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Evaluation of antioxidant, antimicrobial, and lethality Activities of leaf and bark extract of *Alnus nepalensis* D. Don

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Abstract

Herbal medicine has been used for centuries to treat both minor and life-threatening illnesses. This study aims to evaluate the total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), antioxidant potential, and various biological activities of Alnus nepalensis D. Don (Himalayan alder) extracts. Among the tested extracts, the ethanolic bark extract exhibited the highest TPC (300.6 ± 3.12 mg GAE/g) and TTC (120.09 ± 3.15 mg TA/g), while the methanolic leaf extract had the highest TFC (137.21 ± 4.67 mg QE/g). In terms of antioxidant activity, the ethanolic bark extract showed the lowest IC_{50} value (17.55 ± 1.17 µg/mL), followed by the methanolic leaf extract (IC_{50} = 24.03 ± 1.59 µg/mL), with quercetin as the standard (IC_{50} = 3.43 ± 1.61 µg/mL). For antimicrobial activity, the ethanolic bark extract exhibited significant inhibition against Escherichia coli (20 mm) and Klebsiella pneumoniae (19 mm), comparable to the positive control neomycin (28 mm). Notably, the dichloromethane (DCM) bark extract demonstrated the highest zone of inhibition (21 mm) against E. coli. The methanolic leaf extract exhibited a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 0.78 mg/mL and 1.56 mg/mL, respectively, against K. pneumoniae, while the positive control showed MIC = 0.003 mg/mL and MBC = 0.007 mg/mL. In cytotoxicity assessment, the ethanolic bark extract had an LC_{50} value of 80.08 µg/mL, whereas the methanolic leaf extract had an LC_{50} of 468.81 µg/mL. These findings highlight the medicinal potential of Alnus nepalensis, suggesting its potential use as a source of bioactive compounds for developing novel therapeutic agents against infectious diseases.

Keywords

Alnus nepalensis D. Don, Antimicrobial activity, Cytotoxicity, Minimum Bactericidal Concentration (MBC), Minimum Inhibitory Concentration (MIC), Phytochemistry

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1 Introduction

Natural materials, including different parts of plants, animals, and microorganisms, have been used in medicine to heal illnesses since ancient times. Fossil evidence suggests that humans have been using plants as medicine for at least 60,000 years [1]. The bioactive compounds naturally found in medicinal plants help to form various kinds of medicines, dietary supplements, and functional foods [2]. The broad therapeutic application range comprises, ease of accessibility, and safer side effects, natural products are extensively used as active ingredients for drug synthesis [3]. Antioxidant, anticancer, antibacterial, antiviral, and anti-inflammatory properties have been shown by medicinal plants. Natural phytochemicals known as flavonoids and other phenolic compounds are present in the leaves, fruits, barks, stems, and roots of medicinal plants. These properties have led to the use of flavonoids and other phenolic compounds in medicine for a variety of conditions [4]. Oxidative stress, which is caused by oxygen-free radicals, is the primary cause of several degenerative illnesses, such as cancer, gastric ulcers, and atherosclerosis. Medicinal herbs are rich in antioxidants that actively scavenge oxygen [5]. The phytochemicals contained in medicinal plants, such as phenolics, flavonoids, anthocyanins, terpenoids, and tannins, enable the prevention and treatment of disease. Many therapeutic plants are excellent providers of natural antimicrobial agents, offering viable substitutes for bacterial infection treatments of disease [6]. The usage of dietary or pharmaceutical supplements was influenced by an imbalance between reactive oxygen species (ROS) and the body's natural antioxidant capacity, especially during the illness assault [7].

The chemical constituents found in medicinal plants are thought to be antibiotic, antifungal, antiviral, and shielded plants from infections [8]. Primary metabolites are necessary to perform various biological activities such as translocation, photosynthesis, and respiration. Secondary metabolites are those that have biological significance but are not necessary for survival. The production of primary metabolites involves several chemical processes, including methylation, glycosylation, and hydroxylation. Secondary metabolites undoubtedly have more complicated side chains and structural compositions [9, 10].

Phenolic compounds are a large family of secondary metabolites that are primarily present in many higher plant parts including fruits, bark, leaves, and seeds, organs, and microorganisms. Phenolic substances exhibit important biological characteristics that include immunemodulating, antibacterial, anticarcinogenic, antithrombotic, anti-inflammatory, antiulcer, antiartherogenic, antiallergenic, antioxidant, and analgesic effects [11]. Phenolic compounds reduce FC reagents and produce a molybdenum-tungsten blue complex [12].

The fundamental structure of flavonoids is a subclass of phenolic compounds involved in the production of plant colour in flowers, the ability to resist disease, prevent ultraviolet damage, and influence the formation of legume root nodules [13]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) in biological systems, such as superoxide, hydroxyl, and nitric oxide radicals, may oxidize lipids and proteins in cells and damage DNA [14–16]. Antioxidants can lessen oxidative stress, antioxidants have been shown in several studies crucial for preserving human health as well as for diagnosing, treating, and preventing disease. Antioxidants are substances that, in very small amounts found in either naturally occurring food or the human body that delay, regulate, or stop oxidative processes. The process of preventing these antioxidant molecules from oxidizing involves a variety of techniques and actions [17]. The natural antioxidant system found in the human body may scavenge produced radicals by maintaining the equilibrium between oxidation and anti-oxidation. However, exposure to radiation, alcohol, tobacco smoke, or environmental contaminants causes an overabundance of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which disrupts the equilibrium between oxidation and anti-oxidation and causes many degenerative and chronic illnesses [18, 19]. *Alnus nepalensis* D. Don is commonly known as Nepalese alder which belongs to the Betulaceae family that found in gully terrace forests or river beach wetlands throughout a wide range of elevations between around 700-3600 meters [20] and is historically used to treat wounds, cuts, diarrhoea, and dysentery [21].

