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Chemical Characterization of *Corydalis chaerophylla* D.C. Extracts and Preliminary Evaluation of Their *in Vitro* and *in Vivo* Biological Properties

Binita Maharjan,[a] Lok Kumar Shrestha,[b, c] Jonathan P. Hill,[b] Katsuhiko Ariga,[b, d] Shyam Sharan Shrestha,[e] Stefania Sut,[f] Ram Lal Swagat Shrestha,*[a] and Stefano Dall'Acqua*[f]

Genus *Corydalis* is a rich source of isoquinoline alkaloids reported to having potential bioactivities. *Corydalis chaerophylla* collected from Nepal at an altitude of 2400–4800 m was extracted using hexane, methanol and chloroform as solvents. The resulting hexane, methanol and chloroform extracts were subjected to LC–DAD-MS^n analysis to yield fifteen different alkaloids. To assess any potential pharmacological properties, antimicrobial activity against two Gram-positive, two Gram-negative bacterial strains and one fungal strain was assessed, revealing significant inhibitive action of the methanol and chloroform extracts. Of the extracts obtained using chloroform contained the highest content of phenolic compounds at 113 mg GAE/g, while the highest total flavonoid content was found for the hexane extract with a value of 46.45 mg QE/g. The chloroform extract also exhibited a considerable antioxidant activity at IC_{50} value, 261.5 ± 3 μg/mL, for the DPPH assay. Conversely, the methanol extract exhibited the highest LC_{50} value for Brine Shrimp cytotoxicity at 196 ± 3 μg/mL being least potential for the test. The methanol extract was found to be the most active against α-amylase inhibition with an IC_{50} of 51.52 ± 2 μg/mL. In an *in vivo* acute oral toxicity study against mice, methanol and chloroform extracts presented harmful effects with 1000.36 mg/kg BW and 515 mg/kg BW for LD_{50}, respectively. By analyzing all the results of the solvents used, the chloroform extract was found to be the most active, a feature that will be used in future isolation procedures and other pharmacological tests.

Introduction

Plant derived natural products and chemicals obtained from other natural sources as, bacteria, yeast, fungi, and marine species have been, since antiquity, the most important sources for mankind for the development of medicines and remedies, and still in the recent years new bioactive agents have been discovered and brought to the clinical uses. Thus nature is a unique library of bioactive natural compounds and their structural variety, complexity, and propensity for displaying biological activities explain their significant role as pharmacophore and lead molecule sources in medicinal chemistry and thereafter for the development of clinical medications.[1–3] Plants are in many cases rich sources of natural products and the production of bioactive secondary metabolites is strictly related to numerous factors including plant genetics environmental factors. The *Corydalis* genus in particular has been prolific as a source of natural products. There are 470 species in the genus *Corydalis* worldwide, 57 of which are found in Nepal.[4] This genus is abundant in isoquinoline alkaloids, which serve as a chemical defense against different microorganisms and herbivores.[5,6] Although certain species in this genus have been implicated in poisoning cases involving cattle, many others are thought not to be poisonous. *Corydalis* species are extensively employed in conventional Chinese medicine due to their evident antibacterial, antiviral, and anticancer properties.[7–9] In Asian folk medicines, they are used to cure a variety of disorders,[10–12] and research has revealed positive activity

[a] *B. Maharjan, Dr. R. L. Swagat Shrestha*
Department of Chemistry, Amrit Campus, Tribhuvan University, 44613 Kathmandu, Nepal
E-mail: swagatsta@gmail.com

[b] *Dr. L. Kumar Shrestha, Dr. J. P. Hill, Dr. K. Ariga*
Research Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS), 1-1 Namiki, 305-0044 Tsukuba, Ibaraki, Japan

[c] *Dr. L. Kumar Shrestha*
Department of Materials Science, Faculty of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, 305-8573 Tsukuba, Ibaraki, Japan

[d] *Dr. K. Ariga*
Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, 277-8561 Kashiwa, Chiba, Japan

[e] *Dr. S. Sharan Shrestha*
Himalayan Research and Development Center, Nepal, 44613 Kathmandu, Nepal

[f] *Dr. S. Sut, Dr. S. Dall'Acqua*
Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Via Marzolo 5, 35121 Padova, Italy
E-mail: stefano.dallacqua@unipd.it

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against ulcers,[13] breast cancer,[14] and leishmaniasis,[15] and excellent pharmacokinetic properties.[1] *Corydalis* is known to synthesize sanguinarine, alongside a group of anti-inflammatory benzylisoquinoline alkaloid (BIA) compounds referred to as cavidines. Examples of cavidines include apocavidine, cavidine, dehydroapocavidine, and dehydrocavidine.[16] The extract of *Corydalis edulis* (CE), was found to induce insulin exocytosis through a signaling pathway that is dependent on protein kinase C (PKC). Furthermore, CE was observed to specifically activate new protein kinase Cs (nPKCs) and atypical PKCs (aPKCs), while not affecting conventional PKCs (cPKCs), in HIT-T15 cells. CE exhibits promising capacity as a new therapeutic agent for the management of type 2 diabetes mellitus (T2DM) in human subjects.[17]

Corydalis chaerophylla D.C. (*C. chaerophylla*), collected from Nepal, is a glabrous herb found in high-altitude regions of Nepal, India, and Pakistan, flourishing in moist, shaded environments at an altitude of 2400–4800 m. It has been used to treat syphilis, scrofula, dysentery, and diarrhoea.[14] In traditional medicinal use, the juice of *C. chaerophylla* is given for peptic ulcers in doses of about 4 teaspoons, 3 times per day. Juice of the root (dosage: about 6 teaspoons, 3 times per day) is given for indigestion where the root juice is blended in equal amounts with the root juice of *Cyathula capitate* Moq. (N. Kuro).[18] Previous scientific studies of other *Corydalis* species led to the separation of numerous alkaloids that have proven effective in improving immune function, and in inhibiting the growth of tumours, cancer-causing liver viruses, amoebae, and hepatitis.[19–21] However, a review of the literature has revealed that little research has been performed specifically on *C. chaerophylla*. Considering the chemical composition one paper reported the isolation and structural elucidation, from *C. chaerophylla* the alkaloids chaerophylline, corypalmine, berberine, isocorypalmine, corydalmine and bicuculline have been isolated.[22]

To improve the knowledge on Nepalese *C. chaerophylla* and to further explore the potential usefulness as bioactive compound source of this plant here we report a comparative analysis of extracts of *C. chaerophylla*. The proposed approach included a simple spectrophotometrically based screening, liquid chromatography diode array and mass spectrometric detection (LC–DAD-MS) profiling, TPC, TFC, antimicrobial, antioxidant, cytotoxicity, antidiabetic and *in vivo* acute oral toxicity analysis. The present work will become the basis for the further bioassay-guided isolation of bioactive compounds (Figure 1).

Results and Discussion

Yields of Extracts and Phytochemical Screening

The yields of hexane, methanol and chloroform extracts were calculated to be 0.14%, 13.12% and 2.20%, respectively. Phytochemical screening performed on the extracts revealed that alkaloids, flavonoids, glycosides, steroids and terpenoids are present in the hexane extract. The methanol extract contained alkaloids, flavonoids, phenolic compounds, carbohydrates, glycosides, steroids, tannins, terpenoids and saponins, while the chloroform extract contained alkaloids, flavonoids, phenolic compounds, carbohydrates, glycosides, steroids, tannins, and terpenoids, as shown in Table 1.

