Genotoxic and antigenotoxic potential of the *Lagenandra toxicaria* Dalz. rhizome methanol extract using *Allium cepa* assay.

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Research Article

Keywords: *Lagenandra toxicaria*, *Allium cepa* assay, Genotoxicity, Antigenotoxicity, DNA integrity

Posted Date: February 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-179342/v1>

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Abstract

This study is the first ever approach to evaluate the possible genotoxic effect of the *Lagenandra toxicaria* rhizome methanol extract and its antigenotoxic potency against 3% H₂O₂ induced genetic damage on *Allium cepa* root tip model. The assay revealed a significant decrease in mitotic index (MI) and an increase in the percentage of clastogenicity in a time and dose-dependent manner in the roots exposed to *Lagenandra toxicaria* extract at 0.2 mg/ml, 0.5 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml concentration for 1, 2 and 4 hour. The ultra structures of cell surface and biochemical changes of the cells were assessed in four hour treated roots using Field emission scanning electron microscopy (FESEM) and Fourier-transform infrared spectroscopy (FTIR). The higher dose of 10 mg/ml treated roots showed an evident morphological as well as biochemical changes compared to the control. The agarose gel electrophoresis showed the loss of DNA integrity in the roots that were treated with 10 mg/ml extract for four hours, where as the control showed comparatively intact DNA bands. The in situ histochemical staining by Schiff's reagent and nitroblueterasolium (NBT) confirmed the increased lipid peroxidation and free radical generation in four hour treated samples. Subsequently, the possible antigenotoxic potential of the plant extract was explored at its lower doses using H₂O₂ standard assays. The H₂O₂ treatment induced nuclear lesions in $93.45 \pm 2.33\%$ cells and it was seen to be reduced significantly ($50.99 \pm 7.59\%$ and $37.13 \pm 2.66\%$) after the treatment with lower concentration of 0.01 mg/ml and 0.02 mg/ml extract respectively. This suggest that the *Lagenandra toxicaria* rhizome methanol extract acts as antigenotoxic agent at lower doses but at higher doses the extract induces clastogenic effects and thus acts like a janus-faced compound.

1. Introduction

Plants are the major source of traditional medicine, practised since prehistoric times. Each plant has its own chemical constituents and secondary metabolites. In Ayurveda more than 700 plant-based medicines were discussed for promoting the healthy life [26]. Traditional healers use numerous plant based remedies to treat various diseases, of which very few have been scientifically established for their mode of action. The plant based therapeutic modalities are safer and also cost effective than the synthetic drugs. The plant of interest in the present study, *Lagenandra toxicaria* is a herb of the family Araceae. It is endemic to southern India and commonly seen in semi-aquatic regions. The rhizome of the plant is used in the traditional system to treat bilious complaints and also in renal and cardiac ailments. The plant is recognized to be diuretic, carminative and used as tonic [33]. The major storage protein in the members of Araceae is lectin [41] which is used in the development of anticancer therapeutics as it can bind to the cancer cell membranes and/or receptors causing the cell to undergo apoptosis [7]. The rhizome oil is known for its insecticidal activity and the plant juice is known to enhance wound healing. The antibacterial activities of the plant against human pathogens have also been explored [21, 39]. But studies on the genotoxicity of the plant were not carried out. The present study aims to evaluate the genotoxic and antigenotoxic potential of the plant *Lagenandra toxicaria* using *Allium cepa* root tip assay. *Allium cepa* root tip assay is a well known model of genotoxicity studies. Also, it is less expensive,

sensitive and a widely used study model. The larger chromosome of *Allium cepa* which are easily observable under light microscope is an added advantage of this model. Earlier studies report that the effects of genotoxic agents on *Allium cepa* cells and animal cells are similar and comparable [3, 12]. The genotoxic and anti-genotoxic effects of the plant extract has been studied at different doses and different time intervals to establish whether the effect is time and dose dependent. It also enables us to explore the mechanisms through which the extract is acting on the root tips. Induction of oxidative stress is one of the common ways by which a compound causes DNA damage and chromosomal aberrations leading to the cellular damage and apoptosis [11, 31]. Various genotoxic and antigenotoxic bioassays were employed in the present study to unravel these aspects of the plant extracts.

2. Materials And Methods

2.1 Reagents

DNA isolation kit was obtained from Himedia Ltd. Mumbai, India. Schiff's, TTC, NBT, FC reagent, Na₂CO₃, H₂O₂ and Galic acid reagents were obtained from Nice Pvt.Ltd. Kerala. Other chemicals used were all of analytical grade.

