RESEARCH PAPER

Anti-cariogenic potential and comprehensive phytochemical characterization of Achillea millefolium – An In vitro study

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Abstract

Background

Achillea millefolium (*A. millefolium*) is a perennial herb and has gained recognition for its therapeutic properties. It has properties of tissue regeneration, collagen synthesis, and anti-inflammatory effects. This study explores the phytochemical screening, antioxidant activity and anti cariogenic properties of *A. millefolium*, aiming to expand its applications in dentistry.

Materials and Methods

The study utilized methanolic extracts of shade-dried and coarsely ground *A. millefolium* leaves, prepared at room temperature. Phytochemical screening was conducted to identify key bioactive compounds. Antioxidant activity was evaluated using the DPPH radical scavenging assay. Anti cariogenic activity was assessed through the agar gel diffusion method, while biocompatibility was tested using the MTT assay.

Results

Phytochemical analysis of *A. millefolium* revealed the presence of flavonoids, phenolic acids, essential oils, alkaloids, tannins, terpenoids, coumarins, glycosides, and saponins. The extract demonstrated potent antioxidant, anti-inflammatory, anticariogenic and antibiofilm activities. Biocompatibility was confirmed through MTT assay and phase-contrast microscopy. Conclusion

A.millefolium demonstrates significant potential as a therapeutic agent in dental applications. Its antioxidant, anti-inflammatory, anticariogenic and antibiofilm properties underline its efficacy.

Keywords: Achillea, Antioxidants, Dentin, Phytochemicals, Matrix Metalloproteinases

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Introduction

Dental caries remains a prevalent and enduring global health concern, affecting individuals of all age groups and presenting significant challenges to oral health worldwide. [1] Despite considerable advancements in preventive dentistry and the widespread use of fluoride-based treatments, the incidence of dental caries continues to be alarmingly high. This has prompted a growing interest in the development of alternative, natural therapies that are both effective and biocompatible. *Achillea millefolium* (*A. millefolium*), commonly known as yarrow, is a perennial herb belonging to the Asteraceae family, renowned for its extensive use in traditional medicine across various cultures. It is valued for its anti-inflammatory, antimicrobial, and wound-healing properties. [2] *A. millefolium* exhibits broad-spectrum antimicrobial activity, with extracts capable of inhibiting the growth of bacteria and fungi, including antibiotic-resistant strains. [3-4] These properties suggest its potential as an effective agent against cariogenic bacteria responsible for dental caries. Conventional antibacterial agents such as chlorhexidine, fluorides and phenol derivatives are widely used in dentistry for preventing dental plaque biofilm formation and combating cariogenic bacteria. However, these agents often come with undesirable side effects, including enamel staining. [5, 6]

Numerous studies have highlighted the biological activities of plant secondary metabolites, the potential contributions of primary metabolites to such effects remain largely unexplored.[7, 8]One of the major challenges in phytochemical research lies in the identification and quantification of bioactive compounds, especially when present in low concentrations. Moreover, the pharmacological activity observed in medicinal plants may result from complex synergistic or antagonistic interactions among multiple constituents. This study brings a novel approach to the field by evaluating the entire metaboloic pool of *Achillea millefolium*, offering a more holistic understanding of its anti-cariogenic potential within complex biological systems. This study aims to evaluate the anti-cariogenic potential of *A. millefolium* through phytochemical screening and antimicrobial analysis, thereby exploring its application as a natural adjunct in oral health care. By elucidating its bioactive compounds and biological activities, the research aims to validate its traditional medicinal uses and establish its role as an innovative, plant-based therapeutic agent for oral health applications. This study emphasize on its potential to inhibit cariogenic bacteria addressing critical gaps in current dental care solutions.

Materials and Methods

Preparation of *A. millefolium* leaves extract

The leaves were washed with sterile water and shade-dried for 1 week and ground coarsely and extracted with methanol at room temperature. The extracts were combined and concentrated in vacuum at 30 °C. The residue was diluted with water and partitioned against petroleum ether, ethyl acetate and n-butanol and each were concentrated to dryness to obtain powder.

