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Risk of Betel Nut Chewing: Chemical and Toxicological Studies

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Abstract

Last few decades epidemiological reports warn that the development of oral submucous fibrosis – a precancerous state, is universal among betel nut or areca nut users. Betel nut contains arecoline and other microelements which are released into the buccal cavity during the process of areca nut chewing. The present effort was undertaken to find out the correlations between chemical ingredients, like arecoline, magnesium, iron and copper present in betel nut with toxicological incidences in experimental animals. Arecoline was estimated by HPLC (Perkin Elmer, USA) and other micronutrients like magnesium, iron and copper was assessed by AAS (Perkin Elmer, USA). Repeated dose 90-day sub-acute oral toxicity of betel nut water extract (BNE) at the dose of 0.5X10-2 ml/g/day and 1.0X10-2 ml/g/day was performed on Wistar rats as per the Organization for Economic Co-operation and Development (OECD) guidelines No. 408. The result indicates that betel nut contains Group 1 human carcinogen arecoline with significant high level of magnesium, iron and copper. Moreover, consumption of betel nut induces liver damage in rats by indicating significant enhancement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP). It may conclude that chemical ingredients presences in betel nut are harmful for health and it should be restricted.

Keywords: Areca nut, arecoline, alkaloid, fibrosis, HPLC, toxicity

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Introduction

In ancient Indian scripts such as the *Vagbhata* (4th century), and the *Bhavamista* (13th century), the seed or endosperm of betel nut (*Areca catechu* Linn., family: Palmaceae) has been illustrated as a holistic and therapeutic agent.[1] Earlier works indicated it can enhances physical endurances, improves cognitive abilities and protects gum from dental caries and other microbial infections.[2] More than 600 million populations are now habitual consumers of betel nut throughout the world.[3] But, last few decades epidemiological reports warn that the development of oral submucous fibrosis – a precancerous state, is universal among betel nut users.[4] Eventually, the habit of chewing betel nut starts at a young age, and it is being consumed freely by children.[5] Betel nut contains mostly arecoline (1,2,4,5,-tetrahydro-1-methylpyridine carboxylic acid, a pyridine alkaloid) and other microelements which are released into the buccal cavity during the process of betel nut chewing.[6] The World Health Organization (WHO) and International Agency for Research on Cancer (IARC) classified betel nut and arecoline as a Group 1 human carcinogen.[7] Arecoline is a mutagenic in both bacterial and mammalian cells and increases the frequency of micronuclei and chromosomal aberrations in bone marrow cells.[8] A dose dependent relationship was also observed for both frequency and duration of chewing betel nut in the development of oral sub-mucous fibrosis, leukoplakia, and oral cancer.[9] Polyphenols present in nut inhibits the enzyme collagenases and thereby enhances the cross-linkage of collagen.[10] These events may underlie the generation of oral sub-mucous fibrosis in betel nut

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chewers and that could be further enhanced by the release of magnesium, iron and copper salts (which are present in areca nut) into the oral cavity of the chewers. These inorganic salts or ions exaggerated the formation of collagen by oral fibroblasts.[11] Further, adverse health effects are also reported at high rates of usage of betel nut and include cardiac arrhythmia, exacerbation of asthma, infertility, acute psychosis and acute gut upset.[12] Withdrawal symptoms such as mood swings, anxiety, irritability, sleep disturbance and craving were found to be associated with trying to quit the habit of betel nut chewing.[13] Though there are substantial evidences for its adverse effects, but still some workers highlighted its therapeutic applications.[14] Consequently, the present effort was undertaken to find out the correlations between chemical ingredients and toxicological incidences of betel nut chewing in experimental animals.

Materials and Methods

Chemicals and reagents

Betel nut (*Areca catechu* Linn., family: Palmaceae) was purchased from local market in Kolkata. Analytical grade ultrapure fine chemicals like arecoline HBr (Sigma-Aldrich Laborchemikalien, GmbH 31593, SZBB103XV), magnesium (SRL, 1320131, X-737335, 1000 mg/L), iron (Merck, CertiPuR 1.9781.0100, 1000 mg/L), copper (Merck, CertiPuR 1.9786.0500, 1000 mg/L) and other organic solvents were used. Commercial grade ready biological kits (RFCL Limited, India) were used for hematological and clinical biochemical studies.

Animals

Healthy adult male Swiss albino mice (25±5 g) and male Wistar rats (150±5 g) were used in the study. The animals were fed standard rodent diet and maintained according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, 2003).[15] The study was initiated after receiving approval from institutional animal ethics committee (Regn. No. R/N 959/C/06/CPCSEA).

