Effect of *Piper betle* extract on anti-candidal activity, gelation time, and surface hardness of a short-term soft lining material

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The purpose of this study was to evaluate anti-candidal activity, gelation time, and surface hardness of a short-term soft lining material incorporated with varying concentrations of *Piper betle* extract (0.25 to 20% w/w). Agar-diffusion assay was conducted to evaluate an inhibitory effect against *Candida albicans*. The gelation time was assessed and surface hardness was measured at 2 h and 7 days by Shore AO durometer. A soft liner containing at least 5% w/w of P. betle extract was observed the inhibitory effect against *C. albicans*. An increasing of *P. betle* concentrations provided larger inhibition zone. Incorporating 5% w/w of *P. betle* extract into the soft liner did not significantly alter its gelation time and surface hardness (ANOVA; p>0.05). The optimum composition at 5% w/w of *P. betle* extract can be used as an additive in the soft liner to provide the anti-candidal activity without significantly affect these two main properties.

**Keywords**: *Piper betle*, Soft liner, Anti-candidal activity, Gelation time, Surface hardness

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**INTRODUCTION**

Soft lining materials are soft polymers applied to the fitting surface of a denture. The purpose of using these materials is to reduce and distribute occlusal loadings on the underlying mucosal tissues. It is recommended to use in the cases of sharp or atrophied alveolar ridge, thin atrophic mucosa, and recurrent sore spots under denture. Nevertheless, soft lining material is susceptible to the microbial colonization including *Candida albicans*. This can cause the risk of a candida-associated denture stomatitis.

There have been several approaches to develop antifungal effect to denture soft liner. One interesting protocol is incorporation of antimicrobial agents into the materials to prevent fungal accumulation. Many kinds of additives have been applied, including metallic oxides and polyeone, orazole drugs such as nystatin, amphotericin B, miconazole, and ketoconazol. Owing to increasing of antibiotic drug resistances and toxicity, an alternative naturally-derived agents have been developed.

*Piper betle* (Piperaceae), an annual creeper, has been extensively used as traditional herbal medicines in India, China, and countries in South-East Asia, such as Vietnam and Thailand. Its various pharmacological activities were reported as anti-microbial, anti-oxidant, anti-mutagenic, anti-carcinogenic, and anti-inflammatory activities. Furthermore, *P. betle* was reported as a good anti-fungal agent against *C. albicans*. Therefore, an extracted product from *P. betle* was introduced to be an antifungal additive in this study. From the screening study, *P. betle* extract showed antifungal activity against *C. albicans* with the minimum inhibition concentration (MIC) of 3.13 mg/mL, or approximately 0.3% w/w.

Notwithstanding, the incorporation of additive into soft lining materials might alter anti-fungal activity itself and mechanical properties of materials. An optimal condition to maintain material characteristics would be concerned. Thus, the aims of this study were to determine the anti-fungal effect against *C. albicans* of a short-term denture soft liner containing *P. betle* extract and also investigate two main properties of the lining material including gelation time and surface hardness. Our hypotheses were that incorporation of *Piper betle* extract into a short-term soft liner can provide the antifungal activity against *C. albicans* and there would be no significant alteration in gelation time and surface hardness of the material.

**MATERIALS AND METHODS**

**Material preparation**

Temporary acrylic soft lining material (GC Soft Liner™, GC, Tokyo, Japan) was used in this study. Following the manufacturer’s instruction, the standard powder/liquid ratio is 1.22 g/1 g, and it is recommended as a short-term denture soft liner. The soft liner without additive was selected as a negative control. Nystatin oral suspension (Continental Pharm, Bangkok, Thailand) 100,000 IU/mL or 33.3 mg/mL was selected to be a representative of conventional anti-fungal drugs and a positive control.

Extraction product from *P. betle* leaves was prepared from Department of Pharmacognosy and Pharmaceutical...
Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Main chemical composition of this crude contains 4-chromanol (62.33%) and eugenol (17.10%). Its extraction was incorporated into the GC soft liner to get final concentrations from 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 20% w/w.

**Anti-candidal activity testing**

Agar diffusion assay was conducted to evaluate an inhibitory effect against *C. albicans* of the soft liner incorporated with *P. betle* extract.