The 50 g of powdered *A. millefolium* was weighed. Soxhlet extraction using ethanol as a solvent was performed. The solvent was allowed to circulate for several cycles to thoroughly extract the compounds. The solution was filtered using filter paper, and the solvent was evaporated using a rotary evaporator to obtain a concentrated extract. The percentage yield of the extract was subsequently calculated using the formula below:

$$\text{Percentage yield} = \frac{\text{Final weight of the dried extract}}{\text{Initial weight of the powder}} \times 100$$

The extract was redissolved in a small amount of solvent to carry out different qualitative tests for phytochemicals.

1. Phytochemical Profiling of *A. millefolium*

Phytochemical screening of *A. millefolium* from its powdered form following the standard protocols [9] to identify the presence of different classes of compounds including alkaloids, flavonoids, tannins, phenolic acids, saponins, and terpenoids.(Figure 1)

2. DPPH radical scavenging activity - Antioxidant evaluation

The antioxidant activity of extract and Ascorbic acid was measured in terms of electron transfer/hydrogen donating ability, using the DPPH radical method modified by the extract at various concentrations of 20, 40, 60, 80, 100 µg/ml and added to 3.9 ml of DPPH radical solution. The decrease in absorbance at 515 nm was determined continuously at every 1 min with a UV-Visible Spectrophotometer.

3. Anti inflammatory activity of *A. millefolium*

The fraction was dried in a vacuum oven and redissolved in saline. Different concentrations of the fractions were made and added to 1.8 ml of 1 % Bovine Serum Albumin solution. The pH was adjusted to 6.5 using 1N HCl and the solution was incubated at 37 °C for 20 min and then heated to 57 °C for 10 min. After cooling, the absorbance was measured at 660 nm. Aspirin was used as the standard and a solution without sample was considered as the control.

4. Anticariogenic activity of *A. millefolium*

The agar well diffusion method was used to determine the antibacterial activity of *Streptococcus mutans*, and *Shigella sonnei*. Different concentrations of 25µg, 50µg, 75µg and 100µg were incorporated into the wells and the plates were incubated at 37°C for 24 hrs. The streptomycin was used as positive control. Zone of inhibition was recorded in each plate after 24 hours.

5. Anti biofilm activity of *A. millefolium*

The anti-biofilm activity of a test compound was evaluated against *Streptococcus mutans* and *Shigella sonnei* using a 96-well microtiter plate assay at concentrations of 25 µg, 50 µg, 75 µg, and 100 µg, with untreated biofilms serving as the control. Bacteria were initially cultured in vinyl U-bottom 96-well plates, where they were allowed to form biofilms over a 24-48 hour incubation period at optimal growth conditions, such as 37°C. After incubation, the planktonic (free-floating) bacterial cells were carefully washed away using a gentle rinsing buffer like phosphate-buffered saline (PBS), ensuring that only the biofilm adhered to the well surface. To visualize the biofilm, the wells were stained using crystal violet dye, which binds specifically to the biofilm's extracellular polymeric substance (EPS) matrix, providing a visual representation of biofilm density. Once stained, the excess dye was removed, and the wells were rinsed again to remove unbound crystal violet. For quantification, the stained biofilms were solubilized using ethanol or an equivalent solvent to dissolve the crystal violet. The solubilized biofilms were then transferred to a flat-bottom, optically clear 96-well plate to allow for accurate spectrophotometric measurement at a wavelength of 570 nm. This method enabled the quantification of biofilm mass based on absorbance readings. The results were compared between the treated groups at different concentrations (25 µg, 50 µg, 75 µg, and 100 µg) and the untreated control group.