2.2 Plant collection and extraction

The *Lagenandra toxicaria* rhizomes were collected from the markanja village of Sullia taluk, Dakshina kannada district, Karnataka, India (12°34'38.7"N, 75°29'54.8"E). The plant was identified taxonomically and herbarium (AK 08) was deposited in the department of Applied Botany Mangalore University. The rhizomes were cleaned, washed under running tap water, chopped into small pieces and dried in shade. Dried samples were finely powdered using lab mill. The powdered samples were subjected to methanol extraction in a soxhlet apparatus at 60°C [8]. The obtained extracts were concentrated in rotary evaporator and dried in lyophilizer. The extract stock solution of 1 mg/ml and 10 mg/ml were prepared in 1 % DMSO and used for further analysis.

2.3 Genotoxicity test

Healthy onion bulbs were collected. The outer old scales were peeled and the old roots were scraped off. These bulbs were placed on a coupling jar containing double distilled water and kept in dark for 3-4 days to allow the growth of fresh roots. The onion bulbs (n=6) with well developed roots were picked at their peak mitotic period, and were treated with extracts at concentrations (0.2, 0.5, 1, 5, and 10 mg/ml in 1% DMSO) for 1, 2 and 4 hours. The 1% DMSO and water was used as controls, and the 3% H₂O₂ served as the positive control for the genotoxicity [27, 37]. After the treatment, root tips of each treatment were excised, washed with distilled water and transferred to a cool mixture (4-10°C) of ethanol and glacial acetic acid (3:1) fixative and incubated for one hour. These root tips were hydrolyzed with the 1N HCl for about 15 minutes at 55-60°C, then stained with acetocarmine for 5-10 minutes and placed on a microscope slide, root tip region was isolated (M region), covered with a coverslip, squashed and observed under 40x objective lens of the (Olympus CH20i) microscope [29, 43].

The cells were observed for mitotic aberrations in the randomly selected microscopic fields. About 700-800 cells were counted for each treatment and the MI and the cellular aberrations were examined.

The mitotic index was calculated using the formula

See formula 1 in the supplementary files.

The percentage of chromosomal aberration was determined as the ratio of the number of aberrant cells to the total number of observed cells. i.e:

See formula 2 in the supplementary files.

2.3.1 Testing of DNA damage

The DNA was isolated from the treated root tip samples using Himedia plant genomic DNA Miniprep kit MB507-20PR, following the manufacturer's instructions. The isolated DNA samples were loaded into a 2% agarose gel and subjected to electrophoretic separation at 50V and visualised by ethidium bromide staining. The gel pictures were documented using the UVCI-1100 ms major science system, Major science manufacturer, 19959 Sea Gull Way, Saratoga, U.S.A. [14, 15, 28].

2.3.2 Quantitative analysis of total polyphenol content and flavanoids

The quantitative estimation of the polyphenols (n=3) was done using FC reagent spectrometric method [19]. Galic acid was taken as the standard. Absorbance was measured at 735nm. Total flavonoid content (n=3) was estimated using aluminium chloride spectrometric method [10]. Quercitine was taken as the standard and absorbance was measured at 415nm.

2.3.3 Examination of *in situ* lipid peroxidation

After the treatment (n=6) with various concentration of the extract (0.2, 0.5, 1, 5, and 10 mg/ml), 1% DMSO and 3% H₂O₂, the roots were incubated in schiff's reagent for two hours. Then the roots were washed with 0.5% (w/v) K₂S₂O₅ (prepared in 0.05M HCl) solution for 20 minutes, leading to the visualisation of the Malondialdehyde which is a possible product of the lipid peroxidation [5, 37].

2.3.4 Viability test using TTC

The cell viability of the extract treated roots (n=6) was assessed using the 0.5% of 2, 3, 5-Triphenyl tetrazolium chloride (TTC) in phosphate buffer following the procedure of [36]. The images were captured using canon power shot SX400 IS camera

2.3.5 Measurement of superoxide anion radical (O[•])

Measurement of superoxide anion radical was carried out for the root tip (n=6) treated with 0.2, 0.5, 1, 5, and 10 mg/ml of the extract for four hours. 1% DMSO served as a negative control and 3% H₂O₂ was the positive control. The treated roots were kept in 0.1% nitroblue tetrazolium (in 50 mM phosphate buffer pH 7.4) and placed under light until the blue colored formazan precipitate appears. The roots were then washed with 95% alcohol [40, 23].

