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Evaluation of cytotoxic and genotoxic effects of Butyl Hydroxylated Anisole (BHA) on somatic cells of *Allium cepa* L.

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Abstract- Food preservatives are intentionally added in small quantities during processing of food to improve the taste quality and shelf-life of the food. Therefore, we are constantly exposed to different food preservatives in daily life that may or may not show adverse effects. Evaluating the cyto-genotoxicity of food preservatives is necessary. Dose dependent and Time dependent effect of Butyl hydroxylated anisole (BHA), commonly used food preservative was done at two concentrations, 0.01mg/L and 0.04mg/L. The mitotic index had been calculated for the control and treated cells. The mitotic indices at 24hrs were 71.67±1.527 for control and 69.33±2.081 and 35.33±1.527, respectively. The mitotic indices at 48hrs were 54.67±2.081 and 27.67±2.081, respectively. The best results were observed in 72 hrs where the mitotic indices were 48.33±2.88 and 18.33±2.88 at 0.4mg/L. Chromosomal abnormalities like multinucleated cells, laggard and anaphase bridges were observed. The effect of preservative BHA on the somatic cells of *Allium cepa* was cyto-genotoxic.

Key words: Butyl hydroxylated anisole (BHA), onion, mitotic index, genotoxic, chromosomal aberrations.

INTRODUCTION

The genotoxic effect of various chemicals used as food preservatives, cosmetics and drugs has been well studied. Food preservatives are compounds that improve the quality and shelf life of food.[1] Natural food preservatives like salt and sugar were utilized to extend the shelf life of the food by improving their texture, taste, acidity/alkalinity, and consistency.[2] The food industry has changed dramatically in recent years, and the most challenging task is to increase global food quality, which is attained by using chemicals to enhance food flavour, colour and quality. To keep the veggies and fruits fresh for several days, various colouring compounds and waxes were and still being utilised. These compounds have never been used as food ingredient or ingested directly but added as adjuvants.[3] Adeyemo & Farinmade (2013)[4] reported that the present and upcoming generations are on the higher risk for exposure to these chemicals.

Butyl Hydroxylated Anisole (BHA), chemical mixture of 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole molecular formula $C_{11}H_{16}O_2$ has large scale uses in various food industries.[5] It is also the most widely used food antioxidants owing to its low-cost, world-wide availability, stable at high temperature and high performance. It is widely used in cosmetics, food

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packaging, oils, margarines, fat-containing products, rubber industry and petroleum products. At high doses, it causes cancer in rats, mice and hamsters, but it does this exclusively in the forestomach, an organ that humans don't have. In the low proportions used in food preservatives, however, many researchers consider it perfectly safe, especially due to absence of forestomach in humans. In an experiment, the higher dose of BHA was given to the Japanese house musk shrew, lacking fore-stomach, all the experimental model organisms died because of gastrointestinal hemorrhage. Whereas, the lower and mild dosages had effect on 50-60% models showing adenomatous hyperplasia of the lung.[6] There were no reports on BHA causing genotoxic or any adverse effects on human beings, but during current situation various reports are found showing the genotoxic effects.[7] BHA had been also reported as carcinogens by International Agency for Research on Cancer (IARC). The possibility that BHA may be a carcinogenic at normal human dietary levels, which are much lower than the experimental tumor-inducing levels, has also been questioned on the grounds that it is not mutagenic in a variety of test systems.

Genotoxic studies were performed to evaluate the rate of mutation which enhanced due to food preservation addition by food companies using *Allium cepa.*

Allium cepa has been chosen as a model to study the genotoxicity of BHA to test and monitor environmental pollution indicator. It has total number of 16 chromosomes only, thus studies involving chromosomal aberrations can be efficiently done during mitotic stages. The change in chromosomal morphology is due to the genetic changes induced through using food preservatives or any other harmful chemicals. *Allium cepa* test was first performed by Levan in 1938[8] and it was observed that there is similarity with the mammalian test model. Many of the chemical assays are first performed on onion root tips and then further elaboration of work is done using the mammalian system.[9,10] DNA alterations are known to be the early signs of damage in the organisms. The outcomes of these assays provide significant results having relationship with genotoxicity appearance in the mammalian body and more pounced with the children exposed perinatally.[11] In the present study the effect of BHA on the growth of onion root tips and effect on mitotic index and chromosome damage was analyzed.

