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Ingredients Contribute to Variation in Production of Reactive Oxygen Species by Areca Quid

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INGREDIENTS CONTRIBUTE TO VARIATION IN PRODUCTION OF REACTIVE OXYGEN SPECIES BY ARECA QUID

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Areca quid (AQ) chewing has been implicated an independent risk factor for the development of oral cancer. Taiwanese areca quid (AQ) refers to a combination of areca nut (AN), lime, and inflorescence of Piper betle *Linn. (IPB) or* Piper betle *leaf (PBL). Studies of AQ in other countries reported that AN extract combined with lime generates reactive oxygen species (ROS), such as hydroxyl radical ($HO^{\bullet}$), known to be a contributing factor in oral mucosa damage. To determine whether $HO^{\bullet}$ is formed in the oral cavity during AQ chewing, the formation of* meta-*tyrosine (*m-*Tyr) and* ortho-*tyrosine (*o-*Tyr) from* L-*phenylalanine (Phe) was confirmed. It was demonstrated that combined aqueous extracts of AN, lime, metal ions (such as Cu^{2+} and Fe^{2+}), and IPB or PBL produced $HO^{\bullet}$. Thus, the yield of $HO^{\bullet}$ significantly increases when higher amounts of IPB or lime are added and also when Cu^{2+} and Fe^{2+} are increased. Further, the omission of any one of these ingredients significantly reduces the formation of $HO^{\bullet}$. Our results found that chewing AQ with IPB generated significantly higher $HO^{\bullet}$ than chewing AQ with PBL, and may result in greater oxidative damage to the surrounding oral mucosa.*

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The chewing of areca quid (AQ) and tobacco has been closely linked to oral cancer in parts of Southeast Asia and India (IARC, 1985). Oral cancer in Taiwan is the fifth leading cause of cancer death for males (Department of Health [R.O.C.], 2000). Although areca quid (AQ) is not chewed normally with tobacco in Taiwan, a strong link has been found in areca quid (AQ) use with oral cancer (Ko et al., 1995; Yang et al., 2001; Lee et al., 2003). Epidemiological studies demonstrated that areca quid (AQ) chewing was an independent risk factor for oral cancer and the development of oral precancerous diseases (Ko et al., 1995; Lee et al., 2003; IARC, 2003).

In Taiwan, where the practice of betel-quid chewing is widespread, approximately 2 million people chew areca quid (AQ) habitually (Ko et al., 1992). The two major types of areca quid (AQ) are *Lao-hwa* quid, a mixture of tender areca nut (AN), an inflorescence of *Piper betle* Linn. (IPB), and red lime paste, and "betel quid," a mixture of tender areca nut (AN), *Piper betle* leaf (PBL), and white lime paste (Ko et al., 1992; Yang et al., 2001). Under an alkaline condition of pH ≥ 9.5, areca quid (AQ) extract and lime generate reactive oxygen species (ROS) such as hydroxyl radical ($HO^{\cdot}$) (Nair et al., 1987, 1990, 1992, 1995). When catalyzed by metal ions such as Cu^{2+} and Fe^{2+}, $HO^{\cdot}$ yields are increased, and 8-hydroxy-2′-deoxyguanosine (8-OH-dG) is formed in vitro, leading to possible oxidative DNA damage (Nair et al., 1987, 1995; Liu et al., 1996; Chen et al., 2002). Indeed, genetic damage in oral mucosa is known to be associated with oral cancer (Nair et al., 1987, 1990, 1992, 1995; Liu et al., 1996; Chen et al., 2002).

Although the relative risks of oral cancer for the two types of areca quid (AQ) have been studied (Ko et al., 1995; Lee et al., 2003), their relative oxidative effects have not been compared. Two markers of $HO^{\cdot}$, *meta*-tyrosine (*m*-Tyr) and *ortho*-tyrosine (*o*-Tyr) from L-phenylalanine (Phe), were measured in the oral cavity (Nair et al., 1995; Chen et al., 2002). The formation of *m*-Tyr and *o*-Tyr from Phe in vitro has been demonstrated via a metal-catalyzed oxidation (Huggins et al., 1993) or via the $HO^{\cdot}$-generating systems such as ultraviolet (UV) irradiation (Ishimitsu et al., 1990) and ionizing radiation (Karam & Simic, 1990). Therefore, *m*-Tyr and *o*-Tyr formation are useful markers of $HO^{\cdot}$ and have been shown to induce oxidative damage in the human oral cavity (Nair et al., 1995; Chen et al., 2002). In this study, *m*-Tyr and *o*-Tyr were measured in the two types of areca quid (AQ), and $HO^{\cdot}$ was measured and compared in the saliva of their users.

METHODS

Subjects Collection

The present study was reviewed and approved by the Human Study Committee of the Institutional Review Board (IRB) of Kaohsiung Medical University (KMU).

