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ORIGINAL ARTICLE

Systematic Analysis of Bioactive Components of Distinct Medicinal Organs of Mulberry by High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometry and Molecular Docking

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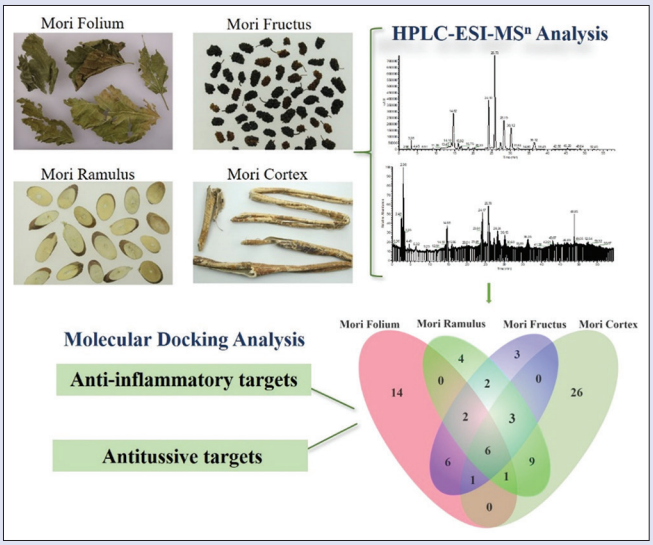
ABSTRACT

Background: Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex are traditional Chinese medicines derived from distinct organs of mulberry (*Morus alba* L.). Intriguingly, their efficacies are different. **Objectives:** In this study, we aimed to systematically analyze the similarities and differences in the chemical composition of the aforementioned four medicines and to explore their bioactive components of anti-inflammatory and antitussive activity. **Materials and Methods:** High-performance liquid chromatography with electrospray ionization mass spectrometry (HPLC-ESI-MS^n) was used to identify the various compounds present in each of the four medicines from the methanolic extracts and different polar fractions of aqueous extracts. Moreover, the identified compounds were docked against anti-inflammatory or antitussive targets by means of the molecular docking tool, molecular operating environment 2018. **Results:** A total of 77 compounds were identified from the four study materials, of which 30 were identified in Mori Folium, 27 compounds in Mori Ramulus, 23 compounds in Mori Fructus, and 46 compounds in Mori Cortex, and they had 14, 4, 3, and 26 compounds that were specific to them, respectively. The results of molecular docking indicated that quercetin, chlorogenic acid, and isoquercitrin, the shared compounds of the four medicines showed strong anti-inflammatory activity. Of note, the top 10% of compounds with better anti-inflammatory activity were mostly identified in Mori Cortex, whereas the least in Mori Fructus. Moreover, out of the top 10% of compounds with better antitussive activity, 11 compounds were identified in Mori Cortex, and only 2 or 3 compounds were identified in the other three medicines. **Conclusion:** The results of this study are consistent with the clinical application of the four study medicines derived from mulberry, which further supports the fact that their efficacy is dependent on their chemical composition.

Key words: Bioactive components, high-performance liquid chromatography with electrospray ionization mass spectrometry, molecular docking, Mori cortex, mulberry

SUMMARY

- The chemical composition and potential compounds present in the four medicinal parts of mulberry were investigated
- A total of 30 compounds were identified in Mori Folium, 27 compounds in Mori Ramulus, 23 compounds in Mori Fructus, and 46 compounds in Mori Cortex
- The top 10% of the compounds with better docking effect of anti-inflammatory and antitussive targets were mostly identified in Mori Cortex, which were consistent with clinical application of the four medicines of mulberry
- It further supported the correlation between chemical composition and pharmacodynamics.



Abbreviations used: HPLC-ESI-MS^n: High-performance liquid chromatography electrospray ionization mass spectrometry; TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; BPI: Base peak intensity; M_{CH}: $CHCl_3$-soluble fraction of mulberry; M_{PE}: Petroleum ether-soluble fraction of mulberry; M_{EA}: EtOAc-soluble fraction of mulberry; M_{BU}: N-BuOH-soluble fraction of mulberry; MOE: Molecular Operating Environment.

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INTRODUCTION

Mulberry (*Morus alba* L.) is a Traditional Chinese Medicinal (TCM) plant, and various versions of Chinese Pharmacopoeia record that its dried leaves, shoots, fruits, and root bark can be used as medicines. Among them, the dried leaves of mulberry known as Mori Folium, clinically used for improving eyesight (i.e., eyes with bloodshot or dry eyes) and as well as for alleviating anemopyretic cold, lung heat disease with dry cough, headache, and vertigo disease. Next, the dried shoot of mulberry, known as Mori Ramulus, used in the treatment of rheumatism, as well as numbness in the shoulder and arm joints. Next, the dried fruits of mulberry, known as Mori Fructus, used in treating in deficiency of liver and kidney, dizziness and tinnitus, palpitations and insomnia, internal heat and intestinal dryness with constipation. Finally, the dried root bark of the mulberry, known as Mori Cortex, used for lung heat disease with panting and cough, as well as for edema and oliguria.[1]

Pharmacological studies have shown that the four distinct parts of mulberry exhibit different bioactivities with regard to lowering of blood sugar and fatty acid components, anti-inflammatory, antioxidant, and antitumor activities, as well as with immunoregulation as they contain inherent bioactive compounds including flavonoids, alkaloids, polysaccharides, coumarins, steroids, benzofuran derivatives, Diels-Alder adducts, terpenes, and volatile oils.[2-5] Analysis of the chemical composition of mulberry has been studied extensively, for example, Zhao *et al.*[6] established fingerprint patterns and common pattern maps of different medicinal parts of mulberry. Their results showed that there were 12 common peaks in Mori Folium, 11 in Mori Ramulus, 8 in Mori Fructus, and 10 in Mori Cortex. Furthermore, Han *et al.*[7] showed that the aforementioned medicines have four common volatile compounds including hexanal, 1-octen-3-ol, methylheptenone, and nonanal. They also identified some unique compounds such as camphor and eugenol in Mori Folium, menthol in Mori Fructus, and terpineol and (-)-4-terpineol in Mori Cortex.

Therefore, the pharmacological effect and clinical application among the distinct medicinal organs of mulberry depends on the similarity and differences in their chemical composition. However, so far, there is lack of comprehensive and systematic analysis with regard to the chemical composition of mulberry. Molecular docking technology has been proven to be an effective method to develop and study the mechanism of curative compounds in TCM.[8,9] In this study, high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI-MS^n) was used to conduct a comprehensive and systematic research on the chemical composition of Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex in order to establish a compound library of distinct medicinal parts of mulberry. In addition, we used molecular docking technology to virtually screen the components responsible for anti-inflammatory and antitussive activity for providing a basis for the quality evaluation and drug design from different parts of mulberry.

