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Nature's healing palette: Unlocking the mysteries of *Cheilanthes bicolor* (Roxb) Fraser-Jenkins: A journey into phytochemical diversity and potent biological actions

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Abstract
The exploration of plants' potential as sources of novel medications, nutritious foods, and nutraceuticals as well as a comprehension of their functions in conventional medicine and human health phytochemical screening is a fundamental step. *Cheilanthes bicolor* is one of the native plants in Nepal, so we need to unlock the hidden potential of this plant. The goal of this study was to assess the phytochemical analysis, antioxidant, and antibacterial of plant extracts (root and frond) in different solvent systems (methanol, 50% methanol, ethyl acetate, dichloromethane, and hexane). Phytochemicals screening revealed the presence of an array of secondary metabolites including polyphenol flavonoids, phenolics, coumarin tannins, terpenoids, glycoside, reducing sugar, etc. in both the frond and root, total phenolic, flavonoid, tannin and sugar content test indicate that all are found to higher in ethyl acetate extract than other extract except total sugar content it was found to be higher in methanol and 50% methanol extracts of frond than root extract. The IC_{50} values from 2, 2-diphenyl -1-picrylhydrazyl (DPPH) radical scavenging assay of extract indicate noteworthy antioxidant potential. Antibacterial teste demonstrated modest activity against Gram-positive bacteria and antifungal activity. Ultra-performance Liquid chromatography-electrospray ionization mass spectrometry (UPLC-MS) analysis of methanol extract allowed the identification of compounds with molecular formulas, ($C_{28}H_{45}O_9$, $C_{22}H_{21}O_{11}$, $C_{17}H_{13}O_6$, $C_{26}H_{43}O_6$, and $C_{27}H_{39}N_4O_2$). Comparison between fronds and roots it seems that frond is more effective than root. As far as we are aware, there has rare investigation of *Cheilanthes bicolor*. Here, many active phytoconstituents were identified during phytochemical research, which might be extracted and studied in future studies to generate strong medications and the current study adds the protective effect of *Cheilanthes* bicolor fronds as a powerful antioxidant and antibacterial agent.

Keywords: Phytochemical screening, Antioxidant, Antimicrobial, liquid chromatography-mass spectroscopy, *Cheilanthes bicolor*

1. Introduction
Nepal is home to a diverse range of medicinal plants that have been used for centuries in traditional medicine practices. For millennia, medicinal plants have been an important part of Nepal's traditional medicine systems. The traditional usage of medicinal herbs is strongly anchored in diverse indigenous cultures and is passed down through generations [1]. Therefore, Nepalese people use medicinal plants in diverse ways for various health issues and to enhance their overall well-being. In Nepal, 1,600 to 1,900 plant species have traditionally been used for medical purposes [2]. Traditional medicinal plant use has a long history and is becoming more popular due to its low risk of adverse effects and simple access to inexpensive costs [3]. Among these plants *Cheilanthes bicolor* (Figure 1) (Roxb) Frans-Jenk, also known as *Aleuritopteris bicolor*, locally known as '*Helsinki'* is a perennial herb that belongs to fern allies of pteridophyte. In Nepal flora, 534 species of pteridophytes are reported [4]. The genus *Cheilanthes* has about 180 to 200 species worldwide [5]. Various species of *Cheilanthes* have widely been recommended as medicines in traditional therapy. Therefore, *Cheilanthes* species have been screened for various biological activities [4]. It is also a well-liked selection for fern fans and gardeners due to its eye-catching fronds and versatility in a variety of growing environments in different foreign countries [6]. But in the case of Nepal, people even my grandparents have been using it for more medicinal purposes than decoration since many years ago without knowing the medicinal constituent in this plant. Therefore, my curiosity drags me to explore the phytochemical constituent of this plant.

