Original Article

Efficacy of Royal Jelly Extract on Inhibition of Candida Albicans Adherence on Various Types of Denture Base Material

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Abstract

Denture stomatitis is a common disease found in 60 percent of denture wearers. The causes are denture trauma and the high concentration of *Candida albicans* adherence on the inner surface of denture. Microbial adherence is the initial stage and the most important process, which causes the disease. The aim of this research was to study the efficacy of crude royal jelly extract on inhibition of *Candida albicans* adherence on 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 concentration 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 37°C for 24 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). Adherence of *Candida albicans* was found on both heat-cured and self-cured denture base acrylic for the negative control group, but was less found on both types of tissue conditioners. In addition, crude royal jelly extract solution at a concentration of 50 mg/mL and 25 mg/mL could significantly inhibited the adherence of *Candida albicans* when compare with the negative controls group ($P < 0.05$). The increase of royal jelly concentration further reduced the adherence of *Candida albicans* on both types of denture acrylic, which was consistent with the SEM result. 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 a baseline information for further development of royal jelly products as an antimicrobial agent especially for those who wear denture.

Keywords: Acrylic resin, *Candida albicans*, Royal jelly, Tissue conditioner

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Introduction

Denture stomatitis is one of the most common diseases in denture wearers, affecting 60 % of the population.[1-5] Denture stomatitis is caused by poor-fitting denture, improper denture border extension, and improper denture cleansing which could lead to microbial adherence and colonization.[6-9] *C. albicans* is often found as the cause of denture stomatitis.[10-12] The adherence of *C. albicans* on the inner surface of denture is the initial stage and the most important process, which causes the disease.[13] However, adherence processes are different depending on the type of denture base material, for example; heat-cured acrylic resin, self-cured acrylic resin and tissue conditioner, they have varying degrees of porosity, surface free energy, hydrophobicity and roughness. Acrylic resin is currently the most widely used denture base material. Introduction of PMMA (Polymethyl methacrylate) for using as 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.[14] Since its introduction, PMMA has been continuously used because of its favorable working characteristics, processing ease, accurate fit, stability in oral environment, good color stability, dimensional stability, superior esthetics, repairing ease and it can be used with inexpensive equipment, however, *Candida* can adhere to the inner surface of PMMA dentures.[15] 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.[16,17] Therefore the difference between heat-cured acrylic resin and self-cured acrylic resin is the activation process that causes free radicals. However, The polymerization of self-cured acrylic is not completed when compared to heat-cured acrylic, hence some unpolymerized monomers is left after the reaction.[18] 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 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. Tissue conditioner has been developed in order to reduce and redistribute occlusal stress especially in patients who have thin, sharp, or badly resorbed residual alveolar ridges or chronic tissue irritation from denture forces that might damage the underlying mucosal tissues.[19] The problems of 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 concentrations of exotoxins and metabolic products produced by the fungal colonies.[20] Unfortunately, conflicting adherence results are reported on tissue conditioner. Some *in vitro* studies reported significant inhibitory effects on *C. albicans*.[21] However, some studies showed only limited antifungal properties and no significant reduction on Candida adherence.[22] *C. albicans* adhere to polymeric surfaces by Van der Waals and electrostatic forces.[5,23,24] 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 intermediated 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 inner surface of denture is the initial stage which causes the denture stomatitis.[13] Thus the prevention of *C. albicans* adherence to acrylic resin could be a possible method for prevention of denture stomatitis.[25]

In general, denture stomatitis often be treated by application of topical antifungal drug, Nystatin which is commonly used for the treatment of local fungal infection is therefore often used for treating this disease. 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.[26] Despite aforementioned benefit, the antifungal medications are chemically synthesized and 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 of the *in vitro* and *in vivo* antibacterial action of Royal jelly, which is a natural product.[27,28] It 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 (Trans-10-hydroxy-2-decenoic acid), which is an efficient bacteriostatic against gram-positive and gram-negative bacterias.[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.

Materials and methods

The sample size calculation

The sample size was calculated using G* Power 3.0 for Windows XP program.[30] 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.

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) and tissue conditioners (Soft liner, GC corporation, Tokyo, Japan / Dura conditioner, Dental Mfg., Worth, IL) were prepared according to the manufacturer's recommended ratio shown in Table 1 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) 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. They were then sterilized by ethylene oxide gas.