2.3.6 FT-IR analysis of treated root tips

From the onion root tips (n=6) subjected to various treatments, root tips of each treatment were dried in an oven for 15 hours at 60°C and the dried samples were finely powdered. The powder was analyzed by FTIR to know the possible differences in the characteristic signal of the biochemical components of the different root samples [30, 37].

2.3.7 Root ultrastructure studies using Field emission scanning electron microscopy (FESEM)

The root tips treated with 1% DMSO (control) and different concentrations of methanol extracts like 1 mg/ml, 5 mg/ml, and 10 mg/ml were excised, washed with PBS and then fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 12 h at 4°C with intermittent stirring. Roots were dehydrated using ethanol series (10%, 20%, 30%, 50% and 70%). Dehydrated roots were gold sputter coated and observed under FSEM at 5 kV voltage following the procedure of Ahmed *et al.* (2017), Shahid, *et al.* (2018) and Shetty *et al.* (2017).

2.4 Examination of antigenotoxicity activity of *Lagenandra toxicaria* methanol extract

The onion roots were grown as mentioned previously. The healthy bulbs (n=6) with sufficient number of roots were treated with the 3% H₂O₂ for 1 hour. Then the roots were incubated with different concentrations of the plant extract (0.04, 0.02, 0.01, 0.005 and 0.0025 mg/ml) for 24 hours to analyse the possible antigenotoxic potential of the extract. The roots treated with the 3% H₂O₂ served as the control. The squash was made and observed under the microscope as mentioned previously.

The antigenotoxicity potency of the plant extract was calculated using the following formula

$$\text{Inhibitory activity (\%)} = \frac{A-B}{A-C} \times 100$$

A=Number of aberrant cells induced by hydrogen peroxide

B=Number of aberrant cells observed after treating hydrogen peroxide treated roots with plant extract

C=Number of aberrant cells observed in 1% DMSO control group

The group treated with one percent DMSO was taken as a negative control. The group treated with 3% H₂O₂ and one percent DMSO was considered as a positive control. The lipid peroxidation of the treated root was evaluated using Schiff's reagent.

2.5 Statistical analysis

The results are presented as mean $\pm$ SD. The data was analyzed and compared with one way ANOVA followed by Newman-Keuls multiple comparison tests (PRISM GraphPad, version 5.0; GraphPad software Inc., San Diego, CA).

3. Results

3.1 Genotoxicity of the plant extract

There was a significant decrease in the root mitotic index (MI) in the roots treated with the extract at the time points 1 hour, 2 hours and 4 hours in all concentrations used. The lowest dose of the extract used, i.e. 0.2 mg/ml brought a significant decrease ($p < 0.05$) in the MI at 2 and 4 hours (Fig. 1) But there was no significant effect upon its 1 hour treatment. The other concentrations such as 0.5 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml induced significant decrease in the MI in a time and concentration dependent manner (Fig. 1) The MI observed for the normal control water 1, 2 and 4 hour was 94.45 ± 0.99 , 96.72 ± 1.69 and 96.56 ± 1.09 respectively, negative control 1%DMSO for 1, 2 and 4 hours was 94.13 ± 2.48 , 96.46 ± 1.41 , and 97.57 ± 0.57 respectively and the MI of the 0.2 mg/ml extract treated root tips was 92.05 ± 2.57 , 83.62 ± 3.26 , and 64.38 ± 3.94 respectively for 1, 2 and 4 hours. Whereas, the 0.5 mg/ml had the MI of 83.13 ± 3.51 , 64.45 ± 4.49 and 51.25 ± 3.68 . The 1 mg/ml treatment displayed a MI of 74.00 ± 3.09 , 46.33 ± 2.74 , 39.32 ± 2.70 for 1, 2 and 4 hours. The 5 mg/ml and the 10 mg/ml concentrations also followed the similar pattern as the previous lower doses displaying a significant decrease in the root MI in a time and dose dependent manner. I.e., 75.44 ± 1.76 , 40.06 ± 2.54 , 31.31 ± 2.81 and 73.56 ± 0.45 , 29.52 ± 5.52 , 14.34 ± 1.82 respectively for 1, 2 and 4 hours.

The various cellular/chromosomal abnormalities such as chromosomal breaks, bridges, sticky metaphase, disturbed anaphase, multinucleated cells, lesions etc., were counted for each treatment and expressed in percentage in Table.1 and Fig. 2. The relevant photographs are given in Fig. 3. Figure 4 shows images of the normal cell cycle stages.