The analysis of phytochemicals and evaluation of biological properties of bark and leaf extracts such as antioxidant, antimicrobial, and toxicity against brine shrimp nauplii have not been well reported yet. So, the proposed research work plays a significant role in fulfilling the research gap. The major phytoconstituents which show richness reported from the genus *Alnus* are shown in Figure 1.

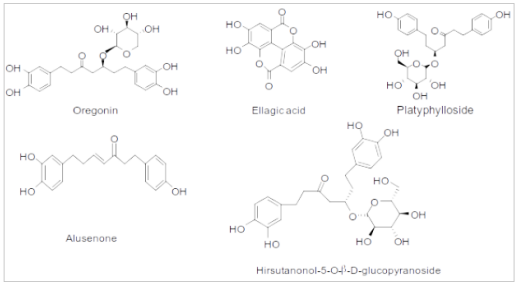

Figure 1: Chemical compounds reported from *Alnus nepalensis* D. Don and *Alnus nitida* D. Don

2 Materials and Methods

2.1 Chemicals

Merck and Scientific Fischer provided analytical grade (extra pure) solvents, such as methanol, ethanol, ethyl acetate, dichloromethane, and hexane. EDTA disodium salt dihydrate was purchased from SRL, and boric acid, calcium chloride fused, and sodium chloride were purchased from Merck. FC reagent and Resazurin (LOBA CHEMI Pvt. Ltd), Mueller Hinton Broth, Nutrient Agar, and Mueller Hinton Agar media were purchased from HieMedia.

2.2 Collection and Identification of the Plant

The bark and leaf parts of *A. nepalensis* D. Don medicinal plant were collected from the Far Western region of Nepal (Doti). The local name, scientific name, parts of medicinal plants used, and their ethnomedical usage are shown in Table 1. The taxonomic identification of *A. nepalensis* D. Don medicinal plant was conducted by National Herbarium and Plant Laboratories, Godawari, and research officers of the herbarium center provided the voucher code 01KATH163159. The Photographs of the selected medicinal plant and the study area are shown in Figure 2 and Figure 3.

Figure 2: *Alnus nepalensis* D. Don.

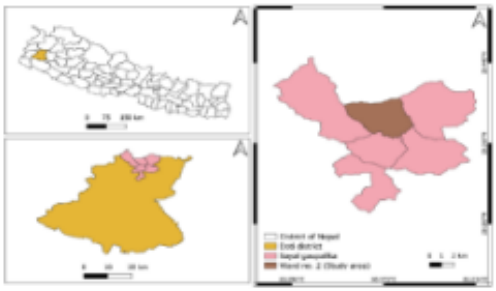

Figure 3: Sample collected cite.

Table 1: Description of *A. nepalensis* D. Don

Scientific Name	Local Name	Family	Parts Used	Traditional Uses	Reference
A. nepalensis D. Don	Utis	Betulaceae	Leaf, Bark	The plant parts have been used to treat wound healing, against dysentery, diarrhea, inflammation, and headaches.	[21]

2.3 Preparation of Extract

The bark and leaves parts of *A. nepalensis* D. Don were washed thoroughly, shade dried, ground into finely divided powder, and immersed in six different solvents, ranging in polarity from more polar to less polar they are; water > methanol > ethanol > ethyl acetate, DCM > hexane. Afterward, each filtrate was dried in a water bath that was kept at a temperature of up to 40 ^{o}C. Finally, they were collected in glass vials and kept at 4 ^{o}C for further analysis. The yield percentage was calculated by using the formula:

$$\text{Yield percentage of crude extract} = \frac{\text{Weight of dried crude extract}}{\text{Weight of sample}} \times 100 \quad (1)$$

2.4 Qualitative Phytochemical Analysis

The phytochemicals present in the crude plant extracts were qualitatively analyzed by following standard protocol [22–24]. The numerous phytochemicals, including glycosides, flavonoids, alkaloids, phenolic compounds, terpenoids, steroids, carbohydrates, saponins, tannins, fixed oils, and lipids, were screened as metabolites.

2.5 Estimation of Total Phenolic Content (TPC)

The Folin-Ciocalteu colorimetric method was used to estimate the total phenolic content of plant extracts as described by Lu et al. [25]. 20 μL of plant extract, 100 μL of 10 % FC reagent (1:10), and 80 μL of 1M Na_2CO_3 were loaded in a 96-well plates in triplicates. The reaction mixture was allowed to in-

cubate at room temperature for half an hour before a deep blue colour was observed. Ultimately, a spectrophotometer was used to measure absorbance at 765 nm. The standard curve was generated which is the standard curve for gallic acid (7.5-100 μg/mL) and the total phenolic content (TPC) was measured in milligrams of gallic acid equivalent (mg GAE/g) per gram of extract dry weight.

2.6 Estimation of Total Flavonoid Content (TFC)

The total flavonoid content was estimated by using aluminium chloride method as described by Ahmed et al. [26]. 20 μL of plant extract, 100 μL of distilled water, and 60 μL ethanol followed by 10 μL of 10% $AlCl_3$ solution and 10 μL (1M) CH_3COOK solution were loaded in 96-well plates in triplicates. The reaction mixture was incubated for 30 minutes at room temperature. Then, absorbance was taken at 415 nm with the help of a spectrophotometer. A quercetin (10-100 μg/mL) standard calibration curve was created, and measured in milligrams of quercetin equivalent per gram of the extract's dry weight (mg QE/g).