Preparations of test drug

Dry betel nuts were cleaned and pulverized into coarse powder. 100 g of powdered nut was submerged into 50 ml of deionized water and incubated for 6 h at 70°C. After cooling the filtrate was collected in a 100 ml volumetric flask. The process was repeated for one time and the volume was made up to the mark at a concentration of 1 g/ml. Finally, the betel nut extract (BNE) were stored on a sterilized container at 4°C.

Chemical analysis

Physicochemical constants

The physicochemical characters like, total ash, acid insoluble ash, alcohol soluble extractive, water soluble extractive, chloroform soluble extractive and pH of betel nut coarse powder was determined according to Indian Pharmacopeia.[16]

Atomic absorption spectrometric study

Magnesium, iron and copper content of betel nut were determined using Flame Atomic Absorption Spectrophotometer (Perkin Elmer, AAS: Model No. AA200, USA) following American Public Health Association method.[17] In brief, 5 g of coarse betel nut powder was heated to ashes in a muffle furnace at 773.15 K and digested with 10 ml of concentrated nitric acid for 2 h. Thereafter, 5 ml of 70% perchloric acid was mixed and heated for another 1 h. A few drops of hydrogen peroxide were then added to discolour the digested material. The, it was dissolved in deionized water and filtered. The filtrate was transferred into a 50 ml volumetric flask. Six replicate digestions were made for each elemental study. The instrumental working conditions were given below [1]:

A blank digest was prepared without sample. Four point calibration graphs were performed separately for magnesium, iron and copper. The prepared concentration of magnesium standard used for the plot varied between 00µg/L and 500µg/L depending on the absorbance of magnesium, while, for iron standard used for the plot varied between 00µg/L and 1000µg/L and copper standard used for the plot varied between 00mg/L and 50mg/L. The coefficient of correlation (r) for standard calibration graph for magnesium was 0.9934, iron 0.9985 and copper 0.9987. The detection limit of this instrument was 0.0001 ppm. The concentrations of elements present in the samples were determined using standard calibration curves. The measured concentration derived as µg/L. Then, it was expressed as ng/mg of betel nut.

Table 1. Flame atomic absorption spectrophotometer instrumental set up condition

Conditions	Magnesium	Iron	Copper
Wave length (nm)	285.21	248.33	324.8
Slit width (nm)	2.7	1.8	1.9
Cathode lamp current (mA)	15 max	30 max	30 max
Flame type	Air/Acetylene	Air/Acetylene	Air/Acetylene
Air flow (L/min)	10.00	10.00	10.00
Acetylene flow (L/min)	2.50	2.50	2.50
Integration time (sec)	3	3	3

Flame Atomic Absorption Spectrophotometer (Perkin Elmer, AAS: Model No. AA200, USA)

HPLC analysis for arecoline

Extraction and estimation of arecoline from betel nut was prepared as described earlier Adhikari *et al.* (2015).[18] In brief, 1 g coarse betel nut powder was mixed with 10 ml of methanol and kept at room temperature for 24 h. In the following day, the suspension was filtered, washed and made to the final volume at 10 ml. Thereafter, the solvent was removed and the gummy extract was dissolved in 2 ml of 0.1N hydrochloric acid and the acidic solution was further washed with 3 ml of chloroform. 1 ml of acid layer was then taken and neutralized it with 1N ammonium hydroxide. Thereafter, 10 ml chloroform was poured on it and mixed. Chloroform fraction was aspirated out and saturated with anhydrous sodium sulphite. Finally, it was carefully taken and evaporated to dry. This eluted material was dissolved in HPLC grade acidic methanol (methanol : HCl = 98 : 2 v/v). The standard calibration of arecoline (Sigma-Aldrich Labrchemikalien, GmbH) was made at the concentration of 12.5 µg/ml, 25 µg/ml and 50 µg/ml and 100 µg/ml. The sample was injected through a 20 µl fixed loop of integrated HPLC containing isocratic Flexer LC pump, PDA detector, C-18 ODS 2 Hypersil column (Perkin Elmer, Model No. AA200, USA). The mobile phase was consisted with potassium dihydrogen phosphate (10 g), phosphoric acid (3 ml), triethylamine (8 ml), acetonitrile (9 ml) and deionized water (980 ml) at pH 5.5. The flow rate was adjusted at 1 ml/min and run time was 10 min. Peak height was used for determination of the concentration of the sample preparation and analyzed using Perkin Elmer Chromera Manager (version 4.1.0.6386) software.