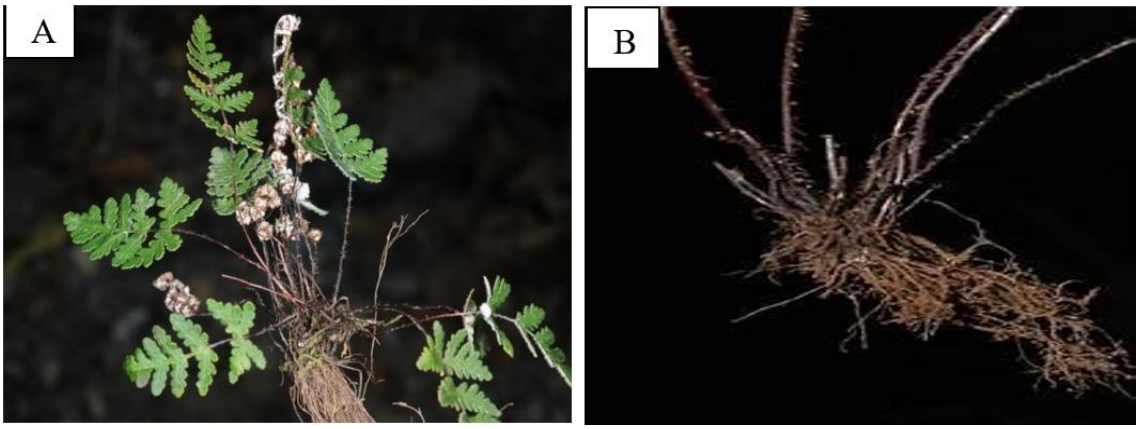

Fig 1: A= Frond, B= Root of *Cheilanthes bicolor*

Behind the scientific logic, the oxidation reaction is necessary for life, but it generates free radicals, which cause oxidative stress and are responsible for the bulk of disease, as well as defective antioxidant defense mechanisms [7]. On another hand, an antioxidant is a molecule that inhibits the oxidation of other molecules. Scientific research now confirms that free radicals play a major role in the development of cancer, heart disease, aging, cataracts, and impairment of the immune system. Antioxidants are abundant in fruits vegetables, spices as well as nuts, grains, poultry, and fish. Antioxidants also have industrial uses, such as preservatives in food and cosmetics and to prevent the degradation of rubber and gasoline. Many medicinal plants contain large amounts of antioxidants, such as phenolics, terpenoids, vitamin C, vitamin E, selenium, β-carotene, lycopene, lutein, and other carotenoids, which play important roles in adsorbing and neutralizing free radicals, quenching singlet, and triplet oxygen, or decomposing peroxides [8]. Moreover, significant problems across various domains not only because of the free radicals but there is a problem in humans because of tiny bacteria. These microscopic agents are responsible for a range of ailments affecting humans, animals, and plants, with outcomes ranging from mild discomfort to severe, life-threatening conditions [9]. Although some infectious diseases have been conquered by modern medicines, new diseases are constantly emerging while others re-emerge in resistant forms. One of the reasons for the development of resistant microbes is the massive, inappropriate, and improper use of antimicrobials in humans, animals, and agriculture. Hence, substantial attention should be focused on the development of new antibiotics for the treatment of infections caused by resistant organisms. Therefore, the search for natural antibiotics with a broad spectrum of activity and new modes of action is most preferred. The plants which are considered useful agents against infectious diseases are interesting to test regarding different etiological agents. Thus, plants with medicinal values should be investigated by modern scientific techniques to establish their safety and efficacy and to determine their potential as a source of future new drugs [10, 11]. Although many types of drugs are available in the market, searching for new classes of drugs is one of the most important challenges currently. Medicinal plants used in traditional medicines could be an alternative source for the development of new drugs [12]. Nepalese medicinal plants could be potentially valuable sources for the development of Phyto-medicines, nutraceuticals or food supplements, and personal care products. However, for the value addition, it is essential to establish phytochemical and pharmacological information as well as the effectiveness and safety of the plants. Therefore, the assessment of such properties remains an interesting and useful task. Pteridophytes are not well investigated phytochemically in comparison to angiosperms. Ferns grow under different habitats under stress conditions and are mainly used in folk medicine and have the potential as a source of economically important products. This prompted us to investigate the pteridophyte. The purpose of this study was to evaluate the total phenolic, total flavonoid, and total sugar contents, antioxidant, and antimicrobial activity of different extracts and UPLC-MS methanol extract, and TLC analysis of ethyl acetate extract. It is found in southeast, northeast Asia and grows in tropical and warm temperate regions. A rhizome is short erect, apex scaly, scales dark brown, bicolor (central region dark brown, margin pale), lanceolate, margin entire, apex cute. Stipe length longer than the lamina is 20-30 cm long [5]. It is used as a medicine to cure fever using whole-part extract. Plant powder mixed with cow's ghee is used as incense to keep off fear in children. Brown stripe is used by the children as nose and ear studs. Rhizomes and leaves are mostly used from a medicinal point of view and used as a general health tonic and antiseptic [5, 13]. Juice of plant, about six teaspoons three times a day is suggested in case of gastric troubles [14, 15]. Various species of *Cheilanthes* have widely been recommended as medicines in traditional medical systems. Therefore, *Cheilanthes* species have been screened for various biological activities. *C. centifolia, C. farinose* and *C. bicolor* are used as a general health tonic and antiseptic, *C. farinose is* mostly used in stomachache and menstrual disorders [16, 4]. Till now only 3-methoxy flavones have been isolated from this plant and not any other related biological activities have been tested. Based on ultra-violate mass spectrometry (UV/MS) and proton nuclear magnetic resonance (1HNMR) the two compounds (1) with Rf value (0.81) Kaempferol 3,5 dimethyl ether and compound (2) with Rf (0.83) value Kaempferol 3-methyl ether have been identified [4].

In this work, we are trying to explore the significant antioxidant agent present in this plant by evaluating the total phenolic, total flavonoid, and total sugar contents. Antimicrobial activity of different extracts by agar well diffusion method in different bacteria and UPLC-MS of methanol extract identify unknown compounds present in the mixture. Mass spectrometry provides accurate mass measurements, fragmentation patterns, and molecular formula information, aiding in the characterization of unknown substances and Thin Layer Chromatogram (TLC) analysis of ethyl acetate extract.

