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Research Article

Evaluation of Genotoxicity and Cytotoxicity of Different Known Anti-Leishmanial Natural Products

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ABSTRACT

Toxins are harmful substances that can cause visible or invisible damage to organisms or illness if absorbed, inhaled, or taken through the skin [1]. Genotoxicity is the term used to describe the characteristics of chemical agents that damage a cell's genetic information, possibly resulting in mutations that cause cancer. Cytotoxicity is the quality of being toxic to cells. This research aimed to determine the cytotoxicity and genotoxicity of plant extracts from *Cannabis sativa, Digera muricata,* and *Saccharum spontaneum.* It was hypothesized that the genotoxicity and cytotoxicity of these plant extracts increase with concentration. Extracts were prepared in ethanol and evaluated for toxicity. Human red blood cells (RBCs) were used to evaluate cytotoxicity, while the Allium cepa test was performed to assess genotoxicity. Cytotoxicity results showed varying levels of RBC toxicity: *D. muricata* at 1000 µg/mL (16.438%), *C. sativa* at 1000 µg/mL (34.017%), and *S. spontaneum* at 1000 µg/mL (16.169%), indicating higher toxicity at increased doses. Genotoxicity results indicated that *D. muricata* caused the highest mitotic phase arrest at higher concentrations, predominantly affecting prophase and metaphase stages. Chromosomal aberrations included breaks, bridges, sticky chromosomes, abnormal spindle fibers, and irregular chromosomal arrangements during metaphase. Among the plants tested, *C. sativa* was slightly more genotoxic compared to *D. muricata* and *S. spontaneum*. Root length inhibition assays supported the cytotoxicity results, with *D. muricata* being the most effective, followed by *C. sativa* and *S. spontaneum*. Overall, the extracts exhibited dose-dependent cytotoxicity and genotoxicity. Further in-vivo testing and isolation of toxic compounds are recommended.

Keywords: Genotoxicity; Cytotoxicity; Leishmania; Anti-Leishmanial; Natural Products

INTRODUCTION

Toxins are harmful substances that cause damage or illness when inhaled, absorbed, or swallowed. Toxicity refers to the degree to which a chemical substance or mixture can damage an organism, affecting various targets, from enzymes to vital organs. Understanding toxicity is crucial for categorizing toxins and comparing their effects, with metrics like LC_{50} and LD_{50} commonly used [1]. Although some natural products exhibit therapeutic activity, they can also have toxic effects [2].

Natural products remain significant in treating diseases, especially in low-income countries where they have reduced negative effects and more compatibility with human body [3]. Some drugs derived from plants can be effective but may also exhibit toxicity, such as cisplatin, known for its antitumor activity but associated with nephrotoxicity and other side effects [4]. Evaluating drug toxicity involves techniques like metabolomics to identify target organs and biochemical mechanisms [5].Herbal medicines have a long history in traditional treatments and are increasingly demanded by patients for their natural constituents. Despite their benefits, some natural products are genotoxic or cytotoxic, necessitating careful evaluation of their safety and efficacy [2].

Genotoxicity refers to the ability of chemical agents to cause DNA damage, potentially leading to cancer. Assays like the Allium cepa test detect DNA damage and chromosomal aberrations. Cytotoxicity, the quality of being toxic to cells, is commonly assessed using assays such as MTT, with natural products sometimes exhibiting both cytotoxic and genotoxic effects [6, 7].

Plants and plant products have been used in medicine for centuries, offering benefits such as low cost and fewer adverse effects compared to synthetic drugs. However, their effectiveness varies, and some can be toxic [3]. For example, certain natural products exhibit anti-leishmanial activity, but their cytotoxic and genotoxic effects must be evaluated to ensure safety [8].

This study evaluates the genotoxicity and cytotoxicity of extracts from *Cannabis sativa, Digera muricata*, and *Saccharum spontaneum* **(Figure 1)**, which are known for their anti-leishmanial properties. Using human red blood cells and the Allium cepa test, the research aims to determine the toxicity levels of these plant extracts.

