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Composition of betel specific chemicals in saliva during betel chewing for the identification of biomarkers

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Abstract

Betel nut chewing causes cancer in humans including strong associations with head and neck cancer in Guam. In the search for biomarkers of betel chewing we sought to identify chemicals specific for the 3 most commonly consumed betel preparations in Guam: nut ('BN'), nut + *Piper betle* leaf ('BL'), and betel quid ('BQ') consisting of nut+lime+tobacco+*Piper betle* leaf. Chemicals were extracted from the chewing material and saliva of subjects chewing these betel preparations. Saliva analysis involved protein precipitation with acetonitrile, dilution with formic acid followed by LCMS analysis. Baseline and chewing saliva levels were compared using t-tests and differences between groups were compared by ANOVA; $p < 0.05$ indicated significance. Predominant compounds in chewing material were guvacine, arecoline, guvacoline, arecaidine, chavibetol, and nicotine. In chewing saliva we found significant increases from baseline for guvacine (BN, BQ), arecoline (all groups), guvacoline (BN), arecaidine (all groups), nicotine (BQ), and chavibetol (BL, BQ) and significant differences between all groups for total areca-specific alkaloids, total tobacco-specific alkaloids and chavibetol. From this pilot study, we propose the following chemical patterns as biomarkers: areca alkaloids for BN use, areca alkaloids and chavibetol for BL use, and areca alkaloids plus chavibetol and tobacco-specific alkaloids for BQ use.

Keywords

betel nut; betel quid; alkaloids; saliva; Guam; biomarkers

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Conflict of Interest: none

1. Introduction

Betel nut is consumed by an estimated 600 million people worldwide with prevalent consumption in India, South East Asia, and Micronesia [1]. Betel nut is traditionally chewed alone or as a betel 'quid' (BQ) consisting of a *Piper betle* leaf, slacked lime, and sometimes tobacco [2]. In Guam, betel nut chewing is widely practiced among many populations (including Chamorros, Yapese, Palauans and Chuukese) and approximately 11% of Guam's population chews betel nut on a regular basis [3].

Epidemiological and animal data have suggested strong associations between betel nut consumption and oral mucosal diseases such as leukoplakia, oral and nasopharyngeal cancer, and oral submucous fibrosis [4, 5]. Based on these and other profound evidence, the International Agency for Research on Cancer has deemed betel nut chewing (with or without tobacco) carcinogenic to humans [6]. In Guam, the incidence of mouth cancer in some Micronesian ethnicities is almost 3 times higher than in Caucasians [7] while the mortality rate of nasopharyngeal cancer is vastly higher on Guam than in the US mainland, where betel nut consumption is virtually non-existent.

Carcinogenic N-nitroso compounds such as the alkaloid metabolites N-nitrosoguvacoline, N-nitrosoguvacine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoproline, N-nitrosodimethylamine, and N-nitrosodiethylamine have been detected in the saliva of habitual BQ chewers in India who were not actively chewing during saliva collection [8-10]. Unpublished data from pilot studies have suggested that regional variations in betel nut alkaloids may exist in betel preparations consumed in Guam (Drs. Wright and Suleman, University of Guam). However, to our knowledge, no salivary profile to date has been established to identify betel compounds specific to varieties consumed in Guam.

Therefore, our study aims were to: 1) identify compounds specific for the 3 most common betel preparations consumed in Guam: red, mature betel nut only ('BN' group), red mature betel nut wrapped in *Piper betle* leaf ('BL' group), and BQ consisting of the young, green betel nut wrapped in *Piper betle* leaf with lime and tobacco ('BQ' group) and; 2) identify compounds that get extracted into the saliva of occasional chewers while chewing these preparations.

