EFFICACY OF ROYAL JELLY EXTRACT ON INHIBITION OF CANDIDA ALBICANS ADHERENCE ON VARIOUS TYPES OF DENTURE BASE MATERIAL

A THESIS

BY

DANAI JAREEMIT

Presented in Partial Fulfillment of Requirements for the

Master of Science in General Dentistry

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AN ABSTRACT

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Denture stomatitis is a common disease found in sixty percent of denture wearers. The causes are denture trauma and a high concentration of *Candida albicans* adherence to the inner surface of the denture. Microbial adherence is the initial stage and most important process, which also causes the disease. The aim of this research was to study the efficacy of crude royal jelly extract on inhibition of *Candida albicans* adherence to various types of denture base material. The specimens of heat-cured acrylic resin, self-cured acrylic resin and tissue conditioners were placed in a various concentrations of crude royal jelly extract solution, using Sabouraud Dextrose Broth as a negative control group and Nystatin as a positive control group. The standard cell suspension was added in each well and incubated at thirty seven°C for twenty four hours. The adherence of *Candida albicans* was determined using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and SEM (scanning electron microscopy). The adherence of *Candida albicans* was found in both heat-cured and self-cured denture base acrylics for the negative control group, but found less on both types of tissue conditioners. In addition, crude royal jelly extract solution at a concentration of fifty mg/mL and twenty five mg/mL could significantly inhibit the adherence of *Candida albicans* when compared with the negative control group ($P < 0.05$). The increase of royal jelly concentration further reduced the adherence of *Candida albicans* on both types of denture acrylics, which was consistent with SEM results. There was no statistical significance ($P > 0.05$) between the type of acrylic resin and the adherence of *Candida albicans*. The results obtained from this research can be used as baseline information for further development of royal jelly products and as an antimicrobial agent, especially for those who wear dentures.

ประสิทธิภาพของสารสกัดจากนมผึ้งในการยับยั้งการเกาะติดของเชื้อแคนดิดาอัลบิแคนส์บนพื้นผิวเรซินอะคริลิกประเภทต่างๆ

บทคัดย่อ

ของ

ดนัย จารีมิตร

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา

ตามหลักสูตรวิทยาศาสตรมหาบัณฑิต สาขาวิชาทันตกรรมทั่วไป

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การอักเสบของเนื้อเยื่อใต้ฐานฟันเทียมเป็นปัญหาที่พบได้บ่อย ประมาณ ร้อยละ 60 ของผู้ใส่ฟันเทียม สาเหตุเกิดจากการกดทับของฐานฟันเทียม และจากการรวมตัวเป็นกลุ่มของเชื้อราแคนดิดา อัลบิแคนส์บนพื้นผิวด้านในของฐานฟันเทียมโดยที่ความสามารถในการเกาะยึดของเชื้อต่อพื้นผิวด้านในของฟันเทียมเป็นขั้นตอนแรก และเป็นขั้นตอนที่มีความสำคัญมากที่สุดในกระบวนการรวมตัวกันของกลุ่มโคโลนีของเชื้อราและการก่อให้เกิดโรค วัตถุประสงค์ของการวิจัยในครั้งนี้เพื่อศึกษาประสิทธิภาพของนมผึ้งที่ความเข้มข้นต่าง ๆ ในการยับยั้งการเกาะติดของเชื้อแคนดิดา อัลบิแคนส์บนพื้นผิวเรซินอะคริลิกประเภทต่างๆ โดยนำชิ้นงานเรซินอะคริลิกชนิดบ่มตัวด้วยความร้อน ชนิดบ่มตัวด้วยปฏิกิริยาเคมี และวัสดุปรับสภาพเนื้อเยื่อ วางลงในเวลเพลทที่มีสารละลายนมผึ้งที่ความเข้มข้นต่างๆ โดยมีอาหารเลี้ยงเชื้อชนิดเหลวเป็นกลุ่มควบคุมผลลบ และสารสารละลายของยานิสเตติน เป็นกลุ่มควบคุมผลบวก จากนั้นนำเชื้อแคนดิดา อัลบิแคนส์ที่ได้เตรียมไว้ ใส่ลงในหลุมทุกๆ หลุม นำไปบ่มที่อุณหภูมิ 37°C, 5% คาร์บอนไดออกไซด์เป็นเวลา 24 ชั่วโมง ตรวจสอบการเกาะติดของเชื้อแคนดิดา อัลบิแคนส์บนพื้นผิวเรซินอะคริลิกด้วยวิธี MTT assay และกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด ผลการทดสอบพบการเกาะติดของเชื้อแคนดิดา อัลบิแคนส์บนพื้นผิวเรซินอะคริลิกทั้งสองประเภท เมื่อทดสอบในกลุ่มควบคุมผลลบ แต่พบการเกาะติดที่น้อยกว่าในวัสดุปรับสภาพเนื้อเยื่อทั้งสองชนิด อีกทั้งยังพบว่าสารละลายนมผึ้งที่ความเข้มข้น ที่ 50 มก./มล และ 25 มก./มล สามารถลดการเกาะติดของเชื้อแคนดิดา อัลบิแคนส์ เมื่อเปรียบเทียบกับกลุ่มควบคุมผลลบ ที่ระดับความเชื่อมั่นร้อยละ 95 และเมื่อสารละลายนมผึ้งมีความเข้มข้นมากขึ้น พบการลดลงของการเกาะติดของเชื้อแคนดิดา อัลบิแคนส์บนพื้นผิวเรซินอะคริลิกทั้งสองประเภท ซึ่งให้ผลสอดคล้องกับกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด ผลการศึกษาไม่พบความสัมพันธ์ระหว่างประเภทการบ่มตัวของเรซินอะคริลิก ต่อการเกาะติดของเชื้อแคนดิดา อัลบิแคนส์ที่ระดับความเชื่อมั่นร้อยละ 95 ผลการทดสอบจากการศึกษาครั้งนี้น่าจะเป็นทางเลือกหนึ่งของการพัฒนารูปแบบผลิตภัณฑ์นมผึ้ง เพื่อยับยั้งการเกาะติดของเชื้อราให้กับผู้ป่วยที่ต้องสวมใส่ฟันเทียมชนิดถอดได้

The thesis titled

"Efficacy of Royal Jelly Extract on Inhibition of *Candida albicans* Adherence on Various Types of Denture Base Material"

by

Danai Jareemit

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in General Dentistry of Srinakharinwirot University

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Danai Jareemit

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CHAPTER 1
INTRODUCTION

Denture wearing may cause various environmental changes in the mouth such as changing pH of saliva, reducing the amount of oxygen in saliva and reducing salivary flow rate. These conditions will then support colonization and biofilm-formation of fungus, thus the patient who wears denture are more susceptible to fungal infection than patient who does not wear denture, especially those wearing acrylic denture. Patients are therefore advised to remake new acrylic denture every 1-2 years depending on their oral hygiene.[1] Regular denture hygiene procedure by brushing can effectively remove dental plaque from the denture. However, in elderlies or patients with hand movement restriction, brushing together with chemical cleansing such as immersion of dentures in a cleansing solution is a more effective strategy to reduce the adherence of dental plaque to the denture base.[2]

Denture stomatitis is one of the most common diseases in denture wearers, affecting 60% of population.[3-7] Denture stomatitis is caused by denture trauma, poor-fitting denture, improper denture border extension, and improper denture cleansing which could lead to yeast cells adherence and colonization.[1,8-10] *Candida albicans* is often found as a cause of denture stomatitis.[11-13] The adherence of *C. albicans* on the fitting surface of denture is the initial stage and the most important process which causes the disease.[14] However, adherence process can be different depending on type of denture base material, for example; heat-cured acrylic resin, self-cured acrylic resin and tissue conditioner. They vary in degree of porosity, surface free energy, hydrophobicity and roughness. Acrylic resin is currently the most widely used denture base material. Introduction of PMMA (Polymethyl methacrylate) as a denture base material dates back to the year 1937 when Dr. Walter Wright clinically evaluated PMMA and found that it fulfilled all the requirements of an ideal denture base material.[15] Since its introduction, PMMA has been continuously used because of it's advantageous properties; user friendly, low cost, acceptability by most patients, it's stability in the oral cavity and aesthetic properties. Candida can adhere to the fitting surface of PMMA dentures.[16] Heat-cured acrylic resin utilizes heat from hot water or ultraviolet light to activate the polymerization process, while self-cured acrylic resin utilizes chemical activator such as

Dimethyl-para-toluidine.[17-18] Therefore the difference between heat-cured acrylic resin and self-cured acrylic resin is the activation process that causes free radicals. Nevertheless, the polymerization of self-cured acrylic is not completed when compared to heat-cured acrylic, hence some unpolymerized monomers is left after the reaction.[19] The consequence is the reduced strength and tissue irritation although self-cured acrylic resin causes less contraction which results in more dimensional accuracy. Self-cured acrylic is suitable for repairing the denture base because of its convenience. It also takes much less time for denture repair and can be done in one visit in dental clinic. In addition, tissue conditioner has been developed in order to reduce and redistributed occlusal stress especially in patients who have thin and sharp of residual alveolar ridges or chronic tissue irritation from denture forces that might damage the underlying mucosal tissues.[20] The problem found when using tissue conditioner is the colonization of *C. albicans* on and within it. Fungal growth is known to destroy the surface properties of tissue conditioner and this may lead to irritation of the oral tissues. This is due to a combination of increased surface roughness and high concentration of exotoxins and metabolic products which produced by the fungal colonies.[21] Interestingly, conflicting adherence results are reported on tissue conditioner. Some *in-vitro* studies reported significant inhibitory effects on *C. albicans*[22] ,whereas some studies showed only limited antifungal properties and no significant reduction on Candida adherence.[23] *C. albicans* adhere to polymeric surfaces by Van der Waals and electrostatic forces.[7,24-25] The development of yeast biofilm on acrylic resin occurs in 3 distinct stages after colonization. The initial stage (up to 11 hours), forming of micro-colonies, the intermediate stage (12 hours to 30 hours), extracellular matrix accumulates over colonies, and the maturation stage (38 hours to 72 hours), forming of biofilm. The forming of yeast biofilm on the fitting surface of denture is the initial stage which causes the denture stomatitis.[14] Thus the prevention of *C. albicans* adherence to acrylic resin could be a possible method for prevention of denture stomatitis.[26]

In general, denture stomatitis is often treated by application of topical antifungal drug. Nystatin is commonly used for the treatment of local fungal infection, therefore it is often used for treating this disease. Despite aforementioned benefit, the antifungal medications are chemically synthesized. They can possibly lead to drug-resistance when used continuously.

