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Research Article

CHARACTERIZATION OF ESSENTIAL OIL, ESTIMATION OF PHENOLIC AND FLAVONOID CONTENT AND BIOLOGICAL ACTIVITIES OF *Ephedra pachyclada* BOISS

Lekha Nath Khanal[1], Khaga Raj Sharma[2], Yuba Raj Pokharel[3], Surya Kant Kalauni[2,*]
[1]Department of Chemistry, Prithvi Narayan Campus, Tribhuvan University, Pokhara, Nepal
[2]Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal
[3]Faculty of Life Science and Biotechnology, South Asian University, New Delhi, India
**Corresponding author: skkalauni@gmail.com*
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ABSTRACT

The northern areas of Nepal are rich in biodiversity and contain a large number of medicinal plant species including the Genus *Ephedra* of evergreen gymnosperm, belonging to the family Ephedraceae. The plants have been used by the peoples of the Himalayan region for the treatment of asthma, blood pressure, and gastritis for many years. This study was aimed for the evaluation of phytochemical, antioxidant, and antimicrobial activities of methanol, ethyl acetate, dichloromethane, and *n*-hexane extracts, and gas chromatography-mass spectrometry (GCMS) profiling of the essential oil of the aerial parts of *Ephedra pachyclada* Boiss from Mustang district of Nepal. Antioxidant activity was evaluated by 2,2-dipheny-1-picrylhydrazyl (DPPH) free radical scavenging assay and antimicrobial activity by the agar well diffusion method. Total phenolics and total flavonoid content were determined by Folin-Ciocalteu and aluminum chloride colorimetric method, respectively. The methanol extract contained the highest total phenolic content of 54.42 ± 1.40 mg GAE/g followed by the ethyl acetate (46.84 ± 0.62 mg GAE/g), DCM extract (19.58 ± 0.24 mg GAE/g), and the lowest TPC was shown for *n*-hexane extract (5.21 ± 1.40 mg GAE/g) of the dry weight. The methanol extract showed the maximum TFC of 33.28 ± 0.48 mg QE/g, followed by ethyl acetate extract (31.73 ± 0.52 mg QE/g), DCM extract (31.64 ± 0.56 mg QE/g), and the least value was obtained for the *n*-hexane extract (21.44 ± 2.91 mg QE/g). The methanol extract showed the highest antioxidant activity with a half-maximal inhibitory concentration (IC_{50}) of 37.81 ± 2.24 µg/mL. The methanol extract showed potent activity against *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) with zones of inhibition of 13 mm and 12 mm respectively. Ethyl acetate extract showed a slight potency against *Staphylococcus aureus* (ATCC 25923) with a zone of inhibition of 9 mm. The essential oil contained diisooctyl phthalate (46.90%), dodecane, 2,6,11-trimethyl-(16.35%), dodecane, 4,6-dimethyl- (11.59%), tetrapentacontane (11.56%), and myrtenol (4.37) as the major compounds. The plant exhibited significant antioxidant and antimicrobial activities which could be used as the source to isolate the active natural antioxidant and antimicrobial agent as the drug candidate in the future drug discovery process.

Keywords: Antimicrobial activity, antioxidant activity, *Ephedra pachyclada,* Mustang district

INTRODUCTION

Medicinal plants have long been used by different indigenous cultures for the treatment of a myriad of health complications around the world for many years. Many modern drugs have been established from the valuable biomedical information after the trial and error of plant-based compounds on human subjects over thousands of years (Li & Weng, 2017). Plants synthesize structurally specialized secondary metabolites containing highly active functional groups like aldehyde, sulfhydryl, epoxides, hydroxyl, and carboxyl which target enzymes, cell receptors, and transporters. They can mediate many cellular chain reactions which are useful in treating many diseases like cancer, diabetes, neurogenetic disorders, etc. Such compounds exhibit pharmacological properties like antiviral, antimicrobial, analgesic, antihypertensive, antitumor, psychoactive, etc. to the host (Mawalagedera et al., 2019). Alkaloids, flavonoids, phenolics, glycosides, tannins, gums, resins, etc. are the important secondary metabolites that are reported in the root, stem, flower, leaf, and barks of several plants of this genus that exhibit numerous pharmacological functions in the human body (Singh et al., 2016). Phenolics and flavonoids have a significant role in defense mechanisms and scavenging various types of free radicals produced in the cells by oxidative stress. They are interesting candidates for pharmaceutical and medical applications due to their substantial antioxidant, antibacterial, cardio-protective, immune defensive, UV-protective, and anti-inflammatory activities (Eugenio et al., 2017; Tungmunnithum et al., 2018).