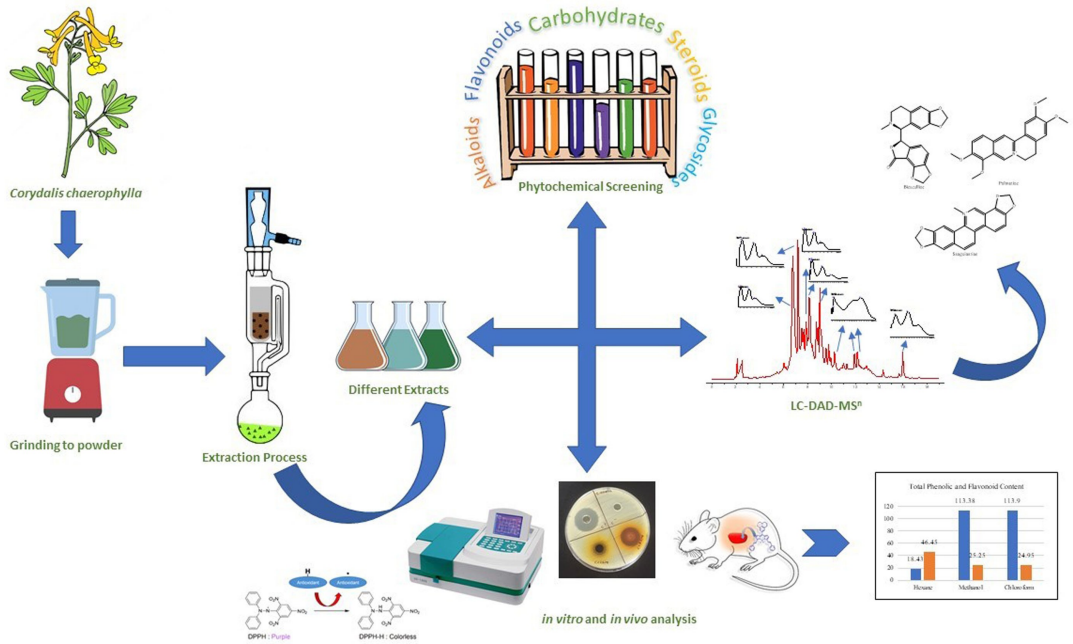

Figure 1. Overview of Research process.

Table 1. Phytochemical screening of extracts of *C. chaerophylla*.

Phytochemicals	Type of Tests	Result in Extracts		
		H	M	C
Alkaloids	Dragendorff″s Test	+	+	+
	Mayer's Test	+	+	+
	Wagner's Test	+	+	+
Flavonoids	Lead Acetate Test	+	+	+
	Shinoda Test	+	+	+
Phenolic Compounds	Ferric Chloride Test	–	+	+
	Lead Acetate Test	–	+	+
	Alkaline Reagent Test	–	+	+
Carbohydrates	Molisch's Test	–	+	+
	Fehling's Test	–	+	+
	Benedict's Test	–	+	+
Proteins	Millon's Test	–	–	–
	Biuret Test	–	–	–
Glycosides	Keller-Killiani Test	+	+	+
	Legal's Test	+	+	+
Steroids	Salkowski's Test	+	+	+
Tannins	Ferric Chloride Test	–	+	+
	Lead Acetate Test	–	+	+
	Alkaline Reagent Test	–	+	+
Anthraquinones	Anthraquinones Test	–	–	–
Terpenoids	Chloroform test	+	+	+
Saponins	Foam test	–	+	–

H: hexane, M: methanol, C: chloroform.

Alkaloid composition studied by Liquid Chromatography Diode-Array Detection and multiple stage mass spectrometry (LC–DAD-MS^n)

The extracts were analyzed combining the Diode-Array detection (DAD) and multiple stage mass spectrometry (MS^n) to obtain structural information on the eluted compounds. The chromatograms obtained from the hexane, methanol and chloroform extracts showed numerous peaks with significant UV absorptions. Based on the UV spectra of the major peaks, the presence of different classes of alkaloids, can be at least preliminary established.[23] Peaks presenting UV max at 296 nm suggest the presence of benzyltetrahydroisoquinoline derivatives, while protoberberinic alkaloids are characterized by the UV with maximum at 425 nm and 345 nm, and phthalide isoquinolinic by the maximum at 320 nm and 290 nm. Furthermore, in the last part of the chromatogram, peaks showing maximum absorption at 325 nm and 283 nm support the presence of benzophenanthridine alkaloids. The Figures 2–4 summarise that the main peaks of the three extracts are clearly supporting the presence of different alkaloids on the basis of their UV spectra.

Interpretation of MS^n spectra, comparison with the relevant literature, and the confirmation obtained with the injection of reference compounds allowed the identification of fifteen different alkaloids (**1**–**15**) that were also quantified on the basis of the LC–DAD in the three extracts. Compounds and amount are summarised in Table 2, structures are reported in Figure 5 and spectra are reported in supplementary materials. From qualitative point of view the use of multiple stage allowed the profiling of the alkaloid content in the different extracts.

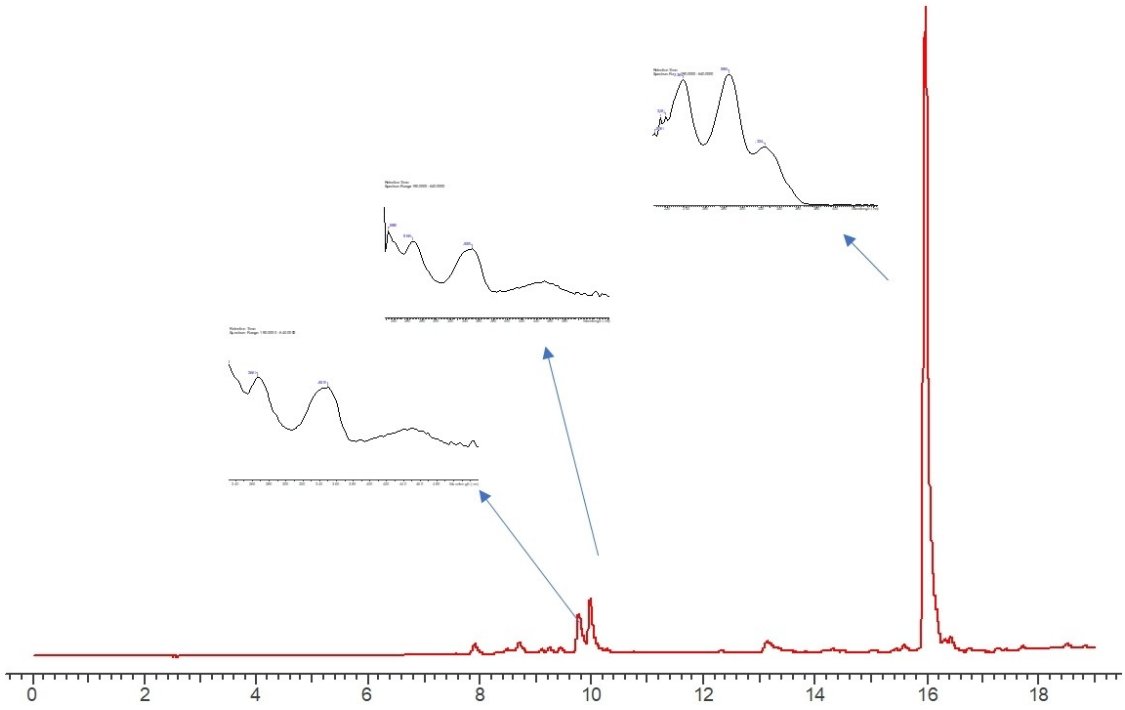

Figure 2. LC–DAD chromatogram (280 nm) of the hexane fraction, the UV spectra ascribable to main classes of isoquinoline alkaloids are shown.