2. Materials and Method

2.1. Chemical and equipment

All the chemicals, solvents, and equipment (like methanol, hexane ethyl acetate DCM Quercetin, gallic acid, aluminum chloride, ascorbic acid, etc.) used were of analytical grade and

equipment used were (like electrical grinder, weighing balance, hot air oven, rotary evaporator, UV-spectrophotometer etc.) provided by RECAST and Department of chemistry (Tribhuvan University).

2.2. Plant Material
Two kilograms of fresh *C. bicolor* were selectively collected from the Lamjung district. The National Herbarium and Plant Laboratories in Godawari provided the plant's botanical identification. Separated its fronds and roots, cleaned in distilled water to get rid of any dust, dried in the shade, and then ground in an electric grinder.

2.3. Extraction
The dried and powdered plant material (100 g) was successively extracted using the Soxhlet extraction method for 7-8 hours with hexane (600 ml), dichloromethane (500 ml), ethyl acetate (400 ml), and methanol (600 ml) until last extract becomes colorless. Following extraction using various solvents, the leftover residue was refluxed with 400 ml of 50% aqueous Methanol for two hours before being allowed to cool and filtered. To achieve solid or semisolid extract, the filtrate was concentrated under a rotatory evaporator at a lower pressure. For further investigation, the dried extract was preserved in the freezer.

2.3. Phytochemical analysis
To identification of specific compounds present in plant extracts like alkaloids, flavonoids, polyphenols, terpenoids, glycosides, and reducing sugar in the different plant extracts was analyzed by reacting with different reagents [10, 11].

2.4. Estimation of total phenolic content (TPC)
The total phenolic content in the plant extracts was estimated by using a phenol reagent (Folin- Ciocalteu) based on a colorimetric method involving oxidation-reduction [17, 18]. In summary of this method, various concentrations (10, 25, 50 and 100 ppm) of gallic acid and the plant extract (at a concentration of 1 mg/mL) were added in 1 mL amounts to 5 mL of FCR (10%) and 1 mL of 7% Na_2CO_3 individually when the mixture turned blue in color, was vigorously mixed and left to incubate for 30 minutes at 40 °C in a water bath. Finally, the absorbance of these solutions was measured at a wavelength of 760 nm using UV-spectroscopy against a blank. The total phenolic content (TPC) was determined from the calibration curve and expressed as milligrams of gallic acid equivalents (GAE) per gram of the dried test material.

2.5. Estimation of total flavonoid content (TFC)
The total flavonoid content in plant extract was determined using an Aluminum chloride colorimetric assay [19]. Like in the TPC test, we prepared the four different concentration (10, 25, 50 and 100 ppm) solutions of sample and Taking 1 mL of quercetin or the extract (at a concentration of 1 mg/mL in methanol), in the test tube containing 4 mL of distilled water. At first, 0.3 mL of 5% $NaNO_2$ was introduced into the test tube. After 5 minutes elapsed, 0.3 mL of 10% $AlCl_3$ was added, followed by the addition of 2 mL of 1 M NaOH after 6 minutes. Immediately, the total volume of the mixture was brought up to 10 mL by adding 2.4 mL of distilled water, after which the mixture was incubated in darkness for 30 minutes. And finally, the absorbance of the test sample was measured at 450 nm by using. a UV-spectrophotometer against a blank solution. After creating the calibration curve, the total flavonoid content (TFC) values were derived and expressed as milligrams of quercetin equivalents (QE) per gram of the dry test material.

2.6. Estimation of Total Carbohydrate/Sugar Content
The total carbohydrate/sugar content in plant extracts was estimated by using anthrone reagents-based colorimetric assay [20]. Glucose solutions were prepared at different concentrations (100, 200, 400, and 600 ppm). For each glucose concentration (2 ml), mixed with distilled water and 8 ml of freshly prepared anthrone reagent (200 mg anthrone in 100 ml ice-cold 95% conc. H_2SO_4) in a 15 ml test tube. The mixture was well shaken, heated for 8 minutes in a boiling water bath, and then rapidly cooled, and its absorbance was measured at 630 nm against a blank without sugar. The average absorbance values for various glucose concentrations were used to create the calibration curve. The plant extract solutions in different concentrations (100, 200, 400, and 600 ppm) in boiling tubes were hydrolyzed with 5 ml of 0.2 N HCl, heated in a boiling water bath for 3 hours, and cooled to room temperature. The resulting solution was neutralized using solid sodium carbonate until effervescence ceased and centrifuged to settle insoluble particles. Anthrone reagent was added to these diluted solutions and heated for 8 minutes, like the glucose process.

2.7. Estimation of Total Tannin Content (TTC)
The total tannin content in plant extract was estimated by using the Folin-Ciocaltue method [21]. Tannic acid and sample solutions were prepared at varying concentrations (10, 25, 50, and 100 ppm). For each tannic acid concentration (1 ml), it was mixed with 8.4 ml of distilled water in a test tube. Subsequently, 0.5 ml of Folin-Ciocalteu's reagent and 0.1 ml of sodium carbonate solution were added. Following thorough shaking, the solution was allowed to sit for 30 minutes, and absorbance was recorded at 700 nm using a reference blank solution.