Preparation of *C. albicans*

The *Candida* strains used in this study was *C. albicans* (ATCC 90028), 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))

Preparation of royal jelly extract

Royal jelly powder (Su Pha Bee Farm, Chiang Mai, Thailand) was extracted with 20 percent ethanol at a concentration of initial solution royal jelly equals 100 mg/mL. 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 and freeze dried (FreeZone 2.5, LABCONCO., USA). 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)

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 Liechtensten	Self-cured	**Powder** Polymethyl methacrylate, Softening agent, Benzoyl peroxide, Catalyst, Pigments **Liquid** Methyl methacrylate, 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

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. After that, the yeast colonies were observed to get the MIC and MFC value.

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. 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.[31] 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 4 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.

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, specimen were washed with PBS and then soaked in 2.5 percent Glutaralaldehyde in PBS for two hours, 25°C, then washed with PBS, dehydrated with Alcohol and sputter coated with platinum for investigation with scanning electron microscopy (JEOL, USA) at a magnification of 400 times.

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.

Results

C. albicans adherence was investigated in four types of denture base materials. The results were statistically analyzed using one-way ANOVA and Welch test. It was found that the groups of denture base material were significantly different on the *C. albicans* adherence. Subsequent Post-hoc test 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 2. We therefore decided to continue the study on the efficacy of various concentration of CRJE on inhibition of *C. albicans* adherence on both acrylic resin materials.

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 fungal inhibition and fungicidal effect of CRJE against *C. albicans* can be expressed in MIC and MFC values, which 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. 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 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 percent reduction in the adherence of *C. albicans* are presented in Table 4, which showed the percent 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 1. This result was consistent with the SEM result. 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. 2).

Table 3 *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 4 *Percent 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
Nystatin 23 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.

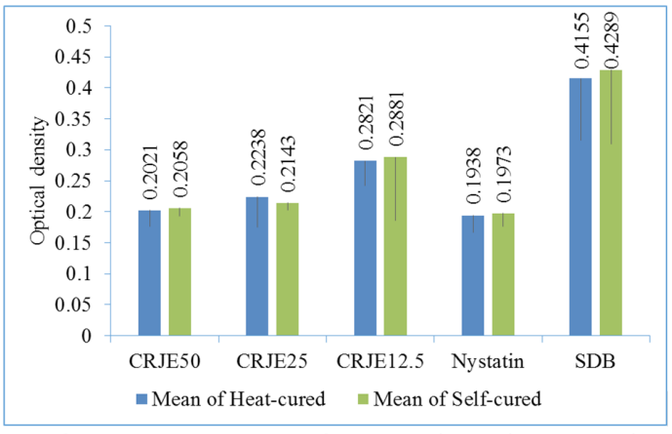

Figure 1 *Mean of the absorbance of CRJE at a concentration of 50 mg/mL (CRJE50), 25 mg/mL (CRJE25), 12.5 mg/mL (CRJE12.5), Nystatin 23 mg/mL (Positive) and SDB (Negative) of heat-cured acrylic resin (H) and self-cured acrylic resin (S).*

