Evaluation of anti-genotoxicity of the leaf extracts of *Morinda citrifolia* Linn.

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ABSTRACT

Morinda citrifolia Linn. (family Rubiaceae) is a small tree occurring in tropical areas of the world. The plant contains several medicinally active components that exhibit the therapeutic effects such as antibacterial, antiviral and anticancer activities. Anti-genotoxic effects of aqueous extracts prepared using dried leaves of *M. citrifolia* was studied. *Allium cepa* root tip meristem cells treated with 7% hydrogen peroxide were used for eliciting anti-genotoxicity. For this purpose experiments were performed with *A. cepa* onion bulbs treated for 24 h with different concentrations (15 or 30 g/L) of aqueous extract with or without pre-treatment (1 h) with 7% hydrogen peroxide. A significant reduction in mitotic index was recorded in treatment groups over negative control. Chromosomal aberrations such as breaks, bridges, stickiness and polar deviations were observed in positive control and treatment groups. The highest (21.48) percentage of chromosomal aberrations was noticed in positive control. A significant reduction in chromosomal aberrations (9.39) was recorded in root tips treated with hydrogen peroxide followed by 15 g/L extract. H_2O_2 induced chromosomal aberrations were reduced due to leaf extract treatment indicates anti-mutagenic potential of the *M. citrifolia*. The observations suggest that *M. citrifolia* aqueous leaf extracts have anti-mitotic and anti-genotoxic effects; consequently oxidative stress induced aberrations due to H_2O_2 are efficiently restored in the extract treated *A. cepa* root meristem cells.

Keywords: *Allium cepa*; chromosome aberrations; hydrogen peroxide; mitotic index; oxidative stress

Higher plants used extensively in traditional medicines are increasingly being screened for their role in modulating the activity of environmental genotoxicants. It is evident from data that out of total chemical structures discovered, a major share was directly derived from higher plants (Kinghorn and Balandrin 1993, Sarkar and Sharma 1996) and several of them can be used as antitumour agents (Mukherjee et al. 2001).

In the past few years, considerable advancements were made in the natural products endowed with antimutagenic and anticarcinogenic properties. Many natural products referred to as dietary chemopreventive compounds offer a great potential in the fight against cancer through a different range of mechanisms including antioxidant, antimutagenic activity, enzyme modulation, gene expression, apoptosis etc (De Flora et al 2001, Webb and Ebeler 2004, Miadokova et al. 2008).

Morinda citrifolia Linn. (family Rubiaceae) popularly known as 'noni' is a medium sized ever green tree distributed throughout the tropics in Southern and Northern hemispheres. All parts of the plant, including leaf, fruit, bark and root contain several pharmacologically active principles that exhibit the therapeutic effects of antibacterial, antiviral and anticancer activities (Wang et al. 2002). In the recent past, noni fruit-juice was approved as a novel food by the European commission. Therapeutic properties of *M. citrifolia* are largely attributed by the anthraquinones present in the plant. Winjsma and Verpoorte (1986) reviewed anthraquinones in Rubiaceae, and various anthraquinone principles present in *Morinda* spp. was documented. About 90% of these compounds occur as derivatives of 9, 10-anthracenedione with several hydroxy and other functional groups such as methyl, hydroxy methyl or carboxyl side chain. Hydroxy anthraquinones are the active principles of many phyto-therapeutic drugs (Westendorf et al. 1990).

Present communication deals with the anti-mitotic and anti-genotoxic effects of aqueous leaf

extracts of *M. citrifolia*. *Allium cepa* root tip based test system was used for the study. The toxicity to root tip cells was developed by treating with H_2O_2. H_2O_2 itself acts as an oxidizing agent that induces oxidative stress (Torbergesen and Collins 2000) and therefore *A. cepa* based anti-genotoxicity test is considered as an easily adoptable method for screening of plants for their mitotic activity (Fiskesjo 1993).

MATERIALS AND METHODS

Plant collection and extraction. Fresh leaves (500 g) of *M. citrifolia* were collected from approximately 3-year-old trees growing in swamps near Aakulam tourist village, Thiruvananthapuram District (latitude 8°31.505'N; longitude 76°53.942'E; altitude 8 m above sea level). Leaves were shade dried and powdered. Leaf powder (15 and 30 g) was subjected to extraction using 1000 mL boiling water for 10 min. Extract was filtered using filter paper (Whatman No. 1) and used for the root-tip treatment.

