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Direct-Acting DNA Alkylating Agents Present in Aqueous Extracts of Areca Nut and Its Products

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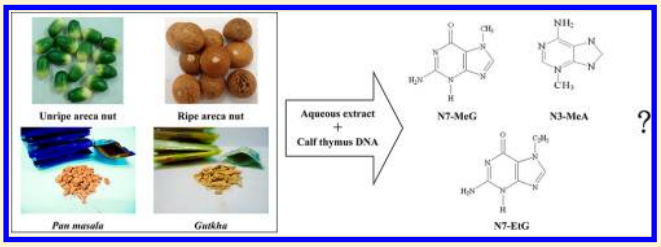
Supporting Information

ABSTRACT: Areca nut is a carcinogen to humans and has been strongly associated with oral premalignant and malignant diseases. Previous studies speculated the presence of unknown direct-acting mutagens present in aqueous extracts of areca nut. We hypothesized whether any direct-acting alkylating agents are present in areca nut and its commercial products. In this study, calf thymus DNA was treated with four different aqueous extracts obtained from unripe and ripe areca nuts or their commercial products, namely, *pan masala* (without tobacco) and *gutkha* (with tobacco). Three N-alkylated purines including *N*7-methylguanine (N7-MeG), *N*3-methyladenine (N3-MeA), and *N*7-ethylguanine (N7-EtG) were detected using sensitive and specific isotope-dilution liquid chromatography–tandem-mass spectrometry (LC–MS/MS) methods. The results showed that four types of aqueous extracts significantly induced the formation of N7-MeG and N3-MeA in a linear dose–response manner. Extracts from unripe areca nut exhibited higher methylating potency than those of ripe areca nut, while *gutkha* had higher methylating potency than *pan masala*. Meanwhile, *gutkha* made with areca nut and tobacco, was the only extract found to induce the formation of N7-EtG. Overall, this study first demonstrated that the presence of direct-acting alkylating agents in areca nut and its commercial products exist at a level that is able to cause significant DNA damage. Our findings may provide another mechanistic rationale for areca nut-mediated oral carcinogenesis and also highlight the importance and necessity of the identification of these direct-acting alkylating agents.



INTRODUCTION

The areca nut (fruit of the *Areca catechu* tree) is the fourth most commonly used psychoactive substance in the world after tobacco, alcohol, and caffeine.[1] It is commonly consumed by Asian populations and Asian communities living in Europe and North America.[2] Areca nut can be chewed alone or with a variety of substances that differ by region. The habit of chewing betel quid (BQ) contains fresh, dried or cured areca nut, catechu (*Acacia catechu*), and slaked lime (calcium oxide and calcium hydroxide) wrapped in a betel leaf (*Piper betle*). In some countries, particularly in India, most habitual chewers of BQ add tobacco. In Taiwan and parts of southern China, the green unripe areca nut is often chewed with betel inflorescence, but tobacco is not added.[3] Areca nut has been also available in commercially prepared forms in the last few decades, especially in India. The product is basically a flavored and sweetened dry mixture of areca nut, catechu, and slaked lime with tobacco (*gutkha*) or without tobacco (*pan masala*).[4] A previous evaluation in 1985 by the International Agency for Research on Cancer (IARC) had found that chewing BQ with tobacco (group 1) is carcinogenic to humans.[5] Recently, the new evaluation goes further to conclude that chewing BQ without tobacco (group 1) and areca nut itself (group 1) are also carcinogenic to humans.[6] Moreover, the use of commercial BQ substitutes (*pan masala* and *gutkha*) has been suggested to pose even higher risks.[2]

There was evidence of mutagenic, genotoxic, and carcinogenic activity of areca nut extracts in a variety of experimental systems.[7] The carcinogenic mechanism has been suggested to involve the nitrosation of alkaloids present in areca nut. Though areca nut-specific nitrosamines (ASNAs) were not directly detected in BQ containing areca nut, endogenous nitrosation of areca nut alkaloids can occur during chewing as supported by the identification of three areca derived *N*-nitrosamines in the saliva of BQ-chewers.[8] Generally, the metabolic activation of *N*-nitrosamines to reactive intermediates by cytochrome P450 enzymes is required to exert their genotoxicity.[9] Of these ASNAs, 3-(methylnitrosoamino)-propionitrile (NMPN) has been shown to be a powerful carcinogen and can form alkylated (i.e., methyl and cyanoethyl) adducts in the DNA of the target tissues in which the tumors developed.[10,11]

Areca nut extract is a complex mixture of many structurally diverse components that makes the attribution of cancer risk to a single agent or group of agents difficult. Aqueous extracts of BQ, areca nut, and *pan masala* have been shown to be

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mutagenic in the Ames *Salmonella* test in the presence or absence of metabolic activation and to induce chromosomal damage in mammalian cells *in vitro*.[6] Particularly, the genotoxic effects of aqueous extracts of *pan masala* on Chinese hamster ovary (CHO) cells were examined.[12] A dose-dependent increase was noted in the frequency of chromosomal aberrations, sister chromatid exchange, and micronuclei in CHO cells cultured without metabolic activation, although metabolic activation markedly inhibited the chromosomal damaging effect, implying the content of direct-acting mutagens. Patel et al.[13] also reported the presence of direct-acting mutagens in dimethyl sulfoxide extracts of *pan masala*. However, the identity of these direct-acting agents remains unknown. Recently, the aqueous extract of areca nut was speculated to induce alkylation lesions since O^6-methylguanine-DNA methyltransferase (MGMT), a repair enzyme for the removal of alkylating DNA adducts, was found to increase with the dose of extract in normal human oral keratinocytes.[14] So far, no alkylating agent in areca nut has been found in the literature.

