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Original Article

Effect of different Vitis vinifera seed extracts on lactobacillus acidophilus and casei bacteria

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Abstract

Introduction: Due to the limitations of chemical antimicrobial methods in the treatment of dental caries, the recent studies have focused on the use of plant-derived antibacterial agents to inhibit tooth decay bacteria. Therefore, the aim of this study was to investigate the effect of Vitis vinifera seed extract (VVSE) on Lactobacillus acidophilus and casei bacteria.

Material & Methods: In this cross-sectional study, the VVSs were dried, the obtained powder was poured into separate containers to prepare aqueous, alcoholic and acetone extracts, and the desired solvents were added. After being placed in the shaker incubator and passing through the filter paper, the solvents were transferred to the plates. After cultivation of Lactobacillus acidophilus and casei bacteria in tubes containing Mueller Hinton Broth, the aqueous, alcoholic and acetone extracts were added to them. A tube with no extract was considered as control. The resulting samples were cultured on chocolate agar medium. The initial concentrations (2, 4 and 8 µg/ml) were not able to inhibit bacterial growth; thus, the higher concentrations were assessed to determine minimum inhibitory concentration (MIC). The data were analyzed using SPSS-17 via Chi-square, Mann-Witney and Kruskal-Wallis. Moreover, α=0.05 was considered significant.

Results: The MIC of aqueous extract was 32 and 64 µg/ml for Lactobacillus acidophilus and casei, respectively. The alcoholic extract stopped the growth of both bacteria at concentration of 128 µg/ml. The MIC of acetone extract was 64 and 128 µg/ml for Lactobacillus acidophilus and casei, respectively. So, aqueous extract was more effective than alcoholic one (p=0.016). However, there was no significant difference between alcoholic and acetone (p=0.1267) as well as aqueous and acetone (p=0.061) extracts.

Conclusion: Antibacterial activity of aqueous extract was more than that of alcoholic and acetone extracts. Alcoholic and acetone extracts had no significant difference in inhibition of bacterial growth. Therefore, it is possible to use aqueous extract of VVSs to control caries.

Keywords: Vitis, Lactobacillus acidophilus, Lactobacillus casei, Anti-bacterial agents

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اثر عصاره های مختلف هسته انگور سیاه بر باکتری های لاکتوباسیلوس اسیدوفیلوس و لاکتوباسیلوس کازئی

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چکیده

مقدمه: به دلیل محدودیت روش های آنتی میکروبیال شیمیایی در درمان پوسیدگی دندان، اخیراً مطالعات بر مواد آنتی باکتریال با منشا گیاهی برای مهار باکتری های عامل پوسیدگی معطوف شده است. هدف از این مطالعه، بررسی اثر عصاره هسته انگور سیاه بر باکتری های لاکتوباسیلوس اسیدوفیلوس وکازئی می باشد.

مواد و روش ها: در این مطالعه توصیفی–مقطعی، هسته انگور vitis vinifera خشک و پودر حاصل برای تهیه عصاره های آبی، الکلی و استونی در ظروف مجزا ریخته و حلال به آن ها اضافه شد. پس از قرار گرفتن درshaker incubator و عبور از کاغذ صافی، به پلیت منتقل شد. پس از کشت دادن باکتری های لاکتوباسیلوس اسیدفیلوس و کازئی در لوله های حاوی محیط کشت مولر هینتون براث، عصاره های آبی، الکلی و استونی به آنها اضافه شد. لوله ای بدون عصاره به عنوان شاهد در نظر گرفته شد. ترکیب حاصل در محیط شکلات آگار کشت داده شد. غلظت های اولیه ۲، ۴ و۸ میکروگرم/میلی لیتر قادر به مهار رشد باکتری ها نشد لذا غلظت های بالاتر به منظور تعیین MIC مورد بررسی قرار گرفت. نتایج به دست آمده در نرم افزار SPSS-17 و با آزمون های Chi-Square,Mann-Witney و Kruskal Wallis مورد تجزیه و تحلیل آماری قرار گرفت.α =0.05 معنی دار تلقی می گردد.

