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RESEARCH ARTICLE

EVALUATION OF CYTOTOXIC EFFECT OF SMOKELESS TOBACCO EXTRACT USING *ALLIUM CEPA* ABBERATION ASSAY

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ABSTRACT

Smokeless tobacco products are not burnt when used. There is an increasing interest and controversy within the public health community about smokeless tobacco. Smokeless tobacco especially the chewing tobacco commonly known as gutka was studied for its genotoxic effect using *Allium cepa* chromosome aberration assay. Gutka is an extremely popular herbal concoction sold throughout subcontinent. Aqueous extract of the selected smokeless tobacco was prepared, EC_{50} value for Allium root growth inhibition was determined (1000ppm) and accordingly different concentration in ppm (100, 300,500,1000,1500,2000, 2500, & 3000 ppm) were selected to study the effect in response to time (24 hrs and 48 hrs) on the mitotic activity of the growing root tips. The mitotic index was the least (2.04) at the highest concentration (3000 ppm) at 48 hrs treatment of gutka than 33.20 (24 hrs) and 36.37 (48 hrs) in the control set using normal water. The genotoxicity of gutka was measured using the frequency of chromosomal aberrations. The high frequency of sticky chromosomes, laggard and multipolarity indicated that smokeless tobacco product like the gutka too can cause abnormal DNA condensation, abnormal chromosome coiling and inactivation of the spindles, having an eugenic potential. The Cytotoxic threshold concentration was estimated to be 500 ppm though no lethal effects were observed.

INTRODUCTION

The use of tobacco is a public health problem worldwide and has a significant impact on socio economic status and environment sustainability. Tobacco is used in various forms and is a global epidemic among the young adults. A study in United States has reported serious health threat and significant implications on nation's public and economic health in the future (Kessler 1995).Some believe that the enormity of the health problems are associated with cigarette smoking, than the smokeless tobacco products generating a controversy within the public health community(Stratton *et al*., 2001). This has resulted in the use of a viable alternative, nicotine containing products with lower mortality and morbidity and with recommendation for the cigarette smokers to switch to smokeless tobacco products (Rodu and Godshall, 2006; Brinson, 2006). In India about 72% of tobacco consumed is in the form of smoked tobacco and rest is used mostly in smokeless form. Smoked tobacco goods are cigarettes, bidis and hookah (water pipe). Smokeless tobacco goods include paan (piper betel leaf filled with sliced areca nut, lime, catechu, and other spices chewed with or without tobacco), gutka (tobacco mixed with areca nut, slaked lime, and catechu), zarda (mixture of tobacco, areca nut, areca catechu, and slaked lime with added fragrance, spices and flavors), khaini (tobacco leaves with slaked lime), mishri (a powder tobacco rubbed on the gums as paste), and kaddipudi(dried tobacco leaves taken orally or with betel leaves) (Nelson *et al*., 2006; Federal Trade Commission, 2007). Gutka is dry, relatively nonperishable commercial preparation containing areca nut, slaked lime, catechu, powdered tobacco, along with certain flavoring and food additives (Gupta, 1984). Gutka is a dried version of paan without the betel leaf, can be preserved and perfumed with chemicals sealed in a plastic or foil pack. It is placed in the cheek lining, savored and then expelled. Around 5 million young Indians are suffering from oral submucosa fibrosis, a disease which is precursor of the oral cancer as a result of increased popularity with habits of chewing pan masala and gutka (Nair *et al*., 2004). A large number of studies have revealed genotoxicity of the tobacco and tobacco containing products consumed by human population (Jyoti *et al.,* 2011). Since the use of animals in toxicology research and testing is a concern for both science and ethics, therefore *Allium cepa chromosomal assay* was considered as a valid alternative. Healthy normal onion cell has 16 chromosomes. This model is simple, cheap, reproducible and effective for evaluating and monitoring cytotoxicity and genotoxicity of any chemicals and mixture substances (Grant, 1978). In the present investigation allium root tips were treated with aqueous extract of gutka of different concentration for 24hours and 48 hours to study the genotoxic effect of gutka on the meristematic cells.

