Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 8 [10] September 2019 : 93-99

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.876
Universal Impact Factor 0.9804
NAAS Rating 4.95

ORIGINAL ARTICLE **OPEN ACCESS**

Radioprotective effect of *Citrullus lanatus* rind extract against X-ray irradiation in the *Allium cepa* assay

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ABSTRACT
The radio protective potential of Watermelon (Citrullus l+anatus) against X-ray irradiation was investigated using Allium cepa assay. A single dose delivery of 200 cGy X-ray was applied to the bulbs in all treatments except for those intended for T_0(negative control). All bulbs was presoaked in distilled water to break dormancy before irradiation. Culture medium for $T_0$and T_+(positive control) was distilled water, while for T_1, was watermelon rind aqueous extract (WMRE) of 1g/mL and for T_2, was Vitamin E d-Alpha-Tocopherol at 1.072mg/ml. The results showed mitotic depression in all irradiated bulbs; being greatest in T_+. Variations in mitotic index was not significant between T_1 and T_2; both being significantly lesser than T_0 but are significantly higher than T_+. A notable array of chromosomal aberration (CA) were observed which include stickiness, micronucleus, c-metaphase, vagrant chromosome, multinucleated cell, bridges, laggards and breaks; they were more frequent in all irradiated groups but with apparent mitigating effects in treatments T_1 and T_2. These results indicate radio protective potential of Watermelon (Citrullus lanatus) .
Keywords: Radioprotective, Watermelon rind, X-ray, Allium cepa, mitotic index, Chromosomal aberration

Received 19.07.2019 Revised 03.08.2019 Accepted 29.08. 2019

INTRODUCTION

Ionizing radiation such as X-ray is widely used in medical diagnostics, cancer-related therapy and various industrial applications [41; 36]. In spite of its usefulness, X-ray can induce harmful effects directly or indirectly on cellular constituents [15]. Known hazards associated with human exposure to ionizing radiation include: induction of cellular death, genetic mutations, and carcinogenesis [41]. Another direct effect of radiation exposure is the generation of excessive reactive oxygen species (ROS) [30].

ROS are among the earliest products formed when ionizing radiation is absorbed by small molecules, primarily water surrounding cellular bio-macromolecules [41, 4]. Martin *et al.* [26] and Dizdaroglu [11] have shown thathydroxyl radicals in particular are highly reactive, affecting DNA molecules resulting to a broad spectrum of structural damage to include oxidative base modification, single strand breaks (SSB), double strand breaks (DSB), cross-links, clustered base damage, and mismatch repair (MMR). These may upset subsequent processes of replication, transcription and translation.

Cells can mitigate increases in the concentration of free radicals through production of natural antioxidants (e.g. superoxide dismutase, glutathione and catalase) that eliminate or minimize free-radical induced damage to cellular structures. This allows cells to enhance DNA repair, reduce the post-irradiation inflammatory response, or even delay cellular division allowing more time for cells to repair or undergo apoptosis [32]. However, as shown in a number of studies, administering of radioprotective agents, can also reduce radiation-related deleterious effects [7, 22, 32, 41]. Intervention like this would enable the cellular defenses to keep up with the generation of free radicals due to radiation exposure.

Presently, the agriculture and food processing industries produce significant amount of solid waste by-products which could be valuable source of antioxidants and potential radioprotective agents. Watermelon (*Citrullus lanatus*) is considered as one of the major under-utilized fruits grown in the warmer parts of the world [39] being primarily utilized in the food industry for its pulp and juice [1, 5] but the rind is often discarded. However, the watermelon rinds are reported to have therapeutic effect and ascribed to as antioxidant [29, 31]. Watermelon rind are known to be a rich source of Citrulline, a non-essential amino acid which is an efficient hydroxyl radical scavenger and a strong antioxidant. The

usage of these wastes as a source of radioprotective agent could also present a solution to the environmental problem. Thus potential of this underutilized agricultural waste as a source of radioprotective agent is therefore considered and addressed in this study.

