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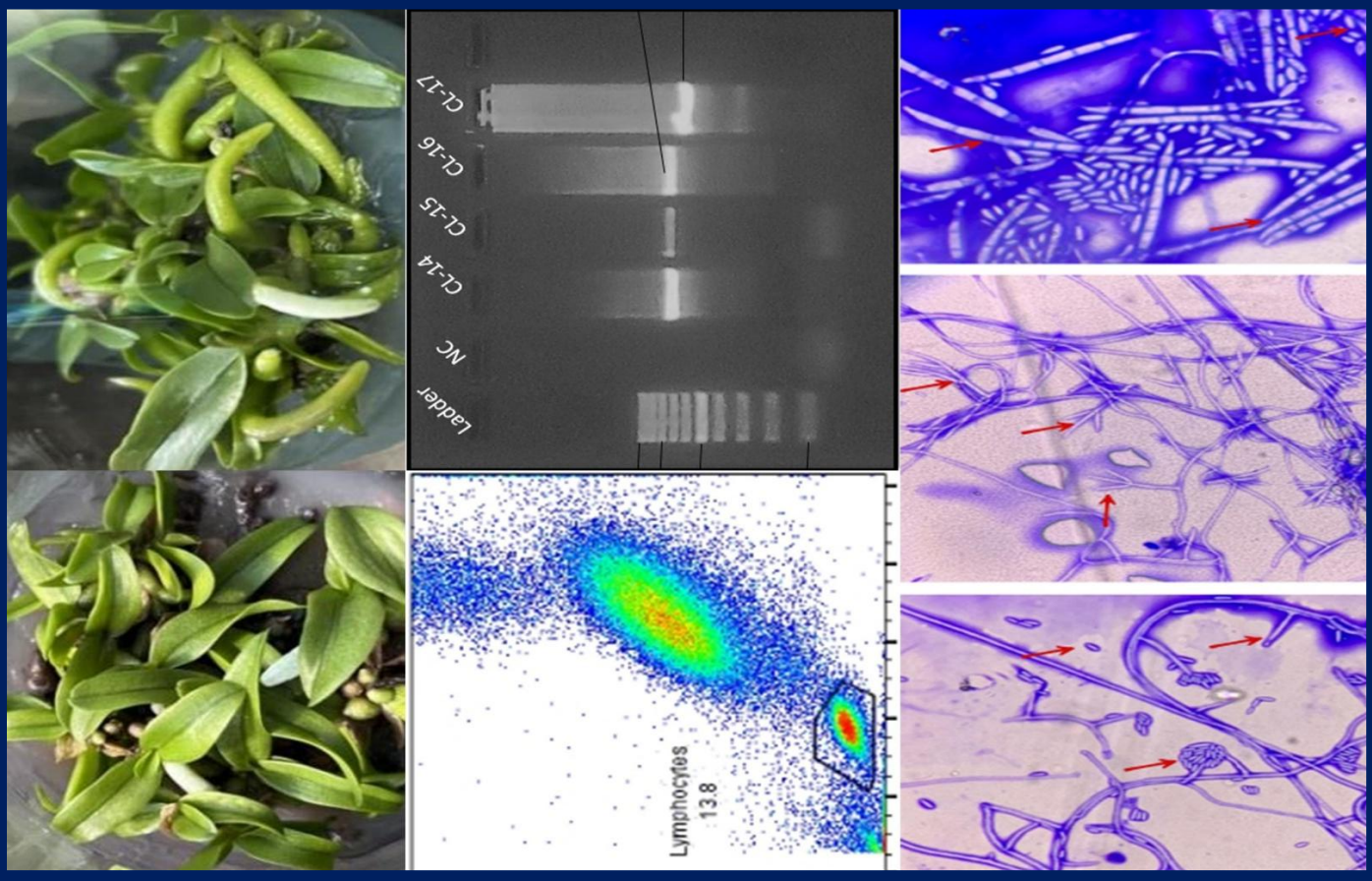

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Research Article

Expression of *Candida intermedia* GXF1 Improves Xylose Transport in *Saccharomyces cerevisiae*

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ABSTRACT

The heterologous expression of the GXF1 xylose transporter from *Candida intermedia* was investigated in *Saccharomyces cerevisiae* DTY165 to evaluate the role of xylose transport in a strain lacking engineered xylose-metabolizing pathways. Introduction of *GXF1* led to increased xylose uptake and improved growth when xylose was used as the sole carbon source, as evidenced by a higher specific growth rate and faster depletion of extracellular xylose compared to the empty vector control. Enhanced intracellular accumulation of xylose further supported the contribution of *GXF1* to transport efficiency. Despite improved xylose uptake, ethanol production from xylose remained low, and only small increases in ethanol levels were observed in both glucose- and xylose-containing media. These findings indicate that improving xylose transport alone is not sufficient to achieve substantial ethanol production in *S. cerevisiae*. Instead, effective conversion of xylose to ethanol will likely require additional engineering of downstream xylose-utilization pathways. Overall, this study highlights the importance of coupling enhanced xylose transport with metabolic pathway optimization for meaningful ethanol production.

Keywords: Bioethanol; *Candida intermedia*; GXF1; *Saccharomyces cerevisiae*; Xylose transport

Introduction

Increased global demand for renewable energy has grown interest in bioethanol as a fuel alternative due to being sustainable and as derived from green masses. While first-generation ethanol derived from corn and sugarcane raises concerns over food security, second-generation bioethanol produced from lignocellulosic biomass offers a non-food-based environmentally friendly option (Karp & Richter, 2011). Nepal, known as an agriculturally resourceful country, produces high quantities of lignocellulosic residues including rice husks, wheat straw and sugarcane bagasse (NV et al., 2006). Such by-products are largely underutilized, and this gives a valuable opportunity as feedstocks for bioethanol production. Effective conversion of plant biomass requires the extraction and hydrolysis of its main biomass which are embedded in the highly recalcitrant structure of the plant cell wall. This complex matrix is predominantly composed of cellulose (40–

50%), hemicellulose (25–35%) and lignin (15–20%) (Jordan et al., 2012; dos Santos et al., 2016). Cellulose is made up of glucose chains, the most abundant sugar in biomass, whereas hemicellulose contains xylose, a five-carbon sugar and the second most prevalent monomer.

Saccharomyces cerevisiae is the primary yeast employed for industrial ethanol production, utilizing glycolysis to convert sugars into pyruvate under anaerobic conditions (Figure 1). This pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase, followed by reduction to ethanol via alcohol dehydrogenase, regenerating NAD^+ in the process—steps that form the core of ethanol fermentation industries (Walker, 1998). *S. cerevisiae* application in industrial sectors is favoured by its several advantageous traits including the Generally Recognized as Safe (GRAS) status, food-grade production, ethanol tolerance, stress resilience, ease of genetic modification and adaptability to large-scale fermentation (Steensels et al., 2014; Walker & Walker, 2018; Parapouli et al., 2020). These kinds of features collectively make *S. cerevisiae* an ideal host for ethanol production and industrial fermentation applications, with strain selection often focusing on ethanol productivity and robustness under stress conditions. Despite being one of the preferred industrial ethanol producers, *S. cerevisiae* lacks a dedicated xylose transport system.

As a result, during lignocellulosic biomass utilization, the pentose fraction of lignocellulosic hydrolysates remains largely underutilized, leading to reduced ethanol yields. Engineering yeast with heterologous xylose transporters from other organisms, therefore, represents a promising strategy to enhance xylose uptake. Beyond ethanol production, improved xylose transport capacity also has broader implications for the biosynthesis of value-added products such as xylitol, xylonate and biopolymers (Qiao et al., 2021; van Arsdale et al., 2024; Regmi & Bhattarai, 2025; Queiroz et al., 2025). Sugar uptake in cells is facilitated by transmembrane transporter proteins, which enable the movement of substrates between the extracellular and intracellular spaces. Among these, the major facilitator superfamily (MFS) is widely distributed across all domains of life and includes a variety of transporters with different mechanisms: uniporters transport a single substrate, symporters couple substrate movement to ions, and antiporters exchange two distinct substrates in opposite directions (Marger & Saier, 1993; Forrest et al., 2011; Reddy et al., 2012; Quistgaard et al., 2016). Within the MFS, several subfamilies contain well-characterized xylose transporters including GAL2, HXT, GXF1 and GXS1 (Hamacher et al., 2002; Maier et al., 2002; Sedlak & Ho, 2004; Leandro et al., 2008; Runquist et al., 2009; Fonseca et al., 2011; Young et al., 2011; Aeling et al., 2012; Young et al., 2012; Young et al., 2014).

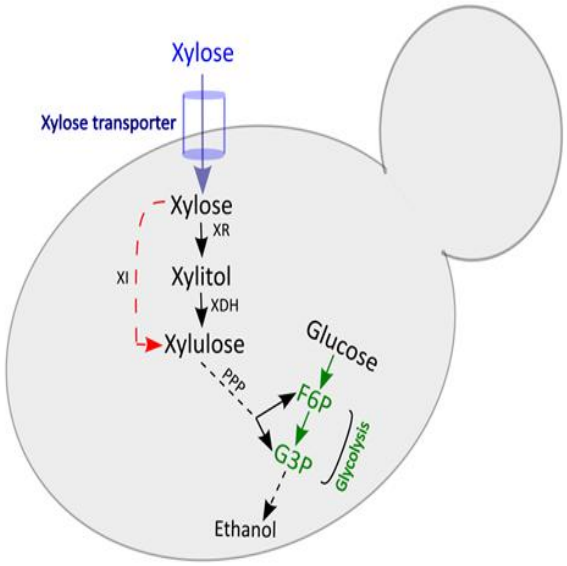

Figure 1: Schematic diagram of xylose metabolism in *S. cerevisiae*. XR: xylose reductase, XDH: xylitol dehydrogenase, XI: xylose isomerase, PPP: Pentose phosphate pathway, F6P: Fructose 6-phosphate, G3P: Glyceraldehyde 3-Phosphate. The red line indicates the heterologous pathway.

In *S. cerevisiae*, native hexose transporters such as Hxt1p, Hxt2p, Hxt4p, Hxt5p, Hxt7p and Gal2p can import xylose, but their low affinity for xylose and strong preference for glucose restrict efficient fermentation of mixed sugars (Farwick et al., 2014; Bracher et al., 2018). To overcome these limitations, heterologous xylose transporters have been expressed in engineered strains, often in combination with downstream metabolic pathway modifications, leading to significant improvements in xylose uptake and utilization. The fungus *Candida intermedia* possesses two different glucose/xylose transporters. GXF1 encodes a glucose/xylose facilitator, whereas GXS1 encodes a glucose/xylose proton symporter, with K_m values of 50 mM and ~0.4 mM respectively (Leandro et al., 2006). Expression of GXF1 could enhance xylose transport kinetics thereby boosting recombinant *S. cerevisiae* growth (Leandro et al., 2006; Runquist et al., 2009; Runquist et al., 2010; Fonseca et al., 2011; Parachin et al., 2011; Young et al., 2011). In *S. cerevisiae*, xylose transport across the plasma membrane is less efficient, posing a major obstacle for biomass conversion (Reznicek et al., 2015). This study specifically evaluates the contribution of the GXF1 membrane transporter to xylose uptake and growth in a non-xylose engineered *S. cerevisiae*, without modification of downstream xylose metabolizing pathways. By isolating the role of transport capacity, the

work aims to clarify how enhanced xylose uptake alone influences cellular growth and ethanol formation.

Materials and Methods

Strains, plasmid and culture conditions

Escherichia coli DH10B was used for plasmid propagation cultured in the Luria-Bertani (LB) medium at 37 °C. *Saccharomyces cerevisiae* DTY165 (leucine auxotroph) was used as host for functional expression studies cultured in YPD or synthetic complete (SC) medium at 30 °C. *E. coli* was gifted by Boles Lab, University of Frankfurt, Germany. And, *S. cerevisiae* strain was gifted by Clemens Lab, University of Bayreuth, Germany. The expression plasmid pGXF1 containing *Candida intermedia* GXF1 gene (1664 bp) and LEU2 selectable marker was gifted by Prof. Dr. Paula Gonçalves (University of Lisbon, Portugal).

Primer design and PCR amplification

Oligonucleotide primers was needed for the amplification and verification of the GXF1 gene. The primer sets (YTUF1F and YTUF1R) and (CiGXFL1 and CiGXFR1) were designed using NCBI primer designing tool. All primers were synthesized by Macrogen (South Korea). Primer sequences and their specific applications are listed in Table 1.

Transformation and verification of transformant

The pGXF1 plasmid was first transformed into *E. coli* competent cells using the heat-shock method (Sambrook & Russell, 2001). Transformants were selected on LB agar supplemented with 100 µg/ml ampicillin. Plasmid DNA was isolated from *E. coli* using an alkaline lysis method (Sambrook & Russell, 2001) for subsequent analysis and yeast transformation. For expression in yeast, isolated pGXF1 plasmid was transformed into *S. cerevisiae* via electroporation (Becker & Guarente, 1991). Transformants were selected by plating on synthetic complete (SC) medium lacking leucine.

Verification of transformation was performed through a multi-step process. Putative *E. coli* transformants were initially confirmed by the presence of plasmid DNA, visualized via agarose gel electrophoresis. This plasmid DNA was used to transform yeast, with successful observations of colonies in SC agar plate without leucine supplement indicates successful transformation. Yeast transformants were further verified by PCR amplification of yeast DNA using the internal GXF1-specific primers (CiGXFL1 and CiGXFR1), with successful transformation confirmed by the appearance of the expected 163 bp amplicon. The presence of GXF1 insert was further confirmed by Southern blot analysis (Southern, 1975) of yeast DNA using a DIG-labeled probe generated with the CiGXFL1 and CiGXFR1 primers set. Briefly, the PCR product was separated by gel electrophoresis, transferred to a nylon membrane, and hybridized with a biotin-labeled GXF1-specific probe. Hybridization was detected using a streptavidin-alkaline phosphatase conjugate and a chromogenic substrate. After confirmation, yeast transformants were subsequently used for functional expression analysis, including growth kinetics on various sugar substrates.

Growth kinetics assay

For growth assay, colonies of pGXF1 transformed (hereafter referred as GXF1 transformed) and empty vector transformed (hereafter referred as control) into *S. cerevisiae* DTY165 were inoculated into YEP medium containing 20 g/l of either glucose or xylose as the sole carbon source. Cultures were incubated at 30°C with shaking (180 rpm). Growth was monitored by measuring the optical density at 600 nm at 3-hour intervals for 36 hours. The specific growth rate (µ/h) was calculated from the exponential phase of growth. For the intracellular xylose accumulation assay, yeast grown in liquid culture was pelleted, and the cells were broken by freezing them, and finally, glass beads were used to vortex to break the cells (Stowers & Boczko, 2007). The concentrations of substrates and products (glucose, xylose and ethanol) were determined using colorimetric assays. For each assay, a standard curve was prepared using known concentrations of the pure compound, and sample concentrations were calculated based on the standard curve. Glucose concentration in the culture medium was quantified using the 3,5-dinitrosalicylic acid (DNS) method for reducing sugar (Miller, 1959). Xylose concentration was specifically quantified using the phloroglucinol assay (Eberts et al., 1979). Ethanol concentration was determined using a potassium dichromate-based method (Seo et al., 2009). All the tests were done in duplicate.

Table 1: Oligonucleotide primers used in the study.

Primer	5'-3' sequence	Restriction sites	Expected size
CiGXFL1	CTTTGCTTCCACCTTCGTTG	-	163 bp
CiGXFR1	AGTGTTGGAGGTCTCGTTGG	-	

YTUF1F	GACTA ***GAGCTCCATATG*** TCACAAGATTCGCATTC	NdeI/SacI	1600 bp
YTUF1R	TACTGT ***GGATCC*** CAT***TCTAGA*** TTAAACCTGTTCGTCGGTG	XbaI/BamHI	

Data analysis

Data are presented as the mean of biological duplicates. Statistical comparisons between the growth rate of control and GXF1 transformed strains were performed using an unpaired two-tailed Student's t-test. In the present study, given the limited number of biological replicates, statistical analysis was used to support observed trends rather than to draw definitive quantitative conclusion. A p-value of ≤0.05 was considered statistically significant.

Results and Discussion

Confirmation of pGXF1 transformation in *E. coli* and *S. cerevisiae*

The successful construction of recombinant *S. cerevisiae* strains expressing the GXF1 transporter was systematically confirmed through a multi-tiered molecular analysis, confirming the presence and maintenance of the expression plasmid.

Confirmation of transformants in *E. coli*

The *E. coli* transformation was observed by the appearance of approximately 60 ampicillin-resistant colonies in LB agar plate containing ampicillin, contrasted with the complete absence of growth in the control plate, and this demonstrated successful selection. Alkaline lysis of plasmid DNA isolation and its gel electrophoresis further confirmed the presence of plasmid DNA. This plasmid DNA was then used to transform *S. cerevisiae* to make transgenic yeast expressing GXF1.

Confirmation of *S. cerevisiae* transformants

The successful transformation of pGXF1 plasmid into leucine auxotroph *S. cerevisiae* DTY165 was first indicated by functional complementation with transformants growing on synthetic medium lacking leucine. This phenotype is contingent upon the presence of the episomal plasmid containing LEU2 selectable marker. Crucially, the control failed to grow under the same selective conditions, confirming that growth was plasmid-dependent. Further molecular analyses confirmed the presence of the plasmid within the yeast cells. Isolation of plasmid DNA was done from the GXF1 transformed yeast cells. The DNA was then used as a template to perform the PCR using two different primer sets as listed in Table 1. The Figure 2 clearly shows the observation of expected size bands using two different set of primers.

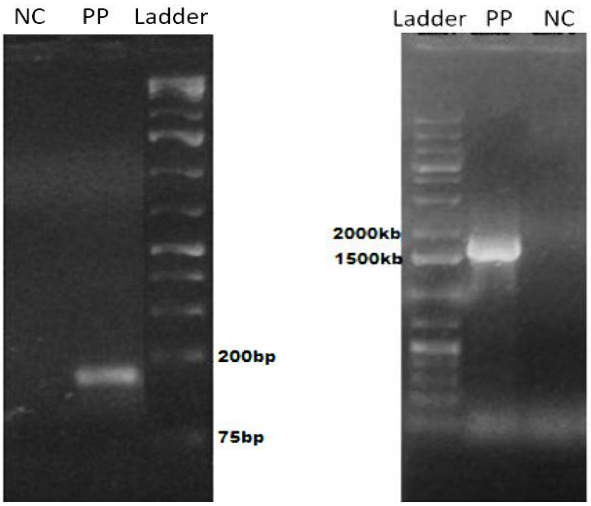

Figure 2: PCR of GXF1 transformed yeast DNA using different primers CiGXFL1 and CiGXFR1, expected size 163 bp (left), and YTUF1F and YTUF1R, expected size ~1600 bp (right). NC, PP, Ladder refers to negative control, PCR product and 1 Kb plus ladder (Thermoscientific) respectively. Lane 2 is the PCR product of GXF1 transformed yeast cells.

Finally, Southern blot analysis using a biotin-labeled GXF1 probe detected a clear hybridization signal in DNA isolated from the GXF1 transformed *S. cerevisiae* strain, while no signal was observed in the control strain (Figure 3).

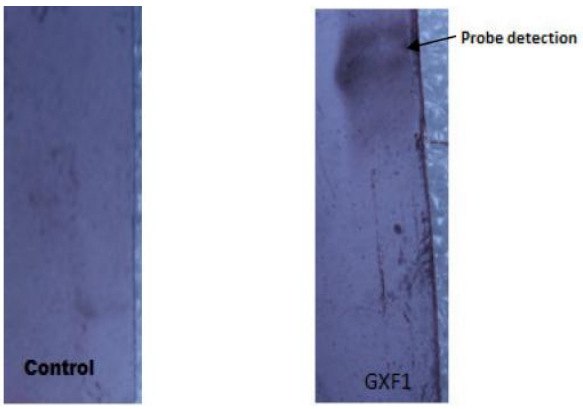

Figure 3: Confirmation of yeast transformation by Southern blotting.

This result confirms the successful introduction of the GXF1 gene into the yeast host. The molecular evidence obtained from Southern blotting complements the

phenotypic selection and supports the identification of the recombinant strain carrying the GXF1 expression plasmid. The episomal plasmid system enabled consistent maintenance of the GXF1 construct during strain validation, providing a stable genetic background for subsequent functional analyses of xylose uptake.

Xylose uptake profile

The growth profile of GXF1-transformed and control *S. cerevisiae* strains on glucose was comparable throughout the cultivation period (Result not shown). A slight advantage was observed in GXF1-transformed cells (μ = 0.019/h) compared to the control (μ = 0.018/h). This minor difference may be attributed to the dual substrate specificity of GXF1, which functions as both a xylose and hexose transporter (Leandro et al., 2006), thereby facilitating glucose uptake in addition to xylose.

Growth in xylose as a carbon source

In contrast, the difference between the strains (GXF1-transformed and control) was more pronounced when xylose was provided as the sole carbon source (20 g/l). Both strains exhibited a brief lag phase of approximately 9 hours, but their growth trajectories diverged significantly thereafter (Figure 4). Control cells displayed negligible further growth, reflecting the inherent inability of wild-type *S. cerevisiae* to efficiently metabolize xylose (Barnett et al., 1983). By comparison, the GXF1-transformed strain showed sustained exponential growth between 9 and 30 hours and reached a higher final biomass yield than the control strain (Figure 4).

The quantitative growth analysis supported this phenotypic difference. The specific growth rate of the GXF1-transformed (μ = 0.022/h) was nearly fourfold higher than that of the control (μ = 0.00572/h), representing a 249.65% increase. An independent t-test confirmed the statistical significance of this difference ($p = 0.0015$, $\alpha = 0.05$), supporting that the observed growth enhancement is associated with GXF1 expression. Thus, the introduction of the GXF1 transporter appears to enhance xylose uptake and utilization, which is associated with improved growth under the conditions tested. These findings suggest that sugar transport represents an important factor influencing xylose utilization and indicate that GXF1 expression can contribute to improved xylose uptake in *S. cerevisiae*. The slight increase in xylose-based growth observed in the control strain may be explained by the promiscuous activity of native hexose and galactose transporters, which can exhibit weak xylose transport activity (Hamacher et al., 2002; Sedlak & Ho, 2004; Young et al., 2011).

Previous studies have reported even higher growth rates following GXF1 expression—for example, an increase from μ = 0.070 to 0.081/h (Parachin et al., 2011). These enhanced performances likely result from additional genetic modifications, including overexpression of non-oxidative pentose phosphate pathway (PPP) genes, co-expression of XR, XDH, and XK, and deletion of GRE3. In contrast, the present study employed a simple haploid laboratory strain without further pathway engineering, which explains the lower, yet still significant, improvement in growth rate (μ = 0.022/h).

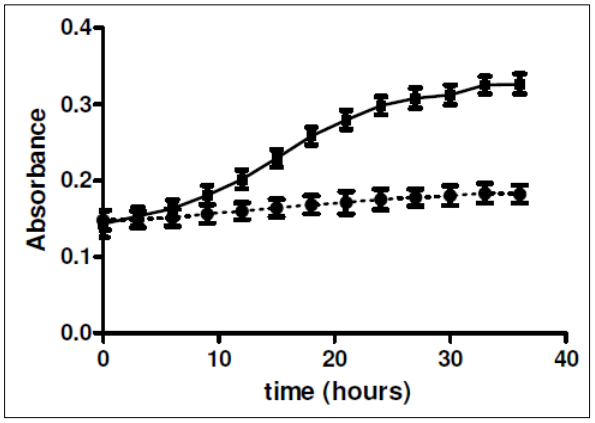

Figure 4: Growth kinetics of GXF1-transformed (solid line) and control (dotted line) *S. cerevisiae* in xylose as a carbon source.

Extracellular and intracellular xylose content

Xylose transport efficiency was evaluated by monitoring both extracellular depletion (Figure 5A) and intracellular accumulation (Figure 5B) over 30 hours using phloroglucinol assay. In both cases, GXF1-transformed *S. cerevisiae* showed markedly improved uptake compared to control cells. Extracellular xylose concentration decreased more rapidly in GXF1 transformants with a 28.3% reduction (19.50 to 13.97 mg/ml) compared to only 12.5% in control cells (19.73 to 18.26 mg/ml), a difference that was statistically significant ($p = 0.015$) (Figure 5A).

Correspondingly, intracellular accumulation was significantly higher in GXF1- transformed strains ($p = 0.035$), increasing by 15.8% (0.152 to 0.176 mg/ml) versus only 5.9% (0.151 to 0.158 mg/ml) in control cells (Figure 5B). A strong negative correlation ($r = –0.99$) between the extracellular depletion and the intracellular accumulation confirmed that xylose transport kinetics were tightly coupled. The plasmid-based expression of a xylose transporter from *Arabidopsis thaliana* increased intracellular xylose accumulation in a strain

not engineered for xylose utilization (Hector et al., 2008).

However, it should be noted that in this study, the decrease in xylose content is not same as the increase in xylose content (Figure 5). Approximately 5.53 mg/ml of xylose was consumed from the medium, whereas only 1.47 mg/ml accumulated intracellularly due to the presence of pGXF1. The portion of xylose not accounted for intracellularly may have contributed, at least in part, to ethanol production or could have been diverted toward other metabolites, such as xylitol (Zhu et al., 2021).

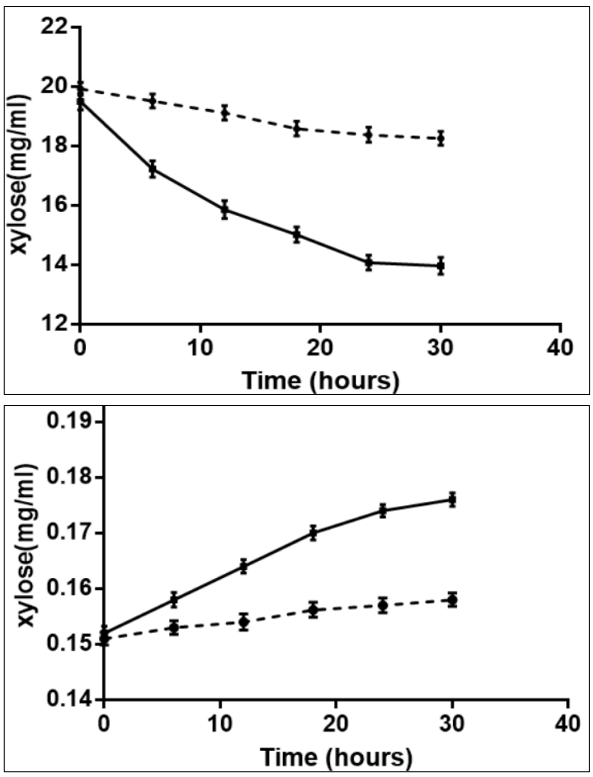

Figure 5: The extracellular **(**upper**)** and intracellular **(**lower**)** xylose concentrations of GXF1-transformed *S. cerevisiae* (solid line) and control (dotted line), measured using the phloroglucinol assay.

Ethanol production

Ethanol production, estimated by the dichromate method, measured at 30 hours, was marginally higher in GXF1-transformed strains compared to controls on both glucose and xylose substrates (Figure 6). On glucose, ethanol titers reached 0.396 mg/ml in the GXF1-transformed versus 0.368 mg/ml in the control cells (7.6% increase). On xylose, ethanol levels were 0.0902 mg/ml and 0.0863 mg/ml respectively representing only a 4.5% improvement. Although GXF1 expression enhanced sugar utilization, the ethanol gains remained modest, particularly under xylose conditions.

Ethanol production was only modestly increased in GXF1-transformed strains, with yields on glucose showing a slight improvement and xylose-derived ethanol remaining nearly fourfold lower (Figure 6). These results suggest that sugar transport is not the only limiting step for ethanol biosynthesis in *S. cerevisiae*. For glucose, this is expected since the yeast already harbors a broad repertoire of efficient hexose transporters (Boles & Hollenberg, 1997; Wieczorke et al., 1999; Diderich et al., 2001). Furthermore, glucose serves as the preferred carbon source and exerts strong regulatory control through carbon catabolite repression, which ensures its prioritized consumption while delaying utilization of other alternative sugars (Gancedo, 1998; Santangelo, 2006; Belinchón & Gancedo, 2007a, 2007b; Conrad et al., 2014).

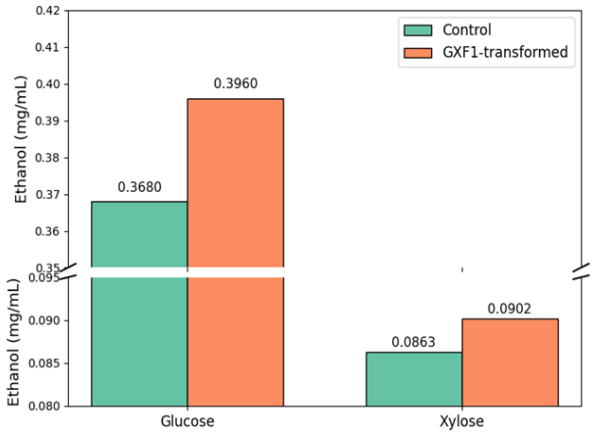

Figure 6: Ethanol production by GXF1-transformed and control yeast using glucose or xylose. The ethanol production was measured by dichromate test.

In the present study, the ethanol yield from xylose was likely constrained by downstream metabolic inefficiencies rather than transport capacity. Previous studies have shown that overexpression of GXF1 accelerates xylose uptake but often diverts carbon into xylitol due to the redox imbalance inherent in the XR/XDH pathway (Runquist et al., 2009; Fonseca et al., 2011). Even after the deletion of the major xylose reductase gene (GRE3) in yeast, residual xylose reductase activity can still sustain xylitol formation, leaving ethanol production largely unaffected. Higher ethanol titers have only been achieved when transport engineering was combined with rational or non-rational metabolic engineering. For example, co-expression of XYL1, XYL2, and XKS1 with a plant xylose transporter enhanced ethanol production to 1.5 g/l (Hector et al., 2008), while introduction of xylose isomerase, xylulokinase, and the *Pichia stipitis* SUT1 transporter, followed by adaptive evolution, yielded up to 0.43 g ethanol per gram xylose (Madhavan et al., 2009). Adaptive laboratory evolution has also proven effective in improving xylose consumption rates in GXF1-expressing strains (Diao et al., 2013). Beyond transporter integration, optimization of the xylose reductase (XR) node, particularly introducing cofactor preference for NADH instead of NADPH, has been

shown to reduce xylitol accumulation and increase ethanol yield (Olofsson et al., 2011). Cofactor balancing is thus a critical strategy, as highlighted in recent studies on tailoring redox metabolism for improved microbial performance (Regmi et al., 2024).

In our study, the absence of a dedicated xylose-metabolizing background strain may have likely further constrained flux through the pentose phosphate pathway, limiting ethanol conversion. Although xylitol accumulation was not quantified, it remains a plausible factor contributing to the modest increase in ethanol observed in GXF1-transformed yeast. Overall, these findings indicate that while GXF1 enhances substrate uptake, ethanol production remains restricted by downstream metabolic bottlenecks. The ethanol concentrations obtained (~0.09 mg/ml on xylose vs. ~0.40 mg/ml on glucose) reflect the long-standing challenge of efficient pentose fermentation in *S. cerevisiae*. Transporter engineering alone, therefore, seems insufficient; and to achieve industrially relevant yields will require integrated strategies that couple transporter enhancement with redox balancing, pathway optimization, and process-level improvements. Such a systems-level approach is essential for unlocking the full potential of xylose fermentation and advancing lignocellulosic bioethanol production using *S. cerevisiae*. Interpretation of these findings should take into account the limited number of biological replicates and the use of colorimetric assays for metabolite estimation. Accordingly, the observed differences are discussed as indicative trends rather than definitive quantitative effects.

Conclusion

Expression of the *Candida intermedia* xylose transporter GXF1 in *Saccharomyces cerevisiae* DTY165 enhanced xylose uptake, intracellular accumulation, and growth on xylose as the sole carbon source. The GXF1-transformed strain exhibited a higher specific growth rate and faster extracellular xylose depletion than the control. However, ethanol production from xylose remained low, indicating that improvement in xylose transport alone is not sufficient to support substantial ethanol formation. The study was conducted in a non-xylose-engineered *S. cerevisiae* background and relied on colorimetric assays for metabolite quantification, which limits quantitative resolution. Nevertheless, these findings demonstrate that enhanced xylose transport can contribute to improved xylose utilization, while highlighting the need for additional engineering of downstream xylose-metabolizing pathways to enable more effective conversion of xylose to ethanol.

Acknowledgements

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Research Article

Spatio-temporal Assessment of the Bagmati River Water Using Real-Time Data

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ABSTRACT

Water pollution and tributaries are deteriorating the water quality of the Bagmati river, especially in Kathmandu valley. Mostly, the water quality of the Bagmati river is characterized using traditional methods, therefore, have been unable to identify sources of pollutants. In this study, real-time continuous monitoring and collecting data in a large volume using sophisticated sensors were performed. The diurnal, spatial, daily and temporal variations of physicochemical water quality parameters were presented for an in-depth understanding of the real status of the Bagmati river. The spatial variation of water quality parameters indicated that conductivity was drastically increased (605.41 ± 8.9 to 1145.30 ± 9.32 µS/cm), and the oxidation-reduction potential (ORP) was excessively reduced (28.09 ± 6.88 to -306.75 ± 4.95 mV) after mixing of the tributaries. The high conductivity (>605.41 ± 8.9 µS/cm and negative ORP values (-40.29 ± 32.14 to -306.75 ± 4.95 mV) are an indication of a sewer connection. Daily, diurnal and temporal variations suggested that the Bagmati river is always polluted with a negative ORP value (<-50 mV) in the daytime, and river quality worsens day to day. In conclusion, it is important to control the mixing of effluent into the Bagmati river and its tributaries to revive the river ecosystem and prevent loss of survives.

Keywords: Oxidation-reduction potential; Real-time data; River water pollution; Temporal variation; Tributary

Introduction

The Bagmati river enters the Kathmandu valley at Sundarijal, passes through a densely populated area, and exits at the Chovar. Rapid, unmanaged urbanization generates excessive solid waste and sewage in core city areas within the Kathmandu Valley (MoUD, 2017). Solid waste and effluent from municipal, hospital and industrial outlets are dumped directly into the river (Bhandari et al., 2021; Pant et al., 2021). The river is converting into one of the easily accessible dumping sites for solid wastes and untreated domestic, industrial and agricultural effluents (Kannel et al., 2007; Mishra et al., 2017; Adhikari et al., 2024). It was reported that the Bagmati river is in pristine condition near the Sundarijal, however, it is critically polluted downstream (Regmi, 2013; Mishra et al., 2017). Tributaries drain into the mainstream, serve as important habitats and

carry pollutants that contribute unique conditions of the mainstream. The tributaries of the Bagmati river are the most promising factors that modify the water quality downstream (Adhikari et al., 2021). The polluted tributaries, such as Monohara, Dhobikhola, Tukhucha, Bishnumati and Balkhukhola, load contaminants into the Bagmati river between the Shankhamul and Balkhu sites. Due to the mixing of effluent and solid waste, water quality of the Bagmati river downstream is worsening day by day (Mishra et al., 2017; Adhikari, 2020), destroying the river ecosystem and harming the health of people living around it.

The assessment of the Bagmati river and its tributaries reported that conductivity increased and oxidation-reduction potential (ORP) decreased continuously downstream of the river. The dissolved oxygen was almost zero, and the ORP decreased from -100 to -263 mV after mixing of the five polluted tributaries (Adhikari et al., 2021). The water quality index (WQI) was very high, indicating extremely polluted water unfit for domestic as well as agricultural and industrial use (Adhikari et al., 2024). The deterioration of river water quality highlights the necessity of continuous monitoring of water quality to generate awareness among people and carry out the treatment of industrial and domestic waste before its disposal into the river water. Pal et al. (2019) suggested the main river and tributaries are equally polluted, hence, need to take action for sustainability of the aquifer. Previous studies used traditional monitoring with limited data points, therefore, it is not possible to predict the time dependency of river water quality. To pinpoint the sources and time dependency of pollutants, instead of traditional water quality monitoring methods, an advanced monitoring system with fine-scale and real-time data logging was used in this study. A detailed assessment including spatial, diurnal, daily and temporal variations of river water quality in the Bagmati river was analyzed by logging a huge data set. This is a very new approach to monitor/characterize water quality parameters. The time dependency data will provide clear insight of real status of river water and the effects of human activities on the degradation of river water quality. Since it is not possible to collect data of all water quality parameters in real-time and on a fine-scale basis, this study considered that conductivity, dissolved oxygen (DO) and oxidation-reduction potential (ORP) are the most useful parameters indicating sewer pollutants.

Materials and Methods

The daily, diurnal and temporal variations of the Bagmati river water quality was monitored at Shankhamul site. The data on daily variation was collected during the pre-monsoon season in May 2024 on the date of 3, 10, 14, 19, and 21 May. Diurnal and temporal variations were conducted on May 21, 2023, and May 19, 2024. The spatial variation of water quality was determined by collecting data from six different positions along the Bagmati river from Shankhamul to Balkhu sites. Data were continuously collected and logged every 10 second from 6 stations. The observation started from the upstream site of the Manohara tributaries at Shankhamul (B-1), the second data was collected from upstream of Dhobikhola at UN park (B-2), the third was after the confluence of the Dhobikhola (B-3), the fourth from Thapathali after the confluence of Tukuchakhola (B-4), the fifth from Kuleshwor after the confluence of Bishnumati river, and sixth from Balkhu after the confluence of Balkhukhola (B-6) (Figure 1). Real-time fine-scale water quality data were measured using an advanced multi parameter analyzer (HANNA instruments, Hi-9829) (Adhikari et al., 2024). This study mainly focuses on the collection of continuous real-time data to understand the time dependency of river water quality and to point out the source of pollutants. Most commonly, water quality can be characterized by measuring physicochemical parameters for example pH, alkalinity, conductivity, ammonia, turbidity, dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD) and heavy metals. The monitoring of all these parameters in real-time basis is tough. Therefore, physicochemical parameters such as temperature, pH, turbidity, total dissolved solids (TDS), oxidation-reduction potential (ORP), dissolved oxygen (DO) and conductivity were measured and logged on a real-time basis. Based on the previous studies (Adhikari et al. 2021, Pant et al. 2021), it is considered that the conductivity and total dissolved solids are useful to predict the level of contaminants, dissolved oxygen, and oxidation-reduction potential are useful to predict the sewer pollution.

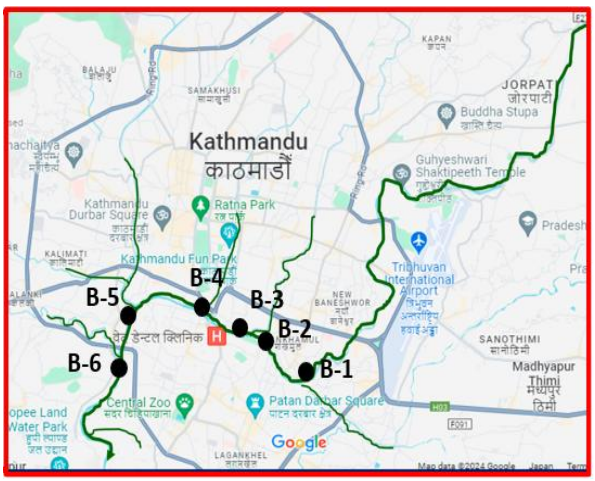

Figure 1: Sampling sites in the Bagmati river, Kathmandu, Nepal.

Results and Discussion

Spatial variation of water quality parameters

The physicochemical characteristics of water were determined along the Bagmati river by collecting data from six different sites. The sampling sites were chosen to determine the source of pollutants and the effect of tributaries on the main river. Data were collected same time but on different days to minimize the effect of time on the analysis. More than 150 data sets were collected from each station. Data recorded from 11:50 to 12:15 in May 2023. The observed water quality parameters are shown in Figure 2. The river water temperature varied from 23.87 ± 0.34 to 28.31 ± 0.19 °C may be due to variation of air temperature on different days (Figure 2a). The observed turbidity was also low, 110.48 ± 2.32 FNU at the B-1 and higher than 167 FNU at other observation sites (Figure 2b). The increase of turbidity in downstream was also reported (Pal et al., 2019). It may be due to the mixing of soil or effluent in the river water (Costa et al., 2015).