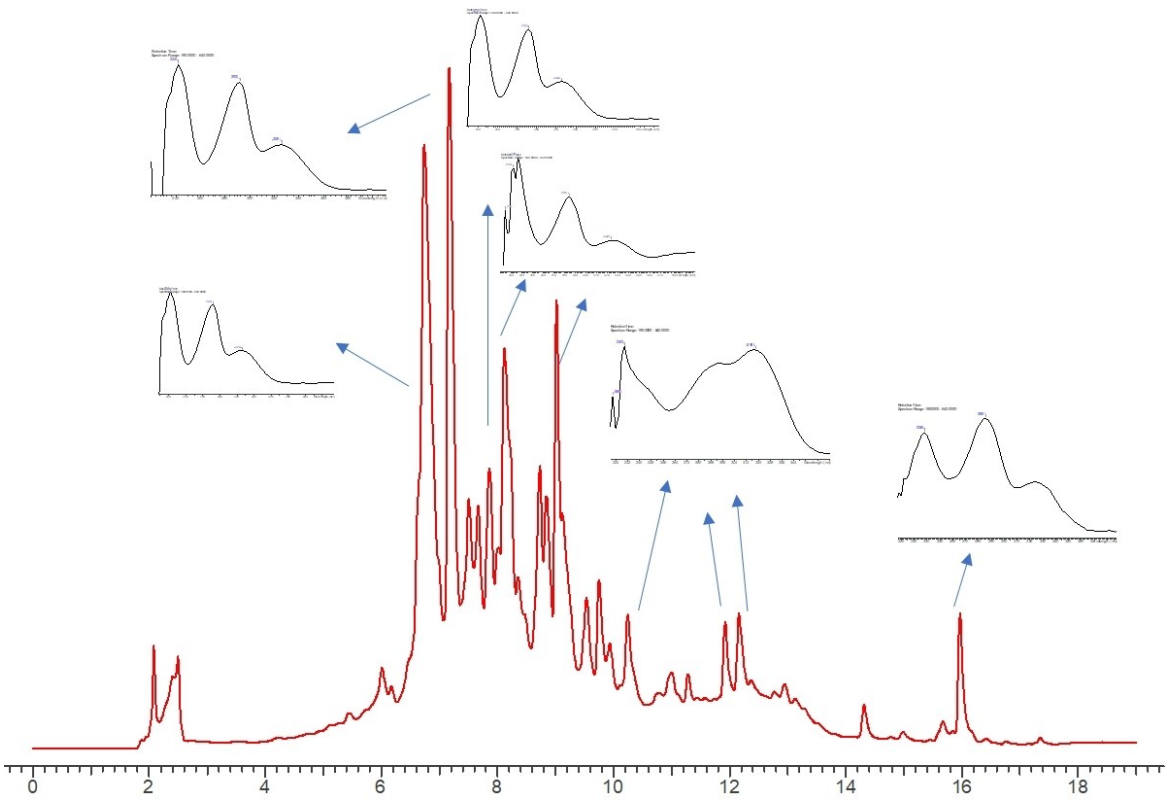

Figure 3. LC–DAD chromatogram (280 nm) of the methanol fraction, the UV spectra ascribable to main classes of isoquinoline alkaloids are shown.

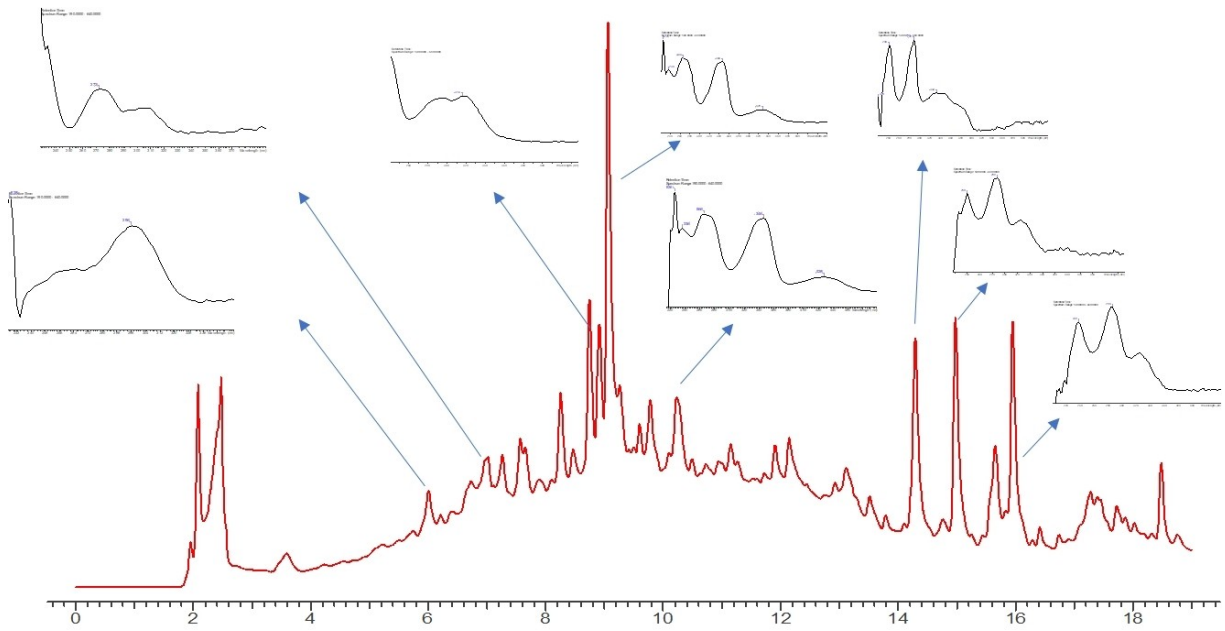

Figure 4. LC–DAD chromatogram (280 nm) of the methanol fraction, the UV spectra ascribable to main classes of isoquinoline alkaloids are shown.

The magnocurarine (**1**) presence was established due to the molecular ion $[M+H]^+$ at *m/z* 314 which due to the loss of the dimethylamino group lead to the ion at *m/z* 269 that further generate the fragment at *m/z* 175 due to the loss of hydroxybenzylic group. N–Me-tetrahydropalmatine (**2**) was assigned on the basis of the molecular ion $[M+H]^+$ at *m/z* 370 and fragments due to methyl loss generating *m/z* 352 and the fragment at *m/z* 190 formed from the N-methyl-isoquinolinium moiety. Similar fragmentation scheme can be observed for the tetrahydro columbamine (**3**) with molecular ion $[M+H]^+$ at *m/z* 342 generating in MS^2 the species at *m/z* 178 ascribable to the methoxy-hydroxy-isoquinolinium ion. Sharing similar structure

Table 2. Compounds observed in LC–DAD-MS of extracts of *C. chaerophylla*.

S.N.	Rt	m/z	Fragments	Compound	Extracts (mg/g)			Reference
					H	M	C	
1.	6.2	314	298.0, 269.0, 237.0, 175.0, 137.0, 107.0	Magnocurarine	–	0.12	0.08	[24]
2.	6.8	370	352.0, 334.0, 320.0, 290.0, 190.0, 175.0, 149.0, 131.0	N–Me-tetrahydropalmatine	0.77	23.31	218.01	
3.	8.53	340	178.0, 163.0	Tetrahydrocolumbamine	2.31	4.10	1.37	[25,26]
4.	8.58	356	192.0, 177.0, 148.0	N–Me-tetrahydrocolumb-amine	0.28	1.10	1.15	
5.	8.65	324	309	Demethylene berberine	4.26	12.48	45.38	
6.	8.84	368	307.0, 190.0	Bicuculline*	17.94	93.24	115.30	
7.	9.04	400	382.0, 355.0, 337.0, 319.0, 279.0	Protoberberine derivative	0.05	4.14	8.28	
8.	9.31	354	338.0, 190.0, 188.0, 149.0, 130	Protopine*	5.08	58.69	99.04	[24,27]
9.	9.34	356	338.0, 190.0	Hunnemanine	0.58	64.29	64.49	
10.	9.4	338	323.0, 294.0, 307.0, 279.0	Jatrorrhizine*	22.04	116.04	63.16	[24,25]
11.	9.5	368	338.0, 353.0, 321.0, 320.0, 307.0, 278.0	Berberastine	3.22	6.38	2.16	[24]
12.	9.76	352	336.0, 321.0, 308.0, 292.0, 278.0, 292.0, 275.0	Palmatine	3.30	8.30	2.09	[25,26]
13.	11.5	366	348.0, 323.0, 307.0, 190.0	Dehydrocorydaline	0.22	0.21	–	[25]
14.	15.8	332	317.0, 304.0, 274.0, 246.0	Sanguinarine*	1.88	2.52	0.36	[24,27]
15.	16.3	334	319.0, 304.0, 275.0, 246.0, 261.0	Dihydrosanguinarine	54.32	1.19	2.06	[24]

H: hexane, M: methanol, C: chloroform; compounds indicated with "*" were also confirmed by standard injection.

