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Chemical and biological research of *Clematis* medicinal resources

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Clematis is a botanical source for various pharmaceutically active components, which has long been used in conventional medicine since the beginning of Chinese civilization. Increasing interest in *Clematis* medicinal resources has led to additional discoveries of triterpenoid saponins, flavonoids, coumarins, alkaloids and many other compounds in various *Clematis* species, and to investigations on their chemotaxonomy, molecular phylogeny and pharmacology. In continuation with our studies on *Clematis* chemistry and biology, we review the chemistry, chemotaxonomy, molecular biology and phylogeny of *Clematis* and their relevance to drug efficacy and drug development. Various databases and technology have been used in literature search in order to characterize the global scientific effort. It is essential to study more species for both the sustainable utilization of *Clematis* medicinal resources and finding novel compounds with potential clinical utility. Systems biology and omics technologies will play an increasingly important role in future medicinal research involving bioactive compounds of *Clematis*.

***Clematis*, chemical components, chemotaxonomy, molecular taxonomy, medicinal resource**

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Clematis is a genus of about 355 species [1] within the eudicot family Ranunculaceae. The genus consists of typically vigorous, woody, climbing vines/lianas, which are mainly distributed in the temperate zone of the north of Earth's equator. *Clematis* is a botanical source of various pharmaceutically active components, which has long been used in conventional medicine since the beginning of Chinese civilization. Roots, rhizomes and stems of some *Clematis* species are used to disperse wind-damp, unclog channels and ease pain. Wei Ling Xian and Chuan Mu Tong, recorded in the Chinese Pharmacopoeia (http://www.chp.org.cn/cms/home/), are the most well-known Chinese herbal medicines obtained from *Clematis* species. The former is from *Clematis chinensis*, *C. hexapetala* and *C. mandshurica*, while the latter from *C. armandii* and *C. montana*. Traditionally, *Clematis* medicine is administered orally to treat sexually transmitted infection, podagra, rheumatoid arthritis, bone disorder, chronic skin diseases, and is used as a diuretic. In folk medicine, *Clematis* is applied to body surfaces for blisters and is also used as a cataplasm to treat purulent infections and ulcers. Different *Clematis* species may have dissimilar drug effects. Though, it is pretty hard to authenticate *Clematis* species only by morphology.

To date, at least 30 *Clematis* species have been characterized for their chemical components. However, it is essential to study more species for both the sustainable utilization of *Clematis* medicinal resources and finding novel compounds with potential clinical utility. In this brief review, we focus on recent progress in phytochemistry and chemotaxonomy of *Clematis*, as well as molecular taxonomy and pharmacology.

1 Methods

Information was withdrawn from Google Scholar and the journal databases Scopus, PubMed and CNKI (http://www.cnki.net).

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2 Chemical components of the genus *Clematis*

2.1 Triterpenoid saponins

Triterpenes are assembled in a four or five ring configuration of 30 carbons with a number of oxygens appended. The aglycone of *Clematis* plants is of five-ring triterpenoid oleanane type, including oleanolic type (Figure 1A), olean-3β, 28-diol type (B), hederagenin type (C) and hederagenin-11,13-dien type (D). The conjugated glycosyl groups include glucose (Glc, D configuration), rhamnose (Rha, L configuration), galactose (Gal, D configuration), arabinose (Ara, L configuration), xylose (Xyl, D configuration) and ribose (Rib, D configuration). Since 2009, 19 novel triterpenoid saponins have been isolated from *Clematis* plants (Table 1). To date, more than 50 oleanolic type prototype saponins, more than 40 hederagenin type prototype saponins and two gypsogenin saponins have been found in *Clematis* plants [2,3]. Most of these saponins are bidesmosidic, i.e. C-3 and C-28 tie with the oligosaccharide chains. The monodesmosidic saponin is less common. Some oligosaccharide chains are substituted with acetyl, caffeoyl (CA), isoferuloyl (IF), *p*-methoxy cinnamyl (MC) and 3,4-dimethoxy cinnamyl (DMC) groups (Figure 1). In addition, more than 20 secondary glycosides have been found [2], which lose their C-28 oligosaccharide chains after hydrolysis.

