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ANTIMICROBIAL EFFECT OF ROSMARINUS OFFICINAL EXTRACTS ON BIOFILM OF SOME IMPORTANT HUMAN BACTERIAL PATHOGENS

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Abstract:

Microorganisms form biofilm from a self-produced extracellular polymeric, substances under which they develop their colony. These biofilms are very tough to eradicate and microorganisms cause various infections. In this study, the anti-biofilm and antibacterial activities of extract Rosmarinus officinalis were examined.

Materials and Methods: Bacterial strains were obtained from standard laboratory and growth and biofilm formation of strains were determined by microtiterplate method. Rosemary extracts were prepared by maceration.

Results: The levels of Minimum Inihibitory Concentration (MIC) was observed ranges from 3.1 to 12.5 ppm. The highest MIC value was observed against Streptococcus pyogenes, Streptococcus pneumoniae and Staphylococcus saprophyticus and The minimum MIC value of rosemary extract concentration in 1/3 ppm that against Staphylococcus aureus. The results of this study showed that the rate of absorption (OD) for biofilm formation of Staphylococcus aureus and Streptococcus pneumoniae in concentrations 50 ppm and 100 ppm rosemary extract to zero value.

Conclusion: Results of this study suggest that the extract of Rosmarinus officinalis may be useful alone to treat bacterial infections.

Introduction

According to World Health Organization(Santos et al, 1995) medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficiency (Ellof, 1998; Nascimento et al, 2000; Mothana et al, 2009). Rosemary plant with the scientific name Rosmarinus officinalis Lamiaceae family, and as a spice and medicinal herb is commonly known in many countries. It has anti-bacterial, antioxidant and anti-mutagenic properties (Campo and Amiot, 2000; Ozcan , 2003; Minnunni et al, 1992).

It has been cultivated since ancient days in England, Germany, France, Denmark and other Scandinavian countries, Central America, Venezuela and the Philippines. Rosemary is one of the ancient cult plants, closely associated with love and marriage, birth and death.Few bacteria live as free floating cells in nutrient rich mediums, and nearly majority of them depend on other microorganisms for energy, carbon and other nutrients and live in microecosystems filled with hundreds of other microorganisms. it has estimated that in the natural world more than 99% of all bacteria exist as biofilms(Costerton et al., 1987). A biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix. Biofilms share an important structural feature: their constituent cells are bound together by an extracellular matrix that mainly consists of macromolecules, including polysaccharides, proteins, and nucleic acids, that are produced by the cells themselves. In this study, the antibacterial and anti-biofilm activities of extract Rosmarinus officinalis were examined.

Material and methods

Bacterial strains and culture conditions:

Bacterial strains were obtained from standard laboratory. Evaluate the antibacterial activity of the plant extracts were investigated using strain of bacteria Streptococcus pyogenes ATCC® 19615™, Streptococcus pneumoniae ATCC 49619, S.saprophyticus ATCC®15305, Proteus mirabilis ATCC 35659, Serratia marcescens ATCC 274, Staphylococcus aureus ATCC® 25923. The typed cultures of bacteria was sub-cultured on Nutrient agar (Oxoid) and stored at 4oC until required for study.

Plant materials:

The leaf of Rosmarinus officinalis were collection in the region of Iran and under appropriate conditions and has been dried in the shade. Samples were crashed and transferred into glass container and preserved until extraction procedure was performed in the laboratory.

Preparation of extracts:

Plants were properly dried and pulverized into a coarse powder. Each of 10 g grinded powders was soaked in 60 ml ethanol 95 %, separately for one day (shaking occasionally with a shaker). After one day of dissolving process, materials were filtered (Whatman no. 1 filter paper) .Then the filtrates were evaporated using rotary evaporator. At last, 0.97 g of dried extracts were obtained and then stored at 40 °C in air tight screw-cap tube.

Minimum Inhibitory Concentration (MIC) of extract:

The broth microdilution method was used to determine MIC. All tests were performed in Mueller Hinton broth supplemented with Tween 80 at a final concentration of 0.5% (v/ v). Briefly, serial doubling dilutions of the extract were prepared in a 96-well microtiter plate ranged from 6.25 ppm to 100 ppm. To each well, 10 µl of indicator solution (prepared by dissolving a 10-mg extract in 2 ml of DMSO) and 10 µl of Mueller Hinton Broth were added. Finally, 10 µl of bacterial suspension was added to each well to achieve a concentration of Under minimum inhibitory 104 (colony forming

unit/ milliliter) CFU/ml and 106 CFU/ml change to 104CFU/ml and 106 CFU/ml. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates, and then they were placed in an incubator at 37°C for 18–24 hours. The color change was then assessed visually. The lowest concentration at which the color change occurred was taken as the MIC value. The average of 3 values was calculated providing the MIC values for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate the visible growth. The microorganism growth was indicated by turbidity.