2.7 Estimation of Total Tannin Content (TTC)

The Folin-Ciocalteu colorimetric method was used to estimate the total tannin content by applying standard protocol [25]. 96-well plates were filled with 10 μL of plant extract and different tannic acid concentrations (7.5-100 μg/mL). The first reading was taken at 725 nm using a microplate reader after adding 70 μL of distilled water and 50 μL of 10% FC reagent. After taking an initial reading, 70 μL of 35 % Na_2CO_3 was loaded. Finally, the 96-well plate was incubated for 30 minutes and consequently, its final absorbance was taken at 725 nm. The TTC was measured as mg TA/g.

Calibration curves were created by plotting the graph of absorbance on the y-axis and the standard concentration on the x-axis. These curves were then used to calculate the concentrations of phenolics, flavonoids, and tannins. R^2, the coefficient of determination, was determined to be between 0.9753 and 0.9997 when the data were fitted linearly.

2.8 Evaluation of Antioxidant Activity

The antioxidant activity of crude plant extract was evaluated by following a standard protocol [27, 28]. The crude plant extract was diluted serially up to 5 μg/mL from 160 μg/mL concentrations but positive control quercetin was serially diluted up to 0.625 μg/mL from 20 μg/mL concentrations. 100 μL of plant extracts and positive control were loaded in 96-well plates in triplicates. After that, the initial reading was taken at 517 nm. Following this, 100 μL of DPPH reagent was loaded into every well and incubated for half an hour. Finally, the final absorbance was taken at 517 nm. Since 50% DMSO and methanol were used as a negative control.

The following formula was used to evaluate radical scavenging activity:

$$\text{Radical scavenging capacity} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100 \quad (2)$$

Where, A_{control} = absorbance of control, A_{sample} = absorbance of sample.

Inhibitory concentration (IC_{50}) was calculated by using GraphPad Prism (version 8.0.2.263).

2.9 Evaluation of Antimicrobial Activity

The agar well diffusion method was applied to perform an antibacterial activity using Mueller Hinton Agar (MHA) plates [29–31]. Mueller Hinton Broth (MHB) was used for the growth of test microorganisms, ATCC 25931 *Shigella sonnei*, ATCC 43300 *Staphylococcus aureus*, ATCC 700603 *Klebsiella pneumonae* and ATCC 25312 *Escherichia coli* respectively and it was incubated at 37 ^{o}C for 24 hrs and its turbidity was maintained by using 0.5 McFarland. 50 μL of plant extract, 50% DMSO as the negative control, and 50% neomycin as the positive control were loaded into each well created by a cork borer. The Petri dishes were then incubated for 18-24 hours at 37 °C after being left for 15 minutes to allow for diffusion. After incubation, the zone of clearance was observed and measured.

2.10 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration were determined by following the standard protocol as described by Sarker et al. [32]. The 0.5 McFarland turbidity culture in MHB was diluted 1:100 to give the bacterial inoculum a final concentration of 106 CFU/mL. 5 μL of bacteria were injected into every well of 96 well plates. A common medication called neomycin served as the positive control. The plate was covered with a sterile lid and incubated for 20-24 hrs at 37 °C. The microtiter plate wells were filled with 0.003% resazurin, and the mixture was incubated for 3 to 4 hours at 37 °C. The colour of the wells with bacterial growth changed to pink, whereas the wells without infection stayed blue. The extract's minimum inhibitory concentration (MIC) was found to be the lowest at which bacterial growth is inhibited. By streaking the contents

of the wells onto nutrient agar plates and incubating them at 37 °C for more than 18 hours consequently, the MIC and MBC of the crude plant extracts were determined.

2.11 Brine Shrimp Lethality Activity (BSLA)

The toxicity of plant extracts was determined by following a standard protocol [33]. The artificial sea salt water was prepared by maintaining a pH of around 8 to 8.5 by adding 1M NaOH. The plant extract was diluted in various concentrations such as 1000, 800, 500, 250, 125, 100, and 10 μg/mL. Then, 4 mL of artificial sea salt water was filled in each test tube. After that, 500 μL of sample, and 10 nauplii were added to each test tube in triplicate. Artificial sea salt water served as the negative control, while potassium dichromate solution was employed as the positive control. The number of dead nauplii in a test tube was counted after 24 hours, and the mortality of nauplii percentage was determined using the following formula:

$$\% \text{ mortality} = \frac{\text{Number of dead nauplii}}{\text{Total number of nauplii}} \times 100$$

Using the Probit value table, the linear equation can be obtained as $Y = mx + c$, where Y is the Probit value at 50% mortality, m is variable, and c is the intercept. x is the lethal concentration and finally, the lethal concentration (LC_{50}) was calculated.

2.12 Statistical Analysis

The Gen5 Microplate reader for Data Collection and Analysis software was used for result processing, followed by Microsoft Excel. The data were reported for TPC, TFC, and TTC as the mean ± standard deviation. For antioxidants, the data were reported as mean ± standard error mean. Inhibitory concentration (IC_{50}) was calculated by using GraphPad Prism (version 8.0.2.263). One-way ANOVA using Tukey's test was used for comparisons, $P < 0.05$ values were regarded as statistically significant.

3 Results

3.1 Percentage Yield

The yield percentage of crude extracts of bark was found to be higher in methanolic extract (19.17 %) followed by ethanol (9.23 %), ethyl acetate (3.56 %), hexane (2.81 %), aqueous (2.80 %), and DCM (2.45 %). Similarly, In the case of leaves, methanolic extract had a higher percentage of yield (10.78 %) followed by ethyl acetate (10.18 %), DCM (8.92 %), ethanol (5.82 %), and hexane (5.42 %) (Figure 4).

