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Original Research Article

Antioxidant Potential and Cytogenotoxicity Activity of Methanol Extract of *Asystasia vogeliana* Benth Leaf

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ABSTRACT

In Nigeria, *Asystasia vogeliana* leaf is used for treatment and management of many human illnesses. The research explored the antioxidant, cytotoxicity and genotoxicity effects of *Asystasia vogeliana* leaf methanol extract (AVLME), in order to establish its anti-malignant potential. Fine powder of the air-dried plant leaves was produced by pulverization. It was extracted using methanol. The filtrate was concentrated to dryness on a rotavap at 40ºC. Antioxidant activity was assessed using Ferric reducing antioxidant potential (FRAP), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and metal chelating assay. Brine Shrimp lethality and *Allium cepa* L. assays were used to investigate the cytotoxicity and genotoxicity activities. The results revealed that the IC_{50} for FRAP, DPPH and metal chelating assays were 129.13 µg/mL, 0.43 mg/mL and 2.17 mg/mL, respectively. Nauplii of Brine shrimp responded to the extract treatment with high lethality in the Brine shrimp assay. Mortality was highest at 0.1 mg/mL concentration of the extract. Lethal Concentration 50 (LC_{50}) of the extract for the Brine Shrimp mortality was evaluated to be 62 µg/mL. Analysis of variance revealed that the mitotic index mean of *A. Cepa* treated with various concentrations of AVLME was significantly difference ($P < 0.05$). The reduction in the mitotic index along with disrupted and degenerated cells showed that the extract has cytotoxicity potential. Chromosome aberration observed in the cells of Allium *cepa* root indicates genotoxicity activity of the extract. Hence, the study shows that the AVLME possesses antioxidant potential and exhibits genotoxicity and cytotoxicity effects toward *A. cepa* root tip cells and Brine shrimp nauplii.

***Keywords*:** Antioxidant, cytotoxicity, genotoxicity, *Asystasia vogeliana*, *Allium cepa*, and Brine Shrimp

Introduction

Recently, the significance of plants and its extracts in contemporary medicine has been reported in many review papers. The literature showed that there is growing attention in the study of natural product chemistry especially phytochemistry[1]. Several factors that contributed to this growing level of interest includes but not limited to the astonishing diversity in biological activities as well as the chemical structure of the majority of secondary metabolites. The usage of modern techniques to detect pharmacologically active phytochemicals, novel methods of isolation, purification, and structurally characterization of these active ingredients, and determination to meet the request for the supply of complex natural products are also part of the factors[1,2].

Asystasia vogeliana (Benth) is an under-shrub straggling green plant with simple, opposite and ovate leaves and it belongs the genus *Asystasia* in the family Acanthaceae. The flowers have brightly-colored bract and are bilaterally symmetry[3]. *Asystasia vogeliana*, among the Eastern Nigeria people, is referred to as "ogwu iba ocha nanya", which literarily translates to drug for treatment[4]. It is also used for management of hepatitis in Nsukka, Nigeria.

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Popoola *et al.*[3] reported that there is an increase in effectiveness of *A. vogeliana* in the treatment of leprosy, chronic fever, malaria and gonorrhea when combine with *Senna alata* (L.) Roxb leaves, *Cymbopogon citratus* Stapf leaves and fruit juice of *Citrus aurantifolia* (Christm.) Swingle. The phytochemical screening of the *A. vogeliana* revealed the presence of saponins, flavonoids, steroids, polyphenols, glycosides, anthraquinones, alkaloids, tannins, and reducing compounds[3,5]. In order, to ascertain the traditional usage, the *in-vitro* cytotoxicity studies of *A. vogeliana* along with *Moringa oleifera* Lam. and *Andrographics paniculata* (Burm. F.) Wall. Ex Nees was carried out against BGC-823 and HeLa cancer cell lines[3]. Also, studies have shown that *A. vogeliana* increased the farmed African Catfish (*Clarias gariepinus*, Burchell) mortality rate compared to *Tephrosia vogelli* (Hook, F) and *Adenia cissampeloides* ((Planch. ex Hook.) Harms, due probably to the quantity and concentration of the phytochemical components present in the *A. vogeliana* leaf extract relative to other extracts[6].

