The Effect of Essential Oils on Microbial Biofilm on Denture Base Surface.

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ABSTRACT

Nowadays, there is a global effort to find solutions to replace the synthetic drugs with the therapeutic power of natural products to reduce the percentage of many side effects resulted from conventional treatment. The main objective of this research is to investigate the antimicrobial action of essential oils against grown microorganisms on the surface of acrylic denture base materials. In terms of methodology, four types of natural oils (Linseed, Harmal, Radish and Black seed) have been used to investigate their antimicrobial effects against the biofilm formation of three types of bacteria and one fungus on the surface of denture base material. Totally, 30 specimens (10mm×10mm×2mm) prepared from the heat cured acrylic resin denture base materials are provided. One-way ANOVA has been utilized in data analysis at P ≤ 0.05 or 0.01, also t-test is used for independent samples. According to the results, all the concentrations of radish oil and black seed oil have shown a significant effect on bacteria. Regarding its effect on fungus, only radish oil has shown a significant effect against Candida albicans. Linseed oil is less effective than other types of essential oils used in this study particularly against Streptococcus pyogenes. As a result, all the tested natural oils have shown antibacterial effect, while only radish oil has shown significant effect against candida albicans.

Keywords: acrylic; antifungal; antibacterial; denture stomatitis; radish oil

How to cite this article: Abdulkareem MM (2020): The effect of essential oils on microbial biofilm on denture base surface, Ann Trop Med & Public Health; 23(S9): SP2394. DOI: http://doi.org/10.36295/ASRO.2020.2394

INTRODUCTION

Denture stomatitis is known as a popular infectious disease, affecting the palatal mucosa while is highly prevalent in denture wearers and characterized by the presence of Candida albicans. However, it is not a specific disease entity like other existed factors such as bacterial infection [1, 2]. According to prior clinical studies, 65% of denture wearers suffer from the problems caused by Candida albicans. Candida albicans is classified into Candida species as the most widely spread fungus in oral cavity. In other words, C. albicans can be seen in the entire of oral mucosal surface, especially palatal mucosa and tongue [3]. In recent years, using antibiotics is a crucial treatment for diverse bacterial diseases and disorders. However, microbial drug resistance is a major difficulty. Different side effects have been commonly occurred after antibiotic usage, thus the case of drug resistance development could be a superior problem [4,5,6]. In order to decrease the dose dependent side effects and drug-resistance development based on the effectiveness maintenance, different approaches have been gone for natural plants to minimize these problems. Medicinal plants are supposed to be a pivotal source of new chemical substances with potential therapeutic influences [7]. Accordingly, many researches are performed to study the impact of black seed extract in inhibiting the C. albicans growth on heat cured acrylic resin relatively due to the active compounds with antimicrobial power of black seed extract [4]. Additionally, some natural oils as ginger oil, nigella sativa oil, flax oil, sesame oil, and sunflower oil have been observed as effective antifungal agents of cold and heat cured acrylic resin denture base materials when immersed for 8hrs in the oils [8]. As a result, it is found that the essential natural oils have antifungal, antiviral and antibacterial action including black seed, radish, linseed, and harmal [9,10,11,12]. Therefore, in this study, the effect of some essential oils has been investigated against microbial biofilm found on denture surface.

MATERIALS AND METHODS

Oil preparation:

In this study, 4 types of oils have been applied as Harmal (Peganum harmala L), Linseed or flax (Linum usitatissimum L), Radish (Raphanus sativus), and Black seed (Nigella sativa) purchased from Hemani International KEFZ Karachi-Pakistan (KEPZ). These oils have been diluted with ethanol to prepare different concentrations of oils (0.25%, 0.5%, 0.75%, 1%, 5%, and 25%).

Microorganisms:

In this study, test microorganisms are three bacterial types Streptococcus pyogenes, Staphylococcus aureus, and Pseudomonas aeruginosa plus one fungus Candida albicans obtained from the central public health laboratory of Kerbala. Test microorganisms have been cultured on various media, bacteria have been cultured on Muller Hinton Broth (MHB) and Sabouraud Dextrose Broth (SDB) for Candida albicans at 37°C for 24h.

Sample preparation:

Thirty specimens have been prepared from the heat cured acrylic resin denture base materials using the hard elastic foil of 2mm thickness that was cut into plastic specimens of (10mm×10mm×2mm) in dimensions based on Agrawal [13]. Flasking and packing has been performed by the conventional method. Curing for the heat cured acrylic resin specimens conducted by placing the clamped flask into the thermostatically controlled water bath for 1.5h at 74°C, then 1h at 100°C based on the manufacturing instructions. After the completion of curing, flasks have been allowed to bench cool, and the acrylic specimens removed from their stone moulds. Also, any flashes of excess resin material have been removed from the specimens using acrylic bur, then specimens stored in distilled water at 37°C in the incubator for 7 days for conditioning. According to AL-Sumaidae, [8] all specimens have been sterilized in autoclave at 15 pound/inch²/121°C for 15 minutes.