Nowadays, the interest in medicinal nature as a source of antimicrobial agents has grown dramatically. Recently, there were sequentially reports the *in-vitro* and *in-vivo* antibacterial action of Royal jelly, which is a natural product.[27-28] Royal jelly is a milky secretion produced by young worker honeybees, containing numerous compounds such as water, proteins, amino acids, minerals and vitamins. It was also found to contain 10-HDA (10-hydroxy-2-decenoic acid), which is an efficient anti-microbial substance against gram-positive and gram-negative bacteria.[29] The aim of this study was to investigate the efficacy of CRJE (Crude Royal Jelly Extract) on the inhibition of *C. albicans* adherence on various types of denture base material.

CHAPTER 2
LITERATURE REVIEW

1. Denture stomatitis

Denture stomatitis is caused by denture trauma or poor-fitting denture, improper denture border extension, imbalance of dental occlusion, Immunodeficiency and allergy to denture base material. It is a common problem found in approximately 60% of denture wearers, especially acrylic resin dentures.[30] The cause of denture stomatitis is colonization and biofilm-formation of *C. albicans* that is associated with denture stomatitis. The majority of which were detected accidentally by dentist. The patients frequently have no symptoms or may have inflammation or sore tissue from other causes, e.g. wearing poor-fitting dentures, soreness when chewing with fungal infection. Consequently, the dentist should find the cause, give advice and prevent or eliminate Candida colonization by removing dentures, applying topical antifungal drugs to oral tissue surface and the fitting surface of dentures, the use of antifungal agents incorporated in tissue conditioner[31], applying topical antifungal agents such as Miconazole, a drug of Azole group to act by disturbing the synthesis of Ergo sterol at the fungal cell membrane, thus causing change in the cell's selective permeability, resulting in dead cells. Another method includes using fungus-killing mouthwash such as chlorhexidine digluconate, which causes nucleoprotein precipitate of microbe, inhibits the growth and changes the cell wall, thus having an effect on killing and inhibiting the fungus. The disadvantages of chlorhexidine are bad taste and the staining of teeth, oral mucosa and acrylic dentures. In addition, Polyene drug, i.e. Nystatin is used as the initial drug to treat local fungal infection.

2. Mechanism of denture stomatitis

This mechanism is associated with the formation of *C. albicans* colony, which may or may not show symptoms. There are many common causes, e.g. wearing poor-fitting dentures, denture base trauma, Immunodeficiency, allergy to acrylic with the changes in oral condition, including the decreasing pH of saliva, decrease in oxygen content and less saliva secretion rate. This is an important part in encouraging the growth more than normal of fungus.[32] In case of traumatic injury of the denture base, there are infection channels through the lining wounds, loss of the preliminary disease prevention system. It is normally found that inflammation of oral tissue usually occurs under the denture base.

Candida adherence on the fitting surface of dentures is the initial stage. It is also the most important process of the formation of fungal colony and pathogenesis.[14] The adherence efficacy varies according to the type of denture base material, including the type of incubation reaction with different impact on physical properties such as Porosity, Surface free energy, hydrophobicity, and roughness. *C. albicans* adhere to polymer used as the denture base material and tissue conditioner with London's vander waals and electrostatic forces.[5,7,24 -25,33] For this reason, inhibiting Candida adherence will help reduce denture stomatitis.

Candida can form Biofilm adhering to the surface of both biotic and abiotic surfaces, including mucosa and acrylic denture base material with various types of different characteristics. Presumably this will affect candida adherence differently.[34-35] As a result, denture is a source of microorganism, and particularly in people with poor denture hygiene.[36] Candida can adhere to the fitting surface of dentures mostly made of polymethyl methacrylate (PMMA), which is a hydrophobic material by binding between hydrophobic surfaces. Candida adherence occurs from hydrophobic interaction and Lewis' acid-base interaction. Hydrophobic interaction comes from London's vander waals and electrostatic forces occurring between the cell surface and the substratum. The cell will overcome the repulsive forces, depending on each microbial type. If the surface is very hydrophobic (Low surface energy), microbial adherence is more.[16] Besides, the microbe can change its own cell surface composition according to the environmental conditions in which it is exposed. The aim is to escape from the immune system of the body. It can also increase adherence on receiver cells of cell membranes in oral epithelium. The adherence efficacy of Candida in the pathogenic form of Hyphae is better than Candida in the form of blastoconidia. This is because the cell surface of Candida Hyphae contains more diverse protein components to adhere to receiver cells of different types of oral mucosa.[5,37]

The development of yeast biofilm on acrylic resin occurs in 3 distinct stages after colonization. The initial stage (up to 11 hours), forming of micro-colonies, the intermediate stage (12 hours to 30 hours), extracellular matrix accumulates over colonies, and the maturation stage (38 hours to 72 hours), forming of biofilm. The study on Biofilm formation of *C. albicans* revealed differences in adhesion of the control group and the experimental group at 12-30 hours (Intermediate Phase).[16] Based on a scanning electron microscope (SEM)

study, it was found that microbial adherence was in the form of hyphae with hydrophobic surface properties and was in pathogenic stage.[37] Hydrophobicity of cell surface of *C. albicans* depends on the strain, medium and condition.[13,34] Moreover, the test revealed that surface free energy and contact angle of denture base material mostly made of polymethyl methacrylate have the properties of large base component (Hydrophobic). This facilitates microbial adherence. Thus, efforts are made to adjust the material to have hydrophilic properties by adding a substance to reduce microbial adherence to the initial material. However, it was found that the results are not sustainable with impact on decrease in mechanical and physical properties.[33,38] Therefore, a coating method has been used instead because of it's advantages, including changing surface specific properties, convenience, possibility of doing every day.[37]

3. Nystatin

This antifungal medication of Polyene group is extracted from a bacterial strain, i.e. *Streptomyces noursei*. The mechanism of Nystatin starts when forming complexes with the ergosterol, a major component of the fungal cell membrane. When present in sufficient concentrations, it forms pores in the membrane that lead to K^+ leakage, acidification, and death of the fungus.[39] Nystatin acts on inhibiting and killing Candida mostly. It is less absorbed through gastrointestinal tract, is expelled in the form of faeces without deformation. It is manufactured in the forms of cream, powder, suspensions (Figure 2.3) and tablet. The drug easily deteriorates if exposed to heat, oxygen and light. Therefore, it should be stored in opaque container with dehumidifier and cover.

Side effects of Nystatin are less common even when used continuously for a long time. Allergic reactions are usually caused by preservatives. According to laboratory studies, the fungus exposed to Nystatin at 9.36 micrograms/mL, i.e. sublethal concentration for 1 hour revealed that microbial adherence on acrylic denture base was reduced by 86.48 %.[39]

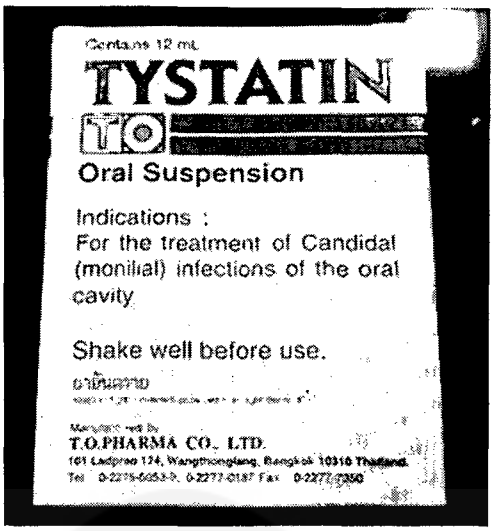

FIGURE 1 Nystatin at 23 mg/mL was used as a positive control.

4. History of denture base materials

In the early days, ivory and wood were used to make the denture base (in 1800). Vulcanized rubber was used as denture base material in 1855. But there were limitations in terms of beauty, unpleasant smell and taste, thus not being popular. Subsequently in 1868, celluloid was discovered, it was produced from the dissolution of nitro cellulose (Pyroxene) by John Wesley Hyatt. Celluloid has proper toughness and flexibility, the color can also be altered as desire. However, the findings indicate that there is much dimensional change, high water absorption and non-durable color, thus phenol formaldehyde resin was suggested as a replacement by Dr. Leo Baekeland in 1909. However, the material was subsequently found to be very brittle, hence cancellation of it's use.

