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Three medicinal *Corydalis* species of the Himalayas: Their ethnobotany, pharmacognosy, phytochemistry and pharmacology

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ABSTRACT

Herbal medicines including the scholarly Bhutanese *Sowa Rigpa* medicine (BSM) contribute significantly to primary health care services and play a considerable part in modern drug discovery. BSM, which was integrated with modern health care system in 1967, currently uses more than 200 species of medicinal plants including three closely related members of the *Corydalis* species; *Corydalis calliantha*, *C. crispa*, and *C. dubia*. Maintaining the quality of these medicinal plants has been always challenging due to adulterants and also due to lack of traditional quality control parameters that could distinguish closely related species. Therefore there is an urgent need to develop reliable analytical methods and quality parameters. Given the significant medicinal properties attributed to the three *Corydalis* species which are extensively used in BSM, a thorough literature review was performed to investigate their existing quality control parameters including: 1) morphological assessment, 2) habitat and ecological evaluation, 3) macroscopical and microscopical examination, 4) physiochemical limit setting and comparative high-performance thin layer chromatography (HPTLC) profiling, and 5) phytochemical and biological activities. The study revealed that there are distinct species-specific features including ecological adaptation, micromorphology, anatomy, phytochemical values and HPTLC profiles presented by each *Corydalis* species, and that these quality parameters support traditional quality screening processes in BSM.

1. Introduction

Herbal plants are used as bulk ingredients in many traditional and indigenous medicines worldwide, which still provide primary health services to 80 % of the world's population (Wangchuk and Tobgay, 2015). In the scholarly Tibetan *Sowa Rigpa* medicine (TSM), more than 1000 medicinal plants have been prescribed for treating various disorders (Phuntshok, 1994; Boesi, 2006). TSM is today practiced worldwide including Asia (Bhutan, Nepal, India, Tibet-China), Russia, Europe (Switzerland and Germany), and North America (USA). The scholarly TSM has been adapted to suit the need of the Bhutanese people and it is locally known as '*nang-pai sman*' (indigenous medicine) or popularly as Bhutanese *Sowa Rigpa* medicine (BSM). Since its introduction by Zhabdrung Ngawang Namgyal in Bhutan in the year 1616, BSM has evolved independently from TSM for many centuries and the differences can be observed in the usage of medicinal plants, current formulations, golden and silver needle acupuncture therapies, and treatment regimens (Wangchuk et al., 2013a). Such deviations are to be expected, as Boesi (Boesi, 2006) stated that the *materia medica* of mainstream "Tibetan medicine should not be considered as either standard or static in time and space, but as a tradition that has been constantly evolving in several geographical and climatic regions with adaptations to local vegetation, culture and foreign influences".

BSM was formally and officially integrated into the modern health care system in 1967 (Wangchuk et al., 2007). Today, both allopathic and traditional medicines in Bhutan are managed by the Ministry of Health and are offered in all public hospitals and health care centers throughout the country. In 2015, there were 30 hospitals, 208 Basic

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Health Units (BHUs), 28 sub-post clinics and 562 outreach clinics in the country (Thinley et al., 2017). There are 65 traditional medicine units attached to all 30 hospitals and 35 Grade I BHUs in the country, which administer free medications to patients (Yeshi et al., 2018). These hospitals and BHUs follow a cross-referral system where patients are referred to both allopathic doctors and traditional physicians especially in the area of chronic diseases. Besides playing a significant role in providing primary health care services to the people, BSM also constitutes an important socio-economic resource for farmers through the collection and cultivation of medicinal plants. More than 600 medicinal plants that are described in the BSM textbooks and pharmacopoeia have been known to grow in Bhutan. Currently, more than 200 of these medicinal plants are used on a daily basis for preparing multi-ingredient formulations at the Manjong Sorig Pharmaceuticals under the Ministry of Health in Bhutan (Wangchuk and Tobgay, 2015). Hence, for patients - its formulations or medicines have become a source of healing; for the BTM practitioners - it is a source of employment; for farmers – it is a source of income; and for the chemist and researchers - it is a potential chemotherapeutic pool waiting for biodiscovery (Wangchuk and Tobgay, 2015). The medicinal plant programs have therefore become one of the sustainable pillars of Gross National Happiness (GNH) in Bhutan.

Since the integration and the expansion of BSM services in the country and due to regulatory requirements of the World Health Organization (WHO) or the Drug Regulatory Authority of Bhutan (DRAB), standardization of both medicinal plants and the formulations/prepared drugs have become a pressing issue to improve the quality, safety and efficacy of the *Sowa Rigpa* medicines (Adams, 2002; Craig, 2011; Kloos, 2015 & 2017). Significant differences in the identification and use of plants across the *Sowa Rigpa* world – covering six countries and an area the size of Europe in Inner Asia (Kloos, 2020) – are well known and documented (Dawa, 1999; Kletter and Krichbaum, 2001; Norbu, 2015). While such variations demonstrate the adaptability of *Sowa Rigpa* to local sociocultural, climatic and environmental conditions, this strength becomes problematic in contexts of standardized health systems and official pharmacopoeias. Scientific studies are essential to improve the quality, safety and efficacy of the medicines and also to build the basis for mutual understanding, respect, and increased collaboration between *Sowa Rigpa* and biomedicine (Wangchuk and Tashi, 2016).

Ethnobotanical identification is the first stage in assuring the quality, safety and efficacy of any herbal medicine. The main pharmacological compendium used in *Sowa Rigpa*, Deumar Tenzin Phuntshog's 18th century *Shel-gong-shel-phreng* (Phuntshok, 1994) describes the ethnopharmacognosy of medicinal plants in detail and provides guidelines for their identification and quality control. However, this traditional method of identification doesn't always distinguish closely related botanical species, which are often used equivalently. For example, it is likely that different *Corydalis* species especially *C. meifolia* var. *sikkimensis* and *C. stracheyi* var. *stracheyi*, which are very similar morphologically to *C. calliantha* and *C. dubia*, may be collected for medicinal use in *Sowa Rigpa* medicines under the same traditional name of *stong-ril-zil-pa* and *re-skon,* respectively (Wangchuk et al., 2016a & 2017). This poses a problem for providing a standard *Sowa Rigpa* care and treatment. It is therefore imperative to ensure the correct botanical identification of not only three *Corydalis* species described here but all the medicinal ingredients used in BSM.

Of more than 32 *Corydalis* species (Papaveraceae) growing in Bhutan (Gierson & Long, 1984), three species of *Corydalis*, namely *Corydalis calliantha* D.G Long, *Corydalis crispa* Prain, and *Corydalis dubia* Prain are currently used in preparing BSM formulations (MoH, 2016). The latter two – *C. crispa* and *C. dubia* – have been classified as endangered species (Lakey and Dorji, 2016). Since these may be also used in other regions with emerging *Sowa Rigpa* industries (Kloos, 2017), like China (Tibet and Inner Mongolia), India, Nepal, Mongolia, and Buryatia, it is likely that *Corydalis* species are traded and, in some instances, even trafficked illegally. The depletion of these species can lead to substitutions or adulterations by other species, which would directly affect the quality of medicines. Therefore, setting standard quality control parameters for authenticating these medicinal plants is vital for providing safe and effective *Sowa Rigpa* health care services. In this article, we have extracted comprehensive information from the available literature on the ethnopharmacogonosy, botanical features, ecological adaptations, physiochemical properties, phytochemical and biological properties of three *Corydalis* species and highlighted the potential parameters that could be used for authenticating and differentiating three *Corydalis* species used in BSM.