Figure 1. Images of studied plants in their natural habitats (a) *Cannabis sativa* (b) *Saccharum spontaneum* and (c) *Digera muricata.*

MATERIALS AND METHODS

Chemicals:

Acetocarmine, fetal bovine serum (FBS), normal HCl, 70% & 90% ethanol, 45% acetic acid, penicillin-streptomycin solution, potassium chloride, Triton X-100, sodium bicarbonate, phosphate-buffered saline, chloroform, ethyl acetate, Hank's balanced salt solution, dimethyl sulfoxide (DMSO), and hydrogen peroxide were purchased from Sigma Aldrich. Triton X-100 was used as a positive control. 96-well plates were used for the experiment under a laminar flow hood (LFH), and for incubation, the CO_2 incubator (MCO-17AIC, Sanyo, Japan) was used.

Preparation of Stock Solutions and Dilutions

110 mg of each plant extract were dissolved in 11 mL of ethanol to create stock solutions. Using the following formula, three different concentrations (1000, 500, and 100 µg/mL) were made:

$$C1V1=C2V2$$

Where $V1$ is the initial volume, $C1$ is the initial concentration, $V2$ is the final volume and $C2$ is the final concentration.

Biological Assays

Chromosomal Aberration Assay (Using Onion Seeds)

The genotoxicity assay on *A. cepa* root meristem was performed according to Bianchi (2016) protocol with minor adaptations [9]. *A. cepa* seeds were sterilized with 70% ethanol, rinsed with distilled water thrice, dried, and germinated in the dark at room temperature (25°C) on filter paper-covered Petri dishes. Each plate initially received 5 mL of water, with additional water added as needed. When the radicles reached 2-3 mm, they were transferred to 3 mL of different drug concentrations (1000, 500 µg/mL) of *Cannabis sativa, Saccharum spontaneum,* and *Digera muricata* for 72 hours. The root tips were then fixed in acetomethanol overnight in a fridge. After removing the fixative, the samples were treated with 90% ethanol for 2 hours and stored in 70% ethanol at 4°C [10].

Following Bianchi (2016), squashes were prepared by treating the root tips with 1N HCl for 1 minute to soften cell walls, staining with acetocarmine for 1 hour, and heating for 5 minutes. The stained root tips were placed on slides with 45% acetic acid and acetocarmine, covered with a glass slip, and gently pressed. Slides were sealed with nail polish. Chromosomal aberrations were observed under a microscope (40X to 100X magnification). At least 100 cells in each mitosis phase (prophase, anaphase, metaphase, telophase) were counted [11]. The mitotic index (MI) was calculated using:

$$\text{Mitotic index } (MI) = \frac{\text{total number of dividing cells}}{\text{total cell number}} \times 100$$

Root Length Inhibition

Methanolic extracts of selected plants were prepared in various concentrations (10,000, 1000, 500, 100, 50, and 25 µg/mL) and stored at 4°C. Healthy A. cepa bulbs were procured, their outer scales removed, and placed in disposable cups with 5 mL of double-distilled water. Prior to use, cups were sterilized with 1N HCl. The bulbs were incubated at room temperature in the dark until roots reached 2-3 cm. Roots were then exposed to different plant extract concentrations for 72 hours under identical conditions [12]. Root length was measured, and the Index of Tolerance (IT) was calculated using:

Index of tolerance (%)
= Mass (length) of roots of experimental group ×100/ Mass (length) of roots of control group

Cytotoxicity Assay

Cytotoxicity assay followed [13] with minor modifications. Fresh human blood cells from volunteers were washed with PBS and centrifuged. Suspensions of 999 µL, 995 µL, and 990 µL of red blood cells were mixed with 1 µL, 5 µL, and 10 µL of each extract (*C. sativa, S. spontaneum, D. muricata*) to achieve final concentrations of 1000 µg/mL, 500 µg/mL, and 100 µg/mL. Samples were incubated at 37°C for 3 hours, then centrifuged at 1000 RPM for 5 minutes. Supernatants were collected and hemoglobin release was measured at 576 nm using

an ELISA plate reader. DMSO was used as a negative control, while 0.5% Triton X-100 served as a positive control with 100% hemolysis. Hemolysis percentage was calculated using:

$$\text{Hemolysis (\%)} = \frac{\text{(OD 576 nm in the sample solution – OD 576nm in PBS)}}{\text{(OD 576 nm in 0.5\% Triton X-100 – OD 576nm in PBS}} \times 100$$

