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## Microbiological safety of areca nut-containing, ready-to-eat chewing substances common among Pakistani paediatric population: A pilot study

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### Abstract

**Objective:** To evaluate microbiological contamination of areca nut-containing, ready-to-eat chewing substances easily accessible to vulnerable paediatric population.

**Methods:** A pilot study was conducted at the Aga Khan University Medical College from June to October 2016 on twelve samples of areca nut-containing chewing substances (four supari, paan masala and gutka each) collected from various localities of Karachi. These were evaluated individually for total colony counts, hygiene indicator organisms, pathogenic organisms, and levels of aflatoxin. Microbial contamination was analysed using pour-plate method. Fungal aflatoxin levels were measured by enzyme-linked immunosorbent assay (ELISA).

**Results:** Wet gutka preparations were contaminated by *Escherichia coli* and *Enterobacteriaceae*. High levels of fungal aflatoxin (range: 0.43-1.84 mg/kg), a proven carcinogen, were identified in all the 12(100%) products. No sample contained pathogenic bacteria. However, 1(8.33%) sample did not meet hygiene criteria cut-off.

**Conclusion:** Habitual use of unhygienic chewing substances containing fungal toxins is a public health concern that needs to be addressed through a preventative, behaviour-changing strategy.

**Keywords:** Areca, Tobacco, Smokeless, Paediatric, Population, Safety, Microbiological. (JPMA 69: 450 2019)

### Introduction

Areca nut, also known as betel nut, is the fruit of *Areca catechu* and is used around the world as a chewing substance, either alone or in combination with other ingredients. It is the fourth most commonly used psychoactive substance in the world and is chewed by approximately 600 million people worldwide.<sup>1,2</sup>

Areca nut is used in many traditional remedies and is credited with having anti-depressant,<sup>3</sup> anti-inflammatory<sup>4</sup> and anti-hyperlipidaemic<sup>5</sup> properties, and cytotoxic, insecticidal and anti-microbial activities,<sup>6</sup> in addition to acting as an analgesic<sup>4</sup> and immunomodulator.<sup>7</sup> Studies have also attributed a variety of negative health outcomes to the use of areca nut-containing products, including development of oral submucous fibrosis (OSF),<sup>8-10</sup> oral,<sup>11,12</sup> pharyngeal and oesophageal,<sup>12</sup> hepatocellular,<sup>13</sup> stomach<sup>14</sup> and cervical cancers,<sup>15</sup> metabolic syndrome and cardiovascular disease.<sup>16</sup> Areca nut-containing products are also often contaminated with aflatoxin, a mycotoxin which is a secondary metabolite produced by certain strains of filamentous fungi such as *Aspergillus (A.) flavus* and *A. parasiticus*.<sup>17</sup>

Long-term exposure to aflatoxin has documented negative health impacts.<sup>18</sup> Moreover, many consumers chew and spit these products out in public spaces. This may pose a public health risk by increasing transmission of various diseases, including tuberculosis, which has a high prevalence in South Asia.<sup>19</sup>

Various commercially available preparations of areca nut mixed with other substances are widely available, and the industry has grown to be worth several hundred million US dollars.<sup>1,19</sup> Some of the chewing substances are commonly available and consumed in South Asia (Annexure). Processed areca nut products have been aggressively marketed since the 1980s.<sup>20</sup> As a result of media portrayal, these products have come to be widely accepted and are especially popular amongst the youth, particularly those of lower socioeconomic status.<sup>20-22</sup> Despite legislation to ban the sale of gutka in the province of Sindh in Pakistan, it is still available in the market.<sup>23,24</sup>

While there have been studies in the past regarding the prevalence of use and effects of areca nut-containing products, no study has investigated the microbial contamination of areca nut-containing products. It is important to determine the possible contaminants in these substances in order to better understand the health risks they pose. This information can also help in educating the population, particularly the youth, on the possible negative effects and create a focus on

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**Annexure:** Areca nut-containing preparations commonly found in South Asia. <sup>1, 21, 33</sup>

Type	Components
Betel quid (paan)	Betel leaf
	Areca nut
	Catechu
	Slaked lime
Supari Mainpuri	May also contain: Tobacco Fragrances Artificial/natural sweeteners
	Roasted areca nut
	Areca nut
	Tobacco
	Slaked lime
Mawa	Camphor
	Cloves
	Areca nut
Paan Masala	Tobacco
	Slaked lime
	Areca nut
Gutka	Artificial flavouring
	Areca nut
	Powdered tobacco
	Slaked lime
	Catechu
	Fragrances

prevention of development of a chewing habit. The current study was planned to assess microbial contamination and aflatoxin concentrations in products easily accessible by youth in urban Pakistan.