Two new saponins from *C. argentilucida* showed noteworthy cytotoxicity against human leukemia cells, hepatocellular carcinoma cells and glioblastoma cells [3]. Triterpene saponins from *C. mandshurica* inhibit human colon cancer cells [4]. Four triterpene glycosides from *C. ganpiniana* showed cytotoxicity against cancer cells and antibacterial activity [5]. However, the full potential of the anticancer activity of *Clematis* saponins cannot be revealed unless

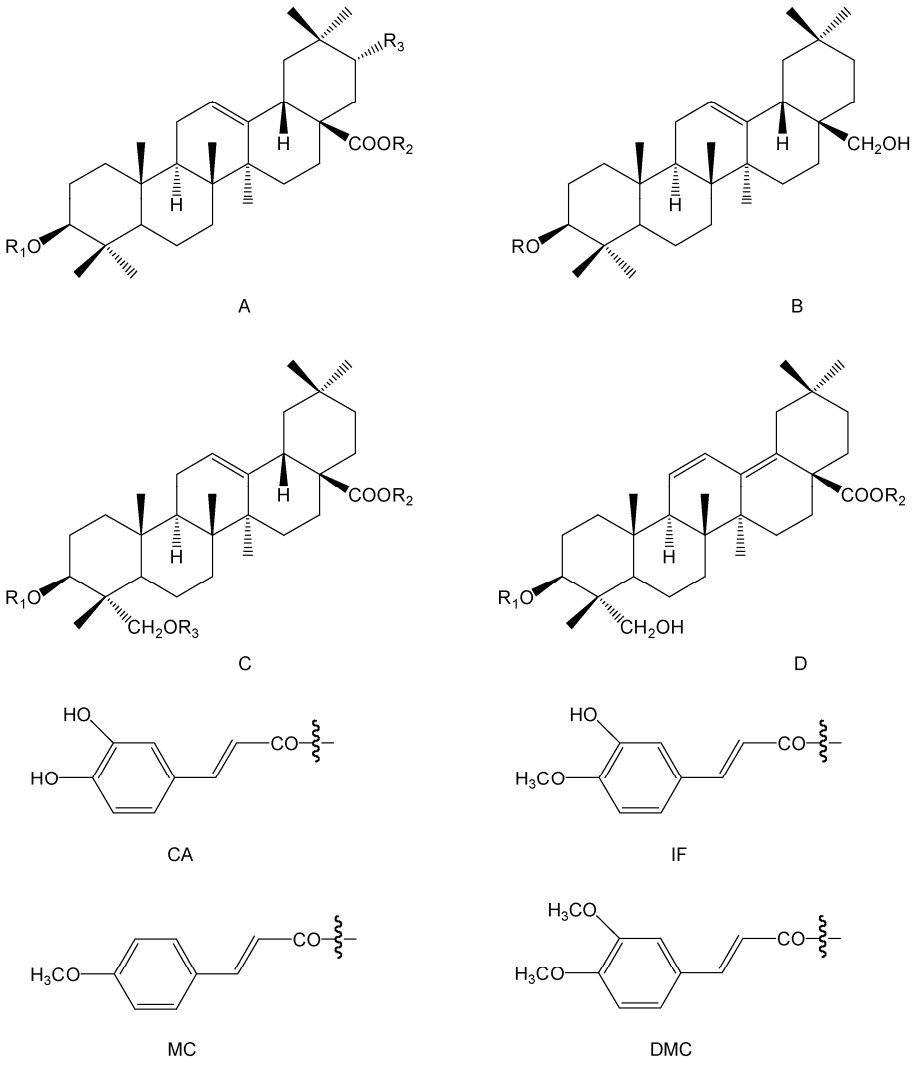

Figure 1 Aglycone and the substituent linked with glycosyl groups of triterpenoid saponins of *Clematis*. A, Oleanolic type; B, olean-3β, 28-diol type; C, hederagenin type; D, hederagenin-11,13-dien type; CA, caffeoyl; IF, isoferuloyl; MC, *p*-methoxy cinnamyl; DMC, 3,4-dimethoxy cinnamyl. Modified from [2].

Table 1 Triterpenoid saponins found in *Clematis* medicinal plants in recent years[a)]

	Compound	Aglycone	Oligosaccharide chain	Species	Tissue	Reference
1	3β-O-[β-D-ribopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl] hederagenin-11,13-dien-28-oic acid	D	R1=Rib(1→3)-Rha(1→2)-Ara-, R2=H	*C. argentilucida*	root	[3]
2	3β-O-{β-D-ribopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-xylopyranosyl} oleanolic acid	A	R1=Rib(1→3)-Rha(1→2)-Glc(1→4)-Xyl-, R2=H, R3=H	*C. argentilucida*	root	[3]
3	3β-O-[β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl]-12-oleanene-3β, 28-diol	B	R= Xyl(1→3)-Rha(1→2)-Glc-	*C. argentilucida*	root	[59]
4	mandshunosides A	C	R1=Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2=H, R3=H	*C. mandshurica*	roots, rhizomes	[4]
5	mandshunosides B	A	R1=Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2=H, R3=H	*C. mandshurica*	roots, rhizomes	[4]
6	3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-ribopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl oleanolic acid 28-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (clematomandshurica saponin E)	A	R1=Rha(1→6)-Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Glc(1→6)-Glc-, R3=H	*C. mandshurica*	roots, rhizomes	[60]
7	clematochinenosides A	C	R1=Glc(1→2)-Rha(1→6)-Glc(1→3)-Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Rha(1→4)-Glc(1→6)-Glc-	*C. chinensis*	roots, rhizomes	[8]
8	clematochinenosides B	C	R1=Glc(1→2)-Rha(1→6)-Glc(1→3)-Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Rha(1→4)-Glc(1→6)-Glc-	*C. chinensis*	roots, rhizomes	[8]
9	clematochinenosides C	C	R1=Rha(1→6)-Glc(1→4)-Glc(1→3)-Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Rha(1→4)-Glc(1→6)-Glc-	*C. chinensis*	roots, rhizomes	[8]
10	clematochinenosides D	C	R1=Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Rha(1→4)-Glc(1→6)-Glc-	*C. chinensis*	roots, rhizomes	[8]
11	clematochinenosides E	C	R1=Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Rha(1→4)-Glc(1→6)-Glc-	*C. chinensis*	roots, rhizomes	[8]

