 http://doi.org/10.18231/j.jooo.2019.014

# Copper containing fungicide – the actual culprit in the pathogenesis of OSMF?

**Anjana Mohan Kumar[1*], Sravani Kota[2], L.K. Chatra[3], Veena KM[4], Prasanna Kumar Rao[5]**

[1]Senior Lecturer, [2]Post Graduate, [3,5]Professor and HOD, [4]Professor, Dept. of Oral Medicine and Radiology, [1]Al Azhar Dental College, Thodupuzha, Kerala, [2-4]Yenepoya Dental College, Yenepoya University, Mangalore, Karnataka, [5]AJ Institute of Dental Science, Mangalore, Karnataka, India

***Corresponding Author: Anjana Mohan Kumar**
Email: anjanamohan99@gmail.com

## Abstract

**Introduction:** To estimate possible role of copper in etiology of oral sub mucus fibrosis.

**Aims and Objectives:** To analyse and compare metal constituents of arecanuts sprayed with copper containing fungicide and that not sprayed with fungicide. To evaluate the effects of arecanuts sprayed with copper containing fungicide on the buccal mucosa of rats.

**Materials and Methods:** Aquous solutions of arecanuts sprayed and not sprayed with fungicide, were prepared and sent to NITK for metal analyses. Buccal mucosa of 6 adult Wistar albino rats, were treated twice daily, for 6 days a week, with topical application of aqueous areca nut extracts, for one year. A control group (n = 6) was treated with distilled water. Biopsy was conducted at the end of six, nine and twelve months. The influence of areca nut on the oral epithelium and connective tissue of the rats were recorded semi quantitatively by light microscopy.

**Results:** Metal analysis showed that the difference of copper content of arecanut treated with fungicide when compared to that of those not treated with fungicide was very meagre. The histopathological report of the tissue treated with arecanut solution showed development of fibrosis even at the 6th month. Though not uniformly progressive, fibrosis was noticed in all the tissues at 6, 9 and 12 months.

**Conclusion:** The copper content of arecanut treated with fungicide and that not treated with fungicide was not very significant. The arecanut treated with copper containing fungicide can induce OSMF like changes in rat mucosa.

**Keywords:** Arecanut, Copper containing fungicide, OSMF, Rat mucosa.

## Introduction

There are an estimated 600 million people who chew betel nuts worldwide making the habit a very popular one. While chewed, various alkaloids and mineral components are released from the areca nut which produces a range of effects on the neurological system, which makes pan chewing addictive.[1]

Despite these general CNS stimulatory effects, arecanut chewing has many adverse effects as well.[2] The most significant adverse effect is oral submucous fibrosis, a potentially malignant disorder of the oral cavity which has been recorded since 1956 from the South East Asian countries, due to high consumption of arecanut in these parts. However, reason for increased prevalence of the disorder during the last two decades is not well explained, as areca nut was in use since many centuries. The increase in prevalence of OSMF in the last two decades is seen to be coinciding with the increased processing and commercialization of areca and its products since early 1980s.[3]

The chemical constituents of the areca have been strongly implicated in the etiopathogenesis of OSMF.[4] Role of elemental copper in the development of OSMF was a recently introduced concept by Trivedi et al in 1997.[5] Copper helps in the up-regulation of lysyl oxidase enzyme, which plays a crucial role in cross linking of collagen and elastin molecules. Copper content of arecanut were significantly higher than in the other chewable nuts.[5] It was viewed by various authors that the copper was incorporated into the arecanut from the copper containing fungicide that is sprayed on the trees to prevent tree rot due to heavy rainfall.[6]

The aim of this study was to analyse and compare metal constituents of arecanuts sprayed with copper containing fungicide and that not sprayed with fungicide and to evaluate the effects of arecanuts sprayed with copper containing fungicide on the buccal mucosa of rats during a period of 6 months, 9 months and 12 months.

## Materials and Methods

The current in-vivo rat study was conducted on 12 Wistar albino rats, obtained from the Animal House (CPCSEA approved, Reg No: 347), after obtaining Institutional Animal Ethical Committee Clearance (IAEC) from the concerned University.

**Inclusion Criteria**

1. Dry exfoliated mature arecanuts sprayed with copper containing fungicide (Bordeaux mixture).

**Exclusion Criteria**

1. Dry exfoliated mature arecanuts which are not sprayed by any fungicide.
2. Commercially available arecanut products.

**Methodology**

Aqueous solution of exfoliated mature arecanuts sprayed with copper containing fungicide and those not sprayed with any fungicide was prepared by crushing the dry arecanuts and grinding it to a coarse powder. 12gms of each powder was weighed and dissolved in 100ml of distilled water over night and then filtered using Whatman filter paper number 41 so that a clear filtrate was obtained.