Ephedra is a genus of gymnosperm seed-bearing plants of the family Ephedraceae that contains about 67 species distributed in the desert areas of Asia, America, North Africa, and Europe (Zhang et al., 2018). *Ephedra* (ephedra major host) known as 'Haoma' was considered a sacred plant in Zoroastrianism in Iran (Jamshidi-Kia et al., 2018).

The different plants of this genus have been used in traditional Chinese medicine against allergies, asthma, colds, coughs, fever, edema, flu, headache, and nasal congestions (Pirbalouti et al., 2013). The ephedra plants are valuable due to the presence of ephedrine and pseudoephedrine alkaloids, which are effective in the treatment of headaches, nasal inflammation, common cold, and bronchial asthma. Moreover, these alkaloids serve as marker compounds in the quality control of the ephedra herb (Minami et al., 2021).

The hydroalcoholic extract of *E. pachyclada* from Iran at the dose of 1000 mg/kg was effective in healing artificially induced ulcers in Wistar rats (Pirbalouti et al., 2013). Quinoline-2- carboxylic acid is an active compound isolated from the plant that exhibited potent antidiabetic activity. The compound was found to have significant activity with an IC_{50} of 9.1 ± 2.3 μg/mL and 15.5 ± 1.9 μg/mL in the α-glucosidase and α-amylase inhibitory activity respectively (Lee et al., 2014).

Essential oils are concentrated hydrophobic liquids containing volatile aromatic compounds isolated from various plants. They are composed of complex mixtures of different compounds like alcohols, hydrocarbons, phenols, aldehydes, ketones, esters, etc., (Rassem et al., 2016). Essential oils provide a benign defense mechanism against pests, pathogens and attract pollinators of the plant. Although the pharmacological properties of ephedra plants are due to the ephedrine-like alkaloids, essential oil compounds also exhibit certain medicinal properties. For example, 2,3,5,6-tetramethylpyrazine present in the essential oil of *E. sinica* is a cardiovascular drug (Wang et al., 2006).

Nepal lies in a unique position of the central Himalayan region having varied geography, high variation in altitude, and climate. It bears an excessive floral variety with more than 7000 and 4000 species of flowering and non-flowering plants respectively. Most of the people residing in remote areas depend on medicinal plants for primary health care as well as annual family income (Gurung & Pyakurel, 2017). *Ephedra pachyclada* Boiss is a perennial, dioecious plant distributed in the mountains and alpine dry zones in the Himalayan region between Iran and Nepal (Motomura et al., 2007). In the Mustang district of Nepal, the plant is known as '**Somlata'** that grows with bluish-green tough and cylindrical leaves of 5 – 50 cm height from big rootstalk. The local medical workers called *Amchis* use the plant against different health complications like asthma, blood pressure, eye problems, and gastric disorder (Chhetri et al., 2006). The increasing global demands of herbal products and subsequent uncontrolled exploitation, lack of conservation, and proper documentation have threatened the future of the country's important medicinal plants. The information of the biological activities and chemical constituents of plants is supportive for the identification of sources of important plant materials. It is valuable to the cultivators, producers, policymakers, exporters, processors, and academicians for the development of natural drugs (Kunwar et al., 2011; Shrestha et al., 2015). The present study is aimed at chemical profiling of essential oil, evaluating the phytochemical screening, estimation of total phenolics, total flavonoids, antioxidant, and antimicrobial activities of *E. pachyclada*. To the best of our knowledge, it is the first scientific study on phytochemical and biological activities which might be helpful to ascertain the traditional knowledge of the plant from the remote area of Nepal.

MATERIALS AND METHODS

Collection of plant and preparation of extracts

The aerial parts of the plant were collected from the Thini village of Mustang district in July 2018. The plant was taxonomically authenticated in the National Herbarium and Plant Laboratories, Lalitpur, Nepal (voucher no: L3 (2075). The shade-dried parts were ground into a fine powder using a mechanical grinder. Extraction of phytochemicals was performed by cold-percolation method from different solvents of different polarities namely, methanol, ethyl acetate, dichloromethane, and *n*-hexane separately. The different extracts were concentrated by using a rotary evaporator (IKA) and stored at 4℃ for use.

