Effect of copper-based fungicide (bordeaux mixture) spray on the total copper content of areca nut: Implications in increasing prevalence of oral submucous fibrosis

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Abstract

**Background:** Potentially malignant disorders like oral submucous fibrosis (OSMF) often precede oral squamous cell carcinoma (OSCC). The rate of transformation of OSMF to OSCC ranges from 3 to 19%. OSMF is etiologically related to chewing of areca nut (betel nut), and the high copper content in areca nut plays an important role in the pathogenesis of the disorder. Even though many studies estimated and confirmed increased copper levels in areca nuts, studies tracing the source of the increased copper content are scarce. Interestingly, on review of agricultural literature, it was found that most of the areca nut plantations in South India commonly use a copper-based fungicide, bordeaux mixture (BM). **Aim:** The aim of the study was to estimate and compare the copper content in areca nuts from plantations with and without copper-based fungicide usage. **Materials and Methods:** Four areca nut plantations from Dakshina Kannada district, Karnataka (group A) and four plantations from Ernakulam district, Kerala (group B) were selected for the study. The plantations from Karnataka used copper-based fungicide regularly, whereas the latter were devoid of it. Areca nut samples of three different maturities (unripe, ripe, and exfoliated) obtained from all plantations were dehusked, ground, and subjected to atomic absorption spectrometry (AAS) for copper analysis. **Results:** There was statistically significant difference in the copper content of areca nuts from both groups. The areca nuts from plantations treated with copper-based fungicide showed significantly higher copper levels in all maturity levels compared to their counterparts in the other group (P < 0.05). **Conclusions:** The high copper content in areca nut may be related to the copper-based fungicide treatment on the palms. These areca nuts with high copper content used in quid or commercial products may be responsible for the increasing prevalence of OSMF.

**Key words:** Areca nut, bordeaux mixture, copper, fungicide, oral submucous fibrosis

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common cancer of the oral cavity and represents about 90% of all oral malignancies.[¹] Several factors like tobacco consumption, areca nut chewing, alcohol

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intake, genetic predisposition, and hormonal factors are suspected as possible risk factors. In southeast Asia, because of cultural, ethnic, and geographic factors, and the prevalence of addictive habits, the frequency of OSCC is high. In India, oral cancer is the most prevalent cancer in males and the third most cancer prevalent in females, indicating that it is a major health problem constituting up to 40% of all cancers.\(^2\)

OSCC is usually preceded by certain warning lesions categorized as potentially malignant disorders (PMD). Oral submucous fibrosis (OSMF) is one of the established PMD found in oral mucosa.\(^3\) The malignant transformation of OSMF ranges from 3 to 19%.\(^4\) OSMF is causally related to chewing of areca nut (betel nut), a habit prevalent among the population groups in southeast Asia.\(^5\) Areca nut is the seed of the fruit of the oriental palm *Areca catechu*. Areca nut palm is a long, slender, single-trunked tree which can grow up to a height of 15 m. The nut is the seed (endosperm) found within the fruit and is mottled gray to brown in color with white markings. The outer surface of the fruit is green in color when it is unripe, which changes to orange-yellow as it ripens.\(^5\) Areca nut has been chewed for hundreds of years and is currently being used by 10% of the world’s population.\(^6\) With the introduction of various commercial areca nut products with or without tobacco in the market, the raw areca nut chewing habit has decreased. Importantly, the increase in prevalence of OSMF in the last two decades or so corresponds to the increased processing and commercialization of areca products since the early 1980s.\(^7\)