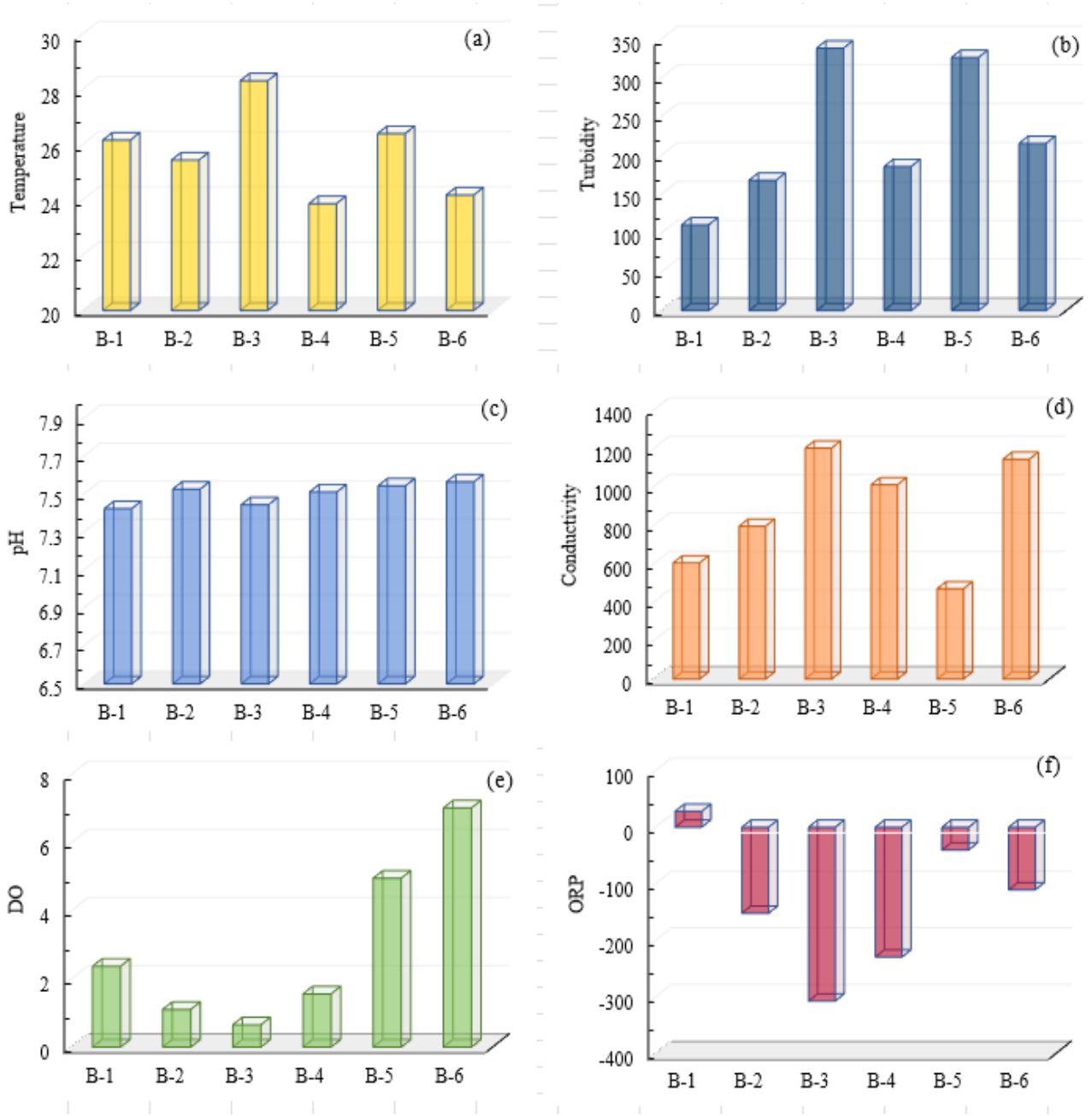

Figure 2: Spatial variation of water quality parameters (a) Temperature (°C), (b) Turbidity (FNU), (c) pH, (d) Conductivity (µS/cm), (e) DO (ppm), and (f) ORP (mV).

The pH of river water was comparatively low (7.42±01) upstream and increased slowly to 7.56 ± 0.01 near the end of the observation at the Bagmati river (Figure 2c). Similar results were reported by Adhikari et al. (2021). Water with pH less than 6.5 could be soft while above 8.5 could be hard and corrosive (Modoi et al., 2014;

WHO, 1999). The slightly alkaline pH of river water may be due mixing of ammonical compounds from domestic and agricultural effluent (Aggarwal & Arora 2012), and/or microbial decomposition of organic matters (Sundararajan et al., 2018). The conductive ions present in the water are related to conductivity. The comparatively low conductivity (470 ± 7.46 µS/cm) was observed at B-5, the conductivity was more than two times at B-3 (1203.24 ± 1.30), B-4 (1012.24 ± 3.60) and B-6 (1145.30 ± 9.32). It was increased almost continuously downstream (Figure 2d). Another vital parameter of the water quality is the dissolved oxygen (DO). The concentration of DO was less than 3 ppm B-1 to B-4, and it was high at B-5 and B-6 (Figure 2e). The oxidation-reduction potential (ORP) is useful to measure the water disinfection potential in wastewater treatment (Suslow et al., 2004; Goncharuk et al. 2010). The reducing agent decreases the ORP value, and the oxidizing agent increases the ORP value. The observed ORP of river water was positive and was 28.09±6.88 upstream at B-1, it was less than -50 mV at other observation sites, and it varied from -40.29 ± 32.14 to -306.75 ± 4.95 (Figure 2f). The 50 to 250 mV ORP is suitable for the nitrification and organic compounds degradation (Suslow et al., 2004; Goncharuk et al., 2010).

The denitrification occurs from +50 to -50 mV and biological phosphorus and sulfide release between -50 and -250 mV of ORP. The positive ORP value at B-1 indicates the possibility of nitrification of river water, however low ORP (< -50 mV) downstream suggests that the river water is extremely polluted with reducing substances from wastewater. The water with low ORP produces biological phosphorus and sulfide, generating a stinky smell that is toxic and responsible for an unhealthy environment around it and for aquatic animals (Suslow et al., 2004; Goncharuk et al., 2010). The low ORP value but high DO concentration at B-5 and B-6 may be due to the dissolution of atmospheric oxygen at the dam at Kuleshwor bridge and at Balkhukhola.

Diurnal variation of water quality parameters

Real-time continuous data on water quality parameters were collected in the pre-monsoon period on 21 May 2023 and 19 May 2024. Diurnal variations of water quality parameters were plotted in Figure 3. The temperature shows similar diurnal variations on both days, increasing slowly with time and decreasing slowly after 14:00. The maximum temperature was observed at 14:00 on both observation days (Figure 3a). The diurnal variations of conductivity on 19 and 21 May were slightly different. It was nearly double on 19 May 2024 than on 21 May 2023. The highest recorded conductivity was about 628 µS/cm, and the lowest value was about 526 µS/cm on May 21. It was 1123 and 780 µS/cm respectively on May 19 (Figure 3b). The pH was slightly alkaline and variation was less pronounced on 21 May (7.43 to 7.56), but it was neutral to slightly acidic and slowly decreased at afternoon on 19 May (7.1 to 6.8) (Figure 3c). Total dissolved solids were about 263 ppm initially and slowly increased with time and reached 360 ppm at evening on 21 May. It was almost two times high on 19 May 2024. The observed TDS was as high as 560 ppm on 19 May 2024 (Figure 3d). The maximum TDS of 612 ppm downstream was reported in previous study (Pal et al., 2019). The previous research concluded that anthropogenic wastes, for example, municipal and industrial, are responsible to increase conductivity and TDS downstream. Dissolved oxygen (DO) in water increases with atmospheric oxygen and photosynthesis, but it is decreased by biochemical and chemical phenomena occurring in water (Athokpam et al., 2014; Aniyikaiye et al., 2019). The dissolved oxygen was high (>6ppm) initially and decreased linearly with time up to 1 ppm at noon, and increased slowly and reached 2 ppm in the evening on 21 May (Figure 3e). It was very low, < 1 ppm, on 19 May in whole observed day though a slightly higher value was observed initially.

A very low concentration of DO indicates that it is almost impossible for aquatic animals to survive on it. The ORP value was initially positive and linearly decreased to negative on 21 May, however, it was always negative on May 19. The decreasing pattern of ORP value was almost similar on both days, though values were different (Figure 3f). The diurnal variation patterns of DO and ORP on 21 May were similar to those of DO and ORP reported by Adhikari et al. (2019). Due to the very low concentrations, the variation of DO on 19 May was not pronounced, but ORP showed a similar trend. All the results suggested that the river water at the Shankhamul region is influenced by local pollutants from human activities. All measured water quality parameters indicated that the river water consists of more contaminants in the year 2024 than in 2023. The comparatively high concentration of conductivity, low concentration of DO, and low values of pH and ORP reveal that the Bagmati river water quality is degrading continuously at an alarming rate.

It suggests the necessity of the implementation of strict rules and regulations to control anthropogenic activities toward river water pollution for the prevention of further degradation of the holy river.

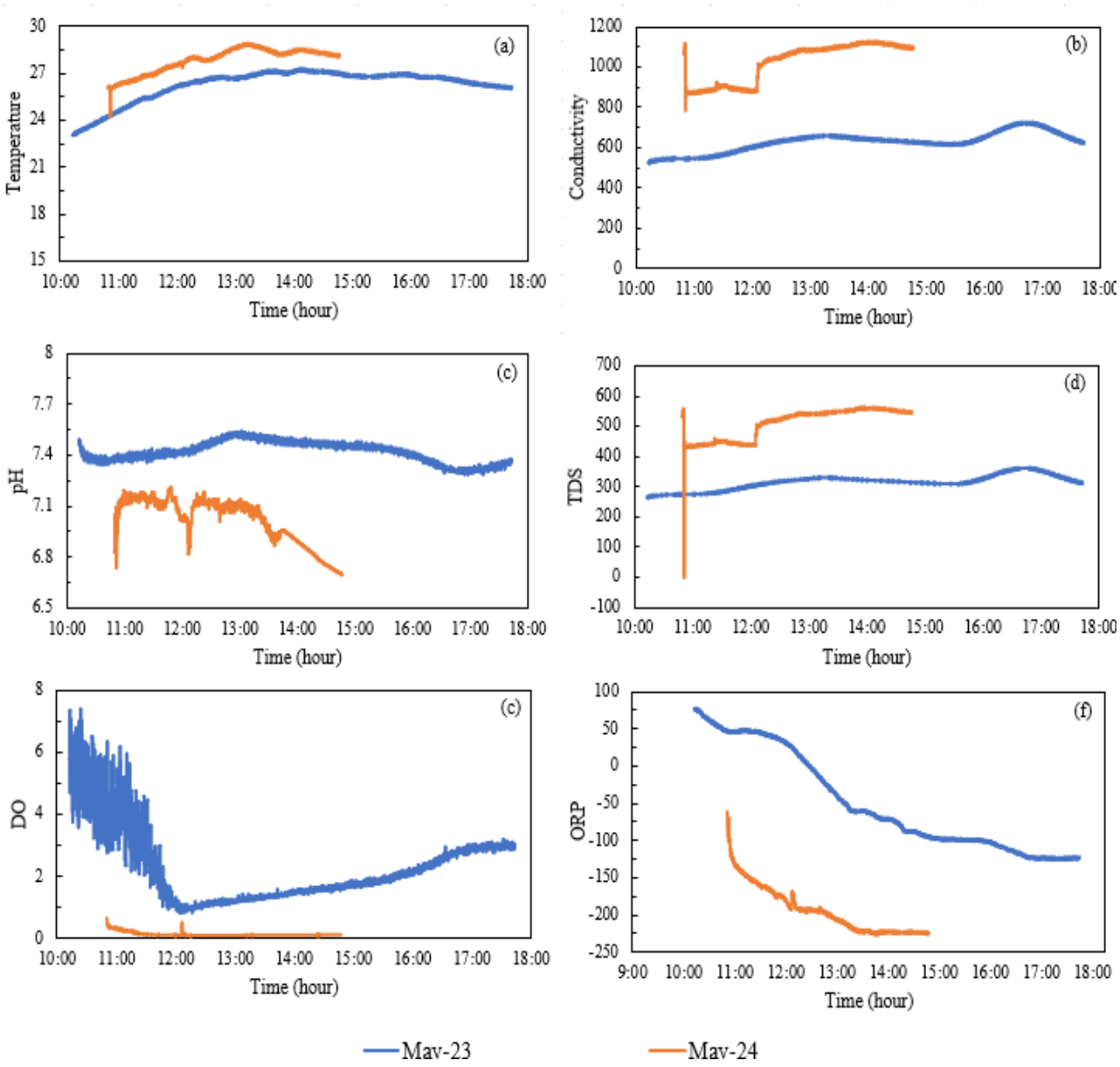

Figure 3: Diurnal variations of water quality parameters (a) Temperature (°C), (b)Conductivity (µS/cm), (c) pH, (d) TDS (ppm), (e) DO (ppm), and (f) ORP (mV) measured on May 21, 2023, and May 19, 2024.

Daily variation of water quality parameters

Daily variations of water quality parameters of the Bagmati river were observed in pre-monsoon (May 2024). The observed temperature, ORP, pH, DO, conductivity and turbidity were plotted in Figure 4. The water temperature ranged from 24 to 29 °C during the pre-monsoon period (Figure 4a). The turbidity was as high as 287 FNU on Day 2, but less than 10 on other days (Figure 4b). The pH was comparatively high on Day 1 and slowly decreased afterward (Figure 4c). The conductivity was as high as 1020 µS/cm on Day 1 and was lowest on Day 2 (413 µS/cm), higher than >850 µS/cm on the other days (Figure 4d).

The dissolved oxygen was less than 0.5 ppm on all days except on Day 2 (2.47 ppm) (Figure 6e). The ORP value was always negative (< -100 mV) except on Day 2 (39.5 mV) (Figure 4f). The observed water quality of Day 2 was different than that of other days. The conductivity was low, dissolved oxygen was high, and the ORP value was positive on this day, although all data was collected on same time. All the parameters indicated that the river water was less polluted on this day. It was observed from the recorded weather system that on Day 2 (10 May), the heavy rainfall occurred from 2:15 to 3:15, and light rain occurred at 5:15. Therefore, it is considered that the heavy rainfall reduced the pollutants and enhanced dissolved oxygen, which enhanced the ORP value on Day 2 (10 May 2024). The dissolved oxygen and ORP values indicated that the water quality of the Bagmati river was always heavily polluted. The very low ORP indicated that the water consists of organic pollutants that consume dissolved oxygen excessively.

The negative ORP value (< -50 mV) suggested that there were excessive amounts of organic contaminants from the sewer which excessively reduced dissolved oxygen. The results suggested that the inflow of domestic effluent continuously loads pollutants in the Shankhamul region. This is the reason why the dissolved oxygen remains low on all days. The analytical results suggest that the Bagmati river water is highly influenced by anthropogenic activities, which is reduced to some extent by heavy rainfall.

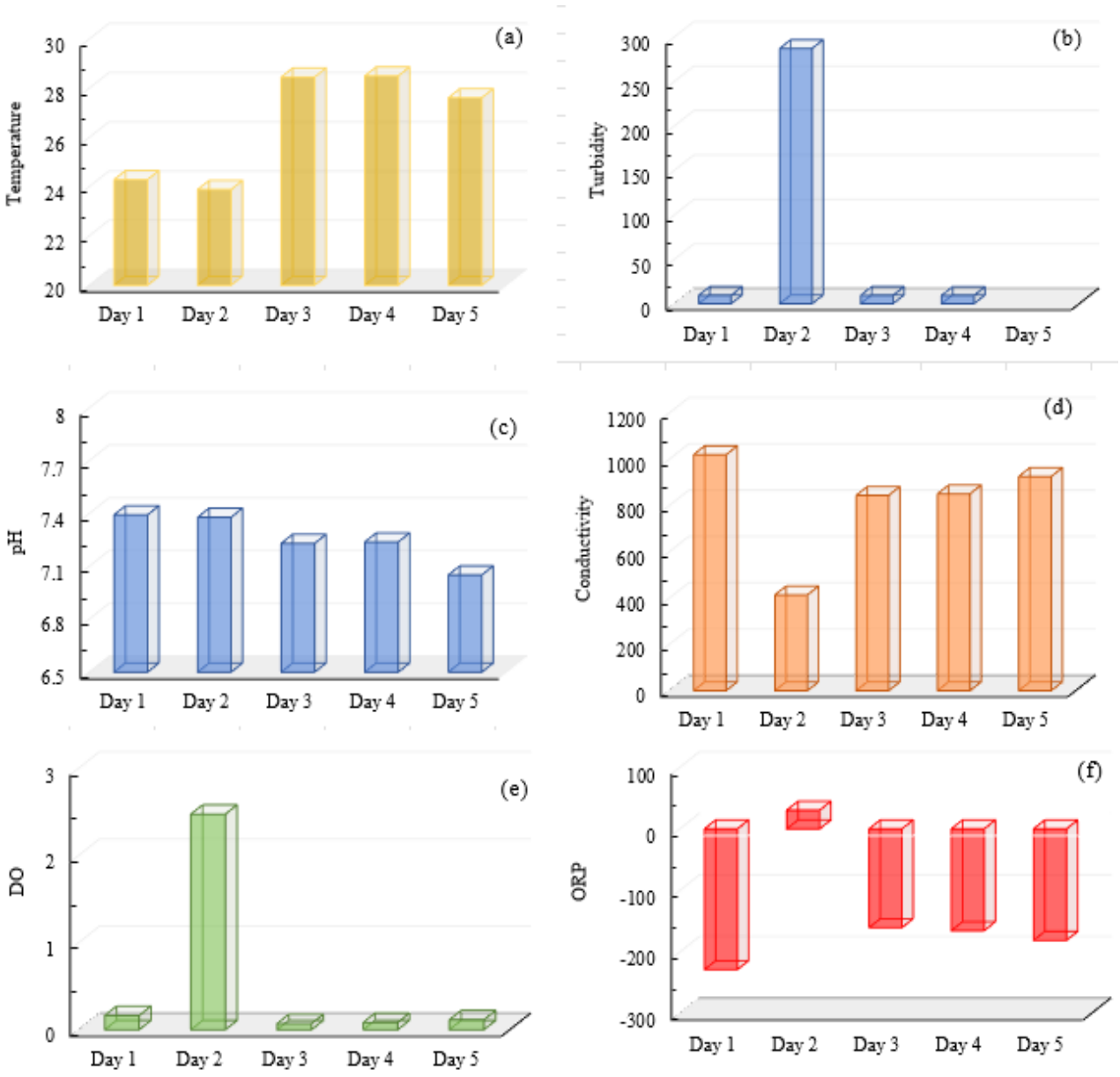

Figure 4: Daily variation of water quality parameters (a) Temperature (°C), (b) Turbidity (FNU), (c) pH, (d) Conductivity (µS/cm), (e) DO (ppm), and (f) ORP (mV) in May 2024 at the Shankhamul site.

Conclusion

This study collected fine-scale real-time data of different water quality parameters along the Bagmati river. The diurnal, spatial, daily and temporal variations of water quality parameters were analyzed. The analytical results suggested that river water was radically changed after mixing of the tributaries. The measured conductivity was 605.41 ± 8.9 µS/cm at Shankhamul before confluence of tributaries, after mixing of the Dhobikhola tributary, it increased to 1203.24 ± 1.3 µS/cm, and it was 1145 µS/cm at the end of the observation at Balkhu. Further, oxygen reduction potential (ORP) was excessively reduced after mixing of tributaries. it was positive and comparatively high (28.09 ± 6.88 mV) at Shankhamul but reduced to a negative value as low as -306 mV after mixing of Dhobikhola, indicating extremely polluted conditions with large amounts of reducing pollutants. The river water quality was more degraded temporally i.e., worse in the year 2024 than in 2023. The diurnal variation

suggested that the daytime increment of contaminants is due to human activities. The excessively negative ORP suggested the polluted river water was responsible for the stinky smell. The daily variation of water quality parameters showed that the monsoon rain reduced pollutants to some extent; however, effluents quickly worsened the quality of the Bagmati River. The contaminants consume almost all the oxygen; hence, living organisms cannot get enough oxygen to survive in it. In addition, it spreads a hazardous fouling smell along the bank of the river. This study investigates the temporal, daily, diurnal, and spatial conditions of the Bagmati River water, useful for the researcher and policy maker to understand the ecological condition of the holy river. Further continuous study is necessary to improve the river water quality by generating awareness and taking action on sewer management.

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Research Article

In vitro Inhibitory Action of Honey Against Extended Spectrum β-Lactamase Producing *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Antibiotic resistant Gram-negative bacteria, in particular, extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* are frequently involved in various human infections. Despite traditional applications of honey, a comprehensive evaluation of antimicrobial activity against ESBL-producing bacteria is lacking. This study aims to evaluate the antibacterial activity of honey against ESBL-producing and non-producing bacteria isolated from clinical samples (urine and sputum) by tube dilution method and the time kill viability assay. The minimum inhibitory concentration (MIC) of honey was found to be 25% (v/v) against most of *E. coli* (13 out of 18 isolates were inhibited by both indigenous and Dabur honey) and 50% (v/v) against most of *K. pneumoniae* (10 and 11 out of 14 isolates inhibited by indigenous and Dabur honey respectively). The MIC of honey for ESBL-producing and non-producing bacteria was found to be almost similar ($p > 0.05$). The complete reduction of bacterial population (8 $\log_{10}$ CFU/ml) was observed after 24 hours of exposure to honey at 50% (v/v) concentration. The honey samples showed bacteriostatic and bactericidal activity against tested isolates of ESBL-producing and non-producing *E. coli* and *K. pneumoniae*. These findings suggested indigenous honey serve as a promising complementary therapeutic agent in managing resistant bacterial infections.

Keywords: ESBL; *E. coli*; Honey; *K. pneumoniae*; Time kill assay

Introduction

Antibiotic resistant bacteria pose a serious threat to public health. The frequencies of bacterial resistance, to all kinds of antibiotics including the last resort drugs, are increasing worldwide. Among them, extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* are major bacterial pathogens being isolated and reported from urine globally (WHO, 2017). The high occurrence of ESBL producing bacteria has not only been reported in clinical settings but also from environmental samples (Bhatt et al., 2007; Talukdar et al., 2013; Zhang et al., 2016;

Khanal et al., 2025). Considering the ever-growing pool of antimicrobial resistance among bacterial pathogens, there is an urgent need for antimicrobial agents which can be used as an alternate therapeutic option against ESBL infection.

Various herbal and animal extracts have shown antimicrobial characteristics against different pathogens (Bajracharya et al., 2008; Stephens et al., 2010; Khan et al., 2013). Moreover, some plant extracts exhibit antibacterial effect against multidrug-resistant human pathogens (Shalayel et al., 2017). At present, considerable focus is being directed toward the use of honey as an antimicrobial agent. This growing interest concerns the adverse effects of antibiotics in humans, as well as the declining effectiveness of conventional antibiotics against the resistant pathogens. Many researchers have reported the antibacterial activity of honey against several pathogenic bacteria those are aerobes, anaerobes, Gram positives, Gram negatives and multidrug-resistant (Kwakman et al., 2008; Al-Waili, 2013; Wasihun & Kasa, 2016). The high reducing sugar, osmotic nature, low pH (Kwakman & Zaat, 2012), ability to produce hydrogen peroxide (H_2O_2), bee defensin-1 (Kwakman et al., 2011), phenolic compounds, methylglyoxal (MGO) (Stephens et al., 2010), glycoprotein (Brudzynski & Sjaarda, 2015), flavonoids (Al-Waili, 2013) and lysozyme (Molan, 1992) are the key factors attributing an inhibitory effect on bacteria. The antimicrobial potency of indigenous honey and Dabur honey against ESBL producing bacteria has not been assessed in Nepal. Investigating this could provide valuable insight into the potential of locally available natural products as alternative and adjunct therapeutic option against multidrug-resistant pathogens. Moreover, such evidence may support the development of cost-effective, sustainable strategies to combat antimicrobial resistance in resource-limited healthcare settings. Thus, this study aimed to investigate the possible application of honey as an effective and safe alternative for the treatment of multiple antibiotic-resistant infections, in particular those caused by ESBL producing *E. coli* and *K. pneumoniae*.

Materials and Methods

Test bacterial pathogens

Two bacterial pathogens, *E. coli* and *K. pneumoniae,* were isolated from non-invasive urine (n = 838) and sputum (n = 34) samples from the patients who were requested for microbiological investigation (culture) during the study period from October 2017 to March 2018. Collected specimens were inoculated in parallel on Blood agar and MacConkey agar and incubated at 37 ºC for 24 hours. The isolated bacterial colonies were sub-cultured on nutrient agar and identified based on their colony morphology, Gram's staining and biochemical characteristics as described by Cheesbrough (2006). The study was conducted after ethical approval from the Institutional Review Committee of Human Organ Transplant Center, Bhaktapur, Nepal (Approval Ref. No. 074/75 dated 2017.09.22).

Antibiotic susceptibility test

Antibiotic susceptibility test of *E. coli* and *K. pneumoniae* was done on Mueller-Hinton agar (MHA) by the modified Kirby-Bauer disk diffusion method. The commercially available antibiotics, meropenem (10 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), piperacillin/tazobactam (100/10 µg), amoxicillin (20 µg), ofloxacin (5 µg) and ciprofloxacin (5 µg) were selected and test results were interpreted according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2016). Control strain of *E. coli* ATCC 25922 was used for quality assurance and comparison. Bacteria showing resistance to at least one of these antibiotics were referred as multidrug resistant bacteria.

Phenotypic test for ESBL production

ESBL producing isolates were screened based on the size of inhibition zone for ceftazidime (< 22 mm) (CLSI, 2016). A phenotypic confirmatory test was carried out by combining disc diffusion method following the standard protocol (CLSI, 2016). The ESBL producing isolates were confirmed by an increase in the diameter of the inhibition zone by ≥ 5 mm due to the combined disc with ceftazidime-clavulanate (30/10 µg) than ceftazidime alone.

Honey sample

Indigenous honey was collected from Natural History Museum, Tribhuvan University whereas Dabur honey was purchased from the local market. The diluted honey samples (50%) were sterilized by using membrane filter of pore size 0.45 µm. Sterility of honey samples was checked by streaking on nutrient agar plate and incubated at 37 ºC for 24 hours and assurance of sterility was confirmed only after 7 days. Sterile honey samples were stored at room temperature until use.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the honey samples was determined using the broth tube

dilution method (Wasihun & Kasa, 2016). Briefly, one milliliter of honey was serially two-fold diluted with nutrient broth so as to maintain v/v concentrations of 50.0%, 25.0%, 12.5%, 6.25%, 3.125%, 1.562%, 0.781% and 0.391% honey in respective tube. Except for the negative control tube, each tube was inoculated with 0.1 ml of the test bacterial culture of standard inoculum that matched 0.5 McFarland turbidity. The presence and absence of growth (assessed by turbidity evaluation with reference to positive control and negative control) was noted following the 24-hour aerobic incubation at 37 °C. The MIC is the lowest concentration inhibit bacterial growth as detected of visible turbidity. The least concentration that showed no visible growth of organisms inoculated by streaking on nutrient agar at 37 °C for 24 hours considered as the minimum bactericidal concentration (MBC) of the honey (Wasihun & Kasa, 2016).

Time kill viability assay

Time kill assay was done for selected ESBL- producer and non-producer isolates of *E. coli* and *K. pneumoniae* as described by Jayaraman et al. (2010) by using MBC concentration (50% v/v) of the honey samples. To a 1 ml volume of 50% diluted honey sample, 100 µl of 10^8 CFU/ml of bacterial isolate was added. A bacterial suspension of 10^8 CFU/ml was used as a control. Bacterial suspension incubated (37 °C) in the presence of honey sample and without honey (control) were sampled out (100 µl) at 0, 2, 4, 6, 8, 10, 12, 18, 24 and 48 hours, and microbial load was determined. The killing rate was determined by plotting log of viable colony counts ($\log_{10}$ CFU/ml) against time (hour). The time kill curve was drawn using Origin-Pro 2019 software.

Data analysis

All the experiments were done triplicate for reproducibility. For the statistical analysis of the results, SPSS version 21 was used. Fisher's exact test was applied to determine the association between different variables and a $p \leq 0.05$ was considered statistically significant.

Results and Discussion

Out of the total 872 clinical samples (urine = 838 and sputum = 34) collected during the study, 70 samples showed growth of either *E. coli* (n = 43) or *K. pneumoniae* (n = 27). The majority of the isolates were resistant to penicillin and fluoroquinolones but susceptible to carbapenems (Figure 1). Among the total isolates, 77.1% (*E. coli*, n = 33 and *K. pneumoniae*, n = 21) were found to be multidrug resistant (Table 1). On primary screening for ESBL, a total of 39 isolates (*E. coli*, n = 23 and *K. pneumoniae*, n = 16) were resistant to ceftazidime (Figure 1) and therefore suspected as ESBL-producers. Among suspected ones, 43.6% isolates (*E. coli*, n = 10 and *K. pneumoniae*, n = 7) were confirmed as ESBL-producers. All ESBL-producing isolates (n = 17) and randomly selected fifteen ESBL non-producing isolates (*E. coli*, n = 8, and *K. pneumoniae*, n = 7) were used as test organisms for evaluation of the antibacterial activity of honey.

The antibacterial activity of honey samples was assessed by determining minimum inhibitory concentration (MIC) (v/v) value. The results revealed that all the test organisms were inhibited either at 50% or 25% (v/v) concentrations of the tested honey samples. The MIC of indigenous honey (Table 2) and Dabur honey (Table 3) was found to be 25% to 50% for the majority of *E. coli* and *K. pneumoniae* isolates, respectively. The indigenous honey showed bacteriostatic activity at a concentration of 25% for most of the *E. coli* isolates (77.8%) and that of 50% for *K. pneumoniae* isolates (78.6%). Similar results were also revealed by the Dabur honey.

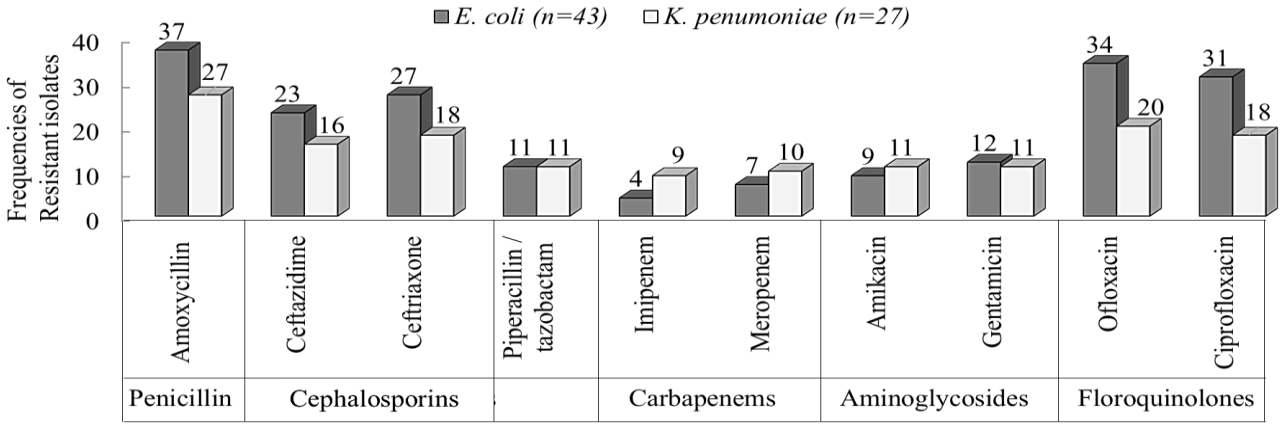

Figure 1: Frequencies of resistant isolates of *E. coli* and *K. pneumoniae* belonging to different classes.

Table 1: Frequencies of multidrug-resistant (MDR) isolates.

	E. coli	*K. pneumoniae*	Total isolate
MDR	33 (76.7%)	21 (77.8%)	54 (77.1%)
Non-MDR	10 (23.3%)	6 (22.2%)	16 (22.9%)
Total isolate	43	27	70

Table 2: Minimum inhibitory concentration (MIC) of indigenous honey against bacterial isolates.

	Bacterial isolate	MIC value of indigenous honey		*p*-value
		25% (v/v)	50% (v/v)	
ESBL producer	*E. coli*	6 (60%)	4 (40%)	0.502
	K. pneumoniae	3 (42.9%)	4 (57.1%)	
ESBL non-producer	*E. coli*	7 (87.5%)	1 (12.5%)	
	K. pneumoniae	1 (14.3%)	6 (85.7%)	

Table 3: Minimum inhibitory concentration (MIC) of Dabur honey against bacterial isolates.

	Bacterial isolate	MIC value of Dabur honey		*p*-value
		25% (v/v)	50% (v/v)	
ESBL producer	*E. coli*	6 (60%)	4 (40%)	0.476
	K. pneumoniae	2 (28.6%)	5 (71.4%)	
ESBL non-producer	*E. coli*	7 (87.5%)	1 (12.5%)	
	K. pneumoniae	1 (14.3%)	6 (85.7%)	

On comparing the bacteriostatic activity of both types of honey on ESBL-producer and non-producer isolates, almost identical activities were observed. Most of the ESBL-producers (52.9%) and non-producer (60%) isolates were inhibited by indigenous honey at 50% and 25% concentration respectively (Table 2). Likewise, Dabur honey at 50% concentration showed inhibitory activity against 64.7% isolates of ESBL-producer and 46.7% non-ESBL isolates (Table 3). The maximum proportion of ESBL isolates and non-ESBL isolates were inhibited at a concentration of 50% and 25% by the two types of honey, respectively. However, the association was statistically insignificant ($p>0.05$). The MBC of both types of honey on tested bacterial isolates was found to be 50% v/v.

The time kill analysis of indigenous and Dabur honey at 50% (v/v) concentration on randomly selected ESBL-producer and non-producer *E. coli* and *K. pneumoniae* during 48 hours is shown in Figure 2 and 3 as time kill curve. The curves were well fitted in the sigmoid curve model. As shown in the curves, both types of honey samples inhibited the growth of all test organisms within 24 hours exposure. Both types of honey showed little effect (<1 $\log_{10}$ CFU/ml reduction) until 12 hours but then after bacterial population decreased rapidly. There was a decrease up to 4 $\log_{10}$ CFU/ml (half of initial population) following 18 hours exposure to the honey samples and complete inhibitions (reduction of total 8 $\log_{10}$ CFU/ml) of bacteria were observed after 24 hours exposure to the honey samples. However, both *E. coli* and *K. pneumoniae* (ESBL and non-ESBL producing) exhibited typical sigmoidal growth curves in absence of honey, with rapid exponential growth during the first 8-12 hours followed by a stationary phase around 9.0-9.1 log CFU/ml (Figure 4).

The primary research question of this study was to understand susceptibility of ESBL-producing bacteria towards the Nepalese honey. The indigenous and Dabur honey samples showed the inhibitory effects either at 25% or 50% v/v concentrations against all the tested isolates of *E. coli* and *K. pneumoniae*. Similar results were also shown by other researchers from different countries (Agbaje et al 2006; Gomashe et al 2014; Ahmed et al 2014). In the present study, the minimum inhibitory concentration for both types of honey against the tested bacteria was observed to be 25-50% v/v. Shah and Williason (2015) found 30-40% v/v concentration of honey were sufficient to inhibit the growth of multidrug resistant bacteria. Likewise, Al Waili et al (2013) investigated antibacterial potential of different types of honey and reported 40% and 40-50% concentrations as MIC for *K. pneumoniae* and *E. coli* respectively. In contrast to this, Tan et al (2009) found that comparatively low concentrations of Tualang and Manuka honey (MIC, 8.75-25%) were inhibitory against wound and enteric microorganisms. This difference in the antibacterial activity of honey over place might be due to the difference in the species of bees and plant sources used for nectar and the difference in the test methods used and test organisms. It may be due to composition of bioactive compounds in honey.

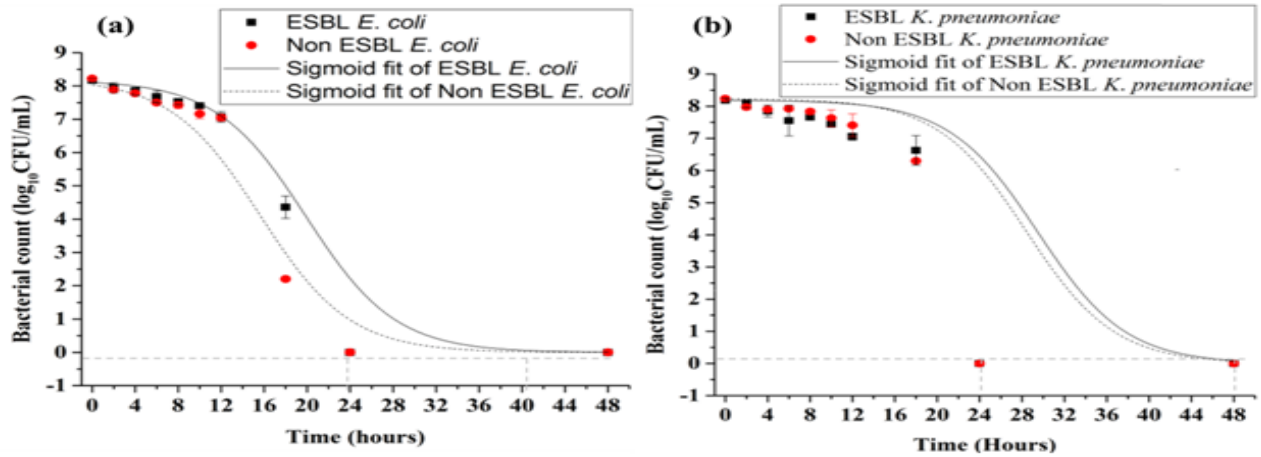

Figure 2: Time kill curve of *E. coli* (a) and *K. pneumoniae* (b) with indigenous honey. Error bars indicate standard deviation from mean. The Sigmoid curve was fitted using OriginPro2019 software. The curve revealed a pattern of slower inhibition rate until approximately 16 hours and then a sharp fall in viable cell numbers. Irrespective of test bacteria, no viable cells were obtained at 24 hours and onwards.

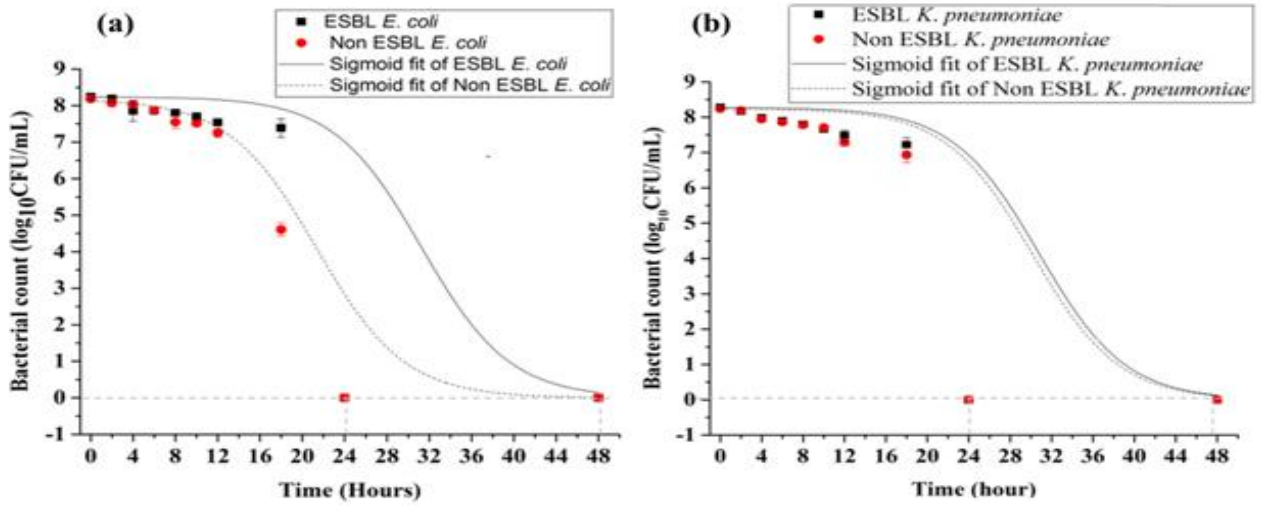

Figure 3: Time kill curve of *E. coli* (a) and *K. pneumoniae* (b) with indigenous honey. Error bars indicate standard deviation from mean. The Sigmoid curve was fitted using OriginPro2019 software. The curve revealed a pattern of slower inhibition rate until approximately 16 hours and then a sharp fall in viable cell numbers. Irrespective of test bacteria, no viable cells were obtained at 24 hours and onwards.