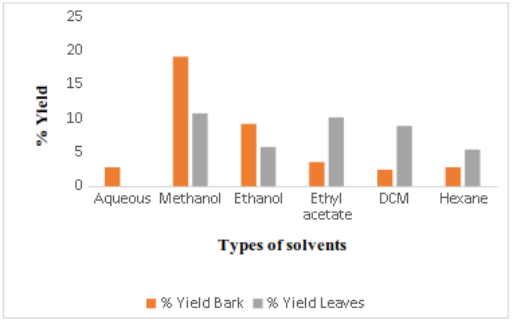

Figure 4: Yield percentage of bark and leaf crude extracts

3.2 Qualitative Phytochemical Analysis

The results of qualitative phytochemical screening of the bark and leaf extract (in different solvents) are shown in Table 2.

Table 2: Qualitative phytochemical screening of plant extracts.

Phytochemicals	Test	Bark extract	Leaf extract
Alkaloids	Dragendorff's test	-	-
Carbohydrates	Molish's test	+	+
Reducing sugars	Fehling's test	-	-
Glycosides	Borntrager's test	+	+
Amino acids	Xanthoproteic test	+	+
Flavonoids	Alkaline reagent test	+	+
Phenols	$FeCl_3$ test	+	+
Tannins	Braymer's test	+	+
Terpenoids	Salkowski's test	+	+
Anthraquinones	Borntrager's test	+	+
Phytosterols	Salkowski's test	+	+

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

3.3 Total Phenolic Content (TPC)

The ethanolic bark extract exhibited the maximum TPC of 300.6 ± 3.12 mg GAE/g. The TPC of hexane bark extract was found to be the lowest which is 53.67 ± 3.59 mg GAE/g. The TPC of bark extracts ranges from 300.6 ± 3.12 mg GAE/g to 53.67 ± 3.58 mg GAE/g (Table 3). The TPC of leaf extracts ranges from 254.6 ± 5.58 mg GAE/g to 73.93 ± 3.63 mg GAE/g. Methanolic leaf extract had the highest TPC which is 254.6 ± 5.38 mg GAE/g and hexane extract of the leaf had the lowest TPC which is 73.93 ± 3.63 mg GAE/g respectively. Ethanolic bark extract had the highest TPC than of methanolic leaf extract. The calibration curve is shown in Figure 5.

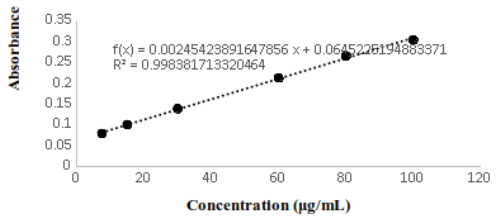

Figure 5: Gallic acid standard calibration curve.

Table 3: TPC for various solvent extracts

Extraction sources	Solvent type	Total phenolic content (mg GAE/g)
Bark	Aqueous	101.8 ± 3.41
Bark	Methanol	292.2 ± 1.74
Bark	Ethanol	300.6 ± 3.12
Bark	Ethyl acetate	124.47 ± 4.20
Bark	DCM	121.53 ± 1.22
Bark	Hexane	53.67 ± 3.59
Leaves	Methanol	254.6 ± 5.58
Leaves	Ethanol	152.47 ± 2.89
Leaves	Ethyl acetate	201.13 ± 3.45
Leaves	DCM	88.47 ± 2.57
Leaves	Hexane	73.93 ± 3.63

3.4 Total Flavonoid Content (TFC)

DCM extract of bark had the highest TFC which is 75.99 ± 4.72 mg QE/g and hexane extract had the lowest TFC which was 50.09 ± 6.31 mg QE/g. TFC of bark extract ranges from 75.99 ± 4.72 mg QE/g to 50.09 ± 6.31 mg QE/g (Table 4). The methanol leaf extract had the highest TFC which is 137.21 ± 4.67 mg QE/g and hexane extract had the lowest TFC 13.12 ± 1.84 mg QE/g. The TFC of leaf extract ranged from 137.21 ± 4.67 mg QE/g to 13.12 ± 1.84 mg QE/g. Among all the extracts, the methanolic leaves extract had the highest TFC, which is 137.21 ± 4.67 mg QE/g than that of bark extracts. The calibration curve is shown in Figure 6.

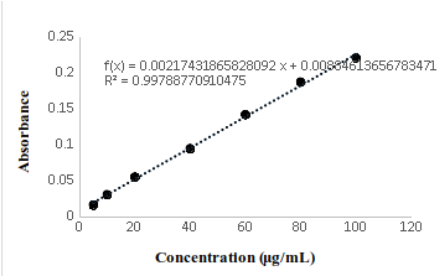

Figure 6: Quercetin standard calibration curve.

Table 4: TFC of bark and leaf extracts

Extraction sources	Solvent types	Total flavonoid content (mg QE/g)
Bark	Aqueous	62.97 ± 1.84
Bark	Methanol	60.40 ± 2.78
Bark	Ethanol	65.24 ± 2.24
Bark	Ethyl acetate	61.76 ± 1.39
Bark	DCM	75.99 ± 4.72
Bark	Hexane	50.09 ± 6.31
Leaves	Methanol	137.21 ± 4.67
Leaves	Ethanol	104.33 ± 1.39
Leaves	Ethyl acetate	111.15 ± 3.87
Leaves	DCM	35.24 ± 2.24
Leaves	Hexane	13.12 ± 1.84

3.5 Total Tannin Content (TTC)

The maximum TTC was found to be 120.09 ± 3.15 mg TA/g, in the ethanolic bark extract. Hexane extract of bark had the lowest TTC which is 8.27 ± 0.45 mg TA/g. The TTC of bark extract ranged from 120.09 ± 3.15 mg TA/g to 8.27 ± 0.45 mg TA/g. The TTC of the methanolic leaf extract was found to be 43.72 ± 3.96 mg TA/g, whereas the lowest TTC was found in ethyl acetate leaf extract which is 8.42 ± 1.31 mg TA/g (Table 5). The TTC ranges from 43.72 ± 3.96 mg TA/g to 8.42 ± 1.31 mg TA/g in leaf extracts. Among all solvent extracts, ethanolic bark extract had the highest TTC, and hexane extract of bark had the lowest TTC. The calibration curve is shown in Figure 7.