RESULTS

Cytotoxicity

Cytotoxicity tests measured the safety and effects of the selected plant extracts on human blood cells. Cells were treated with plant extracts at 1000, 500, and 100 µg/mL concentrations and incubated for 3 hours. Hemolysis was recorded using an ELISA plate reader at 576 nm. The results indicated*:* ***Saccharum spontaneum***: Hemolysis rates of 16.169%, 2.925%, and 0.642% at 1000, 500, and 100 µg/mL, respectively. ***Cannabis sativa***: Hemolysis rates of 34.017%, 1.720%, and 0.719% at 1000, 500, and 100 µg/mL, respectively. ***Digera muricata***: Hemolysis rates of 16.438%, 1.451%, and 0.621% at 1000, 500, and 100 µg/mL, respectively **(Figure 3)**. *Cannabis sativa* showed slightly higher toxicity compared to *S. spontaneum and D. muricata*. Toxicity levels decreased with lower concentrations. Pictortial representation of RBCs hemolysis by *S. spontaneum, C. sativa* and *D. muricata* are shown in **Figure 2 (a), (b), (c)** respectively. The red color of the solution shows more cytotoxic effect of any plant extracts.

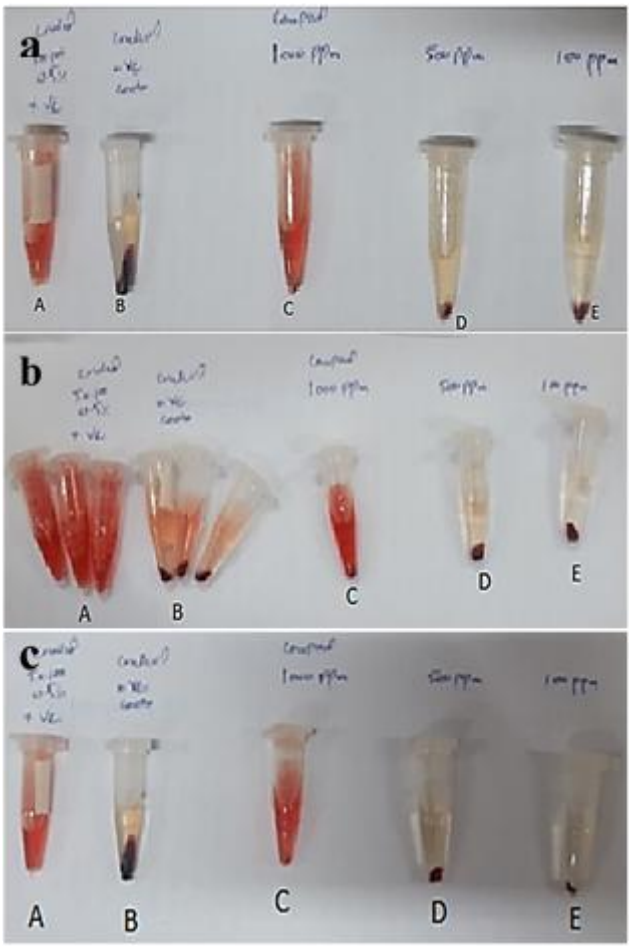

Figure 2. RBCs hemolysis caused by extracts from (a) *S. spontaneum (b) C. sativa* (c) *D. muricata* (A) positive control (B) negative control (C) 1000 µg/mL (D) 500 µg/mL and (E) 100 µg/mL.

