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Investigation of Antioxidant Activity and Cytotoxic Effect of Stem of *Anthocleista djalonensis*

Oyinlade C. Ogundare[1*], S. M. Akoro[2], Abiodun O. Ogunfowora[3] and Adekunle Oladunni[1]

[1]Biochemistry Unit, School of Pure and Applied Sciences, Lagos State Polytechnic, Ikorodu, Lagos State, Nigeria.
[2]Chemistry Unit, School of Pure and Applied Sciences, Lagos State Polytechnic, Ikorodu, Lagos State, Nigeria.
[3]Environmental Biology Unit, School of Pure and Applied Sciences, Lagos State Polytechnic, Ikorodu, Lagos State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author OCO designed the study, compiled the results, performed the statistical analysis and wrote the first draft of the manuscript. Authors OCO and SMA managed the literature searches, wrote the protocol and proofread the manuscript. Authors AOO and AO managed the analyses of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: To investigate the antioxidant and cytotoxic activities of stem of *A. djalonensis*.
Study Design: The study assessed the antiradical potential of the stem bark of *A. djalonensis* and determined the cytotoxic effect of the plant extracts using *Allium cepa* root growth inhibition and Brine Shrimp lethality assays.
Methodology: The stem bark of *A. djalonensis* was extracted in cold solvents using hexane, ethyl acetate, ethanol and water. The free radical scavenging activity of the plants extracts was determined using DPPH scavenging assay. The extracts cytotoxicity was studied by means of *Allium cepa* root growth inhibition and then Brine Shrimp lethality assays using vincristine sulphate as the standard drug.

**Corresponding author: E-mail: ogundareoyinlade@yahoo.com;*

Results: The ethanol extract of *A. djalonensis* showed the highest free radical scavenging activity (14.24 ± 2.80 µg/ mL) while the remaining extracts of the plant exhibited varying free radical scavenging activities (14.24 to 29.91 µg/ mL). The extracts restrained the growth of *A. cepa* after 72 hr of the study at $p< 0.01$. Similarly, the extracts showed varying cytotoxic effect against newly hatched brine shrimps. The ethanol extract had the highest cytotoxic value (IC_{50}) of 296.68 µg/ mL while hexane extract had the lowest (422.80 µg/ mL).
Conclusion: The extracts of stem of *A. djalonensis* are potential antioxidant and cytotoxic agents and may be relevant in the management of free radical provoking diseases such as cancer.

Keywords: Antiradical; DPPH; cytotoxic; Brine Shrimp; Allium cepa.

1. INTRODUCTION

Cancer is one of the major health problems all over the world. It affects all ages in both developing and developed countries. Ferlay et al. [1] reported that new cancer cases in Nigeria are more than 100,000 per annum. Cancer results into serious morbidity and mortality. Although, there are many types of anticancer chemotherapy, some of them are obtained from plants. Plant-derived chemotherapeutics include epipodophyllotoxins (etoposide and teniposide), vinca alkaloids (vincristine, vinblastine and vindesine), taxanes (docetaxel and paclitaxel) and the camptothecin derivatives (camptothecin and irinotecan) [2]. Anticancer chemotherapeutics are scarce, expensive and toxic on both cancer and healthy cells, thereby resulting into different side effects. Therefore, researchers are faced with the challenges of deriving cheap and effective chemotherapy or drugs that are devoid of some of these side effects.