In early 1930s, polymer was widely used in dentistry. Acrylic resin or Polymethyl methacrylate was discovered in 1937 by Dr. Walter Wright and used as denture base because of the advantages, i.e. the color similar to oral tissue, dimensional stability, light weight, not transferring excessive heat or cold, adhering to plastic teeth, low cost, easy to repair, easy to reline. Acrylic denture base can be categorized as heat-cured, using heat from hot water, ultraviolet light or microwave stimulating polymerization and self-cured by chemical activation from adding tertiary amine such as dimethyl-para-toluidine.

The basic difference between heat-cured and self-cured acrylic resin is the method of initiating free radicals. Polymerization of self-cured type is not as complete as heat- cured. Thus there are monomers left by the reaction, problems of reduced strength and tissue irritation.[40] However, contraction is less, hence greater dimensional accuracy. So, it is suitable for denture repairing. Moreover, it takes less time to repair, the repair can be done in one visit in dental clinic.

5. Tissue conditioner

Tissue conditioner is a viscoelastic material used to reline or rebase the acrylic denture base on the fitting surface to help in distributing the chewing forces, absorbing forces or acting as a cushion between the hard denture base and supporting tissue and is used to relieve the pressure transmitted by the denture on the mucosa. This may be called temporary soft liner due to its softness and elastic characteristic in a short time. It can be generally used for not more than 1 week because the material becomes noticeably harder and more porous as plasticizer is lost when used up to such period, which can be a source of *C. albican* adherence.[11]

The powder of tissue conditioner is polyethylene methacrylate, which may have filler and the liquid contains plasticizer and ethanol. Softness of soft lining material is associated with thickness. Therefore, it is recommended to use as much thickness as possible to support the impact of chewing with the typical value, i.e. 0.5-4 mm.

The components in tissue conditioner affecting the growth of *C. albicans* may be present in powder part or liquid part. This depends on the mixing of some substances that inhibit microbial growth. Polymer powder does not affect the growth of microbe. However, Zinc Undecylenate or Azulene mixed in powder part can inhibit the growth of microbe.[41] Liquid part, including both plasticizer and ethanol can inhibit the growth of microbe in the laboratory.[41-43]

Although some materials can have an inhibiting effect on microbial growth due to the components, but the material can naturally absorb water well. As a result, oral fluids are absorbed into the material, thereby encouraging the growth of *C. albicans* despite the material's antifungal agent in the composition. Another important factor is porous and uneven surface compared to heat-cured acrylic resin[44], which facilitates microbial adherence.

6. Royal jelly

Royal jelly is a milky secretion used to feed the larvae of bees and queen bees. Royal jelly is characterized by powder or scales, is produced by young worker honeybees during the age of 7 days. It is expelled from hypopharyngeal glands and mandibular glands at the heads of worker bees. Worker honeybees spit royal jelly out, put it into the tubes of larvae and feed royal jelly to queen bees from being worm larvae into full queens.[45] The bees are usually given royal jelly for the first 3 days only. Only larvae that will grow to be the queens receive royal jelly throughout their life. Therefore this royal jelly is called nectar, queen jelly or queen food. The characteristic of royal jelly is homogeneous, thick liquid in cream or light yellow like white cream similar to yogurt or sweetened condensed milk. It has a pungent odor of phenolic compound, sour taste and is quite spicy.

The main components of royal jelly are water, proteins, fats and sugar as well as vitamins, minerals, amino acids. It also contains enzymes, e.g. glucose oxidase, phosphatase and cholinesterase. Royal jelly is also found to contain peptide with perfectly connected amino acid, i.e. Royal Lysine that is antimicrobial protein.[29] The presence of these components varies by season, area of the bees' growth and growth period. Improper storage also causes changes in the composition of royal jelly.[46]

7. Benefits of royal jelly in the pharmacology

Royal jelly contains (10-HDA) 10-hydroxy-2-decenoic acid. Research revealed that 10-HDA acts on anti-growth of many kinds of bacteria, gram-positive and gram-negative, including typically antibiotic -resistant bacteria. The efficacy of 10-HDA acid is equivalent to approximately 75% of Penicillin. Antibacterial action of royal jelly is maximized within 24 hours after royal jelly collection from the nests. After that, the effect decreases to a constant level. Furthermore, it is found that royal jelly is anti-radioactive, acts on anti-growth and helps inhibit cancer cell proliferation, reduce inflammation of cancer cell.[47]

Considering this antibacterial mechanism of peptide derivative of royal jelly, it is a form of peptide monomers in spirals embedded in the cell membrane acting as a passageway of the cell membrane with the peptide structure resembling a carpet, the ability to act like cleansing solution. This structural characteristic has the length of the derivative and the charge, including the same hydrophilic and hydrophobic properties, thus affecting the cell membrane and membrane core of bacteria.

Regarding the efficacy of royal jelly on inhibition of *C. albicans*, a study was conducted by Ayse Nedret Koc *et al.* on the efficacy of bee products to inhibit *Candida spp.* and *Trichosporon spp*. They studies bee's products included honey, royal jelly, bee pollen and propolis, which were tested for the efficacy to inhibit 40 yeast strain of *C. albicans, C. glabrata, C. krusei and Trichosporon spp.* Broth micro-dilution method was used to evaluate the antifungal efficacy. It was found that MIC (Minimal inhibitory concentration), the lowest concentrations that can inhibit microbial growth are in the range of 5-80% (weight / volume), 0.06-1 mg. / mL, 0.002-0.25 mg. / mL, 0.006-0.1 mg. / mL, and 0.02-96 mg. / mL of Honey, Royal Jelly, Bee Pollen, Propolis and Fluconazole used for comparison, respectively. It can be concluded from the study that bee products have the efficacy on inhibiting the microbe differently. Propolis has more inhibition efficacy than Bee Pollen, Royal Jelly and Honey, respectively.[48]

However, the antifungal mechanism of peptide derivative is not clear yet because fungus has the cell membrane with cell wall containing polysaccharide. In order to make the substance inhibit the action of fungal, it is necessary to permeate through carbohydrate layer to the cell membrane layer. Peptide derivative that has anti-bacterial effect must be anode so as to bind to cathode of the cell membrane. This peptide particle in royal jelly may permanently bind to the cell membrane, which is cathode. This is more effective than binding to the mammalian cell membrane, which is both anode and cathode.

CHAPTER 3
RESEARCH METHODOLOGY

1. Materials and methods

1.1 The sample size calculation

The sample size was calculated using G* Power 3.0 for Windows XP program.[49] This program was created by Cohen's formula (1977). First we select the statistical test used in the F-test. ANOVA: Fixed effects, special, main effects and interaction. Second we select the type of power analysis in the test is Post hoc: Compute achieved power - given α, sample size, and effect size. Then input parameters required for the analysis are: Effect size f, which is selected from the three-level effect size table. Test difference between many means, from the Medium is 0.25, α err prob is 0.05, Total sample size is 400, The number degree of freedom is 12 and the number of groups is 5, then click "Calculate", we will get the sample sizes group 1 and 2 were 8, so the obtained number of sample in each group was eight specimens. Four experimental groups are heat-cured acrylic resin, self-cured acrylic resin, Soft-liner and Dura conditioner. Nystatin (23 mg/mL) (Tystatin Oral Suspension, T.O. Phama Co., Ltd., Thailand) was used as a positive control and SDB (Sabouraud Dextrose Broth) (Himedia, USA) was used as the negative control.

1.2 Preparation of acrylic resin specimens

Brass metal mold was used to fabricate samples of 10 mm in diameter and 3 mm in thickness. A thin layer of Vaseline (Unilever, Thailand) was applied inside the mold as a lubricant, self-cured acrylic resin (ProBase Cold, Ivoclar-Vivadent AG, Liechtenstein) (Figure 3.1.2.1) and tissue conditioners (Soft liner, GC corporation, Tokyo, Japan (Figure 3.1.2.2) / Dura conditioner, Dental Mfg., Worth, IL(Figure 3.1.2.3)) were prepared according to the manufacturer's recommended ratio shown in Table 3.1.2 and placed in the mold, the surface was finished with a flat mirror to obtain a flat surface. The heat-cured acrylic resin (Vertex-Dental, B.V., Netherlands) (Figure 3.1.2.4) was prepare by pouring pink wax into the mold, the pink wax samples were flasked and heat-cured acrylic was packed to obtain heat-cured acrylic samples of the same dimension. The Vaseline on specimens' surface was cleaned off using dishwashing liquid (Sunlight®, Unilever, Thailand) and the specimens were soaked in distilled water for 24 hours to get rid of the residual monomer (Figure 3.1.2.5). They were then sterilized by ethylene oxide gas.