Allium test. To evaluate the anti-mitotic and anti-genotoxic effects of *M. citrifolia* leaf extracts, two different experiments were performed under the same conditions.

The first experiment consists of two treatments. For each treatment eight commercial equal sized *A. cepa* onion bulbs (3–4 g) were used. They were carefully unscaled and placed on top of test tubes filled with distilled water and allowed to germinate in dark. After 24 h, they were removed for each treatment. After that the onion bulbs were placed onto test tubes filled two different concentrations of leaf extract (Ex_1 and Ex_2) respectively and kept for 24 h. After 24 h, the roots were washed and the onion bulbs were placed on top of test tubes filled with water for 24 h.

To perform the second experiment sixteen (8 for each treatment) commercial, equal sized *A. cepa* onion bulbs (3–4 g) were used. They were carefully unscaled and placed on the top of test tubes filled with distilled water and allowed to germinate in dark for 24 h. After 24 h two onions from each treatment with most poorly growing roots were removed. The other onion bulbs were treated with 7% H_2O_2 for 1 h. After H_2O_2 treatment, roots were washed and onion bulbs were treated with two different concentrations (15 or 30 g/L) of extract (HEx_1 and HEx_2) for 24 h. Subsequently the onion bulbs were placed on top of the test tubes filled with water for 24 h.

Onion bulbs allowed to germinate in distilled water for 72 h followed by hydrogen peroxide (7%) treatment for 1 h were used as positive control (C^{+ve}) and onion bulbs germinated in distilled water (72 h) were used as negative control (C^{-ve}). After the completion of treatment (72 h), the roots were counted and their length was measured for each bulb. The roots were fixed in Carnoy's fluid (ethanol: acetic acid, 3:1). After the fixation the roots were hydrolysed in 1 mol/L HCl for 1 min at 60°C and squashed using 2% acetocarmine and examined using light microscope. Mitotic index was expressed in terms of divided cells/total no. of cells counted (Ozmen and Sumer 2004). Chromosomal aberrations were determined by scoring cells with fragments, bridges, sticky chromosomes and polar deviation in randomly picked four zones per slide. Four slides were examined from each treatment group which included 6 onions bulbs.

Statistical analysis of data. Data on root number, root length, mitotic index and different chromosomal aberrations were subjected to statistical analysis. One way ANOVA was performed to determine significance of treatments. The mean separation was performed according to Duncan's New Multiple Range test ($P < 0.05$).

RESULTS AND DISCUSSION

The effects of *M. citrifolia* leaf extracts (15, 30 g/L) on root number and root length were significant ($P < 0.001$). The mean root numbers in negative and positive controls were 19.33 and 13.66, respectively. The mean root length was found to be 1.93 cm and 0.765 cm in negative and positive controls. There was a significant ($P < 0.05$) difference in root number and root length between the control groups and treatment groups (Table 1). The average root length in treatment groups Ex_1, Ex_2 (leaf extract treatment) and HEx_1, HEx_2 (extract treatments after H_2O_2 treatments) were significantly ($P < 0.05$) lower than the negative and positive control. The reduction in root length and root number in treatment groups indicates the antimitotic activity of *M. citrifolia* leaf extracts.

The mitotic index shows that aqueous leaf extracts of *M. citrifolia* had a significantly ($P < 0.05$) reduced mitotic index (Table 2). The mitotic index values in the treated onion root cells were significantly ($P < 0.05$) lower than the MI of negative control. This suggests the suppression of mitotic activities in *A. cepa* by *M. citrifolia* aqueous leaf extract. Since mitotic index is a quantitative esti-

Table 1. Root number and root length of germinated (72 h) onion bulbs in controls and various treatments

Concentrations	Average root number ± SE	Average root length (cm) ± SE
C^{-ve}	19.33 ± 0.66[a]	1.93 ± 0.35[a]
C^{+ve}	13.66 ± 0.95[b]	0.76 ± 0.32[b]
Ex_1	11.66 ± 1.22[b,c]	0.67 ± 0.06[b]
Ex_2	10.66 ± 0.66[b,c]	0.65 ± 0.10[b]
HEx_1	10.66 ± 1.02[b,c]	0.94 ± 0.19[b]
HEx_2	9.83 ± 1.19[c]	0.93 ± 0.23[b]
Main effect $F\ df\ (n-1) = 5$	12.962***	9.331***

C^{-ve} – negative control; C^{+ve} – positive control (7% H_2O_2); Ex_1 – 15 g/L *M. citrifolia* leaf extract; Ex_2 – 30 g/L *M. citrifolia* leaf extract; H 7% H_2O_2, ***$P < 0.001$. Means within column followed by the same letters are not significantly ($P < 0.05$) different as determined by DNMRT

mation of the mitotic activities in an organism or a particular organ, the reduction in mitotic index is due to the constituents in the aqueous extracts, that in turn have cytotoxic effects.