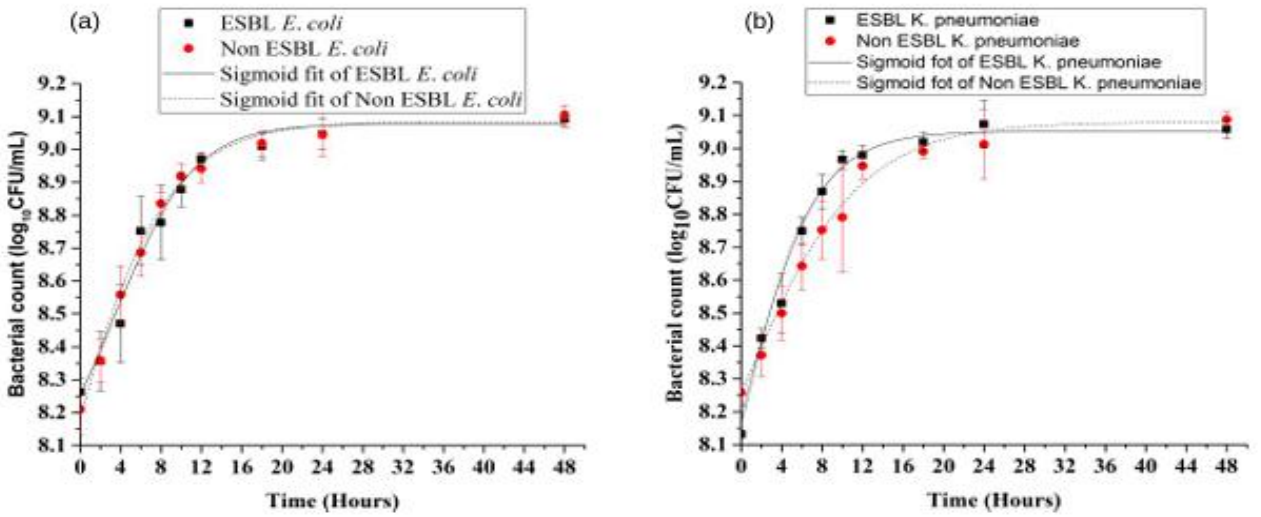

Figure 4: Time kill curve of *E. coli* (a) and *K. pneumoniae* (b) without honey. Error bars represent standard deviation from mean. The well fitted sigmoid growth curve demonstrated exponential growth of bacteria up to the 12 hours of incubation followed by stationary growth after 20 hours.

Antibacterial properties of honey have been well documented along with several compound that contributed to its activity such as H_2O_2, bee defensin-1 (Kwakman et al., 2011), phenolic acids and methylglyoxal (Stephens et al., 2010), glycopeptides (Brudzynski and Sjaarda 2015), flavonoids (Al-Waili, 2013), and lysozyme (Molan, 1992). Antibacterial activity of the test honey samples may also be influenced by osmotic inhibition as the controls like sugar or artificial honey was not tested in this study.

The emergence of ESBL producing bacteria may present an increasing risk of transmission of resistant strains in humans and animals. It is a worrying global public health issue as infections caused by such enzyme-producing organisms are associated with a higher morbidity and mortality and greater economic burden. The problem is clearly severe in developing countries where studies on this subject, drug availability, and its appropriate use are limited and resistance rate is high (Pitout et al., 2005; Ayukekbong et al., 2017; Khanal et al., 2024). In this study, considering insignificant statistical (p>0.05) association between the bacteriostatic activity of indigenous and Dabur honey against ESBL-producing and non-producing bacteria, we found that the antibacterial activity of honey was not affected by ESBL enzymes produced by bacteria. This was in agreement with the study by Kwakman et al. (2008) and Boorn et al. (2010). Similar to bacteriostatic activity, the minimum bactericidal concentration of honey was also found to be 50% v/v for all the tested isolates. This finding was consistent with reports from other researchers in various regions (Al-Waili, 2004; Kwakman et al., 2008; Ahmed et al., 2014; Wasihun & Kasa, 2016). Therefore, it is understood that the same concentration of honey can inhibit both the ESBL-producing and non-producing strains. The study also revealed that around 50% dilution of the honey samples had more antibacterial activity. The honey accumulates maximum H_2O_2 at the concentration of 30-50%, however this declines rapidly at <30% concentration due to the relatively low affinity of glucose oxidase of honey for its substrate, glucose (Schepartz & Subers, 1964). It has been shown that the antimicrobial activity of honey may range from concentrations < 3% to >50% (Wilkinson & Cavanagh, 2005; Al-Waili, 2013; Agbaje et al., 2006).

In order to explore the approximate time taken for inhibition of a given population, we assessed the time kill effect of honey at 50% (v/v) concentration on ESB-producing and non-producing *E. coli* and *K. pneumoniae* for 48 hours. In this study, the initial population of test bacteria was taken 8 $\log_{10}$ CFU/ml which are generally an infective dose to cause the infections (Schmid-Hempel & Frank, 2007). We found that complete inhibition of bacterial population (8 $\log_{10}$ CFU/ml) could be achieved in 24 hours exposure to the honey samples. The result was in accordance with the findings of previous researchers (Al-Maaini, 2011; Kwakman et al., 2008). The potential bioactivity of honey used in this study was equivalent to 30% (w/v) of Manuka honey, and 10-40% (v/v) of Revamil honey (a medical grade honey) (Kwakman et al., 2008; Kwakman et al., 2010; Kwakman et al., 2011). Nishio et al. (2016) found a significant decrease in *S. aureus*, up to 4 $\log_{10}$ CFU/ml, following 4 hours of treatment with the honey. Hashim (2014) found reduction of *E. coli* NCTC 10418 by 2 log and 3 log CFU/ml in 6 hours and 9 hours respectively at 50% (w/v) concentration of Sudanese honey and in 5 hours and 7 hours respectively at 25% (w/v) concentration of Manuka honey. All these results indicated that Gram negative bacteria require more exposure time for the same result. Though the targeted organisms were Gram negative in this study, 2 $\log_{10}$ CFU/ml diminution was observed only after 12 hours exposure to honey.

There were little differences in the killing effect of indigenous and Dabur honey against tested bacterial isolates. The comparatively higher killing rate was observed by indigenous honey (1.5-6 $\log_{10}$ CFU/ml) than Dabur honey (1-3.5 $\log_{10}$ CFU/ml) within 18 hours. Also, high and rapid killing effect of both type of honey was observed in case of *E. coli* isolate. In general, *K. pneumoniae* commonly displayed higher rates of resistance than *E. coli* (Hyle et al., 2005; Mohsen et al., 2016). However, both honeys showed complete inhibitory effect in 24 hours for all isolates indicating requirement of equivalent time of exposure for bactericidal activity. The physicochemical properties of indigenous honey (Shrestha, 2000) and Dabur honey (Shenoy et al., 2012) have been determined and the moisture content, reducing sugar, sucrose, hydroxymethyl furfuraldehyde (HMF) and pH of both honeys were within the range. The low pH, high sugar content, low moisture, elevated HMF or accumulation of H_2O_2 might be responsible for reduction of bacterial survival. Regarding the botanical origin, a variety of natural floras around the museum are speculated to be of polyfloral origin (Shrestha, 2006) and Dabur honey also have the floral source from Himalayas, Nilgiris, and Sunderbans of India (Shenoy et al., 2012).

Although, limitation in terms of geographic variation and sample size, this study clearly showed the antibacterial activity of honey against clinical isolates of *E. coli* and *K. pneumoniae* within 24 hours exposure. More research is needed to establish the potential antimicrobial activity of indigenous honey. As a limitation, the mode of action of honey against different bacterial species was not investigated. Screening of bioactive compounds and understanding their molecular mechanism would be important scientific evidence for alternative therapeutic application of the indigenous honey.

Conclusion

Indigenous honey and Dabur honey have both bacteriostatic and bactericidal activity against ESBL-producers and non-producers when tested *in-vitro*. The MICs of honey against ESBL producers and non-producers are almost similar (25% and 50%) however; it is different according to species of bacteria. The MBC of honey against all the bacterial isolates is identical (50%). Both types of honey reduced the microbial load by 8 $\log_{10}$ CFU/ml in 24 hours of exposure. However, pharmacological standardization and clinical evaluation on the effect of honey are essential before using honey as a preventive and curative measure to common diseases related to the tested bacterial species.

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Research Article

Assessment of *In vitro* Antidiabetic Activity in Selected Species of *Berberis* L.

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ABSTRACT

Species of *Berberis*, especially their roots, have long been used in traditional medicine to manage hyperglycemia. However, only a few species are commonly used for this purpose. Although more than 20 species of *Berberis are* found in Nepal, only two species, namely *B. aristata* and *B. asiatica,* are widely utilized in traditional medicinal practices. The present study aims to evaluate the therapeutic potential of five *Berberis* species (*B. concinna, B. everestiana, B. hamiltoniana, B. insignis,* and *B. jaeschkeana*) by assessing their total phenolic content, total flavonoid content, and *in vitro* α-glucosidase inhibitory activity. On the basis of parameters evaluated, *B. hamiltoniana* emerged as the most promising species exhibiting the highest total phenolic content (66.97 ± 2.56 mg GAE/g) and the lowest half maximal inhibitory concentration (IC_{50} 10.64 µg/ml) against α-glucosidase. Its inhibitory activity surpassed that of acarbose, the standard drug commonly used in the management of hyperglycemia.

Keywords: α-glucosidase; *Berberis* species; Flavonoid; Phenols

Introduction

Diabetes mellitus has emerged as one of the most prevalent metabolic disorders, representing an intimidating challenge to global public health (Karalliedde & Gnudi, 2016). It is classified into four main categories: Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), gestational diabetes, and other specific types of diabetes (American Diabetes Association, 2019; Perišić et al., 2022; Salguero et al., 2023; Ortega et al., 2025). More than 90% of diabetes cases are attributed to T2DM, which is characterized by persistent hyperglycemia resulting from insulin resistance and an inadequate insulin response, accompanied by disturbances in carbohydrate, lipid, and protein metabolism (Weyer et al., 1999; Farzaei et al., 2017). According to the International Diabetes Federation (2025), approximately 11.11% of adults aged 20-79 years, which is nearly 589 million individuals, were living with T2DM globally in 2024.

The federation identifies unhealthy dietary patterns, physical inactivity, obesity, adverse lifestyle behaviors, and rapid urbanization as major contributors to the global rise in T2DM. In the case of Nepalese adults, diabetes is also one of the major health problems with prevalence rates ranging from 6.3% to 8.5% (Sharma et al., 2011; Shakya-Vaidya et al., 2013).

In recent years, there has been increasing interest in alternative therapeutic strategies for diabetes management, particularly using medicinal plants. Plant-derived bioactive compounds exhibit diverse mechanisms of glycemic regulation, including inhibition of carbohydrate-digesting enzymes, enhancement of insulin sensitivity, and reduction of oxidative stress, often with fewer or no side effects (Wei et al., 2023). Furthermore, several herbal remedies have long been used to treat diabetes across various traditional medical systems; however, their active constituents and precise mechanisms of action remain incompletely characterized and have not been systematically integrated into modern medical practice (Prasathkumar et al., 2021; Liu et al., 2024).

Species of *Berberis* L. (Family Berberidaceae) have been widely used as herbal medicines in various traditional medicine systems, including Ayurveda, Sowa-Rigpa (Tibetan-based), Unani, Siddha, homeopathy, naturopathy, and Yogic culture systems. In addition to their medicinal value, these species are utilized as natural dyes, consumed as wild edible fruits, and used in the preparation of wines, pickles, sauces, and coloring agents (Manandhar, 2002; Tutak & Korkmaz, 2012).

Several species of the genus *Berberis* have traditionally been consumed as herbal medicines to treat numerous ailments, including diabetes (Manandhar, 2002; Gaire & Subedi, 2011; Subba & Gaire, 2022). Notably, species like *B. asiatica*, *B. orthobotrys*, and *B. chitria* have been specifically reported for their antidiabetic properties in regions including India and Pakistan (Uniyal et al., 2006; Singh et al., 2017; Dwivedi et al., 2019; Majid et al., 2019)

Experimental and clinical studies have confirmed the antidiabetic efficacy of several *Berberis* species, supporting their traditional use in the management of T2DM or hyperglycemia (Belwal et al., 2020). Phytochemical studies on some of these species have revealed the presence of isoquinoline alkaloids, especially berberine (Hussaini & Shoeb, 1985; Lee et al., 2006; Gomes et al., 2012; Liu et al., 2015), palmatine, columbamine, oxyacanthine, and berbamine (Papiya et al., 2010; Bajpai et al., 2015; Alamzeb et al., 2018). These alkaloids have been reported to exhibit various pharmacological activities, including antidiabetic (Upwar et al., 2011; Mittal et al., 2012; Tiwari et al., 2024; Abid et al., 2025), antioxidant (Alamgeer et al., 2017; Bhatt et al., 2018), and hepatoprotective activities (Tiwari and Khosa, 2010).

In Nepal, the genus *Berberis* comprises 21 species (Adhikari et al., 2012). However, limited information is available regarding the ethnomedicinal uses and therapeutic potential of several of these species. Therefore, the present study aims to evaluate the hypoglycemic potential of extracts from five *Berberis* species: *B. concinna*, *B. everestiana*, *B. hamiltoniana*, *B. insignis* and *B. jaeschkeana*. Among these, *B. concinna* is an endemic species of Nepal. *B. insignis* is confined to eastern Nepal, whereas *B. jaeschkeana* is confined to western Nepal. Similarly, *B. hamiltoniana* occurs in central and western Nepal, while *B. everestiana* is distributed across eastern, central, and western regions of the country (Adhikari et al., 2012). All of these species are being screened for the first time for their *in vitro* antidiabetic potential.

Materials and Methods

Plant materials and sample preparation

Plant samples of different *Berberis* species were collected from various geographical locations across Nepal and identified using the available taxonomic literature. The details of collection sites, including their GPS coordinates, are provided in Table 1, and representative photographs are presented in Figure 1.

Table 1: *Berberis* species collected and their parts used for the study.

S.N.	Species	Parts used	Altitude (m.)	*Locality
1	*B. concinna* Hook.f.	Aerial parts	3692	A
2	*B. everestiana* Ahrendt	Aerial parts	4335	B
3	*B. hamiltoniana* Ahrendt	Aerial parts	2975	C
4	*B. insignis* Hook.f. & Thomson	Bark	2944	D
5	*B. jaeschkeana* C.K. Schneid.	Aerial parts	2975	C

*A: Kharpu Bhanjyang, Rasuwa (26.1689 N; 85.2187 E); B: Timbung Pokhari, Taplejung (27.4268 N; 88.0431 E); C: Patarasi Jumla (29.1906 N; 82.2148 E); D: Sankhuwasbha, Jaljale (27.1962 N; 87.2961 E).

The collected samples were packed in cotton bags at the collection site, thoroughly cleaned to remove adhering soil and debris. Then, shade-dry under proper aeration at room temperature for one week to eliminate excess moisture. Herbaria were prepared from voucher specimens using the method described by Bridson &

Forman (1992). The dried samples were homogenized using an electric grinder and sieved through a 60-mesh screen. The pulverized samples were portioned, vacuum-packed, and stored until further use.

Figure 1: Photographs of different species of *Berberis* L. (A: *B. concinna*; B: *B. everestiana*; C: *B. hamiltoniana*; D: *B. insignis*; E: *B. jaeschkeana*).

Solvent extraction

The extraction of *Berberis* samples was carried out using 80% (v/v) aqueous methanol. The ratio of powdered sample to extraction solvent was kept at 1:10. Initially, the mixture was vortexed and allowed to stand for 24 hours with intermittent vortexing for better infusion. On the subsequent day, the tubes were shaken well and filtered using a Whatman No. 1 filter paper. The residue was re-suspended in the solvent and subjected to extraction for another 24 hours. After 24 hours, the mixture was filtered again. The filtrates were mixed and allowed to evaporate under reduced pressure in a rotary evaporator (RE 100-Pro, Dragon Lab China) at 65 °C. The condensed filtrate was then transferred to pre-weighed petri-plates and allowed to evaporate in a laminar air flow hood until a constant weight was achieved. Thereafter, the dried extracts were scraped off the surface, collected in 2 ml polypropylene tubes, and stored at -20 °C.

Estimation of total phenolic content

The total phenolic content (TPC) was estimated following the method of Ainsworth & Gillespi (2007) with minor procedural modifications. The method involves the color reaction of Folin-Ciocalteu (FC) reagent. The FC reagent (Fisher Scientific, India) was diluted to 10 times the original concentration. The total volume of the reaction mixture was 300 µl. Each well was loaded with 20 µl of plant extract (2.5 mg/ml) and 200 µl of diluted FC reagent and incubated for 5 minutes at 25 °C. Then, 80 µl of 1 M Na_2CO_3 solution was followed by incubation for 25 minutes at 25 °C. Methanol (80%) was used as a blank. Absorbance was measured at 765 nm using an EPOCH 2 microplate reader (Agilent Technologies). The experiment was also carried out using gallic acid solution of different concentrations (10-60 µg/ml) instead of plant extract, and absorbance values obtained at different concentrations of gallic acid were used to obtain a calibration curve. The concentration of phenolic content in the plant extract was determined by using the equation of the standard curve (Figure 2) of gallic acid ($y = 0.006x$, $R^2 = 0.999$). TPC of the plant extract was calculated according to the formula:

$$TPC = (c \times v)/m$$

Where *c* represents the phenolic concentration obtained from the gallic acid calibration curve, *v* is the volume of the extract used, and *m* is the dry weight of the sample.

TPC was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g).

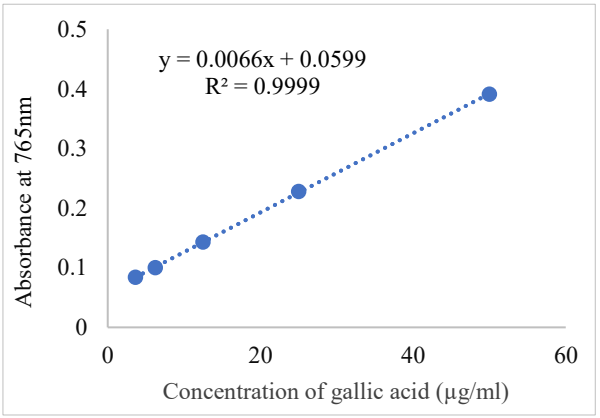

Figure 2: Standard curve of gallic acid.

Estimation of total flavonoid content

Total flavonoid content (TFC) estimation was done by following the protocol of Roy et al. (2011). The plant extracts dissolved in respective solvents at a concentration of 10 mg/ml. Then, 25 µl of each sample solution was separately mixed with 75 µl of 10% aqueous solution of $AlCl_3$, 5 µl of 1 M aqueous solution of CH_3COOK and 140 µl of distilled water. Methanol, instead of plant extract, was used as a blank. After vigorous shaking and subsequent incubation at room temperature for half an hour, absorbance was measured at 415 nm using a spectrophotometer. In a separate

experiment, quercetin solution in methanol in the concentration range 10 to 100 µg/ml was used instead of the plant extract to prepare a standard curve of quercetin. TFC in the extract was determined based on the equation of standard curve (Figure 3) of quercetin ($y = 0.0068x + 0.025$; R^2=0.9962). From this concentration value TFC of the plant extract was calculated by using the formula:

$$TFC = (c \times v)/m$$

Where *c* represents the flavonoid concentration obtained from the quercetin calibration curve, *v* is the volume of the extract used, and *m* is the dry weight of the sample.

The value of TFC was expressed as milligrams of quercetin acid equivalent per gram of dry weight (mg QE/g).

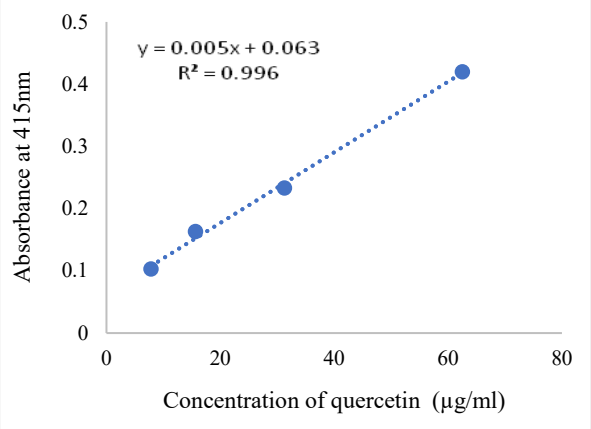

Figure 3: Standard curve of quercetin.

Evaluation of α-glucosidase inhibition activity

The modified protocol of Si et al. (2010) was employed for the α-glucosidase enzyme inhibition assay. One hundred fifty microlitres of p-nitrophenyl α-D-glucopyranoside (PNPG) solution (Sigma Aldrich, Germany) prepared in 0.1 M potassium phosphate buffer (pH 6.4) was pipetted into each well of 96 well Plate. In wells marked as blank and negative control, 10 µL of 80% methanol was added, while in the wells marked as sample, the same amount of either plant extract or acarbose solution (1 mg/ml) prepared in 80% methanol was added. Then, 40 µl of 1 M Na_2CO_3 (British Drug Houses) was added to the wells, representing the blank. The reaction mixture was pre-incubated at room temperature (28°C) for 10 minutes. After pre-incubation, 10 µl of α-glucosidase (Sigma Aldrich, Germany) enzyme solution (0.2 U/ml) prepared in 0.1 M phosphate buffer (pH 6.4) was added, and the plate was incubated at 28 °C for a further 20 minutes. The reaction was terminated by adding 40 µl of 1 M Na_2CO_3 to all the wells except the blank. The absorbance was measured at 405 nm using a microplate reader (EPOCH-2, Agilent Technologies). Inhibition of α-glucosidase activity was calculated by using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \times 100$$

In order to calculate concentrations of plant extracts and acarbose that inhibit the enzyme activity by 50% (i.e., IC_{50} value), an enzyme inhibition assay was carried out using plant extracts and acarbose solution of different concentrations. From the value of enzyme inhibition at different concentrations, IC_{50} was calculated using the following formula.

$$IC_{50} = \text{Exp (Ln (conc pi>50) – ((pi>50 - 50) / (pi>50 - pi<50)) * Ln (conc pi>50 / conc pi<50))}$$

Where, Exp – exponential function; Ln – Natural log; Conc pi>50- extract concentration at which enzyme inhibition is just above 50%; Conc pi<50 - extract concentration at which enzyme inhibition is just below 50%; Pi>50 – observed percentage inhibition value that is immediately above 50%; Pi<50– observed percentage inhibition value that is just below 50%

Data analysis

All experimental data obtained for each sample were the average value of three independent measurements. Values were reported as mean ± standard deviation. Microsoft Office Excel (2007) was used to compute means, standard deviation, and one-way analysis of variance (ANOVA), followed by post hoc analysis by Tukey's test. Percentage values were subjected to log transformation before ANOVA.

Results and Discussion

Total phenolic content

The total phenolic content (TPC) of different species of *Berberis* extract is presented in Figure 4. The highest TPC (66.97 ± 2.56 mg GAE/g) was observed in *B. hamiltoniana* extract, whereas the lowest TPC (5.8±0.04 mg GAE/g) was observed in *B. concinna* extract. The TPC of *B. concinna* was significantly lower, while that of *B. hamiltoniana* was significantly higher compared to other species ($p<0.05$).

Phenolic compounds are widely acknowledged secondary natural metabolites produced biogenetically (Cheynier et al., 2013). Phenol and polyphenols are abundant in fruits, berries, cereals and vegetables, and they play a crucial role in mitigating oxidative stress due

to their redox properties, hydrogen-donating ability and metal-chelating potential (Pandey & Rizvi, 2009; Wang et al., 2011; Wootton-Beard & Ryan, 2011; Zhang & Tsao, 2016; Minatel et al., 2017). Studies have shown that increased intake of polyphenol-rich diet reduces the risk of degenerative diseases like diabetes, cardiovascular diseases, cancer, etc. (Nisar, 2022). As a result measurement of total phenolic and flavonoid content has been a standard practice in studies on medicinal plants and those on functional foods and dietary substances.

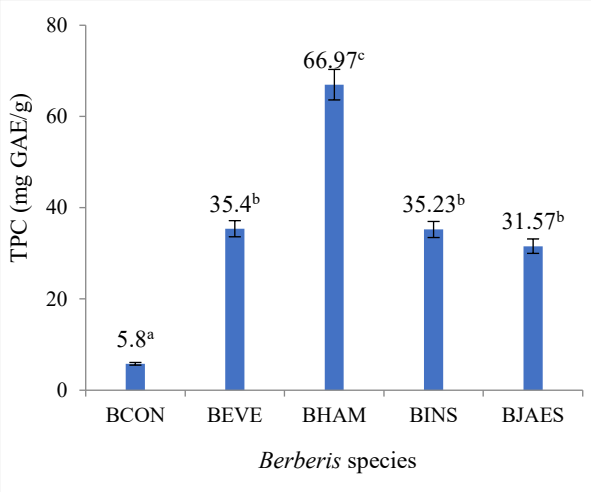

Figure 4: Total phenolic content (TPC) in extracts of different species of *Berberis* (BCON: *B. concinna*; BEVE: *B. everestiana*; BHAM: *B. hamiltoniana*; BINS: *B. insignis*; BJAES: *B. jaeschkeana*). Values are expressed as mean ± SD (n = 3). Means followed by different superscript letters are significantly different at $p < 0.05$ (one-way ANOVA followed by Post-hoc Tukey's HSD test).

Earlier studies have reported the presence of phenols in various parts of *Berberis*, including fruit, leaves, stem bark, and roots (Dhungel et al., 2016; Bhatt et. al, 2018; Awal et al., 2025).The abundance of phenolic content of methanolic extract of *B. hamiltoniana* from the aerial-part is quite higher among the studied taxa of *Berberis*. The elevated phenolic concentration of *B. hamiltoniana* indicates significant variation in accumulation of phenolic among the evaluated species.

Total flavonoid content

The total flavonoid content (TFC) in extracts of different species of *Berberis* is presented in Figure 5. Among the studied species, *B. everestiana* and *B. hamiltoniana* exhibited the highest TFC (7.61 ± 0.15 and 7.46 ± 1.15 mg QE/g resectively), while *B. insignis* showed the lowest (1.89 ± 0.25 mg QE/g). The TFC values in extract of *B. everestiana* and *B. hamiltoniana* were significantly higher ($p \leq 0.05$), while the extract of *B. insignis* was significantly lower ($p \leq 0.05$) than that of rest of the species.

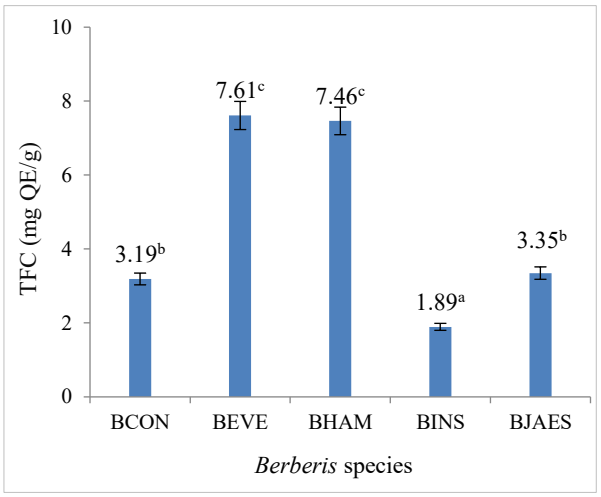

Figure 5: Total flovonoid contentment (TFC) of different *Berberis* species (BCON: *B. concinna*; BEVE: *B. everestiana*; BHAM: *B. hamiltoniana*; BINS: *B. insignis*; BJAES: *B. jaeschkeana*). Values are expressed as mean ± SD (n = 3). Means followed by different superscript letters are significantly different at $p \leq 0.05$ (one-way ANOVA followed by Post-hoc Tukey's HSD test).

Flavonoids are one of the most crucial secondary metabolites belonging to the phenol family (Panche et al., 2016). They are well known for their antioxidant, anti-inflammatory, and antidiabetic properties. The most commonly occurring flavonoids in certain beverages, vegetables, fruits, spices, and soup include quercetin and anthocyanins (Hertog et al., 1993; Pandey & Rizvi, 2009; Panche et al., 2016). Epicatechin, a natural flavonoid present in *Camellia sinensis* (tea), has been reported to mediate the activation of the insulin receptor in T2DM (Ganugapati et al., 2011). Additionally, earlier studies have shown that flavonoids in *Berberis* species contribute significantly to free radical scavenging and enzyme inhibition activities (Imenshahidi & Hosseinzadeh, 2016).

While studying the total flavonoid content in different parts of *Berberis aristata* and *B. thomsoniana*, Bhatt et al. (2018) reported that there is variation in total flavonoid content in species- specific as well as organ-specific manner. The species-specific variation in total flavonoid content in the present study supports the findings of Bhatt et al. (2018). Awal et al. (2025) further reported that flavonoid content decreases while total phenol increases with rising soil moisture and elevation. Since the samples in this study were collected from different elevations, these factors may also have contributed to the observed variations. Additionally, such variations could also be influenced by soil physicochemical properties, biotic and abiotic stresses, thermal exposure, ecological interactions, genetic variation, or differences in metabolite pathways (Andola et al., 2010, 2019; Thompson et al., 2010; War et al., 2012; Cheynier et al., 2013; Gan et al., 2017).

α-glucosidase enzyme inhibition

The ability of crude extracts obtained from different *Berberis* species to inhibit the activity of enzyme α-glucosidase is presented in Figure 6. Among the tested species, the extract of *B. hamiltoniana* exhibited the highest enzyme inhibition (88.01% ± 0.34), whereas *B. everestiana* showed the lowest inhibition (0.76% ± 0.034) under experimental conditions. Two species, *B. concinna* and *B. hamiltoniana,* were further evaluated to determine their maximal half inhibitory concentration (IC_{50}) values.

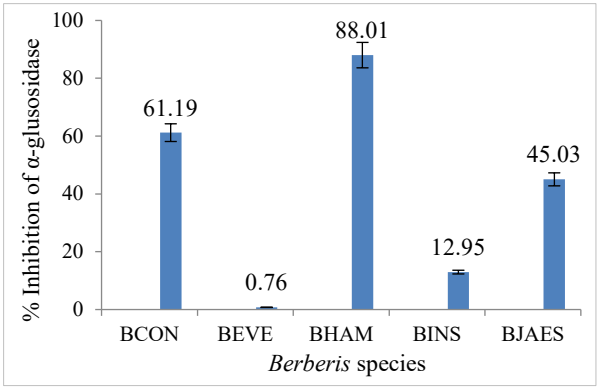

Figure 6: Percentage inhibition of α-glucosidase enzyme by *Berberis* species extract (BCON: *B. concinna*; BEVE: *B. everestiana*; BHAM: *B. hamiltoniana*; BINS: *B. insignis*; BJAES: *B. jaeschkeana*).

The comparison of IC_{50} values of extracts from *B. concinna* and *B. hamiltoniana* with the standard drug acarbose against α-glucosidase is shown in Figure 7. The extract of *B. hamiltoniana* showed the most promising inhibitory potential, exhibiting an IC_{50} value significantly lower than that of acarbose. In contrast, the extract of *B. concinna* showed an IC_{50} value significantly higher than that of acarbose. Based on these screening results, *B. hamiltoniana* (with lowest IC_{50} value against α-glucosidase, i.e., highest inhibition) is identified as the most promising species for further investigation.

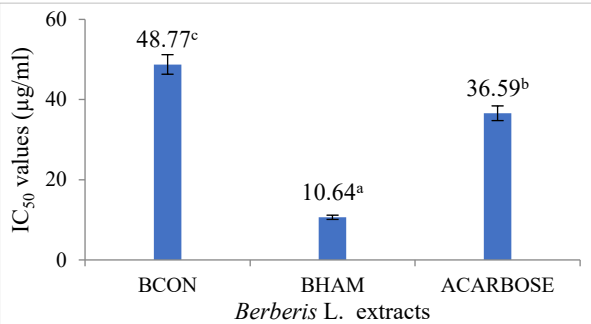

Figure 7: α-glucosidase IC_{50} value of *Berberis* extracts (BCON: *B. concinna* & BHAM: *B. hamiltoniana*). Values are expressed as mean (n = 3). Means followed by different superscript letters are significantly different ($p \leq 0.05$).

Although several *Berberis* species, such as *B. aristata, B. asiatica, and B. orthobotrys,* have been traditionally used to manage diabetes, none of the species examined in the present study has reported medicinal uses. Notably, *B. hamiltoniana* exhibited significant α-glucosidase inhibitory activity, surpassing even that of acarbose. Since the antidiabetic effects of various *Berberis* species have been linked to phytoconstituents such as berberine, palmatine, columbamine, and berbamine (Tiwari et al., 2024; Abid et al., 2025), the hypoglycemic potential observed in *B. hamiltoniana* may be due to the presence of similar or other novel bioactive compounds.

Conclusion

Comparative evaluation of TPC, TFC, and *in vitro* antidiabetic potential of extracts of selected species of *Berberis* from Nepal revealed species specific variations in these parameters. Though none of the species selected for study are used in traditional medicine, their therapeutic potential as revealed by high TPC values and high inhibitory potential against α-glucosidase *in vitro* indicate towards their potential use as substitutes to species of *Berberis* like *B. aristata, B. asiatica* and others used for various therapeutic applications. However, a detailed study on their phytochemical constituents and potential toxicity should be carried out before their use as substitutes to those highly demanded species.

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Research Article

Microbial Fuel Cell for Dairy Waste Treatment and Electricity Generation

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ABSTRACT

Observing waste as a resource sparks interest in recovering dairy wastewater for resilient sustainability. Dairy wastewater, rich in organic content, is very much suitable for microbial fuel cell (MFC) applications. This paper used MFCs to study electricity generation and dairy waste treatment with paneer whey as a substrate and a biocatalyst culture from dairy development corporation (DDC) sewage targeting for lactose-utilizing strains. Bacteria from DDC sewage found to contain *Klebsiella, Pseudomonas, Salmonella*, and *Escherichia coli,* which were identified by biochemical tests. An yeast *Sungouiella pseudointermedia* was identified molecularly by D1D2 primer amplification and sequencing. A dual-chamber MFC with paneer whey, a bacterial consortium from DDC sewage, and alcohol-treated CNT-coated graphite felt electrodes removed 75.71% of total reducing sugar, 61.03% COD, and 48.91% total phosphorus, generating 25.869 W/m^3. A simpler MFC with paneer whey, a bacterial consortium, and 0.1 M phosphate buffer removed 95.06% of total reducing sugar, 58.27% COD, 65.67% total phosphorus and 67.13% ammoniacal nitrogen but had lower power output. Challenges like pH regulation and equipment limitations are key for optimizing MFC performance and adoption. MFCs show promise as sustainable energy recovery and wastewater treatment solutions.

Keywords: Dairy waste treatment; Electric output; Microbial fuel cell; Bio-electricity; Removal efficiency

Introduction

Waste management is a critical global issue that poses challenges in treatment and disposal, especially with the growth of industries and urbanization. There is growing interest in resource recovery from waste for social and environmental sustainability (Adersa et al., 2021). Dairy industries are significant sources of emerging contaminants, such as estrogens, which enter the environment through wastewater effluents from dairy and livestock activities (Dongre et al., 2021). Dairy wastewater contains high levels of biological oxygen demand (BOD) and chemical oxygen demand (COD), along with fats, lactose, detergents, sanitizing agents, and milk constituents like casein, lactose, fat, and inorganic salts. Industries generate various forms of waste that are often disposed of without treatment (Chaudhary, 2017). The highly biodegradable nature of dairy wastewater necessitates urgent treatment. Common treatment methods for dairy wastewater

include aerobic and anaerobic biological treatments like trickling filters, aerobic lagoons, anaerobic lagoons, sequencing batch reactors (SBR), anaerobic sludge blankets, anaerobic filters, and constructed wetlands. Physical and chemical treatments, such as membrane technology and coagulation/flocculation, are also utilized. However, these conventional techniques have drawbacks such as high costs, energy requirements, and sludge generation. The high energy demands of conventional treatment systems have spurred interest in alternative technologies that are cost-effective and energy-efficient. Microbial fuel cells (MFCs) have emerged as a promising option for wastewater treatment due to their ability to generate electricity from organic waste and renewable biomass (Al-saned et al., 2021). MFCs can efficiently convert this organic matter into electricity, addressing both energy shortages and environmental concerns. In an MFC, bacteria break down organic matter, creating a bio-electrochemical system that produces electricity through microbial metabolism. This process involves the oxidation of organics to generate electrons and protons. Bacteria then transfer electrons to the anode using shuttles or matrices, which flow to the cathode through an external circuit. Protons migrate to the cathode, where they combine with electrons and oxygen to form water. The voltage and current required for electricity production stem from the potential difference between the bacteria's metabolism and the electron acceptor. MFCs not only treat dairy waste but also enhance biodegradation while recovering energy in the form of electricity (Dongre et al., 2021).

There are now numerous private dairies of different sizes in and outside the Kathmandu valley. Despite progress, many dairy industries struggle to meet environmental standards and face pressure from community groups and government regulations. Implementing resource efficient cleaner production (RECP) technique can help these industries reduce their environmental impact, increase productivity, and enhance profitability in the long term (Shrestha, 2017). This research aims to develop a MFC to improve the treatment of industrial dairy waste, specifically Paneer whey, by reducing sugar, COD, phosphorus, and nitrogen content. Additionally, the goal is to generate electricity using substrates from dairy waste, potentially addressing fuel and electricity shortages in the future. Managing dairy waste also benefits in controlling shared air pollution.

Materials and Methods

Sample collection

Freshly prepared paneer whey from the Dairy Development Corporation (DDC) in Lainchaur, Kathmandu, was collected in a sterile bottle and utilized as a substrate in a Microbial Fuel Cell (MFC). Furthermore, sewage from DDC was collected to isolate and identify microbes and to use as a culture in treating the whey in MFC. *Lactobacillus* sp. was collected from Biofuel Laboratory of the Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu isolated from dairy product. The samples were stored at 4 °C in the Biofuel Laboratory.

Sample analysis

Various physicochemical parameters of paneer whey were analyzed, including chemical oxygen demand (COD), total phosphorus and ammonia-nitrogen using standard method of water analysis (Aniruddha, 2010). Various bacteria were isolated from sewage sample by streaking serially diluted sample to nutrient agar and incubating at 37 °C for 24 hour. To isolate yeast, potato dextrose agar (PDA) plate was used. Different bacteria or yeast were selected according to their morphology and colour. They were repeatedly subcultured to get pure isolates. Biochemical tests were performed. The bacteria were identified by the Bergey's Manual of Systematic Bacteriology (Bergey & Holt, 1994).

