Physical Properties and Antibacterial Activity of Herbal Tinctures of Calendula (Calendula officinalis L.) and Cashew Tree (Anacardium occidentale L.)

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ABSTRACT

Objective: To evaluate in vitro the pH, °Brix and antibacterial activity of herbal tinctures of calendula (Calendula officinalis L.) and cashew tree (Anacardium occidentale L.) against the following bacterial strains: Streptococcus mutans (ATCC 25175), Streptococcus oralis (ATCC 10557), Streptococcus salivarius (ATCC 9758), Enterococcus faecalis (ATCC 29212) and Eikenella corrodens (ATCC 23834). Material and Method: The strains were reactivated in agar Brain Heart Infusion (BHI) and seeded in blood agar with BHI. Susceptibility tests were performed and the strains were incubated in microaerophilia (37 °C) for 48 h. Chlorhexidine at 0.12% was used as a positive control and distilled water as a negative control. Dilution of the tinctures was carried out from 1:1 (pure form) to 1:64 in alcohol 70%, which is found in their composition, in order to evaluate a possible influence of alcohol on the antibacterial activity of the tinctures. °Brix readings were made by refractometry using an Abbe refractometer and the pH was measured using a digital pH meter. Results: The calendula tincture had pH=5.43 and 18 °Brix and cashew tree tincture had pH=7.2 and 17 °Brix. The cashew tree tincture did not present antibacterial activity against S. oralis, but it inhibited the growth of all other bacteria up to the 1:64 dilution. The calendula tincture presented antibacterial activity against S. oralis up to 1:16 dilution (6.5 mm halo diameter); against S. mutans and S. salivarius up to 1:32 dilution (7 and 7.5 mm halo diameter, respectively); and against E. faecalis and E. corrodens up to 1:64 dilution. Alcohol 70% did not show any antibacterial activity against the bacterial strains. Conclusion: The tinctures of calendula and cashew tree have soluble solids, have different pH values and present a significant antibacterial activity against several bacterial strains, except for the cashew tree tincture against S. oralis, which was ineffective.

Key-words: Microbiology; Phytotherapy; Physical properties; Antimicrobials; Phytotherapeutic Drugs.

INTRODUCTION

Dental biofilm is intimately associated with the onset, dissemination and progression of oral problems such as caries and periodontal diseases. The formation of bacterial biofilm on teeth, restorations, implants or other surfaces exposed in the oral cavity is an omnipresent phenomenon of microbial colonization at interfaces between solid surfaces and biologic fluids [1]. It starts within a few minutes by the adsorption of salivary proteins, glycoproteins and mucins, resulting in an “acquired enamel pellicle”. Shortly after, the first bacteria adhere to start the colonization of this pellicle surface [2]. This process leads to the formation of the dental plaque, a biofilm with multiple layers of bacteria resulting from the growth and co-adhesion of additional bacteria. Maturation of the extracellular matrix encasing the dental plaque is characterized by the formation of a network of water-soluble and non-soluble glucans synthesized by bacterial glycosyl transferases [3]. Therefore, oral biofilm is established as a community structured three-dimensionally and composed by several microbial species that interact and communicate by signal transduction processes [1].

Streptococcus oralis is an acidogenic microorganism present in the initial phase of dental caries. It makes the environment more adequate for plaque colonization by Streptococcus mutans, which is considered as one of the most important pathogens associated with the initiation and progress of dental caries and has a key role in lesion formation [4]. Streptococcus salivarius is one of the main components of biofilms formed on oral epithelium, tongue, dorsal epithelium [3] and corresponds to the most part of total cultivable flora in the oral soft tissues [5]. Enterococcus faecalis is a bacterium usually found in the human gastrointestinal tract, but it is associated with persistent periapical infections and failure of endodontic treatment with calcium hydroxide [6], which suggests resistance to the drug. Eikenella corrodens is an opportunistic bacillus and is found in the endogenous oral microflora (forming a subgingival plaque), the upper airways, and the gastrointestinal and genitourinary tracts. This bacterium may cause infections with slow progression, usually of
polymicrobial nature and frequently associated with a putrid odor, simulating an anaerobic process. It is commonly associated with periodontal problems and may be involved in bacterial endocarditis [7].

