ORIGINAL ARTICLE

Assessment of pH and Copper Content among Raw and Commercial Areca Nut Products, Contributing Factor towards Oral Submucous Fibrosis

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ABSTRACT

Introduction: The increased prevalence of oral submucous fibrosis (OSMF) in the last few years relates to the increased consumption of areca nut(AN) products. OSMF is a premalignant condition and risk to progression to oral cancer is more when AN is chewed along with tobacco. Moreover, high copper content in AN is responsible for fibroblast dysfunction and fibrosis. This study was conducted with aim to assess and compare pH and copper content of raw AN and popular Indian commercial AN based (with and without tobacco) products. **Method:** Six samples each of twelve different brands of AN based commercial products i.e. six without tobacco (pan masala) and with tobacco were analyzed for pH and then the samples were dried, and powdered for estimation of the copper content. **Results:** For the six raw areca nuts (sample 1-6), the pH was found to range from 3.06±1.08 to 5.04±0.81, among the six non tobacco containing samples (sample 7-12), the pH was found to range from 6.03±1.08 to 9.09±0.81, and for six tobacco containing samples (sample 13-18), the pH was found to range from 9.18±0.90 to 11.07±0.09. The mean copper concentration among raw areca nut samples (sample 1-6) was 4.05±0.18 µg/g, among non-tobacco containing samples (sample 7-12) it was 10.17±1.08µg/g and among tobacco samples (sample 13-18),it was 18.09±1.08 µg/g ($p<0.001$). **Conclusion:** High copper content present in quid and commercial AN may be a causative factor for an increased fibrosis in OSMF, our findings need evaluation by further research and standardization.
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INTRODUCTION

Areca nut (AN) or betel nut use remains an integral part of the sociocultural life of Indians (1). AN contains 11 to 26% of tannins , 0.15 to 0.67% of alkaloids namely arecoline, arecaidine , guvacoline and guvacine which are potentially carcinogenic and contributes to substance abuse. It is the fourth most commonly used social drug, ranking next to tobacco, alcohol and caffeine and perhaps the second most consumed carcinogen after tobacco in India. Globally, 600 million people have been reported to consume AN, and around 10 to 20% of population worldwide use betel quid which is a preparation containing mixture of AN and slaked lime to which tobacco can be added, all wrapped in a betel leaf. India and South East Asian countries have been documented to be largest consumers of AN in the world. In India, AN products such as pan masala, gutka, mawa, etc are available in the market for commercial use (3). AN may be used fresh or it may be processed that is dried and cured before use. Commercial or processed AN contain wide variety of additives such as tobacco, spices, catechu, spices and sweeteners which vary according to local preferences. Supari is a packaged AN which is processed and flavoured (4). Pan masala is a mixture of betel leaf with lime, AN, cardamom, clove, mint, tobacco and other ingredients which vary based on the personal preference and geographical location. Mawa is a mixture of small pieces sun cured AN, crushed tobacco leaves and slaked lime. Gutka is a mixture of tobacco, crushed AN, pan masala, spices and other ingredients and is one of most commonly used AN product among youngsters who are fascinated by the colourful packages of gutka hanging in the shop outlets (4). It has been suggested that individuals who consume

AN with tobacco additives report more dependence symptoms when compared to only AN users i.e., without tobacco (5). AN form particularly gutka, pan masala, and Supari mix are also being promoted as a mouth freshener (3). Consumption of AN in India has been reported to be on increase since the last two decades in India due to easy availability of various commercial tobacco products. Secondly, as smoking tobacco has become a social nuisance, therefore most of the people have switched over to products such as pan-masala that contain AN (2).

The increased prevalence of premalignant condition, oral submucous fibrosis (OSMF) in the last few years has been attributed to the increased consumption of AN products. Malignant transformation of OSMF has been estimated to be 7 to 13%, and consumption of tobacco containing commercial AN preparations have been found to have more carcinogenic potential than raw and commercial AN without tobacco. Studies have demonstrated high copper levels in OSMF patients which may promote tissue fibrogenesis through copper dependent enzyme, lysyl oxidase (6). A significant amount of copper, around 302 nmol/g is present in the AN and substantial amount of copper is released into the saliva while chewing AN which is then absorbed into the oral mucosa. Copper is either bound to protein metallothionein or transferred across the basolateral membrane. Glycyl-L-histidyl-L-lysine (GHK), a tripeptide that has a high affinity for copper ions and forms a complex (GHK-Cu) that plays an important role in collagen synthesis, facilitate its crosslinking, and eventually inhibit its degradation (7). Another evidence suggests that trace elements such as copper released during AN chewing play a contributory role in progression of OSMF to oral cancer due to mutagenic effects of copper binding to DNA resulting in p53 tumour suppressor gene aberrations in oral keratinocytes (8). Therefore, the present study was conducted with an aim to determine pH and copper concentrations among raw AN and twelve popular Indian commercial AN based product.