## Materials and Methods

The pilot study was conducted at the Aga Khan University Medical College from June to October 2016 on samples of areca nut-containing chewing substances collected from various localities of Karachi.

Four common variants of supari (areca nut alone), paan masala and gutka were procured from kiosks and hawkers near schools and in the local market.

The substances were evaluated individually for total colony counts, hygiene indicator organisms, pathogenic organisms, and levels of aflatoxin.

Pour-plate technique was used to determine microbial contamination. Samples were dissolved in peptone water and were then serially diluted. The dilutions were plated on nutrient agar to determine total colony count. For the isolation of indicator and pathogenic organisms, MacConkey's Agar was set and incubated at 37°C for 24-48 hrs. In order to detect mould contamination, Sabouraud Dextrose Agar (SDA) was inoculated and incubated at 25°C.

Criteria of the United Kingdom Health Protection Agency (now called Public Health England)<sup>25</sup> were used to

determine the contamination of the ready-to-eat samples by microbes.

Safety criteria requires no growth of pathogenic bacteria within the sample, including *Campylobacter*, *Salmonella*, *Shigella* and *Vibrio species*.

Hygiene criteria means no evidence of contamination by *Enterobacteriaceae*, *Escherichia coli*, or *Listeria species*.

A satisfactory test result indicates good microbiological quality, while an acceptable result reflects a borderline limit of microbiological quality. Unsatisfactory test results indicate that further sampling may be necessary.

Total aflatoxin levels were measured by direct competitive enzyme-linked immunosorbent assay (ELISA) kit (Glory Science Company Ltd., Shanghai, China). Five grams of ground and sieved sample was dissolved in 20ml of 70% methanol and oscillated for 5 minutes. The samples were then filtered through Whatman Filter No.1 paper. Briefly, sample standard and negative controls in duplicates were added to wells coated with coupled antigen, along with anti-aflatoxin antibody conjugate. After 30 minutes of incubation at 37°C, the wells were washed and colour solutions were added. The optical density was measured after 10 minutes at 450nm on iMark™ Microplate Absorbance Reader (BioRad, Hercules, California, USA) and aflatoxin levels were calculated through Logger Pro® software (Vernier, Beaverton, Oregon, USA).

## Results

The 12 samples included four common variants of supari (areca nut alone) (S1-4), paan masala (PM1-4) and gutka (G1-4) (Table-1). PM1, PM2, PM3 and S2, S4 samples had less than one colony forming unit (CFU)/g on aerobic total colony counts. The sample with the highest colony count was G2 with  $2.0 \times 10^3$  CFU/g. Only 1 (8.3%) sample, G4, had growth of *Escherichia coli* and *Enterobacteriaceae* with 2.0

**Table-1:** Samples and their constituents.

Sample ID	Sample constituents and packing form
S1	Supari: sweetened, roasted, small sachet
S2	Supari: unsweetened, roasted, bulk packaged
S3	Supari: sweetened, dry, bulk packaged
S4	Supari: sweetened, wet, bulk packaged
PM1	Paan masala: sweetened, commercially prepared
PM2	Paan masala: unsweetened, contains areca nut, coconut and aniseed
PM3	Paan masala: chocolate coated areca nut
PM4	Paan masala: sweetened mixture without areca nut
G1	Gutka: commercially prepared, imported sachet
G2	Gutka: homemade, wet consistency
G3	Gutka: homemade, dry consistency
G4	Gutka: commercially prepared, local sachet