The negative control showed normal mitotic divisions (Figure 1A). Chromosomal aberrations in terms of breaks, bridges, stickiness and polar deviation (Figure 1) for each group were counted by microscopic observations (Table 3). The lowest percentage (2.86) of aberrant cells was noticed in negative control in contrast to the highest percentage in positive control (21.48). Chromosomal stickiness was a major type of abnormality observed in *A. cepa* cells (Figures 1B and C). The values were increased in groups treated with HEx_2, but in C^{-ve} and Ex_1 the stickiness was reduced.

Chromosomal breaks were found to be the highest in C^{+ve} cells (Table 3). All the other treatments also showed fragments (Figures 1D and E), but it was less when compared with C^{+ve}. Another chromosomal aberration observed in the experiment was chromatid bridges. All the treatments except C^{-ve} showed varying degrees of chromatid bridge formation (Figures 1F and G), but its frequency was increased in C^{+ve} (Figure 1H).

Polar deviation (Figures 1I–K) were also recorded high in C^{+ve} compared to other treatment groups. Other major abnormalities like strap shaped vacuolated nucleus (Figure 1L), binucleated cells (Figure 1M) and cells with disintegrating nucleus which leads to cell death (Figure 1N) were noticed in C^{+ve}. Total chromosomal aberrations showed a significant ($P < 0.05$) decrease in *M. citrifolia* aqueous extract treated groups and groups treated with extract after H_2O_2 treatment. Aberrations in treatment Ex_1 were found significantly lower compared to other treatment groups.

Mitotic index is an acceptable measure of cytotoxicity in all living organism (Smaka-Kinel et al. 1996). The cytotoxicity level can be determined by the decreased rate of mitotic index. In the present study, decreased mitotic index values of *M. citrifo-*

Table 2. Mitotic index in control and various treatments

Concentrations	Total cells ± SE	Dividing cells ± SE	Mitotic index ± SE
C^{-ve}	192.25 ± 12.62[a]	68.00 ± 6.64[a]	35.38 ± 2.81[a]
C^{+ve}	211.75 ± 18.28[a]	53.00 ± 12.13[a,b]	24.21 ± 3.15[b]
Ex_1	195.00 ± 7.90[a]	37.25 ± 2.28[b,c]	19.18 ± 1.37[b,c]
Ex_2	185.75 ± 4.66[a]	30.50 ± 1.50[c]	16.50 ± 1.14[c]
HEx_1	191.25 ± 3.19[a]	40.25 ± 3.75[b,c]	21.10 ± 2.20[b,c]
HEx_2	196.50 ± 7.19[a]	40.75 ± 4.09[b,c]	20.65 ± 1.62[b,c]
Main effect $F\ df\ (n-1) = 5$	0.729^{NS}	4.724*	9.266***

C^{-ve} – negative control; C^{+ve} – positive control (7% H_2O_2); Ex_1 – 15 g/L *M. citrifolia* leaf extract; Ex_2 – 30 g/L *M. citrifolia* leaf extract; H 7% H_2O_2. ^{NS}non significant; *$P < 0.05$, ***$P < 0.001$. Means within column followed by the same letters are not significantly ($P < 0.05$) different as determined by DNMRT