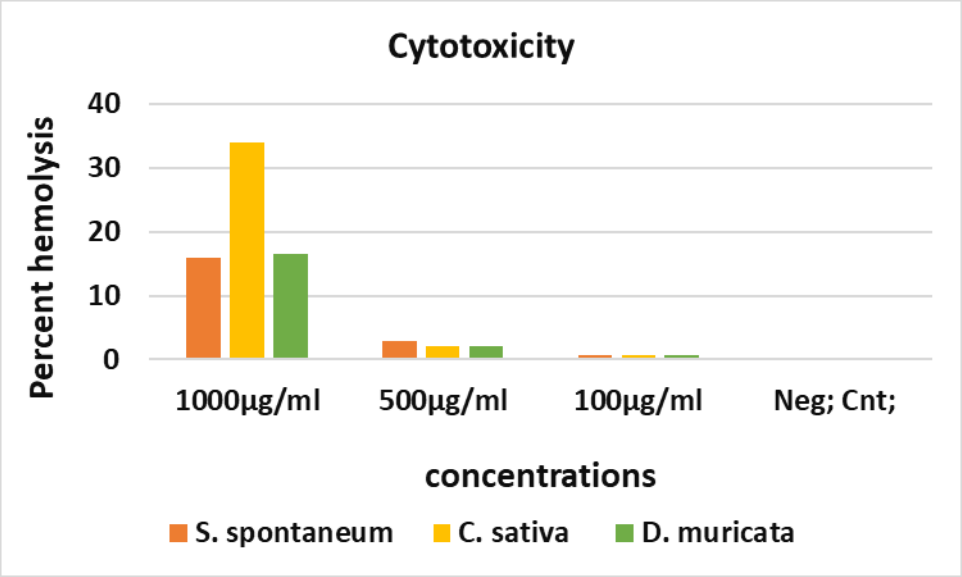

Figure 3. Percent hemolysis of RBC's due to various concentrations of *S. spontaneum*, *C. sativa* and *D. muricata* plant extracts.

Chromosomal Aberration Assay

Onion root tips were exposed to plant extracts at 10,000, 1000, and 500 µg/mL for 72 hours. Chromosomal aberrations (root tip of *A. cepa* meristem cell/tissues) were observed under a microscope and documented.

- ***Digera muricata***:
  - At 10,000 µg/mL: High genotoxicity with de-shaped nuclear membranes and cytoplasmic damage.
  - At 1000 µg/mL: Clear membrane damage with aberrant metaphase and multipolar anaphase stages.
  - At 500 µg/mL: Various aberrations including abnormal anaphase and metaphase **(Figure 4, a, b, c)**.

D. muricata extracts were both cytotoxic and genotoxic, particularly at higher concentrations.

- ***Cannabis sativa***:
  - At 10,000 µg/mL: Aberrations such as broken chromosome bridges and abnormal cytokinesis stages.
  - At 1000 µg/mL: Normal and aberrated cytokinesis stages observed.
  - At 500 µg/mL: Sticky metaphase, cell membrane damage, and chromosomal aberrations.

C. sativa showed significant genotoxicity and cytotoxicity **(Figure 4 d, e,f)**.

- ***Saccharum spontaneum***:
  - At 10,000 µg/mL: Chromosomal aberrations and cell cycle arrest at the prophase stage.
  - At 1000 µg/mL: Cells mainly in normal metaphase and prophase, with some chromosomal aberrations.

S. spontaneum caused both cytotoxicity and genotoxicity, with effects decreasing at lower concentrations **(Figure 4 g, h, i)**.