Screening of medicinal plants for pharmacological activity (anticancer) involves the assessment of their natural antioxidants for cytotoxic effect. Component of cytotoxic plants are then studied for biological activities such as anticancer or antitumor. Methods of determining ability of plant to cause the death of selected cells (cytotoxicity) include both (i) *in vitro:* reduction of yellow tetrazolium 3-(4,5-dimetylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) to a purple formazan dye by mitochondrial dehydrogenase or reductases in the selected cells [3], PC biosensor-based cell attachment assay [4], measurement of enzymes (lactate dehydrogenase or proteases inform of Caspases) that leach out of the membrane [5,6] (ii) *in vivo*: cytotoxicity of tissues of experimental animals abused by carcinogens 1,2-dimethylhydrazine (DMH) or azoxymethane or use of cancer allograft [7,8], Brine Shrimp lethality assay (BSLA) [9] and *Allium cepa* (*A*. cepa) root growth inhibition test [10]. Brine Shrimp lethality assay and *A. cepa* inhibition test are well known simple and cheap methods for assessment of cytotoxicity of anticancer agents and have been used along with other protocols by scientists such as Bagya et al. [11] during preliminary investigation and standardization of medicinal plants for anticancer activity.

Antioxidant phytochemicals are common components of many plant derived materials and are highly useful in the management of numerous diseases. Plant obtained antioxidants are more acceptable than the synthetic ones because of they possess multiple mechanisms of actions and are naturally non-toxic [12]. Screening of medicinal plants for antioxidants properties involves protocols where a synthetic colored radical or redox- active substance is generated. Capability of a biological sample to scavenge the radical or to reduce the redox-active compound is studied using spectrophotometer and appropriate standard inform of Trolox equivalent antioxidant activity (TEAC) or Vitamin C equivalent antioxidant capacity (VCEAC) are used to quantify antioxidant capacity [13]. This kind of determinations include the tendency of the plant extracts to scavenge free radical and determined by means of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) in the DPPH assay, as the stable red color DPPH turns yellow when scavenged by antioxidant in the plant extracts and result to a decrease absorbance value at 517 nm.

A. djalonensis is a medium-sized tree found in the tropics. It is about 30-45 feet high with blunt spines on the branch, pale grey trunk and wide spreading crown. The stem, root-bark and leaf of *A. djalonensis* are used to treat malaria, jaundice, diabetes and abscesses [14,15]. An investigation of the antioxidant and cytotoxic potential of valuable medicinal plants such as *A. djalonensis* stem will provide the scientific basis for the medicinal applications of this plant and enhance the exploitation of the plants material in the management of cancer.

2. MATERIALS AND METHODS

2.1 Chemicals

Chemicals such as 2, 2-diphenyl-2-picrylhydrazyl (DPPH), sodium phosphate, vincristine sulphate and dimethylsulfoxide (DMSO) were purchased from standard reagent vendors such as Sigma-Aldrich.

2.2 Collection of Plant Material

Barks of the stem of *A. djalonensis* were collected from Ejigbo in Osun State, Nigeria in December, 2015, and were identified by a Botanist. The plant bark sample was washed with clean tap water in other to remove contaminants. The clean sample was dried under shade for about two weeks at room temperature and ground into coarse sample by use of grinding mill and the sample was stored at temperature of 4℃ (274 K) for future use.

2.2.1 Preparation of plant extract

Approximately 30 g of powdered plant material was added into four beakers containing 300 mL of hexane, ethyl acetate, ethanol and water respectively. The plant material was soaked with intermittent stirring for 24 hr. The resulted extracts were filtered independently using cotton wool, and then Whatman No. 1 filter papers. They were concentrated in a rotary evaporator under reduced pressure at 60℃ (333.15 K) and the percentage extraction yields for various extracts was calculated.

2.2.2 Determination of DPPH radical scavenging activity

The antioxidative activity of the extract of *A. djalonensis* stem was determined using hydrogen donating or free radical scavenging ability by use of the stable radical 2, 2- diphenyl-2-picrylhydrazyl (DPPH) according to modified protocol of Yu et al. [16]. Briefly, a solution of 0.1 mmol/ L of DPPH in methanol was prepared and 3.5 mL of this solution was mixed with 0.5 mL of extract in methanol at different concentrations (10 – 100 µg/ mL). The reaction mixture was shaken enthusiastically and kept in the dark at room temperature for about 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Methanol was used to zero the spectrophotometer.

$$\% \text{ DPPH radical scavenging activity} = \{(A_{blank} - A_{sample})/A_{blank}\} \times 100$$

Where, A_{blank} is the absorbance of the control, and A_{sample} is the absorbance of the extracts/ or standard. Then, graph of percentage of inhibition against concentration was used to calculate IC_{50}.

