Phytochemical and biological evaluation and isolation of quercetin from hypericum cordifolium flower

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Abstract:*Hypericum cordifolium* is a medicinal plant used in the treatment of various ailments in Nepalese traditional medicine. The medicinal values and bioactive constituents of *Hypericum perforatum*have been well investigated and is used as an alternative medicine in the treatment of depression, cancer, inflammation, bacterial and viral infections. However, scientific reports on Nepalese *H. cordifolium* are very limited. Here we report about the chemical screening of extracts, quality analysis of *H. cordifolium* extract by thin layer chromatography, TLC using *H. perforatum* extract and hyperoside as the standards, estimation of phenolics, flavonoids, tannins and sugars as well as antioxidant, antibacterial and cytotoxic activities of flower extracts. The ethyl acetate extract was fractionated and some phytochemicals were isolated. In our findings, TLC analysis of *H. cordifolium* extract showed the presence of chlorogenic acid, hyperoside and quercetin. Hypericine and rutin were not detected which indicated that the chemotaxonomy of *H. cordifolium* is different from *H. perforatum.* In phytochemical analysis, ethyl acetate extract showed the greater amounts of flavonoids, methanol extract showed the greater amounts of tannins and sugars and 50% aqueous methanol extract showed the greater amounts of phenolics. In DPPH free radical scavenging assay, ethyl acetate (18.79±0.98 µg/ml) and 50% aq. methanol (18.98±0.81 µg/ml) extracts showed the lower IC_{50} values than the methanol extract (27.00±1.03 µg/ml) due to the presence of high amounts of flavonoids and phenolics in these extracts. In antibacterial assay, all extracts showed moderate antibacterial activity against *Staphylococcus aureus* except 50% aqueous methanol extract. In cytotoxicity assay on human bladder carcinoma cell line 5637, ethanol extract showed IC_{50} value of 41.35±5.01 µg/ml. Quercetin was isolated from ethyl acetate extract and characterized by UV and FTIR spectroscopy. The present findings indicated that the flowers of *H. cordifolium* could be the rich source bioactive compounds with antibacterial, cytotoxic and antioxidant activities.

Keywords: Antioxidant; Antibacterial; Cytotoxicity; Phytochemical analysis.

Introduction

The genus *Hypericum* (Hypericaceae) comprises 484 species, distributed in a variety of habitats from tropical, temperate to alpine regions[1]. Many species of *Hypericum* are reported from Nepal. Among them, *H. cordifolium* is the most abundant species distributed around Kathmandu valley at an altitude between 900-2000 m on open slopes. Locally it is called Areli, Areto or Ghod Jatra Phool as it is offered in the religious places in Ghoda Jatra festival. The yellow flowers bloom from March to April[2]. In Nepalese traditional medicine, the juice of *H. cordifolium* is given to menstrual disorder, root juice is used to treat diarrhea, bark juice is applied in the case of back pain and dislocation of bone, flower paste, fresh or dried is eaten with warm water to cure dysentery. The fresh young shoot is eaten as medicine to relieve throat pain and root and flower juice is used to treat fever, pneumonia, diarrhea, dysentery, cough and cold[2]. In our previous study, we have screened the flower extracts for wound healing activities[3]. It was also included in antiviral screening[4]. The leaf extracts were screened for cytotoxicity against Brine shrimps[5] and total phenolic, flavonoid and antibacterial activities were evaluated[6].

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Email: Karmacharyameena@gmail.com
Received: 22 January 2023; Received in revised form: 05 June 2023; Accepted: 09 June 2023.
Doi: https://doi.org/10.3126/sw.v16i16.56765

Among the different species of *Hypericum, H. perforatum* L. commonly known as St. John's wort is one of the thoroughly investigated species because of its wide medicinal values. Extracts of this plant are applied to treat minor burns, wounds, inflammation of the skin and nerve pain externally while internally the extracts are used for the treatment of anxiety and depressive episodes[7-9]. It is used widely in Germany for the treatment of depression as an alternative medicine and prescribed approximately 20 times more often than fluoxetine chloride[10]. It is also sold in Europe and North America to treat mild to moderate depression[11]. Besides its antidepressant activity, several additional pharmacological effects such as antiinfammatory, hepatoprotective, antiviral, antimicrobial, antioxidant, antitumoral and wound-healing activities have been reported[12-14] due to the presence of phytochemicals like naphthodianthrones, acyl-phloroglucinols, favonoids, phenolic acids and xanthones[15,16].

The quality and quantity of *Hypericum* secondary metabolites are responsible for their pharmacological activities and they depend on ecological and environmental conditions where they grow as well as physiological and genetic factors (Košuth *et al*., 2003). *H. cordifolium* of Nepalese origin that grows in unique climatic condition is still under explored. Therefore, the aim of the present investigation is the quality analysis of flower extract by TLC, quantification of phytochemicals in different flower extracts, isolation and characterization of phytochemicals by UV, FTIR spectroscopy and evaluation of antioxidant, cytotoxic, antibacterial activities of different flower extracts.

Materials And Methods

Plant materials

The plant, *H. cordifolium* was collected from the pine forest of Nagarkot area of Bhaktapur district in April 2021. It was authenticated by comparison with the herbarium species deposited at Central Department of Botany, Tribhuvan University. The voucher specimen (HC-21-MR) was deposited at Research Center for Applied Science and Technology, Tribhuvan University. The collected materials were separated into flowers, leaves and stems, shade dried separately and crushed to fine pieces and stored in an air tight container for further use.