(*To be continued on the next page*)

(*Continued*)

	Compound	Aglycone	Oligosaccharide chain	Species	Tissue	Reference
12	clematochinenosides F	A	R1=Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Rha(1→4)-Glc(1→6)-Glc-, R3=H	*C. chinensis*	roots, rhizomes	[8]
13	clematochinenosides G	C	R1=Glc(1→3)-Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Rha(1→4)-Glc(1→6)-Glc-	*C. chinensis*	roots, rhizomes	[8]
14	3β-[(α-L-arabinopyranosyl)-oxy]-olean-12-en-28-oic acid	A	R1=Ara, R2=R3=H	*C. ganpiniana*	roots, rhizomes	[5]
15	hederagenin 3β-O-α-L-arabinopyranoside	C	R1=Ara, R2=R3=H	*C. ganpiniana*	roots, rhizomes	[5]
16	3β-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl oleanoic acid	A	R1=Rha(1→2)-Ara-, R2=H, R3=H	*C. ganpiniana*	roots, rhizomes	[5]
17	α-hederin	C	R1=H, R2=H, R3=H	*C. ganpiniana*	roots, rhizomes	[5]
18	3-O-β-[(O-α-L-rhamnopyranosyl-(1→6)-O-β-D-glucopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→4)-O-β-D-ribopyranosyl-(1→3)-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]olean-12-en-21α-hydroxy-28-oic acid-O-α-L-rhamnopyranosyl-(1→ 4)-O-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (clematichinenoside AR2)	A	R1=Rha(1→6)-Glc(1→4)-Glc(1→4)-Rib(1→3)-Rha(1→2)-Ara-, R2= Rha(1→4)-Glc(1→6)-Glc-, R3=OH	*C. chinensis*	roots	[61]
19	23-O-acetyl-hederagenin-3-O-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-l-arabinopyranoside (parvilobaside A)	C	R1=Rib(1→3)-Rha(1→2)-Ara-, R2= H, R3=Ac	*C. parviloba*	stem	[62]

a) Aglycone: A, oleanolic type; B, olean-3β, 28-diol type; C, hederagenin type; D, hederagenin-11,13-dien type. The locations of R1, R2 and R3 are shown in Figure 1.

much more pharmacological studies are performed.

Traditionally, Wei Ling Xian is used to treat gout, obstinate bi-syndrome, rheumatism, limb numbness, cold pain in the lumbus and knees, and inhibited bending and stretching, etc. Correspondingly, the triterpene saponin AR-6 from *C. chinensis*, a source plant of Wei Ling Xian, has a potential anti-inflammatory effect in rats with collagen-induced arthritis, which inhibits the expression of NF (nuclear factor) -κB p65 subunits, TNF (tumor necrosis factor) -α and COX (cyclo-oxygenase)-2 [6]. Anti-arthritis effects of AR-6 were related to substantial decline of nitric oxide and TNF-α generated by peritoneal macrophages [7]. In addition, AR-6 appreciably reduced the propagation of synoviocyte. Multiple triterpene saponins of *C. chinensis* showed the potential anti-inflammatory activity with inhibitory activities against COX-1 and COX-2 enzymes [8]. Saponins might be responsible for the inhibition effects of *C. chinensis* on the pro-inflammatory and degradative mediators associated with inflammatory arthritis [9]. The saponin fraction from this plant showed the therapeutic effect on monosodium iodoacetate induced osteoarthritis via sheltering articular cartilage [10]. The release of the full potential of *Clematis* saponins calls for more extensive and deeper investigations.

2.2 Flavonoids and anthocyanins

Flavonoids (or bioflavonoids) are a class of plant secondary metabolites. More than 50 flavonoid compounds have been isolated or detected from *Clematis* [2,11–14], the aglycones of which are mainly apigenin，kaempfeml，luteolin and quercetin. Sugar moieties are connected to the aglycone through either the oxygen or the carbon atom. Flavonoids of *Clematis* are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones and their

glycosides, xanthones and anthocyanidins.

