Available online at www.pharmacie-globale.info ISSN 0976-8157

Research Article

PHARMACIE GLOBALE

INTERNATIONAL JOURNAL OF COMPREHENSIVE PHARMACY

PHYTOCHEMICAL, ANTIOXIDANT ACTIVITY AND CYTOTOXICITY OF METHANOLIC EXTRACT OF *Balanites aegyptiaca* (L.) DELILE

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Received: 31 Jan 2016; Revised: 29 Feb 2016; Accepted: 8 March 2016; Available online: 21 March 2016

ABSTRACT

Balanites aegyptiaca (L.) Del. belongs to family balanitaceae. The bark, unripe, fruits, and leaves of this plant are reported to have anthelminthic, antifertility, purgative and antidysenteric Properties. The present study was conducted to investigate the *in-vitro* antioxidant (DPPH assay), cytotoxic (MTT assay) and phytocemical of methanol extract of *B. aegyptiaca* (leaves). Antioxidant activity of the methanol extract of *B. aegyptiaca* (leaves) was screened for its free radical scavenging properties using 2.2Di-(4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH), while propyl galate was used as standard antioxidant and screened for their cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5- diphenyl tetrazolium bromide (MTT). The tested antioxidant activity gave (70 ± 0.07 RSA%) in comparison to the control of propylgalate levels (88 ± 0.07 RSA%), MTT assay verified the safety of the examined extract. The results of Phytochemical screening of *B. aegyptiaca* (leaves) showed that positive results were recorded for terpenoids, alkaloids, saponins and coumarins. Negative results were flavonoids, anthraquinone and tannins.

Keywords: Antioxidant activity, cytotoxicity, *Balanites aegyptiaca* (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.[1]

Balanites aegyptiaca Del. (Zygophyllaceae), known as 'desert date', is spiny shrub or tree up to l0 m tall, widely distributed in dry land areas of Africa and South Asia.[2,3] This tree is native to much of Africa and parts of the Middle East.[4] This is one of the most common trees in Senegal.[5] It can be found in many kinds of habitat, tolerating a wide variety of soil types, from sand to heavy clay, and climatic moisture levels. It is traditionally used in the treatment of various ailments i.e. jaundice, intestinal worm infection, wounds, malaria, syphilis, epilepsy, dysentery, constipation, diarrhea, hemorrhoid, stomach aches, asthma, and fever. It contains protein, lipids, carbohydrate, alkaloid, saponin, flavonoid, and organic acid. In Ayurvedic, Fruits are described to have a bitter sharp taste, and are used in alternative medicine as anthelmintic and analgesic preparation.[6] And in Unani system, it is used for treatment of skin diseases[7] while in Sudanese and Egyptian folk medicines it is used for treatment of jaundice[1] and the fruit (after removal of the apocarps) is used as an oral antidiabetic drug.[8] Its antimalarial and molluscidal activity is well studied.[9] Root is used in various folk medicines for treatment of abdominal pain and as purgative, while the bark is employed as a fish poison. The root, bark, kernel, and fruit have been shown to be lethal to mollusks.[10] In Senegal, Nigeria, Morocco, and Ethiopia, *B. aegyptiaca* is taken as a purgative for colic and stomach ache. In Libya and Eritrea, the leaves are used for cleaning infected wounds.[11] It was also reported to possess immune modulating properties, anti-inflammatory, antinociceptive, antioxidant and hypo-cholesterolemic actions.[12] Hepatoprotective activity of the park as well as fruit pulp of this plant has been reported. Therefore, in view of these reasons, we have selected it to study the *in-vitro* antioxidant (DPPH assay), cytotoxic (MTT assay) and phytochemical potential of its leaves.

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MATERIALS & METHODS

Plant Material and Extraction

Fresh leaves of the plant *Balanites aegyptiaca* were collected in the morning from Khartoum area in the year 2015, dried and powdered. Methanolic extract was prepared by maceration of leaves powder (1000g) with methanol (3L) for 48 hours with intermittent stirring. After extraction, the solvent was filtered and concentrated under reduced pressure. The extract (yield: 27%) obtained was stored at -20°C until being used.

Preparation of the Extract

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

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 Harborne.[13]

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 by Harborne.[13]

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 by Harborne.[13]

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 tube 1 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 by Harborne.[12]

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.

