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Full Length Research Paper

Antimicrobial, antioxidant and phytochemical investigation of *Balanites aegyptiaca* (L.) Del. edible fruit from Sudan

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The methanol extract of the fruit (edible mesocarp) of *Balanites aegyptiaca* (L.) Del., a popular plant from Sudan, widely employed in Sudanese folk medicine was screened for antimicrobial, antioxidant and some active phytochemical compounds. The extract showed the presence of saponin, terpenoids, phenolic compounds and alkaloids. The methanolic extract of the fruits extracted from *B. aegyptiaca* exhibited a strong antioxidant activity in the DPPH assay and a potent capacity in preventing linoleic acid oxidation. Methanol extracts (50 and 100 mg/ml) were tested on several microbial strains using agar-well diffusion, minimum inhibitory concentration (MIC), minimum bacterial concentration (MBC) and minimum fungal concentration (MFC) methods. These experiments were performed on ten bacterial strains (five Gram-positives and five Gram-negatives) and four fungal strains. Methanol extracts, particularly at concentration of 100 mg/ml was found to be active against all bacterial and fungal strains and it was comparable to standard antibiotics Gentamicin and Amphotericin B. These findings support some of the traditional applications of the fruit of *B. aegyptiaca* against microbial aliments. It is therefore recommended that further studies regarding fractionation, separation and purification of these active antimicrobial compounds are required, in addition to toxicological evaluation *in vivo*.

Key words: *Balaneties aegyptiaca*, antibacterial, antifungal, phytochemical, antioxidant, methanol extract.

INTRODUCTION

In recent years, there has been a considerable interest in extracting antioxidants and antimicrobial compounds from natural sources to control human and plant diseases (Tepe et al., 2005). Natural antioxidant inhibited oxidative damage of food products and may prevent inflammatory conditions (Khanna et al., 2007), ageing and neurodegenerative diseases (Fusco et al., 2007). The market constantly addresses its attention to secondary metabolites produced by plants to check their properties and to evaluate their possible use in industry. Also, the scientific interest in these metabolites has increased today with the search of new antimicrobial agents, due to the increasing development of the resistance pattern of microorganisms to most currently used antimicrobial drugs. A considerable number of natural products and medicinal plants contain some active phytochemical ingredients such as phenolics, flavonoids, coumarins, curcuminoids or terpenes which induce different biological activities in animals including antioxidant, anti-

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Abbreviations: DPPH, 1,1-Diphenyl-2-picrylhydrazyl; **BHT,** butylated hydroxytoluene, **IC50,** values corresponding to the amount of extract required to scavenge 50% of DPPH radicals present in the reaction mixture. High IC_{50} values indicate low antioxidant activity; **mg GAE/g,** mg of gallic acid equivalent per g of dry plant extract; **mg QE/g,** mg of quercetin equivalent per g of dry plant extract; **A0 and A'0,** the absorbance values measured at zero time for the test sample and control, respectively; **At and A't,** the corresponding absorbance values measured after incubation for 2 h.

inflammatory and anti-cholinesterase effects (Loizzo et al., 2007).

Balanites aegyptiaca (L.) Del., known as Hegleig is a plant that belongs to the Balanitaceae family. It is an evergreen savanna tree, 4.5 to 6 m high, woody and with small spine scents (Koko et al., 2000). This plant is an indigenous species in Sudan, popular and of great concern, with diverse uses in folk medicine and many other applications (Elfeel and Warrag, 2011). It is widely distributed in arid and semi-arid regions of Sudan. It is estimated that up to one third of total trees population in central parts of Sudan is from this plant (NCR, 2008). It is also distributed in other countries located in the tropical dry belt of North Africa (Mohamed et al., 1999) and dry areas of India and South Asia (Chothani and Vaghasiya, 2011). Almost all parts of this plant are used in traditional medicine. It is traditionally employed in treatment of jaundice, yellow fever, syphilis, diarrhea, epilepsy, cough and wound healing, in addition to its applications as anti-inflamatory, anti-helminthic, insecticidal, anti-ralarial, molluscicidal, anti-fungal, anti-bacterial and even for snake bites (Mohamed et al., 1999; Chothani and Vaghasiya, 2011; Koko et al., 2000; John et al., 1990; Inngerdingen et al., 2004; Kubmarawa et al., 2007; Maregesi et al., 2008).

