

# Cytotoxic, anti-mitotic and cytogenetic effects of the leaves and stems of *Olax subscorpioidea* Oliv. (Olacaceae) against *Artemia salina* nauplii and *Allium cepa* meristematic cells

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## Abstract

The search for novel, safe and effective anticancer agents has been a continuous and recurring objective in cancer research. *Olax subscorpioidea* is a medicinal plant used for ethnomedicinal treatment of cancer in Nigeria. In this study, we investigated the cytotoxic, anti-mitotic and cytogenetic effects of methanol extracts of the leaves and stems of *O. subscorpioidea*. The cytotoxic activities were evaluated against brine shrimps (*Artemia salina*) nauplii and *Allium cepa* roots, while anti-mitotic and cytogenetic effects were assessed on *Allium cepa* meristematic cells. Cytotoxicity assays showed that the extracts demonstrated potent cytotoxic activities, having LC<sub>50</sub> values of 10.7 and 45.2 µg/mL against brine shrimps and IC<sub>50</sub> values in the range of 47.03 – 81.93 µg/mL on *A. cepa* roots. These activities were comparable to that of potassium dichromate and methotrexate used as positive control in each of the assays. Anti-mitotic results indicated that at a concentration of 10 µg/mL, the extracts demonstrated percentage mitotic inhibition (% M.I) of 35.95 – 70.12%, with greater or similar anti-mitotic effects to methotrexate at a concentration of 125 µg/mL, % M.I of 39.04 – 40.64%. Cytogenetic investigation revealed that the extracts showed chromosomal aberrations that were not considerably different from those observed in the negative control group. The current findings suggest that the plant extracts may contain cytotoxic and anti-mitotic compounds. Further research to investigate these extracts in more sophisticated test systems and to isolate and characterize their active principles is ongoing.

**Key words:** *Olax subscorpioidea*, cancer, cytotoxicity, anti-mitotic, *Allium cepa*, Brine shrimp

## Introduction

Cancer constitutes a major obstacle to human development and well-being. Latest statistics indicate that, two out of ten people; one in five men and one in six

women, will develop cancer in their lifetime, while two out of twenty people; one in eight men and one in eleven women, will die from this morbid disease (Bray et al., 2018). Globally, cancer incidence and mortality are estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018 (Bray et al., 2018). According to the World Health Organization (WHO), 30 – 50% of these cases can be prevented and as a result, cancer prevention is

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identified as being central to reducing or reversing the rise in cancer burden (Chan, 2014). This sentiment is shared by the United Nations, which described cancer prevention as the cornerstone of global cancer control (United Nations General Assembly, 2011). The need for improvement in preventive measures is important, particularly in low-income and middle-income countries (LMICs), in which 60% of cancer cases and 75% of cancer deaths were recorded, a disproportionate index as opposed to a mere 5% of global spending on cancer (Farmer et al., 2010).

To complement efforts dedicated towards cancer prevention, there is an urgent need for development of effective and safer treatment interventions for the affected. Currently, the major methods for the treatment of cancer are surgery, radiotherapy and chemotherapy. Each of these methods is plagued with various limitations (Arnon et al., 2001; Brydøy et al., 2007; Davila, 2006). Natural products, either as pure compounds or as standardized extracts, provide great opportunities for the development of alternative treatments for cancer management (Cosa et al., 2006). Folkloric investigation revealed that *Olox subscorpioidea* is used in Nigeria for ethnomedicinal treatment of cancer.

*Olox subscorpioidea* Oliv. is a shrub or tree of the family Olacaceae and is found across Nigeria, Senegal, Zaire and West Cameroun (Oladipupo et al., 2018). Extracts of different parts of *O. subscorpioidea* are used traditionally for treating snake bites, scorpion stings, venereal diseases, infectious diseases, mental illnesses, arthritis, rheumatism, diabetes mellitus, cancer, asthma and as febrifuge (Ibrahim et al., 2008; Soladoye et al., 2010; Sonibare and Gbile, 2008). The antinociceptive, anti-ulcer, anti-depressant, anti-protease and antimicrobial activities of the plant have been confirmed by scientific studies (Adeoluwa et al., 2014; Ayandele and Adebisi, 2007; Oyedapo and Famurewa, 1995; Victoria et al., 2010). Phytochemical investigations have reported the presence of alkaloids, flavonoids, tannins, saponins, and glycosides in the leaves and stem of the plant (Abdulazeez et al., 2015; Ayandele and Adebisi, 2007; Oyedapo and Famurewa, 1995; Victoria et al., 2010). Also, 9 compounds, including 9,12-octadecadienoic acid (*Z,Z*)- (linoleic acid) and *n*-hexadecanoic acid have been reported in the leaves of *O. subscorpioidea* (Oladipupo et al., 2018). In some cancer research, conjugated linoleic acids have been shown to reduce metastasis of cancers to lung tissue and *n*-hexadecanoic acid has been reported to strongly boost metastasis in mouse models of human oral cancer (Cesano et al., 1998; Pascual et al., 2017).

