Efficacy of Four Herbal Extracts on *Actinobacillus Actinomycetemcomitans* and *Prophyromonas Gingivalis*: an in vitro microbiological study

Kafle D,[1] Humagain M,[2] Luintel S,[3] Karki A,[3] Lamichhane J[4]

[1]PhD Scholar, Department of Biotechnology,

[2]Department of Periodontology and Implantology,

[3]Department of Orthodontics,

Kathmandu University School of Medical Sciences,

Dhulikhel, Kavre, Nepal.

[4]Department of Biotechnology,

Kathmandu University School of Science,

Dhulikhel, Kavre, Nepal.

Corresponding Author

Janardan Lamichhane

Department of Biotechnology,

Kathmandu University School of Science,

Dhulikhel, Kavre, Nepal.

E-mail: ljanardan@ku.edu.np

Citation

Kafle D, Humagain M, Luintel S, Karki A, Lamichhane J. Efficacy of Four Herbal Extracts on Actinobacillus Actinomycetemcomitans and Prophyromonas Gingivalis: an in vitro Microbiological study. **Online First.**

ABSTRACT

Background

Periodontitis is probably one of the most common diseases occurring worldwide caused by interaction of anaerobic and aerobic microorganism found in dental plaque. Medicinal plant extracts, rooted in traditions like Ayurveda, provide a promising avenue for antimicrobial study against periodontal pathogens.

Objective

To assess the antimicrobial properties of extracts from indigenous medicinal plants against *Actinobacillus actinomycetemcomitans (ATCC: 29522) and Prophyromonas gingivalis (ATCC: 33277),* two potent periodontal pathogens.

Method

Four medicinal plants grown in different altitudes of Nepal were selected *Tejpatta (Cinnamomum tamala), Vajradanti (Barleria prionitis), Danti (Baliospermum montanum), and Ghotape (Centella asiatica)*, henceforth denoted as GP1, GP2, GP3 and GP4 respectively), and their extracts were prepared using standard biochemical protocol. Phytochemical composition was analyzed through Fourier Transform Infrared Spectroscopy (FTIR) and Liquid Chromatography-Mass Spectrometry (LC-MS). Antimicrobial activity against *A. actinomycetemcomitans* and *P. gingivalis* was assessed through MIC, MBC, and zone of inhibition assays.

Result

All of the four extracts were found to have antimicrobial activities against *A. actinomycetemcomitans* and *P. gingivitis*. The dry extract of *Tejpatta* was the most effective for zone of inhibition with minimal concentration of 187.5 µg/ml for MIC and 750 µg/ml for MBC. Additionally, *Vajradanti* and *Danti* were also effective against *P gingivalis* with the highest zone of inhibition (25 mm) followed by the dry extract of *Tejpatta* (22 mm) at a concentration of 100 mg/ml.

Conclusion

Phytochemical analysis revealed diverse chemical compounds in the plant extracts, indicating a potential therapeutic value. Among the four herbal plants, GP1 *(Cinnamomum tamala)* was found as the most potent against *A. actinomycetemcomitans*. GP2 *(Barleria prionitis)*, GP3 *(Baliospermum montanum)* and GP1 *(Cinnamomum tamala)* displayed significant inhibition zones against both the periodontal pathogens *A. actinomycetemcomitans and P gingivalis.*

KEY WORDS

Actinobacillus actinomycetemcomitans, Periodontitis, Prophyromonas gingivalis, Medicinal plants

INTRODUCTION

Periodontitis is one of the most common diseases globally and is also the most prevalent oral disease.[1] Approximately 24.2% to 63% of people worldwide suffer from periodontal disease.[2] In Asia, the prevalence is 62.4%, while in Nepal, it ranges from 47.5% to 71.6%.[2-4] Periodontitis and dental caries are widespread oral health issues.[5] Periodontitis is more common in adults, whereas dental caries are more frequently seen in children and adolescents.[6] Periodontitis is one of the leading causes of edentulism in humans. Unlike dental caries, periodontitis is a multifactorial disease. Although bacteria are the major etiological agents of periodontitis, the multifactorial theory better explains its development. Interestingly, around 500 species of bacteria have been isolated from dental plaque and calculus.[7] Not all the bacteria are detrimental to periodontal health. Within dental plaque, there are certain bacteria that exist to actually help regulate other harmful bacteria. However, the endotoxins and enzymes released by bacteria can cause damage to both soft tissues (collagen matrix) and hard tissues (alveolar bone), ultimately contributing to the progression of periodontitis.[8]