2.8. Antioxidant Activity: The antioxidant activity was determined by DPPH radical scavenging activity [22, 23, 24]. Serial dilution was used to create test solutions of plant extract at concentrations of 5, 10, 15, and 20 ppm. Subsequently, DPPH was added to each sample in a 1:1 ratio, followed by a 30-minute incubation in darkness. The absorbance of each solution was then measured using a UV-Vis spectrophotometer at 517 mm. Ascorbic acid was used as a standard by using the plot of standard we determined the percentage inhibition of sample.

$$\% \text{ of radical scavenging } = \frac{Ac - As}{Ac} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Where
Ac= Absorbance of control
As = Absorbance of solution
IC_{50} values is the concentration of sample required to scavenge 50% of DPPH radical and was calculated from the plotted graph of radical scavenging activity against the concentration of extract.

2.9. Antibacterial activity
The antimicrobial properties of the plant extracts were determined using the agar-well diffusion method [25,26]. Prepared sterile Mueller-Hinton Agar (MHA) plates were dried to eliminate excess moisture. A sterile cotton swab was immersed in standard inoculums, the excess was removed by

pressing against the tube wall, and cultures of bacterial and fungus (*Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumonia*, and *Escherichia coli* and one fungus *candida albicans*) strains the swab was gently applied across the plates, rotating each time by 60° and tracing along the edges. Inoculated plates were air-dried for a day in a laminar airflow. Wells was introduced in incubated media plates using a sterile cork borer (4mm diameter) and labeled accordingly. Next, 40 µl (16 mg per well) of the plant extract working solution (each concentration 400 mg/mL prepared in 50% DMSO) were carefully pipetted into wells. Ciprofloxacin served as the standard antibiotic in a separate well. The plates were covered and allowed to stand for an hour for extract diffusion into the media. Following this, the plates were incubated at 37 °C overnight (18-24 hours).

2.10 Sephadex LH- 20 column chromatography [27] of ethyl acetate extract of *Cheilanthes bicolor* extract

The ethyl acetate extract (1 g) was chromatographed on a Sephadex LH- 20 columns (18 cm x 0.9 cm I. D.) filled with Sephadex (11.5 cm), equilibrated in methanol, and eluted with methanol. Fractions were collected consisting of 10-12 ml. Each fraction was monitored by TLC (Silica gel GF254) using ethyl acetate: methanol: water 100: 3: 1.5, Methanol: water: formic acid at 10: 8:1 and n - Butanol- Acetic acid-water 4:1:5 as three different solvent systems.

2.11 Thin layer chromatography (TLC) [28]

The ethyl acetate extract and the fractions obtained from the Sephadex LH-20 column were examined by TLC in different solvent systems and spots were visualized in daylight as well as under UV light, both at 254 nm and 366 nm. The solvent systems used for the development of the chromatogram were: Ethyl acetate - Methanol - Water 100: 3:1.5.

2.12 Ultra-performance Liquid Chromatography-Mass Spectrometry analysis [29] of methanol extract.

The UPLC-MS technique offered a straightforward, highly sensitive, and precise method to analyze intricate biological compounds [30]. Therefore, in this research, we tried to use UPLC-MS to find out the possible chemical compound in this plant. Ultra-performance liquid chromatography-electrospray ionization mass spectrometry (UPLC- ESI-MS) measurements were carried out on the Thermo Scientific UHPLC Ultimate 3000 apparatus (Thermo Fisher Scientific, Waltham, MA, USA) consisting of an LPG-3400RS quaternary pump with a vacuum degasser, a WPS-3000RS autosampler, and a TCC-3000 SD column oven. The ESI-q-TOF (Quadrupole Time of flight) Compact (Bruker Daltonics, Bremen, Germany) was connected as the MS detector. The instrument was calibrated with the Tunemix™ (Tunning mixture) (Bruker Daltonics).

3. Results and discussion

The extractive values of the frond in hexane, methanol, 50% methanol, Ethyl acetate, and DCM solvents were found to be 3.94, 5.20, 2.75, 3.90, and 1.50 in a gram respectively. In the case of root 0.90, 3.287, 1.50, 0.78, and 0.67 in a gram respectively. Here we can see polar solvents extract more secondary metabolite than non-polar solvents.

3.1 Phytochemical Analysis

Table 1: Phytochemical screening of frond extracts of *Cheilanthes bicolor*

Extract	Hexane	DCM	Ethyl acetate	Methanol	50% methanol
Alkaloid	-	-	-	-	-
Terpenoids	-	-	+	+	+
Flavonoids	-	-	+	+	+
Polyphenol	-	-	+	+	+
Glycoside	-	-	+	+	+
Reducing Sugar	-	-	-	+	+
Saponin	-	-	-	-	-
Tannins	-	-	+	+	+
Coumarin	-	-	-	-	+
Quinines	-	-	-	+	+
Steroid	-	-	-	-	-

