Phytotoxicity and apoptotic impact assessment of an over-the-counter drug (paracetamol) residue using *Allium cepa* as a bioindicator

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ABSTRACT

Pharmaceutical compounds (PCs) present in the environment can induce adverse toxic consequences on exposed biotic features. Some PCs have been reported to exert fatal impacts on aquatic fauna. However, information on their toxic actions on plants, which are critically essential to ecosystems' health and balance, is scarce and scanty. This study investigated the adverse phytotoxic and apoptotic impacts of a common over-the-counter drug (paracetamol) residue at environmental levels of 0.01 and 0.1 mg/L, using *Allium cepa* (*A. cepa*) as a bioindicator. The inhibition of the germination rate of *A. cepa* root tip was employed as indices (after a 4-day exposure) for assessing paracetamol's phytotoxicity, while deoxyribonucleic acid (DNA) fragmentation assay was used in the apoptotic investigation of paracetamol. It was observed that 0.1 mg/L paracetamol had a phytotoxic impact on the germination rate of *A. cepa* root tips relative to the control ($p<0.05$), while there was no phytotoxic impact exerted by 0.01 mg/L paracetamol. Furthermore, microscopic examination showed irregular prophase in the onion cells. The DNA fragmentation assay revealed the induction of apoptosis by 0.1 mg/L paracetamol, while no apoptosis was induced by 0.01 mg/L paracetamol. Based on the findings from this study, paracetamol can be said to be phytotoxic and bring about DNA damage when exposed to plants at environmental levels. It is therefore recommended that policies be put in place to remediate the environment as regards the removal of paracetamol residues.

Keywords: over-the-counter drug, phytotoxicity, bioindicator, germination rate, apoptosis

INTRODUCTION

Environmental contamination by residues of pharmaceutical compounds (PCs) is unabating due to the health-economic dynamics of demand and supply. Reports (Fatoki *et al.*, 2018, Fekadu *et al.*, 2019, Kovalakova *et al.*, 2020) on findings from the monitoring of PCs worldwide indicated the widespread occurrence of residues of PCs in many fresh surface water river systems and other environmental compartments, although comprehensive information on their occurrence in Nigeria and some African countries are still scanty. They reach the environment from various sources. These sources range from manufacturing and trading industries, post-consumption elimination via sweat, urine, feces, emissions from medical units/hospitals, intensive livestock farming operations, indiscriminate disposal of unused/expired drugs, and effluents from inefficient sewage treatment plants (Deblonde *et al.*, 2011, Puckowski *et al.*, 2016, Olatunji *et al.*, 2017). Discharges from these point sources usually consist of numerous pharmaceutical waste materials which end up in the environment as contaminants (Figure 1).

