Comparative study between pre and post bacterial growth of periodontal infections by treatment with extracts Rue. An in vitro study.

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Abstract
Background/ purpose: Oral diseases are major health problems with periodontal diseases. Oral health is linked to acute and chronic periodontal diseases, which have been linked to anaerobic Gram-negative bacteria. To determine antimicrobial medicinal effect of the extracted Rue which used in treating of periodontal diseases.

Materials and methods: A group of 30 patients undergoing of periodontal diseases were investigated by making growth culture pre and post of their treatment with extracted Rue, or phytochemicals that inhibit the growth of oral pathogens. Then making a comparative between the extracted Rue and 10 types of antibiotics for three types of bacteria Aggregatibacter actinomycetemcomitans, Tannerella forsythia and Porphyromonasgingivalis.

Results: The result show that there is 7(23%), 3(10%) and 3(10%) isolates of each bacteria respectively. The inhibitory effect of extracted Rue show significant effect by inhibiting growth of bacteria in vitro 5(71%),2(67%) and 3(100%). Among 10 type of antibiotic used and compared with extracted Rue showed significant inhibitory effect for A. actinomycetemcomitans. of antibiotic for Chloramphenicol, , Nitrofurantoin, Oxacinil and Lincomycin, while the antibacterial effect on T. forsythia of antibiotic Chloramphenicol, Ampicloxacillin, Carbenicillin and Bacitracin, and The antibacterial effect on P. gingivalis of antibiotic showed significant inhibitory effect on bacterial growth for Chloramphenicol, Ampicloxacillin, Amoxicillin and Bacitracin.

Conclusion: The incidence of oral disease, antibiotics resistance increased by bacteria and adverse affects of some antibacterial agents used in dentistry and financial considerations in developing countries, there is a need for alternative prevention and treatment options that are safe, effective and economical.

Keywords: Extracted Rue; Periodontal diseases; Antibiotics resistant; Gram-negative bacteria; Peganum harmala alkaloid; Pharmacological activity.

INTRODUCTION

Nowadays, bacterial infections especially those caused by multi-drug resistant bacteria have become one of the great challenges for modern healthcare. Therefore, discovering new antibacterial compounds with improved activity is necessary. Majority of scientists define multi-drug resistance as resistance to at least 3 classes of antimicrobial agents

Oral diseases continue to be a major health problem worldwide, among the most important global oral health problems, the primary etiologic agent of periodontal disease is the microbial population of the dental plaque in humans. Periodontal diseases, including gingivitis and periodontitis, are serious infections that, left untreated, can lead to tooth loss. Periodontal disease, which effects the tissue around teeth, is a chronic bacterial infection that affects the gum and bone supporting the teeth. Periodontal disease can affect one tooth or many teeth. It begins when the bacteria in plaque (the sticky, colorless film that constantly forms on teeth) causes the gums to become inflamed 2. With time, if untreated, plaque can spread and grow below the gum line. Toxins produced by the bacteria in plaque irritate the gums. The toxins stimulate a chronic inflammatory response in which the body in essence turns on itself, and the tissues and bone that support the teeth are broken down and destroyed. Gums separate from the teeth, forming pockets (spaces between the teeth and gum) that become infected. There are many forms of periodontitis. The most common ones include the following. Gingivitis, Aggressive periodontitis, Chronic periodontitis, Periodontitis as a manifestation of systemic diseases, Necrotizing periodontal diseases. Periodontal disease is often silent, meaning symptoms may not appear until an advanced stage of the disease. However, warning signs of periodontal disease include the following: Red, swollen or tender gums, bleeding while brushing, flossing, or eating hard food, gums that are receding or pulling away from the teeth, causing the teeth to look longer than before. Loose or separating teeth, pus between gum and teeth, sores in mouth, persistent bad breath, and change in the way of teeth bite. Early diagnosis is important for successful treatment of periodontal diseases. Therefore, it is important that periodontists are also dentists’ experts in the treatment of oral inflammation. Aggregatibacter actinomycetemcomitans, Tannerella forsythia and Porphyromonasgingivalis, are a anaerobic Gram negative bacteria, Aggregatibacter actinomycetemcomitans, is coccoabacillus, is nonmotile, is non–spore forming, and has been strongly implicated in the pathogenesis of periodontitis, mainly in aggressive periodontitis, and in systemic infections such as endocarditis and soft tissue abscesses. Aggressive periodontitis often occurs in young people and causes a rapid loss of attachment and alveolar bone affecting teeth, particularly first molars and incisors. An important virulence factor produced by A. actinomycetemcomitans is the leukotoxin that is genetically composed of a cluster of 4 genes that gives to this species the ability to kill human polymorph nuclear leukocytes.

