Evaluation of Achillea Millefolium as MMP Inhibitor in Human Dentin - an in Vitro Study

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**Abstract:** Matrix metalloproteinases (MMPs), especially MMP-9, are important enzymes that contribute to the breakdown of the collagen matrix in dentin and in turn reduces the durability of restorations. Inhibiting collagen-degrading enzyme can protect collagen fibers within the adhesive layer, thus prolonging stability of the resin dentin bonds. Although they work well, synthetic MMP inhibitors like doxycycline can have unfavorable side effects. *Achillea millefolium*, medicinal herb and also known as yarrow can be considered as potential substitute with anti-inflammatory and antioxidant properties. The purpose of this study was to assess *Achillea millefolium* extract's MMP-inhibitory action on human dentin in vitro, providing a biocompatible means of maintaining the dentin matrix.Dried *Achillea millefolium* leaves were used to prepare an ethanol extract. 15 freshly extracted teeth were collected and stored at 4 ℃. Human dentin samples were demineralized with 10% phosphoric acid and treated with various concentrations (100 µg, 200 µg) of the extract. The enzymatic activity was measured using ELISA, with absorbance values recorded and statistical analysis conducted via ANOVA and post hoc tests.When compared to the negative control group, MMP-9 activity was considerably decreased by both(100 µg, 200 µg) concentrations of *Achillea millefolium* extract. The 200 µg concentration showed a marginally better inhibitory effect than the 100 µg dosageThis study supports *Achillea millefolium* potential use in dental treatments, showing significant reductions in MMP-9 activity

**Keywords:** *Achillea millefolium*, collagen degradation, matrix metalloproteinases,

# Introduction

Commonly referred to as yarrow, Achillea millefolium is well known for its anti-inflammatory, antibacterial, and antioxidant qualities. Its potential as a therapeutic agent has generated interest in dentistry, especially in the treatment of dentin deterioration and dental cavities.[(Goyal & Chauhan, 2020; Puzari et al., 2022)](https://paperpile.com/c/CQh6QV/LPFaX+Vqh16) [(P. Kumar et al., n.d.)](https://paperpile.com/c/CQh6QV/LPnmv)In order to shed light on Achillea millefolium's potential to stop collagen deterioration and enhance dentin preservation in restorative dentistry, this study aims to assess the plant's effectiveness as an MMP inhibitor in human dentin. The medicinal plant Achillea millefolium, has long been utilized for its many therapeutic benefits, such as its anti-inflammatory and wound-healing capabilities.[(P. Kumar et al., n.d.)](https://paperpile.com/c/CQh6QV/LPnmv)These medicinal benefits are facilitated by its bioactive constituents, which include terpenoids, alkaloids, and flavonoids[(Ajay et al., 2023; Chokkattu et al., 2023; Padarthi et al., 2023)](https://paperpile.com/c/CQh6QV/Xa5BK+jSFJx+fHdDE). The possible uses of these substances in dentistry, specifically in the preservation of dental tissues like dentin, have recently been studied by researchers[(Dharman et al., 2023; S. Sindhu et al., 2023; Sreenivasagan et al., 2023)](https://paperpile.com/c/CQh6QV/zHsW7+WuxHB+4ENZd). The deterioration of dentin, the hard tissue beneath enamel, during and after dental operations is one of the main problems in restorative dentistry[(Ramakrishnan et al., 2023; Shenoy & Maiti, 2023; J. S. Sindhu et al., 2023)](https://paperpile.com/c/CQh6QV/P0qP0+QCxVb+AY89B). The organic matrix that makes up dentin is abundant in collagen and is essential to preserving the tooth's structural integrity[(Kasabwala et al., 2021; Rajeshkumar & Lakshmi, 2021; Varghese et al., 2023)](https://paperpile.com/c/CQh6QV/o5tTq+ipqIJ+eJcMY). Dentin may be vulnerable to destruction when exposed during processes such as cavity preparation, mostly because of matrix metalloproteinases (MMP’s) activity.[(de Moraes et al., 2020)](https://paperpile.com/c/CQh6QV/GWimS)Although these enzymes are latent in the dentin matrix, they get activated during restorative operations by the use of adhesive systems, acids, or mechanical preparation [(Keerthana & Ramesh, 2021; Murugesan, 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/CQh6QV/QPCi+UUDV+mFLX). Over time, the dentin-resin bond in restorations deteriorates because MMPs, specifically MMP-2, MMP-8, and MMP-9, tear down the collagen fibers in dentin [(Keerthana & Ramesh, 2021; Murugesan, 2021; Subramanian et al., 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/CQh6QV/QPCi+UUDV+mFLX+8ELa). The connection is weakened by this enzymatic breakdown, which jeopardizes the long-term viability of restorative procedures like crowns and restorations.[(Napoli et al., 2020)](https://paperpile.com/c/CQh6QV/BfTc)To reduce this degradation, synthetic MMP inhibitors like chlorhexidine have been employed.[(Breschi et al., 2010)](https://paperpile.com/c/CQh6QV/txfFh) [(Tjäderhane, 2015)](https://paperpile.com/c/CQh6QV/dEZKQ) However, worries about the possible negative effects of synthetic inhibitors and the need for less toxic, biocompatible alternatives have led to an increase in interest in natural alternatives [(Pranati et al., 2021; Sakthi & et al, 2021)](https://paperpile.com/c/CQh6QV/rrABG+LgB3G). Because Achillea millefolium inhibits MMP activity, it has become a prospective contender and is being studied in the dental area. The capacity of Achillea millefolium extracts to inhibit MMPs in human dentin has been assessed in vitro. This in vitro study aims to evaluate the effectiveness of Achillea millefolium extract as an MMP inhibitor in human dentin. By assessing the inhibitory effects of this natural extract on MMP activity, we can explore its potential application in dental care to prevent the degradation of the dentin matrix and improve the outcomes of dental treatments. The results of this study could provide valuable insights into the development of novel, plant-based therapeutic agents for the management of dental caries and the preservation of dentin integrity.