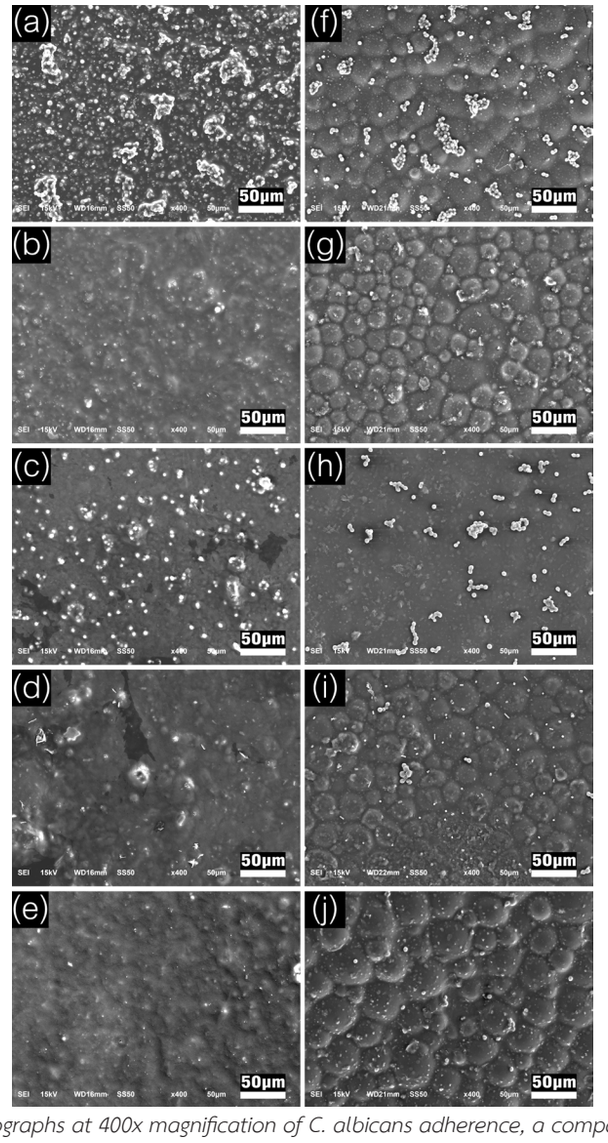

Figure 2 *Scanning electron micrographs at 400x magnification of C. albicans adherence, a comparison between heat-cured acrylic resin after exposure to SDB (a), Nystatin 23 mg/mL (b), CRJE 12.5 mg/mL (c), CRJE 25 mg/mL (d) CRJE 50 mg/mL (e) and self -cured acrylic resin after exposure to SDB (f), Nystatin 23 mg/mL (g), CRJE 12.5 mg/mL (h), CRJE 25 mg/mL (i), CRJE 50 mg/mL (j)*

Discussion

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 microbial adhesion on denture base materials varies, depend on the type of material. This could impact the physical properties such as porosity, surface free energy, hydrophobicity, or roughness.[3,5,22,23] *Candida* can adhere to the inner surface of dentures made of PMMA because it is a hydrophobic material. In addition, epithelial cell can bind easily with the hydrophobic surfaces. Microbial adherence arises from hydrophobic interaction and Lewis acid-base interaction. If the surface is very hydrophobic (low surface energy), microbial adherence is increased.[15] Regarding the study on hydrophobicity, Minagi *et al.*, 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.[32] 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 of tissue conditioners had less *C. albicans* adherence. Probably because of the material components of tissue conditioners react with the microorganisms in the mouth or Plasticizer, such as Benzyl benzoate and Benzyl salicylate is effective in killing fungus.[31,33,34] 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*.[35]

In term of the efficacy of CRJE on inhibition of *C. albicans* (Ayse Nedret Koc *et al.,* 2011), they 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.[36]

The results of the study are consistent with 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. The 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. Also the CRJE processing does not cause result an environmental pollution.

This research result is similar to Moselhy *et al.* which conducted research on the inhibition of microbial, using royal jelly from Egypt and China in experiments done by disc diffusion method. The results showed 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,[37] in which this study 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* were found to be different. A study of Bachnova *et al.* in 2004, which explained 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. The different bee species in each country were different. 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.*[39] 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.[40] 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*).[41]

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, such as creating extracellular components and chemical surface adherence inhibiting effect.[42]

In a study using synthetic substances to inhibit the adherence of *C. albicans* on various surfaces. Lin Zhou *et al.* used Parylene® to inhibit the adherence of *C. albicans* on the surface of denture made of heat-cured acrylic resin. The result showed that Parylene® had the ability to reduce the adherence of pathogenic *C. albicans* on the acrylic resin. Cell counts and XTT assay also showed significant reduction.[43]

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 is consistent with the less adherence of *C. albicans* on heat-cured resin.[44] However, the results from this study found no difference in the adherence of *C. albicans* in both of acrylic resins type. This is probably because of the specimens preparation process

This research is only an in vitro study, therefore the results from this study may differ from results from an experiment conducted in oral condition. This is an important factor encouraging the growth of fungus. Thus more studies are still needed to provide more information regarding the mechanism of the adherence of *C. albicans.* In addition, a study on CRJE pre-coating application to the denture base material could also be done instead of soaking application.

Conclusion

The study concluded that CRJE has the ability to inhibit the adherence of *C. albicans* on the surface of both heat-cured acrylic resin and self-cured acrylic resin. This is an alternative development the product to patients who wear dentures which made from acrylic resin. Especially the elderly or patients with restrictions on the hand movement.

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