MATERIALS AND METHODS

Chemicals and reagents

The dried leaves, shoots, fruits, and root bark from the same tree of *M. alba* L. from mulberry field of Jiangsu University were collected during their traditional harvest period and authenticated by Professor Zhen Ouyang. Methanol, formic acid, glacial acetic acid, petroleum ether, chloroform, and ethyl acetate were of analytical grade and purchased from Sinopharm Group Reagent Co., Ltd. (Shanghai, China), and HPLC-grade methanol and HPLC-grade acetonitrile were purchased from TEAD.

Analysis of methanolic extracts by high-performance liquid chromatography with electrospray ionization mass spectrometry

Preparation of samples

The coarse powder (1.0 g, passed through No. 3 sieve) of Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex were extracted in 20 mL of methanol under reflux at 80°C for 2 h to obtain respective methanolic extracts (M_{Me}). The extracts were filtered through 0.45 μm membranes and then used directly for the HPLC-ESI-MS^n analysis.

HPLC and MS conditions

HPLC was performed using Agilent 1260 (Agilent, USA), and Table 1 shows the experimental conditions applied for the HPLC analysis. The MS conditions were negative ion mode with 3.5 Kv source voltage; sheath gas (N_2) flow rate was 36 au; auxiliary gas (N_2) flow rate was 10 au; collision gas was helium; capillary temperature was 325°C; capillary voltage was −30 V; and full ion scanning mode with scanning range of 50–1,000 m/z. The MS/MS collision energy was 50%. Data processing was conducted by Xcalibur software version 2.0.7 (Thermo Fisher Scientific, USA).

Analysis of different polar extracts by high-performance liquid chromatography with electrospray ionization mass spectrometry

Preparation of samples

The coarse powder (50.0 g, passed through No. 3 sieve) of Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex were thrice extracted in 500 mL of distilled water under reflux at 100°C and 2 h. Subsequently, the combined filtrates were concentrated by a rotary evaporator at 55°C (Buchi-200, BUCHI Labortechnik AG, Switzerland, Germany) to obtain the concentrated solution of each of the study material.

Next, the same volume of petroleum ether (PET), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) was added to each of the solutions in a successive extraction step-by-step for three times and then the extracts were combined separately. Next, the extracts were concentrated under vacuum, which was followed by freeze-drying for 12 h using a vacuum freeze dryer (ALPHA 1–2 LD, Marin Christ, Osterode, Germany). Finally, each of the resultant products were dissolved in an appropriate volume of methanol to obtain the PET-soluble fraction (M_{PE}), $CHCl_3$-soluble fraction (M_{CH}), EtOAc-soluble fraction (M_{EA}), and *n*-BuOH-soluble fraction (M_{BU}). The fractions were filtered through 0.45-μm membrane, and the filtrates were used as samples for HPLC-ESI-MS^n analysis.

High-performance liquid chromatography and mass spectrometry conditions

HPLC analysis was performed using Agilent 1260 (Agilent, USA), and Table 2 shows the MS condition.

Molecular docking studies

The 3D structures of the compounds were searched in the TCM system pharmacology (TCMSP, http://lsp.nwu.edu.cn/tcmsp.php) and PubChem (https://www.ncbi.nlm.nih.gov) databases. The Chem3D Pro 14.0 software (Cambridge Soft, Waltham, MA, USA) was used for the transformation of the standard 3D structure module. The molecular operating environment (MOE 2018, Chemical Computing Group Inc., Montreal, Canada) was used to construct ligand database, and force field was adjusted to Amber 10. The energy and conformation majorization were done to all compounds by MOE Energy Minimize.

Table 1: The high performance liquid chromatography conditions of methanol extracts of the four medicinal organs of mulberry

	Mori Folium	Mori Ramulus	Mori Fructus	Mori Cortex	
HPLC column	Kromasil C_{18} (250 mm × 4.6 mm, 5 μm)				
Solvent A	100% acetonitrile				
Solvent B	0.2% formic acid in ultrapure water				
Eluent gradient	0~5 min, 10%	0~5 min, 15%	0~8 min, 9%	Condition 1	Condition 2
	5~10 min, 10%→13.5%	5~9 min, 15%→20%	8~15 min, 9%→15%	0~5 min, 13%→15%	0~5 min, 16%→18%
	10~15 min, 13.5%→19%	9~20 min, 20%→40%	15~19 min, 15%→22%	5~15 min, 15%→17%	5~10 min, 18%→35%
	15~18 min, 19%	20~25 min, 40→50%	19~30 min, 22%→25%	15~22 min, 17%→22%	10~15 min, 35%→50%
	18~19 min, 19%→22%	25~30 min, 50%→57%	30~40 min, 25%→30%	22~31 min, 22%→34%	15~25 min, 50%→60%
	19~35 min, 22%	30~38 min, 57%	40~50 min, 30%→40%	31~45 min, 34%→46%	25~30 min, 60%→60%
	35~40 min, 22%→30%	38~45 min, 57%→80%		45~50 min, 46%→51%	30~50 min, 60%→80%
	40~50 min, 30%→40%			50~55 min, 51%→55%	
				55~59 min, 55%→65%	
				59~75 min, 65%	
				75~80 min, 65%→80%	
Determine wavelength (nm)	358	320	358	340	
Flow rate (mL/min)	1.0	1.0	0.8	1.0	
Injection volume (μL)	10	10	20	10	

HPLC: High performance liquid chromatography

Table 2: The high performance liquid chromatography conditions of different polar extracts of the four medicinal organs of mulberry