Antimicrobial assay:

A standard microbial density of approximately 1.5×10⁸ has been prepared by matching with Macfrland 0.5. Antimicrobial assay has been performed by disc diffusion method [14]. Then 0.1ml of standard microbial density has been spread on SDA for Candida albicans and MHA for bacteria. On the other hand, Whatman Filter Paper No.1 has been used to prepare discs (6mm) which were sterilized by autoclaving. Discs have been submerged into different concentrations of oils and added to the inoculated plates. Later, plates have been incubated at 37°C for 24h. Inhibition zones have been measured after incubation periods. Two types of controls were used; chlorohexidine (0.2%) and ethanol as a positive control and discs with sterilized distal water as a negative control.

Determination of Minimum Inhibition Concentration (MIC)

The MIC of active oils has been determined against bacteria and candida albicans. Micro titration plate has been applied to delineate the MIC of tested microorganism. 100µl SDB for fungi or MHB for bacteria added in each well of plate. Then, 20µl oil at different concentrations have been added to well with media. After mixing, 20µl microorganism has been added for each well. The plate was incubated at 37°C for 24h including the control plates (negative control) which contained the broth with microbial inoculums without the addition of tested oils. Then visual growth has been recorded as MIC values.

Biofilm forming assay:

PMMA resin samples have been treated with MIC of each essential oil, while other samples have been treated with sterile distilled water based on Sookto with some modifications [15]. All PMMA resin samples have been soaked in 2ml of MIC of oils for 30 min, then washed with phosphate buffer solution. Putting 20ml of the prepared standard microbial density in a screw capped bottle and immersed one acrylic samples in each one. Inoculated PMMA has been agitated for 1hr at 37°C in a shaking incubator (130rpm). Non-adherent cells have been removed from the resin samples by gently dipping into 2ml phosphate buffer solution for 3 times. The remaining adherent cells on the surface of PMMA resin samples have been stained by sufranin stain for 90sec, then examined under optical microscope for biofilm counting.

Statistical test:

One-way ANOVA has been utilized in data analysis at P ≤ 0.05 or 0.01, also t-test is used for independent samples.

RESULTS

All the concentrations of radish oil and black seed oil have shown a significant effect on bacteria (Streptococcus pyogenes, Pseudomonas aeruginosa, and Staphylococcus aureus). Besides, radish oil has shown a significant effect on Candida albicans. On the other hand, there is no important difference between the effects of radish oil on all bacterial types and candida used in this research. There is a significant difference between the action of black seed, linseed and harmal on all bacterial types and candida due to their higher effectiveness against bacteria. The most effective concentrations of radish oil are (5% and 25%), however, black seed oil is the most effective (1% and 5%) against all bacterial types. The effective concentration of radish oil against Candida albicans is 5%. Linseed oil is less effective than other oils types used in this study, especially against Streptococcus pyogenes. The most resistant microorganism to the tested oils (black seed, linseed, and harmal) is Candida albicans (Table 1).
Table 1: Antimicrobial effect of essential oils

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Pseud.</th>
<th>Staphylo</th>
<th>Strepto.</th>
<th>Candida</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>Conc. (%)</td>
<td>Inhibition zone diameter (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radish</td>
<td>1</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>11</td>
<td>.952</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>12</td>
<td>12</td>
<td>11</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>15</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Black seed</td>
<td>1</td>
<td>14</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>6.970</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Linseed</td>
<td>1</td>
<td>13</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>4.387</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Harmal</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4.499</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>12</td>
<td>10</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>15</td>
<td>12</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>0.2</td>
<td>10</td>
<td>12</td>
<td>11</td>
<td>12</td>
<td>2.750</td>
</tr>
</tbody>
</table>

(-) resistant to oil. *: significant differences between essential oils and chlorhexidine at P ≤ 0.05 (horizontal)
*: significant differences between microorganisms for each oil at P ≤ 0.05 (vertical).

The MIC of effective essential oils determined against *Pseudomonas aeruginosa*. Therefore, the value of MIC has shown 1%, 1%, 5%, and 0.25% for black seed oil, linseed oil, harmal oil, and radish oil, respectively. However, the MIC of radish oil on *Candida albicans* is 0.25% (Table 2).