is the compound presenting molecular ion $[M+H]^+$ at *m/z* 356 which in MS^2 form species at *m/z* 192, 177 and 148 corresponding to the formation of the N-methyl-methoxy-hydroxy-dihydroisoquinolinium species and the corresponding loss of methyl group and CO (*m/z* 148) and that was assigned to N–Me-tetrahydrocolumbamine (**4**). The compound presenting molecular ion $[M+H]^+$ at *m/z* 324 that in MS^2 showed the loss of 15 and 17 Da leading to ion species at *m/z* 309 and 307 respectively. The MS^3 fragmentation led to the further loss of 15 Da originating species at *m/z* 294 and 279. MS^4 of the ion at *m/z* 294 generate fragments at *m/z* 269, 266 and 237. The compound can be assigned to a demethylene berberine (**5**) or to its isomer. Peak presenting *m/z* 368 was assigned to bicuculline (**6**) and the typical fragments of the phthalide isoquinoline are the ion at *m/z* 190 formed from the N-methyl-isoquinolinium moiety originated after the bond cleavage with phthalide ring. Fragmentation of the ion at *m/z* 190 show loss of the methyl group generating the ion at *m/z* 175. The identification was confirmed by co-injection of reference compound. The compound (**7**) showed molecular ion $[M+H]^+$ at *m/z* 400 and a loss of water leading to *m/z* 382. Further fragments were revealed at m/z 337, 319 and 289 supporting the presence of a protoberberine derivative bearing a hydroxyl substituent and methoxylation. Structure hypothesis for compound **7** is presented. The benzophenantridin derivative protopine (**8**) *m/z* 354 was identified on the basis of the diagnostic fragments at *m/z* 336 due to water loss and the one at *m/z* 188 and 204 corresponding to the structural cleavage and subsequent loss of water from the isoquinoline fragment. A similar derivative showing the same loss of water and intense fragment at *m/z* 190 presented molecular ion $[M+H]^+$ at *m/z* 356 and was assigned to hunnemanine (**9**) a benzophenantridine derivative.

The protoberberine alkaloid jatrorrhizine (**10**) presented molecular ion $[M+H]^+$ at *m/z* 338 was identified on the basis of the loss of the methyl groups generating *m/z* 323,324 and 307 as well as due to the cleavages that separate the isoquinoline and the benzyl moieties generating small ions at *m/z* 190. The fragmentation in the experimental condition was compared with authentic standard and retention time as well as fragmentation pattern were superimposable. Two more protoberberine derivatives were observed. The alkaloid presenting molecular ion $[M+H]^+$ at *m/z* 368 showed loss of water and of methyl groups and it was assigned to berberastine (**11**). The compound presenting molecular ion $[M+H]^+$ at *m/z* 352 was assigned to palmatine (**12**) due to the loss of the methyl group and the strong MS^2 signal at *m/z* 336 and intense signals at 348 and 307. The peak presenting molecular ion $[M+H]^+$ at *m/z* 366 and fragments at 348, 318 and 307 and 190 was tentatively assigned to dehydrocorydaline (**13**). Two intense peaks at higher retention time were assigned to benzophenantridinic alkaloids namely sanguinarine (**14**) characterized by the molecular ion $[M+H]^+$ at *m/z* 332 and fragment at 317 and intense fragment at 304 as reported in the literature and from the analysis of reference compound. The second presenting molecular ion $[M+H]^+$ at *m/z* 334 and similar pattern of fragmentation but with intense signal in MS^2 at m/z 319 and 304 was assigned to dihydrosanguinarine (**15**).

Thus, the Nepalese *C. chaerophylla*, presented various classes of alkaloids and it can be a valuable botanical source for the extraction of those compounds.

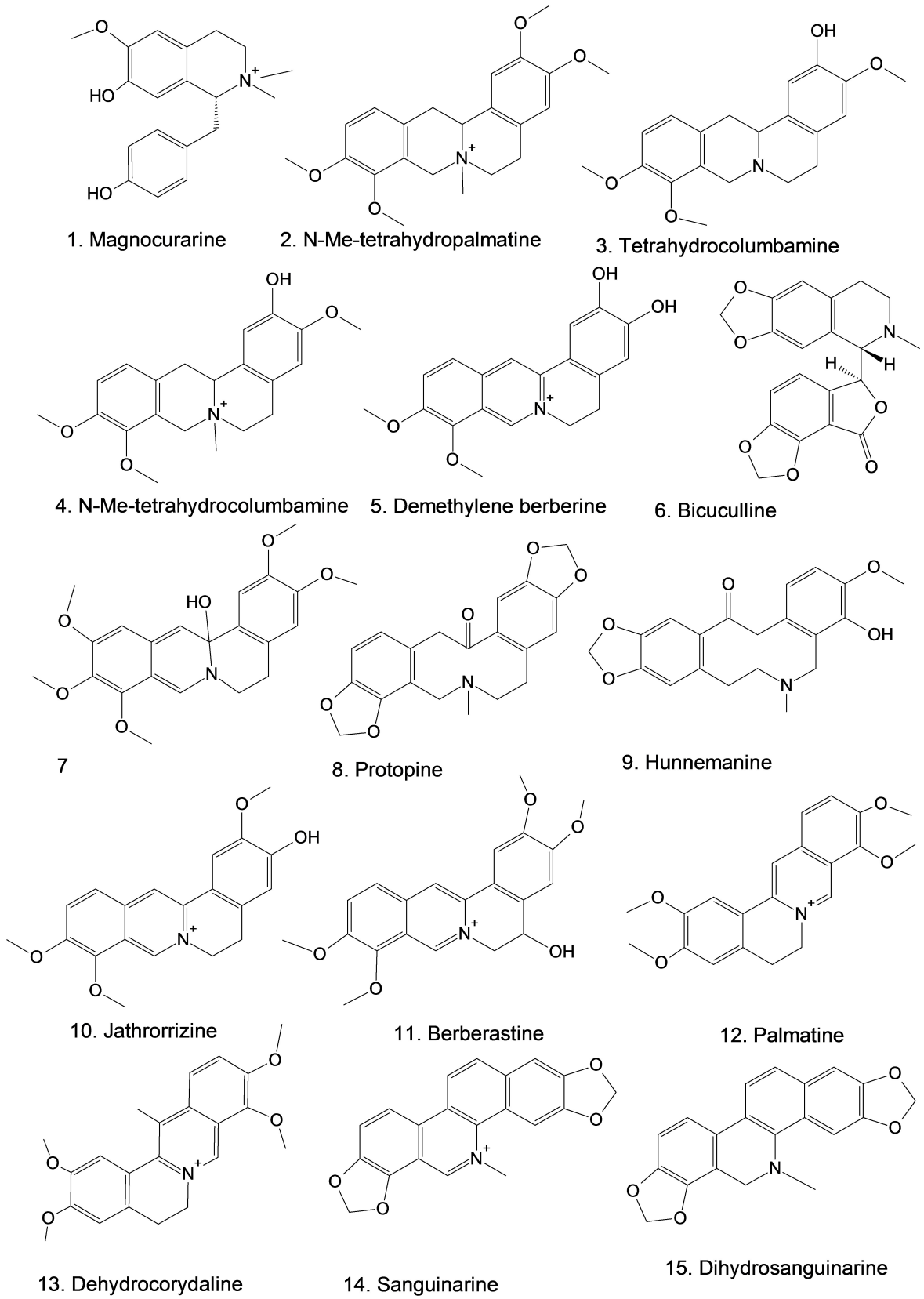

Figure 5. Structures of the identified alkaloids.

The quantitative data revealed that the use of solvents with different polarity can influence the composition of the obtained extract. In particular the nonpolar hexane is less favorable for the extraction of the whole alkaloids but in quite specific way can be used to obtain fraction mostly presenting the more nonpolar compounds as dihydrosanguinarine. The methanol and chloroform extracts on the other hand present larger ability to extract the different alkaloids and efficiently can extract jatrorrhizine, protopine and hunnemanine.

Total Phenolic and Flavonoid Content Analysis

Total phenolic content (TPC) was calculated as a milligram of gallic acid equivalent using the gallic acid calibration curve, and total flavonoid content (TFC) per gram of dried material is expressed as milligrams of quercetin equivalents.[28,29] As can be seen from Figure 6, the methanol and chloroform extracts contain similar concentrations of phenolic and flavonoid compounds. Methanol extract has a TPC of 113.38 mg GAE/g and a TFC of 25.25 mg QE/g. Chloroform extract also has a TPC 113.90 mg GAE/g with TFC 24.95 mg QE/g. However, hexane presented a lower quantity of phenolic (TPC: 18.43 mg GAE/g) but a higher quantity of flavonoids (TFC: 46.45 mg QE/g).

