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Antimicrobial Activity of Traditional Medicinal Plant Extracts against Bacterial and Fungal Strains Causing Dental Caries: An in Vitro Study

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ABSTRACT

Backgrounds: The use of plant extracts or their compounds as antimicrobial agents for oral infections worldwide represents that herbal medicines could be used as an effective alternative method in oral health care. This study aimed to evaluate the antifungal and antibacterial effects of five traditional medicinal plant extracts on standard and clinical strains of bacteria and fungi causing dental caries.

Materials & Methods: Aqueous and methanolic extracts of Zataria multiflora, Lawsonia alba, Zizyphus spina-christi, Myrtus communis, and Citrus aurantium were prepared using maceration method. The minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) of the prepared extracts were evaluated against bacterial (Streptococcus sanguinis and S. mutans) and fungal (Candida albicans, C. krusei, and C. tropicalis) isolates using broth microdilution method.

Findings: Aqueous extracts of the studied plants showed no antimicrobial effects on the studied microorganisms, except M. communis and C. aurantium. The results indicated the antimicrobial potency of the methanolic extract of M. communis (MIC range =2 to 64 µg/mL) against all the studied microorganisms, followed by Z. multiflora (MIC range = 512-2048 µg/mL), L. alba (MIC range = 1024-2048 µg/mL), C. aurantium (MIC range = 1024-4096 µg/mL), and Z. spina-christi (MIC range=2048- >4096 µg/mL). Also, the lowest MMCs against the studied strains were related to the methanolic extract of M. communis (MMC range = 16-512 µg/mL).

Conclusion: The results showed remarkable antimicrobial effects of M. communis extract, which could be a suitable alternative to chemical mouthwashes to prevent and control oral infections.

Keywords: Plant extract, Antimicrobial, Dental caries, Bacterial, Fungal.

CITATION LINKS

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Introduction

Dental caries is the most prevalent disease worldwide. This infection is caused by acid produced by microorganisms in bacterial and fungal communities called dental plaque on tooth surfaces, which causes tooth demineralization [1]**.** *Streptococcus mutans* as a member of the oral bacterial flora is the most important etiologic agent of dental caries, oral infections, periodontal diseases, and extra-oral infections such as bacterial endocarditis [2]. The main pathogenic factor of *S. mutans* is biofilm formation. Glucosyltransferase activity, as one of the main virulence factors, promotes the fermentation of various carbohydrates to form water-insoluble glucans, which are essential for the effective attachment and proliferation of S*. mutans* and other oral bacteria on tooth surfaces and subsequently the early formation of dental plaque [3]. *S. sanguinis* is also a member of the oral bacterial community and a main colonizer of the oral cavity [4]. Among the members of the oral fungal flora*, Candida albicans* is associated with tooth decay, but we now know that other types of *Candida,* like *C. krusei* and *C. tropicalis*, could also contribute to tooth decay [5]. *Candida* species synergistically with *S. mutans* could play a role in dental caries by secreting adhesive factors and forming plaque biofilms on tooth surfaces under certain conditions such as poor oral hygiene, antibiotic therapy, and immunosuppression [6, 7]. *C. albicans* promotes biofilm formation, carbohydrate intake, and adhesion of *S. mutans* [6, 7]. The formation of cavities on the surface of the teeth is a continuous process of mineral loss. This process could be stopped or reversed by observing oral and dental hygiene and using mouthwashes with antimicrobial properties. For instance, certain chemicals like case inphosphopeptideamorphouscalciumphosphate (CPP-ACP), xylitol, fluoride, and chlorhexidine have been shown to prevent tooth decay [8-12]. Although topical fluoride agents could be helpful, using them in inappropriate amounts could have negative effects on the body, such as disturbance of bone homeostasis (skeletal fluorosis) [13]. Moreover, it is possible to prevent plaque biofilm formation by cariogenic agents using antibiotics and antifungal drugs. However, the use of current antibiotics and antifungal drugs might have limitations due to the emergence of resistant strains, interactions with other drugs, and harmful side effects that may prevent their long-term use [14, 15]. Therefore, it is necessary to discover new substances with antimicrobial effects on bacterial and fungal strains responsible for dental caries. In the last decades, the use of herbal remedies in the prevention and treatment of oral infections has received special attention because of their effectiveness, less toxicity, and lack of adverse effects compared with chemical compounds. Therefore, the use of herbal medicines is considered as a very effective alternative method in oral health care [14, 15]. Our country has long been rich in traditional and herbal medicine, and as a result of the abundance and variety of herbs in this vast land, it is worthy to research on medicinal plants. *Zataria multiflora*, *Myrtus communis*, *Lawsonia alba*, *Zizyphus spina-christi,* and *Citrus aurantium* have been considered as traditional medicinal plants since ancient times, and many studies have revealed their antimicrobial activities on important human bacterial and fungal pathogens [16-19]. Thus, we hypothesized that aqueous and methanolic extracts of these five traditional medicinal plants would exhibit antimicrobial effects against bacterial and fungal strains causing dental caries. However, there is no enough evidence about the antimicrobial activities of aqueous and methanolic extracts of these plants on fungal (*C. albicans, C. tropicalis,*

