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In vivo mitodepressive and genotoxic effects induced by herbal toothpowders in *Pisum sativum*

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Abstract: The potential genotoxic effects of aqueous extracts of three herbal toothpowders which are commonly used as teeth cleaning agents to treat a variety of oral conditions was investigated using cytogenotoxicity assay in *Pisum sativum*. The extracts of 1%, 2% and 3% concentrations were tested on root meristems of *P. sativum*. Distilled water was used as negative control. The result showed decrease in mitotic index comparatively with enhanced level of concentrations of herbal toothpowder extracts. The drastic retardation in mitotic index (13.23%) was recorded for 3% herbal powder sample C. A dose-dependent increase of chromosome aberrations was also observed. Abnormalities scored were stickiness, bridges, laggards, vacuolated chromosomes and polar deviations. Result obtained in this study confirmed that the aqueous extracts of herbal toothpowders exerted significant mitodepressive and genotoxic effects.
Keywords: genotoxicity, *Pisum sativum*, herbal toothpowders, mitotic index, chromosome aberrations.

Introduction

Good oral health has a major influence on an individual's quality of life. There exists an increasing global demand for the development of new preventive and treatment methods and products that are also economical, safe, and effective.

Tooth powders are more popular in suburban and rural areas[1]. The constituents of tooth powder and tooth pastes are same except that tooth powders do not contain humectants, water and binding agents[2]. Herbal tooth powder is a tooth-cleaning agent that is almost entirely made from all-natural ingredients. Its purpose is to freshen breath, help heal gums, rid teeth of bacteria, and reduce the amount of inflammation in the mouth. Herbal tooth powder has been around for centuries and many believe it to be an essential part of any teeth-cleaning regimen[3]. Basically herbal tooth powders are considered to be safe for daily use. More over herbal tooth powders are free from Sodium lauryl sulphate, parabens, fluoride source (NaF), chlorine source and sodium saccharin.

The belief that natural plant products are much safer than synthetic drugs has caused exceptional growth in human exposure to natural products, as plants, phyto-therapeutic agents, and phyto-pharmaceutical products. This fact has lead to a resurgence of scientific interest in their biological effects. Based on the long-term use of herbal medicines by humans, one might expect herbs used in traditional medicine to have low toxicity. Nevertheless, some of them can cause adverse effects or have the potential to interact with other medications and lead to toxicity; moreover, there is little information on the potential risk to health of such herbs[4]. Recent investigations have revealed that many plants used as food or in traditional formulation have mutagenic, cytotoxic and genotoxic effects *in vitro* and *in vivo* assays[5].

In screening for genotoxic and cytotoxic effects, extracts of different plant parts have been used, ranging from leafy vegetables, fruits, and underground storage organs to whole plants. Among the plant species, *Alium cepa* and *Pisum sativum* have been used to evaluate DNA damages, such as chromosome aberrations and disturbances in the mitotic cycle. The mitotic index and some nuclear abnormalities are used to evaluate cytotoxicity, genotoxicity and analyze micronucleus to verify mutagenicity, of different chemicals. MI measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be considered as cellular death or delay in the cell proliferation kinetics[6]. In this study, seeds of *P. sativum* were used to assess the cytotoxicity and genotoxicity of aqueous extract of herbal toothpowders.

Materials and Methods

Three brands of herbal toothpowders (Fig. 1) were purchased from the local market of Mumbai. Three different concentrations of each toothpowder were prepared for treatment in distilled water. 1%, 2% and 3% concentrations were prepared by mixing 0.5gm, 1gm and 1.5gm toothpowder in 50 ml distilled water. The above mixture was sonicated at 50% frequency for 20 minutes and then filtered using Whatman filter paper no. 1. The obtained extract was used to treat the *P. sativum* seeds for an hour. Distilled water served as the negative control. After the treatment, the seeds were washed with distilled water and were allowed to germinate for 24 hours on wet blotting paper. The germinated root tips were cut and fixed in Carnoy's fluid. The fixed material was stored in the refrigerator for 24 hr. After fixation, the root tips were transferred to 70% alcohol for further preservation.

Three slides were prepared for each concentration and control. The mitotic index was calculated as the number of dividing cells per 100 observed cells. The slides were examined under the microscope for induction of chromosomal aberrations. The slides with good stages were sealed by wax coating. The microphotographs of the important stages of interest were taken on Olympus light microscope with digital camera on X100 objective. Various types of aberrations induced by each treatment at various stages were recorded.

Fig 1: Three different herbal toothpowders used: A. Toothpowder sample A, B. Toothpowder sample B, C. Toothpowder sample C.

Result and Discussion

Table 1: Cytogenetic analysis of *Pisum sativum* root tips exposed to the treatment of Herbal toothpowder samples with different concentrations.