In this study, we hypothesized whether any direct-acting alkylating agents could be present in aqueous extracts of areca nut and its products (*pan masala* and *gutkha*). For this purpose, calf thymus DNA was treated with these aqueous extracts from areca nut and its products, and the possible resulting alkylated DNA lesions were measured. If direct-acting alkylating agents are present, a number of DNA adducts can be formed including those alkylated at the O^6, N^2, N-7, and N-3 positions of guanine, the N-7 and N-3 positions of adenine, the O^2 position of cytosine, and the O^2 and O^4 positions of thymine as well as the phosphodiester backbone.[15,16] Numerous studies have suggested that O-alkylations (e.g., O^6-alkylguanine and O^4-alkylthymidine) are highly mutagenic and genotoxic, whereas N-alkylations (e.g., *N*7-alkylguanine and *N*3-alkyladenine) are cytotoxic but less mutagenic.[17] Nevertheless, we chose to determine *N*7-alkylguanine and *N*3-alkyladenine in this study since they are the major products of DNA alkylation and abundant enough to be reliably measured. Highly sensitive and specific isotope-dilution liquid chromatography−tandem-mass spectrometry (LC−MS/MS) methods were used to quantify the levels of *N*7-methylguanine (N7-MeG), *N*7-ethylguanine (N7-EtG), and *N*3-methyladenine (N3-MeA) in treated DNA.[18,19] This is the first study demonstrating that areca nut and its products contain direct-acting DNA methylating and/or ethylating agents.

EXPERIMENTAL PROCEDURES

Chemicals. Solvents and salts were of analytical grade. Reagents were purchased from the indicated sources: N3-MeA and calf thymus DNA (Sigma-Aldrich); N7-MeG and N7-EtG (Merck); d_3-*N*3-methyladenine (d_3-N3-MeA) (Cambridge Isotope Laboratories). $^{15}N_5$-*N*7-methylguanine ($^{15}N_5$-N7-MeG), and $^{15}N_5$-*N*7-ethylguanine ($^{15}N_5$-N7-EtG) were synthesized as described previously.[20,21]

Preparation of Aqueous Extracts from Areca Nut and Its Products. Fresh and tender areca nut with husk (unripe areca nut) was purchased from a local shop in Taiwan. Ripe areca nut without husk (ripe areca nut) and two commercial areca nut products (*pan masala* and *gutkha*) was purchased from the local market in India. Fifty grams of areca nut (or *pan masala* or *gutkha*) was ground and suspended in 75 mL of deionized water (DIW). Then, it was stirred for 3 h at 37 °C, and the extract was collected by centrifugation. This extraction procedure was repeated once more by adding 75 mL of water to the residue. Both extracts were pooled, representing 50 g of extracted material in 150 mL of distilled water, filtered under vacuum (qualitative filter paper no. 1, Advantec, Japan), and kept for complete freezing at −80 °C. The filtrate was lyophilized in a Heto FD4 lyophilizer. The yield of dried crude extract calculated as the weight of lyophilized powder of extracts divided by the weight of raw materials was 4.1, 5.7, 7.9, and 7.8% for unripe and ripe areca nuts, *pan masala*, and *gutkha*, respectively. The lyophilized powder was dissolved in DIW to a concentration of 25 mg/mL, referred to as crude aqueous extract.

Since there were trace amounts of N7-MeG originally present in areca nut, *pan masala*, and *gutkha* (unpublished data), the crude aqueous extracts were further purified by C18 cartridges to remove N7-MeG contamination. C18 cartridges (2 g/12 mL, Waters Milford, MA, USA, ref: WAT036915) were activated with 12 mL of methanol and 12 mL of DIW. Twelve milliliters of crude aqueous extract was eluted through the cartridge at a flow rate of about 0.3 mL/min, and the eluate was collected, combined with 12 mL DIW to wash the cartridge, and then dried under vacuum. The residue (polar fraction of extract) was redissolved in 12 mL of 0.1 M potassium phosphate buffer (pH 7.4), referred to as purified aqueous extract (PAE), and kept at −20 °C until further reaction with calf thymus DNA. The procedure has been summarized in Supporting Information (Figure S1).