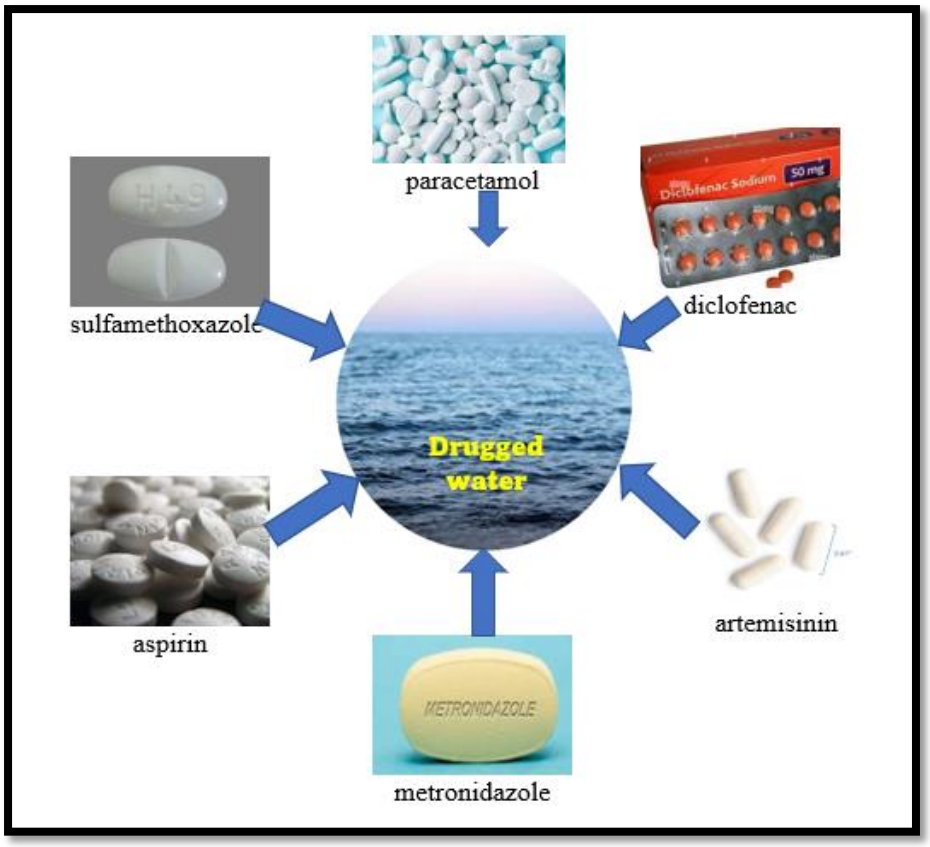

Figure 1: Schematic representation of the presence of selected PCs in aquatic system

Once in the environment, these compounds find their way into different biotic and abiotic features through various routes and pathways, where they are subjected to different fates. Some of these contaminants have been reported to elicit unusual and adverse responses from living organisms in the environment (Mezzelani *et al.*, 2018, Omotola *et al.*, 2022).

Since the detection of PCs in the environment, there have been continuous efforts to monitor their presence and occurrence levels in different environmental compartments. However, investigations on their fate, transformation or depuration, and environmental activities are less conducted, especially in Africa. Unfortunately, this has led to a dearth of information/reports on the ecological impacts of PCs in aquatic ecosystems and other environmental matrices. It is important to understand pharmaceutical bioactivities in the environment in order to ascertain their potential risks and the extent of risks these compounds pose to exposed living entities in the environment. More so, the available few reported data were not measured based on environmentally relevant levels but on discretionary therapeutic concentrations; such data is based mainly on selected fauna as bio-indicators, while data on the ecotoxic actions of PCs on plants are hardly available.

Several faunas has been used in the ecotoxicological characterization of PCs in the environment. Priyan *et al.* (2021) employed the use of *Danio rerio* (zebrafish) to investigate the ecotoxic action of diclofenac in a 4-day exposure study and reported an LC50 value of 156990 µg/L for diclofenac. *Eisenia fetida* (red worm) was used in the ecotoxicity bioassay of sulfamethoxazole (Pino *et al.*, 2015), where the toxic action of concentrations as high as 4000000 µg/L of the compound was assessed. The outcome of this study showed no lethal impact on the red worm after one and two-week-exposure studies to sulfamethoxazole. *Ceriodaphnia dubia* has also been employed in the assessment of the ecotoxic action of ciprofloxacin, returning with an LC50 impact value of 27000 µg/L (Kergaravat *et al.*, 2021). Other reported negative impacts on biotic features arising from exposure to residues of PCs in the environment include hermaphroditic situations in fish due to the presence of 17-β estradiol in the aquatic environment

(Adeogun *et al.*, 2016), gene toxicity, and antibiotic resistance (Beedanagari, 2017, Voigt *et al.*, 2020). However, the concentrations of PCs used in the investigation of ecotoxic actions in most of the earlier reported studies appear too high (up to 1.0×10^6 µg/L) to be reliable (Havelkova *et al.*, 2016, Hernández Martínez *et al.*, 2017). Theses concentrations are at variance with the very low concentration levels often detected in the environment and reported in many monitoring studies. This limits the relevance and reliance of such data for environmental management application/use.