Sr. no.	Conc. of tooth powder extract	Total no. of cells screened	Number of dividing cells				Mitotic index (In %)	Total no. of aberrant cells	Cells with chromosomal anomalies (In %)
			Prophase	Metaphase	Anaphase	Total no. of dividing cells			
1.	Control	509	19	39	68	126	24.75	1	0.19
2.	A- 1%	542	13	31	62	106	19.55	5	0.92
3.	A- 2%	496	13	18	45	76	15.32	8	1.61
4.	A- 3%	506	12	22	48	82	16.20	14	2.76

5.	B- 1%	560	21	30	71	122	21.78	2	0.35
6.	B- 2%	535	22	28	58	108	20.18	5	0.93
7.	B- 3%	513	12	25	52	89	17.34	7	1.36
8.	C- 1%	565	23	33	72	128	22.65	7	1.23
9.	C- 2%	590	12	37	59	108	18.30	12	2.03
10.	C- 3%	582	16	19	42	77	13.23	21	3.60

In this study, toxic effect of herbal toothpowder extracts was evaluated by analyzing root morphology and mitotic cell screening. The higher concentration of toothpowder extracts causes an inhibition of root growth. Morphological deformities observed in treated material were short, bent, spiral and crochet-like roots, in comparison to the control roots. In addition, the toothpowders extracts induced light yellow coloration in the roots. Cytogenotoxicity was estimated by studying cytological parameters such as the mitotic index and chromosome abnormalities. The occurrence of normal dividing cells was prominently recorded in controlled root tips of *Pisum sativum* (Table 1, Fig. 2). However, retardation in mitotic cell division was observed in all the treated samples with the highest in sample C with 3% concentration. Toothpowder sample C showed decrease in the mitotic index at high level as the concentration increases followed by Toothpowder sample A and then Toothpowder sample B. The mitotic index (MI) in *P. sativum* meristematic cells treated with the toothpowder samples A, B and C was significantly decreased at the highest concentration, i.e., 3% as compared to negative control (8.55%, 11.52% and 7.41%, respectively).

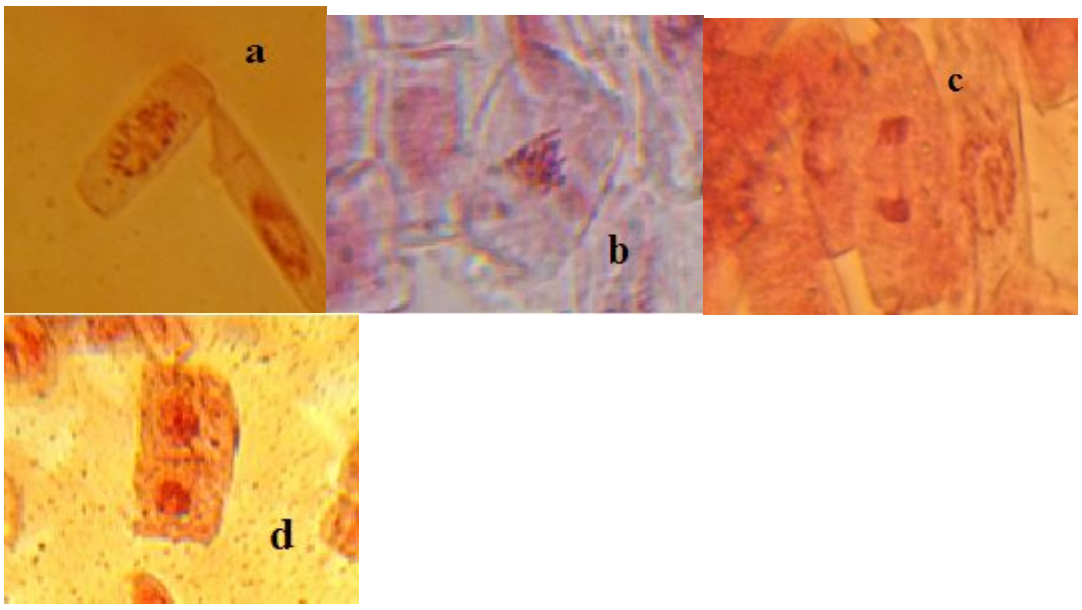

Figure 2: Normal stages of mitosis: a- Normal Prophase, b- Normal Metaphase, c- Normal Anaphase and d- Normal Telophase.