The *C. albicans* (ATCC® 10231™, Manassas, VA, USA) was cultured at 37°C into Sabouraud dextrose broth (Dickinson and Company, Sparks, MD, USA). After 8 h, the turbidity of the suspension was diluted to 1.0 McFarland standard (equivalent to 1.5×10^8 CFU/mL). A hundred microliter (100 µL) of suspension was spread onto Sabouraud agar plates. A 6-mm-diameter wells were created to a depth of 4 mm by a punch hole technique.

Various amount of *P. betle* extract measured by weight were mixed with the GC soft liner liquid in a sterile beaker at room temperature. Subsequently, the prepared liquid was mixed with powder to prepare the soft liner containing *P. betle* extract according to the manufacturer's instruction. Finally, the composition of *P. betle* extract in the soft liner were 0.25, 0.5, 1, 2.5, 5, 10, and 20% w/w.

The punched wells were filled with GC soft liner containing various concentrations of *P. betle* extract. According to the positive control, 20% w/w of nystatin in the soft liner was applied into the well as same manner. GC soft liner without additive was used to be a negative control. All plates were incubated at 37°C for 48 h. The MIC could be defined as the lowest concentration exhibiting clear zone around the well. The mean inhibitory zone (MIZ), in millimeters, for each test punch well was measured a diameter of clear area. Five independent experiments (*n*=5) were performed.

**Gelation time testing**

Viscoelastic property of material was determined using an oscillating rheometer (RHEOPLUS/32 Service V3.40 21000066-36670). The flat plattens were 25 mm in diameter. Frequency of oscillating movement was set at 1 Hz. The stress was applied at 40 Pa in order to control the deformation within linear viscoelastic (LVE) limit.

The GC soft liner was manually mixed with *P. betle* extract at MIC and higher concentration, following the manufacturer’s instructions. The final concentrations of the *P. betle* in the GC soft liner were 5, 10 and 20% w/w. After mixing at room temperature, the test material was placed on the lower plate of the rheometer. Then, the upper plate was lowered on its guide into the position so that the thickness of the material between the two plates was 1 mm. The changes of viscoelasticity with time in varying concentrations of *P. betle* extract were recorded at 37°C. The similar procedure was also performed for GC soft liner with 20% w/w of nystatin and without additive as controls. Five tests were carried out for each composition (*n*=5).

The parameters obtained from the viscoelastic testing compose of complex shear modulus (G*), shear storage modulus (G'), shear loss modulus (G''), and loss tangent (tan δ). To analyze the results, shear modulus values (G*, G', G'') and tan δ were plotted against time (Fig. 1). The gelation time can be defined as a time point when loss and storage modulus are equal (G'=G''). The effect of *P. betle* extract on viscoelastic behavior of the short-term soft liner was also discussed.

**Shore AO surface hardness testing**

According to ISO 10139-1:2018, the surface hardness of a short-term soft lining material can be evaluated by Shore AO hardness durometer. Similar to gelation time testing, final concentrations of *P. betle* extract in GC soft liner were 5, 10 and 20% w/w. They were manually prepared for 30 s at room temperature, and then loaded into a stainless steel mold (55 mm in diameter and 8 mm thickness; Fig. 2). Waiting for 15 min, the specimens were immersed into water bath at 37±1°C for 2 h. Then, the
specimens were removed from the mold and measured the Shore AO hardness. The values were recorded 5 s after loading the durometer. In each specimen, five different loading areas were tested as shown in Fig. 2. Subsequently, the specimens were reimmersed into the water bath at 37±1°C and maintained for 7 days. The Shore AO hardness at 7 days was measured again on the opposite site of the specimen in the same manner. The similar procedure was done for GC soft liner with 20% w/w of nystatin and without additive as controls. Five specimens (n=5) were tested and mean Shore AO values were calculated.

Statistical analysis
All statistical computations were performed by SPSS software (IBM corp. released 2013, IBM SPSS statistics for Windows, version 22.0, IBM, Armonk, NY, USA). The significant level was set at α=0.05.
Shapiro-Wilk test was performed to validate normality of the data and Levene’s test for equality of variances between group data. Comparison in the inhibition zone and gelation time between different compositions were analyzed by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison test or the Games-Howell post-hoc test. Influence of material compositions and immersion times on the surface hardness were evaluated by repeated two-way ANOVA and Pairwise comparisons.

