Journal of
Oral Pathology & Medicine

J Oral Pathol Med (2011) 40: 90–96

doi: 10.1111/j.1600-0714.2010.00921.x

wileyonlinelibrary.com/journal/jop

Antifungal activity of lawsone methyl ether in comparison with chlorhexidine

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OBJECTIVE: The aim of this study was to determine the antifungal activity of lawsone methyl ether mouthwash (LME) in comparison with chlorhexidine mouthwash (CHX) *in vitro* and *in vivo*.

MATERIALS AND METHODS: For *in vitro* study, each mouthwash preparation was added into the inoculum of *Candida*. The turbidity was recorded after incubation at 37°C for 48 h. Candidal culture was performed and the number of colony of *Candida albicans* was recorded. For *in vivo* study, a crossover clinical trial was conducted in 22 HIV-infected subjects and 32 denture wearers. Clinical examination was performed and oral rinse technique was carried out immediately before and 0, 1, 2 h after using each mouthwash. Allergy and subjective assessment of the mouthwashes were recorded. Statistical analysis was performed using one-way ANOVA and linear mixed effect modeling.

RESULTS: *In vitro*, antifungal activity of 0.25% LME was significantly greater than that of 0.12% CHX ($P < 0.05$) and comparable with that of 0.2% CHX. *In vivo*, antifungal activity up to 2 hours of 0.025% LME mouthwash was evidenced in both groups of subjects, although significantly lower than that of 0.12% CHX. No allergic reaction was reported. LME mouthwash was graded to have less bitter taste than that of CHX. Subjects' satisfaction on taste and smell of LME mouthwash was significantly greater than that of CHX ($P < 0.05$).

CONCLUSIONS: Lawsone methyl ether mouthwash possesses potent antifungal activity both *in vitro* and *in vivo*. However, concentration of the mouthwash needs to be adjusted in addition to further clinical trials on long-term use.

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Accepted for publication May 1, 2010

J Oral Pathol Med (2011) **40**: 90–96

Keywords: antifungal activity; *Candida;* chlorhexidine; HIV; lawsone methyl ether; mouthwash

Introduction

Oral candidiasis (OC) caused by *Candida* species is frequently observed in HIV-infected subjects (1–4) and denture wearers (5). The colonization and growth on prostheses by *Candida* species are of clinical importance. A wide variety of agents including both topical and systemic antifungal drugs are available for the treatment of OC (6, 7). However, this opportunistic oral disease has increased in its prevalence despite the therapeutic progress. The relative ease with which OC can be treated contrasts with the high rate of recurrence observed. Many patients with HIV infection suffer from recurrent episodes of OC with a relapse rate higher than 50% in the mouth following successful treatment (8). In HIV-infected individuals with persistent OC, repeated course of antifungal therapy can only obtain a clinical reduction in the disease without a real resolution (9). Thus, control of the symptoms rather than cure may be the goal in the treatment of OC in this patient group.

In developing countries, where antiretroviral therapy is available to a small percentage of HIV-infected population, OC is still a major problem. As HIV-infected individuals are likely to develop OC during the course of the disease, prevention is better than the frequent use of antifungals that may lead to the development of drug resistance. Chlorhexidine (CHX) gluconate at 0.2% concentration has long been shown that it can be used as a mouthwash in the treatment of chronic atrophic and acute pseudomembranous candidiasis (10). A recent study by Nittayananta et al. (11)