Table 2: The MIC of effective essential oil on *Pseudomonas aeruginosa* and *Candida albicans*

<table>
<thead>
<tr>
<th>Studied groups</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Candida albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Black seed oil</td>
<td>Linseed oil</td>
</tr>
<tr>
<td>25%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.75%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.5%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.25%</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(-) No growth; (+) Growth

The result of biofilm counting of *Pseudomonas aeruginosa* and *Candida albicans* growing on denture surface has shown that radish oil is more effective than other oils in reducing the biofilm formation of microorganism on denture base surface. Black seed oil, linseed oil, and harmal oil have also shown significant effectiveness against *Pseudomonas aeruginosa* (Table 3).

Table 3: Biofilm count of *Pseudomonas aeruginosa* and *Candida albicans* on denture base surfaces

<table>
<thead>
<tr>
<th>Studied groups</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Candida albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean biofilm No.</td>
<td>SD.</td>
</tr>
<tr>
<td>Radish oil 0.25%</td>
<td>1.4</td>
<td>1.76</td>
</tr>
<tr>
<td>Black seed oil 1%</td>
<td>3.3</td>
<td>3.01</td>
</tr>
<tr>
<td>Linseed oil 1%</td>
<td>8.4</td>
<td>3.29</td>
</tr>
<tr>
<td>Harmal oil 5%</td>
<td>9.13</td>
<td>3.20</td>
</tr>
<tr>
<td>Control (distilled water)</td>
<td>67.33</td>
<td></td>
</tr>
</tbody>
</table>

SD: standard deviation **: significant differences between essential oils and control at P ≤ 0.01

DISCUSSION
In recent years, a wide spread of multidrug-resistant bacterial pathogens has globally raised a concern in economic effects and public health [16]. Therefore, a growing interest in medicinal plants and natural products has been raised due to the efficiency increment of new plant-derived drugs. Regarding the side effects of conventional medicine, using natural products as an alternative to conventional treatment has been grown in last few decades [17]. Nowadays, there is high attention to replacement of synthetic drugs with the therapeutic power of natural products to reduce the level of many side effect resulted from conventional treatment. One of these medicinal plants is black seed (Nigella sativa) with its extensively usage known as the seed of blessing “Habbatul Barakah” because of its high effectiveness in many ailments [4,18]. The effect of black seed extracts on varies body systems has been studied in vitro or in vivo. Black seed could be consumed as herbal products or medicinal oil for medicinal purposes. It comprises saponin and atsiri oils with antifungal, antimicrobial and antibacterial effects [19, 20, 21, 22, 23]. Indeed, people believe that black seed is a true potential to be discovered thus deserved the name “Natures Miracle” [24]. According to the results of this study, all the concentrations of black seed oil have a significant effect on bacteria (Streptococcus pyogenes, Staphylococcus aureus and Pseudomonas aeruginosa), but no effect on Candida albicans. Black seed could likely represent a promising prophylactic adjunct to conventional chemotherapy to reduce the severity of oral mucositis [25]. Black seed oil could be demonstrated as an effective remedy against certain bacteria types, particularly those with the most resistance to antibiotic drugs. In addition, it prevents undesirable inflammation and improves bone formation and maturation [26, 27]. Antimicrobial effect of black seed (Nigella sativa) oil might be because of the complex chemical structures of seeds, having more than 100 various chemical components comprising abundant sources of all the essential fatty acids thus it majorly used medically [28]. The black seed oil extract has a bactericidal influence on mutans streptococci at a concentration of 10%, and can inhibit the adherence of these microorganisms to tooth surface [17]. Methanolic extracts of black seed have the strongest antifungal effect followed by the chloroform extracts against different strains of Candida albicans [29]. Black seeds cause concentration-dependent inhibition of Staphylococcus aureus, Pseudomonas aeruginosa and E. coli and a pathogenic yeast Candida albicans [30]. It is also studied that additives materials of pure natural oil of black seed (1.5%) and thyme have been recommended to give acceptable properties beside its antimicrobial effect after curing the acrylic resin denture base [9].