Most of the previous ecotoxicity studies were conducted on aquatic fauna, while few studies were on plants. However, these ecotoxicity studies of PCs reported on plants were not all of the environmentally relevant concentrations. Ecotoxicological characterization of PCs as emerging environmental contaminants should not rely on aquatic fauna as the only ecological bio-indicator that could be used to assess the toxic actions of PC residues found in the environment. Plants, including aquatic and marine ecosystem dwellers, are critical components of environmental dynamics involving processes such as atmospheric oxygen regulation through photosynthesis, provision of essentials for conversion into energy in heterotrophs, nutritional sources, and soil runoff regulation. A severe negative effect on plants' health could result in dire consequences on the ecosystem balance and, in a worse scenario, food security; hence, the need for detailed ecotoxicity assessment of PCs in plants. The presence of residues of PCs has also been reported in soils and other compartments (Beausse, 2004, Kümmerer, 2004). For instance, indiscriminate disposal, use of PC-contaminated 'surface waters' for irrigation of farmland soils, liters containing drug residues dropped from treated farm animals, etc., unintentionally expose crops and flora to the contaminated water. In addition, fauna and flora that inhabit contaminated surface waters and soils are exposed possibly to multiple PC residues along with other organic contaminants, as they are released from multiple channels into the environment.

In this study, the ecotoxic actions of a common over-the-counter pharmaceutical drug (paracetamol (Figure 2)) in water were investigated using *A. cepa* as an index plant (bio-indicator). This PC has earlier been detected in Nigerian surface waters (Ebele *et al.*, 2020). Surprisingly, paracetamol residue has also been reportedly present in tap water in Nigeria (Olaitan *et al.*, 2017). Paracetamol contamination is prevalent in Nigeria's water bodies, with the detection frequencies of 100 % conducted in tap water (Olaitan *et al.*, 2017) and surface water (Ebele *et al.*, 2020). *Allium cepa* assay was adopted and modified for the ecotoxicity studies of this group of contaminants (PCs) from its previously established application for toxicants (Fiskesjö, 1988, Fiskesjó, 1993). *Allium cepa* is also an effective test species for deoxyribonucleic acid (DNA) fragmentation assay (Ndlela *et al.*, 2020, Omotola *et al.*, 2021) and for ascertaining chromosomal aberrations induced in plants at different mitotic stages by toxic substances, and for the determination of potential fatal impacts such as apoptosis (Ndlela *et al.*, 2020, Omotola *et al.*, 2021). In this study, environmentally relevant concentrations tested were considered within the 0.01 to 0.1 mg/L range.

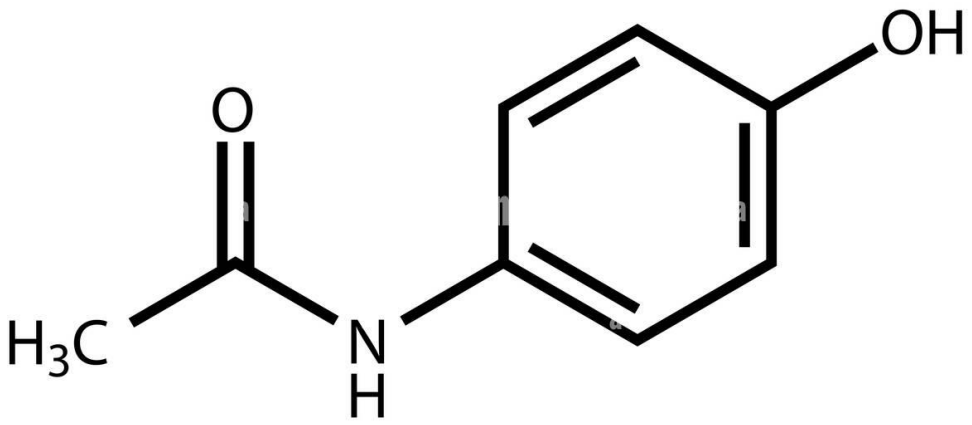

Figure 2: Chemical Structure of paracetamol

MATERIALS AND METHODS

Materials

Neat standard of paracetamol was sourced from Sigma Aldrich, Germany. ApoTarget™ quick apoptotic DNA ladder detection kit (Invitrogen) was procured from South Africa. *Allium cepa* was purchased from South African stores. Other materials and instruments used such as Whatman filter paper and Petri-dishes, sterile surgical blades, light microscope (LEICA DM-750), centrifuge (Thermo Scientific, SL 16R), vortex shaker (Lasec), and incubator (Labnet Accublock mini™) were provided by the Council for Scientific and Industrial Research (CSIR) laboratory, and Genetics Department, Stellenbosch University (SU), Stellenbosch, South Africa. All experiments were carried out in the Council for Scientific and Industrial Research (CSIR) laboratory, Stellenbosch, and Genetics Department, Stellenbosch University, Stellenbosch, South Africa.