2. Materials and methods

A comprehensive and systematic data mining was performed by surveying relevant documents on BSM and *Corydalis* species. The documents were obtained either from the BSM library in Bhutan or were retrieved from several online bibliographic databases including PubMed, Google Scholar and SciFinder Scholar. For online database searches, the keywords such as '*Corydalis crispa*', '*C. dubia*', '*C. calliantha*', 'traditional uses of *Corydalis*', '*Corydalis* trade', 'morphology of *Corydalis*', geo-ecological distribution of *Corydalis*', and the 'phytochemical and biological activities of *Corydalis* species' were used. The existing traditional scholarly BSM textbooks including *Shel-gong Shel-phreng* (Phuntshok, 1994), Plant Monographs (Samten, 2009; Wangchuk and Samten, 2009), and the Handbook on Quality Control Practices (Tashi, 2009) were obtained from BSM library in Bhutan and were consulted for the traditional description of medicinal plants and the traditional uses and the quality parameters for each plant. For each *Corydalis* plant, an ethnobotanical description including distribution, habitat, collection season, parts collected or used and traditional formulations containing it are reported here. The information about the ecological, botanical, phytochemical and biological activities of three *Corydalis* species were obtained from the journal articles, most of which were published by the authors listed in reference section of this article. The extracted information was presented in a table format and was supported by the pictorial representation of the plants.

3. The traditional pharmacopoeial concepts of quality of medicinal plants

The content analysis of the traditional textbooks and pharmacopoeia of BSM revealed that there exist written ethnopharmacognosy protocols for assessing the quality of medicinal plants (Wangchuk et al., 2013a). Most commonly used traditional quality parameters include: a) the observation of macroscopic/morphological features such as shape, size, cutting appearances and texture; b) the organoleptic properties such as color, aroma and taste; and c) the characteristics of the plants such as smooth, heavy, warm, oily, cold, blunt, cool, flexible, fluid, dry, non-oily, hot, light, sharp, coarse and mobile. Often the authenticity and the quality of medicinal plants are determined through taste and smell. BSM distinguishes six different types of tastes such as sweet (*ngar*), sour (*kyur*), salty (*tsha*), bitter (*kha-ba* or *khag-tra*), pungent (*tsha-ba*) and astringent (*ka*). The organoleptic properties and plant attributes rely heavily on the '*gches-pai-yan-lag-bdun*' (translated as 'seven quality attributes of medicinal procedures' or 'seven related branches of quality practices/doctrines') (Wangchuk and Tashi, 2016). These seven quality practices include: 1) correct identification of medicinal plants, 2) collecting medicinal plants from the right natural habitat, 3) following appropriate collection season and time, 4) processing, drying, pre-processing and detoxifying the plants (wherever prescribed), 5) using appropriate BSM recommended drying methods, 6) proper storage, and 7) spiritual empowerment of herbs. To obtain maximum therapeutic benefits, the three *Corydalis* medicinal plants are collected strictly by adhering to these seven related branches of quality control guidelines ('*gches-pai-yan-lag-bdun*).

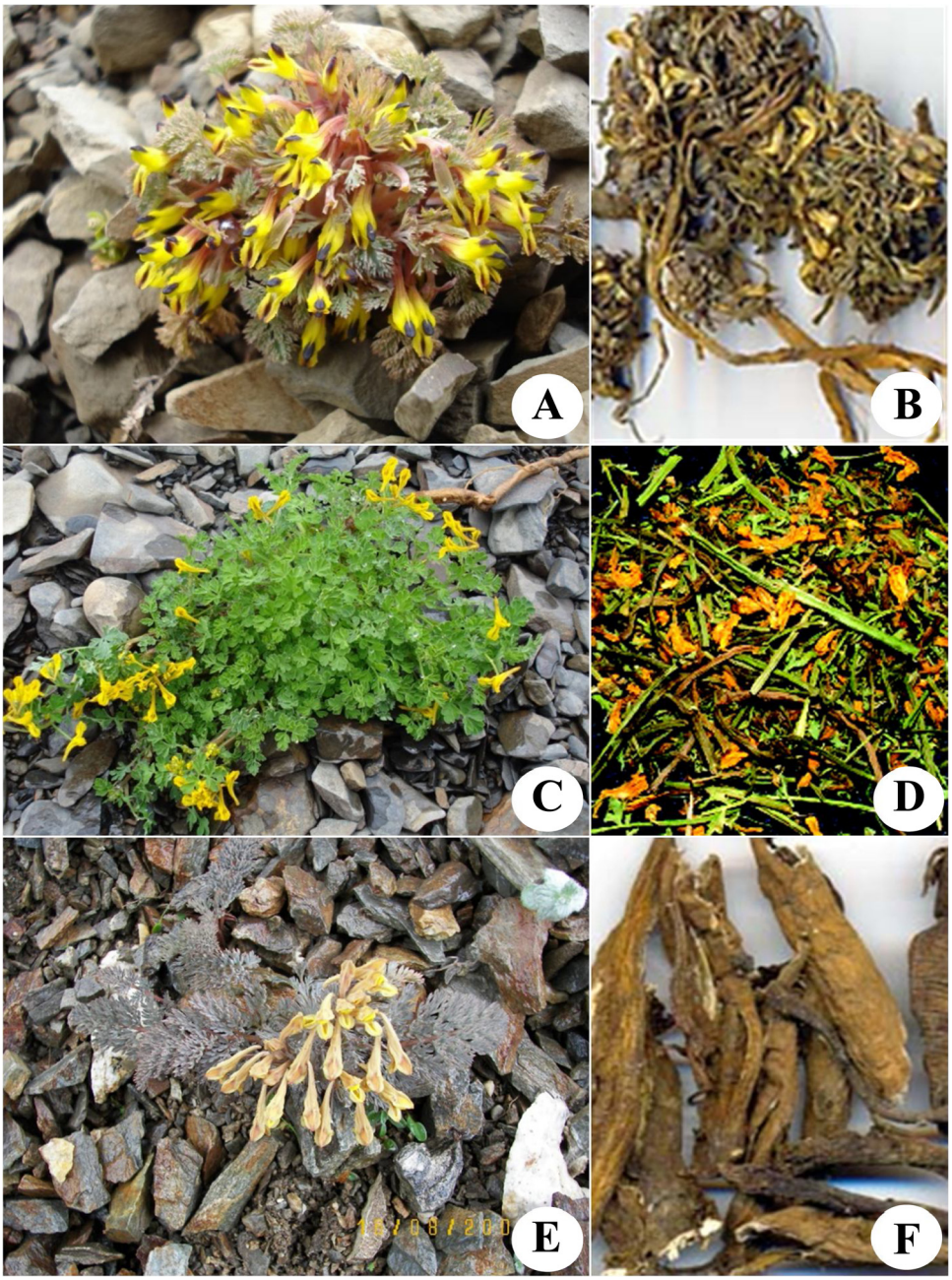

Fig. 1. Live plant and crude drug photos of three *Corydalis* species used in BSM: A. *Corydalis calliantha* in natural habitat; B. *Corydalis calliantha* crude drug (whole plant including the roots, leaves and flowers). C. *Corydalis crispa* in natural habitat; D. *Corydalis crispa* crude drug (consists of stem, leaves and yellow flower); E. *Corydalis dubia* in natural habitat; F. *Corydalis dubia* crude drug (whole dried plant including leaves, flowers and the roots).