Evaluation of Biocompatibility of *A. millefolium*

The biocompatibility of the material was evaluated through an MTT assay, a colorimetric method used to

measure cell metabolic activity, which serves as an indicator of cell viability and proliferation. Cell viability was assessed after a 24-hour incubation period, during which cells were exposed to the test material. The L929 mouse fibroblast cell line, commonly used in biocompatibility and cytotoxicity studies, was selected for this assay. These cells are known for their sensitivity to foreign materials, making them ideal for assessing the compatibility of the test material. L929 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with fetal bovine serum (FBS) and necessary growth factors to ensure optimal cell health. The cells were maintained at standard culture conditions (37°C, 5% CO2) to simulate physiological environments. After 24 hours of exposure to the test material, the MTT reagent was added to the culture wells. MTT is a tetrazolium salt that is reduced to formazan crystals by the mitochondrial enzymes of metabolically active cells. The formazan product was then solubilized, and absorbance readings were taken at a wavelength of 570 nm using a microplate reader. The absorbance values directly correlate with the metabolic activity of the cells, which reflects their overall viability. Higher absorbance indicates greater metabolic activity and cell health, suggesting that the material is non- cytotoxic, while lower absorbance would indicate potential cytotoxic effects.

Results

1. Phytochemical Profiling of *A. millefolium*

The qualitative phytochemical screening presented the significant notable positive results. It confirmed the presence of Coumarins, gylcosides, flavonoids, terpenoids, tannins, saponins, alkanoids, phenolic acids and essential oils [Figure 1(B)].

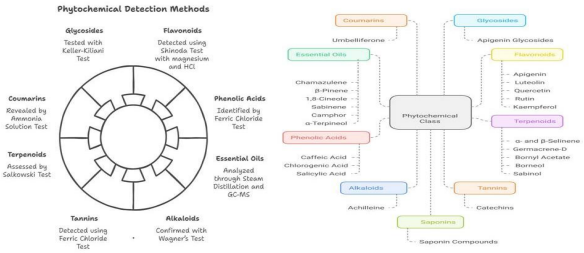

Figure 1: Qualitative analysis of Phytochemicals present in *Achillea millefolium*: (A) Experimental procedures used to detect bioactive compounds (B) Identified bioactive compounds

2. **DPPH radical scavenging activity - Antioxidant evaluation**

The DPPH assay demonstrated the ability of the test compound to scavenge free radicals, highlighting its antioxidant potential. The results revealed a clear dose-dependent increase in radical scavenging activity (RSA) with higher concentrations of the compound. At lower concentrations, the RSA was moderate, but it steadily increased as the concentration rose, ultimately achieving a substantial scavenging effect. (Figure 2) This linear trend indicates a strong correlation between the concentration of the test compound and its antioxidant activity, emphasizing its potency in neutralizing free radicals. Such results are characteristic of substances with significant antioxidant properties, where increased doses effectively enhance their radical-neutralizing capacity.

3. **Anti inflammatory activity of *A. millefolium***

The BSA Denaturation Assay evaluated the ability of the test substance to inhibit protein denaturation, a key event in inflammation. The results indicated a dose-dependent inhibition, with the effect becoming progressively stronger at higher concentrations of the sample.(Figure 2) At lower doses, the inhibition was moderate, but as the concentration increased, the test substance exhibited significantly enhanced anti-denaturation activity. This trend reflects the potential anti-inflammatory properties of the compound, as substances capable of effectively preventing protein denaturation are often associated with the suppression of inflammatory processes.

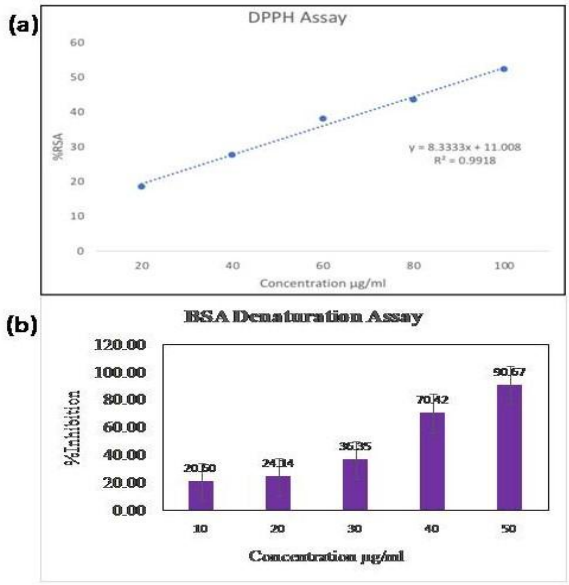

Figure 2: The figure 2(a) shows a linear trend indicates a strong correlation between the concentration of the test compound and its antioxidant activity.The figure 2(b) shows the concentration-dependent reduction in protein denaturation by *A. millefolium*, measured as BSA denaturation assay.