The most common periodontal pathogens so far known are *Actinobacillus actinomycetemcomitans* and *Prophyromonas gingivalis*.[9] *A. actinomycetemcomitans* is frequently associated with aggressive and acute periodontitis, whereas *P. gingivalis* is linked to chronic periodontitis.[10] At present, the cornerstone of periodontal therapy is the control of bacteria using mechanical debridement and the host defense mechanism. Systemic antibiotic therapy has limited application due to side effects and the limited penetration of antibiotics into the crevicular fluid which is the primary site of periodontal pathogens.[11] These pathogens reside in dental plaque, which is a biofilm aggregate of bacteria. Aerobic, anaerobic, and facultative bacteria are found in dental plaque. The subgingival plaque and plaques in deep periodontal pockets primarily harbor anaerobic species of bacteria.[12]

Controlling bacteria and plaque accumulation is extremely difficult due to the diverse makeup of bacterial populations in deep periodontal pockets. Local administration of antibiotics has been attempted but with limited success, mainly due to difficulties in retaining medication in the crevicular fluid.[13] Although localized sustained release of antibiotics may be an efficient means of controlling bacteria, research on the mechanisms involved in establishing sustained release is still in its early phases. Many pharmaceutical agents have adverse effects on the body's normal commensal flora.[14] However, natural products are believed to have specific actions against harmful bacteria while causing less disruption to normal flora. Ayurveda, a traditional medicinal practice particularly prevalent in Nepal and India before modern medicine, is known for its natural remedies. Therefore, this research aims to assess the efficacy of four herbal plant extracts against *P. gingivalis* and *A. actinomycetemcomitans*. The reason for choosing these two periodontal pathogens is because of their potent pathogenicity and involvement on acute as well as chronic periodontitis.[9]

METHODS

Four indigenous medicinal plants traditionally used for oral cure are collected from different locations of Nepal and coded as GP1, GP2, GP3, and GP4, corresponding to Tej Patta (*Cinnamomum tamala*), Vajradanti (*Barleria prionitis*), Daanti (Baliospermum montanum), and Ghotape (Centella asiatica), respectively as shown in (Table 1).

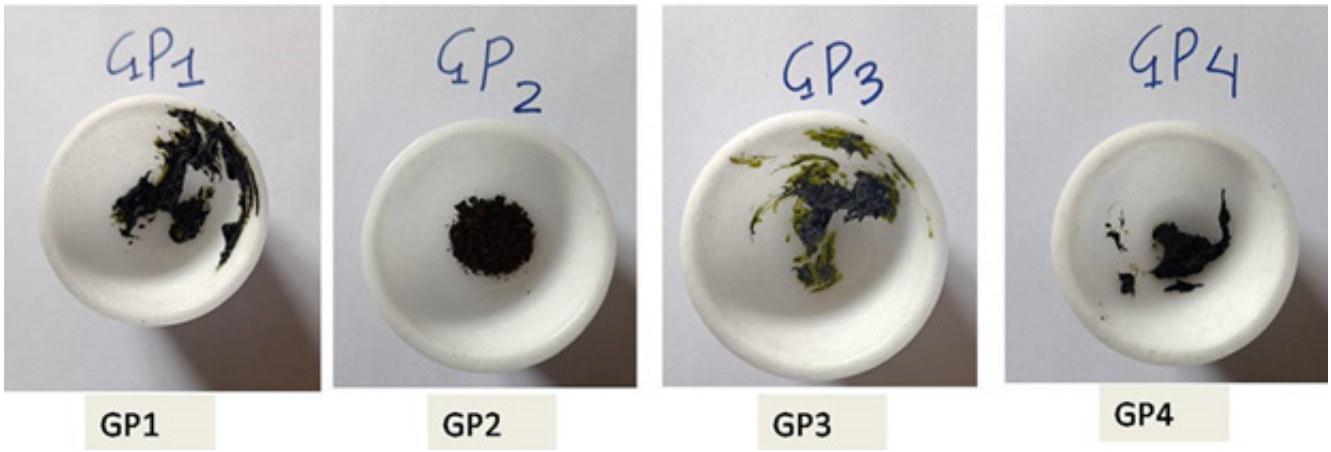
The study was conducted in Department of Biotechnology, Kathmandu University and Dhulikhel Hospital, Kathmandu University Hospital from January 2023 to January 2024. The selected plants are collected, washed and dried at room temperature under the shade. The dried plants were then finely powdered using a mechanical grinder (Fig. 1).

Fifty grams of finely powdered plant samples were weighed into 1000 ml screw-capped reagent bottles of methanol. Maceration was carried out for 4 days at room temperature using methanol as the extraction solvent, maintaining a 1:10 ratio of plant material to the solvent.

