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Phytochemical screening, Cytotoxic, genotoxic and mutagenic effects of the aqueous extract of *Azadirachta indica* leaves

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Abstract
Azadirachta indica is a medicinal plant used to treat a various number of diseased conditions such as malaria, cough, asthma, diarrhea and diabetes. In traditional medicine, the aqueous concoction of the leaves is used to treat malaria. The phytochemical screening of the aqueous extracts from the leaves of *A. indica* have been shown to contain tannins, saponins, terpenoids, glycosides, steroids, flavonoids and glycosides. Previous studies on the phytochemical screening of *Azadirachta indica* leaves have shown the presence of tannins, saponins, terpenoids, glycosides, phytosterols, flavonoids and glycosides. Four different concentrations of the extracts were used to treat the roots of *Allium cepa* seedlings. In the *Allium cepa* assay, the extracts exhibited cytotoxic effects, expressed as the decrease in the root growth length when the concentrations increased. The EC50 was 0.21g/l. The extract induced an increased mitodepressive effect and chromosomal aberrations in the *Allium cepa* cells as the concentrations increased. The results with a p-value < 0.05 were considered as statistically significant compared to the untreated group in distilled water. The observed chromosomal aberrations appeared as c-mitosis, chromosomal fragments, microbridges, bipolar and multipolar anaphases. The aqueous extracts of *A. indica* leaves have also shown to cause mutation in *Salmonella typhimurium* TA 98 and TA 100 strains. Therefore the leaves of A. indica have a genotoxic effect on chromosomes and also can cause mutations in cells. However the potential genotoxicity of the aqueous extract of *Azadirachta indica* leaf in human cells needs has not been established.

Keywords: *Azadirachta indica*, phytochemistry, *Allium cepa*, Ames test.

1. Introduction
Interest on medicinal plants amongst local communities has increased globally because of their value in the treatment of diseases [1-2]. An estimated 80% of the population in Africa relies on medicinal plants to treat their various ailments [3]. This has generated the interest to establish their chemical constituents and therapeutic application during the last few decades. In modern therapeutics, natural products and their derivatives represent more than 50 % of all the drugs [4]. Medicinal plants have therefore played a pivotal role in the health care of many cultures, both ancient and modern [5-9]. *Azadirachta indica is* used to treat malaria in various part of the world [10]. However, though *Azadirachta indica* leaves are beneficial to human health, their potential cytotoxic, teratogenic, genotoxic or carcinogenetic potential may be under-evaluated. It has been reported that some plant secondary metabolites can induce genotoxic or cytotoxic effects [11]. In this study, the aqueous leaf extract of *Azadirachta indica* were used to expose *Allium cepa* meristematic cells (eukaryotic) and *Salmonella typhimurium* (prokaryotic) TA98 and TA100 strains.

2. Material and methods
2.1 Material
Onion seeds (Kenya Highlands), distilled water, petri dishes, filter papers (Whatman N°1), meter ruler, glyphosate, safranin, analytical grade acetic acid, concentrated hydrochloric acid (analytical grade), Ames test (MPF) microplate format kit (Xenometrix), 24-(flat bottomed) and 96-microwell plates (U-shaped).

2.2 Methods
Azadirachta indica leaves were collected in Sokode (Togo) and voucher specimen authenticated at the Botany Department of University of Nairobi (Kenya). The leaves have been dried in a greenhouse of the botany department of Jomo Kenyatta University and pounded to a powder.

2.2.1 Preparation of the extracts

Aqueous extracts were prepared at laboratory temperature by soaking 250 g of the powdered Azadirachta indica leaves in 100 ml of water at + 4 C for three days. After filtration of the plant extracts using Whatman n° 1 filters, the extracts were freeze-dried.

2.2.2 Phytochemical screening

The phytochemical test was performed following the method described by Harborne (1984) [12], Kavit *et al.*, (2013) [13], Wadood *et al.*, (2013) [14] and Yadav and Agarwala (2011) [15]. These tests were based on visual observation of color or precipitate formation after addition of specific reagents. The dry powder of *Azadirachta indica* was soaked in water and subjected to qualitative phytochemical screening for saponins, alkaloids, phenolic compounds and tannins, flavonoids, steroids, glycosides and terpenoids.