General experimental procedure

All the solvents and chemicals used were of analytical grade and purchased from local vender. Gallic acid and precoated Silica gel 60 GF_{254}were purchased from Merck, Daemstadt, Germany, (±)-catechin and DPPH was purchased from Sigma Chemical Company, USA. Sephadex LH-20 was purchased from Pharmacia Biotech, Uppasala, Sweden. Absorbance was measured using Chemito UV-VIS Spectrophotometer. UV spectrum was measured on SHIMADZU UV-VIS UV-1900i spectrophotometer. FTIR was measures using High performance SHIMADZU Fourier Transform Infrared Spectrophotometer.

Extraction

The crushed flower of *H. cordifolium* (50 g) was extracted successively with hexane (300 ml) dichloromethane (300 ml), ethylacetate (200 ml) and methanol (200 ml) by using Soxhlet extractor. The remaining residue after extraction with methanol was dried and refluxed with 50% aqueous methanol (100 ml) for 2 hours then allowed to cool and filtered. The solvents were evaporated under reduced pressure using rotavapour. The concentrated solid or semi-solid mass was kept in freeze for further use. Similarly, 100 mg flower was percolated with 2 ml ethanol at room temperature for 24 hours. The solvent was evaporated and the dried extract was used for the cytotoxicity assay as well as for TLC analysis.

Phytochemical screening

The presence of different classes of natural constituents in different extracts were analyzed by using different specific reagents according to the standard protocol[17].

Quality analysis of ethanol extract

Extracts of *H. cordifolium* and *H. perforatum* (each 50 mg/ml in ethanol) were prepared and the standard hyperoside solution (1 mg/ml ethanol) was prepared for TLC analysis. The extracts were visualized in day light. The extracts and hyperoside were loaded and the TLC plate (Silicagel GF254) was developed with the mobile phase, EtOAc-MeOH –H_2O (100:13.5:10 v/v/v) and spots were visualized under UV light at 254. After that, the plate was sprayed with 1% methanolic diphenyl boryloxy ethylamine followed by 5% ethanolic polyethylene glycol, incubated for 30 minutes and visualized under 366 nm.

Estimation of total phenolic content

The total phenolics content in different extracts was estimated by colorimetric method using Folin-Ciocalteu reagent[18]. The diluted extract (1 mL) was mixed with 5 mL of Folin-Ciocalteu reagent (10% v/v) and 4 mL of sodium carbonate (7% w/v). The mixture was shaken and incubated for 30 min at 40°C in a water bath before absorption was measured at 760 nm. Total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram extract (mg GAE/g extract). All extracts were analyzed in triplicate. This method gives a general measurement of phenolic content, as it is not completely specific for phenolic compounds and not all phenolic compounds exhibit the same level of activity in the assay (Öztürk et al, 2009).

Estimation total flavonoid content

The total flavonoid content in different extract was estimated by Aluminium chloride colorimetric assay[19]. The diluted extract (1 mL) was mixed with 4 mL of double distilled water and 0.3 mL of $NaNO_2$ (5 % w/v). After 5 minutes, 0.3 mL of $AlCl_3$ (10% w/v)and after 1 minute 2 mL of 1 M NaOH was added. The total volume of the mixture was made up to 10 mL by the addition of 2.4 mL double distilled water and mixed thoroughly. Absorbance of the pink color mixture was determined at 510 nm. Total flavonoids content was expressed as catechin equivalents (CE) in milligrams per gram extract (mg CE/g extract). All extracts were analyzed in triplicate

Estimation total tannin content

The total tannin content in different extracts was estimated by using Folin-Ciocalteu method[20]. The diluted extract (1 mL) was mixed with 8.4 mL of double distilled water, 0.5 mL of FC reagent (10% v/v) and 0.1 mL of sodium carbonate solution (7% w/v). The mixture was then shaken well and the allowed to stand for 30 minutes and then absorbance was taken at 700 nm. Total tannins content of the extracts was expressed as mg tannic acid equivalents (TAE) per gram of dry extract (mg/g). All extracts were analyzed in triplicate.

Estimation total sugars content

The total carbohydrate/sugar content indifferent extracts was estimated by colorimetric assay using anthrone reagents[21]. The diluted extract (2 mL) was mixed with 8 mL of freshly prepared anthrone reagent (200 mg of anthrone in 100 mL ice-cold 95% conc. H_2SO_4). The mixture was shaken well and heated for 8 minutes at boiling water bath. Then cooled rapidly and the absorbance was measured at 630 nm. Total sugar content of the extracts was expressed as mg glucose equivalents (GE) per gram of dry extract (mg/g). All extracts were analyzed in triplicate.

Determination of antioxidant activity

Antioxidant activity of extracts was assessed using DPPH free radical[22]. A mixture of 2.5 ml of DPPH (0.10 mM in methanol) and 0.5 ml of extract was kept in the dark for 30 min. Its absorbance was then read at 517 nm. A control was prepared by mixing 0.5 ml methanol and 2.5 ml 0.1 mM methanolic DPPH solution. DPPH radical scavenging activity (%) was calculated as follows:

$$\%\ \text{of radical scavenging} = \frac{Ac - As}{Ac} \times 100$$

Where, Ac= Absorbance of control and As = Absorbance of solution.

