



## Research Article

## Antimicrobial activities of ethanolic extraction from leaves of medicinal plants

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## Abstract

Medicinal plants, rich in phytochemicals such as glycosides, phenols, tannins, and alkaloids, are highly valued for their therapeutic properties. This study was a cross-sectional descriptive study conducted between December 2023 and March 2024 to explore the antimicrobial properties of plant extracts against different bacterial strains. In this study, 400 grams of leaves from each of four different plants samples (*Ocimum tenuiflorum*- Holy Basil, *Artemisia vulgaris*- Mugwort, *Azadirachta indica*-Neem, and *Mentha arvensis*- Basil) were collected from Thankot, Kathmandu and laboratory processing was done in Microbiology Laboratory of Padmakanya Multiple Campus. All the test bacteria were collected from Central Department of Microbiology, Tribhuvan University. To determine antimicrobial activities, 200gm of each plant leaves were processed for ethanolic extraction (Soxhlet extractor). Phytochemical analysis of glycosides, alkaloid, flavonoids, steroids, saponins, reducing sugar and phenol and tannins of each plant leaves were done. From ethanolic plant extracts, antimicrobial activity was assessed through the Agar Well Diffusion method and Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC) were also carried out. In this study, Phytochemical analysis showed seven phytochemical compounds found in all leaf extracts except alkaloid in Mugwort and Basil and reducing sugar in Neem. From ethanol extracts, Mugwort extract showed the greatest inhibition zone (26mm) against *Staphylococcus aureus*, whereas Holy Basil extracts showed no inhibition zone against any test bacteria. MIC values varied between 12.5 and 50mg/ml, while MBC values fell within the range of 12.5 to 100mg/ml showing extensive antimicrobial efficacy. This result indicated that ethanol extract of the medicinal plants was effective against test bacteria. Thus, ethanol extracts have potential for developing targeted antimicrobial drugs.

**Introduction**

Plants are used globally for medicinal purposes and plants in Nepal have been reported to be used by the indigenous community especially in mid-hill and mountainous areas. (Kunwar *et al.*, 2022). But, nowadays due to the development of Ayurvedic medicines and its remarkable cure ness to fatal diseases, people are more concerned about those medicinal species. In the context of Nepal, out of 8737 plants, 1762 are being used as medicinal plant (Kunwar *et al.*, 2022).

Antimicrobial agents may be either bactericidal (kills bacteria) or bacteriostatic (inhibits bacterial growth) (Pankey & Sabath, 2004). The antimicrobial agents work by disrupting (1) building of cell walls (2) maintaining plasma membrane (3) producing nucleic acids (4) functioning of ribosomes (5) synthesizing folate (Neu & Gootz, 1996). Understanding the effectiveness of the locally available plants from their antimicrobial perspectives are vital to use them in

home remedy and to explore their pharmaceutical uses. The pharmaceutical usefulness of the plants is the function of the phytochemicals present in the plants. Phytochemicals are chemical constituents which are present in plants. They include alkaloids, glycosides, saponins, essential oils, mucilage, tannins, phenol, etc. (Oladeji *et al.*, 2020). These phytochemicals help the plant in growing, propagating color, odor, flavor, etc. (Molyneux *et al.*, 2007). Plants like Holy Basil (Nepali name: Tulsi), Mugwort (Nepali name: Titipate), Neem and Basil (Nepali name: Babari) are most widely produced as well consumed worldwide. They are also being used as medicinal plants from ancient times (Petrovska, 2012). They are equally common in case of Nepal. Also, many studies have proven that most of the plants possess at least one medicinal property. They may be either antibacterial, antifungal, antiviral, anticancer, antiseptics, insecticidal, etc. (Vozza Berardo *et al.*, 2024).

Medicinal herbs can significantly contribute to addressing the challenges currently being experienced

worldwide. Ethanolic extraction has been effectively trialed on different bacteria (*Escherichia coli* and *Staphylococcus aureus*) and fungi (*Aspergillus niger*, *Candida albicans*) (Jeyaseelan & Jashothan, 2012), (Gizaw *et al.*, 2022). Regrettably, there has been limited exploration of the antimicrobial properties from plants against pathogenic organisms. Therefore, the current in vitro study aimed to evaluate the antibacterial properties of medicinal herbs against test bacteria affecting humans.