and *C. krusei*) and bacterial (*S. sanguinis* and *S. mutans*) isolates. Considering the high incidence of oral infections, especially dental caries, the current survey was conducted to investigate the antimicrobial effects of aqueous and methanolic extracts of these five medicinal herbs (including *Z. multiflora, M. communis, L. alba, Z. spina-christi,* and *C. aurantium*) on fungal and bacterial isolates.

Objectives: The main aim of the present investigation was to assess the antimicrobial activities of five traditional medicinal plant extracts on standard and clinical bacterial and fungal strains causing dental caries. Also, determining the minimum inhibitory concentration (MIC) of these extracts against standard and clinical isolates and determining the extract with the highest antimicrobial effect on the studied standard and clinical strains using broth microdilution method were the other objectives of the study.

Materials and Methods

Plant materials: The leaves of *C. aurantium, Z. multiflora, M. communis, L. alba*, and *Z. spina-christi* were collected from seed culture in pots for 2 months in a greenhouse located in Rasht, northern Iran. All of the studied plants were identified and verified in the Pharmacognosy Department of the School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran.

Preparation of extracts: The collected leaves were cleaned with water, dehydrated under shade, minced, powdered using a mechanical grinder, and filtered through a 20-mesh sieve. The extraction process was conducted using 96% methanol (v/v) (for alcoholic extracts) and distilled water (for aqueous extracts) by maceration method. The extraction process was carried out for 72 hours at room temperature with mild shaking. Then each suspension was filtered through Whatman filter paper No.1, concentrated at 37 °C for 48 hours using a rotary evaporator (Heidolph, Germany), and stored at refrigerator temperature in a tightly-closed sterile tube until used [20, 21].

Microorganisms and growth conditions: The standard strains used in this study were *C. albicans* ATCC®10231™, *C. tropicalis* ATCC®14056™, *C. krusei* ATCC®34135™, *S. mutans* ATCC® 35668 ™, and *S. sanguinis* ATCC®10556 ™. Also, 10 clinical isolates of each standard species were obtained from dental plaque swabs of healthy human volunteers. For isolation and identification of bacteria, swab samples were inoculated in sheep blood agar plates. The plates were incubated overnight at 37 °C. The isolates were subjected to Gram staining, catalase assay, fermentation, and biochemical tests [22]. For fungal agents, specimens were cultured on SC media (SDA: Sabouraud dextrose agar + chloramphenicol) from Merck (a company in Germany). Furthermore, sequencing method was applied for fungal identification. In short, DNA was purified using a high pure PCR template preparation kit from Roche (a company in Germany). A fragment of the ITS gene was amplified using special primers called ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). PCR reactions were performed under the following conditions: a hot-start step at 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 45 s and a final extension step at 72 °C for 5 min [23]. PCR products were tested by sequencing in one direction using a forward primer from Bioneer (a company in South Korea). The results were checked visually using Chromas software Version 3.5.1 and deposited to GenBank.

The type of each isolated organism was determined by comparing it to known sequences in GenBank using a search tool from the National Center for Biotechnology Information.