Biofilm formation assay in presence of the biocides:

After performing the procedure described above, the microplate was covered and incubated aerobically for 24 h at suitable temperature. At first, the OD (Optical Density) was measured (600 nm) by using an automated ELISA counter, then, the content of each well of the microplate was aspirated and each well was washed three times with 250 µL of sterile physiological saline.

The remaining attached bacteria were fixed with 200 µL of 99% methanol per well and after 15 minute all of the wells were emptied and left to dry. Then, each well was stained for 5 minute with 0.2 mL of 2% crystal violet. Excess stain was rinsed off by washing the plate slowly with distilled water. After the plate was air dried, the dye bound to the adherent cells was resolubilized with 160 mL of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 492 nm by using an automated ELISA counter.

Statistical Analysis:

All experiments and measurement were repeated at least three times. Statistical analyses were performed using SPSS and Excel 2010 software. All experimental results were analyzed using mean descriptive statistics and the correlation-coefficient. A value of $P<0.05$ was regarded as statistically significant.

Result:

The results of rosemary extract in the Human Pathogens shown that the minimum inhibitory concentration of rosemary extract concentration 3.1 ppm that against Staphylococcus aureus has been viewed as the most inhibitory concentration 12.5ppm that S.pyogenes bacteria, Streptococcus pneumoniae and Staphylococcus aureus is inhibited

Table1. Antimicrobial susceptibility, MIC extract plant for Standard bacteria

Antibiotic resistant	MIC(ppm) Rosmarinus officinalis	Bacterial code
E,CE,TE	3.1	S.aureus
-	12.5	S.pyogenes
E,CE,CF	12.5	S.pneumoniae
E,CF,TE	12.5	S.saprophyticus
E,TE	6.25	P.mirabilis

E= Erythromycin, CE= Cefixime, CF= Ceftazidime, TE= Tetracyclin

Tabel2. Satureja extract intensity pattern of deterrence against human pathogens in different concentrations

100ppm	50ppm	25ppm	12.5ppm	6.25ppm	3.1ppm	Bacterial cod
-	-	-	-	-	+	S.aureus
-	-	-	+	++	++	S.pyogenes
-	-	-	+	++	++	S.pneumoniae
-	-	-	+	++	++	S.saprophyticus
-	-	-	-	+	++	P.mirabilis

++ Represents enormous growth of microorganisms
+ Represents the low growth of microorganisms
- Represents absence growth of microorganisms

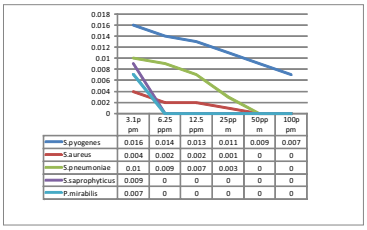

Figure1.The effects of different concentrations of extract plant o the biofilm formation of bacterial

The results of this study showed that the rate of absorption (OD) for biofilm formation of Staphylococcus aureus and Streptococcus pneumoniae in concentrations ppm50 and ppm100 rosemary extract to zero value If the biofilm formation of Streptococcus pyogenes in concentrations equal to 0.009 and 0.007 ppm50 and ppm100's Nano, while the biofilm P.mirabilis and S.saprophyticus concentration ppm 25, ppm 50 and unformed ppm100(Figure1).

Discussion:

Natural products derived from medicinal plants are abundant source of biologically active compounds. Many plant compounds have been used for development of new antimicrobial agents (Cowan, 1999). However, few plant extract have been investigated for their antibiofilm activity. In the east and the excellent effect of different concentrations of alcohol extract of rosemary, Hypericum and Carthamus on different growth stages of E. coli were examined The results showed that in the early hours of the three concentrations (2.0, 3.0 and 4.0 grams per ml) were observed in E. coli in the presence of alcoholic extract of rosemary grow less than the other two species and in the least effect of alcoholic extract of Hypericum. Three hours later, the changes will reverse and reduce the growth of E. coli in the presence of alcoholic extracts of Hypericum extracts are more than others (Mashreghi and momtazi, 2012). In another study of the effects of rosemary extract on pathogenic microorganisms such as Leuconostoc mesenteroides, listeria monocytogenes, Staphylococcus aureus, Streptococcus mutans and Bacillus cereus, the results showed that the minimal inhibitory concentration for various bacteria and from 0.60% for Bacillus cereus started and the 0.1% for the Leuconostoc mesenteroides(Campo and Amiot, 2000). The study Okoh have studied the antibacterial effects Rosemary Rosemary results showed that the concentration of the antimicrobial effects of good (Okoh et al, 2010). The study of Gachkar 2007, essential oils extracted by hydrodistillation from Cuminum cyminum and Rosmarinus officinalis were characterized by means of GC and GC–MS. C. cyminum and R. officinalis contained α-pinene (29.1%, 14.9%), 1,8-cineole (17.9%, 7.43%) and linalool (10.4%, 14.9%), respectively, as the major compounds. C. cyminum oil exhibited stronger antimicrobial activity than did R. officinalis oil against E. coli, S.aureus and L. monocytogenes (Gachkar et al, 2007). The study of Wang, GC–MS analysis of the essential oil resulted in the identification of 19 compounds, representing 97.97% of the oil, the major constituents of the oil were described as 1,8-cineole (27.23%), α-pinene (19.43%), camphor (14.26%), camphene (11.52%) and β-pinene (6.71%)(Wang et al, 2008). Viuda-Martos study results showed that the inhibitory zone diameter rosemary essential oil in concentrations of 25, 50 and 100 times the diameter of inhibition zone with 10.88 ± 0.03, 0.21 ± 15.81, 17.23 ± 0.9 mm against Streptococcus xylosus created, the inhibitory zone 12.51 ± 0.87, 17.26 ± 0.61 and 23.53 ± 0.79 mm against Staphylococcus carnosus, the inhibitory zone 11.69 ± 0.55 , 21.19 ± 0.39, 1.67 ± 28.47 mm against the bacteria Enterobacter gergoviae, the inhibitory zone 11.75 ± 0.58, 12.93 ± 0.71, 18.07 ± 0.83 mm m against Enterobacter amnigenus, Inhibition zone 12.23 ± 0.77, 16.45 ± 0.50, 20.17 ± 0.79 mm and the diameter of the inhibitory Lactobacillus sakei 11.94 ±0.29, 15.61 ± 0.74, 18.82 ± 0.73 mm against bacteria Lactobacillus curvatus created (Viuda-Martos et al., 2008). The study of Bozin, the essential oils of rosemary (Rosmarinus officinalis L.) and sage (Salvia officinalis L.) were analyzed by means of gas chromatography–mass