Flavonoids were the major vigorous compounds in four preparative extracts of *C. terniflora*, which might be responsible for reducing sensitivity to painful stimuli and anti-inflammatory effect in rats with carrageenan-induced persistent non-bacterial prostatitis [15]. Anti-inflammatory, pain-relieving and fever-reducing effects of the aqueous extract of *C. brachiata* leaf in male rats may be due in part to the flavonoids [16]. Two flavone C-glycosides from *C. rehderiana* showed potent antioxidant activities [12]. Studies of pharmacological effects of *Clematis* flavonoids are still in the burgeoning stage.

Twenty-four lignans isolated from *Clematis* are mainly eupomatene lignans, cyclolignans, monoepoxylignans, bisepoxylignans and lignanolides [2]. Six coumarin compounds have been isolated [2,17]. Eleven alkaloids isolated from *Clematis* fall into two categories: aporphine and terpenoid alkaloid. Two aporphine alkaloids showed potent antifungal activities [18]. A mannose-binding lectin was separated from *C. Montana* and showed antiviral and apoptosis-inducing activities [19]. Many other compounds were also isolated from various *Clematis* species, such as phenolic glycosides [20], volatile oils, triterpenes, steroids, organic acids and their derivatives [21], macrocyclic compounds and others [22]. Their functions and pharmacological effects await further studies.

3 Chemotaxonomy of *Clematis*

The genus *Clematis* was founded by Linnaeus (1753) with only nine species. The following taxonomic studies increased the number of species: Johnson [23] and Grey-Wilson [24] independently summarized 314 and 297 species respectively; Wang and Li [1] depicted 355 species in the new classification of the genus, with around 150 species scattered in China. Four subgenera and ten sections are possibly defined based on a number of essential morphological characters of vegetative and floral organs. The four subgenera are (1) *Cheiropsis* Peterm.: section *Cheiropsis* DC.; (2) *Clematis* Keener & Dennis: sect. *Clematis* Tamura, *Meclatis* Baill., *Fruticella* Tamura, *Naraveliopsis* Hand.-Mazz, *Viticella* DC. and *Tubulosae* Decne.; (3) *Viorna* Gray: sect. *Viorna* Prantl and *Archiclemaits* Tamura; and (4) *Atragene* Torr. & Gray: sect. *Atragene* DC. A consensus has not been reached on taxonomy, origin and systematic location of this genus.

According to pharmacophylogeny, plants with close genetic relationship are similar in systematic classification and chemical components [25,26]. A chemosystematic study of sect. *Viorna*, subsect. *Viornae* was performed using flavonoid distribution patterns [27]. The close relationship between *C. viorna* and *C. reticulata* inferred from their form and structure and geographical allocation was corroborated by the resemblance of their flavonoid profiles. The supposition that *C. glaucophylla* recently derived from *C. versicolor* was propped up by their alike flavanoid contours, and the flavonoid multiplicity of *C. pitcheri* samples parallelled their morphological diversity. Flavonoids were used as chemical markers to argue the taxonomy of Ranunculaceae [28]. Feng et al. [29] compared the total flavonoid content of above-ground parts of 11 *Clematis* species. *C. apiifolia* leaves had the highest (5.581%) total flavonoid while *C. huchouensis* had only 0.195%. The amount of flavones in different species varied much. The cluster analysis based on the flavonoid content set apart the species of the subgenus *Clematis* and those of the subgenus *Viorna*, except *C. terniflora* and *C. huchouensis*. The aerial parts of *C. apiifolia* and *C. finetiana* could be used for flavonoid drug development.

Plant saponins have been used as chemotaxonomic markers to differentiate various taxa [30–32]. Saponins have been separated from a range of *Clematis* species [2,11], some of which were used for the chemosystematic study. Muhammad Ishtiaq et al. [33] analyzed 15 populations, representing 12 species of sections *Rectae*, *Clematis*, *Meclatis*, *Tubulosae* and *Viorna*, with HPLC (High-performance liquid chromatography) linked with diode array detector and ESI (electrospray ionization)-MS (mass spectrometry) [34]. A cluster tree was constructed in terms of the distribution of nine saponins and their peak area in the respective species. Three clades were identified. Clade I includes species of sect. *Rectae* of subgenus *Clematis*, *C. finetiana*, *C. armandii*, *C. chinensis* and *C. terniflora*, which share five saponins: Huzhangoside B (HGB), Clematichinenoside C (CCC), Seiboldianoside A (SDA), Huzhangoside D (HGD) and Clematichinenoside B (CCB). Clade II has *C. apiifolia*, *C. argentilucida*, *C. ganpiniana* and *C. peterae*, which belong to sect. *Clematis* and share saponins CCB, HGD, CCC and HGB. *C. henryi* of subgenus *Viorna* shares HGD and Clemochinenoside A (CCA) with *C. heracleifolia* of subgenus *clematis* and they were grouped together in clade III. This illogicality implies that more saponins and/or other secondary metabolites should be used in the chemotaxonomic analysis. *C. huchouensis* of subgenus *clematis* was quite different in total flavonoid [29] and saponin composition, which may have divergent secondary metabolic pathway.