IC_{50}values is the concentration of sample required to scavenge 50% of DPPH radical. It was calculated from the plotted graph of radical scavenging activity against the concentration of extracts. The percentage scavenging was then plotted against concentration and IC_{50} value of the extracts determined graphically.

Determination of cytotoxic activity

The ethanol extract of *H. cordifolium* was evaluated against Human bladder carcinoma cell line 5637 (ATCC HTB-9) cultured in RPMI (Roswell Park Memorial Institute) 1640 medium with 10% FCS (fetal calf serum). Cell viability was analyzed using the colorimetric WST-8 (water soluble tetrazolium salt-8) Cell Viability kit from Promokine (PromoCell GmbH, Heidelberg, Germany). Briefly 100 µl of a cell suspension was seeded into 96 well microtiter plate with 3000 cells/well. After twenty-four hours of incubation at 37ºC, the cells were treated with ethanol extract of *H. cordifolium* of different concentrations (250, 125, 62.5, 31.25 and 15.625 µg/ml) and incubated for next 72 hours. Etoposide was used as a positive control and DMSO was used as a solvent control. At the end of the incubation time, the medium was removed and the cells were washed with PBS and

stained with 10 µl WST-8 solution and again incubated for four hours at 37 ºC. All experiments were carried out twice with six replicates for each tested concentration. The absorbance was measured at 450 nm with an ELISA plate reader. The percentage growth values were calculated by the equation,

$$\% \text{ Growth} = \frac{ODt - ODc,0}{ODc - ODc,0} \times 100$$

where ODt is the mean absorbance of the treated cells, ODc is the mean absorbance of the control, ODc,0 was the mean absorbance at the time the extract was added. The IC_{50} value was calculated by linear regression using MS Excel.

Determination of antimicrobial activity

The antimicrobial activity of the extracts were evaluated by agar well diffusion method[23] against one Gram positive bacteria *Staphylococcus aureus*(ATCC 25923) two Gram negative bacteria *Escherichia coli* (ATCC 25922) and *Klebsiella pneumonia*(ATCC 14028) and an yeast *Candida maltosa* by agar well diffusion method. An aliquot of 10 µL of sample of each concentration (200 mg/mL prepared in 50% DMSO) was introduced into each well (diameter 4 mm) so that the exact amount of extracts in each well were 2 mg. Negative control experiments were performed using equivalent volume of 50% DMSO and positive control experiments were performed by use of a standard antibiotic, neomycin (10 µg/well). At the end of the incubation period, the clear inhibition zones of bacterial growth around the wells were observed in the presence of different extracts. Inhibition of the bacterial growth in the presence of extracts was measured in the form of zone of inhibition (ZOI).

Fractionation of ethyl acetate extract

The ethyl acetate extract (2 g) was chromatographed on a sephadex LH- 20 column (18cm×0.9cm I. D, filled with 50 g Sephadex) and eluted with methanol. Sub-fractions were collected consisting of 10-12 ml. Each sub-fraction was monitored by TLC (Silica gel GF_{254}) using solvent system, ethyl acetate-methanol-water (100:3:1.5 v/v/v). The sub-fractions were pooled into four major fractions on the basis of TLC character, fraction F-1, F-2, F-3 and F-4. The fraction F-4 (0.5 g) was further chromatographed on a sephadex LH-20 column (18cm×0.9cm I.D, filled with 50 g Sephadex) and eluted with methanol. Sub-fractions of 4-5 mL were collected. Each sub-fraction was monitored by TLC (Silica gel GF_{254}) using solvent system, toluene-ethyl acetate-formic acid (5:4:1v/v/v).The sub-fractions were pooled into four major fractions, F-4-A, F-4-B, F-4-C and F-4-D on the basis of TLC character. The fractions F-4-A and F-4-D showed single spot on TLC. They were purified by re-crystallization and purity of F-4-D was checked by TLC in solvent systems, ethylacetate-methanol-water, EMW (100:13.5:10) chloroform-acetic acid-water, CAW (10:9:1) and toluene-ethylacetate-formic acid, TEF (10:8:1). The UV and FTIR spectra were recorded.

Results

Extractive values in different solvents

Extraction with solvents of different polarities provided different amounts of extracts. The yields of the various extracts are shown in Table 1. The yield depends on the nature of the solvent applied. The highest amount of extract was obtained with methanol. As methanol is a highly polar solvent, the hot methanol extracted almost all polar compounds.

Phytochemical screening

The results of the phytochemical screening of the hexane, dichloromethane, ethyl acetate, methanol and 50% aqueous methanol extracts of flowers of *H. cordifolium*are shown in Table 1 which revealed that all the tested phytochemicals were absent in hexane extract. As hexane is a non-polar solvent, it can extract only non polar compounds like hydrocarbons and fatty acids. In our screening, dichloromethane extract showed the presence of phenolics. It is well known that the *Hypericum* species are rich in acylphloroglucinols[24] which are non-polar phenolic compounds. Only terpenoids, phenolics, flavonoids and tannins were present in ethyl acetate extract. In methanol and 50% aqueous methanol extracts, in addition to terpenoids, phenolics, flavonoids and tannins, phytochemicals like saponins, glycosides and reducing sugars were also present. The results of chemical screening of the extracts are in agreement with the results reported for other species of *Hypericum*[25,26] with phenolics and flavonoides were the main constituents.