The dividing cells with abnormalities were more in the treated root tips than in the control root tips (Table 1). 3% concentration of the water extract for all three toothpowder samples has induced highest number of abnormal cells; however, this number was not significantly different from that of 2% concentration. Among the three different concentrations of water extract, 1% induced the least number of abnormal cells. In fact the linear correlation was noted in number of dividing abnormal cells with successive enhancement in the studied concentrations. The chromosomal aberrations observed were chromosome breaks, stickiness, and polar deviations. The different types of abnormal cells induced were vacuolated cells at interphase (Fig. 3j), sticky metaphase (Fig. 3a & 3b), lagging chromosomes (Fig. 3g, 3h & 3i), and anaphase bridges (Fig 3c-3f). In addition to the chromosome fragments, polar deviations (unusual directions of chromosome movement) were also observed (Fig. 3k). Few incidences of occurrence of aberrant cells were also observed in control root tips which may be attributed to the spontaneous process. There was a linear relationship between macroscopic and microscopic parameters for all the extracts. The 3% extract of toothpowder sample C has prominent inhibitorier and mitodepressive effects (3.60% aberrations) than all the other extracts, followed by 3% concentration of toothpowder sample A with 2.76% occurrence of structural anomalies.

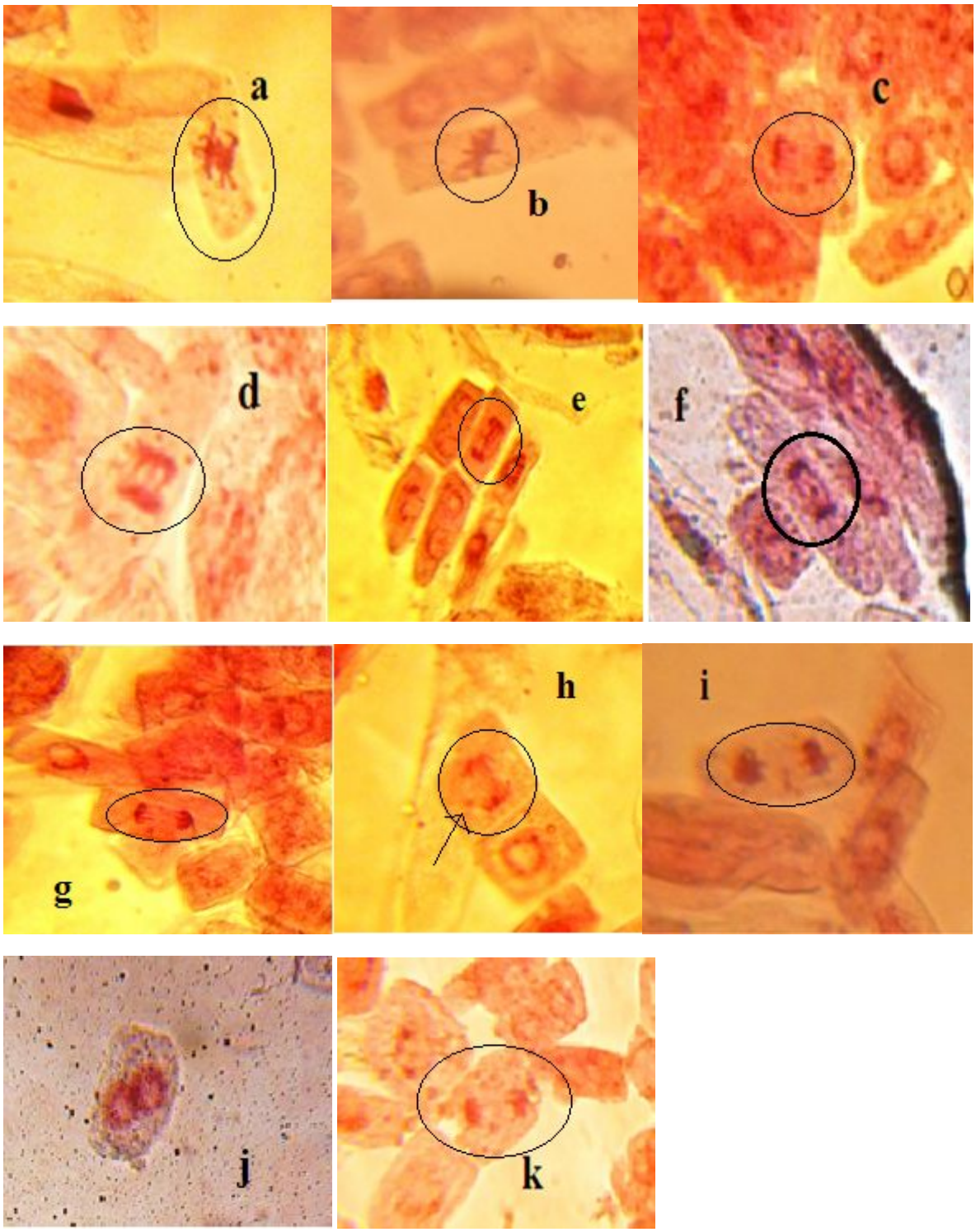

Figure 3: Induced Chromosomal aberrations as observed in *Pisum sativum* by treatments of studied Toothpowder samples. a, b- Sticky chromosomes, c, d, e, f- Chromosomal bridges, g, i- Laggards, h- Laggard and chromosome bridge, j- Vacuolated chromosome and k- Polar deviation.