_Calendula officinalis_ L. is an annual herbaceous plant of European origin adapted to Brazil and belonging to the _Asteraceae_ family. It is widely employed in phytotherapy because of its antiinflammatory, healing and antibacterial properties [8]. Flavonoids are among the most widely researched secondary metabolites of this plant species, and are in great part responsible for its biological activities, specially the rutin heterosidic flavonoid [8]. Regarding the substances that produce the therapeutic effects attributed to _Calendula officinalis_ L., it has been confirmed the presence of flavonoids, flavones/flavonols and xanthones, catechins, flavonones and alkaloids. There is also evidence of phenol in its constitution [9].

_Anacardium occidentale_ L. (cashew tree) belongs within the _Anacardiaceae_ family and is originally native to northeastern Brazil, in the area between the Atlantic rain forest and the Amazon rainforest. This evergreen tree has several edible pear-shaped “pseudo-fruits” or “false fruits” known as “cashew apples”, which are actually floral peduncles, and small bean-shaped, grey “true fruits” that are adhered to the lower end of the cashew apples and are known as “cashew nuts”. These parts are used in the treatment of several inflammatory conditions (e.g.: arthritis) [10], fever, pain [11] and asthma [12]. Antimicrobial action against the rotavirus that causes diarrhea [13] and against _Candida albicans_, _Trichophytion rubrum_ and _Cryptococcus neoformans_ [14] has been investigated. Brazilian studies have identified several chemical complexes, such as carotenoids (β-carotene, α-carotene, β-cryptoxanthin, 9-cis- +13-cis-b-carotene, auroxanthin, 5,8-epoxy-cryptoxanthin, 5,8-epoxy-lutein, z-carotene) and ascorbic acid [15].

Research on the antibacterial action of these two plants is still limited and there is need to widen the knowledge about their medicinal properties and possible applications, looking forward to contributing to the development of new phytotherapeutic products in Dentistry. The aim of this study was to evaluate _in vitro_ the pH, “Brix and antibacterial activity of herbal tinctures of calendula (_Calendula officinalis_ L.) and cashew tree (_Anacardium occidentale_ L.) against several bacterial strains.

**MATERIAL AND METHODS**

An inductive approach with descriptive statistics and direct laboratory documentation technique was adopted. The bacterial strains used in this study were obtained from Laboratory of Reference Materials of the National Institute for Health Quality Control - Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro, RJ, Brazil) and provided by the Laboratory of Oral Microbiology of the Tropical Medicine Center (NUMETROP) of the Health Sciences Center of the Federal University of Paraíba (UFPB). The following bacterial strains were used: _S. mutans_ (ATCC 25175), _S. oralis_ (ATCC 0557), _S. salivarius_ (ATCC 9758), _E. faecalis_ (ATCC 29212) and _E. corrodens_ (ATCC 23834). Because of its scientifically confirmed antibacterial action, 0.12% chlorhexidine gluconate was used as a positive control, while distilled water as a negative control.

The tinctures tested in this study were purchased ready-to-use from a prescription pharmacy (Vita Flora Farmácia de Manipulação, João Pessoa, PB, Brazil). According to the technical report, both tinctures were diluted 20% in alcohol 70%. In addition to this initial dilution, a serial dilution of the tinctures was performed starting from 1:1 (“pure” form) to 1:64 (6th dilution) in alcohol 70%, which is routinely used in the preparation of phytotherapeutic tinctures.

The bacteria were reactivated in Brain Heart Infusion agar broth (BHI Agar; Difco®, São Paulo, SP, Brazil) and seeded on blood agar in Petri dishes using disposable swabs and following the visual pattern of the McFarland scale. Bacterial aggregate inocula were immersed in test tubes containing saline (one tube per bacterial strain) and left to grow until reaching a turbidity corresponding to 0.5 on the McFarland scale, which indicates the existence of approximately 1.5 x 10^8 colony forming units per milliliter (CFU/mL).

