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CHROMOSOME ABERRATION STUDY OF *ISODON NILGHERRICUS* (BENTH.) H. HARA EXTRACT USING *ALLIUM CEPA* ASSAY

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ABSTRACT

Isodon nilgherricus (Benth.) H. Hara belongs to a genus used in traditional Chinese folk medicine for treatment of respiratory and gastrointestinal bacterial infections, and as anti-inflammatory and anti-tumor agents. Mitotic cell division inhibition and chromosome aberration induction are widely used as indicators of cytotoxicity and genotoxicity which helps to identify mutagenic hazards associated with high dosage and long term use of the plant. Hence, effects of *I. nilgherricus* extract were investigated using root meristems of *Allium cepa*. Different concentrations of the extract for varying time durations were analyzed. Studies indicated slight decrease in mitotic index while abnormality percentage increased with increasing concentrations. Major aberrations observed includes nuclear lesions, micronucleus, scattered metaphase, stickiness, c-metaphase, disturbed metaphase, shift in microtubule organizing centre, polyploid cell, diagonal anaphase, chromosome bridges, stellate anaphase, chromosome laggards, multipolar anaphase, ring chromosome, ball anaphase and giant cell. Results showed that the extract possessed significant genotoxic effect. All values were statistically significant at $p<0.05\%$. Since mitotic index and abnormality percentage observed were dose and time dependent, it may be concluded that judicious use of the plant is essential for safe administration in herbal preparations while the genotoxic effect should be exploited in the development of chemotherapeutic drugs.

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INTRODUCTION

The medicinal use of plants is probably as old as human kind itself. More than 150,000 plant species have been studied, and many of them contain therapeutic substances (Ishii *et al*., 1984; Hoyos *et al*., 1992). The use of medicines is being increased day by day as new diseases of unknown origin are engulfing the changing environment. Development of new medicines has always targeted traditional/folk medicines which explores the bountiful resources of nature. The universality and efficacy of traditional medicine/medicinal herbs is evident in their continued use and dependence up till the present day by a significant portion of the world's population.

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However, the historic role of medicinal herbs in the treatment and prevention of diseases and in the development of pharmacology do not assume their safety for uncontrolled use by an uninformed public (Mathews *et al*., 1999). Injuries and even death resulting from misuse, contamination and/or adulteration of medicinal herbs have always been a major concern (De Smet *et al*., 1997). Hence, a toxicity analysis is constantly needed before conducting further research on a medicinal herb. The genus *Isodon* which have long been used in Chinese traditional medicines are natural drug resources with antitumor, antibacterial, and anti-inflammatory activities, and are known for being rich in ent-kaurane diterpenoids. A number of these isolated diterpenoids have been found to have potent antitumor activities with low toxicity. They are therefore being studied as candidates for anticancer drugs (Sun *et al*., 2001; Fujita and Node, 1984).

Table 1. Mitotic index and abnormality percentage of *Allium cepa* root meristems exposed to *Isodon nilgherricus* extract and control solutions

Extract	Time duration (h)	Total cells	Dividing cells	Chromosomal aberrations	Mitotic index ± SE	Abnormality % ± SE
NC	½	1000	809	0	80.9±0.46^{a}	0±0.00^{f}
	1	1000	814	0	81.4±0.26^{a}	0±0.00^{f}
	2	1000	818	0	81.8±0.18^{a}	0±0.00^{f}
	3	1000	808	0	80.8±0.23^{a}	0±0.00^{f}
	½	1000	281	411	28.1±0.14^{f}	41.1±0.06^{a}
PC	1	1000	272	489	27.2±0.11^{f}	48.9±0.11^{a}
	2	1000	266	545	26.6±0.11^{f}	54.5±0.15^{a}
	3	1000	241	604	24.1±0.09^{f}	60.4±0.14^{a}
	½	1000	714	356	71.4±0.14^{e}	35.6±0.09^{b}
E1	1	1000	709	362	70.9±0.11^{e}	36.2±0.07^{b}
	2	1000	701	368	70.1±0.11^{e}	36.8±0.06^{b}
	3	1000	690	379	69.0±0.09^{e}	37.9±0.06^{b}
	½	1000	734	312	73.4±0.11^{d}	31.2±0.09^{c}
E2	1	1000	727	322	72.7±0.06^{d}	32.2±0.07^{c}
	2	1000	719	329	71.9±0.09^{d}	32.9±0.06^{c}
	3	1000	718	335	71.8±0.03^{d}	33.5±0.06^{c}
	½	1000	762	263	76.2±0.06^{c}	26.2±0.06^{d}
E3	1	1000	756	273	75.6±0.09^{c}	27.3±0.07^{d}
	2	1000	751	279	75.1±0.06^{c}	27.9±0.03^{d}
	3	1000	748	297	74.8±0.06^{c}	29.7±0.06^{d}
	½	1000	786	223	78.6±0.09^{b}	22.3±0.03^{e}
E4	1	1000	777	237	77.7±0.09^{b}	23.7±0.07^{e}
	2	1000	773	242	77.3±0.03^{b}	24.2±0.06^{e}
	3	1000	769	258	76.9±0.06^{b}	25.8±0.06^{e}

NC - negative control (distilled water); PC - positive control (0.01% methyl parathion); Extract at different concentrations: E1 - 0.1%, E2 - 0.05%, E3 - 0.01%, E4 - 0.005%; SE - standard error. Means within a column followed by the same letters are not significantly different at $p< 0.05$ as determined by Duncan's multiple range test.

