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PHYTOCHEMICAL, ANTIOXIDANT AND ANTIMICROBIAL ANALYSIS OF *BAMBUSA TULDA* ROXB. *AND BAMBUSA NUTANS* WALL. EX MUNRO

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Bamboo shoots possess high nutritional contents and medicinal properties. Around 53 species of bamboos are disseminated throughout the country between elevations varying from 70m-4,000 masl. The aim of this study is to conduct phytochemical evaluation, quantitative analysis with assessment of antioxidant and antimicrobial potential of two Nepalese *Bambusa* species by the application of different analytical methods. Methanol seems to be the better solvent than ethanol for extraction of compounds from leaves and stem of *B. tulda* and *B. nutans.* Spectrophotometric assay was utilized to measure the amount of phenolic, flavonoid, carbohydrate, proteins and chlorophylls. Antimicrobial efficiency of extracts was analysed against *Streptococcus mutans*, *Bacillus subtilis, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans* using agar well diffusion method. Methanolic stem extract of *B. nutans* exhibited the highest antioxidant activity using DPPH essay with IC_{50} value of 384.14 µg/ml alongside highest antibacterial activity with 7.5 mg/ml of extract concentration; whereas, the minimum inhibitory concentration (MIC) was 3.75 mg/ml. *Streptococcus sps.* was the most susceptible bacterial strain. None of the extracts inhibited efficacy against *Candida albicans*. In conclusion, *B. tulda* and *B. nutans* contain phytosterols and phenols which inhibit the growth of *Streptococcus* sps. and also, it can be used in pharmaceuticals and nutraceuticals due to antimicrobial and antioxidant activities.

Keywords: Bamboos, Phytochemical Analysis, Antioxidant Activity, Antimicrobial Screening, Qualitative Analysis

Bamboos are woody perennial tall grasses belonging to the grass family Poaceae in the subfamily *Bambusoideae*. With the likely presence of numerous phytochemical constituents, bamboo is also one of the promising plants for the production of high-value therapeutics. Macwan *et al.* (2010) found that methanolic bamboo extract shows free radical scavenging, metal chelating and nitric oxide scavenging capacity. Current studies on bamboo leaves showed the presence of phenolic acids in the form of flavonoids that have antioxidant capabilities (Sujarwo 2010). In addition, bamboo extracts have demonstrated antimicrobial activities (Güllüce 2003, Nazreen *et al.* 2012) also reported that bamboo leaves have been used in traditional Chinese medicine for treating fever and detoxification for over 1000 years.

Bambusa is a genus containing large bamboos of up to 26m in height, as well as several smaller species of only 10m or less. These clump-forming bamboos are similar to *Dendrocalamus* species, but they are generally smaller, with straighter culms and thicker culm walls. Among them, *B. nutans* are the commonest cultivated bamboos in the hills of central and west Nepal, not found in the Terai. Used for the weaving of rough baskets and mats as the branches are small and the poles split easily; these species are reputed to be resistant to termite attack and tolerate dry sites well. On the other hand, *B. tulda* are rare in the Himalayas but occasionally found in the Terai. It can be distinguished from *B. nutans* by the larger, more prominent, leaf sheath auricles. As they are very thick-walled, they are used for constructional purposes whereas leaves can be used for fodder. On top of that, their dense surface roots and large mass of rhizome system, these species; in combination with trees having roots to greater depth can potentially function as a vital element of bio-engineering approach in conservation of Nepalese soil from erosion due to the water and

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strong winds; rendering a cost effective method of slope stabilization.

Bamboo has been associated with native lifestyle since primeval time in Nepal. Traditional medicinal practices in Nepal have demonstrated that plants are the sources of many effective medicinal therapeutics. Quantitative analysis of the *B. tulda* and *B. nutans* for phytochemical screening is followed by cold extract procedure in ethanolic and methanolic solvents (Upreti *et al*. 2016). The susceptibility of *Staphylococcus aureus, Streptococcus sps.*, *Klebsiella pneumoniae, Pseudomonas* sps*., Bacillus subtilis* to the methanolic extract as well as minimum inhibitory concentration (MIC), studied by using modified agar well diffusion method, was employed for screening of antimicrobial activity of extracts as illustrated by Rios *et al.* (1988), Perez *et al.* (1990) and Thapa *et al. (*2018) .