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MATERIALS AND METHODS

The habit of chewing of gutka is prevalent among both young and old in the population. For the study Gutka sold under the brand name Kolhapuri was used to prepare aqueous extract of

3000ppm concentration as stock solution and small onion bulbs of average size (2cm – 4cm) were selected for the chromosome aberration assay. The loose outer scales of onion were carefully removed and the dry bottom plates were scraped away without destroying the root primordial. The onion bulbs were allowed to produce roots in tap water for 3 days. On the third day all the bulbs were transferred to the test solutions (250,500, 750, 1000, 1250 ppm) and EC_{50} value for inhibition of root growth was determined. Later according to EC_{50} value different dilutions from the stock test solutions were made (100, 300,500, 1000, 1500, 2000, 2500 & 3000 ppm) and tested for the genotoxic effect. For each concentration six onions were set up. The rooted bulbs were directly exposed to the gutka extract for 24 hours and 48 hours. After treatment, the root tips were fixed immediately in aceto-alcohol (1:3), the slides were prepared for microscopic examination using 5-6 root tips from each bulb. The root tips were hydrolyzed in 1 N HCl at 60^0 C for 3-5 min, then squashed in 2% acetocaramine and observed first under low power than under high power (45X) of the microscope. Later, the chromosomal aberration stages were photographed. The mitotic index, phase index and chromosome aberrations were determined by examination of at least 1000 cells. The number of dividing cells was scored and the mitotic indices calculated for treated and control (Balog 1982). Data pooled was statistical analyzed using One Way ANOVA; Correlation and linear regression analysis (SPSS 15.0) to evaluate the relationship of different concentration of the smokeless tobacco (gutka) extract time period on the mitotic index values.

RESULTS AND DISCUSSION

Global adult tobacco survey of 2009 – 2010 reports that 53.5% of Indians use gutka products constituting 66.2% of men and 40% of women. Gutka use is assumed to be leading to an increased risk of cancer of the gums, mouth, throat, lung, liver, stomach, prostate and oesophagus. India has 75,000 to 80,000 new cases of oral cancer per year, the world's highest incidence, according to the World Health Organization (WHO). Since 2013, gutka a kind of smokeless tobacco is banned by government of Maharashtra. In the present study the genotoxic effect of the aqueous extract of gutka on the growing root tips of onion with respect to the concentration – time response relationship was studied. Certain changes were observed during the onion root development in the experimental samples, the growth of roots in control set was much faster than in all the treated sets irrespective of the gutka concentration. The length of root decreased as concentration of the experimental gutka solution increased. The colour of control root was white, and the roots that were treated appeared brownish as concentration of the experimental gutka solution increased. EC_{50} value is the concentration that produces 50% decrease in root growth. The root tips were initially exposed to different concentrations of gutka aqueous extract (250, 500, 750, 1000 & 1250 ppm) for 48 hours. The EC_{50} value was found to be 1000ppm. This value was used to design the experimental concentration of 100, 300, 500, 1000, 1500, 2000, 2500 & 3000ppm for further study. The result revealed that most of the chromosomal aberrations observed were at metaphase and at anaphase, and very few at prophase and telophase. In all the concentration range lower than the 1000 ppm, aberration observed were vacuolated nuclei and sticky chromosomes at metaphase (100 ppm);vagrant chromosome, irregular metaphase, arrested metaphase and sticky chromosome (300 ppm); spindle abnormalities in anaphase and metaphase with chromosomal break, sticky chromosome and multi vacuolated nuclei (500 ppm); and in 1000 ppm vagrant chromosome, spindle aberration were observed. Adhesion of the centromeres of one or more chromosomes to the outer layer of the plasma and movement of the others towards the equatorial plate led to the appearance of such lagging chromosomes (Amer and Ali, 1968). Disturbed metaphase and ana-telophase observed might be due to the disturbance of the spindle apparatus. The chromosomal damage produced by chemicals may be due to their effect on DNA (Grant, 1978).