The antioxidant properties of plants are directly correlated with the phenolic and flavonoid content they possess. These substances function as reducing agents, providing hydrogen atoms, and possess the ability to scavenge free radicals.[30,31] The plant extracts of *C. chaerophylla* exhibit a noteworthy abundance of phenolics and flavonoids, which are likely to play a substantial role in the antioxidant properties. Due to the aforementioned characteristics, it is probable that this plant species has been employed in various traditional herbal remedies. The amount of phenols and flavonoids are also influenced by the polarity of the solvents employed during the extraction process.[32]

Antimicrobial Screening Analysis

The area around an antimicrobial disk where germs do not proliferate is known as the zone of inhibition.[33] To estimate the antimicrobial activity of plant extracts, the diameter of the zone of inhibition (ZOI) formed on certain bacterial and fungal cultures was measured. Using the method described in the succeeding section, the ability of various fractions of *C. chaerophylla* extracts to inhibit bacterial and fungal growth at a fixed dose (200 mg/mL) was evaluated. The results were represented in terms of the diameter of the zone of inhibition (table 3).

Hexane, methanol, and chloroform extracts of *C. chaerophylla* were evaluated for their capacity to inhibit the growth of two Gram-positive bacteria, *Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 6538P, and two Gram-negative bacteria, *Escherichia coli* ATCC 8739 and *Klebsiella pneumonia* ATCC 700603, and a fungal culture, *Candida albicans* ATCC 2091. The zone of inhibition (ZOI) was measured in centimeters.

The antimicrobial properties of hexane, methanol and chloroform extracts at concentration of 200 mg/mL were investigated and results are shown in Table 3. Methanol and chloroform extract showed comparable ZOI in all microbes used relative to the positive control Kanamycin 5 mg/mL. Whereas, the hexane extract showed antimicrobial activity against *Staphylococcus aureus* and *Candida albicans* only. The observed potent antimicrobial activity exhibited by the extracts from *C. chaerophylla* suggests that these plant extracts has the potential to serve as valuable antimicrobial agents. Furthermore, methanol and chloroform extracts may serve as a promising starting point for the isolation and identification of exciting antimicrobial compounds.

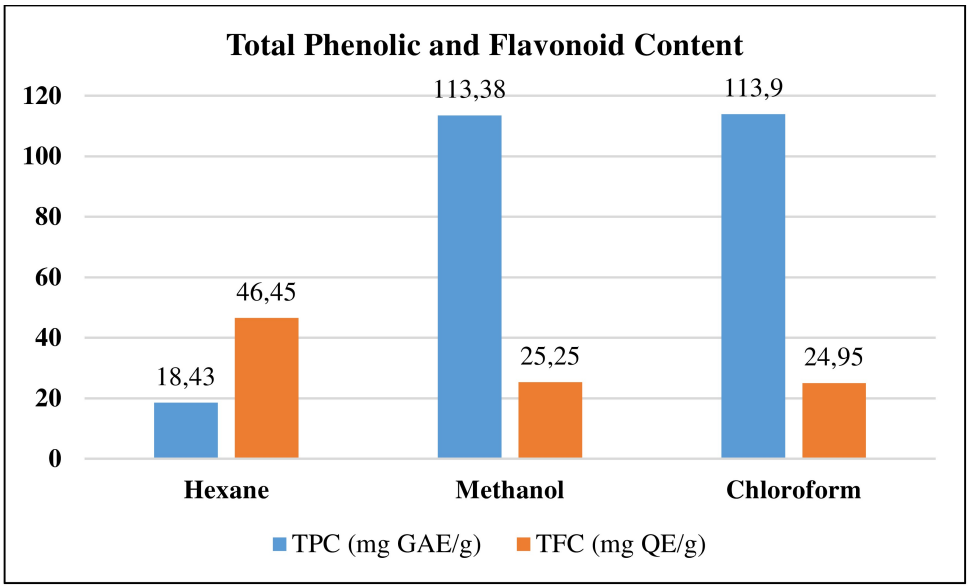

Figure 6. Total phenolic (TPC) and total flavonoid (TFC) content of the extracts of *C. chaerophylla*.

Table 3. Antimicrobial activity of extracts of *C. chaerophylla*.

Bacterial/Fungal strain	Reference culture	Type	Positive control (c+) cm	Negative control (c−) cm	Hexane Extract ZOI (cm)	Methanol Extract ZOI (cm)	Chloroform Extract ZOI (cm)
Escherichia coli	ATCC 8739	Gram−ve	2.6	0	0	2.5	1.8
Klebseilla pneumoniae	ATCC 700603	Gram−ve	1.8	0	0	1.2	1.7
Bacillus subtilis	ATCC 6051	Gram+ve	2.6	0	0	1.2	1.7
Staphylococcus aureus	ATCC 6538P	Gram+ve	2.6	0	1.3	2.0	2.3
Candida albicans	ATCC 2091	Fungi	2.4	0	1.5	2.0	2.3

Kanamycin was used as positive control at concentration of 5 mg/mL.

Antioxidant Screening Analysis

The DPPH results showing the percentage scavenged obtained by the absorbance at 517 nm of each extract is shown in Table 4.

The IC_{50} values of hexane, methanol, and chloroform extracts of *C. chaerophylla* were found to be 1586±3 μg/mL, 758.3±3 μg/mL, and 261.5±3 μg/mL, respectively. According to the data, hexane extract was shown to be the least effective at scavenging DPPH, methanol revealed moderate scavenging property whereas chloroform extract was found to be most effective. The observed DPPH radical scavenging activity can be attributed to the greater amount of phenolic compounds in the methanol and chloroform extracts compared to the hexane extract. These phenolic compounds are known to inhibit or prevent the detrimental effects of oxidative stress.[34] Phenolic compounds exhibit reducing and antioxidant properties through the hydrogen-donating ability of their hydroxyl groups.[35]

Table 4. IC_{50} values for DPPH assay of different extracts at of *C. chaerophylla*.

Extracts	Concentration (μg/mL)	Percentage Scavenged	IC_{50} (μg/mL)
Hexane Extract	4000	69.80	1586±3
	2000	59.24	
	1000	37.35	
	500	21.29	
	250	14.36	
Methanol Extract	1000	64.72	758.3±3
	500	54.02	
	250	25.19	
	125	8.73	
	62.5	3.87	
Chloroform Extract	1000	64.26	261.5±3
	500	57.13	
	250	39.23	
	125	26.33	
	62.5	15.25	

Reference compounds used in the assay: Ascorbic acid IC_{50} 12 μg/mL.

In vivo Brine Shrimp Lethality Analysis

The fact that the mortality rate of brine shrimp nauplii caused by *C. chaerophylla* increases with increasing concentration suggests that the hexane, methanol, and chloroform extracts contain cytotoxic agents that show dose dependent effect.

The lethality concentration (LC_{50}) of the three extracts, hexane, methanol and chloroform, were calculated as 112.3±3 μg/mL, 196.0±3 μg/mL and 153.9±3 μg/mL, respectively (Table 5). This result indicates that the hexane, methanol and chloroform extracts of *C. chaerophylla* are all active against brine shrimp lethality *in vivo* test. However, the hexane extract exhibited the lowest LC_{50} value for Brine Shrimp cytotoxicity at 112.3 mg/mL hence, the most active. The lethality of the three plant extracts against brine shrimps suggests that there is evidence of strong cytotoxic and perhaps anticancer constituents within the plant. Since the data were obtained at extract concentrations lower than 1000 μg/mL, all the extracts can be considered to be cytotoxic.[36]

Antidiabetic Analysis

In this study, the in vitro α-amylase inhibitory assay was employed to evaluate the antidiabetic properties of the extracts derived from *C. chaerophylla*.