Incubation of Calf Thymus DNA with Aqueous Extracts. To 50-μg aliquots of calf thymus DNA dissolved in 0.1 M potassium phosphate buffer (pH 7.4) were added 50 to 500 μL of the PAE and 0.1 M potassium phosphate buffer was added to a final volume of 1 mL and incubated at 37 °C for 24 h. The untreated calf thymus DNA (50 μg) was prepared by adding 0.1 M potassium phosphate buffer (pH 7.4) to a final volume of 1 mL and incubated at 37 °C for 24 h. The control PAE was also prepared by adding 0.9 mL of 0.1 M potassium phosphate buffer, pH 7.4, to 100 μL of the PAE solutions and also incubated at 37 °C for 24 h. After incubation, the DNA solutions (or the control PAE) were spiked with 376 fmol of $^{15}N_5$-N7-MeG, 240 fmol of $^{15}N_5$-N7-EtG and 2690 fmol of d_3-N3-MeA, and subjected to neutral thermal hydrolysis at 100 °C for 30 min to release *N*7-alkylguanines and N3-MeA by the cleavage of the N-glycosidic bond. The DNA hydrolysates were loaded onto a Sep-Pak C18 cartridge (100 mg/1 mL, Waters) preconditioned with 1 mL of methanol and 1 mL of DIW. The column was then washed with 1 mL of DIW and eluted with 1 mL of 40% (v/v) methanol. The eluate containing *N*7-alkylguanines and N3-MeA was divided into two equal aliquots and dried under vacuum. The first aliquot was redissolved in 200 μL of 96% (v/v) acetonitrile with 0.1% formic acid for N7-MeG and N7-EtG analysis.[18] The second aliquot was redissolved in 200 μL of 3% (v/v) methanol/0.1% (v/v) trifluoroacetic acid for N3-MeA analysis.[19] The procedure has been summarized in Supporting Information (Figure S2).

Determination of N7-MeG, N7-EtG, and N3-MeA in DNA Hydrolysates by LC-MS/MS. N7-MeG and N7-EtG were quantified by using LC-MS/MS with online solid-phase extraction (SPE) as described previously by Chao et al.[18] Briefly, after automatic sample cleanup, LC-MS/MS analysis was performed using an Agilent 1100 series HPLC system (Agilent Technology) interfaced with an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems) with electrospray ion source (ESI). The samples were analyzed in the positive ion multiple reaction monitoring (MRM) mode, and the transitions of the precursors to the product ions were as follows: m/z 166→149 (quantifier ion) and 166→124 (qualifier ion) for N7-MeG, m/z 171→153 for $^{15}N_5$-N7-MeG, m/z 180→152 (quantifier ion) and 180→135 (qualifier ion) for N7-EtG, and m/z 185→157 for $^{15}N_5$-N7-EtG. Limits of quantification (LOQs) in the sample matrix were determined by using a signal-to-noise ratio of 10. With the use of isotope internal standards and online SPE, this method had a high sensitivity with LOQs of 1.5 fmol and 0.8 fmol on-column for N7-MeG and N7-EtG, respectively, which corresponds to 0.19 and 0.08 μmol adducts/mol guanine when using 10 μg of DNA per injection.

The N3-MeA concentrations in DNA hydrolysates were analyzed using the same LC-MS/MS with online SPE as described above. Detection was performed in the positive ion MRM mode for quantitation of N3-MeA as recently reported by Chao et al.[19] Optimal MRM conditions were obtained for 3 channels: m/z 150→123

(quantifier) and 150→108 (qualifier) for N3-MeA, and m/z 153→126 for the internal standard d_3-N3-MeA. With the use of isotopic internal standards and online SPE, this method exhibited a LOQ of 40 fmol for N3-MeA on-column, which corresponds to 4.1 μmol adducts/mol adenine when using 10 μg of DNA per injection.

For each analyte in DNA hydrolysates, the peak identity was also confirmed by comparing the peak area ratios (quantifier ion/qualifier ion) with those of the calibrators. As an acceptance criterion, ratios in DNA samples should not deviate by more ±25% from the mean ratios in the calibrators.

RESULTS

To investigate the presence of direct-acting DNA alkylating agents in areca nut and its products, calf thymus DNA (50 μg) was individually incubated with four different purified aqueous extracts (PAEs) obtained from unripe and ripe areca nut, *pan masala* and *gutkha*, for 24 h at 37 °C. Figures 1 and 2 show, as an example, N7-MeG and N3-MeA formations in DNA treated with the PAE of ripe areca nut, as measured by online SPE LC-MS/MS. It was noted that there was an endogenous N7-MeG present in untreated DNA with a trace amount (Figure 1A, normally ranged from 260 to 270 fmol in 50 μg untreated DNA), and no N7-MeG was detected in PAE of ripe areca nut (Figure 1B). When DNA was treated with 100 μL of ripe areca nut extract, a large excess of N7-MeG (2400 fmol) was formed (Figure 1C). As for N3-MeA, N3-MeA was initially not detected in both untreated DNA and PAE from ripe areca nut (Figure 2A and B). After incubation with PAE from ripe areca nut, a considerable amount of N3-MeA (220 fmol) was observed (Figure 2C). Figure 3 shows the N7-EtG formation that was detected only in the DNA treated with PAE from *gutkha*, and similarly no N7-EtG was initially detected in both untreated DNA and PAE of *gutkha*. Table 1 summarized the *N*-alkylpurines detected in untreated DNA, PAEs, and DNA treated with four different PAEs. It is important to note that no *N*-alkylpurines were measured in the PAEs, obtained from the crude aqueous extracts. In all cases, significantly higher levels of N7-MeG and N3-MeA were observed in DNA treated with four PAEs, while N7-EtG formation was only observed in the DNA treated with *gutkha* aqueous extract.