During maceration, occasional shaking at 150 rpm was performed. The content of each bottle was filtered through Muslin cloth and further filtered thrice using Sartorius grade 292 filter papers. The solvent was removed by rotatory evaporation under reduced pressure to obtain the crude extract. The concentrated crude extracts were stored at -20°C until further use. Dry extracts were suspended in High-performance liquid chromatography (HPLC) grade methanol for most experiments. For antibacterial activity testing, the extract was dissolved in Dimethyl sulfoxide (DMSO). The phytochemical composition of the plant extracts was determined using Fourier Transform Infrared (FTIR) Spectroscopy at Kathmandu University. Liquid Chromatography–Mass Spectrometry (LC-MS) was performed at IIT Mumbai with reference number EXT.SAIF-Mumbai.REF.8659/2022-12-05/HRLCMS to separate, identify, and quantify known and unknown compounds in the plant extracts. Antimicrobial activity of the plant extracts were assessed against *Actinobacillus actinomycetemcomitans* (ATCC 29522) and *Prophyromonas gingivalis* (ATCC-33277) purchased from Global Bioresource Center, ATCC, USA. Both aerobic and anaerobic cultures were conducted at the Department of Microbiology, Dhulikhel Hospital, Kathmandu University Teaching Hospital. The aerobic culture was carried out using the blood agar method. Anaerobic culture was performed on sheep blood agar plates with vitamin K supplement. The qualitative estimation of antimicrobial potential of the plant extracts was determined using the agar well diffusion method. A

Table 1. **Details of Medicinal Plants and Sample Collection Sites.**

Sample Code	Common Name	Scientific Name	Sample Collection Site	Altitude	Latitude	Longitude
GP1	Tejapatta	*Cinnamomum tamala*	Dhulikhel	1550 m	27°37'0''N	85°33'0''E
GP2	Vajradanti	*Baleria prionitis*	Gosaikunda	4421 m	28°5'3''N	85°24'31''E
GP3	Danti	*Baliospermum montanum*	Syangja	800 m	27°58'59.99" N	83° 46' 0.01" E
GP4	Ghotape	*Centella asiatica*	Syangja	800 m	27°59'59.4"N	83°46'58.0"E



Graph 1. **Illustrating MIC and MBC of different plant extract against Actinobacillus actinomycetemcomitans**

concentrated stock solution of the plant extract (12,000 µg/mL) was prepared using 2% Dimethyl sulfoxide from Merck Life Science Pvt. Ltd. (India). In 96-well microplates by Tarsons (India), 150 µL of BHI broth enriched with 1 µg/mL Vitamin K1 was dispensed. The plant extract underwent sequential two-fold dilution within the microplate, yielding final concentrations ranging from 6000 to 93.75 µg/ml. Bacterial strains (1-2×108 CFU/ml) were introduced, and incubation occurred in an anaerobic environment at 37°C for 48-72 hours within the Anaoxomat® III Jar System. Sterility control, growth control, and negative control with 2% DMSO were implemented. The microdilution procedure was conducted in triplicate, and observations were made to determine Minimum Inhibitory Concentration (MIC).

Following MIC, a 10 µL sample was taken from the 96-well microplate at the MIC concentration and two dilution steps above and below. These samples were evenly spread onto Mueller-Hinton Agar enriched with 1 µg/mL Vitamin K1. The culture plates were placed in the Anaoxomat® III Jar System at 37°C for 3-7 days. Minimum Bacterial Concentration (MBC) was determined based on the lowest concentration of the plant extracts that exhibited no discernible bacterial growth on the agar plates. These experiments were conducted in triplicate for reliability and consistency.

Agar plates were prepared with Mueller-Hinton and Anaerobic Blood Agar, both enriched with 1 µg/mL Vitamin K1. Inoculated with microbial culture (1-2×108 CFU/mL), the plates were air-dried, and wells were created. Plant extract solutions with concentrations of 50 mg/mL, 70 mg/mL, and 100 mg/mL were introduced into the wells. These solutions were prepared by dissolving the plant extracts in DMSO. DMSO served as a negative control, while positive controls included Amoxicillin/Clavulanic acid and Tetracycline antimicrobial susceptibility discs from Oxoid Ltd. (United Kingdom). The agar plates were incubated anaerobically at 37°C for 2-3 days. Tests were conducted in triplicates, and resulting inhibition zones were measured using the HiAntibiotic Zone Scale from Hi Media Laboratories Pvt. Ltd. (India).

RESULTS

The phytochemical analysis of the four selected indigenous medicinal plant extracts revealed the presence of several chemical compounds, including alkenes, carbonyl compounds, and aromatic compounds (Table 2). Additionally, Tejpatta exhibited alcohol and alkyl halides, Vajradanti contained aldehyde and carboxylic acid, Danti was found to possess nitrile, amides, and alkyl halides, while Ghotape was found to possess tannins, steroids, terpenoids, cardiac glycosides. These findings indicated the diverse chemical composition of these traditional remedies, suggesting potential for therapeutic uses.