3.1.1 Plant extract induced DNA damage, *in-situ* lipid peroxidation and accumulated free radical.

The genomic DNA damage analysis was carried out to examine the possible mechanism of the plant extract induced genotoxicity. The isolated DNA from the negative control (1%DMSO), positive control (3% H_2O_2) and 0.2, 0.5, 1, 5, and 10 mg/ml treated root samples for one hour and four hours were subjected to electrophoretic separation. (Fig. 5) An evident DNA damage was seen in the four hour treated roots at 5 mg/ml and 10 mg/ml. whereas only a slight DNA damage was seen in the roots treated with 10 mg/ml extract for one hour.

The *in situ* histochemical localisation of the lipid peroxides were observed by the Schiff's staining of the treated roots. Roots treated for four hours for all extract concentrations developed a prominent pink colour whereas roots treated for one hour showed relatively less colour (Fig. 6) The accumulation of reactive oxygen species in the extract treated roots were visualised by NBT staining. A characteristic blue colour was observed in the four hour treatment which increased with the increase in the dosage of treatment (Fig. 7).

3.1.2 Polyphenol and flavanoid estimation

The *Lagenandra toxicaria* methanol extract had 64.89 ± 1.8147 mg GAE/g of polyphenol content and 39.09 ± 1.975 mg QE/g flavonoid.

3.1.3 SEM analysis

Root surface ultrastructure after the four hour treatment with the extract and the control 1% DMSO was observed under scanning electron microscope. The SEM images revealed a high degree of alterations in the surface morphology at the higher doses, root surface tissue shows fissures and cells were crumbled (5 mg/ml and 10 mg/ml) in comparison with the control. (20 μ M and 10 μ M scale) (Fig. 8)

3.1.4 TTC cell viability test

The cell viability of the plant extract treated samples were checked and compared with the controls (normal control: water, negative Control: 1%DMSO and positive control: 3% H₂O₂). The one hour and two hours of treatment of the root tip with different concentrations of the extract showed no effect on the cell viability and were all stained pink as in the negative control (Fig. 9). But the 10mg/ml treatment for 4 hours resulted in a moderate loss of cell viability showing less staining in comparison with the negative control (Fig. 9).

3.1.5 FTIR analysis

Both the extract treated and control samples were subjected FTIR spectroscopy to examine the possible changes in the biochemical components of the roots. The obtained spectral peaks were assigned to their characteristic organic molecules and the changes in the peak positions due to the chemical shift were noted. The Peaks at the 1033 cm^{-1} and 1244 cm^{-1} were due to symmetric and asymmetric stretching of P = O. The 1545 cm^{-1} and 1633 cm^{-1} positioned peaks were because of the amide II and amide I of the beta sheets and alpha helix of the secondary structure of proteins respectively. 1735 cm^{-1} placed peak is due to ester C = O stretching. 2926 cm^{-1} placed peak is due to the C-H asymmetric stretch of the methyl group of lipids and a broad peak at 3302 cm^{-1} was assigned to the N-H stretching of proteins (Fig. 10).

3.2 The antigenotoxicity potential of the plant extract

The *Lagenandra toxicaria* plant extract showed a potential antigenotoxic activity. Genotoxicity was induced using 3% H₂O₂ in the root tips. Roots treated with 3% H₂O₂ for one hour showed a higher clastogenicity of 93.45 ± 2.33 . Whereas the control roots (water and 1% DMSO) showed only 4.66 ± 1.48 and 5.07 ± 1.78 clastogenicity respectively. The post treatment of the 3% H₂O₂ treated roots with the 0.01mg/ml and 0.02 mg/ml plant extract for 24 hour significantly decreased the clastogenic effects of the H₂O₂ treatment. The 0.005 mg/ml, 0.01 mg/ml and 0.02 mg/ml treatments showed a decreased clastogenicity with increased percentage of inhibition in a dose dependent manner i.e 10.73 ± 6.66 , 43.31 ± 13.56 and 63.28 ± 7.77 respectively. The post treatment of 0.0025 mg/ml extract did not show any evident percentage inhibition and the higher concentration of 0.04 mg/ml treatment for 24 hour resulted in the total disruption of the root tip cells and hence they could not be counted (Fig. 11 and Table 2). The 3% H₂O₂ induced cellular abnormalities were observed to be restored after the treatment with the plant extract at 0.01 and 0.02 mg/ml (Fig. 12). Lipid peroxidation test was done using Schiff's reagent and it showed a pink color in 3% hydrogen peroxide-treated roots. Whereas the color intensity decreased in 0.01 mg/ml and 0.02 mg/ml post treated roots indicating the decreased lipid peroxidation. But the 0.04 mg/ml post treatment resulted in the enhanced pink colour indicating that at the higher concentrations the extract itself acts like a pro-oxidant (Fig. 13).