	M_{PE}	M_{CH}		M_{EA}		M_{BU}
		Mori Ramulus	Mori Folium, Mori Fructus, Mori Cortex	Mori Ramulus	Mori Folium, Mori Fructus, Mori Cortex	
HPLC column	Kromasil C_{18} (250 mm × 4.6 mm, 5 μm)					
Solvent A	100% acetonitrile					
Solvent B	0.2% formic acid in ultrapure water					
Eluent gradient	0~5 min, 13%→15%	0~5 min, 15%	0~5 min, 16%→18%	Same as the conditions of Mori Ramulus part of M_{CH}	Same as the conditions of M_{PE}	0~5 min, 15%→18%
	5~15 min, 15%→17%	5~9 min, 15%→20%	5~10 min, 18%→35%			5~9 min, 18%→20%
	15~22 min, 17%→22%	9~20 min, 20%→40%	10~15 min, 35%→50%			9~20 min, 20%→40%
	22~31 min, 22%→34%	20~25 min, 40%→50%	15~25 min, 50%→60%			20~25 min, 40%→50%
	31~45 min, 34%→46%	25~30 min, 50%→57%	25~30 min, 60%			25~30 min, 50%→55%
	45~50 min, 46%→51%	30~38 min, 57%				
	50~55 min, 51%→55%	38~45 min, 57%→80%				
	55~60 min, 55%→65%					
Determine wavelength (nm)	320	320	340	340	340	340
Flow rate (mL/min)	1.0	1.0	1.0	1.0	1.0	0.8
Injection volume (μL)	20	10	10	10	10	10

HPLC: High performance liquid chromatography, M_{PE}: Petroleum ether-soluble fraction of mulberry, M_{CH}: Chloroform - soluble fraction of mulberry, M_{EA}: Ethyl acetate -soluble fraction of mulberry, M_{BU}: *n*-butanol -soluble fraction of mulberry

According to the TTD database (http://db.idrblab.net/ttd/) and related literature, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, interleukin (IL)-1 β, and IL-6 were used as anti-inflammatory targets.[10-12] The X-ray crystallographic structures of the aforementioned proteins (PDB codes: 3HR4, 2AZ5, 1ALU, 3O4O, 1F0J, and 2R4S) were downloaded from the Protein Data Bank (PDB, www.rcsb.org). MOE 2018 was used to preprocess the original structure of the target protein, force field was set to Amber 99, hydrogen atoms were first added, all monosaccharide, and water molecules were removed, and energy optimization was done to all compounds.

Docking was done in MOE-Dock module by the settings of placement function (Triangle Matcher) and scoring function (London dG). The rest of the parameters were kept as default, and then, all atoms were docked. The ligand and the receptor were semi-flexibly docked with their associated scores indicated based on their free energy of binding and their subsequent interactions with the surrounding amino acids. The docking scores of all the compounds with 4 anti-inflammatory targets and 2 antitussive targets were obtained. The lower the score, the more stable the ligand binds to the receptor. Notably, the binding activity of the compound to the target was evaluated by the scores and the number of combinative amino acid residues.

RESULTS

Analysis of methanolic extracts via high-performance liquid chromatography with electrospray ionization mass spectrometry

The chemical composition of M_{Me} of the study materials was analyzed using HPLC-ESI-MS^n. According to the results, most of the compounds had a better response under ESI negative ion mode conditions [Figures 1 and 2]. According to the retention times of each chromatographic peak, ion peak, and main fragment peak information, combined with relevant literature data and the database of natural products, such as TCMSP, TCM, Mass Bank and SciFinder, and so

on, a total of 42 compounds were identified in methanol extracts of the study materials [Table 3]. These included 27 flavonoids, 5 phenolic acids, 3 Diels-Alder-type adducts, 2 stilbenes, 4 2-arylbenzofurans, and 1 triterpene. Among them, 16 compounds were identified in Mori Folium, 13 compounds in Mori Ramulus, nine compounds in Mori Fructus, and 26 compounds in Mori Cortex.

Analysis of different polar extracts via high-performance liquid chromatography with electrospray ionization mass spectrometry

A total of 50 compounds were identified from different polar fraction of water extracts through HPLC-MS^n. In the case of M_{PE} [Figures S1 and S2, Table 4], seven compounds were identified, of which 2, 2, 4, and 5 compounds were, respectively, identified from Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex. In addition, for the M_{CH} [Figures S3 and S4, Table 5], 6 compounds were identified, of which 3, 2, 4, and 2 compounds were respectively identified from Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex. Furthermore, 28 compounds were identified in M_{EA} [Figures S5 and S6, Table 6], of which 9, 10, 12, 10 compounds were respectively identified from Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex. Moreover, 18 compounds were identified in M_{BU} [Figures S7 and S8, Table 7], of which a total of 6, 4, 7, and 5 compounds were respectively identified from Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex.

Importantly, our results showed that four of these compounds were identified in each of the four medicines, namely, 9, 10, 11-trihydroxy-12-octadecenoic acid; eicosanoic acid; isoquercitrin; and mothaxanthin-3-*O*-β-D-glucoside. Moreover, 12, 3, 8, and 10 characteristic compounds were respectively identified in Mori Folium, Mori Ramulus, Mori Fructus, and Mori Cortex. Of note, 2-arylbenzofuran and flavonoid compounds were mainly identified in

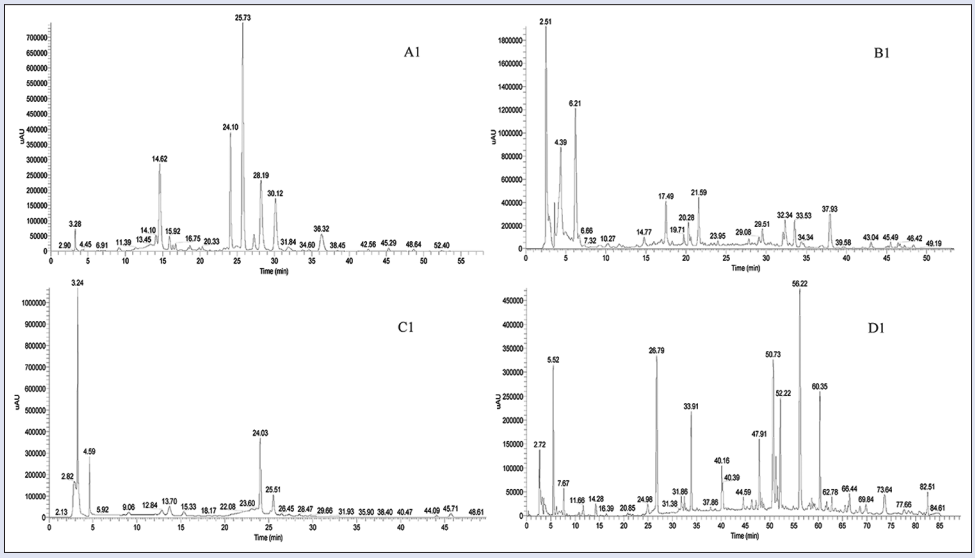

Figure 1: The high-performance liquid chromatography chromatogram of M_{ME} of Mori Folium (A1), Mori Ramulus (B1), Mori Fructus (C1) and Mori Cortex (D1)