In terms of their antibacterial properties, the plant extracts were evaluated for minimal inhibitory concentration (MIC), minimal bacterial concentration (MBC), and zone of inhibition (ZOI) against *Actinobacillus actinomycetemcomitans* and Prohyromonas gingivalis, a significant contributor to periodontitis. The results for MIC, MBC and ZOI are shown in table 3, 4, Graph 1 ,2,3 and 4.

Table 2. Major phytochemical compounds found in plant extracts

Plant	Scientific name	Compounds	Test done
GP1	Cinnamomum tamala	Alkaloids, flavonoids, alcohol and alkyl halides	FTIR, LC-MS
GP2	Baleria prionitis	Alkaloids, flavonoids, aldehyde and carboxylic acid	FTIR, LC-MS
GP3	Baliospermum mon-tanum	Alkaloids, flavonoids nitrile, am-ides, and alkyl halides	FTIR, LC-MS
GP4	Centella asiatica	Alkaloids, flavonoids, tannins, steroids, terpenoids, cardiac gly-cosides	FTIR, LC-MS

Table 3. Aggregatibacter actinomycetemcomitans (b) ATCC 29522

Ex-tract ID	MIC	MBC	ZOI				
			100 mg/ml	70 mg/ml	50 mg/ml	AMC	TETR
GP1	187.5 µg/ml	750 µg/ml	20 mm	12 mm	12 mm	32 mm	50 mm
GP2	750 µg/ml	750 µg/ml	20 mm	16 mm	12 mm		
GP3	375 µg/ml	750 µg/ml	22 mm	22 mm	20 mm		
GP4	750 µg/ml	750 µg/ml	20 mm	20 mm	16 mm		
Note:BHI,VITAMIN K1, ANBA							

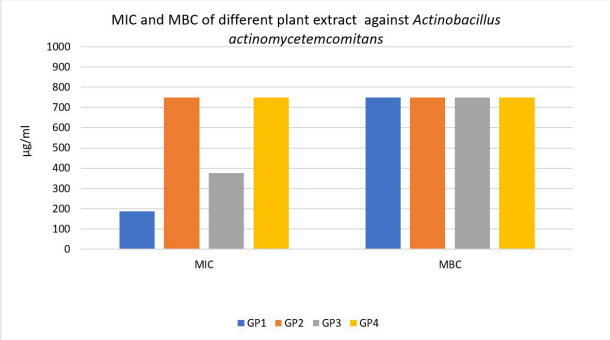
Table 4. Porphyromonas gingivalis ATCC 33277

Extract ID	MIC	MBC	ZOI				
			100 mg/ml	70 mg/ml	50 mg/ml	AMC	TETR
GP1	750 µg/ml	3000 µg/ml	15 mm	12 mm	10 mm	56 mm	40 mm
GP2	750 µg/ml	3000 µg/ml	25 mm	24 mm	20 mm		
GP3	750 µg/ml	3000 µg/ml	25 mm	22 mm	20 mm		
GP4	750µg/ml	1500µg/ml	18mm	14mm	14 mm		
Note: BHI,VITAMIN K1, ANBA							

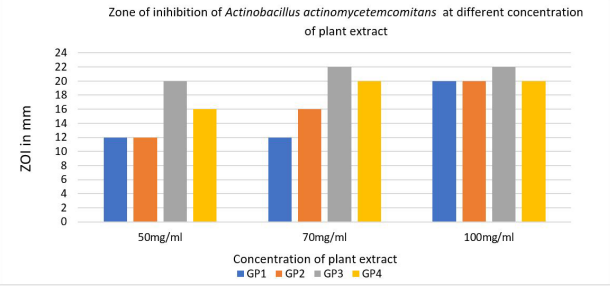
In the present study, all tested samples were effective in inhibiting *A. actinomycetemcomitans* and *P. gingivalis* growth with MIC and MBC values ranging from 187.5 µg/ml to 750 µg/ml and 750 µg/ml to 3000 µg/ml respectively (Table 3). Among the four different plant extracts, GP1 exhibited the lowest MIC and MBC values of 187.5 µg/ml and 750 µg/ml, respectively against *A. actinomycetemcomitans* (Graph 1). GP2, GP4 showed similar MIC and MBC value of 750 µg/ml and 750 µg/ml. The MIC and MBC values of GP3 had 375 µg/ml and 750 µg/ml, respectively.