Toxicological animal studies

Acute oral toxicity

Single dose toxicity was done on adult Swiss albino mice according to Organization for Economic Co-operation and Development (OECD) guidelines No. 423 (2001)[19] adopted for acute toxicity in animals (limit test). Betel nut water extract (BNE) was administered to overnight fasted mice (n=6) by oral route in a single dose and observed for 3 days. The maximum dose was restricted to 2 g/kg body weight. The rate of mortality and 50% lethal dose concentration up to 72 h was recorded.

Sub-chronic oral toxicity

Repeated dose 90-day oral toxicity of betel nut water extract (BNE) were performed on Wistar rats as per the OECD guidelines No. 408 (1998).[20-21] From a total of 30 rats (average body weight 150 g), 10 animals each were randomly assigned to the control and the two treatment groups. The rats in the two treatment groups were administered BNE at the dose of (i) 0.5×10^{-2} ml/g/day (250 mg/kg/day) and (ii) 1×10^{-2} ml/g/day (500 mg/kg/day) by gavages for a period of 90 days. Control animals received deionized water at the dose of 1×10^{-2} ml/g/day along with standard laboratory diet for 90 days. During the period of BNE administration, the animals were observed closely for signs of any toxicity. Cage side behavioural observations, food and water consumption, body weight, and urinalysis were conducted as a part of the regular check up of the animals.[21] At the end of 90 days, the overnight fasting animals were sacrificed under

deep anesthesia (intraperitoneal thiopentone sodium, 40 mg/kg). After euthanizing, blood samples were collected by cardiac puncture. Hematological investigations were performed for the measurement of hemoglobin (Hb) levels, total count of red blood cells (RBC) and white blood cells (WBC). Biochemical parameters measured were glucose, total protein, albumin, total cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine in serum.

Statistical Analysis

All group values are expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance followed by Tukey's multiple comparison tests for comparison between treatment groups. Statistical significance was set at $P<0.05$.

Results

Chemical analysis

Total ash content showed within the limit, but acid insoluble ash values, alcohol solubility and water solubility were lower than the limit. The samples also showed acidic properties in water extraction [Table 2]. Chemical analysis revealed betel nut contains 428.54 ng arecoline per mg of dry nut, which was nearly 0.042%. The mean retention time was detected at 2.58 min [Fig. 1].

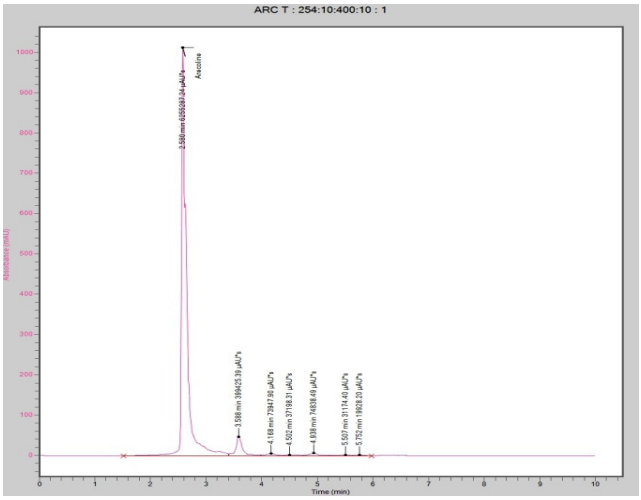


Figure 1. HPLC chromatogram of betel nut water extract for arecoline.

Toxicological studies

In acute oral toxicity studies up to the dose of 2 g/kg BNE did not showed any mortality in mice, though there was profuse salivation noted after ingestion. There was no significant differences in body weight among BNE treated rats, compared to control group [Fig. 2]. The hematological parameters of 90-days are shown in Table 3. No compound related (arecoline or any other compounds) effects were shown in red blood cells, white blood cells, hemoglobin values, that clearly indicate no adverse effect was observed in blood borne parameters. Examination of the serum biochemical values of BNE treated and control groups are