Table 2
Percentage of the nuclear lesions in antigenotoxicity study.

Treatment	Total cells Mean $\pm$ SD (n = 6)	Total number of nuclear lesions Mean $\pm$ SD (n = 6)	Percentage of nuclear lesions Mean $\pm$ SD (n = 6)	Percentage of inhibition Mean $\pm$ SD (n = 6)
Water control	192.33 $\pm$ 3.51	9 $\pm$ 3	4.66 $\pm$ 1.48	
Negative control (1% DMSO)	203 $\pm$ 5.29	10 $\pm$ 3.79	5.07 $\pm$ 1.78	
3% H ₂ O ₂ + 1% DMSO	200 $\pm$ 15.62	187 $\pm$ 10.01	93.45 $\pm$ 2.33	
3% H ₂ O ₂ + 2.5mg/L	202 $\pm$ 13.08	184 $\pm$ 10.69	91.28 $\pm$ 0.73	4.41 $\pm$ 3.76 ^a
3% H ₂ O ₂ + 5mg/L	218 $\pm$ 29.05	180 $\pm$ 25.01	82.09 $\pm$ 0.69	10.73 $\pm$ 6.66 ^a
3% H ₂ O ₂ + 10mg/L	215 $\pm$ 13.89	110 $\pm$ 24.01	50.99 $\pm$ 7.59	43.31 $\pm$ 13.56 ^b
3% H ₂ O ₂ + 20mg/L	201 $\pm$ 23.64	75 $\pm$ 13.75	37.13 $\pm$ 2.66	63.28 $\pm$ 7.77 ^c

4. Discussion

The plant system is known to be an efficient bioindicator of various genomic alterations such as chromosomal aberrations, sister chromatid exchange and DNA strand breaks induced by any genotoxic agent. Currently, the genotoxicity and antigenotoxicity assay is widely used for the evaluation of any harmful effects of environmental pollutants and synthetic drugs [20, 22, 37 & 38]. Thorough screening of any plant extract for its possible genotoxic and anti-genotoxic potential is important as these extracts with genome modifying capabilities can make way for the emergence of new drugs and therapeutic approaches to treat diseases such as cancer. In this study, we analysed the genotoxic and antigenotoxic potential of the extracts of *Lagenandra toxicaria* using a well established plant model namely *Allium cepa* bioassay. The onion roots possess relatively large-sized cells which are mitotically active having monocentric chromosomes (2n = 16) that stain well with aceto-orcein. The mutagenic responses across plants, mammals and humans are similar and hence the results obtained in this model can be extrapolated to higher organisms [12].

4.1 Genotoxicity assay

A genotoxic compound can alter the DNA of an organism thereby changing the structure and function of its chromosome. These alterations in turn can disturb the cell cycles affecting their MI. MI is an accepted measure of cytotoxicity. A decrease in MI can be a measure of the level of cytotoxicity of the compound. A decrease in the MI below 50% (in comparison with the negative control) is known to be sublethal for the organism where as a decrease below 22% cause lethal effect on the test organism [32, 27]. In this study, the MI of the roots decreased significantly in all concentrations of the extract except for the lowest dose at lower incubation period (0.2 mg/ml for 1 hour). A decrease in MI at higher concentrations like 10 mg/ml of the plant extract is comparable to the decreased MI caused by H_2O_2 . The decrease in MI suggest the anti-proliferative activities of the plant extract and this could be due to DNA replication failure at the S phase or the cell cycle arrest at the G2 phase or reduction in the synthesis of ATP and nucleoproteins preventing the cell from proceeding to the mitosis [25, 35]. The chromosomal aberrations like sticky metaphase, chromosomal bridges, clumped chromosome, multipolar anaphase and nuclear lesions were also increased in a time and dose dependent manner (Table 1 & Fig. 3). According to Rajeshwari *et al.* (2015) such chromosomal aberrations were due to the depolymerization of spindle fibers resulting in shifting of poles. A toxicity of an irreversible type can cause these chromosomal stickiness and the formation of chromatin bridges are usually the product of an unequal chromatin exchange during the translocation processes leading to a structural chromosomal aberration [44, 27]. Earlier studies suggest that naturally occurring bioactive compounds like flavanoids, polyphenols, alkaloids and tannins when present in abundance act as prooxidants and damage the DNA and hinder the cell proliferation mechanism [17]. This oxidative stress can also cause lipid peroxidation, leading to tissue damage. The lipid peroxidation can alter the cell membrane dynamics causing cell injury both in plants and animals. Lipid peroxidation in both plants and animals generates a small molecular weight compound known as the melandialdehyde (MDA) [13, 16]. These aldehyde moieties present on the cell surface can be detected by the schiff's reagent [42]. Hence *in situ* histochemical localization of the MDA was carried out in the roots treated with the plant extracts for different time intervals. The test revealed that the four hour treatment of the plant extracts induced high degree of lipid peroxidation in a dose dependent manner.