2.2.2.1 Test for saponins

Saponins were tested by adding 1 ml of water to 1 ml of *A. indica* leaf aqueous extract in a test tube. The suspension was shaken for 5 minutes and allowed to stand for 10 minutes. A 2cm layer of foam was taken as the indicator for the presence of saponins.

2.2.2.2 Test for tannins

Ferric chloride test was used. In this test, 0.5ml of 5% ferric chloride solution was added to 0.5ml of the sample solution. A dark-green color indicated the presence of tannins.

2.2.2.3 Test for flavonoids

To 1 ml of leaf extract, 3 drops of ammonia solution was added followed by 0.5ml of concentrated HCl. The formation of a pale brown coloration indicated the presence of flavonoids.

2.2.2.4 Test for glycosides

Keller-Kiliani test was used. Glycosides were tested by adding 1 ml of 3.5% ferric chloride in acetic acid to 1ml of the sample solution followed by careful drop-wise addition of 1.5ml concentrated sulfuric acid by the sides of the test tube to form a separate layer at the bottom. A brown ring at the interface due to the presence of de-oxy sugar is characteristic of cardenolides and a pale green color in the upper layer due to the steroidal nucleus was taken as the indicator for the presence of cardiac glycosides.

2.2.2.5 Alkaloids

The Mayer's test was carried out to determine the presence of alkaloids.

In this test, 1ml of Mayer's reagent (potassium mercuric iodine) was added to 1ml of the leaf extract and observed for a white precipitate, which is a positive indicator for the presence of alkaloids**.**

2.2.2.6 Phytosterols

Crude extract was mixed with 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A red coloration indicated the presence of phytosterols.

2.2.2.7 Terpenoids

3 ml of chloroform and 2 ml of concentrated sulfuric acid (H2SO4) were added to 2 ml of the extracts. A reddish brown color interface indicated the presence of terpenoids [16-17].

2.3 Tests for cytotoxicity and genotoxicity

This method was described by Fiskesjo [18].Onion seeds were obtained and broadcast on water moistened filter papers in petri dishes. After 96 hours, the germinated seeds were transferred to four different concentrations of the *A. indica* leaf extracts and *Azadirachta indica* stem barks. 50 rooted seeds per petri dish were exposed to 0.125, 0.25, 0.5 and 1 g/l of the aqueous extract of *A. indica*. Glyphosate was used as positive control [19] and the untreated group was transferred to distilled water. The treatment duration was 48 hours. The tests were done in triplicate.

2.3.1 Cytotoxic effect of the aqueous extracts on *Allium cepa* roots

The roots lengths of the *Allium cepa* seedlings were measured for each treatment group and compared to the untreated group. The root lengths were measured using a meter ruler. The EC_{50} was determined from the linear regression standard curve obtained by plotting the values of the root lengths against the different concentrations of the treatment. The EC_{50} was expressed as the concentration at which the root growth of the untreated group is reduced by 50 %.

2.3.2 Genotoxicity assay

The treated rooted seedlings were fixed by complete immersion in Carnoy fixative solution (Ethanol: Acetic acid 3:1) for 24 hours, without removing the roots. After their washing in distilled water, the seedlings where hydrolyzed in 1N HCl at 60 °C for 8 minutes [20]. The seeds were then washed with distilled water. About 2 mm of the root tips were cut and each placed on a microscopic slide. One drop of safranin dye was added to the root tips, and then covered with a coverslip. The preparation was squashed by applying pressure on the root tip to spread the onion cells. The slides were mounted on a light microscope and the observation was done at 1000 X magnification after applying oil immersion on top of the coverslip. 1000 cells were scored per slide and a total of 3000 cells per treatment group. The total number of dividing cells in prophase (P), metaphase (M), anaphase (A) and telophase (T) was counted. The mitotic index (M.I) was determined as the number of dividing cells in a total number of 1000 scored cells. This is illustrated by the formula:

$$M.I = \frac{P + M + A + T}{1000}$$

2.3.3 Mutagenicity test

The Ames mutagenicity test [22] was used. TA 98 and TA 100 *Salmonella typhimurium* strains were removed from the - 80 °C deep freezer and allowed to thaw at laboratory temperature. 50 ml of growth medium was added to the TA 98 and TA 100 vials and the bacterial pellets disrupted using a sterile pipette tip. From each vial, 25 ml of suspension was transferred each to three 50 ml tubes labeled respectively 'TA 98', 'TA 100' and 'negative control'. 10 µl of ampicillin and 10 ml of growth medium were added to the tubes. The tubes were incubated overnight at 37°C in a shaker incubator for 16 hours. After incubation, the optical densities of the colonies were determined at a wavelength of 260 nm using a spectrophotometer. An optical density < 2 for bacterial cultures indicates a contamination, and would result in stopping the experiment and the preparation of another culture. For the negative control, the optical density should be less than 0.05. After recording the optical density values, the serial dilution of the test compound *Azadirachta indica* leaves

aqueous extract was performed on a 96-well plate. Concentrations of 1, 0.5, 0.25, 0.125, 0.0625 and 0.0325 µg/µl were prepared. The 1 µg/µl solution was prepared by dissolving 0.05 g of the leaves powder in 50 ml of distilled water. The different concentrations of the extracts were then transferred to a 24-well plate. The negative control was made of distilled water (solvent) and the positive control of Nitroquinoline-N-Oxide 50 µg/ml and nitrofluorene 50µg/ml. A 10 % of the TA98 culture was prepared by adding 0.7 ml of the exposure medium (Xenometrix) to 6.3 ml of overnight culture. For TA 100, 0.35 ml of the exposure medium was added to 0.65 ml of the overnight culture, making a concentration of 20 %. The negative control, the six concentrations and the positive control were added to the wells in triplicates. 2.6-2.8 ml of an indicator medium (Xenometrix) was added to the wells. The contents of the 24 wells were transferred each to 48 wells of 96-well plates, hence a total of 12 X 96 wells plates per *Salmonella typhimurium* strain. For instance, aliquots of the negative control will be used to fill the wells A to D, from 1 to 12. After 2 days, the plates were scored and the number of revertant colonies recorded. The least deviation in color from blue to yellow indicated a positive result.

3. Results

The phytochemical screening performed on *A. indica* leaf aqueous extract was based on visual observation of coloured complex formation. The screened phytochemical classes were present in in the samples as indicated in Table 1. The screened phytochemical classes were saponins, tannins, alkaloids, steroids, glycosides phytosterols and terpenoids. Table 1 shows the presence of the screened phytochemical classes in the aqueous *Azadirachta indica* leaf extract.

Table 1: Phytochemical screening of *A. indica* stem barks methanol extracts

Phytochemical classes	Test	Result
Saponins	Froth test	+
Tannins	Braemer's test	+
Flavonoids	Ammonia test	+
Glycosides	Keller-Killiani test	+
Alkaloids	Mayer's test	+
Phytosterols	Salkowski test	+
Terpenoids	Acetic acid-sulfuric acid test	+

The cytotoxic effect of the leaf of *A. indica* was expressed in the reduction of root growth of *Allium cepa* seedling exposed to four different concentrations of its aqueous extracts. The percentage of the root growth of treated *Allium cepa* seedlings was expressed in percentage, in relation to the growth of the untreated seedlings. The percentage of root growth was determined using the formula:

$$\text{Percentage Root growth} = \frac{\text{Root length of treated group}}{\text{Root length of the untreated group}} \text{ X } 100$$

Table 2: Cytotoxicity of *A. indica* aqueous extracts on *Allium cepa* roots. RG: Root growth (in percentage of the control). P-Values < 0.05 were considered statistically significant compared to the negative control. AIAE: *A. indica* aqueous extracts.