This microorganism produces adhesions used in early events of infectious processes by attaching to specific receptors on oral mucosa and dental plaque on dental surfaces and epithelial cells using its own extracellular polymer. The bacterial ability of adherence to oral surfaces is an important property of biofilm-forming bacterium production which is a pili- or fimbriamediated process. In addition, this microorganism also produces a polysaccharide, poly-β-1,6-N-acetyl-D-glucosamine, which is also associated with adherence and invasion. A. actinomycetemcomitans produces rough and smooth colonies which are characterized for producing fimbriae, and smooth colonies produce extracellular microvesicles that are associated with the attachment process.
Rue (Syrian Rue, or Peganum harmala), with small, brown seeds contains harmine and other harmala alkaloids, are commonly used as a short-acting reversible Mono Amine Oxidase Inhibitor \(^{11}\). The plant is usually used orally as mouthrinse with seeds as an extracted Rue. One of the most striking features of this plant is indeed the strong, aromatic, bitter or acrid scent, By analyzing the medicinal uses and risks of rue, one aspect became very clear the energy cures can also kill the bacteria, it depends only on the amount of concentration of extracted Rue that used. 

The dry seeds contain significant amounts of harmine and harmaline (respectively at the concentration of 5.6% and 4.3 %). Harmaline and harmine are very potent inhibitors of monoamine oxidase \(^{12}\).

Antimicrobial properties of extracted Rue have been investigated in order to suggest them as potential tools to overcome the microbial drug resistance. As a medicinal herbal treatment was recommended in very different diseases, but here in our study, were using the extracted Rue as antibacterial, antimicrobial, and antifungal, and anti-inflammatory herbal treatment in the infected oral cavity as mouthrinse, and helps to protect against free radicals. The appropriate dose of rue depends on several factors such as the user's age, health, and several other conditions.

Chemical analysis of the rue herb has shown the presence of several elements in it, more than 15 compounds in Rue have been identified as having in vitro antibacterial and antifungal activity. 

One report suggests that extracts of Rue graveolens demonstrated inhibitory effects against gram positive organisms such as Staphylococcus aureus, Streptococcus pyogenes, Listeria monocyctogenes, and Bacillus subtilis \(^{13}\). Other researchers have found that a number of components of rue interfere directly with DNA replication, thereby preventing the propagation of some viruses. 

The aim of this research is to study the antimicrobial and antibacterial and antifungal effects of medicinal extracted Rue from seeds in treating of periodontal diseases (An in vitro study).

### MATERIALS AND METHODS

#### Identification of isolates:
Identification of Bacterial Isolates were identified to the level of species in the diagnostic microbiology lab. College of dentistry/ Kufa university \(^{16,17}\).

#### Antibiogram test

In vitro susceptibility tests were performed on Mueller-Hinton agar by the disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI).

After streaking the colony on Mueller-Hinton agar, the antibiotic disks was added after 15 minutes on the plates by sterile forceps. Then, the plates were incubated at 37°C for overnight (24-48 hours) in anaerobic jar. The results were read according to CLSI standards as in table 1.

<table>
<thead>
<tr>
<th>Antibiotic types</th>
<th>Abbreviation</th>
<th>Dosages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>C</td>
<td>10μg</td>
</tr>
<tr>
<td>Ampicillin, Cloxacillin (Ampicloxacillin)</td>
<td>APX</td>
<td>(25 μg, 5μg respectively) 10μg</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>MET</td>
<td>30μg</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>PY</td>
<td>25μg</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>AX</td>
<td>25μg</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>F</td>
<td>300μg</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>OX</td>
<td>5μg</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>L</td>
<td>10μg</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>DA</td>
<td>10μg</td>
</tr>
<tr>
<td>Bactracin</td>
<td>B</td>
<td>10 units</td>
</tr>
</tbody>
</table>

The result show that there is 7(23%) isolates of each bacteria respectively.

### Preparation of extract Rue

Peganum harmala (Syrian Rue) seeds, we used 5000 gm in 25000 ml of distal water after boiling at 100 °C for 30 mints, and then take the extracted Rue only by filtration the fluid with concentration of 20% \(^{11}\). A group of 30 patients with different ages and genders undergoing of periodontal diseases were investigated by making growth culture pre and post of their treatment with Rue extracts. Harmine belongs to the family of β-carboline alkaloids, its chemical name is 7-methoxy-1-methyl-9H-pyrrole[3,4-b]indole, and its molecular formula is \(C_{13}H_{12}N_2O\). It has a molecular weight of 212.25 and a melting point of 261° C \(^{18}\). The chemical structure of harmine is shown as follows figure 1.