Screening for antimicrobial activity: Minimal inhibitory concentrations (MICs) were determined using the method suggested by the Clinical and Laboratory Standards Institute (CLSI) [24, 25] with some minor modifications. Firstly, an overnight culture of each bacterial and fungal strain was prepared. To determine the antimicrobial activities of the plant extracts against all the studied microorganisms, the concentration of each plant extract was diluted two-fold from 4096 to 2 µg/mL. The working inoculums containing 1×10^5 CFU/mL of bacteria and yeasts were added to plant extracts in each well of 96-well micro titration plates (Sigma, USA). Suspensions in a total volume of 100 µL were prepared using RPMI 1640 medium (GIBCO, UK) for fungal agents and Mueller-Hinton broth medium (Merck, Germany) for bacterial isolates and incubated at 37 ˚C for 24-48 hrs with 5 to 10% CO_2 in the atmosphere. Microwells containing uninoculated medium and also medium with inoculum but without extract were considered as negative and positive controls, respectively. Each assay was performed in duplicate. Visually, the lowest concentration of the plant extract that was able to inhibit the growth of 99% of the inoculum with no visible growth was considered as the MIC of the extract.

To determine the minimum microbicidal concentrations (MMCs), 10 µL of each well showing no visible growth (containing MIC or higher than the MIC) was sub-cultured on Mueller-Hinton agar supplemented with 5% sheep blood for bacteria and SDA for yeasts to determine the minimum bactericidal concentrations (MBCs) and the minimum fungicidal concentrations (MFCs), respectively. The lowest concentration of the plant extracts that yielded no more than 4 colonies on the agar, indicating a mortality rate of 99.9% of microbes, was considered as the MMC of the extract.

Statistical analysis: All experiments were performed in duplicate, and data were calculated as means ± standard deviations. Mann-Whitney U test was performed to compare MIC and MMC levels of the plant extracts against standard and clinical bacterial and fungal strains. To determine any significant difference in MIC or MMC levels between the plant extracts, Friedman test was used. Post hoc analysis with Wilcoxon signed-rank tests and Bonferroni correction was applied to detect the plant extract with a significantly different MIC or MMC level compared to the other extracts. A p value < .05 was considered as a statistically significance level. Statistical analysis was performed using SPSS™ software, Version 21.0 (IBM Corp., Armonk, NY, USA) [26].

Findings

In the current investigation, the antimicrobial activity of aqueous and methanolic extracts of five medicinal plants (*C. aurantium, Z. multiflora, M. communis, L. alba*, and *Z. spina-christi)* was evaluated against clinical and standard strains of *C. albicans, C. krusei, C. tropicalis, S. sanguinis,* and *S. mutans*. None of the aqueous extracts of the studied plants showed antimicrobial effects on the studied microorganisms at the concentrations tested, except *M. communis* with MIC and MBC values of 64 and 1024 µg/mL against two strains of S*. mutans* and *S. sanguinis* and MIC and MFC values of 256 and 4096 µg/mL against *C. albicans* strains, respectively. Also, the MIC and MFC values of *C. aurantium* aqueous extract against *C. albicans* were calculated to be 512 µg/mL, and the rest of the studied isolates were resistant to this aqueous extract.

Moreover, the methanolic extract of *Z. spina-christi* had antimicrobial activity only against the standard strains of *S. mutans* and *S. sanguinis* studied (MIC= 2048 µg/mL), and the rest of the studied bacterial isolates were

resistant to this methanolic extract. On the other hand, among the fungal isolates, only *C. albicans* strains were susceptible to the methanolic extract of *Z. spina-christi,* and the other yeasts showed antifungal resistance to this extract.

The results of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of methanolic extracts of the five medicinal plants against clinical and standard *S. mutans* strains are shown in Table 1. Also, Table 2 shows the antifungal effects of the five traditional medicinal plant extracts on clinical and standard *C. albicans* strains. The MIC and MBC/MFC values of each extract were almost the same for all the studied bacterial and fungal strains. The results showed that the methanolic extracts of four plants, including *Z. multiflora*, *M. communis, L. alba*, and *C. aurantium,* inhibited the growth of all the studied bacterial and yeast strains with MIC values ranging from 2 to 4096 µg/mL.