MATERIALS & METHODS

The fresh bulbs of *Allium cepa*, of medium sized were taken from the local market. Butylated Hydroxy Anisole (BHA), chemical formula $C_{11}H_{16}O_2$, molecular weight 180.24 g/mol, procured from LOBA CHEMIE. For preparation of Carnoy's fixative chemicals taken from SRL was glacial acetic acid and ethyl alcohol in 1:3 ratios. For the hydrolysis of cells HCl was purchased from Thomas Bakers and the stain acetocarmine was from LOBA CHEMIE.

Raising fresh tips and treatment with BHA

The experimental set up was designed with slight modification of method given by Fiskesj (1988)[12]. The first two days onion root tip were grown on fresh water and then followed by treatment with BHA for 24 hours, 48 hours and 72 hours, respectively. To obtain fresh root tips, the dried roots were removed using sharp blade without damaging the meristematic part. The onion bulb was then placed on the coupling jar filled with water in a way that meristematic part of bulb is immersed in water. After 48 hours 2-3 cm long fresh onion root tips emerged out, the bulbs were then transferred at three different concentration (0, 0.01 mg/L and 0.04 mg/L) of BHA and after 24hrs 5-8 root tips were excised and fixed in Carnoy's fixative after 48hrs again 5-8 tips were excised and fixed in Carnoy's fixative and after 72 hours of treatment the left-over roots were excised and fixed.[12] After 24 hours treatment with Carnoy's fixative the tips were transferred to 70% alcohol at 4°C.[13]

Squash preparation of treated root tips

Stored onion root tips were placed at room temperature then the translucent area at the tip of roots (2-3 mm) was excised on the slide and treated with 0.1 N HCl for 2-3 minutes in case the tip is hard. This step is not required if tip cells separate with slight pressing with thumb. Then 2-3 drops of lukewarm acetocarmine stain were placed above the root tip for approximately 2 minutes. The root tip was covered with coverslip and with the help of blotting sheet the excess stained was removed. The slide was placed under 2-3 layers of blotting sheets and pressed using thumb, the onion root tip cells spread on the slide and further spreading of the cells is done by gentle heating and tapping. For each sample 3 slides were prepared and observed under Nikon Eclipse E200 with digital camera microscope at 40X and 100X magnification and 3 pictures were taken randomly from each slide for

the calculation of mitotic index and study of chromosomal morphology.

Mitotic index was calculated for three different concentrations of BHA using the formula:

$$Mitotic\ index\ MI = \frac{number\ of\ dividing\ cells}{total\ number\ of\ cells} x\ 100$$

Different types of aberrations were observed as an indicator of the specificity in the mutagenic action of food preservatives.

RESULT & DISCUSSION

The dose-dependent reduction in mean root length has been observed at higher concentrations significantly differ from the control but it may not be significant within the same group. In control, the roots of the onion bulbs were growing well hydroponically and morphologically it appeared long, healthy, shiny, fast growing and dense roots whereas treated root tips were less dense, slow growing and lesser number of root tips (Fig 1).

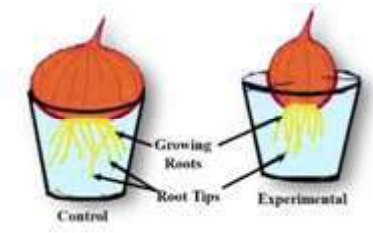

Figure 1- Experimental Set-up

Doses between 0.04 mg/L for 72 hrs showed significant reduction in growth when compared to control but insignificant when compared with each other whereas concentration of 0.04 mg/L was observed to be insignificant. The calculated mitotic indices have been tabulated for control and the treated cells (Table 1).