4. Ethnopharmacognosy of three *Corydalis* species

The main *Sowa Rigpa* medicinal ingredient textbook (*Shel-gong-shel-phreng*) authored by Deumar Tenzin Phuntshog (Phuntshok, 1994) describes the ethnopharmacognosy of medicinal plants including three *Corydalis* species in detail and provides guidelines for their identification and quality control. *C. calliantha* (Fig. 1A) is known as *stong-ril-zil-pa* in *Sowa Rigpa.* The crude drug (Fig. 1B) of *C. calliantha* comprises the whole plant including the roots, leaves, and flowers. The root of the crude drug is slightly bitter in taste. The therapeutic indications of this plant include treating malaria, coughs and colds, and biliary inflammation (Wangchuk et al., 2010).

Corydalis crispa (Fig. 1C), locally known as *ngo-ba-sha-ka*, is indicated for treating infections in the blood, liver and bile and as a febrifuge (Wangchuk et al., 2012a). The crude drug (Fig. 1D) of *C. crispa* consists of stem, leaves, and yellow flower and therefore appears greenish-yellow in color. The crude drug is brittle and has an aromatic smell with a slightly bitter taste. *Corydalis dubia* (Fig. 1E), which is known as *re-skon,* is therapeutically indicated for detoxifying impure blood, alleviating fever arising from infectious liver, heart, lung, pancreas and kidney, neuralgia and against complicated disorders due to a disturbance of the three primary energies according to the principles of BSM (*rLung*, *mKhris-pa*, *Bad-kan*) (Wangchuk et al., 2012b). The crude drug (Fig. 1F) derived from *C. dubia* also comprises of whole dried plant including leaves, flowers and the roots. Roots dominate the crude drugs composition. Traditionally, the inner part of the *C. dubia* root is removed and only the root bark is present in the dried crude drug. While the dried leaves and flowers are very fragile and black in color, the roots are wrinkly and mostly appear yellowish-brown in color. The fractured size of the root pieces varies from 4–6 cm in length. The crude

drug was described to be odorless with a bitter taste.

Interestingly, these plants are mixed with other natural ingredients (herbs, minerals or animal parts) and are made into multi-ingredient formulations. These three *Corydalis* species are used for preparing 26 multi-ingredient formulations (Tenzin, 2007). According to the 'Traditional Medicine Formulary Book' (Tenzin, 2007), *C. calliantha* is used in preparing at least three multi-ingredient formulations (including *Klu-bdud-18, Sman-sna-phyema,* and *Sman-grub*. While *C. dubia* is used for making six formulations including *Gyu-ril-13, Hong-len-9-pa, Khrag-smen-11, Rma-sman-reg-pa-bde-ster, Sman-sna-phyema,* and *Sman-grub*), *C. crispa* is used in preparing 21 multi-ingredient formulations (including *A-gar-35, Bol-sman-7-pa, Brag-skya-5-thang, Ghi-vang-9-pa, Gong-sman-a-ru-18, Gtso-bo-8-pa, Gtso-bo-25, Gu-yu-28, Gyu-ril-13, Klu-bdud-18, Man-ngag-bsil-sbyor, Rga-lo-sman-dmar, Ru-rta-6-pa, Ru-rta-13, Se-'bru-kun-phan-bde-byed, Seng-ldeng-23, Skyu-ru-25, Spang-rgyan-15, Spos-dkar-10-pa, Thang-chen-25,* and *Ya-sman-rdo-rje-rab-'jom*).

5. Botanical description of three *Corydalis* species and their ecological distribution

The plant ecology, distribution, descriptions and their association with other species were obtained from the Flora of Bhutan (Grierson and Long, 1984). *C. calliantha* is endemic to Bhutan and found in alpine habitats (4000–4600 meters above sea level-masl) including cliffs, damp screes and river banks of locations like Lingzhi, Gasa, Dagala and Bumthang. *C. crispa* (local name *ba-sha-ka*) is endemic to western Bhutan and the Chumbi/Phari valley of Tibet (near tri-junction of Bhutan, Tibet and Sikkim). In Bhutan, this plant grows in the damp mountain screes and gravelly places of Naro, Wasa La, Lingshi and Yalela at an altitude range of 3350–4570 masl. *C. dubia* (local name *re-skon*) is distributed in Bhutan (Lingzhi and Bumthang), Sikkim and Chumbi (Tibet/China) and grows at altitudes of 4500–4900 masl around alpine screes slopes. Live *C. calliantha*, *C. crispa,* and *C. dubia* are very distinct species and their comparative botanical descriptions are provided in Table 1.

6. Pharmacognosy of three *Corydalis* species

While there exists a proper traditional ethnopharmacognosy protocol for the identification and quality control of medicinal plants used in BSM, it is essential to corroborate this traditional protocol with botanical and pharmacognostic parameters. Botanical description of plants is helpful for describing only the live plant species. It cannot distinguish the dried crude drugs of the plants especially if the plants are closely related. For this reason, it is necessary to develop pharmacognostic parameters that could distinguish the plant parts at the microscopic and chemical levels or perform genetic analysis. Pharmacognostical parameters include evaluations of plant anatomy and powder features using microscopy techniques, physiochemical properties using natural product protocols, chemical bands/spots using HPTLC profiling, and the phytochemical isolation and identification using the mass spectrometry and Nuclear Magnetic Spectroscopy techniques.

6.1. Plant anatomy of three Corydalis species using microscopy technique

The medicinal plant monographs (Wangchuk and Samten, 2009; Samten, 2009) was consulted and the transverse section (TS) of stems (*C. crispa*) and the roots (*C. calliantha* and *C. dubia*) reproduced using a plant microtechnique (Johansen, 1940) and a Novex Microscope K-Range (Holland). As reported in the medicinal plants monograph, the TS sections were photographed under microscope, and distinct anatomical features were found as shown in Fig. 2.

Corydalis calliantha: The TS of the root is irregular in outline. The epidermis is single layered. 3–4 layers of elongated parenchyma cells are present just below the epidermis. The dumbbell shaped vascular bundles are present in the center. The phloem and xylem are of usual type. In a few sections, root hairs were seen arising from the main part of the root.

Corydalis crispa: The TS of the stem has a hexagonal shape due to the presence of ridges covered with thick cuticles and a single layer of epidermal cells of various shapes and sizes. At every ridge, there is a well-developed vascular bundle embedded in the cortex. The stem has hollow pith in the center. A few unicellular warty trichomes are also visible on the surface. The epidermis is followed by 15–20 layers of parenchyma. At every ridge, there is a patch of thick-walled sclerenchyma cells. On the phloem region, towards the periphery, patches of lignified fibrous cells are also present. The six vascular bundles are of usual type but the cambium ring is not clear.