Their antibacterial potency was quantitatively confirmed by an inhibition zone absence or presence all over the disc, loaded with the extract. All herbal products were found to have variable antimicrobial activity against *A. actinomycetemcomitans*. In this study, GP3 extract was reported to be the most significant against *A. actinomycetemcomitans*. Mean zone of inhibition after 24 hours incubation of GP3 plant extract was 22 mm, 22 mm, 22 mm at a concentration of 100 mg/ml, 70 mg/ml, 50 mg/ml respectively. Details of other plant extracts are shown in table 3, graph 2.

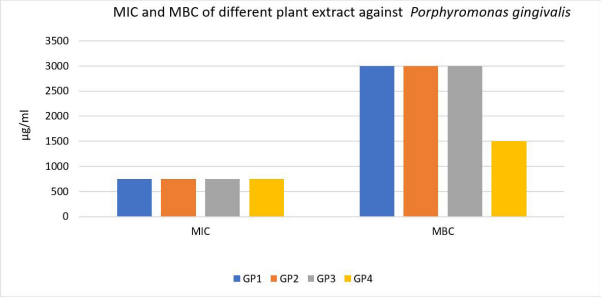
The effect of plant extracts on *P. gingivalis* is shown in table 4. MIC and MBC were similar in all the four samples except GP4 which had MBC of 1500 µg/ml (Graph 3). In term of zone of inhibition Gp2 and Gp3 had higher inhibitory zone



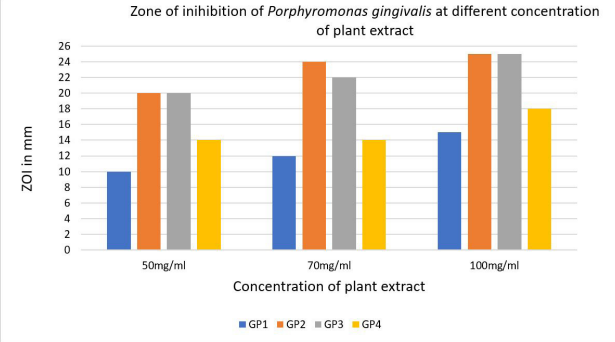
Graph 1. Illustrating MIC and MBC of different plant extract against *Actinobacillus actinomycetemcomitans*



Graph 2. Zone of inhibition of Actinobacillus actinomycetemcomitans at different concentration of plant extract



Graph 3. Graph illustrating MIC and MBC of different plant extract against Porphyromonas gingivalis



Graph 4. Zone of inhibition of Porphyromonas gingivalis at different concentration of plant extract.

(25 mm, 22 mm and 20 mm) at the concentration of 100 mg/ml, 70 mg/ml, 50 mg/ml respectively followed by GP4 and GP1 (Graph 4).

DISCUSSION

Periodontitis, commonly referred to as pyorrhea or gum disease, is a widespread oral health problem that affects individuals of all ages globally especially adults.[1] While there are no specific primary etiological agents solely responsible for periodontitis, *P. gingivalis* and *A. actinomycetemcomitans* stand out as particularly potent and virulent bacteria associated with the disease. It is an established fact that periodontitis is a multifactorial disease. The accumulation of dental plaque with periodontal pathogen, lack of mechanical debridement and poor oral hygiene leads to rapid periodontal breakdown. Moreover, there are systemic links to periodontitis as well. The degraded body defense mechanism, such as in case of diabetes mellitus, immune deficiency syndromes etc. have high risk of having periodontal diseases. In addition to that, genes, stress and occlusal factors should also be taken into consideration.

Periodontal microorganisms thrive in the oral cavity, specifically in dental plaque, and play a pivotal role in bone and soft tissue breakdown mediated through endotoxins, acid and alkaline phosphatases, lipases etc. While bacterial factors are the primary cause of periodontitis it is important to acknowledge the other contributory elements like genetics, immune system, dietary habits, oral hygiene practices, host factors and deleterious habits like smoking, chewing tobacco, etc.[15-17]

In our study, we have taken the extracts of Vajradanti, Danti, Ghotape and Tejpatta to see the effectiveness against potent periodontal bacteria *P. gingivalis* and *A. actinomycetemcomitans*. Nepal, a country known for its diverse flora and indigenous knowledge of medicinal plants, there is a wealth of botanical resources spread across different ecological zones.[18,19] There is high potential that these huge resources can be used for naturopathy. Most of the herbal extracts contain secondary metabolites like tannins, terpenoids, alkaloids, and flavonoids, which have demonstrated antimicrobial properties in laboratory studies.[20,21]