![Figure (1): The chemical structure of harmine](image)

Harmine and its analogs thereof are widely distributed in nature. Numerous researches on the synthesis of harmine derivatives have been reported since harmine was first isolated from Peganum harmala. Current research shows, harmine hydrochloride having a wide range of pharmacological effects of antibacterial, antifungal, and anti-inflammation.

### Preparation of dried filter paper discs

Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven. Dispense 0.005 ml (5 microliter) of solution using sterile micropipette tips.

### RESULTS:

All oral diseases, the incidence of those that have a microbial etiology is greatest in all parts of the world. Numerous traditional medicinal plants have been evaluated for their potential application in the prevention or treatment of oral diseases. Many studies investigating the activity of traditional medicinal plants against oral pathogens. In our study, (An in vitro study).

We were using the medicinal extracted Rue as a mouth rinse for 10 days, 3 times daily with concentration of 20%, in order to prove the effectiveness of this extracted as antibacterial, antimicrobial, antifungal, and anti-inflammatory agents without any side effects. Then making a comparative between the extract Rue and 10 types of antibiotics for three types of bacteria Aggregatibacter actinomycetemcomitans, Tannerella forsythia and Porphyromonas gingivalis.

The result show that there is 7(23%), 3(10%) and 3(10%) isolates of each bacteria respectively. The inhibitory effect of extract Rue show significant effect by inhibiting growth of bacteria in vitro 5(71%),2(67%) and 3(100%). Among 10 type of antibiotic used and compared with extract Rue showed significant inhibitory effect for A. actinomycetemcomitans, of antibiotic for Chloramphenicol, Ampicloxacillin, Nitrofurantoin, Oxacillin and Lincomycin, while the antibacterial effect on T. forsythia of antibiotic Chloramphenicol, Ampicloxacillin, Carbenicillin and Bactracin, and antibacterial effect on P. gingivalis of antibiotic showed significant inhibitory effect on bacterial growth for Chloramphenicol, Ampicloxacillin, Amoxicillin and Bactracin, the results show in table 1, and figure 2 below.
Table (2): The percentage of bacterial isolates of three type of bacteria (*A. actinomycetemcomitans, T. forsythia* and *P. gingivalis*) out of 30 patients before and after treatment with extracted Rue.

<table>
<thead>
<tr>
<th>Type of bacteria</th>
<th>No. of isolates before treatment</th>
<th>Percentage of isolated bacteria out of 30 patients (%)</th>
<th>No. of isolates after treatment</th>
<th>Percentage of isolates after treatment for each type of bacteria (%)</th>
<th>No. of clear growth after treatment</th>
<th>Percentage of clear growth after treatment for each type of bacteria (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>7</td>
<td>23</td>
<td>2</td>
<td>29</td>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td><em>T. forsythia</em></td>
<td>3</td>
<td>10</td>
<td>1</td>
<td>33</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td>3</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>100</td>
</tr>
</tbody>
</table>

The antibacterial effect on *A. actinomycetemcomitans* of antibiotic showed significant inhibitory effect on bacterial growth for Chloramphenicol, Ampicloxacillin, Nitrofurantoin, Oxacillin and Lincomycin, while it resist for Metronidazole, Carbenicillin, Amoxicillin, Clindamycin and Bacitracin as in figures 3, 4.

The antibacterial effect on *T. forsythia* of antibiotic showed significant inhibitory effect on bacterial growth for Chloramphenicol, Ampicloxacillin, Carbenicillin and Bacitracin, while it resist for Metronidazole, Amoxicillin, Nitrofurantoin, Oxacillin, Lincomycin and Clindamycin as in figures 5, 6.

Figure (2): Antibacterial activity of 10 type of antibiotics compared with Rue extract in the growth of *A. actinomycetemcomitans*.

Figure (3): a culture of *A. actinomycetemcomitans* show resistant for Metronidazole and Carbenicillin and sensitive for Ampicloxacillin, Oxacillin and Lincomycin compared with sensitivity for Rue extract disc in the center.

Figure (4): Antibacterial activity of 10 type of antibiotics compared with Rue extract in the growth of *T. forsythia*.

Figure (5): a culture of *T. forsythia* show resistant for Metronidazole, Carbenicillin, Ampicloxacillin, Oxacillin and sensitive for Chloramphenicol and Bacitracin compared with sensitivity for Rue extract disc in the center.