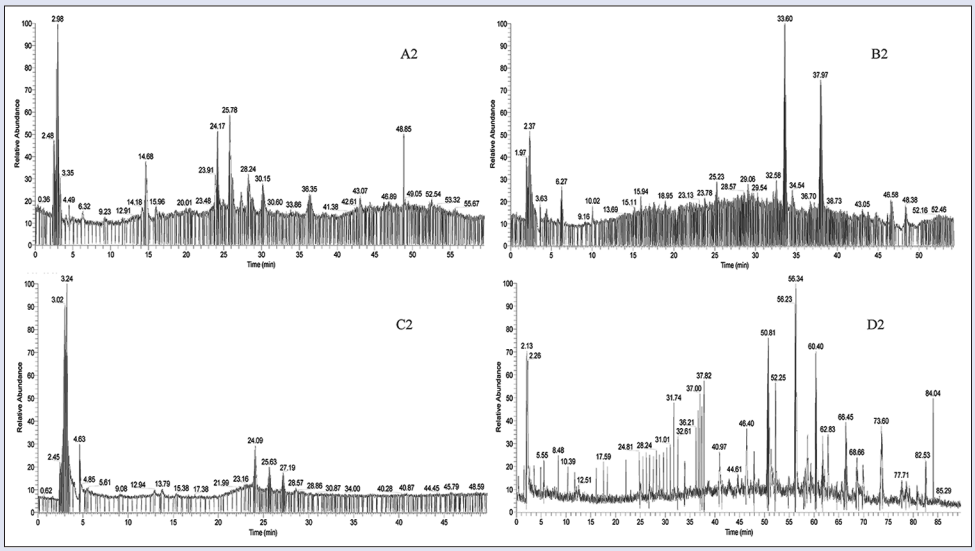

Figure 2: The base peak intensity chromatogram of M_{ME} of Mori Folium (A2), Mori Ramulus (B2), Mori Fructus (C2) and Mori Cortex (D2)

Table 3: Identification results of the compounds in methanolic extracts of the four medicinal organs of mulberry

Serial number	Molecular ion/[M-H]⁻	Fragment ion	Putative ID	Formula	Found
			Flavonoids		
1	609.11	300.90	Rutin	$C_{27}H_{30}O_{16}$	A, B, C
2	300.80	206.79/79.82	Quercetin	$C_{15}H_{14}O_{9}$	A, B, C
3	463.05	300.99	Isoquercetin	$C_{31}H_{20}O_{12}$	A, C
4	593.17	284.91	Kaempferol -3-O- rutin glycoside	$C_{27}H_{30}O_{15}$	A, C
5	447.08	283.93/284.89	Astragalin	$C_{21}H_{20}O_{11}$	A, C
6	302.97	285.04/124.94	Dihydromorin	$C_{15}H_{12}O_{7}$	B, D
7	693.35	582.23/354.20	Sanggenone G	$C_{40}H_{36}O_{11}$	B, D
8	691.24	581.34	Mulberrin K	$C_{40}H_{36}O_{11}$	B, D
9	421.21	299.26/244.08	Cudraflavanone C	$C_{25}H_{26}O_{6}$	B, D
10	423.25	297.22	Sanggenol A	$C_{25}H_{28}O_{6}$	B, D
11	625.25	300.89	Quercetin -3,7-di-O-β-D- glucopyranose	$C_{27}H_{30}O_{17}$	A
12	609.00	447.13	Kaempferol -3,7-di-O-β-D- glucopyranose	$C_{27}H_{30}O_{16}$	A
13	625.04	299.94/300.94	Quercetin -3-O-β-D-pyranoside-(1,6)-β-D-glucopyranoside	$C_{28}H_{32}O_{16}$	A
14	505.02	300.93	6”-acetyl isoquercitrin	$C_{23}H_{22}O_{13}$	A
15	514.83	378.82/447.10	6”-O-crotonyl astragalin	$C_{25}H_{24}O_{12}$	A
16	488.99	284.97	6”-acetyl astragalin	$C_{23}H_{22}O_{12}$	A
17	300.92	228.96/124.95	Morin	$C_{15}H_{10}O_{7}$	D
18	325.12	253.06	Moracin O	$C_{19}H_{18}O_{5}$	D
19	563.16	463.22	Kuwanol A	$C_{34}H_{28}O_{8}$	D
20	710.16	647.26/625.22	Sanggenone T	$C_{40}H_{40}O_{12}$	D
21	581.23	471.22/361.17	Mulberrin Y	$C_{34}H_{32}O_{9}$	D
22	707.21	369.17/353.40	Sanggenone C	$C_{40}H_{36}O_{12}$	D
23	758.85	695.08	Mulberrin W	$C_{45}H_{42}O_{11}$	D
24	693.18	567.30	Mulberrin O	$C_{40}H_{38}O_{11}$	D
25	707.21	638.28/597.39	Sanggenone D	$C_{40}H_{36}O_{12}$	D
26	419.11	310.09/364.18	Cyclomulberrin	$C_{25}H_{24}O_{6}$	D
27	419.27	297.13	Morusin	$C_{25}H_{24}O_{6}$	D
28	760.65	389.08/692.48	Sanggenol M	$C_{45}H_{46}O_{11}$	D
29	839.14	419.01	Moracin dimer	$C_{50}H_{46}O_{12}$	D
30	437.06	124.84/311.05	Mulberrin U	$C_{26}H_{30}O_{6}$	D
			Phenolic acid		
31	352.96	190.89	Chlorogenic acid	$C_{16}H_{18}O_{9}$	A, B, C
32	191.00	172.85	Quinic acid	$C_{7}H_{12}O_{6}$	A, B
33	352.92	190.85/172.87	Cryptochlorogenic acid	$C_{16}H_{18}O_{9}$	A, C
34	353.12	178.90/172.95	Neochlorogenic acid	$C_{16}H_{18}O_{9}$	A, C
35	352.98	190.92/178.94	1-caffeoylquinic acid	$C_{16}H_{18}O_{9}$	A, C
			2-arylbenzofurans		
36	561.28	451.24/439.24	Mulberrofuran G	$C_{34}H_{26}O_{8}$	D
37	579.20	469.20	Mulberrofuran C	$C_{34}H_{28}O_{9}$	D
38	579.24	469.11	Mulberrofuran J	$C_{34}H_{26}O_{8}$	D
39	457.08	325.24	Mulberroside C	$C_{24}H_{26}O_{9}$	D
			D-A adducts		
40	691.42	581.29/353.15	Kuwanon G	$C_{40}H_{36}O_{11}$	B, D
41	759.40	581.21/353.28	Kuwanon H	$C_{45}H_{44}O_{11}$	B, D
42	353.23	124.99/227.08	Albanin A	$C_{20}H_{18}O_{6}$	D
			Stilbenes		
43	567.36	405.18/243.08	Mulberroside A	$C_{26}H_{32}O_{14}$	B, D
44	243.05	224.98/228.05	Oxyresveratrol	$C_{14}H_{12}O_{4}$	B, D
			Triterpene		
45	469.22	359.17	Limonin	$C_{26}H_{30}O_{8}$	D

A, B, C and D represented the compounds were identified in Mori Folium, Mori Ramulus, Mori Fructus and Mori Cortex, respectively. And the same as below

M_{EA} and M_{BU}, whereas the fatty acids were mainly identified in M_{PE} and M_{CH}.

