB1
Ovarian cancer	GNAS, HSP90AB1
Urothelial cancer	CASP9, MAPK10
Lung cancer	PLAU
Melanoma	LCK
Prostate cancer	TP53
Thyroid cancer	UBE2I
Breast cancer	CASP9

* The genetic prognostic factors were represented in their official gene symbols.

the hallmarks of cancer [29,30]. It can be observed from Figure 4 that the five dominant ingredients modulate 19

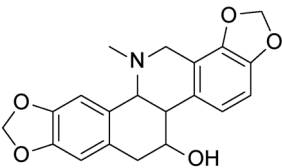
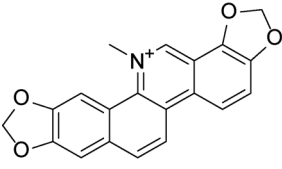
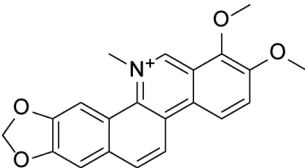
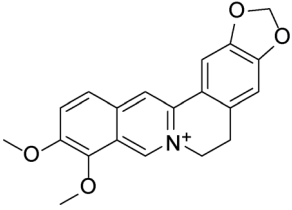
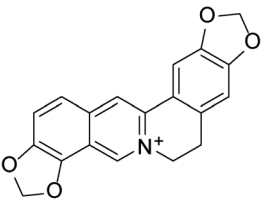
cancer-associated targets, and subsequently regulate the 18 cancer-associated pathways. These pathways are

crucial in the cell proliferation, apoptosis, cancer angiogenesis and cancer metastasis [22–26,31–42]. From Figure 4, we can also see that cell proliferation was regulated by all the five alkaloids, while berberine and sanguinarine can modulate the apoptosis process. Chelerythrine, coptisine and sanguinarine can inhibit cancer metastasis through four pathways, and chelidonine, sanguinarine and chelerythrine can exert anti-angiogenesis activity. Considering the abundant content of the five alkaloids in *C. majus*, chelidonine, sanguinarine, chelerythrine, berberine and coptisine are therefore identified as the key anticancer components of *C. majus*. The structures of these alkaloids were presented in Table 2.

Bioassays and literature survey to verify the prediction

Our systems pharmacology study revealed that *C. majus* is a potent anticancer medicinal herb that inhibits proliferation, metastasis and angiogenesis, as well as induces apoptosis in cancer cells. Plenty of literatures have validated our results. Our computational results have shown that *C. majus* might be effective on several types of cancers (Table 1). Chelerythrine, the main component of *C. majus*, was reported to induce apoptosis in renal cancer cell lines [43]. Chelidonine has also been reported to exert antiproliferative activity against breast cancer cells [44]. Besides, we also found that the potential anti-colorectal

Table 2 Key anticancer ingredients in *C. majus*

Name	Structure	Number of cancer-related genes
Chelidonine		4
Sanguinarine		12
Chelerythrine		6
Berberine		9
Coptisine		3

cancer activity of sanguinarine, another key component of *C. majus*, was reported. The anticancer activity was mediated by inducing apoptosis in HT-29 human colon cancer cells [45].

We also performed bioassays to validate part of our predictions. Based on our prediction, chelidone is the key component in *C. majus* that exerts anticancer activity, but its biological data are limited. Thus, we conducted a bioassay to show chelidone's antiproliferative activity

on the B16F10 melanoma cell line. With the elevation of chelidone's concentration, the percentage of B16F10 cells in G1/M phase increased (Figure 5), while the G1 phase and S phase were insignificantly influenced. The results indicated that chelidone could induce G2/M phase cell cycle arrest in B16F10 melanoma cell line, which was consistent with our computational analysis that chelidone and its derivatives may affect the cell cycle pathway.