MATERIALS AND METHODS

Raw mature AN obtained from three plantations of Dakshina Kannada district, Karnataka, India, which are used in the commercial preparations were dehusked and assessed. The study included a total of n = 18 samples, the calculated power of the study was 80%, six samples each of twelve different brands of AN were randomly divided by lottery method into three groups- raw AN(A), commercial (processed) AN preparations without tobacco(B) and commercial AN preparations with tobacco(C). They were purchased at local retail outlets in Bengaluru city, India , were personally collected from the shops due to the absence of a national level manufacturer and assessed. The samples were selected on the basis of popularity among the local people representing a large and uniform sample pool and were assigned unique identity numbers. Some of the common smokeless tobacco products (SLT) like Gutkha, Manipuri tobacco, khaini, snus, and mawa available in South Indian market, containing differing compositions of areca nut, and slaked lime besides tobacco were evaluated. Samples of the same batch numbers were mixed together to obtain a representative sample of that product. The ethical clearance was obtained from the institutional ethical committee (Ethical Clearance Approval Number: FDS/EC/2014-2016/PGST, dated: 25.08.2016). All procedures followed were carried out according to principles of Helsinki Declaration of 1964 and later versions.

For pH assessment, samples of each group were extracted with 20 ml distilled water using a mechanical shaker. The clear supernatant was used for pH analysis by laboratory grade pH meter (Systronics Digital pH Meter MK-VI) and the results were expressed as mean pH±SD. Measurements for pH to a precision of at least two decimal places were done at room temperature. Then the samples were dried, powdered and sent to Ramaiah Drug testing laboratory, for estimation of the copper content using atomic absorption spectrometry (9)

The data was statistically analysed using Statistical Package for Social Sciences (SPSS) version 20 (IBM SPSS Statistics Inc., Chicago, Illinois, USA) Windows software program. Descriptive statistics that include mean and standard deviation of parameters was used and paired sample t-test was applied to calculate difference between the mean of the parameters assessed in this study. $p \leq 0.05$ was considered as statistically significant.

RESULTS

18 samples were divided into three groups- raw AN(A), commercial AN preparation without tobacco(B) and commercial AN preparation with tobacco(C). Among the six raw AN (sample 1-6), the pH was found to range from 3.06±1.08 to 5.04±0.81 with a mean pH of 4.05. Among the six commercial AN samples without tobacco (sample 7-12), the pH was found to range from 6.03±1.08 to 9.09±0.81 with a mean pH of 7.02. Sample 2 had the highest value of 9.09 and sample 5 gave the lowest value of 6.03. Among the six tobacco containing commercial AN samples (sample 13-18), the pH was found to range from 9.18±0.90 to 11.07±0.09 with a mean pH of 10.08. Sample 9 had the highest value of 11.07 and sample 7 gave the lowest value of 9.18. The mean concentration of triplicate readings of copper in all 18 samples is expressed in µg/g along with the corresponding standard deviation (SD). The mean copper concentration among raw AN samples (sample 1-6) was 4.05±0.18 µg/g, among non-tobacco containing commercial AN samples (sample 7-12) it was 10.17±1.08µg/g. and among tobacco containing commercial AN samples (sample 13-18), it was

18.09±1.08 µg/g. Statistically significant difference was observed in the copper content of raw and commercial areca nut products (P< 0.001). Table I shows pH and copper content values among commercial and raw AN product.