Materials and Chemicals

Lao-hwa quid and betel quid samples were simultaneously obtained from two different vendors during the August areca quid (AQ) season in Kaohsiung

City, Taiwan. Phe (Ajinomoto, Japan) was obtained from the China Chemical Company (Taiwan). The *m*- and *o*-Tyr and *para*-tyrosine (*p*-Tyr) were purchased from Sigma (Sigma Chemical Company, St. Louis, MO). All other chemicals were of analytical grade, and solutions were prepared with double-distilled water.

Analytical Quantification

Multiple level calibrations with *m*-, *o*-, and *p*-Tyr standards were performed by linear regression. The concentration of *m*- and *o*-Tyr ranged from 0.1 to 100 μ*M*, and peak areas were increased linearly. The correlation coefficients (*R*) of *m*- and *o*-Tyr were .998 and .999, respectively. The concentrations of *m*- and *o*-Tyr (means ± standard deviation [SD]) in AQ samples were estimated from peak areas by comparison with *m*- and *o*-Tyr standards.

m- and *o*-Tyr Formation From Phe in *Lao-hwa* Quid and Betel Quid Samples

Samples of different compositions of *Lao-hwa* quid and betel quid were prepared for analysis of *m*- and *o*-Tyr. Five quids of *Lao-hwa* quid and betel quid were prepared with Phe and transition metal ions, such as Cu^{2+} and Fe^{2+}, in 100 ml of 37°C distilled water and powdered in a blender. The solution was incubated in a shaker bath at 37°C for 2 h and used to determine pH. A reaction was commenced by adding lime to each 100-ml sample, and stopped by adding 2 ml glacial acetic acid. After centrifugation (8000 × g) and filtration through a 0.45-μm membrane filter, the solution was frozen and lyophilized immediately. The lyophilized samples were stored at −20°C prior analysis by high-performance liquid chromatography (HPLC).

m- and *o*-Tyr Formation From Phe in Human Saliva During Areca Quid Chewing

Fifteen healthy volunteers (8 males and 7 females, aged 25–30 yr) with no previous history of habitual areca quid (AQ) use were recruited, and randomly divided into three groups. The first group chewed 2 quids of *Lao-hwa* with 20 mg Phe ($n = 5$) added. The second group chewed 2 quids of betel quid with 20 mg Phe ($n = 5$) added. The third group chewed gum with 20 mg Phe ($n = 5$). Saliva samples were collected from each subject after 15 min of chewing to determine pH. The saliva samples were deproteinated by adding 25% trichloroacetic acid, and supernatant was frozen and lyophilized immediately. The lyophilized samples were prepared at −20°C prior to analysis by high-performance liquid chromatography (HPLC).

Analysis of *m*-, *o*-, and *p*-Tyr Formation

The formation of *m*-, *o*-, and *p*-Tyr was measured according to methods described in a previous study, with minor modifications (Nair et al., 1995). Samples were separated from Lichrospher RP-18(e) (5 μm, 4.0 mm × 250 mm; Merck, Germany) with aqueous solutions containing 0.5% acetic acid and

0.1% NaCl at a 1 ml/min flow rate. The effluent was monitored with excitation at 275 nm and emission at 305 nm using a fluorescence detector (Jasco FP-1520, Japan). Analyses carried out in four samples of each group and their mean concentrations (means ± SD, $n = 4$) are shown in the Results section.

The occurrence of *m*- and *o*-Tyr in human saliva was confirmed by liquid chromatography–mass spectroscopy (LC-MS). Analytes (*m*- and *o*-Tyr) were separated on a C-18 analytical column (3 μm, 4.0 mm × 55 mm; Merck, Darmstadt, Germany). The mobile phase of high-performance liquid chromatography (HPLC) was delivered using two micro pumps (PE series 200, Perkin-Elmer, Elmer, Norfolk, CT) with 5% acetonitrile in 0.1% formic acid at a flow rate of 0.6 ml/min. An API 3000 mass spectrometer (PE-SCIEX, Concord, Ontario, Canada) was used in the present study. A TurboIon Spray source was operated in positive ionization mode with typical settings as follows: nebulizer gas 10, curtain gas 10, CAD gas 4, drying gas 8 L/min, electrospray voltage 4500 V, ring voltage 300 V, orifice voltage 30 V, collision energy 30 eV, and a drying temperature of 400°C. Qualitation of each analyte was achieved using the product's ion scan mode with a precursor ion of *m*/*z* 182 selected.

Statistical Analysis

The general linear model (GLM) procedure with statistical multiple comparison by Tukey's method was used for the primary analysis. Group means are presented with SD. The differences in means ± SD between groups and subsets were assessed by one-way analysis of variance (ANOVA) or Mann–Whitney nonparametric test. The p value for significance was set at $p < .05$. The SPSS v11.0.1 software package (SPSS, Inc.) was employed for statistical analysis of all data.