Table 1) Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of methanolic extracts of the five traditional medicinal plants against clinical and standard *S. mutans* strains

S. mutans Strains	*Myrtus communis*		*Zataria multiflora*		*Zizyphus spina-christi*		*Lawsonia alba*		*C. aurantium*	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
1	8	64	512	512	>4096	>4096	1024	2048	1024	2048
2	16	128	512	1024	>4096		2048	4096	2048	4096
3	4	32	512	1024	>4096		2048	>4096	2048	>4096
4	4	32	1024	1024	>4096		2048	4096	2048	4096
5	2	16	2048	2048	>4096		2048	>4096	2048	>4096
6	8	64	1024	1024	>4096		2048	4096	2048	4096
7	8	32	512	1024	>4096		2048	2048	4096	2048
8	8	64	512	1024	>4096		2048	2048	2048	2048
9	8	64	512	512	>4096		2048	4096	2048	4096
10	4	16	512	1024	>4096		2048	4096	2048	4096
Standard strain	16	128	512	1024	2048	4096	1024	1024	1024	1024

Table 2) Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of methanolic extracts of the five traditional medicinal plants against clinical and standard *C. albicans* strains

C. albicans Strains	*Myrtus communis*		*Zataria multiflora*		*Zizyphus spina-christi*		*Lawsonia alba*		*C. aurantium*	
	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
1	32	256	2048	>4096	2048	>4096	2048	>4096	2048	>4096
2	64	512	2048	4096	2048	>4096	2048	>4096	2048	>4096
3	16	128	2048	4096	1024	4096	1024	4096	1024	4096
4	32	128	1024	4096	2048	>4096	1024	4096	4096	4096
5	64	256	2048	>4096	1024	2048	1024	2048	1024	2048
6	64	256	1024	4096	2048	>4096	2048	4096	2048	4096
7	64	512	2048	>4096	1024	4096	1024	4096	1024	4096
8	32	128	2048	>4096	2048	4096	1024	4096	1024	4096
9	64	256	2048	>4096	2048	4096	2048	4096	4096	4096
10	16	64	2048	>4096	1024	4096	1024	4096	1024	4096
Standard strain	32	256	2048	4096	1024	4096	1024	4096	1024	4096

Among the tested methanolic extracts of plants, the highest antimicrobial effect against all the studied bacterial and yeast strains was related to *M. communis* with MIC values ranging from 2 to 64 µg/mL ($p<$ .05). The results indicated the antimicrobial potency of *M. communis* methanolic extract (MIC range=2 to 64 µg/mL) against all the studied microorganisms, followed by *Z. multiflora* (MIC range= 512-2048 µg/mL), *L. alba* (MIC range= 1024-2048 µg/mL), *C. aurantium* (MIC range= 1024-4096 µg/mL), and *Z. spina-christi* (MIC range= 2048->4096 µg/mL). *S. mutans* strains were the most susceptible strains to *M. communis* with MIC values ranging from 2 to 16 µg/mL.

Also, the lowest MMCs against the studied strains were related to the methanolic extract of *M. communis* (MMC range = 16-512 µg/mL). All the tested strains were killed by methanolic extracts with MMCs approximately equal to or 2-8 times higher than their corresponding MICs. In the present study, no statistically significant difference in antimicrobial susceptibility was found between the fungal and bacterial

isolates collected from healthy volunteers and the corresponding standard strains. The MIC and MMC ranges of methanolic extracts of *M. communis* and *Z. multiflora* were significantly lower (p< .001) for standard and clinical bacterial strains than for standard and clinical fungal strains. However, the MIC ranges of methanolic extracts of *L. alba* and *Z. spina-christi* were significantly lower (p< .001) for standard and clinical fungal strains than for standard and clinical bacterial strains. The MMC ranges of methanolic extract of *Z. spina-christi* were significantly lower (p< .02) for standard and clinical fungal strains than for standard and clinical bacterial strains.

The MIC and MMC ranges of methanolic extract of *M. communis* compared to methanolic extracts of *Z. multiflora* (p= .005), *Z. spina-christi* (p= .004), and *L. alba* (p= .005) were significantly lower for standard and clinical bacterial and fungal strains.

Discussion

The most common oral disease is dental caries. It is an avoidable disease, but the formation of lesions may be prevented by prompt identification of risk factors [1]. The application of herbal remedies in the successful caries prevention has received special attention in recent years so that they have almost replaced antibiotics, antifungals, and chemical compounds due to their good therapeutic potential, fewer adverse effects, variety of effective compounds, and lower economic costs [15]. On the other hand, to prevent the emergence of fungal resistance, it is essential to use novel alternative molecules which are more effective than conventional antifungals.