Antioxidants are any molecule that possesses the ability to chelate or bind metal ions in redox reaction or molecule capable of neutralizing the negative effects of free radicals' cellular entity. Naturally, humans possessed well-coordinated antioxidant defense systems that protect the body generally against free radicals' damage. Kaliora *et al.*[7] stated that the antioxidant system essentially comprises of two main types, endogenous and exogenous, which function together to counteract free radicals for proper physiological function. Natural antioxidants obtainable from plant extracts and natural product derivatives are directly involved in neutralizing the free radicals generated through various metabolic processes. Such free radicals destroy many fundamental building blocks of all tissues such as lipids, proteins, RNA, DNA and mitochondria, and this could lead to the development of various health complications. Most of the degenerative diseases like

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multiple sclerosis, arthritis, Parkinson, osteoporosis, Alzheimer, rheumatoid arthritis, and cancer that affect humanity arise from injurious free radical reactions. Majolo *et al.*[8,9] reported the significance involvement of various naturally occurring antioxidant compounds in controlling degenerative diseases associated with immune system decline, atherosclerosis, cell aging and others. Nowadays, the global increase in the mortality rate is attributed to upsurge in various degenerative diseases. Several researchers have suggested that ailments are caused through oxidative stress due to increase in the generation of free radicals[10,11]. Studies have also shown that many antioxidant agents possess and exhibit various biological activities, including but not limited to antimutagenic, antibacterial, anti-inflammatory, antiatherosclerosis, anticancer, antitumor, and antiviral activities.

The Brine Shrimp Lethality Assay (BSLA) is commonly used as a means for the initial evaluation of toxicity. This assay can easily be used to assess the antineoplastic properties and plant extract cytotoxicity. BSLA is a very simple biological assay, which is cost effective and not time consuming. The result of BSLA is an indication of many other pharmacological activities aside cytotoxicity and anticancer[12]. Though the mechanism of action of the isolated compounds cannot be determined through this assay, yet it remains the most preferred preliminary assay for evaluating the toxicity of extracts and most especially isolated compounds. Plant extracts that give positive toxicity results against Brine Shrimp are most likely to contain an anti-cancerous tumor agent and a good source of the agent for further research[13,14]. Similarly, *Allium cepa* root tip bioassay is a standard assay procedure used for investigation of potential genotoxic effects of biological agents[15]. Several researchers have used the bioassay to reveal the effect of toxic substances at the chromosome level through the manifestation of chromosomal aberration[14,15,16]. This bioassay has been mostly used to evaluate DNA damages caused by toxic agents because *Allium cepa* has a high rate of proliferation and high dividing prominent cell percentage[14,16].

Currently, there is a shortage of information on the toxicity of *A. vogeliana* apart from the reports of Popoola *et al.*,[3] and Uno *et al.*,[6] which discussed the *in-vitro* cytotoxicity of three different plants and acute toxicity of ichthyotoxic plants, respectively. There is no scientific information on the *A. vogeliana* leaves anti-malignant potential; we therefore, aimed to establish, in the current study, the efficacy of *A. vogeliana* leaves as anticancer agent. Two assay techniques, Brine shrimp lethality assay and *Allium cepa* root tip bioassay, which are known methods of ascertaining cytotoxicity and DNA damages caused by the toxic agent were adopted.

Materials and Methods

Collection and Identification of Asystasia vogeliana

Asystasia vogeliana leaves were collected fresh around April, 2021 from the neighborhood of Asherifa (GPS location: Latitude 7º 29'42.6"N Longitude 4º 31' 14.2"E), Old Ibadan Road, Ile-Ife, Nigeria. The plant was identified, authenticated and deposited for voucher number (18310) at Ife Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria.