RESULTS

Evaluation of anti-candidal activity
The anti-candidal activity of the short-term soft liner with P. betle extract was illustrated by inhibition zone as shown in Fig. 3. Means of the inhibition zones were measured and shown in Table 1. Negative control and GC soft liner with P. betle 0.25, 0.5, 1 and 2% w/w did not show any inhibitory effect against C. albicans. The inhibition zone was clearly observed about 7.58±0.79 mm at the concentration at least 5% w/w of P. betle extract. Furthermore, higher concentration (10 and 20% w/w) of P. betle extract showed significantly larger inhibition zone (p<0.05). Accordingly, MIC of the P. betle extract in GC soft liner was 5% w/w. However, no significant difference of inhibition zones between same concentration 20% w/w of P. betle extract and nystatin was found (p>0.05).

Gelation time testing
As mentioned previously, the MIC against C. albicans of P. betle extract in the soft liner was 5% w/w. Consequently, three effective concentrations (5, 10, and

Table 1  Mean±the standard deviation (SD) of Inhibition zone in the different test samples

| Samples                        | Inhibition zone (Ø, mm) |
|--------------------------------|-------------------------|
| GC soft liner                  | ND                      |
| GC soft liner+P. betle 0.25% w/w| ND                      |
| GC soft liner+P. betle 0.5% w/w | ND                      |
| GC soft liner+P. betle 1.0% w/w | ND                      |
| GC soft liner+P. betle 2.5% w/w | ND                      |
| GC soft liner+P. betle 5.0% w/w | 7.58±0.79               |
| GC soft liner+P. betle 10% w/w  | 10.43±1.15              |
| GC soft liner+P. betle 20% w/w  | 16.40±1.11              |
| GC soft liner+nystatin oral suspension 20% w/w | 16.59±0.85 |

Uncertain inhibition zone or less than 6 mm was defined as “not detected, ND.”
20% w/w) of *P. betle* extract and nystatin (20% w/w) were selected to evaluate the gelation time compared to GC soft liner without additive. The gelation times of GC soft liner with different composition of additives are shown in Fig. 4.

The gelation time of GC soft Liner without additive (control) was 91.7±2.3 s. The incorporation of 10 and 20% w/w of anti-fungal additives significantly altered gelation time of this material. The soft liner containing 20% w/w of nystatin oral suspension showed slowest gelation time (200.3±12.8 s) and its gelation time was statistically significant difference when compared to control (*p*<0.05). While, the *P. betle* extract slightly extended the gelation time; 98.8±6.9 s, 112.6±3.9 s, and 118.2±2.4 s for 5, 10, and 20% w/w, respectively. We found that incorporation of 10 and 20% w/w of *P. betle* extract into the soft liner provided significantly longer gelation times when compared to the control (*p*<0.05). However, no significant difference was found between the gelation times of GC soft liner with 5% w/w of *P. betle* extract and control (*p*>0.05).

Besides the gelation time, viscoelastic behavior can be also investigated by the oscillating rheometer. Three parameters were determined. The change in *G'*, *G''*, and tan δ against time were plotted in Fig. 5.

Regarding GC soft liner with no additive, its storage modulus (*G'*), and loss modulus (*G''*) increased exponentially and then increased gradually. For loss tangent (tan δ), a decreasing with time was noted. Remarkably, the incorporation of additives into the soft liner led to change in the pattern of *G'*, *G''*, and tan δ especially in the GC soft liner with 20% w/w nystatin oral suspension. For the *P. betle* extract, dose dependent effect was found. Higher concentration of *P. betle* extract in the soft liner provided slower increasing rate of the *G'* and *G''* and decreasing rate of tan δ. Fortunately, the soft liner with only 5% w/w of *P. betle* extract showed trend in these parameters as same as the control GC soft liner.

**Shore AO surface hardness testing**

Three different concentrations (5, 10, and 20% w/w) of *P. betle* extract into GC soft liner were also determined the
Denture soft liners are clinically used for therapeutic purposes to improve adaptation of the denture and promote recovery of traumatized tissue. However, it could be a reservoir of oral microflora including fungi, due to no prevention effect against microbial accumulation. Several agents have been developed to be anti-fungal additives either synthetic or natural antifungal agent into soft liners for controlling microbial attachment and colonization. The soft lining materials with antifungal additives could provide a great advantage for patients with poor compliance. In this method, the denture liners can serve as a vehicle for drug delivery system in the oral cavity. Consequently, this approach can reduce frequency of application during treatment.