Antioxidant activity of plant extract

DPPH radical scavenging assay: The DPPH radical scavenging was determined according to the method of Shimada[14], with some modification. In 96-wells plate, the test sample was 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 sample was 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 the sample was determined in comparison with a DMSO treated control group and Propyl Gallate (PG). All tests and analysis were run in triplicate.

Cytotoxicity Screening

Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of *B. aegyptiaca* (leaves).

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 Formosan 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.[15]

Preparation of Extract Solution: Using a sensitive balance 5 mg of the 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°C. The cells were subcultured twice a week.

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:

$$(Cells/ml)\ N = \frac{Number\ of\ cells\ counted \times dilution\ factor\ \times 104}{4}$$

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 the extract were prepared. All wells in rows A, 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, 125µg/ml. 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 below:

$$\%\ cell\ inhibition = \frac{A_c - A_t}{A_c} \times 100$$

Where, A_t = Absorbance value of test compound, A_c = 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

Phytochemical Screening of *B. aegyptiaca* extract

The Phytochemical analysis of crude methanolic extract of *B. aegyptiaca* performed for phytocompounds like steroids or terpenoids, alkaloids, flavonoids, coumarins, saponins, tannins and anthraquinone. The positive results were recorded for terpenoids, alkaloids, saponins and coumarins. Negative results were anthraquinone and tannins, as shown in Table **1**.

Table 1. Preliminary Phytochemical Screening *B. aegyptiaca* extract

Scientific Name	Part Used	Unsat. Sterol and/or Triterpenes	Alkaloids	Flavonoids	Tannins	Saponins	Anthraquinone glycoside	Coumarins
B aegyptiaca	**Leaves**	+	+	+	-	+	-	+

+ = Present   - = Absent

Antioxidant activity of *B. aegyptiaca* extract

To explain the health benefits attributed to both plants focused in this present work, antioxidant activity tests was carried for methanolic extract of *B. aegyptiaca* (leaves) through DPPH. The result of DPPH of methanol extract of *B. aegyptiaca* (leaves), the reading and propyl gallate was used as standard drug level. The tested antioxidant activity gave (74 ± 0.08 RSA %) in comparison to the control of propylgalate levels gave (86 ± 0.06 RSA%) as shown in Table **2**. The results showed high antioxidant activity against the DPPH free radical (74 ± 0.08 RSA%). This table indicates the anti DPPH of methanol extract of *B. aegyptiaca* (leaves) the reading and propyl gallate was used as standard drug level. The tested antioxidant activity gave (74 ± 0.08 RSA %) in comparison to the control of propylgalate levels gave (86 ± 0.06 RSA %).

Table 2. Antioxidant activity of *B. aegyptiaca* leaves

Name of plant	Part	% RSA* ± SD (DPPH)
B aegyptiaca	leaves	74 ± 0.08
*Control	PG	86 ± 0.06

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

Cytotoxicity assay of *B. aegyptiaca* extract

Interestingly, the cytotoxicity assays were conducted in this study to evaluate the methanolic extract of *B. aegyptiaca* (leaves) for its cytoxtoxicty effect by using MTT-assay include (vero cell line). The result of MTT assay verified the safety of the examined extract. The maximum concentration used was 500 µg/mL. When this concentration produced less than 50% inhibition, the IC50 cannot be calculated, as shown in Table **3**. This table indicates the % inhibition of vero cell line growth in vitro by methanolic extract of *B. aegyptiaca* (leaves). MTT colorimetric assay was used. Reading in triplicate for different concentrations 125-500 µg/mL.

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

Name of plant (part)	Concentration (µg/ml)	Absorbance	Inhibition (%) ± SD	IC50 (µg/ml)
B aegyptiaca (leaves)	500	2.24	34.8 ± 0.05	< 100
	250	2.86	16.0 ± 0.06	
	125	3.19	8.4 ± 0.02	
	*Control	0.16	94.4 ± 0.01	

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

CONCLUSION

This result enhances the ethno botanical uses of the plant as a drug source of various ailments. Further investigations regarding the mode of action and other related pharmacological studies such as *in-vivo* investigation, drug formulation and clinical trials are highly recommended.

ACKNOWLEDGEMENTS

I gratefully acknowledge the Medicinal & Aromatic Plants Research Institute, Khartoum, Sudan for providing necessary facilities to carry out this work.

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