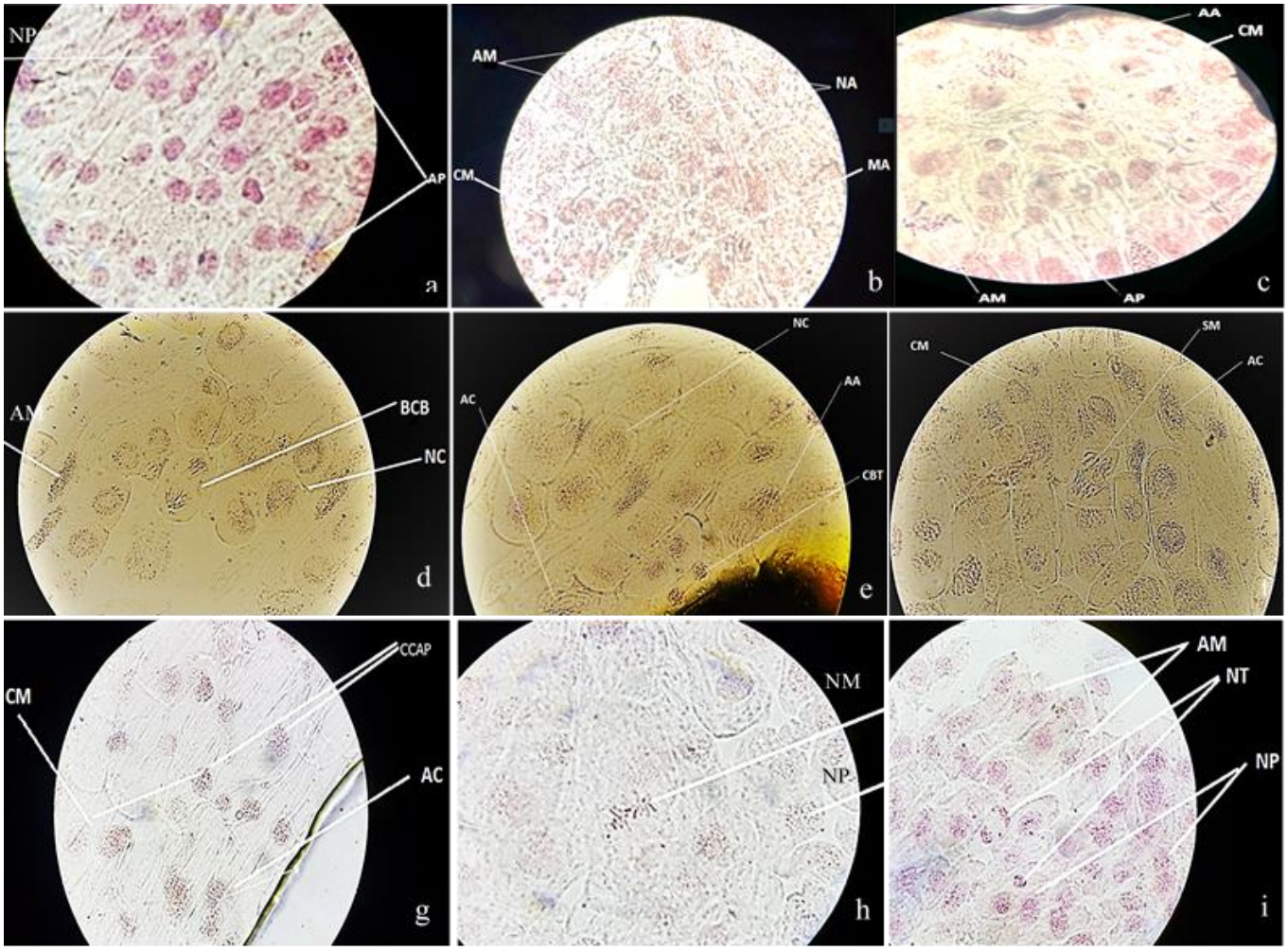

Figure 4. Microscopic analyses of chromosomal damages in *A. cepa* root meristem cell treated by *D. muricata, C. sativa, S. spontaneum* at various concentrations 10,000µg/ml, 1000µg/ml, and 500µg/ml at 72h, respectively. Where NP (normal prophase), AP (aberrated prophase), AM (abnormal metaphase) CM (cell membrane damage), MA (multipolar anaphase), AA (abnormal anaphase), BCB (broken chromosome bridge anaphase), NC (normal cytokinesis), AC (aberrated chromosome), NC (normal cytokinesis) CBT (chromosome bridge telophase) and AA (aberrated anaphase), SM (sticky metaphase), CCAP (cell cycle arrest prophase), NM (normal metaphase), NT (normal telophase).

Mitotic Index (MI):

The mitotic index was calculated to determine the percentage of dividing cells in each phase of A. cepa root cells.

- ***D. muricata***: At high concentrations, 40% of cells were in normal prophase and 60% in abnormal prophase.
- ***C. sativa***: At high concentrations, 24% of cells were in normal cytokinesis, with various other stages showing aberrations.
- ***S. spontaneum***: At high concentrations, 50% of cells were in normal prophase and 50% in abnormal prophase.

D. muricata and *S. spontaneum* were more genotoxic compared to C. sativa at the same concentrations **(Table 1).**

Table 1. The mitotic index (MI) of onion root tip cells treated with different concentrations of *D. muricata, C. sativa* and *S. spontaneum* crude methanolic extract.

	Percentage MI of treated *A. cepa* meristem tissue with *D. muricata*								
Conc. (µg/ml)	**NM**	**AM**	**NP**	**AP**	**NT**	**AT**	**NA**	**AA**	**cytokines**
10,000	0	0	40	60	0	0	0	0	0
1000	0	8	40	12	0	0	28	0	0
500	0	4	68	16	0	0	0	8	0
	percentage MI of treated *A. cepa* meristem tissue with *C. sativa*								
10,000	0	24	20	22	0	0	2	8	24
1000	0	24	24	20	8	8	0	8	8
500	0	24	40	36	0	0	0	0	0
	Percentage MI of treated *A. cepa* meristem tissue with *S. spontaneum*								
10,000	0	0	50	50	0	0	0	0	0
1000	8	0	80	10	0	0	0	2	0
500	0	28	44	8	16	0	0	0	0

Root Length Inhibition

This assay measured the impact of plant extracts on the root length of onion seedlings.

