SUMMARY

'Gudakhu' is a paste like tobacco preparation. Its use is highly prevalent in the state of Orissa, India and other neighbouring states. During use it is rubbed with a finger tip over teeth and gums for a sufficiently long time. Some persons prefer to keep it in the gingival area for some time and swallow the extract mixed with saliva, while others spit it away intermittently. The ingredients of gudakhu are tobacco (10%), lime (7%), red-soil (28%), molasses (35%) and water (20%). The lime used here is slaked lime. The red-soil contains oxides of iron, aluminium and several other elements. Finely cut tobacco (Nicotiana tabacum) of the chewing variety is used for its preparation. The molasses is of poor quality and is pyrolysed by heating before use.

In the present investigation potential genotoxic and cytotoxic effects of 'gudakhu' and its ingredients were evaluated. Although lot of works have been done on carcinogenic and mutagenic capacity of varieties of smokeless tobacco our knowledge on such a potential of gudakhu is extremely limited. The evaluation was done in whole mammal system (mice) as well as in habitual users. Section 2 and 3 deal with the mice experiments, while effect on habitual users has been dealt with in section 4.

In mice experiments were conducted to evaluate:

(a) Effect of acetone extract of gudakhu.
(b) Effect of water extract of gudakhu.
(c) Effect of water extract of different ingredients (tobacco, lime, molasses, and red-soil) and of acetone extract of tobacco.
(d) Modulating effect of human (non-user) saliva on chromosome damaging effect induced by acetone extract of gudakhu.
In mice the assays followed were:

(a) Chromosome aberration in bone marrow cells.
(b) Micronucleus test in bone marrow cells.
(c) Micronucleus test in regenerated hepatocytes.
(d) Micronucleus test in peripheral blood erythrocytes.
(e) Sister chromatid exchanges in bone marrow cells.
(f) Spermatocyte chromosome analysis.
(g) Sperm morphology assay.
(h) Sperm count assay.

However, all the assays could not be conducted for each type of extract, for acetone extract all assays were followed. Further, single treatment regimen as well as chronic treatment regimen (once daily for 4, 8, 12 and 16 weeks) was followed; the latter was done particularly to simulate human exposure.

Healthy adult inbred Swiss mice were used. Gudakhu of 'Samalestari' brand purchased from the local market was tested. Mice always received the extract by gastric intubation. In case of acetone extract the dried extract suspended in water was fed to mice; while for water extract, the extract was filtered and the filtrate was used. As the mice received the extracts via oral route age and sex matched untreated mice served the purpose of negative control which was run in parallel.

Cyclophosphamide (CP) and mitomycin C (MC) treated mice were used as positive control. For spermatocyte chromosome analysis MC was used, while for all other assays CP was used.
5.1 EFFECT OF ACETONE EXTRACT OF GUDAKHU(AEG)

In bone marrow cells the frequency of breaks increased in a dose-dependent manner following single treatment. Similarly a dose-effect relationship was also marked when the animals were treated with AEG once, twice or thrice spaced 4 h. However, the frequency of breaks decreased with increase of the sampling interval. Repeated daily treatment for 8 and 12 weeks for both the doses (150, 300 mg/kg), and for 16 weeks for the higher dose yielded significantly high breakage frequencies; however, the value for week 4 did not exhibit any significant change.

The frequencies of Mₙ in bone marrow polychromatic erythrocytes (PCE) increased significantly over the control for two higher dose levels following single treatment and also in all the test weeks in the repeated treatment regimen. A dose-related increase in the incidence of MNPCEs was also marked; however, there was no significant different among the values. The incidence of MNNCEs remained in the control range except at week 16. PCE/NCE ratio did not also differ significantly over the control value indicating very little cytotoxic effect.

The frequencies of MN in regenerated hepatocytes increased significantly, compared to the control value, in all test weeks (8, 12 and 16 weeks) following repeated daily treatment. A distinct positive linear relationship was revealed with the time-course of treatment of AEG (8, 12, and 16 weeks).

A significantly elevated incidence of MN, in comparison to control, was marked in peripheral erythrocytes of mice in all the test weeks (1-4). A time-course-response analysis of the data revealed a very good positive correlation between the incidence of MN and time-course of treatment.
The frequencies of SCEs increased greatly in all the test weeks (4, 8, 12 and 16) and for all the dose levels. The values for single and chronic treatment did not differ markedly; however, lower dose induced less effect.