Many studies have investigated the chemical components and bioactivities of *Radix et Rhizoma Clematidis* (Wei Ling Xian), *Caulis Clematidis Armandii* (Chuan Mu Tong) and other *Clematis* species. Triterpenoid saponins are rich in many species (Table 1) and give multiple pharmacological effects. However, raw drugs from other species are also found on the market without verification of efficacy, which may be abused clinically. It is therefore obligatory to resolve the distribution and the contents of the pharmaceutical ingredients in the approved *Clematis* species for standardization and quality control. Sun et al. [35] developed a straightforward and precise method with HPLC and evapo-

rative light scattering detection to concurrently resolve five triterpenoid saponins of *Clematis*. The chemical outlines were used to identify the botanical origin of ten *Clematis* samples. The contents of HGD and 3-O-α-L-ara(2-1)-α-L-rha(4-1)-β-D-rib pulsatiloside C were much higher in *C. chinensis* than in other species, which match its efficacy in clinical use. However, these saponins were not found in the above-ground part of *C. manshurica*, suggesting that the aerial fraction of this plant is not suitable as a source of Wei Ling Xian. The saponins were abundant in *C. ganpiniana* and *C. apiifolia var. obtusidentata*, which are promising pharmaceutical resources. No triterpenoid saponins were spotted in *C. armandii*, which agrees with previous chemical studies [36]. Thus its drug effects might be associated with other compounds. Five saponins in the roots and stems of *C. ganpiniana*, *C. apiifolia* and *C. apiifolia var. obtusidentata* were more abundant than those in the whole plants except for CCB and CCC of *C. apiifolia*, which suggests that root and stem might be a better medicinal part than the leaves.

The research group led by Cheng YY and Xiao PG performed a series of *Clematis* studies. A simple and fast gradient HPLC-MS method was built up and validated for parallel determination of CCB, HGD, CCC and HGB in *Radix Clematidis* and related species [37]. Optimally, the saponins were well separated in 25 min. The samples hold a wide range (0.0652–41.7 mg/g) of the four analytes. *Radix Clematidis* of Tianmu mountain, Zhejiang, China has the highest saponin content (41.7 mg/g), which was about seven times that in the commercial *Radix Clematidis* (China market) (5.80 mg/g). This could be caused by different harvest time and places, suggesting that the source of *Radix Clematidis* should be defined and curbed. The advantages of this method are good linearity, high sensitivity, precision, accuracy and shorter time, which are obliging in the quality control of *Clematis* medicine. Triterpenoid saponins were spotted by qualitative HPLC-MS/MS of root and rhizome of 18 *Clematis* samples and the whole plant of *C. puberula var. ganpiniana* and *C. terniflora* [38]. The HPLC-MSn identified 17 oleanolic acid or hederagenin saponins, which were used for phylogenetic relationship inference. Three branches were identified based on the 17 peaks. The first branch comprised *C. lasiandra* (subgenus *Viorna*), *C. finetiana*, *C. terniflora* (whole plant), *C. grandidentata* and the outgroup species *R. sieboldii*. The next branch contained *C. chinensis* and other seven species of subgenus *Clematis*. The third cluster consisted of *C. chinensis* and other five species of subgenus *Clematis*, which are closer to the second cluster than to the first one. The two *C. chinensis* samples did not bunch together, which might be due to different collection time and area. The similar reason is valid for two samples of *C. uncinata*. HGB could be unequivocally identified in all taxa. All 17 compounds were present in *C. puberula var. ganpiniana*, *C. apiifolia* and *C. argentilucida*. Further comprehensive study must incorporate more species to check the appropriateness of these molecules for chemotaxonomy of the whole genus. Flavonoids and lignans are important medicinal compounds, which should be further examined to see if they reveal any additional information about the relationships within *Clematis*.