Study of lactose utilization and molecular analysis of lactose utilizing yeast isolate

Lactose utilizing yeast isolate was identified by culturing in lactose as sole carbon source which gave growth with bubble formation was molecularly analyzed. Genomic DNA was extracted from the isolate using DNA isolation kit (Promega). PCR was performed (Biorad thermo cycler) using forward primer NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse primer NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') obtained 680 bp fragment. A 25 µl reaction volume with master mix 12.5 µl (2x), $MgCl_2$ 1 µl (25 mM), forward and reverse primer each of 1.5 µl (10 pmol), DNA templete 1 µl (45 ng) nuclease free water (7.5 µl) was used. The PCR conditions were, initial denaturation: 96 °C, 2 min followed by 35 cycles of denaturation: 96 °C, 45 sec; annealing: 52 °C, 45 sec; extension: 72 °C, 2 min; final extension: 72 °C, 10 min and holding at 4 °C (Cocolin et al., 2002). The products were then sequenced in Excelris lab, Ahamdabad, India followed by phylogenetic tree construction using neighbor-joining algorithm in MEGA6.

Microbial fuel cell construction and operation

A dual-chambered microbial fuel cell (MFC) was built with Nafion117 as the Proton Exchange Membrane

(PEM) and graphite felt as both the cathode and anode. The anodic substrate in the MFC was filtered paneer whey supplemented with 5% mixed culture from sewage of DDC or single isolated cultures. A closed circuit was set up using an external 1000 Ω resistor, and the power produced in the MFC was measured with a multi-meter. The MFC was operated for 5-8 days, and samples from the anode were tested daily for physicochemical parameters to assess removal efficiency.

MFC was run with different bacterial and yeast samples in single and mixed cultures, different buffers, chemical oxidizers and modification in electrode coated with CNT composite. The generated power was calculated in terms of Open Circuit Voltage (OCV), Closed Circuit Voltage (CCV), current and power.

Removal of physicochemical parameters after MFC operation

Before starting the MFC operation, the physicochemical parameters of the anodic substrate were measured. After each MFC operation with various catholyte and electrode modifications measurements of total reducing sugar, COD, total phosphorus, and Ammoniacal nitrogen were conducted as stated in Aniruddha, P., 2010. The removal efficiency was calculated by determining the difference between the initial and final concentrations of these parameters and using the formula:

$$\text{Removal efficiency (\%)} = \frac{(\text{Final Conc.} - \text{Initial Conc.})}{\text{Initial Conc.}} \times 100$$

Cyclic voltammetry (CV)

The Hokuto-Denko HA151 Potentiostat (Central Department of Chemistry, Tribhuvan University, Nepal) was utilized in conjunction with the workstation for CV measurements, which were conducted in a three-electrode configuration. A platinum electrode served as the counter electrode, and it was pretreated by washing with distilled water. The reference electrode used was calomel (mercury chloride). Measurements were carried out within the range of -1 V to +1 V. Characterization through cyclic voltammetry (CV) was performed at a scan rate of 0.1 V/sec, with data collected at 10 mV intervals to achieve stable current values. CV was repeated for up to 10 cycles (Molina et al., 2011).

Results and Discussion

Sample analysis and characterization of isolated microbes

The physicochemical analysis of paneer whey included testing for six parameters: pH, temperature, total COD, total phosphorus, and ammoniacal nitrogen using standard method of water analysis (Aniruddha, 2010). They were found to be 5.37, 75 °C, 6891.6 mg/l, 0.23 mg/l and 0.03 mg/l respectively. The biological analysis of the wastewater sample focused on isolating and identifying bacteria biochemically or molecularly. Table 1 showed that paneer whey was found to be of acidic nature which may be primarily caused by the presence of lactic acid bacteria, and the acidic agents used to coagulate milk also contribute to this acidity. Since the whey was freshly collected, the recorded temperature was 75 °C, because during the paneer making process, milk is heated and whey being a liquid retains some heat. Simultaneously, with reference of COD value obtained, the whey was organically rich. However, whey has the potential to be utilized in various fields. According to Patowary et al. (2016), paneer whey with a pH of 5.4 ± 0.3 and rich in COD of upto 65000 ± 3.54 mg/l as per concentration strategy adopted which when discharged to water bodies, it increases the nutrition, that can lead to eutrophication. The phosphorus and ammoniacal nitrogen found in the whey may be due to microbial metabolism of organic compound and acidic pH also contribute to its release. The purpose of determining the parameters was to assess the organic content and nutrient availability in the substrate for use in microbial fuel cells to support the metabolic needs of the microbial community.

Table 1: Biochemical identification of bacteria isolated from DDC sewage.

Tests	*Klebsiella* sp.	*Salmonella* sp.	*Pseudomonas* sp.	*Escherichia* sp.
Gram reaction	Gram negative	Gram negative	Gram negative	Gram negative
Indole test	Negative	Negative	Positive	Positive
Methyl red test	Negative	Positive	Negative	Positive
Voges -Proskaur test	Positive	Negative	Negative	Negative
Citrate test	Positive	Positive	Positive	Negative
Catalase test	Positive	Positive	Positive	Positive
Nitrate test	Negative	Positive	Positive	Positive
Urease test	Positive	Negative	Negative	Negative
Motility test	Non-motile	Motile	Motile	Motile

Upon the bacterial isolation from DDC sewage, four bacteria were biochemically identified of the genera *Klebsiella* sp., *Pseudomonas* sp., *Salmonella* sp. and *Escherichia* sp. (Table 1). The bacteria such as *E. coli, Klebsiella* sp., and *Pseudomonas* sp. are frequently present in wastewater, as reported by Chahal et al. (2016). The purpose of using sewage for bacterial source was because the diverse microbial community in wastewater enhances substrate utilization, maximizes electron donor availability for electricity generation, and promotes synergistic interactions among microbes, ultimately improving the overall performance of microbial fuel cells (MFCs). Furthermore, these bacteria have the ability to adapt to changing environments, making them advantageous for MFC modifications.

In the study by Gunawardena et al. (2008), yeast such as *Saccharomyces cerevisiae* were utilized as a biocatalyst in a glucose-powered microbial fuel cell (MFC), achieving a maximum power output of 146.71 ± 7.7 mW/m^3. So, whether or not the yeast performs well as a biocatalyst, in our research, we isolated and tested the isolated yeast for its ability to ferment lactose. Among the isolated yeast strains, one yeast is found capable to utilize lactose as it showed growth and bubble formation in the media with lactose as sole carbon source (Figure 1) which was molecularly identified as *Sungouiella pseudointermedia* (Figure 2).

Figure 1: Analysis of yeast for lactose utilization; Control (left), Yeast sample (right).

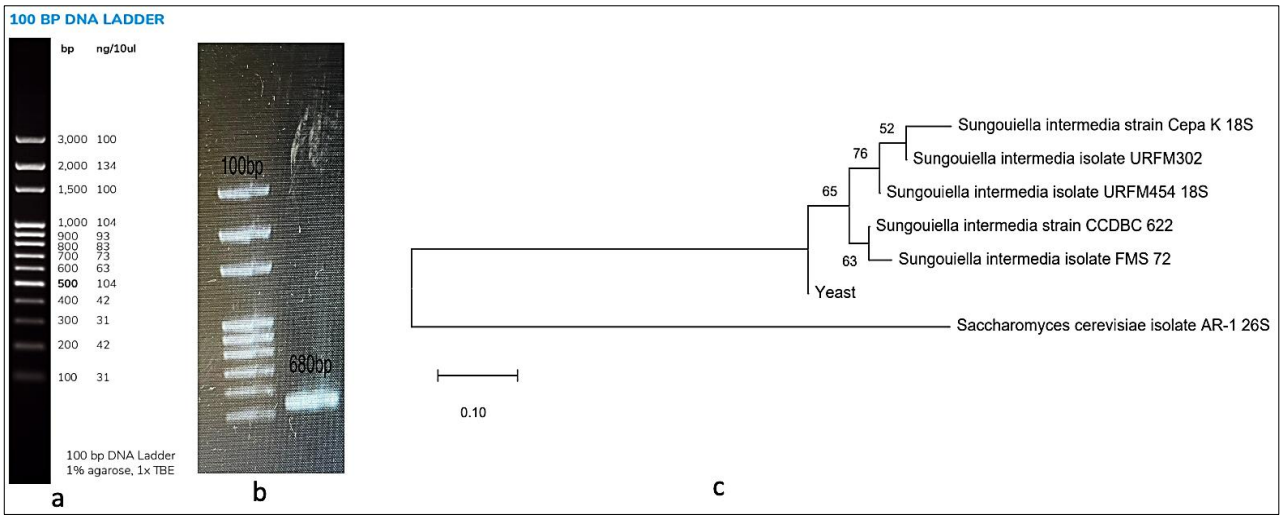

Figure 2: Molecular analysis of yeast by 16S rRNA region amplification; a: marker image, b: gel picture (lane 1:100 bp marker, lane 2: 680 bp PCR product) and c:Phylogenetic tree construct using MEGA 12.

MFC performance in terms of electric output and removal efficiency

The modification in the biocatalyst microbes used, catholyte, and electrode resulted in the optimal configuration for the operation of a microbial fuel cell (MFC) with paneer whey as the anolyte (Table 2). MFCs with mixed bacteria/yeast consistently showed better power output than those with single bacteria due to synergistic effects between species (Han et al., 2024). This was also supported by the study of Ren et al. (2021), where a mixed culture of *Saccharomyces cerevisiae* and *Bacillus subtilis* generated more power than when operated alone. A mixed consortium indeed generated a higher power output than an MFC operated with a single microbe. Despite the mixed consortium generating high power, the power output was overshadowed when the bacterial/yeast consortium was mixed with *Lactobacillus* sp. This may be due to the synergy of *Lactobacillus* sp. with the mixed bacterial/yeast consortium, and *Lactobacillus* sp. which degraded lactose present in paneer whey.

Mixed bacterial/yeast consortium of DDC waste with catholyte 0.1 M phosphate buffer, 0.1 M potassium ferricyanide, and the electrode graphite felt coated with CNT in ethanol (Table 2) generated maximum power of 25.869 mW/m^3. According to Gunawardena et al. (2008), addition of electron mediators like methylene blue and ferricyanide in *Saccharomyces cerevisiae*-based fuel cell showed improved performance, resulting in a maximum power generation of 0.1467 W/m^3 and a maximum open circuit voltage (OCV) of 383.6 mV under 1 KΩ resistance.

The electron acceptor used in the catholyte was potassium ferricyanide, and the buffering solutions were phosphate buffer and sodium acetate. Phosphate buffer helps maintain a suitable pH for electricity-generating bacteria and increases solution conductivity. According to Guerrero et al. (2010), potassium ferricyanide has the potential for a maximum OCV of 710 mV with a maximum power density of 0.92 mW/m^3 using a resistor of 5 KΩ when anaerobic sludge is used. However, in our study, the electric output was comparatively low, which may be due to various influencing factors such as the microbial strain used, their source, external resistor, physicochemical parameters, operating conditions, etc. Graphite felt coated with absolute alcohol-treated CNT stands out among other electrode modifications in terms of power generation. CNT, due to its high surface volume-to-volume ratio, has high electrical conductivity and offers a promising future for improved electrode-microbial interaction. The alcohol treatment helps disperse CNTs evenly, improves their adhesion to the electrode surface, and reduces the internal resistance of the MFC (Basheer et al., 2019).

According to Erbay et al. (2015), microbial growth over the CNT results in excellent charge transfer characteristics due to π-π stacking between the carbon atoms of the graphite and the pili of microbes. This could be the reason behind the maximum power output.

Table 3 revealed the removal efficiencies of MFC at different conditions of operation. Removal efficiencies were found not corelated with electrical performance. This may be due to high lactose utilizing properties of isolated yeast with no recorded electrical performance.

The cyclic voltammogram (CV) exhibited 3 anodic peaks (Figure 3), with two peaks observed between 0.3 V and 0.1 V, likely corresponding to metal oxides in the graphite electrodes. The third peak at 0.5 V indicated the redox peak of electroactive oxide/hydroxide in the carbon electrode. The corresponding cathodic peak was observed at 0.1 V to -0.5 V. The separation between the anodic and cathodic peaks suggested a quasi-reversible system, possibly due to the presence of metal oxide. No distinct cathodic peak was observed for the metal oxide. In the first cycle, the anodic current was influenced by surface activation above 0.2 V. Subsequent cycles showed minimal differences between the fourth and tenth cycles compared to the first cycle, indicating electrode degradation with cycle number, although the degradation was not significant.

Conclusion

In summary, this research underscores the remarkable potential of two-chamber electron-mediated Microbial Fuel Cells (MFCs) utilizing graphite felt electrodes and paneer whey substrate for both bioelectricity generation and dairy waste treatment. The optimized MFC configuration, featuring paneer whey as the anolyte, 0.1 M phosphate buffer with potassium ferricyanide as the cathodic solution, and a specialized bacterial consortium, showcased outstanding performance metrics, including substantial reductions in various pollutants and an impressive maximum power output of 25.869 mW/m^3. Moreover, while simpler MFC setups exhibited effective pollutant removal capabilities, they yielded comparatively lower power outputs. Notably, our findings highlight the superiority of mixed bacterial/yeast cultures over single-strain counterparts, emphasizing the importance of optimizing buffer composition and electron acceptors for enhanced power generation.

Additionally, the promising results obtained with electrodes coated with CNT composite suggest exciting possibilities for further improving power generation efficiency. Addressing challenges such as pH maintenance and equipment limitations will be crucial for maximizing MFC performance and facilitating broader adoption. With continued investment and refinement, MFCs hold significant promise as both sustainable energy recovery solutions and efficient wastewater treatment methods, contributing to a more environmentally sustainable future.

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We would like to acknowledge University Grants Commission, Nepal for partial support as M. Sc. Thesis support grant to Miss Rosy Chaulagain for this work and the assistance provided by the staffs and resources at Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Table 2: Electric output of MFC with different configurations.

MFC Configuration **Anolyte pH: 5.4** **Catholyte pH: 7.6** **Operating temperature: Room temperature (approx. 25°C)**		**Maximum OCV (mV)**	**Maximum CCV (mV)** (External resistor: 1000 Ω)	**Maximum Current (mA)**	**Maximum Power (mW/m³)**
Anolyte: Paneer whey Catholyte: Phosphate Buffer Electrode: Graphite felt	Biocatalyst *Mixed Bacterial /yeast Consortium of DDC waste* *Lactobacillus spp*. only *Klebsiella spp.* only Mixed Bacterial/yeast consortium of DDC waste + *Lactobacillus spp.*	394.4 390.7 509.4 528.4	157.1	0.060	13.510
Anolyte: Paneer whey Electrode: Graphite Felt Biocatalyst: Mixed bacterial/yeast consortium of DDC waste + *Lactobacillus spp.*	Catholyte 0.1M Sodium acetate Phosphate buffer 0.1M Phosphate buffer + 0.1M Potassium ferricyanide 0.1M Sodium acetate + 0.1M Potassium ferricyanide	429.4 478.2 609.5 515.4	191.2	0.0815	22.261
Anolyte: Paneer whey Biocatalyst: Mixed bacterial/yeast consortium of DDC waste+ *Lactobacillus spp.* Catholyte: 0.1M Phosphate buffer + 0.1M Potassium ferricyanide	Electrode CNT + Ethanol	633	203.7	0.0889	25.869

Table 3: Performance of MFCs in terms of removal efficiency with various configurations.

MFC configuration: Dual Chambered **Anolyte pH: 5.4** **Catholyte pH: 7.6** **Operating temperature: Room temperature (approx. 25°C)**		**Removal (%)**		
		COD	Total Phosphorus	Ammoniacal Nitrogen
Anolyte: Paneer whey Catholyte: 0.1 M Phosphate Buffer Electrode: Graphite felt	Biocatalyst Mixed bacterial/yeast Consortium of DDC waste *Lactobacillus* *Klebsiella* Mixed bacterial/yeast consortium of DDC waste + *Lactobacillus spp.*	50 41.72 60.68 58.27	54.20 36.58 49.88 65.67	63.50 67.13 33.98 67.13
Anolyte: Paneer whey Electrode: Graphite felt Biocatalyst: Mixed bacterial/yeast consortium of DDC waste + *Lactobacillus spp.*	Catholyte 0.1M Sodium acetate 0.1M Phosphate buffer + 0.1M Potassium ferricyanide 0.1M Sodium acetate + 0.1M Potassium ferricyanide	65.51 55.17 53.79	60.38 58.59 53.30	- - -
Anolyte: Paneer whey Biocatalyst: Mixed bacterial consortium of DDC waste + *Lactobacillus spp.* Catholyte: 0.1M Phosphate buffer + 0.1M Potassium ferricyanide	Electrode CNT treated with Ethanol	61.03	48.91	- - -

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Research Article

In vitro Micropropogation of Endangered Orchid *Aerides odotrata* Lour. from Seed Culture

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ABSTRACT

Aerides odorata Lour. is an endangered orchid, famous for its appealing, fragrant blooms and its epiphytic growth. This study examined the effects of culture medium composition, plant growth regulators, and organic additives on the *in vitro* growth of *Aerides odorata* Lour. Full-strength MS medium enriched with 10% coconut water produced the highest seed germination rate (85%), showing earlier protocorm and shoot development in comparison to other strength of MS medium and medium with plant growth regulators. Shoot multiplication was found a notable increase with 2.0 mg/l of BAP, yielded the highest shoot count (13.4 per explant), whereas shoot elongation was most effective in MS medium with 1.0 mg/l of BAP and 0.1 mg/l of NAA. The effective of root number induction was showed the highest in MS medium with NAA and IBA at 3 mg/l, while root length induction was in MS medium with IBA at 3 mg/l, highlighting its prolonged auxin effectiveness. The results emphasize the combined effect of coconut water, BAP and IBA in enhancing effective micropropagation of *A. odorata*, offering a dependable method for the conservation and extensive propagation of this orchid species.

Keywords: *Aerides*; Auxin; Coconut water; Cytokinin; Micropropogation

Introduction

Orchids (Orchidaceae) are among the most varied families of flowering plants globally, with an estimated 31,480 species spread across 758 genera (Elliott et al., 2025). They have inhabited almost every environment from sea level to mountain peaks, exhibiting impressive morphological and ecological modifications, which makes them essential elements of worldwide plant diversity (Kindlmann & Rocamora, 2023). In Nepal, the orchids exhibits considerable diversity because of the country's diverse topography and climate. Nepal's orchid diversity comprises around 501 species within 108 genera, found in both tropical and alpine regions (Shrestha et al., 2022).

Aerides odorata Lour. is a critically endangered orchid species, recognized for its beautiful, aromatic blooms and epiphytic nature (Huda et al., 2021). It is extensively found throughout subtropical and tropical region in Nepal, China, India, Bhutan, Bangladesh, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia,

Indonesia and Philippines. In Nepal, *A. odorata* thrives in mixed forests at low to mid elevations (200–1100 m) and often develops as an epiphyte on the trunks and branches of trees (Rajbhandari & Rai, 2017; Huda et al., 2021). It has thick and branching stems with elongated leaves. This blooms from late spring to summer, yielding clusters of fragrant flowers that are frequently utilized decoratively. *odorata* has been utilized for addressing skin diseases, wounds, fevers, ear infections, digestive issues and inflammatory ailments (Singh et al., 2001). Phytochemical investigations have revealed biologically active secondary metabolites, including flavonoids, alkaloids, phenolic compounds, terpenoids, and glycosides, which play a role in its known antioxidant, antimicrobial and cytotoxic characteristics (Hossain, 2011; Pant & Raskoti, 2013; Saifur et al., 2025). In Nepal, wild orchids have been collected for medicinal, horticultural and commercial purposes for a long time, with more than 60 species traditionally used to address issues like wounds, fever, bone fractures and skin ailments (Subedi et al., 2013). Despite the scarcity of specific ethnomedicinal documentation for *A. odorata* in Nepalese customs, regional research from South Asia shows that this plant is utilized typically for healing wounds and as a tonic, incorporating different parts of the plant in traditional therapies (Panda & Mandal, 2013; Teoh, 2016). Initial phytochemical assessments of *A. odorata* leaf extracts have identified the existence of alkaloids, coumarins, flavonoids, glycosides, phenols, and terpenoids, substances frequently linked to medicinal benefits (Katta et al., 2019). Later research also indicated antioxidant and liver-protective properties, implying that the species contains bioactive substances with medicinal potential.

Though varied and valuable, orchids encounter major conservation issues around the world. Many species are at risk due to threats such as habitat loss, climate change, illegal collection and overexploitation putting orchids in danger (Gale et al., 2018; Hinsley et al., 2018). *Aerides odorata* is classified as endangered category by the IUCN because of habitat loss and decreasing wild populations. In Nepal, the unlawful collection and sale of orchids for traditional medicine and commerce have intensified population pressures, underscoring the necessity for conservation strategies and sustainable management (Subedi et al., 2013)

Materials and Methods

Explant source and surface sterilization

Mature unopened capsules of *Aerides odorata* Lour. were collected from naturally growing plants in the Makwanpur district of Hetauda, Nepal. To remove surface contaminants and debris, the capsules were first rinsed under running tap water for 15–20 min after being agitated in 0.01% (v/v) Tween 20 solution for 30 min on a rotary shaker. Subsequent surface sterilization was performed under aseptic conditions in a laminar airflow cabinet. The capsules were briefly immersed in 70% (v/v) ethanol for 30 s and flamed to eliminate superficial microorganisms, followed by treatment with 1% (w/v) sodium hypochlorite solution for 8 min for further disinfection. After sterilization, the capsules were thoroughly rinsed 4–5 times with sterile distilled water to remove any traces of sterilizing agents. Finally, the disinfected capsules were longitudinally opened using a sterile scalpel, and the immature seeds were carefully inoculated onto the prepared culture media under aseptic conditions in culture jar.

Culture media and growth regulators

The basal culture medium comprised Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 3% (w/v) sucrose as a carbon source and solidified with 0.8% (w/v) agar. The pH was carefully adjusted to 5.8 ± 0.02 prior to sterilization. The medium was autoclaved at 121 °C under 1.05 kg/cm² pressure for 15–20 min. For seed germination and protocorm development, the MS medium was supplemented with various concentrations and combinations of plant growth regulators (PGRs) and coconut water. To induce shoot formation and shoot proliferation, cytokinins including 6-benzylaminopurine (BAP; 0.5-2.0 mg/l) and kinetin (Kn; 0.5-2.0 mg/l) were incorporated at different levels. For the root induction medium was supplemented with auxins such as α-naphthaleneacetic acid (NAA; 0.5-4.0 mg/l), indole-3-butyric acid (IBA; 0.5-4.0 mg/l), and indole-3-acetic acid (IAA; 0.5-4.0 mg/l), either individually or in selected combinations. All chemicals and media constituents were procured from standard commercial suppliers and used without further modification.

Culture conditions

All cultures were maintained in a controlled growth room at 25 ± 2 °C under a 16 h light/8 h dark photoperiod. Illumination was supplied by cool white fluorescent lamps. The relative humidity of the culture room was regulated at around 60-70% to ensure stable growth conditions.

Data analysis

Observations of seed germination, protocorm development, shoot induction and multiplication, as well as root formation, were systematically recorded at

regular intervals typically every 4 weeks. For each treatment, 10-15 explants were cultured, and all treatments were performed in triplicate to ensure reproducibility. Quantitative data were expressed as mean ± standard deviation (SD). Statistical comparisons among treatments were carried out using Duncan's multiple range test (DMRT) at a significance level of $p \leq 0.05$.

Results and Discussion

Effect of media composition on seed germination

The response of mature seeds of *Aerides odorata* Lour. varied markedly with the composition of the culture medium. Nine different media formulations, including full strength MS (FMS), half strength (HMS), quarter strength MS (QMS), and media supplemented with or without plant growth regulators and organic additives, were evaluated for seed germination, protocorm development, and shoot initiation.

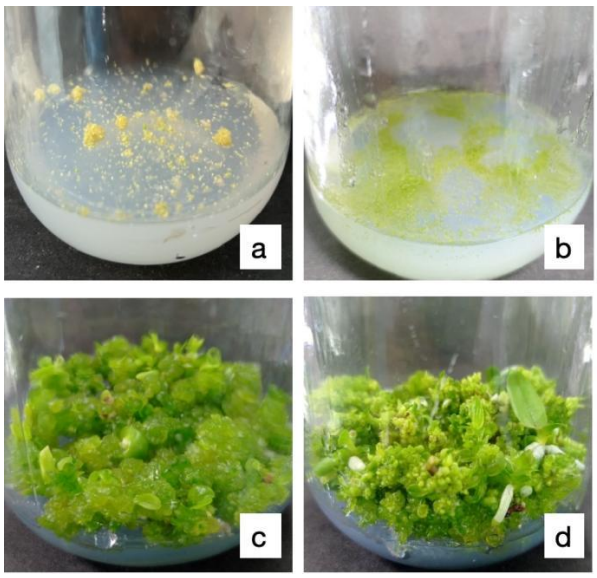

Figure 1: Seed germination stages in MS medium. a) Initiation of seed germination in MS medium with 10 % CW; (b) Protocorm like bodies; (c) Protocorm formation in MS medium with 10 % CW; (d) Shoot initiation.

Among all treatments, FMS supplemented with 10% (v/v) coconut water (CW) exhibited the most rapid and efficient response which is 85 % of total germination (Figure 1a &b, Figure 2). This results is consistent with the previous studies reporting enhanced seed germination in the presence of 10 % coconut (Thapa et al., 2020; Pant et al., 2020). Coconut water contains natural cytokinins which stimulate cell division and differentiation of the embryo. Initial seed germination was observed within 4 weeks of culture, followed by FMS where initiated of seed germination took place after 5 weeks of culture. In quarter-strength MS medium supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA, seed germination was initiated only after 8 weeks of culture (Figure 3) and seed germination rate is only 39.33 % (Figure 2). As the concentration of MS salts decreased from FMS to quarter-strength MS (QMS), a consistent delay in developmental milestones was recorded. For instance, in basal media without additives, germination initiation shifted from 5.0 weeks (FMS) to 6.6 weeks (QMS). Furthermore, the integration of synthetic plant growth regulators (0.5 mg/l BAP + 0.1 mg/l NAA) did not enhance the growth rate compared to CW (Figure 2). Similar inhibitory effects of full concentrations MS salts have been reported on seed germination of some orchid species (Joshi et al., 2023; Azad et al., 2025).

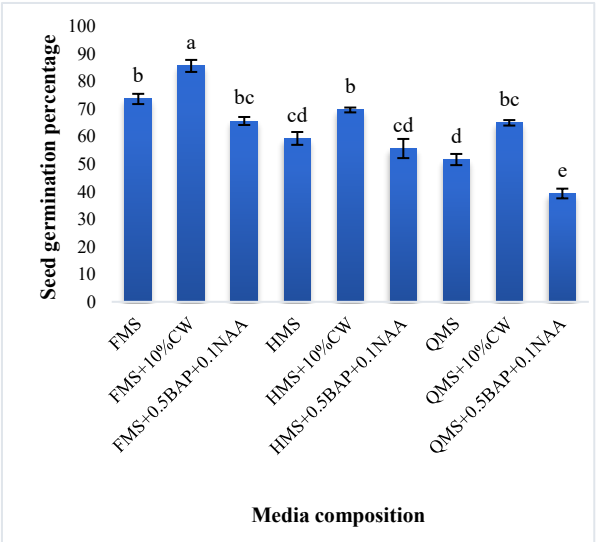

Figure 2: Seed germination percentage of *A. odorata* in different media composition.

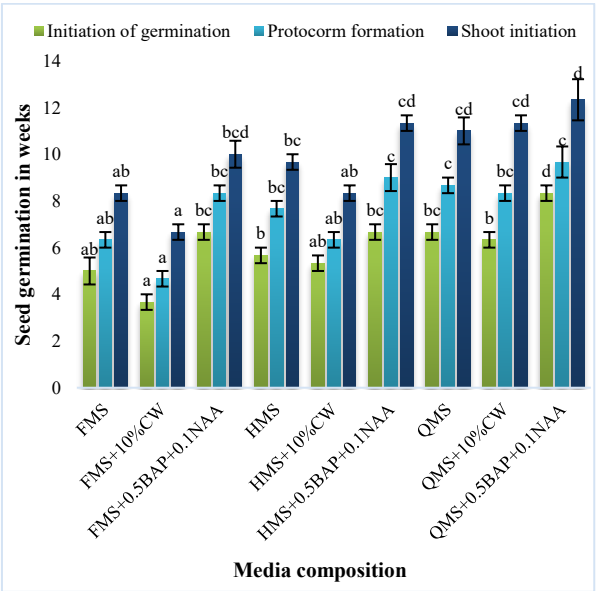

Figure 3: Seed germination of *A. odorata* in different media composition.

Effect of media composition on protocorm formation and shoot initiation

The most rapid and efficient response of protocorm formation (Figure 1c) and shoot initiation (Figure 1d) was also observed with FMS supplemented with 10% (v/v) coconut water (CW). The effect of coconut water aligns with its known content of natural cytokinins, vitamins, and organic compounds that enhance cell division and morphogenesis (Nambiar et al., 2012; da Silva et al., 2017). Initiation of protocorm formation was started in 4.67 days while shoot initiation was started in 6.67 days (Figure 3). Media supplemented with plant growth regulators showed a reduced and delayed response compared to organic additive-based media. In quarter-strength MS medium supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA, seed germination was initiated only after 8 weeks of culture (Figure 3). Protocorm formation was observed at approximately 9 weeks, while shoot initiation required up to 12 weeks (Figure 3). Moreover, this treatment exhibited the lowest overall response.

Overall, the results clearly indicate that medium composition with coconut water significantly influences *in vitro* seed germination and subsequent developmental stages in *A. odorata.* This results is consistent with the previous studies where in the presence of coconut water enhance the seed germiantion and protocorm formation (Maharjan et al., 2020; Pant et al., 2022).

Effect of media composition on shoot induction

Shoot number of Aerides odorata was significantly affected by the type and concentration of plant growth regulators. Among all treatments, MS medium supplemented with 2.0 mg/l BAP produced the highest number of shoots (13.4 ± 0.30 shoots per explant), followed by MS + 1.5 mg/l BAP (12.1 shoots) and MS + 1.0 mg/l BAP (11.2 shoots) (Figure 4 & Figure 6a) after 12 weeks of culture. Coconut water supplementation also enhanced shoot proliferation (9.0 shoots) compared with the control MS medium (6.8 shoots). In contrast, kinetin containing media resulted in comparatively lower shoot numbers (3.4–7.2 shoots), with the minimum response observed at 0.5 mg/l Kn. The addition of low NAA (0.1 mg/l) to BAP containing media moderately influenced shoot number but did not surpass the proliferation achieved with 2.0 mg/l BAP alone.

The superior performance of BAP over kinetin observed in this study is consistent with numerous recent reports indicating that BAP is more effective in stimulating axillary bud break and shoot multiplication in orchids due to its higher cytokinin activity and stability *in vitro* (Pant & Gurung, 2005; da Silva et al., 2025;). Similar dose-dependent effectively increases in shoot proliferation with increasing BAP concentrations have been reported in *Dendrobium*, *Cymbidium* and *Vanda* species (Tao et al., 2011; Lukatkin et al., 2019). The enhanced shoot number at higher BAP levels may be attributed to increased meristematic activity and suppression of apical dominance, which promotes lateral bud proliferation (Su et al., 2011). Overall, the results demonstrate that BAP at 2.0 mg/l is optimal for maximizing shoot multiplication in *A. odorata*, while kinetin is comparatively less effective for shoot proliferation in this species.

Shoot length varied significantly among the different media compositions tested (Figure 5), indicating that the nature and concentration of plant growth regulators substantially influenced shoot elongation in Aerides odorata. The longest shoots (3.8 ± 0.14 cm) were obtained on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA (Figure 6b), significantly producing greater elongation than all other treatments ($p \leq 0.05$). Moderate shoot growth was observed on MS supplemented with 10% coconut water (~2.3 cm), MS + 2.0 mg/l BAP (~2.1 cm), and MS + 1.5 mg/l BAP (~2.0 cm), while media containing kinetin exhibited comparatively limited elongation. The lowest shoot lengths (0.4-0.6 cm) occurred in MS + 0.5-2.0 mg/l Kn treatments (Figure 5). These results are consistent with multiple recent studies reporting that shoot proliferation and elongation in orchids are highly responsive to the type and balance of cytokinins and auxins. Similarly, Bhattacharjee & Islam (2014) and Talukder et al. (2003) reported BAP and NAA enhanced shoot elongation in *Vanda* and *Dendrobium*, consistent with the present study.

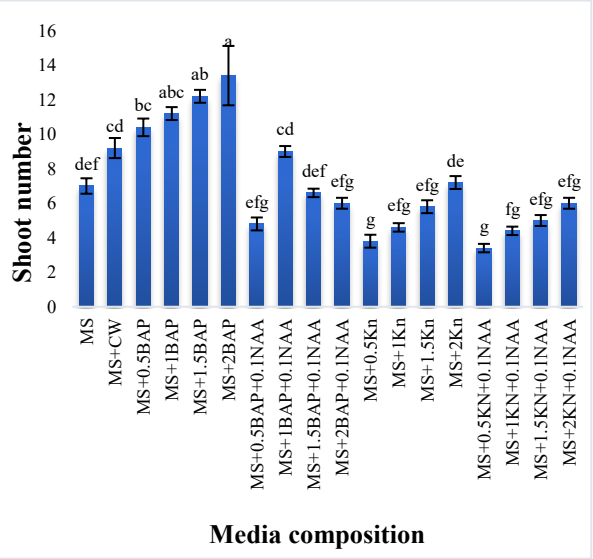

Figure 4: Number of shoot proliferation in different media composition.

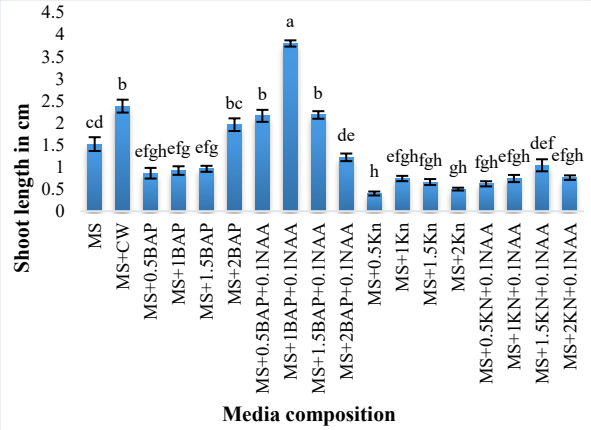

Figure 5: Shoot length in different media composition.

In vitro root formation using plant growth regulators

The present study demonstrated that root induction in *Aerides odorata* was maximized at 3 mg/l NAA and 3 mg/l IBA, with both treatments producing significantly higher root numbers compared to IAA (Table 1). These findings are consistent with previous reports in orchids, where NAA has been shown to stimulate root primordia formation (Thiyam et al., 2025; Mirani et al., 2017), while IBA provides sustained auxin activity leading to vigorous root development (Frick & Strader, 2018; Kaushik & Shukla, 2020). The comparatively weaker response to IAA corroborates earlier observations that natural auxins are less stable *in vitro* (Nissen & Sutter 1990). Thus, synthetic auxins, particularly NAA and IBA, remain the most effective regulators for *in vitro* rooting of *A. odorata*.

Table 1: Effect of different media composition on root number and root length induction of *A. odorata*.

Media Combination	Root Number (Mean ± SE)	Root Length (cm) (Mean ± SE)
MS + 0.5 NAA	2.0 ± 0.0566^{bc}	0.64 ± 0.0072^{f}
MS + 1.0 NAA	2.2 ± 0.0358^{bc}	1.14 ± 0.0313^{de}
MS + 2.0 NAA	2.6 ± 0.0438^{b}	1.68 ± 0.0256^{cd}
MS + 3.0 NAA	3.0 ± 0.0800^{ab}	2.20 ± 0.0247^{c}
MS + 4.0 NAA	2.4 ± 0.0438^{bc}	1.20 ± 0.0179^{de}
MS + 0.5 IAA	1.8 ± 0.0358^{c}	0.44 ± 0.0145^{f}
MS + 1.0 IAA	2.0 ± 0.0566^{bc}	1.06 ± 0.0145^{e}
MS + 2.0 IAA	2.4 ± 0.0716^{bc}	2.04 ± 0.0440^{cd}
MS + 3.0 IAA	2.6 ± 0.2683^{b}	1.52 ± 0.0119^{d}
MS + 4.0 IAA	2.2 ± 0.0669^{bc}	1.24 ± 0.0308^{de}
MS + 0.5 IBA	2.2 ± 0.0358^{bc}	1.70 ± 0.0247^{cd}
MS + 1.0 IBA	2.8 ± 0.0358^{ab}	2.42 ± 0.0409^{bc}
MS + 2.0 IBA	3.0 ± 0.0566^{ab}	2.94 ± 0.0308^{b}
MS + 3.0 IBA	3.2 ± 0.1315^{a}	3.86 ± 0.0762^{a}
MS + 4.0 IBA	2.4 ± 0.0716^{bc}	3.68 ± 0.0296^{a}

Root length in *Aerides odorata* was significantly influenced by both the type and concentration of auxin incorporated into the culture medium. Among the treatments evaluated, IBA at 3 mg/l consistently produced the longest and most well-developed roots (Figure 6c), whereas NAA resulted in intermediate root elongation and IAA produced comparatively shorter roots. The superior performance of IBA observed in the present study aligns with earlier findings in orchids and other micropropagated species, where IBA has been reported to be more effective than other auxins in stimulating *in vitro* rooting and enhancing root elongation (Rafique et al., 2012).

Figure 6: Shoot and root induction. (a) shoot formation; (b) shoot elongation in terms of length; (c) root formation and root length.

The enhanced rooting response under IBA may be attributed to its physiological behavior within plant tissues. IBA is known to function as a relatively stable auxin precursor that can be converted into the biologically active form, IAA, thereby providing a more sustained auxin supply during root initiation and elongation (Frick & Strader, 2018). Exogenous supplied IAA is more susceptible to rapid degradation through conjugation and oxidative inactivation pathways, which can limit its persistence and reduce its effectiveness in supporting prolonged root growth under *in vitro* conditions (Hayashi et al., 2021). From a practical perspective, the use of IBA at 3 mg/l in rooting media is therefore recommended to obtain robust and elongated roots prior to acclimatization, which is critical for improving survival and establishment under *ex vitro* conditions.

Conclusion

The present study establishes an efficient and reproducible *in vitro* regeneration protocol for *Aerides odorata* Lour., highlighting the critical influence of medium composition and growth regulator balance on morphogenetic responses. Full-strength MS supplemented with 10% (v/v) coconut water significantly enhanced seed germination, accelerated protocorm formation, and promoted earlier shoot initiation compared to basal and PGR supplemented media, confirming the stimulatory role of organic additives during early orchid development. For shoot

multiplication, MS medium containing 2.0 mg/l BAP produced the highest shoot number, demonstrating the superior cytokinin efficiency of BAP over kinetin. However, optimal shoot elongation was achieved with 1.0 mg/l BAP in combination with 0.1 mg/l NAA, indicating that a balanced cytokinin auxin interaction improves shoot quality. Root induction was maximized with 3 mg/l NAA and IBA, and IBA (3 mg/l) produced the greatest root length, supporting its sustained effectiveness in promoting root elongation. Collectively, the optimized combinations identified in this study provide a robust micropropagation system for *A. odorata*, facilitating large scale propagation, conservation, and commercial utilization of this ornamental orchid.