The present study investigates the *in vitro* cytotoxic, anti-mitotic and cytogenetic properties of the methanol extracts of the leaves and stems of *O. subscorpioidea* against *Artemia salina* nauplii and *Allium cepa* meristematic cells.

## Materials and methods

### Collection and authentication of plants

Fresh leaves and stems of *O. subscorpioidea* were collected from Sango-ota, Ogun State and were authenticated

by Mr. Oyebanji at the Herbarium of the Department of Botany, University of Lagos, Akoka. Specimens of the plant were deposited and allotted voucher number LUH: 7562.

### Preparation and extraction of plants

The collected plant materials were shade dried and pulverised. Each of the pulverised plant parts (250 g) was extracted by maceration for 3 days with 2 L of methanol. The macerates were filtered and the filtrates concentrated *in vacuo* on a rotary evaporator at 40 °C. The obtained concentrates were oven-dried at 40 °C to yield *O. subscorpioidea* leaves methanol (OSLM) and *O. subscorpioidea* stems methanol (OSSM) extracts.

### Brine shrimp lethality assay (BSLA)

Brine shrimp Hatch Mix (San Francisco Bay Brand, Inc., U.S.A.) containing 21 g of brine shrimp eggs and salt was poured into a small plastic container (hatching chamber) and 1 L of water was added. The hatching setup was left at room temperature for 48 h to allow the shrimp to hatch and mature as nauplii (larva). After hatching, the larvae were separated from the shells and un-hatched eggs by drawing through a Pasteur pipette. Stock solutions (100 µg/mL) of the extracts were prepared by dissolving 5 mg of each extract in small portion of sea water and making up to 50 mL with the same solvent. From the stock, working solutions (50, 25, 10 and 5 µg/mL) were prepared by dilution with sea water. Five mL of each solution was placed in a test tube. Ten nauplii were added to each test tube with a Pasteur pipette. A negative control was set up for sea water. Potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) was used as a positive control and was prepared in the same concentrations as the test extracts. Each concentration was tested in triplicate. The experiment was maintained at room temperature for 24 h. After 24 h, the numbers of surviving shrimps were counted, the % mortalities were calculated and negative control correction was applied using the Schneider-Orelli's formula (Schneider-Orelli, 1947):

$$\text{Corrected \% mortality} = \frac{\text{test \% mortality} - \text{control \% mortality}}{100 - \text{control \% mortality}} \times 100$$

The LC<sub>50</sub>, lethal concentration that kills 50% of the shrimps, was determined for each test sample from the plot of % mortalities against the log of concentrations.

### Viability test of *Allium cepa*

Equal-sized onion (*A. cepa*-2n=16) bulbs of the purple variety were used for this study and procured from Oyingbo market in Lagos, Nigeria. They were air-dried for more than two weeks before the start of experiment. The viability assay was carried out according to the method of Fiskesjo (1993). Outer scales of the dried onion bulbs were removed before use and also dry brownish roots at the bottom were carefully scraped away without damaging the ring of root primordial.

The peeled bulbs were immersed into distilled water for 48 h to determine the viable ones. The water was changed daily and those onions that sprouted properly were used to carry out the experiment while those with poor or no growth were discarded.

#### Root growth inhibition assay

The root growth inhibition assay was performed from 24 to 48 h exposure tests as previously described (Samuel et al., 2010). The viable onion bulbs were exposed directly to 10, 25, 50 and 100 µg/mL aqueous solutions of the extracts. Methotrexate (MTX), prepared in concentration of 125 µg/mL was used as positive control while distilled water served as negative control. Each concentration was set-up in 5 replicates. The test solutions (30 mL each) were replaced every 24 h with fresh solutions. At the end of each exposure period, the length of the root bundle was measured for the onions in each group, and the root growth in each group was expressed as a percentage of the root growth in the negative control group. The IC<sub>50</sub>, the concentration which inhibited root growth by 50%, was determined for each test sample from the plot of root growths % against the log of concentrations.