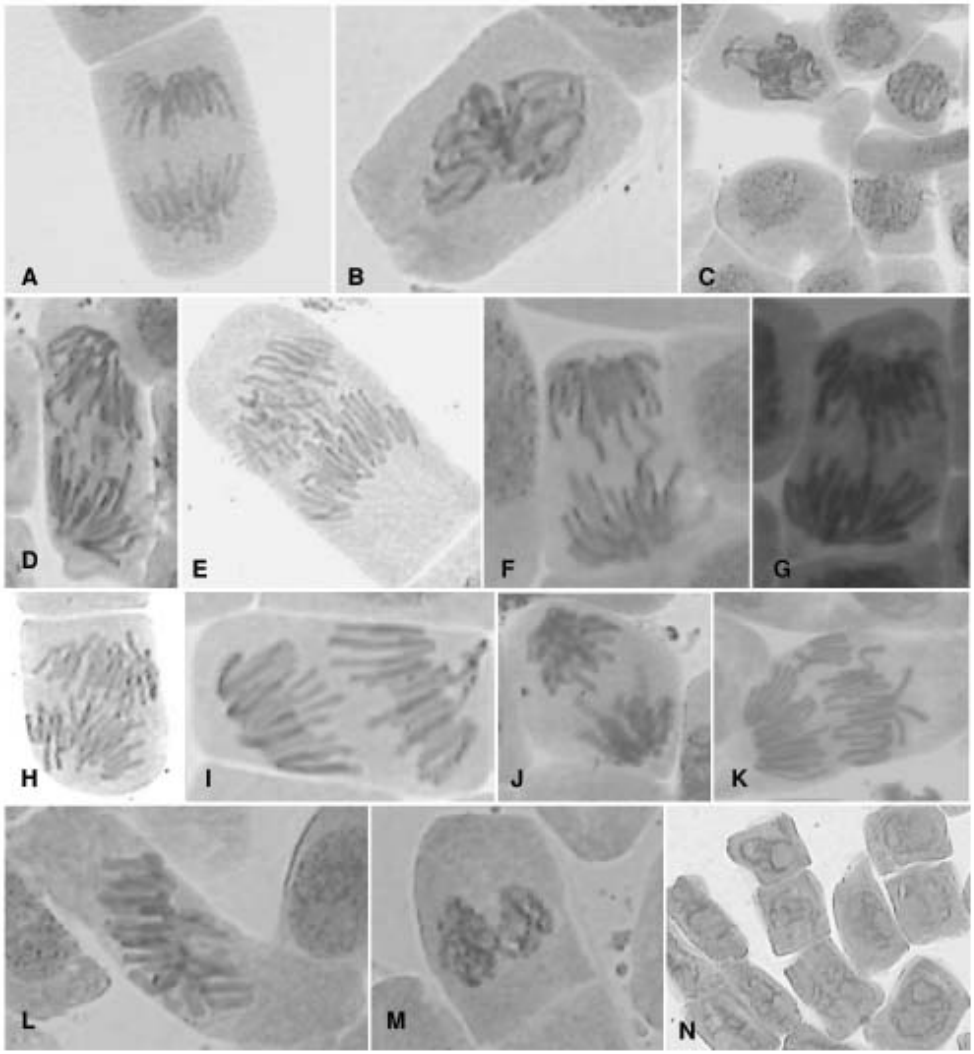

Figure 1. Mitotic aberrations in *A. cepa* root cells. A – normal anaphase in C^{-ve} treated cells; B – metaphase clumping in HEx_1 treated cells; C – chromosomal stickiness in metaphase, HEx_2 treated root tips of *A. cepa*; D – tropokinesis with chromosomal fragments in C^{+ve} treated cells; E – chromosomal fragments in HEx_2 treated cells; F – chromosomal bridges in Ex_2 treated cells; G – chromosomal bridge in HEx_1 treated cells; H – tropokinesis with multiple bridges in C^{+ve} treated cells; I – polar deviation in anaphase, C^{+ve} treated cells; J – polar deviation in Ex_2 treated cells, K – multipolar segregation in C^{+ve} treated cells; L – strap shaped nucleus with vacuoles Ex_1 treated cells; M – binucleate cell without normal division in C^{+ve} treated cells, N – nuclear disintegration leading to cell death in C^{+ve} treated cells

lia leaf extract explained as the extract suppresses the cell proliferation and prevent chromosomal aberration in the organism. In substantiation, immuno-modulating effects and anticancer activity (Hirazumi and Furusawa 1999) of *M. citrifolia* is available. The group of compounds that are responsible for the therapeutic properties in this plant is anthraquinones. Among the different anthraquinones damnacanthal present mainly in the root demonstrated anticancer activity (Hiramatshu et al. 1993). In the present investigation, in addition to the changes in mitotic index, aberrations were also observed in positive control group. It may be possible that the cells undergo a state of oxidative stress due to the exposure to H_2O_2. Active oxygen (O) forms emerging under the oxidative stress are known to affect the cytoskeleton structure (Egorova et al. 2001).