MATERIAL AND METHODS

Preparation of treatments

Watermelon rind extract (WMRE)

The watermelons (var. round, seeded and red flesh) used in this study were purchased from the local grower in Wao, Lanao del Sur, Philippines. The melons were washed thoroughly with distilled water and sliced to remove the red flesh. The rinds were then processed in a blender with distilled water in a ratio of 1:1 (g/ml), then filtered with a Watman filter paper (no. 4) to separate the pulps from the extract. The extract was stored in tinted bottles that were thoroughly cleaned and dried prior to use. Rind extracts were prepared on a daily basis to ensure freshness.

Vitamin E supplement solution

Vitamin E supplement Myra E 400 soft gel capsule (d-Alpha-Tocopherol) were purchased from the local pharmacy. The capsule of Myra E containing 400 UI of Vitamin E which is equivalent to 268 mg were opened and diluted to 250ml of distilled water to produce a 1.072mg/ml emulsion. These process was also done on a daily basis.

Test procedure

Pre-treatment of *Allium cepa* L.

Commercial onions *Allium cepa* L. (2n=16) approximately of equal sizes were used. Before the bulbs were used, the dried outer scales were removed and the base scraped to remove dead tissues exposing root primordia to promote the emergence of new roots. Five bulbs of *A. cepa* were used for each treatment. The bulbs were placed in a small 50 mL plastic cups with the basal end dipping in distilled water for 24 hours to break dormancy.

Irradiation

The bulbs were assigned by following the treatments listed in Table 1. With the exception of the negative control group (T_0), all bulbs were then placed inside transparent zip lock cellophane bags and exposed to 200 cGy X-ray at single dose delivery, 10 cm distance from the source using the High voltage X-ray generator, Analog Series KB-500 (Apothecaries co.). The irradiation was performed by a qualified radiology technologist at an Imaging facility in Wao District Hospital. After the irradiation, the bulbs were cultured in their respective treatments.

Table 1. Experimental groups and their treatments

Treatment groups	Treatment	Replication
T_0- Negative control	Distilled water	5
T_+ - Positive control	200 cGy;Distilled water	5
T_1 - Vitamin E	200 cGy; Vitamin E	5
T_2 - WMRE	200 cGy; Watermelon rind extract	5
The water, vitamin supplement and the WMRE were replaced every day for 3 to 4 days until 1-3 cm roots emerge from the bulbs.		

Staining and slide preparation

Roots were harvested only between 10:30-11:30 AM/PM, fixed in Farmer's fluid (3:1 absolute ethanol and glacial acetic acid) for 24 hours and stored in 70% ethanol at 4^{o}C in the refrigerator until further examination. Staining and slide preparation followed the modified procedure of Guerra and Souza [18]. One thousand cells per bulb (or 5000 cells per treatment) were examined and scored to determine the following: mitotic cell count for mitotic index; chromosomal aberrant (CA) cell count for aberration index; mitotic phase frequencies; and different types of abnormality frequencies in different mitotic phases.

Statistical analysis

All statistical analysis were calculated using the IBM SPSS software Ver. 25.0 (IBM Corp. 2017) with the level of significance established at α=0.05. ANOVA or One way analysis of variance were conducted followed by Dunnet's multiple comparison test and Tukey HSD test to identify the significant differences among experimental groups.

RESULTS AND DISCUSSION

Effect of X-ray on Mitotic index (MI), number of Chromosomal aberration (CA), Aberration Indexes (AI) and normal mitotic cell distribution across different mitotic phases.

The X-ray induced variations in Mitotic index (MI) on *Allium cepa* observed in this study (Table 2) indicates mitotic depression in all irradiated bulbs with the positive control (T_+: 9.1; SD+0.32) registering significantly the greatest decrease relative to the unirradiated Negative control (T_0: 13.58; SD+0.7). For bulbs receiving post irradiation treatment with Vitamin E (T_1: 10.42; SD+0.34), and WMRE (T_2 10.28; SD+0.34), MI were also significantly lower relative to the T_0 Negative control but both values were significantly higher than the T_+MI value. When compared, variance in MI values betweenT_1 and T_2were not significant. The Ratio between the number of mitotic cells to the total number of examined cells (Mitotic index) is a useful measure of cellular proliferation which can be used in predicting the overall survival response to any cellular treatment [34]. A decrease in the mitotic index could be interpreted as cellular death a phenomenon which can be attributed to X-ray induced inhibition of certain types of nuclear proteins that are essential in the mitotic cycle [21, 14, 2, 8]. What this study shows is the potential of WMRE to some extent as a radioprotective agent which is relatively comparable to Vitamin E.