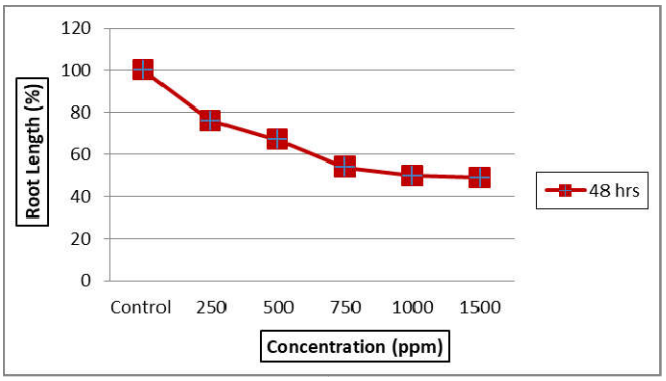
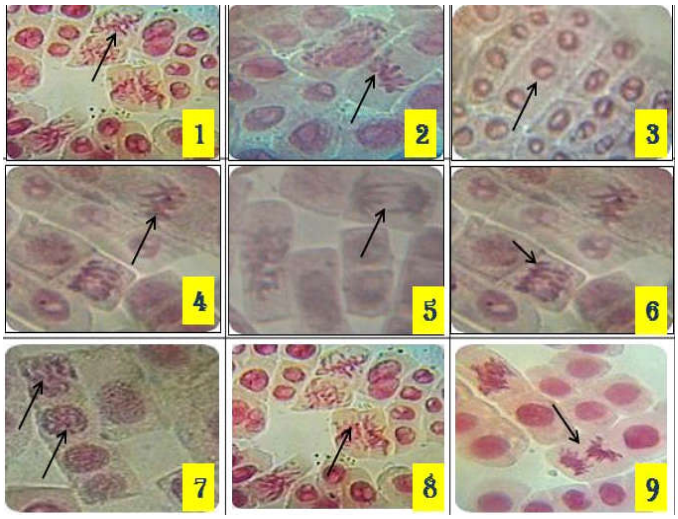


Fig 1. Inhibition of root growth in *Allium cepa* exposed to extract of Smokeless Tobacco

While in concentrations higher than 1000 ppm spindle disturbance at prophase, vagrant chromosome, chromosomal bridge, vacuolated nuclei (1500 ppm); multi vacuolated nucleus, vacuolated nuclei with vagrant anaphase (2000 ppm); multi polar anaphase and spindle disturbance at prophase (2500 ppm) and abnormality in cell shape, chromosomal break, chromosomal bridge and s- shape nucleus (3000ppm) were observed (Plate No.1). The aberrations like chromosome bridge single or multiple bridge in anaphase was observed in the meristematic cells treated with smokeless tobacco extract of concentration above 1000ppm. This may be the result of chromosome breakage and reunion (Badr, 1992). Similarly aberration like chromosome stickiness was observed in root tips treated with 300ppm. Such stickiness of chromosomes may be due to enlargement of inter chromosomal chromatin fibres (Chuvhan *et al*, 1986). The stress in anaphase movement may result in fragmentation of chromosome (Khanna & Sharma, 2013). The duration of time (24 hours compared to 48 hours) has positive influence on the chromosomal aberrations as the frequency of aberration increased with time. The mitotic index was highest in the control 33.20 and 36.37 for 24 hours and 48 hours respectively than the treated ones. The mitotic index reflects the frequency of cell division and regarded as an important parameter in evaluating the rate of root growth. The mitotic index values progressively decreased as the concentration of gutka and period of treatment increased indicating a concentration-time response relationship. The gutka exhibited a strong mito-depressive effect on the mitosis of *A. cepa* (Fig. 2). It was observed that with increasing concentration of the smokeless tobacco extract, the mitotic index was the least. There was 90 % and 94.4% decrease in the mitotic index after 24 hours and 48hours respectively as compared to the normal (control) treatment. Such decrease in mitotic index is reported to be the result of cytotoxic effects (Smaka –Kincl *et al.,* 1996).



1) Disordered metaphase; 2) C-Metaphase; 3) Vacuolated Cell; 4) Laggard; Multiple Bridge; 5) Anaphase with Multiple Bridge; 6) Alignment at Anaphase; 7) Sticky Prophase and Irregular Prophase; 8) Aberration in Telophase; 9) Single Bridge with Vagrant Chromosome

Plate 1. Chromosomal aberration in *Allium cepa* root tips treated with different concentration of smokeless tobacco extract

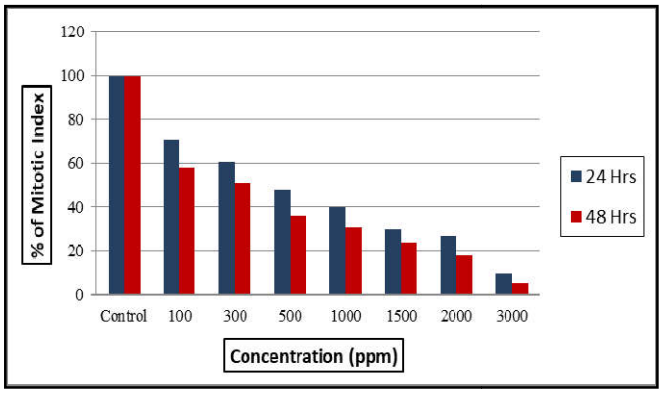


Fig. 2. Influence of smokeless tobacco extract in different concentration for varied time duration on mitotic index (%) in *Allium cepa* root tips