Table 2. Physico-chemical evaluation of betel nut

	Mean ± SD	Pharmacopeia's Limit Value
Total ash (%)	1.78 ± 0.062	Up to 3
Acid insoluble ash (%)	0.325 ± 0.009	Above 0.4
Alcohol soluble extractive (%)	15.94 ± 0.058	Above 19
Water soluble extractive (%)	14.69 ± 0.071	Above 10
Chloroform soluble extractive (%)	2.14 ± 0.015	-
pH	4.79 ± 0.036	-
Iron (µg/g)	49.78 ± 0.27	75
Magnesium (µg/g)	1.65 ± 0.008	2.8
Copper (µg/g)	7.75 ± 0.043	18
Arecoline (ng/mg)	428.54±5.98	-

N=6 in each test; iron, magnesium and copper are estimated using Flame Atomic Absorption Spectrophotometer (Perkin Elmer, USA) and arecoline is estimated using HPLC (Perkin Elmer, USA)

shown in Table 4. Ingestion of BNE significantly reduces fasting blood glucose level, serum protein and albumin concentration as compared to control. Moreover, serum AST, ALT and alkaline phosphatase was significantly and dose dependently elevated, but there was no change in cholesterol, urea and creatinine level.

Discussion

The alkaloid, arecoline is the main ingredient and responsible for all functional abilities of betel nut. Recent studies confirmed its genotoxic, mutagenic and carcinogenic effects and alarmed for pathogenesis of oral mucosal disease. The current study is mainly focused on

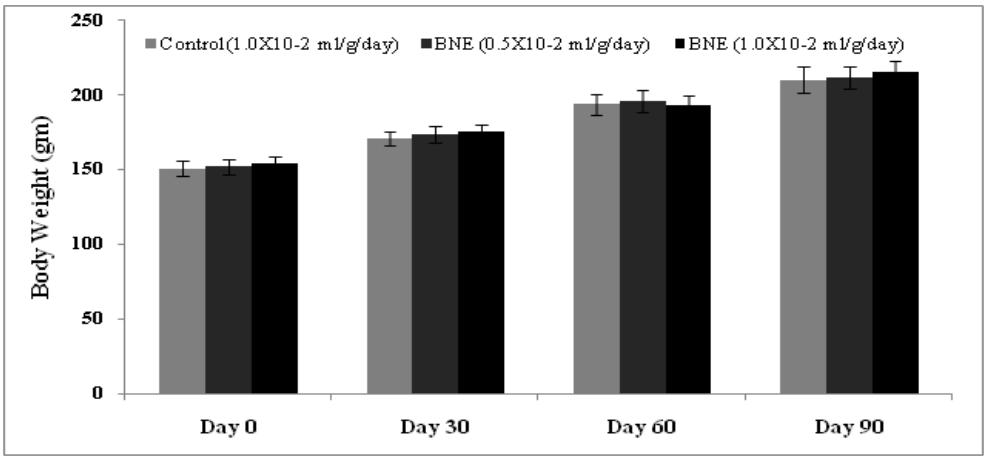


Figure 2. Effect of oral ingestion of betel nut water extract on body weight.

Table 3. Effect of oral ingestion of betel nut water extract on hematological profile during 90-days repeated dose toxicity studies in Wistar rats

	Limit	Control (1.0×10^{-2} ml/g/day)	BNE (0.5×10^{-2} ml/g/day)	BNE (1.0×10^{-2} ml/g/day)
Hb%	11-18	14.26 ± 0.38	14.05 ± 0.29	14.21 ± 0.29
RBC ($\times10^{6}$/mm^{3})	7-10	6.21 ± 0.53	6.16 ± 0.68	6.19 ± 0.68
WBC ($\times10^{3}$/mm^{3})	6-17	7.93 ± 0.68	7.86 ± 0.53	7.81 ± 0.53
Neutrophil (%)	9-34	26.84 ± 3.16	25.45 ± 3.74	24.82 ± 3.90
Lymphocytes (%)	65-85	68.82 ± 5.52	68.50 ± 4.91	67.36 ± 4.72
Eosinophil (%)	0-6	3.74 ± 1.15	3.88 ± 1.15	3.97 ± 1.24
Monocytes (%)	0-5	1.95 ± 0.81	1.93 ± 0.62	1.90 ± 0.83

Each value is a mean ± SD for n=10. Control rat receive distilled water. BNE means betel nut water extract. All changes are statistically not significant in comparison to control.