The BHA at recommended dose (0.01 mg/L) and higher dose (0.04 mg/L) in comparison with the control setup reveals very prominent chromosomal aberrations at recommended dosage but at higher doses most of the cells arrested at prophase stage. The mitotic index was high in controlled setup and gradually it decreased with increase in dosage as well as time of the treatment (Figure 2). The observation indicated the inhibitory effect of BHA on the onion mitotic cells both in terms of division and chromosomal aberrations. The chromosomal aberrations observed were chromosomal bridges, breaks, laggards and multipolar nuclei (Figure 3).

Table 1: The mitotic index MI of onion root tips treated with three different concentrations of BHA treated for 24, 48 and 72 hours.

Concentration of BHA	Mitotic Index (%)		
	24 hours	48 hours	72 hours
Control	71.67±1.527	67.00±1.732	82.33±2.081
0.01 mg/L	69.33±2.081	54.67±2.081	48.33±2.88
0.04 mg/L	35.33±1.527	27.67±2.081	18.33±2.88

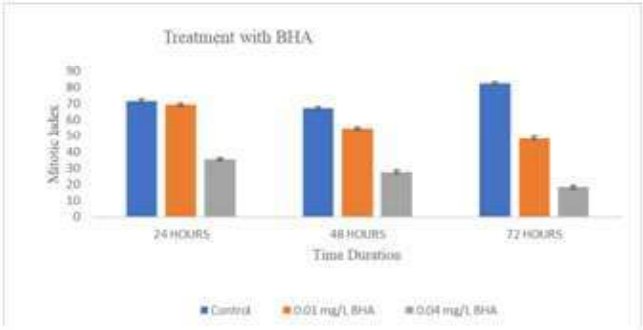

Figure 2- Bar chart showing the mitotic index of root tip cells treated with Butyl hydroxylated anisole (BHA) for different time duration

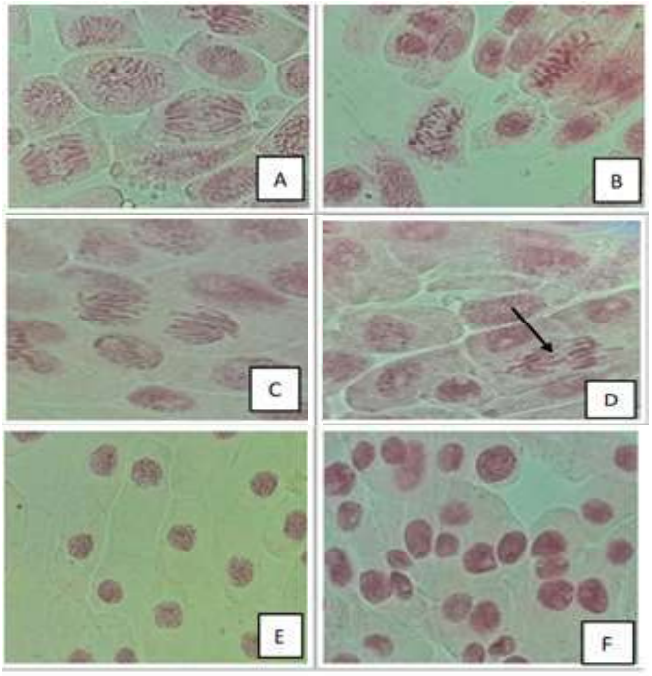

Figure 3- The mitotic cell of onion root tip (A) The untreated root tips showing normal dividing cells after 72 hrs (B) The root tips treated with 0.01 mg/L BHA for 24 hrs showing metaphase and anaphase (C) The root tip from the same onion bulb as shown in B at 48 hrs showing less dividing cells (D) One of the anaphase cell showing laggards (E& F) The root tip cell treated with 0.04 mg/L BHA after 24 and 72 respectively showing complete absence of dividing cell or at the interphase.