The hexane, methanol, and chloroform extract of *C. chaerophylla* were found to have an IC_{50} values of 1156±3 μg/mL, 51.52±2 μg/mL, and 93.13±2 μg/mL, respectively (Table 6). From the data, methanol extract was found to have more significant activity for α-amylase inhibition than the other extracts with hexane extract being least active. The enzyme α-amylase is accountable for the process of hydrolyzing the 1,4-glucosidic bond found in substances such as starch, glycogen, and oligosaccharides. One of the techniques employed in the management of diabetes involves the inhibition of specific enzymes responsible for facilitating the breakdown of starch, hence reducing glucose absorption[37] thereby preventing

Table 5. LC_{50} values for Brine Shrimp Lethality Assay of different extracts of *C. chaerophylla*.

Extracts	Concentration (μg/mL)	Percentage Mortality	LC_{50} (μg/mL)
Hexane Extract	1000	100	112.3 ± 3
	500	100	
	250	80	
	125	56.67	
	62.5	43.33	
Methanol Extract	1000	100	196.0 ± 3
	500	96.67	
	250	56.67	
	125	46.67	
	62.5	43.33	
Chloroform Extract	1000	100	153.9 ± 3
	500	100	
	250	100	
	125	43.33	
	62.5	23.33	

Table 6. IC_{50} values for α-amylase inhibition of different extracts of *C. chaerophylla*.

Extracts	Concentration (μg/mL)	Percentage α-amylase inhibition	IC_{50} (μg/mL)
Hexane Extract	5000	70.31	1156 ± 3
	2500	51.17	
	1250	41.83	
	625	31.48	
	312.5	22.19	
Methanol Extract	500	65.17	51.52 ± 2
	250	60.19	
	125	51.30	
	62.5	39.34	
Chloroform Extract	500	70.93	93.13 ± 2
	250	62.04	
	125	41.65	
	62.5	38.61	

Acarbose was used as the standard and IC_{50} was 6.1 ± 0.1 μg/mL.

postprandial hyperglycemia conditions. Further studies are needed to assess the potential antidiabetic properties of the identified alkaloids.

In vivo Acute Oral Toxicity Study

The median lethal doses (LD_{50}) of the active ingredient in *C. chaerophylla* extracts were found to be > 2000 mg/kg BW for hexane extract,[57–59] 1000.36 mg/kg BW for methanol extract, and 515 mg/kg BW for chloroform extract for mice under environmental conditions (Table 7).

The data obtained reveal that hexane extract can be considered safe or less harmful, showing no *in vivo* toxicity. In contrast, methanol extract is slightly hazardous, with chloroform extract being moderately hazardous. The different toxicities of the extracts are probably due to their variable compositions.

Conclusions

This work is focused on investigating phytochemicals from *C. chaerophylla* to establish their relative *in vitro* and *in vivo* biological properties. Extraction of plant materials using hexane, methanol and chloroform to give extracts, and their phytochemical screening have been performed. LC–DAD-MS^n of the three extracts indicated the presence of fifteen different alkaloids. Hexane extract contained high amount of dihydrosanguinarine, the spectrophotometrical assays indicated a larger flavonoid component over phenolic content. In contrast, methanol and chloroform extracts present high amount of Jatrorrhizine and Bicuculline while the methanol one also presents high amount of N–Me-tetrahydropalmatine. The methanol and chloroform extract in the spectrophotometrical measurements showed a more significant phenolic component over flavonoids. Methanol and chloroform extracts exhibited significant ZOI against all tested pathogens in antimicrobial assay. Methanol extract exhibited moderate results for DPPH scavenging while the chloroform extract is more active. All three extracts presented cytotoxic activity against Brine Shrimp demonstrating > 1000 μg/mL LC_{50}. The methanol and chloroform extracts can be considered as an important inhibitor of α-amylase in antidiabetic activity due to the measured IC_{50} values. Furthermore, the *in vivo* acute oral toxicity in mice revealed a harmful effect of methanol and chloroform extract when swallowed.

Experimental Section

Collection of the Plant Materials

C. chaerophylla plants were collected in Phulchowki, Lalitpur, Nepal, at an altitude between 2400 and 2700 m (27.5711° N, 85.4056° E)

Table 7. Median Lethal Dose (LD_{50}) of different extracts of *C. chaerophylla*.

Extracts	LD_{50} (mg/kg BW)	Hazard Statement	Remarks
Hexane	> 2000 mg/kg BW	May be harmful if swallowed	No death at 2000 mg/kg
Methanol	1000.36 mg/kg BW	Harmful if swallowed	Death with convulsion
Chloroform	515 mg/kg BW	Harmful if swallowed	Death with convulsion

and identified by Mr. Ganga Datt Bhatt, Research Officer, National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal (Voucher specimen 901).

Extraction

The plant materials so collected were cleaned, dried in the shade and processed into a powder. The powdered *C. chaerophylla* plant material was treated with hexane. The hexane extract was then concentrated using a rotary evaporator. The plant residue remained after the hexane extraction was extracted using methanol employing cold percolation and the Soxhlet process. The resulting methanol extract solution was concentrated using a rotary evaporator. The crude methanol extract was then treated with 7% citric acid, neutralized with NH_4OH, and extracted using chloroform.

Phytochemical Screening

The extracts, namely hexane, methanol, and chloroform, were subjected to analysis in order to determine the presence of various chemical constituents including alkaloids, flavonoids, phenolic compounds, carbohydrates, proteins, glycosides, steroids, tannins, anthraquinones, terpenoids, and saponins. This analysis was conducted following the standard protocol as described in references.[38,39] The qualitative findings are represented by the symbols (+) and (−), denoting the presence and absence of phytochemicals, respectively in Table 1.

LC–DAD-MS^n

Dried extracts obtained after solvent removal of the hexane, chloroform and methanol plant extracts, were exactly weighted (20 mg) and dissolved in methanol by sonication and then analysed by LC–DAD-MS^n. An Agilent 1260 chromatograph equipped with autosampler and diode array (DAD) detector was used for chromatographic separation, while Varian Mass spectrometer (MS) Ion Trap, model MS500, was used operating in positive ion mode with electrospray (ESI) ion source. For the separation, agilent SBC18 4.6×50 mm (1.8 micron) was used. As eluent mixtures of three different solvents in gradient mode was used, as reported in the Table 8.

The flow rate was set to 750 μL/min. Diode array spectra were acquired in the 200–600 nm range. After the column, the flow was split using a passive T junction allowing detection by DAD and MS. Spectra were acquired using the TDDS function of the instrument that allows the fragmentation of detected ionic species. Alkaloids were detected in positive ion mode, phenolics were detected in negative ion mode. Berberine, protopine, and bicuculline were used as reference alkaloids. Rutin, quercetin, catechin, epicatechin, chlorogenic acid and gallic acid were used as reference phenolic compounds. All compounds were also used for quantitative purposes by generating calibration curves.[24–27,40]

Table 8. Ternary gradient.

Min	Water 1% formic acid	Acetonitrile	MeOH
0	95	5	0
2.5	85	15	0
12	80	18	2
15.5	50	40	10
19	20	70	10
21	0	85	15
23	0	85	15
24	0	100	0
26	0	100	0
27	95	5	0

Total Phenol Content Assay

The total concentration of phenolic compounds in the hexane, chloroform and methanol extracts was determined using the Folin-Ciocalteu method at 765 nm. Estimated total phenolic contents are given as Gallic acid equivalents per gram of dried extract.[29,41,42]

Total Flavonoid Content Assay

Total flavonoid concentration in the extracts of *C. chaerophylla* was ascertained using the aluminium chloride colorimetric method at 415 nm using methanol as control and Quercetin was used as a standard. The flavonoid concentration was calculated as mg of equivalent quercetin per gram of dried material.[41,43]

Antimicrobial Activity

Preparation of microbial culture media

The liquid broth (LB) media was prepared by dissolving 13 g of LB powder (Himedia research Laboratories Pvt. Ltd., India) in 1 L of water. The mixture was autoclaved at 15 psi pressure at 121 °C for 25 minutes. The sterilized media was cooled down to 40–50 °C, followed by transferring into sterilized 15 mL falcon tubes (5 mL each). The prepared media was used to co-culture bacterial seed culture in each tube separately and was incubated for 24 hours.