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Research Article

Detection and Expression of blaKPC-2 and fimH Genes in *Klebsiella pneumoniae* Clinical Isolates

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ABSTRACT

Klebsiella pneumoniae cause several serious infections, pneumonia, urinary tract infections and bloodstream infections, especially in a immune-compromised patient. There has been rise in antibiotics resistant *K. pneumoniae* in recent years. This study aimed detection and expression of blaKPC-2 and fimH genes in carbapenemase producing *K. pneumoniae* that can form biofilms in clinical samples. A total of 608 clinical samples were processed for *K. pneumoniae* using standard microbiological procedure. Antibiotic resistance was checked using the modified Kirby-Bauer disc diffusion method, and carbapenemase production was tested using a modified carbapenem inactivation method in conjunction with EDTA modified carbapenem inactivation method. In order to detect the presence and expression of resistant genes, bacterial DNA and RNA were extracted. A total of 30 isolates were positive for *K. pneumoniae* among which 21 (70%) were multi-drug resistant and 18 (85.71%) were carbapenemase producers in MDR isolates. A total of 14 isolates (66.67%) produced biofilm, of which 6 (28.57%) were strong, 6 (28.57%) were moderate and 2 (9.52%) were weak. Both blaKPC-2 and fimH genes were detected in 19 isolates (90.47%) and were actively expressed in 16 (76.19%). These findings show the blaKPC-2 and fimH genes are common in clinical isolates and are actively being expressed. In conclusion, there is urgent need to control highly resistant *K. pneumoniae.*

Keywords: blaKPC-2; Biofilm; Carbapenem resistance; fimH; *Klebsiella pneumoniae*

Introduction

Klebsiella pneumoniae is a Gram-negative pathogenic bacterium that causes different infections such as pneumonia, urinary tract infections, wound infections, bacteremia and sepsis. The infections can be serious particularly in immune compromised individuals (Mohamudha et al., 2016; Paczosa & Mecsas, 2016).

Infections by multi-drug resistant (MDR) strains of *K. pneumoniae* are increasing global threats evident by limited available treatments (Pitout et al., 2020). In a meta-analysis done in Nepal, a pooled prevalence of 64% MDR *K. pneumoniae* was reported, which is alarming issue (Odari & Dawadi, 2022). Misuse, overuse and underuse of antibiotics in humans and animals, in addition to unregulated prescription of antibiotics and lack of well-equipped hospitals and clinics may be the driving factors for increasing antimicrobial resistance rates in Nepal (Dahal & Chaudhary, 2018; Acharya & Wilson, 2019; Pokharel & Adhikari, 2020; Rijal et al., 2021). Different mechanisms such as biofilm formation, production of β-lactamases, enzymatic modifications, efflux pumps and reduction of membrane permeability are responsible for developing multi-drug resistant *K. pneumoniae* (Li et al., 2024).

Carbapenems are the most common lastline drug to treat infections caused by MDR *K. pneumoniae*. However increasing rate of carbapenem resistant strains of *K. pneumoniae* is of great concern (Nordmann et al., 2009; Pitout et al., 2015). In 2017, WHO listed carbapenem resistant *K. pneumoniae* in high priority (critical) for which development of new antibiotics is required. *K. pneumoniae* become resistant to carbapenems because of three mechanisms: production of *K. pneumoniae* carbapenemase, efflux pumps and reduction in cell membrane permeability particularly due to ompK35 and ompK36 porins mutation. Carbapenemase enzymes belong to ambler class A (KPC), class B (NDM, IMP, VIM) and class D (OXA-48) (Alizadeh et al., 2020; Li et al., 2025).

Mere detection of resistance genes like blaKPC-2 and blaVIM, as well as virulence factors such as fimH and rmpA, do not show the real impacts of antibiotic resistant strains (Bush & Bradford, 2016; Flores-Mireles et al., 2015). How these strains act clinically depend on gene expressions? In fact, the expression of several genes affects the virulence and resistance level. Hence, we can't ignore the gene expression as it could compromise identification of clinical risk and limit the definite prediction of treatment outcomes (Rasko & Sperandio, 2010).

In addition, the co-expression of resistance and virulence factors, have not been studied enough. This lack of data has constrains on the development of targeted effective infection control and treatment methods (Hall-Stoodley et al., 2004; World Health Organization, 2023). This study aims to detect expression of blaKPC-2 and fimH genes in carbapenemase producing *K. pneumoniae.*

Materials and Methods

Study site and duration

This study was conducted at the Department of Microbiology of the Annapurna Neurological Institute and Allied Sciences (ANIAS) and the Department of Molecular Biology of Annapurna Research Center (ARC), Maitighar, Kathmandu from April to October 2025. Ethical approval was obtained from the Institutional Review Committee (Reg no: 2025/04) at Annapurna Neurological Institute. Written informed consent was obtained from all participants.

Identification of *Klebsiella pneumoniae* from clinical samples

A total of 608 non-duplicate samples including sputum, urine, wound swab, cerebrospinal fluid (CSF) and endotracheal tubes were processed and cultured using standard microbiological techniques. *K. pneumoniae* isolates were identified by studying characteristics on blood agar and MacConkey agar, performing Gram staining and biochemical tests (catalase test, oxidase test, urease test, triple sugar iron agar test and IMViC test).

Antibiotic sensitivity testing

Modified Kirby Bauer Disc diffusion technique was used for antibiotic sensitivity testing of isolated *K. pneumoniae.* In this study, following antibiotics were used: Amikacin (AK), Levofloxacin (LE), Cefixime (CFM), Cefepime (CPM), Cotrimoxazole (COT), Cefotaxime (CTX), Ofloxacin (OF), Gentamicin (GEN), Meropenem (MRP), Piperacillin + Tazobactam (PIT), Doxycycline (DO), Polymyxin B (PB), Clindamycin (CL), Tigecycline (TGC), Ciprofloxacin (CIP), Ceftazidime (CAZ), Linezolid (LZ), Ceftriaxone (CTR) and Nitrofurantoin (NIT). MDR is acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012).

Identification of carbapenemase producers

Meropenem resistant isolates were screened for carbapenemase production modified carbapenem inactivation method (mCIM) in combination with modified EDTA carbapenem inactivation method (eCIM) described by Tsai et al. (2020).

Biofilm formation test

Isolates forming biofilm were screened using the Congo red agar (CRA) method (Freeman et al., 1989).

Extraction of nucleic acid

Alkaline hydrolysis method was used for the extraction of plasmid DNA from isolates (Sambrook & Russell, 2001). The extracted plasmid DNA was subsequently used as templates for amplification of blaKPC-2 gene. For the amplification of fimH gene, chromosomal DNA was extracted using the Quick-DNA/RNA Miniprep Kit (ZYMO Research, USA). Finally, total RNA was extracted from the isolates using Vision Purify Viral RNA Extraction Kit (Vision Biotechnology, Turkey) to detect gene expression.

Gene identification

blaKPC-2 and fimH genes were amplified by PCR using primers, forward: 5'-GATACCACGTTCCGTCTGG-3' and reverse: 5'-GCAGGTTCCGGTTTTGTCTC-3' (Hindiyeh et al., 2008) for blaKPC-2 gene, and forward: 5'-GTGCCAATTCCTCTTACCGTT-3' and reverse: 5'-TGGAATAATCGTACCGTTGCG-3' (Hojati et al., 2015) for fimH gene.

For amplification of each gene, 5 µl DNA template was added to 10 µl mixture containing 6.5 µl master mix, 2.5 µl nuclease free water and 0.5 µl each of respective reverse and forward primers. PCR was performed in Proflex Thermocycler using optimized conditions (Table 1).

The PCR products were resolved by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide run at 70 V for 40 minutes, followed by analysis in gel doc system.

cDNA synthesis and gene expression

The cDNA from isolated RNA was synthesized using the Vision cDNA Synthesis Kit (Vision Biotechnology, Turkey), following manufacturer's instructions. cDNA was used as a template for amplification of blaKPC-2 and fimH genes using specific primers and optimized conditions as explained above. The amplified products were documented using agarose gel electrophoresis and gel doc system.

Table 1: Optimized conditions for amplification of blaKPC-2 and fimH genes.

Gene	Optimized conditions for PCR					Length of PCR product (bp)
	Initial denaturation	Denaturation	Annealing	Extension	Final extension	
blaKPC-2	94°C for 3 min	94°C for 1 min (35 cycles)	60°C for 1 min (35 cycles)	72°C for 1 min (35 cycles)	72°C for 5 min	246
fimH	96°C for 3 min	96°C for 30 sec (35 cycles)	64°C for 1 min (35 cycles)	72°C for 1 min (35 cycles)	72°C for 5 min	164

Results and Discussion

Out of 608 total clinical samples, 150 were cultured positive among which 30 (20%) samples tested positive for *K. pneumoniae.* In a subset of 30 isolates, 21 (70%) *K. pneumoniae* were found to be MDR and 18 (85.7%) isolates were confirmed to be carbapenemase producers by mCIM and eCIM methods. Consistent with our study finding, the recent study conducted in Nepal reported 73.5% MDR *K. pneumoniae* (Neupane et al., 2025). Meanwhile, Pyakurel et al. (2021) reported 77.6% carbapenemase producing *K. pneumonia*, which is higher than our findings.

Out of 21 MDR isolates, 14 (66.67%) showed biofilm production on CRA, which is lower than finding of Pradhan et al. (2019) (Figure 1). Among the 21 isolates, 19 (90.47%) isolates were found to be harbouring fimH gene. fimH gene was detected in 6 strong biofilm forming isolates, 5 moderate and 2 weak biofilm forming isolates. Meanwhile, fimH gene was also detected in 6 non-biofilm forming isolates (Figure 2). Presence of fimH genes only does not necessarily produce biofilms, there are so many other genes responsible for it. These results showed a significant role of fimH genes in production of biofilms, which is consistent with several other studies (Alwan et al., 2024; Ashwath et al., 2022). However, no statistically significant association was observed between biofilm formation strength and either fimH gene detection ($p = 0.710$) or fimH gene expression ($p = 0.660$) (Table 2). These findings suggest that although fimH is widely distributed among clinical isolates, its expression alone may not determine biofilm phenotype, highlighting the involvement of additional virulence determinants in biofilm formation.

Among 21 MDR isolates of *Klebsiella pneumoniae*, the blaKPC-2 gene was detected in 19 (90.47%) isolates, while 16 (76.19%) demonstrated gene expression. Phenotypic carbapenemase production was observed in 18 isolates, whereas 19 (90.47%) and 16 (76.19%) were positive for blaKPC-2 gene detection and expression respectively. This detection rate is higher than the recent report in Nepal (Khadka et al., 2025). No statistically significant association was observed between

carbapenemase production and the presence of the blaKPC-2 gene (p = 0.544), indicating that gene carriage alone does not necessarily correspond to phenotypic resistance. However, a significant association was found between carbapenemase production and blaKPC-2 gene expression (p = 0.001), suggesting that transcriptional activation of this carbapenemase gene plays a crucial role in resistance phenotype (Table 3). These findings indicate that while the gene is widely distributed among *Klebsiella pneumoniae* isolates, blaKPC-2 expression is strongly correlated with phenotypic carbapenemase activity, highlighting the importance of transcriptional regulation in antimicrobial resistance.

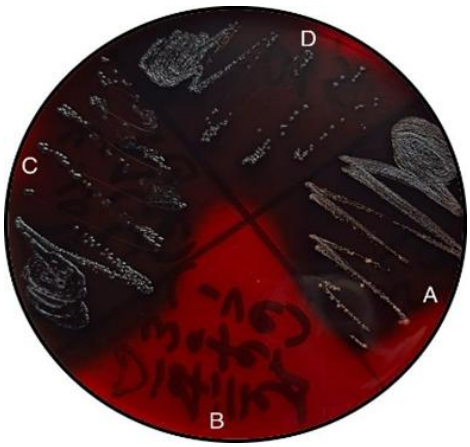

Figure 1: Biofilm Production on CRA: A: Positive control (*E. coli* ATCC 25922), B: Negative control (Distilled water), C&D: Samples (Strong category).

The co-existence of blaKPC-2, and fimH expression and biofilm formation highlights a concerning convergence of resistance and virulence factors in *K. pneumoniae* isolates. Such findings could assist infection control efforts, antimicrobial resistance regulation, and therapy.

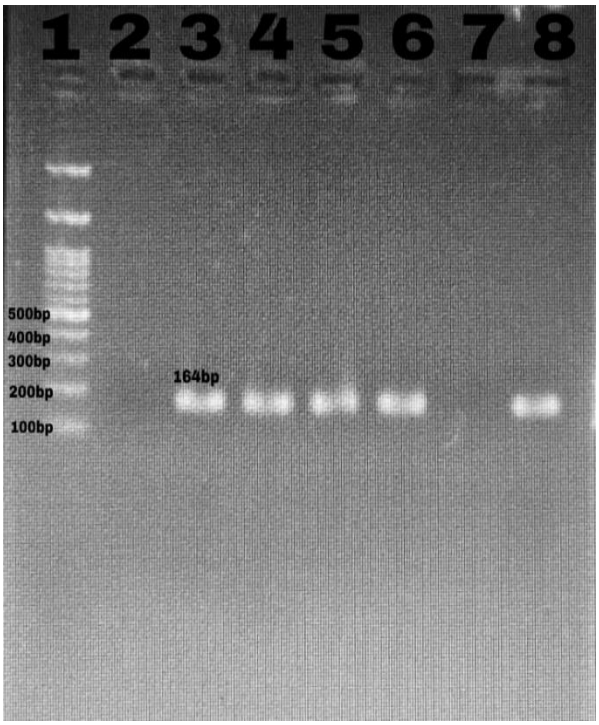

Figure 2: Gel image showing fimH gene expression. 1: Ladder (100 bp); 2: Negative control; 3: Positive control; 4, 5, 6 & 8: Samples (fimH +ve); 7: Blank

Table 2: Pattern of fimH gene detection, gene expression on congo red test (Biofilm production).

		fimH gene detection				fimH gene expression			
		Positive	Negative	Total	p-value	Positive	Negative	Total	p-value
Congo Red Test	Strong	6	0	6	0.71	4	2	6	0.66
	Moderate	5	1	6		5	1	6	
	Weak	2	0	2		1	1	2	
	No-biofilm	6	1	7		6	1	7	
	Total	19	2	21		16	5	21	

Table 3: Pattern of carbapenemase production test for blaKPC-2 gene detection and expression.

		blaKPC-2 gene detection				blaKPC-2 gene expressionn			
		Positive	Negative	Total	p-value	Positive	Negative	Total	p-value
Carbapenemase production test	Positive	16	2	18	0.544	16	2	18	0.001
	Negative	3	0	3		0	3	3	
	Total	19	2	21		16	5	21	

Conclusion

This study investigated blaKPC-2 and fimH genes expression in carbapenemase producing *K. pneumoniae*. The study revealed high prevalence of both genes and most of the gene-positive isolates were also found to possess active expression of both genes, along with co-expression in a majority of isolates. In addition, carbapenemase production was also common among these isolates, and nearly all of them were also multidrug-resistant. The association of resistance and

virulence genes along with a high prevalence of carbapenem resistance and biofilm formation ability among these isolates indicates a serious clinical problem and highlights the need for strict infection control and judicious use of antibiotics in Nepal.

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Research Article

Isolation and Preliminary Characterization of *Fusarium* sp. from Diseased Cardamom and Screening of Bacterial Antagonists

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ABSTRACT

Fusarium species are frequently associated with plant diseases affecting economically important crops, including cardamom (*Amomum subulatum* Roxb.). In this study, a fungal isolate was recovered from diseased cardamom stems collected in Panchthar district, Nepal, and characterized using morphological observations and ITS rDNA sequencing. The isolate was identified as *Fusarium* sp. isolate CS2 showing similarity to members of *Fusarium* species. A wheat seed assay demonstrated fungal colonization, but no visible disease symptoms were observed on wheat seedlings under the tested conditions, likely reflecting that wheat is not the natural host of the isolate. Three bacterial isolates were screened for antagonistic activity against the fungal isolate using dual culture assays. Among them, isolate BGNC-B10, tentatively identified as a putative *Exiguobacterium*-like bacterium based on partial 16S rRNA gene sequencing, showed moderate inhibition of fungal growth, reaching 35.97 ± 2.6% inhibition at Day 6. Inhibition declined to approximately 15% at later incubation times, and the treatment effect was not statistically significant ($p = 0.092$). The isolate also produced extracellular protease on skim milk agar. These findings indicate BGNC-B10 exhibits moderate *in vitro* antagonistic activity against *Fusarium* isolate associated with diseased cardamom. However, pathogenicity toward cardamom was not confirmed.

Keywords: Biological control; Cardamom disease; Exiguobacterium-like bacterium; *Fusarium* sp.; Protease activity

Introduction

Fungal phytopathogens are implicated as major causes of loss of yield in crops across the world, with *Fusarium* being the most destructive pathogen. These pathogens infect crops like cereals, vegetables, fruits, and spice crops. Some of these fungi produce mycotoxins that can pose a threat to food security and international trade (Djemouai et al., 2023). Large cardamom (*Amomum subulatum* Roxb.) is an important high-value spice crop, in Nepal. However, its productivity is threatened by leaf

blight and decline diseases associated with members of the *Fusarium graminearum* species complex (FGSC) and several other diseases. Several fungal diseases, including *Fusarium*-associated blight, have been reported as major constraints to cardamom production in Nepal (Belbase et al., 2018). Generally, diseases in cardamom are managed primarily through the use of chemical fungicides. However, their excessive and repeated application has raised concerns regarding the development of resistant pathogen populations, environmental contamination and potential risks to human health, highlighting the need for more sustainable and environmentally friendly management as alternative strategies. Biological control using antagonistic microorganisms represents a sustainable and environmentally friendly strategy for managing diseases caused by *Fusarium* species, and several bacterial genera, including *Bacillus*, *Pseudomonas* and *Streptomyces*, have been extensively studied as potential biocontrol agents (Baffoni et al., 2015). Reported biocontrol mechanisms include antibiotic production, competition for nutrients and space, and the secretion of cell wall–degrading enzymes (Valiz et al., 2017). Despite its metabolic versatility and adaptability to diverse environments, the genus *Exiguobacterium* remains relatively underexplored. Genomic studies indicate that members of this genus can tolerate multiple environmental stresses and produce metabolites with ecological and pharmaceutical importance (Su et al., 2021). Recent studies have demonstrated the antifungal and plant growth–promoting potential of *Exiguobacterium*. For example, *E. acetylicum* SI17 reduced fruit rot severity in litchi (Huang et al., 2024), whereas glacier-derived *Exiguobacterium* sp. KRL4 produced antimicrobial secondary metabolites (Tedesco et al., 2021). Additionally, airborne bacterial communities containing *Exiguobacterium* have shown both biocontrol and plant growth–promoting activities (Guardado-Fierros et al., 2025). Together, these findings suggest that *Exiguobacterium* is a relatively underexplored genus with potential applications in biological control. This study was designed to address this knowledge gap. Specifically, we aimed to: (i) isolate and identify a *Fusarium* species associated with diseased cardamom stems in eastern Nepal; (ii) screen bacterial isolates for antagonistic activity *in vitro*; (iii) characterize the most active bacterial isolate, BGNC-B10, using partial 16S rRNA gene sequencing; and (iv) evaluate enzyme activity that may contribute to its antagonistic potential. To our knowledge, this study represents one of the first reports describing the isolation of a *Fusarium* species associated with diseased cardamom stems and the preliminary evaluation of bacterial antagonists from the cardamom ecosystem in Nepal.

Materials and Methods

Sample collection, pathogen isolation and identification

Cardamom (*Amomum subulatum* Roxb.) stems showing necrotic lesions were collected from plantations in Panchthar District, eastern Nepal (Figure 1). Five symptomatic plants were sampled. Tissue pieces from affected stems were surface-sterilized with 70% ethanol for 30 second, rinsed twice with sterile distilled water, and aseptically placed on potato dextrose agar (PDA; HiMedia, India) (Aneja, 2003). Plates were incubated at 25 ± 2 °C for 5–7 days. Emerging fungal colonies were purified using the hyphal-tip method. Several fungal colonies were obtained from infected tissues, and one representative isolate (CS2) with typical *Fusarium*-like morphology was selected for further characterization. Colony morphology, including pigmentation, texture, and growth rate, was recorded after 7 days of incubation on PDA. Microscopic features were examined using lactophenol cotton blue–stained slides under a compound microscope (40× magnification). Diagnostic characteristics such as hyphal septation, conidiophore structure, arrangement of phialides and conidial morphology were recorded. Identification was performed using standard taxonomic descriptions (Leslie & Summerell, 2006).

Figure 1: Cardamom (*Amomum subulatum* Roxb.), a large cardamom, showing leaf blight and decline symptoms. Field view of affected plants with brown, necrotic and drying leaves in contrast to adjacent healthy foliage (F), Collected samples from symptomatic plants used for laboratory isolation and further study (S).

For molecular identification, genomic DNA was extracted using the phenol–chloroform method (Sambrook & Russell, 2001). The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using primers ITS1 and ITS4. PCR products were sequenced at the Nepal Academy of Science and Technology (NAST), Khumaltar, Nepal, and compared

with sequences in the NCBI GenBank database using BLAST (Altschul et al., 1990).

Pathogenicity assay

Pathogenicity of the isolate was evaluated using a wheat seed assay. Surface-sterilized wheat seeds were placed on moist sterile tissue in sterilized containers and inoculated with a 5-days-old fungal culture broth containing mycelial fragments. Control seeds received sterile water. Containers were incubated at 25 ± 2 °C, and seed germination and symptom development were recorded over 10 days. In the wheat seed assay, fungal colonization was observed around inoculated seeds, but germination and seedling growth were similar to the control and no disease symptoms were detected after 10 days.

Screening and identification of bioactive biocontrol bacteria against the CS2 phytopathogen

A bacterial isolate, designated BGNC-B10 (NT), originally observed as a contaminant during fungal culturing, was isolated and purified. In addition, two bacillus-like isolates (GR and S3a) were recovered from rhizosphere soil of healthy cardamom plants. All three isolates were screened for antifungal activity against the CS2 phytopathogen using dual culture assays on PDA.

For molecular identification, genomic DNA was extracted from bacterial isolates using the phenol–chloroform method (Sambrook & Russell, 2001). The 16S rRNA gene was amplified by PCR with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). Amplified products were sequenced at the Nepal Academy of Science and Technology (NAST), Khumaltar, Nepal, and compared with sequences in the NCBI GenBank database using BLAST (Altschul et al., 1990).

Dual culture assay

The antagonistic activity of BGNC-B10 was tested using the dual culture method. A 5-mm mycelial disc of *Fusarium* sp. (CS2) was placed on two sides of a PDA plate 5 mm from the edge of a PDA plate, while BGNC-B10 was streaked at center. Control plates contained the fungal disc without bacterial inoculation. Plates were incubated at 28 °C, and radial growth of the fungal colony was measured at 6, 11, and 16 days after inoculation. Colony diameter was determined as the mean of two perpendicular measurements. The percentage inhibition of fungal growth was calculated using the formula described by Pandey et al. (1982). All assays were performed in triplicate:

$$\text{Inhibition (\%)} = ((C - T) / C) \times 100$$

Where C = colony radius in control, T = colony radius in treatment.

Enzyme activity assay

Extracellular protease activity of BGNC-B10 was tested on skim milk agar medium (Hankin & Anagnostakis, 1975). Fresh bacterial cultures were spot-inoculated onto skim milk agar plates (3% skim milk) and incubated at 30 ± 2 °C for 24–48 h. Protease production was indicated by the formation of clear zones around bacterial colonies due to casein hydrolysis. All assays were performed in triplicate. This activity is associated with antifungal mechanisms through degradation of structural proteins in fungal cell walls (Gupta et al., 2002).

Data analysis

All assays were performed in triplicate (n=3). Mean values of fungal growth diameters were used to calculate inhibition percentages. Data were analyzed by two-way ANOVA (additive model) to test the effects of treatment and incubation time. Statistical analyses were carried out in IBM SPSS version 21.0 (IBM Corp., Armonk, NY, USA). Differences were considered significant at $p < 0.05$.

Results and Discussion

Pathogen identification and pathogenicity assessment

Molecular analysis of the fungal isolate recovered from symptomatic cardamom stems showed 96% ITS sequence similarity to *Fusarium graminearum* in BLAST searches. Microscopic examination revealed hyaline, septate, branched hyphae and abundant canoe-shaped macroconidia with three to five septa formed in sporodochial clusters (Figure 2). These morphological characteristics are consistent with the genus *Fusarium*. However, ITS similarity below ~98–99% is generally insufficient for reliable species-level identification within the genus. Therefore, the isolate is conservatively designated here as *Fusarium* sp. isolate CS2, and additional multilocus sequencing (e.g., TEF1-α, RPB1, or RPB2) would be required for definitive species identification.

A wheat seed plate assay was conducted to evaluate the pathogenic potential of the isolate. In the inoculated treatment, visible cottony mycelial growth developed around the seeds, indicating fungal colonization (Figure 3). However, germination and early seedling growth were comparable to the water-treated control, and no visible disease symptoms were observed on germinated seedlings even after 10 days of incubation. These results indicate that although the isolate was able to colonize the seed surface, pathogenicity on wheat seedlings was not confirmed under the assay conditions.

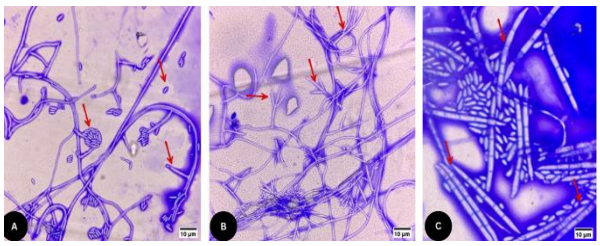

Figure 2: Microscopic characteristics of the *Fusarium* isolate recovered from infected cardamom stem. (A–B) Septate hyphae with branched conidiophores producing phialides. (C) Canoe-shaped, multi-septate macroconidia formed in sporodochial clusters. Stained with lactophenol cotton blue, observed under light microscope at 40× magnification.

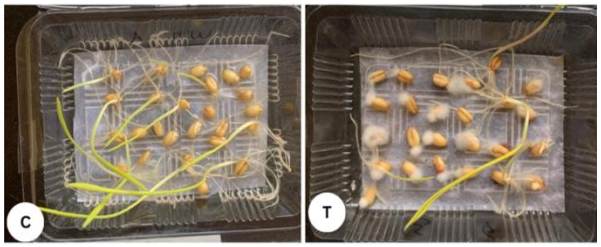

Figure 3: Wheat seed assay showing surface colonization by the Fusarium isolate at day 10. Control seeds treated with sterile water (C), Seeds inoculated with the *Fusarium* isolate (T).

Screening and genetic identification of bioactive biocontrol bacteria against the CS2 phytopathogen

Three bacterial isolates were evaluated for antagonistic activity against CS2, BGNC-B10 (a contaminant isolate) and two bacillus-like isolates (S3a and GR) obtained from rhizosphere soil of healthy cardamom plants. Dual culture assays showed that BGNC-B10 and S3a inhibited fungal growth, whereas GR did not (Figure 4). Both BGNC-B10 and S3a were subjected to molecular characterization. The 16S rRNA gene of BGNC-B10 was amplified successfully (Figure 5) and sequenced in forward and reverse directions. BLAST analysis of the forward sequence showed 95.05% similarity with *Exiguobacterium acetylicum* (38% query coverage), while the reverse sequence showed 92.66% similarity (59% query coverage). Because the sequence similarity (95.05%) and query coverage were relatively low, the isolate is conservatively referred to as a putative *Exiguobacterium*-like bacterium (BGNC-B10) pending further sequencing and taxonomic confirmation. Sequencing of isolate S3a did not yield usable data, and this isolate was excluded from further study. Additional sequencing of both isolates is in progress.

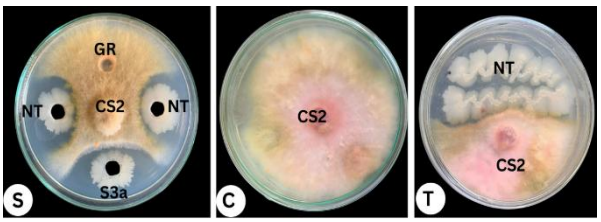

Figure 4: Screening of antifungal activity of bacterial isolates (NT, GR, and S3a) against the phytopathogen CS2. Screening plates showing NT, GR, and S3a (S), Control (CS2 alone) (C); Treated (CS2 challenged with BGNC-B10 (NT) (T).

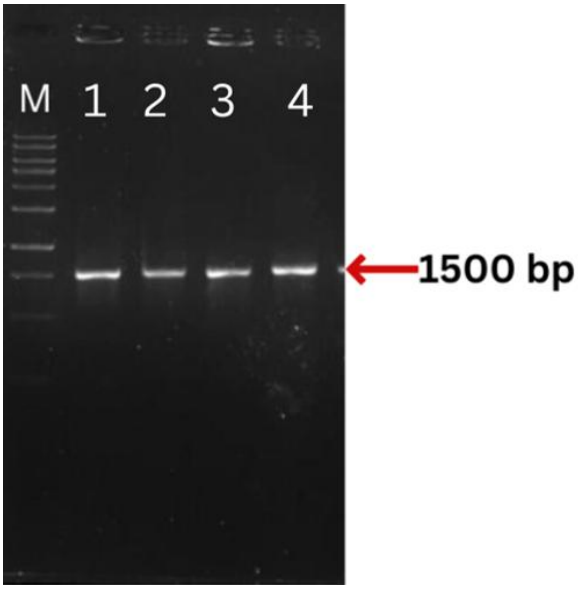

Figure 5: Agarose gel electrophoresis showing PCR amplification of the 16S rRNA gene (~1.5 kb) from bacterial isolates. DNA molecular weight marker (M), BGNC-B10 (lane 1 and 2) and S3a (lane 3 and 4).

Effect of bacterial isolates on the growth of CS2 phytopathogen

Dual culture assays showed partial suppression of fungal growth in the presence of BGNC-B10 compared with the control. Maximum inhibition occurred at Day 6 (35.97 ± 2.6%), followed by a decline to approximately 15% at later time points. Statistical analysis indicated that incubation time had a significant effect on fungal growth, whereas the treatment effect itself was not statistically significant ($p = 0.092$) (Figure 6). At Day-6, average inhibition was 35.97 ± 2.6%. Inhibition decreased to 15.95 ± 7.2% at Day-11 and 15.47 ± 4.5% at Day-16 (Table 1). Statistical analysis using two-way ANOVA (additive model) indicated a significant effect of incubation time ($F = 46.60$, $p = 0.0017$), whereas the treatment effect was not significant ($F = 4.45$, $p = 0.092$).

Table 1: Inhibition of fungal growth by BGNC-B10 in dual culture assays (mean ± standard deviation, n = 3).

Time after inoculation (days)	Inhibition (%) ± SD
6	35.97±2.6
11	15.95±7.2
16	15.47±4.5

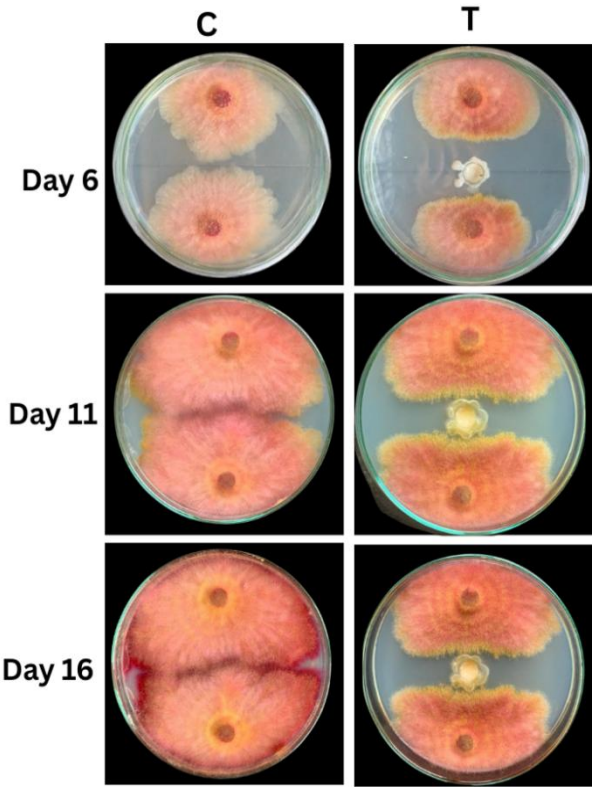

Figure 6: Dual culture assay between the phytopathogen isolate CS2 and the biocontrol agent BGNC-B10. Fungal growth was recorded at 6, 11, and 16 days after inoculation. Control (pathogen alone) (C) and Treated (pathogen with BGNC-B10) (T).

Protease activity of *Exiguobacterium* sp. BGNC-B10

Protease activity of BGNC-B10 was confirmed on skim milk agar. Clear zones were observed around bacterial colonies within 24 to 48 h of incubation, indicating casein hydrolysis (Figure 7).

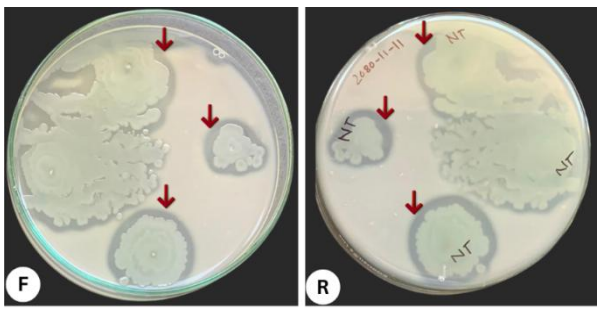

Figure 7: Protease activity of the putative *Exiguobacterium* sp. isolate BGNC-B10 (NT) on skim milk agar. F, front view; R, reverse view of the culture plate. Clear zones surrounding colonies (red arrows) indicate casein hydrolysis and extracellular protease production. NT, isolate BGNC-B10 used in antagonism assays.

The fungal isolate recovered from diseased cardamom stems was identified as *Fusarium* sp. isolate CS2 based on morphological characteristics and ITS rDNA sequencing. Although BLAST analysis showed similarity to members of the *Fusarium graminearum* species complex, the relatively low ITS similarity prevented reliable species-level identification. Furthermore, pathogenicity toward cardamom was not confirmed in this study. A wheat seed assay demonstrated fungal colonization but did not produce disease symptoms. Because wheat is not the natural host of cardamom-associated *Fusarium* isolates, this assay cannot establish pathogenicity according to Koch's postulates. Therefore, the causal relationship between this isolate and cardamom disease remains to be confirmed through inoculation experiments on cardamom plants under controlled conditions.

Dual culture assays indicated that *Exiguobacterium* sp. BGNC-B10 moderately inhibited fungal growth during early incubation. Although BGNC-B10 produced extracellular protease, fungal cell walls primarily consist of chitin and glucans rather than proteins. Therefore, protease activity alone cannot fully explain the observed antagonism. It is possible that additional mechanisms such as competition for nutrients, production of diffusible metabolites, or volatile compounds may contribute to fungal suppression. Similar antifungal activity has been reported for other *Exiguobacterium* isolates (Huang et al., 2024; Tedesco et al., 2021; Guardado-Fierros et al., 2025). Because all experiments were conducted *in vitro,* greenhouse or field studies are needed to evaluate its potential role in disease management. Future studies should investigate additional mechanisms of antagonism including cell-free culture filtrate assays, volatile inhibition assays, and metabolite profiling using LC–MS. Such analyses would provide a more comprehensive understanding of the antifungal activity of BGNC-B10.

Conclusion

A *Fusarium* isolate (CS2) was recovered from diseased cardamom stems in eastern Nepal and identified as Fusarium sp. based on morphological characteristics and ITS rDNA sequencing. A wheat seed assay showed fungal colonization but no disease symptoms under the tested conditions. Pathogenicity toward cardamom was not tested in this study and remains to be confirmed. In dual culture assays, the bacterial isolate BGNC-B10, tentatively identified as *Exiguobacterium* sp., showed moderate inhibition of fungal growth during early incubation. The detection of extracellular protease suggests that enzymatic activity may contribute to the observed antagonism. However, the absence of chitinase and cellulase activity indicates that the

antagonistic mechanism may be limited. These results suggest that *Exiguobacterium* sp. BGNC-B10 may have potential as a bacterial antagonist against *Fusarium*-associated fungi under laboratory conditions. Because the present study was conducted in vitro, further work including detailed molecular identification, metabolite analysis, and greenhouse or field experiments is required to evaluate its possible role in disease management of cardamom.

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Research Article

Cellular Immune Response Evaluation in Nepalese Patients with Cutaneous Leishmaniasis

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ABSTRACT

Cutaneous leishmaniasis (CL) remains a clinically and immunologically heterogeneous disease influenced by parasite species and host immune responses. In this study, seventeen CL cases were assessed through lesion characteristics, histopathological analysis and Kinetoplast DNA (kDNA) nested Polymerase Chain Reaction (PCR)figure. Lesions mainly appeared on exposed body parts, especially the face (64.70%) and most patients had a single lesion. kDNA PCR confirmed CL in 58.82% (n=10) of cases, identifying *Leishmania donovani* (720 bp) in 5 cases and *L. major* (560–590 bp) 5 cases as the circulating species. Immunological analysis showed reduction in T-lymphocytes in CL patients compared to Healthy Controls (HC), largely due to a significant decrease in $CD8^+$ T cells (p=0.036), while $CD4^+$ T cell levels remained stable. Further stratification based on CL PCR positivity showed that reductions in T-lymphocytes (p=0.045) were confined to patients with confirmed *Leishmania* infection, again attributed mainly to decreased $CD8^+$ T cells (p=0.040). No notable changes were observed in B cells, NK cells, or NKT cells. These findings highlight CL infection positivity status-dependent variations in host immune responses and emphasize the importance of $CD8^+$ T-lymphocytes in CL pathogenesis and disease outcome.

Keywords: Cutaneous leishmaniasis; $CD8^+$ T cells; Immune response; kDNA PCR

Introduction

Cutaneous leishmaniasis (CL) is a vector-borne disease caused by *Leishmania* parasites and typically presents with ulcerative or non-ulcerative lesions on the skin. Although the infection was once limited to endemic regions like the South and Central America, Northern Africa, the Middle East and parts of Asia (Kim et al., 2025; Scott & Novais, 2016), it has begun to emerge in previously non-endemic countries, including Nepal (Bhattarai et al., 2025; Pandey et al., 2021; Rai et al., 2023)

CL lesions often heal on their own, but in some cases, they progress into chronic, disfiguring forms. Due to their clinical similarity to conditions such as cutaneous malignancies, discoid lupus erythematosus, lupus vulgaris, and sarcoidosis, these lesions are frequently misdiagnosed, leading to suboptimal therapeutic interventions(Ermertcan, 2018). To enhance diagnostic precision, molecular techniques like PCR have been adopted. PCR-based tests provide faster and more sensitive, species-specific identification of *Leishmania* than conventional methods such as microscopy or culturing the parasite. In Nepal, *L. donovani* and *L. major* are the main etioligical agent causing CL (Rai et al., 2023).

In human leishmaniasis, T-lymphocytes play a key role in establishing protective immunity but they are also involved in sustaining the disease and contributing to the pathology of leishmaniasis. Both $CD4^+$ and $CD8^+$ T cells contribute to immune defense by producing key cytokines that activate monocytes and macrophages (Brelaz-de-Castro et al., 2012). In addition to cellular immunity, B cells have been shown to participate actively in the immune response to *Leishmania* infection, with increased frequencies of $CD19^+$ B lymphocytes and plasma cells as reported in cutaneous leishmaniasis patients presenting with lymphadenopathy (Bomfim et al., 2007).

Only limited data are available on the immunological events associated with human cutaneous leishmaniasis in Nepal. Immunophenotypic evaluation of the lymphocyte is helpful in demonstrating the host immune response. This study aimed to characterize the peripheral immune cell profiles particularly T-cell subsets and B cell subsets in patients with active cutaneous lesions and in healthy controls using flow cytometry, and to assess whether alterations in these immune cell populations are associated with disease activity and potentially influenced by infection with *Leishmania* parasite.

Materials and Methods

Study site

Samples were collected from two hospitals in Nepal: Sukraraj Tropical and Infectious Disease Hospital (STIDH), Teku, Kathmandu and Nepalgunj Medical College and Teaching hospital (NGMCTH), Kohalpur, Banke. These hospitals were selected as they serve as major referral centers, covering cases from the eastern to the western regions of Nepal, thereby ensuring a wide geographic representation.

Ethical approval and collection of biospecimen

The ethical approval for the research involving human participants was granted from Nepal Health Research Council (NHRC) (Reg. No. 45/2018). Anonymity of the patients has been secured in this research according to the NHRC guidelines. Informed consent was obtained before the sample collection from all participants and/or their legal guardians for publication of their clinical information, and laboratory findings.