The purpose of this research was to extract the compounds from leaves and stems of *B. nutans* and *B. tulda* found in Nepal with solvents of different polarity to perform qualitative assay as well as to determine the phytochemical constituents, antioxidant and antimicrobial activity. Only a few researches have so far reported on the phytochemical constitution, antioxidant and antimicrobial activity of bamboos in Nepal. The results of this study will provide basis for further research and use of these bamboo species in the benefit of the mankind.

MATERIALS AND METHODS

Collection of samples and processing: In this study, healthy leaves and stem segments (2cm in length) of *B. tulda* and *B. nutans*, were collected from Sarlahi (Latitude: 27.0° Longitude: 85.52°) and Udayapur district (Latitude: 26.89°, Longitude: 86.70°) of Nepal (Fig. 1). Leaves and stems were examined for any flaws, washed with distilled water then shade dried in 27° C.

The extraction of the samples was carried out from modified cold extraction procedure (Upreti *et al.* 2016). Shade dried samples were cut into small pieces and powdered using a blender. 5gm of powdered sample was

Figure 1: Map of the study sites.

dissolved in 50ml of solvent at 1:10 (w/v) ratio and then loaded in an orbital shaker at 80 rpm for 48 hours to ensure maximum extraction. The solution was filtered through Whatman filter paper no.1 and concentrated further using a Soxhlet apparatus to obtain semi-solid materials. The semi solid extracts were further dried by using rotary evaporator to remove remaining solvents and obtained dry powder extract, which was used for quantitative and phytochemical analysis and antimicrobial screening. Fresh leaves of bamboo samples were carefully separated and then washed with distilled water and immediately ground using a mortar and pestle in order to estimate its carbohydrate and protein contents.

Phytochemical Analysis: The phytochemical analysis to establish the presence or absence of alkaloids, flavonoids, glycosides, phenol, saponin, triterpenes, starch, tannins, proteins, carbohydrate, quinone, resins and sterols were performed following the standerd protocols (Tongco *et al.* 2014, Manohari *et al.* 2016, Banu *et al*. 2015) with few minor modifications.

Total Phenolic Content: Total Phenolic Content of ethanolic and methanolic extract was measured using spectrophotometric assay according to the method described by Singleton *et al.* (1999). A calibration curve was prepared using Gallic acid (GA) as standard and the absorbance was measured at 765 nm.

Total Flavonoid Content: To determine Total Flavonoid Content of ethanolic and methanolic extracts as per aluminium chloride, colorimetric assay procedure described by Quettier *et al*. (2000) was used. A calibration curve was prepared using rutin solution as standard. UV spectrophotometer was used to measure the absorbance at 510nm.

Chlorophyll Content: Chlorophyll content of fresh and uninfected leaves was determined using protocol reported by Wu *et al.* (2002) and Sumanta *et al.* (2014).Before homogenizing in a mortar with different extracts (80% acetone and DMSO), samples were properly cleaned and shredded. Chlorophyll-a, chlorophyll-b, and carotenoid contents were measured using UV spectrophotometer.

Carbohydrate Content:

Estimation of total soluble sugars and starch: Total soluble sugar (TSS) present in fresh leaves was measured following the method of Dubois *et al.* (1956) whereas, starch content was estimated according to the method reported by McCready *et al*. (1950). Absorbance was measured at 490nm and the standard curve was plotted using the known concentration of glucose. The quantity of TSS was expressed in mg/g fresh weight of ethanolic extract while the starch content was calculated in terms of glucose equivalent.

Total protein content: Protein Content in fresh leaves was determined by Lowry *et al.* (1951) method. Absorbance was measured at 750nm. The standard curve of bovine serum albumin (BSA) was used to express total protein content in mg/gm fresh weight of tissue.