RESULTS

Quantifying Areca Quid Samples

According to an IARC report, habitual areca quid (AQ) users chewed an average of 5–20 quids a day (IARC, 1985). Each sample in this in vitro study contained 5 quids to simulate the chewing habit. Each *Lao-hwa* sample (5 quids) dissolved in 100 ml water contained a mean concentration of 0.15 g/ml of areca nut (AN), 0.02 g/ml of IPB, and 0.02 g/ml of red lime. On the other hand, the betel quid sample (5 quids) dissolved in 100 ml water contained areca nut (AN) (0.15 g/ml), PBL (0.05 g/ml), and white lime (0.005 g/ml) (Table 1).

Formation of *m*- and *o*-Tyr From Phe in *Lao-hwa* Quid Groups 1–6

In the *Lao-hwa* quid groups (groups 1–6), significant differences in *m*- and *o*-Tyr from Phe were observed in all variations of ingredients (Figure 1). In *Lao-hwa* quid group 1 [AN (0.15 g/ml) + IPB (0.02 g/ml) + red lime (0.02 g/ml)], the reaction generated 68.01 ± 7.99 μ*M* *m*-Tyr and 65.51 ± 6.59 μ*M* *o*-Tyr at pH 8.85. With the addition of Cu^{2+} (2 m*M*) under the same condition, the formation

TABLE 1. Mean Concentration of Ingredients Contained in *Lao-hwa* Quid and Betel Quid From Two Different Vendors in Kaohsiung Region of Taiwan

Ingredient	Mean concentration (g/ml) of 5 quids
Lao-hwa quid sample	
AN[a]	0.15
IPB[b]	0.02
Red lime	0.02
Betel quid sample	
AN	0.15
PBL[c]	0.05
White lime	0.005

Note. Different types of *Lao-hwa* quid and betel quid were mixed with 100 ml of distilled water and powdered in a blender for 5 min.

[a] Areca nut (AN).

[b] Inflorescence of *Piper betle* Linn. (IPB).

[c] Piper betle leaf (PBL).

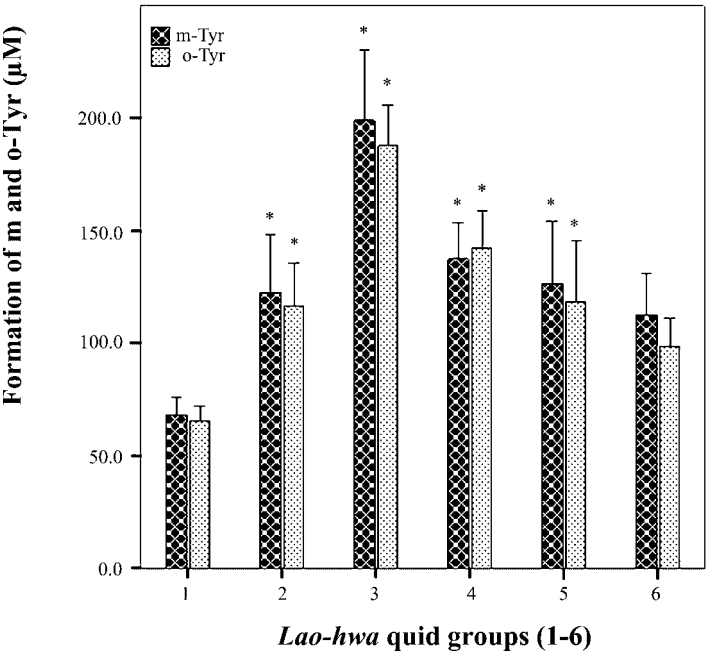


FIGURE 1. Formation of *m*- and *o*-Tyr from Phe with various *Lao-hwa* quid ingredients (1–6): 1, AN + IPB + red lime; 2, AN + IPB + red lime + Cu^{2+} (2 m*M*); 3, AN + IPB + red lime + Cu^{2+} (5 m*M*); 4, AN + IPB + red lime + Fe^{2+} (5 m*M*); 5, AN + 5 × IPB (0.10 g/ml) + red lime; 6, AN + IPB + 5 × red lime (0.10 g/ml); means ± S.D. ($n = 4$) are presented. An asterisk denotes significance at $p < .05$ as compared with group 1.

of *m*- and *o*-Tyr was significantly higher in group 2 (pH 8.82) than in group 1. The higher concentration of Cu^{2+} (5 m*M*) was found to increase *m*- and *o*-Tyr formation significantly at pH 8.84 (group 3 vs. group 1). The presence of Fe^{2+} (5 m*M*) also enhanced *m*- and *o*-Tyr formation at pH 8.95, as seen in group 4. Compared with group 1 (IPB = 0.02 g/ml), a significant increase in the formation of *m*-Tyr and *o*-Tyr was observed for group 5 (IPB = 0.08 g/ml) at pH 8.42. In addition, group 6 (pH = 10.93), exposed to four-fold concentrations of red lime (0.08 g/ml), exhibited no marked change.

Formation of *m*- and *o*-Tyr From Phe in *Lao-hwa* Quid Groups A–D

The omission of any one of the ingredients [areca nut (AN), IPB, or red lime] from the *Lao-hwa* quid mixture significantly reduced the formation of *m*- and *o*-Tyr (Figure 2). The absence of red lime in the reaction significantly reduced the formation of *m*- and *o*-Tyr, as shown in group B (pH 4.93). In the absence of IPB, group C (pH 9.46) demonstrated a significant decrease in the formation of *m*- and *o*-Tyr. Formation of *m*- and *o*-Tyr at pH 11.89 was significantly decreased when the mixture did not contain areca nut (AN) (group D).