Calf thymus DNA was further incubated with 50, 100, 200, and 500 μL of PAEs for 24 h at 37 °C. As shown in Figure 4, linear dose−response relationships were obtained for the formations of N7-MeG and N3-MeA in DNA with increasing amounts of PAEs from unripe areca nut, ripe areca nut, *pan masala*, and *gutkha*. In these samples, methylation occurred more at the N-7 position of guanine (N7-MeG) than at the N-3 position of adenine (N3-MeA). For example, treatment of calf thymus DNA to 500 μL of aqueous extract from ripe areca nut produced 10400 fmol of N7-MeG, which was 8-fold higher than the corresponding level of 1290 fmol for N3-MeA (Figure 4B). Meanwhile, the N7-EtG formation was also found to be directly proportional to the amount of *gutkha* extract (Figure 5).

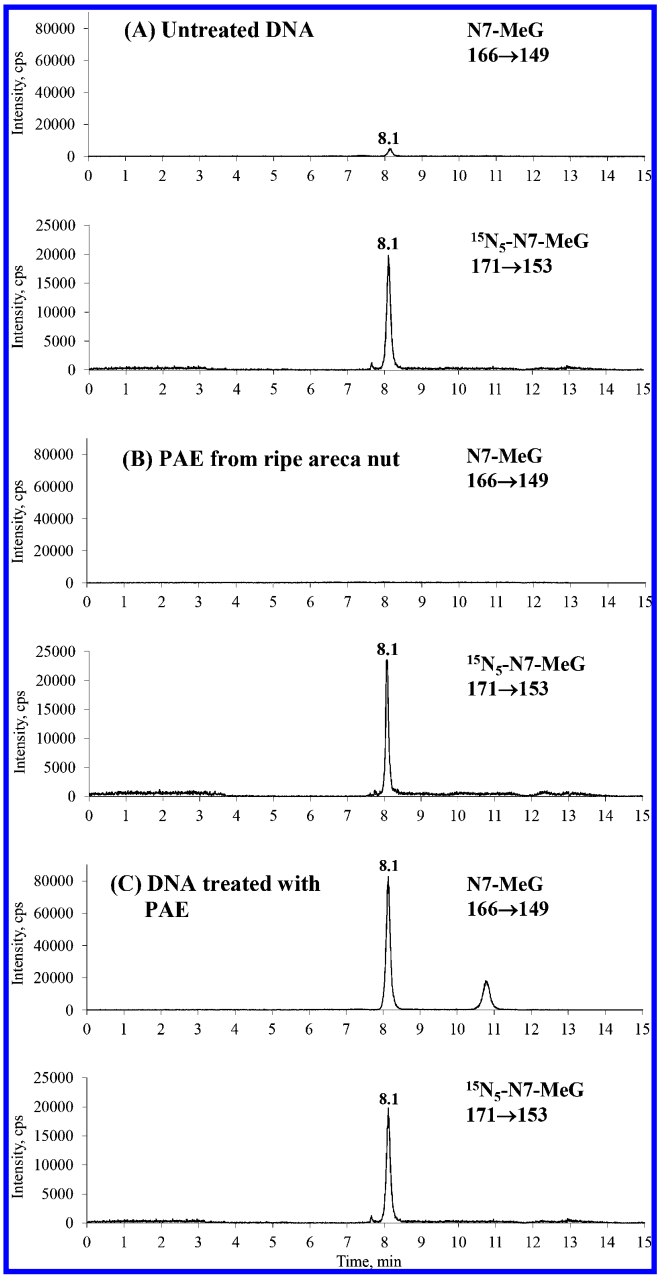

Figure 1. N7-MeG in calf thymus DNA (50 μg) treated with the PAE of ripe areca nut by online SPE LC-MS/MS. Typical MRM ion chromatograms for (A) untreated calf thymus DNA, (B) PAE from ripe areca nut, and (C) calf thymus DNA treated with 100 μL of PAE from ripe areca nut (MRM transitions m/z 166 to 149 for N7-MeG and m/z 171 to 153 for the internal standard $^{15}N_5$-N7-MeG, respectively).