Antioxidant Activity: The DPPH (2, 2-diphenyl-1-picryl-hydrazylhydrate) radical scavenging activity of the bamboo leaf extract was determined following the protocol of Tekao *et al.* (1994). For the assay, DPPH solution of 100µM, Stock solution of 1mg/ml ascorbic acid and test solution of 200, 400, 600, 800 and 1000µg/ml of extracts were prepared. From each test solution, 1ml of aliquot was taken in a test tube and 1ml of DPPH solution was added. Only methanol solvent was used as control and it was used for the baseline correction. All the mixtures were incubated in

dark for 30 min before measuring absorbance at 517nm using UV-visible spectrophotometer (UV-1800). The experiment was carried out in triplicate for each sample to calculate percentage inhibition and IC_{50} value.

Antimicrobial Screening: Modified agar well diffusion method was employed for screening of antimicrobial activity of extracts as illustrated by (Rios *et al.* 1988, Perez *et al.* 1990). Dry methanolic extracts were dissolved in 10% DMSO at the concentrations of 7.5. 3.175, 1.875, and 0.9375 mg/ml for further analysis. In addition, the lowest concentration of extracts exhibiting the potential to inhibit the growth of test microorganisms was noted as the minimum inhibitory concentration (MIC).

Standardized inoculum of Bacterial and fungal samples of corrected turbidity of McFarland turbidity standard of 0.5 (10^6 colony forming units (CFU) per ml) was used to inoculate Muller Hinton agar (MHA) and Sabouraud Dextrose Agar (SDA) plates then incubated at 37°C for 24 hours and 36°C for 48 hours, respectively. Gentamycin (10µg/disc) and fluconazole (30µg/disc) were included as positive control while 10% DMSO was used as negative control.

Test organisms: The investigated pathogenic bacterial strains *Staphylococcus aureus, Streptococcus sps.*, *Klebsiella pneumoniae, Pseudomonas* sps*., Bacillus subtilis* were provided by the Department of Biotechnology, Kathmandu University, Nepal. *Candida albicans* was obtained from Department of Microbiology, Dhulikhel Hospital, Dhulikhel, Kavre, Nepal. Microorganisms were maintained at 4°C in agar slants and broths for further use.

Statistical Analysis: All the determinations were conducted at least three times. The statistical mean was calculated with ± SD using Excel 2016 (Microsoft Corporation, Redmond).

RESULTS AND DISCUSSION

Phytochemical Analysis: Eleven parameters of BNL, BNS, BTL and BTS were analysed using methanolic and ethanolic extract for the presence or absence of the specific compound. The test results were summarized in table 1.

Preliminary phytochemical screening of extracts was done based on the intensity of colour change observed during the experiment. Tests showed the strong presence of flavonoids and glycosides in leaves and stem of both species. Similarly, results revealed that the absence of alkaloids, starch, tannins, proteins, carbohydrates, resins and sterols in *B. nutans* and *B. tulda*; though, the quantitative analysis suggested the presence of extractable proteins, carbohydrates and starch in shoots of *B. nutans* and *B. tulda* (Nongdam 2014). Since both solvents used are polar, they might not be able to properly solvate the bulkier and less polar compounds. Triterpenes are less polar compounds, but capable to be soluble in polar solvents as well, due to the presence of some polar functional groups -hydroxyl and carbonyl groups. Saponins, as expected, are very hydrophilic – should dissolve in polar solvents, but the presence of saponin was less noticed, may be due to evaporation of the volatile phytochemicals during shade drying before extraction (Joseph *et al.* 2014).

Phenolic compounds- consist one or more hydroxyl groups in their aromatic benzene ring, mainly protect plant under biotic and abiotic environmental stress, dissolve in a polar solvent, the inconsistent presence in all extract may be due to exposure in the different levels of stress (Nazreen *et al.* 2011).