2. Methods

2.1 Saliva collection

Fifteen occasional male betel chewers (ages 22-31y) were recruited in Guam via flyers distributed in different villages and by word of mouth. Information regarding age, weight, ethnicity, health condition, and typical betel chewing habits were obtained by interviews with study personnel. Participants were randomized evenly to one of the 3 betel preparation groups (BN, BL, BQ) and signed an informed consent before entering the study. The WIRB approved the protocol of this study. After up to a 7-day washout, saliva was collected before (baseline) and during the participant's betel chewing episode, which averaged 2-15 minutes depending on the participant's habit. Saliva samples were immediately centrifuged,

aliquoted, then kept at -80°C prior to shipment to the University of Hawaii Cancer Center (UHCC) laboratory. Participants received a \$50 gas card upon study completion as compensation for their time and participation.

2.2 Sample preparation

At the UHCC laboratory, a 100 µL aliquot of thawed saliva was mixed with 10 µL of internal standard (IS) solution and 100 µL of acetonitrile in a 1.5 ml microcentrifuge tube then vortexed at high speed to precipitate proteins. The mixture was then diluted with 800 µL of 0.1% formic acid in MeOH:H₂O (v:v = 1:1) and centrifuged at 13,500 × g for 5 min. The supernatant was transferred to HPLC vials and subjected to LCMS analysis.

All betel material (red mature betel nut, young green betel nut, *Piper betle* leaf, lime, tobacco) required for chewing and chemical analysis was obtained locally in Guam from one distributor at the beginning of the study and stored in a cooled environment until all participants completed the study. All betel preparations were prepared by one of the study personnel on the day of and immediately prior to the chewing episodes. For the BN group, one red mature betel nut was cut in half and the hard/crunchy portion was chewed. For the BL group, one red mature betel nut (similar to BN group) was cut in half and wrapped in one *Piper betle* leaf so that the hard/crunchy portion was chewed. For the BQ group, one whole young green betel nut wrapped in one *Piper betle* leaf with tobacco (from one cigarette) and one-half teaspoon liquid lime (purchased locally) was chewed. The liquid lime was made from powdered slacked lime.

Extractions of the red mature betel nut, young green betel nut, *Piper betle* leaf, and tobacco were carried out using thawed (not dried) material. Tobacco (obtained from one cigarette after removing the cigarette wrapper) and one *Piper betle* leaf were cut into small pieces and extracted with 25 ml PBS buffer by stirring at room temperature for 30 min. The young green betel nut and the center of the red mature betel nut were extracted with 25 ml PBS buffer by continuous grinding with a mortar and pestle for 30 minutes. The resulting liquid was collected and subjected to LCMS analysis.

2.3 Chemicals

Arecoline hydrobromide, guvacine hydrochloride, nicotine, cotinine, hydroxycotinine, NNN, NAT, NNK, 4-(Methyl-d₃-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK-d₃), nicotine-d₄, cotinine-d₃ and eugenol and o-eugenol were purchased from Sigma-Aldrich (St. Louis MO), Guvacoline hydrobromide, N-nitrosoguvacoline, Arecaidine Hydrobromide, Arecodine-d₅, hydrobromide salt were purchased from Medical Isotopes (Pelham, NH). All solvents were of LCMS grade and were purchased from Fisher Scientific (Waltham MA).

2.4 Standards

Standard stock solutions of arecaidine, arecoline, guvacoline, guvacine, nicotine, cotinine, hydroxycotinine, N-nitrosoguvacoline, NNK, NNN, NAT, eugenol, and o-eugenol were prepared in MeOH at concentrations of 10 µg/mL. The IS solution consisting of arecoline-d₅, nicotine-d₄, cotinine-d₃, NNK-d₃ was prepared in MeOH at concentrations of 10 µg/mL. Calibrator solutions were made by mixing 100 µL standard stock solutions with 10 µL IS

solution and 900 μL 0.1% formic acid in $\text{MeOH:H}_2\text{O}$ (1:1). Calibration curves were generated by serially diluting standard stock solutions in 0.1% formic acid in $\text{MeOH:H}_2\text{O}$ (1:1) to obtain working concentrations of 0.1, 1, 10, 100, 200, 500, 1000, and 5000 ng/mL .