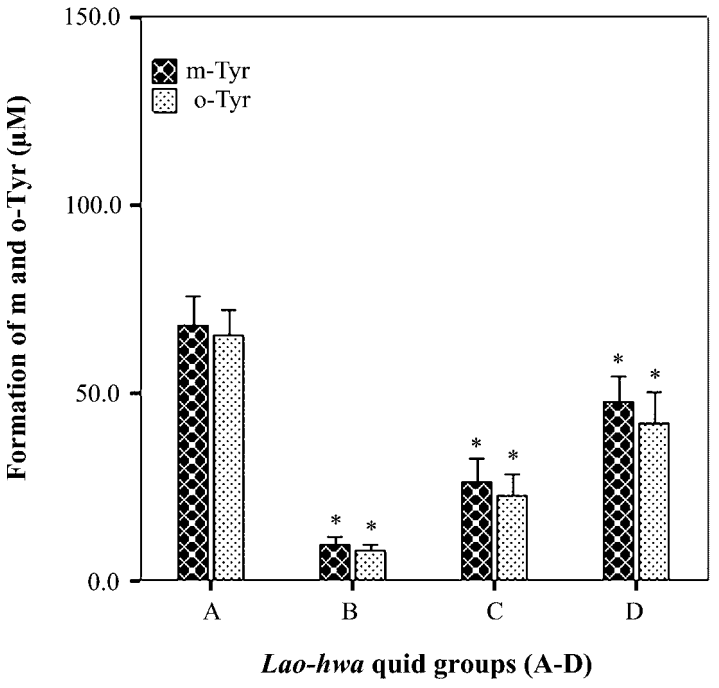


FIGURE 2. Formation of *m*- and *o*-Tyr from Phe with various *Lao-hwa* quid ingredients (A–D): A, AN + IPB + red lime; B, AN + IPB; C, AN + red lime; D, IPB + red lime; means ± SD ($n = 4$) are presented. An asterisk denotes significance at $p < .05$ as compared with group A.

Formation of *m*- and *o*-Tyr From Phe in Betel Quid Groups 1–6

The significant differences in *m*- and *o*-Tyr formation arising from various compositions of betel quid are shown in Figure 3. Betel quid group 1 [AN (0.15 g/ml) + PBL (0.05 g/ml) + white lime (0.005 g/ml)] generated 9.91 ± 5.83 μ*M* *m*-Tyr and 7.36 ± 3.52 μ*M* *o*-Tyr at pH 9.45. When Cu^{2+} (2 m*M*) was added under the same conditions (group 2, pH 9.46), the formation of *m*-Tyr was significantly higher, but that of *o*-Tyr was not (group 2 vs. group 1). Higher concentrations of Cu^{2+}(5 m*M*) significantly increased the formation of *m*- and *o*-Tyr at pH 9.50 (group 3 vs. group 1). The addition of Fe^{2+}(5 m*M*) also significantly increased *m*- and *o*-Tyr formation at pH 9.54 (group 4 vs. group 1). When 0.05 g/ml of PBL was added in group 5 (pH 9.14), the concentration of *m*- and *o*-Tyr fell below detection limits (0.01 μ*M*). The levels of *m*- and *o*-Tyr were significantly elevated with a twofold increase in the concentration of white lime (0.01 g/ml) at pH 11.38 (group 6 vs. group 1).

Formation of *m*- and *o*-Tyr From Phe in Betel Quid Groups A–D

Exclusion of either white lime or areca nut (AN) from the reaction mixture significantly reduced the formation of *m*- and *o*-Tyr (Figure 4). In the absence