The solutions were sent to Department of Chemical Engineering, National Institute of Technology Karnataka, Suratkal and metal analysis was conducted using Atomic Absorption Spectroscopy (AAS). After analysis, only the

solution of areca nut sprayed with copper containing fungicide is taken for the study.

12 adult Wistar albino rats, of either sex weighing 175-200 g, were divided into two groups (control and test) of six each. On the control group 2 drops of distilled water was dropped on their mucosa using a dropper and on the test group 2 drops of the prepared aqueous solution of arecanut was dropped. The procedure was done twice daily, for six days a week, for a period of one year, and the rats were closely observed during this period.

At the end of 6, 9 and 12 months, 2 rats from each group were selected randomly and sacrificed and biopsy was carried out. The obtained specimens were sent to the general pathology department for histopathological examination. The specimens were examined under light microscopy for atrophic epithelium, partial or complete loss of rete ridges, juxta-epithelial hyalinization, inflammation, edema and accumulation of dense bundles of collagen fibres subepithelially.

The results were then subjected to statistical analysis using SPSS software version 14. Chi square test was used for the statistical analysis and wherever necessary Fisher's exact test was used because of low sample size. P value of <0.05 was considered as statistically significant.

## Results and Observations

Table 1 shows comparison of metal constituents of arecanut treated with fungicide and that not treated with fungicide. The copper content in the arecanut treated with fungicide was seen to be 1.77ppm where as the copper content in arecanut not treated with fungicide was 1.36ppm. The analysis of other metals like iron, zinc, manganese, magnesium and potassium showed that the level of these metals were lesser in the arecanut treated with fungicide when compared with the arecanut not treated with fungicide. The level of potassium appeared to be significantly low in the arecanut treated with fungicide. The level of nickel and sodium appeared to be higher in the arecanut treated with fungicide.

Table 2 shows inter group comparison of variables in test with control. The parameters tested are the various histological criteria for sub mucous fibrosis, which includes epithelial atrophy, loss of rete pegs, subepithelial hyalinization, inflammation and edema and fibrosis. Mild atrophy was seen in 3 test rats and moderate atrophy in 3 rats. No loss of atrophy was noticed in the 6 control rats. Mild loss of rete pegs were noted in 4 and moderate in 2 test rats. No loss was noted in the 6 control rats. Mild sub epithelial hyalinization was seen in all 6 test rats whereas no hyalinization was observed in rest of the test rats as well as all the six control rats. No inflammation and edema were noted in 3 test rats and 6 control rats, while mild changes seen in 2 and moderate changes seen in 1 test rat respectively. Mild fibrosis was seen in 3 test rats whereas moderate fibrosis was seen in 3 test rats. Control rats did not show any fibrosis at all. All the parameters recorded were statistically significant except inflammation and edema.

Table 3 shows inter group comparison of all the parameters between control and each test group. All the groups showed a P value which was < than 0.05 suggesting that all the values were significant except the inflammation and edema in the 6 months to control which could not be compared as no inflammation and edema had developed in the time frame of biopsy of that group.

Table 4 shows intra group comparison between the samples in the test group i.e at the end of 6 months, 9 months and 12 months. Comparison of all the variables was done in the group but none of P values obtained from any of the groups showed any statistically significant value. Intra group comparison of sub epithelial hyalinization could not be done.

**Table 1:** Comparison of metal constituents of arecanut treated with fungicide and that not treated with fungicide.

| S. No | Parameter (ppm) | Sample A- areca nut with fungicide | Sample B –areca nut without fungicide |
|---|---|---|---|
| 1 | Copper | 1.77 | 1.36 |
| 2 | Iron | 0.49 | 0.53 |
| 3 | Cadmium | BDL | BDL |
| 4 | Zinc | 0.16 | 0.43 |
| 5 | Nickel | 0.04 | BDL |
| 6 | Lead | BDL | BDL |
| 7 | Manganese | 0.25 | 0.36 |
| 8 | Magnesium | 49.45 | 59.21 |
| 9 | Chromium | BDL | BDL |
| 10 | Cobalt | BDL | BDL |
| 11 | Mercury | BDL | BDL |
| 12 | Arsenic | BDL | BDL |
| 13 | Potassium | 40.5 | 142.4 |
| 14 | Sodium | 5.20 | 3.10 |