#### Cytological and anti-mitotic studies

After 24 and 48 h of exposure, roots from each bulb were harvested for cytological study. The root tips were fixed in freshly prepared acetic:ethanol (1:3, v/v). The fixed root tips were transferred to a glass slide, hydrolysed with a solution of 1 N HCl for 3 min and the excess acid was blotted out neatly with a filter paper. The hydrolysed root tips were squashed on the slide, and macerated with a dissecting needle to enhance stain uptake and ensure spreading of the cells in a monolayer for easy microscopic examination. Two drops of acetic orcein stain was added to the squashed tip and left for 30 min to allow the stain penetrate the cells thoroughly (Fiskesjo, 1993). After staining, the slide was gently covered with a cover slip allowing the stain to spread evenly over the square parts of the cover slip and the excess stain was blotted out with a filter paper. The square edges of the cover slip were sealed with clean fingernail polish to prevent drying out of the preparation by the heat of the microscope as suggested by Grant (1982). The slides were viewed under a microscope using X100 objective. The different stages of cell division were counted and recorded. Scoring of the cells was taken from five microscopic fields for each of the different test sample and approximately 500 cells were observed in each slide. Photomicrographs of the cells were taken with Digital Microscope (DB2-180M).

#### Statistical analysis of data

All values are reported as the mean ± S.E.M. (standard error of mean). The values of the variables were analysed for statistically significant differences using the Student's t-test on Microsoft Excel 2010® Data Analysis tools. Differences were considered significant at  $P < 0.05$ .

## Results

#### Brine shrimp lethality assay

The results of root growth inhibition study of the extracts of *O. subscorpioidea* leaves and stems using *A. cepa* assay are as presented in Fig. 2, which shows the root growth (%) of *A. cepa* after 24 and 48 h treatment with the extracts at different concentrations and methotrexate. When compared to the negative control group, the extracts significantly inhibited root growth of *A. cepa* ( $P < 0.05$ ), with IC<sub>50</sub> values of 47.03 and 60.16 µg/mL for OSLM, while OSSM showed IC<sub>50</sub> values of 71.87 and 81.93 µg/mL after 24 and 48 h exposure, respectively. These activities were comparable to that of methotrexate which gave 33.2 and 45.6% root growths at 125 µg/mL for the same treatment periods.

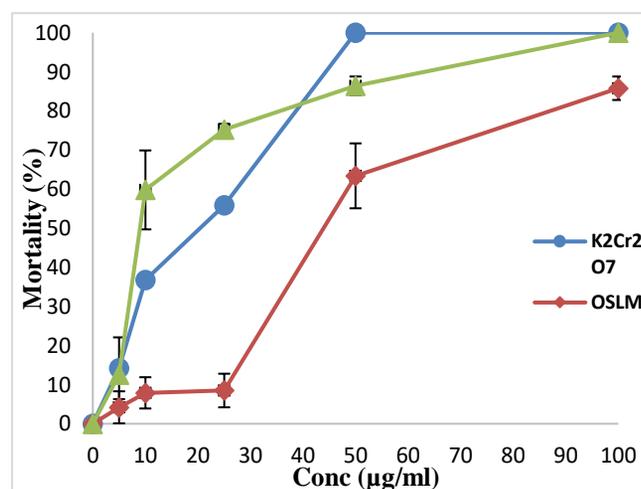


Fig. 1. Cytotoxic effect of *O. subscorpioidea* leaves (OSLM) and stems (OSSM) and Potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) on Brine shrimps.