DISCUSSION

Epidemiological evidence indicated that the chewing of BQ, with or without tobacco, is closely linked to oral carcinogenesis. Oral squamous cell carcinoma has been the third most common malignancy in developing countries, consisting of ~50% of all malignancies in some nations of South Asia.[22] Evidences also link BQ chewing to the risk of esophageal[23] and hepatic carcinogenesis.[24] Areca nut is reported to contain more than four alkaloids including arecoline, arecaidine, guvacoline, and gvacine, which can give rise to areca nut-specific nitrosamines (ASNAs) with the presence of nitrite species in the oral cavity.[8] These ASNAs require metabolism to form the reactive species that can covalently bond to DNA forming adducts in humans. However, this study further demonstrated that the presence of direct-acting (without metabolism) methylating agents existing in the aqueous extracts of either green unripe areca nut or ripe areca nut reacted with DNA to form methylated purines (Table 1). Evidently, regardless of fruit maturity, green unripe areca nut or ripe areca nut contains direct-acting methylating agents and so do its manufactured

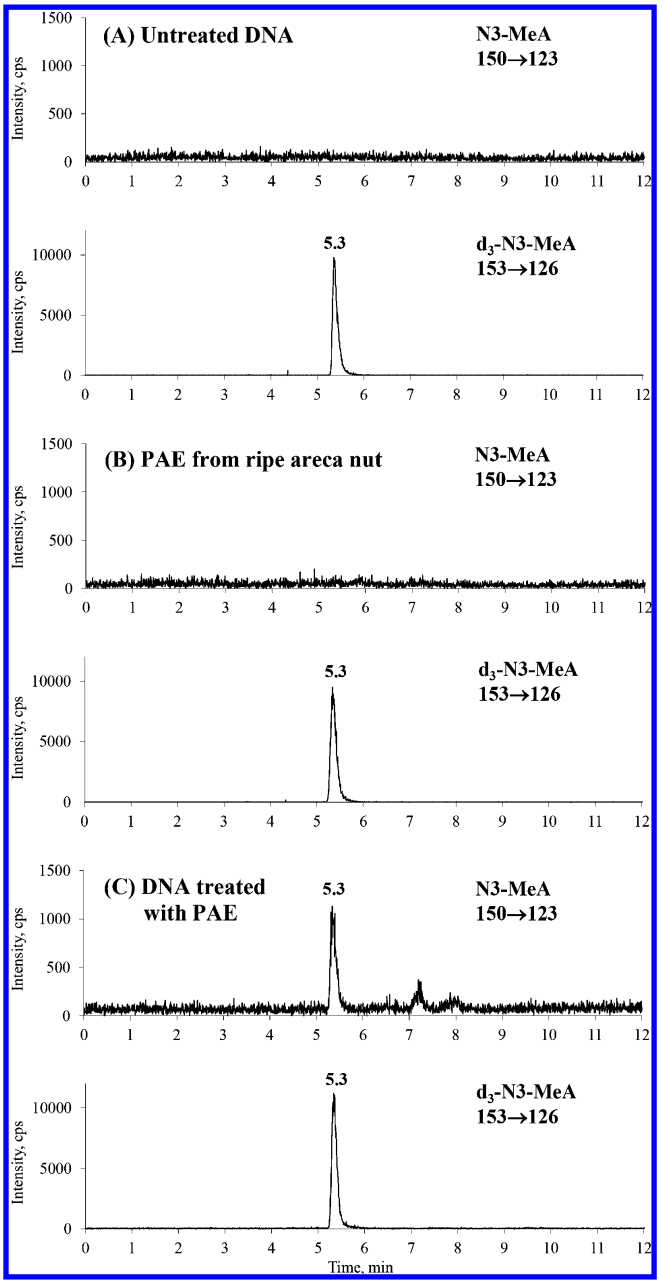

Figure 2. N3-MeA in calf thymus DNA (50 μg) treated with the PAE of ripe areca nut by online SPE LC-MS/MS. Typical MRM ion chromatograms for (A) untreated calf thymus DNA, (B) PAE from ripe areca nut, and (C) calf thymus DNA treated with 100 μL of PAE from ripe areca nut (MRM transitions m/z 150 to 123 for N3-MeA and m/z 153 to 126 for the internal standard d_3-N3-MeA).