Preparation of Asystasia vogeliana leaf extract

The leaf of *A. vogeliana* was harvested and air-dried for two weeks. The dried leaf was pulverized using a grinder (Qasa grinder, QBL-1861A, Qlink Corp, China) into a fine powder. The powdered leaf sample (1248 g) was soaked for 48 h in methanol (6.0 L) with intermittent mixing and then filtered. The residue was extracted again in the same volume of methanol and left for another 48 h. The filtrates were pooled and concentrated into dryness *in-vacuo* on a rotary evaporator (Buchi Rotavapor RH, Buchi Labortechnik Ltd, Switzerland) at 40ºC. Percentage yield was evaluated with the equation (i).

$$\text{Percentage yield} = \frac{Extract\ Weight}{Plant\ Material\ Weight}\ x\ 100\% \quad \text{(i)}$$

Antioxidant assays

DPPH radical scavenging assay

The method used to evaluate the ability of AVLME to scavenge DPPH free radical is a modified protocol earlier described by Blois[17]. Working DPPH reagent (0.1 mg/mL DPPH dissolved in methanol) prepared just before use was mixed with 100 µl of graded concentration of AVLME (0.05 – 0.5 mg/mL). The reaction mixture in test tubes was incubated in the absence of light for half-an-hour at 25ºC. Thereafter, at wavelength of 517 nm, changes in colour development were monitored using Biobase UV-visible spectrophotometer (BK-UV1600, Biobase Group, Shandong, China). Glutathione was employed as standard radical scavenger while extract was replaced with distilled water to serve as blank. The AVLME DPPH radical scavenging potential was obtained using equation (ii).

$$\text{DPPH radical scavenging (\%)} = \frac{Absorbance\ of\ blank - Absorbance\ of\ sample}{Absorbance\ of\ blank}\ x\ 100\% \quad \text{(ii)}$$

Ferric reducing antioxidant potential assay

The ferric reducing potential of the leaf methanol extract of *A. vogeliana* was assessed based on the Oyaizu[18] procedure with little modification. Aliquots of 0.2 mL of *A. vogeliana* leaf extract at different concentration (0.0 – 500 µg/mL) was added to 0.5 mL 20 mM phosphate buffered (pH 6.6) in a test-tube. After shaken the mixture, 0.5 mL potassium ferricyanide solution (1% w/v) was added and then incubated in a water bath for 20 min at 50ºC. Thereafter, 0.5 mL 10% TCA was added. The final reaction mixture was subjected to centrifugation in Biobase low speed centrifuge Model LC-HD at 4000 xg for 15 min. One milliliter of supernatant aliquot was mixed on the vortex mixer with distilled water (1 mL) and 0.1% ferricchloride solution (0.2 mL). Reading of absorbance was done at 700 nm wavelength in the Biobase UV-visible spectrophotometer (BK-UV1600). The assay was carried out in triplicate and distilled water was in the blank in place of extract.

Ferrous ion chelating activity assay

The ferrous ion chelating potential of *A. vogeliana* leaf methanol extract was evaluated according to the principle of iron-ferrozine complex development procedure. Dinis, *et al.*,[19] described procedure was slight amended and adopted for this assay. One hundred microliter containing varying final concentrations of *A. vogeliana* leaf methanol extract was pipetted into 96 well microtitre plates. Ferrous chloride (2 mM, 100 µL) and 5 mM ferrozine (100 µL) was added. The microtitre plate was vortexed and incubated in a dark room at 27ºC for 20 min. The absorbance of the reaction mixture was read at a wavelength of 560 nm. The blank, which is without the extract, contained distilled water. EDTA, a standard chelating agent, was in the positive control experiment. Ability of *A. vogeliana* leaf methanol extract to chelate metal was calculated in percentage using the equation iii below.

$$\text{Metal chelating activity (\%)} = \frac{Absorbance\ of\ blank - Absorbance\ of\ sample\ X\ 100\%}{Absorbance\ of\ blank} \quad \text{(iii)}$$

Brine shrimp lethality assay (BSLA)

The assay was carried out according to the procedure of Meyer *et al.*,[15] which was modified slightly. Brine shrimp nauplii (*Artemia salina*) were produced from Brine shrimp eggs placed in a container filled with a sterilized brine solution, which was aerated continuously during the 48 h incubation. After hatching, ten shrimp larvae were harvested from the brighter portion of the culture vessel and were placed in each of the triplicate bottles per concentration using a plastic Pasteur pipette. Each bottle contains 4.5 mL sterilized artificial seawater. To each experiment, a different volume of *A. vogeliana* leaf methanol extract from stock concentration was mixed with 4.5 mL salt water to give the final graded concentration between 200 to 1000 µg/mL and the volume in the bottles was made the same (5 mL) with salt water. The experiment was continued in an illuminated room at 27 ºC for a whole day after which the non-responding larvae were counted. The assays were carried

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out alongside control (vehicle treated) of the extract and different concentrations of potassium dichromate ranging from 20 µg/mL to 100 µg/mL were employed as the positive control. The percentage of deaths at each extract concentration and control was calculated. The reduction in the number of shrimp present compared with that of control indicated that the extract is toxic. The result was interpreted using a Probit analysis package to determine the LC_{50} values of the extracts.