Similar trends (Table 2) were also observed for chromosomal aberration (CA) and Aberration Indexes (AI) with irradiated bulbs all having higher CA frequencies and AI values relative to the Negative control (T_0: CA, 5 cells; AI 0.1 at SD+0.07). However, under the CA and AI criteria, irradiated bulbs given the post irradiation treatments in T_1 (Vitamin E: CA 27 cells; AI 0.54 at SD+0.11) and T_2 (WMRE: CA 39 cells; AI 0.78 at SD+0.13) appear to show significant recovery from radiation induced injury relative to Positive control (T_+: CA 86 cells; AI 1.72 at SD+0.32). The information provided by the induced chromosomal aberration and the effects of treatments on their frequencies are important bases in the interpretation of the radioprotective potential of certain substances [9]. The significant spike in the number of CA in the irradiated groups compared to the Negative control proves further the already well understood genotoxicity of X-ray such as on biosynthesis of nucleic acids (DNA and RNA), other nucleo-proteins and spindle fibers which are required in mitotic cycles. This interference then results to various chromosomal disturbances both of structure and behavior [2, 33, 28, 3, 21].

Chromosomal aberrations induced by X-ray irradiation

X-ray induces a wide range of chromosomal aberrations affecting all stages of mitosis in the *Allium cepa* root cells. It must be noted that the observed chromosomal aberrations do naturally occur but in very low frequency. This could also be observed in the Negative control group (T_0) shown in Table 3. In this study, the predominant CA observations were seen in Metaphase and Anaphase (Table 3). This is probably due to the limitations of the light microscopy. In all treatments, T_+ the positive control topped the scores consistently in each category of CA; T_0 the negative control the lowest. Moreover, these X-ray induced radiation injury appears to be mitigated by WMRE and Vitamin E treatments as shown in the low CA values for these treatments in Table 3 when compared to the positive control (T_+).Stickiness, micronuclei and bridges were more frequent than c- metaphase, vagrant chromosomes, Multi-nucleated cells, breaks and laggards in these three treatments (Figures 1).

Chromosome stickiness and anaphase bridges were the most commonly observed CA (Table 3). Chromosome stickiness indicates chromatin dysfunction and is a sign of highly toxic activity on chromosomes which is often irreversible leading to cell death. Stickiness could be the result of radiation action on chromosomal fibers which leads to the entanglement of chromatin threads or may be due to the radiation effect on the process of DNA depolymerization which makes the chromosome surface appear sticky [25, 27, 40, 10, 14]. Chromosome bridges are usually observed in anaphase and in rare cases telophase stage and are formed by the breakage and fusion of chromosome and chromatids, consequently increasing the risk of aneuploidy [23, 17].

In this study, micronucleus were fewer compared to chromosome stickiness and anaphase bridges and comes next in the observed order of decreasing CA frequency. This CA is considered as a biomarker of genotoxic damage because they increase with exposure to clastogenic and aneugenic agents [13, 24] and generally appears at the end of telophase and are also often observed in interphase. The presence of this CA suggests that some acentric fragments or lagging chromosome failed to incorporate into either of the daughter nuclei during telophase of the mitotic cells. The subsequent forming of membrane surrounding those lagging chromatin matter gives rise to these abnormal nuclei. Their formation also implies loss of genetic material and DNA breakage [37]. There were also chromosomal laggards and breaks constituting 12% of the total CA (Table 3). Lagging chromosomes arise from chromosomes that failed to move to either of the poles because of spindle disturbances or chromosomal breaks. Likewise, these could represent some of the acentric chromosome fragments as noted by Turkoglu [38].

The c-Metaphase were among the less observed CA in this study. They were scored on cells containing some condensed chromosomes that were randomly distributed. The formation of this CA indicates inhibitory activity of radiation on the synthesis of the spindle fiber arresting metaphase-anaphase transition. This results to increase risk of aneuploidy [23]. Multinucleated cells were also less frequent among the scored cells. They could be distinguished by the presence of two or more nuclei. This type of

CA may arise because of radiation effect that causes failure in cytokinesis or rendering the cells to have multipolar spindle which leads to the pulling of chromosomes in many directions forming a cell with multiple nuclei [35]. Vagrant chromosome are caused by disturbance in spindle formation that may result to uneven distribution of chromosome with paired chromatids which came from non-disjunction of chromatids in anaphase. Vagrant chromosome being the least of the scored CA (Table 3) also causes some sort of c-mitotic effect implying risk of aneuploidy [16, 19, 6].