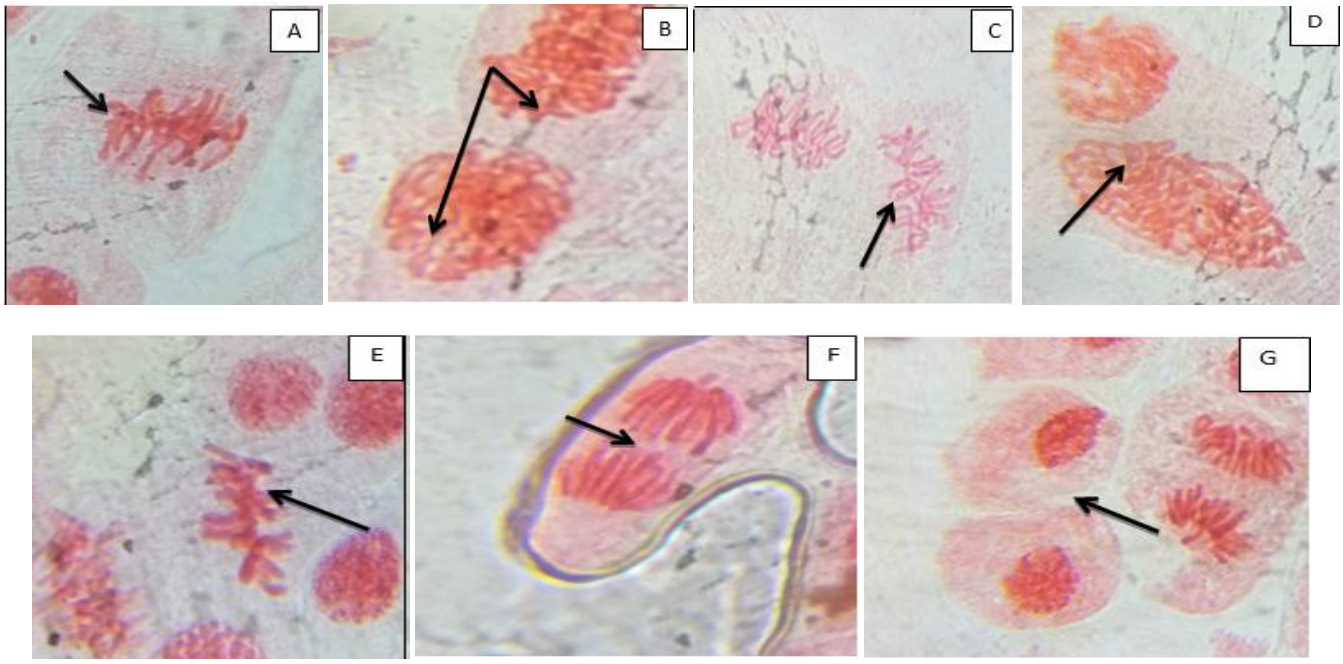
Conc. (g/l)	AILA		
	Mean root length (cm) ± SE	RG% of control	p-value
0	3.47 ± 0.02	100	-
0.125	2.5 ± 0.05	72.05	0.0002
0.25	1.8 ± 0.06	51.87	0.0002
0.5	1.3 ± 0.07	37.46	0.0002
1	1.05 ± 0.12	30.26	0.0006
EC50	0.51 g/l	-	

The chromosomal aberrations are abnormal chromosomal formation occurring during cell division. Chromosomal abnormalities were observed after exposing the Allium seedlings to *Azadirachta indica* leaf aqueous extracts. The four concentrations of extract used were 0.125 g/l, 0.25 g/l, 0.5 g/l and 1 g/l. Table 3 shows the mitotic index of the normal cells of *Allium cepa* seedlings meristems and also the number of abnormal mitotic cells exposed to the different concentrations of *A. indica* in a total number of 1000 dividing cells. Glyphosate was used as positive control.