2.3 Determination of Cytotoxic Activity

2.3.1 *Allium cepa* root growth inhibition test

The tendency of the extracts of *A. djalonensis* to inhibit the growth of onion (*A. cepa*) bulbs was assessed according to the modified protocol of Khade et al. [17]. Healthy onion bulbs that were virtually of the same sizes were purchased from a local market in Ikorodu, Lagos State, Nigeria. The brown scales and roots of the bulbs were removed leaving the root primordial. The bulbs were placed in contact with distilled water in different 50 mL beakers and kept in the dark at room temperature for 48 hr. The roots (eight in numbers) which had grown to about 3 cm after 48hr were selected and measured using a ruler. The sprouted bulbs were then separately treated in triplicates with standard drug (vincristine sulphate) and extracts at concentrations of 1- 20 mg/ mL.in beakers and kept in the dark at room temperature for 72 hr. After the treatment period, the length of the grown onion bulbs were measured and compared with the control (onion bulbs that were treated with distilled water only).

2.3.2 Brine Shrimp lethality bioassay

Brine Shrimp lethality bioassay is a general bioassay used to specify cytotoxicity, pharmaceutical and pesticidal actions. Varied concentrations (10 µg/ mL to 1000 µg/ mL) of extracts of stem of *A. djalonensis* and control (vincristine sulphate) were diluted with clean sea water (hatching medium for the brine shrimps) in test tubes containing ten healthy newly hatched brine shrimps (nauplii). The live healthy nauplii which are constantly moving were counted after 24 hr. The experiment was conducted in triplicates and the average percentage of lethality of the nauplii was calculated at each concentration and analyzed using ED50plus V1.0 software to determine the LC_{50} of various extracts (concentration that causes death of 50% population of nauplii).

2.4 Statistical Analysis

All analyses were carried out in triplicates. Data were presented as mean ± S.E.M and compared using One Way Analysis of Variance (ANOVA). All data were considered statistically significant at $p< 0.01$. ED50plus V1.0 software was used to determine the inhibition and lethality of fifty percent population.

3. RESULTS AND DISCUSSION

3.1 Results

The percentage extraction yields for hexane, ethylacetate, ethanol and water extracts of stem bark of *A. djalonensis* were 2.07%, 4.43%, 10.89% and 10.23% respectively. Fig. 1 shows the percentage DPPH radical scavenging activity of hexane, ethylacetate, ethanol and water extracts of stem bark of *A. djalonensis*. Among the extracts, the ethanol extract possessed the highest free radical scavenging activity. The IC_{50} of hexane, ethyl acetate, ethanol and water extracts was found to be 29.91± 2.04, 28.32± 0.31, 14.24± 2.80 and 26.66± 3.14 µg/ mL respectively (Fig. 2). The higher the IC_{50} value then the lower the free radical scavenging activity.

Table 1 reflects a significant ($p< 0.01$) inhibition of root of onion bulbs (*A. cepa*) after 72 hr study. There were some notable morphological changes (short, bent and twisted roots) in the understudied roots of onion bulbs within the tested concentrations of extracts of *A. djalonensis* and the standard drug (vincristine sulphate). There was no root growth at all when the extracts and standard drug were used at concentration of 20 mg/ mL.

Fig. 3 shows the effect of different concentrations of extracts of *A. djalonensis* and the standard drug (vincristine sulphate) on the brine shrimp nauplii. The LC_{50} of the tested samples was calculated using the curve of concentration of the extract versus average percentage (%) lethality. The lower LC_{50} means higher toxicity. Among the extracts, the ethanol stem of *A. djalonensis* showed most potent activity with LC_{50} value of 296.68 µg/ mL, while hexane extract had the lowest value (422.80 µg/ mL). The standard drug showed maximum livability (minimum lethality) of nauplii (100% viability) when the concentration was 250 µg/ mL and had an LC_{50} of 2.75 µg/ mL.