Molecular docking of anti-inflammatory and antitussive compounds

Table 8 shows the molecular docking scores obtained upon docking tested compounds against iNOS, TNF-α, IL-6, and IL-1 β as anti-inflammatory targets. In general, higher docking scores imply stronger activity. Among the 77 compounds docked, chlorogenic acid, mulberroside A, sanggenone C, and morin have been previously reported to inhibit the activity of iNOS.[13-15] Nonetheless, in this study, the results of molecular docking scores showed that cathafuran B, 6”-acetyl astragalin, quercetin, astragalin, 4-benzoic acid 4-*O*-β-D-glucopyranoside, 6”-*O*-crotonyl astragalin, and cudraflavanone C had stronger binding ability to iNOS targets as compared with chlorogenic acid, mulberroside A, sanggenone C and morin. Notably, among the top 10% of the compounds docked, 4 compounds were identified in Mori Folium, 2 compounds in Mori

Table 4: Identification results of the compounds in petroleum ether-soluble fraction of mulberry of the four medicinal organs of mulberry

Serial number	Molecular ion/[M-H]$^-$	Fragmention	Putative ID	Formula	Found
			Aliphatic acids		
1	311.12	293.15\223.11	Eicosanoic acid	$C_{20}H_{40}O_2$	B, C, D
2	329.25	229.12\211.14	9,10,11-trihydroxy-12-octadecenoic acid	$C_{18}H_{34}O_5$	A, C
3	327.15	171.09\229.04	Tri-hydroxy-octadecadienoic acid	$C_{18}H_{32}O_5$	A, C
			Flavonoids		
4	285.07	270.06\227.03	Ethyl palmitate	$C_{18}H_{36}O_2$	D
5	293.23	274.98	Hydroxy-octadecatrienoic acid	$C_{18}H_{30}O_3$	D
6	325.23	307.21\263.10	Heneicosanoic acid	$C_{21}H_{42}O_2$	D
			Other		
7	313.19	295.23\183.12	Dimethyl (methylenedi-4,1-phenylene) biscarbamate	$C_{17}H_{18}N_2O_4$	B, C, D

Table 5: Identification results of the compounds in chloroform - soluble fraction of mulberry of the four medicinal organs of mulberry

Serial number	Molecular ion/[M-H]$^-$	Fragment ion	Putative ID	Formula	Found
			Aliphatic acids		
1	327.26	170.98\228.84	Octadecadienoic acid	$C_{18}H_{32}O_5$	A, C, D
2	329.16	229.09\293.19	9, 10, 11-trihydroxy12-octadecenoic acid	$C_{18}H_{34}O_5$	B, C, D
3	329.15	170.89\229.06	9, 12, 13-trihydroxy-10-octadecenoic acid	$C_{18}H_{34}O_5$	C
			Flavonoids		
4	579.06	417.11	Naringin	$C_{27}H_{32}O_{14}$	A
5	419.03	404.15	Cyclomulberrochromene	$C_{25}H_{22}O_6$	B
6	285.07	270.06\227.03	Dihydrokaempferol	$C_{15}H_{12}O_6$	C
			Coumarin		
7	417.24	180.94\165.88	Syringaresinol	$C_{22}H_{26}O_8$	A

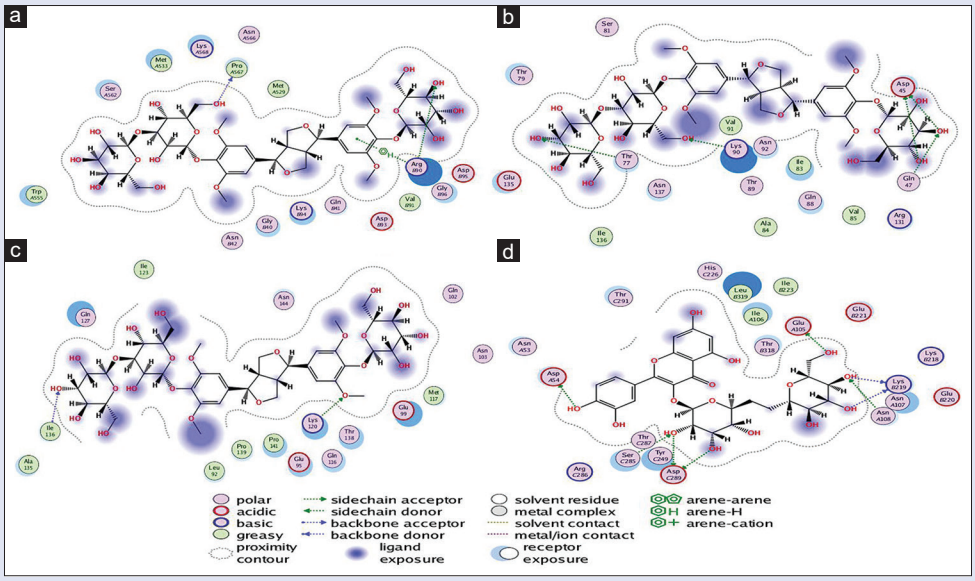

Figure 3: Representation of the best pose of compounds docked with anti-inflammatory target. (a) Cathafuran B docking with inducible nitric oxide synthase target. (b) Cathafuran B docking with tumor necrosis factor-α target. (c) Cathafuran B docking with interleukin interleukin-6 target. (d) Rutin docking with interleukin-1 β target

Ramulus, 2 compounds in Mori Fructus, and 3 compounds in Mori Cortex. Consequently, cathafuran B showed the strongest binding ability for iNOS [Figure 3a], as it formed three hydrogen bonds with two amino acid residues (Pro A567 and Arg B90) present in iNOS target.