- ***D. muricata***:
  - Complete inhibition of root growth at 1000 µg/mL.
  - Maximum root growth at 25 µg/mL **(Figure 6 a)**.
- ***C. sativa***:
  - 100% inhibition at 10,000 µg/mL.
  - No inhibition at concentrations below 25 µg/mL **(Figure 6 b)**.
- ***S. spontaneum***:
  - No root growth at 10,000 µg/mL.
  - Increased root growth with decreased concentrations, with the highest growth at 25 µg/mL **(Figure 6 c)**

Overall, these plant extracts were highly toxic at higher concentrations (1000 and 10,000 µg/mL) but non-toxic at concentrations below 25 µg/mL. Among the three plants, *D. muricata* was the most toxic to onion root growth **(Figure 5)**.

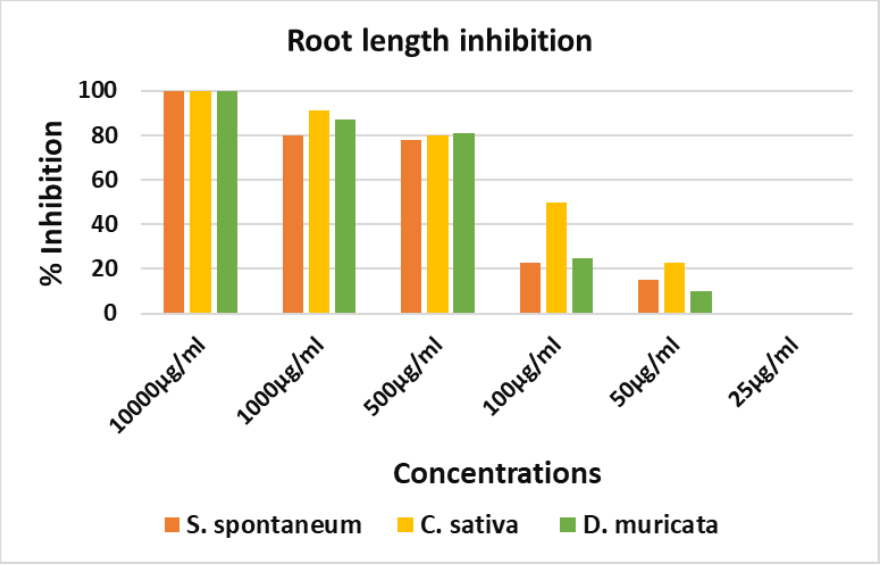

Figure 5. Percent inhibition of roots length of the A. cepa treated with different concentrations (10000, 1000, 500, 100, 50, 25µg/ml) of *S. spontanum. C. sativa and D. muricata* plant extracts.