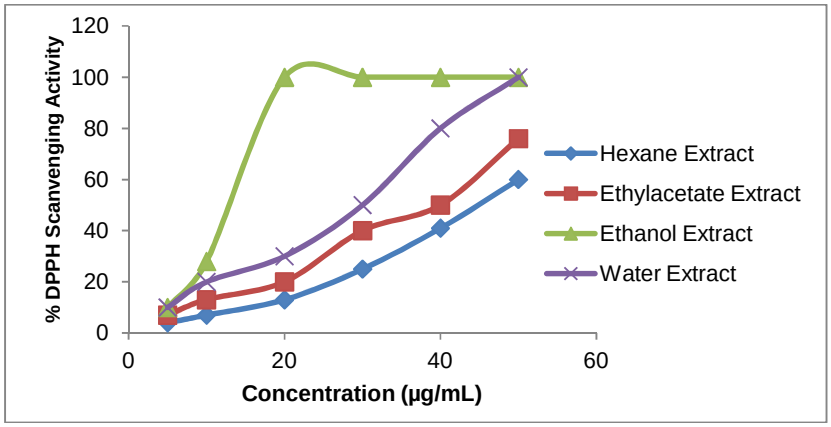

Fig. 1. DPPH radical scavenging activity of *A. djalonensis*

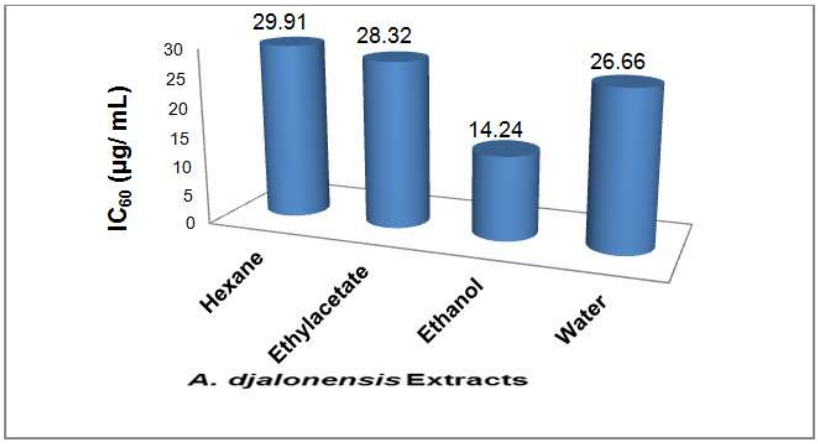

Fig. 2. IC_{50} of *A. djalonensis* using DPPH radical scavenging activity

Table 1. Inhibition of growth of root of *A. cepa*

Groups	Concentration (mg/ mL)	Root length (cm)		
		0 hr	48 hr	72 hr
Control	1	3.33±0.15	3.70±0.10	4.30±0.20
(Water)	5	3.31±0.14	3.93±0.06	4.33±0.21
	10	3.35±0.10	3.97±0.12	4.30±0.16
Vincristine sulphate	1	3.36±0.10	2.73±0.12*	2.90±0.20*
	5	3.30±0.21	2.47±.0.15*	2.20±.0.02
Hexane Extract	10	3.32±0.30	2.27±0.06*	2.10±0.10*
	1	3.20±0.20	3.46±0.15	3.67±0.15*
	5	3.23±0.05	3.13±0.15*	3.17±0.12
Ethylacetate	10	3.33±0.10	3.03±0.15*	2.97±0.21*
Extract	1	3.17±0.32	3.40±0.36	3.60±0.30*
	5	3.14±0.21	3.03±0.12*	3.00±0.00
Ethanol Extract	10	3.17±0.40	2.97±0.06*	2.93±0.06*
	1	3.27±0.16	2.90±0.10*	3.17±0.03*
	5	3.33±0.12	2.63±.0.25*	2.33±0.12
Water Extract	10	3.23±0.16	2.46±0.11*	2.17±0.10*
	1	3.33±0.06	3.57±0.06*	3.73±0.06*
	5	3.23±0.11	3.03±0.25*	3.13±.0.03
	10	3.20±0.20	2.90±0.10*	2.87±0.23*