Antioxidant Activity:DPPH assay is routinely employed in laboratories for determining the free radical scavenging potential of purified phenolic compounds and natural plant extracts. This assay measures the compound for its ability to act as free hydrogen donor. DPPH is a relatively stable free radical

Table 1: Phytochemical analysis of stem and leaf extracts

Tests	Ethanolic Extract				Methanolic Extract			
	BNL	BNS	BTL	BTS	BNL	BNS	BTL	BTS
Alkaloids	-	-	-	-	-	-	-	-
Flavonoids	+	+	++	++	-	++	+	++
Glycosides	++	++	++	++	+	++	++	++
Phenols	-	-	-	+	+	-	+	+
Saponin	+	-	+	+	-	-	-	-
Triterpenes	-	+	+	+	-	+	-	+
Starch	-	-	-	-	-	-	-	-
Tannins	-	-	-	-	-	-	-	-
Proteins (Ninhydrin)	-	+	-	-	-	-	-	-
Carbohydrate	-	-	-	+	-	-	-	-
Quinone	+	+	+	-	+	+	-	-
Resins	-	-	-	-	-	-	-	-
Sterols	-	-	-	-	-	-	+	-

aBNL: B. nutans Leaf, BNS: B. nutans Stem, BTL: B. tulda Leaf and BTS: B. nutans Stem.
b+: Present, ++: Strongly present, -: Absent.
Mean ± standard deviation.

as it can accept an electron or hydrogen radical. Presence of antioxidant molecules neutralizes DPPH free radicles converting them from violet colour to colourless (Amarowicz *et al.* 2003).

Phenolic compounds such as phenolic acids, flavonoids and tannins are the most important secondary metabolites in plant responsible for antioxidant activity. Figure 3 showed that the free radical scavenging capacity of bamboo stem extract is higher than that of leaf extract and the percentage inhibition is directly proportional to concentration. BNS showed the highest scavenging activity with IC_{50} value of 384.14 µg/ml, while lowest activity was shown by BTL extract with an IC_{50} value of 1588.33 µg/ml. This signifies that bamboo leaves contain a remarkable amount of secondary metabolites responsible for free radical scavenging in comparison to its leaves (Govindan *et al.* 2019).

Chlorophyll Content: Chlorophyll-a pigment is mainly involved during the conversion of light energy into chemical energy while chlorophyll-b act as an accessory pigment helps indirectly during photosynthesis. Located in chromoplast, the colour providing pigments to vegetables or fruits are carotenoids (Sumanta *et al.* 2014).

The Figure 4 represents computed chlorophyll content of fresh leaves of BN and BT which indicated the presence of higher amount of chlorophyll content in BN than in BT. Chlorophyll-a is present abundantly than chlorophyll-b and carotenoid, may be due to its main involvement in photosynthesis. Since the role of carotenoid is to provide colour to fruits and vegetables which contents might be less in leaves (Macwan *et al.* 2010).

Total Phenolic and Flavonoid Content: The antioxidant activity of phenolic compound and flavonoid is due to their redox properties. Their free radical scavenging activity is facilitated by their hydroxyl group, which can be used as a basis for screening of antioxidant activity. The result obtained is shown in following graph, where amount of total phenol is expressed as mg gallic acid equivalent (GAE) per gram dry extract (mg GAE/g dry weight) – highest in

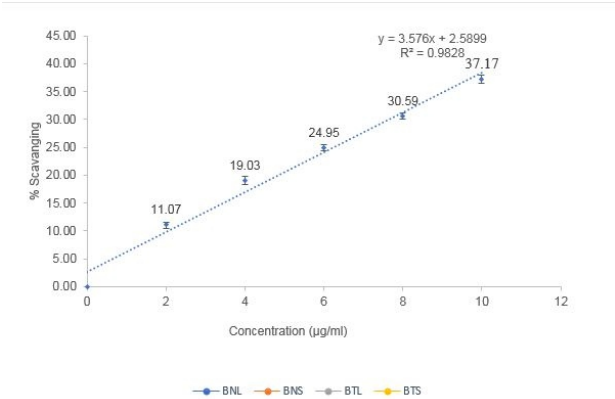

Figure 2: Standard Curve of Ascorbic Acid.