Corydalis dubia: The TS of the root is circular in outline with 2 − 3 layered elongated cork cells. Elongated and thick-walled phellogen (sometimes single layered) is present just below the cork cells. Parenchyma cells filled with starch grains are present next to phellogen. The vascular bundle is located towards the center and is made up of the

Table 1
Comparison of ecological and botanical characters of three *Corydalis* species.

Feature	*Corydalis calliantha*	*Corydalis crispa*	*Corydalis dubia*
Plant height (cm)	8 − 22	10 − 23	10 − 18
Stem type	Bipinnatisect (2–4 × 1.5 − 2 cm)	Decumbent and much-branched	Erect, glaucous, 20 − 60 cm
Root type	Taproot	Fibrous root	Rootstock
Leaf type	Leaves smaller, liner-elliptic, shorter, 0.13 − 0.22 × 0.05 − 0.08 cm	Numerous, ovate, finely biternatisect, obovate, mucronate, 0.1 − 0.2 cm broad	Oblancealate, 0.2 − 0.5 × 0.05 − 0.1 cm. 3 − 6 pairs of leaflets.
Petiole length (cm)	1 − 2.5 (main petioles longer, 0.3 − 0.9)	1 − 2.5	3 − 5
Leaf blade length (cm)	4 − 6 × 1.5 − 2.2	2 − 3 × 2 − 2.5	1 − 2
Leaf color	Pale green with red tint	Green	Grey
Number of flowers	4 − 9	10 − 20	18 − 24 or many
Flower color	Yellow sometimes tipped red	Yellow	Yellow
Petal and petal length (cm)	Petals larger, upper 1.6 − 2.0 cm, broad deflexed spur 0.3 − 0.5 cm	Outer petals broad rounded crests; upper petal 1 − 1.4 cm, curved spur 0.5 − 0.8 cm.	Upper petal 1.5 − 1.8 cm, spur 0.7 − 0.9 cm with low crest.
Inflorescence type	Short condensed racemes	Dense rounded racemes 2 − 4 cm	Spike-like cluster
Spur shape and length (cm)	Broad deflexed; 0.3 − 0.5	Upwardly curve; 0.5 − 0.8	Down-curved and somewhat swollen at the apex; 0.3 − 0.6
Bract shape and length (cm)	1.1 − 1.7	Lower bract: pinnatisect (5–7), upper bract: linear (0.2 − 0.4)	Upper bracts shorter and with fewer teeth; lower bracts larger, 1 − 1.2
Nectariferous gland	Absent	Present	Absent
Capsule shape	Absent	Narrowly obtuse	Narrowly obovoid
Capsule length (cm)	Absent	0.7 − 1.0 × 3	1.2 − 2.0
Sepal type	Larger, ovate, acute, serrated margins	Absent	Small and deciduous

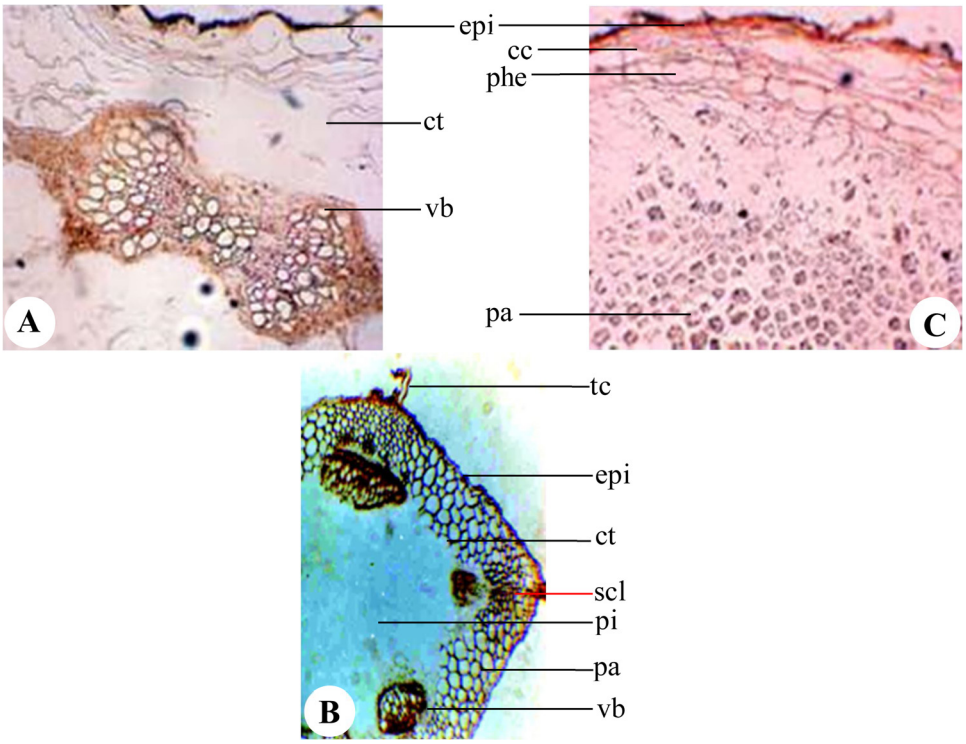

Fig. 2. Microscopic characteristics under Novex microscope K-Range x10: A. *Corydalis calliantha* TS root (epi – epidermis; ct – cortex; vb – dumbbell-shaped vascular bundle); B. *Corydalis crispa* TS stem (tc – trichome; epi – epidermis; ct – cortex; pi – pith; pa – parenchyma cells; vb – vascular bundle; scl – sclerenchyma cell); C. *Corydalis dubia* TS root (epi –epidermis; cc – cork cell; phe – phellogen; pa – parenchyma cell filled with starch grains).

usual cells. The phloem is arranged in the peripheral region and the xylem towards the center.

6.2. Morphology of powdered crude drugs of three Corydalis species using microscopy

In BSM, crude drugs are first ground into a powder, which is then mixed well to prepare various formulations. The medicinal plant monographs (Wangchuk and Samten, 2009; Samten, 2009) have previously described various cellular features including stomata, tracheids, vessels with spiral and scalariform thickening, fibers, parenchyma cells, starch grains, and crystals that were present in the powdered materials from three *Corydalis* species. Using the same protocol, the microscopical characteristics of crude powdered drugs of *C. crispa*, *C. calliantha* and *C. dubia* were reproduced using a high magnification Novex Microscope K-Range (Holland) (Fig. 3). The microscopical analysis showed the presence of similar cellular features including anisocytic stomata, pollen grains, spiral vessels with spiral and scalariform thickening, fibers and tracheids. The parenchyma cells were filled with starch grains and the crystals were absent. Fig. 3 shows the selected cellular components present in the powdered crude drug. Only *C. calliantha* presented a unique kind of vessel that distinguished it from the other two plants (Fig. 3 cal4).