4. **Anticariogenic activity of *A. millefolium***

The *A. millefolium* extract demonstrated anti cariogenic activity against the selected bacteria. As shown in Figure 3, higher concentrations of *A. millefolium* extract (75 and 100 µg/mL) resulted in larger inhibition zones, indicating enhanced antibacterial activity.

The comparison between the effects of the yarrow extract on both types of bacteria is presented in the graph in Figure 3. At higher concentrations, the inhibition zones for *Streptococcus mutans* (Figure 3(a))

are slightly larger than those for *Shigella sonnei* (Figure 3(b)), suggesting that yarrow might be slightly more effective against *Streptococcus mutans* under these conditions.

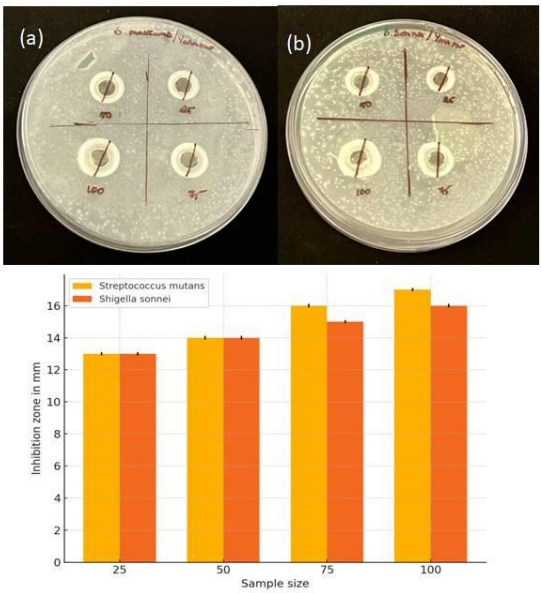

Figure 3. The figure illustrates the comparative antimicrobial efficacy demonstrated by inhibition zones formed around various antimicrobial agents against *Streptococcus mutans* and *Shigella sonnei* [Figure 3(a) and 3(b)] The inhibition zones, measured in millimeters (mm), are depicted as clear circular areas around the test agents on agar plates. The graph depicts each bar correspond the antimicrobial treatment, with standard deviations shown as error bars. Statistical significance between groups is denoted ($p < 0.05$).

5. Anti biofilm activity of *A. millefolium*

The bacterial biofilm formation under various conditions is depicted in Figure 4. The control group images illustrate biofilm formation in the absence of any treatment, characterized by thick, dense structures with intense staining, showcasing the bacteria's natural ability to form biofilms. With increasing concentrations of the test substance, a gradual, concentration-dependent reduction in biofilm formation is evident. The biofilms became less dense and lighter in staining; reflecting diminished bacterial adherence and extracellular matrix production. At the lowest concentration tested, there was a slight reduction in biofilm formation compared to the untreated group, although biofilms were still present. Progressive reductions in biofilm density and staining intensity were observed at intermediate concentrations. At the highest concentration, a marked reduction in biofilm formation was seen in most bacterial species, as evidenced by significantly lighter staining, indicating that the test compound effectively inhibited biofilm development.

