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Full Length Research

Antioxidant activity, phytochemical screening and cytotoxicity of ethanolic leaves extract of *Antigonon leptopus*

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The leaves of *Antigonon leptopus*, belonging to the family Polygonaceae. *A. leptopus* is used as a treatment for cough and throat constriction in Sudan and considered as one of the important medicinal plants in their folk-medicine. The present study investigated the *in-vitro* antioxidant (DPPH assay), phytochemical screening and cytotoxicity (MTT) of ethanol extract of *A. leptopus* (leaves). The ethanol extract of *A. leptopus* (leaves) was screened for detect their free radical scavenging properties using 2.2Di (4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH), while propyl galate was used as a standard antioxidant and screened for their cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). Ethanol extracts of *A. leptopus* (leaves). The tested antioxidant activity gave (89 ± 0.04 RSA%) in comparison to the control of propylgalate levels (72 ± 0.01RSA%), Primary phytochemical screening on leaves of *A. leptopus* appeared the rich of alkaloids, saponins, tannins, unsaturated sterol and/or triterpenes, flavonoids, glycosides and coumarins. And MTT assay appeared the safety of the extract.

Key words: Antioxidant activities (DPPH), phytochemical screening and cytotoxicity (MTT), *Antigonon leptopus* (leaves).

INTRODUCTION

Medicinal plants are still invaluable source of safe, less toxic, lower price, available and reliable natural resources of drugs all over the world. People in Sudan and in other developing countries have relied on traditional herbal preparations to treat themselves. Therefore, it is useful to investigate the potential of local plants against these disabling diseases (Amaral *et al.*, 2006; Koko *et al.*, 2008).

Reactive oxygen species (ROS) have been implicated in the induction of various types of oxidative damage to biomolecules that results against, cancer, neuro-degenerative diseases, atherosclerosis, malaria, several pathological events in living organisms and different other diseases associated with our life-style (Shahidi and Naczk, 1995; Halliwell *et al.*, 1992). These molecules can induce changes in different biological tissues and cell biomolecules such as lipids, proteins, DNA or RNA. Free radicals can also affect food quality; reducing its nutritional content and promoting the development of food deterioration (Nickavar and Aboalhasani, 2009).

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Antigonon leptopus. (Family: Polygonaceae) or coral vine is native Mexico and commonly found in tropical Asia, Africa, the Caribbean and the Americas (Raju *et al*, 2001). It is commonly grown in gardens and often run wild. It is a climbing vine; stems slender. Leaves are alternate, cordate-ovate or triangular, entire, acute to acuminate. Flowers are bright pink, in panicled racemes that terminate in tendril. Fruits of 1-seeded, hard nut let, 3-gonous, biconvex, compressed (Madhava Chetty *et al*., 2008). Traditionally, *A. leptopus* have been used to treat diabetes, asthma, liver and spleen disorders, cough and throat constriction (Cheryl A Lans, 2006; Idu and Onyibe, 2007; Mitchell and Ahmad, 2006). The present study was conducted to investigate the *in-vitro* antioxidant (DPPH assay), phytochemical screening and cytotoxicity (MTT) of ethanol extract of *A. leptopus* (leaves) in Sudan.

MATERIALS AND METHODS

Plant materials

The *A. leptopus* leaves were collected from Central Sudan (Khartoum) between January 2013 and February 2013. The *A. leptopus* leaves was identified and authenticated by the taxonomists of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI).

Preparation of crude extracts

Extraction was carried out for the fruits of *A. leptopus* leaves by using overnight maceration techniques according to the method described in Harbone (1984). About 50 g round material was macerated in 250 ml of ethanol for 3 h at room temperature. Occasional shaking for 24 h at room temperature was performed and, the supernatant was decanted. Thereafter, the supernatant was filtered under reduced pressure by rotary evaporatorion at 55°C. (Virtis, USA). The extracts were kept /stored were required at 4°C until the time of they were required.

Antioxidant activity of *Antigonon leptopus* leaves extract

DPPH radical scavenging assay: The DPPH radical scavenging was determined according to the method of Shimada *et al*. (1992) with some modification. In 96-wells plate, the test samples were allowed to react with 2.2Di (4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical (DPPH) for half an hour at 37°C. The concentration of DPPH was kept as 300 µM. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group and Propyl Gallate (PG). All tests and analysis were run in triplicate.