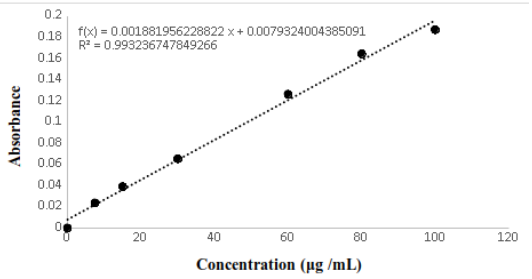

Figure 7: Tannic acid standard calibration curve

Table 5: Total tannin content of bark and leaf extracts.

Extraction sources	Solvent type	Total tannin content (mg TA/g)
Bark	Aqueous	74.03 ± 5
Bark	Methanol	86.15 ± 4.73
Bark	Ethanol	120.09 ± 3.15
Bark	Ethyl acetate	28.88 ± 3.03
Bark	DCM	71 ± 6.01
Bark	Hexane	8.27 ± 0.45
Leaves	Methanol	43.73 ± 3.96
Leaves	Ethanol	28.42 ± 1.72
Leaves	Ethyl acetate	8.42 ± 1.31
Leaves	DCM	15.85 ± 0.69
Leaves	Hexane	9.79 ± 0.95

3.6 Antioxidant Potential

Hexane and ethyl acetate extracts had the highest IC_{50} which is > 500 μg/mL and 247.9 ± 1.73 μg/mL respectively while ethanolic extract had the lowest which is 17.55 ± 1.17 μg/mL (Figure 8). The ethanolic extract had more antioxidant potential than all crude bark extracts. The IC_{50} of the standard quercetin compound was found to be 3.43 ± 1.61 μg/mL. Which is five times less than ethanolic extract. The methanolic extract of the leaf had the lowest IC_{50} 24.03 ± 1.59 μg/mL among all leaf extracts. However, the hexane and ethyl acetate extracts of the leaf exhibited the highest IC_{50} which are > 500 μg/mL and 464.7 ± 0.22 μg/mL respectively. The methanolic leaf extract had greater antioxidant potential and the hexane extract had the lowest antioxidant potential. The IC_{50} of methanolic leaf extract was eight times greater than the IC_{50} of standard quercetin. Compared to the crude leaf

extracts, the ethanolic extract of bark had the highest antioxidant potential. The percent inhibition against the different concentrations of the plant extract is shown in Figure 13.

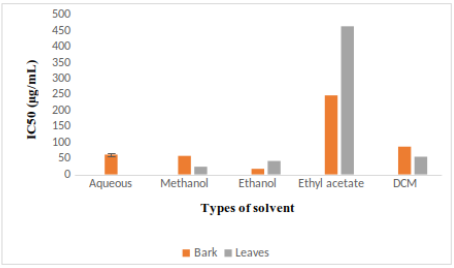

Figure 8: Bar diagram showing antioxidant potential (IC_{50}) of bark and leaf crude extracts in different solvents at various concentrations.

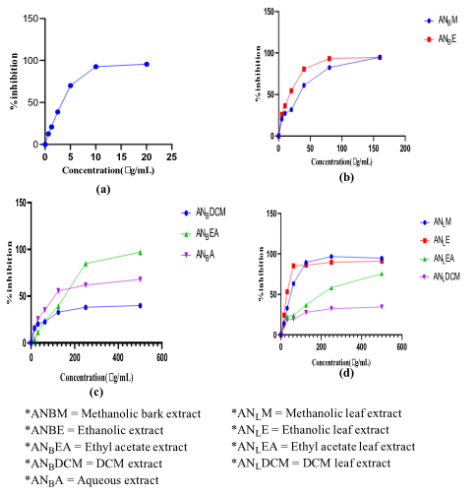

Figure 9: A plot of % inhibition against the concentration of (a) quercetin (b) methanolic and ethanolic bark extracts (c) DCM, ethyl acetate & aqueous bark extracts (d) methanol, ethanol, ethyl acetate, and DCM leaf extracts

The IC_{50} values of various extracts of bark and leaf and standard quercetin as a reference compound are shown in Table 6.

Table 6: The antioxidant capacity (IC_{50}) of the aqueous, methanol, ethanol, ethyl acetate, DCM, and hexane crude extracts of bark and leaf.

Part	Plant extracts	IC_{50} (μg/mL)
Bark	Aqueous	62.16 ± 3.66
Bark	Methanol	58.06 ± 0.22
Bark	Ethanol	17.55 ± 1.17
Bark	Ethyl acetate	247.9 ± 1.73
Bark	DCM	87.16 ± 0.87
Bark	Hexane	> 500
Bark	*Quercetin	3.43 ± 1.61
Leaf	Methanol	24.03 ± 1.59
Leaf	Ethanol	42.07 ± 2.17
Leaf	Ethyl acetate	464.7 ± 0.22
Leaf	DCM	55.44 ± 1.70
Leaf	Hexane	> 500
Leaf	*Quercetin	3.43 ± 1.61

Note: - *Quercetin = positive control

3.7 Antimicrobial Activity

The antimicrobial activity in terms of the zone of inhibition of various crude extracts of bark and leaf against *K. pneumoniae*, *E. coli*, *S. sonnei*, and *S. aureus* respectively are shown in Table 7. The ethanolic bark extract had a strong antibacterial activity with ZOI of 20 mm, 15 mm, 14 mm, and 19 mm respectively against *Escherichia coli*, *Shigella sonnei*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*. This was quite close to the positive control, namely Neomycin 24 mm for *Staphylococcus aureus* and *Shigella sonnei* and 28 mm for *E. coli* and *Klebsiella pneumoniae* respectively. Among all extracts of bark, DCM extract shows very potent activity against *E. coli* with ZOI 21 mm (Neomycin control 28 mm). However, hexane extract had no zone of inhibition against all four bacteria which is 9 mm. This was far from the ZOI of positive control which are 25 mm for *Klebsiella pneumoniae*, 22 mm for *E. coli*, 20 mm for *Shigella sonnei*, and 24 mm for *Staphylococcus aureus* respectively.