Sample preparation

A 1000 ppm stock solution of each PC was prepared in HPLC grade methanol. The stock solutions were stored at 4 ºC in the refrigerator. A second stock solution of 10 ppm of paracetamol was prepared in tap water from where subsequent dilutions were carried out.

Allium cepa assays

Allium cepa root tip assay (onion root)

The onion root length and chromosomal abnormality assay were carried out according to the method described by Barbério (2013). This method was also employed recently by Omotola *et al*. (2021). This method is summarized using a flow chart picture in Figure 3.

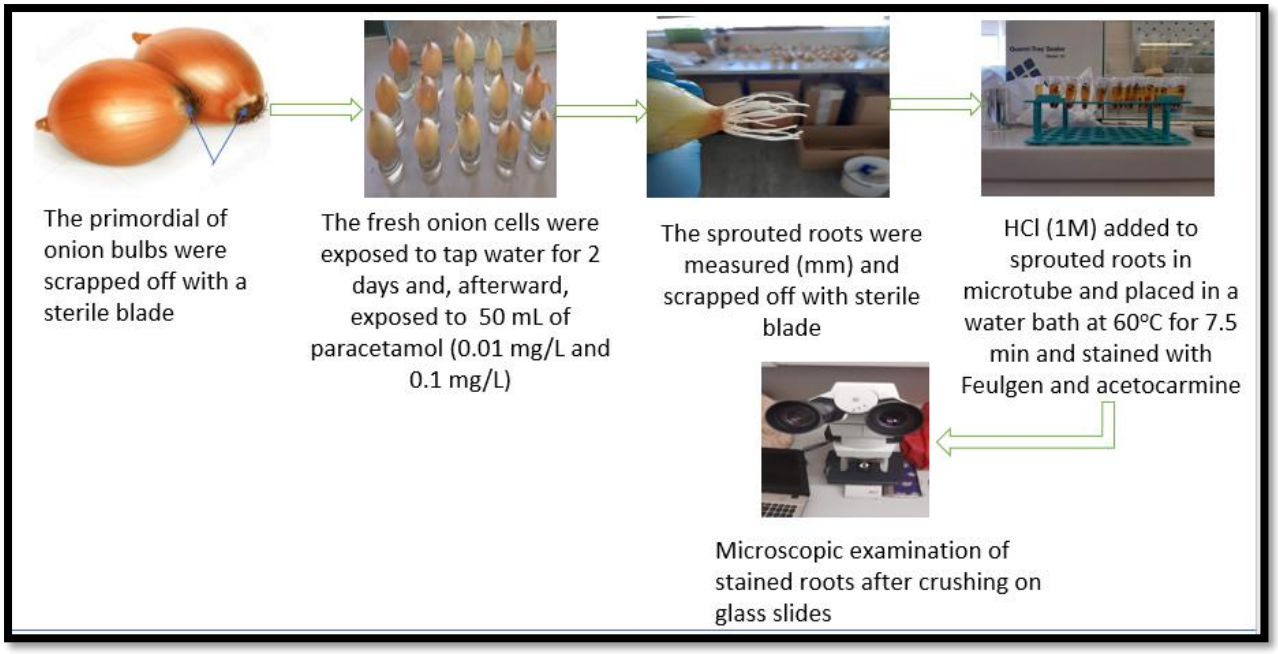

Figure 3: A flow chart picture of *A. cepa* root tip assay procedure

Onion (*Allium cepa*) root DNA apoptosis

Apoptotic effects of paracetamol on *A. cepa* root cells were investigated using a DNA fragmentation assay. Apoptosis, for which DNA fragmentation is an indicator, is described as a form of cell death in

an active and controlled manner that eliminates unwanted cells (Zhang and Xu, 2000, Rahbar Saadat *et al.*, 2015) that can be induced by exposure to toxic chemicals. The ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit (Invitrogen) was used according to the manufacturer's instructions.