Table 2. Summary of Mitotic cell count and Mitotic indexes (MI), Total chromosomal aberrations and Aberration indexes (AI), and Total normal mitotic cells and their distribution in different mitotic stages.

Experimental groups	No. of examined cell	Normal interphase	Mitotic cells	MI (±SD)	C.A	AI (±SD)	Normal Prophase	Normal Metaphase	Normal Anaphase	Normal Telophase	Total normal mitotic cell
Negative control	5000	4321	679	13.58±0.60	5	0.1±0.07	317=47%	189=28%	101=15%	67=10%	674=100%
Positive control	5000	4524	455	9.1±0.64 ◆	86	1.72±0.32 ◆	86=22%	172=44%	70=18%	62=16%	390=100%
WMRE	5000	4478	514	10.28±0.34 ◆■	39	0.78±0.13 ◆■	155=32%	188=39%	77=16%	63=13%	483=100%
Vitamin E	5000	4474	521	10.42±0.45 ◆■	27	0.54±0.11 ◆■	189=38%	185=37%	71=14%	54=11%	499=100%

◆Significant vs Negative control. ■Significant vs Positive control. P<0.05 (Tukey HSD test and Dunnett's Multiple comparison test)

Table 3. Summary of Mitotic aberration types and their percentages

Experimental groups	Stages of mitosis	Chromosome bridge	Sticky chromosome	Micronucleus	Breaks and laggards	c-Metaphase	Multi-nucleated cells	Vagrant chromosome	**Total aberrant**
Negative Control	Interphase	-	-	-	-	-	-	-	-
	Prophase	-	-	-	-	-	-	-	-
	Metaphase	-	2	-	-	-	-	-	2
	Anaphase	2	-	-	1	-	-	-	3
	Telophase	-	-	-	-	-	-	-	-
	Total	**2**	**2**	**-**	**1**	**-**	**-**	**-**	**5**
Positive	Interphase	-	-	21	-	-	-	-	21
	Prophase	-	9	-	-	-	-	-	9
	Metaphase	-	8	-	-	6	-	3	17
	Anaphase	13	1	-	7	-	6	1	28
	Telophase	8	-	-	3	-	-	-	11
	Total	**21**	**18**	**21**	**10**	**6**	**6**	**4**	**86**
WMRE	Interphase	-	-	8	-	-	-	-	8
	Prophase	-	4	-	-	-	-	-	4
	Metaphase	-	6	-	-	4	-	1	11
	Anaphase	7	-	-	2	-	3	-	12
	Telophase	3	-	-	1	-	-	-	4
	Total	**10**	**10**	**8**	**3**	**4**	**3**	**1**	**39**
Vitamin E	Interphase	-	-	5	-	-	-	-	5
	Prophase	-	2	-	-	-	-	-	2
	Metaphase	-	4	-	-	3	-	-	7
	Anaphase	5	2	-	4	-	2	-	13
	Telophase	-	-	-	-	-	-	-	-
	Total	**5**	**8**	**5**	**4**	**3**	**2**	**0**	**27**
Total aberrant		**38**	**38**	**34**	**18**	**13**	**11**	**5**	**157**