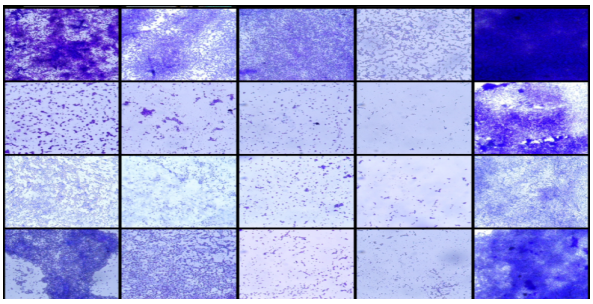

Figure 4: Anti biofilm activity of *A. millefolium* at different concentrations control, 25, 50, 75, 100 µg/ml

6. Evaluation of Biocompatibility of *A. millefolium*

The extract does not significantly reduce cell viability even at higher concentrations. The *A. millefolium* treated cells retain their normal morphology, similar to the control cells. The phase contrast microscopy images are presented in Figure 5 which collectively suggest that the *A. millefolium* extract is biocompatible with L929 fibroblast cells. The Graph illustrates the cell viability data of *A. millefolium* at different concentrations. This indicates that the plant extract is non-toxic to these cells and could be considered safe for further studies or applications related to fibroblast health and tissue repair.

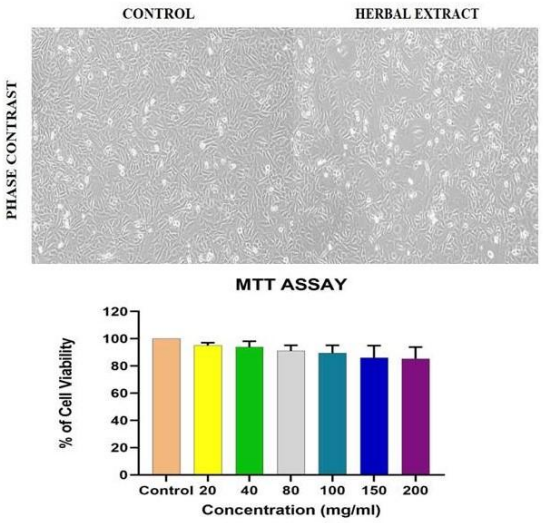

Figure 5: Phase contrast microscopy images shows biocompatibility with L929 fibroblast cells

Discussion

Advancements in the underlying science of caries initiation and lesion dynamics have laid the foundation for redefining our approach to the understanding and management of dental caries. While the treatment of dental diseases is costly across all countries, preventive measures remain simple, cost-effective, and highly efficient. The antimicrobial properties of plants are being widely studied around the globe, driven by the growing issue of multidrug resistance in conventional pharmaceuticals. Plant-derived compounds that can either suppress the growth of microbial pathogens or eliminate them, while remaining non-toxic to host

cells, are potential candidates for the development of new anti cariogenic agents.

In the present study, crude extracts of *A. millefolium* showed anticariogenic activity against *Streptococcus mutans*, and *Shigella sonnei* were tested. Consequently, these extracts were further evaluated for antioxidant, anti inflammatory, antibiofilm properties, biocompatibility and underwent preliminary phytochemical screening.

The phytochemical screening reveals a rich composition of bioactive compounds in *A. millefolium*. These include flavonoids, alkaloids, sesquiterpene lactones, tannins, and essential oils. The presence of these compounds is primarily responsible for the plant's medicinal properties. [9-11] Flavonoids such as apigenin and luteolin, and essential oils containing chamazulene and camphor, are especially recognized for their pharmacological effects. [12]

Reactive oxygen species (ROS) are primarily generated in the human body as byproducts of the mitochondrial electron transport chain. These molecules play critical roles in cellular processes. When ROS interact with lipids, proteins, or nucleic acids, they can induce oxidative stress, potentially leading to cellular damage and contributing to various pathophysiological conditions. Flavonoids, acting as exogenous antioxidants, play a key role in protecting cells from oxidative stress by interacting with reactive species. They are oxidized by free radicals to form more stable, less reactive compounds, thereby reducing the potential for cellular damage caused by oxidative reactions. [13] The antioxidant activity of *A. millefolium* is attributed to its high content of phenolic compounds and flavonoids. In a similar study the extracts of *A. millefolium* exhibit significant free radical scavenging activity, making it a potential natural source of antioxidants. [14] This study also highlights that the high content of phenolic compounds and flavonoids contributes to strong antioxidant effects, positioning it as a potential agent in managing oxidative stress-related conditions.