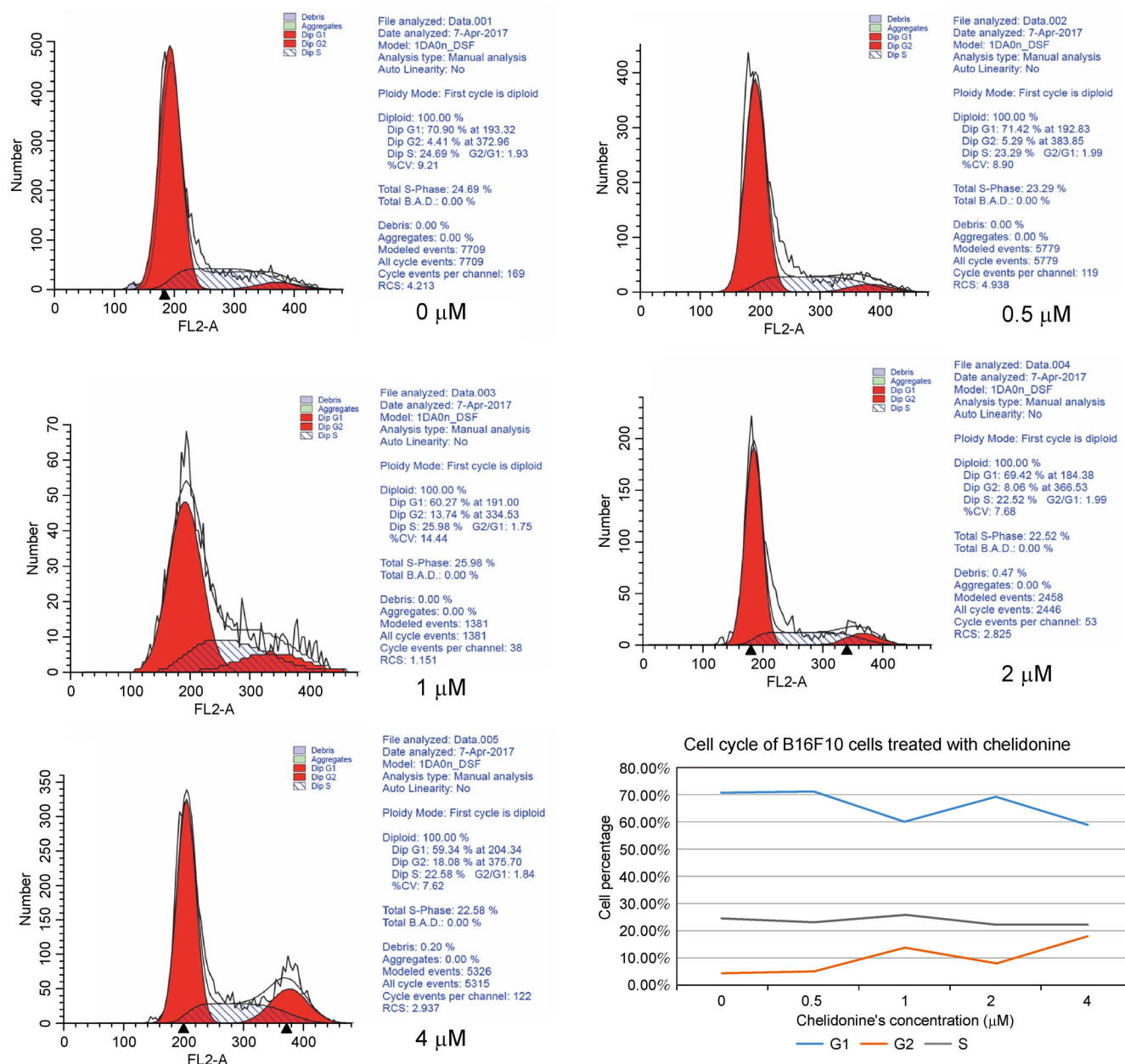


Figure 5. Chelidone induced cell cycle arrest in B16F10 melanoma cell line. The B16F10 cells were treated with 0.5 μM, 1 μM, 2 μM and 4 μM chelidone for 24 hours and one control group without chelidone. The line chart in the lower right represented the B16F10 cells percentage in different cell cycle phases. The blue line represented G1 phase, orange line represented G2 phase and grey line represented S phase.

DISCUSSION

Key anticancer components of *C. majus*

C. majus consists of multiple ingredients, but there are several components that might be essential to the anticancer activity. We hereby discussed the MoA of *C. majus* based on the CTIs and gene ontology. The detailed interaction network is presented in Figure 4.

Sanguinarine. Sanguinarine is an antimicrobial and antioxidant agent firstly discovered in the root of *Sanguinaria canadensis* L. Recent studies stated that sanguinarine exerts both antiproliferative and apoptotic effects in cancer cell lines [46,47]. Our study indicated that sanguinarine interacts with 18 crucial pathways, which are highly correlated with cell proliferation, metastasis and apoptosis. Although sanguinarine was experimentally acknowledged to interact with classic cancer-related targets, such as the tumor protein 53 (TP53) [32], peroxisome proliferator-activated receptor (PPAR) [38] and p38 α (MAPK14) [40], other putative targets were also predicted to be associated with cancers. The overexpression of urokinase, encoded by gene PLAU, was highly correlated with cancer metastasis [33,34], and inhibition of urokinase can successfully suppress the metastasis [31]. In our study, hydroxysanguinarine and dihydrosanguinarine, derivatives of sanguinarine that coexisted in *C. majus*, were predicted to act on the urokinase. Thus, *C. majus* might also prevent the tumor invasion and metastasis.