**Table-2:** Microbial analysis of supari, paan masala, and gutka samples.

Sample	Total Colony Count* (CFU/g)	<i>E. Coli</i> (CFU/g)	<i>Enterobacteriaceae</i> (CFU/g)‡	<i>Listeria sp.</i> (CFU/g)‡	<i>Salmonella sp.</i> (CFU/g) <sup>Δ</sup>	<i>Shigella sp.</i> (CFU/g) <sup>Δ</sup>	<i>Campylo-bacter sp.</i> (CFU/g) <sup>Δ</sup>	<i>Vibrio sp.</i> (CFU/g) <sup>Δ</sup>
S1	4.0 x 10 <sup>5</sup>	NG	NG	NG	NG	NG	NG	NG
S2	<1 x 10 <sup>6</sup>	NG	NG	NG	NG	NG	NG	NG
S3	2.0 x 10 <sup>4</sup>	NG	NG	NG	NG	NG	NG	NG
S4	<1 x 10 <sup>6</sup>	NG	NG	NG	NG	NG	NG	NG
PM1	<1 x 10 <sup>6</sup>	NG	NG	NG	NG	NG	NG	NG
PM2	<1 x 10 <sup>6</sup>	NG	NG	NG	NG	NG	NG	NG
PM3	<1 x 10 <sup>6</sup>	NG	NG	NG	NG	NG	NG	NG
PM4	6.7 x 10 <sup>9</sup>	NG	NG	NG	NG	NG	NG	NG
G1	6.0 x 10 <sup>6</sup>	NG	NG	NG	NG	NG	NG	NG
G2	2.0 x 10 <sup>3</sup>	NG	NG	NG	NG	NG	NG	NG
G3	6.0 x 10 <sup>5</sup>	NG	NG	NG	NG	NG	NG	NG
G4??	3.4 x 10 <sup>8</sup>	2.0 x 10 <sup>6</sup>	1.6 x 10 <sup>6</sup>	NG	NG	NG	NG	NG

\*Total (Aerobic) Colony Counts (to investigate spoilage) are regarded as "unsatisfactory" if >10<sup>7</sup> gram negative bacilli or >10<sup>8</sup> lactic acid bacteria

†Levels of *E. Coli* are regarded as "satisfactory" if colony counts are <20, "borderline" at 20 - <10<sup>2</sup>, and "unsatisfactory" if >10<sup>2</sup>

‡Levels of *Enterobacteriaceae* are regarded as "satisfactory" if colony counts are <10<sup>2</sup>, "borderline" at 10<sup>2</sup> - <10<sup>4</sup>, and "unsatisfactory" if >10<sup>4</sup>

#Levels of *Listeria sp.* are regarded as "satisfactory" if colony counts are <10, "borderline" at 10 - <10<sup>2</sup>, and "unsatisfactory" if >10<sup>2</sup>

ΔAny growth is considered an "unsatisfactory", no growth is a "satisfactory" result

\*\*Samples with an overall grade of "unsatisfactory" in the hygiene criteria<sup>25</sup>

NG: No Growth

**Table-3:** Total aflatoxin concentrations in studied chewing substances.

Sample ID	Aflatoxin concentration mg/kg
S1	1.35*
S2	1.25*
S3	1.4*
S4	1.35*
PM1	1.78*
PM2	1.34*
PM3	1.84*
PM4	1.74*
G1	0.93*
G2	1.21*
G3	1.18*
G4	0.43*

\*Samples with aflatoxin levels above permissible range as determined by European Food Safety Authority i.e. <8-10 μg/kg.<sup>38</sup>

x 10<sup>6</sup> CFU/g and 1.6 x 10<sup>6</sup> CFU/g respectively, and it did not meet the cut-off for the hygiene criteria. No sample had growth of pathogenic bacterial organisms and as such met the outlined safety criteria (Table-2).

The highest concentration of aflatoxin was found in PM3 at 1.84 mg/kg. The lowest concentration was in G4 at 0.43mg/kg (Table-3).

## Discussion

The only sample in the study (G4) that did not meet the cut-off for the hygiene criteria was a locally prepared commercial gutka sachet. Although presence of hygiene

indicator organisms in ready-to-eat food is not inherently a hazard, it can be indicative of poor practice, including poor quality of raw materials or food components, undercooking, cross-contamination, poor cleaning, and poor temperature and time control. Based on the culture results of these products, it can be concluded that although hygiene standards may be compromised, these products are less likely to cause gastrointestinal infections.