یافته ها: MIC عصاره آبی برای لاکتوباسیلوس اسیدوفیلوس و کازئی به ترتیب ۳۲ و ۶۴ میکروگرم بر میلی لیتر به دست آمد. عصاره الکلی در غلظت ۱۲۸ رشد هر دو باکتری را متوقف کرد. و MIC عصاره استونی برای لاکتوباسیلوس اسیدوفیلوس ۶۴ و برای کازئی ۱۲۸ میکروگرم بر میلی لیتر بود. عصاره آبی نسبت به الکلی موثرتر (p=0.016) بود. در حالی که بین عصاره های الکلی و استونی(p=0.1267) و آبی و استونی (p=0.061) تفاوت معناداری وجود نداشت.

نتیجه گیری: خاصیت آنتی باکتریال عصاره آبی هسته انگور سیاه بیش از عصاره الکلی و استونی بود. عصاره های الکلی و استونی در مهار رشد باکتری تفاوت معنی داری نداشتند. بنابراین ممکن است بتوان از عصاره آبی هسته انگور سیاه برای کنترل پوسیدگی استفاده کرد.

واژگان کلیدی: انگور سیاه ،لاکتوباسیلوس اسیدوفیلوس، لاکتوباسیلوس کازئی، عوامل آنتی باکتریال

Introduction

Dental caries and periodontal diseases are caused by microorganisms present in the dental plaque. Clinical control of these diseases is achieved by reducing the microbial load in the dental plaque biofilm. [1] Streptococcus mutans bacterium causes dental caries [2], while lactobacilli spread dental caries. [3] These microorganisms appear in the early life of children and are found in large quantities in saliva, dorsal surface of tongue, mucous membrane, hard palate and in small quantities in dental surfaces. Most species found in dental caries belong to the acidophilus and casei species. [4] One of the methods to prevent dental caries is

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the use of chemical antimicrobial agents such as chlorhexidine or sodium fluoride to inhibit the growth and formation of biofilm created via caries-forming microorganisms in the oral cavity. [5] These chemical antimicrobial agents have a number of limitations. [6] For example, fluorosis may occur through ingestion of large amounts of fluoride in food or drinking water [7] and the most important side effect of chlorhexidine is tooth discoloration. [8] In recent years, more attention has been paid to plant-derived antimicrobial compounds as an alternative to commonly used chemicals in the prevention of caries. [9]

The Vitis vinifera seed extracts are rich in polyphenolic compounds and are commonly found in edible and non-edible plants, drinks and plant foods. Their beneficial effects on health include antioxidant, anti-cancer and anti-inflammatory properties. [10] Eating foods or drinks rich in polyphenols is also beneficial for oral health. This extract prevents the formation of biofilms containing periodontal pathogens and occurrence of periodontal diseases. In recent years, the antimicrobial and antiplaque activities of plant polyphenols have been investigated in numerous in vitro studies. [11]

Given that the early childhood caries as the most common chronic disease is a major problem in many developing countries, it seems that the grape seed extract (GSE) based on its antimicrobial properties can be used to prevent the occurrence of these caries. [12,13] Zhao et al. in 2014 evaluated the effect of GSE on the formation of enamel caries lesions and concluded that this extract had a dose-dependent effect on the growth inhibition and biofilm formation of Streptococcus mutans. [14] Moreover, in 2008, Furgia et al. suggested the strong antiplaque effect of GSE-amine fluoride combination. [15] Although lactobacilli are involved in caries spread, so far, no study has been performed on the effect of GSE on this bacterium. Hence, the aim of this study was to evaluate the antimicrobial effect of GSE (Vitis vinifera) against Lactobacillus acidophilus and casei bacteria.

Materials & Methods

In this experimental study, the effect of GSE was evaluated on Lactobacillus acidophilus and casei with Ethics Committee code of MUBABOL.REC1395.162. Vitis vinifera grapes were purchased from Qazvin vineyard. The Vitis vinifera named grapevine (Tak) in Iran belongs to the family Vitaceae and has different genus. [16] The seeds of grapes were separated (6 kg) and dried at room temperature away from sunlight.