Table 1:Yield and screening of H. cordifolium extracts.

Extracts	Hexane	CH_2Cl_2	EtOAc	MeOH	50% aq. MeOH
Yields in g	1.92	4.92	3.26	9.35	2.00
Alkaloids	-	-	-	-	-
Terpenoids	-	-	+	+	+
Flavonoids	-	-	+	+	+
Phenolics	-	+	+	+	+
Glycosides	-	-	-	+	+
R.Sugars	-	-	-	+	+
Saponins	-	-	-	+	+
Tannins	-	-	+	+	+
Coumarins	-	-	-	+	+
Quinones	-	-	-	+	-
Steroids	-	-	-	-	-

Quality of analysis of *H. cordifolium* and *H. perforatum* ethanol extracts

There was a visual difference between two extracts. The extract of *H. perforatum* looked reddish brown and *H. cordifolium* looked pale yellow (Fig 1 A). The presence of chlorogenic acid (blue fluorescence spot, R_f0.12), hyperoside (yellow spot, R_f0.46) and quercetin (yellow spot, R_f0.75) in ethanol extract of *H. cordifolium* was confirmed by comparing the TLC with standard hyperoside along with *H. preforatum* extract which is known to contain chlorogenic acid, hyperoside and quercetin. Hypericin, a red fluorescence spot (R_f 0.62) was not detected in *H. cordifolium* extract. The TLC plates visualized under UV lamp at 254 nm and after spraying with 1% methanolic diphenyl boryloxy ethylamine followed by 5% ethanolic polyethylene glycol and visualized under 366 nm are shown in Fig 1 B and C.

Phytochemical information is necessary to assess the quality of the herb. Presence of hypericin and pseudohypericin result in red solutions with organic solvents[27]. The present study intended to determine the quality of *H. cordifolium* ethanol extract by simple and quick TLC techniques which allowed the detection of key marker compounds. For routine quality control, detection of major compounds seems to be sufficient. In TLC, the phytochemical compositions of two species were found to be different (Fig 1B, C). According to the European Pharmacopoeia 8.0 Hyperici herba monograph[28],on chromatogram, blue band of chlorogenic acid, two yellow bands of rutin and hyperoside, two red bands of pseudohypericin and hypericin and a yellow band of quercetin have to be visible. Absence of hypericin in indicated that the quality of *H. cordifolium* is different from *H. perforatum*. European Pharmacopoeia[29] specifies that the total hypericin content of Hyperici herba should be 0.08%, the flavonoids should be minimum 6% expressed as rutoside and hyperforin should be maximum 6%. Hypericine is present only in those *Hypericum* species whose aerial parts bear dark glands[30] and the level of accumulation depends on species and plant parts. It was reported that in many *Hypericum* species like *H. calycinum, H. cardiophyllum* and *H. xylosteifolium,* hypericine was absent[26]. However, the TLC assay only provides qualitative information about the phytochemicals.

Total phenolic content

The total phenolic content in different extracts was calculated from regression equation of calibration curve ($Y = 0.011x + 0.021$; $R^2 = 0.994$) and was expressed as mg of GAE per gram of dry extract. The TPC values ranged from 158.22 (dichloromethane extract) to 228.19 (50% aq. methanol extract) mg GAE/g dry extract. The ethyl acetate extract (227.17 mg GAE/g extract) and 50% aqueous methanol extracts contained nearly the same amounts of phenolics. The results are shown in Table 2.

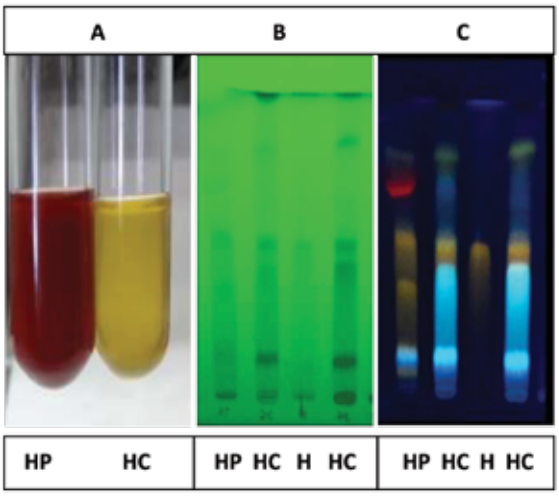

Figure 1: A: Appearance of extracts of H. perforatum (HP) and H. cordifolium (HC).

B: Chromatogram visualized under UV 254 nm along with hyperoside (H).

C: Chromatogram visualized under UV 366 nm along with hyperoside (H).

Total flavonoid content

The total flavonoid content in different extracts was calculated from the regression equation of calibration curve (Y =0.002x+0.002; R^2=0.999) and expressed as mg catechin equivalent (CE) per gram of dry extract. The total flavonoid content ranged from 227.4 (50% aq. methanol extract) to 306.41 (ethyl acetate extract) mg CE/g dry extract. However, methanol extract contained nearly the same amounts of flavonoids (231.5 mg CE/g extract) as 50% aq. methanol extract. The total flavonoid content of plant extracts are shown in Table 2.