2.5 LCMS analysis

LCMS analysis was carried out on a model Accela ultra HPLC system coupled to a Q Exactive Orbitrap Mass Spectrometer and a CTC PAL autosampler (all from ThermoFisher, San Jose, CA). 10 μL of the above mixture was injected into a Kinetex C18 column ($150 \times 3 \text{ mm}$, 2.6 μm , Phenomenex, Torrance CA) with a Phenomenex UHPLC C18 pre-column (3.0 mm i.d.). Gradient elution was performed at a flow rate of 300 $\mu\text{L/min}$ using 10 mM ammonium hydroxide in H_2O (A) and 10 mM ammonium hydroxide in MeOH (B) as follows: 0-15.0 min linear gradient from 65% A to 20% A; 15.0-20.0 min hold at 20% A; 20.0-20.1 min increase to 65% A then equilibrate for 5 minutes. Total HPLC time including equilibration was 25 minutes. Mass analysis was performed under positive and negative electrospray full scan mode, the conditions are as follows: (+) ESI spray voltage 4.5 kV , capillary transfer temperature 350 $^\circ\text{C}$, (–) ESI spray voltage 3.5 kV , capillary temperature 320 $^\circ\text{C}$; other parameters are: HESI heater temperature 200 $^\circ\text{C}$, sheath gas flow rate 30 unit; auxiliary gas 5 unit, in-source CID 5 eV , scan range 100 ~ 1000. AGC target $1\text{e}6$, maximum injection time 100 ms , resolution 35,000, microscan 1. Quantitation of all analytes was performed with Xcalibur™ software by extracting the within 5 ppm of the calculated exact masses as follows: Arecoline and Arecoline- d_5 at +ESI $[\text{M}+\text{H}]^+$ 156.10191 and 161.13329; Guvacoline at +ESI $[\text{M}+\text{H}]^+$ 142.08626, Guvacine at +ESI $[\text{M}+\text{H}]^+$ 128.07061, Arecaidine at +ESI $[\text{M}+\text{H}]^+$ 142.086, NNN at +ESI $[\text{M}+\text{H}]^+$ 178.09749, NAT at +ESI $[\text{M}+\text{H}]^+$ 190.09749, NNK and NNK- d_3 at +ESI $[\text{M}+\text{H}]^+$ 208.10805 and 211.12688, Nicotine and Nicotine- d_4 at +ESI $[\text{M}+\text{H}]^+$ 163.12297 and 167.14808, Cotinine and Cotinine- d_3 at +ESI $[\text{M}+\text{H}]^+$ 177.10224 and 180.12107, N-nitrosoguvacoline at +ESI $[\text{M}+\text{H}]^+$ 171.07642, Hydroxycotinine at +ESI $[\text{M}+\text{H}]^+$ 193.09715, and chavibetol at –ESI $[\text{M}-\text{H}]^-$ 163.07590.

2.6 Statistical evaluation

Student's paired t-tests were used to analyze differences between paired baseline and chewing saliva levels and a one-way ANOVA was used to determine differences of levels during chewing between the 3 betel preparation groups. Analyses were determined using Excel (Microsoft, Seattle, WA) and a p-value of <0.05 was considered significant.

3. Results

After several attempts we were able to optimize our fast and simple method to simultaneously detect 12 betel specific and tobacco specific alkaloids from saliva specimens and extracts of the red mature betel nut, young green betel nut, *Piper betle* leaf, and tobacco. The carboxylic alkaloid acids arecaidine and guvacine were not detected in our initial attempt that applied an alkaline liquid-liquid extraction at pH 10 using a mixture of dichloromethane/isopropanol/ammonium hydroxide (78:20:2, v/v/v). This was because these alkaloids were retained in the aqueous layer. In our second attempt we treated the diluted (10 $\times$) saliva with 10% perchloric acid, perchloric acid followed by sodium hydroxide, or

acetonitrile and found that the perchloric treatments degraded the guvacine, arecaidine, n-nitrosoguvacoline and NAT standards while the acetonitrile treatment did not.