Table 1
Percentage of clastogenicity in genotoxic study.

Treatment	Time (h)	Total cells $\pm$ Mean $\pm$ SD (n = 6)	% Clastogenicity $\pm$ Mean $\pm$ SD (n = 6)
Water control	1	190.67 $\pm$ 6.43	4.18 $\pm$ 1.35
	2	201 $\pm$ 7.549	2.82 $\pm$ 0.32
	4	189.33 $\pm$ 8.50	4.40 $\pm$ 1.12
Negative control (1% DMSO)	1	196 $\pm$ 23.39	5.01 $\pm$ 1.54
	2	220 $\pm$ 26.29	3.32 $\pm$ 1.40
	4	178 $\pm$ 17.44	4.85 $\pm$ 0.56
0.2mg/ml	1	190 $\pm$ 11.53	23.67 $\pm$ 4.86
	2	216 $\pm$ 18.36	24.91 $\pm$ 3.43
	4	209 $\pm$ 23.58	33 $\pm$ 4.17
0.5mg/ml	1	184 $\pm$ 18.52	25.11 $\pm$ 4.41
	2	214 $\pm$ 13.89	39.72 $\pm$ 5.69
	4	180 $\pm$ 16.82	41.99 $\pm$ 8.33
1mg/ml	1	192 $\pm$ 30	39.64 $\pm$ 7.95
	2	208 $\pm$ 17.44	46.27 $\pm$ 2.15
	4	199 $\pm$ 12.17	49.3 $\pm$ 1.78
5mg/ml	1	207 $\pm$ 19	38.75 $\pm$ 5.38
	2	233 $\pm$ 26.06	50.38 $\pm$ 1.05
	4	220 $\pm$ 13.08	61.67 $\pm$ 3.98
10mg/ml	1	191 $\pm$ 9.17	46.28 $\pm$ 1.75
	2	208 $\pm$ 13.11	61.31 $\pm$ 2.53
	4	206 $\pm$ 12.77	71.01 $\pm$ 0.73
Positive control (3% H_2O_2)	1	180 $\pm$ 17.44	93.35 $\pm$ 2.45
	2	213 $\pm$ 6.08	92.77 $\pm$ 1.54
	4	178 $\pm$ 19.08	97.89 $\pm$ 0.88

The affect of plant extracts on the (total genomic) DNA integrity was assessed by agarose gel electrophoresis. This revealed that the four hour incubation of onion roots with the plant extracts adversely affected the DNA integrity in a dose dependent manner. And the maximum loss of DNA integrity was observed in 10mg/ml treatment for four hours. These increased DNA damage and lipid peroxidation is attributed to the free radical generation due to the presence of higher concentration of plant polyphenols, flavanoids and tanin etc., [2, 18]. In agreement with the previous studies [40, 23] the NBT free radical assay also suggested that the free radicals were generated in a dose dependent manner (Fig. 7). Later, the treated and the control roots were subjected to SEM analysis to study the possible alterations in the surface morphology of the roots. The roots treated with higher doses of extract for longer duration showed a characteristic morphological difference in comparison to the control groups (Fig. 8). Each type of chemical bond that is present in a cellular macromolecule exhibits its own vibrational properties. And under the influence of oxidative stress these characteristic vibrational modes of a biomolecule will be altered. These alterations in the cellular macromolecules can be analysed by the FTIR technique. The current study employed the FTIR method to check the possible differences in the nature of bio-molecules among the control and the treated samples. The various peaks obtained for different macromolecules were observed at same wave numbers as mentioned in the previous reports (Fig. 10) [30, 37] The peak appeared at the 1633 cm^{-1} in control samples represents the α -helices and β sheets that constitutes the secondary structures of the proteins, and that peak was shifted slightly to 1631 cm^{-1} among the treated groups. This chemical shift suggests the altered α -helices and β sheets among the treated samples and also shifting of peaks in treated groups compared with the control group at 1033 cm^{-1} and 1244 cm^{-1} indicate damage in the phosphate backbone (Fig. 10).