The susceptibility test was performed using the agar dilution method to verify the presence of external or internal contaminations (impure strains), and the strains were incubated at 37 °C in microaerophilic for 48 h. Hand calipers were used to measure the zones of bacterial growth inhibition formed around the strains. The measurements were made in duplicate and the data were analyzed descriptively, considering as results the arithmetic means of the diameter of the zones of bacterial growth inhibition in millimeters.

pH was measured with an pH meter (Analyser® model 300; São Paulo, SP, Brazil) calibrated at room temperature (25 °C). Total soluble solids contents (°Brix) were measured with a handheld refractometer (model N1, Atago Co. Ltd, Honcho, Itabashi-ku, Tokyo; range: 0 to 32 Brix, precision: 0.2%). All measurements were made in triplicate and averaged to obtain the arithmetic means.

**RESULTS**

Data from the antibacterial activity of the calendula and cashew tree tinctures against the five bacterial species are presented in Tables 1 and 2, respectively. Table 3 presents the °BRIX and pH results.

**DISCUSSION**

The antibacterial agents most frequently used
for prevention and treatment of oral diseases, including
cetylpyridinium chloride, chlorhexidine, amine fluorides
or products containing these agents, are reported to
present toxicity and cause pigmentation of teeth and
microbial resistance as a result of prolonged daily use
[4,16]. Traditional plants used for health care are an
important source of new compounds to serve as the base
of modern pharmacology, since a great part of synthetic
drugs is directly or indirectly derived from medicinal
plants [17].

In this way, studies using phyto-pharmaceutical
drugs or medicinal products derived from plant parts
should receive special emphasis in both social and
scientific contexts, being an alternative for the treatment
of different diseases and conditions in several health
areas. Studies assessing the antimicrobial activity of
phytotherapeutic products are important predecessors of
more specific and accurate evaluations.

In this study, the antimicrobial action of herbal
tinctures of calendula (Calendula officinalis L.) and
cashew tree (Anacardium occidentale L.) was evaluated
against reference strains of S. mutans (ATCC 25175), S.
oralis (ATCC 0557), S. salivarius (ATCC 9758), E. faecalis
(ATCC 29212) and E. corrodens (ATCC 23834) because
despite microorganisms have an important participation in
the oral microflora, have specific behaviors, and
represent a diverse group of bacteria with different site
predilection. The idea was to provide an overview of the
antimicrobial action of the two herbal tinctures against
important pathogens of the oral microbiota. The agar
diffusion method was used because it is usually
employed in studies assessing the antimicrobial action of
artificial or natural products [18-20].

The 0.12% chlorhexidine digluconate was used
as a positive control because it has a recognized efficacy
against a wide range of microorganisms and has long
been considered as a gold standard antimicrobial agent.
Its cationic nature provides connection with ammonium-
based compounds of the bacterial cell surface (teichoic
acid phosphate groups in Gram-positive bacteria and
lipopolysaccharides in Gram-negative bacteria) and able
to alter the bacterial integrity. Alteration of the
permeability of the cytoplasmic membrane produces
precipitation of cytoplasmic proteins, alters the cell
osmotic balance, affects cell metabolism, growth and
division, inhibits cell membrane ATP-ase and the
anaerobic processes [21], acting as a scientifically proven
antimicrobial agent.