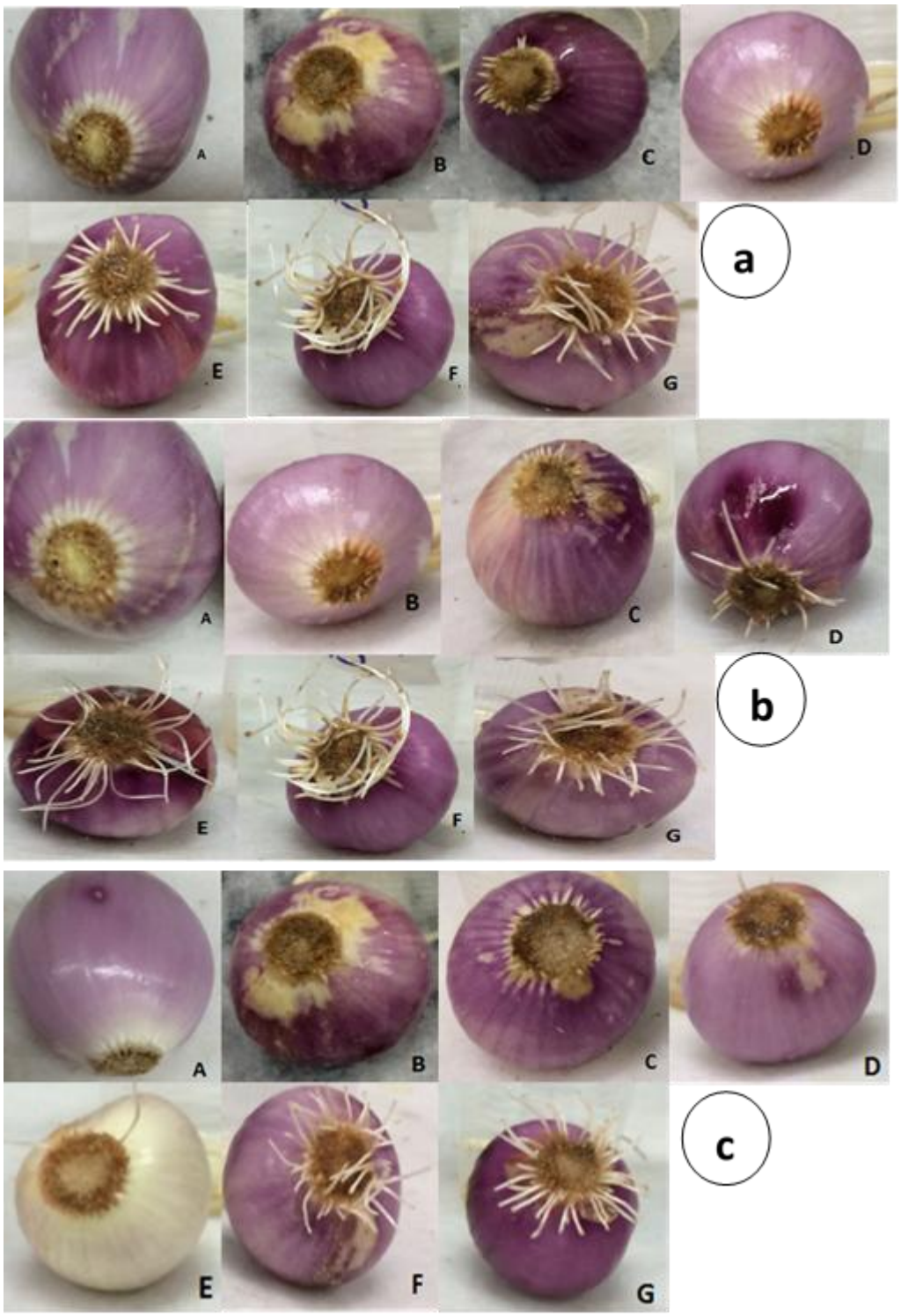

Figure 6. Showing the root length of the onion bulbs at six different concentrations i.e., (A) 10000 µg/mL, (B) 1000 µg/mL, (C) 500 µg/mL, (D) 100 µg/mL, (E) 50 µg/mL, (F) 25 µg/mL, of the plant extract of (a) *D. muricata*, (b) *C. sativa,* and (c) *S. spontaneum* after 72 respectively, (G) negative control exposed to only tap water.

DISCUSSION

Toxins are harmful substances that cause damage or illness when inhaled, absorbed, or swallowed. These toxins range from large to small chemical compounds, affecting various targets such as enzymes [1].

Using plants and plant products to treat diseases is an ancient practice. The main advantages of plant-based medicine are its perceived efficacy, low cost, and minimal serious adverse effects [3]. This practice remains essential for about 75-80% of the global population, especially in developing countries, due to its compatibility with the human body, fewer side effects, and cultural acceptance [2]. However, a significant drawback is that plant-based treatments can be cytotoxic and genotoxic, affecting both normal and cancerous cells. The sensitivity of cells to these treatments depends on the drug's selectivity. Rapidly dividing cancer cells are particularly sensitive, but normal functional cells are also affected. Genotoxic agents can cause DNA damage, leading to mutations and potentially cancer. Not all genotoxic substances are mutagenic, though all mutagens are genotoxic [14].