Table 2: Phytochemical screening of root extracts of *Cheilanthes bicolor*

Extract	Hexane	DCM	Ethyl acetate	Methanol	50% methanol
Alkaloid	-	-	-	-	-
Terpenoids	-	-	+	+	+
Flavonoids	-	-	+	+	+
Polyphenol	-	+	-	+	+
Glycoside	-	-	+	+	+
Reducing Sugar	-	-	-	+	+
Saponin	-	-	-	-	-
Tannins	-	-	-	+	+
Coumarin	-	-	-	-	+
Quinines	-	+	-	+	+
Steroid	-	-	-	-	-

(+) = presence where (-) = absence

3.2 Estimation of Total Phenolic Content (TPC)

The result of the total phenolic content (Table 3) showed that the total phenolic content in the ethyl acetate extracts was found to be relatively high (147.30±1.23 mg GAE) in comparison to other extracts therefore, the TPC order is ethyl acetate > 50% Methanol > Methanol. Similarly, between the

frond and rood ethyl acetate extracts, the frond extract has a significant amount of phenolic (147.30±1.23 mg GAE/g) than the root extract (135.65±0.21 mg GAE/g). Fig 2 tries to compare frond and root extracts in different solvents, we can conclude that the frond has more total phenolic compound than the root i.e., more efficient in antioxidant properties than the root.

Table 1: Total phenolic content in root and frond extract of *C. bicolor*

Extract	Total phenolic content (mg gae/g extract)	
	Frond	Root
Ethyl Acetate	147.30±1.23	135.65±0.21
Methanol	94.48±0.68	83.11±1.23
50% methanol	112.18±1.73	60.79±1.67

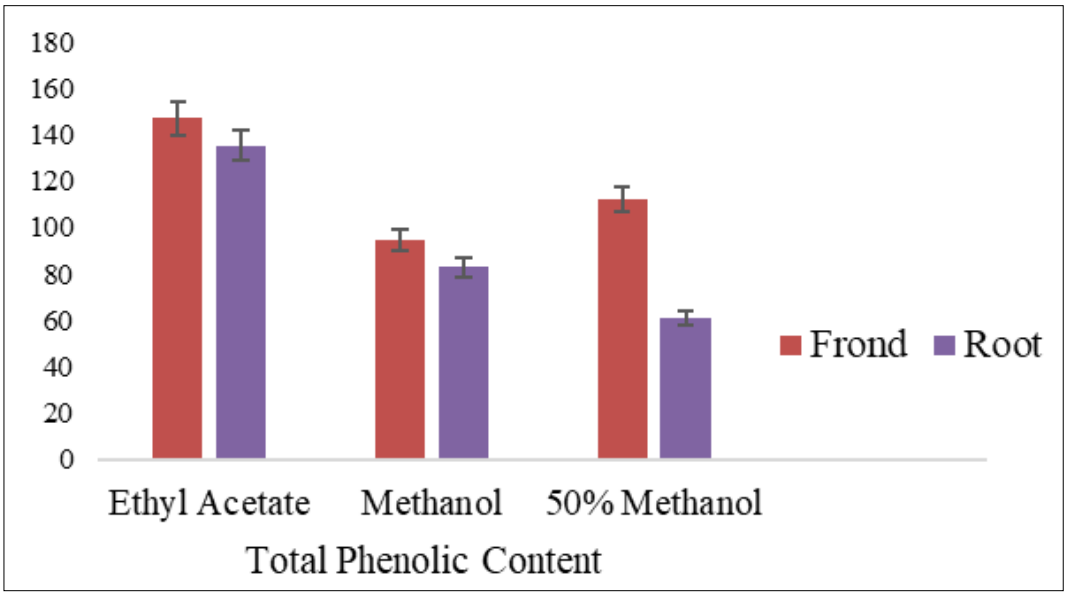

Fig 2: Total phenolic content in root and frond of *C. bicolor*.

3.3 Estimation of total flavonoid content (TFC):

Here the result of total flavonoid content (Table 5) revealed that same trend as the total phenolic content of the ethyl acetate extract showed a significant amount of TFC (82.13±5.61 CE/g) than another solvent extract like 50% methanol extract (77.50±5.7 CE/g) so order of TFC content in the different solvent extract is same as TPC i.e., Ethyl acetate > 50% Methanol > Methanol. Here also compared the TFC between the frond and root (Fig 3), but the frond extract has a higher amount of TFC than the root in each extract (i.e., the Ethyl acetate extract of the frond contains (82.13±5.61 CE/g) whereas, the ethyl acetate of root extract (73.41±2.95 CE/g))

Table 4: Total flavonoid content in root and Frond extract in *C. bicolor*.