Isolation and GCMS analysis of essential oil

The essential oil was isolated from the aerial parts of the plant by using a Clevenger's apparatus. The powdered plant material (70 g) was put in a round bottom flask containing 500 mL of distilled water and heated for about 3 hours. A light brown colored oil having a sharp smell was obtained in the Clevenger's apparatus. The oil was collected, dehydrated over anhydrous Na_2SO_4, and stored in an airtight container in the refrigerator at 4℃ (Baharum et al., 2010). The GCMS analysis of the essential oil was performed by using a Shimadzu QP-2010 GCMS system with an AOC-20i autosampler. The sample was injected at 220 ℃, the oven temperature was 80℃ for two minutes, heated at the rate of 6℃/minute up to a constant temperature of 280℃ for 5 minutes. Helium was used as the carrier gas that flowed at a constant rate of 1.03 mL/minutes for an injection volume of 1 μL. The mass spectra were recorded by 70 eV at the scan interval of 0.5 seconds. The fragments with m/z of 50 -500 were analyzed. The compounds in the oil were recognized by comparing the mass spectra with those from the NIST05 library.

Phytochemical screening

The presence of different phytochemicals in the methanol extract of the plant was assessed by standard protocols (Bora et al., 2019; Tiwari et al., 2021). Tests were accomplished for alkaloids, polyphenols, flavonoids, terpenoids, saponins, coumarin, reducing sugars,

glycosides, carotene, tannins, anthracenes, and phytosterols.

Estimation of total phenolic and total flavonoids

The total phenolic content (TPC) in different extracts of the plant was determined by the Folin-Ciocalteu method (Ainsworth & Gillespie, 2007; Pawar & Dasgupta, 2018) with slight modifications. Gallic acid solutions of 20, 40, 50, 60, and 80 µg/mL were prepared from a stock solution of 1 mg/mL. The microplate bores were filled with 20 µL of gallic acid or the extract (5 mg/mL), 100 µL of Folin-Ciocalteu reagent (FCR),1:10 diluted with distilled water, and 80 µL of 1N sodium carbonate in triplicates. Distilled water with FCR and Na_2CO_3 solution was taken as a blank. The mixture was incubated for about 25 minutes in dark at lab temperature and the optical density was recorded at 765 nm using a microplate reader (Bio Tek Multimode reader). The TPC was calculated and expressed as milligrams gallic acid equivalents per gram (mg GAE/g) of the dry plant material from the standard calibration curve.

The total flavonoid content (TFC) in different extracts of the plant was determined by the aluminum chloride colorimetric method (Makhubu et al., 2019; Pawar & Dasgupta, 2018). Quercetin standard solutions of 10, 20, 30, 40, 50, 60, and 80 µg/mL were prepared from the stock solution (1 mg/mL) in methanol. An aliquot of 130 µL of quercetin solution of each concentration, 5 µL of $AlCl_3$, 5 µL of potassium acetate, and 60 µL of ethanol was filled into the wells of 96-well plate microplate reader in triplicates. Similarly, 20 µL of extract (5 mg/mL), 110 µL of distilled water, 5 µL of $AlCl_3$, 5µL of potassium acetate, and 60 µL of ethanol are fed into the wells of microplate reader in triplicates. The mixture was incubated for 30 minutes at dark, and absorbance was taken at 415 nm with a microplate reader against the blank containing all except the quercetin or the extract. The TFC was calculated and expressed as milligrams quercetin equivalent per gram (mg QE/g) of the dry extract from the standard curve.

***In vitro* antioxidant activity**

The extracts of the plant in different solvents were evaluated for their antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay method (Brand-Williams et al., 1995; Khadayat et al., 2020; Liu et al., 2009) with slight modifications. Each of the plant extracts was dissolved in 50% dimethyl sulphoxide (DMSO) to prepare the test solutions of 500, 250, 125, 62.5, 31.25, 15.62, and 7.8 µg/mL. The aliquots of 100 µL of 0.1 mM of DPPH in methanol were added into 100 µL of test solutions in a 96-well plate in triplicates. Ascorbic acid was taken as a positive control. The microplate was incubated for 30 minutes in dark at lab temperature and the absorbance was recorded at 517 nm against blank using a microplate reader. The results were processed by using Gen5 Microplate data collection and analysis software and then by Microsoft Excel 2016. The free radical scavenging activity of the extracts was calculated by using the formula,

$$\text{Percentage scavenging} = \frac{\text{The absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} X\ 100$$

The half-maximal inhibitory concentration (IC_{50}) values were calculated by using the Graph Pad Prism 8 software.