The methanolic, ethanolic, and ethyl acetate

extracts of leaves show good antibacterial activity against *Klebsiella pneumoniae*, *E. coli*, and *Shigella sonnei* with ZOI 21, 11, and 15 mm respectively (Neomycin positive control 25 mm and 24 mm). The DCM and hexane extracts of leaves had less potent antimicrobial activity against *Klebsiella pneumoniae*, *E. coli*, *Shigella sonnei*, and *Staphylococcus aureus* respectively. The ZOI shown by the bark and leaf extracts of the plant is shown in Figures Figure 10 and Figure 11.

3.8 MIC and MBC

The MIC and MBC of ethanolic bark and methanolic leaf extracts are illustrated in Table 8. The methanolic leaf extract and the ethanolic bark extract were employed for the evaluation of MIC and MBC against *Klebsiella pneumoniae* and *Staphylococcus aureus*, respectively. The ethanolic bark extract shows minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Klebsiella pneumoniae*, which are 3.12 mg/mL and 1.56 mg/mL, respectively. The MBC of ethanolic bark extract against *Klebsiella pneumoniae* and *Staphylococcus aureus* were found to be 3.12 mg/mL and 6.25 mg/mL, respectively.

The methanolic leaf extract shows MIC 1.56 mg/mL and 0.78 mg/mL against *Staphylococcus aureus* and *Klebsiella pneumoniae*, respectively. The MBC of the methanolic leaf extract against *Klebsiella pneumoniae* and *Staphylococcus aureus* was found to be 1.56 mg/mL and 3.12 mg/mL, respectively. The methanolic leaf extract exhibited the lowest MIC and MBC against *Klebsiella pneumoniae*, which are 0.78 and 1.56 mg/mL, respectively. In conclusion, methanolic leaf extract had the lowest MIC and MBC against *Klebsiella pneumoniae*.

The MIC and MBC of methanolic leaf and ethanolic bark extracts against *Klebsiella pneumoniae* and *Staphylococcus aureus* were evaluated using nutrient agar plates, and the captured images are displayed in Figure 12.

Table 7: ZOI shown by crude bark and leaf extracts against *Klebsiella pneumoniae, Escherichia coli, Shigella sonnei*, and *Staphylococcus aureus*.

Part	Plant Extracts	Bacteria Used	ZOI shown by the sample (mm)	ZOI shown by the positive control Neomycin (mm)
Bark	Methanol	*Klebsiella pneumoniae*	17	28
		Escherichia coli	18	28
		Shigella sonnei	14	24
		Staphylococcus aureus	12	24
	Ethanol	*Klebsiella pneumoniae*	19	28
		Escherichia coli	20	28
		Shigella sonnei	15	24
		Staphylococcus aureus	14	24
	Ethyl acetate	*Klebsiella pneumoniae*	9	25
		Escherichia coli	9	22
		Shigella sonnei	14	20
		Staphylococcus aureus	13	24
	DCM	*Klebsiella pneumoniae*	11	28
		Escherichia coli	21	28
		Shigella sonnei	12	20
	Hexane	*Klebsiella pneumoniae*	9	25
		Escherichia coli	9	22
		Shigella sonnei	9	20
		Staphylococcus aureus	9	24
Leaves	Methanol	*Klebsiella pneumoniae*	21	25
		Escherichia coli	9	22
		Shigella sonnei	14	24
		Staphylococcus aureus	9	24
	Ethanol	*Klebsiella pneumoniae*	13	26
		Escherichia coli	11	24
		Shigella sonnei	9	20
		Staphylococcus aureus	9	20
	Ethyl acetate	*Klebsiella pneumoniae*	9	25
		Escherichia coli	9	22
		Shigella sonnei	15	24
		Staphylococcus aureus	9	24
	DCM	*Klebsiella pneumoniae*	9	26
		Escherichia coli	9	24
		Shigella sonnei	9	20
	Hexane	*Staphylococcus aureus*	9	20
		Klebsiella pneumoniae	9	26
		Escherichia coli	9	24
		Shigella sonnei	9	20
		Staphylococcus aureus	9	20

Table 8: MIC and MBC of methanolic leaf and ethanolic bark extracts against *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Plant Extracts	Klebsiella pneumoniae	Staphylococcus aureus		
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Ethanol-B	1.56	3.12	3.12	6.25
Methanol-L	0.78	1.56	1.56	3.12
Positive Control	0.003	0.007	0.007	0.015

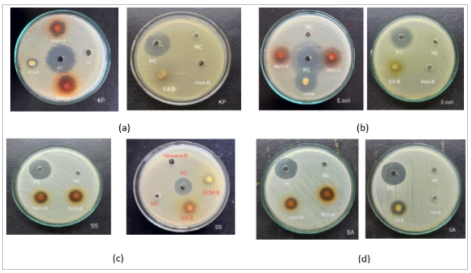

Figure 10: ZOI shown by the bark extract against the organism used (a) *Klebsiella pneumoniae* (b) *Escherichia coli* (c) *Shigella sonnei*.