For this study, 17 patients presented with cutaneous lesions with complaints of non-healing wounds were included. The sampling was done under sterile condition. Some tissue aspirate and freed tissue were withdrawn and collected in Rosewell Park Memorial Institute (RPMI) medium from all patients. Irrespective to treatment and duration of lesion appearance, 5 ml peripheral blood samples was drawn using a syringe with 22 gauze needle and collected in EDTA collection tube from 12 patients. After the collection, the samples were transported to Infectious and Viral Disease Research Laboratory (IVDRL) at Central Department of Biotechnology, Tribhuvan University under ambient condition for further processing.

DNA extraction and polymerase chain reaction

The DNA from the clinical specimen was extracted by using DNA extraction Kit (Quick-DNA™ Universal Kit, Zymo Research) according to the manufacturer's instructions. For the PCR assay, a kinetoplast minicircle DNA (kDNA) nested PCR was performed using CSB2F and CSB1R primers for first round PCR (PCR-1), and 13Z and LiR primers for second round PCR (PCR-2) as described by Noyes et al. (Noyes et al., 1998). The PCR-1 consists of 94 °C for 2 min, followed by 40 cycles of amplification (94°C for 30s, 54°C for 60s, 72°C for 90s), and final extension at 72°C for 10 min. In the PCR-2, 1 µl (1:10) diluted PCR-1 product was used as template and same PCR conditions of PCR-1 were used, except annealing temperature which was increased from

54 to 56 °C and extension time was 40s. PCR amplicons were analyzed on 1.5% agarose gels. The 100 bp DNA ladder (Solis Biodyne) was used as the DNA molecular marker.

Peripheral blood immunophenotyping

Fresh blood was collected from clinically diagnosed CL patients and healthy controls (HC). Fifteen microliter of T cell panel antibody cocktail (BD Biosciences, USA) and 15 µl of B cell panel antibody cocktail panel (BD Biosciences, USA), were mixed separately (Table 1) with 200 µl of fresh blood and incubated in dark for 15 mins at room temperature. Then, 2 ml of 1:10 diluted BD FACS lysing solution (BD Biosciences, USA) was added, vortexed briefly and incubated for 12 minutes at ambient temperature. After that, the tubes were centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and 2 ml of 1X Phosphate buffer saline (PBS) was added and mixed and centrifuged at 1200 rpm for 5 minutes. Again, the same procedure was repeated, supernatant was discarded and the cells were resuspended in 400 µl PBS. For unstained control, the same procedure was followed except the addition of antibodies.

Table 1: Cell markers and tagged fluorochromes of the T and B cell panel.

Channel	Fluorochromes	T-Cell panel	B-Cell panel
FL1	FITC	CD3	CD3
FL2	PE	CD8	CD56/16
FL3	PerCP	CD45	CD45
FL4	APC	CD4	CD19

Compensation of FL channels in flow cytometer

The four FL channels in the BD FACS Calibur machine are FL-1, FL-2, FL-3, FL-4 which collects the emission wavelength from the fluorochromes FITC, PE, PerCP and APC respectively. The compensation of the channels was done by using BD calibrate beads. Compensation was done by aligning the cells in their respective quadrant by manipulating the voltages, current and compensation of different channels. When compensation was performed properly, the further processing of samples were done with the same parameters.

Acquisition of stained cells and analysis

After the completion of compensation of FL channels, the processed cells were acquired. The cells immunophenotypic and morphometric parameters were determined by flow cytometry (FACSCalibur-BD), using the software CELLQuest alies™ (BD Bioscience) for acquisition (50,000 events/tube) and FlowJo software version 10 for analysis of data. The statistical analyses were performed in statistical software R version 4.5.0.

Results and Discussion

Clinical presentations of cutaneous lesions

Out of 17 patients, 8 patients were histo-pathologically confirmed as CL and other 9 patients were diagnosed clinically by the characteristics of lesions. The lesions were found in various parts of the body like face, neck and limbs (Figure 1). Most of the patients (64.70%) had lesions on their face followed by neck (17.64%), lower limb (11.76%) and upper limb (7.69%). Most of the patients had single lesion (n = 14), three patients had multiple lesions; two patients had 2 lesions and one patient had 3 lesions and the lesions were most common in the facial parts. These findings suggest that the exposed body parts are prone to the bite of sandfly (Ghimire et al., 2018).

Figure 1: Clinical feature of CL patients: Nodulo-ulcerated lesion on face (left), Nodulated lesion of upper lip (middle) and Ulcerated lesion of right thigh with central depression (right).

Molecular confirmation: PCR positive CL cases

Molecular level confirmation using kDNA nested PCR revealed that 10 patients (58.82%) were CL positive while histopathological analysis showed LD bodies in 8 cases. Two samples tested negative by microscopy were found to be positive by PCR. Diagnosis of CL with conventional technique is challenging due to extremely diverse clinical presentation and the involvement of different *Leishmania* species (Bailey & Lockwood, 2007; de Monbrison et al., 2007); however, PCR offers a solution, it provides much higher sensitivity than other diagnostic methods. (Pourmohammadi et al., 2010). Moreover the PCR approach makes possible the fast identification at the *Leishmania* species and subspecies level compared to conventional techniques like microscopy and parasite culture (Yehia et al., 2012). Based on comparing the PCR-2 amplicon band size with 100bp DNA ladder, it was found that the band size of 720 bps was comparable with that of *L. donovani* (Bastola et al., 2020) in 5 cases and band size of 560-590 bps was comparable with that of *L. major* (Oryan et al., 2013) in 5 cases (Figure 2). PCR-negativity in 7 samples may reflect low or resolved parasitic burden.

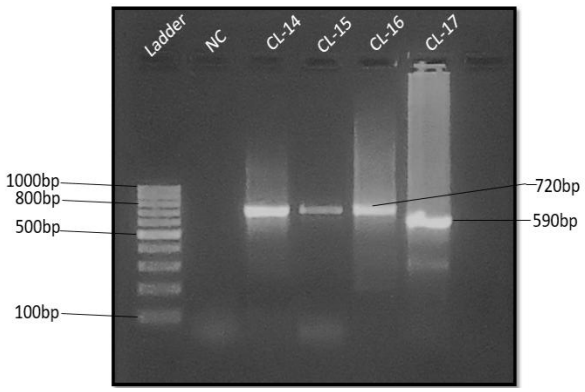

Figure 2: PCR-2 amplicon in 1.5% agarose gel electrophoresis. Ladder: 100 bp ladder, NC: Negative control, CL-14,15,16,17: CL samples.

Analysis of T-Cell and B-Cells population: A flow cytometry study

T-Cell population

Fresh blood samples were collected and stained with T cell panel from 12 CL patients (9 PCR positive and 3 PCR negative). Samples from the remaining five patients were unavailable for the study. The gated mean lymphocyte population of the CL patients in the FSC vs. SSC plot was 21.9 ± 7.73%. The cells positive for CD45 and CD3 were identified as T cells and mean percentage were of 60.5 ± 9.61%. The gated population of the T cells were further categorized to T sub-type cells into $CD4^+$ and $CD8^+$ T cells, the mean percentage of which were respectively 52.00 ± 7.17% and 37.59 ± 6.06% (Table 2 and Supplementary Table 1). Shapiro–Wilk normality testing indicated that all immune cell variables followed a normal distribution in both groups ($p > 0.05$), and none of the percentages are close to 0 or 100 (Table 2), validating the use of parametric Welch's t-statistical analyses. Representative acquisition of flow cytometry is below in Figure 3.

Similarly, the fresh blood samples collected and stained from 6 healthy controls with T cell panel antibodies showed the 68.0 ± 5.48% frequency of T cells and the relative proportion of $CD4^+$ and $CD8^+$ cells among the T cells in healthy control population were 43.4 ± 10.02% and 49.3 ± 10.12% respectively (Table 2 and Supplementary Table 2).

To compare T-lymphocytes between independent two-sample CL patients and healthy individuals, boxplots were generated, and Welch's t-test p-values were calculated. A p-value less than the significance level of 0.05 was considered statistically significant difference in mean between the groups. As depicted in Figure 4, the relative proportion of T-lymphocytes among total lymphocytes in the peripheral blood of CL patients and healthy controls was visualized using box plots. The total lymphocytes in CL patients were found to be higher compared to those in Healthy controls (21.92 ± 7.73% vs. 15.6 ± 3.71%, $p = 0.032$) suggesting immune activation and inflammation and the T-lymphocytes in CL patients were found to be lower compared to those in HC (60.49 ± 9.61% vs. 67.97 ± 5.48%, $p = 0.053$). The percentage of $CD4^+$ T cells was not significantly different among the CL patients and HC. However, there was approximately 24% reduction in $CD8^+$ T cells in CL patients compared to HC (37.59 ± 6.06% vs. 49.28 ± 10.12%, $p = 0.036$).

We infer the reduction in T cell compartment was contributed by the reduction in $CD8^+$ T cells. This observation further strengthens the possibility of protective function of $CD8^+$ cells in cutaneous leismaniasis. $CD8^+$ T cells actively move from blood to infected skin and they participate in parasite killing and tissue inflammation (Bertho & Coutinho, 2005) leading to lower circulating $CD8^+$ T cells and T lymphocytes in peripheral blood.

Table 2: Summary statistics and normality assessment of immune cells (T cells) in peripheral blood from CL patients and healthy controls.

Cells	CL patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
Lymphocytes	21.9	7.73	20.9	15.6	3.71	15.8	0.584	0.795
T-lymphocytes	60.5	9.61	63.3	68.0	5.48	68.6	0.126	0.949
$CD8^+$ T cells	37.6	6.06	35.8	49.3	10.12	50.5	0.794	0.714
$CD4^+$ T cells	52.0	7.17	52.7	43.4	10.02	40.2	0.877	0.183

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous leishmaniasis, SD= Standard deviation, HC= Healthy control

It has been shown that the host immune cells are involved in the progression or healing of the CL lesions primarily based on the types of the immune cells activated. Th1 mediated immune response is considered protective while Th2 mediated immune response support parasite growth and dissemination (Ajdary et al., 2000). Th1 mediated arm of adaptive immune response involve the potent cytokines such as IFN-γ produced by Th1 cells and $CD8^+$ cells which activate macrophages to phagocytose the parasites effectively. The mechanisms by which $CD8^+$ cells limit bacterial and viral infection is well understood.

However, their effector roles in parasitic diseases like cutaneous leishmaniasis is gradually being unfolded (Novais & Scott, 2015).

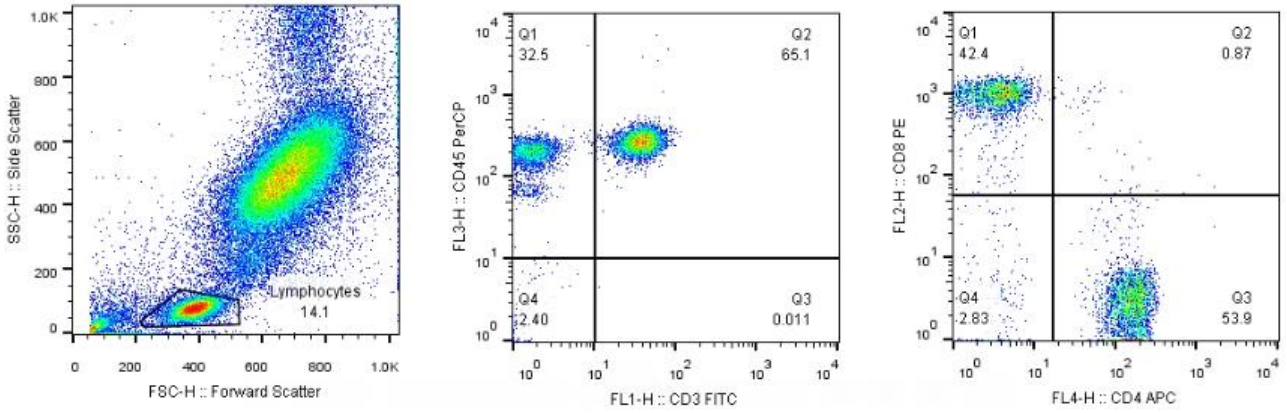

Figure 3: Gating strategy for identification of T cells, CD4+ and CD8+ cells from peripheral blood sample.

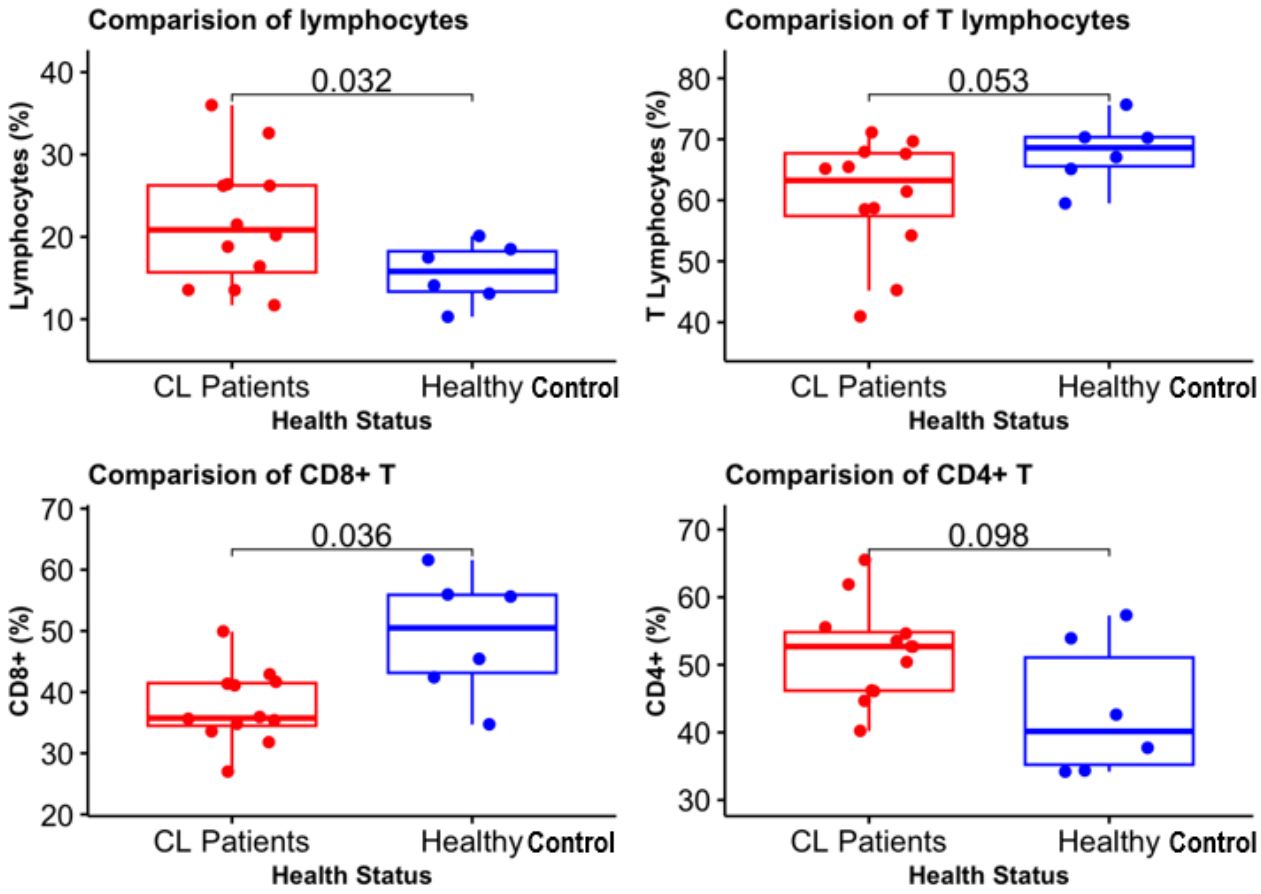

Figure 4: Box plot showing the comparison of T-lymphocytes and its subsets in the peripheral blood of CL patients and Healthy controls (HC) with Welch t-test p-value. Percentage of T-lymphocytes staining positive for $CD45^+$ $CD3^+$ were calculated out of the total lymphocyte gate. Similarly, percentages of $CD4^+$ and $CD8^+$ T-lymphocytes were calculated relative to the gated population of T-lymphocytes (n = 12 for CL, n = 6 for HC).

In our study, we reported decrease in $CD3^+$ T-lymphocytes in patients with active CL disease compared to healthy controls. In a study done by Campanelli et al, there were not significant differences in $CD3^+$ T cells, $CD4^+$ T cells or $CD8^+$ T cells in the peripheral blood of CL patients and the healthy control (Campanelli et al., 2006). In another study done in Brazil by Af et al, it was shown the T cells decreased in active CL cases by approx. 3% compared to control patients which later increased upon chemotherapy. In the same study, the authors did not find significant differences in the $CD4^+$ and $CD8^+$ T cells between active CL cases and controls, which contrasts with our findings. However, in the same study upon chemotherapy, both $CD4^+$ and $CD8^+$ cells were found to be increased which suggest of protective functions of $CD4^+$ and $CD8^+$ T cells (Af et al., 2011). Discrepancy in the composition of immune cells compared to other studies might be due to sampling time as blood was withdrawn irrespective of treatment and also might be because of inherent pathological mechanism associated with different *Leishmania* spp. that cause cutaneous

leishmaniasis. In the previous studies (Af et al., 2011; Campanelli et al., 2006), *L. vianna brazilensis* was implicated in the active CL cases while in our study we found the CL cases could be attributed to infection by *L. donovani* and *L. major*. The reduced $CD4^+$ T-cell proportion relative to $CD8^+$ T cells observed in healthy controls may be related to the inclusion criteria, as controls were defined by the absence of leishmaniasis without assessment of other health factors.

B-Cell population

Fresh blood samples from 9 CL patients and 5 Healthy control (HC) were stained for B cell panel antibodies. The lymphocytes plotted in the FSC vs. SSC further identified as the population of leukocytes as $CD45^+$ cells. B cells ($CD19^+CD3^-$) and T cells ($CD19^-CD3^+$) were identified in CD19 vs. CD3 plot. Likewise, the NK cells and NKT cells in the gated lymphocyte population was identified as NK cells ($CD56^+CD3^-$) and NKT cells ($CD56^+CD3^+$) in the CD56 vs. CD3 plot (Figure 5). Supplementary Table 3 and Table 4 showed the percentage of T cells, B cells, NK cells and NKT cells in CL patients and HC respectively. The frequency of T cells (59.4 ± 6.05%), B cells (9.4 ± 4.21%), NK cells (14.2 ± 7.38%) and NKT cells (3.6 ± 2.57%) were identified in CL patients (Table 3). Shapiro–Wilk testing indicated normal distribution for NK cells, NKT cells, and T cells in both groups ($p > 0.05$), but for B cell CL patients, data deviated from normality ($p = 0.036$). So, to compare two independent sample means, the Welch t-test for NK cells, NKT cells, and T cells, and for B cell data, we use non-parametric Mann-Whitney U-test. The average percentage of B cells in CL patients (9.4 ± 4.21%) was not significantly different from those in HC (8.8 ± 3.96%). Similarly, the NK cells constituted 14.2 ± 7.38% in CL patients which was not statistically different from 17.8 ± 8.73% in the HC. The NKT cells constituted a small portion with an average of 3.6 ± 2.57% in the CL patients and 3.7 ± 1.49% in the HC. Although NK cells, NKT cells and B lymphocytes showed minor variations between groups, these differences were not statistically significant.

In contrast, T cells ($CD3^+$ T) cells differed significantly between CL patients and healthy controls ($p = 0.024$) (Figure 6), highlighting a predominant alteration in the T-cell compartment during CL. The absence of significant differences in B cells, NK cells, and NKT cells significant alteration in T cells in our study suggests that CL predominantly alters the T-cell compartment, consistent with the central role of T-cell mediated immunity in intracellular parasite control. CL is not mainly a humoral (antibody-driven) disease. So B-cell percentages often remain stable (Novais & Scott, 2015). NK cells are rapidly and transiently activated during infection, provide an early source of IFN-γ, and then their activity often wanes. Consequently, detectable alterations in peripheral NK or NKT cell levels may not always be observed in blood samples (Bogdan, 2012). Furthermore, the duration of lesion appearance was not taken into account during sample collection, which may have reduced the ability to detect minor variations in these immune cell population ability.

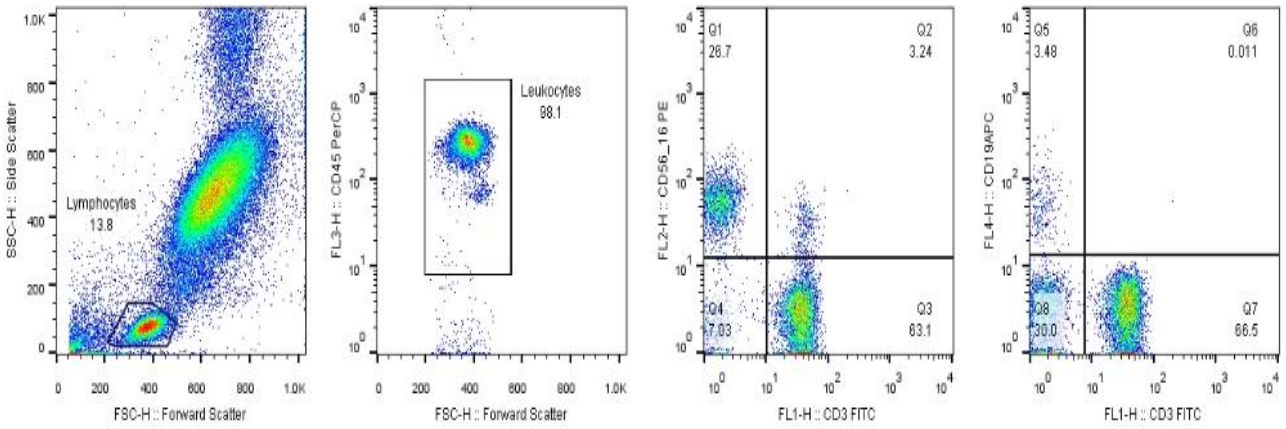

Figure 5: Gating for identification of T cells, B cells, NK cells and NKT cells.

Table 3: Summary statistics and normality assessment of immune cells (NK, NKT, T and B) in peripheral blood from CL patients and healthy controls.

Cells	CL patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
NK cells	14.2	7.38	13.2	17.8	8.73	16.2	0.479	0.513
NKT cells	3.6	2.57	2.6	3.7	1.49	3.2	0.100	0.456
T cells	59.4	6.05	58.5	65.7	3.09	66.8	0.864	0.429
B cells	9.4	4.21	8.2	8.8	3.96	9.5	0.036	0.977

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous Leishmaniasis SD = Standard deviation, HC = Healthy control

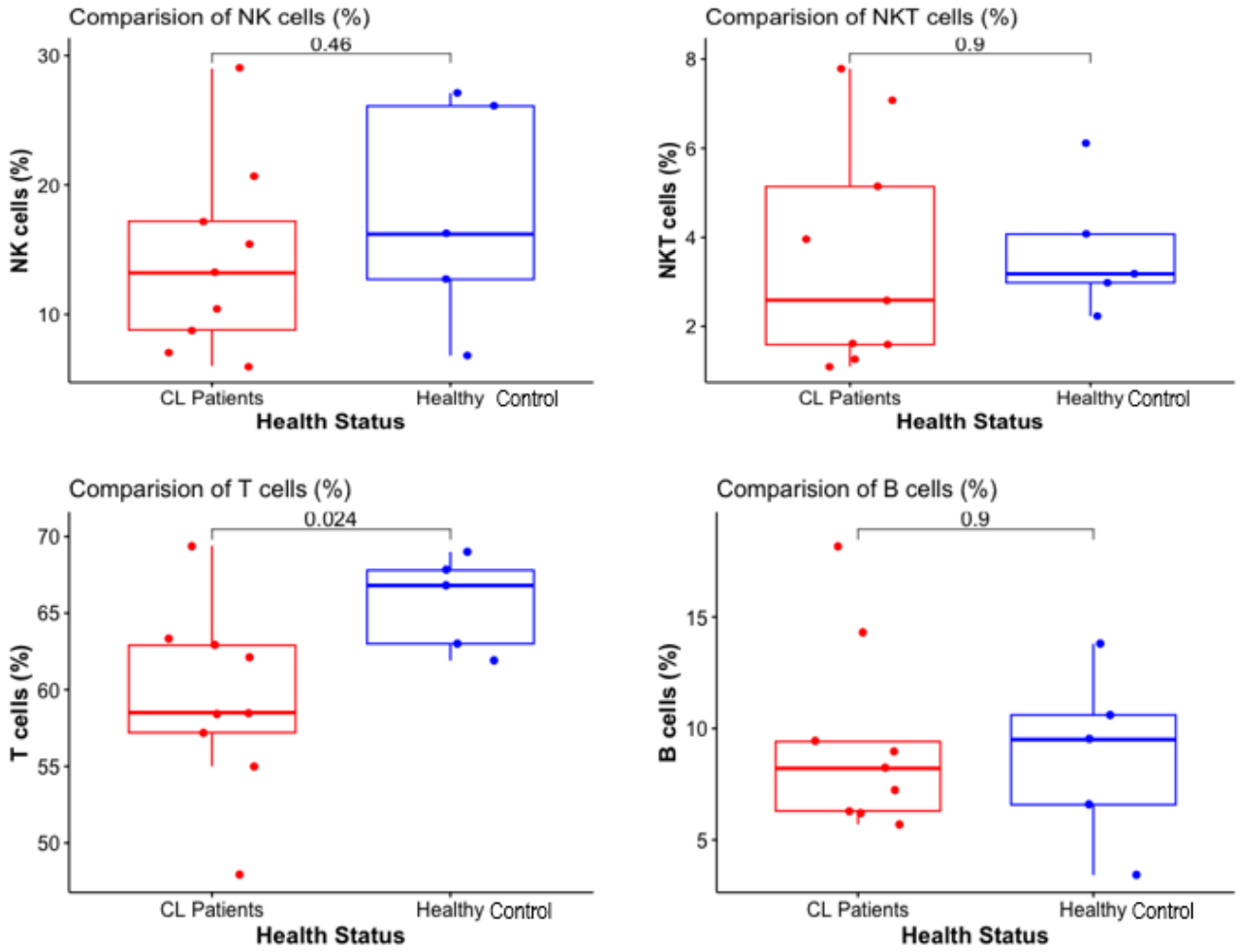

Figure 6: Comparison of the immune cells in the peripheral blood of CL patients and Healthy Controls (HC). Peripheral blood samples were stained for B cell panel the data was acquired in BD FACS Calibur. The relative percentages of NK cells ($CD3^{-}CD16/56^{+}$) and NKT cells ($CD3^{+}CD16/56^{+}$), T cells ($CD19^{-}CD3^{+}$) and B lymphocytes ($CD3^{-}CD19^{+}$) were calculated out of the total lymphocyte gate (n = 9 for CL and n = 5 for HC).

Comparison of cell population between PCR +ve and PCR –ve samples

Interestingly, when we further analyzed the relative proportion of T cell subsets based on PCR positivity for *Leishmania* spp. DNA, there was a significant reduction of T-lymphocytes ($p = 0.045$) in patients who tested positive for *Leishmania* spp. DNA by PCR compared to healthy controls (Table 4). We could infer that reduction in T-lymphocytes was contributed by the significant reduction in $CD8^{+}$ T-lymphocytes ($p = 0.040$) but not by $CD4^{+}$ T-lymphocytes (Figure 7). However, no significant difference was found in any T cell subsets when PCR negative samples were compared with both PCR positive samples and healthy control (Table 5). The lack of significant T-cell changes in PCR-negative individuals likely reflects low or absent parasitic burden, indicating that systemic T-cell modulation is mainly associated with active infection when parasite antigens stimulate immune responses (Novais & Scott, 2015). This further supports the *Leishmania* infection-dependent nature of $CD8^{+}$ T-cell reduction observed in PCR-confirmed cases. Additionally, the limited sample size of PCR-negative cases may have reduced the statistical power to detect minor immunological differences. The Shapiro–Wilk test was performed to assess normality. All variables were normally distributed except $CD8^{+}$ T cells in PCR-negative patients, deviated from normality ($p = 0.034$). Welch's t-test was used for mean comparisons when the normality assumption was satisfied; otherwise, the nonparametric Mann–Whitney U test was applied.

As observed previously, when the patient samples were analyzed without subdivision into PCR +ve or PCR –ve, no significant difference could be observed in the relative proportion of B cells, NK cells or NKT cells between CL patients and HC (Table 6), while significant changes observed in T cells ($p = 0.024$). The similar result was obtained when further compared with PCR+ CL patients and HC (Figure 8). There is only one case with PCR negative for B-cell panel so further comparison could not be made. Moreover, small study population included in the study may have limited the ability to detect subtle changes in these cell populations. To the best of our knowledge, this is the first study the immune cells in peripheral blood from cutaneous leishmaniasis by the application of flow cytometry.

Table 4: Summary statistics and normality assessment of immune cells (T cells) in peripheral blood from PCR positive CL patients and healthy controls.

Cells	PCR positive CL patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
Lymphocytes	22.0	8.84	21.5	15.6	3.71	15.8	0.367	0.795
T-lymphocytes	58.9	10.29	61.4	68.0	5.48	68.6	0.382	0.949
$CD8^+$ T	37.9	5.70	35.6	49.3	10.12	50.5	0.099	0.714
$CD4^+$ T	51.2	6.37	52.7	43.4	10.02	40.2	0.925	0.183

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous leishmaniasis, SD = Standard deviation, HC = Healthy control

Table 5: Summary statistics and normality assessment of immune cells (T cells) in peripheral blood from PCR negative CL patients and healthy controls.

Cells	PCR negative CL patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
Lymphocytes	21.8	4.04	20.2	15.6	3.71	15.8	0.332	0.795
T-lymphocytes	65.4	6.04	68.0	68.0	5.48	68.6	0.270	0.949
$CD8^+$ T	36.7	8.40	41.4	49.3	10.12	50.5	0.034	0.714
$CD4^+$ T	54.3	10.49	52.7	43.4	10.02	40.2	0.747	0.183

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous leishmaniasis, SD = Standard deviation, HC = Healthy control

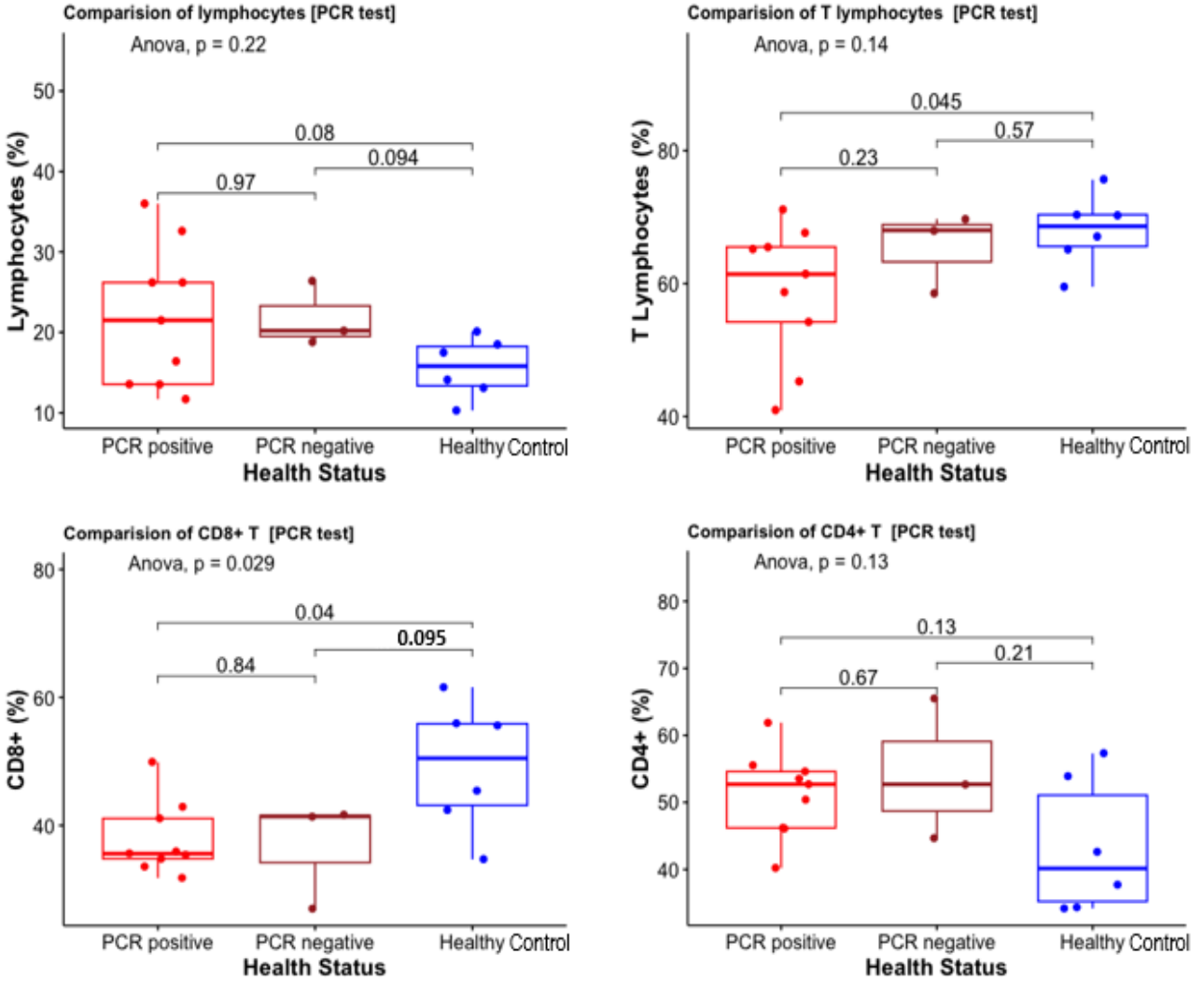

Figure 7: Comparison of T-lymphocytes and its subsets in the peripheral blood of patients with PCR positive (CL PCR +), PCR negative (CL PCR -) and Healthy Controls (HC). Peripheral blood samples were stained with T cell antibody panel and the data was acquired in BD FACS Calibur. Percentage of T-lymphocytes staining positive for CD45+CD3+ were calculated out of the total lymphocyte gate. Similarly, percentages of CD4+ and CD8+ T-lymphocytes were calculated relative to the gated population of T-lymphocytes. (n = 9 for CL PCR +, n = 3 for CL PCR -, n = 6 for HC).

Table 6: Summary statistics and normality assessment of immune cells (NK, NKT, T and B) in peripheral blood from PCR positive CL patients and healthy controls.

Cells	CL Patient			HC			Normality test (p-value)	
	Mean	SD	Median	Mean	SD	Median	CL patient	HC
NK cells	13.8	7.79	11.8	17.8	8.73	16.2	0.288	0.513
NKT cells	3.0	2.17	2.1	3.7	1.49	3.2	0.120	0.456
T cells	59.7	6.41	60.3	65.7	3.09	66.8	0.823	0.429
B cells	9.8	4.32	8.6	8.8	3.96	9.5	0.103	0.977

Normality test: Shapiro-Wilk test of normality; CL = Cutaneous Leishmaniasis, SD= Standard Deviation, HC=Healthy Control

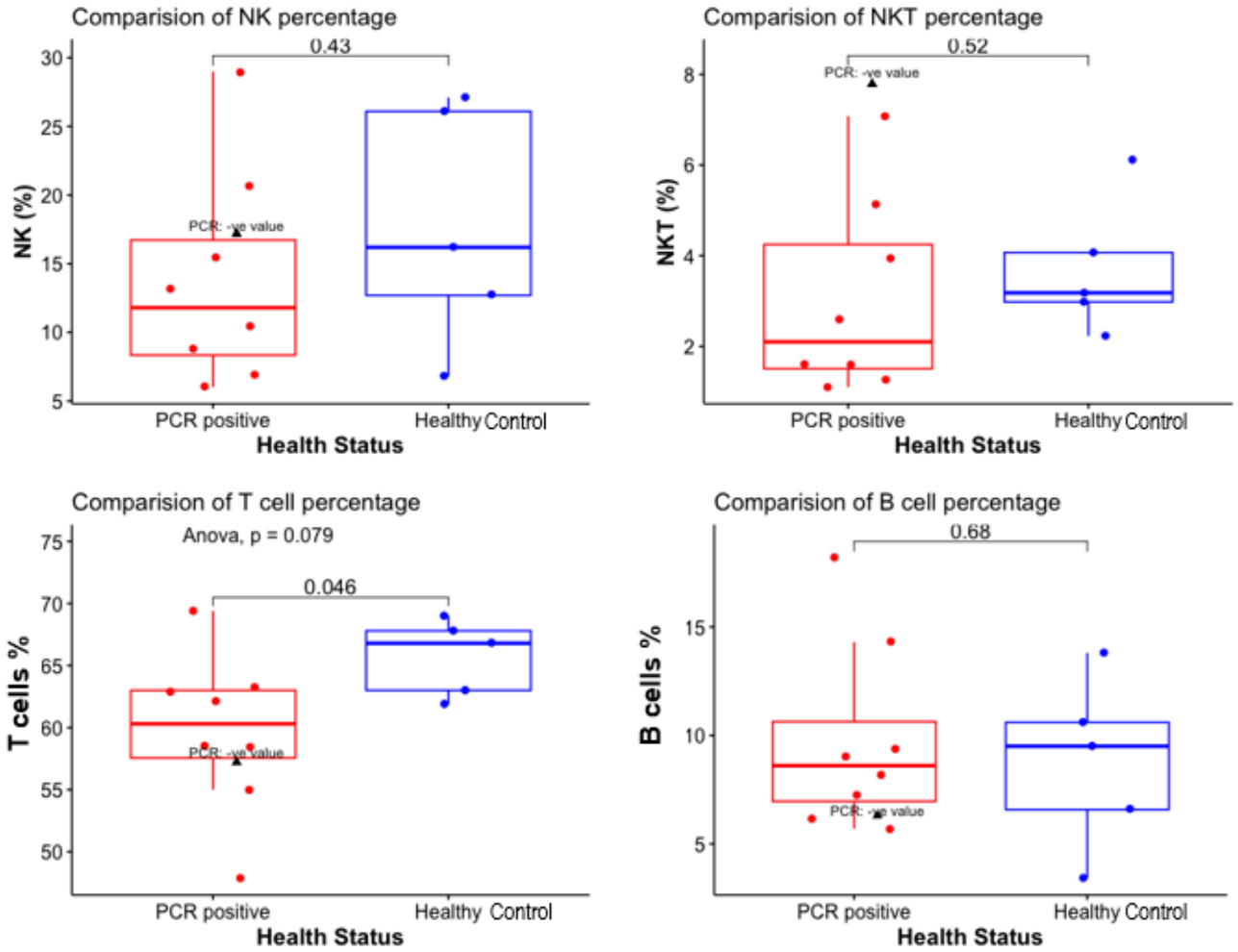

Figure 8: Comparison of the immune cells in the peripheral blood of CL patients with PCR positive (CL PCR +) and Healthy Controls (HC). Peripheral blood samples were stained for B cell panel the data was acquired in BD FACS Calibur. The relative percentages of (A) B-lymphocytes ($CD3^-CD19^+$) (B) NK cells ($CD3^-CD16/56^+$) and (C) NKT cells ($CD3^+CD16/56^+$) were calculated out of the total lymphocyte gate. (n = 8 for CL PCR +, n = 1 for CL PCR -, n = 5 for HC). PCR-negative single case value is represented by small black triangle.

Conclusion

This study highlights the clinical and immunological complexity of cutaneous leishmaniasis in the study population. Molecular analysis confirmed *L. donovani* and *L. major* as the circulating species, emphasizing the coexistence of multiple etiological agents. Immunophenotyping revealed a significant reduction in $CD3^+$ and $CD8^+$ T-lymphocytes in active and PCR-positive CL cases, suggesting an important protective role of $CD8^+$ T cells in controlling infection. In contrast, $CD4^+$ T cells, B cells, NK cells, and NKT cells showed no significant differences between patients and controls. Overall, these findings indicate active immune responses specific to *Leishmania* infection and reinforce the relevance of T-cell–mediated immunity in CL pathogenesis. Further studies with larger cohorts are needed to better define immune alterations and their implications for diagnosis, prognosis, and therapeutic strategies.