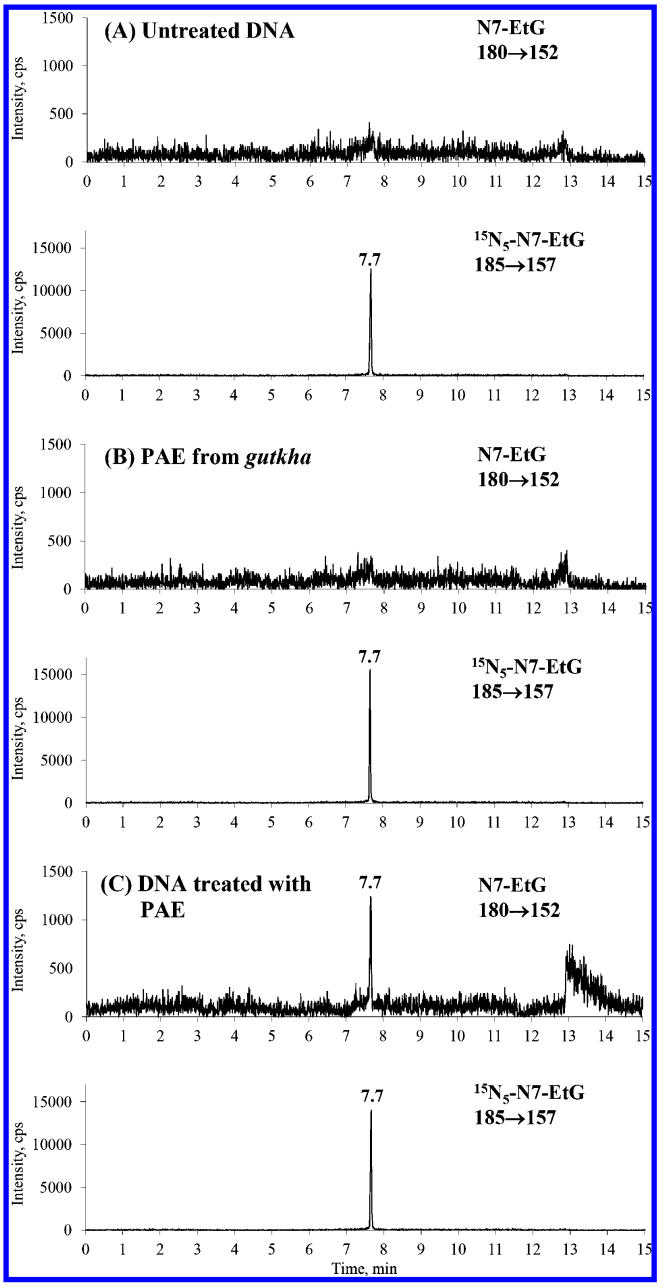

Figure 3. N7-EtG in calf thymus DNA (50 μg) treated with the PAE of *gutkha* by online SPE LC-MS/MS. Typical MRM ion chromatograms for (A) untreated calf thymus DNA, (B) PAE from *gutkha*, and (C) calf thymus DNA treated with 100 μL of PAE from *gutkha* (MRM transitions m/z 180 to 152 for N7-EtG and m/z 185 to 157 for the internal standard $^{15}N_5$-N7-EtG).

products (*pan masala* and *gutkha*). The identity of this direct-acting methylating source is unknown and is currently being studied.

The levels of N7-MeG and N3-MeA formed were directly proportional to the dose of aqueous extracts (Figure 4). Extracts from unripe areca nut had higher methylating potency (ability to induce the formation of methylated purines) than those extracts from ripe areca nut, while for commercial BQ substitutes, *gutkha* had higher methylating potency than *pan masala*. It was interesting to note that the ratio of relative yields of N7-MeG and N3-MeA were similar in all DNA treated with four different extracts and found to be 7.4−8.6:1, which was comparable to those achieved following *in vitro* methylation of DNA with known direct-acting methylating agents (e.g., methyl methanesulphonate and *N*-methyl-*N*-nitrosourea).[17] Meanwhile, direct-acting methylating agents can also form O^6-methylguanine that is highly mutagenic leading to a G-to-A transition mutation during DNA replication.[17] Interestingly, such G-to-A transition mutations were observed in the *p53* gene for Thai BQ-chewers who neither smoked nor drank alcohol.[25] Furthermore, it is known that alkylation agents of the S_N1 (unimolecular nucleophilic substitution) type alkylate both oxygens and nitrogens in nucleic acid, whereas S_N2 (bimolecular nucleophilic substitution) reagents mainly alkylate

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Table 1. *N*-Alkylpurines Detected in Untreated Calf Thymus DNA (50 μg), PAEs (100 μL), and Calf Thymus DNA (50 μg) Treated with 100 μL of PAEs

	untreated DNA (fmol)			PAEs (fmol)			DNA treated with PAEs (fmol)		
test materials	N7-MeG	N3-MeA	N7-EtG	N7-MeG	N3-MeA	N7-EtG	N7-MeG	N3-MeA	N7-EtG
unripe areca nut	270 ± 16[a]	N.D.[b]	N.D.	N.D.	N.D.	N.D.	4300 ± 460	520 ± 87	N.D.
ripe areca nut	260 ± 21	N.D.	N.D.	N.D.	N.D.	N.D.	2400 ± 410	240 ± 64	N.D.
pan masala	270 ± 27	N.D.	N.D.	N.D.	N.D.	N.D.	1700 ± 180	200 ± 56	N.D.
gutkha	260 ± 14	N.D.	N.D.	N.D.	N.D.	N.D.	5200 ± 410	550 ± 76	19 ± 2.8

[a]Data are the mean ± SD (n = 3). [b]N.D. = not detected; LOQs were 1.5 fmol for N7-MeG, 0.8 fmol for N7-EtG, and 40 fmol for N3-MeA.