Phytochemical Screening: Phytochemical screening is of great importance in providing us with information about chemicals found in the plant in term of their nature and range of occurrence. This information would enable us to correlate between the nature and range of occurrence of these chemicals and biological assays held to investigate a certain bioactivity of the mentioned plant. In this study the preliminary phytochemical screening was conducted according to the method described by Harbone (1984).

Preparation of the extracts: 10 mg of the powdered leaves of *A. leptopus* were refluxed with 100 ml of ethanol 80% for 4 hours. The cool solution was filtered and enough ethanol 80% was passed through the volume of the filtrate 100 ml. This prepared extract (PE) was used for the various tests.

Test for unsaturated sterols and triterpenes: 10 ml of the prepared extract (PE) was evaporated to dryness on a water bath and the cooled residue was stirred several times with petroleum ether to remove most of the coloring materials. The residue was then extracted with 20 ml of chloroform. The chloroform solution was dehydrated over sodium sulphate anhydrous. 5 ml of chloroform solution was mixed with 0.5 ml acetic anhydride followed by two drops of conc. Sulphuric acid. The gradual appearance of green, blue pink to purple color was taken an evidence of the presence of sterol (green to blue) and or triterpenes (pink to purple) in the sample .

Test for alkaloids: 7.5 ml of (PE) was evaporated to dryness on a water bath. 5 ml of HCl (2N) was added and stirred while heating on the water bath for 10 minutes, cooled filtered and divided into two test tubes. To one test tube few drops of Mayer's reagent were added. While to the other tube few drops of Valser's reagent were added. A slight turbidity or heavy precipitate in either of the two test tubes was tanked as presumptive evidence for the presence of alkaloids.

Test for flavonoids: 17.5 ml of the (PE) was evaporated to dryness on a water bath, cooled and the residue was defatted with petroleum ether and the defatted residue was dissolved in 30 ml of ethanol (80%) and filtered. The filtrate was used for the following tests: (A) To 3 ml of the filtrate in a test tube 1 ml of 1% aluminum chloride solution was in methanol was added. Formation of yellow color indicated the presence of Flavonoids, (Flavones and / or chalcone). (B) To 3 ml of the filtrate in a test tube1 ml of 1% potassium hydroxide solution was added.

A dark yellow color indicated the presence of the Flavonoids compounds (flavones or flavanones) chalcone and/or flavonol. (C) To 2 ml of the filtrate 0.5 ml of magnesium turnings were added. Producing of defiant color to pink or red was taken as presumptive evidence that flavanones were present in the plant sample**.**

Test for Tannins: 7 ml of the (PE) was evaporated to the dryness on water bath. The residue was extracted several times with n-hexane and filtered. The insoluble residue was stirred with 10 ml of saline solution. The mixture was cooled, filtered and the volume of the filtrate was adjusted to 10 ml with more saline solution. 5 ml of this solution was treated with few drops of gelatin salt reagent. Formation of immediately precipitate was taken as an evidence for the presence of tannin in plant sample. To another portion of this solution, few drops of ferric chloride test reagent were added. The formation of blue, black or green was taken as an evidence for the presence of tannins.

Test for saponins: 1 g of the original dried powdered plant material was placed in a clean test tube. 10 ml of distilled water was added and the tube was stoppered and vigorously shaken for about 30 seconds. The tube was then allowed to stand and observed for the formation of (honeycomb). The appearance of honeycomb, which persisted for least an hour, was taken as an evidence for the presence of Saponins.

Test for anthraquinone glycosides: 10 g of the powdered plant sample were boiled with 10 ml of 0.5N KOH containing 1 ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with10 ml of benzene. 5 ml of the benzene solution was shaken with 3 ml of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of Anthraquinones was indicated if the alkaline was found to have assumed pink or red color.

Test for coumarins: 3 g of the original powdered plant sample was boiled with 20 ml of distilled water in a test tube and filter paper was attached to the test tube to be saturated with the vapor after a spot of 0.5N KOH put on it. Then the filter paper was inspected under UV light, the presence of coumarins was indicated if the spot has found to be absorbed the UV light.