6.3. Physiochemical parameters of three Corydalis species

Physiochemical parameters comprises evaluation for percentages of: foreign matter, moisture content, total ash, acid insoluble ash, water soluble ash, water soluble extractive and alcohol soluble extractive values, and loss on drying of crude drugs. While all these physiochemical parameters are considered important, only foreign matter and moisture content analysis are used in the routine quality control of herbal drugs in Bhutan. These parameters have previously been described in the medicinal plants monographs (Wangchuk and Samten, 2009; Samten, 2009) and are compiled here for comparison (Table 2). The values given in Table 2 are the 'average percentage' of three

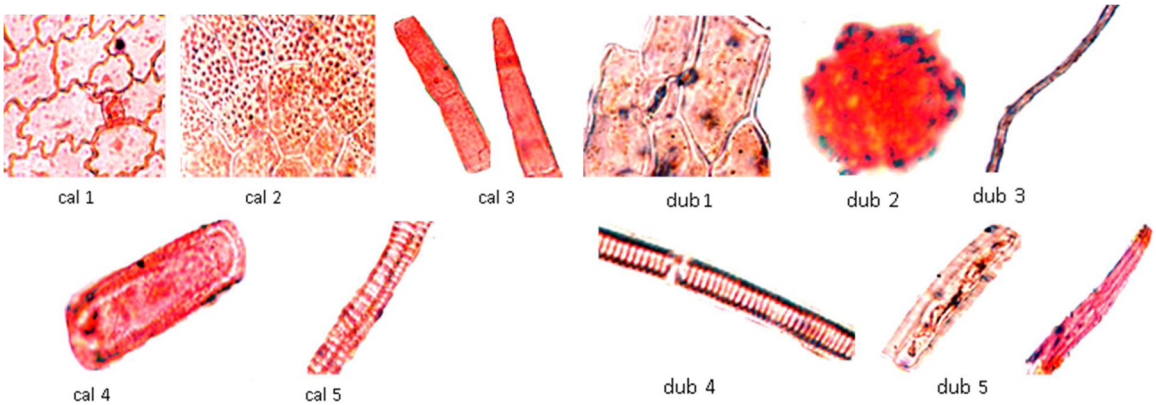

Fig. 3. Isolated elements of powdered crude drug: cal 1. Anisocytic stomata; cal 2. Starch grain; cal 3. Different types of tracheids; cal 4.Unique vessel (characteristic of this plant); cal 5. Ladder type vessel of *Corydalis calliantha*. dub 1. Anisocytic stomata; dub 2. Pollen grain; dub 3. Fiber; dub 4. Spiral vessel; dub 5. Different types of tracheids of *Corydalis dubia*.

Table 2
Percentage of ash and extractive values of three *Corydalis* species.

Parameters	*Corydalis calliantha* (≤ %)	*Corydalis crispa* (≤ %)	*Corydalis dubia* (≤%)
Foreign matter	2.0	2.0	2.0
Total ash	15.0	15.6	12.0
Acid-insoluble ash	3.0	1.98	5.0
Water-soluble ash	8.0	2.0	2.0
Alcohol-soluble extractive	15.0	7.0	4.0
Water-soluble extractive	36.0	25.4	12.0
Loss on drying	10.0	10.1	11.0

biological replicate plant samples. These parameters are standard parameters for monitoring the quality of herbal drugs. While less than 2% foreign matter is an acceptable limit for the crude plant drugs (Anonymous, 1990 & 2000; WHO, 2002), less than 10 % of moisture content is regarded as an acceptable limit for the plant drugs. Moisture content is an important quality parameter for herbal materials since the presence of high moisture in the crude drug could easily lead to the growth of microorganisms such as fungi and bacteria. The parameter of acid-insoluble ash largely depends on the amount of fine soil and sand particles present in the crude drug, which may have carried over from collection from the field or introduced during transportation.

7. High performance thin layer chromatography (HPTLC) profile of three *Corydalis* species

At Manjong Sorig Pharmaceuticals (MSP), where the quality of medicinal plants are constantly monitored before preparing multi-ingredient formulations, High Performance Thin Layer Chromatography (HPTLC) has recently replaced the traditional thin layer chromatography fingerprinting techniques. HPTLC is used for the rapid separation and visualization of chemical bands and its fingerprint is used as the principal means for identification and quality control of herbal drugs. Although, the HPTLC fingerprint/profiles has not been published in the two volumes of medicinal plants monographs (Wangchuk and Samten, 2009; Samten, 2009), which includes three *Corydalis* species, the profile for each *Corydalis* species exist. Their HPTLC profile have been reproduced using previously described method (Reich and Schibli, 2006). The profile of three *Corydalis* species are shown in Fig. 4.

The comparative HPTLC finger print profiles of *Corydalis crispa* and *Corydalis dubia* were very similar and showed four common bands at 366 nm at R_f 0.10, 0.46, 0.56, 0.82 of deep skyblue, skyblue, yellow-white, and red respectively. A large yellow band was observed at R_f 0.92, which was more pronounced in *Corydalis dubia* than in *Corydalis crispa* extract (marked with a yellow line in Fig. 4A) and which could be protopine. Protopine was isolated as the major chemical constituents of *C. crispa* and *C. dubia* and was observed to be the same fluorescent color under UV. *C. calliantha* as well as *C. dubia* have at around R_f 0.59 a second yellow-white-green (band marked with a red oval) (Fig. 4A), which is missing in *C. crispa*. In *Corydalis calliantha,* only two of the bands described above at R_f 0.10 and 0.56 were observed. However, two additional major bands of pale green and pink were present at R_f 0.18 and 0.92 (marked with a blue oval in Fig. 4A) respectively. Under UV light of 254 nm wavelength, many bands were not distinctly visible (Fig. 4B) as under 366 nm. Only one band at around Rf 0.59 (band marked with a red oval) that was common in *C. calliantha* and *C. dubia* was distinct.

8. Phytochemical constituents of three *Corydalis* species

Other than the information published by the first author of this article, there is no other literature that reported on the phytochemical and biological activities of three *Corydalis* species described here. A preliminary evaluation of crude extracts of these three *Corydalis* species for their major phytochemical contents showed that all three species contain alkaloids (Wangchuk et al., 2011). A total of 15 isoquinoline alkaloids (see Fig. 5 for their structures) have been isolated from these three *Corydalis* species. From *C. calliantha*, four known alkaloids including protopine (**1**), stylopine (**2**), cheilanthifoline (**3**) and scoulerine (**4**), were isolated (Wangchuk et al., 2010).