Antimicrobial activity

Microorganisms

The American Type Culture Collection (ATCC) bacteria were used for the test. The pure cultures from the Muller Hinton Agar (MHA) were sub-cultured and stored at 4°C. Both Gram-negative and Gram-positive bacteria were used (Table 1).

Table 1 List of bacterial strains used

Microorganisms	Type	ATCC
Klebsiella pneumonia	Gram-negative	700603
Escherichia coli	Gram-negative	25922
Salmonella typhi	Gram-negative	14028
Staphylococcus aureus	Gram-positive	25923

Agar well diffusion assay

The bacterial susceptibility of the extracts of the plant was assessed by the agar well diffusion method (Murray et al., 2007; Nasir et al., 2015). The bacteria were incubated in Muller Hinton Broth (MHB) overnight to adjust the turbidity equal to 0.5 McFarland's standard. The test bacteria were grown on the surface of a sterile agar medium at 37°C. The wells of 6 mm diameter were bored at equivalent distances on the surface of MHA plates with a sterile cork-borer. The wells were fed with 20 µL of the plant extracts (50 mg/mL) in 30% DMSO, neomycin (1 mg/mL), and the solvent as a negative control carefully, and incubated for 24 hours at 37°C. On the next day, the plates were taken out, and the clear region around the wells that corresponds to the zone of inhibition (ZOI) was measured with a scale and recorded.

RESULTS AND DISCUSSION

Chemical constituents identified by GCMS analysis

The mass spectra data for each peak of GC analysis were compared with the NIST05 library and the components in the essential oil of *E. pachyclada* were identified for each of the chromatograms. The compounds in the essential oil with their retention time and relative percentage are presented in Table 2. The molecular structures of the major compounds are shown in Figure1.

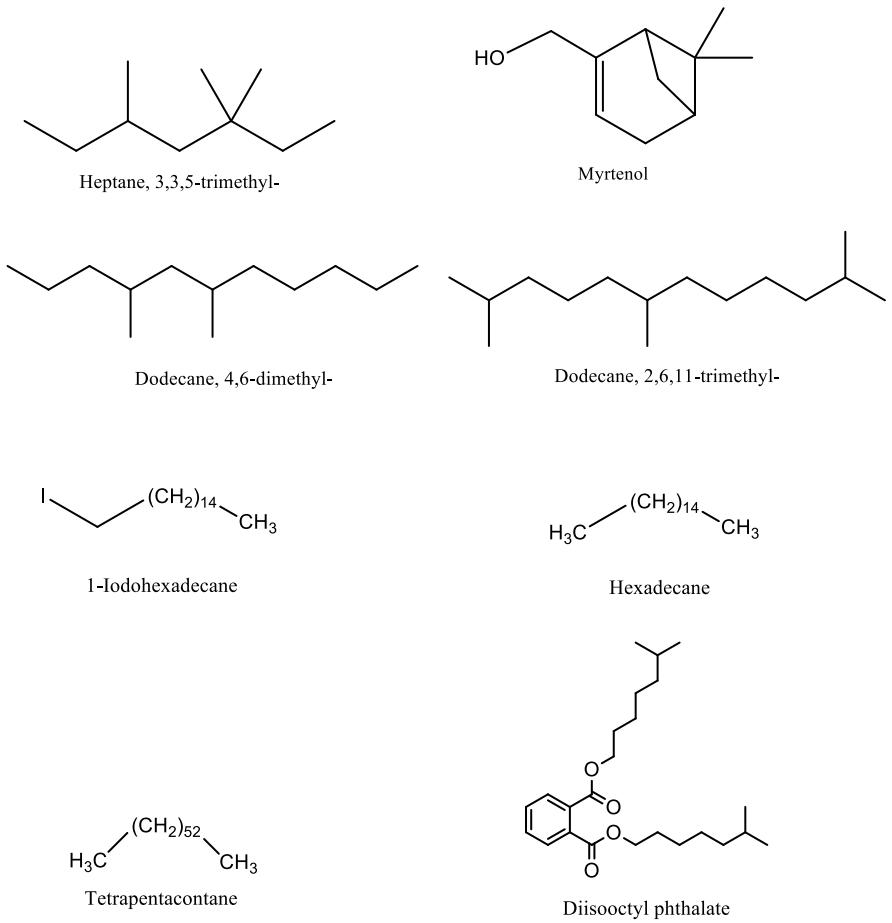


Figure 1 Molecular structures of the identified compounds in the essential oil of *E. pachyclada*