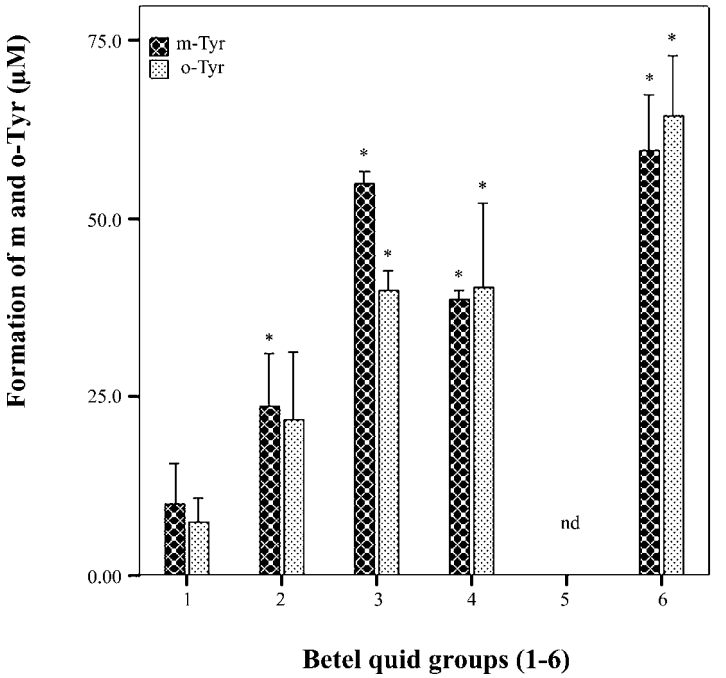


FIGURE 3. Formation of *m*- and *o*-Tyr from Phe with various betel quid ingredients (1–6): 1, AN + PBL + white lime; 2, AN + PBL + white lime + Cu^{2+} (2 m*M*); 3, AN + PBL + white lime + Cu^{2+} (5 m*M*); 4, AN + PBL + white lime + Fe^{2+}(5 m*M*); 5, AN + 2 × PBL (0.10 g/ml) + white lime; 6, AN + PBL + 3 × white lime (0.015 g/ml); means ± SD ($n = 4$) are presented. Nondetection (nd) indicates the *m*- and *o*-Tyr concentrations below detection limit (0.01 μ*M*). An asterisk denotes significance at $p < .05$ as compared with group 1.

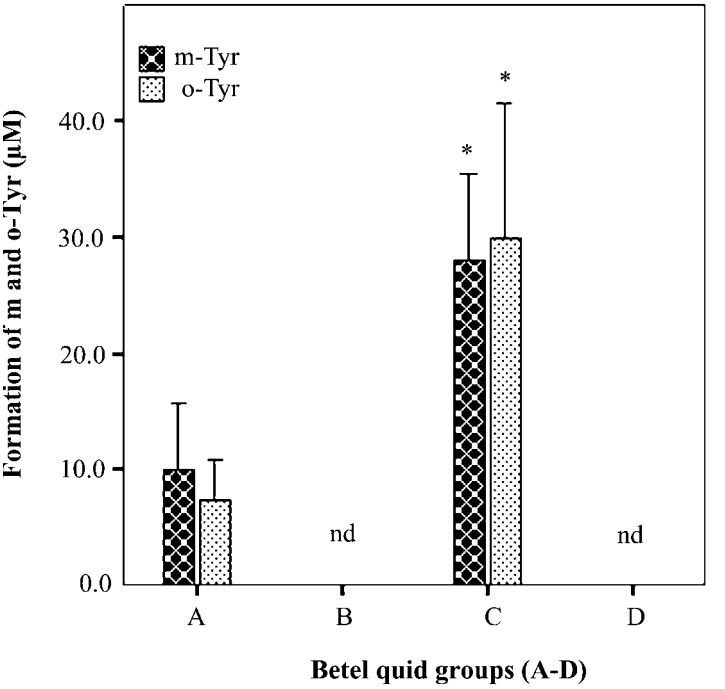
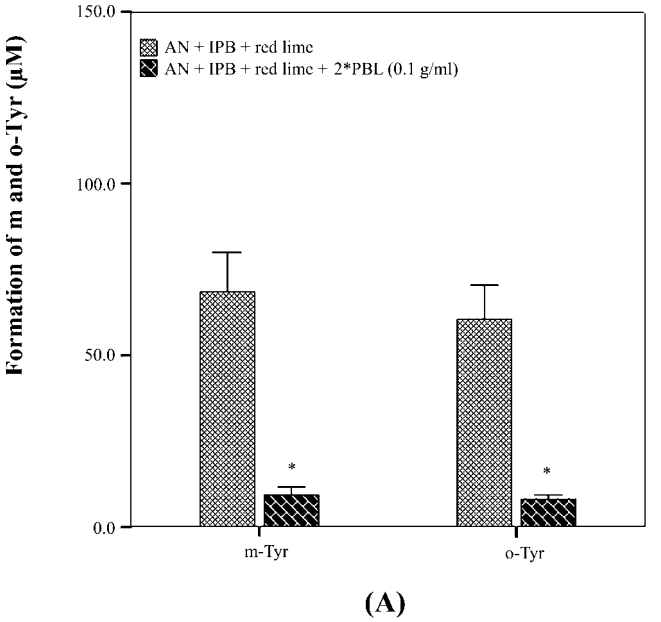


FIGURE 4. Formation of *m*- and *o*-Tyr from Phe with various betel quid ingredients (A-D): A, AN + PBL + white lime; B, AN + PBL; C, AN + white lime; D, PBL + white lime; means ± SD ($n = 4$) are presented. Nondetection (nd) indicates the *m*- and *o*-Tyr concentrations below detection limit (0.01 μ*M*). An asterisk denotes significance at $p < .05$ as compared with group A.

of white lime, the formation of *m*- and *o*-Tyr was below detection limits, as shown in group B. However, group C, which was not exposed to PBL, exhibited significantly higher levels of *m*- and *o*-Tyr formation than group A, which was exposed to PBL. Even with higher alkaline levels (group D, pH 12.18), exclusion of areca nut (AN) reduced *m*- and *o*-Tyr formation to below detection limits.

