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A LABORATORY STUDY ON ANTIBACTERIAL EFFECT OF ACHILLEA MILLEFOLIUM HYDROALCOHOLIC EXTRACT ON DECAYING MICROORGANISMS

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Habibeh Zare
Department of biology, payamenoor university(pnu), iran
Department of Biology, Faculty of Science, Shiraz University, Iran
habibehzare1354@gmaiil.com

Resumen: Antecedentes y objetivos: La necesidad de tratamientos tradicionales y de extracción de medicamentos a partir de materiales naturales y plantas medicinales está aumentando diariamente en el mundo. En este estudio experimental, el extracto hidroalcohólico se preparó a partir de Salvia officinalis y Achillea millefolium por maceración y su efecto antibacteriano sobre Streptococcus mutan, Lactobacillus rhamnosus y Actinomyces viscosus se evaluó mediante la macrodilución de Broth (un método mediante el cual los valores de los materiales antibacterianos se diluyen en Caldo). Los resultados fueron analizados y comparados por el método de Mann Whitney.

Hallazgos: La concentración inhibitoria mínima de Salvia officinalis y Achillea millefolium se obtuvo como 6.25 y 50 mg / ml para Streptococcus mutan, 1.56 y 12.5 mg / ml para Lactobacillus rhamnosus y 12.5 y 50 mg / ml para Actinomyces viscosus.

Conclusión: Broth macrodilution método mostró que tanto Salvia officinalis y Achillea millefolium extractos tenido un efecto inhibidor del crecimiento en las tres bacterias. Este efecto fue significativamente mayor para Salvia officinalis que Achillea millefolium y ambos extractos tuvieron un efecto bactericida en el rango de concentración estudiado.

Palabras clave: microorganismos en descomposición, extracto de plantas, actividad antibacteriana, Achillea millefolium, Streptococcus mutan, Lactobacillus rhamnosus, Actinomyces viscosus.

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Abstract: Background and objectives: The need to traditional treatments and extract medicine from natural materials and medicinal plants is increasing daily in the world. For this purpose, the present study aimed at investigating the antibacterial effect of Salvia officinalis and Achillea millefolium on decaying microorganisms.Method: in this experimental study, the hydroalcoholic extract was prepared from Salvia officinalis and Achillea millefolium by maceration method and their antibacterial effect on Streptococcus mutan, Lactobacillus rhamnosus, and Actinomyces viscosus was evaluated by Broth macrodilution (a method by which the values of antibacterial materials are diluted in Broth). The results were analyzed and compared by Mann Whitney method.

Findings: The minimum inhibitory concentration of Salvia officinalis and Achillea millefolium extract was obtained as 6.25 and 50 mg/ml for Streptococcus mutan, 1.56 and 12.5 mg/ml for Lactobacillus rhamnosus, and 12.5 and 50 mg/ml for Actinomyces viscosus.

Conclusion: Broth macrodilution method showed that both Salvia officinalis and Achillea millefolium extracts had a growth inhibitory effect on all three bacteria. This effect was significantly more for Salvia officinalis than Achillea millefolium and both extracts had a bactericidal effect on the studied concentration range.

Keywords: decaying microorganisms, plant extract, antibacterial activity, Achillea millefolium, Streptococcus mutan, Lactobacillus rhamnosus, Actinomyces viscosus

1. INTRODUCTION

Tooth decay is an infectious-microbial disease destroying the calcareous texture of tooth. Restorative and symptomatic treatment disregarding the underlying cause of the disease leads to failure (Theondor MR, Harold OH, Ward J, Swift JR. (2006). Although tooth decay is probably the most common chronic disease in the world (Theondor MR, Harold OH, Ward J, Swift JR. 2006), no plan was conducted for eradicating this microbial disease such as Smallpox and Poliomyelitis.

The dentistry care cost was 70.3 billion dollars in the USA in 2003 (Theondor MR, Harold OH, Ward J, Swift JR. 2006). The complications of this disease are losing teeth, pain, and beauty deficits. The major etiologic factors known for tooth decay are Streptococcus mutan, Lactobacillus rhamnosus, and Actinomyces viscosus (Theondor MR, Harold OH, Ward J, Swift JR. (006).

Treatment and prevention of decay by antibiotics and steroids change the oxidation-resuscitation of Saliva, weaken the Lysozyme activity, facilitate the allergic reactions, and reduce the body resistance to pathologic factors (Walsb 1997).

On the other hand, traditional medicine and herbal medicines in different fields of medical sciences were welcomed due to the use of plants as medicine since many centuries ago (Fabrican, Farnsworth 2001). Using traditional medicine is one of the methods to achieve new medicines. There are currently 119 medicines with plant origin and only 90 types were obtained among 250000 known types (Farnsworth NR 1990). However, the systematic search for the effective components of these plants and all diseases is very time-consuming, costly, and impossible (Farnsworth NR 1990). Thus, relying on traditional teachings is one of the acceptable strategies in the world to discover and use herbal medicines; the traditional medicine of Iran is one of the old bases of medical sciences containing valuable data on using plants in treatments. Oral and dental health was discussed in traditional medicine of Iran and oral-dental diseases were included in treatment books. The treatment of oral and dental diseases is divided into three main parts of measure and nutrition, taking medicine, and using tools.