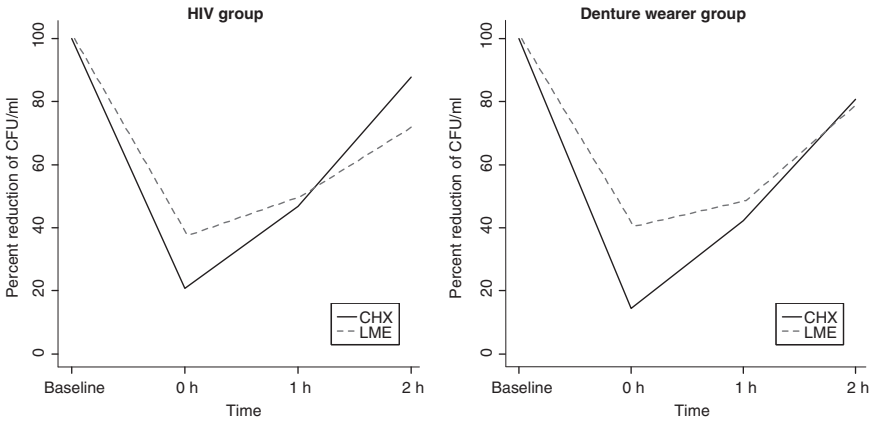

Figure 1 Antifungal activity of 0.025% lawsone methyl ether mouthwash (LME) vs. 0.12% chlorhexidine mouthwash (CHX) determined by geometric mean of percent of reduction in colony forming unit (CFU/ml) of *Candida* isolated from HIV-infected subjects and denture wearers after using the mouthwashes.

reported that 0.12% CHX may be a useful mouthwash in the maintenance of OC-free period among HIV-infected subjects. However, bitter taste and tooth staining of CHX may cause poor compliance.

Lawsone methyl ether (2-methoxy-1,4-napthoquinone) (LME) isolated from *Impatiens balsamina* L. (12, 13) and *Swertia calycina* (14) was found to be a group of active constituents exhibiting potent antifungal and antibacterial activities (12, 15–17). The value of both minimal inhibitory concentration and minimal fungicidal concentration of LME against *Candida* was 1.25 μg/ml (12). Another study reported that the minimal amount of LME required for inhibiting the growth of *Candida albicans* was 0.4 μg (14). A previous study on cell culture revealed that LME exhibits anti-allergic and anti-oxidative activities without the toxicity to cells (18).

As antifungal activity of LME has been reported, the agent might be used as an intervention after successful treatment of OC to prolong the time for relapse and to reduce antifungal drug use. Thus, the purpose of this study was to determine the antifungal activity of LME mouthwash *in vitro* and *in vivo* and compare with that of CHX.

Materials and methods

Preparation of 0.5% LME mouthwash

Lawsone methyl ether potassium salt was performed by the method previously described (19). LME mouthwash was prepared as follows: LME potassium salt (0.5 g) was dissolved in purified water (25 ml), and then glycerol (20 ml) was added and mixed well, and dissolved l-menthol (0.02 g) and peppermint oil (0.05 g) in absolute ethanol (20 ml) and added into the solution of LME potassium salt. The sufficient water was added to make a total volume of 100 ml.

Determination of antifungal activity of 0.25% LME mouthwash in vitro

Candida albicans was *in priori* isolated from 51 HIV-infected subjects. The inoculum of 1×10^6 to 5×10^6 cell/ml was prepared by isolating colonies of *C. albicans* from each stock with RPMI-1640 using 0.5 McFarland. The adjusted inoculum of 0.5 ml was placed in four test tubes. Each mouthwash (0.5%LME, 0.24% and 0.4%CHX) of 0.5 ml volume was then added into each test tube except the control one. All test tubes were incubated at 37°C for 48 h. The turbidity of each tube was compared with its control and recorded. The antifungal activity of each mouthwash was performed by sub-culturing 100 μl aliquots from each tube onto Sabouraud's dextrose agar (SDA) plates. The plates were incubated at 37°C for 48 h before the number of colony was counted. The number of colony growth was used as the assessment of the antifungal activity. In addition, 100 μl aliquots from the control tube was poured on SDA plates and kept in the same incubator for the same period of time to confirm the medium sterility and viability of the tested organism.

Determination of antifungal activity of 0.025% LME mouthwash and 0.12% CHX mouthwash in vivo

Subjects

A crossover clinical trial was performed in two groups of adults. The first group consisted of HIV-infected subjects previously diagnosed as seropositive for antibody to HIV, using a particle agglutination test for antibodies to HIV (SERODIA®-HIV; Fujirebio Inc., Tokyo, Japan) and an enzyme-linked immunosorbent assay (ELISA) (Enzygnost® Anti-HIV1/2 Plus; Behring, Behringwerke AG, Marburg, Germany). They came for regular treatments at the Internal Medicine Clinic at Songklanagarind Hospital and Hat Yai Regional Hospital. The second group was comprised of denture wearers who came for routine dental care at the Dental Hospital at Prince of Songkla University in southern Thailand. Other inclusion criteria common to both groups were: (i) no current use or history of antifungal therapy within the last month; (ii) no history of allergy to mouthwash; (iii) able to use a mouthwash properly and (iv) willing to provide informed consent.