Recent evidence suggests that the high copper content in areca nut play a key role in the pathogenesis of OSMF.\(^8,9\) On chewing raw areca nut or its commercial products, significant amounts of soluble copper is released into saliva, which is absorbed into the oral mucosa.\(^10\) Copper gains entry into the connective tissue and acts by upregulating lysyl oxidase (LOX) activity, which is the key enzyme in the collagen metabolic pathway. Thus, increased level of soluble copper could act as an important risk factor in OSMF as it stimulates fibrogenesis through enhanced LOX activity. Furthermore, copper-dependent LOX facilitates cross-linking of collagen and, eventually, makes it resistant to proteolysis.\(^11,12\) Several authors have reported increased serum and tissue copper levels in OSMF patients.\(^8,9,11\)

The high copper content in areca nut has been reported by several studies.\(^10,13,14\) In our previous study, we observed increase in copper content as the areca nut matures.\(^15\) However, studies tracing the source of the increased copper content are scarce in the literature. Interestingly, on review of literature, it was found that the areca nut plantations in South India commonly use a copper-based fungicide, bordeaux mixture (BM), on areca nut palms.\(^16-18\) Copper sulfate is the main ingredient in BM.\(^17,18\) This prompted us to consider the possibility of copper from BM gaining entry into the areca nut. Hence, strongly suspecting exogenous copper entering the nut, we planned to estimate the copper levels in areca nut from plantations with and without BM application.

**MATERIALS AND METHODS**

Four areca nut plantations from Dakshina Kannada district, Karnataka with 20 years history of BM application were selected in one group (group A). Four plantations without BM usage for the last 25 years were identified in Ernakulam district, Kerala and constituted the other group (group B) in the study. Areca nuts were obtained from each plantation and were categorized depending on the degree of maturity into unripe (green), ripe (yellow-orange), and exfoliated mature nuts [Figures 1–3]. The unripe and ripe areca nuts were plucked from the palms, whereas the exfoliated ones were collected from the surroundings.

All areca nuts were dehusked, and the nut was ground and homogenized using a mortar and pestle and was stored in clean polythene bags. Test portions were dried and then ashed at 450°C, with initial temperature not higher than 100°C which was gradually increased (≤50°C/h). Then 5 ml of 6 M hydrochloric acid (HCl) was added to the ash and the solution was evaporated to dryness. The residue was dissolved in 0.1 M nitric acid (HNO3) and the resulting solution was analyzed using flame atomic absorption spectrometry (FAAS). The spectrophotometer was
standardized using the standard solution of the element being analyzed and acidified deionized water was aspirated to zero the instrument. Acetylene flame was used and sample extracts were aspirated. The absorption of radiation by copper from the sample solutions was measured in wavelengths and its concentration was read off by the instrument. The obtained results were expressed in mg/kg.

The data obtained were subjected to statistical analysis and basic variation statistical values (arithmetic mean, standard deviation, standard error, maximum and minimum values) were calculated. The differences between two groups were determined by independent sample t-test and values were compared at 0.05% significance level. Plantation-wise comparison within both groups was carried out by one-way analysis of variance, and difference between three maturity levels was checked by paired sample t-test.

RESULTS

The copper content was determined in the 72 samples obtained from eight plantations. The average copper content of areca nuts at three maturity levels from the plantations of two groups is shown in Table 1. This analysis has yielded very useful information regarding the copper content in areca nuts from plantations with and without BM application.

- Average copper content in both groups: The average copper content of areca nuts at three maturity levels from the plantations of two groups is shown in Table 1. Among the plantations with BM application (group A), copper content was least in the unripe areca nut (3.64 ± 0.84 mg/kg) and highest in the exfoliated mature areca nut (8.77 ± 0.65 mg/kg). The plantations without BM usage (group B) also showed similar pattern with the lowest copper content in unripe nuts (2.32 ± 0.48 mg/kg) and the highest in exfoliated mature areca nuts (4.74 ± 0.77 mg/kg). Ripened areca nut showed intermediate values in both groups (7.84 ± 0.81 mg/kg) and (3.54 ± 0.89 mg/kg), respectively [Table 1].
- Difference in copper content at three levels of maturity: Copper content of areca nuts increased with maturity in both groups. Also, the difference in copper content among the three stages of maturity was statistically significant in group A and B (P < 0.05) [Tables 2 and 3].
- Difference in copper content between the two groups: Importantly, the copper levels in areca nuts from the plantations with BM application were significantly higher than the ones from the other group. Moreover, the difference in copper content was statistically significant in areca nuts of all three
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**Table 2: Paired samples test - group A**