Onion roots exposed to paracetamol for 48 hr were harvested in 1.5 mL Eppendorf tubes and rinsed with phosphate-buffered saline (PBS). The mixture was centrifuged at 500 x g for 5 minutes for the effective separation of the PBS (with other debris) from the sample. Following careful removal of the PBS, 35 µL of the lysis buffer (composed of phenol and guanidine isothiocyanate), as provided by the manufacturer, was added to the roots and then gently ground with a micro-pestle and pipetted (Note that root material should not be excessive to allow for effective lysis). After sufficient pipetting, 5 µL enzyme A solution was added (the enzyme nucleophilically attacks and cleaves a target protein) to the crude lysate and vortexed with a Lasec Vortex Genie 2 to ensure homogeneity in the distribution of the enzyme A with the sample. The resultant mixture was thereafter incubated at 37ºC in a Labnet Accublock mini™ incubator for 10 minutes. This was followed by DNA extraction, precipitation with alcohol, and cleaning to confirm the presence and quality of the extracted DNA.

DNA extraction involves breaking cells open to release the DNA by washing samples with PBS and adding lysis buffer to lyse the cells to separate the DNA from proteins and other cellular debris. This DNA extraction step was followed by the addition of 5 µL enzyme B and incubation at 50 ºC for 30 min until the lysate became clear. About 5 µL ammonium acetate solution and 100 µL of absolute ethanol, which had been kept at -20 ºC in the freezer prior to analysis, were added to the mixture. The resultant mixture was then vortexed and then stood for 10-15 min to allow for precipitation. Samples were subsequently centrifuged at 12,000 rpm for 10 min to collect the precipitated DNA, followed by the discarding of the supernatant. The DNA pellets were washed with 0.5 mL of 70 % cold ethanol, and this mixture was re-centrifuged at 12,000 rpm for another 10 min. The supernatant was discarded, and DNA pellets were air-dried for 10 min at room temperature. The DNA was resuspended in 30 µL of DNA suspension buffer. The samples were imaged on a 1.2 % agarose gel made up of 1 x Tris borate EDTA, TB-EDTA, running buffer, stained with Gel Red nucleic acid stain (Thermo-Fischer Scientific) after electrophoresis for two hours at five volts per cm, using a transilluminator (Bio-Rad). The flow chart picture for the DNA fragmentation test of the treated onion root tip procedure is presented in Figure 4.

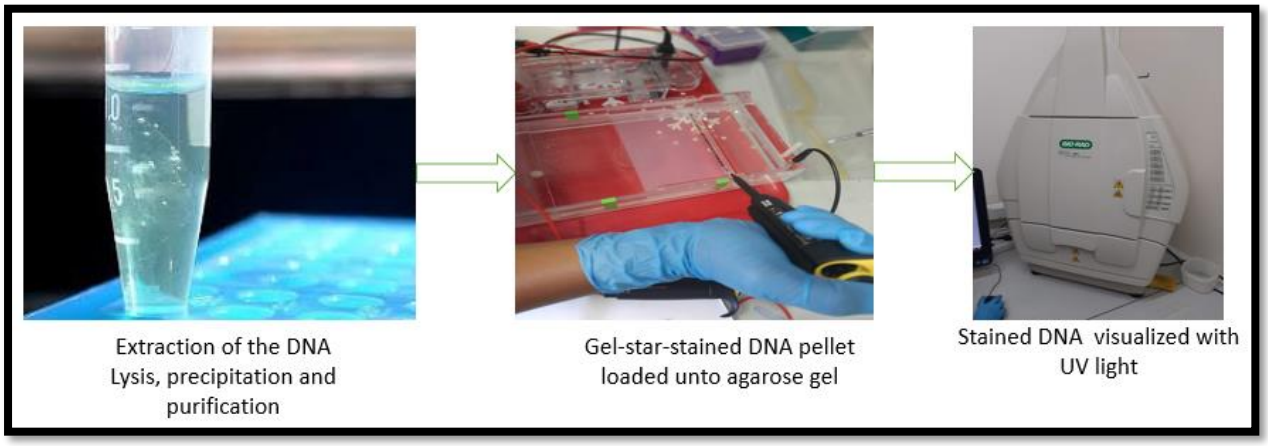

Figure 4: A flow chart picture for DNA fragmentation test of treated onion root tip procedure