Similarly, chlorogenic acid, sanggenone G, sanggenone C, morin, and quercetin have been demonstrated to inhibit the activity of TNF-α;[16-18] however, based on the results of molecular docking scores in this study, cathafuran B, mulberrin K, mulberrin Y, mulberrin O, mulberrofuran G, cyclomulberrochromene, and moracin dimer displayed stronger binding ability to TNF-α than that of the five compounds reported. Nevertheless, among the top 10% of the compounds, that is, 7 compounds, only 1 compound was identified in Mori Ramulus, whereas 6 compounds were identified in Mori Cortex. Cathafuran B showed the best binding ability to

Table 6: Identification results of compounds in ethyl acetate-soluble fraction of mulberry of the four medicinal organs of mulberry

Serial number	Molecular ion/[M-H]$^-$	Fragment ion	Putative ID	Formula	Found
Flavonoids					
1	463.10	301.01	Isoquercitrin	$C_{31}H_{20}O_{12}$	A, B, C, D
2	463.07	301.01	Morin-3-O-β-D- glucoside	$C_{31}H_{20}O_{12}$	A, B, C, D
3	300.94	150.97	Quercetin	$C_{15}H_{14}O_9$	B, C, D
4	302.95	285.04	Dihydromorin	$C_{15}H_{12}O_7$	B, C, D
5	287.09	258.97\243.06	Dihydrokaempferol	$C_{15}H_{12}O_6$	B, D
6	447.09	284.90	Astragalin	$C_{21}H_{20}O_{11}$	B, C
7	449.13	287.01	Dihydrokaempferol -7-O- glucoside	$C_{21}H_{20}O_{11}$	B, C
8	447.08	284.95\165.00	Kaempferol -7-O- glucoside	$C_{21}H_{20}O_{11}$	A, C
9	625.90	300.91	Quercetin -3-O-β-D- pyranose -(1-6)-β-D-glucopyranoside	$C_{28}H_{32}O_{16}$	B
10	327.20	291.15\170.90	Cudraxanthone S	$C_{18}H_{16}O_6$	B
11	609.05	300.96	Rutin	$C_{27}H_{30}O_{16}$	C
12	505.03	300.91	6”- acetyl isoquercetin	$C_{23}H_{22}O_{13}$	C
13	302.94	284.87	Taxifolin	$C_{15}H_{12}O_7$	D
14	300.99	228.93\256.82	Morin	$C_{15}H_{10}O_7$	D
2-arylbenzofurans					
15	307.04	289.10\234.97	Moracin D	$C_{19}H_{16}O_4$	A
16	307.04	289.03\234.98	Moracin G	$C_{19}H_{16}O_4$	A
17	309.24	291.10\183.05	Moracin C	$C_{19}H_{18}O_4$	A
18	241.07	196.94\199.15	Moracin M	$C_{14}H_{10}O_4$	A
19	457.02	325.04	Mulberroside C	$C_{24}H_{26}O_9$	D
20	325.03	253.02	Moracin O	$C_{19}H_{18}O_5$	D
Phenolic acids					
21	353.03	190.96	Chlorogenic acid	$C_{16}H_{18}O_9$	C
22	353.92	172.95\178.89	Neochlorogenic acid	$C_{16}H_{18}O_9$	C
23	352.94	190.97	Cryptochlorogenic acid	$C_{16}H_{18}O_9$	C
Coumarins					
24	338.91	176.90	5- hydroxycoumarin -7-O-β-D-furan glycoside	$C_{15}H_{16}O_9$	A
Stilbenes					
25	242.97	224.92	Oxyresveratrol	$C_{14}H_{12}O_4$	B, D
Aliphatic acid					
26	311.13	293.17\182.97	Eicosanoic acid	$C_{20}H_{40}O_2$	A

TNF-α [Figure 3b], which was due to its bonds formed with several surrounding amino acids including five hydrogen bonds formed with Lys90, Asp45, Gln47, and Thr77.

According to the literature, chlorogenic acid and sanggenone G and C show good inhibitory against IL-6.[16,19] In this study, cathafuran B, moracin dimer, isoquercitrin, and mulberrin U exerted stronger binding ability to IL-6 targets than them. Among the top 10% of the compounds with better binding ability to IL-6, 3 compounds were identified in Mori Folium, 2 compounds in Mori Ramulus, 2 compounds in Mori Fructus, and 6 compounds in Mori Cortex. Cathafuran B bonded to Lys120 and IIe136 in IL-6 with two hydrogen bonds, which had the best docking activity [Figure 3c].

Previous reports indicate that sanggenone C, quercetin, and moracin M show good inhibitory activity against IL-1 β;[16,17,20] however, our results showed that rutin; quercetin-3-*O*-β-D-py ranosyl-(1,6)-β-D-glucopyranoside; 6”-*O*-crotonyl astragalin; cyclomulberrochromene; and mulberrin Y combined strongly with the target protein as compared with quercetin, moracin M, and sanggenone C. Of note, out of the top 10% of the compounds screened, five compounds were identified in Mori Folium, 3 compounds in Mori Ramulus, 3 compounds in Mori Fructus, and 3 compounds in Mori Cortex. Among them, rutin had the best binding ability with IL-1 β [Figure 3d]. It formed eight hydrogen bonds with several surrounding amino acids including Asp AS4, Asp C289, Glu A105, Lys B219, Ser C285, and Asn A108.

Table 9 shows the results of molecular docking with phosphodiesterase (PDE) 4B and β2 adrenergic receptors as antitussive targets. Among the 77 compounds screened, previous reports indicate that moracin M and sanggenone C are potential inhibitors of PDE 4B;[21] however, the results of our study showed that quinic acid, cathafuran B, mulberrin U, isoquercitrin, ethyl palmitate, sanggenon G, and cryptochlorogenic acid binding to the target protein better than PDE 4B and β2 adrenergic receptors. Among the top 10% of the compounds screened, 3 compounds were identified in Mori Folium, 2 compounds in Mori Ramulus, 2 compounds in Mori Fructus, and 6 compounds in Mori Cortex. Consequently, quinic acid had the best binding stability with PDE 4B target with five hydrogen bonds through Asn 283, Glu 304, Met 347, and Asp 346 [Figure 4a].

The results of molecular docking with β2 adrenergic receptors showed that the top 10% of the compounds with better docking capability were mostly flavonoids that were identified in Mori Cortex. Furthermore, mulberrin U was the best in docking as it forms two hydrogen bonds with Gly A41 and Thr B165 in β2 adrenergic receptor target [Figure 4b]. Of note, mulberrin U was only identified in Mori Cortex.