Isodon nilgherricus (Benth.) H. Hara belonging to this genus is thus chosen for the present study. Although plant extracts have been used in the treatment of diseases according to knowledge accumulated over centuries, scientific research has shown some substances present in these medicinal plants to be potentially toxic and carcinogenic (De Sa Ferreira and Ferraro Vargas, 1999). Investigation of traditionally used medicinal plants is thus valuable on two levels: firstly, as a source of potential chemotherapeutic drugs and secondly, as a measure of safety for the continued use of medicinal plants (Vershaeve *et al*., 2004). *Allium* test, a standardized test for cyto-genotoxicity monitoring (Fiskesjö, 1985), is important in this aspect since it combines toxicity and genotoxicity analyses. The chromosome aberration assay is one of the few direct methods capable of measuring mutations in systems exposed to putative mutagenic or carcinogenic substances (Rank *et al*., 2002; Leme *et al*., 2008). Moreover, plant genotoxicity assays are relatively inexpensive, fast, give reliable results and the chemicals which cause chromosomal aberration in plant cells produce aberrations in cultured animal cells that are frequently identical (Grant, 1978; Ma *et al*., 1994). Hence, genotoxic screening of *I. nilgherricus* extract using *Allium cepa* chromosome aberration assay is being conducted.

MATERIALS AND METHODS

Germplasm collection of *Isodon nilgherricus* was made from Munnar in Idukki district of Kerala (10°6'0"N, 77°4'0"E, 1602 m), identified taxonomically and the voucher specimen herbarized. Fresh aqueous extracts were prepared by grinding the leaves in distilled water. The lowest concentrations of the extract *viz*., 0.1%, 0.05%, 0.01%, and 0.005% (w/v; E1, E2, E3, E4) were chosen after preliminary toxicity analysis for *Allium cepa* assay. Distilled water and an organophosphorus pesticide, methyl parathion (0.01%), were taken as the negative (NC) and positive control (PC), respectively.

Uniformly sized germinated bulbs of *A.cepa* with healthy roots (1–2 cm) were collected at the peak mitotic period (9 am– 10 am), washed in distilled water, and kept in different concentrations of the extract. Root tips cut from the samples at different time intervals of 1/2 h, 1 h, 2 h and 3h were washed in distilled water and immediately fixed in modified Carnoy's fluid for 1 h. Mitotic squash preparation was done with the help of improved techniques (Sharma and Sharma, 1990). Hydrolysis with 1 N HCl and staining with 2% acetocarmine was carried out. Mitotic index and abnormality percentage were calculated. All the slides were scanned and tabulated, and photomicrographs were taken with a Leica ICC 50 digital camera attached to a Leica DM 500 research microscope. Data obtained on mitotic index and abnormality percentage were statistically analyzed. One way ANOVA and Duncan's Multiple Range test was performed to determine mean separation and significance of treatments using SPSS version 20, SPSS Inc., Chicago, USA. Each data point represents the arithmetic mean ± standard error of at least three independent experiments.

RESULTS AND DISCUSSION

Genotoxic screening of *Isodon nilgherricus* on *Allium cepa* revealed the toxic effect of the extract. Mitotic index and abnormality percentage was found to be dose and time dependent. Studies revealed a slight decrease in mitotic index with increasing concentrations and time durations when compared to the negative control, distilled water which shows that the extract is mildly cytotoxic. The abnormality percentage showed an increase with increasing concentrations and time durations but was less than the positive control, methyl parathion. This indicated that the plant extract is genotoxic but not much harmful as the positive control especially at lower concentrations. Both clastogenic and non-clastogenic abnormalities were observed. The major