From *C. crispa,* nine known isoquinoline alkaloids including compounds **1**-**2**, coreximine (**5**), 13-oxocryptopine (**6**), 13-oxoprotopine (**7**), sibiricine (**8**), bicuculline (**9**), ochrobirine (**10**) and rheagenine (**11**) were isolated (Wangchuk et al., 2012a). Similarly, seven known isoquinoline alkaloids including compounds **1**, **3**, **4**, **9**, corydecumbine

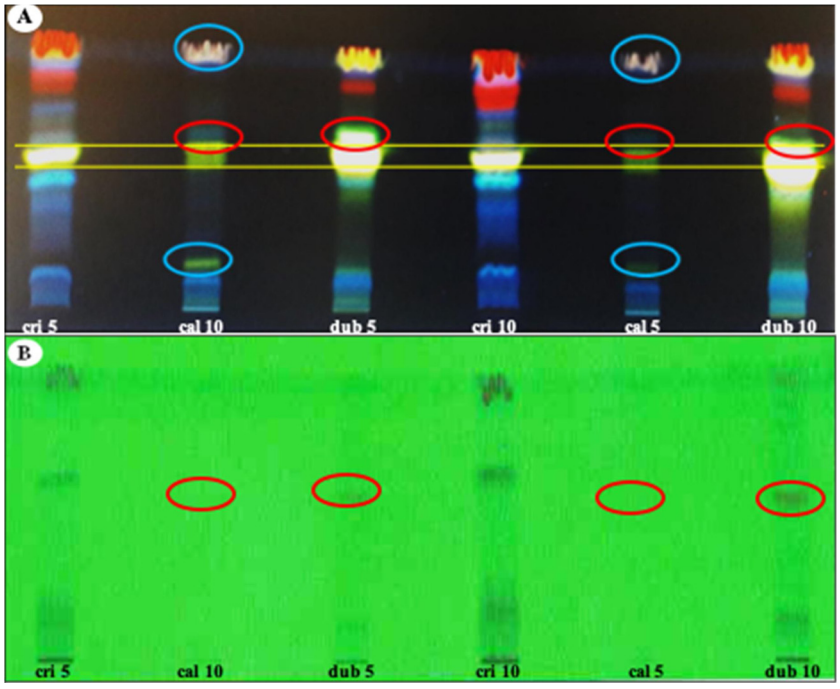

Fig. 4. Chromatograms obtained from separation of methanolic extracts of three *Corydalis* sp. using solvent system: methanol:chloroform (3:7) at two different dilutions (5 µl, 10 µl) under UV light of wavelength (**A**) 366 nm and (**B**) 254 nm: cri 5. *Corydalis crispa* (5 µl); cal 10. *Corydalis calliantha* (10 µl); dub 5. *Corydalis dubia* (5 µl); cri 10. *Corydalis crispa* (10 µl); cal 5. *Corydalis calliantha* (5 µl); dub 10. *Corydalis dubia* (10 µl).

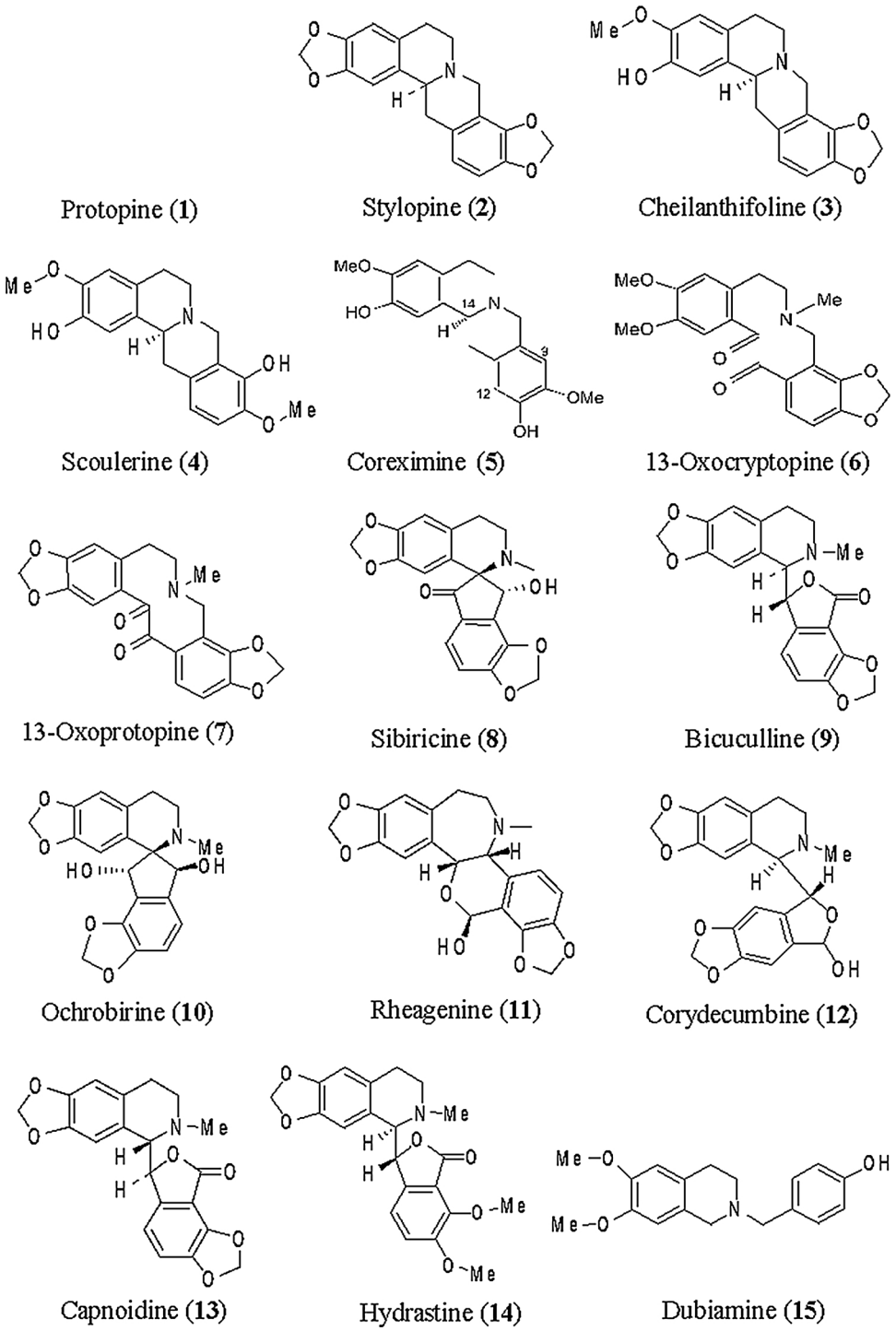

Fig. 5. Structures of compounds isolated from three *Corydalis* species. Compounds **1**-**4** were isolated from *Corydalis calliantha* (Wangchuk et al., 2010). Compounds **1**-**2** and **5**-**11** were isolated from *C. crispa* (Wangchuk et al., 2012a). Compounds **1**, **3**, **4**, **9** and **12**-**15** were isolated from *C. dubia* (Wangchuk et al., 2012b). Compound **15** was identified as a new isoquinoline alkaloid and its name was derived from the species name of a plant *C. dubia*.

(**12**), capnoidine (**13**), hydrastine (**14**), and a new isoquinoline alkaloid, which was named as dubiamine (**15**) (Fig. 5), were isolated from *C. dubia* (Wangchuk et al., 2012b). The name of a new isoquinoline alkaloid (dubiamine) was derived from the plant species name, *C. dubia*. Protopine (**1**) was present as a major alkaloid in all three *Corydalis* species namely *C. calliantha, C. crispa,* and *C. dubia*. While stylopine (**2**) was a common compound produced by *C. crispa* and *C. calliantha*, cheilanthifoline (**3**) and scoulerine (**4**) were produced by both *C. calliantha* and *C. dubia*.