It was reported the total phenolic content of the methanol extract of the aerial parts of *H. foliosum* was 174.13±2.90 mg GAE/g extract[31]. The ethanol-water extract of the flowers of *H. perforatum* contained total phenolics 371±49 mg GAE/g extract and total flavonoids 160±7 mg CE/g extract[32]. From the reported data on other species, it was found that the extracts of *H. cordifolium* contained relatively greater amounts of phenolics and flavonoids compounds.

Total tannin content

The total tannin content in different extracts was calculated from the regression equation of calibration curve (y=0.003x+0.008; R^2 = 0.996) and expressed as mg tannic acid equivalent (TAE) per gram dry extract. The total tannin content in different extracts ranged from 278.08 (50% aq methanol extract) to 621.32 (methanol extract) mg TAE/g dry extract. The results are shown in the Table 2.

Total carbohydrate/sugar content

The total sugar content in different extracts were calculated from the regression equation of calibration curve (y=0.0085x + 0.0077; R^2 = 0.9983) and expressed as mg glucose equivalent (GE) per gram of dry extract. The total sugar content ranged from 105.71 (50% methanol extract) to 178.08 (methanol extract) mg GE/g dry extract. The total carbohydrate/ sugar contents in plant extracts are given in Table 2.

The tannin and sugar content in *H. perforatum* were determined by the method described in the European Pharmacopoeia and found that the tannin content varies from 6.2-9.0% and sugar content varies from 19 to 25% by weight in dry herbal extracts[33]. In our findings, tannins and sugars were estimated by spectrophotometric method using tannic acid and glucose as the standard. Although the methods applied were different, tannins and sugars were detected in *H. cordifolium* which is in agreement with the literature.

Table 2: Total phenolic, flavonoid, tannin and sugar in various extracts, antioxidant and cytotoxic activity.

Extracts	CH_2Cl_2	EtOAc	MeOH	50% aq. MeOH	EtOH
Total phenolic content (mg GAE/g dry extract (Mean ± S.D) (n=3)	158.22 ± 0.74	227.17 ± 0.736	199.28 ± 0.576	228.19 ± 0.639	-
Total flavonoids content (mg CE/g extract) (Mean ± S.D) (n=3)	-	306.41 ± 0.954	231.5 ± 0.935	227.4 ± 0.993	-
Total tannins content (mg TAE/g extract)(Mean ± S.D)(n=3)	-	298.72 ± 0.89	621.32 ± 0.537	278.08 ± 0.712	-
Total sugar content (mg GE/g extract) (Mean ± S.D)(n=2)	-	-	178.08 ± 0.712	105.71 ± 0.531	-
IC_{50} µg/ml against DPPH free radical assay(Mean ± S.D)(n=2)	-	18.79±0.98	27.00±1.03	18.98±0.81	-
IC_{50} µg/ml against bladder carcinoma (Mean ± S.D)(n=2)	-	-	-	-	41.35 ± 5.01

DPPH free radical scavenging activity

In DPPH radical scavenging assay, ethyl acetate and 50% aqueous methanol extracts showed nearly similar IC_{50} values (18.79 and 18.89 µg/ml) and methanol extract showed somewhat higher IC_{50} value (27.0 µg/ml) but these values are relatively higher than the IC_{50} value of ascorbic acid (11.0µg/ml). The results are presented in Table 2. The IC_{50} values of ethyl acetate (2.63±0.01

µg/ml), methanol (3.63±0.01 µg/ml) and methanol-water (4.71±0.04 µg/ml) extracts of *H. perforatum* in DPPH free radical scavenging assay were reported[34]. In general, extracts with higher radical scavenging and antioxidant activities showed a higher phenolic content. This is in agreement with our findings and a strong correlation was observed between the phenolics and flavonoids content and antioxidant activities.

Cytotoxic activity

In cytotoxicity assay, it was observed that the ethanol extract at 250, 125, 62.5, 31.25 and 15.625 µg/ml concentrations significantly decreased the viability of human bladder carcinoma cell line 5637 after 72 hours. Etoposide, a semi-synthetic derivative of podophyllotoxin from the rhizome of *Podophyllum peltatum* was used as a positive control. The methanol extract showed the IC_{50} value of 41.35 ± 5.01 µg/ml where as the standard etoposide has IC_{50} value of 0.67± 0.03 µM.

It is well known that *Hypericum* species are rich in acylphloroglucinols which showed significant cytotoxicity on different cancer cell lines[35,36,37]. Hyperforin inhibits tumor cell growth in vitro[38]. It was reported that phenols, anthraquinonoids, xanthones, benzophenone, phloroglucinol and flavonoids isolated from *H. sampsonii* showed selective cytotoxic activities against cancer cells[39]. The cytotoxicity of ethanol extract of *H. cordifolium* could be due to the presence of various classes of phytochemicals as shown by chemical screening.

Antimicrobial activity

In antibacterial assay, all the tested extracts showed only narrow spectrum of activities. Except 50% aq. methanol extract, other extracts showed weak to moderate antibacterial activity against *S. aureus* with inhibition zone ranged from 9-18 mm. The methanol extract also showed moderate activity against *E. coli.* The tested extracts did not show activity against *K. pneumonia* and *C. albicans*. The results are shown in Table 3.

It was reported that the main antibacterial component from *H. perforatum* was hyperforin[40]. *H. perforatum* and other species have been investigated for their antimicrobial activity and found that the extract was active against gram-positive bacteria but not against *C. albicans*[41]. This is in agreement with our findings. It was reported that the *Hypericum* species containing considerable amounts of acylphloroglucinol derivatives showed antibacterial, cytotoxic and antioxidative activity[42,43]. The observed biological activities of *H. cordifolium* extracts could be due to the presence of phytochemicals like acylphloroglucinol, phenolics and flavonoids.