Effects of IPB and PBL on the Formation of *m*- and *o*-Tyr From Phe

The *Lao-hwa* quid group [AN (0.15 g/ml) + IPB (0.02 g/ml) + red lime (0.02 g/ml)] generated 68.59 ± 11.33 μ*M* *m*-Tyr and 60.67 ± 9.63 μ*M* *o*-Tyr. The formation of *m*- and *o*-Tyr was significantly inhibited by adding twofold amounts of PBL (0.1 g/ml) (Figure 5A). On the other hand, the betel quid group containing areca nut (AN) (0.15 g/ml), PBL (0.05 g/ml), and white lime (0.005 g/ml) generated 11.84 ± 2.66 μ*M* *m*-Tyr and 14.04 ± 4.59 μ*M* *o*-Tyr. The addition of fivefold amounts (0.1 g/ml) of IPB significantly increased formation of *m*- and *o*-Tyr (Figure 5B).



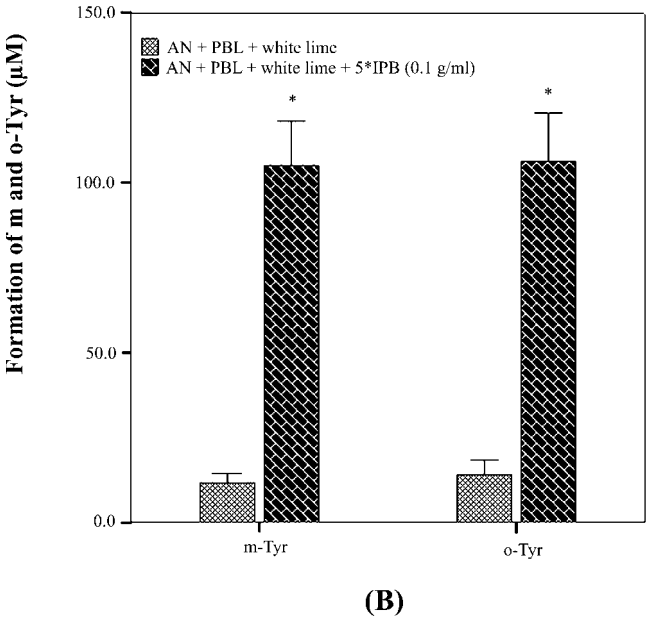


FIGURE 5. The effects of PBL and IPB on the formation of *m*- and *o*-Tyr from Phe in two types of AQ. (A) Effects of *Piper betle* leaf (PBL) on the *Lao-hwa* quid. (B) Effects of inflorescence of *Piper betle* Linn. (IPB) on the betel quid; means ± SD ($n = 4$) are presented. An asterisk denotes significance at $p < .05$.

Formation of *m*- and *o*-Tyr From Phe in Human Saliva During Areca Quid Chewing

The formed *m*-, *o*-, and *p*-Tyr in the saliva samples collected after areca quid (AQ) chewing were separated using HPLC and then detected through a fluorescence detector (Figure 6). Human saliva, especially from *Lao-hwa* quid chewers with 20 mg Phe, contained high concentrations of *m*- and *o*-Tyr (Figure 6B). On the other hand, a small amount of *m*- and *o*-Tyr was detected in human saliva after chewing betel quid with 20 mg Phe (Fig. 6C). No *m*- and *o*-Tyr was detected above the detection limit of 10 n*M* in the human saliva of subjects who chewed gum with 20 mg Phe (Figure 6D). The concentrations of *m*- and *o*-Tyr detected in subjects who chewed *Lao-hwa* quid are given in Table 2. Further, these levels were significantly higher than in the subjects who chewed betel quid. The presence of *m*- and *o*-Tyr in the human saliva of subjects was confirmed by LC-MS, as described in the Methods section (data not shown).

DISCUSSION

Studies have shown that areca quid (AQ) combined with lime will result in the formation of ROS such as $HO^{\cdot}$ (Nair et al., 1987; Stich & Anders, 1989). $HO^{\cdot}$ is formed by the auto-oxidation of polyphenols of areca quid (AQ) in the presence of transition metals via Haber–Weiss or Fenton reactions. In the presence of $HO^{\cdot}$-generating systems, *m*- and *o*-Tyr formation from Phe was proposed as a useful marker to measure $HO^{\cdot}$-induced tissue damage (Nair et al., 1995). In accordance with this system, our study demonstrated that aqueous extracts of Taiwanese areca quid (AQ) are capable of producing $HO^{\cdot}$ at alkaline pH conditions.