Our study shows that the essential oil contained diisooctyl phthalate (46.90%), dodecane, 2,6,11-trimethyl- (16.35%), dodecane, 4,6-dimethyl- (11.59%), tetrapentacontane (11.56%), and myrtenol (4.37) as the major compounds. We detected ester (46.90%) and hydrocarbons (39.5%) as the major compounds in the essential oil of *E. pachyclada*. Kobaisy et al., (2005) reported the presence of different compounds in the essential oil of three species of ephedra from Italy. *E. distachya* sample contained an ester, ethyl benzoate as a major component (46.9%) together with benzaldehyde (8%), and cis-calamene (3.6%). *E. fragilis* contained (E) phytol (10.1%), pentacosane (5.2%), and 6,10,14-trimethyl-2-pentadecanone (5.3%). Similarly, the oil of *E. major* contained eugenol (4.3%), α-terpineol (3.7%) and methyl linoleate (3.5%) as the main constituents. The essential oil of *E. intermedia* collected from Iran contained 2-ethyl-pyrazine (67.37%), γ-elemene (9.21%), benzyl acetate (9.10%) and 2-methyl-butyl acetate (5.28%) (Ni et al., 2019).

Table 2 List of compounds detected in the essential oil of *E. pachyclada*

Peak	Retention time	Area%	Names/Molecular formulae
1	6.521	2.31	Heptane, 3,3,5-trimethyl-/($C_{10}H_{22}$)
2	8.664	4.37	Bicyclo[3.1.1]hept-2-ene-methanol,6,6-dir (Myrtenol)/ ($C_{10}H_{16}O$)
3	10.347	11.59	Dodecane, 4,6-dimethyl- /$C_{14}H_{30}$
4	18.983	16.35	Dodecane, 2,6,11-trimethyl-/($C_{15}H_{32}$)
5	23.608	2.67	1-Iodohexadecane/$C_{16}H_{33}I$)
6	26.609	4.25	Hexadecane/($C_{16}H_{34}$)
7	29.589	11.56	Tetrapentacontane /($C_{54}H_{110}$)
8	31.690	46.90	1,2-Benzenedicarboxylic acid, diiosoctyl ester/($C_{24}H_{38}O_4$)

Phytochemical screening

The methanol extract of the plant was verified for the presence of different phytochemicals. The extract showed the presence of 11 phytochemicals out of 12 tested. Polyphenols, glycosides, reducing sugars, and phytosterol was found in excess whereas terpenes were absent as shown in Table3. These phytochemicals play a significant role in the biological activities of the plant. Most of the plant species contain a variety of phytochemicals like steroids, terpenoids, flavonoids, carbohydrates, etc. which have diverse physiological activities in the human body and, consequently, they can be used for the development of natural drugs for different health complications (L. Zhang et al., 2020). In addition to ephedrine alkaloids, ephedra plants contain polysaccharides, flavonoids, tannins, and other important phytochemicals. These compounds exhibit antioxidant, antimicrobial, anti-inflammatory, anticancer, and hepatoprotective properties (Zhang et al., 2018).

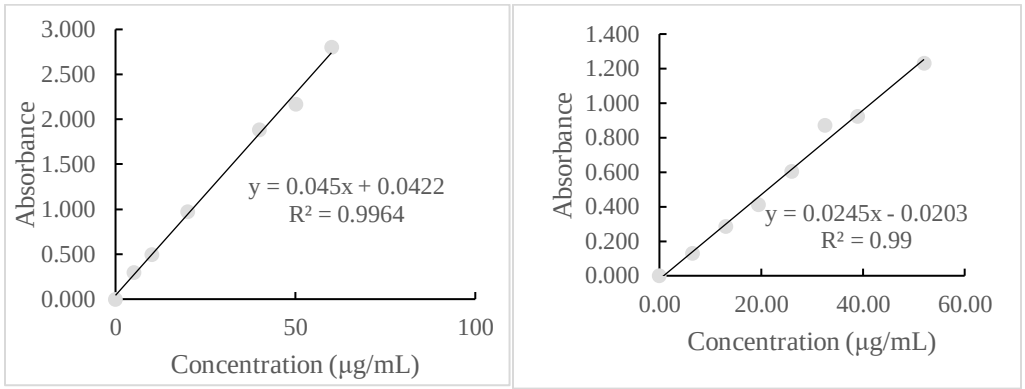
Table 3 Results of phytochemical screening