9. Biological activities of the crude extracts and their isolated compounds of three medicinal *Corydalis* species

There is only limited literature that has reported the biological activities of the crude extracts and their isolated compounds of the three *Corydalis* species, which are presented in Table 3. All three species were reported to be good antimalarial agents against *Plasmodium falciparum* antifolate sensitive TM4/8.2 strain and the multidrug resistant K1CB1 strain (Wangchuk et al., 2010, 2011; Wangchuk et al., 2012a & 2012b).

Table 3
Major biological activities of compounds isolated from three Corydalis species.

Plants	Model/method	Tested	Biological activity	IC_{50} [a] values	References
Corydalis calliantha	*Plasmodium falciparum* strain: TM4/8.2 (a wild type chloroquine and antifolate sensitive strain)	PT CF	Antiplasmodial activity	1.50 μg/mL 0.90 μg/mL	(Wangchuk et al., 2010; Yeshi et al., 2017a)
	Plasmodium falciparum strain: K1CB1 multidrug resistant strain (chloroquine and antifolate resistant)	PT CF	Antiplasmodial activity	1.51 μg/mL 1.22 μg/mL	
	DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical	CAE CME	Antioxidant activity (DPPH)	18.7 μg/mL 10.0 μg/mL	
Corydalis crispa	TNF-alpha production in LPS activated THP-1 cells.	PT	Anti-inflammatory activity	467 pg/mL	(Wangchuk et al., 2012a)
	Plasmodium falciparum strain: TM4/8.2 (a wild type chloroquine and antifolate sensitive strain)	CM PT PT, OB	Antiplasmodial activity Anti-acetylcholinesterase activity	5.56 μg/mL 1.45 μg/mL 0.14 nmol	
	Plasmodium falciparum strain: K1CB1 multidrug resistant strain (chloroquine and antifolate resistant)	CM PT PT, OB NT	Antiplasmodial activity Anti-acetylcholinesterase Antioxidant activity	6.87 μg/mL 1.38 μg/mL 0.14 nmol NT	
Corydalis dubia	Acetylcholinesterase (AChE)	SL	Anti-acetylcholinesterase activity	0.0015 nmol	(Wangchuk et al., 2012b, 2013b, 2016b, 2018; Shepherd et al., 2017; Yeshi et al., 2017a & 2017b)
	DC assay		Immunomodulatory activity	Strongly upregulated	
	Plasmodium falciparum strain: TM4/8.2 (a wild type chloroquine and antifolate sensitive strain)	SL	Antiplasmodial activity	5.4μM	
	Plasmodium falciparum strain: K1CB1 multidrug resistant strain (chloroquine and antifolate resistant)	SL	Antiplasmodial activity	3.1μM	
	Trinitrobenzenesulfonic acid (TNBS; Sigma Aldrich) induced male mice model (C57BL/6) of acute colitis	CD	Anti-inflammatory activity	**P = 0.006	
	LPS-activated THP-1 human monocytic cells	ME CE	TNF-α inhibitory activity	236 pg/mL 258 pg/mL	
	DPH (2,2-Diphenyl-1-picrylhydrazyl) free radical	CAE CME	Antioxidant activity (DPPH)	17.5 μg/mL 9.6 μg/Ml	

IC_{50}[a], 50 % inhibitory concentration; SL/IA[b], suppression level/ inhibitory activity, AChE[c] MIR, Acetylcholinesterase inhibitory activity minimum inhibitory requirement; SOCS[d], Significant overall clinical score; NT, not tested; PT, protopine; CF, cheilanthifoline; SL, Scoulerine; CD, capnoidine; PT, protopine; CM, coreximine; OB, ochrobirine; 13-OP, 13-oxoprotopine; SP, stylopine; CME, crude methanol extract; CAE, crude aqueous extract; CE, chloroform extract.

The crude extracts of *C. crispa* and *C. dubia* showed significant anti-inflammatory activity by inhibiting the TNF-α production in LPS activated THP-1 cells (Wangchuk et al., 2013b) and also demonstrated moderate antimicrobial activities with MIC values in between 300 − 700 μg/mL as highlighted in Table 3 (Wangchuk et al., 2011). The crude extracts of *C. calliantha* and *C. dubia* showed moderate antioxidant activities (Yeshi et al., 2017a & 2017b). The chloroform extract of *C. crispa* showed significant antiparasitic activity against *T. brucei rhodesiense* (which causes sleeping sickness) with IC_{50} values of 4.6 μg/mL (Wangchuk et al., 2011).

The 15 compounds that were isolated from three *Corydalis* species were found tested against different bioassay targets including: 1) antimalarial activity against *P. falciparum* antifolate sensitive TM4/8.2 and the multidrug resistant K1CB1 strains, 2) acetylcholinesterase activity, 3) anthelmintic activity against *Trichuris muris* and *Schistosoma mansoni*, 4) anti-inflammatory activity against chemical colitis, 5) immunomodulatory activity against dendritic cells (Table 3). For examples, cheilanthifoline, scoulerine and protopine were reported to be the best antiplasmodial compounds against *P. falciparum* with IC_{50} values in between 2.78–4.29 μM and these compounds were identified as novel drug lead compounds against *P. falciparum* antifolate sensitive TM4/8.2 strain and the multidrug resistant K1CB1 strain (Wangchuk et al., 2010, 2012a & 2012b).

Of four alkaloids (13-oxoprotopine, stylopine, ochrobirine and scoulerine) tested, scoulerine significantly inhibited acetylcholinesterase with a minimum inhibitory requirement of 0.0015 nmol, which is twofold better than that of galanthamine (0.003 nmol), the currently used standard drug to treat Alzheimer's disease (Wangchuk et al., 2016b). Scoulerine also strongly up-regulated several important genes and proteins including *MHC-I*, *CD80* and *CD86*, suggesting that this compound would potentially make a good immunologic adjuvant for a $CD8^+$ T cell vaccine (Wangchuk et al., 2018). Capnoidine significantly improved the health of the colitic mice induced by a chemical, trinitrobenzenesulfonic acid (TNBS) (Shepherd et al., 2018). None of the alkaloids obtained from *Corydalis* species showed significant anthelmintic activity (Wangchuk et al., 2016c).

10. Plant specific features and the potential checklist for authenticating three *Corydalis* species

In this study, three very closely related *Corydalis* species were prepared: *C. calliantha, C. crispa* and *C. dubia* and a few plant characteristics identified that are unique to each species. These plant-specific traits/characteristic/features that can be used as checklist for distinguishing three *Corydalis* species are given in Table 4. Plant characteristics that were common to all three species were not listed in this table. For example, all three *Corydalis* species showed strong antimalarial and anti-inflammatory activities and therefore these biological activities were not listed in Table 4.