Therefore, in the applied method we eliminated the organic extraction solvents and instead included acetonitrile and a weakly acidic formic acid (HCOOH) to our 10× diluted saliva, which assisted in increasing the solubility of the free carboxylic acids (-COOH functional group) arecaidine and guvacine. This treatment was followed by direct injection into the LCMS system and resulted in the best recoveries and lowest CVs (data not shown).

Characteristics of the participants are shown in **Table 1**. The 15 participants ranged in age from 22 to 31 years old and most began chewing at a young age (mean 14 y). Chewing was a habitual habit for most – ranging from constant chewing to 1×/week with the typical preparation consisting of betel nut, lime, and tobacco.

The areca alkaloids guvacine, arecoline, guvacoline, and arecaidine were found in the betel nut extracts (**Table 2**) with arecoline (339.1 ug/g) and guvacine (1,871.4 ug/g) having the highest concentrations in the young and mature betel nut extracts, respectively; guvacine levels were 6× higher in the mature versus young betel nut extracts (1,871.4 ug/g vs. 302.9 ug/g) and arecaidine levels ca. 2× higher in the young versus mature betel nut extracts (93.2 vs. 47.7 ug/g). Chavibetol was the major constituent of and exclusively found in the *Piper betle* leaf extract at a concentration of 39,873.8 ug/g. Nicotine was the main alkaloid in the tobacco extracts (15,454.8 ug/g) with relatively negligible amounts detected in the nut and leaf extracts (0.1 – 1.4 ug/g). N-nitroso compounds were not found in either the nut or leaf extracts and N-nitrosoguvacoline was not found in any betel extract material although trace levels of cotinine (41.6 ug/g), hydroxycotinine (2.1 ug/g), NNK (0.4 ug/g), NNN (1.3 ug/g), and NAT (2.9 ug/g) were detected in tobacco extracts. **Figure 1** shows the structures of alkaloid and nitroso compounds detected in saliva samples and extracts of the betel nuts, *Piper betle* leaf, and tobacco.

Guvacine, arecoline, guvacoline, arecaidine, nicotine, cotinine, and hydroxycotinine were detected in saliva samples from all 3 betel preparation groups whereas chavibetol was detected only in saliva from BL and BQ chewers (**Table 3**). Tobacco specific nitrosamine compounds NNK, NNN, and NAT were below detection limits at baseline and found only in saliva chewing samples of the BQ group at concentrations of 4-17 ng/mL. Significant mean increases ($p < 0.05$) from baseline to during chewing were observed for guvacine (BN and BQ groups; 51 to 201,200 ng/mL and 35 to 37,009 ng/mL, respectively), arecoline (all groups, 181 to 31,575 ng/mL (BN), 39 to 17,867 ng/mL (BL), and 159 to 46,246 ng/mL (BQ)), guvacoline (BN group; 211 to 21,862 ng/mL), arecaidine (all groups; 390 to 1,887 ng/mL (BN), 43 to 621 ng/mL (BL), and 218 to 12,480 ng/mL (BQ)), nicotine (BQ group; 922 to 105,342 ng/mL), and chavibetol (BL and BQ groups; <20 to 27,032 ng/mL and <20 to 13,615 ng/mL, respectively). Interestingly, cotinine and hydroxycotinine levels in the BN and BL groups were significantly higher at baseline than during chewing.

Among the saliva levels during chewing, we also found significant differences between the 3 betel preparation groups for total areca alkaloids (guvacine, arecoline, guvacoline, and

arecaidine; $p=0.045$), total nicotine alkaloids (nicotine, cotinine, hydroxycotinine, N-nitrosoguvacoline, NNK, NNN, and NAT; $p<0.001$) and chavibetol ($p=0.006$) (Table 3).