Chelerythrine. Chelerythrine is another substantial alkaloid existing in *C. majus* that exhibits selective inhibition against protein kinase C [48]. Chelerythrine was reported to exert cytotoxic effect against human prostate cancer cells [49] and induce cell cycle arrest in the human leukemia HL-60 cell lines [50]. In our study, chelerythrine was predicted to interact with urokinase and heat shock protein (HSP) 90 β [39], both of which were overexpressed in cancer cells. Therefore, chelerythrine might suppress cancers by interacting with urokinase and HSP 90 β . Our study also indicated the interaction between chelerythrine and the c-Jun protein, which was encoded by the proto-oncogene Jun [35]. Chelerythrine might inhibit the c-Jun protein to suppress tumors.

Chelidonine and derivatives. Chelidonine and its derivatives including homochelidonine, isochelidonine, methoxylchelidonine and 6-oxochelidone were reported to manifest antiproliferative activities against tumor cells in a cellular level, but the MoA was still uncertain. However, the experimental data of chelidonine in the molecular level was very limited. In our study, we predicted top 20 targets for chelidonine and its derivatives. Ten CTI pairs, which we believed might explain the potential anticancer mechanism of chelidonine, were

identified. It was predicted by bSDTNBI method that chelidonine and three derivatives interacted with androgen and estrogen receptors, which are highly associated with hormone-dependent cancers. Apart from the nuclear receptors, methoxylchelidonine was predicted to interact with cyclin-dependent kinase 2 (CDK2), which is highly related to cell division [36]. Chelidonine and derivatives might induce cell cycle arrest in cancer cells.

Berberine and coptisine. Berberine and coptisine also predominantly exist in *C. majus*. The two alkaloids have been extensively studied on their anticancer activities. Berberine can suppress cell invasion, induce apoptosis and arrest cell cycle in plenty cancer cells [51]. Similar pharmacological activities were also observed in coptisine against cancer cells [52,53].

Potential antineoplastic mechanisms of *C. majus*

From our study, we can see that *C. majus* might exert positive therapeutic activities against 13 types of cancers through several cancer-related pathways. We discussed three major pathways here.

Cell cycle arrest. The cell cycle pathway is the biological process of DNA replication and cell division, which takes place in almost all types of cells. Five targets were enriched in the cell cycle pathway, namely SMAD3, CHEK1, CDK2, GSK3B, and TP53. More than one-third compounds (16 out of 44) from *C. majus* interact with these targets, ten of which are alkaloids. It is interesting that five targets distribute in the G1 phase and S phase of cell cycle, so the cell division might be arrested in the G2/m phase.

Apoptosis induction. Inducing apoptosis in certain cancer cells has been a hotspot in oncology research and drug development. Our study found that seven compounds interact with four targets in the apoptosis process, including TNF, CASP9, TP53 and NFKB1. *C. majus* might suppress the tumor cells by inducing apoptosis. Eight compounds from *C. majus* explicitly or putatively interacted with these targets above, inducing programmed cell death in cancer cells. Several researches verified *C. majus*' antiproliferative activity on diverse cancer cells by inducing apoptosis. In a study, methanol extract of *C. majus* inhibited cytotoxicity towards human promyelocytic leukemia HL-60 cell lines in a dose-dependent pattern [54]. Another study showed that *C. majus* extract exerts both cell cycle arrest and apoptosis activity against six cancer cell lines [55].

Signal transduction. Cell cycle arrest and apoptosis induction were perceived to be the key mechanisms that *C. majus* exerts antineoplastic activity against cancer cells. Besides, *C. majus* might also suppress cancer in other pathways. A research reported that the PI3K-Akt signaling pathway was activated in the early stage of

tumorigenesis [41], and this cascade was considered to be a promising target for cancer treatment [37]. *C. majus* may act on the PI3K-Akt signaling pathway and affecting the downstream signaling pathways, including the NF- κ B signaling pathway and VEGF signaling pathway, and suppress angiogenesis and cell proliferation. Moreover, *C. majus* remarkably influenced the MAPK signaling pathway, including the extracellular-signal regulated kinases (ERK) subfamily and p38 subfamily, and subsequently led to cell cycle arrest and apoptosis. In addition, the focal adhesion pathway, regulation of actin cytoskeleton pathway and epithelial cell signaling pathway are high correlated with cancer metastasis. *C. majus* might inhibit metastasis through interacting with these pathways.