The dried seeds were powdered by electric grinder, the resulting powder was collected in a glass container, 150 g of this powder was poured into separate calibrated containers to prepare aqueous, alcoholic (70% ethanol) and acetone extracts, and then each of the solvents was added to a volume of 200 ml.

These containers were transferred to the shaker incubator for 72 hours, the containers were removed, the solutions were filtered using a filter paper and poured into the plates placed in the oven to evaporate the solvents and finally, the extract-containing plates were sterilized in a furnace to eliminate any contamination.

Lactobacillus acidophilus (ATCC 1643) and Lactobacillus casei (ATCC 1608) bacteria were purchased as lyophilized powders from Iran Scientific and Industrial Research Organization. At first, these lyophilized bacteria were active. They were then transferred to Muller Hinton agar-containing plates using a sterilized swap, and the cultures were incubated at 37 ° C for 24 hours to allow the bacteria to grow fully and obtain a single colony. Four to six colonies of each bacterial culture were inoculated with 5 ml of Muller Hinton medium using a sterilized swap and incubated at 37 ° C until the bacteria reached exponential growth phase after 4-6 hours. The turbidity of each tube was compared with that of a 0.5McFarland standard.

Determination of minimum inhibitory concentration (MIC) of extracts was performed by macrodilution according to the CLSI standard (2017). Each of the aqueous, alcoholic and acetone extracts was poured into separate tubes at concentrations of 2, 4 and 8 µg/ml. For each bacterium, one tube with no extract was considered as control. At this stage, to determine the effect of the extracts, the bacteria were cultured using loops on plates containing chocolate agar medium in the presence of flame to prevent contamination.

The plates were transferred to the incubator and after 48-72 hours, the results of bacterial growth were examined so that the lack of bacterial growth was considered as an indicator of the inhibitory effect of the extract on bacterial growth. The inhibitory effect was not observed in any plates. Since the above concentrations could not inhibit bacterial growth; thus, the higher concentrations of the extract were used (16, 32 and ... 128) to achieve a concentration of the extract that inhibited bacterial growth. The experiments were

duplicated to ensure the accuracy of the results. The data were collected, coded and analyzed using SPSS via Chi-Square, Mann-Witney and Kruskal Wallis in order to determine the significant differences in the efficacy of extracts on bacteria with significant difference of α =0.05.

Results

In the measurement using quantitative macrodilution technique, the lowest MIC was related to the aqueous extract of Vitis vinifera seed for Lactobacillus acidophilus with 32 µg/ml (Table 1).

Table 1. MIC of aqueous, alcoholic and acetone extracts for Lactobacillus acidophilus and casei bacteria (µg / ml)

Extract *Bacterium*	Aqueous	Alcoholic	acetone	*Pvalue*
Acidophilus	32	128	64	0.08
Casei	64	128	128	0.08
P-value	0.33	1	0.33	-

Statistical analysis via Chi-Square test

Overall, comparison of Lactobacillus acidophilus and casei via Mann-Witney test exhibited no significant difference in terms of their resistance to extracts (p=0.1). Total comparison of all three aqueous, alcoholic and acetone extracts via Kruskal Wallis test showed a significant difference in terms of the efficacy of these extracts (p=0.019) (Table 2).

Table 2. MIC comparison of aqueous (1), alcoholic (2) and acetone (3) extracts

MIC Mean±SD (median)	Extract
48±18.475[a] (48)	Aqueous
128±33.0[b] (128)	Alcoholic
96±36.950[ab] (128)	Acetone
0.019	P- value

Statistical analysis via Kruskal-Wallis test

In addition, comparison of aqueous and alcoholic extracts of Vitis vinifera seed via Mann-Witney test represented that the aqueous extract inhibited bacterial growth more effectively than alcoholic extract (p=0.016). However, comparison of alcoholic and acetone extracts displayed no significant difference in efficacy (p=0.127). There was no significant difference in the degree of inhibitory effect on the growth of these bacteria between aqueous and acetone extracts (p=0.061). The comparison results of different extracts for one bacterium and two bacteria in each extract are presented in table 1 through Chi-square test.