S. No	Phytochemical	Abundance	S. No	Phytochemical	Abundance
1	Polyphenols	++	7	Tannins	+
2	Alkaloids	++	8	Coumarin	+
3	Glycosides	++	9	Saponins	+
4	Reducing sugars	++	10	Carotene	+
5	Flavonoids	+	11	Phytosterol	++
6	Terpenes	-	12	Anthracene	+

Note: (++) = present, (+) = slightly present, and (-) = absent

Total phenolic and flavonoid contents

The TPC was determined from the linear equation of the standard curve (R^2 = 0.9964) prepared by plotting concentration against absorbance as Figure 2(a). TPC was expressed in terms of mg GAE/g of the dry weight. Similarly, TFC was calculated from the quercetin standard curve (R^2 = 0.99) and expressed as mg QE/g of the dry extract as Figure 2(b). The TPC and TFC of different extracts of *E. pachyclada* are shown in Table 4. Methanol extract shows the maximum phenolic content of 54.42 ± 1.40 mg GAE/g, followed by the ethyl acetate (46.84 ± 0.62 mg GAE/g), and DCM extract (19.58 ± 0.24 mg GAE/g), and the lowest TPC was shown for *n*-hexane extract (5.21 ± 1.40 mg GAE/g) of the dry weight. A similar trend of variation was observed for TFC also. The methanol extract showed the maximum TFC of 33.28 ± 0.48 mg QE/g, followed by ethyl acetate extract (31.73 ± 0.52 mg QE/g), DCM extract (31.64 ± 0.56 mg QE/g), and the least value was obtained for the n-hexane extract (21.44 ± 2.91 mg QE/g).



(a) (b)

Figure 2 (a) Gallic acid calibration curve (b) Quercetin calibration curve

Similarly, the estimation of TPC and TFC of seven ephedra species showed a similar variation to our results. The sample, which contained higher phenolics, was found to contain higher flavonoids also. In the tested species, *E. alata* contained the highest amount of TPC of 53.3 ± 0.1 mg GAE/g and TFC 2.8 ± 0.0 mg QE/g of dry weight (Ibragic & Sofić, 2015). The TPC of methanolic extract and aqueous extracts of *E. alata* were 47.62 and 19.175 mg GAE/g of the dry weight respectively which is comparable to that of our result (Al-Snafi, 2017).

Table 4 TPC and TFC of different extracts of *E. pachyclada*

Extracting solvents	TPC (mg GAE/g)	TFC (mg QE/g)
Dichloromethane	19.58 ± 0.24	31.64 ± 0.56
Ethyl acetate	46.84 ± 0.62	31.73 ± 0.52
n-Hexane	5.21 ± 1.49	21.44 ± 2.91
Methanol	54.42 ± 1.40	33.28 ± 0.48

Note: Vales are mean ± SD, (n = 3)

The TPC and TFC of the extracts of *E. alata* from Palestine prepared using different solvents were dissimilar. The extract of 80% methanol was reported the (TPC = 101.2 ± 0.9 mg/g, TFC = 9.8 ± 0.5 mg/g), 100% ethanol extract (TPC = 40.9 ± 0.2 mg/g, TFC = 19.5 ± 0.3 mg/g), and the water extract (TPC = 30.9 ± 0.5 mg/g, TFC = 4.2 ± 0.1 mg/g) (Al-Rimawi et al., 2017). These extracts contained the nearly same quantity of phenolics and lower flavonoid contents than our results. The polar solvents were more effective for the extraction of phenolic and flavonoids that are also polar, and the amount of the phytochemicals varies with species, collected part, solvents, geographical location, season, maturity of the plant, etc. The TPC of the methanolic extracts of the wild and callus culture of *E. pachyclada* was evaluated by the Folin-Ciocalteu reagent method. The wild sample was found to contain 454.5 ± 62.24 µmol eq catechin/g of dry material which is higher than that of callus culture with 108.77 ± 17.15 µmol eq catechin/g of dry material (Parsaeimehr et al., 2010).