Extract	Total flavonoid (mg qe/g extract)	
	Frond	Root
Ethyl acetate	82.13±0.61	73.41±1.95
Methanol	52.43±1.64	41.75±0.12
50% methanol	77.50±1.52	48±1.01

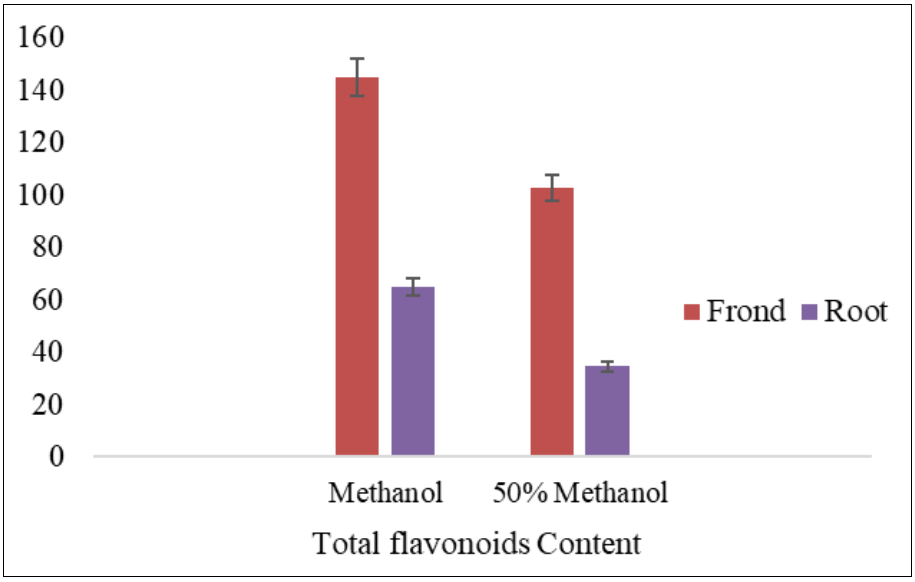

Fig 3: Total flavonoids content in *C. bicolor*

3.4. Determination of total tannin content

The result showed that (Table 5) the total tannin content we found that a higher amount of tannin in ethyl acetate extracts (75.96±1.41 mg TAE/g) of the frond part than in other extracts. The order of tannin content in the frond is Ethyl acetate > Methanol > 50% Methanol. The same pattern (like

root extracts) fallowed here. We compared the total tannin content between the frond and root (Fig 3) in a bar graph of two extracts and the result showed that the ethyl acetate of the frond contains more tannin than that of the same solvent system of root extract.

Table 2: Total tannin content in Root and leaves extract in *C. bicolor*.

Extract	Total tannin content (mg tan/g extract)	
	Frond	Root
Ethyl acetate	75.96±1.41	72.39±0.23
Methanol	47.55±1.87	39.30±1.65
50% methanol	31.73±0.84	34.53±2.09

The presence and concentration of tannins in plants can significantly impact their nutritional value, palatability, and medicinal properties. In herbal medicine, tannins are often associated with various therapeutic effects, such as anti-inflammatory and antimicrobial properties.

3.5 Estimation of total sugar content

To explore the sugar content (table 6) of the frond and root of this plant we chose only two extracts methanol, and the 50% methanol result is shown in Table 7. Between them, methanol (144.93±1.54 mg GE/g extract) extract of the frond contains a significant amount of sugar than 50% methanol (102.67±1.73 mg GE/g extract). And comparison to the frond and root (Fig 4) frond contains a substantial amount of sugar (144.93±1.54 mg GE/g extract) than the root extract (82.69±1.5 mg GE/g extract) in the case of methanol extract. Same result for 50% methanol extract.

Table 3: Total sugar content of methanol and 50% methanol leaves and root extract of *C. bicolor*

Extract	Total sugar content (mg glucose/g extract)	
	Frond	Root
Methanol	144.93±1.54	82.69±1.50
50%methanol	102.67±1.73	64.70±0.35

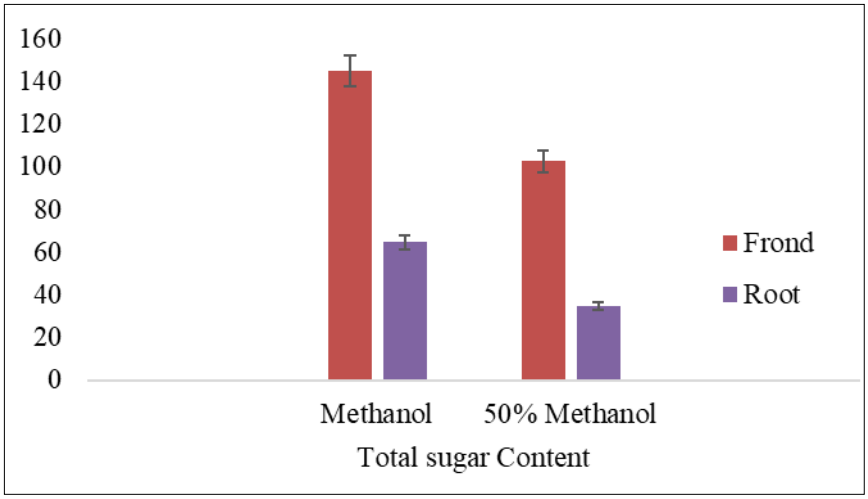

Fig 1: Total Sugar content in *Cheilanthes* bicolor

3.6 DPPH Radical Scavenging Assay

The results of the DPPH assay revealed (Table 7) that the IC_{50} of the ethyl acetate extract (31.6 µg/mL) was found to be slightly lower than 50% methanol extract of frond (33.9 µg/mL) but relatively higher than that of ascorbic acid (11.0 µg/mL). The lower the IC_{50} value, the higher the antioxidant activity. Therefore, the ethyl acetate and 50% methanol extract of the frond showed good antioxidant activity relative to the ethyl acetate extract of the root. On the other hand, in comparison between the ethyl acetate extract of the frond and the root of this plant, the frond showed a lower IC_{50} value (31.6 µg/mL) compared to the root's IC_{50} value (38.6 µg/mL).