Scientific studies have revealed that the leaves, stem, and roots of Baleria prionitis possess antibacterial and anti-inflammatory properties. Although number of applications and researches have been done on *B. prionitis*, the unique feature of this plant available in Nepal is its availability in high altitude and extremophilic weather condition. It is our belief that this species found in Nepal has more medicinal properties compared to the species found in other part of the world. Apart from this, we have chosen other three plants used for toothache traditionally. *Cinnamomum tamala*, commonly known as Indian bay leaf or Tejpat, is a versatile and aromatic spice that holds a significant place in the culinary and medicinal traditions of the Indian subcontinent. This evergreen tree, native to the Indian subcontinent, is characterized by its glossy, elliptical leaves, which exude a warm and spicy fragrance when crushed. Beyond its culinary significance, C tamala also possesses various therapeutic properties, making it an essential ingredient in traditional Ayurvedic and herbal medicine systems.[22] Another plant selected in our study is Danti, scientifically known as Baliospermum montanum, is a versatile herb with a rich history in traditional healing, especially in Ayurveda. Extracting beneficial compounds from Danti involves methods such as cold pressing for oil, maceration for tinctures, and steam distillation for essential oils. It shows various therapeutic properties as it possesses alkaloids, flavonoids nitrile, amides, and alkyl halides.[23,24] Ghotape, scientifically known as Centella asiatica, is a remarkable herb deeply rooted in the annals of Ayurveda, India's ancient system of natural medicine. This versatile plant, native to various parts of Asia, including India, has a rich history of traditional use. It's adaptogenic and healing properties have earned it the moniker "herb of longevity." It is believed to have potential to enhance cognitive function, facilitate wound healing, alleviate anxiety, and improve skin health. In recent years, C. asiatica has garnered attention in dentistry for its antimicrobial properties, showing promise in combatting dental caries and periodontitis. This fact has been proved in our study. A similar study done by our team has found potent activity against S mutans as well. The phytochemical analysis of the four indigenous medicinal

plant extracts, uncovered a diverse array of major compounds. alkenes, carbonyl compounds, and aromatic compounds were present in all extracts. Specifically, C. tamala exhibited alcohol and alkyl halides, while *B. prionitis* contained aldehyde and carboxylic acid. *B. montanum* was found to possess nitrile, amides, and alkyl halides, and C. asiatica showed the presence of alkaloids, flavonoids, tannins, steroids, terpenoids, and cardiac glycosides. Similar types of results were also obtained by Hassan et al., Sharma et al., Johnson et al., and Arumugam et al.[25-28]

Current study showed the potential antibacterial capabilities of the four herbal extracts against *A. actinomycetemcomitans*, with GP1 (C. tamala) as the most effective, requiring lower concentrations (187.5 µg/ml for MIC and 750 µg/ml for MBC) to inhibit bacterial growth. The results of inhibition zone assays show GP3 (*B. montanum*) is one of the effective plants against *A. actinomycetemcomitans*. Similarly, *B. prionitis* and *B. montanum* are found to be most effective against *P. gingivalis* with highest diameter of zohe of inhibition among four herbal extracts. Despite these findings, the observed variability among the extracts necessitates exploration into influencing factors such as geographical origin and cultivation conditions.

The study done by Gupta et al. has found the *B. prionitis* mouth is as effective as chlorhexidine mouth wash.[29] Till today, 0.2% chlorhexidine is taken as gold standard chemical agent for plaque control. In a recent publication the contribution of *B. prionitis* has been acknowledged in an article called *B. prionitis* : a journey form Ayurveda to modern medicine by Pandey et al.[30]

In our study, *Barleria prionitis* exhibited significant antibacterial activity against periodontal pathogens which is likely attributed to its elevated levels of alkaloids, flavonoids, aldehyde, and carboxylic acid. A study done by Sawarkar et al. in 2016 showed that the ethanolic extract of *B. prionitis* was highly effective against different oral micro bacteria.[31]

A study done on cinnamon by Wang et al. has found that cinnamon oil is effective against *P. gingivalis* which is similar to our study. In our study, the MIC of cinnamon is very low as compared with other plant extracts. This fact has been well reviewed by Yanakiev recently.[32] The effect on different species of cinnamon on controlling periodontal disease has been well studied by Mosaddad et al. who have similar finding like of ours.[33]

In our study, *B. montanum* was found to exhibit good effectiveness to both the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis*. The flavonoids and terpenoids of *B. montanum* are the main components to have antimicrobial activity which is reviewed in an article by Radhakrishna et al.[34] It has also found to possess antibacterial and antifungal properties.[23]

This research also explored the chemical composition and antimicrobial potential of Tejpatta, Vajradanti, Danti, and Ghotape plants which are abundantly available in Nepal. While the study provides promising results, several considerations and limitations must be acknowledged. The variability among extracts necessities further investigation into influencing factors, including geographical variations and cultivation conditions. Current focus only on periodontal pathogens need to be evaluated in detail. Additionally, the study does not delve into potential side effects or cytotoxicity, emphasizing the importance of safety assessments before integrating these remedies into modern healthcare practices. The research opens avenues for the integration of indigenous medicinal plants into oral health practices. The antimicrobial potency demonstrated against *A. actinomycetemcomitans* and *P. gingivalis* suggests a potential role of natural products on controlling periodontists and gingivitis. However, the complex nature of oral health and the multifactorial causes of periodontitis necessitate comprehensive studies to establish the safety, efficacy, and broader applications of these plant extracts.