Cytotoxicity screening: Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of the studied *N. sativa* (seeds).

Microculture tetrazolium (MTT) assay

Principle: This colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, blue colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

Preparation of extracts, solutions: Using a sensitive balance 5 mg of *A. leptopus* leaves extract were weighed and put in eppendorf tubes. 50 µl of DMSO were added to the extract and the volume was completed to 1 ml with distilled water obtaining a concentration of 5 mg/ml. The mixture was vortexed and stirred by magnetic stirrer to obtain a homogenous solution.

Cell line and culturing medium: Vero (Normal, African green monkey kidney) cells were cultured in a culturing flask containing a complete medium consisting of 10% fetal bovine serum and 90% minimal essential medium (MEM) and then incubated at 37^{o}C. The cells were subcultured twice a week.

Cell line used: (RAW 264.7) Establish from an ascites of a tumour induced in a male mouse by intraperitoneal injection of Abselon Leukaemia virus (A-MuLV). Cells with pinocytose neutral red and phagocytose zymosan. Cells capable of antibody-dependent lysis of sheep erythrocytes and tumour targets. Growth inhibited by LPS (0.5ng/ml).

Cell counting: Cell counts were done using the improved Neubauer chamber. The cover slip and chamber were cleaned with detergent, rinsed thoroughly with distilled water and swapped with 70% ethanol, then dried. An aliquot of cell suspension was mixed with equal volume of 0.4% trypan blue in a small tube. The chamber was charged with cell suspension. After cells had settled, the chamber was placed under light microscope. Using 40 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating cells:

$$(\text{Cells/ml})\ N = \frac{\text{number of cells counted} \times \text{dilution factor} \times 10^4}{4}$$

Procedure: The monolayer cell culture formed in the culturing flasks was trypsinized and the cells were put in centrifuging tube and centrifuged for 5 minutes separating the cells from the supernatant that flicked out. 1 ml complete medium was added to the cells and all the cell suspension was contained in a basin. In a 96- well microtitre plate, serial dilutions of each extracts were prepared. 3 duplicated concentrations for each extracts that is, 6 wells for each of 8 extracts. All wells in rows A,

Table 1. Antioxidant activity of *A.leptopus* (leaves).

No	Name of plant	Part	%RSA* ± SD (DPPH)
1	*A. leptopus*	leaves	89 ± 0.04
2	*Control	PG	72 ± 0.01

Key: RSA* = Radicals scavenging activity *Control = P.G = Propyl Gallate .

Table 2. Preliminary Phytochemical Screening analysis of *A.leptopus* (leaves) extract.

No.	Tested	*A. leptopus* (leaves)
1	Unsaturated Sterol And/or Triterpenes	+
2	Alkaloids	+++
3	Flavonoids	+
4	Tannins	++
5	Saponins	+++
6	Anthraquinone glycoside	+
7	Coumarins	+

Key: (+): Low concentration (++): Moderate concentration (+++): High concentration

B and C were used in addition to first 4 wells from each rows D, E and F. The first 2 wells of row G were used for the negative control and the first 2 wells of row H were used for the positive control Triton X. 20 µl complete medium pipetted in all wells in rows B, C and mentioned wells of rows E and F. Then 20 µl from each extracts were pipetted in rows A and B and first 4 wells of rows E and F. 20 µl taken from row B were pipetted and mixed well in row C from which 20 µl were taken and flicked out. The same was done from E to F. After that 80 µl complete medium were added to all used wells. Then adjusting the cell account to 3000 cell/well, 100 µl of cell suspension were added completing all wells to the volume 200 µl. Now, we have duplicated three concentrations 500, 250, 125 µg/ml for each extract. Then the plate was covered and incubated at 37°C for 96 hours.

On the fourth day, the supernatant was removed from each well without detaching the cells. MTT suspension stock (5 mg/ml) prepared earlier in 100 ml phosphate buffer solution (PBS) was diluted (1:3.5) in a culture medium. To each well of the 96-well plate, 50 µl of diluted MTT were added. The plate was incubated for further 4 hours at 37°C. MTT was removed carefully without detaching cells, and 100 µl of DMSO were added to each well. The plate was agitated at room temperature for 10 minutes then read at 540 nm using microplate reader. The percentage growth inhibition was calculated using the formula as shown:

$$\% \text{ cell inhibition} = 100-\{(Ac-At)/Ac\} \times 100$$

Where,
At = Absorbance value of test compound;
Ac =Absorbance value of control.