Table 3: Genotoxicity of *A. indica aqueous* extracts on *Allium cepa* meristematic cells. The mitotic index is calculated as the number of mitotic dividing cells in a total number of 1000 cells scored.

Sample	Concentration (g/l)	Mitotic index	Abnormalities				
			C. B	C-M	S. A	B. A	F
	0 (Distilled water)	123.33 ± 7.57	0.33 ± 0.58	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
AILW	0.125	79.67 ± 2.52	2.67 ± 1.53	4.33 ± 0.58	2.66 ± 2.08	0 ± 0.00	0 ± 0.00
	0.25	89.67 ± 7.5	5 ± 1.73	9 ± 2	3.33 ± 0.58	0 ± 0.00	2 ± 1
	0.5	78.66 ± 5.86	7.66 ± 2.52	16.67± 4.5	6.66 ± 1.53	4.33 ± 0.58	4.33 ± 0.58
	1	58.66 ± 4.16	20.33 ± 7.64	22.33 ± 4.5	10.3 ± 1.53	10 ± 2	10 ± 2
Glyphosate	0.125	68 ± 4.58	8 ± 3.05	3 ± 0.94	3 ± 1.05	3 ± 2.08	0 ± 0.00
	0.25	47 ± 4	6 ± 2.08	7 ± 2.52	7 ± 2.52	5 ± 2.08	3 ± 2.08
	0.5	29 ± 4.36	5 ± 1.15	12 ± 2	5 ± 3.21	4 ± 1.53	2 ± 1.53
	1	18 ± 6.08	0 ± 0.00	8 ± 2.08	1 ± 0.58	0 ± 0.00	2 ± 1.53

Photographs were obtained from the most representative normal mitotic stages and also different types of chromosomal aberrations were observed and scored. They are presented respectively in the Figures 1and 2. The observed aberrations were: C-mitosis (c-mit), chromosome fragments (F), bridges (B), bipolar (B. A), multipolar anaphases (M. A) and sticky chromosomes (S).



A, C, E: Metaphase; B, D: Prophase; F: Anaphase; G: Telophase

Fig 1: Photographs of normal cells in different mitotic stages observed after treatment of *Allium cepa* seeds with different concentrations of medicinal plant extracts and distilled water as negative control. Magnification 1000 X