# Materials and methods

## Preparation of *Achillea millefolium* Extract

Fresh leaves from mature yarrow plants were collected to ensure the highest concentration of bioactive compounds. They were washed thoroughly to remove any dirt or contaminants with sterile water, and then air-dried in a shaded area to preserve the integrity of the phytochemicals. The dried leaves were then finely ground into a powder using a mechanical grinder. This powder was subjected to ethanol extraction, a process that effectively isolates the active components, particularly flavonoids and tannins, known for their potential MMP inhibitory properties. The ethanol extraction was carried out by mixing the powdered leaves with ethanol in a specific ratio, followed by continuous stirring for 48 hours at room temperature to maximize the extraction efficiency. The mixture was then filtered to remove any solid residues, and the filtrate was concentrated using a rotary evaporator at reduced pressure to remove the ethanol, yielding a concentrated guava extract. This concentrated extract was stored at -20°C to preserve its bioactivity until further use in the experiments.

## Collection and Preparation of Human Dentin Samples

Human dentin samples were meticulously prepared to provide a reliable substrate for the in vitro analysis.15 freshly extracted premolars for orthodontic purpose were collected and stored at 4℃ until use. The informed consent was taken from the patients and with ethical approval, ensuring compliance with ethical standards in research. Once collected, the teeth were cleaned to remove any remaining soft tissue and then sectioned using a diamond saw. The dentin was separated from the enamel and pulp, and then it was ground into a fine powder using a mechanical mill. This process ensured uniformity in the dentin samples, providing a consistent surface area for the subsequent experimental treatments.

## Demineralization of Dentin Powder

The powdered dentin was subjected to a demineralization process to expose the collagen matrix, which is the substrate for MMP activity. This was achieved by mixing the dentin powder with 10% phosphoric acid, a common demineralizing agent in dental research, which effectively dissolves the mineral components of dentin, primarily hydroxyapatite. The demineralization process was carefully monitored to ensure complete removal of the mineral content while preserving the organic matrix.After demineralization, the acidic environment was neutralized by adding 1 M sodium hydroxide (NaOH) to the mixture. This step was critical to restore the pH to neutral (approximately 7.0), creating an environment similar to physiological conditions, which is necessary for accurate assessment of MMP activity. The neutralized dentin powder was then thoroughly rinsed with distilled water to remove any residual acid or base and dried under sterile conditions.

## Experimental Group Design

The study was designed to evaluate the inhibitory effects of *Achillea millefolium* extract on MMP activity in human dentin, with four distinct groups established for comparison. The first group, serving as the positive control, was treated with chlorhexidine, a well-known synthetic MMP inhibitor commonly used in dental research to validate the experimental model. The other two groups, designated as test groups, were treated with different concentrations of *Achillea millefolium* extract-100 µg, 200 µg, respectively. These concentrations were selected based on preliminary studies that indicated their potential effectiveness in inhibiting MMP activity. The fourth group served as a negative control, receiving no treatment to establish the baseline MMP activity in the absence of any inhibitors. Each group of dentin powders was incubated in phosphate-buffered saline (PBS) at 37°C for 24 hours, a temperature that simulates the human body's conditions. This incubation period allowed the extract or control solutions to interact with the exposed collagen matrix, facilitating the evaluation of their effects on MMP activity.