The exclusion criteria were: (i) history of local radiation therapy on head and neck region; (ii) pregnant woman and (iii) severely ill subject who could not cooperate with the study procedures of using the mouthwash.

Sample size calculation

As this was a crossover study, the hypothesis was tested on the same subjects with a meaningful intra-subject difference of one-third of the standard deviation. The formula for calculation of sample size was $n = (1.96 \times S/d)^2$ where n = sample size required S/d = ratio between the standard deviation of the difference and the level of change considered as importance.

Assuming that S/d being 3 (or important change is as small as one-third of the standard deviation), the required sample size was 34.6 or 35 subjects.

In practice, we could recruit up to 22 HIV-infected subjects and 32 denture wearers.

History taking and study procedure

History taking and oral examination were performed in all subjects. Clinical diagnosis of HIV-related oral lesions was made according to the criteria classified by the EC-Clearinghouse (20). The following data were recorded; age, gender, marital status, education, income, smoking habit, alcohol consumption, systemic disease, current drug use, presence of oral symptom, presence of oral lesions, oral hygiene, type of denture, and number of teeth.

Before using the mouthwash, the oral rinse technique (21) was performed in each subject to determine the CFU/ml of *Candida* as baseline data. The subjects were then randomized to receive either 0.025% LME or 0.12% CHX. They were advised to rinse their mouth with 10 ml of the mouthwash for 1 min and then spit it out. After using the mouthwash, the oral rinse technique to obtain specimen for culture was performed again immediately (T = 0), at 1 h (T = 1 h) and 2 h (T = 2 h), respectively. A questionnaire to assess subjects' satisfaction using the mouthwash was given for immediate assessment. After 1 week, the subjects were appointed to come for the same procedures but switching to the other mouthwash.

Allergy and subjective assessment of 0.025% LME in comparison with 0.12% CHX

Subjects were asked to observe and record allergy for each mouthwash at four different time points; within 30 min, 24 h, 48 h and 72 h after using the mouthwash. Feeling of burning sensation and feeling of bitter taste were assessed using a visual analog scale (VSA). Subjects' satisfaction using each mouthwash was graded in terms of taste and smell.

Ethics

The study protocol was approved by the research committee at the Prince of Songkla University and at the Ministry of Public Health. All information about the patients and their identity was anonymous. Subjects were given both verbal and written information about the nature of the study and written consent was obtained. They were allowed to leave the study at any time during the procedures.

Data entry and statistical analysis

Data were computerized in SPSS 16 (SPSS Inc., Chicago, IL, USA) and cleaned, coded and analyzed using R program. For the *in vitro* study, the distribution of colony of *Candida* recovered was broken down by mouthwash type in a table. One-way ANOVA test was carried out to examine whether they had the same population mean. If the overall differences were significant in the ANOVA test, comparison between possible pair of preparation was further made with *P*-value adjustment for multiple comparisons using Bonferroni's method (22).

For the *in vivo* study, geometric mean of CFU/ml reduction by group of subjects and mouthwash preparation was plotted against time of specimen collection (T = baseline, 0, 1 and 2 h after mouth washing). As the tests were repeated in the same subjects over time and across preparations, mixed effects Poisson regression was used. As our main interest was in the reduction in CFU/ml from baseline, the $\log_e$ (baseline CFU/ml) was inserted into the model as an offset term. In other words, we predicted reduction from baseline, and not absolute value. The random effects part denotes variation among subjects under study in each group, which is not the main interest of this study and therefore not displayed. The fixed part of the model consists of variables, the effects of which were fixed to all subjects. These variables were: whether they were in HIV-positive 'group', 'time' of specimen collection (baseline as the referent group), and 'treatment' with 0.025% LME vs. 0.12% CHX. As the effect of the mouthwash may change over time and vary across the group of subjects, two interaction terms i.e., time × treat and group × treat were added. Package 'lme4' (23) was used. Significance of the interaction term was tested by comparing models with and without the terms. Significance of time*treat would indicate that the better mouthwash preparation had inconsistent level of superiority over time. Significance of the group*treat would mean that the superiority of one mouthwash over the other is not consistent across the groups of subjects. All significant levels were set at $P < 0.05$.