DISCUSSION

Identification of compounds from the four medicinal parts of mulberry

A total of 77 compounds were identified from the four medicinal parts of mulberry based on HPLC-MS^n analysis. Of them, 42 were flavonoids,

Table 7: Identification results of compounds in *n*- butanol-soluble fraction of the four medicinal organs of mulberry

Serial number	Molecular ion/[M-H]⁻	Fragment ion	Putative ID	Formula	Found
			Flavonoids		
1	315.02	152.92\151.78	Isorhamnetin	$C_{16}H_{12}O_7$	A, C
2	463.07	300.94	Isoquercitrin	$C_{31}H_{20}O_{12}$	B, C
3	625.10	463.01\300.99	Quercetin -3-O-β-D- pyranose -(1-6)-β-D-glucopyranoside	$C_{23}H_{22}O_{13}$	A
4	609.11	477.12	Kaempferol -3,7-di-O-β-D- glucopyranose	$C_{27}H_{30}O_{16}$	A
5	315.02	178.95\164.95	3'- methoxy-quercetin	$C_{16}H_{12}O_7$	A
6	625.10	463.02\301.07	Quercetin-3, 7-di-O-β-D- pyranose	$C_{27}H_{30}O_{17}$	A
7	609.18	301.10	Rutin	$C_{27}H_{30}O_{16}$	D
8	611.18	300.94	Kaempferol -3-O-β-D- pyranose -(1,6)-β-D- glucopyranoside	$C_{28}H_{32}O_{16}$	D
9	593.21	284.91	Kaempferol -3-O-rutin glycoside	$C_{27}H_{30}O_{15}$	D
10	284.77	190.85	Kaempferol	$C_{15}H_{10}O_6$	C
			Coumarin		
11	904.34	845.94	Liriodendrin A	$C_{40}H_{56}O_{23}$	B
12	471.11	178.86	5- hydroxyl coumarin -7-O-β-D- furacinyl-(1-6)-O-β-D- glucoside	$C_{20}H_{24}O_{13}$	B, D
13	190.89	172.93\126.86	Scopoletin	$C_{10}H_8O_4$	B, C
			Aliphatic acid		
14	190.89	84.90\173.03	Quininic acid	$C_7H_{12}O_6$	C
15	352.99	190.8 8\172.92	Chlorogenic acid	$C_{16}H_{18}O_9$	C
			D-A adducts		
16	477.07	315.01	Guangsangon L	$C_{27}H_{24}O_8$	A
			2-arylbenzofurans		
17	378.74	284.80	Cathafuran B	$C_{24}H_{26}O_4$	C
			Others		
18	299.94	270.92\235.01	Benzaldehyde-4-O-α-L-rhamnopyranoside	$C_{13}H_{16}O_8$	D

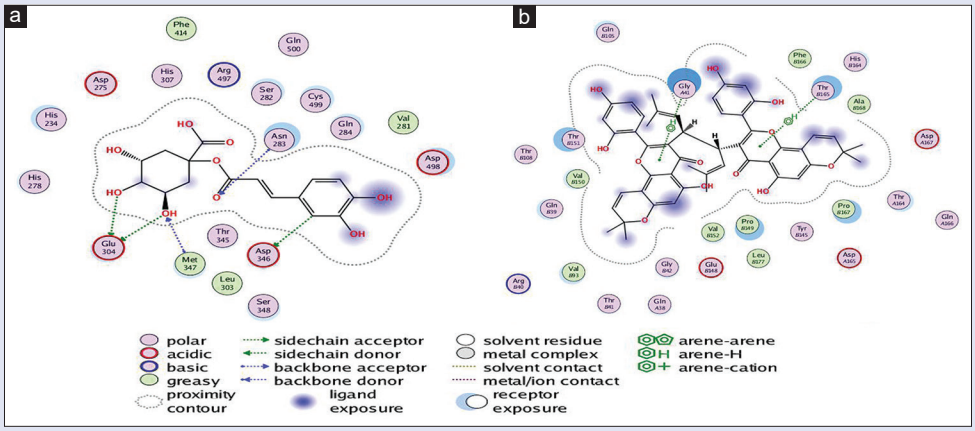

Figure 4: Representation of the best pose of compounds docked with antibechic target. (a) Quinic acid docked with PDE 4B target. (b) Muberrin U docked with β2 adrenergic receptors target

9 were 2-aryl benzofurans, 7 were fatty acids, 5 were coumarins, 5 were phenolic acids, 4 were Diels-Alder-type adducts, 2 were stilbenes, 1 was triterpene, and 2 were other species. Among these compounds, thirty were identified in Mori Folium, 27 compounds in Mori Ramulus, 23 compounds in Mori Fructus, and 46 compounds in Mori Cortex, and they have 14, 4, 3, and 26 exclusive compounds, respectively [Figure 5]. Moreover, it was found that Mori Ramulus and Mori Cortex shared the most similar chemical composition with 19 compounds, which corroborate the results of Zhao *et al.*[6]

The differences in types and content of active compounds are generally considered to be the key factors for the multiple efficacies of TCM. In this study, the differences in the chemical composition of the four medicines can be mainly attributed to flavonoids, Diels-Alder adducts, and 2-arylbenzofuran, which is consistent with a previous report.[22] These compounds were obtained from the same source, but they demonstrated different efficacies. In this regard, further explanation could be made from the analysis of content determination and metabolic network pathways in future works.

Analysis of molecular docking with anti-inflammatory targets

According to the modern pharmacological research, four mulberry medicines all have anti-inflammatory activity. Therefore, to explore the anti-inflammatory effects through molecular docking of the various compounds identified in the aforementioned medicines, iNOS, TNF-α, IL-6, and IL-1 β were selected as the targets. The results showed that the

Table 8: Docking scores of the compounds bonded with inducible nitric oxide synthase, tumor necrosis factor-alpha, interleukins-6 and interleukins-1 (top 10%)

Compounds	Score
iNOS	
Cathafuran B	−7.99
6”-acetyl astragalin	−7.94
Quercetin	−7.82
Astragalin	−7.72
Benzoic acid 4-O-β-D-glucopyranoside	−7.52
6”-O-crotonoyl astragalin	−7.46
Cudraflavanone C	−7.40
TNF-α	
Cathafuran B	−8.00
Mulberrin K	−7.59
Mulberrin Y	−6.94
Mulberrin O	−6.92
Mulberrofuran G	−6.91
CycloMulberrochromene	−6.89
Morusin dimer	−6.83
IL-6	
Cathafuran B	−8.45
Moracin dimer	−7.24
Isoquercetin	−7.20
Mulberrin U	−7.09
Chlorogenic acid	−6.90
Limonin	−6.90
Kaempferol -7-O- glucoside	−6.89
IL-1β	
Rutin	−9.68
Quercetin-3-O-β-D-pyranosyl-(1,6)-β-D-glucopyranoside	−8.94
6”-O-crotonoyl astragalin	−8.80
Cyclomulberrochr-omene	−8.73
Mulberrin Y	−8.61
Quercetin	−8.23
6”-acetyl isoquercitrin	−8.20