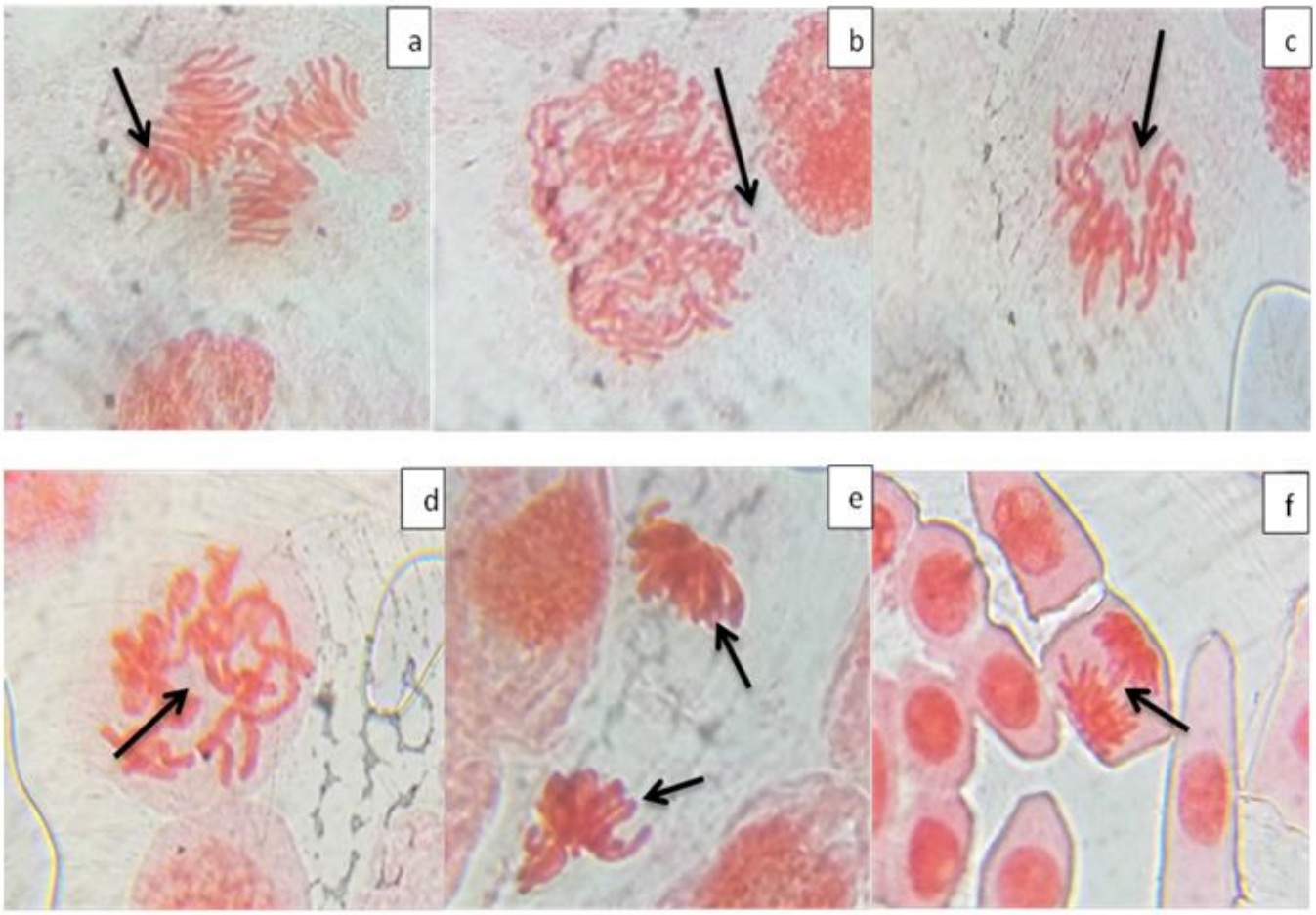


Fig 2: Representative photographs of abnormal chromosomes in treated *Allium cepa* seeds root cells a: Bipolar anaphase (BA); b: Fragment (F); c: Multipolar anaphase (MA); d: C-mitosis (CM); e: Sticky anaphase (SA); f: Bridge (B). Magnification 1000X

In the Ames test, a change in color from blue to yellow has been observed, indicating a change in the bacteria metabolism. The change in color is due to the change in pH as a result of histidine synthesis in the mutated bacteria strains.

Figure 3 is a graph illustrating the number of revertants in function of the concentration of *A. indica* leaf extract.

The values of the revertant colonies were statistically significant at 95% confidence interval using the unpaired

Student t-test.

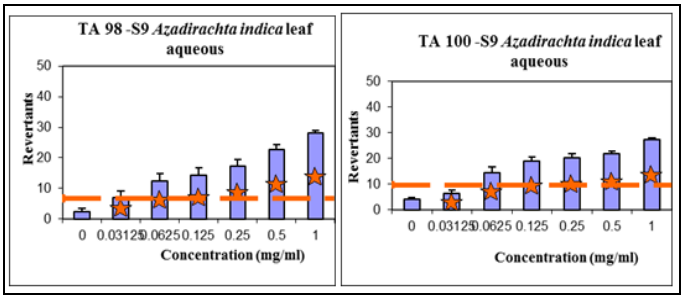


Fig 3: Graph showing the number of revertant *Salmonella typhymurium* TA 98 and TA 100 per concentration of aqueous *A. indica* leaf extract

4. Discussion

A number of medicinal plants have been tested for genotoxicity and some of them revealed to have a genotoxic potential [23]. The screened phytochemicals: Tannins, Saponins, Flavonoids, Alkaloids, Glycosides, phytosterols and terpenoids in this study showed to be present for all the antimalarial plant extracts. Biu *et al*., (2010) [24] found that the stem barks of *P. curatellifolia* contained saponins, alkaloids, tannins, glycosides, flavonoids, terpenoids and phytosterols.