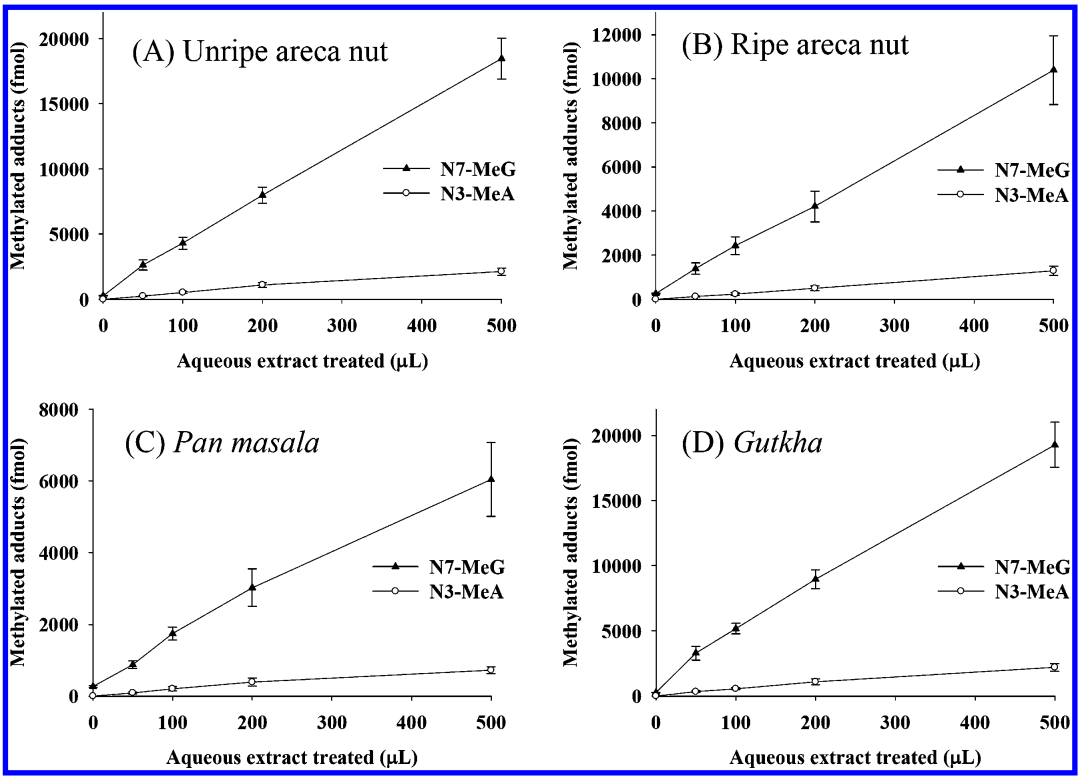

Figure 4. Dose-dependent formations of N7-MeG and N3-MeA following the treatment of calf thymus DNA (50 μg) with PAEs from (A) unripe areca nut, (B) ripe areca nut, (C) *pan masala*, and (D) *gutkha*. The error bars represent standard deviation of the mean (n = 3).

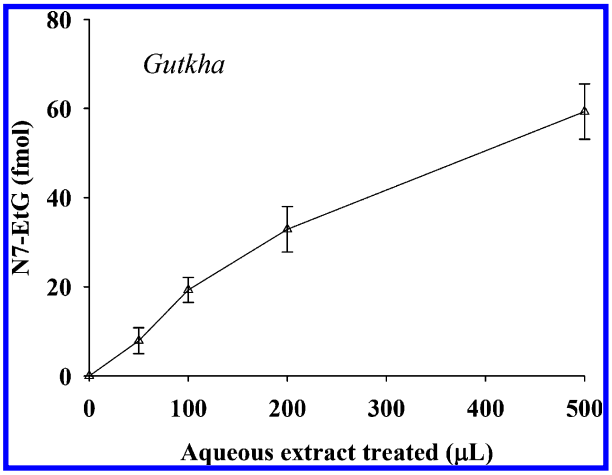

Figure 5. Dose-dependent formation of N7-EtG following the treatment of calf thymus DNA (50 μg) with PAE from *gutkha*. The error bars represent standard deviation of the mean (n = 3).

nitrogens.[16] It is suggested that O^6-alkylguanine residues in treated DNA should be also quantified in a future study; the ratio of *N*7-alkylguanine and O^6-alkylguanine would give useful information on the nucleophilic substitution mechanism (S_N1 or S_N2) to assist in identifying these direct-acting alkylating agents and provide some insights as to their carcinogenic potency.