Chromosomal aberrations are changes in chromosome structure resulting from a break or exchange of chromosomal material. Most chromosomal aberrations observed in the meristem cells of *A. cepa* are lethal, but there are many corresponding aberrations that are viable and can cause genetic effects (Askin and Aslanturk 2007). Chromosomal stickiness was a major type of abnormality observed in *A. cepa* cells. Chromosomal stickiness values were 0.5% in negative control and 3.25% in posi-

Table 3. Chromosomal aberrations in controls and various extract treated *A. cepa* root tips

Concentrations	Dividing cells	Fragments	Bridges	Stickiness	Polar deviation	Aberrant cells	Percentage of aberrations
C^{-ve}	68.00 ± 6.64^{a}	1.00 ± 0.40^{b}	0.00 ± 0.00^{a}	0.50 ± 0.28^{c}	0.25 ± 0.25^{b}	1.75 ± 0.62^{d}	2.86 ± 1.23^{d}
C^{+ve}	53.00 ± 12.13^{a,b}	4.00 ± 0.57^{a}	0.75 ± 0.25^{a}	3.25 ± 0.47^{b}	2.50 ± 0.50^{a}	10.50 ± 0.86^{a}	21.48 ± 2.76^{a}
Ex_1	37.25 ± 2.28^{b,c}	2.00 ± 0.40^{b}	0.25 ± 0.25^{a}	0.75 ± 0.25^{b,c}	0.25 ± 0.25^{b}	3.25 ± 0.62^{c,d}	8.65 ± 1.52^{c}
Ex_2	30.50 ± 1.50^{c}	1.75 ± 0.25^{b}	0.50 ± 0.28^{a}	1.75 ± 0.47^{b,c}	1.00 ± 0.57^{b}	5.00 ± 0.71^{b,c}	16.51 ± 2.38^{a,b}
HEx_1	40.25 ± 3.75^{b,c}	1.00 ± 0.00^{b}	0.50 ± 0.28^{a}	1.75 ± 0.25^{b,c}	0.50 ± 0.28^{b}	3.75 ± 0.63^{b,c,d}	9.39 ± 1.59^{c}
HEx_2	40.75 ± 4.09^{b,c}	1.25 ± 0.47^{b}	0.50 ± 0.28^{a}	2.00 ± 0.57^{a}	0.75 ± 0.50^{b}	4.50 ± 0.50^{b}	11.31 ± 1.44^{d}
Main effects $F\,df\,(n-1)=5$	4.724**	8.087***	1.067^{NS}	5.800**	5.049**	20.284***	11.835***

C^{-ve} – negative control (distilled water); C^{+ve} – positive control (7% H_2O_2); Ex_1 – 15 g/L *M. citrifolia* leaf extract; Ex_2 – 30 g/L *M. citrifolia* leaf extract; H – 7% H_2O_2. ^{NS}non significant, $^{**}P < 0.005$, $^{***}P < 0.001$. Means within a column followed by the same letters are not significantly ($P < 0.05$) different as determined by DNMRT

tive control. These values increased in HEx_2 but in all other treatment groups, stickiness values were decreased significantly ($P < 0.05$). Other frequently observed aberrations in positive control were breaks, chromatid bridges and polar deviations.

As such *M. citrifolia* aqueous extracts have protective effect against the oxidative damage induced by H_2O_2. This protective effect may be due to the anthraquinones present in *M. citrifolia* leaves (Sang et al. 2001, Zin et al. 2006). Recent studies revealed the antigenotoxic effects of noni juice against the chromosomal aberration and sister chromatid exchange induced by a chemotherapeutic agent, mitomycin C (MMC) in human lymphocytes (Treetip et al. 2008).

The percentage of suppression of mitotic aberrations by *M. citrifolia* leaf extract on H_2O_2 induced chromosomal aberrations in all the groups is an indicative of its anti-mutagenic potential on *A. cepa* test system. The effect of *M. citrifolia* leaf extract on the reduction of total number of aberrations induced by H_2O_2 was statistically significant when compared with positive control (H_2O_2 treatment). The present finding implies that pre-treatment of *M. citrifolia* leaf extract has a strong inhibitory role against the oxidative stress induced mutagenic action of hydrogen peroxide in the cells.

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