Root length is expressed as inhibition of growth of root of the onion bulbs (A. cepa) and expressed as Mean ± S.E.M. (*) indicates level of significance of root growth inhibition at P< 0.01 compared with the control. Where Mean ± SEM = Mean values ± Standard error of means of three replicates

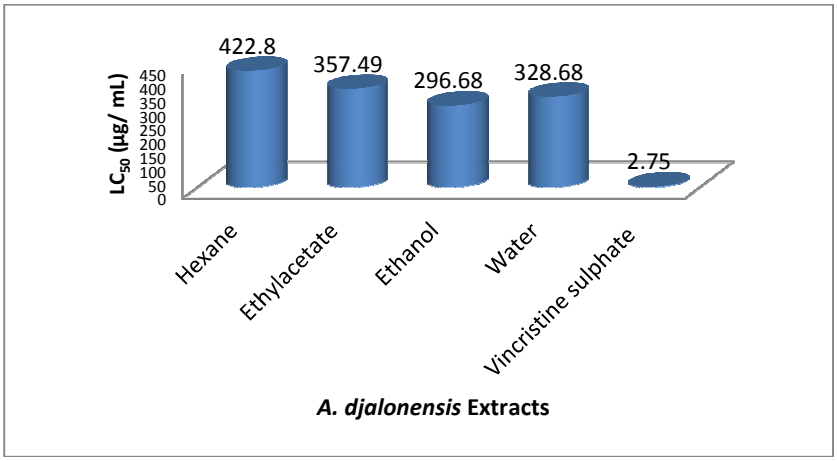

Fig. 3. LC_{50} of extracts of *A. djalonensis* using brine shrimp lethality assay

3.2 Discussion

The effect of extracting solvents on characteristic properties of bioactive compounds obtained from plants cannot be overemphasized. This is a manifestation of the concentration and composition of an extract as may be reflected by the value of extraction yields. There was a disparity in the percentage extraction yields of hexane (2.07%), ethylacetate (4.43%), ethanol (10.89%) and water (10.23%) extracts of stem bark of *A. djalonensis*. Report from Iloki-Assanga et al. [18] has proven that a variation of chemical compounds in plant extracts and their biological activities could be due to the nature and type of selected extraction solvents. Flavonoid, phenolic, saponnin, alkaloid and tannin are soluble in polar solvents such as methanol, ethanol and water. Despite the high polarity of water, alcohols like methanol and ethanol dissolve many more polar compounds than water. Thus, ethanol extracted more of these polar compounds from the stem of *A. djalonensis* than water, thereby increasing their concentrations in ethanol extract of stem of

A. djalonensis than the water extract. This research is in accordance with the previous reports in which chemical compounds like flavonoids, phenolic, sterols and alkaloids were dissolved by ethanol while water dissolved alkaloids glycosides, but ethylacetate could dissolve sterols than alkaloids [19,20].