The research has been able to find out the antimicrobial properties of plant extracts however still there need to work a lot before bringing into clinical practice. The cytotoxicity test as well as clinical trials should be done before product formulation.

CONCLUSION

Tejpatta (*Cinnamomum tamala*) exhibited the highest effectiveness, requiring lower concentrations to hinder bacterial growth. In addition, Danti (Baliospermum montanum) and Vajradanti (*Barleria prionitis*) demonstrated higher inhibition zones across different concentrations, indicating its potency against A. Actinomecemcomitans and *P. gingivalis*. So it can be concluded that C tamala, B montanum and B prionitis can have the future application in controlling periodontitis. This study can be an excellent starting point in connecting indigenous Ayurveda into modern medicine.

ACKNOWLEDGEMENTS

We would like to acknowledge the help of Mr. Rupesh Kumar Jha for assisting plant collection and laboratory testing.

REFERENCES

1. Petersen PE, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C. The global burden of oral diseases and risks to oral health. *Bulletin of the world health organization*. 2005;83:661-9.
2. Trindade D, Carvalho R, Machado V, Chambrone L, Mendes JJ, Botelho J. Prevalence of periodontitis in dentate people between 2011 and 2020: A systematic review and meta-analysis of epidemiological studies. *J Clin Periodontol.* 2023 May;50(5):604-626. doi: 10.1111/jcpe.13769. Epub 2023 Jan 20. PMID: 36631982.
3. Rajkarnikar J, Acharya J. Prevalence and severity of periodontal diseases among Nepalese adults-a hospital based study. *J Coll Med Sci Nepal.* 2014;10(1):11-6.
4. Goel K, Sharma S, Baral DD, Agrawal SK. Current status of periodontitis and its association with tobacco use amongst adult population of Sunsari district, in Nepal. *BMC Oral Health*. 2021;21(1):66.
5. os T, Abajobir AA, Abate KH, Abbafati C, Abbas KM, Abd-Allah F, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet.* 2017;390(10100):1211-59.
6. Frencken JE, Sharma P, Stenhouse L, Green D, Laverty D, Dietrich T. Global epidemiology of dental caries and severe periodontitis - a comprehensive review. *J Clin Periodontol.* 2017 Mar;44 Suppl 18:S94-S105. doi: 10.1111/jcpe.12677. PMID: 28266116.
7. Moore WE, Moore LV. The bacteria of periodontal diseases. *Periodontol 2000*. 1994 Jun;5:66-77. doi: 10.1111/j.1600-0757.1994.tb00019.x. PMID: 9673163.
8. Nusrat F, Rahman MT, Karim MM. Bacterial endotoxins in periodontal health and diseases. *Biocell.* 2023;47(1):81-9.
9. Genco R. Consensus report. Periodontal diseases: pathogenesis and microbial factors. *Ann Periodontol.* 1996;1(1):926-32.
10. Claesson R, Johansson A, Belibasakis GN. Age-Related Subgingival Colonization of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Parvimonas micra-A Pragmatic Microbiological Retrospective Report. *Microorganisms*. 2023 May 29;11(6):1434. doi: 10.3390/microorganisms11061434. PMID: 37374936; PMCID: PMC10301537.
11. Kapoor A, Malhotra R, Grover V, Grover D. Systemic antibiotic therapy in periodontics. *Dent Res J (Isfahan).* 2012;9(5):505-15.
12. Chenicheri S, R U, Ramachandran R, Thomas V, Wood A. Insight into Oral Biofilm: Primary, Secondary and Residual Caries and Phyto-Challenged Solutions. *Open Dent J*. 2017;11:312-33.
13. Budală DG, Luchian I, Tatarciuc M, Butnaru O, Armencia AO, Virvescu DI, et al. Are Local Drug Delivery Systems a Challenge in Clinical Periodontology? *J Clin Med*. 2023;12(12).