Based on the medical treatment methods in traditional medicine of Iran, finding new medical references on this subject seems essential. Thus, in this study two plants of Salvia officinalis and Achillea millefolium that were among the most used plants were selected for presenting a solution with the minimum complications for controlling decay especially in those with dry mouth or uncontrollable decay due to

radiotherapy by studying the antimicrobial effect of these plants on tooth decay. Furthermore, the recent studies investigated the antimicrobial effects of these plants (La Gow 2005).

Salvia officinalis is a more well-known plant with more antimicrobial studies. This plant was considered as the plant positive control in this study (Hayouni, Chraiaf, Abedrabba, Bovix , Leveau , Mohammed and et al. 2008). No study was conducted on the antimicrobial effect of Salvia officinalis and Achillea millefolium except one study on the antimicrobial effect of Salvia officinalis only on Streptococcus (16).

Since these plants are local to Iran and can be extracted, this study examined their antibacterial effect particularly on the bacteria with proved anti-decay such as pain, and beauty deficits. The major etiologic factors known for tooth decay are Streptococcus mutan, Lactobacillus rhamnosus, and Actinomyces viscosus.

2. METHOD

This laboratory study was conducted in two steps:

2.1. Extracting

It was carried out by maceration method. Firstly, the aerial parts in plants were weighted as dry by digital balance (Lib ROR AEU-210) for 50 grams and secondly they were placed inside Erlenn after powdering. After that, 1500 cc of the solvent (50% ethanol (96%) and 50% water) was poured on each sample to completely cover the powder. After covering the tip of Erlenns by aluminum sheet, they were placed on shaker (Heidolph unimax 2010) with 90 rpm speed for 48 hours. When the solvent and plant were homogenized, the solvents were smoothed by filter paper (Watmann 0.5 mm). Then, the solvents were placed in a device called rotary evaporator (Heidolph WD 2000) to separate the solvent from the extract. The obtained pure extract was kept in sterile vials in refrigerator for conducting microbial experiments.

2.2. Testing the inhibitory activities of extracts

Standard strains were prepared as Lipophilic for Streptococcus mutan (ATCC: 35668), Lactobacillus rhamnosus (ATCC: 7469, PTCC: 1637), and Actinomyces viscosus (ATCC: 15987) from the reference centers of [AmericanATCC: type of culture collection] and [PTCC: Persian type of culture collection]. In order to prepare bacteria from lipophilic samples, firstly the samples were cultivated in liquid medium as overnight at 30-35 degrees of centigrade.After creating opacity in liquid medium, the samples were isolated on soild medium [for Actinomyces viscosus BHI agar (Spain, CONDA), Streptococcus mutan blood sheep 5 % + BHI agar (Spain, CONDA), and for Lactobacillus rhamnosus (Germany, Merck) of MRS Agar to ensure their purity.Then, the antibacterial effect of each extract on these three bacteria was studied by Broth macrodilution method based on CLSI (Clinical Laboratory Standards Institute, 2006, M7-A4.USA).Firstly, 800 mg/ml stock solvent was prepared from each extract and sterilized by filter (0/22 um Millipore filters). Then, the extracts were diluted in 11 tubes containing liquid medium (for Thyioglycollate Actinomyces viscosus (USA, Difco), Medium for BHI Streptococcus mutan (Spain, CONDA), broth for Lactobacillus rhamnosus (Germany, Merck) by serial dilution method (1/2 dilution) (There was finally 500 ml of medium and extract in each tube). After preparing the bacteria suspension based on McFarland 0.5 tube (McFarland is a chemical suspension that its opacity rate can be compared to microbial suspension). Through this, the number of bacteria in each milliliter of suspension can be estimated (Faddin 2000), $1.5x10^8$ CFU(s) (colony forming unit) per milliliter was obtained and diluted by medium as 1/100 to obtain $1.5x10^6$ CFU per milliliter. Then, 500 microliter of bacteria suspension was added to each tube. Finally, the concentration range of tubes was 200-0.15 mg/ml. Then, heating was performed for 20 hours at 37 degrees of centigrade. After the occurrence of growth, the opacity in tubes was studied to evaluate the growth or lack of growth in bacteria. The concentration of the first tube in which no growth was observed was considered as the minimum inhibitory concentration of bacteria growth by that extract. Then, the tubes with no growth were cultivated in Agar solid medium. The first plate with no growth was considered as the minimum inhibitory concentration of extract for that bacterium. The controls were as follows:

Medium and extract without bacteria -> lack of growth, medium, distilled water with bacteria -> growth, medium and Chlorhexidine (positive control) with bacteria -< lack of growth.

In addition, heating on Actinomyces viscosus and Streptococcus mutan was performed in anaerobic jar adjacent to CO2. These stages were repeated three times for the two extracts and for all three types of bacteria. The results were analyzed and compared by Mann-Whitney ($p<0.05$).