Table 4: Percentage radical scavenging and IC50 values for DPPH radical scavenging of different extracts of and standard ascorbic acid at a different concentration.

Concentration (µg/mL)	% Free radical scavenging			
	Ethyl acetate (Frond)	50% methanol (Frond)	Ethyl acetate (Root)	Ascorbic acid
5	21.7	20.80	19.85	37.97
10	25.27	25.5	22.81	52.15
15	28.72	27.7	23.55	64.85
20	29.96	28.0	25.77	77.18
IC_{50}(µg/mL)	31.6	33.9	38.6	11.0

The phenolic compounds containing free hydrogen are mainly responsible for antioxidant activity. The high antioxidant activity of ethyl acetate extract can be correlated to the high phenolic, flavonoid, and tannin content in these extracts. The DPPH assay is based on the capability of an antioxidant compound or Phyto-constituents to donate a hydrogen radical or an electron to a stable DPPH radical having a deep violet color. When it gets either a hydrogen radical or an electron in the presence of an antioxidant agent (Free radical scavenger), DPPH radicals get reduced to corresponding hydrazine, DPPH-H form, and the solution gets decolorized from its initial deep violet to light yellow color. The extent of the decrease in the absorbance is measured spectrophotometrically and is proportional to the concentration of the antioxidant. The measurement of the scavenging of DPPH radical allows one to determine exclusively the intrinsic ability of the substance to donate either hydrogen radical or electrons to DPPH solution in a

homogenous system.

3.7. The Antibacterial Susceptibility Test

The result of antibacterial activity (Table 8) of the frond extract of the plant has a zone of inhibition. The result was not as excited as we thought among all frond extracts except methanol extract, all the tested extracts showed very weak antifungal activity against *Candida albicans* ZOI is 4 mm for all extracts. Ethyl acetate and hexane extracts showed weak antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* ZOI is 9 and 7 respectively. The activity of ethyl acetate extract showed a little bit more antibacterial activity this may be possibly due to the presence of the flavonoids, terpenoids, polyphenols, tannins, and other phytochemicals in comparison to other extracts.

Table 5: Antibacterial screening result of different extracts of *C. bicolor*

Zone of inhibition (ZOI in mm) of Plant extract							
Name of bacteria	**Hexane**	**DCM**	**Ethyl acetate**	**Methanol**	**50% methanol**	**NC**	**PC**
S. aureus	7	-	9	-	-	2	34
K. pneumonia	-	-	-	-	-	2	34
S. Typhi	-	-	-	-	-	2	34
E. coli	-	-	-	-	-	2	34
C. albicans	4	4	4	-	4	2	34

PC = Positive Control (Ciprofloxacin) and NC = Negative Control (50% DMSO).

Sephadex LH- 20 Column Chromatography of Ethyl Acetate Extract of *C. bicolor*

The Ethyl acetate extract of (1g) was purified on a Sephadex LH- 20 column using ethyl acetate-methanol- water (100:3:1.5) as a solvent system. The fraction F5(Test tube number 35,39) showed a single spot-on TLC with an Rf value of 0.63) and it was visualized on UV- 336 nm (Fig 5).

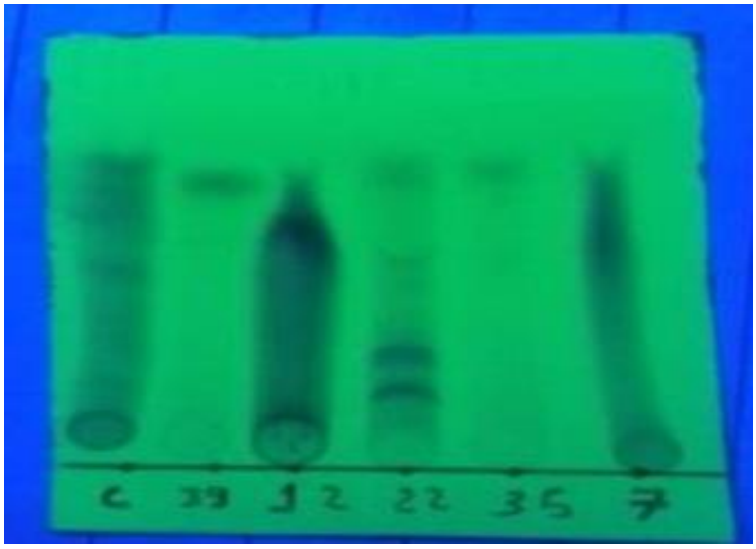

Fig 5: Thin layer chromatogram of different fractions from Sephadex LH 20 column visualized at UV- at 366 nm and UV-254 nm in solvent system ethyl acetate: methanol: water (100:3:1.5)

Ultra-Performance Liquid Chromatography/Mass Spectrometry (UPLC-MS) analysis of methanol extract of *Cheilanthes bicolor* frond:

Because of frond parts showed more TPC, TFC as well and antioxidant properties, we decided to check different compounds present in the front part, so used the mass spectrometry technique to determined possible compound on it.