Proteins and enzymes are the center players of the physiological metabolic pathways of plant cells. Proteins can be biomarkers for cataloging of botanical medicines. Two dimensional gel electrophoresis of leaf protein was useful for examination of the systematic relationship of section *Clematis* [39]. Significant variety was found among the 1085 spots scored: only 255 spots were shared by all. Nine proteins were only present in *C. chinensis* and absent in *C. finetiana* and *C. armandii*, although they are closer to each other than to other taxa. *C. apiifolia*, *C. peterae*, *C. argentilucida* and *C. ganpiniana* form a group. The genetic distance 0.4–0.45 and 0.25–0.55 was observed at intra-subsection level in *Clematis* and *Rectae* respectively. Proteomics is a capable taxonomic tool and the differentially expressed proteins provide hints for deciphering the distinction in metabolic pathway of different species. In addition, Shi et al. [40] suggested that the macrocyclic glucosides from *C. chinensis*, *C. armandii*, *C. hexapetala* and *C. mandshurica* are useful for the chemotaxonomy of sect. *Clematis*. Even with these studies, the relationships among *Clematis* species have not been resolved. Molecular methods have to be combined with morphology and chemotaxonomy in the elucidation of the pharmacophylogeny of *Clematis*.

4 Molecular taxonomy and molecular phylogeny

RAPD (random amplification of polymorphic DNA) is a type of PCR reaction, and the DNA sections that are amplified are haphazard [41]. The random primers of 8–12 bp and the genomic DNA template are used in the PCR. By resolving the PCR product patterns, a semi-distinctive profile can be garnered. Among 59 species and 24 varieties in six sections of three *Clematis* subgenera in Yunnan of Southwest China [42], 56 are endemic to China and 16 are only found in Yunnan. RAPD was used to study 12 *Clematis* species of Yunnan [43]. Selected ten RAPD primers were used in the random amplification and 89 polymorphic DNA bands were generated. The 12 species can be unambiguously identified. More importantly, the cluster tree basically mirrored the systematic relationship of the involved species. The subgenus *Cheiropsis*, the most ancient group, are basal to other taxa. *C. buchananiana*, *C. connata* and *C. ranunculoides* of subgenus *Viorna* are basal to subgenus *Clematis*. *Caulis clematidis armandii* (Chuan Mu Tong) is a traditional Chinese herbal medicine. The botanical origins of Chuan Mu Tong are the dried stems of *C. armandii* and *C. montana*. Chuan Mu Tong has the effects of heat-clearing, dieresis, activating blood to dredge vessels, and thus is

used to treat edema, gonorrhea, dysuria, rheumatoid arthritis, amenorrhea and hypogalactia [44]. Due to the morphological resemblance, it is hard to distinguish *C. armandii* and *C. montana* from other *Clematis* species. The following species are also marketed in China as Chuan Mu Tong [44]: *C. finetiana*, *C. kerriana*, *C. argentilucida*, *C. peterae*, *C. apiifolia var. obtusidentata*, *C. tangutica*, *C. meyeniana* and *C. uncinata* of subgenus *Clematis*, *C. leschenaultiana*, *C. lasiandra*, *C. henryi*, *C. chrysocoma*, *C. pogonandra* and *C. trullifera* of subgenus *Viorna*, and *C. macropetala* of subgenus *Atragene*. The distribution area of these species is less extensive than that of *C. montana* and *C. armandii*, and some endangered species should be protected [45]. Moreover, although these species are similar to raw plants of Chuan Mu Tong in drug efficacy, they are sometimes used for specific purposes in some regions and their pharmaceutical ingredients have not been investigated comprehensively. Other plants (e.g. *Aristolochia* and *Akebia*) were also mistakenly used as Chuan Mu Tong, which may cause serious toxicity [44]. Guo et al. [46] established a molecular method to discern *Caulis clematidis armandii* from its frequent contaminants. The RAPD-based dendrogram illustrated that evolutionarily ancient *C. montana* is basal to other taxa and *C. urophylla* and *C. lasiandra* of subgenus *Viorna* are basal to the seven species of subgenus *Clematis*, which is not incongruent with RAPD results of Pu et al. [43]. *C. armandii* is closer to *C. finetiana* than to *C. argentilucida*, *C. peterae var. trichocarpa* and *C. apiifolia*, which well agrees with the chemotaxonomic results of Muhammad Ishtiaq et al. [33]. Since *C. montana* and *C. armandii* are only distantly related, it is crucial to carry out a detailed study to uncover the physical basis of their common drug efficacy. The sequences of two RAPD fragments specific for *C. armandii* and *C. montana* were determined and PCR primers were designed for sequence characterized amplified region (SCAR) markers [46]. The two SCAR markers were specific to both *C. armandii* and *C. montana* but were not present in other species, which can be used in quality control of Chuan Mu Tong. RAPD was also used to study the hybrid origin of descendants from crosses of *C. tubulosa* and *C. brevicaudata* [47].