Higher plants such as *P. sativum* are accepted as admirable genetic models to evaluate genotoxic effects. Results of the current study reflected the utility of root tips cells of *P. sativum* for monitoring the genotoxic effects of herbal toothpowder extracts. Cytotoxicity assay in *Pisum sativum* enabled the assessment of different genetic endpoints, which are mitotic index and chromosome aberration. Mitotic index is used as an indicator of cell proliferation biomarkers which measures the proportion of cells in the mitotic phase of the cell cycle. Hence, the decrease in the mitotic index in *P. sativum* somatic cells could be interpreted as retardation in mitosis or cellular death. The depression of mitotic index reflects negative impacts on somatic growth at initial stages as a primary effect in the development of plant[7].

Chromosome bridges indicate that the clastogenic effects are caused by chromosome breaks. The chromosomal stickiness was common in occurrence in this study (Fig. 3a & 3b). Chromosomal stickiness may result due to improper folding of chromosome fibers which makes the chromatids connected by means of sub-chromatid bridges[8]. However, this stickiness may be interpreted as a result of depolymerisation of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fiber units of chromatids and the stripping of the protein covering of DNA in chromosomes[9]. In many studies sticky chromosomes indicated a highly toxic, irreversible effect, probably leading to cell death[10].

Another noticeable abnormality was the occurrence of chromosome bridges. Chromosome bridges were commonly observed during anaphase (Fig. 3c-3f). The bridges noticed in the cells were probably formed by breakage and fusion of chromatids or sub-chromatids[11]. Chromosome bridges may be caused by stickiness of chromosomes which blocks their separation and free movements and thus they remained connected at both the ends[12].

Chromosomal aberrations are changes in chromosome structure resulting from a break or exchange of chromosomal material. Our results showed among other aberrations, induction of sticky chromosomes, bridges at different stages of mitotic division in the pea root cells. In general, whenever chromosome aberrations occurred, there are always growth restrictions to certain extent and it was also evident in present study. Most of these aberrations are lethal which can cause somatic or inheritable genetic effects[13].

There is a consensus that many of the components of medicinal plants bind onto tubulin and either inhibit tubulin assembly or cause the depolymerization of already assembled microtubules. The spindle so disturbed according to him, is reduced and may eventually disappear, resulting in the blockage of cell division at prophase or even metaphase and their separation at anaphase. He also speculated that there may be a possible amplification of anti-spindular effects where two or more anti-spindle compounds may be present in the same medicinal plant since different binding sites in tubulin may exist for different anti-spindle compounds[14]. Anaphase bridge is one of the 3 types of aberrations that are lethal to the cell, the other two being dicentric and the ring chromosomes[15]. Bridges cause structural chromosome mutations that include duplications or deletions in DNA double strand[16].

Lagging chromosomes (Fig. 3g-3i) have been a regular feature of many cytotoxicity and genotoxicity studies with medicinal plant extracts[17]. One paper was of the opinion that such chromosomes have the potentials to form micronuclei[18]. The presence of such nuclei is a manifestation of the efforts of a main nucleus to eliminate excess DNA in an attempt to restore the normal ploidy condition[17]. A positive result in *Allium* test system should be taken to indicate a potential biological hazard and that false negative have been shown to rarely occur in either the *Allium* test or other similar plant tests[19]. Thus, the occurrence of these chromosomal anomalies in *Pisum sativum* in the present study is an indication of toxic potentials of water extracts of herbal toothpowders.

Conclusion

In this study, we have used aqueous extracts of three herbal toothpowders. Herbal toothpowders are a complex mixture of biologically active compounds. Retardation of mitotic index and insertion of chromosomal structural changes are the clear indications of the ability of some constituents in herbal toothpowder to exhibit its impact at cytotoxic and genotoxic level. However, some of the constituents in the extract contain metabolic components like flavonoids, saponins, tannins which can be cytotoxic and genotoxic, others can be cytoprotective and/or antigenotoxic. From the present study it appears that the herbal toothpowder aqueous extracts, which are used as teeth cleaning agents in rural India, clearly exhibits mitodepressive effects at 3% and 2% concentrations. Therefore, it is necessary to take precautions when using these extracts for daily oral health. In addition, further cytogenetic studies dealing with clastogenicity and genotoxicity of these extracts with more comprehensive genotoxicity assessment in animal model may reveal further interesting results for its safe suitability for human health.

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