It is difficult to confront our results of
antimicrobial activity with the literature in view of the
lack of studies evaluating the same tinctures against
the same bacteria and using the same methodology. This
fact emphasizes the importance of this study for future
research that supports the clinical use of the calendula
(Calendula officinalis L.) and cashew tree (Anacardium
occidentale L.) tinctures. It is important to highlight that
the diameter of the microbial growth inhibition zones
produced by the tinctures were relatively close to those
produced by the positive control (0.12% chlorhexidine),
especially the cashew tree tincture, which inhibited
bacterial growth up to the 1:64 dilution, except for S.
oralis.
The initial formulation of hydroalcoholic extracts is made with alcohol 70% and for this reason it was used for dilution of the samples [19]. Additionally, its interaction with bacteria was evaluated, as reported elsewhere [19], in order to verify whether alcohol had any action against the bacterial strains, reducing the chances of research bias. The results ruled out this hypothesis, demonstrating bacterial resistance to alcohol and indicating that the antimicrobial effect of the tinctures seems to be not influenced by this substance [19]. If this hypothesis were not rejected, it would be impossible to attribute the antibacterial action to the phytotherapeutic compound, since it could be caused by the alcohol 70% present in the tincture formulation or by the association of tincture with alcohol 70%.

Refractometry or °Brix is a physical method for measuring the quantity of soluble substances (sugar salts, proteins, acids and other) in a water solution [22]. A high level of sugars in the formulations is directly related to their cariogenic potential. In the present study, the calendula and cashew tree tinctures presented 17 and 18 °Brix respectively, which corresponds to 17 and 18 g of solids dissolved in 100 g of solution. Speaking generally, the higher this value, the greater the possibility of the tincture to present a sweet nature. It should be pointed out that such values are lower than those found in mouthwashes available on the Brazilian market, such as Clinerize (23.7%), Listerine Cool Citrus (22.7%), Prevident 220 (20.9%) and Equate (20%) [23].

A pH value equal or lower than 5.5 is considered critical for dissolution of dental enamel, but the mineral loss may start with a higher pH value [24]. Therefore, prolonged use of mouthwashes with pH below this value may be potentially harmful to the dental tissues. This is important when using calendula tinctures, which have low or very close pH values. In a previous study [9], a *Calendula officinalis* L. tincture presented a pH value of 4.79, which is lower than the one obtained in the present study (5.43). On the other hand, the cashew tree tincture had a neutral pH (7.20), which is not aggressive to enamel (low pH values).

Increased acidity of the medium reduces the antibacterial action of basic substances such as streptomycin and, on the other hand, creates favorable conditions for a more intense activity of acid substances like penicillin [25]. This could have a negative influence on the antimicrobial activity of the calendula tincture.

The physicochemical properties of the products may influence on their diffusion in culture media and interfere in the results of antimicrobial evaluation [20]. Other factors that can affect the pharmacological properties of phytotherapeutic drugs must also be considered, such as the age of the plant, site and season of plant collection, quality of the used solvent, type of packaging (hermetically sealed or not), as well as product evaporation during storage possibility increasing the concentration of the individual components. Additionally, tannin contents may vary significantly with the used extraction method [26]. This is a limitation to the study because the tinctures were purchased from a prescription pharmacy, which hampers obtaining information on the plants. Additionally, dilution in alcohol in the “pure” form at 20% might have influenced the results by underestimating the diameters of the bacterial growth inhibition zones. Consequently, some of these factors may have acted to alter the antimicrobial properties of the tinctures, but might as well explain the different pH found in a previous study, even though they are within the normal range of pH values [9].

There are important limitations in the *in vitro* experimental models with planktonic cells, but their results cannot be overlooked because of their importance for the preliminary evaluations of antimicrobial agents against some specific bacterial species [27].

Considering that there is a growing interest for natural therapies like phytotherapy and an emphasis to use phytotherapeutic agents in public health treatments, the promising results of this study suggest further research with the calendula and cashew tree tinctures for a better understanding of their antimicrobial activity and their potential clinical use in Dentistry.

**CONCLUSION**

The calendula (*Calendula officinalis* L.) and cashew tree (*Anacardium occidentale* L.) tinctures have different pH values, possess soluble solids in their composition and present a significant antimicrobial activity against several oral bacterial strains. Antimicrobial activity was not verified only for the cashew tree tincture against *S. oralis*. In view of the above findings, further studies with different methodologies are recommended in order to corroborate the promising results of this experiment.

**REFERENCES**

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