| Paired differences | Mean | Standard deviation | Standard error mean | 95% confidence interval of the difference | t | df | Sig. (2-tailed) |
|-------------------|------|--------------------|---------------------|----------------------------------------|---|----|----------------|
|                  |      |                    |                     | Lower | Upper |
| Pair 1            |      |                    |                     |       |       |
| Unripe-ripe       | −4.199 | 0.441              | 0.127              | −4.480 | −3.919 | −32.963 | 11 | 0.000 |
| Pair 2            |      |                    |                     |       |       |
| Unripe-exfoliated | −5.126 | 0.818              | 0.236              | −5.645 | −4.606 | −21.711 | 11 | 0.000 |
| Pair 3            |      |                    |                     |       |       |
| Ripe-exfoliated   | −0.927 | 0.655              | 0.189              | −1.343 | −0.511 | −4.904  | 11 | 0.000 |

**Table 3: Paired samples test - group B**

| Paired differences | Mean | Standard deviation | Standard error mean | 95% confidence interval of the difference | t | df | Sig. (2-tailed) |
|-------------------|------|--------------------|---------------------|----------------------------------------|---|----|----------------|
|                  |      |                    |                     | Lower | Upper |
| Pair 1            |      |                    |                     |       |       |
| Unripe-ripe       | −1.219 | 0.643              | 0.186              | −1.627 | −0.811 | −6.572  | 11 | 0.000 |
| Pair 2            |      |                    |                     |       |       |
| Unripe-exfoliated | −2.425 | 0.462              | 0.133              | −2.719 | −2.131 | −18.173 | 11 | 0.000 |
| Pair 3            |      |                    |                     |       |       |
| Ripe-exfoliated   | −1.206 | 0.408              | 0.118              | −1.465 | −0.946 | −10.229 | 11 | 0.000 |

**Table 4: Comparisons between two groups - Student’s t-test**

| Levene’s test for equality of variances | t | df | Sig. (2-tailed)* | Mean difference | Standard error difference | 95% confidence interval of the difference | Lower | Upper |
|----------------------------------------|---|----|-----------------|-----------------|--------------------------|----------------------------------------|-------|-------|
| Unripe                                 | 9.445 | 0.006 | 4.755 | 22 | 0.000 | 1.323 | 0.278 | 0.746 | 1.899 |
| Equal variances assumed                | 4.755 | 17.486 | 0.000 | 1.323 | 0.278 | 0.737 | 1.908 |
| Equal variances not assumed            | 0.394 | 0.537 | 12.448 | 22 | 0.000 | 4.303 | 0.346 | 3.586 | 5.019 |
| Ripe                                  | 12.448 | 21.811 | 0.000 | 4.303 | 0.346 | 3.585 | 5.020 |
| Equal variances assumed                | 0.016 | 0.902 | 13.826 | 22 | 0.000 | 4.023 | 0.291 | 3.420 | 4.627 |
| Equal variances not assumed            | 13.826 | 21.334 | 0.000 | 4.023 | 0.291 | 3.419 | 4.628 |

*The mean difference is significant at the 0.05 level

maturity levels (P < 0.05) [Table 4].

- **Difference in copper content among plantations within groups:** On plantation-wise comparison within groups, all four plantations of group A had similar copper levels. All four plantations of group B also showed uniform copper levels, even though the copper levels in this group were comparatively less.

Thus, within the groups, there was no statistically significant difference in copper content between plantations (P > 0.05) [Tables 5 and 6].