Figure (6): Antibacterial activity of 10 type of antibiotics compared with Rue extract in the growth of *P. gingivalis*. 
The antibacterial effect on *P. gingivalis* of antibiotic showed significant inhibitory effect on bacterial growth for Chloramphenicol, Ampicloxacillin, Amoxicillin and Bacitracin, while it resist for Metronidazole, Carbencillin, Nitrofurantoin, Oxacillin, Lincomycin and Clindamycin as in figure 7.

**Figure (7):** a culture of *P. gingivalis* show resistant for Metronidazole, Ampicloxacillin, and Bacitracin and sensitive for Chloramphenicol and Amoxicillin, compared with sensitivity for extracted Rue disc in the center.

**DISCUSSION**

In recent years, an explosive spread of multi-drug resistance bacterial pathogens has become a serious concern worldwide in terms of public health and economic effects. Over the past two decades, natural compounds with antibacterial, antifungal and anti-inflammatory properties have received considerable attention as new therapeutic agents, source of pharmaceutical natural products, for the treatment of periodontal infections. Periodontal diseases are polymicrobial infections and are the most common chronic inflammatory disorders. Periodontitis is induced by a specific group of Gram-negative anaerobic bacteria and is the major cause of tooth loss in adults. Given the incidence of oral disease, increased resistance by bacteria to antibiotics.

In this study, we investigated the potential of the antibacterial, antimicrobial, antifungal and anti-inflammatory activity of extracted Rue from *P. harmala*, in this project, we were evaluated that by use it as in vitro study and in vivo study to treating an infectious mouth clinically as periodontal diseases. Rue has been used as a medical preparation and has a variety of roles, probably because of its varied chemical composition. Rue benefits and medicinal uses antimicrobial and anti-bacterial. One of other important features of *P. harmala* alkaloids is their bactericidal activity were compared with another common antibiotics in its effectiveness against some bacterial defect, so that kill and prevent bacterial infections. The chemicals in rue help decrease and reduce swelling as inflammation. It has been reported that harmine as a highly aromatic planar alkaloid exerts its antibacterial activity through intercalate with DNA, thus, this antibacterial mechanism must be considered for active extract of *P. harmala*.

Harmine in its chemical structure considered as pharmacological importance and therapeutic potentials with tricyclic beta-carboline alkaloid having a core indole structure and a pyridine ring, that was originally isolated from seeds of *Peganum harmala* in 1847 are known to be strong inhibitors of which metabolizes catecholamine neurotransmitters. Harmine has been commonly used for ritual and medicinal preparations in the Middle East, Central Asia and South America, and widely distributed in nature. Harmine have antimicrobial, antiplasmodial, antifungal, antitumoral, antimutagenic, cytotoxic and hallucinogenic properties. Beta-carboline compounds act as inverse agonists at the benzodiazepine site of the gamma-aminobutyric acid type A receptors and have actions entirely opposite to those of the anxiolytic benzodiazepines. These compounds are also associated with the potentiation of monoaminergic pathways through inhibition of A or B, blockade of reuptake sites and direct activation of monoamine receptors.

Antibiotics have been continuously to be the only effective treatment of periodontal infections caused by periodontal bacteria in biofilm. However, sufficient evidence exists that antibiotic resistance has increased in the periodontal flora over the last decades. This resistance due to widespread use of these antibiotics.