aberrations observed includes nuclear lesions, micronucleus, scattered metaphase, stickiness, c-metaphase, disturbed metaphase, shift in microtubule organizing centre (MTOC), polyploidy, diagonal anaphase, chromosome bridges, stellate anaphase, chromosome laggards, multipolar anaphase, ring chromosome, ball anaphase and giant cell. This shows that *I. nilgherricus* extract has more of non-clastogenic activity. All values were statistically significant at $p< 0.05\%$. Chromosome abnormalities can be mainly classified into clastogenic and non-clastogenica berrations. Clastogenecity is attributed to the direct action in chromosomes. The major clastogenic aberrations were nuclear lesions, stickiness, chromosome bridges, ring chromosome and giant cell. The occurrence of nuclear lesions in *A. cepa* root meristematic cells may be due to the disintegration of portion of nuclear material by the action of the plant extracts (Mercykutty and Stephen, 1980). Chromosome stickiness is regarded as a physiological effect caused by the affected proteins of the chromosome. It may cause incomplete separation of daughter chromosomes as a result of the cross-linkage of chromoproteins (Kong and Ma, 1999; Tkalec *et al*., 2009). Liu *et al*. (1992) suggested that sticky chromosomes reflect a highly toxic effect, usually of an irreversible type, and probably lead to cell death. Chromosome bridges may be due to chromosomal stickiness and the subsequent failure of free anaphase separation.

These bridges are usually formed by joined sister chromatids that stay together until late anaphase or telophase (Gömürgen, 2005; Türkoglu, 2008). Raghuvanshi and Singh (1976) suggested that the formation of ring chromosome may be due to telomeric losses. Giant cells can be induced either by physical means or by employing chemicals, which are capable of affecting the cell cycle especially in the 'S' phase. The cell division is completely arrested and cell expansion seems to be generated, as a result of which the cells become large giant cells. The frequency of giant cells seems to be increased depending upon the dosage and duration of the treatment with cytotoxic agents (Verma and van Huyste, 1971). Non-clastogenicity or physiological aberrations are attributable to spindle abnormalities. The major non-clastogenic aberrations include micronucleus, scattered metaphase, c-metaphase, disturbed metaphase, shift in microtubule organizing centre, polyploidy, diagonal anaphase, stellate anaphase, chromosome laggards, multipolar anaphase and ball anaphase. Micronucleus may originate from a lagging chromosome at anaphase or from a chromosome fragment (Badr and Ibrahim, 1987), which may lead to loss of genetic material and have been regarded as an indication of mutagenicity of their inducers (Ruan *et al*., 1992). Chromosome scattering is attributed to the interference of the leaf extracts with tubulin or polymerization of the microtubular subunits forming the spindle apparatus (Mathur and Chua, 2000). C-mitosis is one of the consequences of inactivation of spindle apparatus connected with the delay in the division of centromere (Gömürgen, 2000). Disturbed metaphase occurs due to the loss of activity of microtubules in spindle fibers (Pickett-Heaps and Spurck, 1982; Saleem *et al*., 1993). In plants, the absence of organelles such as the centrosome has led to the belief that MTOCs originate on the nuclear envelope and are transported to specific intracellular locations by microtubule proteins (Asada and Collings, 1997; Baluska *et al*., 1997). Alternatively, spontaneous and *de novo* assembly of such MTOCs may occur in the cell by enhanced microtubule stability (Cyr and Palevitz, 1995). In the present study, the chemical principles present in the extract might have affected the stability of microtubules thereby causing a shift in MTOC. Polyploidy is a numerical chromosomal abnormality. Minija *et al*. (1999) attributed polyploidy to be due to the inhibition of spindle mechanism. Abnormal movement of chromosomes in different directions was also observed which may be due to severe disturbances in the spindle mechanism (Minija *et al*., 1999). According to Saggoo *et al*. (1991), lagging of chromosome is due to abnormal spindle activity. The failure of normal organization and function of spindle apparatus may lead to formation of laggards (Patil and Bhat, 1992). Ball anaphase is the stage in mitosis in which sister chromatids separate into a hollow ball of chromosomes that results from the early cleavage divisions in some aberrant cells (Morgan, 2006).

In spite of the efficacy of the medicinal herbs in the treatment of various kinds of ailment, the unrefined nature of the preparations and the lack of standard prescriptions on dosage constitute a major setback in the use of herbs in medicare. This can lead to complications in human system resulting from bioaccumulation of plant ingredients due to overconsumption of the herbs (Okafor, 1987). Other causes of complications include uptake of toxic plant ingredients, and possible herb/herb and herb/drug interactions (Okafor, 1987; Mathews *et al*., 1999). Higher plants provide reliable bioassays for monitoring and testing genotoxins (Grant, 1999), with the *Allium* test being particularly sensitive and reproducible (Fiskesjö, 1985). In addition, this system has been used as an indicator of the chromosomal alterations in order to warn people about product consumption (Vicentini *et al*., 2001). Several researchers have performed the *in vitro* animal test together with the test system of *A. cepa*, obtaining similar results (Chauhan *et al*., 1999; Vicentini *et al*., 2001; Teixeira *et al*., 2003), providing valuable information for the human health. Studies conducted revealed that *I. nilgherricus* extract possessed genotoxic and mild cytotoxic activity but was dose and time dependent. Thus, it may be concluded that judicious use of the plant is essential for the safe administration in the form of herbal preparations as emphasized by its cytotoxic effect while the genotoxic effect should be exploited in the development of chemotherapeutic drugs.

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