Results

Antifungal activity of 0.25% LME in comparison with 0.12% and 0.2% CHX in vitro

All tubes with the mouthwash were clear compared with the controls. Positive culture of *Candida* was found in 4 of 51 plates (8%) of 0.25% LME, 13 of 51 plates (25%) and 5 of 51 plates (10%) of 0.12% and 0.2% CHX, respectively. The mean numbers of *Candida* on culture of each mouthwash were 0.84 (range 0–24), 3.08 (range 0–40), 0.35 (range 0–13), respectively. Antifungal activity was found to be significantly different between the two concentrations of CHX ($P < 0.05$). However,

Table 1 Number and mean of colonies of *Candida* grown on 100 μl of each mouthwash culture

Type of mouthwash	*Number of candidal isolate tested*	*Range number of colonies*	*Mean ± S.D. of colonies*
0.25% LME	51	0–24	0.84 ± 3.74[a]
0.12% CHX	51	0–40	3.08 ± 8.34
0.2% CHX	51	0–13	0.35 ± 1.85[a]

LME, lawsone methyl ether mouthwash; CHX, chlorhexidine mouthwash.
One-way ANOVA F-test *P*-value < 0.05.
[a]Rows having the same superscript denote no significant difference at *P*-value of 0.05 with adjustment for multiple comparison using Bonferroni's method.

no significant difference was observed between the antifungal activity of 0.2% CHX and 0.25% LME ($P > 0.05$) (Table 1).

Antifungal activity of 0.025% LME in comparison with 0.12% CHX in vivo

Twenty-two HIV-infected subjects (age range 30–56 years, mean age 39 years) and thirty-two subjects with denture (age range 22–87 years, mean age 54 years) were enrolled. Demographic data of the two groups are shown in Table 2.

Figure 1 shows reduction in CFU/ml overtime by both mouthwashes in HIV-infected subjects and denture wearers. The immediate effect of both mouthwashes is shown by distinct depression of the lines at 0 h, which was followed by slow gain at 1 h and 2 h after application of the mouthwash. Of interest, 0.025% LME showed potent antifungal activity in both HIV-infected subjects and denture wearers, although significantly lower than that of 0.12% CHX.

The results of mixed effects Poisson regression (only the fixed part) are summarized in Table 3. The final model had all terms except 'visit1' being highly statistically significant. Visit1 = LME was not significant indicating that the order of using LME before CHX and vise versa in the same person did not have any effect on the test results. In other words, there was no 'carry-over' effect of the first mouthwash to the second. All main effects (with no interaction term) of 'times' were significant suggesting that in the CHX group, subsequent CFU/ml was lower than the initial one. The interaction term between time and LME, and HIV and LME was all positive suggesting that LME had less inhibitory effect, especially among the HIV group.

Allergy and subjects satisfaction on 0.025% LME in comparison with 0.12% CHX

No subjects reported any allergic reactions due to the mouthwashes. VSA of burning sensation and feeling of bitter taste during and after using the mouthwash showed no significant difference between the two mouthwashes. When degree of bitter taste was compared, more subjects scored that 0.025% LME was significantly less bitter than 0.12% CHX ($P < 0.05$). Satisfaction of subjects on taste and smell of 0.025% LME was shown to be significantly better than 0.12% CHX ($P < 0.05$).