Table 3. Antibacterial activity of extracts.

Extract	Bacteria	Zone of inhibition
Hexane	*S. aureus*	17.0 mm
Dichloromethane	*S. aureus*	9.0 mm
Ethyl acetate	*S. aureus*	18.0 mm
Methanol	*E. coli*	14.0mm
	S. aureus	14.0 mm
Ampicilin 10 µg/well	*S. aureus*	33.0 mm
	E. coli	14.0 mm

Characterization of compounds

Repeated column chromatograph of the ethylacetate extract over Sephadex LH 20 yielded fraction F-4-A, yellow amorphous solid (A). It was purified by re-crystallization. It showed single spot on TLC (R_f 0.57 in TEF). The UV spectrum showed the absorption bands at 268 and 340 nm (Fig 2). This is the characteristics UV-VIS absorption profile of flavonoid structure. The FTIR spectrum showed the broad peak between 3400-3000 cm^{-1}, a strong peak at 1650 cm^{-1} and a peak at 1600 cm^{-1}.

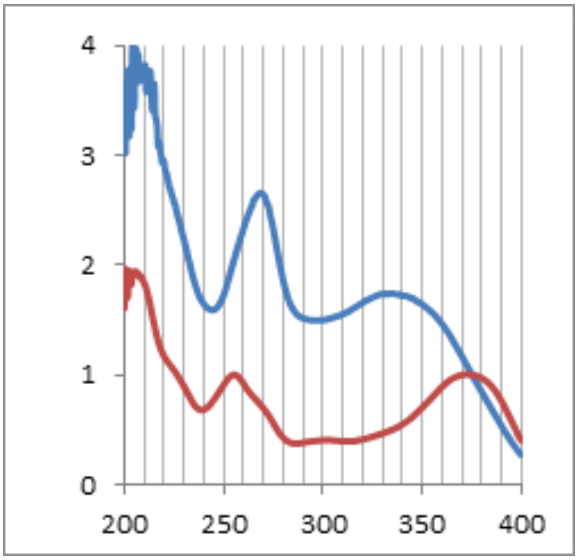

Figure 2: UV spectrum of compound A (above) and compound D (below).

Similarly, fraction F-4-D yielded yellow amorphous solid (D). It was purified by recrystallization and purity was checked by TLC, R_f 0.87 in CAW; 0.46 in TEFand 0.76 in EMW[44]. The UV spectrum showed the absorption bands at 255 and 375 nm (Fig 2). The FTIR spectrum showed the broad peak between 3400-3000 cm^{-1}, a strong peak at 1650 cm^{-1} stretching and a peak at 1600 cm^{-1}.

The UV-VIS absorption spectrum of both compounds were characteristic of flavonoid structure. In compound D, Band I, which appeared at 375 nm is considered to be associated to the B-ring cinnamoyl system. Band II, which appeared at 255 nm is caused by electron transition of benzoyl group of A-ring. These λ^{max} values of compound D match with quercetin[44,45]. In FTIR spectrum, the broad peak between 3400-3000 $cm^{-1}$is due to the O-H stretching, a peak at 1450 cm-1 is due to O-H bending of alcohols and a peak at 1350 cm-1 is due to the O-H bending of phenols. These indicated the presence of hydroxyl group. A strong peak at 1650 $cm^{-1}$and a peak at 1150 cm^{-1}indicated the presence of C=O group. A peak at 1600 cm^{-1} indicated the presence of phenyl ring skeleton. These IR stretching frequencies of compound D match with quercetin[46]. Thus, by comparing the R_f values, λ^{max} values and IR stretching frequencies with quercetin, compound D was identified.

Conclusions

The quality of *H. cordifolium* ethanol extract was determined by simple and quick TLC technique which allowed the detection of key marker compounds. The phytoconstituents of *H. cordifolium* was found to be different from *H. perforatum*. Estimation of the contents of total phenolics, flavonoids, tannins and sugars indicated the extracts are rich source of these phytochemicals. Evaluation of antioxidant, cytotoxicity and antibacterial activities revealed that the extracts exhibited good biological activities and supports the traditional use of *H. cordifolium* in the treatment of infections and inflammation. Isolation of some phytochemicals and characterization of quercetin from the ethyl acetate extract indicated that *H. cordifolium* could be the potential source of bioactive compounds. As there is a great chemical diversity, detailed phytochemical investigation on *H. cordifolium* of Nepalese origin is necessary.

Acknowledgements

We are grateful to UGC, Bhaktapur for faculty research grant (2076/78), Dr. Michal Glensk, Department of Pharmacognosy and Herbal Medicines, Faculty of Pharmacy, Wroclaw Medical University for his help to provide TLC results, Dr. Martina Wurster, Institute of Biochemistry, University of Greifswald, Germany for cytotoxicity assay and Prof. Dr. Rameshwar Adhikari for recording UV and FTIR spectra.

References

1. Crockett, S. L., & Robson, N. K. 2011. Taxonomy and chemotaxonomy of the genus Hypericum. *Medicinal and aromatic plant science and biotechnology*. **5**(Special Issue 1),: 1.