### Methods

The cross-sectional descriptive study was done. The convenience sampling was done for collecting the medicinal plants. For this study, 400gm of each of 4 different medicinal plant leaves which were Holy Basil (Tulsi- *Ocimum tenuiflorum*), Mugwort (Titipate- *Artemisia vulgaris*), Neem (*Azadirachta indica*) and Basil (Babari- *Mentha arvensis*) were collected from Thankot, Kathmandu in sterile Ziplock bags. All the collected samples were transported to the laboratory of Padma Kanya Multiple Campus for laboratory processing.

### Laboratory Processing of the Sample

The healthy plant was chosen. The unwanted parts of plants like roots, rhizomes, stems, etc. were removed and required parts i.e., leaves were chopped into 3-5 cm pieces. The samples (200gm) were left to dry for approximately one week in room temperature. The samples were rotated two times daily to speed up the drying process (Khanal, 2020). The fully plant leaves (100gm) were placed into waterproof containers.

Then dried samples were grinded in powder which were used for ethanolic extraction. The dried plant powder (100gm) was measured and placed in a clean and dry thimble of the Soxhlet extractor. It was subsequently packed with a 250ml dry round bottom flask. Ethanol was gradually poured from the top opening of the Soxhlet extractor, measuring 150ml. Then it was ultimately equipped with a condenser. The heating mantle was used to heat the flask. The solvent vapor entered the cylinder via the side tube and condensed upon entering the condenser. The siphon tube is used to transfer the solution into the round bottom flask. The procedure is carried on for 3-4 hours until the solvent color is seen in the siphon (Giovanni Appendino, 2008).

### Preparation of stock and working solution

The ethanolic extraction from every medicinal plant was carefully moved into a sterile 20ml screw cap test tube that was clean and dry. Then, 9ml of DMSO was added into each test tube and was mixed by vortexing to create a uniform mixture with a concentration of 1g/10ml (stock solution) and 100mg/ml (working solution) (Yadav & Agarwala, 2011).

### Collection of Standard Cultures

Five different species of bacteria were chosen for the study. All the test bacteria which were *Staphylococcus*

*aureus*, *Escherichia coli*, *Bacillus spp.*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and ATCC bacteria (*Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922) were collected from the Central Department of Microbiology, Tribhuvan University. All test bacteria were then sub-culture by streaking them on Nutrient Agar plates and confirmed from identification by Bergey's manual of determinative bacteriology (Bergey & Breed, 1957). Before performing antibacterial activity of extracts against test bacteria, antibiotic susceptibility test of test bacteria was done (CLSI, 2007).

### Qualitative Phytochemical Analysis from ethanol extraction

The ethanol extraction was used for the detection of phytoconstituents present in each plant extract by standard methods (Thangjam *et al.*, 2020).

#### Keller-Kalani test (Test for glycosides)

In the test tube, 2ml of crude extract was mixed with 2ml of glacial acetic acid, then a small amount of ferric chloride solution and 1 ml of sulfuric acid were added. A brown ring indicated the presence of glycosides (Akerele *et al.*, 2011).

#### Wagner's test (Test for alkaloids)

In the test tube, 1ml of crude extract was combined with 1ml of Wagner's reagent (dilute iodine solution). The presence of alkaloid was verified by the reddish-brown precipitate (Yadav & Agarwala, 2011).

#### Test for Phenols and Tannins

Crude extract was combined with a 2% solution of ferric chloride in a 2ml mixture. The presence of phenols or tannins was shown by a blue-green or black color (Sirikantaramas *et al.*, 2005).

#### Alkaline reagent test (Test for flavonoids)

The crude extract was mixed with 1-2ml of 2% NaOH. The appearance of a bright yellow color changed to colorless when a small amount of diluted acid was added, showing the presence of flavonoids (Yadav & Agarwala, 2011).

#### Test for Steroids

The crude extract was combined with 2ml of chloroform and concentrated Sulfuric acid was added in a sideways manner. The presence of steroids was confirmed by the appearance of a red color in the chloroform layer below (Kancherla *et al.*, 2019).

#### Test for Saponins

The crude extract was combined with 5ml of distilled water in a test tube and vigorously shaken. The presence of saponins was verified by the formation of foam (Devmurari, 2010).

#### Benedict's Test

The crude mixture was placed in a sterile and dry test tube then 2 ml of Benedict's reagent was added to the mixture and placed in a hot water bath for 5 minutes.

A brick-red color indicated the existence of reducing sugar. (Dahal, 2022).