Median fold changes from baseline to levels during chewing ranged from 4,473-40,473 $\times$ (guvacine), 138-927 $\times$ (arecoline), 74-1.6 $\times 10^6 \times$ (guvacoline), and 5-125 $\times$ (arecaidine), data not shown.

4. Discussion

Approximately 11% of Guam's population chews betel nut on a regular basis [3] with reasons attributed to its perceived traditional, social, and health benefits [1]. In addition, according to our participants betel is chewed as a means to prevent boredom, as a force of habit, to relax, and/or due to peer pressure. While all but one participant was generally aware of the hazards linked to betel chewing, most participants chewed betel habitually - as often as every 10 minutes. This habitual chewing may be due to the addictive nature of betel nut [11] and/or tobacco [12], which was included in the betel preparation habitually consumed by all our participants, or due to reasons stated previously.

Extracts from our young betel nut extracts revealed four main alkaloids in the order of abundance: arecoline>guvacine>guvacoline>arecaidine, which is in agreement with a previous report [13]. However, unlike Wang et al. [13] who detected exclusively arecoline in mature betel nut at 1550 ug/g fresh weight, more than twice our values (595.3 ug/g, Table 2), our mature betel nut extracts also contained alkaloids guvacine, guvacoline, and arecaidine, which may be due to seasonal and/or geographical variations as previously suggested [14] and reported [15] or due to methodological differences. Our alkaloid profile for *Piper betle* leaves was also similar to that of Wang et al [13], which contained guvacine, arecoline, arecaidine, and guvacoline (Table 2) except that we also detected chavibetol, which was highly concentrated in our leaf extracts (3.9%). This is within the concentration range of 1.51% to 4.44% found by Musa [16], but much higher compared to Rathee [17], whose analysis of three *Piper betle* varieties revealed concentrations ranging from 0.1% to 0.21%.

The differences in concentrations could be due to methodological differences or system detection sensitivities and necessitates further evaluation using a larger number of samples. In this pilot study our goal was exclusively to identify specific compounds present in betel preparations commonly consumed by betel-chewing individuals in Guam (i.e. BN, BL, and BQ) and identify these compounds when extracted into saliva while chewing these betel preparations. These findings are needed to provide leads for future biomarker studies that require the identification of betel specific compounds that are secreted into saliva after chewing in order to be used in future metabolic, cross sectional and/or cessation studies.

As expected, nicotine was the main alkaloid in the tobacco extracts with negligible amounts being detected in the betel nut and leaf extracts.

Chavibetol was found exclusively in chewing saliva samples of subjects in the BL and BQ groups. This was expected given that this allyl benzene is found in betle leaves [16] (and to our knowledge not found in betel nuts), which is a component of the BL and BQ

preparations but is absent in the BN preparation. Salivary chavibetol has been found to protect against photosensitization-mediated lipid peroxidation in rat liver mitochondria [18] and, thus, may have important implications as a bioactive compound. However, to our knowledge no other study has examined chavibetol levels in saliva of individuals chewing different betel preparations.

The higher baseline levels of cotinine and hydroxycotinine in the saliva of individuals from the BL and BN groups may be due to the dilution of these compounds during betel chewing as individuals in these groups habitually included tobacco in their preparation and, therefore, had cotinine in their saliva probably due to the secretion from the circulation.

Approximately 70-80% of nicotine, the principal alkaloid occurring in tobacco [12], is converted to cotinine and hydroxycotinine. The elimination half-lives of cotinine and hydroxycotinine are approximately 20 hours [19] and 7 hours [20], respectively. Most participants stated a minimum two-day abstinence from betel chewing prior to the study. However, it is possible that our participants did not abstain from betel chewing or from use of tobacco products for the stated duration and, therefore, the detected nicotine and resulting cotinine and hydroxycotinine at baseline and during chewing in the BN and BL groups (who did not consume tobacco during the chewing trial) may be due to the remainder of tobacco use from days preceding the chewing trial, as stated previously. It is also possible that the metabolism of our participants may be different from the smokers/tobacco users in the elimination/infusion studies [19, 20]. The tobacco specific nitrosamines NNK, NNN, and NAT found mostly in the saliva during chewing samples of the BQ group are of significance due to their reported carcinogenic properties [12].