Qualitatively the effects on the meiotic chromosomes were analyzed under two general heads: structural aberrations comprising chromosome breaks, chromatid breaks, fragments and exchanges and univalent formation. Two different doses of AEG following chronic treatment for 4, 8, and 12 weeks exhibited identical results; and the data showed significant increase over the control in all the test weeks (4, 8, 12 and 16). However, time-course-effect relationship was not marked. AEG also elevated the incidence of univalent formation significantly over the control value following 8, 12 and 16 weeks of daily treatment for 300 mg/kg dose, but for 12 weeks of treatment for 150 mg/kg dose.

Vasa differential sperm exhibited numerous types of abnormalities in their head morphology in AEG treated animals. The frequency of mis-shapen sperm (considering all abnormal types together) increased greatly and significantly in all test weeks (4, 8, 12 and 16) following repeated daily treatment; the frequency for the lower dose (150 mg/kg) after 4 weeks of chronic treatment remained at the control range.

Haemocytometric count of epididymal sperm revealed significant decrease, compared to the control value, following 12 and 16 weeks of repeated treatment for both the doses of AEG.

5.2 EFFECT OF WATER EXTRACT OF GUDAKHU

For water extract of gudakhu (WEG) the animals were treated once daily for different weeks (4, 8, 12 and 16), and the following assays were conducted.
(a) Chromosome aberration assay in bone marrow cells.

(b) Spermatocyte chromosome analysis.

(c) Sperm morphology assay.

(d) Sperm count assay.

In addition, short term test (treated once and sampled at 24 h post-treatment) was conducted for chromosome aberrations in bone marrow cells.

Both single and chronic treatment of WEG revealed significantly higher breakage frequencies of CAs, compared to the control, in bone marrow cells, and breakage incidence showed a strong positive correlation with the time-course of treatment.

Similarly in spermatocytes significantly higher incidences of breakages, and a positive correlation between frequencies of breakages and time-course of treatment were noted. Univalent formation also increased greatly for weeks 12 and 16.

Significant increase, compared to the control value, in the incidence of mis-shapen sperm was recorded in all the test weeks. The epididymal sperm count decreased appreciably following chronic treatment of WEG.

All the assays followed here have clearly demonstrated genotoxic and cytotoxic potential of both organic solvent and water extracts of gudakhu. A comparison of the data for AEG and WEG in relation to consumption of gudakhu revealed higher effectiveness of WEG. In AEG series each mouse received an extract of 250 mg of gudakhu (for 300 mg/kg dose), while in WEG series a mouse received an extract of 42 mg of gudakhu (for 625 mg/kg dose). The data on breakage and total aberrations for 24 h following single
treatment clearly indicate that water extract is a more effective clastogen. So far tobacco content is concerned 250 mg of gudakhu contains 25 mg of tobacco, while 42 mg of gudakhu contains only 4.2 mg of tobacco. So in AEG series though a mouse received tobacco nearly 6 times the amount received by a mouse in WEG series but less effect was noted, compared to WEG treated series. Possibly other ingredients which do not come in acetone extract contribute a lot in inducing genotoxicity. It may also happen that water facilitates better extraction of tobacco specific genotoxins.

Data on regenerated hepatocytes indicate accumulation of clastogenic factor(s) and/or preclastogenic damages in the hepatocytes. Persistence of micronucleated RBCs in peripheral circulation is also documented.

5.3 EFFECT OF INGREDIENTS OF GUDAKHU

Attempts were made to find out if a particular ingredient or some ingredients is/are responsible in causing genotoxicity. For that separately each ingredient (tobacco, red-soil, lime and molasses) was tested by extracting in water; for tobacco, however, both water and acetone extracts were tested.

The following assays were conducted for each of the ingredients:

(a) Chromosome aberrations in bone marrow cells.

(b) Spermatocytes chromosome analysis.

(c) Sperm morphology assay.

(d) Sperm count assay.

In addition, for tobacco SCE assay was conducted.
All four ingredients of known weight were taken separately and homogenized in distilled water and then filtered. The filtrate was diluted with an appropriate amount of water and fed to mice through gavage. Acetone extract of known weight of tobacco was prepared in the same manner as done for gudakhu.

Both water extract and acetone extract of tobacco increased the frequency of chromosome aberrations, including and excluding gaps over the control value in both single and repeated treatment regimens except at week 4, water extract was slightly more effective than the acetone extract.

More than two fold increase, over the control value, was marked in SCE frequency following chronic treatment.