#### Determination of Antibacterial Activity of plants by Agar Well Diffusion

The antimicrobial effects of the medicinal plants were assessed using the Agar Well Diffusion technique. 3-4 well-isolated bacterial colonies were inoculated in Nutrient Broth and incubated for 4 hours. Then, the turbidity of broth was compared with standard of 0.5 MacFarland. A sterile cotton swab was immersed in the broth and swirled against the test tube wall to eliminate any extra inoculum, then inoculum was lawn cultured on Muller Hinton Agar (MHA). An aseptic punch was used with a sterile cork-borer to create a circular well with a 6mm diameter, then 70  $\mu$ L of ethanolic extracts were distributed into the wells. For negative control and positive control, DMSO and ofloxacin were distributed into the wells respectively. All the plates were incubated at 37°C for 24hrs. Then, zone of inhibition was observed and measured (Raut *et al.*, 2014).

#### Determination of MIC and MBC

The ethanolic plant extract of each plant leaves were serially diluted in nutrient broth to determine MIC and MBC. A set of 11 dry screw capped test tubes were taken and labelled as 1 to 11. Test tube no 1 was taken as positive control where only ethanolic extract was taken (without bacterial broth). Similarly, test tube no 11 was taken as negative control where only nutrient broth was taken (without plant extract). So, 2ml of nutrient broth were taken in test tubes no. 1. without bacterial broth while 2 ml of ethanol extract and 2ml of bacterial broth were added and mixed in test tube labelled no 2. The solution was mixed well and 2 ml of

solution was transferred to test tube no 3. This process was continued up to test tube no. 10. Finally, 2 ml of solution was discarded from test tube no 10. Hence, the serial dilution was done in which each test tube contained equal volume (2 ml) of solution in decreasing concentration from 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.390625 and 0.1953125 respectively. All the test tubes were incubated at 37°C for 24 hours. Then, the turbidity in the test tubes was observed by comparing with positive and negative control i.e., tube tubes no 1 and 11. The results were interpreted on the basis of the lowest concentration that showed the lack of turbidity and determined as Minimum Inhibitory Concentration (MIC). For the determination of MBC, the samples from each test tubes were further sub cultured on NA plates. The plates were incubated at 37°C for 24 hours and the lowest concentration that showed no growth was considered as the MBC (Owuama, 2017).

#### Quality Control in the Laboratory

Agar well diffusion method, MIC and MBC of test bacteria were compared to ATCC bacteria (*Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922) for quality control.

#### Data Analysis

The experiment's data was entered in MS excel and further analysis was done. The data obtained from antimicrobial assays (zone of inhibition, MIC and MBC values) were analyzed descriptively. The data were then presented in tables and charts. No any other statistical tests were applied as only single measurements were recorded. It was however interpreted with previously published studies.

## Result

**Table 1:** Phytochemical Analysis of *Artemisia vulgaris* (Titipate), *Mentha arvensis* (Babari), *Ocimum tenuiflorum* (Tulsi) and *Azadirachta indica* (Neem)

S. No	Test	<i>Artemisia vulgaris</i> (Titipate)	<i>Mentha arvensis</i> (Babari)	<i>Azadirachta indica</i> (Neem)	<i>Ocimum tenuiflorum</i> (Tulsi)
1	Glycoside	+	+	+	+
2	Alkaloid	-	-	+	+
3	Phenol and Tannin	+	+	+	+
4	Flavonoids	+	+	+	+
5	Steroids	+	+	+	+
6	Saponins	+	+	+	+
7	Reducing Sugar	+	+	-	+