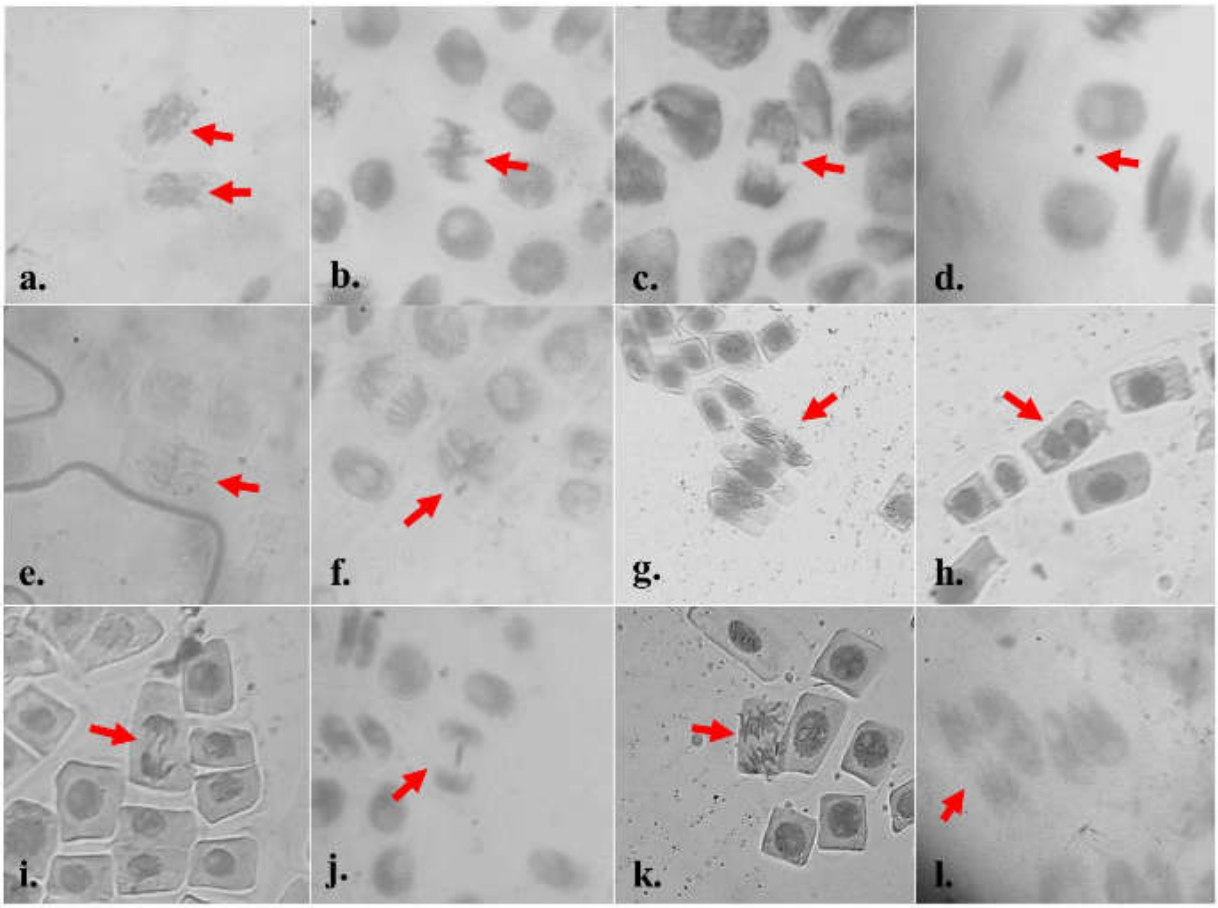

Figure 1. X-ray induced Mitotic aberration observed in *Alium cepa*: a. Stickiness in prophase; b. Stickiness in metaphase; c. Stickiness in anaphase; d. Micronucleus; e. c-Metaphase; f. Vagrant chromosome in metaphase; g. Vagrant chromosomes in anaphase; h. Multinucleated cell; i. Chromosomal bridge in anaphase; j. chromosomal bridge in telophase; k. chromosomal laggards and breaks anaphase; l. chromosomal laggards and breaks telophase.

CONCLUSION

Based on the present study, radiation injury in cells more specifically, cytotoxicity and genotoxicity due to X-ray irradiation has been clearly demonstrated. This significantly reduced the mitotic activity and induced a wide range of chromosomal aberration affecting all stages of mitosis in *Allium cepa* root cells. But this study also shows that WMRE can mitigate this radiation induced injury and is comparable the capacity of Vitamin E. Further evaluation of different WMRE concentration and radiation dosage using more comprehensive genotoxicity assessment that utilize animal model may provide more interesting results that can be used for future human well-being.

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CITATION OF THIS ARTICLE

J. M. Fabrigar and H Porquis. Radioprotective effect of *Citrullus lanatus* rind extract against X-ray irradiation in the *Allium cepa* assa. Bull. Env. Pharmacol. Life Sci., Vol 8 [10] September 2019: 93-99