The incidence of CAs in spermatocytes increased significantly at 8, 12 and 16 weeks and the values were about 10 times the control one. Univalent formation also increased after prolonged treatment. Vasa differential sperm exhibited significantly high incidence of abnormal head morphology. In no test week the sperm count revealed any appreciable deviation from the control value.

Water extract of molasses also showed significant increase in chromosome aberrations, excluding and including gaps, in bone marrow cells over the control value both for single and repeated treatment regimens for the higher dose. But for the equivalent dose of gudakhu there was no such significant difference from the control. Repeated treatment of the higher dose, in meiotic cells, also exhibited significantly higher incidence of both structural aberrations and univalent formation at 8, 12 and 16 weeks of treatment. Sperm head abnormality increased appreciably in all the test weeks, but sperm count data failed to exhibit marked deviation from the control.
Aqueous extract of red-soil in equivalent amount present in gudakhu extract also induced CAs in bone marrow cells at 24 h and at 12 and 16 weeks following daily repeated treatment. The frequencies of breaks in spermatocytes exhibited appreciable increase after 12 and 16 weeks of daily treatment. The incidence of abnormality in sperm head morphology increased following longer time-course of treatment. A tendency of decrease in the sperm count was noticed.

Neither break-type nor total aberrations increased significantly for two doses of lime tested in single treatment regimen in bone marrow cells. The higher dose which is three times the lower one increased significantly the breakage frequencies following longer time-course of treatment and the data exhibited a positive correlation with the time-course of treatment.

Structural changes of meiotic chromosomes and univalent formation in spermatocytes also increased following longer time-course of treatment. Frequency of sperm with abnormal head morphology increased with the increase of course of treatment. However, sperm count data showed a tendency of decrease following increase of the treatment course.

Analysis of CAs, SCEs and sperm head abnormality distinctly indicate the genotoxic potential of acetone extract of tobacco. A comparative analysis of the data for acetone extract of gudakhu (AEG) and acetone extract of tobacco (AET) with respect to tobacco consumption revealed higher effectiveness of tobacco when applied alone. In AEG series for 300 mg/kg dose a mouse received an extract of 250 mg of gudakhu which contains 25 mg of tobacco. But in case of AET series for 8 mg/kg dose a mouse received an extract of 8 mg of dry tobacco. So, the amount of tobacco a mouse
received in AEG series is 3 times more than that in AET series, but the effects induced by AEG is relatively less.

The clastogenic efficiency of molasses as indicated from the data is attributed to the pyrolysed products present in it.

Lime extract is alkaline in nature. At alkaline pH tobacco specific phenolics are supposed to be converted into potent clastogens.

Red-soil contains a number of oxides including $\text{Al}_2\text{O}_3$ and $\text{Fe}_2\text{O}_3$. In single treatment regimen there was no marked genotoxicity, but prolonged treatment schedule probably causes heavy release of $\text{Al}^{3+}$ and $\text{Fe}^{3+}$ and other metallic ions in the acidic pH of the stomach which may be responsible for causing aberrations.

5.4 EFFECT OF SALIVA IN MODULATING GUDAKHU INDUCED GENOTOXICITY

During use of gudakhu lot of saliva is secreted, and some users swallow the saliva mixed gudakhu and its extract; besides, saliva mixed gudakhu extract is absorbed by the buccal mucosa cells. To know if human saliva has any modulating role on the genotoxic effect induced by gudakhu extract we performed this experiment. Saliva from four non-users were collected. They had no habit of drinking, smoking or taking betel-quid or any other tobacco preparation. Similarly saliva was collected from one habitual gudakhu user who used to practise gudakhu for nearly last 20 years and 15 times a day.

Five different groups of mice were used for the purpose. Group-I served as negative control. Mice of Group-II received daily undiluted and UV-exposed non-user's saliva at the rate of 0.125 ml. Group-III mice received daily
acetone extract of 125 mg of gudakhu. Each of Group-IV mice was fed with an extract of 125 mg of gudakhu extract suspended in 0.125 ml of non-user's saliva. Group-V mice were fed with the user's saliva at the rate of 0.125 ml/day/animal. The daily treatment was continued for 4, 8 and 12 weeks; 24 h after the final treatment the animals were killed and their bone marrow cells, testes, vasa differentia and epididymes were processed.