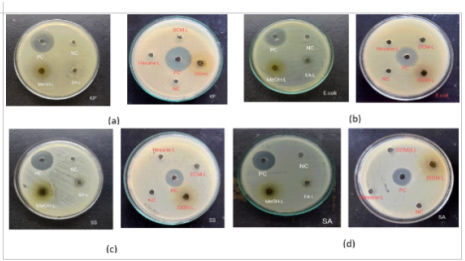

Figure 11: ZOI shown by the leaf extracts against the microorganisms (a) *K. pneumoniae*, (b) *E. coli*, (c) *S. sonnei*, and (d) *S. aureus*

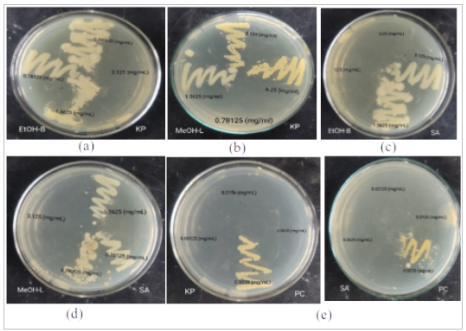

Figure 12: MIC and MBC shown by (a) ethanol-bark extract, (b) methanol-leaf extract, (c) ethanol bark extract (d) methanol leaf extract (e) positive control against *Klebsiella pneumoniae*, and *Staphylococcus aureus*

3.9 Toxicity Analysis

The percentage mortality shown by the plant extract against the Brine shrimp nauplii is displayed in Table 9. The ethanolic bark extract showed the lowest LC_{50} of 80.08 μg/mL. The methanolic leaf extract had the highest LC_{50} of 468.81 μg/mL. The potassium dichromate served as the positive control and 50% DMSO served as the negative control. All nauplii were dead in the positive control (10 nauplii), and the same number of nauplii survived in the 50% DMSO solution. The LC_{50} of crude plant extracts.

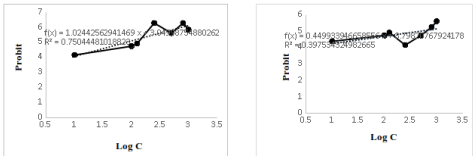

Figure 13: A plot Log C against Probit for the determination of LC 50 of ethanolic bark and methanolic leaf extract

4 Discussion

The Genus *Alnus* is an alder plant mostly found in Nepal's mountainous regions, it is also found in China, India, and Korea. The majority of its quite extensive applications are for the treatment of fever, diarrhoea, dysentery, inflammatory diseases, skin diseases, elephantiasis, stomachaches, and wound healing. Numerous biological activities, including antidiabetic, antioxidant, hepatoprotective, and antifungal effects, are performed by *A. nepalensis* D. Don.

In previous studies, 423.49 ± 0.89 mg GAE/g was the TPC of methanolic leaf extract and 408.88 ± 2.08 mg GAE/g was the flavonoid content [34]. However, in this study, 254.6 ± 5.58 mg GAE/g TPC and 137.21 ± 4.67 mg QE/g TFC respectively. Methanolic extract of the leaf contains maximum phenolic and flavonoid levels. TPC and TFC of bark extracts were compared with *Alnus nitida*. According to a previous study on *Alnus nitida* (Bark), methanolic bark extract had TPC 631.5 ± 1.7 mg GAE/g and ethanolic bark extract had TPC 607 ± 1.97 mg GAE/g respectively. Similarly, 221.5 ± 2.5 mg QE/g was the reported TFC of methanolic extract of this plant [35]. However, in this study, the ethanolic bark extract had a higher phenolic level which is 300.6 ± 3.12 mg GAE/g. However, the flavonoid level was higher in the DCM extract which is 75.99 ± 4.72 mg QE/g. Methanolic leaf ex-

tract had maximum TPC and TFC which is 254.6 ± 5.58 mg GAE/g and 137.21 ± 4.67 mg QE/g respectively. In comparison with both bark and leaf different extracts, ethanolic bark extract contained a high quantity of phenolics. But, methanolic leaf extract had a high amount of flavonoids. The overall phenolic and flavonoid contents of medicinal plants might vary depending on factors such as geographical range, plant collecting time, and climate fluctuation. Phenolics and flavonoids were directly correlated with antioxidant properties. High amounts of phenolics and flavonoid phytoconstituents correlated with antioxidant properties.

Phenolic compounds are directly correlated with tannins. Ethanolic bark extract had a high TTC which is 120.09 ± 3.15 mg TA/g. In the case of leaves, methanolic extract had a high TTC which is 43.73 ± 3.96 mg TA/g. In the previous study, methanolic leaf extract had IC50 which is 4.838 μg/mL [34]. However, in this study, IC50 of methanolic leaf extract was found as 24.03 ± 1.59 μg/mL. Ethanolic bark extract had IC50 39.5 ± 2.11 μg/mL in the previous study [35]. In this study, IC50 of ethanolic bark extract found as 17.55 ± 1.17 μg/mL. In comparison with leaves and bark, ethanolic bark extract possesses good antioxidant potential. TPC and TTC values in bark extract were higher in ethanolic extract which was positively correlated with the IC50 of ethanolic bark extract. The climatic conditions and other environmental factors, antioxidant capacity, TPC, TFC, and TTC were considerably different [36]. Plant extracts are more effective antioxidants because they have a special functional group of secondary metabolites [37, 38].