**DISCUSSION**

Fruit rot disease (Koleroga or Mahali) is a common...
In our study, copper content was found to increase significantly with maturity in both groups. Jayalakshmi and Mathew\cite{20} have mentioned about the difference in moisture content in the unripe and ripe areca nuts. According to their observation, the moisture content in unripe areca nut was about 69.4–74.1% and that of ripe ones was 38.9–56.7%, suggesting that the moisture content was getting reduced as the nut matured. In our study, the unripe nuts which are rich in moisture content had the least copper levels, whereas the exfoliated mature nuts with relatively less moisture content showed higher values of copper for a given weight.

Importantly, in the present study, the copper content in areca nuts from plantations with BM application was significantly higher than from the plantations without fungicide treatment. There was statistically significant difference in the copper content of areca nuts of all three maturity levels with the corresponding ones from the other group. A study by Aikpokpodion et al.\cite{21} in cocoa plantations concluded that copper fungicides applied on cocoa pods contribute to the total copper residue in cocoa beans. A study by Nzegbule\cite{22} which estimated the copper content in cocoa seed of plants with BM treatment concluded that there is an 87% increase in the copper content of cocoa seeds from those plantations. Similarly, a study by Jung\cite{23} which evaluated the effect of BM spraying on grape plants also showed elevated copper levels in the grapes. These three studies were in agreement with our finding of increased copper content in nuts from plantations with BM application.

Spraying of BM on areca bunches and tender nuts may enable soluble copper to permeate the cuticle and gain access into the areca nut. Copper from soil is another possible route through which it can gain entrance into areca nut. It is noteworthy that soil of plantations with BM application will have high residual copper content.\cite{24} When a soil is rich in bio-available copper, it is mostly transported via the plant root and there is a tendency of its accumulation in the nuts.\cite{25} In the present study, there was no significant difference in the copper content among plantations within groups A and B. Uniformity of the copper levels within both groups suggested that the plantations of each group were homogenous in nature. Also, the plantations within both groups were exposed to similar natural geographic features, except for BM spraying. The weather and rainfall pattern in the two groups were similar. Hence, it is logical to assume that BM spraying is predominantly responsible for the increased copper levels, rather than other environmental factors.

### Table 5: Comparisons between plantations in group A - one-way ANOVA

|             | Sum of squares | df | Mean square | F     | Sig. |
|-------------|----------------|----|------------|-------|------|
| Unripe      |                |     |            |       |      |
| Between plantations | 2.755          | 3  | 0.918      | 1.486 | 0.290|
| Within all plantations | 4.943          | 8  | 0.618      |       |      |
| Total       | 7.698          | 11 |            |       |      |
| Ripe        |                |     |            |       |      |
| Between plantations | 3.224          | 3  | 1.075      | 2.190 | 0.167|
| Within all plantations | 3.926          | 8  | 0.491      |       |      |
| Total       | 7.150          | 11 |            |       |      |
| Exfoliated  |                |     |            |       |      |
| Between plantations | 1.978          | 3  | 0.659      | 2.011 | 0.191|
| Within all plantations | 2.623          | 8  | 0.328      |       |      |
| Total       | 4.601          | 11 |            |       |      |

### Table 6: Comparisons between plantations in group B - one-way ANOVA

|             | Sum of squares | df | Mean square | F     | Sig. |
|-------------|----------------|----|------------|-------|------|
| Unripe      |                |     |            |       |      |
| Between plantations | 1.193          | 3  | 0.398      | 2.415 | 0.142|
| Within all plantations | 1.318          | 8  | 0.165      |       |      |
| Total       | 2.511          | 11 |            |       |      |
| Ripe        |                |     |            |       |      |
| Between plantations | 2.688          | 3  | 0.896      | 1.208 | 0.367|
| Within all plantations | 5.931          | 8  | 0.741      |       |      |
| Total       | 8.619          | 11 |            |       |      |
| Exfoliated  |                |     |            |       |      |
| Between plantations | 2.803          | 3  | 0.934      | 1.981 | 0.196|
| Within all plantations | 3.774          | 8  | 0.472      |       |      |
| Total       | 6.577          | 11 |            |       |      |