Cytotoxic and genotoxic effects of Asystasia vogeliana

Allium cepa bioassay was employed to investigate the root growth inhibition and the antimitotic activity of *A. vogeliana* leaf methanol extract. Onion bulbs used were obtained from a local market (GPS location: Latitude 7º 29' 20.8"N Longitude 4º 33' 12.1"E) in Ile-Ife, Nigeria and were air-dried at room temperature for fourteen days and the bulbs outer dry scales were gently removed. The onion bulbs were allowed to grow in double distilled water for two days for the root to develop. The three best root-growing bulbs were later treated for 24 h with different concentration (0.050 to 0.50 mg/mL) of the extract. Also, a different set of bulbs were used for negative control and were placed in distilled water. Thereafter, the root lengths of the bulbs for each concentration were taken and the mean of the root lengths were calculated. The cytological examination of *A. cepa* root tip cells was carried out with the modified methods of Chukwujekwu *et al.* [20] described by Azeez *et al.*[21]. The chromosome aberrations were documented and the mitotic index was expressed as shown in the equation (iv).

$$Mitotic\ index = \frac{Number\ of\ dividing\ cells}{Total\ number\ of\ cells} \quad \text{(iv)}$$

The chromosome aberration frequency in percentage was calculated following the equation (v) below:

$$Chromosome\ aberration\ frequency = \frac{Number\ of\ aberrant\ cells}{Total\ number\ of\ cells}\ x\ 100 \quad \text{(v)}$$

Statistical Analysis

Results were subjected to statistical analysis performed with GraphPad Prism Version 6.0 (Graphpad Software Inc., San Diego, California). The experimental values were shown as Mean ± Standard Error of Mean where n=3.

Results and Discussion

Globally, plants usages as a source of extracts with medicinal and physiological potential is well documented. Various parts of plants have one or more phytochemicals that have been implicated in treatment or management of a particular illness or disease. The side effects of these extracts are often not noticed when used because they are not pronounced as that of synthetic drugs. People use herbal products for their healthcare needs without prescription. It has been reported that there is always a correlation between the medicinal plant phytochemical constituents and the biological or pharmacological activities they elicit[22].

Phytochemical constituents of *Asystasia vogeliana* leaf was extracted with methanol, a polar and volatile organic solvent. The methanol extract obtained from the leaf of *A. vogeliana* was 99.8 g, which represented 7.99% of the starting plant materials. Methanol has been reported as one of the best extraction solvents because of its volatility at room temperature. In addition, methanol has the potential to extract both lipophilic and hydrophilic plants metabolites. Truong *et al.*[23] identified methanol among other solvents used as the most effective solvent that produced the highest percentage of extract and also the highest phenolic and flavonoid content as well as antioxidant activity from *Severinia buxifolia*. Borges *et al*,[24] report on the effect of methods and solvents of extraction on the biological activities of *Acacia dealbata* and *Olea europaea*, also supported methanol as a good extraction solvent.

Three different *in-vitro* antioxidant activity assays were used to assess the *A. vogeliana* methanol extract ability to protect cells against both exogenous and endogenous oxidant. The role played by free radicals in biological damages either in-vivo or in-vitro is enormous. DPPH assay is the commonest and simplest method of evaluating the potential of antioxidant agent from natural sources to scavenge free radical[25]. The DPPH radical scavenging showed that the extract possessed a quantifiable amount of antioxidant. The DPPH radical scavenging activity of *A. vogeliana* leaf extract was concentration dependent (Figure 1). The increase in concentration is proportionate to rise in percentage scavenging activity. The inhibition percentage of both glutathione and *A. vogeliana* leaf extract increased across the concentration gradient (Figure 1). Antioxidants present in the biological extract scavenge free radicals by hydrogen donation, which is noticed in the DPPH radical colour changing from purple to yellow after the reaction. The degree of the reaction is hinged on the potential of the antioxidant to donate hydrogen. Likewise, the antioxidant ability of a compound or an extract depends on its ability to donate an electron[26]. The IC_{50} (0.44 ± 0.07 mg/mL) indicated measurable antioxidant potential which was significantly different from glutathione. DPPH radical scavenging ability of *A. vogeliana* methanol extract was dose-dependent (Figure 1).