The anti-inflammatory effects can make it useful in the treatment of inflammatory conditions and diseases. In this study *A. millefolium* has demonstrated notable anti-inflammatory properties, might be largely due to its sesquiterpene lactones and flavonoids. These compounds inhibit the production of pro-inflammatory cytokines and enzymes such as COX-2, which play a key role in the inflammatory response.[15] Studies have also shown that *A. millefolium* extracts reduce the expression of inflammatory cytokines like IL-1β and TNF-α, which are implicated in various inflammatory diseases.[16]

The anticariogenic activity of *A. millefolium* is particularly important in the context of oral health. The antimicrobial properties can help in inhibiting the growth of cariogenic bacteria. In this study anticariogenic activity was observed against *Streptococcus mutans* and *Shigella sonnei*. Additionally, the presence of tannins contributes to the reduction of dental plaque formation and tooth decay, making it a valuable natural agent in dental care.[17] *A. millefolium* exhibits broad-spectrum antimicrobial activity against a variety of pathogens including bacteria, fungi, and viruses. The essential oils and phenolic compounds are primarily responsible for this activity.[18] Studies have shown that it is effective against common bacteria such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus,* and *Candida albicans.*[19] Studies have demonstrated that *A. millefolium*'s bioactive compounds, such as sesquiterpene lactones and flavonoids, effectively inhibit the growth of various bacteria, including *Staphylococcus aureus* and *Bacillus cereus*.[20] It also impairs biofilm formation, which is critical in preventing infections and promoting oral health. Research shows that *A. millefolium* extracts reduce the production of acid by oral bacteria, which are significant factors in the development of dental caries. [16]

Biocompatibility is an essential consideration when assessing the potential of *A. millefolium* for medicinal and therapeutic use. Studies show that *A. millefolium* compounds have low cytotoxicity and good biocompatibility with human cells. [21] The antibacterial insights from the in-silico docking study on Murraya koenigii against Porphyromonas gingivalis support the present findings on Achillea millefolium, suggesting that phytochemicals such as flavonoids and alkaloids may exhibit significant anti-cariogenic activity through targeted antimicrobial mechanisms and inhibition of pathogenic biofilm formation. [22]

This makes it a promising candidate for long-term therapeutic use. This study has confirmed that *A. millefolium* does not induce significant toxicity in human fibroblasts and epithelial cells, further supporting its safety profile. This study shows that the plant extracts are generally safe and non-toxic to human cells at therapeutic concentrations. This makes it a promising candidate for dental use. *A. millefolium,* characterized by a rich phytochemical composition and broad spectrum bioactivity, presents substantial potential for therapeutic applications. Its potent antioxidant, anti-inflammatory and anticariogenic combined with high biocompatibility, render it a promising natural agent in cariology.

Limitation

The phytochemical variability can affect the consistency and reproducibility of its therapeutic effects. Differences in harvesting times and processing methods (e.g, drying, extraction) can lead to variations in the concentration and potency of bioactive compounds.

Conclusion

A. millefolium or yarrow has antioxidant properties that combat free radicals, protecting cells and promoting overall health. Its anticariogenic effects inhibit cariogenic bacteria, aiding in oral health by reducing plaque and cavities. It also exhibits anti-inflammatory properties, further enhancing its health benefits and

potential applications. The biocompatibility ensures safe interaction with living tissues, making it a versatile and safe ingredient. Phytochemical analysis of *A. millefolium* revealed the presence of flavonoids, phenolic acids, essential oils, alkaloids, tannins, terpenoids, coumarins, glycosides, and saponins. While *A. millefolium* shows great promise as a versatile therapeutic agent, addressing the current limitations and expanding research into its clinical applications, bioavailability, and safety will be crucial for its integration into modern healthcare. By overcoming these challenges, *A. millefolium* can be effectively harnessed to provide natural and effective treatments as an anticariogenic agent.

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