Comparison with other similar work

Recently, a systems pharmacology research on the antitumor mechanism of *C. majus* was reported [56]. Compared with that report, we noticed that they collected 442 known targets of *C. majus*, greater than 111 known targets that we have retrieved in this work. However, we found that the 442 targets came from multiple species; most of them are unrelated to human cancers. Besides, the SysDT method [57] they applied for target prediction requires negative samples to build a predictive model, while negative samples are usually rarely reported. Our bSDTNBI method, on the other hand, needs no negative samples to build a promising network inference model. In addition, the chelidonine, which is the most abundant ingredient in *C. majus*, was perceived as an unessential component in their report. However, our study elucidated that chelidonine is a promising anticancer ingredient in *C. majus* by both computational method and bioassay.

CONCLUSIONS

In this study, the bSDTNBI method, integrated with pathway enrichment, network analysis and cancer biomarker analysis, was successfully used to decipher the antineoplastic mechanism of *C. majus* at the molecular level. Systems pharmacology approach was proven to be a useful tool in understanding the pharmacological activity of multi-component TCM.

MATERIALS AND METHODS

Data collection and preparation

The chemical components of *C. majus* were collected from three TCM databases, namely TCM Database@-Taiwan, TCMSP and TCMID, as well as research literatures reporting *C. majus* composition. The com-

pounds were converted into canonical SMILES via our in-house script integrated with Python, OpenBabel toolkit [58] and Schrödinger 2015 package [59].

The known CTI pairs were collected from four databases including ChEMBL, BindingDB, IUPHAR/BPS Guide to PHARMACOLOGY and PDSP Ki Database. The interaction pairs were exacted only when the data met below four criteria: (i) the IC_{50} , EC_{50} , K_i , K_d or potency values $\leq 10 \mu M$; (ii) the target protein was labelled as “reviewed” in the UniProt Database [60]; (iii) the target proteins originate from *Homo sapiens*; (iv) duplicate CTIs were removed.

Chemical space analysis

We conducted a chemical space comparison between chemical ingredients of *C. majus* and compounds from the bSDTNBI model, to analyze the drug-like properties of those ingredients. The molecular weight and logP values were calculated by Schrödinger 2015 package.

Prediction of putative targets

Our bSDTNBI method was applied for target prediction. Based on our previous study [9], three parameters, α , β and γ , were set to 0.41, 0.06 and -0.51 , respectively. Our previous study also showed that the inference method manifested better performance when the Klekota-Roth (KR) fingerprint [61] was selected to generate substructures for each compound.

Canonical SMILES format was firstly converted to KR fingerprint via PaDEL-Descriptor [62]. The top 20 putative targets were predicted for each compound by bSDTNBI method. The putative targets were then converted to their official gene symbols for further pathway enrichment analysis.

Pathway enrichment analysis

After removing duplicates, the known and putative targets were merged for pathway enrichment analysis. The pathway enrichment analysis was conducted by DAVID v6.8 server (<https://david.ncifcrf.gov/>), an online functional annotation tool. The 173 targets were uploaded onto DAVID v6.8 online server. “*Homo sapiens*” was selected as background species for gene annotation. “KEGG_PATHWAY” was selected for functional annotation.

Compound-target network construction

We constructed a binary network (Figure 3) including 44 compounds and 173 targets to illustrate the CTI of *C. majus*. We also constructed a tripartite network (Figure 4)

to illustrate the relationship between the key anticancer components, targets and cancer-related pathways. The tripartite network was prepared via Cytoscape 3.6.0 [63].

Cell culture and treatments

The melanoma cell line B16F10 was purchased from the Cancer Cell Repository of Shanghai Cell Bank, Shanghai, China, which was maintained in DMEM, supplemented with 10% FBS, 100 units per milliliter penicillin and streptomycin. B16F10 cells were cultured at 37°C in 5% CO₂ and the medium was replaced as required.

Analysis of cell cycle

B16F10 cells were treated with DMEM or drugs for 24 h, then collected and washed with PBS. To analyze cell cycle, B16F10 cells were stained with PI (BD Biosciences, Franklin Lakes, NJ, USA; cat no. 556547). Fluorescence was measured by a FACSCalibur flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

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COMPLIANCE WITH ETHICS GUIDELINES

The authors Xinzhe Xiao, Zehui Chen, Zengrui Wu, Tianduan Wang, Weihua Li, Guixia Liu, Bo Zhang and Yun Tang declare that they have no conflict of interests.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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