The cytotoxic threshold is the concentration causing 50% mito-depression as compared to the control. This concentration was estimated to be 500 ppm of smokeless tobacco extract, though no lethal effect was observed. It has been reported that decrease in the mitotic index may be due to the result of the suppressive effect of the extract on DNA and nucleoprotein synthesis (El-Ghamery *et al* 2000). The mitotic phase index increased with the increasing concentration of the smokeless extract (Fig 3 and 4). The prophase index increased significantly and showed a strong correlation when exposed to 24 hrs (r 0.954, P <0.05). Similar effect was observed when other mitotic phases were evaluated, it appear to be significant though a weak correlation was observed for metaphase (r 0.9409; P <0.05), anaphase (r 0.9174; P<0.05) and the telophase (r 0.8574; P<0.05). Moreover a strong correlation was also observed between the prophase index and the time period of 48 hrs (r 0.9717, P <0.05), but a weak correlation for other mitotic phases, metaphase (r 0.9657; P <0.05), anaphase (r 0.9318; P < 0.05) and the telophase (r 0.9579; P < 0.05). (Scolnic and Halazonetis (2000) have reported that there exist a control point between prophase and metaphase that prevents the entry into the anaphase in cells treated with chemicals. The appearance of sticky chromosomes, arrested metaphase, disturbed spindle and vagrant chromosomes, all such aberrations can be considered as conditions that are in agreement with Scolnic and Halazonetis (2000).

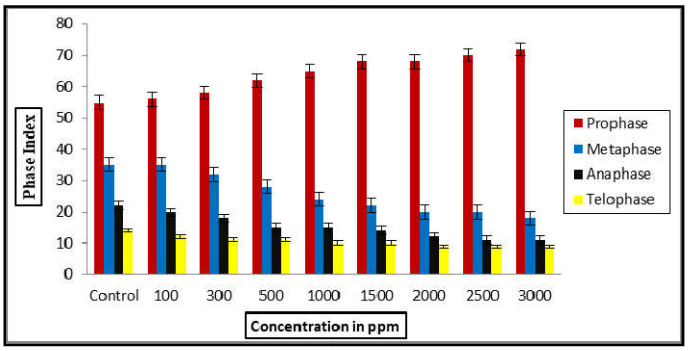


Fig. 3. Percentage mitotic phase index in *Allium cepa* root tips induced by different concentrations of smokeless tobacco extract exposed for 24 hrs

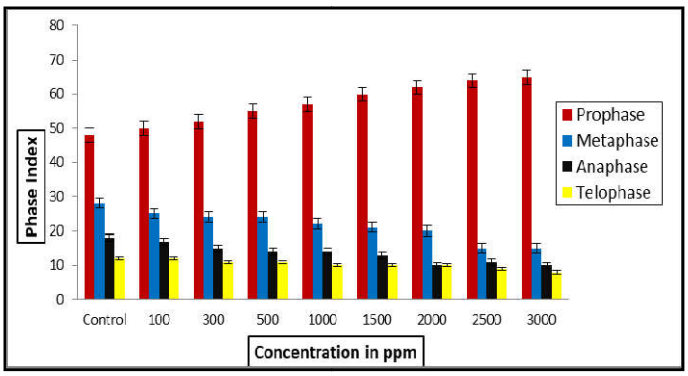


Fig. 4. Percentage mitotic phase index in *allium cepa* root tips induced by different concentrations of smokeless tobacco extract exposed for 48 hrs

Conclusion

The result of our study clearly indicates the cytotoxic effect of the aqueous extract of smokeless tobacco, gutka. The cytotoxic effect observed depends both on the concentration and the exposure time period. All the concentrations, except 100ppm exhibited an inhibition in the mitotic division of the Allium root tip cells. The mito-depressive effect observed in the present investigation suggests that Gutka is strong mitotic inhibitor and could give rise to mitotic abnormalities with increase in concentrations. The genotoxic effects of the Gutka established in this study indicates that the gutka contain toxic substances, which may constitute a risk to the human health.

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