Furthermore, extract potential to serve as antioxidant against oxidative stress can be detected if the extract could reduce the ferric ion to ferrous ion. This ferric reducing activity is based on the fact that the extract can reduce ferricyanide (Fe^{3+}) to produce ferrocyanide (Fe^{2+}) using its reduction potential. Sujith *et al.*[27] showed that *Anacylus pyrethrum* roots possess antioxidant ability because the root extract was able to reduce ferric ion in a dose-dependent mode. Similarly, *A. vogeliana* methanol extract was found to possess ferric reducing potential that was increasing as the extract concentration rises in this present study (Figure 2).

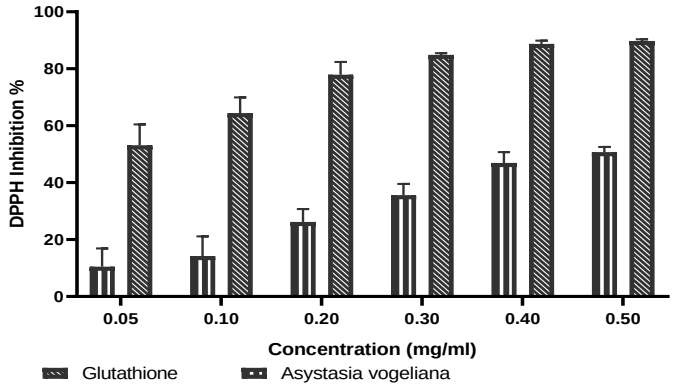

Figure 1: *Asystasia vogeliana* leaf methanol extract ability to scavenge DPPH radical

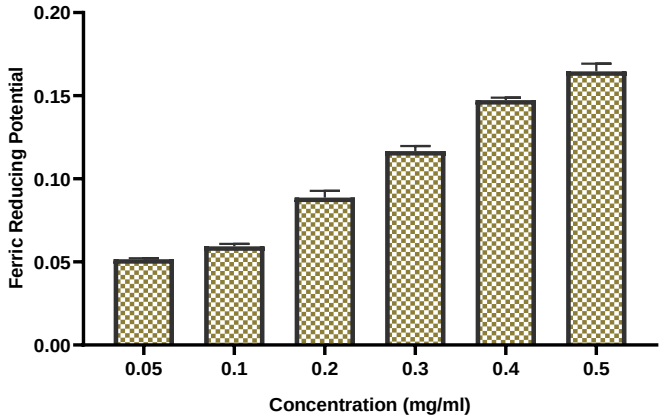

Figure 2: Ferric reducing antioxidant potential activity of *A. vogeliana* leaf methanol extract.

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The metal chelating assay also collaborate the result of DPPH and ferric reducing potential assays. Chelation or deactivation of transition metals is also an essential mechanism of determining the antioxidant capacity of a compound or chemical isolate from natural. The way out to evade the development of reactive oxygen species, that is associated with redox active metal catalysis, is to chelate metal ions in the system. The transition metal ion like ferrous ion has the aptitude to transfer lone electrons owing to this, it permits the creation and spread of many radical reactions, even beginning from non-reactive radicals[28]. Chelating agents may also function as secondary antioxidants since they decrease redox potential, thereby stabilizing the oxidized forms of metal species. Yamaguchi et al.[29] noted that in the presence of chelating agents, the development of ferrous-ferrozine complex is interrupted, causing a reduction in the red colour of the complex. In metal chelating assay, the ability of methanol extract of *A. vogeliana* leaf to chelate metal was shown to be concentration dependent (Figure 3). The extract chelating ability increased with the extract concentration increase. The metal chelating activity assay result is shown in Fig. 3. *Asystasia vogeliana* leaf methanol extract affected the production of ferrous-ferrozine complex, indicating that it has chelating action and the ability to capture ferrous ion ahead of ferrozine.