5. Conclusion

This study was performed in preparation for future projects geared towards identifying specific biomarkers in human body fluids that reflect the type and extent of betel chewing. Our results indicate that the combination of areca alkaloids (arecoline, guvacine, and arecaidine), chavibetol, and tobacco-specific alkaloids (nicotine, cotinine, hydroxycotinine, NNK, NNN, and NAT) are promising compounds to identify BQ chewers. The pattern of salivary areca alkaloids combined with chavibetol but with the absence of tobacco alkaloids could identify BL chewers while salivary areca alkaloids alone could identify BN chewers. Betel chewing has been shown to contribute to head, neck, and nasopharyngeal cancers. Our findings will be most helpful in assisting with compliance during cessation programs, which are urgently needed to reduce the burden of these cancers in regions with high betel consumption including Guam.

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Abbreviations

BL group	red, mature betel nut wrapped in <i>Piper betle</i> leaf
BN group	red, mature betel nut only
BQ group	betel quid consisting of young, green betel nut wrapped in <i>Piper betle</i> leaf with lime and tobacco
IS	internal standard
NAT	<i>N</i> -nitrosoanatabine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNK-d₃	4-(Methyl-d ₃ -nitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosonornicotine
UHCC	University of Hawai'i

REFERENCES

1. Paulino Y. Areca (Betel) Nut Chewing Practices in Micronesian Populations. *Hawaii J Public Health*. 2011; 3(1):19–29. [PubMed: 25678943]
2. Jeng JH, Chang MC, Hahn LJ. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. *Oral Oncol*. 2001; 37(6):477–92. [PubMed: 11435174]
3. Paulino, Y. Betel nut chewing in Micronesian populations. Achievement Rewards for College Scientists Selection Meeting; Honolulu, HI.. 2008;
4. Thomas S, Kearsley J. Betel quid and oral cancer: a review. *Eur J Cancer B Oral Oncol*. 1993; 29B(4):251–5. [PubMed: 11706417]
5. Ko YC, et al. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J Oral Pathol Med*. 1995; 24(10):450–3. [PubMed: 8600280]
6. IARC, IARC. Smokeless Tobacco and Some Tobacco-specific N-Nitrosamines. Vol. 89. World Health Organization; Lyon: 2007. Monographs on the evaluation of carcinogenic risks to humans..
7. Haddock RL. Oral cancer incidence disparity among ethnic groups on Guam. *Pac Health Dialog*. 2005; 12(1):153–4. [PubMed: 18181481]
8. Nair J, et al. Tobacco-specific and betel nut-specific N-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid. *Carcinogenesis*. 1985; 6(2):295–303. [PubMed: 3971493]
9. Nair J, et al. Endogenous nitrosation in the oral cavity of chewers while chewing betel quid with or without tobacco. *IARC Sci Publ*. 1987; (84):465–9. [PubMed: 3679424]
10. Bhide SV, et al. N-nitrosamines in the saliva of tobacco chewers or masheri users. *Food Chem Toxicol*. 1986; 24(4):293–7. [PubMed: 3732974]
11. Chu NS. Effects of Betel chewing on the central and autonomic nervous systems. *J Biomed Sci*. 2001; 8(3):229–36. [PubMed: 11385294]
12. Hukkanen J, Jacob P 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev*. 2005; 57(1):79–115. [PubMed: 15734728]
13. Wang C-K, Lee W-H, Peng C-H. Contents of phenolics and alkaloids in Areca catechu Linn. during maturation. *J Agric Food Chem*. 1997; 45(4):1185–1188.
14. Huang JL, McLeish MJ. High-performance liquid chromatographic determination of the alkaloids in betel nut. *J Chromatogr A*. 1989; 475(2):447–450.
15. Yuan J, et al. Alkaloids from areca (betel) nuts and their effects on human sperm motility in vitro. *J Food Sci*. 2012; 77(4):T70–8. [PubMed: 22515256]