High levels of aflatoxin levels were detected in all the samples tested in the study. In a previous study, aflatoxin B1 levels in areca nut samples imported to Pakistan between 2010 and 2011 were measured, and contamination within the range of 11.7 to 262.0 μg/kg was identified.<sup>26</sup> The difference between these values and those identified in the current study may in part be due to different methods of analysis and preparations considered. Asghar et al.<sup>26</sup> used thin layer chromatography compared to ELISA, and used areca nut before it was incorporated into its final marketed mixture. Contamination by other constituents and handling of samples could also affect results. The high aflatoxin levels detected in the current study was despite little to no fungal growth on cultures. There are various possible explanations for this paradoxical finding. A possible factor is roasting of areca nut samples before being incorporated into the final commercial mixtures; while this would kill contaminating fungi, the preformed toxin is heat-resistant and would remain.<sup>27</sup> Dry food products

are regularly contaminated with *Aspergillus* during storage, leading to a build-up of aflatoxin within these products prior to consumption. Chronic aflatoxin exposure has long been known to lead to the development of hepatocellular carcinoma, and most likely accounts for a large percentage of hepatocellular carcinoma cases in the developing world.<sup>18</sup> It has also been suggested that aflatoxin also has a link with cancer of the gallbladder.<sup>28</sup> Furthermore, aflatoxin exposure may have an impact on malnourishment in children,<sup>29</sup> which is a formidable issue in developing nations such as Pakistan, and may also lead to adverse effects in pregnancy, such as low birth weight.<sup>30</sup> Exposure at high doses can lead to the development of aflatoxicosis, which leads to severe liver damage.

The antimicrobial effect of other ingredients often mixed with areca nut or areca nut itself may also play a role in the low fungal and microbial growth in some products. For example, calcium hydroxide, the main constituent of slaked lime, has been shown to have a wide range of antimicrobial activity due to its high potential of hydrogen (pH).<sup>31</sup>

This is a pilot study limited by a small sample size. While the results of this study cannot be generalised due to the sample size and limited sensitivity of culture methods,<sup>25,32</sup> the absence of pathogens does not render these items safe for consumption. The carcinogenic nature and contamination of the constituents can lead to cancer<sup>20</sup> and other chronic diseases.<sup>16</sup>

The ready availability of areca nut products and subsequent development of addiction is widespread. One of the most at-risk populations to whom various areca nut-containing products are often marketed is the paediatric population.<sup>21</sup> The addictive properties and low prices of these products ensure that once children begin consuming them, it is very difficult to stop. In a Karachi slum population, the main gutka users identified were adolescent, unmarried males.<sup>22</sup> A cross-sectional study found that 79.8% of school-going children in Karachi consume areca nut products on a regular or semi-regular basis. Of these, 40.8% self-reported as chewing these products regularly, while 39% reported chewing occasionally.<sup>33</sup> Although there is awareness of negative health outcomes associated with chewing, the habit continues to be pervasive. Almost all (~99%) school-going children interviewed in Mahmoodabad and Chanesar Goth, Karachi, knew that chewing supari had associated negative health effects, while 60% knew that paan masalas were harmful.<sup>34</sup> Psychosocial transmission of the paan-chewing habit also plays a role in development of a

chewing habit. Paan chewing by parents has shown to be significantly associated with eventual paan-chewing by their offspring<sup>35</sup> and 84% of school-children in Mahmoodabad and Chanesar Goth, reported that they used these substances in full knowledge of their families.<sup>34</sup>

Children should ideally be the focus of preventative measures as it is important to stop the development of a chewing habit in its early stages to prevent addiction and subsequent adverse health outcomes. As study similarly suggested educating the youth about the adverse effects associated with the use of mainpuri, as the average age for development of oral squamous cell cancer in mainpuri users is 16.4 years.<sup>36</sup> To address this major public health concern, innovative large-scale awareness campaigns and behaviour change interventions<sup>37</sup> are required across homes, schools and communities.

## Conclusion

While none of the tested samples of areca nut-containing chewing substances contained pathogenic bacteria, all samples had high levels of aflatoxin contamination. This pilot study needs to be extended to a larger sample size to evaluate the microbiological safety of other areca nut-containing products that are commonly used by children.

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