Areca quid (AQ) ingredients and chewing methods vary widely by country and season (Wei & Chung, 1997; Gupta & Warnakulasuriya, 2002). In India, for example, areca quid (AQ) generally contains areca nut (AN), lime, and tobacco, with or without PBL, whereas in Taiwan and Papua New Guinea, IPB is used instead of tobacco (Thomas & MacLennan, 1992; Yang et al., 2001). In Papua New Guinea, a paste of areca quid (AQ) with lime and IPB is used. This form elevates the salivary pH level, creating an alkaline condition conducive to stimulating ROS generation, such as $HO^{\cdot}$, in the corner of the mouth. The formation of ROS suggests a possible mechanism for oral cancer (Thomas & MacLennan, 1992). In this in vivo study, the human saliva from *Lao-hwa* quid chewed with IPB displayed significantly higher amounts of *m*- and *o*-Tyr than for betel quid chewed with PBL. The presence of *m*- and *o*-Tyr in human saliva, confirmed by LC-MS, may implicate the presence of $HO^{\cdot}$. In vitro studies also demonstrated that *Lao-hwa* quid containing IPB generated higher $HO^{\cdot}$ than betel quid containing PBL. These results are compatible with the study by Chen et al. (2002), which indicated that chewing areca quid (AQ) containing IPB generated a higher concentration of *m*-Tyr than chewing areca quid (AQ) containing PBL.

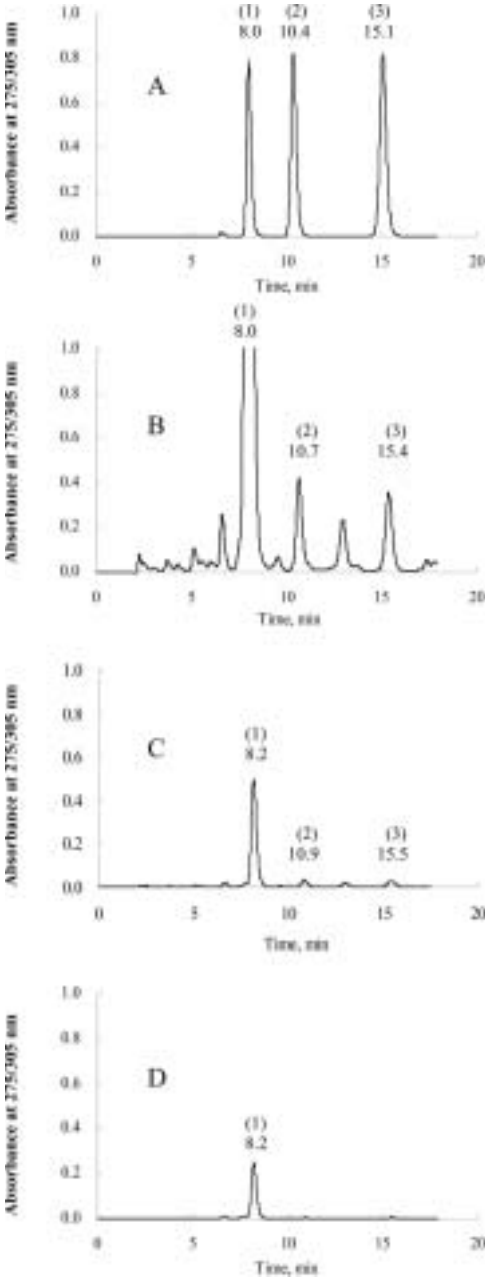


FIGURE 6. (A) HPLC fluorescence chromatograms of *p*-, *m*-, and *o*-Tyr standards. (B) Human saliva created by chewing *Lao-hwa* quid with 20 mg Phe. (C) Human saliva created by chewing betel quid with 20 mg Phe. (D) human saliva created by chewing gum with 20 mg Phe. Peaks: (1), *p*-Tyr; (2), *m*-Tyr; (3), *o*-Tyr.

TABLE 2. Formation of *m*- and *o*-Tyr (Means ± SD) in Human Saliva From Phe After Chewing *Lao-hwa* Quid, Betel Quid, and Chewing Gum

			Formation of Tyr (n*M*)	
	n	pH[a]	m-Tyr	o-Tyr
Chewing 2 quids of *Lao-hwa* containing AN (0.06 g/ml) + IPB (0.008 g/ml) +red lime (0.008 g/ml)	5	7.57 ± 0.22	809 ± 38[c]	643 ± 88[c]
Chewing 2 quids of betel quid containing AN (0.06 g/ml) + PBL (0.02 g/ml) + white lime (0.002 g/ml)	5	8.32 ± 0.26	187 ± 60	178 ± 38
Chewing gum	5	6.87 ± 0.34	nd[b]	nd

[a] The pH level of human saliva was assessed after chewing AQ for 15 min.
[b] nd: *m*- and *o*-Tyr concentrations below detection limits (10 n*M*).
[c] Significant at $p < .05$ as compared with the subjects chewing betel quid, by Mann–Whitney nonparametric test.