All the concentrations used in this study showed to decrease the mitotic index in the meristem tic cells of *Allium cepa* seed roots. The EC 50 indicates the concentration that reduced by 50 % the root growth of the negative control. The inhibition of the root growth is an indication that *A. indica* aqueous extracts contain compounds which interfere with cell division as the roots growth as the meristem tic cells actively divide. All the values of the root lengths of A. *indica* leaves were statistically significant at 95 % confidence interval (p-values < 0.05) compared to the negative control treated with distilled water. The M.I is an indication of the frequency of mitotic division and has shown to be reduced while the concentrations increased. The dividing cells scored were in prophase, metaphase, anaphase and telophase. The mean value for the mitotic index of the untreated group was 94.33 (or 9.44 % in percentage) and it decreased when the dose of the extract increased. From the lowest concentration used (0.125 g/l), the M. I was reduced by 35.4 % whereas at the highest concentration (1 g/l) the M.I was reduced by 52.4 %. The concentrations of 0.25 and 0.5 g/l reduced the mitotic index extracts showed to reduce the frequency of mitosis in the meristem tic cells of *Allium cepa* seedlings roots as the dividing cells were less frequent than those which were untreated, showing that the mitodepressive effect is due to the treatment with the aqueous extract of *A. indica* leaves.

The chromosomal abnormalities observed appeared as chromosomal bridges, C-mitosis, multipolar anaphase, sticky chromosomes and chromosome fragments. The chromosomal bridges, multipolar and sticky chromosome were observed in anaphase cells. The negative control group exhibited no aberrant cell division while aberrations were observed in the treated cells. The most common chromosomal aberration was c-mitosis. The C-mitosis occurs when the mitotic spindle fails to form or is destroyed, which leads to the scattering of the chromosomes in a random fashion inside the cell (Figure 2.a). The chromosome bridges (Figure 2.f) and sticky chromosomes (Figure 2.e) reveal a risk of lethality to the cells.

In this study, glyphosate was used as positive control according to de Souza, *et al*., 2010 [20].

The reduction of the mitotic index in the treated root tips may be an indication of the inhibition of DNA synthesis [25] or the arrest of one or more mitotic stages [26], or the cell cycle might be blocked in the G2 stage, preventing the cells to enter the mitosis [27]. The extract has the ability to slow down cell progression through the hindering of DNA replication.

The inhibition of the mitotic activity caused by the leaves of *A. indica* aqueous extracts is an indication that the extract contains cytotoxic substances.

For a substance or a xenobiotic to be carcinogenic it should induce repeated mutations in the exposed cells. The Ames test is an assay using prokaryotic systems such as various strains of *E. coli* or *Salomonella typhimurium* to monitor genotoxic compounds that lead to mutations in the DNA by using the bacteria as model. It is aimed at giving some indication about whether the medicinal plant extracts are mutagenic and can increase the risk of carcinogenicity. The Ames test showed a significant mutagenic activity in *Salmonella typhimurium* TA 98 and TA 100 strains. However the tests did not reveal the evidence of carcinogenic potential. As such, the comet assay should be performed on human normal cell lines should confirm whether the medicinal plant extract can cause breakages in human DNA. The mouse lymphoma TK assay should assess the ability of *Azadirachta indica* leaf aqueous extracts to induce cancer.

5. Conclusion

The *Azadirachta indica* leaf aqueous extract is potentially genotoxic by reducing the frequency of mitosis and inducing chromosomal aberrations in *Allium cepa* meristematic cells. However its mutagenicity needs to be fully established by a battery of tests to confirm that it can cause mutations in mammal or human cell DNA. Therefore it is crucial to assess the genotoxic and mutagenic potential of medicinal products, and before their use.

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7. References

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