Qualitatively the non-user's saliva showed a trend of increase of CAs in bone marrow metaphases, but the increase over the control was not so marked. But user's saliva yielded a five-fold increase of chromosome aberrations following 4 weeks of treatment and the increase was more with the increase of course of treatment. AEG also revealed significantly higher frequency of breaks particularly after 8 and 12 weeks of daily treatment. In mice treated with non-user's saliva plus gudakhu extract the frequency of chromosome aberrations at week 4 was close to that obtained with saliva or gudakhu extract alone. But the values for weeks 8 and 12 reduced remarkably, compared to the additive value, though not to the values for gudakhu or saliva alone.

Almost the same trend was found in the meiotic cells. As for sperm morphology the mixture of saliva and gudakhu extract induced less effect than the additive value at week 8, but the values for gudakhu extract, saliva and saliva plus gudakhu extract for week 4 were very close to the control value. The sperm count data did not show any appreciable variation from the control value.

Reduction in the incidence of chromosome aberrations and mis-shapen sperm in general following treatment of saliva plus gudakhu extract, compared to the additive values for gudakhu and non-user's saliva, supports
the earlier findings. Probably the enzymes and other proteins present in the saliva react antagonistically causing reduction of the effect. However, gudakhu user's saliva yielded high incidence of genotoxicity. It may be due to the fact that the saliva is loaded heavily with genotoxic agents and lose the inactivating capacity, or the genotoxic compounds released from tobacco may escape inactivation by factor(s) present in the saliva.

5.5 EFFECT OF GUDAKHU PRACTICE IN HABITUAL USERS

Study was also done on possible genotoxic effect of gudakhu use in habitual users in vivo. For this the micronucleus test was conducted in the buccal mucosa cells which are directly exposed to the agent; an attempt has been made to analyze micronuclei in the peripheral blood erythrocytes too of the habitual users. A total of 120 (59 males and 61 females) habitual gudakhu users were sampled. Their age ranged from 17 to 70 years with a mean age 39.38 years, and the history of using gudakhu varied from 1 to 42 years. None of the users had any habit of drinking, smoking or taking betel-quid or any other tobacco preparation. At the time of sampling it was made sure that they were not exposed to X-ray or medicinals for therapeutic purpose, or had no bacterial/viral infection at least 1 month prior to sampling. Sampling was also done from 102 non-users from the same localities whose age varied from 16 to 65 years. They were also non-smokers, non-drinkers, non-betel chewers, and served the purpose of control.

Exfoliated cells of buccal mucosa were scraped out from both the cheeks of habitual users and non-users and smears were prepared. The Feulgen stained smear counter stained with light green were examined for presence of MN.

Peripheral blood from finger tip was also collected from each of the habitual users and non-users who
provided buccal smear, and smear was prepared. Giemsa stained smears were examined for presence of MN in erythrocytes.

Analysis of MN in buccal mucosa cells: Micronuclei of varied sizes were noted in exfoliated cells. Some cells were found to contain two nuclei of normal size, nucleus with a blebbing, fragmented nucleus (Karyorrhexis) or condensed chromatin; all these were considered (separately from the micronucleated cells) together under the category nuclear anomaly for convenience in quantitative analysis.

Data on both micronucleated buccal mucosa cells and nuclear anomaly for users were sorted out on the basis of sex (male and female), years of use (≤5, 6-10, 11-20 and >20 years) and frequency of daily use (≤5, 6-10 and >10 times a day). Both years of use and frequency of daily use showed a strong positive correlation with the frequency of micronucleated cells, but sex had no such effect. Similarly an analysis among the non-users revealed absence of any influence of the age of the donors on the frequency of the micronucleated cells. However, the incidence of nuclear anomaly remained almost unchanged indicating little influence of years of use or frequency of daily use.

Analysis of MN in peripheral blood erythrocytes: Here both polychromatic and matured erythrocytes were considered. Almost the same trend as observed in the case of buccal mucosa cells was noted here. An increase in the incidence of micronucleated erythrocytes was noted, though not so markedly, with the increase of years of use or frequency of daily use.

The incidence of micronucleated buccal mucosa cells clearly demonstrates genotoxic potential of gudakhu use. The interesting point is that its frequency increases with
years of use as well as frequency of daily use. We do not like to attach much importance on the frequency of micronucleated erythrocytes at this stage because we failed to restrict our scoring among polychromatic erythrocytes only; in human micronucleated erythrocytes are known to be screened out by the spleen. More work is needed in this line.

Thus, gudakhu, like other tobacco preparations, is demonstrated to be highly genotoxic not only in experimental animals but in habitual users also.