Medicinal herbs have been interesting because of its safe and affordability, especially in developing countries. Herein, P. betle was used as the antifungal additive. It has been reported as a good antifungal agent since it contains bioactive components such as 4-chromanol, eugenol, allylpyrocatechol, and hydroxychavicol. According to previously study, P. betle used in this study contains 4-chromanol as the active compound to be responsible for the antifungal activity. Its MIC against C. albicans ranged from 1.56 to 3.13 mg/mL. In this study, the MIC of P. betle extract combined with GC soft liner was 5% w/w. Accordingly, its anti-fungal effect seemed to be subsided after incorporating process. This finding corresponded to previous studies that reported a decrease in antifungal properties of drugs when they were loaded into denture liners. Moreover, the soft lining material was also claimed to be a drug carrier for a sustained delivery system. The anti-fungal agents were gradually released from the soft liner molecules and maintained the effective dose for a period of time.

As mention previously, the incorporation of additive should not alter material properties. The gelation time is one crucial property of denture soft liners. It determines working time, manipulation after mixing, and adaptation between the supporting mucosa and the denture fitting surface. Regard to the result, it was found that both antifungal additives could delay the gel formation period of GC soft liner, particularly for 20% w/w of nystatin. For P. betle extract, higher concentration of P. betle extract in the soft liner provided longer gel formation. Since the P. betle extract is viscous substance, the addition of such additive into the soft liner would decrease powder to liquid ratio. According to a previous study, lower powder to liquid ratio reduced diffusion coefficients and provided less polymer entanglement, resulting in longer gelation times. Interestingly, the incorporation of less amount of additive (the soft liner with 5% w/w of P. betle extract) did not exhibit significant alteration in the Shore AO hardness at initial stage (2 h) and late stage (7 days) as shown in Fig. 6. All tested materials and control showed significantly increases in the Shore AO hardness values after aging process. However, at both initial and late stages, no significant difference of shore AO hardness in the groups of 20% w/w of nystatin and 5% w/w of P. betle when compared to the control GC soft liner. Though, there was a significant alteration of the material’s surface hardness in 10 and 20% w/w of P. betle groups at both initial and late stages when compared to the control GC soft liner.

**DISCUSSION**

Denture soft liners are clinically used for therapeutic purpose to improve adaptation of the denture and promote recovery of traumatized tissue. However, it could be a reservoir of oral microflora including fungi, due to no prevention effect against microbial accumulation. Several agents have been developed to be anti-fungal additives either synthetic or natural antifungal agent into soft liners for controlling microbial attachment and colonization. The soft lining materials with antifungal additives could provide a great advantage for patients with poor compliance. In this method, the denture liners can serve as a vehicle for drug delivery system in the oral cavity. Consequently, this approach can reduce frequency of application during treatment.

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composition with 5% w/w of \textit{P. betle} extract.

Interestingly, the soft liner containing 20% w/w of nystatin had significant longer gelation time than the control GC soft liner, while it had no difference in Shore AO hardness. This phenomenon is mainly contributed to the different in polarity between aqueous solution of nystatin oral suspension and PEMA composition of GC soft liner, so it will not compatible to each other\textsuperscript{30). This incompatibility resulted in no softening of the lining material and interrupted the gelation process. Finally, the hypotheses can be supported by the short-term soft liner combined with 5% w/w of \textit{P. betle} extract. It could be an optimal concentration that promotes inhibitory effect against \textit{C. albicans} without dramatic change in the material properties including the gelation time and surface hardness. The limitation of this study is the difference in the amount of soft liner used in the experiment and clinical situation. Hence, further investigation should be carried.

Moreover, drug releasing profile, water sorption, and color stability should also be investigated before clinical use.

CONCLUSION

\textit{P. betle} extract incorporated into GC soft liner could promote anti-fungal activity against \textit{C. albicans}. Additionally, an application of the short-term soft liner containing \textit{P. betle} extract with an optimal concentration at 5% w/w showed no alteration of the material properties as both gelation time and surface hardness. This could be a noteworthy herbal substance to serve as an anti-fungal agent.

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