## Measurement of MMP Activity

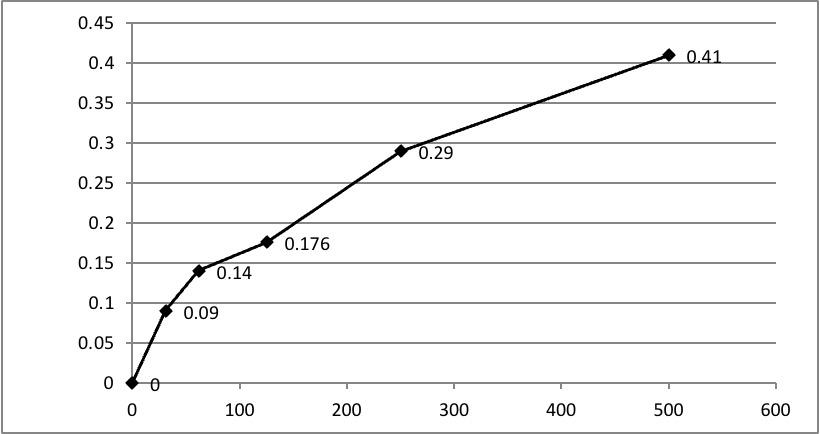
To quantitatively assess MMP-9 activity in the treated dentin samples, commercially available enzyme-linked immunosorbent assay (ELISA) kits specific to MMP-9 were utilized. ELISA is a sensitive and specific method for detecting and quantifying proteins, making it ideal for measuring MMP activity in this context. After the 24-hour incubation, the supernatants from each sample group were collected and subjected to the ELISA procedure. The assay involved the binding of MMP-9 present in the samples to specific antibodies coated on the microplate wells, followed by a series of reactions that produce a color change proportional to the amount of MMP-9. The intensity of the color was measured as absorbance using a microplate reader at a specific wavelength. The absorbance values were recorded, providing a quantitative measure of MMP-9 activity in each sample group.

## Data Analysis:

Statistical Analysis: The data obtained from the ELISA assay were analyzed for mean and standard deviation (SD) values. The statistical analysis aimed to compare the MMP-9 activity between the different study groups. One-Way ANOVA: A one-way analysis of variance (ANOVA) was used to determine whether there were statistically significant differences between the groups. This test helps compare multiple groups to see if the mean MMP activity differs significantly between the treatments. Significance Level: The significance level was set at p < 0.05. If the p-value obtained from the ANOVA analysis is below this threshold, it indicates that there is a statistically significant difference between the group suggesting that the treatment (Achillea millefolium extract or doxycycline) had measurable effect on MMP inhibition compared to the control group.This methodology ensures a controlled and precise evaluation of the potential MMP-inhibitory properties of Achillea millefolium in human dentin, offering valuable insights into its potential use in restorative dentistry to preserve the dentin matrix.

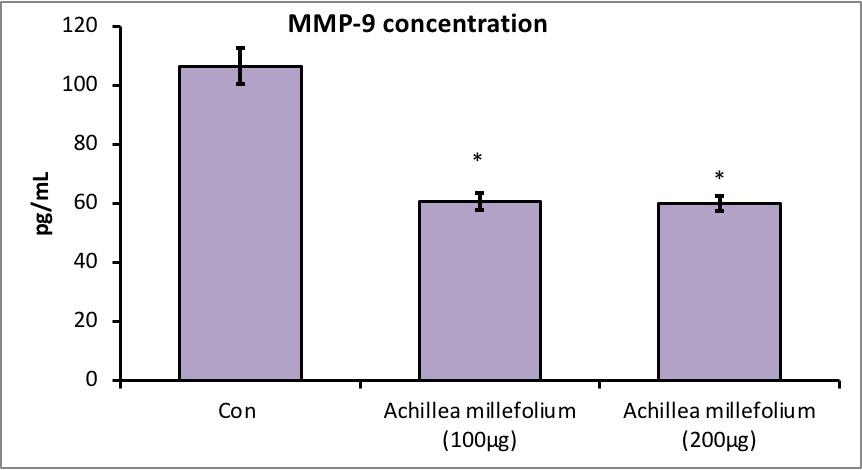
# Results

The dose-dependent inhibition of MMP-9 was evident in this study, highlighting that higher concentrations of *Achillea millefolium* result in more significant inhibition of MMP-9 activity in the dentin samples. Standard curve for MMP-9 activity or concentration was established (Figure 1). The key purpose of such a standard curve is to allow for the determination of MMP-9 concentrations in unknown samples by comparing their measured responses to this curve. The x-axis represents the known concentrations of MMP-9 which were used to generate the standard curve. These concentrations are plotted to establish a relationship between MMP-9 levels and absorbance values. The y-axis represents the absorbance measured at a specific wavelength for each corresponding MMP-9 concentration. The absorbance reflects the enzymatic activity, which is used to quantify MMP-9 concentrations in unknown samples by comparison with the curve.