Mitotic division is a mandatory process of living cell and involves highly controlled steps.[14] The complete cell division is very precisely regulated in the presence of essential nutrients and other factors. Any change in condition results in halting and/or alterations in the cell division. Food preservatives which have been added in the food to increase quality, texture, taste and colour directly or indirectly causes alteration in the cell division cycle which results into chromosomal aberrations. First-tier bioassay are always easier, inexpensive and reliable in nature by involving plant system. The result of plant root tip system and mammalian culture shows positive correlation when treated with the same chemicals hence plant root tips assays are preferred.[15] The experiment results of onion root tips using various food preservatives shows warning signals which should be marked as potential health poisons.[16] Various experiments have been conducted using variety of food preservatives has been found to be genotoxic in nature.[17-19] The experiment performed by Kumar and Srivastava in 2011[17] showed inhibitory effect of the sunset yellow colour and boric acid on *Trigonella foenum-graecum* root tips. Similar results were obtained while working with Monosodium Glutamate at higher concentration affecting the cell growth, chromosomal aberrations and change in the root tips colour. Sodium metabisulphite and Potassium metabisulphite tested on the root tips of *Vicia faba* resulted decrease of mitotic index and chromosomal abnormalities.

Length of growing root tips has also been considered as perfect parameter as there is direct interaction of dividing cells with the changed environment as well as nutrients which causes significant modification in root length.[20,21] The elongation zone of the root tips grows due to expansion of cells and cellular differentiation.[22] During growth of elongation zone there is substantial increase in water uptake, sugar synthesis, plasma membrane elasticity, tonoplast elasticity and nitrogen metabolism. Any disturbance during cell division causes halting of lipid synthesis and increase in deposition of toxins which causes reduction in cell wall expansibility, loss of homeostatic regulations, enhanced cell necrosis, root growth inhibition and cellular toxicity.[23] Even shortening and decaying of roots has been reported to be evidence for cytotoxicity.[24]

Turkoglu (2007)[25] also reported that BHA exposure leads to inhibition of MI and DNA synthesis and the effect is more pronounced at higher concentrations. C-mitosis is the resultant of inhibition of spindle formation observed under the treatment of colchicines.[26] Colchicine acts as spindle poison with weak toxic effects and causes aneuploidy.[27] Failure in chromosome separation during anaphase leads to multipolarity and unequal translocation while anaphase bridge formation occurs due to fusion and breakdown of chromosomes and chromatids. Spindle formation disturbance results into multipolarity which mostly occurs when cells are treated with BHA.[10,15] Stickiness in chromosomes is further aggravated at higher chemical dosage which might be due to change in chromosomal proteins resulting in toxic effect ultimately causing cell death. Treatment with high concentration of BHA retards the movement of chromosome to opposite poles resulting in formation of laggards. Nitrites at lower concentration results in abnormalities while at higher concentrations and exposure for more duration leads to the swollen root tip cell and restriction of chromosomes at prophase. Chromosome breakage was also frequently reported in all groups which might involves an involvement of DNA distortion. BHA treated cells shows binucleated and lobulated nucleus which is an indicative of death process. Recommended dose of BHA shows low abnormalities because of the antioxidative properties and has been used to prevent rancidity in edible fats and oils and in fat containing foods. Kahl and Kappus (1993)[28] reported that BHA have less or no toxic effects but abnormalities at higher concentrations show genotoxic effects.[29-31]

CONCLUSION

The experimental outcome suggests that *Allium cepa* assay is significant and can be used for genotoxicity studies. On the basis of chromosomal aberrations like laggards, bridges and multipolar nuclei, BHA can be considered as toxic chemical. As BHA is commonly used for various purposes, it should be considered as major concern related with the health of organisms. For gaining proper knowledge about the mode of action of BHA more experimental studies should be conducted and till the time the basis of action is understood it must be used in limited doses.

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