Preparation of MH media plates and antimicrobial assay

The Mueller -Hinton Agar (MHA) plates were prepared by dissolving 39 g of MH agar powder (Himedia Research Laboratories Pvt. Ltd., India) in 1 L of water. The mixture was autoclaved at 15 psi pressure at 121 °C for 25 minutes. The sterilized media was cooled down to 40–50 °C, followed by transferring into Petri dishes (25 mL each). The prepared media plates were stored in refrigerator until used. The prepared media plates were labeled properly with sample names and 150 μL liquid bacterial suspension were spread with the help of sterile cotton swab on the surface of media plates. The wells were made on the surface of agar and the samples 200 mg/mL (100 μL) and standard kanamycin 5 mg/mL (10 μL) were loaded in the prepared well. The media plates were then incubated for 24 hours at 37 °C. The antimicrobial test results were observed after 24 hours.[44–46]

Antioxidant Activity

Antioxidant assays were performed against DPPH assay at 517 nm using and methanol as a control.[47] A calibration curve was also constructed. The IC_{50} value (Inhibitory concentration of 50%) is the effective sample concentration that must be present for the sample to scavenge 50% of the DPPH free radicals. Using the dose inhibition curve in the logarithm range, we were able to determine the IC_{50} values by plotting the extract concentration vs. the corresponding scavenging action.[48–50]

Table 9. Classification of substances according to the guidelines of the Globally Harmonized system of classification and labeling of chemicals (GHS), third edition[57]

Ranges (mg/kg)	Category	Classification	Hazard statement
>2000 mg/kg	Category 5	Not classified	May be harmful if swallowed
$>300 \leq 2000$ mg/kg	Category 4	Dangerous	Harmful if swallowed
$>50 \leq 300$ mg/kg	Category 3	Toxic	Toxic if swallowed
$>5 \leq 50$ mg/kg	Category 2	Very toxic	Fatal if swallowed
<5 mg/kg	Category 1	Highly toxic	Fatal if swallowed

Brine Shrimp Lethality Assay

Lethality experiments using brine shrimp (*Artemia salina*, fairy shrimp, or sea monkeys) were carried out. The nauplii of brine shrimp were exposed for 24 hours to solutions containing varying concentration of each extracts from *C. chaerophylla*. The number of motile nauplii was used to determine efficacy of the three extract. The Brine Shrimp Lethality Assay indicates that tested extract are active if the LC_{50} value is lower than 1000 μg/mL.[51–55]

In vitro inhibition of α-amylase

In this experiment, the 3,5-dinitrosalicylic acid (DNSA) technique was used to inhibit α-amylase. *C. chaerophylla* extracts were diluted to a minimum concentration of 10% DMSO. The material was combined with buffer, NaCl, and DMSO at a pH of 6.9 to provide a wide range of concentrations. A 200 μL aliquot of this mixture was then added to the α-amylase solution and the mixture was incubated for 10 minutes at 30 °C. After that, 200 μL of the 1% starch solution was added to each tube, and the tubes were left to set for 3 minutes. Addition of DNSA reagent (200 μL), detained this process. The combined sample was heated in an 85–90 °C water bath for 10 minutes. After the combined sample had cooled to room temperature, it was diluted with distilled water (5 mL). A UV spectrophotometer was used to measure the sample's absorbance at 540 nm and compared to the blank solution. Phosphate buffer pH 7.4 (200 μL), was used in place of the plant extract to produce a blank with 100% enzyme activity.[56] A plot of extract concentration against percentage of α-amylase inhibition was constructed to obtain the IC_{50} values, which show the concentration at which α-amylase inhibition is 50%.

In vivo assays

All the experimental protocols were reviewed and approved with reference number 312079/80.

In vivo Acute Oral Toxicity Study

Based on the Chemical Testing Guidelines for Acute Oral Toxicity, the OECD's Acute Toxic Class Method 425 was applied. In the Natural Product Research Laboratory (NPRL), Thapathali, Nepal pharmacology lab, experiments were conducted on mice. The subjects were fasted for twelve hours prior to commencement of the experiment. The body weight of the mice was measured just prior to provision of the extracts. Two groups of animals were randomly assigned. The first (control) group received physiological saline, the second group received the extract through an orogastric tube at a dose of 2000 mg/kg of body weight, as reference Table 9 was used to classify effects.

Clinical observations of the subjects were conducted four times per day, with close attention paid to their behavior, general health, nasal mucosa, alterations to their skin and fur, respiratory frequency, somatomotor activity, and any potential occurrence of symptoms such as tremors, convulsions, diarrhoea, fatigue, salivating, low response to stimuli, sleep, light sensitivity, and coma. Abdominal palpation was also performed. The experimental group was given 2000 mg/kg of seed oil following 48 hours of clinical observation, during which there were no indicators of harm. The statistical test "t-Test for Independent Groups" was implemented in STATISTIC V. 7.0 for Windows; P values of 0.005% were considered relevant. The mice were cremated humanely at the conclusion of the experiment.[57–59]

Author Contributions

Conceptualization: B. Maharjan, R. L. Shrestha, S. Sut, S. Dall'Acqua; Lab Work: B. Maharjan, S. Sut, S. Dall'Acqua, J. P. Hill, K. Ariga; Manuscript preparation: B. Maharjan, S. Sut, S. Dall'Acqua and R.L. Shrestha; Review and Editing: S. S. Shrestha, R. L. Shrestha, J. P. Hill, K. Ariga; Supervision: S. Dall'Acqua and R. L. Shrestha.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: *Corydalis chaerophylla* · extraction · chemical analysis · bioactivities