**Figure 1:** The graph is used to establish a standard curve for MMP-9 activity or concentration. The key purpose of such a standard curve is to allow for the determination of MMP-9 concentrations in unknown samples by comparing their measured responses to this curve.

In the control group, which received no inhibitor, the MMP-9 concentration was measured at 106.5 pg/mL, representing the natural and unregulated activity of the enzyme. However, when treated with 100 µg of *Achillea millefolium* extract, the concentration of MMP-9 significantly dropped and demonstrated a notable 45.8% inhibition of enzyme activity. This indicates that the extract effectively slows down the breakdown process.Interestingly, when the dosage was increased to 200 µg, the MMP-9 concentration only slightly decreased further, resulting in a 46.6% inhibition. The similar levels of inhibition at both doses suggest that the extract is highly effective even at lower concentrations, but increasing the dose does not lead to a proportionally greater reduction in enzyme activity.



**Figure 2:** The x-axis represents the different concentrations of *Achillea millefolium* including the control group. The y-axis represents the concentration of MMP-9 (in pg/ mL), indicating the enzymatic activity level in each group. The values reflect the effectiveness of *Achillea millefolium* as an inhibitor of MMP-9 activity compared to the control.

Achillea millefolium demonstrates a dose-dependent inhibitory effect on MMP-9 concentrations, with higher dosages correlating with progressively lower MMP-9 levels (Table 1). The standard error of the mean (SEM) values provides a quantitative assessment of the precision of the mean estimates, with relatively low SEMs signifying robust statistical reliability and confidence in the reported data.

**Table 1:** The table provides a summary of the mean MMP-9 concentrations and their corresponding standard error of the mean (S.E.M) for the control group and the two test groups treated with *Achillea millefolium* extract at concentrations of 100 µg and 200 µg.

|  | **Concentration** | ***Achillea millefolium* (100µg)** | ***Achillea millefolium* (200µg)** |
| --- | --- | --- | --- |
| Mean | 106.5 | 60.7 | 59.9 |
| S.E.M | 6 | 3 | 2.5 |