Different techniques are used for the rapid screening of *in vitro* antioxidant activity. The antioxidant activity of stem bark of *A. djalonensis* was determined from the decolourisation of DPPH solution by addition of free radical species or antioxidants from the plant. Most antioxidants donate hydrogen ions in other to scavenge the DPPH [21], thereby causing a change in the DPPH colouration in a concentration dependent manner. This is important as the extracts demonstrate the ability to prevent the harmful role of free radicals in different diseases including cancer. Our results showed that the ethanol extract of stem of *A. djalonensis* possessed the highest free radical scavenging activity (14.24± 2.80 µg/ mL) when compared to other extracts (Fig. 1). This value was impressive compared to the free radical scavenging activity of butylated hydroxyl toluene (BHT) (18.5± 0.19 µg/ mL) as reported by Perumal et al. [22] and other extracts of stem bark of *A. djalonensis.* The sequence of free radical scavenging activities of the plant stem bark and BHT is ethanol extract (14.24± 2.80 µg/ mL) > BHT (18.5± 0.19 µg/ mL) > water extract (26.66± 3.14 µg/ mL) > ethyl acetate extract (28.32± 0.31 µg/ mL) > hexane extract (29.91± 2.04 µg/ mL). The higher the IC_{50} the lower the radical scavenging activity. Moreover, this result indicates the proton-donating ability of the extracts chemicals which serve as free radical inhibitors or scavengers and effective antioxidants. Total phenolic and flavonoid contents of *A. djalonensis* like any other medicinal plants have been shown to have a close relationship with the radical scavenging and antioxidant activities [23,24]. A similar report was made by Johnson and Ayoola [24] as the ethanol extract of *A. djalonensis* removed the free radicals released by DPPH. Therefore, the various extracts of *A. djalonensis* obtained in this study might contain natural antioxidants (phenolic and flavonoid) whose concentrations and antioxidant activities vary according to the polarity of the extracting solvents in a way that the ethanol extract compounds possess a substantial free radical activity in relation to BHT.

Cytotoxic effect represents a wide range of pharmacological activities of the bioactive natural products. Reports from Mesi (Dizdari) and Kopliku [25] and Del Socorro et al. [26] showed that most cytotoxic compounds are known to possess pesticidal and most times anticancer or antitumor activities. The cytotoxic property of *A. djalonensis* stem might be due to the presence of valuable chemicals such as saponnin, tannin, volatile oil and alkaloid as reported by Johnson and Ayoola [24] which may be responsible for the cytotoxic and anticancer effects.

Root growth in onion bulbs and other plant results from expansion of cells at a section of cellular differentiation which is usually an elongation zone at the root tips [27]. The graded concentrations of chemical substances in extracts of stem bark *A. djalonensis* and standard anticancer drug impeded the growth of onion roots at varied degrees through the arrest of cellular expansion or differentiation in onion root cells [28], thereby revealing the toxicity of the plant extracts especially in the arrest of growth as commonly observed in investigation of plants for cytotoxic and preliminary anticancer studies. Medicinal plants such as *Aloe vera* and *Rosmarinus officinalis* have been implicated in the inhibition of growth of onion bulbs and proven anticancer remedy [10,28,29].

In addition, the extracts of *A. djalonensis* stem bark showed cytotoxic effect on newly hatched artemia (brine shrimps) in a concentration dependent manner. The ethanol extract was the most cytotoxic (LC_{50} = 296.68 µg/ mL) in contrast to hexane extract (422.80 µg/ mL) and other extracts. Meyer et al. [30] described crude plant extract as cytotoxic (active) if it has an LC_{50} value greater than 1000 µg/ mL and non-toxic (inactive) at LC_{50} lesser than 1000 µg/ mL Report from Olowa and Nuñeza [31] revealed that extracts of *Lantana camara, Chromolaena odorata*, and *Euphorbia hirta* were cytotoxic LC_{50} values. However, since there is a high correlation between the toxicity of plant, drug or its agents using Brine Shrimp lethality assay and biological activities, stem bark of ethanol extract of *A. djalonensis* may be a superior anticancer agent as reported in the case of secretion from *Commiphora swynertonii* by Kalala et al. [32].

4. CONCLUSIONS

The ethanol extract of stem bark of *A. djalonensis* possessed a superior antioxidant activity and cytotoxic effect than the hexane, ethyacetate and water extracts as a result of its ability to scavenge free radicals (DPPH), inhibit

the growth onion bulbs and arouse the death of newly hatched brine shrimps (nauplii). Therefore, the stem bark of *A. djalonensis* contains chemotherapeutic agents that may be used to manage free radical provoking diseases such as cancer.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that there are no competing interests.

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