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Supplementary Table 1: Percentage of T-lymphocytes in the fresh blood samples drawn from patients.

Sample No.	PCR	% of Lymphocytes	% of T-lymphocytes	% of $CD8^+$ T cells	% of $CD4^+$ T cells	Ratio (CD4/CD8)
CL5	+	36	65.5	33.6	61.9	1.84
CL8	+	16.4	45.2	35.9	53.5	1.49
CL10	+	26.2	40.9	49.9	40.2	0.81
CL12	+	32.6	67.6	31.8	54.6	1.72
CL13	+	11.7	54.2	35.4	52.7	1.49
CL14	+	13.55	65.1	35.6	55.5	1.57
CL15	+	13.53	71.1	41.1	46.2	1.12
CL16	+	26.2	61.4	42.9	50.4	1.17
CL17	+	21.5	58.7	34.8	46.1	1.32
CL4	-	18.8	58.5	27	65.5	2.43
CL9	-	20.2	69.7	41.7	52.7	1.26
CL11	-	26.4	68	41.4	44.7	1.08
Mean		21.92	60.49	37.59	52.00	1.44
SD		7.73	9.61	6.06	7.17	0.43

Supplementary Table 2: Percentage of T-lymphocytes in the fresh blood drawn from healthy controls.

Sample no.	% of Lymphocytes	% of T-lymphocytes	% of $CD8^+$ T cells	% of $CD4^+$ T cells	Ratio (CD4/CD8)
HC1	13.1	75.6	34.7	57.3	1.65
HC2	20.1	67.0	45.4	42.6	0.94
HC3	14.1	65.1	42.4	53.9	1.27
HC4	18.5	70.2	55.6	37.7	0.68
HC5	10.3	70.4	56	34.4	0.61
HC6	17.5	59.5	61.6	34.2	0.56
Mean	15.60	68.0	49.3	43.4	0.95
SD	3.71	5.48	10.12	10.02	0.43

Supplementary Table 3: Percentage of T cells, B cells, NK cells and NKT cells in the fresh blood samples drawn from CL patients.

Sample No.	PCR	% of NK cells	% of NKT cells	% of T cells	% of B cells
CL5	+	8.8	1.1	69.4	18.2
CL8	+	29	5.14	47.9	8.21
CL12	+	6.01	2.59	58.5	9
CL13	+	20.7	7.08	55	5.7
CL14	+	6.98	3.95	63.3	9.41
CL15	+	13.2	1.61	62.9	6.17
CL16	+	15.4	1.59	62.1	7.22
CL17	+	10.4	1.26	58.4	14.3
CL 11	-	17.2	7.79	57.2	6.3
Mean		14.2	3.6	59.4	9.4
SD		7.38	2.57	6.05	4.21

Supplementary Table 4: Percentage of T cells, B cells, NK cells and NKT cells in the fresh blood samples drawn from Healthy Controls

Sample No.	% of NK cells	% of NKT cells	% of T cells	% of B cells
HC1	16.2	4.07	67.8	9.5
HC2	26.1	3.18	61.9	3.42
HC3	12.7	6.11	66.8	6.58
HC4	6.81	2.98	69	10.6
HC5	27.1	2.23	63	13.8
Mean	17.8	3.7	65.7	8.8
SD	8.73	1.49	3.09	±3.9

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Research Article

Screening of Vip Gene in *Bacillus thuringiensis* Isolated from Different Geographical Areas of Nepal and Vip Protein Effect on Fall Armyworm

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ABSTRACT

Incautious use of chemical pesticides in the agricultural field has led to detriment of lands and decline soil fertility. It also poses human health risk and environmental degradation. *Bacillus thuringiensis*, a spore forming soil bacterium, predominantly produces insecticidal proteins, is a promising solution for sustainable pest management in agriculture. The present work aims to screen Vip genes in isolated stock samples of *B. thuringiensis* and to show the insecticidal effect of Vip 3 protein on Fall armyworm larva. Fifty *B. thuringiensis* isolates along with two commercial strains, *B. thuriengiensis* subsp. *galleriae* HD8 (BGSC 4G1) and *B. thuriengiensis* subsp. *morrisoni* HD12 (BGSC 4K1), were used for screening of vegetative insecticidal protein coding genes (Vip1, Vip2 and Vip3) by conventional PCR and sequencing. Gram staining was performed for bacterial morphology and only bipyramidal shaped protein crystals were visualized during Coomassie Brilliant Blue staining. Among 50 crystal-forming isolates, 90% strains were found to carry the Vip3 gene but none of the isolates were found to possess the Vip1 and Vip2 genes. However, Vip2 gene was detected in reference strains (BGSC 4G1 and BGSC 4K1). The pairwise comparisons and phylogenetic tree analysis of Vip3 genes from five Bt isolates showed 100% sequence similarities with worldwide distributed *B. thuringiensis* Vip3 gene. Ammonium sulfate precipitation was followed by partial purification of Vip3 protein. SDS-PAGE analysis revealed the molecular weight of the Vip3 protein around 90 KDa. From bioassay of Fall armyworm, lethal concentration (LC_{50}) of Vip3 protein of reference sample BGSC 4G1 was 16.2607 ± 5.4239 µg/ml and isolate Bt Kasara showed 19.1268 ± 8.289 µg/ml in 7 days. Protein of BGSC 4G1 showed strong effectiveness (needed 17.63% less protein) as compared to Bt Kasara Vip3 protein. Therefore, Vip3 protein showed a potential biopesticidal effect and it may be utilized as a sustainable pest management approach.

Keywords: *Bacillus thuringiensis*; Biopesticides; Pest management; Vegetative insecticidal protein

Introduction

The development of insect resistance to synthetic pesticides and their chemical hazards to the environment has led to the extensive interest in *Bacillus thuringiensis* (Bt) in pest-controlling technologies (Ibrahim et al., 2010; Watkins et al., 2012). *B. thuringiensis* (Bt) is a Gram-positive, motile, rod and spore-forming bacterium belonging to the family Bacillaceae widely known for bioinsecticides. *B. thuringiensis* was first discovered in 1901 by Shigetane Ishiwatari as a cause of sudden-collapse disease in silkworm larvae, later named by German scientist Ernst Berliner (Hannay & Fitz-James, 1955), reisolated from flour moth larvae in the state of Thuringia (Milner, 1994).

Many species of *B. thuringiensis* have shown broad-spectrum activity against different orders of insects, including Lepidoptera, Coleoptera, Diptera, and Hemiptera, as well as some nematodes and mosquitoes (El-Gaied et al., 2020; Ma et al., 2023; Şahin et al., 2018). Crystal (Cry or Cyt) protein (Bechtel & Bulla, 1976; Labaw, 1964), also known as (σ-endotoxins) and vegetative insecticidal protein (Vip) (Yu et al., 1997) are two major proteins secreted by *B. thuringiensis* during their respective stationary and vegetative growth phases. The vegetative insecticidal protein has distinct genomic sequences and binding sites with Cry protein (Aswathi et al., 2024; Yan et al., 2020). The family of vegetative insecticidal protein is classified into four subfamilies, Vip1, Vip2, Vip3 and Vip4, within which a total of more than 100 types, based on the amino acid sequence homology (Gupta et al., 2021). Subfamily Vip1 and Vip2 toxin form a heterodimer and their combination exhibits activity within Coleoptera and Hemipteran insect orders, whereas Vip3 is effective against many species of Lepidoptera (Syed et al., 2020). Among the Vip family, Vip3 proteins are the most widely studied and present in greater parts than others. Vip3 is divided into three classes (Vip3A, Vip3B and Vip3C), and their classes are subdivided again into 10 Vip3A (Vip3Aa to Vip3Aaj), three Vip3B (Vip3Ba, Vip3Bb, and Vip3Bc) and one Vip3Ca subclasses (Chakrabarty et al., 2020; Estruch et al., 1996; Gupta et al., 2021; Palma et al., 2012). Vip4, on the other hand, is the least characterized, and its pesticidal activity has been under research. The lethal effectiveness of Bt protein is based on the specificity of ligand-receptor binding (Wang & Cheung, 1994). The crystalline form of protein undergoes protease activation, following conformational changes, which drives pore formation and destruction of the larval midgut membrane (Bravo et al., 2007; Kumar et al., 1996). Normally, Vip3 protein (around 90 KDa) is in an inactive state. During activation, trypsin-like enzymes in the midgut of insect, cut the protoxin at 198 amino acid residues, releasing nearly 20 kDa N-terminal and 65 kDa C-terminal as minor and major fragments (Chakrabarty et al., 2020; Jiang et al., 2020; Núñez-Ramírez et al., 2020). The specific receptor present in the microvilli of epithelial cells can recognize the activated protein (Lee et al., 2003). The membrane pore formation process is still unclear; the fibroblast growth factor receptor (Fgfr) mediated endocytosis probably the cell lethality has been reported (Jiang et al., 2018).

The use of Bt protein, especially, cry protein is one of the sustainable approaches to developing a safer way of agricultural reform. The worldwide use of biopesticides has already been for a century (Nester et al., 2002), Bt protein is used as a major biopesticide (Kumar et al., 2021), accounting for 90% of biopesticides from Bt protein of total biopesticides in the United States (Sanchis & Bourguet, 2008). Besides the broad action of Cry protein against insects, the development of insect resistance to Bt crystal protein (Tabashnik, 1994; Van Rie & Ferré, 2000) and against a group of commercial pesticides (Washim et al., 2024), the researcher has increasing interest in the vegetative insecticidal protein. Vip protein has a crucial role in resistance pest management since it harbors activity against a target group of insects, along with a group of insect-resistant pesticides and Bt crystal protein-resistant pests.

In Nepal, the use of pesticides in agriculture has a long history and plays an important role in the commercial production of agricultural commodities. Vegetables are the major produce with nearly 90% use out of the national average of 396 gm of a.i/ha total pesticide use (Parajuli et al., 2021). Similarly, use of pesticides is increasing about 20% per year, with insecticides sharing the second highest imported pesticides, that is 33% (Ghimire & GC, 2018).

However, due to the poor integrated pest management strategy, the haphazard use of chemical pesticides is increasing day by day. Large amounts of pesticide residues have been reported, especially in plant-based food (Nyaupane, 2022), which directly affects human health and livelihood (Karki & Dangol, 2023). So, this situation redirects to an alternative search for sustainable agriculture practices. The application of Bt protein as a biopesticide in Nepal is lagging behind despite its worldwide use. One of the reason behind this is, the research and development of the biopesticides and its industrial production is less prioritized by the government and concern bodies. However, small-scale research is conducted in different institutions and research academics. Bioassay and lethal dose

determination of different insects using chemical pesticides and biopesticides have been showed in the previous studies (Limbu et al., 2020; Sharma et al., 2022). Till now, research on the bioassay of Fall armyworms using Vip protein in Nepal has not been conducted yet. The study of Vip3 protein isolated from a diverse altitude ranging from 75-5050 meters (Rana et al., 2002) is one of the works demonstrating the specific insecticidal effect of the protein. Approaching its very distinct features, here we try to figure out the screening, characterization, and sequencing of the Vip3 gene encoding a protein of *B. thuringiensis* and its bioassay. Extracted Vip3 protein may be utilized as a sustainable pest management approach, contributing to reduce environmental impact, and exploited as a safer alternative to chemical pesticides.

Materials and Methods

Bacillus thuringiensis isolates and growth conditions

A total of 50, *Bacillus thuringiensis* lyophilized stock which were previously collected and biochemically characterized samples from six geographical regions of Nepal (Figure 1 and Figure 2) along with two references *B. thuriengiensis* subsp. *galleriae* HD8 and *B. thuriengiensis* subsp. *morrisoni* HD12 (BGSC 4G1 and 4K1), kindly provided by Prof. Dr. Zeigler (*Bacillus* Genetic Stock Center, Columbus, Ohio, USA) were used for this study. All stocks were revived in T3 agar media (per liter: 3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 0.05 M sodium phosphate [pH 6.8], and 0.005 g of MnCl; Travers et al., 1987).

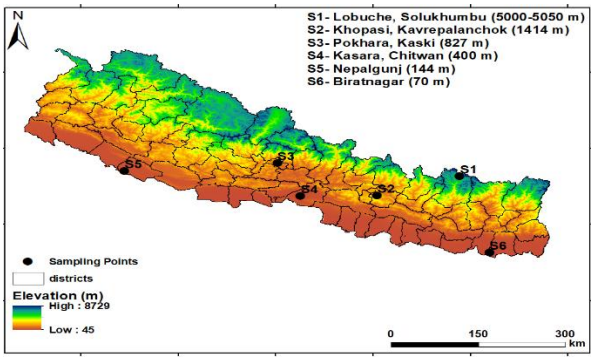

Figure 1: Map of Nepal showing six different geographical regions from where samples were collected. It covered an altitude from 70 to 5050 m asl.

Gram staining and coomassie brilliant blue (CBB) staining

Gram staining of Bt samples was performed thoroughly by smear preparation, CV-I complex formation using crystal violet and iodine, followed by decolorization with acetone and safranin as a counter stain. Bt spore crystal was observed by the Coomassie Brilliant Blue staining (CBB) technique. For this, loopful colonies of Bt isolates were inoculated in 50 ml T3 broth media and incubated at 110 rpm in a 30°C shaking incubator for 5 days. After incubation, 5 µl spore crystal mixture was transferred onto a glass slide, then it was air dried and heat-fixed. The slides were flooded with 0.133% CBB stain in 50% acetic acid CBB stain for 1 min, followed by rinsing with destaining solution (10% acetic acid, 50% methanol, and water). The type of spore crystal was visualized under the microscope.

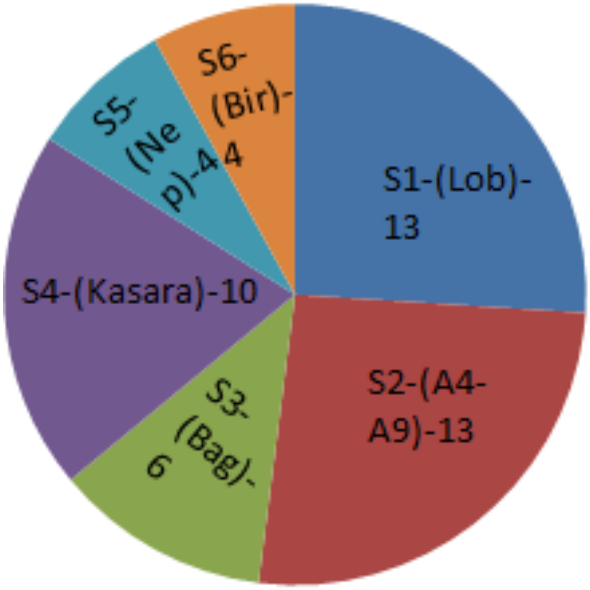

Figure 2: Pie chart showing the total number of samples and their first name of sample code from the respective region (S1 to S6) according to Figure 1.

DNA extraction and PCR amplification of Vip gene

The boiling method was used for DNA extraction of all samples (Abdelhai, 2016). Brielfly, overnight cultures of *B. thuringiensis* were prepared in Luria Bertani (LB) broth, and it was centrifuged at 13,000 rpm for 5 min. Pellets were resuspended in sterile distilled water and left to boil (100 °C) for 10 min. The suspension was again centrifuged at 10000 rpm for 2 minutes and the collected supernatant was stored at 4 °C for further use.

PCR amplification of the Vip gene from the selected *Bacillus* isolates was performed using three primer sets: Vip1, Vip2, and Vip3 consisting reference primer sequences, Vip1 forward-5' TTATTAGATAAACAACAACAAGAATATCAATC TATTMGNTGGATHGG 3', Vip1 reverse-5' GATCTATATCTCTAGCTGCTTTTTCATAATCTS ARTANGGRTC 3', Vip2 forward-5' GATAAAGAAAAAGCAAAAGAATGGGRNAARR A 3', Vip2 reverse-5' CCACACCATCTATATACAGT

AATATTTTCTGGDATNGG 3' and Vip3 forward-5' TGCCACTGGTATCAARGA 3', Vip3 reverse-5' TCCTCCTGTATGATCTACATATGCATTYTTRTT RTT 3' (Hernández-Rodríguez et al., 2009). The PCR reaction mixture was prepared in a final volume of 20 µL consisting of 2X master mix, 10 pM/µl of primer concentration, and maintaining 100 ng of template. An Applied Biosystem PCR thermocycler is used to carry out PCR amplification. PCR condition set up for the *Vip* gene was initial denaturation of 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 51°C for 1 minute, extension at 72°C for 1.5 minutes, and one cycle of final extension at 72°C for 10 minutes. Expected product sizes, 585 bp, 845 bp, and 1621 bp for Vip1, Vip2 and Vip3 genes respectively were analyzed under 1.5% agarose gel electrophoresis.

Sequencing of Vip3 gene

Out of 50 samples, five strains from five geographical regions were taken for sequencing of the Vip3 genes. Sequencing was performed in a 3500xL Genetic Analyzer (Applied Biosystems) in the Molecular Biology Unit Laboratory of Nepal Academy of Science and Technology. Both obtained forward and reverse Sequences were aligned and edited using BioEdit. The sequence homology was determined by NCBI nucleotide BLAST. The phylogenetic tree was constructed using Mega 11 (Bootstrap 1000 repetition), the Neighbor-joining (NJ) method.

Extraction and purification of protein

Vegetative insecticidal protein was purified using a slightly modified method described by (Estruch et al., 1996). Selected Bt strains were grown in Terrific broth media (12% tryptone, 2.4% yeast extract, 0.04% glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) for 16 hours with 30°C shaking incubation. After the day, the protein suspension was separated at 5000 g for 20 minutes. Ammonium sulfate (80% W/V saturation) was added to the supernatant at 4°C with continuous stirring (magnetic) for protein precipitation, and the precipitate was collected again through centrifugation at 5000g for 15 min. After centrifugation, the pellets were resuspended in 1 ml of 100 mM Tris-HCl buffer (pH 7.5). Dialysis against 20 mM pH 7.5 Tris-HCl buffer was followed at 4 °C overnight to remove especially the salts and small molecules.

Analysis of Vip protein

Partially purified proteins were separated based on their molecular weight by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Invitrogen Mini Gel Tank System. A separating gel (12%) and 5% stacking gel were used for Electrophoresis. Before loading, Protein samples were heated at 95 °C for 10min, and prepared in sample loading buffer containing 0.01% bromophenol blue. Electrophoresis was performed using 1X SDS-PAGE running buffer at 120 volts for 2 to 3 hours. After completion of electrophoresis, proteins were stained with 0.1% CBB, and a destaining solution was applied to remove the background stain.

Preparation of protein standard and detection of extracted protein concentration

The concentration of protein was determined by the Biuret method. A total of six stocks of concentration of BSA from 0.5 mg/ml to 10 mg/ml were taken for standard preparation. For the Biuret test, 25 µl of protein (partially purified protein for the test sample and BSA for standard) and 125 µl of Biuret reagent were mixed, and it was incubated for 30 min at room temperature in the dark conditions. Tris buffer with biuret reagent was taken for the blank. After incubation, absorbance was read at 540 nm. The concentration of test samples was calculated using a standard equation.

Bioassay of insects

Fall armyworm (FAW) eggs were collected from a laboratory of the Entomology Division, Nepal Agriculture Research Council (NARC), Khumaltar, Lalitpur. Eggs were reared for up to 3 days until the larvae reached their third instar phase by feeding on fresh leaves of maize. Third-instar larvae were used for the assay.

The *in vitro* bioassay against FAW was performed by taking the reference from different sources (Estruch et al., 1996; Maheesha et al., 2022; Sharma et al., 2022). Different concentrations of protein were prepared: 10 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml for activity against larvae.

Fresh Maize leaves were dipped into 100 ml of a particular concentration of protein solution for 30 seconds. Treated leaves were air-dried for a few minutes and placed into bioassay containers containing tissue at the bottom. Larvae were allowed to feed on protein-treated maize leaves. For control larvae, protein untreated fresh leaves were feeded accordingly. This process was repeated up to 6-7 days at 24-hour intervals. Old leaves were removed daily, and freshly treated leaves were provided for feeding. Containers were kept well-labeled, provided with proper ventilation, and

maintained at 30 °C room temperature. Mortality was recorded after 6 days.

Results and Discussion

Staining of vegetative cells and crystal protein

Gram staining of all 50 samples showed Gram-positive bacteria. Rod-shaped purple-coloured bacteria were observed under a 100X (immersion oil) microscope. Figure 3 shows the Gram staining and crystal staining of the *Bacillus thuringiensis* bacterium. All 50 isolates of *B. thuringiensis* form crystal protein during sporulation. Under microscopic observation, released crystals could be distinguished from spores since they stained purple and displayed a unique diamond shape, while spores remained white and elliptical in appearance (Figure 3).

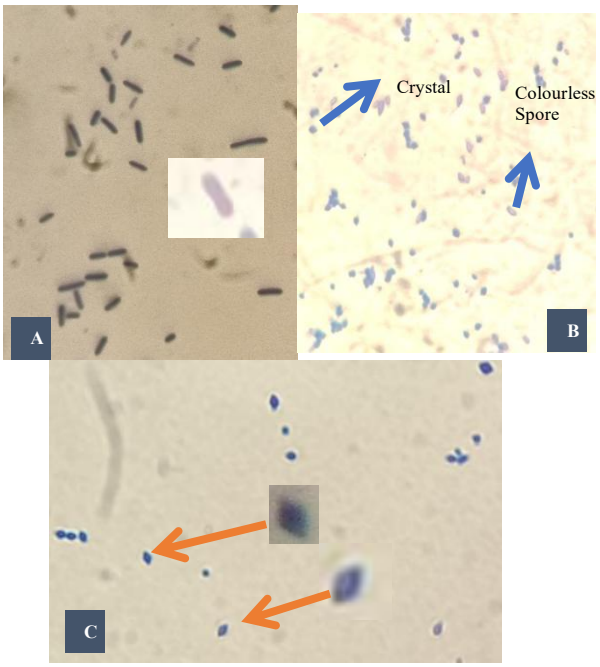

Figure 3: Gram staining and spore staining of *Bacillus thuringiensis* isolates. Rod shaped bacterium (A), Spore protein mixture (B) and Bypiramidal shaped crystal structure of Bt protein (C).

PCR amplification of the vegetative insecticidal protein (VIP) gene

A total of 45 samples, along with two reference strains 4G1 and 4K1, showed the Vip3 gene positive among all 52 (50 isolates and 2 references) samples tested, with the amplicon around 1621 bp (Figure 4). The Vip1 gene was amplified only in two commercial strains of BGSC 4G1 and 4K1 around the product size of 585bp, but none of the Bt strains were found to carry the Vip2 gene. The occurrence of the Vip3 gene has been found in around 90% of total samples.

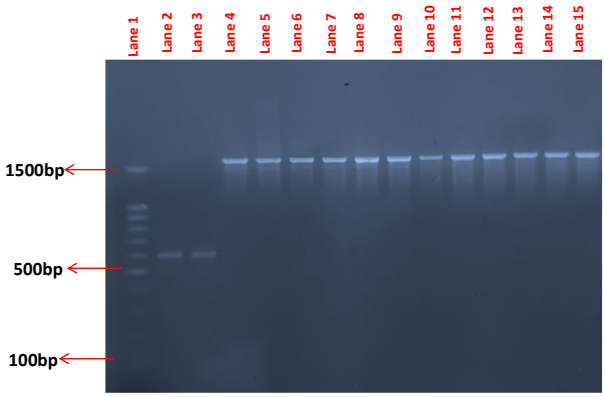

Figure 4: Screening of Vip genes. Lane 1; 100 bp Promega DNA ladder, Lane 2 and 3; 585 bp amplicon of vip1 gene, Lane 4 to lane 15 amplified PCR product of Vip3 gene size around 1621 bp.

Construction of a phylogenetic tree of the Vip 3 protein gene

The Vip3 gene from five Bt strains (Bt Nep LA, Bt Lob 10 LA, Bt A9 b LA, Bt Kasara, Bt Bir) showed a 100% match with Bt strains reported across various regions worldwide, including MZ191101.1 *B. thuringiensis* Vip3 gene India, CP001910.1 *B. thuringiensis* Vip3 gene China, KY780302.1 *B. thuringiensis* Vip3 gene Pakistan, MH318013.2 *B. thuringiensis* Vip3 gene Turkey, DQ426899.1 *B. thuringiensis* Vip3 gene Thailand, as shown in Figure 5.

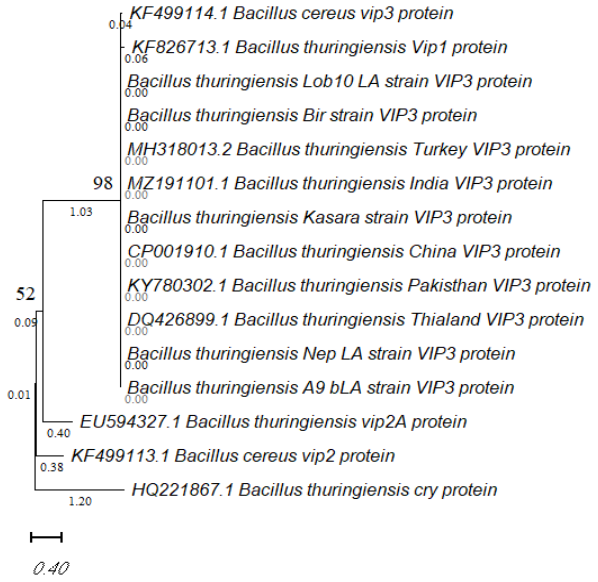

Figure 5: Phylogenetic tree analysis based on Vip3 gene of five Bt strains (Bt Nep LA, Bt Lob 10 LA, Bt A9 b LA, Bt Kasara, Bt Bir) constructed using neighbor joining method of Mega 11 software with 1000 bootstrap values.

Concentration and molecular weight determination of extracted protein

The concentration of different proteins was calculated using a BSA standard curve from the Biuret test. The

concentration of six Bt isolates (Bt Nep LA, Bt Bag LA, Bt Lob 10 LA, Bt A9 b LA, Bt Kasara, Bt Bir) along with reference strain 4G1 (Table 1) were calculated using BSA as a standard (y = 0.0636x + 0.1116, with the correlation coefficient (R^2 = 0.9903). Among the six Bt isolates from six geographical regions, the highest concentration of Vip3 protein was found in the isolate of Bt Kasara, 9.566 mg/ml, with the lowest concentration 2.71 mg/ml from isolates Bt Lob 10 LA (Table 1). Isolate Bt Kasara was selected for the bioassay to compare activity with reference strain 4G1 of 10.242 mg/ml protein concentration (Table 1).

Table 1: Concentration of protein from different isolates and positive control.

Sample	Concentration (mg/ml)	Bacterial type	Geographical region
Bt Nep LA	7.33	Isolate	Nepalgunj
Bt Bag LA	9.25	Isolate	Pokhara, Kaski
Bt Lob 10 LA	2.71	Isolate	Lobuche, Solukhumbhu
Bt A9 b LA	9.17	Isolate	Khopasi, Kavrepanchok
Bt Kasara	9.566	Isolate	Kasara, Chitwan
Bt Bir	3.11	Isolate	Biratnagar
4G1	10.242	Reference strain	
4K1	9.581	Reference strain	

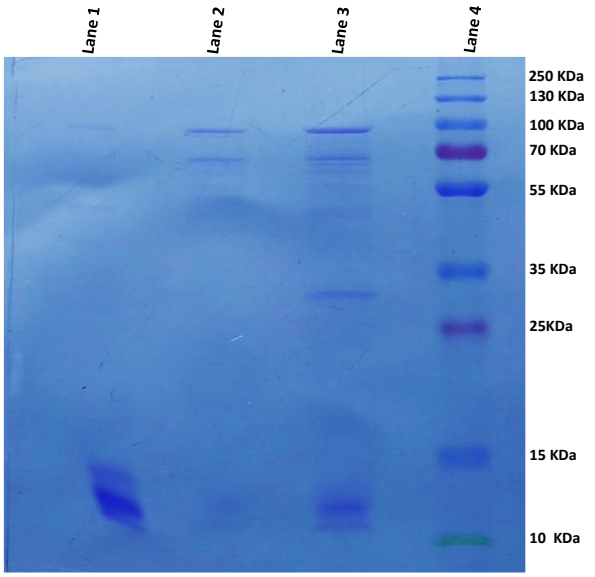

Figure 6: SDS PAGE of BT Vip3 protein. Lane 1 to 3 : Vip 3 protein of BT Bir, BT Kasara, and BT reference strain 4G1 respectiveyly. Lane 4; protein marker (Thermofisher Scientific prestained PAGE Ruler).

In our result, the molecular weight of the Vip3 protein is found around 90 kDa, which is shown in Figure 6. Lanes 1 and 2 are the Vip3 protein band of isolates Bt Bir and Bt Kasara, and lane 3 is the band from the protein extract of the 4G1 reference strain. The size was compared with the protein marker (Thermofisher Scientific prestained PAGE Ruler. This preliminary protein could be useful for further characterization and bioassay of the Vip protein against different lepidopteran pests.

Bioassay of Vip protein

The lethal concentration (LC_{50}) is the concentration of a compound that causes the death of *50%* population of test animals. From probit analysis, the Lethal Concentration of Bt Kasara Vip3 protein was determined as 19.1268 µg/ml and 16.2607 µg/ml for BGSC 4G1 at 7 days (Table 2). Bt Kasara, with an average mortality 60% and 64% respectively (data not shown). The detailed study to determine the LC50 value of Vip3 protein from all isolates is underway.

Table 2: Toxicity of Vip3 protein and LC_{50} determination.

S.N.	Bt Strain	LC_{50} (µg/ml)
1.	Bt Kasara	19.1268±8.289
2.	BGSC 4G1	16.2607±5.4239

Discussion

In the present study, the majority of Bt strains were dominated (in all isolates we used in our study) by bipyramidal-shaped crystal protein. But the crystal protein shape may vary between the initially and most studied bipyramidal (Hannay & Fitz-James, 1955) to spherical, cuboidal, and rectangular shapes (Handayani et al., 2025). Among the three primers tested for screening of Vip genes, the presence of the Vip3 gene was found more prevalent than the Vip1 and Vip2 genes. This denotes that the Vip3 gene has a high abundance among other genes. Although we have samples from different geographic regions, there is a huge difference (nil to 90%) in the distribution of Vip1/Vip2, and Vip3 genes. This frequency may vary due to the difference in environmental conditions of collected sources, like altitude elevation, texture and nutrition of the soil, aeration, temperature, etc (X. Yu et al., 2011). Some research already showed that the frequency of the Vip3 gene is much higher, about 49.8% than Vip1 and Vip2 which was 10% (Hernández-Rodríguez et al., 2009; R. Shingote et al., 2013). Also, the newly designed primer helps to provide the robustness of the primers to amplify and differentiate among different subclasses of the Vip family. Based on the sequences of the Vip3 region, we can assume a broad geographic distribution of Bt strains found in Nepal. Variation in Vip gene sequences leads to variation in amino acid sequences, which influences the level of toxicity in the Vip protein (Li et al., 2007).

Although the research on *B. thuriengiensis* has been reported in Nepal (Limbu et al., 2020; Parajuli et al., 2015), the extensive and systematic analysis in distribution and diversity of Vip gene is not highly studied. So it might be the potential work to determine the sequence similarity in Vip genes present in Bt strains of Nepal.

In this study, we use partially purified Vip3 protein for characterization and bioassay. The fully purified protein has a high concentration and higher binding affinity, so even at low concentration, it can be more effective against the target. The variation in protein profiling may depend on the diversity of the soil sample collection. As our results showed that the highest protein concentration was 9.566 mg/ml, which was the Bt strain from an altitude of 400 m asl. A similar range of proteins was extracted from the remaining isolates from 144 m asl to 1414 m asl height (Table 1, Figure 1). Similarly, the lower concentrations ranged from 2.71 mg/ml to 3.11 mg/ml, which were geographic elevations from 70 and 5050 m asl (Table 1, Figure 1).

Protein profiling of various *Bt* isolates helps in studying the diversity of insecticidal proteins and their functionality. The vegetative insecticidal protein of Bt is secreted during the vegetative growth phase (Jiang et al., 2023). The Vip3 gene encodes a protein of around 90 kDa (Chakrabarty et al., 2020) having 787 to 789 amino acids (Syed et al., 2020). SDS-PAGE confirmed the presence of Vip3 protein in the *Bt* isolates, showing visible bands at ~ 90 kDa similar to the reference strain 4G1 Figure 5. The Vip3 proteins can be cleaved into N-terminal and C-terminal fragments of about 20 kDa and 66 kDa, similar to the Vip3Aa type protein responsible for specificity and toxicity. The deletion of amino acids from the N-terminal enhances the toxicity of Vip3 protein, and this can be achieved by the trypsinization of the protein (Jiang et al., 2023). This divergence in protein profile might be because of Bt strains from different geographic soil samples. As many researchers showed that protein profile is also dependent on the Bt strains of diverse geography (Baranek et al., 2023; Maheesha et al., 2022; Nair et al., 2018).

Bioassay results revealed that Vip protein from isolate Bt Kasara showed a lethal concentration (LC_{50}) of 19.1268±8.289 µg/ml at 7 days, which was 17.63% greater concentration needed to affect fall armyworm larvae than that of the control strain 4G1. The lethal concentration of insecticidal protein depends on the percentage of mortality rate, the type of protein and purification methods we use, the stages of larval stage, the group of insect species, and the type of diet provided for the insect (Lone et al., 2016). A previous study demonstrated a large range of (0 to 100%) mortality rate on *Plutella xylostella* larvae at 25 µg/ml using a spore crystal mixture of different Bt isolates (Navya et al., 2021). *B. thuringiensis* Bt294 Vip3A toxin's LC_{50} values of 187.1 ng/cm^2 at 7 days against third instar larvae of *Spodoptera exigua* when they use artificial diet as a supplement (Nutaratat et al., 2023). Similarly, 100% mortality was found using *E. coli* AB88 (pCIB7104) cloned Vip3A protein treated in *Agrotis ipsilon* larvae, and while using neonates of fall armyworm (*S. frugiperda*) as a host, they also showed 100% mortality at 140 ng per cm^2, pCIB 7105 Vip3A clone protein concentration (Estruch et al., 1996). A very low (4 ng/cm^2) Vip3A protein concentration needed for Fall armyworm gut paralysis and above 40 ng/cm^2 for larval death (Yu et al., 1997). These results suggest that if we use purified cloned Vip3 protein, they have high activity against the target at low concentration since it has a strong affinity to bind at specific sites. Although our isolates showed less activity compared to the reference, if we go through further characterization and purification processes, then we can figure out the lowest concentration of Vip3 protein needed for different lepidopteran insects. However, it is necessary for extensive research on biopesticides to protect against environmental detriment and the development of sustainable agricultural practices.

Conclusion

From this study, we concluded that Vip3 gene has abundantly present in the *Bacillus thuriengiensis* strains native to different geographical soil of Nepal. The partially purified Vip 3 protein is effective against one of the insects of lepideptera order. Our preliminary result above supports the development of reproducible and validated Vip protein which can be used as a sustainable biopesticides.

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Review Article

Plant-based Functional Foods from South Asia – Importance and Requirement for Better Healthcare: A review

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ABSTRACT

Functional foods incorporating plant-derived bioactive compounds offer potential health benefits by providing nutrition in the form of polyphenols, dietary fiber, and vitamins. South Asia is rich in biodiversity and ethnobotanical heritage, and has the potential to provide a wide range of plants with high nutritional value. The existing knowledge and utilization of plants from South Asia that have been incorporated into functional food formulations, with specific emphasis on Nepal, is very limited. Cereals, millets, fruits, and medicinal plants have long been used in households to treat various health conditions, but their rich phytochemical and dietary fiber content remains incompletely understood. Plants such as Moringa, Ginger, and Amla have been successfully incorporated into bakery products, gummies, beverages, and dairy products. Additionally, several underutilized plants in Nepal and the region show potential for future use in functional food development. This review highlights the potential of plants in South Asia and functional food formulations based on these plants.

Keywords: Functional food; Healthcare; Medicinal plant; Nutraceuticals; Phytochemicals

Introduction

Functional foods offer vital nutrients and health advantages that we might not get from our regular diet. Chronic conditions including diabetes, heart disease, and obesity are becoming more common worldwide, and are particularly pronounced in South Asia. As a result, there is greater interest in functional foods, which incorporate bioactive ingredients that confer health benefits beyond their basic nutritional value (Sahidi, 2009; Granato, 2010). Young children's consumption of unhealthy foods has also increased dramatically, and awareness of the importance of eating nutritious snacks has been gradually limited (Pries et al., 2019). Rich sources of bioactive compounds including flavonoids, phenolic acids, carotenoids, alkaloids and dietary fibers, have attracted particular attention to plant-based functional foods because they help prevent disease and promote health (Sahidi & Ambigaipalan, 2015). These substances have antibacterial, anti-inflammatory,

antidiabetic and antioxidant properties that are crucial for lowering the risk of chronic illnesses (Pandey & Rizvi, 2009).

Plants have long been used for food and medicinal purposes in South Asia. Many medicinal herbs that offer practical advantages are incorporated into traditional dietary systems, including Ayurvedic and indigenous food practices (Baliga et al., 2011). Nowadays, a large number of these plants are being studied scientifically and used in contemporary functional food products. The development of functional foods using traditional plants such as amla, moringa, turmeric and millets has attracted greater attention. Because of their rich nutritional and phytochemical content, these plants have been used in cereal-based foods, beverages and baked goods (Leone et al., 2015; Barbhai et al., 2021).

Despite these advances, many plants from Nepal and the Himalayan region remain underutilized and unknown for use in functional food development due to a lack of scientific research and validation of available compounds. Many medicinal plants are traditionally used as vegetables and have high pharmacological activity (Sharma et al., 2023), whereas some of the plants are being used by the community people in their daily life for a variety of sicknesses (Shrestha et al., 2021). Also, the proper amalgam of traditional knowledge of utilizing the medicinal herbs (Lamichhane et al., 2014) with added knowledge of the secondary metabolites can assure the benefits of using plants in functional foods. The separation and extraction of existing secondary metabolites in plants can be used to produce health beneficial functional foods that can impact the personal health with specificity. This review examines the use of South Asian and some specific Nepali plants in functional food formulation and identifies future opportunities for research and development.

Methods

This review was conducted following PRISMA guidelines to ensure a systematic and transparent selection of relevant studies (Page et al., 2021). Literature searches were conducted using Google Scholar, PubMed, and Science Direct databases. Keywords used included functional food, medicinal plants, South Asia, food formulation, and phytochemicals. Studies published between 2000 and 2025 were included. Studies were selected based on relevance to plant-based functional food formulation in South Asia. Data extracted included plant species, bioactive compounds, and functional food applications.

Results and Discussion

Global functional food, functional components and their health benefits

It highlights the diversity of commercially available functional foods developed by incorporating bioactive compounds shows the options to enhance nutritional value and promote targeted health benefits (Table 1).

Table 1: Global functional foods and their key bioactive compounds (Arshad et al., 2025).