- indicated absent

+ indicated present

**Table 2:** Antimicrobial Activity of *Artemisia vulgaris* and *Mentha arvensis* from ethanolic plant extract

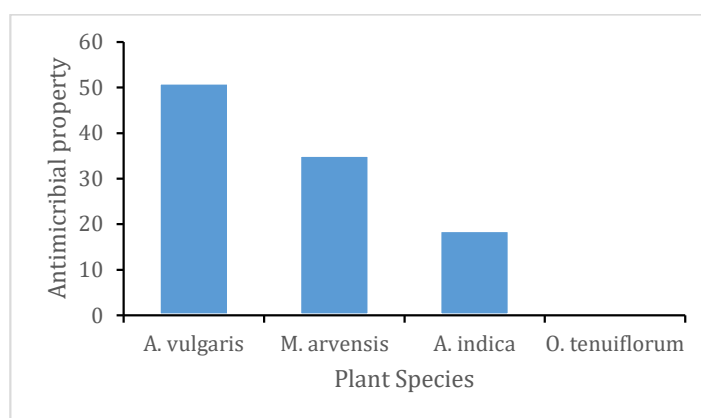
Samples	Test bacteria	Zone of Inhibition		
		100%	10%	1%
<i>Artemisia vulgaris</i>	<b>Gram positive bacteria</b>			
	<i>Staphylococcus aureus</i> ATCC 29213	-	-	-
	<i>Staphylococcus aureus</i>	14mm	26mm	-
	<i>Bacillus</i> spp.	10mm	12mm	-
	<b>Gram negative bacteria</b>			
	<i>Escherichia coli</i> ATCC 25922	-	-	-
	<i>Escherichia coli</i>	-	-	-
	<i>Klebsiella pneumoniae</i>	-	16mm	-
<i>Pseudomonas aeruginosa</i>	-	12mm	-	
<i>Mentha arvensis</i>	<b>Gram positive bacteria</b>			
	<i>Staphylococcus aureus</i> ATCC 29213	-	-	-
	<i>Staphylococcus aureus</i>	-	13mm	-
	<i>Bacillus</i> spp.	9mm	15mm	-
	<b>Gram negative bacteria</b>			
	<i>Escherichia coli</i> ATCC 25922	-	-	-
	<i>Escherichia coli</i>	-	-	-
	<i>Klebsiella pneumoniae</i>	-	-	-
<i>Pseudomonas aeruginosa</i>	-	14mm	-	

**Table 3:** Antimicrobial Activity of *Azadirachta indica* and *Ocimum tenuiflorum* from ethanolic plant extract

Samples	Test bacteria	Zone of Inhibition		
		100%	10%	1%
<i>Azadirachta indica</i>	<b>Gram positive bacteria</b>			
	<i>Staphylococcus aureus</i> ATCC 29213	12mm	-	-
	<i>Staphylococcus aureus</i>	10mm	11mm	-
	<i>Bacillus</i> spp.	-	-	-
	<b>Gram negative bacteria</b>			
	<i>Escherichia coli</i> ATCC 25922	-	-	-
	<i>Escherichia coli</i>	-	-	-
	<i>Klebsiella pneumoniae</i>	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	
<i>Ocimum tenuiflorum</i>	<b>Gram positive bacteria</b>			
	<i>Staphylococcus aureus</i> ATCC 29213	-	-	-
	<i>Staphylococcus aureus</i>	-	-	-
	<i>Bacillus</i> spp.	-	-	-
	<b>Gram negative bacteria</b>			
	<i>Escherichia coli</i> ATCC 25922	-	-	-
	<i>Escherichia coli</i>	-	-	-
	<i>Klebsiella pneumoniae</i>	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	

**Table 4: Determination of MIC and MBC from ethanolic plant extract**

Test Organism	<i>Artemisia vulgaris</i>		<i>Mentha arvensis</i>		<i>Azadirachta indica</i>		<i>Ocimum tenuiflorum</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<b>Gram positive bacteria</b>								
<i>Staphylococcus aureus</i> ATCC 29213	12.5	25	25	50	50	50	25	50
<i>Staphylococcus aureus</i>	25	50	50	50	25	50	50	100
<i>Bacillus</i> spp.	25	25	25	25	12.5	12.5	50	50
<b>Gram negative bacteria</b>								
<i>Escherichia coli</i> ATCC 25922	12.5	25	50	50	25	50	25	50
<i>Escherichia coli</i>	25	50	25	50	25	50	25	50
<i>Klebsiella pneumoniae</i>	12.5	25	12.5	12.5	12.5	25	25	25
<i>Pseudomonas aeruginosa</i>	25	25	25	25	25	50	25	50



**Fig. 1. Comparative study of antimicrobial activity of ethanolic extraction of leaves of medicinal plant**

**Discussion**

From the phytochemical tests, Keller-Kalani test was performed for the test of glycosides. All 4 medicinal plants showed the present of glycosides. For the test of alkaloids, Wagner’s test showed that only *Azadirachta indica* and *Ocimum tenuiflorum* had alkaloids. Similarly, phenol and tannins, steroid and saponins test were present in 4 medicinsiral plants. All medicinal plants showed positive result for reducing sugar by Benedict’s test except *Azadirachta indica*. Similar study was done by Das *et al.*, (2014) which showed the presence of alkaloids, flavonoids, tannins, glycosides in the ethanol extract which was quite similar to this study except absence of alkaloid. Similarly, the study of Thakur *et al.*, (2021) observed the presence of phenolics and alcohols which were effective against liver diseases. Also, Hikaambo *et al.*, (2022) observed the presence of flavonoids, phenolic compounds, tannins, saponins, steroids and alkaloids were present in ethanol extract of *Azadirachta indica*.