Earlier studies showed a resistance of periodontal flora to penicillin and tetracycline. Recent studies have found that periodontal microorganisms, in patients with periodontal infections, exhibit moderate susceptibilities to clindamycin, metronidazole and amoxicillin. In our study, according to isolated gram-negative bacteria from patients mouth, amazing results were getting, as showed in figures 2 and 3, as the antibacterial effect on *A. actinomycetemcomitans* of antibiotic showed significant inhibitory effect on bacterial growth for Chloramphenicol, Ampicloxacillin, Nitrofurantoin, Oxacillin and Lincomycin, while it resist for Metronidazole, Carbencillin, Amoxicillin, Clindamycin and Bacitracin. While the another flora of biofilm, which is *Tannerella forsythia* gave the results as in figures 4 and 5, that explained the antibacterial effect on *T. forsythia* of antibiotic showed significant inhibitory effect on bacterial growth for Chloramphenicol, Ampicloxacillin, Carbencillin and Bacitracin, while it resist for Metronidazole, Amoxicillin, Nitrofurantoin, Oxacillin, Lincomycin and Clindamycin. The final microorganism in biofilm of periodontal disease, which cultured from patients mouth is *Porphyromonas gingivalis*. The results were very clear in figures 6 and 7, that showed the antibacterial effect on *P. gingivalis* of antibiotic showed significant inhibitory effect on bacterial growth for Chloramphenicol, Ampicloxacillin, Carbencillin and Bacitracin, while it resist for Metronidazole, Amoxicillin, Nitrofurantoin, Oxacillin, Lincomycin and Clindamycin. From these results in our study, we will improved and explained that the sensitivity of extracted Rue in all the growth of these 3 types of gram-negative microorganisms with high percentage as explained in table 7, in comparison to the 10 types of antibiotics. Also in our study, we will improved that the resistance of these 3 types periodontal flora to metronidazole, while Chloramphenicol, Augmentin antibiotics have significant inhibitory effect on bacterial growth of these 3 types periodontal flora, as the same inhibitory effect of extracted Rue in both *T. forsythia* and *P. gingivalis*, while less than inhibitory effect of extracted Rue in *A. actinomycetemcomitans*, and this very clear in figures 2, 4 and 6. While Bacitracin has significant inhibitory effect on *T. forsythia* and *P. gingivalis*. Amoxicillin has significant inhibitory effect on *P. gingivalis* only, and so on the other types of antibiotics as explained in the figures above. There are many factors that influence the increased resistance of organisms in biofilm to antibiotics, such as types of species, antibiotic molecules, biofilm growing in different habitat. There is one important mechanism of resistance, by the slower rate of growth of bacterial species in biofilm makes them less susceptible to many antibiotics. Also, other parameters, such: nutritional status, temperature, pH and prior exposure to sub effective concentrations of antimicrobial agents, can cause varied response to antibiotics within a biofilm. For many years, it has been shown that organisms growing in biofilm are more resistant to antimicrobial agents than the same species growing in a planktonic state.
properties and close proximity of the bacterial cells within the biofilm appears to be an excellent environment for horizontal gene transfer, which can lead to the spread of antibiotic resistance genes amongst the biofilm inhabitants 41. For these reasons, there is a need for alternative natural agents such as extracted Rue as we used.

However, the susceptibility of a given organism to extracted depends on numerous factors; the most important ones are the properties of the extracted Rue and the microorganism itself. Definite results, found in the study described above. The mechanism of action of extracted Rue on bacteria cells is entirely explained 21.

Many studies have investigated the efficacy of such plant derived medicines on periodontal pathogens. These studies may be explained by the differences between the types and numbers of bacterial isolates tested in each study, and the method used, including the criteria for determining the Minimum Inhibitory Concentrations. Also, recent isolates may exhibit an increased resistance against the antimicrobial compounds, which possibly derives from their recent interactions with host cells 42. Also, the susceptibility of bacteria to any mouthrinse depends on their growing form, planktonic or biofilm forms.

In fact, FINE et al 43 demonstrated that mouth washes, especially essential-oil containing mouthrinse, kill almost all organisms in the planktonic form, while effects on the biofilm forms of the organisms were more variable. This study supports the observation that resistance to antimicrobial agents is affected by biofilm formation.

Therefore, the use of extracted Rue mouthrinse may have benefits on plaque reduction 44, 45, affecting bacteria growing in supragingival biofilm, but also the subgingival microflora, reducing the levels of total anaerobic bacteria, through the disruption of the nearby supragingival plaque. However, mouthrinse penetrate the subgingival area only minimally. The crevicular fluid outflow would dilute the subgingivally applied antiseptics within minutes 46. It may however, partially reach the subgingival bacteria, with a mean penetration of 70% on the total pocket depth. mouthwash is microbiologically safe, with no change in the bacterial composition of supragingival plaque 47, and no evidence of antimicrobial resistance 48.

As demonstrated in this study, there is considerable evidence of that extracted Rue have strong antibacterial activity against periodontal pathogens and economical. However, numerous factors can modify this efficacy, including the complexity of the subgingival biofilm, the constituents and doses used of extracted Rue and the method of administration of the agent as mouthrinse.

Therefore, in order to develop these new products into solutions or gels for subgingival irrigation commercially, further studies are required to establish them therapeutic or preventive benefits for periodontitis, either alone or in combination with conventional therapies, that can help to reduce the overall burden of oral diseases worldwide. In particular, studies with adequate statistical power, blinding, standardization of purified compounds, and quality control would be for great value. However, its antibacterial activity on periodontal bacteria is controversial. Thus, our research team is currently employed to investigating the extracted Rue, which containing beta-carboline alkaloids of Peganum harmala seeds by testing their antibacterial and antifungal activities on periodontal pathogens in vitro and in vivo.

Conflicts of interest
The authors declare no conflicts of interest related to this study.

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REFERENCES