ITS (internal transcribed spacer) locates between nuclear ribosomal RNAs (rRNA) of a precursor transcript, which proved valuable for defining relationships among congeneric species and closely related genera [41]. ITS sequences were PCR-amplified from leaf genomic DNA of eight *Clematis* species of Zhejiang, China [48]. The ITS region varied between 534 and 561 bp, with 50 variable sites and 22 parsimony informative sites. On the ITS-based phylogenetic tree, *C. patens* is closer to *C. patens ssp. Tientaiensis* than to *C. finetiana* and *C. chinensis* in one group, while *C. henryi* of subgenus *Viorna* is closer to *C. lasiandra* (subgenus *Viorna*) than to *C. apiifolia* and *C. uncinata* of subgenus *Clematis* in the other group. ITS2 is a part of ITS, which is shorter and easier to amplify. The ITS2 region of Wei Ling Xian and its adulterants varied between 220 and 230 bp [49]. On the ITS2-based phylogenetic tree, *C. chinensis* is closer to *C. hexapetala* than to *C. mandshurica*. These official species of Wei Ling Xian form one cluster, while *C. lasiandra* of subgenus *Viorna* is closer to *C. pogonandra* (subgenus *Viorna*) than to *C. uncinata* (subgenus *Clematis*), which does not contradict with ITS results [48]. With ITS2, it is also easy to differentiate Wei Ling Xian and plants of *Sarcandra*, *Podophyllum*, *Paeonia* and *Smilax*, which were inadvertently or deliberately used as Wei Ling Xian in China markets [49]. ITS2 was also used to identify Chuan Mu Tong, its adulterants (e.g. *Aristolochia* and *Akebia*) and closely related species [50]. With ITS2, it is also easy to differentiate Chuan Mu Tong and other *Clematis* species. However, ITS2 cannot distinguish *C. aethusifolia* and *C. pogonandra* (subgenus *Viorna*), *C. leschenaultiana* and *C. siamensis* (subgenus *Viorna*), and *C. tangutica* and *C. tibetana* (subgenus *Clematis*).

Due to low resolving power, the actin I intron is not suitable for phylogenetic study of *Clematis* [51]. The nuclear (nr) ITS and five chloroplast (cp) DNA markers were used to deduce the evolutionary relationship of 33 *Clematis* species and related taxa [52]. The traditional subgenus *Viorna* and sect. *Atragene* (a subgenus in Wang and Li's system of 2005) were monophyletic, whereas taxa of subgenus *Clematis*, as well as those of subgenus *Cheiropsis*, did not form a clade. Substantial morphological *divergence* and meager nucleotide replacements within *Clematis* imply the recent radiation of the genus. Based on 75 species, sequences of the nrITS, the cp *atpB-rbcL* spacer, *psbA-trnH-trnQ* spacer and *rpoB-trnC* spacer regions were analyzed using parsimony, maximum likelihood and Bayesian inference methods [53]. Analyses of the combined data set by the three methods engendered similar trees. Ten major clades with various support values were found. The molecular inferences do not support prior morphology-based infrageneric classifications but suggest momentous evolutionary convergence in reproductive and vegetative characters in *Clematis*. Several branches represent geographically distinct groups. *C. montana*, *C. chrysocoma* and *C. fasciculiflora* of southwestern China, belonging to subgenus *Cheiropsis*, form clade X and are basal to other clades. *C. rehderiana*, *C. leschenaultiana*, *C. ranunculoides*, *C. siamensis* and *C. lasiandra*, distributed in southern, eastern and southeastern Asia and members of subgenus *Viorna*, form clade VIII and are basal to subgenera *Clematis* and *Atragene*. The members of subgenus *Clematis*, *C. delavayi*, *C. peterae*, *C. heracleifolia*, *C. pinnata*, *C. brevicaudata*, *C. taiwaniana* and *C. apiifolia*, are in clade VII, while *C. patens* is in clade VI. The raw species of Wei Ling Xian and their relatives, *C. armandii* and its relatives with similar drug efficacy are in clade V. Interestingly, *C. akebioides*, *C. tangutica*, *C. tibetana*, *C. orientalis* and *C. serratifolia* of clade I are closer to clade II (subgenus *Atragene*) than to other members of subgenus *Clematis*. However, relation-

ships among some closely related species are not resolved, e.g., *C. tangutica* and *C. tibetana*, *C. apiifolia* and *C. taiwaniana*, *C. brachiata* and *C. strigillosa*, and *C. uncinata* and *C. meyeniana*, suggesting that these taxa may have diverged recently. Bayesian dating suggests a fairly ancient origin of the genus in the Oligocene (25.99 million years ago, mya) and a quite recent species radiation in the Miocene (7.81 mya). Geologic and climatic changes in the late Tertiary to Quaternary (2.588 mya-now) might be important for the speciation of *Clematis*, especially in East Asia. Long-distance spreading of the fruits by wind, water or animals and significant environmental adaptability might explain the current world-wide distribution and high species diversity.