fungal infection seen in areca nut palms, which is characterized by rotting and extensive shedding of the immature nuts. The fungus causing fruit rot of areca nut is Phytophthora arecae.\cite{16-18} BM (1%) is very effective in controlling this fungal infection in plantations.\cite{17,18} It is prepared by mixing copper sulfate, calcium hydroxide (slaked lime), and water at a proportion of 1:1:100.\cite{19} The freshly prepared fungicide is sprayed on all areca bunches and tender nuts three times in a year, once just before the onset of southwest monsoon and the rest at 40 days intervals.\cite{19}

The chemical reaction involved in BM is

\[ \text{CuSO}_4 + \text{Ca(OH)}_2 \rightarrow \text{Cu(OH)}_2 + \text{CaSO}_4 \]

Subsequent to spraying the mixture, the resultant copper hydroxide of the reaction yields soluble copper. The fungicidal action of the mixture is believed to be due to the toxicity of soluble copper on the fungal spores.\cite{19}
It is quite clear from the above observations that the copper-based fungicide (BM) plays an important role in the total copper content of the areca nut. Even though the usage of BM in areca nut plantations dates back to late 1920s, widespread usage with appropriate spraying equipment was initiated in 1970s. This fact is not documented in agriculture literature. We got this crucial information on interviewing some senior areca nut farmers during the process of sample collection. This suggests that the BM-induced increase in copper content of areca nuts corresponds to that time period. Importantly, commercial areca nut products industry saw a sharp growth from 1980s, especially due to widespread advertisements and advances in packaging. These small sachets of products also increased their transportability and decreased the cost of single purchase.\(^{[25]}\)

Most of the plantations in South India use BM, and hence, majority of the raw areca nuts available for the preparation of commercial products are rich in copper. Understandably, the highly concentrated commercial areca nuts will have still higher copper content, as we reported in our previous study.\(^{[19]}\) Thus, exogenous copper from BM and increased processing could synergistically increase the total copper content of the commercial areca nut products and this high copper concentration may be related to the increasing prevalence of OSMF.

CONCLUSION

By adopting environment-friendly copper-free fungicidal alternatives, areca nut with lesser copper content may be produced. The maximum permissible copper content for raw areca nut used in commercial products has to be evaluated and curtailed by further research and standardization. Further studies including more plantations from different locations and additional parameters like copper content in soil are also recommended.