Table 4. Effect of oral ingestion of betel nut water extract on blood profile during 90-days repeated dose toxicity studies in Wistar rats

	Limit	Control (1.0×10^{-2} ml/g/day)	BNE (0.5×10^{-2} ml/g/day)	BNE (1.0×10^{-2} ml/g/day)
Glucose (mg/dl)	50-110	72.9 ± 6.15	65.8 ± 5.32	60.3 ± 6.02*
Total Protein (g/dl)	5.6-7.6	7.22 ± 0.27	6.71 ± 0.22	6.48 ± 0.18*
Albumin (g/dl)	2.8-4.8	3.96 ± 0.08	3.35 ± 0.06*	3.12 ± 0.05*
Total Cholesterol (mg/dl)	25-135	82.6 ± 6.96	79.3 ± 7.18	76.4 ± 6.23
ALT (IU/L)	12-45	23.5 ± 4.06	39.6 ± 5.01*	46.5 ± 5.38*
AST (IU/L)	12-45	24.1 ± 4.13	41.7 ± 5.15*	45.9 ± 5.29*
ALP (IU/L)	45-110	56.4 ± 3.22	76.8 ± 6.22*	82.7 ± 6.15*
BUN (mg/dl)	15-21	17.24 ± 1.03	18.16 ± 2.74	18.24 ± 2.01
Creatinine (mg/dl)	0.2-0.8	0.48 ± 0.06	0.54 ± 0.08	0.51 ± 0.09

Each value is a mean ± SD for n=10. Control rat receive distilled water. BNE means betel nut water extract. ANOVA followed by Tukey's multiple comparison is used. * indicates $P < 0.05$.

the quantification of arecoline and other micronutrients, magnesium, copper and iron in betel nut through sensitive tools. The concentration of arecoline reported in areca nut was 0.042% of its dry weight. The concentration of arecoline in betel nut varies with geographical conditions and other environmental factors. In other reports the concentration of arecoline in processed nuts has been suggested to 0.012-1.4% of its dry weight.[7]

It has also been reported that the presence of high amount of magnesium, copper and iron in betel nut. Magnesium with its carbonate salts in alkaline medium fastens the collagen formation in oral tissues, resulting in the progress of fibrosis or tumour growth.[22] Similarly, iron plays a major role in generation of free radicals in oral cavity and thus promotes tissue damage. The copper is released during chewing of betel nut has brought in direct contact with the mucosal keratinocytes. It has been reported on copper induced mutagenesis through the p53 aberrations in oral sub-mucous fibrosis.[11] In this study, betel nut possesses all these three harmful heavy metals with significant high amount.

The therapeutic oral dose of arecoline in human is 5 mg/kg. But, recent studies point out that betel nut is harmful and hazardous for human health. In the present study, oral ingestion of high dose of BNE (2 g/kg) in mice showed profuse salivation and neurological motor disturbances for a short period (2 h), but no mortality reported within the stipulated time frame, i.e., up to 3 days.

There is limited evidence that aqueous extracts of betel nut are carcinogenic to animals. It has been reported, arecoline causes neurotoxicity through enhancement of oxidative stress and suppression of the antioxidant protective system.[23] In a repeated dose 28 days oral toxicity study, aqueous extract of betel nut showed enhancement of liver enzymes and creatinine in blood.[24] In the present effort, BNE was tested for repeated dose 90-day oral toxicity study in Wistar rats. The body weights depend on food consumption and metabolic changes occurred during the drug treatment. Hence, any toxic signs of test drug may reflect on the body weight within this period of study. Both the groups gained weight during the study period and it remained fairly constant throughout the experimental period. Intake of food and water is an important subject of behavior study. The rats administered with BNE showed slight depression and profuse salivation for first few minutes, there after they showed normal. Previous studies revealed that betel nut alkaloids have anti-muscarinic effects and also have GABA-ergic actions.[12] The average food and water intake was more over same in all groups and there was no evidence of any effect of treatment with BNE.

Prolonged 90-days ingestion of BNE significantly lowered fasting blood glucose level. Earlier it has been reported that betel nut has hypoglycemic action.[1] Moreover, BNE progressively damaged liver cells and that was evident by elevations of liver enzymes AST, ALT and alkaline phosphatase in serum and reversed effects were noted in protein and albumin. These findings clearly indicated BNE has hepatotoxic properties and that may be due to acceleration of oxidative stress caused by arecoline. Previous studies corroborated these findings.[25] Although, BNE could not damage renal functions as evident by no alteration in the concentration of blood urea nitrogen and creatinine in rat.

From the above findings it may conclude that betel nut contains several known chemical ingredients that have hazardous biological actions in health, particularly in oral hygiene and metabolic functions — and therefore it should be restrict for oral use in any form.

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Conflict of Interest

Nil

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