In the current investigation, the antimicrobial activity of aqueous and methanolic extracts of five medicinal plants was evaluated against standard and clinical bacterial and fungal strains causing dental caries. Consistent with previous studies, the results indicated that the most significant antimicrobial effects against all the studied bacterial and yeast strains were exhibited by the methanolic extracts of *M. communis* and *Z. spina-christi*. These extracts were found to be more effective compared with their aqueous counterparts. A study performed by Dib et al. (2021) found that the methanolic and aqueous extracts of *M. communis* had inhibitory activity against cariogenic microorganisms (*Eikenella corrodens, Aggregatibacter actinomycetemcomitans,* and *Porphyromonas gingivalis*), and the antibacterial activity of the methanolic extract was higher than that of the aqueous extract [27]. This may be due to the improved solubility of active compounds in organic solvents such as methanol and ethanol [28]. Consistent with the present study results, Nayf and Salman (2021) reported that the aqueous extract of *M. communis* leaves had potential antimicrobial activity against bacterial cariogenic agents [29].

In agreement with the present study results, previous studies have indicated that methanolic extracts of *M. communis* and *Z. multiflora* have significant antifungal activity against *C. albicans* (ATCC 10231), *C. glabrata* (ATCC 28838), and *C. tropicalis* (ATCC 13801) [30, 31]. Another study showed that the oil obtained from the leaves of *M. communis* had significant antifungal activity against different *Candida* species [32]. However, in a study conducted by Mert et al. (2008), no antifungal activity against *C. albicans* (ATCC 10239) was found for n-hexane, ethyl acetate, methanol, and aqueous extracts of *M. communis* leaves [33]. Differences in results may be due to differences in methodologies and changes in the chemical structure of medicinal plants in different geographical areas [34].

The antimicrobial activity of *M. communis,*

Z. spina-christi, and *Z. multiflora* against bacterial and fungal strains causing dental caries may be associated with their active components. In fact, leaves of *M. communis* and *Z. spina-christi* contain tannins, flavonoid glycosides, and polyphenolic compounds, and leaves of *Z. multiflora* contain rosmarinic acid and thymol, which are extracted exclusively with methanol [35]. So far, 15 new phenolic compounds have been identified in the chromatographic (HPLC) profile of myrtle (*M. communis*) leaves [36]. On the other hand, herbal components are influenced by geographical parameters, such as latitude, altitude, temperature, climate, soil texture relative to the percentage of clay, silt or sand, constituents (P, K, Al, Ca, Fe), and pH [37].

Living microorganisms in dental plaque are more resistant to chemical antimicrobial agents than standard bacteria commonly used for *in vitro* susceptibility testing [38]. However, in the current study, no increase in resistance to plant extracts was observed in fungal and bacterial isolates collected from volunteers with respect to their equivalent standard strains. This is because herbal antimicrobials could inhibit the growth of microorganisms by a variety of mechanisms and are different from those currently used to treat resistant microbial strains with significant clinical benefits [39].

The present study findings suggest that the use of methanolic extract of *M. communis* as a mouthwash is useful for controlling oral infections. Also, it could be used as an oral antimicrobial agent to prevent or treat dental infections.

There are some limitations in the present study. For example, the anti-biofilm activity of the studied plant extracts was not investigated. Besides, the antimicrobial activity of different parts of *M. communis* plant could be investigated in future studies. Also, different extraction methods, solvents, and conditions could be tested. Additionally, it is useful to identify the chemical properties and toxicity of bioactive compounds derived from *M. communis* leaves. Furthermore, conducting *in vivo* studies and clinical trials are suggested.

Conclusion

The results showed remarkable antimicrobial effects of *M. communis* extract, which could be a suitable alternative to chemical mouthwashes to prevent and control oral infections.

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Ethical permissions: The study was approved by the Research Ethics Committee of Guilan University of Medical Sciences (the number of ethics committee protocol: IR.GUMS.REC.1401.556).

Authors` contributions: Raheleh Sheikhi: conceptualization, methodology design, and project administration. Zahra Rafat: conceptualization, methodology design, project administration, writing the original draft, resources, visualization, data curation, writing, reviewing, and editing. Davoud Roostaei: methodology design and investigation. Nasrin Sharifi: statistical analysis. Hamid Neshandar Asli: methodology design and investigation. Rasoul Naseri: investigation. All authors contributed to the article and approved the submitted version.

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