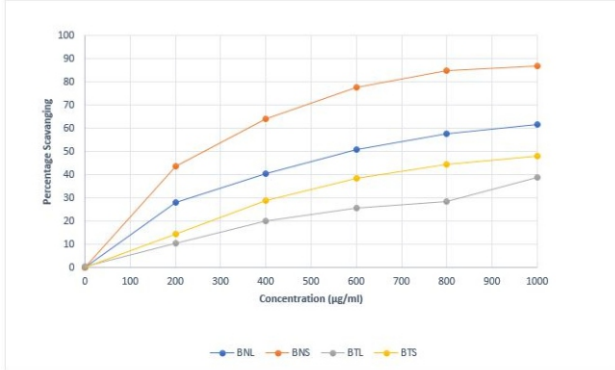

Figure 3: Antioxidant Activity of the extracts as per DPPH Assay expressed in percentage

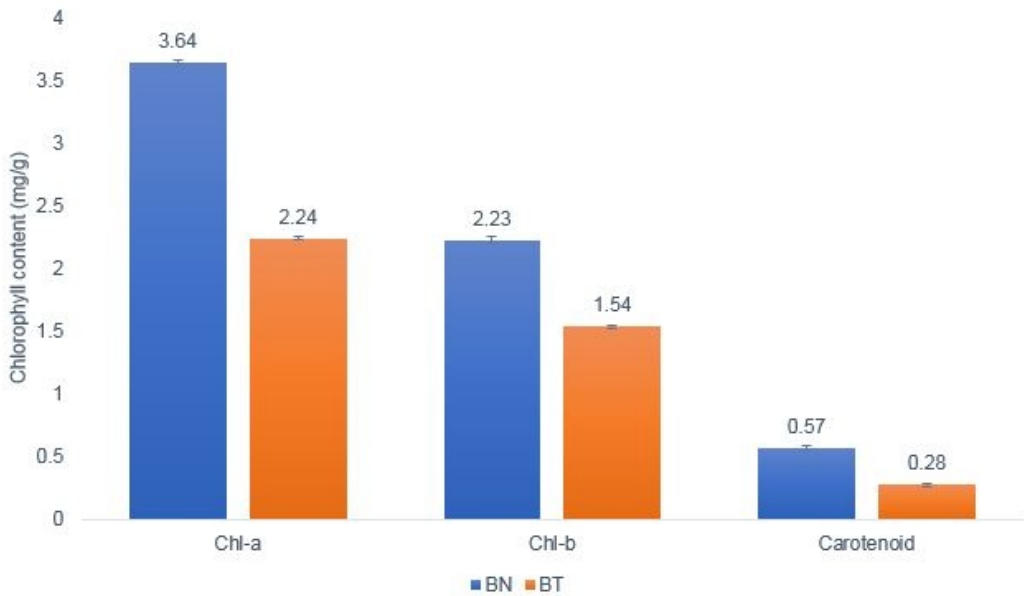

Figure 4: Content of Chlorophyll-a, Chlorophyll-b and Carotenoids in the fresh leaves of BN and BS expressed in mg/g

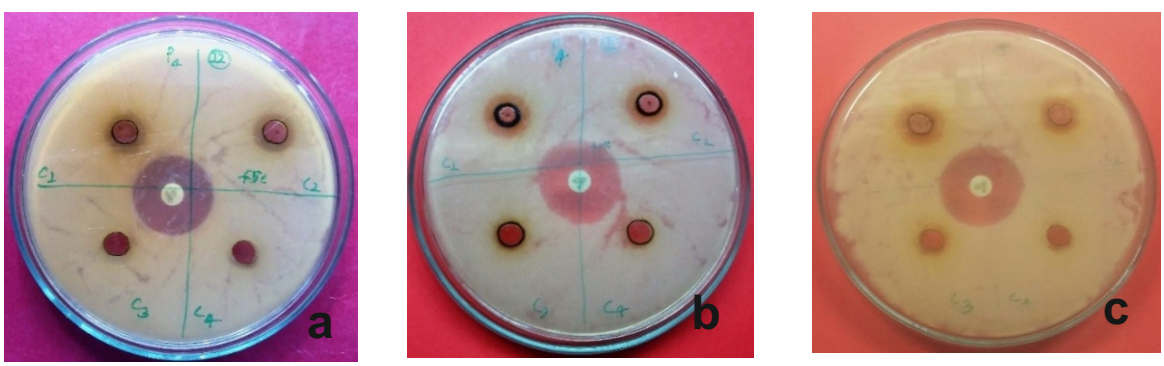

Figure 5: Zone of inhibition from antibacterial activity assay (a) *B. tulda* leaf extract against *Streptococcus sps.* (b) *B. tulda* stem extracts against *Streptococcus sps*. (c) *B. nutans* stem extract against *B. subtilis*