FIGURE 2 Self-cured acrylic resin (ProBase Cold Ivoclar-Vivadent, AG, Liechtenstein)

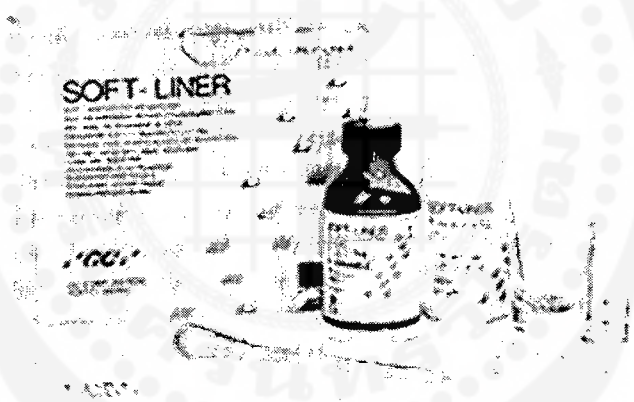

FIGURE 3 Tissue conditioners (Soft liner, GC corporation, Tokyo, Japan)

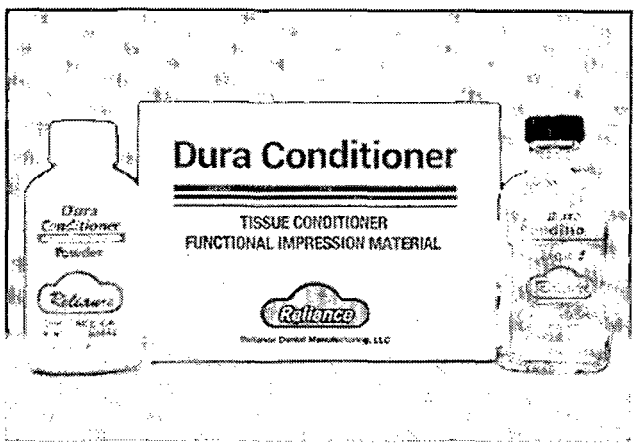

FIGURE 4 Tissue conditioners (Dura conditioner, Dental Mfg., Worth, IL)

FIGURE 5 Heat-cured acrylic resin (Vertex-Dental, B.V., Netherlands)

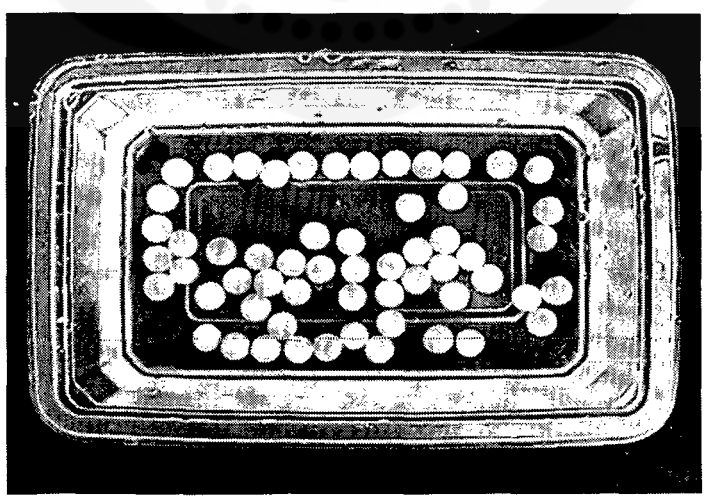

FIGURE 6 The specimens were soaked in distilled water for 24 hours to get rid of the residual monomer.

TABLE 1 Samples of two acrylic resins and two tissue conditioners

Products	Manufacturers	Polymerization Method	Composition
Heat-cured acrylic resin			
Vertex Rapid Simplified Powder Lot.XR135P03 Liquid Lot.XR15L01	Vertex- Dental B.V. Netherlands	Heat-cured	Powder Polymethyl methacrylate, Accelerator, Color agents Liquid Methyl methacrylate, Cross linker, Accelerator
Self-cured acrylic resin			
ProBase Cold Pink NO.5 Powder Lot. R82188 Liquid Lot. S03282	Ivoclar-Vivadent AG Liechtenstein	Self-cured	Powder Polymethyl methacrylate, Softening agent, Benzoyl peroxide, Catalyst, Pigments Liquid Methylmethacrylate, Dimethacrylate, Catalyst
Tissue conditioners			
1.Soft-liner (Soft denture reline material) Powder Lot.1406101 Liquid Lot.1406052	GC corporation Tokyo, Japan	Self-cured	Powder Polymethyl methacrylate Liquid Butylphthalyl butylglycolate, Ethanol
2.Dura conditioner (Reliance) Powder Lot.022305 Liquid Lot.062309	Dental Mfg.Co. Worth, IL	Self-cured	Powder Polymethyl methacrylate Liquid 2-Ethylhexyl diphenyl phosphate, Bis(2-Ethylhexyl) phenyl phosphate, Triphenyl phosphate

1.3 Preparation of *C. albicans*

The *Candida* strains used in this study was *C. albicans* (ATCC 90028) (Thai Can Biotech, BKK) (Figure 3.1.3), which cultured on SDA (Sabouraud Dextrose Agar) (Himedia, USA) by incubation at 37°C for 24 hours. Then the colonies were grown in SDB and incubated at 37°C for 24 hours and the cell suspension was adjusted to 0.5 McFarland ($1x10^6$ CFU (Colony forming unit))

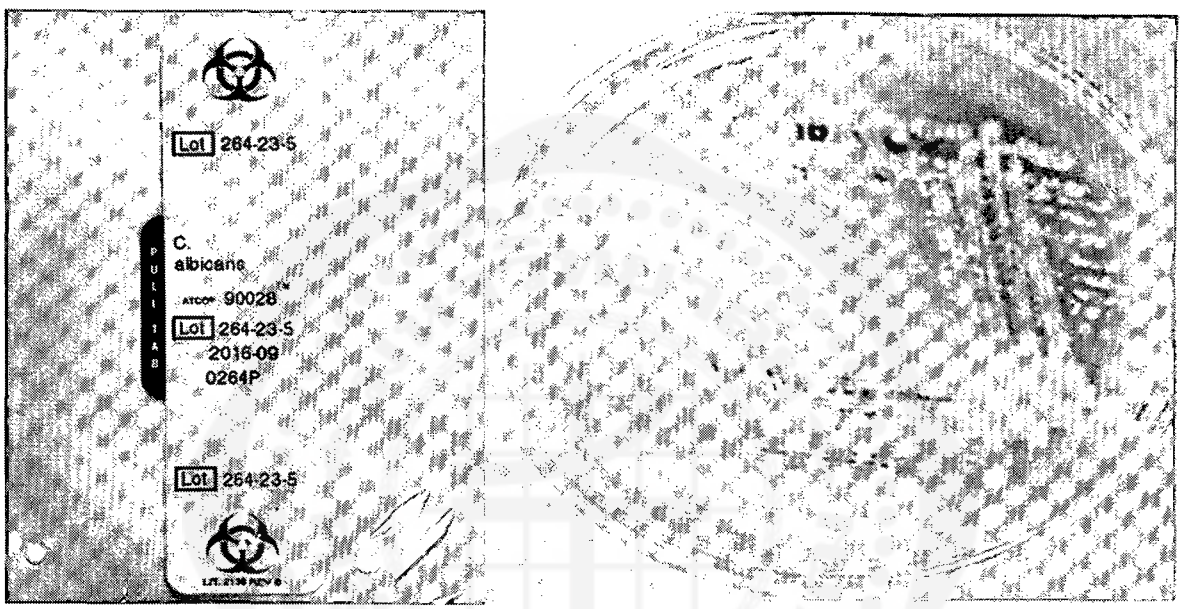

FIGURE 7 *C. albicans* (ATCC 90028), which cultured on SDA (Sabouraud Dextrose Agar) by incubation at 37°C for 24 hours.

1.4 Preparation of royal jelly extract

Royal jelly powder (Su Pha Bee Farm, Chiang Mai, Thailand) (Figure 3.1.4.1) was extracted with 20 percent ethanol at a concentration of initial solution royal jelly equals 100 mg/mL (Figure 3.1.4.2). The supernatant was collected after centrifugation (TOMY® MX-160, American Laboratory Trading, USA) at a temperature of 4°C, 10,000 rpm for 10 minutes (Figure 3.1.4.3) and freeze dried (FreeZone 2.5, LABCONCO., USA) (Figure 3.1.4.4). Then CRJE (Crude Royal Jelly Extract) was weighed and dissolved in SDB for the initial concentration of 100 mg/mL. The clear solution was sterilized through a membrane filter paper with a pore size of 0.20 micrometers. (Minisart®, Sigma-Aldrich Pte Ltd., Singapore) (Figure 3.1.4.5)

FIGURE 8 Royal jelly powder (Su Pha Bee Farm, Chiang Mai, Thailand)

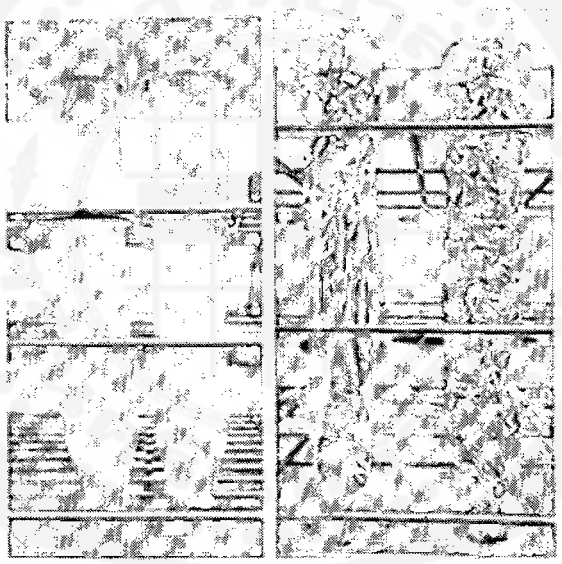

FIGURE 9 Royal jelly powder was extracted with 20 percent ethanol at a concentration of initial solution royal jelly equals 100 mg/mL