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Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Lawoyin JO, Lawoyin DO, Aderinokun G. Intra-oral squamous cell carcinoma in Ibadan: A review of 90 cases. Afr J Med Med Sci 1997;26:187-8.
2. Elango JK, Gangadharan P, Sumithra S, Kurikose MA. Trends of head and neck cancers in urban and rural India. Asian Pac J Cancer Prev 2006;7:108-12.
3. Meghji S, Warnakulasuriya S. Oral submucous fibrosis: An expert symposium. Oral Dis 1997;3:276-91.
4. Murty PR, Bhonsle RK, Pindborg JJ, Daftary DK, Gupta PC, Mehta FS. Malignant transformation rate in oral submucous fibrosis over a 17-year period. Community Dent Oral Epidemiol 1985;13:340-1.
5. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Betel-quad and Areca-nut Chewing and Some Areca-nut-derived Nitrosamines. Lyon, France: IARC Scientific Publications; 2004.
6. Boucher BJ. Pan without tobacco: An independent risk factor for oral cancer. Int J Cancer 2001;91:592-3.
7. Gupta PC, Sinor PN, Bhonsle RB, Pawar VS, Mehta HC. Oral submucous fibrosis in India: A new epidemic? Natl Med J India 1998;11:113-6.
8. Trivedy CR, Warnakulasuriya KA, Peters TJ, Senkus R, Hazarey VK, Johnson NW. Raised tissue copper levels in oral submucous fibrosis. J Oral Pathol Med 2000;29:241-8.
9. Trivedy C, Meghji S, Warnakulasuriya KA, Johnson NW, Harris M. Copper stimulates human oral fibroblasts in vitro: A role in the pathogenesis of oral submucous fibrosis. J Oral Pathol Med 2001;30:465-70.
10. Trivedy C, Baldwin D, Warnakulasuriya S, Johnson N, Peters T. Copper content in Areca catechu (betel nut) products and oral submucous fibrosis. Lancet 1997;349:1447.
11. Trivedy C, Warnakulasuriya KA, Hazarey VK, Tavassoli M, Sommer P, Johnson NW. The upregulation of lysyl oxidase in oral submucous fibrosis and squamous cell carcinoma. J Oral Pathol Med 1999;28:246-51.
12. Rajalatha P, Vali S. Molecular pathogenesis of oral submucous fibrosis - a collagen metabolic disorder. J Oral Pathol Med 2005;34:321-8.
13. Trivedy C, Warnakulasuriya S, Peters TJ. Areca nuts can have deleterious effects. BMJ 1999;318:1287.
14. Gopalani C, Rama Satari BV, Balasubramanian SC. Nutritive Value of Indian Foods. National Institute of Nutrition. Revised ed. Hyderabad, India: Indian Council of Medical Research; 1989.
15. Mathew P, Austin BD, Varghese SS, Manojkumar. Estimation and comparison of copper content in raw areca nuts and commercial areca nut products: Implications in increasing prevalence of oral submucous fibrosis (OSMF). J Clin Diag Res 2014;8:247-9.
16. Saxty MN, Hegde RK. Control of fruit rot or koleroga disease of arecanut (areca catechu L). Tropical Agriculture 1988;65:150-2.
17. Nayaka S, Singh PK, Upreti DK. Fungicidal elements accumulated in Cryptothecia punctulata (Ascomycetes lichen) of an arecanut orchard in South India. J Environ Biol 2005;26:299-300.
18. Kurian A, Peter KV. Commercial Crops Technology. Vol. 8. Horticulture Science Series. New Delhi, India: New India Publishing Agency; 2007.
19. Rangaswami G, Mahadevan A. Disease of Crop Plants in India. 4th ed. New Delhi, India: Prentice-Hall of India Private Limited; 2006.
20. Jayalakshmi A, Mathew AG. Chemical Composition and Processing. In: Bavappa KV, Nair MK, Kumar TP, editors. The Arecaanut Palm, Kerala: Central Plantation Crops Research Institute 1982. p. 225-44.
Mathew, et al.: Effect of copper-based fungicide spray on the total copper content of arecanut

21. Aikpokpodion PE, Lajide I, Aiyesanmi AF. Impacts of Cu-Based Fungicide on Copper Residue and Mineral Elements Distribution in Cocoa Beans and Pods. World J Agric Sci 2013;9:10-6.

22. Nzegbule EC. Assessment of copper levels in the soil and vegetation following repeated application of Bordeaux mixture to a cocoa plantation in south eastern Nigeria. Niger Agric J 2003;34:97-102.

23. Jung SM, Nam JC, Huh YY, Roh JH, Park KS, Lim TJ. Effect of bordeaux mixture spray on fruit quality of ‘kyoho’ grape and copper accumulation in the soil. Acta Hort (ISHS) 2013;1:259-63.

24. Aikpokpodion PE. Assessment of heavy metal pollution in fungicide treated cocoa plantations in Ondo State, Nigeria. J Appl Biosci 2010;33:2037-46.

25. Sushma C, Sharang C. Pan masala advertisements are surrogate for tobacco products. Indian J Cancer 2005;42:94-8.