Ecologically, all three *Corydalis* species grow in distinct and unique habitat and this parameter is listed in Table 4. This ecological parameter is commonly used by BSM physicians to locate and collect each plant species. Botanically, these three species have distinct characteristics and can be identified morphologically by the color of their flowers and leaves, and the types of their roots. For example, while the color of the leaf of *C. calliantha* is pale green with a red tint on the edges/ridges, *C. crispa* is green, and that of *C. dubia* is grey. *C. calliantha* has a larger yellow flower with red tipped petals. *C. crispa* has a yellow flower with a rounded crest of petals and has a nectariferous gland. *C. dubia* has a pale yellow flower with a slightly ash-colored appearance from outside. Interestingly, it is easy to differentiate these three *Corydalis* species by their root types. While *C. calliantha* has a taproot system, *C. crispa* has fibrous roots, and *Corydalis dubia* presents long rootstock. Pharmacognostically, the crude drug of *C. calliantha* is easily disguishable from the

Table 4
Distinguishing/unique features of three *Corydalis* species (A checklist of distinct parameters).

Parameters	Distinctive features	*Corydalis calliantha*	*Corydalis crispa*	*Corydalis dubia*
Ethnopharmacognosy	Used in *Sowa Rigpa* herbal medicine	Stong-ril-zil-pa (local name). Roots bitter in taste.	Ngo-ba-sha-ka (local name). Aromatic smell with yellow flower and mildly bitter taste	Re-skon. Orderless with bitter taste
Ecological	Habitat	Endemic to Bhutan. Grows around cliffs, damp screes and river banks	Grows around damp mountain screes and gravelly places of Bhutan, Sikkim and Tibet (China)	Grows around alpine slopes with scree. Distributed in Bhutan, Sikkim and Tibet (China)
	Root type	Taproot	Fibrous root	Rootstock
	Leaf type	Leaves smaller, ultimate segments liner-elliptic, shorter, 0.13 − 0.22 × 0.05 − 0.08 cm	Numerous, ovate, finely biternatisect but terminal leaflet larger than laterals; segments obovate, 0.1 − 0.2 cm broad.	3 − 6 pairs of leaflets and a terminal leaflet, often deeply 3-lobed; acute; segments oblancealate, 0.2 − 0.5 × 0.05 − 0.1 cm,
	Leaf color	Pale green with red tint	Green	Grey
	Number of flowers	4 − 9	10 − 20	18 − 24 or many
	Inflorescence type	Short condensed racemes	Dense rounded racemes 2 − 4 cm	Spike-like cluster
	Capsule shape	Absent	Narrowly obtuse	Narrowly obovoid
	Capsule length (cm)	Absent	0.7 − 1.0 × 3	1.2 − 2.0
	Sepal type	Larger, broadly ovate, acute with serrated margins	Absent	Small and deciduous
Pharmacognostic	Anatomy of root transverse section	Dumbbell shaped vascular bundle present	Absent	Absent
	Morphology of crude powder	Cylindrical-shaped vessel	Absent	Absent
	Alcohol soluble extract	≤ 15 %	≥ 7%	≤ 4%
	Water soluble extract	≤ 36 %	≤ 25 %	≤ 12 %
HPTLC profile	Chromatogram bands at 366 nm UV light	6 coloured bands/spots. The major center band is light green colour and the top end band (R_f 0.92) is milky white in color.	14 coloured bands/spots. The major center band is one-layered white color and top end band (R_f 0.92) is red in color.	11 coloured bands/spots. The major center band is two-layered bright white color and the top end band (R_f 0.92) is yellow in color.
	Chromatogram bands at 254 nm UV light	1 dark band/spot	4 coloured bands/spots	2 dark bands/spots
Phytochemical	Isoquinoline alkaloids	Total 4 alkaloids isolated and all of them were common to other two plant species.	Total 9 alkaloids isolated with 6 alkaloids (13-oxoprotopine, 13-oxocryptopine, coreximine, rheagenine, ochrobirine, sibiricine) present only in this species.	Total 8 alkaloids with 4 alkaloids (capnoidine, hydrastine, corydecumbine, dubiamine) present only in this species.
Pharmacological	Anti-*Trypanosoma brucei rhodesiense* activity	Not tested	Strongly active (4.6 μg/mL)	Not active
	Antibacterial activity	Not active	Moderately active against, *Vibrio cholerae, Bacillus subtilis* and *Staphylococcus epidermidis* (MIC = 625 μg/mL)	Moderately active against MRSA (MIC = 625 μg/mL)
	Antifungal activity	Not tested	Moderately active against *Candida albicans* (MIC = 625 μg/mL)	Not active

other two species based on the dumbbell shaped vascular bundle and cylindrical-shaped vessel present in the T.S of root and powder, respectively.

The HPTLC profile of the crude extracts of three *Corydalis* species showed that each plant has distinct chromatograms, some common to all three species and few different from each other (Table 4). This HPTLC profiling techniques is commonly used in Bhutan to monitor the quality of medicinal plants by comparing the chromatogram profile with the authentic samples. The phytochemical isolation and pharmacological testing parameter procedings are very expensive and time consuming and therefore cannot be used as quality control parameters for monitoring the quality of medicinal plants on a daily basis. However, high performance liquid chromatography and metabolomics profiling techniques, which are currently not available at MSP, can be employed for daily screening of medicinal plants for their quality. Table 4 could be used as a checklist for discriminating three *Corydalis* species used in BSM and TSM.

11. Conclusion and future requirements

While ecological adaptation (habitat) and morphological features (color, smell, taste, appearances, and shapes) are traditionally used in BSM for the identification and authentication of *Corydalis* plants, three main pharmacognostic parameters including anatomy, physiochemical properties and HPTLC profiles are used for supporting traditional quality control methods and the authentication of these three *Corydalis* species used in BSM. For resource-constrained organizations, especially those involved in non-profit production of BSM, detailed routine microscopical, physiochemical, phytochemical and pharmacological analysis would be lengthy and expensive. We recommend that only selected parameters including at least morphology, foreign matter, moisture content, loss on drying and HPTLC, HPLC be used for the routine quality assessment of medicinal plants. The parameters listed in Table 4 could be used as a checklist for the authentication and differentiation of these three closely related *Corydalis* species.

This review found that the scientific biological activity studies supported some of the claims/indications of ethnopharmacological uses of the three *Corydalis* species in BSM. Their findings can be useful for the authentication and the quality control of *Corydalis* species, which are used in *Sowa Rigpa* medicine worldwide. However, other quality parameters including limits for: 1) microbial and pest contaminations, 2) heavy metals pollution, and 3) the nutritional content remains unexplored and must be determined for all three *Corydalis* species. More detailed clinical trial on BSM formulations that contain *Corydalis* species are needed to provide a scientific basis for the treatment of a broad range of disorders expounded by the BSM/TSM pharmacopoeia.

Author contribution

PW designed and performed the study, analysed the data, and wrote the manuscript. KY collected data, translated the traditional uses of plants in BSM, carried out pharamacognostic studies, analysed data and wrote the manuscript. TJ collected general information on botanical identification of the plants. SCM, SK and ASN cross-checked the pharmacognostic data, Wylie transliteration of *Sowa Rigpa* terms, and wrote the manuscript. CV assisted in analysing the data, and wrote the manuscript. Samten and Tashi helped in identifying and authenticating plant samples and carried out microscopical and physiochemical studies.

Declaration of Competing Interest

The authors declare that we have no conflicts of interest in this study.

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