Table I: showing pH values and copper content among raw areca nut and commercial areca nut products

Sample ID	pH (Mean ±SD)	Copper (µg/g)
Raw areca nut samples		
A1	4.15±1.08	3.70±0.18 µg/g
A2	5.04±0.81	4.17±0.09 µg/g
A3	4.05±0.18	4.70±1.08 µg/g
A4	4.05±0.72	4.14±1.17 µg/g
A5	3.06±1.08	4.05±1.26 µg/g
A6	5.04±0.09	3.15±1.08 µg/g
Non tobacco containing commercial areca nut products		
B7	7.02±1.08	10.08±1.17 µg/g
B8	9.09±0.81	10.17±0.09 µg/g
B9	6.03±0.18	9.09±1.08 µg/g
B10	7.02±0.72	11.07±1.17 µg/g
B11	6.03±1.08	9.05±1.26 µg/g
B12	7.03±0.09	10.26±1.08 µg/g
Tobacco containing commercial areca nut products		
C13	9.18±0.90	18.09±1.08 µg/g
C14	10.08±0.81	18.01±0.18 µg/g
C15	11.07±0.90	18.09±0.09 µg/g
C16	10.17±0.81	17.10±0.27 µg/g
C17	10.08±0.18	18.01±0.09 µg/g
C18	10.08±0.81	17.01±0.36 µg/g

DISCUSSION

pH of AN and its products has been reported to increase the addictiveness of the substance. Among tobacco containing products, the bioavailability of nicotine the addictive chemical is increased by adding alkalizing ingredients such as ammonium compounds (particularly diammonium phosphate and ammonium hydroxide) which increase the pH of tobacco. At alkaline (high) pH (pH>8.0) nicotine is unionized and rapidly absorbed across the biological membranes when compared to an acidic (low) pH (10). The pH of SLT products varies; it is highest for tobacco-based products and lowest for plain raw areca nut. In the present study pH varied between 3.06 to 11.07, lowest for plain AN with a mean pH of 4.05, a mean pH of 7.02 among non - tobacco containing AN products and a mean pH of 10.08 among tobacco containing AN based commercial products. Different manufacturing methods, additives used and moisture contamination has been found to be responsible for difference in pH among SLT products. Addition of tobacco additives such as ammonium compounds, diammonium phosphate and ammonium hydroxide to AN preparation has been found to increase the alkalinity or pH of AN, thereby increasing the rate of absorption of nicotine through oral mucosa. Though other factors also affect the rate of nicotine absorption, manipulating pH has been studied to be a primary determinant of speed of nicotine absorption in the oral cavity (11,12). In the present study it was observed that tobacco containing commercial AN preparations had higher pH value, and increase in pH was associated with higher copper content in the product. Furthermore, there is increase in the production of reactive oxygen species (ROS) such as superoxide, hydroxyl and hydrogen peroxide free radicals in the alkaline pH of the saliva (13). Another study by Nair et al concluded that another component slaked lime when added to AN increase the pH in the oral cavity, and leads to generation of ROS (14). ROS have the potential to cause oxidative DNA and chromosomal damage in the buccal mucosal cells of habitual users of AN preparation especially at the sites where the betel quid is placed (14,15). ROS have been implicated as a causative factor in the genesis of oral submucous fibrosis and oral cancer. Studies have observed increase in salivary pH levels of AN chewers and OSMF patients and production of ROS is increased in the presence of alkaline salivary pH (10). Chang et al stated that AN extracts or ingredients exert cytotoxic and genotoxic effects on different types of cells. They reported that AN extract-induced unscheduled deoxyribonucleic acid synthesis of gingival keratinocytes due to free radical reactions. Hence Vitamin C , glutathione , and N-acetyl-L-cysteine have an important role in chemoprevention of betel quid induced oral mucosal lesions (16).

According to Food and Agriculture Organization of the United Nations (FAO) and WHO, recommended daily intake of copper is 0.5 mg/kg bw/day, i.e., 500 µg/kg body weight (17). As copper content in AN has been reported to be much higher than that of most other nuts consumed by humans and is responsible for fibrogenic, mutagenic and toxic effects of AN (3,7). To this consideration, copper content in raw and processed AN was assessed in the present study. Variations in the copper concentrations in AN has been attributed to the stages of maturity, and cultivation in variable climatic conditions and soil in different geographical locations (3). Alexander et al in their study found that most of the AN plantation in South India use copper-based fungicide, bordeaux mixture (BM) which increases the levels of copper in the AN (3). In the present study, the mean copper content among mature raw AN was 4.05±0.18 µg/g. It is found that as the nut matures, the moisture content decreases and the copper content increases as it mineralizes. Unripe nuts with high moisture content had lower copper levels when compared to the exfoliated mature nuts (18, 19). Trivedy et al , Mathew et al, and Shakyaet al found that copper content increased significantly in commercial products than the raw AN (8, 11, 18).