16. Musa TA, et al. Determination of 4-Allyl Resorcinol and Chavibetol from Piper betle Leaves by Subcritical Water Extraction Combined with High-Performance Liquid Chromatography. *Food Anal Methods*. 2014; 7(4):893–901.
17. Rathee JS, et al. Antioxidant activity of piper betel leaf extract and its constituents. *J Agric Food Chem*. 2006; 54(24):9046–54. [PubMed: 17117789]
18. Mula S, et al. Inhibitory property of the Piper betel phenolics against photosensitization-induced biological damages. *Bioorg Med Chem*. 2008; 16(6):2932–8. [PubMed: 18207412]
19. Jacob P 3rd, et al. Minor tobacco alkaloids as biomarkers for tobacco use: comparison of users of cigarettes, smokeless tobacco, cigars, and pipes. *Am J Public Health*. 1999; 89(5):731–6. [PubMed: 10224986]
20. Benowitz NL, Jacob P 3rd. Trans-3'-hydroxycotinine: disposition kinetics, effects and plasma levels during cigarette smoking. *Br J Clin Pharmacol*. 2001; 51(1):53–9. [PubMed: 11167665]

Highlights

- Predominant compounds in betel nuts and saliva from those chewing the nuts were guvacine, arecoline, guvacoline, and arecaidine
- The predominate compound in the Piper betle leaf and saliva from those chewing the leaf was the allyl benzene compound chavibetol
- We found salivary chemical patterns during chewing to identify the 3 most common betel preparations consumed in Guam
- We observed significant increases in salivary baseline levels during chewing for predominant betel and nicotine alkaloids, as well as chavibetol

Figure 1.
Structures of alkaloid and nitroso compounds detected in betel nuts, *Piper betle* leaf, tobacco, and saliva samples.

Table 1

Characteristics of betel chewers

ID	BMI (kg/m ²)	Betel Chewing Habits		Typical duration of chewing	Age commence chewing	Amount of time chewed on a regular basis
		Typical preparation	Frequency of chewing			
BN01	36.8	Betel quid - everything	every 10 min constant		12y	7y
BN02	19.9	nut, lime tobacco	10×/day	5 min	8y	14y
BN03	35.5	nut, lime tobacco	10×/day	20 min	7y	10y
BN04	26.3	nut, lime tobacco	6×/day	10 min	15y	8y
BN05	27.6	nut, lime tobacco	10×/day	2 min	18y	4y
BL01	27.6	nut, tobacco	3×/day	5 min	18y	5y
BL02	26.0	nut, lime, tobacco, peppermint	5×/day	10 min	13y	7y
BL03	30.9	nut, lime, betel leaf, tobacco	no response	5 min	16y	7y
BL04	35.9	everything, betel quid	1-2×/day	4 min	16y	8y
BL05	22.1	young nut, cigarette, lime	1×/week	5 min	27y	2y
BQ01	28.8	nut, lime, tobacco	20×/day	10 min	6y	15y
BQ02	48.5	nut, lime, tobacco, clover seeds	6×/day	2 min	16y	4y
BQ03	22.6	nut, lime, tobacco	2-6×/day	3 min	14y	6y
BQ04	33.8	all types except leaf	constant	10-15 mi	14y	7y
BQ05	32.9	young nut, lime, tobacco, betel leaf	10×/day	2 min - 1 hr	12y	16y

Table 2

Predominant chemicals specific for components of the betel preparations used in the chewing study