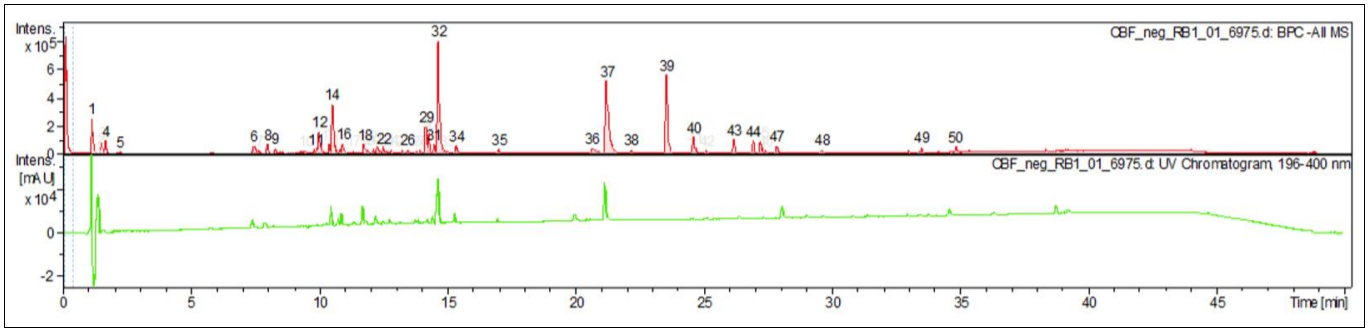

Fig 6: UPLC-MS Chromatogram in negative ion mode

The chemical composition of the methanol extract was determined using the LC-ESI-MS technique. In negative ionization mode, altogether fifty peaks were seen (Fig 6). The chromatograms were recorded in negative ionization modes

are shown in this spectrum. Based on the different mass peaks we calculated the molecular mass and isolated the molecular formula of five compounds present in the front of this plant (Table 9). This successful isolation and molecular formula determination represent a significant milestone in drug discovery, potential therapeutic applications, and advancing our understanding of plant chemistry. This discovery opens new avenues for exploring the compound's pharmacological properties and may contribute to the development of novel drugs and nutraceuticals. We need furthermore research for confirmation in the future.

Table 9: Molecular mass and calculated molecular formula in UPLC-MS

Retention time (minute)	Molecular mass measured calculated	Molecular mass Obtained	Molecular Formula
10.44-10.69	525.3072	525.3069	$C_{28}H_{45}O_9$
14.55-14.83	461.1089	461.1079	$C_{22}H_{21}O_{11}$
21.12-21.52	313.0718	313.0726	$C_{17}H_{13}O_6$
23.46-23.72	451.3065	451.3076	$C_{26}H_{43}O_6$
23.46-23.72	451.3079	451.3076	$C_{27}H_{39}N_4O_2$

4. Conclusion

Phytochemicals screening of frond and roots extracts indicated that *Cheilanthes bicolor* plants are rich in secondary metabolite therefore, this plant contains a significant amount of total phenolic content, total flavonoids content, total sugar content, and total tannin content. However, comparison between frond and root frond part is more efficient than that of root parts. The radical scavenging activity of the extracts [DPPH (1, 1-diphenyl-2- picrylhydrazyl) method]. Plant extracts showed effective inhibition activity against the DPPH radical. Again, in this case, also frond is a more effective antioxidant than the root of this plant. The antibacterial activity extracts of frond against four bacteria, *Staphylococcus aureus, Salmonella typhi, Klebsiella pneumonia, and Escherichia coli,* and one fungus *Candida albicans*, indicate that different extracts did not have significant activity against bacteria and fungus activity. This might be because of different circumstances like poor methods.

The TLC test (single spots in TLC with R_f values of 0.63) suggested that it could have different compounds. From UPLC-ESI-MS analysis of methanol extract confirms that five compounds are present with molecular formulas $C_{28}H_{45}O_9$, $C_{22}H_{21}O_{11}$, $C_{17}H_{13}O_6$, $C_{26}H_{43}O_6$, and $C_{27}H_{39}N_4O_2$. ^{1}HNMR, ^{13}CNMR, and ESI-TOFMS will be carried out further in order to characterize the compounds above possible reported molecular formula. In this work, we revealed that *Cheilanthes bicolor* possesses promising antioxidant and somehow antibacterial properties. The findings of this study suggested that the plant *Cheilanthes bicolor* used in the ethnomedicine in *Lamjung* district could be an important source of bioactive constituents that can be used in the formulation of phytopharmaceuticals, used in primary health care and allowed five compounds from the UPLC-ESI-MS, could be the novel compound.

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6. Conflict of interests

There is no conflict to declare.

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