2. Manandhar, N. P. 2022. N.P. Plants and People of Nepal. Timber Press, Portland. USA.

3. Zimmermann-Klemd, A. M, Konradi, V., Steinborn, C., Uecker, A., Falanga, C. M., Woelfle, U., Huber, R., Juergenliemk, G., Rajbhandari, M., Gruendemann, C.2019. *Journal of Ethnopharmacology.* **235**: 415-423.

4. Taylor, R.S., Manandhar, N.P., Hudson, J.B., Towers, G.H. 1996. Antiviral activities of Nepalese medicinal plants. *Journal of Ethnopharmacology*. **5**: 157-63.

5. Basyal, D., & Bhandari, N. L. 2019. Phytochemical screening and cytotoxicity evaluation of ethanolic extract of *hypericum cordifolium* (choisy) leaves. *Journal of Nepal Chemical Society*. **40:** 19-24.

6. Shresta, S., Bhandari, S., Aryal, B., Marasini, B. P., Khanal, S., Poudel, P., Rayamajhee, B., Adhikari, B., Bhattarai, B. R., & Parajuli, N. 2021. Evaluation of phytochemical, antioxidant and antibacterial activities of selected medicinal plants. *nepla. Journal of Biotechnology*. **9**: 50–62.

7. Sakar, M. K., & Tamer, A. Ü. 1990. Antimicrobial activity of different extracts from some *Hypericum* species. *Fitoterapia*. **61**: 464-466.

8. Butterwerck, V., Jurgenliemk, G., Nahrstedt, A., & Winterhoff, H. 2000. Flavonoids from *Hypericum perforatum* show antidepressant activity in the forced swimming test. *Planta Medica*. **66**: 3–6.

9. Barnes, J., Anderson, L. A., & Phillipson, J. D. 2001. St John's wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties. *Journal of pharmacy and pharmacology*. **53**: 583-600.

10. Greeson, J. M., Sanford, B., & Monti, D. A. 2001. St. John's wort (*Hypericum perforatum*): a review of the current pharmacological, toxicological, and clinical

literature. *Psychopharmacology*. **153**: 402-414.

11. Lawvere, S., & Mahoney, M. C. 2005. St. John's wort. *American family physician*. **72**: 2249-2254.

12. Nahrstedt, A., & Butterweck, V. 1997. Biologically active and other chemical constituents of the herb of Hypericum perforatum L. *Pharmacopsychiatry*. **30**: 129-134.

13. Schempp, C. M., Pelz, K., Wittmer, A., Schöpf, E., & Simon, J. C. 1999. Antibacterial activity of hyperforin from St John's wort, against multi resistant Staphylococcus aureus and gram-positive bacteria. *The Lancet*. **353**: 2129.

14. Schempp, C, M., Winghofer, B., Ludtk, R., Simon-Haarhaus, B., Schopf, E., Simom, J. C. 2000. Topical application of St. Jone's wort (*Hypericum perforatum* L.) and its metabolite hyperforin inhibits the allostimulatory capacity of epidermal cells. *British Journal of Dermatology*. **142**: 979-984.

15. Nahrstedt, A., & Butterweck, V. 2010. Lessons learned from herbal medicinal products: the example of St. John's wort. *Journal of Natural Products*. **73**: 1015-1021.

16. Velingkar, V. S., Gupta, G. L., & Hegde, N. B. 2017. A current update on phytochemistry, pharmacology and herb–drug interactions of *Hypericum perforatum*. *Phytochemistry Reviews*. **16**: 725-744.

17. Culie, I. 1982. Methology for analysis of vegetable drugs, Practical manuals on industrial utilization of medicinal and aromatic plant, Bucharest. *Phytochemistry*. **63**: 97-104

18. Waterhouse, A. 2002. Determination of total phenolics. In: Current protocols in food analytical chemistry (Ed Wrolstad, R. E). John Wiley and Sons, New York, Units I1.1.1-I1.1.8.

19. Woisky, R. G., & Salatino, A. 1998. Analysis of propolis: some parameters and procedures for chemical quality control. *Journal of apicultural research*. **37**: 99-105.

20. Katoch, R. 2011. Methods for Nutritional Quality Evaluation of Food Materials. In: Analytical Tech- niques in Biochemistry and Molecular Biology. Springer, New York, NY, pp 251-322.

21. Hedge, J.E. and Hofreiter, B.T. 1962. In: Carbohydrate Chemistry. Whistler R.L. BeMiller, J.N., Academic Press, New York, pp. 420.

22. Brand-Williams; W., Cuveiler, M. E., Berset, C. 1995. Use of a free radical method to evaluate antiox- idant activity. *Food Science and Technology.* 28: 25-30

23. Bauer, A. W., Kirby, M. K., Sherris, J. C., Turck, M., 1996. Antibiotic susceptibility testing by standard single disc diffusion method. *American Journal of Clinical Pathology*. **45**: 493- 496.

24. Schmidt, S., Jürgenliemk, G., Skaltsa, H., & Heilmann, J. 2012. Phloroglucinol derivatives from *Hypericum empetrifolium* with antiproliferative activity on endothelial cells. *Phytochemistry*. **77**: 218-225.