Gutkha is a mixture of areca nut and other ingredients with a large quantity of tobacco, while *pan masala* is a similar product made without tobacco. It was of interest that the aqueous extract of *gutkha* was the only extract exhibiting significant ethylating potency in a linear dose−response manner (see Table 1 and Figure 4). Previously, several reports have suggested that there could be uncharacterized ethylating agents present in cigarette smoke based on the observation of increased levels of *N*3-ethyladenine and N7-EtG in the urine of smokers as compared with nonsmokers.[21,26,27] The presence of direct-acting ethylating agents in cigarette smoke was further confirmed by treating calf thymus DNA directly with cigarette smoke,[28] showing that the formation of N7-EtG was highly correlated with the number of cigarettes treated. Although the identity of the direct-acting ethylating agent also remains unknown, the authors postulated that the ethylating agent could be generated by interactions between the diverse array of

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chemicals that are present in cigarette smoke. Nevertheless, in this study, we suggested that the direct-acting ethylating agent in cigarette smoke could also originate from unburned tobacco because ethylating potency was only found in aqueous extracts of *gutkha* (with tobacco), not in *pan masala* (without tobacco). To test our hypothesis, calf thymus DNA was further treated with aqueous extracts of unburned cigarette tobacco (2R4F from the Kentucky Tobacco Research and Development Center). The results showed that the incubation of DNA with aqueous extracts of unburned tobacco resulted in an increased amount of N7-EtG formation, indicating the presence of direct-acting ethylating agents in unburned tobacco (manuscript in preparation).

However, the high temperature (100 °C for 30 min) might play a role in activating the active agent of the areca nut causing the alkylation because a neutral thermal hydrolysis step following incubation was done in the presence of PAE. In trying to clarify the issue of high temperature, we therefore conducted further experiments in which the treated DNA was washed free of PAE before neutral thermal hydrolysis and then measured for N-alkylated purines (the detailed description of the experimental procedure is provided in Supporting Information, Figure S3). In the case of PAE from unripe areca nuts, the results showed that at the same dose (100 μL of PAE) a comparable amount of N7-MeG (3500 fmol) was formed, while a significantly less amount of N3-MeA was found (240 fmol) as compared to our original reported data without washing DNA (see Table 1: 4300 fmol for N7-MeG and 520 fmol for N3-MeA). The relatively low amount of N3-MeA measured (only 46% of that detected in treated DNA without washing) could be because the N3-MeA formed was more unstable and prone to spontaneous depurination during the incubation of DNA with PAE for 24 h (i.e., half-life at 37 °C, N3-MeA, 38 h; and N7-MeG, 105 h),[29] and the base adducts released in the reaction solution could have been removed during the DNA washing process. On the basis of the above findings, we can ensure that these methylating agents are capable of causing DNA alkylation at 37 °C and that the high temperature might not be a significant factor in alkylation.

Preliminary characterization of these direct-acting alkylating agents in four crude aqueous extracts (from unripe and ripe areca nuts, *pan masala*, and *gutkha*) was attempted in our study. As shown in Supporting Information, chromatographic separation of crude aqueous extracts was performed on a C18 column by HPLC (Table S1, Supporting Information). HPLC fractionation yielded 12 fractions from each crude aqueous extract, and each fraction was then individually incubated with DNA and analyzed by LC-MS/MS. As expected, the results showed that the direct-acting methylating potency was found in four crude aqueous extracts, while ethylating potency was only observed in *gutkha* (Table S2, Supporting Information). Furthermore, the alkylating potency of either methylating or ethylating was only found in the most polar fraction (fraction 1) among 12 fractions, suggesting that these direct-acting alkylating agents present in areca nut and its commercial products are highly hydrophilic (water-loving) and easily extracted by saliva, allowing them to be readily in contact with oral tissues.

In conclusion, we have used sensitive isotope-dilution LC-MS/MS methods for the detection and accurate quantitation of alkylated purines in DNA that has been exposed to aqueous extracts of areca nut and its products *in vitro*. Our results demonstrated the presence of direct-acting alkylating agents in areca nut and its commercial products, implying that BQ-chewers are exposed to alkylating agents regardless of their capacity for metabolic activation. Therefore, the ability of an individual to reduce the harmful effects following exposure to these alkylating agents may be dependent on his or her ability to detoxify the reactive metabolite or repair the DNA damage, which will be ultimately affected by the amount of BQ consumed and the length of time of BQ usage. Although these direct-acting methylating and ethylating agents have yet to be identified, our study showed that these alkylating agents are present at levels that are sufficient to cause DNA damage and could potentially have adverse implications to human health, particularly in the case of the development of oral cancer for BQ-chewers.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Schemes for the preparation of PAEs from areca nut and its products, and determination of *N*-alkylpurines in DNA samples; calf thymus DNA treated with the PAE from unripe areca nut followed by washing DNA free of PAE prior to neutral thermal hydrolysis; and preliminary characterization of direct-acting alkylating agents in four crude aqueous extracts (from unripe and ripe areca nut, *pan masala* and *gutkha*) by HPLC fractionation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

BQ, betel quid; ESI, electrospray ionization; LC-MS/MS, liquid chromatography−tandem mass spectrometry; LOD, limit of detection; MRM, multiple reaction monitoring; N3-MeA, *N*3-methyladenine; N7-EtG, *N*7-ethylguanine; N7-MeG, *N*7-methylguanine; PAE, purified aqueous extract; SPE, solid-phase extraction; ASNAs, areca nut-specific nitrosamines

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