4.2 Antigenotixic/genoprotective effects of *Lagenandra toxicaria*

At higher doses the plant extracts induce genotoxicity but it is also a well established fact that these same compounds at a lower concentration can act as an anti-genotoxic agent that protect the DNA from oxidative damage and keep the cell intact [4, 6]. Hence it is important to note that these two contradictory outcomes are the results of difference in the compound concentration. Therefore, it is reasonable to analyse a plant extract for its antigenotoxic potential at a lower dose. The present study showed that the *Lagenandra toxicaria* plant extract did have antigenotoxic potential at its lower doses (0.01 mg/ml & 0.02 mg/ml) as it displays the chemoprotective activity against the H_2O_2 induced cytological anomalies, mainly the nuclear lesions (Fig. 12). The free radicals generated as a result of H_2O_2 treatment will target the proteins, lipids and carbohydrates and further lead to damage and dysfunction of the cell membranes, enzymes and genetic material. The treatment with 3% H_2O_2 exerts a high oxidative stress leading to the high percentage of nuclear lesions [27]. However, the counter treatment of the H_2O_2 treated root tips with lower doses (0.02 mg/ml) of plant extracts were seen to affectively reverse the actions of H_2O_2 as evident by the decrease in the percentage of nuclear lesions from 93.45 ± 2.33 to 37.13 ± 2.66 (Table 2). The compounds with such bimodal activities having genotoxic effect at higher doses and genoprotective properties at lower doses are referred to as 'Janus-faced compounds' [37]. The crude

extracts are complex mixtures of phytochemicals which work mostly synergistically either as a geno-protective or genotoxic substance. At lower concentration polyphenols, flavanoids, tannins and alkaloids shows antigenotoxic property [27, 37, 24]. The observed decrease in the H₂O₂ induced genotoxicity after the extract modulatory treatment is probably due to the presence of antioxidants in the extract that counters the actions of free radicals. This could also be due to the lower concentration of polyphenols in lower dose treated groups. The presence of polyphenols can be one of the factors that contribute to the antigenotoxic potential of the extract. A study on *Escherichia coli* has found that the polyphenols like the vanillin, cinnamaldehyde, coumarin and tannic acid are involved in the regulation of DNA replication and repair systems after the free radical attack. Another possible explanation for the action of extract is that it can induce the expression of the DNA repair related gene such as the DNA glycosylases which can alkylate the DNA bases and activate the DNA repair systems [9, 27].

5. Conclusion

Our experimental data confirmed the genotoxic and antigenotoxic properties of *Lagenandra toxicaria* rhizome methanol extract. It is found that the plant extract induced the cell clastogenicity and genotoxicity in a time and dose dependent manner. Auto oxidation of polyphenols will trigger oxidative stress and free radical generation depending on different conditions. These phenolic compounds are known for their potential to form complexes with the metal ions such as copper, zinc and iron that are the possible source of hydroxyl radical genesis. These could be the reason for the genotoxic potential of the extract at higher doses. But at lower concentrations, the extracts seem to be geno-protective as shown by the anti-genotoxicity studies. Also the decreased levels of lipid peroxidation of the 3% H₂O₂ treated roots after the subsequent treatment with the extract unfolds the potential of these extract to be a promising genoprotective agent at lower doses. The study presents preliminary data on the potential of the *Lagenandra toxicaria* plant extract as genotoxic and genoprotective agent and further studies are will unravel the molecular mechanisms that underlie these observed phenomenon. However, at higher concentrations the plant extract has proved to be genotoxic.

Declarations

Conflicts of interest

Authors declare there are no conflicts of interests.

Acknowledgement

This work was supported by Directorate of minorities Karnataka Ph.D fellowship (DOM/FELLOWSHIP/CR-2/2017-18).

I thank full to Mangalore university DST-PURSE and USIC.