Yunnan, China has the most *Clematis* species in the world and Hengduan Mountains of northwestern Yunnan are viewed as centers of origin, differentiation and endemism of the genus [42]. ITS and three cp markers, same as those of Xie et al. [53], were PCR amplified to study the phylogenetic relationship of *Clematis* [54]. Seventy-three species (132 samples) cluster into 18 clades. *C. potaninii* and *C. fasciculiflora* of subgenus *Cheiropsis* are basal to other taxa. Clade XV containing *C. montana*, *C. gracilifolia*, *C. venusta* and *C. chrysocoma* is close to clade XIV of subgenus *Clematis*. Clade XIII containing *C. connata*, *C. rehderiana*, C. lasiandra and clades XII and X containing other members of subgenus *Viorna* cluster together. Clade VIII represents subgenus *Atragene* and is closer to clade VII containing members of *Meclatis*, subgenus *Clematis*, which matches the results of Xie et al. [53]. *C. hexapetala*, *C. terniflora*, *C. armandii*, *C. uncinata*, *C. crassifolia* and *C. patens* cluster in clades IV and V. This study also suggests a recent species radiation. Most involved species are from southwestern China, particularly Hengduan Mountains of northwestern Yunnan. The massive diversification of modern *Clematis* occurred in Himalaya-Hengduan region during the uplift of the Qinghai-Tibetan Plateau and was manipulated by the global geologic and ecologic alterations during the late Tertiary [53]. Many plant groups underwent adaptive radiation, differentiation and speciation after migrating to this region, e.g. *Pedicularis* [55], *Aconitum*, *Corydalis*, *Primula*, *Silene*, *Rhododendron* [56], etc. Jiang [54] found that relationships among some closely related species of Hengduan Mountains are not decided, e.g., *C. tangutica*, *C. akebioides* and *C. tibetana* of subgenus *Clematis*, *C. ranunculoides* and *C. yuanjiangensis* of subgenus *Viorna*, suggesting that these taxa might be the result of rapid radiation and speciation. *C. peterae* and *C. gouriana* of southwestern China, as well as *C. lasiandra* and *C. pseudootophora* of Subgenus *Viorna*, might also come into being from species radiation. Morphology has diverged significantly in these pairs of species, but the molecular marker sequences have not yet diverged dramatically. It is motivating to study whether the chemical profile has also diverged or not, which could be helpful in choosing the source plant for the specific medicinal use. Moreover, natural hybridization may be common in *Clematis*, since artificial hybridization among closely related species or even among sections is easy and widely used in horticulture [23,47]. Many cultivars are obtained by hybridization and possess unique pharmaceutical components [13,14]. Due to the conflicting position of *C. delavayi* in the cpDNA and ITS trees, Miikeda et al. [52] suggested a hybrid origin of this species in the early evolution of the genus. Based on the incongruent position in the cpDNA and ITS trees, Jiang [54] inferred that *C. delavayi*, *C. peterae*, *C. parviloba* (subgenus *Clematis*), *C. connata*, *C. rehderiana* and *C. ranunculoides* (subgenus *Viorna*) might have a hybrid origin. ITS and *psbA-trnH-trnQ* can be used as DNA barcodes in the authentication of *Clematis*.

5 Conclusions

Clematis is a botanical source for various pharmaceutically active components, which has been used in conventional Chinese medicine for many centuries. Increasing interest in *Clematis* medicinal resources has led to additional discoveries of triterpenoid saponins, flavonoids, coumarins, alkaloids and many other compounds in various *Clematis* species, and to investigations on their chemotaxonomy, molecular phylogeny and pharmacology. However, around 90% of all 355 species have not been explored in phytochemistry, molecular biology and pharmacology, which is a gold mine of myriad medicinal compounds. Chemotaxonomy tries to classify and spot plants by discovering differences and similarities in their biochemical compositions, which is of great help in pharmaceutical resource discovery. However, the results of chemotaxonomy have to be cross-examined with those of molecular taxonomy and traditional morphology-based classification, in order to streamline the quality control and guarantee the authenticity of plant materials used in the clinical setting, research laboratory and pharmaceutical industry. More importantly, chemical and biological research of *Clematis* medicinal resources should not be restricted in the above aspects, e.g. the biosynthetic pathway of secondary metabolites has not been studied; the regulation of *Clematis* biological processes at genomic level, epigenomic level [63], transcriptional and post-transcriptional levels, and translational and post-translational levels is totally unknown, although it is essential for the conservation, sustainable development and utilization of the *Clematis* medicinal resources. The biological and chemical studies of *Taxus* [57] and *Polygonum* [58] with the use of omics technologies provide a paradigm of the active integration of various state-of-the-art methodologies into the early stage of the drug research and development. Systems biology and omics techniques will play an increasingly significant role in future medical research involving bioactive compounds of *Clematis*.

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