Lethality of Brine shrimp test has been used as preliminary protocol to assess the toxicity of biological agents suspected to be toxic [12,14,15]. Table 1 showed the effects of *A. vogeliana* leaf methanol extract against the *Brine Shrimp* nauplii and revealed varying level of lethality. The degree of lethality was commensurate to the extract concentration (Figure 4). The maximum and minimum concentrations of the leaf extract tested gave the highest and lowest of the mortality rate, respectively. The Brine Shrimp LC_{50} value is 62 µg/mL. Meyer *et al*,[15] and Khan *et al*.[30] considered LC_{50} values, in toxicity assessment of plant phytochemical components using Brine shrimp lethality assay, lower than 1000 µg/mL as bioactive and value higher as non-toxic. The LC_{50} value obtained in the present study is comparable to the value (68.80 µg/mL) reported by Ikpefan *et al*.[31] *Euphorbia graminea* Jacq methanol extract, which was considered cytotoxic. The level of mortality showed by *A. vogeliana* methanol leaf extract is also within the range (39.25 µg/mL – 138.56 µg/mL) reported by Adelegan *et al*.[32] for *Olax subscorpioidea* ethanol leaf extract and its various fractions. Hence, the methanol leaf extract of *A. vogeliana* could be considered to contain a bioactive agent with potential cytotoxic activity.

The reduction in the mitotic index recorded in *A. cepa* root tip cells treated with different concentration of extract was concentration dependent. This reduction could be attributed to the inhibition of cell growth and cell death. In addition, the inhibition of DNA synthesis as well as the blockage of G_2 phase in the cell cycle was indicted for the reduction in the mitotic index[33,34]. The leaf methanol extract of *A. vogeliana* reduced the mitotic index of the actively dividing meristematic cells of *A. cepa*. The results revealed that there was a reduction in cellular activities as the concentrations of the leaf extract increases. There was about 400% reduction in the mitotic index (from 59.5% to 13.9%) at 500 µg/mL, which is the highest concentration tested (Table 2). The mitotic index noticed in all the extract concentration tested was significantly lower than the mitotic indices obtained for control. At higher concentrations (300 µg/mL and above), *A. vogeliana* leaf extract showed sub lethal to lethal activity. According to Iqbah *et al*.[35] a reduction in the mitotic index of less than 22% when compare with control, would have a mortal effect on the test organism. The clumpy or sticky chromosomes documented in this study indicated the genotoxic effect of the extract on the *Allium cepa* cells. This could be attributed to the excessive chromosome contraction coupled with partial dissolution of nucleoproteins, which consequently could cause irreparable cell death[33]. Moreover, the interactions among different chemical compounds in the plant might not be unconnected with the genotoxic and cytotoxic activities of the *A. vogeliana* leaf extract reported in this study. Chromosomal aberrations observed include clumped metaphase and anaphase, disrupted chromosomes and cells as shown in the photomicrographs (Figure 5). Celik[36] stated that chromosome aberrations may be associated with the interactions of different chemical agents in the plant extract tested and the plant DNA. These observations showed that the extract was both cytotoxic and genotoxic.

Table 2: The mitotic index after 24 hours in *Allium cepa* root cells treated with *A. vogeliana* leaf methanol extract different concentrations

Concentration of *A. vogeliana* (µg/mL)	Mitotic index (%)
0	59.5^{g}
50	36.4^{a}
100	34.4^{b}
200	31.6^{c}
300	22.7^{d}
400	15.7^{e}
500	13.9^{f}

The different letters show that there is a significant difference in the values ($P < 0.05$)