In vitro antioxidant activity

The plant was found to exhibit a moderate antioxidant activity on examination with DPPH radical scavenging assay. Only the methanol extract showed the highest antioxidant activity with a half-maximal inhibitory concentration (IC_{50}) of 37.81 ± 2.24 µg/mL, while the others exhibited low activity as shown in Table 5. Ascorbic acid was taken as a positive control had the highest activity with an IC_{50} value of 6.39 ± 0.29 µg/mL. The extracts of *n*-hexane, DCM, and ethyl acetate showed weak scavenging power in comparison to that of methanol extract.

Table 5 DPPH radical scavenging (IC_{50} values) of different extracts of *E. pachyclada*

S. No	Extracts	IC_{50} (µg/mL)
1	Methanol extract	37.81 ± 2.24
2	Ethyl acetate extract	230.30 ± 4.75
3	Dichloromethane extract	249.97 ± 17.65
4	*n*-Hexane extract	278.93 ± 23.52
5	*Ascorbic acid	6.39 ± 0.29

Note: Values are mean ± SD, (n = 3), * Positive control

Our result agrees with the result obtained by Ghasemi et al., (2014). The aqueous extract of the plant showed significant antioxidant activity with an IC_{50} value of 55.53 ± 0.05 µg/mL. Similarly, the ethanol extract of *E. procera* from Iran also exhibited a substantial activity on DPPH scavenging assay with an IC_{50} of 56 µg/mL (Dehkordi et al., 2015). The antioxidant activity of different extracts was correlated with the polarity of the extracting solvent. The polar solvent, methanol can pick up the polar phenolic and flavonoid compounds showed relatively higher radical scavenging activity than the extracts of less polar solvents. The antioxidant activity of plants can be enzymatic and nonenzymatic. The enzymes and different compounds as well as polar secondary metabolites synergically comport the antioxidant defense system in the plants (Pisoschi et al., 2016). The plot of percentage scavenging activity of different extracts and ascorbic acid shows a proportionate variation with concentration in Figure 3.

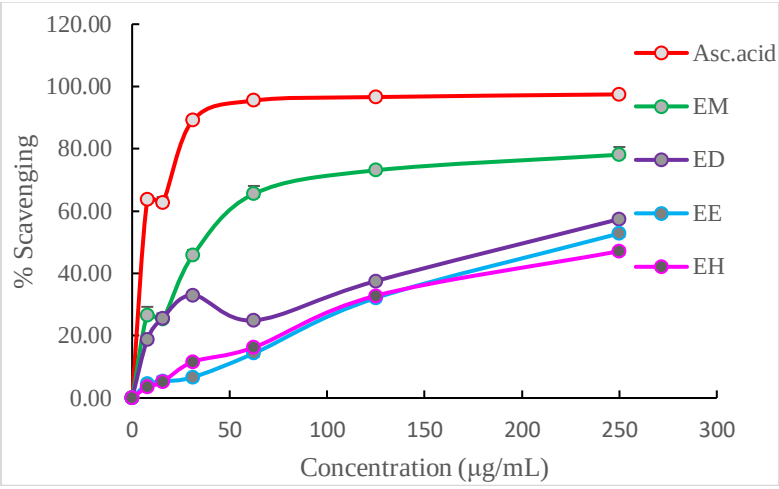


Figure 3 Percentage inhibition of ascorbic acid and different extracts of *E. pachyclada* (Note: EM= methanol extract, ED= DCM extract, EE= ethyl acetate extract, EH= n- hexane extract)

The antioxidant power of phenolic compounds depends on the structure of the aromatic ring, position, and number of -OH groups. The OH- group forms the free radical which is resonance stabilized by the aromatic ring of the molecule (Zeb, 2020). Flavonoids transfer electrons or hydrogen atoms to reactive oxygen and nitrogen species, chelate metal catalysts that generate free radicals, activate antioxidant enzymes as well as inhibit the actions of free radical generating enzymes (D'Amelia et al., 2018).

Antibacterial activity

The *E. pachyclada* plant collected from the Mustang district of Nepal exhibited a mild antibacterial activity towards some of the bacterial strains on the Agar well diffusion method. The Figure 4 shows that the plant was found effective against the two bacteria only. The methanol extract showed potent activity against *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) with the zone of inhibitions of 13 and 12 mm respectively. Ethyl acetate extract showed a slight potency against *Staphylococcus aureus* (ATCC 25923) with a zone of inhibition of 9 mm as shown in Table 6.