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Figures

Figure 1

The percentage mitotic index of various treatment groups i.e. 1% DMSO negative control, 3% H₂O₂ positive control and different concentrations of the *Lagenandra toxicaria* extract for 1h, 2h and 4h are shown in the bar graph. The results represented are the average of six individual replicates expressed in mean $\pm$ SD (n=6). The data were analysed by the one-way ANOVA and Newman-Keuls Multiple comparison test. Bars with different superscript letters a, b, c, d, e, f, and g indicate significant difference (p<0.05) across the treatments.

Fig. 2: a, b and c represents % Clastogenicity

Figure 2

The figure displays the percentage of Clastogenicity observed after different control and experimental treatments with the extracts. a) 1 hour, b) 2 hour c) 4 hour. The results represented are the average of six individual replicates expressed in mean $\pm$ SD (n=6). The data were analysed by the one-way ANOVA and Newman-Keuls Multiple comparison test. Bars with different superscript letters a, b, c, d, e, f, and g indicate significant difference (p<0.05) across the treatments.

Figure 3

The various types of chromosomal aberrations and cellular clastogenicity observed after the experimental treatments are displayed in the photo. The cells were observed under the light microscope (400X magnification) and photographed. (A, B, C) Bridged anaphase; (D, E) Disturbed telophase; (F, G, H, I, J) Loss of chromosome in anaphase (K, L, M, N, O, P) Distorted metaphase; (Q) Nuclear lesions; (R, L) Giant cell with strap nuclei.

Fig.4: Normal cell cycle stages

Images were captured under 400X magnification. Bar line represents 20µm

Figure 4

Microscopic images of the normal cell cycle stages a) prophase, b) metaphase, c) anaphase, d) telophase. (400x magnification)

Figure 5

DNA integrity study DNA was isolated and gel electrophoresis was done for different concentration of extract, 1% DMSO (negative control) and 3% H₂O₂ (positive control) treated onion root for 1 and 4 hour.

Fig. 6: Shows in-situ lipid peroxidation level using Schiff's stain

Figure 6

Melanoldehyde detection assay Onion roots were treated with different concentration of extract (0.2, 0.5, 1, 5 and 10 mg/ml), 1% DMSO and 3% H₂O₂ (positive control) for 1, 2, and 4 hour. After the treatment, roots were incubated in to Schiff's stain and photographed.

Figure 7

Free radical detection assay Accumulation of free radical examined in different concentration of extract treated root, 1% DMSO and 3% H₂O₂ for 4hour using Nitro blue tetrazolium stain.

Fig. 8: Ultra structures of the root tips examined using FESEM

Figure 8

Ultra structure of the root examined using field emission scanning electron microscopy. Different concentration (1 mg/ml, 5 mg/ml and 10 mg/ml) of extract treated onion root and 1%DMSO (negative control) treated onion root tip images were captured using field emission scanning electron microscopy.

Figure 9

Cell viability assay by Triphenyl tetrazolium chloride: Roots were treated with different concentration of the extract, 1%DMSO and 3% H₂O₂ for 1, 2 and 4 hour. After the treatment, roots were stained in TTC and photographed.

Figure 10

Fourier-transform infrared spectroscopy (FTIR) analysis: Chemical shift examined in different extract treated roots, 1%DMSO and 3% H₂O₂ in 4 hour using Fourier-transform infrared spectroscopy.

Figure 11

Anti-genotoxic potential of the *Lagenandra toxicaria* extract: Graph represents the percentage of lesions in the root tips treated with 3% H₂O₂ and the percentage of lesions after the 24 hours modulatory treatment with plant extract. The 1%DMSO served as the negative control. The data is represented in mean $\pm$ SD and analysed by one-way ANOVA and Newman-Keuls post test.). Column with different superscript letters (a, b, c, d and e) are significantly different ($p < 0.05$)

Figure 12

The picture shows the clastogenicity formed under the influence of 3% H₂O₂ for 1 hour and the effect of modulatory treatment (24 h) in lowering the observed number of clastogenicity. Images were photographed under 400X magnification. Table 2: Shows percentage of the nuclear lesions in antigenotoxicity study. Percentage of nuclear lesions (Mean $\pm$ SD) in Control (1% DMSO) and low concentrations of the *Lagenandra toxicaria* extract treated onion root tip. Column with different superscript letters are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by the Newman-Keuls Multiple comparison test.

Figure 13

Schiff's staining represents level of lipid peroxidation in the root treated with different lower concentration of the extract in antigenotoxicity study.