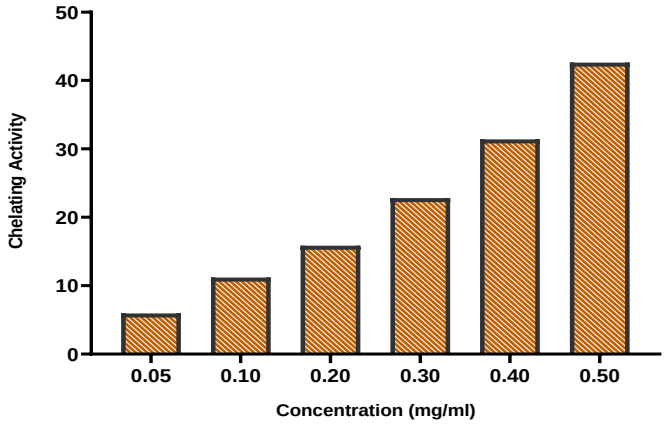

Figure 3: Metal chelating activity of *A. vogeliana* leaf methanol extract.

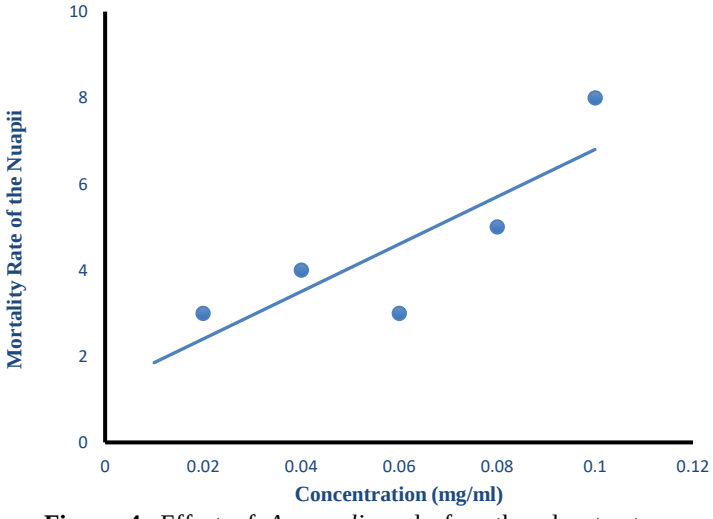

Figure 4: Effect of *A. vogeliana* leaf methanol extract different concentrations on Brine Shrimp growth inhibition

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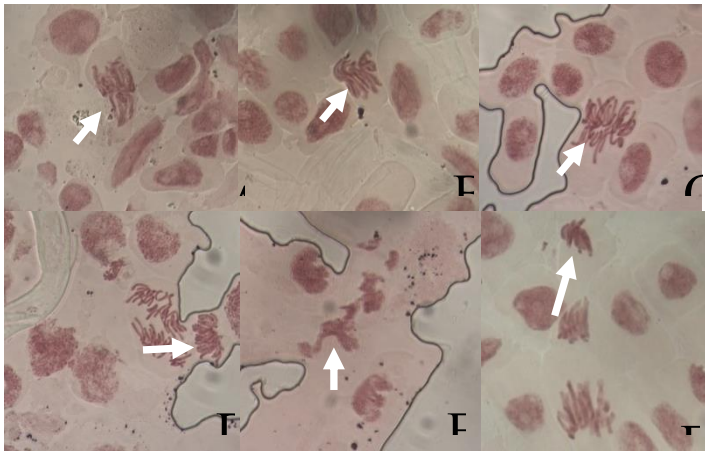

Fig. 5: Cell divisions in *A. vogeliana* leaf methanol extract treated *Allium cepa* root tip cells
Clumped metaphase at 50 µg/mL; B- Clumped metaphase at 100 µg/mL; C- Spindle disturbance at 200 µg/mL; D- Clumped metaphase at 300 µg/mL; E- Disrupted cells at 400 µg/mL; F- Clumped metaphase at 500 µg/mL.

Conclusion

The antioxidant, cytotoxic and genotoxic activities of the leaf methanol extract of *A. vogeliana* were explored. The extract showed a measurable amount of antioxidant activity and showed cytotoxic effect on Brine Shrimp nauplii. *Allium cepa* assay revealed both cytotoxicity and genotoxicity of the extract, which was concentration-dependent. Hence, the study concludes that the leaf methanol extract of *A. vogeliana* possesses antioxidant, genotoxic and cytotoxic activities. Further studies needed may include fractionation of the methanol extract, bioactivity-guided isolation of the bioactive components and usage of various cancer lines to authenticate the anti-malignant activity of the plant.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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