A prior study depicts that methanolic leaf extract of *A. nepalensis* D. Don was subjected to the antimicrobial assay against *A. baumannii* with ZOI 14.66 mm and *P. mirabilis* with ZOI 15.50 mm [34]. However, the methanolic leaf extract of this plant in this study possesses better antimicrobial activity against *Klebsiella pneumoniae* with ZOI 21 mm. No antimicrobial assay was carried out for the *A. baumannii* and *P. mirabilis* bacteria. Instead, this antimicrobial assay was carried out against *Klebsiella pneumoniae*, *Escherichia coli*, *Shigella sonnei*, and *Staphylococcus aureus* respectively. Methanolic leaf extract had ZOI 14 mm against *Shigella sonnei*. Ethanolic leaf extract of this medicinal plant possesses 13 mm ZOI against *K. pneumoniae*. 15 mm was the ZOI of ethyl acetate extract against *Shigella sonnei*. None of the remaining leaf extracts inhibit bacterial growth. In bark, ethanolic bark extract had good antimicrobial activity against *E. coli* with ZOI 20 mm, and *K. pneumoniae* with ZOI 19 mm. DCM extract had maximum ZOI which is 21 mm against *E. coli*. Hexane has no inhibition against subjected bacteria. Ethanolic extract of bark and methanolic extract of leaf showed good antimicrobial activity against *Klebsiella pneumoniae* and *Staphylococcus aureus* respectively. Not only phenolics and flavonoids but other phytochemicals such as vitamins, carotenoids, Saponins, enzymes, and minerals showed antimicrobial activity [39, 40]. Ethanolic bark extract and methanolic leaf extract of this medicinal plant showed good antimicrobial activity against *K. pneumoniae* and *S. aureus*, so, these samples were subjected to determine MIC and MBC. The ethanolic bark and methanolic leaf extract medicinal plant had good MIC and MBC which are 1.56 mg/mL and 3.12 mg/mL for bark and for leaf 0.78 mg/mL and 1.56 mg/mL respectively against *K. pneumoniae*. In comparison to ethanolic bark extract, methanolic leaf extract had potent MIC and MBC against *K. pneumoniae*. A lethality experiment was conducted on ethanolic and methanolic extracts of bark and leaves and LC50 of ethanolic bark extract was found to be 80.08 μg/mL and LC50 for methanolic leaf extract was found to be 468.81 μg/mL. In comparison between them, ethanolic bark extract had a potent lethal activity as compared to methanolic leaf extract.

Table 9: The number of survived nauplii after treatment with methanolic leaf and ethanolic bark extracts and their percentage mortality

Plant Extract	Concentration (g/mL)	Total No. of Survived Nauplii	% Mortality
Ethanol-Bark Extract	10	24	20
	100	18	40
	125	16	46.66
	250	3	90
	500	8	73.33
	800	3	90
	1000	6	80
Methanol-Leaf Extract	10	22	26.66
	100	18	40
	125	16	46.66
	250	24	20
	500	18	40
	800	12	60
	1000	8	73.33

Table 10: LC50 of ethanolic bark and methanolic leaf plant extracts

Plant Extracts	Linear Regression Equation	LC50 (g/mL)
Ethanol-B	$5.0 = 1.0244x + 3.05$	80.08
Methanol-L	$5.0 = 0.4499x + 3.79$	468.81

5 Conclusion

The present study highlights *Alnus nepalensis* D. Don as a rich source of bioactive phytochemicals, including carbohydrates, glycosides, amino acids, flavonoids, phenols, tannins, terpenoids, anthraquinones, and phytosterols. Among the tested extracts, the ethanolic bark extract exhibited the highest total phenolic content (TPC) among bark extracts, while the methanolic leaf extract had the highest TPC among leaf extracts. The dichloromethane (DCM) bark extract showed the highest total flavonoid content (TFC) among bark extracts, whereas methanolic leaf extract had the highest TFC among leaf extracts. Similarly, tannin content was highest in the ethanolic bark and methanolic leaf extracts. Overall, bark extracts demonstrated higher phenolic and tannin content, while leaf extracts were richer in flavonoids.

In terms of bioactivity, the ethanolic bark extract exhibited the strongest antioxidant potential among the bark extracts, whereas the methanolic leaf extract showed the highest antioxidant activity among leaf extracts. Hexane extracts of both bark and leaves demonstrated the weakest antioxidant potential. The ethanolic and DCM extracts of bark exhibited good antimicrobial activity against *Escherichia coli*, whereas the methanolic leaf extract showed significant antimicrobial activity against *Klebsiella pneumoniae*. Furthermore, the methanolic leaf extract demonstrated superior MIC and MBC values compared to the ethanolic bark extract. The ethanolic bark extract exhibited higher toxicity, as indicated by a lower lethal concentration, compared to the leaf extract. These findings suggest that *A. nepalensis* D. Don possesses significant antioxidant, antimicrobial, and toxicological properties, making it a promising candidate for the discovery of natural antibacterial compounds. Further research is required to explore its pharmacological potential and underlying mechanisms, particularly in the development of novel therapeutic agents.

Abbreviations

- DMSO: Dimethyl sulfoxide
- GAE/g: Gallic acid equivalent per gram
- QE/g: Quercetin equivalent per gram
- TA/g: Tannic acid equivalent per gram
- TPC: Total phenolic content
- TFC: Total flavonoid content
- TTC: Total tannin content
- IC50: Half-maximum inhibitory concentration
- DPPH: 2,2-diphenyl-1-picrylhydrazyl
- ZOI: Zone of inhibition

- MIC: Minimum inhibitory concentration
- MBC: Minimum bactericidal concentration
- LC50: Lethal concentration 50%

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Conflicts of Interest

The authors declare that there are no conflicts of interest associated with the publication of this research paper.

Author's Contribution

- Dipak Raj Jaishi: Performed laboratory work, writing, review, editing, and original draft.
- Indra Ojha: Writing, review, and editing
- Dinesh Raj Ojha and Govinda Bhattarai: Contributed equally
- Khaga Raj Sharma: Writing, review, editing, supervision, and conceptualization

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