RESULTS

Phytotoxic inhibition of root growth of *Allium cepa* bioassay

The test solution of paracetamol was investigated for its phytotoxic characteristics at 0.01 mg/Land 0.1 mg/L concentrations using *A. cepa* bioassays. The root length assay relies on evaluating the root length

of *A. cepa* as an indicator. This is to assess the susceptibility of plants exposed to xenobiotics by evaluating the physical impacts on their root growth. The ratio of the root length after exposure (RLAE) to the root length before exposure (RLBE) with respect to the test solutions was used as indices for the phytotoxicity. According to Figure 5, the results from the root length assay for 0.01 mg/L PC test solutions ranged from a low ratio value of 1.58±0.30 for paracetamol (0.1 mg/L) to a high ratio value of 2.50±0.83 for paracetamol (0.01 mg/L).

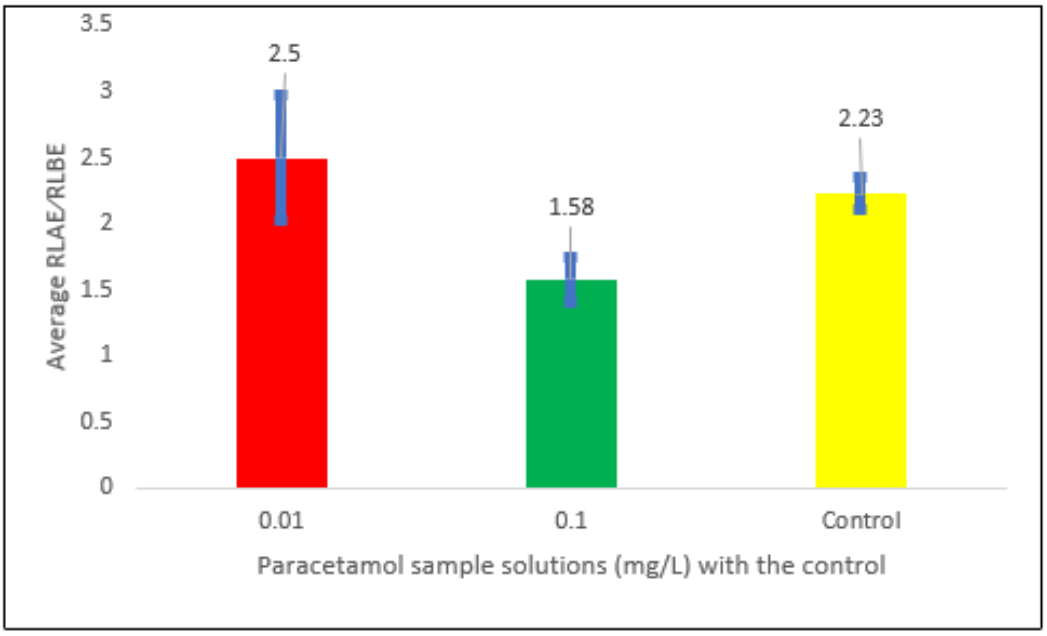

Figure 5: Result from *A. cepa* root tip bioassay

Under controlled conditions, it is expected that the root length should double after two days of exposure (ratio of root length, RRL ≥ 2). Analysis of variance indicated that only higher concentrations (0.1 mg/L) of paracetamol (1.58±0.30) exerted a significant negative impact ($p<0.05$) on the onion root length after exposure relative to the control. Based on the result obtained, it can be concluded that paracetamol exerted a phytotoxic impact on onion root tip growth. However, there is still a need for a more cut-cross evaluation of toxic ecological impacts of toxicants on the wider flora variety.

Allium cepa root tip microscopic examination

Microscopic assessment of *Allium cepa* cells during mitosis was also conducted to ascertain the genetic impacts of the test samples of paracetamol. These impacts were established by different chromosomal aberrations induced in the onion cells after the exposure study. The micrograph (Figure 6) showed that a chromosomal aberration was exerted on all the onion cells exposed to 0.1 mg/L concentration of paracetamol.