Table 2 Demographic data and characteristics of subjects enrolled

Variables	*Subjects*	
	HIV-infected subjects(n = 22)	*Denture wearers (n = 32)*
Age		
Age range (year)	30–56	22–87
Mean age (year)	39	54
Gender		
Male	10 (45%)	6 (19%)
Female	12 (55%)	26 (81%)
Marital status		
Single	4 (18%)	7 (22%)
Married	16 (72%)	25 (78%)
Divorce	1 (5%)	0 (0%)
Widow	1 (5%)	0 (0%)
Education		
None	1 (5%)	1 (3%)
Primary level	5 (23%)	7 (22%)
Secondary level	11 (50%)	2 (6%)
Polytechnic level	2 (9%)	6 (19%)
Bachelor degree	3 (13%)	16 (50%)
Income (Baht)/month		
< 5000	5 (23%)	4 (13%)
5000–10 000	10 (45%)	10 (31%)
10 001–20 000	5 (23%)	10 (31%)
> 20 000	2 (9%)	8 (25%)
Smoking habit		
Smoker	7 (32%)	3 (9%)
Non-smoker	15 (68%)	29 (91%)
Alcohol consumption		
Drinker	6 (27%)	2 (6%)
Non-drinker	16 (73%)	30 (94%)
Presence of systemic diseases		
Yes	22 (100%)	15 (47%)
No	0 (0%)	17 (53%)
Current drug taken		
Yes	18 (82%)	12 (38%)
No	4 (18%)	20 (62%)
Oral hygiene		
Good	0 (0%)	5 (16%)
Fair	12 (55%)	21 (66%)
Poor	10 (45%)	6 (18%)
Presence of oral symptom		
No	17 (77%)	28 (88%)
Pain	0 (0%)	0 (0%)
Dryness	3 (14%)	1 (3%)
Burning sensation	1 (5%)	2 (6%)
Pain and dryness	1 (5%)	0 (0%)
Pain and burning sensation	0 (0%)	1 (3%)
Number of teeth		
Mean	25	20
Range	5-32	0–31

Discussion

Our *in vitro* study demonstrated that 0.5%LME mouthwash exhibits potent antifungal activity against *C. albicans*. The activity was significantly greater than that of 0.12% CHX ($P < 0.05$) and comparable with that of 0.2% CHX. The results of the *in vivo* study were consistent with these findings. Antifungal activity of 0.025% LME mouthwash was distinct in both HIV-infected subjects and denture wearers, although significantly lower than that of 0.12% CHX. To our knowledge, this is the first study attempted to determine antifungal activity of the active constituent extracted from *I. balsamina* L. on oral *Candida* both *in vitro* and *in vivo*.

Table 3 Final model predicting $\log_e$ CFU/ml of *Candida* in various treatments, group of patients and time based on mixed model Poisson regression

Variables	*Estimate*	*SE*	*P-value*
1st visit1 = LME vs. CHX	0.1	0.0776	0.23
T = 0 vs. baseline*	−1.92	0.0004	< 0.001
T = 1 h vs. baseline*	−1.14	0.0003	< 0.001
T = 2 h vs. baseline*	−0.75	0.0002	< 0.001
Treat = LME vs. CHX	−0.28	0.0003	< 0.001
Group = HIV vs. denture	0.1	0.0789	0.23
T = 0:treat = LME	1.21	0.0005	< 0.001
T = 1:treat = LME	0.68	0.0004	< 0.001
T = 2:treat = LME	0.42	0.0004	< 0.001
Treat = LME:Group = HIV	−0.08	0.0007	< 0.001

LME (0.025%), lawsone methyl ether mouthwash; CHX (0.12%), chlorhexidine mouthwash.
Baseline level was the referent group.

A previous study revealed that 2-methoxy-1, 4-naphthoquinone was the compound responsible for antifungal activity of LME (14). It has been reported that lawsone and LME were detected as the major naphthoquinones found in *I. basamina* leaves, while bilawsone was a minor one. In addition, a mixture of 50% v/v chloroform in methanol is a suitable solvent for the naphthoquinone extraction (24). A study *in vitro* by Panichayupakaranant and Reanmongkol, (19) reported that 0.5% LME preparation in oral base has antifungal activity similar to 1% clotrimazole cream. A study of its toxicity was performed in rats, and no skin rash was observed at 24 h and 72 h after application or even when it was applied for 5 days (19).

Chlorhexidine gluconate in a concentration of 0.2% is widely prescribed in dentistry as an antiseptic mouthwash due to its broad spectrum antimicrobial activity including *C. albicans* (25). Little is known about the mechanism of action against *Candida*. Ultrastructural studies of *Candida* exposed to CHX have shown coagulation of nucleoproteins with inhibition of budding and cell wall changes. These may lead to death of some cells while cells with previously protruding buds survived revealing both fungicidal and fungistatic effects of the mouthwash (26). It has been proposed that the fungicidal activity of CHX may be due to its penetration of the region of the glycerol moieties with subsequent breakdown of the permeability barrier and leaking of cytoplasmatic contents (27). A previous study demonstrated that exposure of the oral mucosa to CHX devastates the normal ultrastructure of epithelial cells and their receptors for microbes (28). The efficacy of CHX may depend on its pharmacodynamic associated with binding to the oral mucosa. It has been shown that after rinsing with 10 ml of 0.2% CHX for 1 min, approximately 30% of the drug may be retained in the mouth for up to 24 h (29–31). As the mode of action of LME is not clearly understood, ultrastructural studies of *Candida* exposed to this compound should be performed to determine how the compound affects the organism.