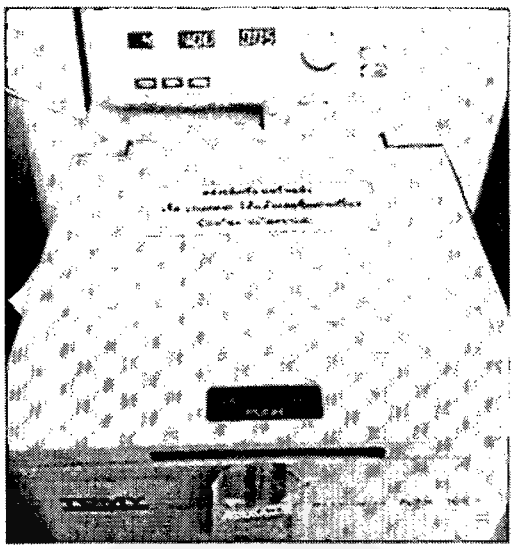

FIGURE 10 The centrifugation (TOMY® MX-160, American Laboratory Trading, USA) at a temperature of 4°C, 10,000 rpm for 10 minutes.

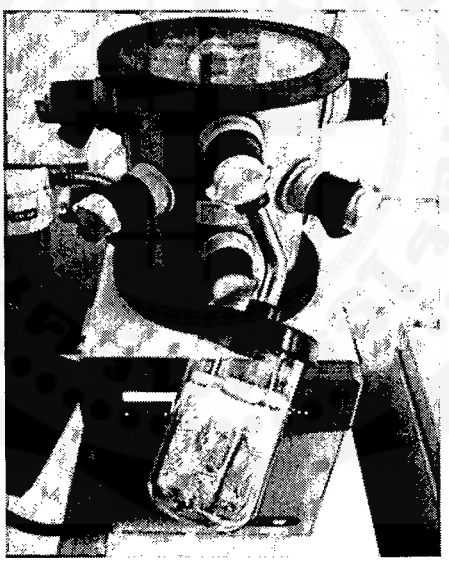

FIGURE 11 The supernatant was freeze dried (FreeZone 2.5, LABCONCO., USA)

FIGURE 12 The solution was sterilized through a membrane filter paper with a pore size of 0.20 micrometers. (Minisart®, Sigma-Aldrich Pte Ltd., Singapore)

2. MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration)

The sterile CRJE solution was diluted by Two-fold dilution at a concentration of 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and 3.125 mg/mL respectively; at the volume of 1 mL. *C. albicans* suspension 1 mL was then added. So, a final concentration of CRJE in the treatment group was 50 mg/mL, 25 mg/mL. 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.0625 mg/mL, respectively. A negative control group was used SDB 2 mL. These tubes were incubated at 37°C temperature for 24 hours (Figure 3.2). The lowest concentration tube which does not has planktonic cell suspension is the MIC value (The lowest concentration of CRJE that inhibits growth of *C. albicans*). After that, the tube which are clear solution were streaked on the agar plate. These plates were incubated at 37°C temperature for 24 hours. The lowest concentration plates which does not has yeast colonies is the MFC value (The lowest concentration of CRJE that has fungicidal effect)

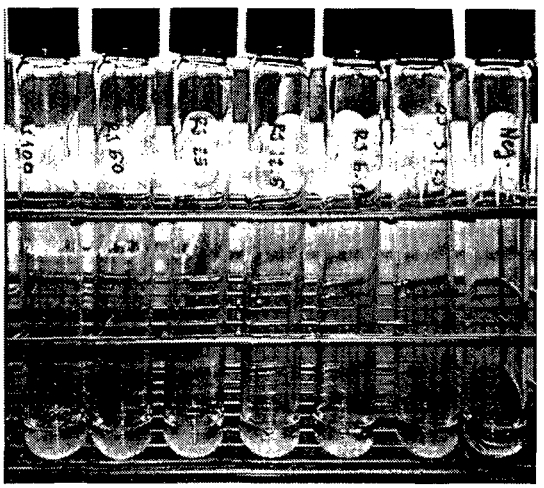

FIGURE 13 Two-fold dilution of sterile CRJE solution.

3. Adhesion assay and analysis

The candida adherence to the acrylic resin specimens was assayed in Broth dilution method and MTT assay. Each specimen was placed in a well containing 500 µl of CRJE at a concentration of 12.5 mg/mL, 25 mg/mL and 50 mg/mL, SDB used as negative control group and Nystatin of concentration 23 mg/mL used as positive control group. (Figure 3.3) The 500 µl of the standard cell suspension was then added in each well and incubated at 37°C for 24 hours to allow the cells to attach to the surface of the specimens.[41] After the incubation, the specimens were washed in a standard manner by dipping in sterile PBS (Phosphate Buffered Saline) to remove loosely attached cells, then placed in a new 24-well plates with a 600 µl volume of SDB and a 150 µl volume of MTT Stock. Shake and then incubated at 37°C for four hours to find the Formazan purple crystals stuck on the specimens. The specimens were placed in a new 24-well plates with a 700 µl volume of DMSO (Dimethyl sulfoxide) to dissolve the crystals and then into the shaker (Rocker-Shaker®, Biosan, Latvia) for 15 minutes. It has a purple solution, were determined in terms of optical density at a wavelength of 570 nm using DMSO solution as a blank to analyze and use this data to calculate the percentage reduction of Candida adherence from the formula.

$$\% \text{ of Reduction} = \frac{\mathbf{OD570\ (Mean)SDB - OD570\ (Mean)CRJE}}{\mathbf{OD570\ (Mean)SDB}} \times \mathbf{100}$$

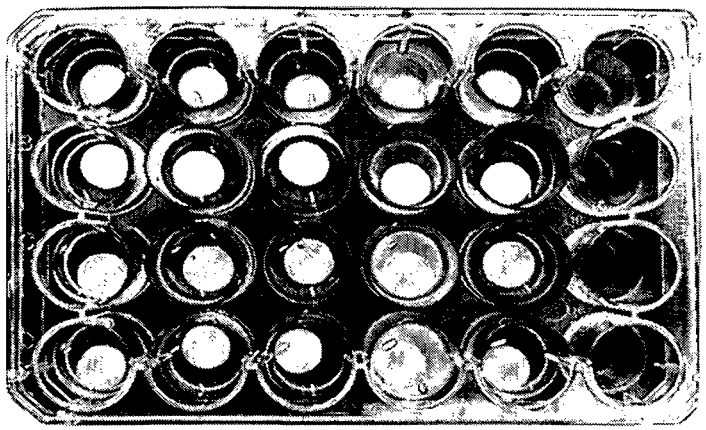

FIGURE 14 Each specimen was placed in a well containing 500 µl of CRJE at a concentration of 12.5 mg/mL, 25 mg/mL and 50 mg/mL, SDB used as negative control group and Nystatin of concentration 23 mg/mL used as positive control group.

4. Scanning electron microscopy for *C. albicans*-attached specimens

C. albicans were adhered to the acrylic resin as described above and were incubated at 37°C for 24 hours, these samples were subsequently processed for scanning electron microscopy (SEM). Briefly, samples were washed with PBS and then placed in fixative (2.5 percent Glutaralaldehyde in 0.1 M Phosphate buffer saline (PBS)) for two hours, 25°C. The samples were rinsed in PBS. The samples were subsequently dehydrated in a series of ethanol washes (30% for 5 minutes, 50% for 5 minutes, 70% for 5 minutes, 100% for 3×5 minutes), then treated with Hexamethyldisilazane (HDMS), and finally air dried in a desiccator. The specimens were coated with platinum. After processing, samples were observed in a scanning electron microscopy (JEOL, USA) (Figure 3.4) at a magnification of 400 times. The images were processed for display using Photoshop software.

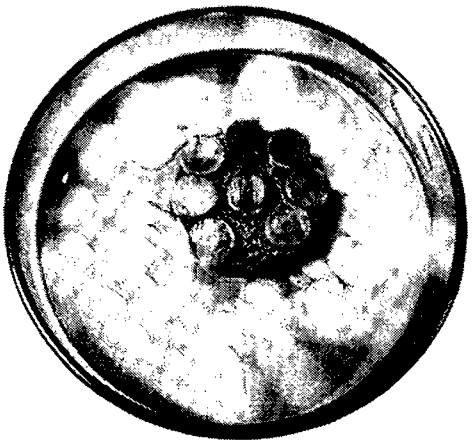

FIGURE 15 The specimen were sputter coated with platinum for investigation with scanning electron microscopy.