3. FINDINGS

In Broth macrodilution, the amount of MIC and MBC per mg/ml and their significance level ($p<0.05$) for each extract on three bacteria species were shown in Table 1 (small amounts of MIC and MBC mean the antibacterial effect).

In case of Streptococcus mutan, the amount of MIC and MBC for Salvia officinalis was 6.25 and 50 mg/ml which is significantly less than MIC and MBC for Achillea millefolium as 500 and 200 mg/ml. in case of Lactobacillus, the amount of MIC and MBC for Salvia officinalis was1.56 and 12.5 and for Achillea millefolium was 12.5 and 12.5 mg/ml. the amount of MIC for Salvia officinalis was significantly less than Achillea millefolium but MBC had no significant difference. In case of Actinomyces viscosus, the amount of MIC and MBC for Salvia officinalis was 12.5 and 12.5 which is significantly less than Achillea millefoliumas 50 and 100 mg/ml.

Tooth decay is the destructive disease of tooth hard texture. While the etiology of this disease is multifactor, it was proved to be microbial plaque-dependent diseases (Prabu , Gnanamani , Sadulla 2006). Decay increases at the presence of Acidogenic bacteria such as Streptococcus mutan diseases (Prabu , Gnanamani , Sadulla 2006). Streptococcus mutan is the first and most important microorganism in plaque with proved anti decay (Fejerskov , Kidd 2008). This microorganism has a basic role in beginning of decay (Summitt, Robbins, Hilton, Schwartz 2006) and is observed in all waste lesions of enamel and ivory rot. Lactobacillus has a role in decay progress and Actinomyces plays a role in root decay lesions (Fejerskov , Kidd 2008). Thus,

Removing the bacterial base of tooth decay is one of the factors to remove this infection. In recent years, searching for new antimicrobial substances of plants was considered due to the increase of pathogenic microorganisms' resistance to the current medicines and side effects of antibiotics. In this study, the antibacterial effects of hydroalcoholic extract of Salvia officinalis and Achillea millefolium on three main bacteria causing tooth decay were studied by Broth macrodilution method.

Based on Table 1, both studied extracts had an antibacterial effect on the three above-mentioned bacteria. The inhibitory effect of Salvia officinalis was significantly more than Achillea millefolium for all three bacteria. Salvia officinalis is a more well-known plant on which more antimicrobial studies were conducted (Weckesser, Engel, Haarhaus, Wittmer, Pelz, Schmepp 2007). Its function on Anorexia, oral inflammation, and sweating increase was confirmed by herbal medicine references (La Gow 2005).

In previous studies, the effect of hydroalcoholic extract of Salvia officinalis leaf on Collagenolin Porphyromonas gingivillis activity was shown (Kemper 2003) and the wide antibacterial and antifungal effect of Salvia officinalis on a wide range of bacteria such as Pseudomonas and Aspergillus was reported (1121). The findings of this study on the bactericidal effect of Salvia officinalis were consistent to the above-mentioned studies.

In the study of Sanei et al **(16)**, the effect of the extract of some plants including Salvia officinalis on a number of oral pathologic bacteria such as Staphylococcus aureus, Streptococcus Salivares, and Streptococcus mutan was studied. Salvia officinalis with concentrations of 4.8 mg/ml had a growth inhibitory effect on this microorganism at 60 and 90 seconds. However, in the present study, the effect of Salvia officinalis and Achillea millefolium on all decaying microorganisms was studied. It has more totality than the study of Sanei et al.

Achillea millefolium is one of the plants that were used since the past in treating the wounds, digestive problems, and infectious diseases and even in reducing the blood fat. Chamazulen, Caryophyllene, 1-8 Cineol, and Flavonoids like Epigene and Rutin are the

components found in Achillea millefolium (La Gow 2005).

Achillea millefolium had an inhibitory and bactericidal effect on the studied strains that was consistent with the results of Candan et al (Candan, Unlu, Tepe, Daferera, Polissiou, Sokmen and et al 2003.) On the antibacterial effect of Achillea millefolium essence. The presence of Caryophyllene and 1-8 Cineol compounds in Achillea millefolium and Salvia officinalis is of great importance discussing the probability of the effect of these compounds on bacteria. Thus, since in the results of this study the hydroalcoholic extract of Salvia officinalis and Achillea millefolium had a growth and bactericidal inhibitory effect on the bacteria causing tooth decay, it is suggested to study the antibacterial effect of their common effective materials including 1-8 Cineol and Caryophyllene in the future. On the other hand, due to the importance of traditional treatments and the need to extract medicine from plants and microbial resistance to chemical medicine including antimicrobial mouthwash from these plants seem useful and essential.

4. CONCLUSION

1. Both extracts of Salvia officinalis and Achillea millefolium had growth inhibitory effect on all three bacteria species.
2. The inhibitory effect of Salvia officinalis was significantly more than Achillea millefolium.
3. In the studied concentration area, both extracts had bactericidal effect on all three bacteria.

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