**BDL**= Below Detection Level (<0.0001ppm)

**Table 2:** Inter group comparison of variables in test with control

| Variable | Group | Nil | Mild | Moderate | P value |
|---|---|---|---|---|---|
| Atrophy of epithelium | Test | 0 | 3 | 3 | 0.002* |
| | Control | 6 | 0 | 0 | |
| Loss of rete pegs | Test | 0 | 4 | 2 | 0.002* |
| | Control | 6 | 0 | 0 | |
| Sub epithelial hyalinization | Test | 0 | 6 | 0 | 0.002* |
| | Control | 6 | 0 | 0 | |
| Inflammation & oedema | Test | 3 | 2 | 1 | 0.135 |
| | Control | 6 | 0 | 0 | |
| Fibrosis | Test | 0 | 3 | 3 | 0.002* |
| | Control | 6 | 0 | 0 | |

**Table 3:** Inter group comparison between control and each test group

| Variable | Group | P value |
|---|---|---|
| **Atrophy of epithelium** | 6 months to control | 0.036* |
| | 9 months to control | 0.036* |
| | 12 months to control | 0.018* |
| **Loss of rete pegs** | 6 months to Control | 0.036* |
| | 9 months to control | 0.018* |
| | 12 months to control | 0.018* |
| **Sub epithelial hyalinization** | 6 months to control | 0.036* |
| | 9 months to control | 0.036* |
| | 12 months to control | 0.036* |
| **Inflammation & oedema** | 6 months to control | - |
| | 9 months to control | 0.018* |
| | 12 months to control | 0.250 |
| **Fibrosis** | 6 months to Control | 0.036* |
| | 9 months to Control | 0.036* |
| | 12 months to Control | 0.018* |

**Table 4:** Intra group comparison

| Variable | Group | P value |
|---|---|---|
| **Atrophy of epithelium** | 6 months and 9 months | 0.167 |
| | 9 months and 12 months | 0.5 |
| | 6 months and 12 months | 0.5 |
| **Loss of rete pegs** | 6 months and 9 months | 0.5 |
| | 9 months and 12 months | 0.833 |
| | 6 months and 12 months | 0.5 |
| **Sub epithelial hyalinization** | 6 months and 9 months | - |
| | 9 months and 12 months | - |
| | 6 months and 12 months | - |
| **Inflammation & oedema** | 6 months and 9 months | 0.135 |
| | 9 months and 12 months | 0.368 |
| | 6 months and 12 months | 0.5 |
| **Fibrosis** | 6 months and 9 months | 0.167 |
| | 9 months and 12 months | 0.5 |
| | 6 months and 12 months | 0.5 |

## Discussion

In the present study 14 elements were estimated since these elements were seen in a higher concentration in the arecanut when compared to the rest of the metal constituents as estimated by The International Agency for Research on Cancer monographs and Zaidi et al.[4,7] These metals were – copper, iron, cadmium, zinc, nickel, lead, manganese, magnesium, chromium, cobalt, mercury, arsenic, potassium and sodium; of which the levels of only magnesium and potassium were seen to be higher in comparison with that of the metal constituents shown in the IARC monographs. The rest of the 12 metals estimated in the present study had a lesser value.

The concentration of only sodium and nickel from the arecanuts treated with fungicide were seen to be more than that from arecanuts not treated with fungicide. Level of potassium was seen to be considerably lower.

The role of various metal elements of arecanut on humans are not clear. Manganese generates Reactive oxygen species that may result in neurotoxicity.[8] Poor arsenic metabolism in female chewers may be responsible for their greater arsenic induced skin lesions. Nickel and chromium induce oxidative stress and are known to be genotoxic.[9] Chromium interacts directly with DNA, forming Cr-DNA adducts causing DNA damage. Nickel cause damage to DNA through inhibition of repair enzymes. Cadmium induces DNA strand breaks, DNA–protein cross-links, oxidative DNA damage and chromosomal aberrations, at high concentrations which are cytotoxic.[10]

A study conducted in Taiwan suggested that betel nut chewing in Taiwanese patients with type II diabetes mellitus was associated with hypertension.[10] The level of sodium is seen to be increased in the arecanut metal analysis which maybe one of the etiological factors for hyper tension. Level of potassium in arecanut not treated with fungicide is seen to be extremely high (142.4ppm). Hence relevance of various metal components in arecanut has to be further studied.