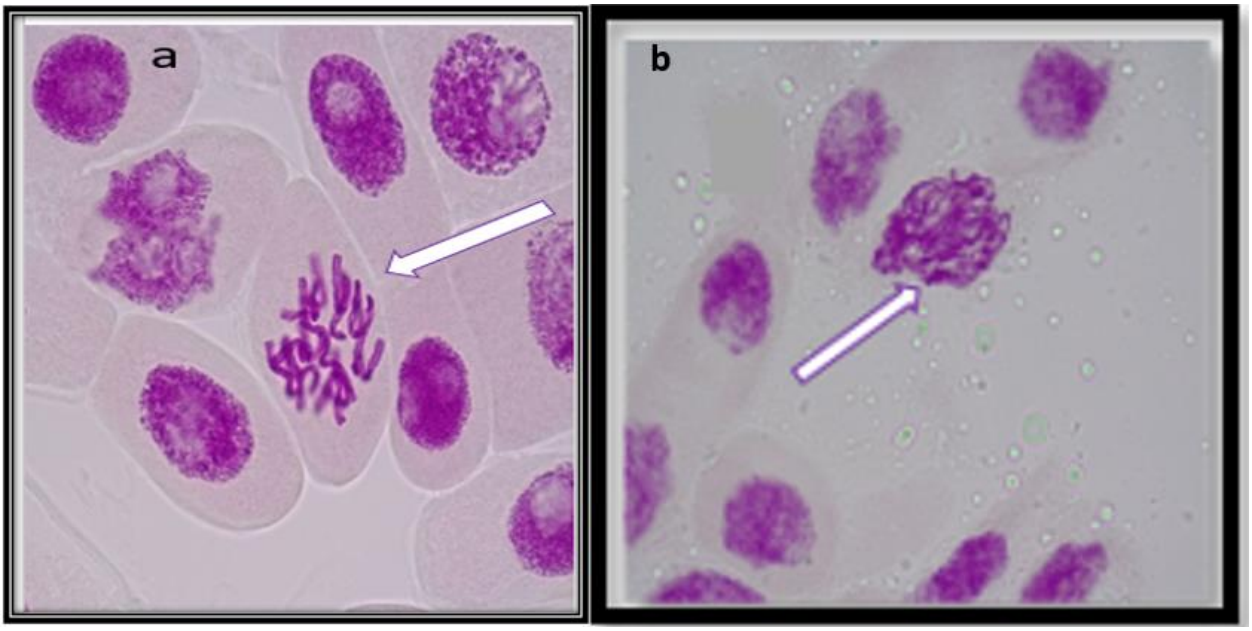

Figure 6: Micrographs of exposed onion cells to the control (tap water (a)) and 0.1 mg/L paracetamol (b), respectively.

Microscopic examination of the exposed onion cells to 0.1 mg/L paracetamol includes irregular prophase while the control sample returned with normal metaphase. Structural chromosome abnormalities sometimes result in the loss of genetic materials (Theisen & Shaffer, 2010), thereby resulting in gene rearrangements. These rearrangements may alter the dosage of genes expressed within the affected chromosomal segment, resulting in fatal consequences eventually. Results from this study revealed the toxicity of paracetamol at the molecular (microscopic) level, and this corroborated the fact that paracetamol is indeed toxic, both at the macro and microscopic levels.

DNA fragmentation assay

DNA fragmentation generally precedes apoptosis (a form of cell death) in an active and controlled manner that eliminates unwanted cells (Zhang & Xu, 2000; Rahbar Saadat *et al.*, 2015), which sometimes takes place in living organisms exposed to toxic chemicals (Ndlela *et al.*, 2020). Ecotoxicity assessment of paracetamol was assessed using indices such as *A. cepa* apoptosis after the chromosomal aberration and reduction in root tip length in *A. cepa* bioassay. A further assessment of the cell stress through the use of the DNA laddering kit was conducted. Gel laddering relies on the ability of DNA molecules to travel through gel based on their size. Apoptosis results in DNA fragmentation, and therefore, smaller DNA fragments migrate further through the gel as opposed to larger and more intact DNA (bands) in the presence of a charge. The migration of same-sized fragmented DNA particles results in the formation of charge-dependent bands. In the event where no banding is observed, complete smearing is indicative of necrosis. Results from this assay (Figure 7), however, indicated that most of the compounds caused cell stress at 0.1 mg/L.