#### Root growth inhibition assay

The results of root growth inhibition study of the extracts of *O. subscorpioidea* leaves and stems using *A. cepa* assay are as presented in Fig. 2, which shows the root growth (%) of *A. cepa* after 24 and 48 h treatment with the extracts at different concentrations and methotrexate. When compared to the negative control group, the extracts significantly inhibited root growth of *A. cepa* ( $P < 0.05$ ), with IC<sub>50</sub> values of 47.03 and 60.16 µg/mL for OSLM, while OSSM showed IC<sub>50</sub> values of 71.87 and 81.93 µg/mL after 24 and 48 h exposure, respectively. These activities were comparable to that of methotrexate which

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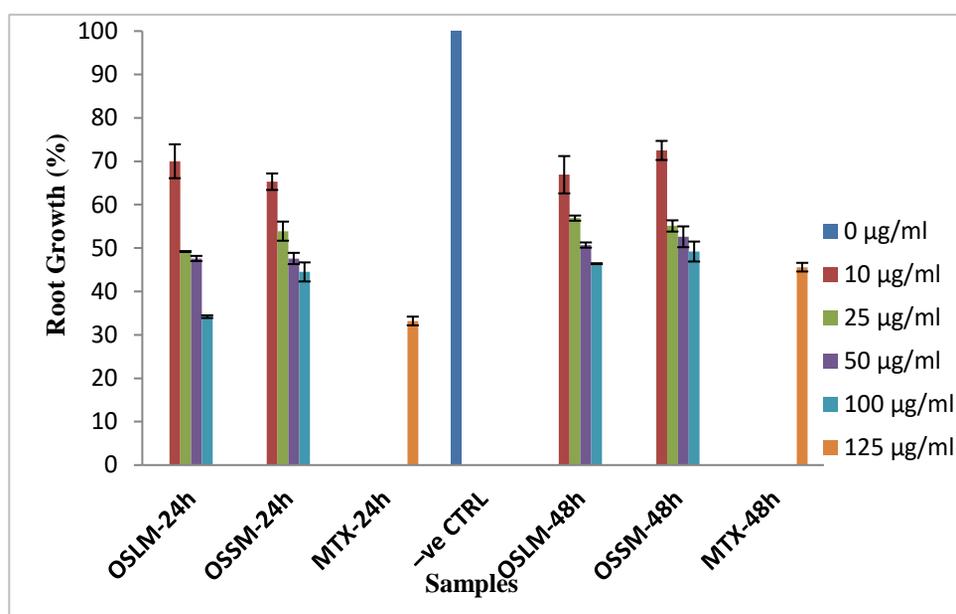


Fig. 2. Root growth (%) of *A. cepa* treated with *O. subscorpioidea* leaves (OSLM) and stems (OSSM), methotrexate (MTX) and distilled water (-ve CTRL) after 24 and 48 h.

#### Cytological and anti-mitotic studies

The results of cytological and anti-mitotic assays of the extracts of *O. subscorpioidea* leaves and stems are as presented in Fig. 3 and Fig. 4. Additionally, Fig. 3 shows the mitotic inhibition (%) of the extracts and methotrexate at different concentrations on *A. cepa* after 24 and 48 h

exposure while Fig. 4 give some representative photomicrographs of meristematic cells in *A. cepa* treated with the extracts and methotrexate at the end of the treatment period. Moreover, Table 1 shows the chromosomal aberrations consisting anaphase bridges (AB), laggards (L), vagrants (V), sticky or clumped chromosomes (SCC), other abnormal chromosomes (OAC) and total aberrations (TA).