In this study, we examined the cytotoxic and genotoxic activities of three medicinal plants: *Digera muricata*, *Saccharum spontaneum*, and *Cannabis sativa*. *Digera muricata*, from the Amaranthaceae family, is an annual plant used extensively in traditional medicine. Its extracts contain numerous phytochemical constituents, demonstrating various activities like diuretic, antibacterial, antifungal, and antioxidant properties [15]. *Saccharum spontaneum* (Poaceae family) is a perennial grass found mainly in tropical Asia. Its leaves and stalks contain lignin, proteins, carbohydrates, and amino acids, while its roots contain starch and polyphenolic compounds. *Cannabis sativa*, native to South and Central Asia, is cultivated worldwide. It has multiple uses, including fiber, medicine, food, and recreational drugs [16].

Cytotoxicity

Previous studies have investigated the molecular mechanisms of hemolysis, identifying that interactions between molecules and RBCs result in increased membrane pore formation, leading to cell swelling and bursting [17]. Toxicity assessments of plant extracts help evaluate their safety [18].For example, acetogenins from *Annona muricata* and *Annona squamosa* were found more toxic to mammalian cells than the standard drug glucantime [19]. Our results showed that at higher doses (1000 µg/ml), *D. muricata* (16.438%), *C. sativa* (34.017%), and *S. spontaneum* (16.169%) are more cytotoxic compared to lower doses (100 µg/ml), which exhibited minimal toxicity: *D. muricata* (0.621%), *C. sativa* (0.719%), and *S. spontaneum* (0.642%) **(Figure 3)**.

Chromosomal Aberration Assay

The roots of *Allium cepa* were exposed to different concentrations of plant extracts (10,000, 1000, and 500 µg/ml) of *D. muricata*, *S. spontaneum*, and *C. sativa*. Observations under a microscope revealed various chromosomal aberrations, including sticky chromosomes, disturbed metaphase, chromosome breaks, and chromosomal bridges at anaphase and telophase. Normal phases such as prophase, metaphase, and cytokinesis were also noted. These results align with [9, 20, 21]. Higher concentrations (10,000 µg/mL) of *D. muricata* caused significant cell cycle arrest with 40% normal prophase and 60% abnormal prophase. *C. sativa* at high concentrations showed 24% normal cytokinesis and various stages of cell arrest. *S. spontaneum* also demonstrated a substantial cell cycle arrest at higher concentrations **(Figure 4)**. Overall, the plant extracts showed varying degrees of toxicity across different mitotic phases, with *C. sativa* being slightly more genotoxic than *D. muricata* and *S. spontaneum*.

Root Length Inhibition

The average length of *A. cepa* roots treated with different concentrations of plant extracts was measured after 72 hours. High concentrations (10,000 µg/ml) resulted in null root growth, while lower concentrations showed the highest growth. Negative controls (tap water) had fresh, well-grown roots **(Figures 5 and 6)**. The results showed a decreasing percentage of root length with increasing concentrations, indicating the presence of toxic substances in the plant extracts affecting root growth. These substances interfere with cell division and elongation processes [22]. Similar findings were reported by [23]. Among the three plants tested, *D. muricata* was the most effective in inhibiting root length, followed by *C. sativa* and *S. spontaneum*. All plants showed dose-dependent activity, with higher concentrations increasing toxicity. In conclusion, our study confirmed that the selected plant extracts exhibit cytotoxic and genotoxic properties at higher concentrations, affecting both cell division and root growth in *A. cepa*.

CONCLUSION

This study evaluated the cytotoxicity and genotoxicity potentials of plant extracts from *Cannabis sativa*, *Digera muricata*, and *Saccharum spontaneum*. The results indicated that at high concentrations, all three plant extracts were hemolytic to human red blood cells, while at low concentrations, they were not hemolytic. Among the plants tested, *C. sativa* exhibited slightly higher toxicity to human erythrocytes compared to *D. muricata* and *S. spontaneum*. Furthermore, *C. sativa* showed greater genotoxicity than the other two plants. At the highest concentrations, all three plant extracts caused nearly 100% inhibition of root length, with *D. muricata* being the most effective. Based on these findings, we conclude that *C. sativa* is the most cytotoxic and genotoxic, followed by *D. muricata* and *S. spontaneum*. We strongly recommend isolating and purifying the specific toxic substances from these plants and further evaluating their toxicity levels in vivo.

Data availability

All data is available in the manuscript.

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Competing interests

The authors declare no competing interests.

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