5. Statistical analysis

The adherence of *C. albicans* on acrylic resin surfaces were analyzed using one-way and two-way ANOVA (analysis of variance). The analysis was done with a statistic package for social science (SPSS for Windows® version 22). For all of the statistic analysis, a *P*-value below 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

1. The adherence of *C. albicans* on various types of acrylic resin surface

C. albicans adherence was investigated in four types of denture base materials, which are Heat-cured acrylic resin, Self-cured acrylic resin, Dura conditioner and Soft-liner. The results were statistically analyzed using one-way ANOVA and Welch test (Table 4.1.2). It was found that two groups of denture base material were significantly different on the *C. albicans* adherence. Subsequent Post-hoc test (Table 4.1.3) revealed that both types of acrylic resin had more *C. albicans* adherence while both of tissue conditioners had less *C. albicans* adherence as shown in Table 4.1.1. Therefore, the efficacy of various concentration of CRJE on inhibition of *C. albicans* adherence on both acrylic resin materials was further studied.

TABLE 2 Adherence of *C. albicans* in SDB to different types of denture base material

Denture materials	OD_{570} (Mean ± SD) (n=8)
Heat-cured acrylic resin	0.4155 ± 0.0996 [a]
Self-cured acrylic resin	0.4289 ± 0.1191 [a]
Dura conditioner	0.1255 ± 0.0103 [b]
Soft-liner	0.1196 ± 0.0090 [b]

The groups with the same superscript are not significantly different at P = 0.05.

TABLE 3 Effect of various types of acrylic resin on the adherence of *C. albicans* compared by one-way ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.719	3	.240	34.540	.000
Within Groups	.194	28	.007		
Total	.913	31			

df, Degree of freedom.

Test of Homogeneity of Variances by Levene Statistic sig =.000

Robust Tests of Equality of Means by Welch sig =.000

TABLE 4 Multiple Comparisons by Post-hoc test shown that both types of acrylic resin had differed *C. albicans* adherence compare with both of tissue conditioners.

		Mean Difference			95% Confidence Interval	
(I) Type	(J) Type	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Heat-cured acrylic resin	Self-cured acrylic resin	-.013375	.058675	.996	-.18459	.15784
	Dura conditioner	.290000*	.037855	.000	.16539	.41461
	Soft-liner	.295875*	.037810	.000	.17126	.42049
Self-cured acrylic resin	Heat-cured acrylic resin	.013375	.058675	.996	-.15784	.18459
	Dura conditioner	.303375*	.045167	.001	.15445	.45230
	Soft-liner	.309250*	.045130	.001	.16032	.45818
Dura conditioner	Heat-cured acrylic resin	-.290000*	.037855	.000	-.41461	-.16539
	Self-cured acrylic resin	-.303375*	.045167	.001	-.45230	-.15445
	Soft-liner	.005875	.005182	.676	-.00922	.02097
Soft-liner	Heat-cured acrylic resin	-.295875*	.037810	.000	-.42049	-.17126
	Self-cured acrylic resin	-.309250*	.045130	.001	-.45818	-.16032
	Dura conditioner	-.005875	.005182	.676	-.02097	.00922

*. The mean difference is significant at the 0.05 level.

2. MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration) value

The fungal inhibition and fungicidal effect of CRJE against *C. albicans* can be expressed in MIC value, which is the lowest concentration tube which does not has planktonic cell suspension (Figure 4.2.1) and MFC values, which is the lowest concentration plates which does not has yeast colonies (Figure 4.2.2). MIC and MFC were 12.5 mg/mL and 50 mg/mL respectively. Therefore, the concentration of CRJE which used in this study were 12.5, 25 and 50 mg/mL.

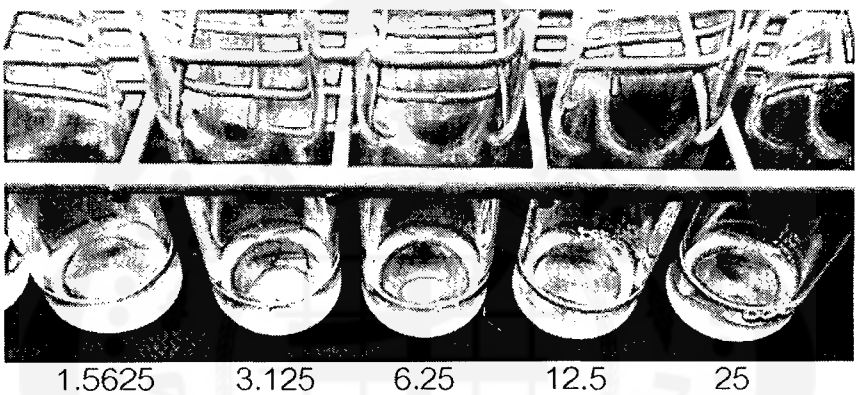

FIGURE 16 MIC value was 12.5 mg/mL, which is the lowest concentration tube which does not has planktonic cell suspension.

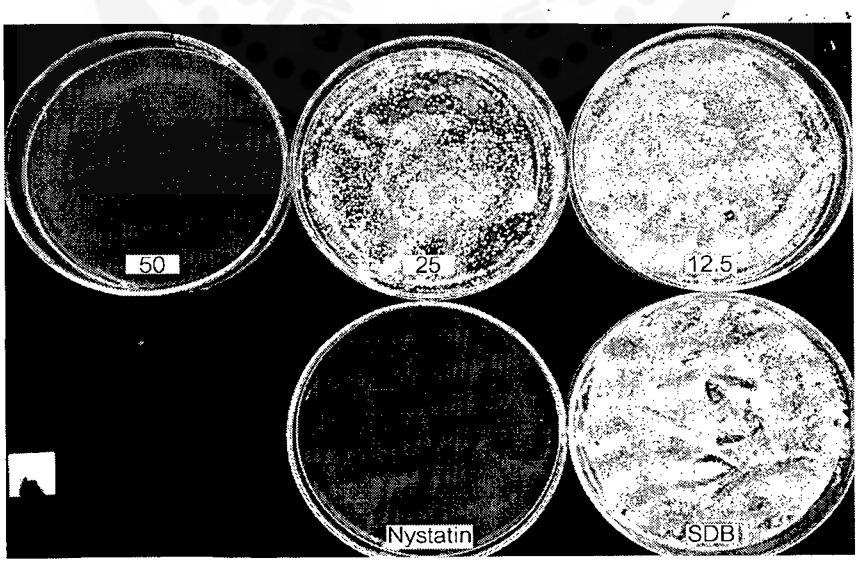

FIGURE 17 MFC value was 50 mg/mL, which is the lowest concentration plates which does not has yeast colonies.

TABLE 5 MIC and MFC values (mg/ml) of CRJE on *C. albicans*

	MIC	MFC
Experiment	12.5	50

3. Efficacy of CRJE on inhibition of *C. albicans* adherence on various types of acrylic resin surface

To determine whether the correlation between the types of cured acrylic and concentration of CRJE solution affect the adherence of *C. albicans*, two-way ANOVA and Levene's Test were used. Tests of Between-Subjects Effects showed no correlation between the type of acrylic resin and the concentration of CRJE solution (P=0.993). Type of acrylic resin did not significantly affected the adherence of *C. albicans* (P >0.05). In contrast, the concentration of CRJE significantly affected the adherence of *C. albicans* (P <0.05).

The adherence of *C. albicans* to the acrylic resin, following a 24-hour exposure to various sublethal concentrations of CRJE are presented in Table 4.3 which shows CRJE at a concentration of 50 mg/mL, 25 mg/mL and positive control group could significantly inhibit the adherence of *C. albicans* when compare with the negative controls group (P < 0.05). The percentage reduction in the adherence of *C. albicans* are presented in Table 4.4, which showed the percentage reduction of *C. albicans* of CRJE at a concentration of 50 mg/mL, 25 mg/mL and 12.5 mg/mL and a positive control group compared to negative controls group on both of acrylic resins type. As mentioned above, we found that CRJE at a concentration of 50 mg/mL can inhibit the adherence of *C. albicans* up to half when compared with the negative control group and CRJE at a concentration between 25-50 mg/mL have 46.15-52.03 percent reduction compared to Nystatin 23 mg/mL which has 53.37-54.01 percent reduction. This reduction was concentration-dependent, since higher concentrations resulted in higher blockage of adherence on both of acrylic resin type as shown in Figure 4.1 and Figure 4.2. This result was consistent with the SEM result. SEM visualization of the acrylic resin specimens with Candida biofilms show yeast forms on top of the surface where remnants of exopolymeric material (dehydrated because of SEM procedures) within irregularities of the specimens. We found the adherence of *C. albicans* on specimens in CRJE at 3 concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL and positive control group were decreased compared to the negative control group. When the concentration of CRJE increased, the adherence of *C. albicans* reduced for both of acrylic resins type as shown in scanning electron micrographs (Fig 4.3).