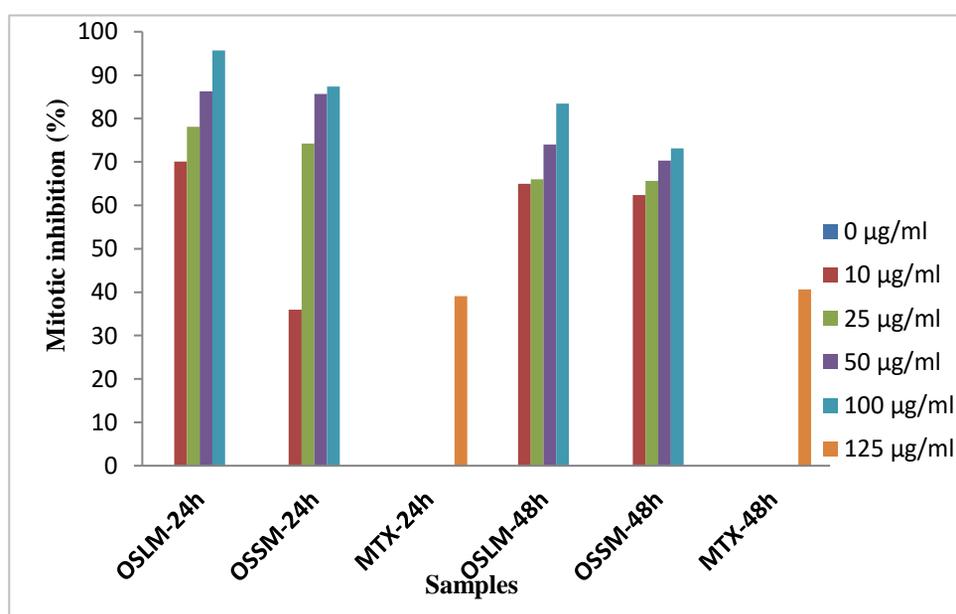


Fig. 3. Mitotic inhibition (%) of *O. subscorpioidea* leaves (OSLM) and stems (OSSM) and methotrexate (MTX) after 24 and 48 h.

Table 1. Chromosomal aberrations in cells of *A. cepa* treated with *O. subscorpioidea* leaves (OSLM) and stems (OSSM) and methotrexate (MTX).

Sample	Chromosomal aberrations						
	Conc ( $\mu\text{g/mL}$ )	AB	L	V	SCC	OAC	TA
Control	–	$0 \pm 0$	$0 \pm 0$	$5 \pm 0$	$0 \pm 0$	$0 \pm 0$	$5 \pm 0$
MTX	125	$2 \pm 2$	$6 \pm 3$	$14 \pm 0$	$9 \pm 2$	$8 \pm 2$	$38 \pm 8$
OSLM	10	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$
	25	$0 \pm 0$	$0 \pm 0$	$1 \pm 1$	$0 \pm 0$	$1 \pm 1$	$1 \pm 1$
	50	$1 \pm 0$	$0 \pm 0$	$0 \pm 0$	$1 \pm 0$	$0 \pm 0$	$1 \pm 1$
OSSM	100	$1 \pm 1$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$1 \pm 1$
	10	$1 \pm 1$	$0 \pm 0$	$0 \pm 0$	$1 \pm 1$	$1 \pm 1$	$2 \pm 1$
	25	$1 \pm 1$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$1 \pm 0$	$2 \pm 1$
	50	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$
	100	$1 \pm 1$	$1 \pm 1$	$0 \pm 0$	$0 \pm 0$	$0 \pm 0$	$2 \pm 0$

Values are mean  $\pm$  S.E.M (n=5). Values with asterisk (\*) are significantly different from control ( $p < 0.05$ ). AB: anaphase bridges; L: laggards; V: vagrants; SCC: sticky or clumped chromosomes; OAC: other abnormal chromosomes