Statistical analysis

All data were presented as means ± S.D. Statistical analysis for all the assays results were done using Microsoft Excel program. Student t test was used to determine significant difference between control and plant extracts at level of $P < 0.05$.

RESULTS AND DISCUSSION

The leaves of *A. leptopus* (Family: Polygonaceae) were screened for antioxidant screening for their free radical scavenging properties using 2.2Di (4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH), while propylgalate was used as standard antioxidant, phytochemical screening and screened for their cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT).

Tables 1, 2 and 3 showed the antioxidant activity, preliminary phytochemical screening and cytotoxicity screening of ethanolic *A. leptopus* (leaves) investigated in this study.

Antioxidant activity of *A. leptopus* (leaves) extract

As shown in **Table 1,** the results of antioxidant activity *A. leptopus* (leaves) showed high antioxidant activity against the DPPH free radical (89 ± 0.04 RSA%).

Table 1 indicates the anti DPPH of ethanol extract of *A. leptopus* (leaves) the reading and propyl gallate was used as standard drug level. The tested antioxidant activity gave (89 ± 0.04 RSA %) in comparison to the control of propylgalate levels gave (72 ± 0.01 RSA %). This result was similar to that reported by Onanong *et al.*

Table 3. Cytotoxicity of *Antigonon leptopus* extracts on normal cell lines (Vero cell line) as measured by the MTT assay.

Name of plant (part)	Concentration (µg/ml)	Absorbance	Inhibition (%) ± SD	IC_{50} (µg/ml)
Antigonon leptopus (leaves)	500	0.717	59.80 ± 0.41	
	250	1.118	45.99 ± 0.28	289.60
	125	1.853	36.79 ± 0.14	
*Control		0.0014	95.60 ± 0.01	

Key: *Control = Triton-x100 was used as the control positive at 0.2 µg/mL.

(2011) which appeared 89±36 although using different part (flower), extract substance (methanol) and place (Tailand).

Phytochemical analysis of *A. leptopus* (leaves)

The Phytochemical analysis of crude ethanolic extract of *A.leptopus* performed by the method described earlier and then and then analyzed for phytocompounds like steroids or terpenoids, alkaloids, flavonoids, coumarins, saponins, tannins and anthraquinone as present in Table 2. The results explain extract have high concentration of saponins and alkaloids compounds; moderate concentration of tannins, compoundes; low concentration of cumarins, anthraquenones, flavonoids, unsaturated sterol and/or triterpenes compounds. This result was similar to that reported by (Johnson Marimuthu @ Antonisamy, 2012) except absence of alkaloids because using different part (flower), extract substance (methanol) and place (India).

Cytotoxicity assay of *A. leptopus* (leaves) extract

The maximum concentration used was 500µg/mL. When this concentration produced less than 50% inhibition, the IC_{50} cannot be calculated (Table 3).

Table 3 indicates the % inhibition of Vero cell line growth *in vitro* by ethanolic extract of *A. leptopus* (leaves). MTT colorimetric assay was used. All reading in triplicate for different concentrations 125- 250 -500 µg/mL.

Interestingly, the cytotoxicity assays were conducted in this study to evaluate the ethanolic extract of *A.leptopus* (Leavs) their cytoxtoxicty effects by using MTT-assay include (RAW 264.7). The result of MTT assay appeared the moderate of the examined extract in Table 3.

Conclusion

From complete investigation about antioxidant and pharmaceutical screening studies of *A. leptopus* leaves it can be recommended that extracts could be used as a easily available foundation of natural antioxidants, which can be used as supplement to aid the therapy of free radical mediated diseases such as cancer, diabetes, inflammation, etc., diabetes swelling. Phytochemical studies of *A. leptopus* (leaves) extracts revealed that this plant contains Triterpenes, alkaloids, Tannins, saponins, glycosides and Coumarins which are very importance for pharmaceutical uses. Further studies are needed on the isolation and elucidation of their chemical structures of antioxidant components in this *A. leptopus*.

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