The copper content of arecanut was significantly higher than the other chewable nuts and chewing arecanut for 5–30 minute significantly increases soluble copper in whole mouth fluids.[5] It was also suggested that the copper was incorporated into the arecanut from the copper containing fungicide that is sprayed on the trees.[6] Studies tracing the source of the increased copper content are scarce in the literature. However on review of literature, it was found that the areca nut plantations in South India commonly used a copper based fungicide, Bordeaux mixture, on arecanut palms to prevent tree rot.[12] Copper content of the soil where Bordeaux mixture was sprayed was seen to be higher and it is postulated that the copper can be absorbed into the palm through the roots.[13] An increase in copper content was also noted as the nuts matures.

The present study estimated copper content of exfoliated mature arecanuts treated with fungicide and of those not treated with fungicide. The analysis revealed that the copper content in the arecanuts treated with fungicide (1.77ppm) was not very high compared to those not treated with fungicide (1.36ppm), the difference being a meagre 0.41ppm. This result is not in agreement with a study conducted by Mathew et al(2015)[11] which showed that copper content in exfoliated mature nuts treated with fungicide was about 8.77 ± 0.65mg/kg and that of the nuts not treated with fungicide had 4.74 ± 0.77mg/kg. These values were significantly higher than the concentration of copper in the arecanuts used in this study.

Variations in the concentrations of copper in this study may be attributed to, different geographical location, degree of maturity of the nuts, small sample size of arecanuts used (12gms – 3-4nuts) as compared to the sample size in the earlier study (12 arecanuts) and also the procedure of making the arecanut solution. No standardised procedure for preparation of arecanut solution was available in the literature.

The results obtained from the present in vivo animal study showed OSMF-like lesions in different degrees in the group treated with areca nut extracts. The histologic changes observed included atrophic epithelium, loss of rete ridges, inflammation, accumulation of dense bundles of collagen fibers, juxta-epithelial hyalinization. Histopathologic findings of the study bear a close resemblance to the characteristic features of OSMF as seen in humans and also as recorded in the literature.

Monitoring clinical changes by visual examination was not possible owing to the small size of the rat oral cavity even though a slight degree of difficulty in mouth opening was felt in the later months of the study. However, on histological evaluation, a significant amount of fibrosis was noted in the arecanut treated group, a finding, consistent with the pathognomic histopathological feature of OSMF. Although these changes were not uniformly progressive in all the samples, it bore a close resemblance to the histological traits of OSMF seen in humans.

In the current study it was seen that within the six months of treatment with aqueous areca solution there was development of moderate atrophy of epithelium, mild loss of rete pegs, mild sub epithelial hyalinization, and moderate levels of fibrosis. But no inflammation was seen. This suggests that arecanut treated with copper containing fungicide, without any other additives like lime or tobacco is capable of causing OSMF like changes in rat buccal mucosa, though the degree of progression was not similar to that which occurs in humans. This finding is in agreement with various other studies.[13,14] Though OSMF is considered multifactorial in origin, arecanut chewing is one of its main causes.

The histopathological report of the test rats in the ninth month showed only mild changes in all the parameters. Epidemiological studies suggest that prolonged intake of areca nut leads to higher grades of OSMF, which is not in agreement with the present study.[15] But in vivo experimental data on the ability of the areca nut extract to produce OSMF are meagre. In a study conducted by Sirat et al in 1962 as part of the attempt at induction of submucous fibrosis in animal models pointed out that tissue changes brought about in animal systems by a test substance cannot be identically correlated to those in man.[16] Differences in species, size of tissue, and response to inciting stimuli may have caused a variation in the expression in the tissues. This variation in the histopathology may also be due to the body immune mechanisms trying to wall off the inflammatory process and other contributory additive factors.

In the twelfth month, the histopathological report of one of the rats showed mild changes in all parameters where as the second rat showed moderate changes in all the parameters except inflammation which was nil. This suggested that longer duration of arecanut chewing can increase fibrosis formation, which is in agreement with a study conducted by Maria et al in 2015.[17] But Shah et al in

1998 also stated that the total duration of the chewing habit was not significantly correlated to OSF.[18]

The severity of fibrosis in the mucosa of test rats at the end of twelve months, treated with same amount of solution, showed mild and moderate levels of fibrosis, suggesting that individual variations in tissue response to external stimuli also plays a role in the pathogenesis.

It was postulated by Caniff et al. that areca nut products exacerbate the submucosal fibrosis initiated by chronic inflammation and that chronic inflammatory cell infiltrate is a common feature in all grades of OSMF.[19] But in severe grades of the disease it shows a reduced presence as a result of the stabilisation of the lesion and reduction in levels of pro-inflammatory mediators. Similar findings were noticed in our study. The rats with mild fibrosis showed inflammatory cell infiltrate whereas the moderate stage rats did not have any inflammatory cell infiltrate.