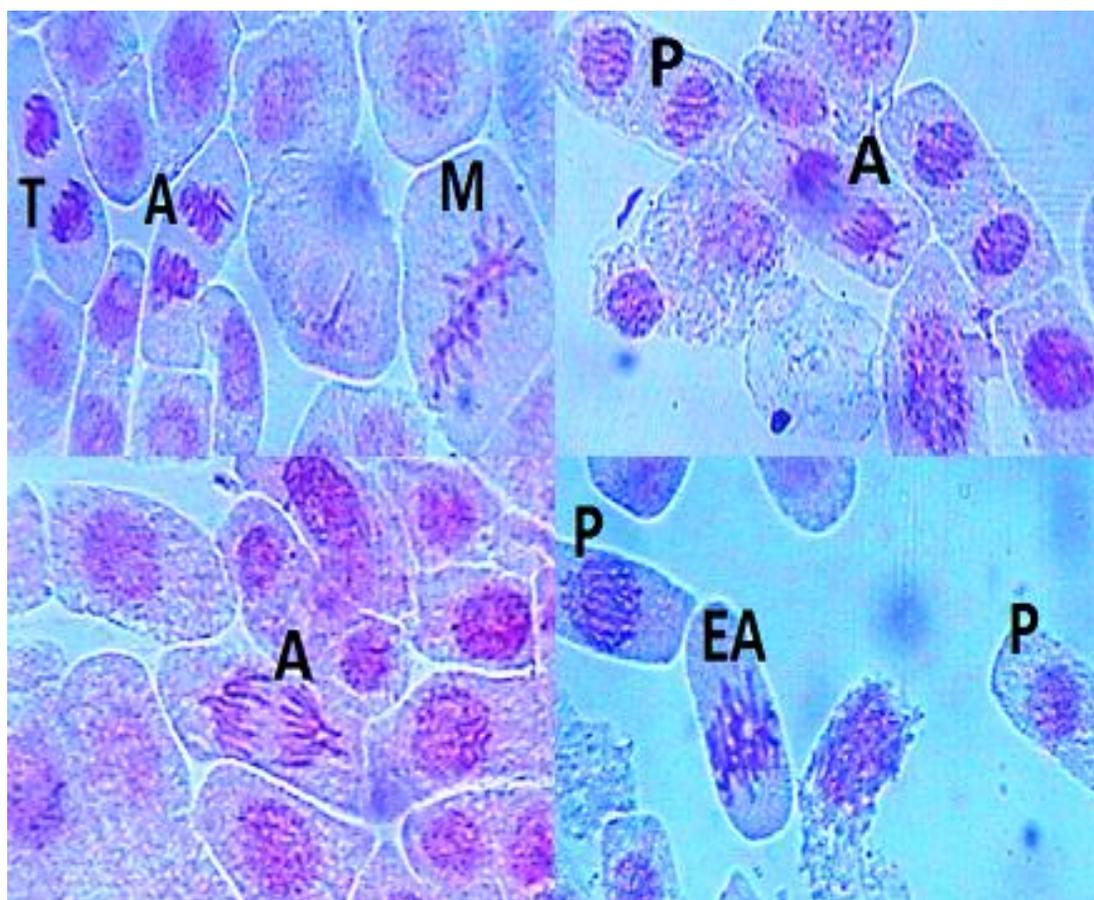


Fig. 4. Representative photomicrographs of *A. cepa* meristematic cells showing telophase (T), anaphase (A), metaphase (M), prophase (P), and early anaphase (EA). Magnification X100.

## Discussion

Despite considerable progress in the understanding of the molecular basis of cancer, the ideal anticancer drug still remains elusive; thus, necessitating the continual and keen search for novel agents that could be useful in cancer management. As part of our ongoing research into the anticancer potentials of some indigenous plants that have ethnomedicinal use for cancer management, in this study, we investigated the cytotoxic, anti-mitotic and cytogenetic effects of methanolic extracts of *O. subscorpioidea* leaves and stems against brine shrimps and *A. cepa* meristematic cells.

The results of the evaluation of the cytotoxicity of the plant extracts by brine shrimp lethality assay (BSLA) are as presented in Fig. 1. The extracts showed a concentration-dependent lethality against brine shrimp. Extract of the stems, LC<sub>50</sub> of 10.7 µg/mL, showed higher potency than the leaves extract and potassium dichromate with LC<sub>50</sub> of 45.2 and 13.0 µg/mL respectively. In a similar study, 5-Fluorouracil (5-FU), one of the most frequently used anticancer drugs, gave an LC<sub>50</sub> of 363 µg/mL on brine shrimps (Lawal et al., 2018). Our results revealed that the extracts of *O. subscorpioidea* leaves and stems showed greater potency than 5-FU. The BSLA is widely used for preliminary screening of plants for anticancer activity. LC<sub>50</sub> values lower than 1000 µg/mL are regarded as considerably potent in this assay (Meyer et al., 1982).

The cytological and anti-mitotic effects of the plant extracts were investigated against *A. cepa*. Since *A. cepa* cell cycle is completed in 24 h (Rank, 2003), the study was carried out at 24 and 48 h to investigate the mitotic and phase indexes in 2 consecutive cell cycle periods. The results of the root growth inhibition assay are as presented in Fig. 2. The extracts demonstrated a significant ( $P < 0.05$ ) concentration- and time-dependent inhibition of *A. cepa* root growth when compared to the negative control. The leaves extract, IC<sub>50</sub> of 47.03 – 71.87 µg/mL, showed stronger inhibitory activity than the stems extract, IC<sub>50</sub> of 60.16 – 81.93 µg/mL across the 48 h period. At 100 µg/mL, both extracts (root growth of 34.2 – 49.2%) exerted comparable activity to the anticancer drug used as positive control, methotrexate (root growth of 33.2 – 45.6% at 125 µg/mL).