TABLE 6 Adherence of *C. albicans* to denture acrylic after exposure to 12.5 mg/mL, 25 mg/mL, 50 mg/mL, Nystatin 23 mg/mL and SDB when comparing between Heat-cured acrylic resin, Self-cured acrylic resin

	OD_{570} (Mean ± SD) (n=8)	
	Heat-cured acrylic resin	Self-cured acrylic resin
CRJE 50 mg/mL	0.2021 ± 0.0250*	0.2058 ± 0.0124*
CRJE 25 mg/mL	0.2238 ± 0.0491*	0.2143 ± 0.0117*
CRJE 12.5 mg/mL	0.2821 ± 0.0396	0.2881 ± 0.1022
Nystatin 23 mg/mL	0.1938 ± 0.0271*	0.1973 ± 0.0204*
SDB	0.4155 ± 0.0996	0.4289 ± 0.1191

** $P < 0.05$ significantly differences compare, CRJE: Crude Royal Jelly Extract, Nystatin: positive control group, (SDB) Sabouraud Dextrose Broth: negative control group*

TABLE 7 The Percentage reduction of *C. albicans* adherence in CRJE at a concentration of 12.5 mg/mL, 25 mg/mL, 50 mg/mL, Nystatin 23 mg/mL and SDB when comparing between Heat-cured acrylic resin and Self-cured acrylic resin

	Reduction in adherence (%)	
	Heat-cured acrylic resin	Self-cured acrylic resin
CRJE 50 mg/mL	51.36	52.03
CRJE 25 mg/mL	46.15	50.04
CRJE 12.5 mg/mL	32.10	32.82
Nystatin23 mg/mL	53.37	54.01
SDB	0	0

CRJE: Crude Royal Jelly Extract, Nystatin: positive control group, SDB (Sabouraud Dextrose Broth): negative control group.

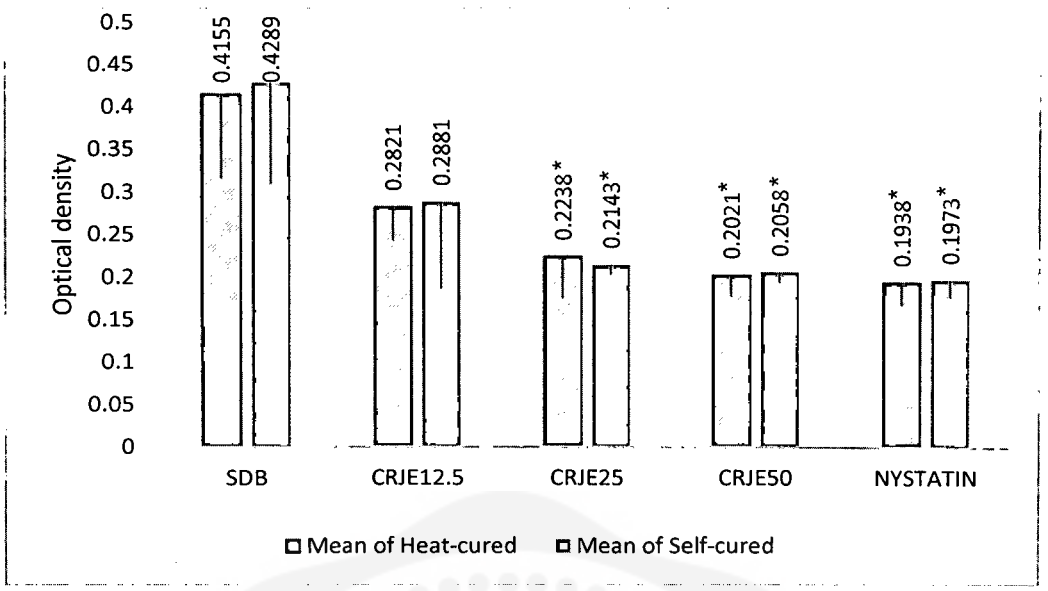

FIGURE 18 Mean of the absorbance of CRJE at a concentration of 50 mg/mL (CRJE50), 25 mg/mL (CRJE25), 12.5 mg/mL (CRJE12.5), Nystatin 23 mg/mL (Positive) and SDB (Negative) of heat-cured acrylic resin and self-cured acrylic resin. Asterisks indicate significant differences (* $P < 0.05$ *significantly*) versus negative control group (SDB).

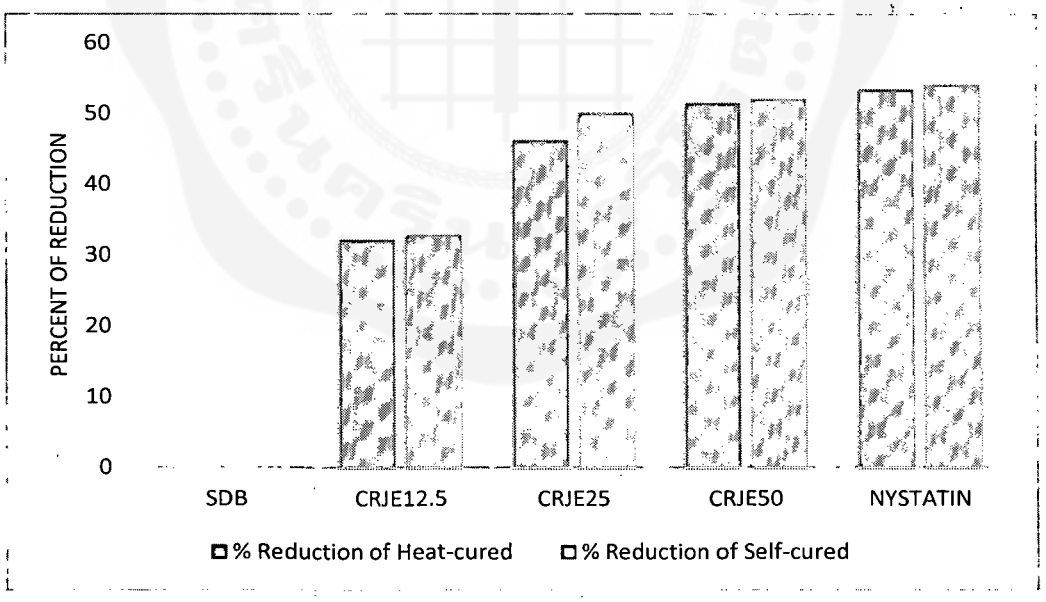

FIGURE 19 The Percentage reduction of CRJE at a concentration of 12.5 mg/mL (CRJE12.5), 25 mg/mL (CRJE25), 50 mg/mL (CRJE50), Nystatin 23 mg/mL (Positive) and SDB (Negative) of heat-cured acrylic resin and self-cured acrylic resin.

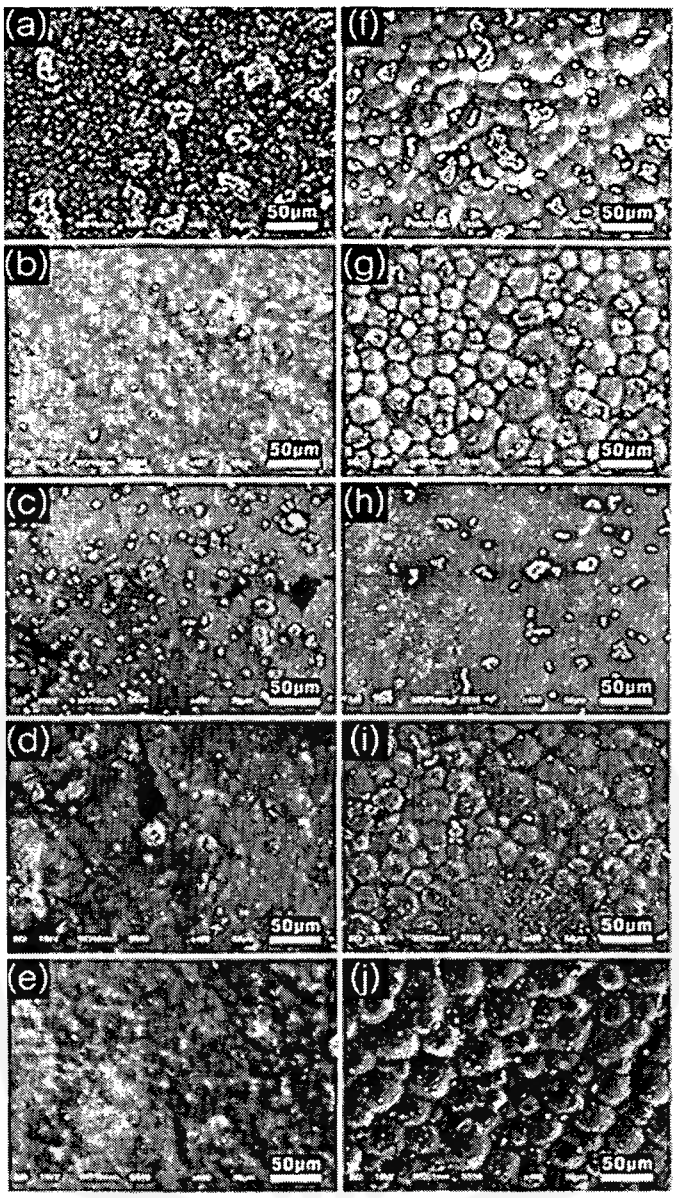

FIGURE 20 Scanning electron micrographs at 400x magnification of *C. albicans* adherence, a comparison between heat-cured acrylic resin after exposure to SDB (a), Nystatin 23 mg/mL (b), CRJE 12.5 mg/mL (c), CRJE 25 mg/mL (d) CRJE 50 mg/mL (e) and self-cured acrylic resin after exposure to SDB (f), Nystatin 23 mg/mL (g), CRJE 12.5 mg/mL (h), CRJE 25 mg/mL (i), CRJE 50 mg/mL (j)