Product/Functional food	Type	Key bioactive compounds	Company brand	Regional availability	Reported health benefits
Special K Fortified cereal	Cereals	Added vitamins and minerals (Iron, B vitamins)	Kelloggs	Global	General micronutrient fortification
GT's synergy Kombucha	Kombucha	Live cultures, Tea polyphenols	GT's Living Foods	USA, EU, APAC	Gut health, antioxidants
Quest protein bars	Snack Bar	(Whey/Milk isolates), Fiber	Quest Nutrition	USA, EU	Muscle maintenance, Satiety
Oatly (fortified oat milk)	Plant-based milk	Added calcium, vitamin D, and B12	Oatly	Global	Dietary alternative with fortification
Alrpo (Fortified Plant Drink)	Plant-based beverage	Vitamins and minerals (Ca, B12, D)	Alpro (Danone)	EU, UK, APAC	Dietary alternative with micronutrients
Optimum Nutrition Gold Standard Whey Protein	Sports nutrition	Whey protein isolate/concentrate	Optimum nutrition (Glanbia)	Global	Muscle recovery and performance
Olly (multi-vitamin gummies)	Dietary Supplements/ Gummies	Vitamins (Multi-blends)	Olly	USA, online global	General wellness, Sleep/beauty formulation
Beyond Burger (plant-based patty)	Plant-based meat	P/Rice proteins; Fortified iron	Beyond meat	Globally	Protein alternative with lower saturated fat

Most functional food products are found in the United States and European markets. Giant multinational companies and brands such as Nestlé, Kellogg, and Cadbury appear to have advanced in functional food production.

Many of these products rely on micronutrient fortification, such as iron, calcium, and vitamins B12 and D, to address dietary deficiencies and support nutritional adequacy, particularly among populations adopting plant-based diets. Fermented beverages like kombucha provide live microbial cultures and tea-derived polyphenols that may contribute to gut health and antioxidant activity. At the same time, high-protein functional foods, including protein bars and whey-based supplements, are formulated to support muscle maintenance, recovery, and safety (Arshad et al., 2025). Nutricosmetic ingredients such as resveratrol, β-sitosterol, and hyaluronic acid support skin health. Cardiovascular functional foods are positioned as a distinct category. A notable innovation depicted is the use of a jelly formulation for sleep support, incorporating bioactive compounds such as melatonin, theanine, and γ-aminobutyric acid to promote relaxation and sleep quality. Table 2 represents how different food matrices can be tailored to deliver specific functional ingredients for targeted physiological outcomes (Yuan et al., 2024).

Table 2: Key bioactive compounds and health benefits (Yuan et al., 2024).

Functional food category	Health function / Target	Key bioactive compounds
Nutricosmetics	Skin and beauty health	Resveratrol, β-sitosterol, Hyaluronic acid
Cardiovascular Health	Heart health and cholesterol regulation	Lycopene, β-carotene, Astaxanthin
Cognitive Health	Brain function and memory support	Phycoerythrin, Selenium
Immunity	Immune system enhancement	β-glucan, phycocyanin, Human milk oligosaccharides
Intestinal Health	Gut microbiota balance and digestion	Inulin, Polysaccharides, Probiotics
Sleep Support	Sleep quality and relaxation	Melatonin, Theanine, γ-aminobutyrate
Weight Management	Fat metabolism and body weight control	Cellulose, Capsaicin, Curcumin
Oral Care	Dental and oral health	Peptides, Enzymes, Sugar alcohol
Energy	Energy metabolism and alertness	Taurine, Caffeine, Vitamin B3
Physical Performance	Athletic performance and endurance	Menaquinone-7, Selenoproteins, Ginsenosides

Table 3: Fruits and their major bioactive compounds.

Fruit	Common Name	Major bioactive compound
Phyllanthus emblica	Amla	Vitamin C, gallic acid, ellagic acid, flavonoids
Syzygium cumini	Jamun	Anthocyanins, ellagic acid, jamboline
Punica granatum	Pomogrante	Punicalagin, ellagic acid, anthocyanins
Psidium guajava	Guava	Vitamin C, carotenoids, polyphenols
Mangifera indica	Mango	Mangiferin, carotenoids, vitamin C
Carica papaya	Papaya	Carotenoids, papain, flavonoids
Morus alba	Mulberry	Anthocyanins, resveratrol
Ziziphus mauritiana	Indian jujube	Phenolics, flavonoids, vitamin C
Aegle marmelos	Bael	Marmelosin, tannins, flavonoids
Vitis vinifera	Grape	Resveratrol, anthocyanins
Litchi chinensis	Litchi	Polyphenols, vitamin C

Functional food formulation using South Asian plants

Fruits-based functional foods

Due to their high antioxidant content and market appeal, fruit-based functional foods are among the most extensively produced items. Because of its potent antioxidant properties and remarkably high vitamin C content, amla (*Phyllanthus amlica*) is one of the most significant functional food plants in South Asia (Baliga & Dsouza, 2011). Although amla has previously been used as a refreshing beverage, its nutritional value is even greater. Amla has been shown to confer significant health benefits in nutraceutical products, confectionery, and functional beverages.

The anti-diabetic effects of jamun (*Syzygium cumini*) have also been well investigated. It has been demonstrated that its bioactive substances, such as ellagic acid and anthocyanins, enhance glucose metabolism (Ayyanar & Babu, 2012). Because of its antioxidant and cardioprotective qualities, pomegranate (*Punica granatum*) has been added to functional beverages (Martos et al., 2010). Because of its high phenolic and vitamin C content, guava (*Psidium guajava*) is another significant functional fruit (Thaipong et al., 2006). We have already found the market filled with fruit-based drinks that claim to provide additional nutritional value. Table 3 and Table 4 show fruits and their potential use in the formulation of functional food.

Table 4: Functional food properties and functional food products of the fruits.

Fruits	Reported functional properties	Functional food products/Formulation	References
Phyllanthus emblica	Antioxidant, antimicrobial, immunomodulatory	Functional beverages, fortified juices, gummies, nutraceutical powders	(Baliga & Dsouza, 2011); (Nile et al., 2018)
Syzygium cumini	Antidiabetic, antioxidant, anti-inflammatory	Functional juices, jams, fermented beverages, powders	(Ayyanar & Babu, 2012); (Rizvi et al., 2022)
Punica granatum	Cardioprotective, antioxidant, anti-inflammatory	Functional beverages, fortified juices, nutraceutical drinks	(Martos et al., 2010); (Gullon et al., 2020)
Psidium guajava	Antioxidant, immune-supportive	Functional juices, fruit blends, fortified beverages	(Thaipong et al., 2006); (Joseph & Priya, 2011)
Mangifera indica	Antioxidant, anti-inflammatory	Functional juices, dried fruit snacks, fortified dairy products	(Masibo & He, 2009)
Carica papaya	Digestive aid, antioxidant	Functional juices, fermented beverages, fruit powders	(G et al., 2013) (Rivera-Pastrana et al., 2010)
Morus alba	Antidiabetic, antioxidant	Functional teas, juices, powders	(Katsube et al., 2006); (Chen et al., 2021)
Ziziphus mauritiana	Antioxidant, anti-inflammatory	Functional beverages, dried functional snacks	(Akassh et al., 2020)
Aegle marmelos	Digestive aid, antimicrobial	Functional beverages, herbal drinks	(Gupta et al., 2026); (Khanal et al., 2023)
Vitis vinifera	Cardioprotective, antioxidant	Functional juices, nutraceutical drinks, fermented beverages	(Martin et al., 2020); (Jiridi et al., 2019); (Majeed et al., 2023)
Litchi chinensis	Antioxidant, anti-inflammatory	Functional beverages, dried nutraceutical fruits	(Prasad et al., 2009); (Emanuele et al., 2017)

Cereals, millets and medicinal plants-based functional foods

Because of their high fiber and phytochemical content, traditional grains and millets have drawn interest as functional dietary ingredients. Due to its high calcium, phenolic, and dietary fiber content, finger millet (*Eleusine coracana*) is a good candidate for the creation of functional foods (Chandrasekara & Shahidi, 2010). Rutin, found in buckwheat (*Fagopyrum esculentum*), has cardio-protective and antioxidant qualities (Sofi et al., 2022). Beta-glucan, which is found in barley, lowers cholesterol and enhances cardiovascular health (Raj et al., 2023).

According to Baraniak & Dobrowolska (2010), amaranth's high protein and antioxidant content have also led to its application in functional foods. Medicinal plants, including ginger, turmeric, and moringa, have been used extensively in functional food items. Fortified beverages and baked goods have been made with *Moringa olifera*, which is high in vitamins, minerals, and antioxidants (Leone et al., 2015). Curcumin, which has anti-inflammatory and antioxidant qualities, is

found in turmeric (Hewlings & Kalman, 2017). Ginger's digestive and antioxidant qualities have led to its use in functional beverages (Shaukat et al., 2023). The antidiabetic properties of fenugreek have led to its usage in functional foods (Sarker et al., 2024). Turmeric and ginger are being consumed along with milk and water to relieve a sore throat and during colds. Meanwhile, holy basil is already consumed as a plant that can boost immunity. Ashwangandha, on the other hand, is used in the form of powder or paste for cognitive support, inflammation, and immunity boost. Cold extraction of Chirayata with laung (*Clove*) and dalchini (*Cinnamon*) is used to stimulate digestive fire, and it improves digestion. Other combinations of Chirayata with honey and other herbs have proved to cure fever and liver disorders (Chauhan & Chauhan, 2019).

Table 5: Millet, cereals, and medicinal plants and their major bioactive compounds.

Plant	Common name	Category	Major bioactive compounds
Eleusine coracana	Finger millet (Ragi)	Millet	Polyphenols, dietary fibre, and calcium
Panicum miliaceum	Proso millet	Millet	Phenolics, flavonoids
Setaria italica	Foxtail millet	Millet	Phenolic acids, flavonoids
Pennisetum glaucum	Pearl millet (Bajra)	Millet	Polyphenols, dietary fiber, and iron
Fagopyrum esculentum	Buckwheat	Pseudocereal	Rutin, flavonoids
Avena sativa	Oats	Cereal	Beta-glucan, phenolics
Oryza sativa (brown rice)	Brown rice	Cereal	Gamma-oryzanol, phenolics
Hordeum vulgare	Barley	Cereal	Beta-glucan, tocopherols
Triticum aestivum	Whole wheat	Cereal	Phenolic acids, fibre
Moringa oleifera	Moringa	Medicinal plant	Flavonoids, phenolics, vitamins
Tinospora cordifolia	Guduchi	Medicinal plant	Alkaloids, glycosides
Withania somnifera	Ashwagandha	Medicinal plant	Withanolides
Ocimum tenuiflorum	Holy basil (Tulsi)	Medicinal plant	Eugenol, flavonoids
Curcuma longa	Turmeric	Medicinal plant	Curcumin
Zingiber officinale	Ginger	Medicinal plant	Gingerols, shogaols
Swertia chirayita	Chirayita	Medicinal plant	Xanthones, flavonoids

Conclusion

The formulation of functional foods incorporating medicinal plants and traditional plants from South Asia represents an expanding area of research that bridges traditional ethnobotanical knowledge with modern food science and innovation. This review demonstrates that a wide range of plants, such as *Curcuma longa, Withania somnifera, Ocimum tenuiflorum, Tinospora cordifolia, Emblica officinalis* and *Moringa oleifera,* have been successfully incorporated into various functional food matrices, including beverages, dairy products, bakery products, confectioneries and nutraceutical formulations. Among fruit-based functional foods, *Phyllanthus emblica* (amla) exhibits the most extensive product diversification, appearing in functional beverages, fortified juices, gummies and nutraceutical powders, and is reported to have antioxidant, immunomodulatory and antimicrobial activities. *Syzygium cumini* (jamun) has shown particular promise for antidiabetic applications, with anthocyanins and ellagic acid contributing to improvements in glucose metabolism in functional juices, jams and fermented beverages. In the cereals and millets category, *Eleusine coracana* (finger millet) has been formulated into functional porridge, malt beverages and bakery products, leveraging its polyphenol and dietary fiber content for antidiabetic and antioxidant applications. *Avena sativa* (oats) and *Hordeum vulgare* (barley) have been incorporated into functional breakfast cereals and beverages, with beta-glucan content providing clinically validated cholesterol-lowering and prebiotic effects. Among medicinal plants, *Moringa oleifera* has been widely incorporated into fortified biscuits, beverages and powders, with its flavonoid and phenolic content supporting antioxidant and anti-inflammatory properties. *Curcuma longa* (turmeric) and *Zingiber officinale* (ginger) have been incorporated into functional drinks and fortified foods, with curcumin and gingerols providing anti-inflammatory and digestive health benefits. *Withania somnifera* (ashwagandha) and *Ocimum tenuiflorum* (tulsi) have been utilized in

functional beverages and nutraceutical formulations for adaptogenic and antimicrobial properties, respectively. South Asia has a long history of traditional medicinal use and remarkable biodiversity, particularly in countries such as Bangladesh, Sri Lanka, Nepal, and India. Only a small percentage of the bioactive plant species have been investigated for the production of functional foods, despite their availability. While many native plants have not yet been investigated for use in functional food applications, most current formulations focus on well-known herbs such as turmeric, ginger, amla, and moringa.

Future studies should concentrate on the systematic investigation of underutilized plants for their use in functional foods, especially in Nepal and other Himalayan regions. In addition to the extraction, integration, and improvement of phytochemical stability, clinical studies should be conducted. Overall, South Asia is a highly promising region for the development of plant-based functional foods owing to its rich biodiversity, robust traditional knowledge systems, and growing consumer demand for natural, health-promoting food products. Incorporating scientifically validated traditional medicinal plants into functional food formulations has the potential to improve public health and to support sustainable agriculture, economic growth, and the global functional food market. Unlocking the full potential of South Asian medicinal herbs as excellent functional food components may require ongoing research and innovation.

Table 6: Millet, cereals, and medicinal plants and their functional properties.

Plant	Reported functional properties	Functional food products/ formulations	References
Eleusine coracana	Antidiabetic, antioxidant, hypoglycemic	Functional porridge, malt beverages, bakery products	(Devi et al., 2011); (kaur et al., 2024)
Panicum miliaceum	Antioxidant, cardioprotective	Functional porridge, ready-to-eat snacks	(Saleh et al., 2013);
Setaria italica	Antidiabetic, antioxidant	Functional noodles, bakery products	(Chandrasekara & Sahidi, 2011)
Pennisetum glaucum	Antioxidant, anti-anemia	Functional breads, fermented foods	(Taylor et al., 2006)
Fagopyrum esculentum	Antioxidant, cardioprotective	Functional noodles, bakery products	(Paul et al., 2021); (Gimenez-Bastida & Zielinski, 2015)
Avena sativa	Cholesterol-lowering, prebiotic	Functional breakfast cereals, beverages	(Rasane et al., 2013)
Oryza sativa	Antioxidant, antidiabetic	Functional rice products, nutraceutical foods	(Goufo & Trindade, 2014)
Hordeum vulgare	Hypocholesterolemic, prebiotic	Functional beverages, bakery products	(baik & Ullrich, 2008)
Triticum aestivum	Antioxidant, digestive health	Functional breads, cereals	(Fardet, 2010)
Moringa oleifera	Antioxidant, anti-inflammatory	Fortified biscuits, beverages, powders	(Leone et al., 2015); (Pareek et al., 2023)
Tinospora cordifolia	Immunomodulatory, antidiabetic	Herbal beverages, nutraceutical drinks	(Jabiullah et al., 2018)
Withania somnifera	Adaptogenic, anti-stress	Functional beverages, capsules	(Singirala et al., 2025)
Ocimum tenuiflorum	Antioxidant, antimicrobial	Functional herbal teas, beverages	(Kumari et al., 2024)
Curcuma longa	Anti-inflammatory, antioxidant	Functional drinks, fortified foods	(Hewlings & Kalman, 2017)
Zingiber officinale	Anti-inflammatory, digestive aid	Bakery and functional beverages	(Mao et al., 2019); (Shaukat et al., 2023)
Swertia chirayita	Anti-inflammatory, digestive aid	Herbal functional beverages	(Dey et al., 2020); (Joshi & Dhawan, n.d.)

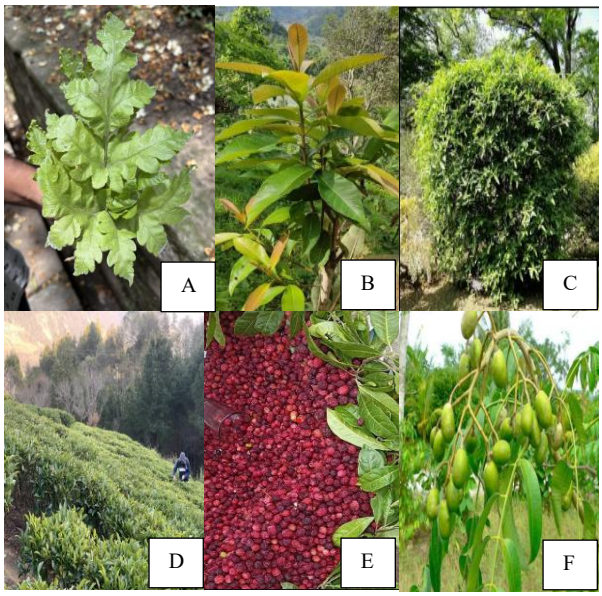

Figure 1: A: *Dryoathyrium boryanum* (Kalo neuro); B: *Diploknema butyracea* (Chiuri) (Dahal et al., 2021); C: *Zanthoxylum armatum* (Timur) (Dahiya et al., 2025); D: *Camellia sinensis* (Tea); E: *Myrica esculenta* (Kafal) (Bhatt et al., 2023); F: *Choerospondias axillaris* (Lapsi) (Labh & Shakya, 2016).

Research gap and future opportunities

Although many South Asian plants have been demonstrated to have strong bioactive properties, only a limited number have been used in functional food formulations. Tea (*Camellia sinensis*) can be a potent functional food ingredient because of the high content of catechins. Dark chocolates contain several health-promoting bioactive components; therefore, the addition of tea to food such as chocolate can enhance the nutritional value. Many plants, such as *Myrica esculenta*, *Diploknema butyracea,* and *Ficus auriculata,* remain underexplored for food applications despite their nutraceutical potential. Fruits such as Aiselu (*Rubus ellipticus),* Lapsi *(Choerospondias axillaris) are* already being consumed by most of the populations in Nepal. Hence, the addition of these fruits not only fulfil the quality of taste but also the nutritional value of the functional food products. Medicinal plants such as *Dryoathyrium boryanum* is considered important plants in the context of the formulation of functional food because of their high flavonoid content and also because they have already been consumed as a staple vegetable around communities in Nepal. Since ancient times, people have used the edible *Zanthoxylum armatum* (Timur) plant for cough, cholera, fever, itching, piles, leucoderma, rheumatism, tonic, indigestion, and toothache (Kumar & Gupta, 2020). Future research should focus on developing functional foods using underutilized plants from Nepal and the Himalayan region. See Table 7 for more underutilized plants that can add nutritional value to the plant.

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Review Article

In vitro Antibacterial Activity of Medicinal Plants of Nepal: A Comprehensive Review

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ABSTRACT

Antimicrobial resistance is a growing challenge in medicine. Medicinal plants are used as antibiotic medicines for primary healthcare. This review systematically compiled and analyzed published *in vitro* studies on the antibacterial activity of medicinal plants of Nepal. A comprehensive search on PubMed, NepJOL and Google Scholar identified 27 original studies in Nepal, approximately 120 plant species from 100 genera and over 50 families. Leaves were most frequently tested (66.7%), followed by roots/rhizomes (40.7%), fruits/seeds/flowers (33.3%), bark (22.2%), and whole plant (18.5%). Methanol (51.9%) and ethanol (33.3%) were commonly used extraction solvents, with cold maceration being predominant method. Plant extracts demonstrated activity against both Gram-positive and Gram-negative bacteria, with Gram-positive strains generally more susceptible (~70% of studies). The largest ZOI reported was 31 mm for an ethyl acetate extract of *Terminalia bellirica* fruit against *Pseudomonas aeruginosa*. MIC values ranged from tens to several hundred µg/ml. However, methodological heterogeneity, limited MIC/MBC reporting and under-representation of multidrug-resistant bacteria highlight the need for standardized and reproducible studies to identify promising species for antibacterial drug development.

Keywords: Antibacterial activity; Antimicrobial resistance; Ethnomedicine; Medicinal plants

Introduction

Antimicrobial resistance (AMR) poses a severe threat to global health. Resistant infections are estimated to cause thousands of deaths annually worldwide and could reach millions per year by 2050 if current trends continue (Parajuli et al., 2024). The South Asian region, including Nepal, is particularly vulnerable due to high population density, substantial infectious disease burden and widespread antibiotics use. In Nepal, multiple interconnected factors contribute to the emergence and spread of AMR (Shrestha et al., 2010; Shakya et al., 2012; Devkota et al., 2018; Dhungana et al., 2019; Niroula et al., 2020; Shrestha et al., 2023;

Tiwari et al., 2024; Acharya et al., 2025a, 2017; Adhikari et al., 2025; Sharma et al., 2025). The country has approximately 0.17 physicians per 1,000 population, far below the World Health Organization recommendations, leading many individuals to seek antibiotics directly from pharmacies or informal drug vendors (Pokharel & Adhikari, 2020). The vendors frequently dispense antibiotics without prescription and at inappropriate doses or durations. Studies suggest that more than half of antibiotic use in Nepal occurs without medical supervision, creating strong selective pressure on bacterial populations (Rijal et al., 2021).

Nepal has recently endorsed a National Action Plan on AMR emphasizing 'One Health' surveillance and laboratory capacity strengthening. However, persistent community-level practices and limited healthcare resources indicate that complementary strategies are required. In this context, Nepal's exceptional botanical diversity and long-standing ethnomedicinal traditions offer significant potential. Historically, approximately 60–80% of rural Nepalese relied on medicinal plants for primary healthcare (Chaudhary et al., 2025). Many of these plants contain bioactive secondary metabolites with documented antimicrobial properties.

While numerous *in vitro* studies have screened Nepalese medicinal plants for antibacterial activity, the findings remain dispersed across the literature. A comprehensive synthesis is therefore needed to evaluate the strength of existing evidence, identify promising plant taxa, and highlight research gaps relevant to AMR mitigation. This review aims to compile systematically and analyze critically the published *in vitro* antibacterial studies on Nepalese medicinal plants.

Methods

Search strategy and data sources

A comprehensive literature search was conducted to identify studies reporting the antibacterial activity of medicinal plants from Nepal. The electronic databases PubMed**,** NepJOL (Nepal Journals Online)**,** and Google Scholar were systematically searched. The following combinations of keywords were used:"medicinal plants" OR "traditional medicine" AND "Nepal" AND "antibacterial" OR "antimicrobial activity" AND "*in vitro*" OR "disc diffusion" AND "zone of inhibition".

Inclusion criteria: Studies were included if they-

1. used plant material collected in or traditionally used in Nepal;
2. prepared plant extracts using any solvent or method; and
3. evaluated antibacterial activity against bacterial strains using *in vitro* assays.

Exclusion criteria: Studies were excluded if they-

1. did not assess antibacterial activity;
2. were reviews or lacked original experimental data;
3. failed to report essential methodological details.

Study selection

All retrieved articles were screened based on titles and abstracts for relevance. Full texts of potentially eligible studies were then assessed according to the predefined inclusion and exclusion criteria. Only studies meeting all inclusion criteria were included in the final analysis (Figure 1).

Data extraction

Data were extracted on study details (authors, year, study type), botanical information (species, plant part, family), extraction (solvent, method, concentration), test organisms (species, strain), and quantitative results (zone of inhibition or MIC/MBC values). Commonly cited ATCC or reference strains (e.g. *E. coli* ATCC 25922, *S. aureus* ATCC 25923) were recorded when specified.

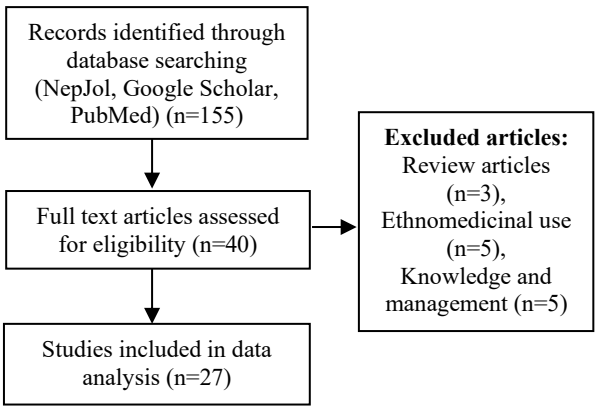

Figure 1: Flowchart for inclusion of published articles.

Results and Discussion

A total of 27 original research articles evaluating the antibacterial activity of Nepalese medicinal plants were included in this review (Appendix 1). Collectively, these studies screened approximately 120 plant species representing nearly 100 genera of over 50 families, reflecting the rich ethnobotanical diversity of Nepal. The most frequently represented families were Fabaceae (≈11.7%), Asteraceae (≈10%), Lamiaceae (≈8.3%), Zingiberaceae (≈6.7%), Euphorbiaceae (≈5.8%) and Rutaceae (≈5%), collectively accounting for nearly half of all investigated species. At the genus level, most genera were represented by a single species, with only a few, including *Ocimum*, *Curcuma*, *Terminalia* and *Zingiber*, examined in multiple studies.

This indicates limited replication and suggests that the current research landscape remains largely exploratory.

Plant parts used

Leaves were the most commonly studied plant part, reported in 66.7% of the studies, followed by roots and rhizomes (40.7%), fruits, seeds, or flowers (33.3%), bark (22.2%), and whole plant (18.5%) (Table 1). The predominance of leaves likely reflects their ease of collection, sustainability, and high concentration of bioactive secondary metabolites. Nevertheless, roots, bark and fruits often demonstrated stronger antibacterial activity, suggesting that phytochemical content and antibacterial potency can vary substantially across plant parts. The diversity of plant parts used across studies introduces variability in the observed antibacterial outcomes, complicating direct comparisons between extracts.

Extraction methods and solvents

Cold maceration or percolation was the most frequently employed extraction method, used in 55.6% of studies. Soxhlet extraction and post-extraction fractionation were each applied in 22.2% of studies. Studies utilizing Soxhlet extraction or fractionation often reported stronger antibacterial activity, highlighting the influence of extraction efficiency on bioactive compound yield. Solvent choice also played a critical role in determining activity. Methanol was the most commonly used solvent (51.9%), followed by ethanol (33.3%). Non-polar or semi-polar solvents such as chloroform, hexane, and ethyl acetate were used in 25.9% of studies, whereas aqueous extracts were less common (14.8%) (Table 1). Extracts prepared with organic solvents consistently exhibited moderate to strong antibacterial activity, whereas aqueous extracts generally showed weak or inconsistent effects.

Table 1: Summary of included studies on antibacterial activity of medicinal plants.

Variables	Parameter	Number of studies (n)	Percentage (%)
Plant parts used	Leaves	18	66.7
	Roots/Rhizomes	11	40.7
	Bark	6	22.2
	Fruits/Seeds/Flowers	9	33.3
	Whole plant	5	18.5
Extraction methods	Cold maceration/percolation	15	55.6
	Soxhlet extraction	6	22.2
	Fractionation of crude extracts	6	22.2
Extraction solvents	Methanol	14	51.9
	Ethanol	9	33.3
	Aqueous	4	14.8
	Non-polar/semi-polar solvents*	7	25.9
Bacterial spectrum tested	Gram-positive only	3	11.1
	Gram-negative only	3	11.1
	Both Gram-positive and Gram-negative	15	55.6
	Not clearly specified	6	22.2
Antibacterial assessment	ZOI reported	27	100
	MIC and/or MBC reported	11	40.7
	ZOI only (no MIC/MBC)	16	59.3
Antibacterial outcome	Any antibacterial activity observed	27	100
	Strong–moderate activity with organic solvents	23	85.2
	Weak/inconsistent activity with aqueous extracts	4	14.8
Susceptibility trend	Gram-positive > Gram-negative bacteria	~19	~70

*Includes chloroform, hexane, ethyl acetate, acetone and other related solvents.
Multiple responses per study led to the total percentages exceeding 100%.

Bacterial spectrum

All included studies evaluated at least one Gram-positive and one Gram-negative bacterium (Table 1). The most frequently tested indicator organisms were *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923), each appearing in over 70% of studies. Other commonly tested bacteria included *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella* species. Gram-positive strains such as

Bacillus subtilis and *Streptococcus* spp. were also assessed in some studies, and a few studies included multidrug-resistant or clinical isolates. The selection of these pathogens aligns with both standard microbiological practice and the clinical relevance of these organisms in the Nepalese context.

Antibacterial activity

The antibacterial activity of plant extracts, as measured by disc diffusion assays, varied widely (Table 1). Weak activity corresponded to inhibition zones of 7–10 mm, moderate activity to 10–20 mm, and strong activity to zones exceeding 20 mm. The largest inhibition zone reported was 31 mm, observed for an ethyl acetate extract of *Terminalia bellirica* fruit against *Pseudomonas aeruginosa*. Other extracts with notable activity included *Morus australis* root (25 mm against *K. pneumoniae*), *Glycyrrhiza glabra* root (23 mm against *S. aureus*), and *Quercus infectoria* galls (23 mm against *S. typhi*). Overall, Gram-positive bacteria were more susceptible than Gram-negative bacteria, with approximately 70% of studies reporting higher inhibition zones against Gram-positive strains. This susceptibility pattern is consistent with structural differences in bacterial cell walls that influence permeability to plant-derived compounds.

Several plant extracts consistently exhibited strong antibacterial activity. The ethyl acetate extract of *Terminalia bellirica* fruit showed the highest inhibition (31 mm) against *P. aeruginosa*, with MIC values ranging from 200 to 800 µg/mL. *Morus australis* root methanol extract demonstrated 25 mm inhibition against *K. pneumoniae*, while *Glycyrrhiza glabra* root ethanol extract produced 23 mm inhibition against *S. aureus*. Extracts of *Quercus infectoria* galls, *Cinnamomum tamala* leaves, and *Mallotus japonicus* stems also showed notable antibacterial activity (Table 2).

Table 2: Extract of plant showing strong antibacterial activity (ZOI: zone of inhibition, MIC: minimum inhibitory concentration).

Plant (Family)	Part	Solvent	Target organism	ZOI (mm)	MIC (µg/ml)
Terminalia bellirica (Combretaceae)	Fruit	Ethyl acetate	*Pseudomonas aeruginosa*	31	200–800 (varied by strain)
Morus australis (Moraceae)	Root	Methanol	*Klebsiella pneumoniae*	25	100–500
Glycyrrhiza glabra (Fabaceae)	Root	Ethanol	*Staphylococcus aureus*	23	250
Quercus infectoria (Fagaceae)	Galls	Methanol	*Salmonella enterica* Typhi	23	180
Cinnamomum tamala (Lauraceae)	Leaf	Ethanol	*Escherichia coli*	21	–
Mallotus japonicus (Euphorbiaceae)	Stem	Methanol	*Pseudomonas aeruginosa*	20	320

MIC and/or MBC values were reported in 11 studies (40.7%). Potent extracts exhibited MICs in the low tens to several hundreds of micrograms per milliliter, while aqueous extracts often demonstrated MICs above 1000 µg/mL or were not reported. In general, strong disc diffusion activity corresponded to low MICs, confirming that extracts demonstrating large inhibition zones also possessed measurable bacteriostatic or bactericidal potency. Differences in methodology, such as agar dilution versus broth-based MIC determination, precluded quantitative aggregation of MIC values across studies.

This review provides a comprehensive synthesis of in vitro antibacterial studies on Nepalese medicinal plants, highlighting both the potential and limitations of the current evidence base. The identification of antibacterial activity in extracts from approximately 120 plant species underscores the rich ethnobotanical resources of Nepal and supports traditional medicinal use (Ashraf et al., 2023; Joshi et al.,2019). The concentration of research on families such as Fabaceae, Asteraceae, and Lamiaceae likely reflects their known phytochemical richness, including flavonoids, alkaloids, terpenoids, and phenolics, which contribute to antibacterial activity (Ashraf et al., 2023). However, most species were assessed in only a single study, limiting reproducibility and preventing robust comparison of antibacterial potency across taxa, suggesting that medicinal plant research in Nepal remains largely exploratory.

Plant part selection emerged as a significant determinant of antibacterial outcomes. Leaves were the most frequently studied, likely due to ease of collection, sustainability, and high concentrations of bioactive metabolites (Pokharel & Adhikari, 2020). Nevertheless, roots, bark, and fruits often exhibited stronger antibacterial effects, indicating part-specific

distribution of phytochemicals (Shrestha et al., 2025). This variability complicates direct comparisons between studies and highlights the need for careful standardization of plant material in future research.

Extraction methodology and solvent choice were critical factors influencing reported activity. Studies employing polar organic solvents, particularly methanol, ethanol, and ethyl acetate, consistently reported stronger antibacterial effects compared to aqueous extracts (Basnet et al., 2020; Baral et al., 2021). This suggests that many antibacterial compounds in Nepalese medicinal plants are moderately polar and poorly extracted by water. While cold maceration was the most common extraction method, Soxhlet extraction or post-fractionation often yielded higher activity, emphasizing the importance of extraction efficiency (Shrestha et al., 2025). However, few studies systematically compared solvents or optimized extraction conditions, limiting understanding of true extract potency.

The observed variation in antibacterial potency among these extracts is fundamentally linked to their diverse mechanisms of action against bacterial cellular targets. For example, the high efficacy of *Terminalia bellirica* and *Quercus infectoria* (Table 3) is likely mediated by high concentrations of hydrolyzable tannins and phenolics. These compounds act by complexing with bacterial cell walls and inactivating membrane-bound enzymes through non-specific forces such as hydrogen bonding. In contrast, members of the Lamiaceae and Fabaceae families, rich in lipophilic terpenoids and alkaloids, may exert their effects by increasing the permeability of the cytoplasmic membrane, leading to the leakage of essential intracellular ions and ATP.

The bacterial spectrum tested primarily included standard indicator strains, with *Escherichia coli* and *Staphylococcus aureus* appearing in over 70% of studies (Pokharel et al., 2020). Gram-positive bacteria were generally more susceptible than Gram-negative bacteria, consistent with structural differences in bacterial cell walls affecting permeability to phytochemicals (Parajuli et al., 2024). While a few studies included multidrug-resistant or clinical isolates (Timilsina et al., 2024), this area remains underexplored, limiting translational relevance in the context of Nepal's growing antimicrobial resistance burden.

Methodological heterogeneity represented a major limitation. The heavy reliance on disc diffusion assays, often without standardized extract concentrations or positive controls, restricted quantitative comparison. MIC and MBC values, the most clinically relevant indicators of antibacterial potency, were underreported (Shrestha et al., 2025). Differences in plant species, plant parts, extraction methods, and bacterial strains further constrained cross-study synthesis. Collectively, these factors indicate that while many Nepalese plants show measurable antibacterial activity, the current evidence is insufficient to support direct application for antimicrobial therapy.

Overall, these findings highlight the need for a more systematic and targeted research approach. Future studies should prioritize repeated evaluation of promising plant species, standardize extraction and assay protocols, and include both standard and clinically relevant multidrug-resistant bacterial strains. Integrating ethnobotanical knowledge with pharmacological and microbiological investigation will be essential to identify reproducible and potent antibacterial agents. With such approaches, Nepalese medicinal plants hold considerable potential as sources of new antibacterial compounds that could contribute meaningfully to combating antimicrobial resistance.

Conclusion

Nepalese medicinal plants have considerable antibacterial potential. However, the current evidence is constrained by methodological heterogeneity and limited quantitative reporting. Addressing these limitations will be essential to harness medicinal plants as credible contributors to antibacterial discovery and AMR mitigation in Nepal.

Recommendations

Future research on Nepalese medicinal plants should adopt standardized extraction and antibacterial testing methodologies to ensure reproducibility and comparability across studies. Quantitative measures should be prioritized to provide clinically relevant assessments. Promising plant species should be re-evaluated across multiple studies to confirm consistent activity, while investigations should also include clinically relevant and multidrug-resistant bacterial strains. Finally, an integrated approach combining ethnobotanical knowledge with microbiological and pharmacological analyses will be essential to identify bioactive compounds, optimize extraction processes, and evaluate their potential for therapeutic application.

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Appendix 1: Published articles included for review.

S.N.	Title	Author et al
1	Antibacterial activities of medicinal plants of Nepal	(Pokharel et al., 2008)
2	Antidiabetic and Antimicrobial Properties of Some High-Altitude Medicinal Plants of Nepal	(Regmi et al.,2019)
3	Antimicrobial Activity of Some Medicinal Plants from East and Central Part of Nepal	(Subba & Basnet, 2014)
4	Assessment of phytochemical content, antioxidant and antibacterial activities of three medicinal plants of Nepal	(Subba, B., Basnet, P., & Sharma, S, 2015)
5	Assessment of Phytochemical, Antioxidant and Antimicrobial Activities of Some Medicinal Plants from Kaski District of Nepal	(Khanal et al., 2020)
6	Assessment of phytochemicals, antimicrobial, antioxidant and cytotoxicity activity of methanolic extract of *Tinospora cordifolia* (Gurjo)	(Shrestha & Lamichhane, 2021)
7	Phytochemical screening, Free radical scavenging, and In-vitro Anti-bacterial activity study of Chloroform, Acetone and Methanol extracts of selected medicinal plants of Nepal	(Karki et al., 2021)
8	Evaluation of Antibacterial Activities of Medicinal Plants	(Maharjan et al., 2013)
9	Evaluation of Antibacterial Activity of Some Traditionally Used Medicinal Plants against Human Pathogenic Bacteria	(Marasini et al., 2015)
10	Evaluation of Antibacterial Properties of Some Medicinal Plants Used for the Treatment of Respiratory Tract Infections in Nepal	(Kunwor & Thapa, 2013)
11	Evaluation of Antioxidant, Antimicrobial, and Cytotoxic Activities and Correlation with Phytoconstituents in Some Medicinal Plants of Nepal	(Ranabhat et al., 2022)
12	Evaluation of Phytochemical, Antioxidant and Antibacterial Activities of Selected Medicinal Plants	(Shresta et al., 2021)
13	Exploring the Potency of Medicinal Plants in Central Nepal's Highlands: A Comprehensive Analysis of Antioxidant, Antibacterial Properties, and Toxicity	(T. Shrestha et al., 2023)
14	In Vitro Antimicrobial Activity of Some Medicinal Plants against Human Pathogenic Bacteria	(Manandhar et al., 2019)
15	Antimicrobial and Antioxidant Activity of Some Indigenous Plants of Nepal	(Subba & Basnet. 2014)
16	Phytochemical extraction and antimicrobial properties of different medicinal plants	(Joshi et al.,2011)
17	Phytochemical Screening and Anti-Microbial Properties of Medicinal Plants of Dhunkharka Community, Kavrepalanchowk, Nepal	(Adhikary, 2010)
18	Phytochemical Screening and Antimicrobial Activities of Some Selected Medicinal Plants of Nepal	(Paudel & Gyawali, 2010)
19	Phytochemical Screening and Biological Activity Analysis of Some Selected Medicinal Plants of Ilam District of Nepal	(Basnet, 2020)
20	Phytochemical profiling using HRLCMS and evaluation of antioxidant and antibacterial activities of Nepalese medicinal plants	(Neupane & Lamichhane, 2020)
21	Phytochemical screening, free radical scavenging and in-vitro antibacterial activity of ethanolic extracts of selected medicinal plants of Nepal and effort towards formulation of antibacterial cream from the extracts	(Tiwari et al.,2021)
22	Preliminary Study on the Antibacterial Activities and Antibacterial Guided Fractionation of Some Common Medicinal Plants Practices in Itum Bahal, Kathmandu Valley of Nepal	(Bhandari et al., 2023)
23	Screening of Antioxidant, Antibacterial, Anti-Adipogenic, and Anti-Inflammatory Activities of Five Selected Medicinal Plants of Nepal	(Lamichhane et al., 2023)
24	Study of phytochemical, anti-microbial, anti-oxidant, and anti-cancer properties of *Allium wallichii*	(Bhandari et al., 2017)
25	Study of Phytochemical, Antioxidant and Antimicrobial Activity of *Artocarpus heterophyllus*	(Thapa et al., 2016)
26	Chemical Profiling and Biological Activities on Nepalese Medicinal Plant Extracts and Isolation of Active Fraction of *Nyctanthes arbor-tristis*	(Khadka et al., 2024)
27	Antimicrobial Potential of Three Nepalese Medicinal Plants Against Multidrug Resistance *Escherichia coli* Isolates From Normal Individuals	(Timilsina et al., 2024)