## Conclusion

Copper content of arecanut treated with fungicide and that not treated with fungicide was not very significan.t Arecanuts alone without any other ingredients can induce fibrotic changes in the rat epithelium.

**Conflict of Interest:** None.

## References


1. Lingappa A, Nappalli D, Sujatha GP, Shiva Prasad S. Areca nut: To chew or not to chew? *e-J Dent* 2011;3:46-50.
2. Anjana Mohan Kumar, Kota Sravani, Veena K.M, Prasanna Kumar Rao J, Laxmikanth Chatra, Prashanth Shenai, et al. Arecanut and its effects on the human body. *Am J Oral Med Radiol* 2015,2:24-8.
3. Mathew P, Austin R D, Varghese S S, Manojkumar. Estimation and Comparison of Copper Content in Raw Areca Nuts and Commercial Areca Nut Products: Implications in Increasing Prevalence of Oral Submucous Fibrosis. *J Clin Diagn Res* 2014;8:247-9.
4. Betel quid and areca nut chewing, IARC Monographs 2004;85:1-349.
5. 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.
6. Khan S, Chatra L, Prashanth SK, Veena KM, Rao PK. Pathogenesis of oral submucous fibrosis. *J Cancer Res Ther* 2012;8:199-203.
7. Zaidi J.H., Arif M., Fatima I. and Qureshi I.H. Radiochemical neutron activation analysis for trace elements of basic ingredients of pan. *J Radioanal Nucl Chem* 2002,253:459-64.
8. Al-Rmali S W, Jenkins O R, Haris I P. Betel quid chewing as a source of manganese exposure, total daily intake of manganese in a Bangladeshi population. *BMC Public Health* 2011;11:85-6.
9. Vela D. Desai, M. V. Sunil Kumar, Renuka J. Bathi, Isha Gaurav, Rajeev Sharma. Molecular Analysis of Trace Elements in Oral Submucous Fibrosis and Future Perspectives. *Universal Res J Dent* 2014;4:27-32.
10. Chin-Hsiao TSENG. Betel Nut Chewing Is Associated with Hypertension in Taiwanese Type 2 Diabetic Patients. *Hypertens Res* 2008;31:3.
11. Mathew P, Austin DR, Varghese S S, Manojkumar A D. Effect of copper‐based fungicide (bordeaux mixture) spray on the total copper content of areca nut: Implications in increasing prevalence of oral submucous fibrosis. *J Int Soc Prevd Community Dent* 2015;5:283-9.
12. Aikpokpodion P E, Lajide L and Aiyesanmi A F. Impacts of Cu-Based Fungicide on Copper Residue and Mineral Elements Distribution in Cocoa Beans and Pods. *World J Agricultural Sci* 2013;9:10-6.
13. Maher R, Lee A, Warnakulasuriya KA, Lewis JA, Johnson NW. Role of areca nut in the causation of oral submucous fibrosis: a case-control study in Pakistan. *J Oral Pathol Med* 1994;23:65-9.
14. Rajendran R. Bulletin of the World Health Organization. 1994;72:985-96.
15. Roberts N, Kamath VV, Stelur K. Permeability of rat oral mucosa to arecanut and pan masala extracts: An experimental study. *J Orofac Sci* 2013;5:32-6.
16. Sirsat SM, Khanolkar VR. S.M.F of palate & pillar of fauces. *Ind J Med Sci* 1962;16:189-97.
17. Maria S, V. V. Kamath, P. S. Krishnanand, R. Komali. Sprague-Dawley rats are a sustainable and reproducible animal model for induction and study of oral submucous fibrosis. *J Orofacial Sci* 2015,7:13.
18. Shah N, Sharma PP. Role of chewing and smoking habits in the etiology of oral submucous fibrosis: A case control study. *J Oral Pathol Med* 1998;28:475-9.
19. Caniff JP, Harvey W. The etiology of OSMF; The stimulation of collagen synthesis by extracts of arecanut. *Int J Oral Surg* 1981;10:163-7.


**How to cite this article:** Kumar AM, Kota S, Chatra LK, Veena KM, Rao PK. Copper containing fungicide – the actual culprit in the pathogenesis of OSMF? *J Oral Med, Oral Surg, Oral Pathol, Oral Radiol* 2019;5(2):52-6.