CHAPTER 5
DISCUSSION AND CONCLUSION

The objective of this study was to study the efficacy of CRJE on inhibition of *C. albicans* adherence on various types of denture base materials as a possible method to treat and prevent denture stomatitis in denture wearers. The amount of microbial adhesion on each denture base materials varies and this could impact the physical properties of denture base such as porosity, surface free energy, hydrophobicity, and roughness.[5,7,23-24] Candida can easily adhere to the fitting surface of denture base made of PMMA because of it's hydrophobic property. In addition, epithelial cells can bind easily with the hydrophobic surface. Microbial adherence occurs from hydrophobic interaction and Lewis' acid-base interaction. Thus the more hydrophobic the surface (low surface energy), the more microbial adherence.[16] A study on hydrophobicity, Minagi *et al.*, in 1985, concluded that there was a higher adherence of micro-organisms to the material which had a surface free-energy closest to that of the specific organism and that hydrophobic interaction is significantly important in the initial attachment of yeasts to polymeric surfaces.[50] The adherence of *C. albicans* to a surface is classically considered to be a two-stage process. The initial interactions between the two surfaces are non-specific and reversible, although the secondary phase is caused by specific intermolecular interactions. The initial adherence of *C. albicans* to surface involves a thermodynamic approach to adhesion, which describes the adhesion of *C. albicans* to the surface in term of surface free energy of the surfaces and *C. albicans*. In addition, the hydrophobicity of the microorganism is a reason for high adherence and also for electrostatic interactions between surfaces. The second phase of the adhesion process involves specific adhesion-receptor interactions. The microorganism carries adhesins that bind stereo chemically to complementary receptors on the surface. This stage is necessary for the tight binding of the microorganisms to the surface, which permits colonization.[8] Other factors contributed to the adherence of *C. albicans* to denture surface include surface roughness, strain variability, concentration of microorganism, culture conditions and viability of *C. albicans*. The cytotoxic effect from residual monomer is greater in self-cured acrylic resins than heat-cured acrylic resins, but in this study we reduced the level of residual monomer by 24 hrs. water storage.

SEM observations of samples showed *C. albicans* with highly dense biofilm mass (Cotton-like mass) on the surface of both acrylic resins after *C. albicans* suspension for 24 hrs. When *C. albicans* was treated with CRJE or Nystatin, cells appeared scattered and no biofilm mass was observed. Scanning electron micrographs also showed the brittle fractured surfaces with small pits and revealed less porous surface in heat-cured acrylic resin when compare to self-cured acrylic resin. When comparing properties and surface characteristics of heat-cured acrylic resin and self-cured acrylic resin, we found that heat-cured acrylic resin has less surface roughness than self-cured acrylic resin which led to less adherence of *C. albicans* on heat-cured resin.[3] However, the results from this study found no difference in the adherence of *C. albicans* on both types of acrylic resins. This could be the result of specimen preparation process, since the material surfaces were produced in a brass metal mold, the variability of surface roughness was not examined.

It was found that when the culture was in a state without any treatment, both types of acrylic resins had more *C. albicans* adherence and both tissue conditioners had less *C. albicans* adherence. This could be explained by some components of tissue conditioners reacted with the microorganisms. Another reason is the plasticizer in tissue conditioner, such as Benzyl benzoate and Benzyl salicylate is effective in killing fungus.[41,42,43] Unlike the results from previous study done by Hema *et al.*, in 2011, they did not find that tissue conditioners (Viscogel and GC soft) can inhibit the adherence of *C. albicans*.[12]

In term of the efficacy of CRJE on inhibition of *C. albicans*, this study used the concentration of CRJE between MIC and MFC values, which were 12.5, 25 and 50 mg/mL. We found no correlation between the type of acrylic resin and the concentration of CRJE solution but the concentration of CRJE significantly affected the adherence of *C. albicans*. The reduction of *C. albicans* adherence was concentration-dependent, since higher concentration resulted in higher blockage of adherence on both of acrylic resin type. This result was consistent with the SEM result.

Ayse Nedret Koc *et al.*, in 2011, evaluated the ability of honeybee products including royal jelly to inhibit the growth of 40 yeast strains of *C. albicans*, *C. glabrata*, *C. krusei*, and *Trichosporon spp.* Using the broth microdilution method, minimal inhibitory concentration ranges 0.06-1 µg/mL had antifungal activities.[48] A study of Hassan *et al.*, in 2015, compare an antifungal activity of hydroalcoholic extract of Iranian propolis and royal jelly against *Rhizopus oryzae*. The mean of MIC and MFC values of royal jelly on *Rhizopus oryzae* were 100 ± 34 and 133 ± 46 mg/mL respectively.[51]

A study on the ability of other natural extracts that inhibit the adherence of *C. albicans* by Taweechaisupapong *et al.*, in 2006, they studied an Inhibitory effect of *Streblus asper* leaf-extract on adherence of *C. albicans* to denture acrylic, using various sublethal concentrations of *Streblus asper* leaf ethanolic extract. The experiments were performed on self-cured acrylic resin by Broth dilution method, combined with A colorimetric tetrazolium assay using XTT ((2, 3)-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-(12)-2H-tetrazolium hydroxide). The results showed that the *Streblus asper* leaf-extract concentration of 31.2 mg/mL, 62.5 mg/mL and 125 mg/mL, led to a significant reduction ($P < 0.05$) of *C. albicans* compared to the control group. The reduction was concentration-dependent, since higher concentrations resulted in higher blockage of adherence. The *Streblus asper* leaf-extract could affect the cell wall of fungi, creating extracellular components and chemical surface adherence inhibiting effect.[52]

The results of the study led to an acception of the hypothesis that CRJE can inhibit the growth of *C. albicans* and adherence of *C. albicans* on both type of acrylic resins. CRJE at a concentration between 25-50 mg/mL can significantly inhibit the adherence of *C. albicans*, this is approximately equivalent to Nystatin 23 mg/mL, thus it could be an alternative anti-fungal product to Nystatin. An advantage of a CRJE such as, unlike synthetic drugs, the royal jelly is a natural product that cause less allergic reaction and contains no chemicals that are harmful to humans. In addition, CRJE processing does not cause an environmental pollution. This research result is similar to Moselhy *et al.*, in 2013, which conducted a study on the inhibition of microbial, using royal jelly from Egypt and China in experiments done by disc diffusion method. The results showed an effective inhibition against bacteria. It also has anti-fungal effect includes *Aspergillus fumigant*, *Aspergillus niger*, *C. albicans* and a *Syncephalastrum racemosum*. The best concentration of royal jelly to inhibit *C. albicans* is 15 mg/mL.[53] They also found that when the concentration is higher, there was also a much wider zone of inhibition. However we noticed that the concentration of the royal jelly extract from many studies that can inhibit the growth of pathogenic *C. albicans* are different. Bachnova *et al.*, in 2014, explained that the efficacy of royal jelly solution cannot be compared with the results of other studies because of many factors including microorganisms of different species and the different environment and culture. And more importantly, the bee species in each country were different.[54]

Several studies have revealed specific components of RJE, including 10-HDA, Royalisin and Jelleines, are the main antimicrobial biaoctives, they have a significant antibacterial potential. A study of RJE was conducted by Takenaka *et al.*, in 1986, described the antibacterial and antifungal effects of 10-HDA against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*.[55] One of the studies that evaluated the antibacterial activity of Royalisin has been reported against *B. subtilis*. This inhibition was equal to that of tetracycline at 50 µg/mL.[56] Whereas Jelleines are small peptides, which have antimicrobial properties against several gram-positive cocci (*S. aureus*, *S. saprophyticus*, and *B. subtilis*) and gram-negative rods (*E. coli*, *E. cloacae*, *K. pneumoniae*, and *P. aeruginosa*), as well as yeast (*C. Albicans*).[57]

This study used ethanol as an extraction solvent as it has been shown as standard in many studies. Besides, ethanol is relatively safe and cheap, but the addition of alcohol solution to extract RJ may increase protection against microbial growth. So, the CRJE was Freeze-dried until alcohol was removed completely.

Although some authors point out that *C. albicans* has a main role in the development of denture stomatitis[11-13], others[58,59] consider the amount of micro-organisms such as *Streptococcus*, *Staphylococcus*, *Neisseria Lactobacillus*, *Bacteroides* and *Actinomices* a more important aetiological factor than the normal level of *C. albicans*. So antibacterial activity effect of CRJE should be more studies.

This research is only done *in-vitro*, therefore the results from this study may differ from results from an experiment conducted *in-vivo* because the oral cavity is an environment extremely rich in saliva and nutrients, which is an important factor encouraging the growth of fungus. Another important reason why *in-vitro* studies may have differ results compare with those observed in clinically is that *in-vitro* studies are frequently performed with single-species biofilm, which is less complex communities compare with multi-species biofilms. Thus more studies are still needed to provide more information regarding the mechanism of the adherence of *C. albicans* , The cytotoxicity test of CRJE to detect allergy or toxic to human epithelial and fibroblast cells need to be concerned before applying in the mouth to support the safety of royal jelly to use. In addition, a study on CRJE pre-coating application to the denture base material could also be done instead of soaking application.

We concluded that CRJE has an ability to inhibit adherence of *C. albicans* on the surface of both heat-cured acrylic resin and self-cured acrylic resin. Furthermore, these findings suggest that the action mechanisms of CRJE for the inhibition of *C. albicans* adherence are unknown and certain components of CRJE, when identified and studied in much more detail, might be used as complementary medicine. The results could be used to develop an alternative product for the patients who wear denture which made from acrylic resin. Especially for elderlies or patients with restriction on the hand movement.

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