ethanolic extract of BNS, whereas lowest amount of phenol is found in methanolic leaf extract of BLT. Similarly, amount of total flavonoid is expressed as mg rutin equivalent (RU) per gm dry extract (mg RU/g dry weight) – highest in methanolic extract of BTL and lower in ethanolic extract of BTS.

The results revealed that both species contain greater phenolic content in stem than in leaves; whereas, the leaves contained higher level of flavonoids in comparison to stem. It can be explained as phenolic compounds are more efficiently solvate in an ethanolic solvent than in methanolic solvent while flavonoids do just the opposite i.e. favours methanol as a superior solvent than ethanol.

Total of 89.764 mg/gm carbohydrate exists in

Table 2: Total phenolic and flavonoid content in the fresh leaves and stem of *B. nutans* and *B. tulda*.

Species	Total Phenolic Content (mg GAE/g dry weight)		Total Flavonoid Content (mg RU/g dry weight)	
	Ethanolic Extract	Methanolic Extract	Ethanolic Extract	Methanolic Extract
BNL	34.02±0.765	23.9±0.245	79.56±3.15	91.11±5.18
BNS	40.25±0.296	28.137±0.8	62.67±1.33	87.33±3.05
BTL	34.725±0.53	16.37±0.413	82.44±2.77	91.11±5.18
BTS	37.7±0.963	26.14±0.67	68.67±3.71	105.33±6

*BNL: B. nutans Leaf, BNS: B. nutans Stem, BTL: B. tulda Leaf and BTS: B. nutans Stem.
Mean ± standard deviation.

Table 3: Total Carbohydrate and Protein Content in the fresh leaves of *B. nutans* and *B. tulda*

Species	Total Carbohydrate Content (mg/g)		Total Protein Content (mg/g)
	Total Starch Content	Total Soluble Sugar	
BN	42.933±4.97	46.771±2.44	54.49±0.357
BT	37.544±3.75	27.371±0.86	58.99±0.423

BN: B. nutans, and BT: B. tulda.
Mean ± standard deviation.

Table 4: Antimicrobial Screening (methanol extracts of *B. tulda* leaf and stem)

Pathogen	Inhibition zone diameter (mm)								Standard
	Concentrations of Crude (Leaf) Extract				Concentrations of Crude (Stem) Extract				
	7.5	3.75	1.875	0.9375	7.5	3.75	1.875	0.9375	
K. pneumoniae	-	-	-	-	-	-	-	-	24
B. subtilis	-	-	-	-	-	-	-	-	26
Pseudomonas sps.	4			-	4	-	-	-	22
Streptococcus sps.	6	4.8	-	-	8	7	-	-	26
S. aureus	-	-	-	-	-	-	-	-	18
C. albicans	-	-	-	-	-	-	-	-	19

*positive control: Gentamycin (10µg/disc) and fluconazole (30µg/disc)

Table 5: Antimicrobial Screening (methanol extracts of *B. nutans* leaf and stem)

Pathogen	Inhibition zone diameter (mm)								Standard
	Concentrations of Crude (Leaf) Extract				Concentrations of Crude (Stem) Extract				
	7.5	3.75	1.875	0.9375	7.5	3.75	1.875	0.9375	
K. pneumoniae	-	-	-	-	5	-	-	-	24
B. subtilis	-	-	-	-	8	7	-	-	26
Pseudomonas sps.	5	-	-	-	5	-	-	-	22
Streptococcus sps.	5	-	-	-	-	-	-	-	26
S. aureus	-	-	-	-	5	-	-	-	18
C. albicans	-	-	-	-	-	-	-	-	19

*positive control: Gentamycin (10µg/disc) and fluconazole (30µg/disc)

BNL, while in BTL only 64.915 mg/gm carbohydrate. The reported level of carbohydrates in shoots of BN and BT were 3.3% and 6.92%. Protein content was present higher in BTL than in BNL. The reported level of protein in shoots of BN and BT 2.84% were and 3.69% (Chongtham *et al.* 2011, Nongdam *et al.* 2014).