In our *in vitro* study, antifungal activity of 0.12% CHX was found to be less potent than that of 0.2% CHX. This finding was consistent with a previous *in vitro* study by Pizzo and Giuliana (32). Another study reported that the mouthwash containing triclosan and sodium fluoride was found to decrease the *Candida* count more than the placebo ($P = 0.005$) (33). However, due to its good substantivity, CHX mouthwash is still considered to be the most effective chemical antimicrobial agent. The *in vitro* study by Pizzo and Giuliana (32) suggested that CHX mouthwash may represent an appropriate to conventional antifungal drugs in the management of OC. Unfortunately, brown staining of the teeth and oral mucosa usually occurs following the application of the mouthwash (31, 34). In addition, many patients may find its initial taste unpleasant and repeated use often produces taste disturbances, which may last for several hours. Desquamative lesions and soreness of the oral mucosa have also been reported (31). Thus, LME may be developed as an alternative mouthwash for prophylaxis of OC to avoid such unwanted effects. However, the fact that 0.025% LME was useful but significantly inferior to 0.12% CHX indicates the need for concentration adjustment of this alternative mouthwash.

In our study, we did not assess antifungal activity of LME in comparison with other antifungal drugs such as nystatin or fluconazole, because a previous study revealed that nystatin was not effective in preventing OC *in vivo* (35). In contrast, fluconazole, a bis-tri-azole antifungal agent, was shown to be effective in preventing clinical episodes of OC compared to placebo and no treatment (35). However, there is a concern that prolonged use of fluconazole increases the risk of developing azole-resistant *C. albicans* (36). Thus, although the use of fluconazole reduces the risk of OC in patients with advanced HIV, it is not recommended as primary prophylaxis because there is potential for resistant *Candida* organisms to develop as well as the cost of prophylaxis.

As OC is frequently observed in HIV-infected subjects and denture wearers, the cost of prophylaxis seems to be a particular concern and may have a major impact on the choice of treatment in the resource poor-setting. The cost-effectiveness of prophylaxis of HIV-related opportunistic infections varies widely (37). In general, azoles are the more expensive compounds, with ketoconazole being cheaper, but with more side-effects. One trial by Nyst et al. (38) reported the cost of gentian violet, nystatin and ketoconazole in Africa. Gentian violet is much cheaper than ketoconazole (0.5 US $/30 ml vs. 13–17 US$/10 tablets) and nystatin oral suspension (of which four bottles of 2.4 million units are necessary per treatment course at 4–5 US$/bottle). In our study, the cost of 0.025% LME mouthwash was 0.5 US$/30 ml, which is comparable to that of 0.12% CHX mouthwash.

Thus, in a resource-poor setting, there is an urgent need for less expensive antifungal drugs that can be used as prophylaxis of OC. Also, there is a strong need for more research to be performed on the treatment and prevention of OC in children, as it is reported that OC is the most frequent fungal infection in children and adolescents who are HIV positive. Clearly, more research on the effectiveness of less expensive

interventions needs to be carried out in developing countries. Development of drug resistance to *Candida* remains under-studied and more works must be carried out in this area.

In conclusion, our study demonstrated that LME possesses potent antifungal activity against oral *Candida*. As an ideal antimycotic for treating OC is not yet available, LME may be an appropriate alternative mouthwash in prophylaxis of OC among HIV-infected individuals, denture wearers or other immuno-compromised patients who are at risk of developing OC. The proper concentration of LME mouthwash, however, requires further research.

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Acknowledgements

This study was supported by Junior Project grants from the Faculty of Dentistry, Prince of Songkla University and by a Prince of Songkla University grant, Prince of Songkla University, Hat Yai, Thailand. The authors wish to thank all subjects included in the study for their kind cooperation. The generosity of Colgate-Palmolive (Thailand) Limited is acknowledged for providing their product gift set with compliments to the subjects.