In a similar manner to their inhibitory activity, the plant extracts demonstrated a significant concentration- and time-dependent anti-mitotic effect. Anti-mitotic effect was estimated using mitotic index (M.I), a ratio of the number of cells undergoing mitosis to the total number of cells. The negative control, distilled water, showed M.I values of 35.24 – 35.45. The mitotic indices were significantly lower ( $P < 0.05$ ) in groups treated with *O. subscorpioidea* extracts and methotrexate, having M.I values of 1.53 – 22.67 and 21.58 – 21.04 respectively across the 48 h period. The results showed that the leaves extract; percentage mitotic inhibition (% M.I) of 64.98 – 95.68%, demonstrated higher anti-mitotic activity than the stems extract; % M.I of 35.95 – 87.40%. It was also observed that, at the lowest concentration, 10 µg/mL, both extracts, % M.I of 35.95 – 70.12%, showed greater or

comparable anti-mitotic effects to methotrexate, 39.04 – 40.64% at 125 µg/mL across the treatment periods. The lower mitotic indices and high percentages of mitotic inhibition suggest that the plant extracts may inhibit cell proliferation by blocking cells in the mitotic phases of the cell cycle. This anti-mitotic activity of the plants extracts indicates a possible mechanism of cytostatic and cytotoxic actions similar to that of some existing plant-derived anticancer drugs, such as taxanes (paclitaxel and its derivatives) and vinca alkaloids (vinblastine, vincristine and their derivatives) (Stanton et al., 2011).

Chromosome aberrations, including anaphase bridges; laggards; vagrants; and sticky or clumped chromosomes were observed across the extracts, methotrexate and negative control groups. The aberrations observed in the extract-treated groups are comparable to the negative control group, indicating that they could have occurred spontaneously from errors in the normal processes of the cell.

## Conclusion

Cancer remains a menacing disease to humanity. The need for novel effective and safer anticancer agents is still very important. This study investigated the cytotoxic, anti-mitotic and cytogenetic effects of methanol extracts of *O. subscorpioidea* leaves and stems. Comparative assessment of cytotoxic, anti-mitotic and cytogenetic results indicates that the plant extracts showed comparable effect to methotrexate, a well-known anticancer agent, even at lower concentrations. These findings may support the ethnomedicinal use of this plant in cancer treatment.

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## Резиме

**Цитотоксични, анти-митотични и цитогенетски ефекти на листови и стебла на *Olax subcorpioidea* Oliv. (Olacaceae) против *Artemia salina* ларви и *Allium cepa* меристемски клетки**

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**Клучни зборови:** *Olax subcorpioidea*, канцер, цитотоксичност, анти-митотичен, *Allium cepa*, солени ракчиња

Пребарувањето на нови, безбедни и ефективни антиканцерогени агенси е континуирана и повторлива цел во истражувањата на канцерогени состојби. *Olax subcorpioidea* е лековито растение што се користи за етнофармаколошки третман на канцерогени состојби во Нигерија. Во оваа студија, ги испитувавме цитотоксичните, анти-митотичните и цитогенетските ефекти на метанолни екстракти од листовите и стеблата на *O. subcorpioidea*. Цитотоксичните ефекти беа евалуирани со примена на солени ракчиња од видот *Artemia salina* и *Allium cepa* корени, додека анти-митотичните и цитогенетските ефекти беа проценети врз меристемски клетки од *Allium cepa*. Испитувањата за цитотоксичност покажаа дека екстрактите манифестираат силна цитотоксична активност со вредност на LC<sub>50</sub> од 10,7 и 45,2 µg/mL против солени ракчиња и IC<sub>50</sub> вредности во опсегот од 47,03 - 81,93 µg/mL на корените на *A. cepa*. Добиените резултати се споредливи со оние за калиум дихромат и метотрексат што се користени како позитивни контроли. Процентуалната митотична инхибиција (% M.I) од 35,95 - 70,12% за екстрактите во концентрација 10 µg/mL покажаа дека имаат споредлив антимиотични ефекти како и позитивната контрола метотрексат, што покажа вредност од 39,04 - 40,64% при концентрација од 125 µg/mL. Цитогенетското испитување покажа дека екстрактите предизвикуваат хромозомски аберации кои не се разликуваат во голем степен од оние забележани во негативната контролна група. Тековните наоди сугерираат дека испитуваните екстракти може да содржат цитотоксични и анти-митотични соединенија меѓутоа испитувањето на овие екстракти со примена на пософистицирани тест системи за изолирање и карактеризирање на нивните активни принципи е сèуште во тек.