Tobacco added to the quid mixture is found to rise copper levels manifold. Studies have documented that trace metals like zinc, copper , and iron have a major role towards initiation of OSMF (19,20,21). Heavy metals get in cooperated in SLT by use of pesticides,

insecticides, and soil fertilizers. In addition, it has been revealed that uptake of metals like cadmium and lead by tobacco plants is a result of atmospheric pollutants and addition of various taste enhancing and flavouring ingredients such as mint, saffron, etc., might raise the heavy metal content in tobacco products (21,22). Furthermore, during the processing of AN for commercial purpose, AN becomes more concentrated with mineral and chemical constituents as the moisture content is reduced by drying or roasting which further causes volumetric shrinkage of AN. As a result, individuals will be consuming commercial AN which is high in copper content when compared to raw AN. As a result, copper is released in saliva and absorbed by the oral mucosa during chewing eventually resulting in OSMF (4, 22,23).

Raja et al observed that 50 % of patients with OSMF had basal salivary copper concentrations higher than normal subjects, and buccal absorption of soluble copper may have a contributory role in development of OSMF in Asians who have a habit of chewing AN regularly (24). Similarly Mohammed et al in their study found higher mean salivary copper levels of 27.023 µg/dl in OSMF patients when compared to 8.393 µg/dl for non-OSMF individuals ($p < 0.005$) (25). Trivedy et al suggested copper as an initiating factor of OSMF as it promotes increased fibrogenesis or collagen formation by upregulation of lysyl oxidase activity (8). Moreover, high levels of copper in saliva are mutagenic for oral epithelial cells thereby increasing the malignant transformation rate of OSMF (8). Cheng et al reported the role of genetic factors in the development of OSMF. They suggested that betel quid induced changes in the extracellular matrix through upregulation of transforming growth factor (TGF-β1), plasminogen activator inhibitor-1 (PAI-1), cystatin, LOX , tissue inhibitors of metalloproteinases (TIMPs) and metalloproteinases (MMPs). Interactions of genetic and environmental factors like betel quid, tobacco , alcohol ,etc , along with other factors such as production of ROS, mucosal trauma, cytotoxicity and genotoxicity result in epithelial and fibroblastic changes thereby contributing towards development of OSMF (26) .

Literature revealed studies which have reported the association between serum copper concentrations and risk of metabolic syndromes which include abdominal obesity, dyslipidemia, glucose intolerance and hypertension (27,28,29). Chen et al suggested that excess copper intake may induce oxidative damage and has an adverse effect on the immune system by decreasing the number and percentage of circulating neutrophils. In their study it was observed that serum copper was positively associated with triglycerides ($p = 0.01$), total cholesterol ($p = 0.002$), low density lipoprotein cholesterol ($P \leq 0.001$), and lipoprotein (a) ($p = 0.01$), and was also positively associated with heme oxygenase-1 ($p = 0.03$) and negatively associated with monocyte chemotactic protein (MCP)-1 ($p = 0.003$) and interleukin (IL)-8 ($P = 0.03$). The findings suggested that high copper intake by women results in increase of serum lipid levels and oxidative stress biomarkers, and decrease in inflammatory responses (27). In another study, Tinkov et al suggested that higher levels of serum copper and iron levels may cause obesity related disturbances such as high blood pressure, higher body fat percentage, serum triglyceride levels and insulin resistance (28). Lin et al stated that betel nut chewers with both tobacco/alcohol use have a high risk of metabolic syndrome, and they found serum triglycerides levels >150 mg/dL in the chewers with both tobacco/alcohol use. The findings suggested that tobacco smoking along with betel nut chewing has synergistic effect on increasing serum triglyceride levels (28). Similarly in the present study commercial areca nut products containing tobacco increase the copper levels in saliva during chewing, and high levels of copper plays an important contributory role in upregulation of collagen synthesis leading to oral submucous fibrosis.

Smaller sample size is the limitation of the present study, that provides an impetus to conduct further research with a much larger sample size on SLT products from different geographical locations, as well as should focus more on the carcinogenic nitrosamines and mutagens present in tobacco products.

CONCLUSION

Our analysis suggests that when compared to raw AN, commercial AN preparation has high copper content and has more potential to increase pH levels in the oral cavity, and its subsequent absorption by the oral mucosa on chewing may be responsible for inducing the submucous fibrosis. However, the maximum permissible copper content in commercial and raw AN product needs to be further validated by more experimental studies.

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