IL: Interleukins; TNF-α: Tumor necrosis factor-alpha; iNOS: Inducible nitric oxide synthase

Table 9: Docking scores of the compounds bonded with phosphodiesterase 4B and β2 adrenergic receptors (top 10%)

Compounds	Score
PDE 4B	
Quinic acid	−7.99
Cathafuran B	−7.10
Mulberrin U	−7.09
Isoquercitrin	−7.05
Ethyl palmitate	−7.02
Sanggenon G	−6.98
Cryptochlorogenic acid	−6.97
β2 adrenergic receptors	
Mulberrin U	−9.76
Cathafuran B	−9.21
Cudraflavanone C	−9.17
Sanggenone D	−8.87
Morusin dimer	−8.72
Mulberrin W	−8.40
Limonin	−8.22

PDE: Phosphodiesterase

shared compounds, namely, quercetin, chlorogenic acid, and isoquercitrin all had good docking effects on the aforementioned anti-inflammatory targets, which is consistent with the pharmacological activities of their monomeric compounds,[14,23,24] as well the four medicines. Out of the top 10% of the compounds (7 compounds) with better docking effects on TNF-α and IL-6, 6 of them were identified in Mori Cortex. Mori Fructus had less of those compounds, which is consistent with the fact that it is not mainly used as anti-inflammatory drugs in clinical treatment, while Mori Cortex was.[25]

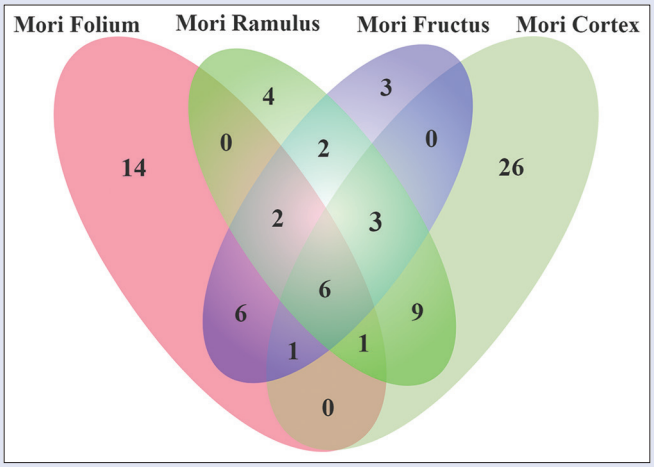

Figure 5: Venn diagram of the number of compounds that identified in Mori Folium, Mori Ramulus, Mori Fructus and Mori Cortex

The top 10% of the compounds docked into the binding sites of IL-1 β showed lower scores than that of other targets, which indicated that they were well bound. Out of the 10% of them, five compounds were identified in Mori Folium. Moreover, rutin, a shared compound identified in both Mori Folium and Mori Fructus, exerted 8 hydrogen bonds with 6 surrounding amino acid residues of IL-1 β, which implied that it is an excellent potential anti-inflammatory agent. Of note, recent pharmacological studies showed that rutin can effectively reduce the level of proinflammatory cytokines such as IL-1 β and TNF-α, regulate inflammation,[26,27] and play a protective role in liver and kidney poisoning,[26] nerve injury and optic neuropathy,[28] cardiovascular,[29] breast tissue,[30] and so on, which further corroborated our research results. Interestingly, cathafuran B showed good binding ability to amino acid residues of iNOS, TNF-α, and IL-6 which revealed that it is a potential anti-inflammatory agent among the compounds tested; nonetheless, there are only few studies conducted to enumerate its efficacy.

Analysis of molecular docking with antitussive targets

PDE 4B and β2 adrenergic receptors are frequently used as cellular targets for the treatment of pulmonary inflammation;[31,32] hence, the two were chosen as antitussive targets. In this study, among the top 10% of the compounds with better docking effect on PDE 4B and β2 adrenergic receptors, 11 compounds were identified in Mori Cortex, especially quinic acid and mulberrin U, which had the best binding activity with PDE 4B target and β2 adrenergic receptors, respectively, but only 2 or 3 compounds were identified in Mori Folium, Mori Ramulus, and Mori Fructus. This result is consistent with the traditional efficacy of Mori Cortex as the “antitussive and anti-asthmatic” medicine.[1] For instance, Cai *et al.*[32] found that the water extracts and 95% ethanol extracts of Mori Cortex inhibited the activity of PDE4. Wang *et al.*[33] observed that the decoction of Mori Cortex has good effects of relieving cough and asthma, eliminating phlegm, and its effective fraction is present in 30% ethanolic extract. Moreover, total flavonoids,[34] mulberroside A,[35] and acetone extracts (identified as nonflavonoids)[36] of Mori Cortex, have antitussive and anti-asthmatic effects. Based on the abovementioned findings, this study further

highlights the ingredients with the effectiveness as an antitussive for Mori Cortex.

CONCLUSION

In this study, we systematically analyzed the chemical constituents of four medicinal parts of mulberry. A total of 77 compounds were identified from M_{Me} and different polar fractions of water extracts, out of which 42 were flavonoids and 9 were 2-arylbenzofurans. Notably, thirty compounds were identified in Mori Folium, 27 compounds in Mori Ramulus, 23 compounds in Mori Fructus, and 46 compounds in Mori Cortex, and they had 14, 4, 3, and 26 compounds that were specific to them, respectively. Comparatively, Mori Ramulus and Mori Cortex had more similar chemical composition. The top 10% of the compounds with better docking effect to anti-inflammatory activity targets were mostly identified in Mori Cortex and the least were in Mori Fructus. Furthermore, the top 10% of compounds with better antitussive activity were 11 compounds identified in Mori Cortex, and only 2 or 3 compounds were identified in other three medicines. These results corroborate clinical application of the four medicines, which further explained the correlation between chemical composition and pharmacodynamics. The results of this study laid the foundation for pharmacodynamics research on distinct medicinal organs of mulberry, and also provided new insights in the research of many TCMs with different efficacies but with same origin.

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Conflicts of interest

There are no conflicts of interest.

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