# Discussion

The study aimed to evaluate the potential of *Achillea millefolium* extract as a matrix metalloproteinase (MMP) inhibitor in human dentin, offering a natural alternative to synthetic MMP inhibitors. The results demonstrate that *Achillea millefolium* has significant MMP-inhibitory properties, with both tested concentrations (100 µg and 200 µg) showing a reduction in MMP-9 activity when compared to the negative control. This finding is consistent with previous research on the plant's bioactive compounds, such as flavonoids and phenolic acids, which are known to exhibit strong antioxidant and enzyme-inhibitory effects. The higher concentration (200 µg) showed a slightly stronger inhibitory effect than the lower concentration (100 µg), indicating a dose-dependent responseCurrently, chlorhexidine is the most explored MMPs inhibitor. It functions as a non-specific inhibitor and alters their three-dimensional structure and chelate the metal ions (Ca²⁺, Zn²⁺) required for activating their enzymatic activity. In a study, Breschi et al observed that chlorhexidine preserves the stability of the resin-dentin bond for only up to 6 months but after1 year it starts degrading. [(Breschi et al., 2010)](https://paperpile.com/c/CQh6QV/txfFh) Tjäderhane et al explained that chlorhexidine is water soluble and has a large molecule which may leach out of the hybrid layer.[(Tjäderhane, 2015)](https://paperpile.com/c/CQh6QV/dEZKQ) Recent in vitro studies have also shown it’s cytotoxicity effect in deciduous teeth.[(Tu et al., 2015)](https://paperpile.com/c/CQh6QV/Xktza) Benzalkonium chloride is another popular synthetic MMP inhibitor. But it also inhibits enzyme activity immediately and over extended periods but eventually it showed increased gelatinolytic activity and decline in bond strength.[(Sabatini & Pashley, 2015)](https://paperpile.com/c/CQh6QV/VOe1y) Barcellos et al used Zinc salts as MMP inhibitor and observed 6 month lasting bond strength.[(Barcellos et al., 2016)](https://paperpile.com/c/CQh6QV/rOM6l) In another study Almeida et al observed high solubility of zinc salts which may undergo high solubility in oral cavity over a period of time.[(Almeida et al., 2017)](https://paperpile.com/c/CQh6QV/QcARa) Additionally, synthetic inhibitors are effective as an MMP inhibitor, but their drawbacks, such as cytotoxicity and interference with resin bonding, necessitate alternative solutions.Doxycycline, a well-established synthetic MMP inhibitor, served as the positive control in this study. In this study, the doxycycline-treated group exhibited the lowest levels of MMP-9 activity, confirming its efficacy in inhibiting MMPs in dentin. While *Achillea millefolium* did not completely match the potency of doxycycline, it nonetheless provided a substantial level of inhibition, especially at the higher concentration. This comparison highlights the potential of *Achillea millefolium* as a natural alternative or adjunct to synthetic MMP inhibitors in dental applications, offering a less toxic and more biocompatible option. Other plant-based extracts have been explored for their MMP-inhibitory effects in dentistry. [(G. B. Kumar et al., 2019)](https://paperpile.com/c/CQh6QV/wnWd3)Green tea polyphenols (particularly epigallocatechin gallate, or EGG) have shown promising results as natural MMP inhibitors.[(Carvalho et al., 2016)](https://paperpile.com/c/CQh6QV/tK5DT) Both plant extracts function through antioxidant mechanisms, which reduce the oxidative activation of MMPs, and direct inhibition of the enzyme activity. However, while green tea extract is well-established and widely studied in this context, *Achillea millefolium* is relatively new to dental research. In addition to green tea, propolis extract, a resinous substance produced by bees, has been studied for its ability to inhibit MMPs. Propolis has shown promise due to its rich composition of flavonoids and phenolic compounds, which are also present in *Achillea millefolium.* Oliveira et al, investigated the effect of Brazilian green propolis and found that it significantly reduced MMP activity, especially MMP-2 and MMP-9[(de Oliveira et al., 2023; Przybyłek & Karpiński, 2019; Zulhendri et al., 2022)](https://paperpile.com/c/CQh6QV/HTZxN+afzLt+pEhp4) suggesting that its effects are comparable to those of *Achillea millefolium.*One of the primary advantages of using natural plant extracts like *Achillea millefolium* over synthetic MMP inhibitors is the reduced risk of toxicity and adverse side effects. Synthetic inhibitors like chlorhexidine and doxycycline, while effective, have been associated with cytotoxic effects on dental pulp cells and other tissues when used over extended periods.[(Tu et al., 2015)](https://paperpile.com/c/CQh6QV/Xktza) In contrast, *Achillea millefolium* has a long history of safe use in herbal medicine, suggesting it may be a safer alternative for long-term use in dental treatments. The fact that plant-based inhibitors are more biocompatible makes them particularly attractive for applications in restorative dentistry. The promising results of this in vitro study indicate that *Achillea millefolium* could be integrated into dental materials or used as an adjunctive treatment in cavity preparations to protect the dentin matrix. For example, incorporating this extract into adhesive systems could enhance the longevity of resin-based restorations by preventing MMP-mediated degradation of the dentin-resin bond. Similarly, it could be used as a rinse or treatment following cavity preparation to stabilize the exposed collagen network before placing a restoration. Further research should focus on the formulation of such products and their clinical efficacy in real-world settings. While the study offers important insights, it also has limitations. Being an in vitro study, the results may not fully replicate the complex environment of the oral cavity, where factors such as saliva, oral microbiota, and mechanical forces could influence the efficacy of *Achillea millefolium* as an MMP inhibitor. Future in vivo studies are needed to confirm these findings and evaluate the extract's long-term effects on dental restorations. Additionally, exploring the mechanism of action in more detail, such as whether the extract directly inhibits MMP catalytic activity or works through other pathways, could provide valuable information for optimising its use. Moreover, this study focused on MMP-9, but other MMPs like MMP-2 and MMP-8 also play a role in dentin degradation. Investigating the effect of *Achillea millefolium* on a broader spectrum of MMPs could provide a more comprehensive understanding of its potential benefits in dentistry.

# Conclusion

This study demonstrated a dose-dependent inhibition of MMP activity by *Achillea millefolium* extract, with results comparable to those of known synthetic MMP inhibitors such as doxycycline. This positions *Achillea millefolium* as a promising natural alternative for dental treatments, potentially offering benefits such as lower toxicity and better biocompatibility.

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