25. Jurgenliemk, G., Nahrstedt, J. 2002. Phenolic compounds from *Hypericum perforatum*. *Planta Medica.* **68**: 88–91.

26. Cirak, C., Radusieneb, J., Jakstasc, V., Ivanauskasc, L., Seyisd, F., Yayla, F. 2016. Secondary metabolites of seven *Hypericum* species growing in Turke. *Pharmaceutical Biology.* **54**: 2244-2253.

27. Frommenwiler, D. A., Reich, E., Sudberg, S., Sharaf, M. H., Bzhelyansky, A., & Lucas, B. 2016. St. John's wort versus counterfeit St. John's wort: an HPTLC study. *Journal of AOAC International*. **99**: 1204-1212.

28. European Pharmacopoeia. 2014. Supplement 8. 0 W/ 8. 1 and 8. 2. Worldwide Book Service, London.

29. European Pharmacopeia. 2017. English hardcover edition supplements 9.0, 9.1 and 9.2 European directorate for the quality of medicines and health care, France.

30. Lu, H. F., Shen, Z. G., Li, J. Y. H. and Hu, Z. H. 2001. The patterns of secretory structure and their relation to hypericin content in *Hypericum*. *Acta Botanica Sinica.* **43**: 1085–1088.

31. Rainha, N., Lima, E., Baptista, J., Rodrigues, C. 2011. Antioxidant properties, total phenolic, total carotenoid and chlorophyll content of anatomical parts of *Hypericum foliosum*. *Journal of Medicinal Plants.* **5**: 1930-1940.

32. Makarova, K., Sajkowska-Kozielewicz, J. J., Zawada, K., Olchowik-Grabarek, E., Ciach, M. A., Gogolewski, K., & Gambin, A. 2021. Harvest time affects antioxidant capacity, total polyphenol and flavonoid content of Polish St John's wort's (*Hypericum perforatum* L.) flowers. *Scientific reports*. 11: 1-12.

33. Eggelkraut-Gottanka, S. V., Abed, S. A., Müller, W., & Schmidt, P. C. 2002. Quantitative analysis of the active components and the by-products of eight dry extracts of *Hypericum perforatum* L.(St John's wort). *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*. **13**: 170-176.

34. Öztürk, N., Tunçel, M., & Potoğlu-Erkara, İ. (2009). Phenolic compounds and antioxidant activities of some *Hypericum* species: A comparative study with *H. perforatum*. *Pharmaceutical biology*. **47**: 120-127.

35. Schmidt, S., Jürgenliemk, G., Skaltsa, H., & Heilmann, J. 2012b. Phloroglucinol derivatives from *Hypericum empetrifolium* with antiproliferative activity on endothelial cells. *Phytochemistry*. **77**: 218-225.

36. Hu, L. H., & Sim, K. Y. 1999. Cytotoxic polyprenylated benzoylphloroglucinol derivatives with an unusual adamantyl skeleton from *Hypericum sampsonii* (Guttiferae). *Organic letters*. **1**: 879-882.

37. Zhou, Z. B., Zhang, Y. M., Pan, K., Luo, J. G., & Kong, L. Y.

2014. Cytotoxic polycyclic polyprenylated acylphloroglucinols from *Hypericum attenuatum*. *Fitoterapia*. **95**:1-7.

38. Schempp, C, M., Kirkin, V., Simon-Haarhaus, B., Kersten, A., Kiss, J., Termeer, C., C., Gilb, B., Kaufmann, T., Borner, C., Sleeman, J. P., Simon, J. C. 2002. Inhibition of tumour cell growth by hyperforin, a novel anticancer drug from St. John's wort that acts by induction of apoptosis. *Oncogene.* **21**: 1242-50.

39. Chen, Q., Di, L., Zhang, Y., Li, N. 2020. Chemical constituents with cytotoxic and anti-inflammatory activity in Hypericum sampsonii and the antitumor potential under the view of cancer-related inflammation, *Journal of Ethnopharmacology*. **259**: 112948.

40. Bystrov N. S, Chernov B. K, Dobrynin V. N, Kolosov M. N. 1975. The structure of hyperforin. *Tetrahedron Letter.* **32**: 2791-94.

41. Unal, E., Mavi, A., Kara, A. A., Cakir, A., Sengul, M., Yildirim, A. 2008. Antimicrobial and antioxidant activities of some plants used as remedies in Turkish traditional medicine. *Pharmaceutical Biology*. **100**: 80-86.

42. Heilmann J., Winkelmann K., and Sticher, O. 2003. Studies on the antioxidative activity of phloroglucinol derivatives isolated from *Hypericum* species. *Planta Medica.* **69**: 202-206.

43. Stojanovic, G., Dordevic, A., Smelcerovic, A. 2013. Do Other *Hypericum* Species Have Medical Potential As St. John's Wort (*Hypericum perforatum*) **20**: 2273-2295.

44. Harborne, J. B. 1973. *Phytochemical methods*. Chapman and Hall Ltd, London.

45. Stefova, M., Kulevanova, S., & Stafilov, T. 2001. Assay of flavonols and quantification of quercetin in medicinal plants by HPLC with UV-diode array detection. *Journal of liquid chromatography & related technologies*. **24**: 2283-2292.

46. Shi, G. Q., Yang, J., Liu, J., Liu, S. N., Song, H. X., Zhao, W. E., & Liu, Y. Q. 2016. Isolation of flavonoids from onion skins and their effects on K562 cell viability. *Bangladesh Journal of Pharmacology*. **11**:S18-S25.