Antimicrobial activity of ethanol extracts of four medicinal plants (*Ocimum tenuiflorum*- Tulsi, *Artemisia vulgaris*- Titipate, *Azadirachta indica*- Neem and *Mentha arvensis*- Babari) were determined against 5 different

test bacteria i.e., Gram positive (*Staphylococcus aureus*, *Bacillus* spp.) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*). From the experiment of antimicrobial activity from ethanolic extraction, it was observed that the highest zone of inhibition was shown against *Staphylococcus aureus* by *Artemisia vulgaris* from 10% concentration which was 26mm. Similar study was done by Hiremath *et al.*, (2011) who showed the antimicrobial activity of ethanol extract of *Artemisia vulgaris* against both Gram positive and Gram negative bacteria. On the other hand, *Ocimum tenuiflorum* didn’t show any ZOI against both Gram-positive and Gram-negative bacteria. However, Srichok *et al.*, (2022) concluded that *Ocimum tenuiflorum* doesn’t show any ZOI against any Gram negative but showed ZOI against Gram positive bacteria only. It was noted that the ethanol extract primarily exhibited inhibition zones against Gram-positive bacteria exclusively as Gram positive bacteria possess a dense peptidoglycan layer that is highly susceptible to ethanol extracts. However, Gram negative bacteria possess lipopolysaccharides that serve as a barrier against ethanol extracts. In the same way, Gram negative bacteria possess efflux pumps that remove antimicrobial compounds from the cell.

**Photographs**



**Photograph 1:** *Artemisia vulgaris* (Titipate),



**Photograph 2:** *Mentha arvensis* (Babari),



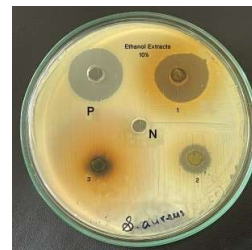
**Photograph 3:** *Ocimum tenuiflorum* (Tulsi)



**Photograph 4:** *Azadirachta indica* (Neem)



**Photograph 5:** Soxhlet extractor



**Photograph 6:** Antimicrobial Activity



**Photograph 7:** Phytochemical Analysis

The MIC and MBC values ranged from 12.5 to 100mg/ml. It was quite in contrast to the study of (Haile & Jiru, 2022) which showed 12.5 mg/ml MIC and MBC value for *Staphylococcus aureus* and *Pseudomonas aeruginosa*, 6.25 mg/ml and 12.5 mg/ml MIC and MBC value respectively for *Escherichia coli* for *Artemisia afra* leaves. The contrast may be due to the geographical origin, climatic condition or different bacterial strains.

Presence of phytochemicals in the plants are controlled by anthropogenic and environmental factors, thus future study should include samples from widespread areas. Furthermore, exploring for the additional groups of phytochemicals might add value in future studies. Antimicrobial activities were only performed using Agar Well Diffusion Method. The process can be further done using other methods. Furthermore, studies can be carried out to determine the antifungal activity.

**Conclusion**

Hence, plant leaves were processed for ethanol extraction (Soxhlet extractor). Ethanol extraction was used for phytochemical analysis, MIC, and MBC, and for antimicrobial activity testing through Agar Well Diffusion method. Phytochemical analysis showed seven phytochemical compounds found in all leaf extracts except alkaloid in Titipate and Babari and reducing sugar in Neem. From ethanol extracts, Titipate extract displayed the greatest zone of inhibition (26mm against *Staphylococcus aureus*), whereas Tulsi extracts showed no inhibition against the bacteria tested. The MIC values varied between 12.5 and 50mg/ml, while the MBC values ranged from 12.5 to 100mg/ml, showing wide antimicrobial efficacy. This result indicated that the ethanolic extract of the medicinal plants were effective against test bacteria.

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## CRedit Author Statement

**ATM:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing- Original Draft, Writing- Review & Editing, Visualization, Project administration, Funding acquisition; **AS:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing-Original Draft, Writing- Review & Editing, Visualization, Project administration; **RM:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing-Original Draft, Writing- Review & Editing, Visualization, Supervision, Project administration, Funding acquisition; **Conflict of interest:** The author declares that there is no conflict of interest.

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