Discussion

In the current study, the antibacterial property of Vitis vinifera seed extract was investigated using macrodilution method. The results indicated that the aqueous extract was more effective than alcoholic extract in inhibiting bacterial growth. However, there was no such a significant difference between the alcoholic and acetone extracts as well as the aqueous and acetone extracts. There were some limitations such as unavailability of grapes when needed. Moreover, because the compounds and consequently the properties of grape seed extract are influenced by the region in which it grows; therefore, comparing the result of this study with similar ones might not very reliable. In addition, to our best knowledge, this was the first study to investigate the effect of grape seed extract on Lactobacilli; hence, it was impossible to compare it with other studies. Studies mentioned below have shown that Vitis vinifera seed extract can be effective in preventing dental caries. Zhao et al. in 2014 explained that 4-µg/ml concentration of GBE inhibited the growth and biofilm formation of Streptococcus mutans. [14] Considering the results of the present study, concentrations of 2 and 8 µg/ml in addition to 4 µg/ml were used to evaluate the effect of concentration on the potency of the extract and none of them had inhibitory effects.

Swadas et al. in 2016 also evaluated and compared the anti-Streptococcus mutans activity of Vitis vinifera seed extract with chlorhexidine at different concentrations. They found that the GSE as a natural antibacterial compound had inhibitory effects on Streptococcus mutans at concentrations of 250 and 500 mg/ml.[17] Since, so far, no study has examined the effect of GSE on lactobacillus, there has been no similar study to compare. It can be hoped that using GSE-containing products in combination with other caries-preventing agents can achieve more favorable results in its preventing. The preventive effects of GSE combined

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with amine fluoride on dental plaque formation and oxidative damage caused by oral bacteria were evaluated by Furiga et al. in 2014 and concluded that the GSE-amine fluoride combination had high antioxidant activity and capacity in in vitro study. [15]

Review of recent studies on optimizing the structure of different solvents to extract the active ingredient of the GBE quantitatively and qualitatively has suggested that this issue has not been thoroughly investigated. [18] Thus, in the ongoing study the antibacterial properties of aqueous, alcoholic and acetone extracts of Vitis vinifera seed were evaluated and the role of each solvent was compared. Although several studies indicated more efficacy of methanol and ethyl acetate solvents in the extraction of phenolic compounds from GSE [18, 19], the aqueous and ethanol solvents were selected for their non-toxicity to human health in the present study. [20] Besides, the acetone extract was compared for a more detailed examination.

Each of these extracts was able to inhibit the growth of Lactobacillus acidophilus and casei at different concentrations. According to the results of the present study, water was more effective than alcohol in extracting the active ingredient of Vitis vinifera seed. Thus, the aqueous extract compared to alcoholic one is a more potent inhibitor. These results are inconsistent with those of Bucic-Kojic et al. In 2009 who compared the efficacy of aqueous and ethanol solvents in extracting polyphenols from grape seeds and indicated that the best result was when using ethanol 50%. [20]

In addition, Li et al. (2008) compared the extracted phenolic compounds after using different solvent systems. Based on their results, the highest efficacy was related to acetone: aqueous solvent (70:30) and the lowest one was for aqueous solvent. [21] This difference in results could be due to the in vitro differences from the bacterial culture stage to the extraction or other stages. According to the current results, the Vitis vinifera seed extract was a good compound for preventing caries. It seems that further studies are needed to select the most effective solvent system. Vitis vinifera seed essence appears to have stronger antibacterial properties than its extract, requiring further investigation.

Conclusion

All three aqueous, alcoholic and acetone extracts indicated antibacterial activity against Lactobacillus acidophilus and casei. The stronger antibacterial effect of the aqueous extract may be due to the fact that the active ingredient of Vitis vinifera seed is extracted more effectively in the presence of aqueous solvent. It can be hoped that this herbal compound can be used as a preventive agent for tooth decay.

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Conflict of interest: There is no conflict of interest to declare.

Author's contribution

Khodadadi E. developed the study concept and design, Rajabnia M. and Moghadamnia AK. performed experimental study, Zarei M. collected data and performed the manuscript, Khafri S. performed interpretation of data and statistical Analysis.

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