spectrometry and assayed for their antimicrobial and antioxidant activities. Antimicrobial activity was tested against 13 bacterial strains and 6 fungi, including Candida albicans and 5 dermatomycetes. The most important antibacterial activity of both essential oils was expressed on Escherichia coli, Salmonella typhi, S. enteritidis, and Shigella sonei. A significant rate of antifungal activity, especially of essential oil of rosemary, was also exhibited (Bozin et al., 2007). The results indicated that the rosemary extracts showed antibacterial activity, according to Weckesser et al. (2007), mainly against the Gram-positive bacteria (S.aureus and B. cereus). The extracts also exhibited an effect against the Gram-negative bacteria (E. coli and P. aeruginosa). The study of J.Jordan, that, the essential oil yield, volatile profile and antimicrobial activity of individual Rosmarinus officinalis L. shrubs growing wild in the different bioclimatic areas of the province of Murcia (Spain) were studied. Determination of the diameter of inhibition in Salmonella typhimurium pointed to a positive contribution effect of eucalyptol and α-pinene. A high proportion of α-pinene increases the effectiveness of the oil against Staphylococcus aureus, while the presence of eucalyptol, as the most abundant compound, considerably decreases the efficiency of rosemary oil. In contrast, the efficacy of these oils against Listeria monocytogenes and Escherichia coli was not affected by this condition. As regards the minimum inhibitory (MIC) and bactericide (MBC) concentrations, the strong activities exhibited by these essentials oils (<0.5 µL/mL) did not allow the chemotypes and antibacterial activities to be differentiated (J.Jordan et al., 2013). The study of Varposhti, biofilm formation of P. aeruginosa strain 214 was determined in presence of three plant extracts, Cyclamen coum, Dianthus orieltalis and Origanum majorana, and Zataria multiflora Bio essential oil. Minimum Biofilm Inhibitory Concentrations (MBICs) were determined by microdilution techniques and XTT assay. The C. coam extract and Z. multiflora Bio essential oil inhibited biofilm formation completely at concentrations<0.062 mg/ml and 4 7l/ml, respectively. The D. orientalis and O. majorana extracts did not inhibit biofilm formation at the used concentrations (0.003 – 8 mg/ml) (Varposhti et al., 2013). Antibiofilm activity of Cyclamen hederifolium extract on Methicillin-Resistant Staphylococcus aureus (MRSA) has been reported by Quave et al. (Quave et al., 2008). The study of Shafiei, the resulr showed that one and three day old biofilm are effected by either. ciprofloxacin or n-butanolic C. coum extract. However, n-butanolic C. coum extract in combination with ciprofloxacin was significantly more effective against P. aeruginosa biofilms(Shafiei et al., 2014). Anti biofilm effect of various plant extracts against biofilm of human pathogenic bacteria has been reported by workers (Essawi and Srour, 2000).

Biofilm is the one of the major virulent factor of most of the pathogenic microorganism Therefore, the developments of effective and safe medicine particularly plant extracts with antimicrobial properties have recently received growing interest from both academic and industrial sectors. The present study demonstrated the effective biofilm inhibition of S.pyogenes, S.Pneumoniae, S.saprophyticus, P.mirabilis, S.marcescens, S.aureus by plant extracts coated with biopolymer. Further study will helpful to understand molecular mechanism of anti-biofilm effect of chitosan coated extracts.

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