Radish known as Raphanus Sativus are a crop grown in Yuma area as a winter vegetable production in the colors of white, red, purple or black and in the forms of long, cylindrical or round. The oil of radish seeds has been used in health applications [31]. Radish oil possessed the highest antibacterial and antifungal effects against Candida albicans and Pseudomonas aeruginosa in all the concentrations applied in this study align with Anonymous [32] reporting that radish has raphanin which is antibacterial and antifungal and inhibits the growth of Staphylococcus aureus, Streptococci, pneumococci and E. coli. The cysteine-rich peptides (Rs-AFP1 and Rs-AFP2) isolated from radish has shown a substantial antifungal activity against several fungal species with MIC. Both Rs-AFPs are among the most potent antifungal characterized proteins [33]. Gutiérrez and Perez [10] have reported that radish contains Caffeic acid with antifungal properties in vitro against Helminthosporium maydis. Moreover, the roots and leaves of Radish are also used for cancer treatment or as antimicrobial and antiviral agents. Radish releases biocidal compounds majorly isothiocyanates which is produced when the enzyme degradation of glucosinolates presented in plant cell. The highest fungicidal activity has depended on the concentration of isothiocyanates [34]. Also, the presence of Myrosinase in radish has been confirmed beside the conformation of Myrosinase with antimicrobial and antmutagenic properties [35]. However, the findings of the current study over the antibacterial activities of radish oil have not been correlated with the study by saeed and tariq in 2006 [14], showing no effect of radish on bacterial growth.

Linseed or flax (Linum usitatissimum L., the Linaceae family), as a source of fiber and oil, is highly used as crop plant in textile and food industries [36]. Linseed oil has a favorable fatty acid composition with high linolenic acid content. Linseed oil includes nearly 60% α-linolenic acid with high omega-3 fatty acid [37], with proof of digestibility, immune enhancing, bioactive peptides, anticancer, antioxidant activities, antimicrobial, anti-proliferative, antiallergic, anti-Parkinson’s, and antihypertensive [38]. Linseed oil has high levels of linolenic (53.21%) followed by oleic (18.51%), and linoleic (17.25%), while the dominant saturated acids are palmitic (6.58 %) and stearic (4.43%) [11]. Other study concluded that linseed oil is individually a good antimicrobial agent and confirming its potency to synergize the antimicrobial effects when analyzed in combination with gemifloxacin [39]. In the current research, linseed oil is ineffective against Candida albicans, mightily because of the differences in fungus cell wall structures and protein synthesis, these results are in agreement with Gaafar et al, in 2013 [40].

Harmal (Peganum harmala L.) is known as Syrian rue, Wild rue and Harmal. P. In Middle East (Egypt and Iran) Harmala extracts are taken as a major medicinal development source due to having many pharmacological activities. For a long time, P. harmala has been applied in traditional medicines as a pain tranquilizer and as antiseptic agent. Harmal has antitumor, antileishmanial, antioxidant, antifungal, antiviral, antibacterial, antidiabetic, insecticidal and cytotoxic activities [16, 41]. Also, it could be supposed as a source of antibacterial compounds for treatment of infections caused by multi-drug resistant (MDR) bacterial pathogens [42]. The histological examination of organs and tissues of treated rats given intramuscularly aqueous extract of Iraqi Peganum harmala are normal, proving that the extract has low level toxicity [43]. Peganum harmala L., enriched by alkaloids up to 4%, is the only species with wild growing in the middle
and north parts of Iraq. Also, harmal as a potential source of antimicrobial drug against the four urinary pathogens *S. aureus*, *E. coli*, *Proteus mirabilis* (*P. mirabilis*), and *P. aeruginosa* [44]. Few studies have reported various pharmacological and biological activities of *Peganum harmala* L. such as antifungal, antibacterial and MAO inhibition (Monoamine oxidases), thus the smoke of its seeds is traditionally applied as a disinfectant [12, 45, 46]. Following the outcomes of this research, *Peganum harmala* L. have the capability to inhibit the growth of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes* at 5% and 25% concentrations. These findings are in conformity with Al-Izzy in 2010 [12], who found that *Peganum harmala* L. extract (aqueous and alcoholic) is very effectual on *Lactobacilli* and *Candida* in vitro. It could be due to the presence of principle alkaloids including its capability to intercalate with the DNA of microorganisms including peganein, harmine, harmaline, and harmalol [16]. It has been reported that harmane as a highly aromatic planar alkaloid exerts its antibacterial activity through the intercalation with DNA [47], thus, this antibacterial mechanism should be taken for active extract of *P. harmala* root and seed. The observed antibacterial activity of *P. harmala* could be based on the high quantity of polyphenols known to possess efficient antibacterial activities [16].

**CONCLUSION**

The aforementioned tested natural oils are effective antibacterial agents. Despite their effect on bacteria, only radish oil has shown significant effect against *Candida albicans*. As a result, Radish oil is more effective than other oils in reducing the biofilm formation of microorganism on denture base surface. Black seed oil has also shown significant influence against *Pseudomonas aeruginosa*.

**ACKNOWLEDGMENT**

The author is very grateful to Professor Dr. Ali Al-janabi and Mrs. Amera Mohammed for their unlimited assistance and support during this research.

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