Compound (µg/g)	Mature betel nut ^{1,2}	Piper betle leaf ²	Young, green betel nut ³	Tobacco ³
Guvacine	1,871.4	11.8	302.9	<0.43
Arecoline	595.3	9.5	339.1	<0.43
Guvacoline	315.5	1.8	283.1	<0.43
Arecaidine	47.7	4.0	93.2	<0.43
All Areca Alkaloids	707.5	6.8	254.6	
Nicotine	0.1	1.4	0.1	15,454.8
Cotinine	<0.04	<0.05	<0.02	41.6
Hydroxycotinine	<0.36	<0.54	<0.19	2.1
N-nitrosoguvacoline	<0.36	<0.54	<0.19	<4.26
NNK	<0.36	<0.54	<0.19	0.4
NNN	<0.36	<0.54	<0.19	1.3
NAT	<0.36	<0.54	<0.19	2.9
All Nicotine Alkaloids	0.1	1.4	0.1	2,583.9
Chavibetol	<72.58	39,873.8	<38.62	<852.66

values are from analyses of materials used in the study for chewing; tobacco was obtained from one cigarette with wrapper removed as present in

NNK=4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN=N-nitrosornicotine; NAT=N'-nitrosoanatabine
numbers with "<" indicate below detection limit

¹ BN group=red, mature nut only;

² BL group=red, mature nut+P. betle leaf;

³ BQ group=betel 'quid' consisting of green, young nut+P. betle leaf+lime+tobacco

Table 3
Mean saliva concentration of compounds specific for each betel preparation after extraction from saliva of betel chewers before (baseline) and during chewing

Compound	BN group (n=5)			BL group (n=5)			BQ group (n=5)		
	before chewing (ng/mL)	during chewing (ng/mL)	<i>I</i> p	before chewing (ng/mL)	during chewing (ng/mL)	<i>I</i> p	before chewing (ng/mL)	during chewing (ng/mL)	<i>I</i> p ²
Guvacine	51±18	201,202±38,326	0.006	5±4	92,592±36,169	0.06	35±26	37,009±5,782	0.003
Arecoline	181±88	31,575±6,118	0.007	39±19	17,867±6,356	0.05	159±103	46,246±14,369	0.03
Guvacoline	211±100	21,862±5,621	0.02	2±2	9,961±4,139	0.07	190±143	8,351±3,014	0.05
Arecaidine	390±106	1,887±280	0.003	43±35	621±213	0.03	218±109	12,480±3,043	0.02
All Areca Alkaloids ^{<i>I</i>}	833±251	256,525±48,889	0.006	88±56	121,040±46,808	0.06	602±347	104,086±23,576	0.01
Nicotine	461±189	32±11	0.08	1,079±846	70±45	0.28	922±627	105,342±24,157	0.01
Cotinine	244±36	86±13	0.003	158±19	53±26	0.02	195±79	480±87	0.13
Hydroxycotinine	49±9	15±4	0.006	27±9	8±6	0.04	54±30	38±5	0.65
N-nitrosoguvacoline	0.2±0.1	<0.1	-	<0.1	<0.1	-	<0.1	<0.1	-
NNK	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	4±2	0.06
NNN	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	9±2	0.02
NAT	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	17±4	0.01
All Nicotine Alkaloids ²	754±195	134±22	0.03	1,264±852	132±48	0.24	1,171±722	105,890±24,256	0.01
Chavibetol	<20	<20	-	<20	27,032±5,388	0.01	<20	13,615±4,970	0.03

Mean±standard error; BN group=red, mature nut only; BL group=red, mature nut+leaf; BQ group=betel ‘quid’ consisting of green, young nut+leaf+lime+tobacco values expressed as mean±SE

NF=not found; NNK=4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN=N-nitrosanornicotine; NAT=N’-nitrosoanatabine

^{*I*} p-values for difference within betel chew groups between baseline and chewing levels as determined by paired t-tests; bolded values are significant (p<0.05)

² p-values for difference of saliva concentrations during chewing between betel chew groups as determined by single-factor ANOVA

^{*I*} Guvacine+Arecoline+Guvacoline+Arecaidine

² N-nitrosoguvacoline+NNK+NNN+NAT