The major differences in HO· formation may be produced by different ingredients in *Lao-hwa* quid and betel quid. The ingredients of the *Lao-hwa* quid chewed in Taiwan include areca nut (AN), IPB, and red lime. Thomas and MacLennan (1992) found that a combination of areca nut (AN), IPB, and powdered lime is required for generation of HO· during the areca quid (AQ) chewing process. Our in vitro studies showed that the omission of any one of areca nut (AN), IPB, or red lime from the *Lao-hwa* quid significantly reduced the formation of *m*- and *o*-Tyr, in accordance with previous studies (Nair et al., 1987, 1990, 1995). When fourfold amounts (0.08 g/ml) of IPB were added to *Lao-hwa* quid, *m*- and *o*-Tyr formation was enhanced significantly. On the other hand, higher amounts of IPB (0.1 g/ml) in betel quid mixtures [areca nut (AN) + PBL + white lime] also significantly enhanced *m*- and *o*-Tyr formation. Thus, IPB as an ingredient seems to contribute to HO· formation.

The preparation of *Lao-hwa* quid in Taiwan is somewhat dissimilar from other countries in that fresh IPB is added; IPB contains a high concentration of safrole (15.4 mg/g), 9.7 mg/g hydroxychavicol (HC), and 2.5 mg/g eugenol (Hwang et al., 1992). These ingredients may contribute to formation of safrole (420 μ*M*) in human saliva (Wang & Hwang, 1993). Safrole is metabolized to dihydroxychavicol (DHAB) and induces oxidative DNA damage (Lee-Chen et al., 1996; M. J. Chang et al., 2002). On the other hand, safrole is a documented animal liver carcinogen and generates DNA adducts (8-OH-dG) that have been shown to produce oxidative DNA damage in the rat liver (Liu et al., 1999). The safrole–DNA adducts derived from IPB may contribute to oral carcinogenesis in areca quid (AQ) chewers (Chen et al., 1999). HC is the major urinary metabolite of safrole in humans and rats, and the minor metabolite is eugenol (Benedetti et al., 1977; Klungsoyr & Scheline, 1983). Both HC and eugenol may oxidize to form DNA adducts (8-OH-dG) and induce oxidative

damage in culture cells (Lee-Chen et al., 1996; Bodell et al., 1998; Chen et al., 2000; Jeng et al., 2004). These properties may at least partially explain the increased $HO^{\cdot}$ exposure and oxidative damage from IPB use. Accordingly, epidemiological studies also suggested that chewing areca quid (AQ) with IPB increases the potential for oral cancer (Ko et al., 1995; Lee et al., 2003).

The PBL of betel quid, sometimes a substitute for IPB, is composed of a small amount of HC and eugenol, but without safrole (Hwang et al., 1992; Chen et al., 2002). The high concentration of *m*- and *o*-Tyr in *Lao-hwa* quid groups may result from higher concentrations of safrole, HC, and eugenol. These agents known to yield $HO^{\cdot}$ formation are found in IPB in greater quantities than in PBL. Recent studies also indicated that HC of PBL extract is an efficient scavenger of ROS (H_2O_2, $O_2^{\cdot-}$, and $HO^{\cdot}$) at low concentrations, whereas at high concentrations (HC > 0.1 m*M*), HC may induce oxidative damage (M. C. Chang et al., 2002). Our results also demonstrate that a significant decline in *m*- and *o*-Tyr formation occurs with the addition of PBL (0.1 g/ml) to the *Lao-hwa* quid mixture [areca nut (AN) + IPB + red lime]. The betel quid group without PBL significantly generates higher levels of *m*- and *o*-Tyr formation than betel quid with PBL.

Transition metal ions such as $Cu^{2+}$and Fe^{2+} are known to enhance the production of $HO^{\cdot}$ by areca quid (AQ) (Nair et al., 1987, 1990). Consistent with this, our studies showed that the formation of $HO^{\cdot}$ is enhanced by both $Cu^{2+}$and Fe^{2+}, where Cu^{2+} is more effective. Our results also concur with a previous study that revealed that the addition of Cu^{2+} to an areca quid (AQ) mixture had generated higher amounts of *m*- and *o*-Tyr than addition of Fe^{2+} (Nair et al., 1995). In contrast, Nair et al. (1987) found ROS formation was enhanced in the following order: $Fe^{2+} > Fe^{3+} > Mg^{2+} > Cu^{2+}$. According to previous studies, $Cu^{2+}$and Fe^{2+} levels are higher in the oral epithelial and connective tissues of AQ chewers (Paul et al., 1996; Trivedy et al., 2000). Since transition metal ions such as $Cu^{2+}$and Fe^{2+} have been shown to play an important role in accelerating oxidative damage (Nair et al., 1987; Liu et al., 1996), the effect of $Cu^{2+}$and Fe^{2+} on the formation of $HO^{\cdot}$ during areca quid (AQ) chewing merits further elucidation.

In conclusion, these in vitro studies indicate that the generation of $HO^{\cdot}$ was inhibited by the addition of PBL. In contrast, the markedly increased oxidative effects of human saliva after chewing areca quid (AQ) with IPB are likely risk factors for oral mucosa damage. Therefore, the difference in risks between areca quid (AQ) with and without IPB implies IPB might induce additional oxidative damage on oral mucosa tissue. The oxidative damage produced by $HO^{\cdot}$ during areca quid (AQ) chewing is probably implicated in the development of oral cancer.

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