[1] L. M. M. Marques, D. R. Callejon, L. G. Pinto, M. L. de Campos, A. R. M. de Oliveira, R. Vessecchi, A. Adhikari, R. L. S. Shrestha, R. G. Peccinini, N. P. Lopes, *J. Pharm. Biomed. Anal.* **2016**, *131*, 464–472.
[2] D. G. I. Kingston, *J. Nat. Prod.* **2011**, *74*, 496–511.
[3] J. Chen, W. Li, H. Yao, J. Xu, *Fitoterapia* **2015**, *103*, 231–241.
[4] G. H. Dar, S. T. Koul, A. R. Naqshi, A. A. Khuroo, A. H. Malik, *Taiwania* **2011**, *56*, 305–308.
[5] W. Majak, Y. Bai, M. H. Benn, *Biochem. Syst. Ecol.* **2003**, *31*, 649–651.
[6] T. Schmeller, B. Latz-Brüning, M. Wink, *Phytochemistry* **1997**, *44*, 257–266.
[7] X. Liu, H. Zheng, R. Lu, H. Huang, H. Zhu, C. Yin, Y. Mo, J. Wu, X. Liu, M. Deng, D. Li, B. Cheng, F. Wu, Y. Liang, H. Guo, H. Song, Z. Su, *Front. Microbiol.* **2019**, *10*, 1–18.
[8] B. Tian, M. Tian, S. M. Huang, *Pharm. Biol.* **2020**, *58*, 265–275.
[9] B. Zhang, R. Huang, J. Hua, H. Liang, Y. Pan, L. Dai, D. Liang, H. Wang, *Phytomedicine* **2016**, *23*, 1599–1609.
[10] Z. Z. Ma, W. Xu, N. H. Jensen, B. L. Roth, L. Y. Liu-Chen, D. Y. W. Lee, *Molecules* **2008**, *13*, 2303–2312.
[11] H. L. Li, W. D. Zhang, R. H. Liu, C. Zhang, T. Han, X. W. Wang, X. L. Wang, J. B. Zhu, C. L. Chen, *J. Chromatogr. B* **2006**, *831*, 140–146.
[12] C. Cheng, J. Qian, Z. Wang, W. Li, C. Huang, M. Chen, Y. Dong, L. Lian, W. Sun, *Biomed Res. Int.* **2019**, *2019*, 1–9.
[13] R. L. Shrestha, A. Adhikari, B. P. Marasini, R. N. Jha, M. I. Choudhary, *Phytochem. Lett.* **2013**, *6*, 228–231.
[14] N. Sivakumaran, S. R. Samarakoon, A. Adhikari, M. K. Ediriweera, K. H. Tennekoon, N. Malavige, I. Thabrew, R. L. S. Shrestha, *Biomed Res. Int.* **2018**, *2018*, DOI 10.1155/2018/3171348.
[15] D. R. Callejon, T. B. Riul, L. G. P. Feitosa, T. Guaratini, D. B. Silva, A. Adhikari, R. L. S. Shrestha, L. M. M. Marques, M. D. Baruffi, J. L. C. Lopes, N. P. Lopes, *Molecules* **2014**, *19*, 5692–5703.
[16] D. S. Bhakuni, R. Chaturvedi, *J. Nat. Prod.* **1983**, *46*, 466–470.
[17] J. Zheng, Y. Zhao, Q. Lun, Y. Song, S. Shi, X. Gu, B. Pan, C. Qu, J. Li, P. Tu, *Sci. Rep.* **2017**, *7*, 1–11.
[18] N. P. Manandhar, *Contrib. to Nepal. Stud. J.* **1993**, *20*, 184–196.
[19] E. K. Jeong, S. Y. Lee, S. M. Yu, N. H. Park, H. S. Lee, Y. H. Yim, G. S. Hwang, C. Cheong, J. H. Jung, J. Hong, *Rapid Commun. Mass Spectrom.* **2012**, *26*, 1661–1674.
[20] N. V. Khodorova, A. L. Shavarda, M. Lequart-Pillon, J. C. Laberche, O. V. Voitsekhovskaja, M. Boitel-Conti, *Phytochemistry* **2013**, *92*, 60–70.
[21] N. Muhammad, R. L. Shrestha, A. Adhikari, A. Wadood, H. Khan, A. Z. Khan, F. Maione, N. Mascolo, V. De Feo, *Nat. Prod. Res.* **2015**, *29*, 430–437.
[22] R. N. Jha, M. B. Pandey, A. K. Singh, S. Singh, V. P. Singh, *Nat. Prod. Res.* **2009**, *23*, 250–255.
[23] A. W. Sangster, K. L. Stuart, *Chem. Rev.* **1965**, *65*, 69–130.
[24] Y. Tian, C. Zhang, M. Guo, *BMC Complement. Altern. Med.* **2017**, *17*, 460.
[25] M. Sun, J. Liu, C. Lin, L. Miao, L. Lin, *Acta Pharm. Sin. B* **2014**, *4*, 208–216.
[26] X. Wei, H. Shen, L. Wang, Q. Meng, W. Liu, *J. Anal. Methods Chem.* **2016**, *2016*, DOI 10.1155/2016/9752735.
[27] M. Tian, C. Yang, J. Yang, H. Dong, L. Liu, Y. Ren, Z. Wang, *Molecules* **2019**, *24*, 3463.
[28] K. H. Miean, S. Mohamed, *J. Agric. Food Chem.* **2001**, *49*, 3106–3112.
[29] S. Lallianrawna, R. Muthukumaran, V. Ralte, G. Gurusubramanian, N. S. Kumar, *Sci. Vis.* **2013**, *13*, 149–156.
[30] A. Wojdyło, J. Oszmiański, R. Czemerys, *Food Chem.* **2007**, *105*, 940–949.
[31] N. Phuyal, P. K. Jha, P. P. Raturi, S. Rajbhandary, *Sci. World J.* **2020**, *2020*, DOI 10.1155/2020/8780704.
[32] L. Jing, H. Ma, P. Fan, R. Gao, Z. Jia, *BMC Complement. Altern. Med.* **2015**, *15*, 1–12.
[33] M. Nisar, H. Shah, I. Khan, Fazal-Ur-Rehman, M. S. Khan, S. K. Marwat, Z. R. Niazi, A. Ullah, *Asian J. Chem.* **2013**, *25*, 1069–1072.
[34] L. L. Mensor, F. S. Menezes, G. G. Leitao, A. S. Reis, T. C. dos Santos, C. S. Coube, S. G. Leitao, *Phyther. Res.* **2001**, *15*, 127–130.
[35] A. Aberoumand, S. S. Deokule, *Pakistan J. Nutr.* **2008**, *7*, 582–585.
[36] L. F. Olowa, O. M. Nuñeza, *Int. Res. J. Biol. Sci.* **2013**, *2*, 74–77.
[37] L. Sun, Y. Wang, M. Miao, *Trends Food Sci. Technol.* **2020**, *104*, 190–207.
[38] E. Iqbal, K. A. Salim, L. B. L. Lim, *J. King Saud Univ. - Sci.* **2015**, *27*, 224–232.
[39] K. S. Banu, L. Cathrine, *Int. J. Adv. Res. Chem. Sci.* **2015**, *2*, 25–32.
[40] X. Cheng, Y. Shi, S. Zhen, H. Sun, W. Jin, *J. Chromatogr. Sci.* **2010**, *48*, 441–444.
[41] V. Banothu, C. Neelagiri, U. Adepally, J. Lingam, K. Bommareddy, *Pharm. Biol.* **2017**, *55*, 1155–1161.
[42] M. O. Agbo, P. F. Uzor, U. N. Akazie-Nneji, C. U. Eze-Odurukwe, U. B. Ogbatue, E. C. Mbaoji, *Dhaka Univ. J. Pharm. Sci.* **2015**, *14*, 35–41.
[43] S. Oksana, B. Marian, R. Mahendra, S. H. Bo, *J. Med. Plants Res.* **2012**, *6*, 2526–2539.
[44] R. K. Das, *Stud. Fungi* **2018**, *3*, 293–301.
[45] S. Manandhar, S. Luitel, R. K. Dahal, *J. Trop. Med.* **2019**, *2019*, DOI 10.1155/2019/1895340.
[46] A. Klančnik, S. Piskernik, B. Jeršek, S. S. Možina, *J. Microbiol. Methods* **2010**, *81*, 121–126.
[47] A. K. Pandey, M. Mohan, P. Singh, U. T. Palni, N. N. Tripathi, *Food Biosci.* **2014**, *7*, 80–87.
[48] R. L. Shrestha, A. Adhikari, *J. Pharmacogn. Phytochem.* **2017**, *6*, 568–570.
[49] L. U. Khan, R. A. Khan, S. Khan, S. A. Bano, F. Fasim, B. Uzair, *Int. J. Pharmacol.* **2017**, *13*, 1000–1009.
[50] V. Sharma, A. Agarwal, U. Chaudhary, M. Singh, *Int. J. Pharm. Pharm. Sci.* **2013**, *5*, 317–320.
[51] S. Islam, S. Rahi, H. K. Koli, I. Jerin, S. A. Sajib, K. F. Hoque, A. Reza, *Malaya J. Biosci.* **2018**, *5*, 1–13.
[52] M. Asaduzzaman, S. Rana, S. M. R. Hasan, M. Hossain, N. Das, *Int. J. Pharma Sci. Res.* **2015**, *6*, 1179–1185.
[53] C. N. S. Antonio, B. de S. Elnatan, F. aacute bio G. R. Marcos, R. J. R. A. Maria, N. B. Paulo, S. dos S. R. P. H eacute lcio, M. de M. Selene, O. dos S. F. Raquel, S. de P. C. Carolina, *African J. Pharm. Pharmacol.* **2016**, *10*, 346–355.
[54] S. A. Gadir, *J. Chem. Pharm. Res.* **2012**, *4*, 5145–5148.
[55] Q. S. Sarah, F. C. Anny, M. Misbahuddin, *Bangladesh J. Pharmacol.* **2017**, *12*, 186–189.
[56] O. O. Olubomehin, K. A. Abo, E. O. Ajaiyeoba, *J. Ethnopharmacol.* **2013**, *146*, 811–814.
[57] R. Ranjitkar, D. P. Bhandari, L. Bhandari, *J. Plant Resour.* **2019**, *17*, 82–85.
[58] OECD, *Guidel. Test. Chem.* **2022**, 26.
[59] D. Kumar, P. Sharma, K. Nepali, G. Mahajan, M. J. Mintoo, A. Singh, G. Singh, D. M. Mondhe, G. Singh, S. K. Jain, G. K. Gupta, F. Ntie-Kang, M. J. Mintoo, D. M. Mondhe, F. Ntie-Kang, *Heliyon* **2018**, *4*, e00661.

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