Antimicrobial Screening: Of all the pathogenic bacteria investigated, the antimicrobial screening revealed prominent activity against *Streptococcus sps.* for stem extract of *B. tulda* at the minimum concentrations of 7.5 mg/ml and 3.75 mg/ml with the zone of inhibition of 8 mm and 7mm respectively. In addition, this very extract demonstrated moderate activity against *Pseudomonas* sps. as shown in Table 4. The stem extract of *B. nutans* was highly effective against *B. subtilis* with a zone of 8mm at minimum concentration of 3.75 mg/ml. Similar yet limited activity against *K. pneumoniae, Pseudomonas* sps*., and S. aureus* with zones of 5mm respectively at the minimum concentrations of 7.5 mg/ml (Table: 5). In order to discover minimum concentration of extracts required to inhibit and/or eliminate the bacterial growth minimum inhibitory concentration (MIC) was determined.

Leaf extracts of both species of bamboos exhibited significant activity against *Pseudomonas* sps. and *Streptococcus sps.* at minimum concentration of 7.5 mg/ml. However, the zone of inhibition of *B. tulda* leaf extract against *Streptococcus sps.* was found to be 4.8 mm at the minimum concentration of 3.75 mg/ml. None of the extracts showed any activity against *C. albicans*.

Bamboo extracts contain a large number of bioactive compounds such as tannins, terpenoids, polyphenols, and flavonoids majorly responsible for antibacterial activity (Fernandez *et al.* 1996, Ouattara *et al.* 2011; Tiara *et al.* 2018). However, antibacterial effect and function vary with the extract concentration and type of bacteria, so they should apply reasonably. Under different pH values, there is a noticeable difference in the antibacterial effect. It is a subject requiring further study as to how to give full attention to the antibacterial effect of effective factors in bamboo extracts to achieve the best antibacterial effect.

Additionally, different type of extracts and different concentration gave different inhibition activities. Increasing the concentrations of all extract was directly correlated with the increasing inhibition activities. Thus, the higher the concentration of extract used, the higher the inhibition activity (Fig. 5). Increasing concentrations would result in the higher composition of bioactive compounds in the extract, so the ability to inhibit bacterial growth was also getting stronger (Tiara *et al.* 2018). The antibacterial

activity of Bamboo extracts also varied with different parts of the plant itself, in this case leaf and stem (Tables 4 and 5). Further, the solvent i.e. 10% DMSO did not contribute to any of the antimicrobial activity as indicated by the negative control

CONCLUSION

This study unveiled the presence of a wide range of phytochemicals in *B. tulda* and *B. nutans* growing in Nepal. Alongside the determination of the antioxidant activity of both Bamboo extracts, the study also demonstrated its antimicrobial activity and the MIC. The quantities of phenols, flavonoids, chlorophyll and nutrients such as carbohydrates and proteins present in the extracts were evaluated. While other Bamboo extracts showed notable inhibition of test bacteria, stem extract of *B. nutans* showed broad and highest antibacterial activity. Present evidences strongly support the fact that these two bamboo species hold the potential to render essential elements for further development such as dietary and therapeutics in developing countries like Nepal where majority of the population is poverty-stricken. Furthermore, this analysis will provide a contribution for future phytochemical and pharmacological studies which contribute in pharmaceutical industries in Nepal.

It is our radiant sentiment to place on record our best regards as well as the deepest sense of gratitude to Department of Biotechnology, Kathmandu University and Department of Microbiology, Dhulikhel Hospital for providing us lab facilities essential to carry out this research. We are also indebted to President of Tarai Madhesh Chure Conservation Broad for providing us financial assistance. We would also like to express our appreciation to Ms. Sudina Bhuju, Mr. Anil Upreti, and Mr. Santosh Dhamala for providing constant support throughout the study.

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