Table 6 Results of antibacterial test of different extracts of *E. pachyclada*

Microorganisms	Zone of Inhibition (ZOI) in mm				
	n-hexane extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	*Neomycin
Klebsiella pneumonia	-	-	-	-	15
Escherichia coli	-	-	-	13	15
Salmonella typhi	-	-	-	-	16
Staphylococcus aureus	-	-	9	12	17

Note: *Positive control (1mg/mL), concentration of the extracts = 50 mg/mL

Our result is in the agreement with the antibacterial activities exhibited by the plant against different nosocomial bacteria. The methanol extract at different concentrations was active against *Escherichia coli* (PTCC 0157), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (PTCC 1053), *Serratia marcesens* (PTCC 1111), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella dysenteriae* (PTCC 1118) (Dosari et al., 2016). The quinaldic acid isolated from the plant exhibited potent activity against *C. difficile* ATCC 9689, and *Clostridium perfringens,* and but was inactive against the tested *Lactobacillus acidophilus* (ATCC 4356), *Bifidobacterium bifidum* (ATCC 29521), and *L. casei* (ATCC 393) (C.-H. Lee & Lee, 2009). A. Parsaeimehr et al., (2010), evaluated the antimicrobial activity of methanol extracts of the wild and callus cultures of the plants from Iran. The test was performed against five Gram-negative, three Gram-positive, and two fungal strains by the disc diffusion method. *Pseudomonas aeruginosa* (PTCC 1074), *Staphylococcus aureus* (PTCC 1112), and *Candida albicans* (PTCC 5027) showed the maximum activity at the concentrations of 0.5 to 4 mg/mL.

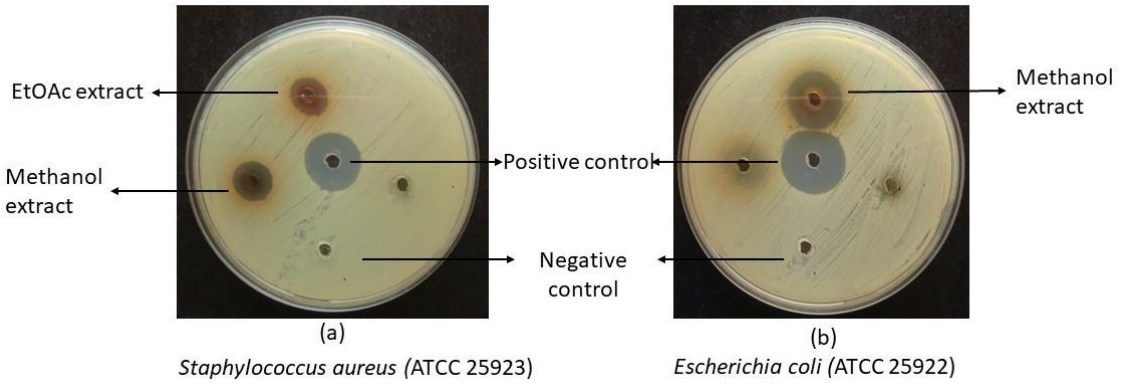


Figure 4 Antibacterial test slides of *E. pachyclada* extracts

CONCLUSIONS

This research attempts a comprehensive study of the phytochemical, antioxidant, and antimicrobial activities of *E. pachyclada* collected from the Mustang district of Nepal for the first time. The essential oil of the plant contained diisooctyl phthalate, dodecane, 2,6,11-trimethyl-, dodecane, 4,6-dimethyl-, tetrapentacontane, and myrtenol as the major compounds. The methanol extract of the plant which contained a relatively higher proportion of TPC and TFC exhibited higher antioxidant activities in comparison to other extracts. It exhibited a significant antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli*. The ethyl acetate extract showed moderate activity against *Staphylococcus aureus* whereas dichloromethane and *n*-hexane extracts were inert towards the tested bacteria. Our study highlights the use of suitable solvents for the extraction of phytoconstituents that exhibit better pharmacological activity. Our results indicated the possibility of isolating important bioactive molecules that could be developed for natural medicines. This study authenticates the traditional use of the plant by the local people against different ailments.

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AUTHOR CONTRIBUTIONS

SKK conceptualized the research project; LNK performed the experiments, analyzed the data, and prepared the manuscript. YRP and KRS. reviewed the work and the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

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

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