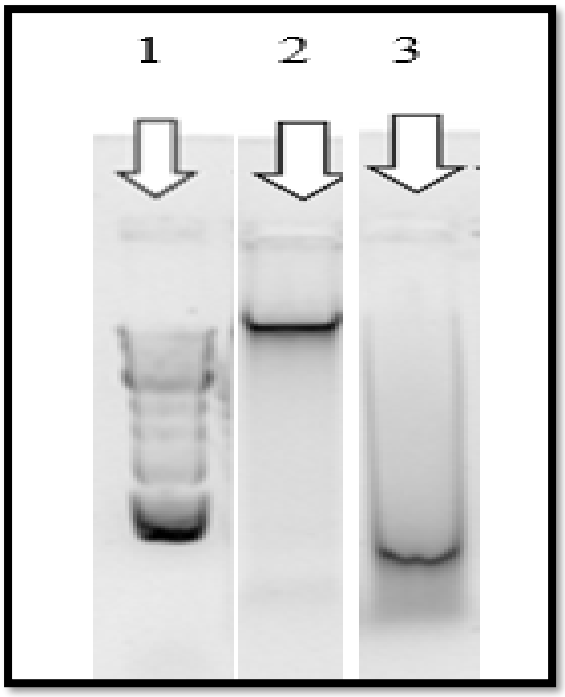

Figure 7: Apoptosis gel for *Allium cepa* exposure to paracetamol

Lane 1 represents the molecular weight marker; Lane 2 represents the 0.01 mg/L paracetamol sample, while Lane 3 represents the 0.1 mg/L paracetamol sample. Based on the banding patterns, as shown in Figure 7, the 0.01 mg/L paracetamol indicated no apoptosis or necrosis, while 0.1 mg/L paracetamol showed apoptotic or fragments of damaged DNA. It can be concluded that a higher concentration of paracetamol (0.1 mg/L) induced more apoptosis in the onions as compared to the lower concentrations where the DNAs were intact. Thus, the DNA fragmentation assay provided information on the apoptotic and necrotic impacts of the paracetamol samples based on the level of smearing and fragmentation, which can be representative of necrosis and apoptosis, respectively (Ndlela *et al.*, 2020).

The ecotoxicity of substances or matrices using *A. cepa* has been previously documented. However, ecotoxicity studies of PCs using *A. cepa* as bioindicators are relatively few. One of such few studies was recently reported by omotola et al. (2021). Omotola and team members reported that lamivudine had a quantifiable adverse impact on *A. cepa*. A similar study on ciprofloxacin reported that 10 mg/kg of ciprofloxacin induced adverse effects in *A. cepa* after a-16 day exposure (Parente *et al.*, 2018). The results from the study by Parente et., 2018 cannot be compared to the present study, as the concentration (10 mg/kg) employed by Parente and colleagues is much higher than that used in this study. Further, Magdaleno et al. (2014) survey revealed the ecotoxicity of hospital wastewater in Argentina. Magdaleno and team members reported that 40 % and 55 % of the samples employed were genotoxic to *A. cepa*. To this end, *A. cepa* has proven to be an effective bioindicator in ascertaining the toxic impacts of pollutants.

CONCLUSION

The assessment of *A. cepa* at a macroscopic, microscopic genetic aberration and cell stress level through the assessment of DNA laddering provides detail on the impacts of paracetamol on biotic components. Only 0.1 mg/L concentration of paracetamol indicated inhibitory growth (phytotoxic effect) at macroscopic assessment through root length measurements. A further assessment of the cell stress through the use of the DNA laddering kit, however, indicated that most of the compounds caused cell stress at 0.1 mg/L. Microscopic assessment of *A. cepa* cells during mitosis was also conducted to ascertain the genetic impacts of these emerging contaminants. Traditionally, the nuclei of mitotic cells in the tip of the onion exposed to test compounds can indicate adverse impacts through the assessment

of normally dividing cells. Based on the known stages of cell division, compounds with adverse impacts would cause anomalies in the cell replication process, which can be correlated to cell death. This provides insight into the sensitivity of *A. cepa*, not only as an agricultural representative but also as a cost and time-effective indicator organism. However, sensitivity to environmental toxins at low or environmentally relevant concentrations varied.

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