14. Neu HC, Gootz TD. Antimicrobial chemotherapy. Medical Microbiology 4th ed. 1996.
15. Tungare S, Paranjpe AG. Diet and nutrition to prevent dental problems. 2018.
16. Werneck RI, Mira MT, Trevilatto PC. A critical review: an overview of genetic influence on dental caries. *Oral Dis.* 2010 Oct;16(7):613-23. doi: 10.1111/j.1601-0825.2010.01675.x. PMID: 20846151.
17. Cogulu D, Saglam C. Genetic aspects of dental caries. *Front Dent Med.* 2022;3:1060177.
18. Kunwar RM, Bussmann RW. Ethnobotany in the Nepal Himalaya. *J Ethnobiol Ethnomed*. 2008 Dec 2;4:24. doi: 10.1186/1746-4269-4-24. PMID: 19055723; PMCID: PMC2639547.
19. Singh AG, Kumar A, Tewari DD. An ethnobotanical survey of medicinal plants used in Terai forest of western Nepal. *J Ethnobiol Ethnomed*. 2012 May 16;8:19. doi: 10.1186/1746-4269-8-19. PMID: 22591592; PMCID: PMC3473258.
20. Cowan MM. Plant products as antimicrobial agents. *Clin Microbiol Rev*. 1999 Oct;12(4):564-82. doi: 10.1128/CMR.12.4.564. PMID: 10515903; PMCID: PMC88925.
21. Lewis K, Ausubel FM. Prospects for plant-derived antibacterials. *Nat Biotechnol.* 2006 Dec;24(12):1504-7. doi: 10.1038/nbt1206-1504. PMID: 17160050.
22. Thakur S, Chaudhary G. Review based upon ayurvedic and traditional uses of Cinnamomum tamala (Tejpatta). *Int J Pharm Sci Rev Res*. 2021;68:71-8.
23. Sewwandi SL, Peiris RM. A Review on Pharmacological activities and Medicinal properties of Baliospermum montanum. GSC Biol Pharm Sci. 2023;24(2):215-9.
24. Rout SP, Acharya R, Dash S. A Critical Review On Danti (Baliospermum Montanum Willd.) With Special Reference To Ayurvedic Nighantus (Lexicons). *Eur J Biomed*. 2017;4(4):348-53.
25. Hassan W, Kazmi SZ, Noreen H, Riaz A, Zaman B. Antimicrobial activity of Cinnamomum tamala leaves. *J Nutr Disord Ther.* 2016;6(2):2161-0509.1000190.
26. Sharma P, Sharma GN, Shrivastava B, Jadhav HR. Evaluation of antioxidant potential of *Barleria prionitis* leaf and stem. *AJPCT.* 2014;2(10):1177-86.
27. Johnson M, Wesely E, Hussain MZ, Selvan N. In vivo and in vitro phytochemical and antibacterial efficacy of Baliospermum montanum (Wïlld.) *Muell Arg Muell Arg Asian Pac J Trop Med.* 2010;3(11):894-7.
28. Arumugam T, Ayyanar M, Pillai YJK, Sekar T. Phytochemical screening and antibacterial activity of leaf and callus extracts of Centella asiatica. *Bangladesh J Pharmacol.* 2011;6(1):55-60.
29. Gupta D, Gupta RK, Jain A, Bindhumadhav S, Garg P, Chaturvedi S, et al. Assessment of Effectiveness of *Barleria prionitis* on Oral Health. *Pharmacognosy Res.* 2016 Jul-Sep;8(3):169-72. doi: 10.4103/0974-8490.181456. PMID: 27365983; PMCID: PMC4908843.
30. Pandey K, Gupta H, Kamble B. *Barleria prionitis*: Journey from Ayurveda to modern medicine. *Nat Prod J.* 2018;8(2):109-30.
31. Sawarkar HA, Kashyap PP, Pandey AK, Singh MK, Kaur CD. Antimicrobial and cytotoxic activities of *Barleria prionitis* and Barleria grandiflora: A comparative study. *Bangladesh J Pharmacol*. 2016;11(4):802-9.
32. Yanakiev S. Effects of Cinnamon (Cinnamomum spp.) in Dentistry: A Review. *Molecules*. 2020 Sep 12;25(18):4184. doi: 10.3390/molecules25184184. PMID: 32932678; PMCID: PMC7571082.
33. Mosaddad SA, Hussain A, Tebyaniyan H. Green Alternatives as Antimicrobial Agents in Mitigating Periodontal Diseases: A Narrative Review. *Microorganisms.* 2023 May 11;11(5):1269. doi: 10.3390/microorganisms11051269. PMID: 37317243; PMCID: PMC10220622.
34. Radhakrishna S, Kumari S. GCMS Analysis of total terpenoids from Baliospermum montanum and its antimicrobial activity. *J Adv Res Appl Sci.* 2018;5(3):94-101.