The mesocarp of *B. aegyptiaca* fruit basically contains 1.2 to 1.5% proteins, 35 to 37% sugars and 15% organic acids. Chemical analysis of *B. aegyptiaca* revealed other compounds such as 3-rutinoside and 3-rhamnogalactoside, diosgenin and a mixture of 22R and 22S epimers of 26-(O-β-D-glucopyranosyl)-3-β-[4-O-(β-D-glucopyranosyl)-2-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyloxy]-22,26-dihydroxyfurost-5-en(Chothani and Vaghasiya, 2011). It is known that the curative effect of any medicinal plant is related to its active phytochemical constituents (Henna et al., 2010; Abdallah 2011). Saponin from the edible fruit mesocarp of *B. aegyptiaca* was found having a larvacidal effect against mosquito's larvae (Zarroug et al., 1990). Moreover, previous studies on the pulp and kernel showed the presence of sterols, terpene, saponins, tannins, alkaloids and resins (Abdel-Rahim et al., 1986). This study deals with antimicrobial activity of the desert dates or the edible fruit mesocarp of *Balanites aegyptiaca* (L.) Del. growing in Sudan (Sudanese variety).

MATERIALS AND METHODS

Collection and extraction of plant materials

Fruits (desert dates) of *B. aegyptiaca* were obtained from Sudan, from Ed-Damazin district, south east Sudan, after scientific authentication. Collected fruits (Figure 1) were properly washed in water, rinsed using distilled water and dried in shade for about one week. Five hundred gram (500 g) of fruits (edible mesocarp) was macerated in 1000 ml methanol absolute for up to 72 h. Then filtered twice with filter paper (Whatman No. 1). The filtrate (Methanol extract) was evaporated using a rotary evaporator (at 40°C) to give semi-solid residues and transferred to an oven to dry (60°C, for up to 48 h) to obtain 54.6 g dry methanol extract. Afterward, dry residues were reconstituted with methanol to prepare the required doses (50 and 100 mg/ml) and kept in a refrigerator for further investigation.

Phytochemical analysis

Preliminary phytochemical tests for the methanol extract of *B. aegyptiaca* fruits have been conducted using standard assays as mentioned by Edeoge et al. (2005) and Abdallah (2009), to investigate tannins, saponin, flavonoids, terpenoids, phenolic compounds, alkaloids and anthraquinones. Total phenolic content (TPC) was also determined using the Folin-Ciocalteu method (Waterman and Mole, 1994), adapted to a microscale. Briefly, 10 µl of diluted sample solution was shaken for 5 min with 50 µl of Folin-Ciocalteu reagent. Then 150 µl of 20% Na_2CO_3 was added and the mixture was shaken once again for 1 min. Finally, the solution was brought up to 790 µl by the addition of distilled water. After 90 min, the absorbance at 760 nm was evaluated using a spectrophotometer SmartSpecTm3000 (Bio-Rad; Hercules, CA, USA). Gallic acid was used as a standard for calibration. The phenolic content was expressed as milligram of gallic acid equivalent per gram of dry sample (mg GAE/g) using the linear equation based on the calibration curve. For determination of total flavonoids content in the extract, spectrophotometrical assay was employed, using a method based on the formation of a complex flavonoid–aluminium, having the maximum absorption at 430 nm. The flavonoids content was expressed in mg of quercetin equivalent per gram of the dry plant extract (mg QE/g) (Quettier-Deleu et al., 2000).

Antioxidant testing

The DPPH radical scavenging activity assay

This assay was carried out as described by Kirby and Schmidt (1997). Briefly, 1 ml of 4% (w/v) solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in ethanol was mixed with 500 µl of sample solutions in ethanol (different concentrations). The mixture was incubated for 20 min in the dark at the room temperature. The blank reaction only contained the solvent (CH_3OH) which was used for the extraction. Scavenging capacity was read spectro-photometrically by monitoring the decrease of the absorbance at 517 nm. Lower absorbance of the reaction mixture indicates a higher free radical scavenging activity. Ascorbic acid was used as a standard. The percent DPPH scavenging effect was calculated using the following equation: DPPH scavenging effect (%) = (control- sample/control) × 100. Tests were carried out in triplicate.

β-Carotene bleaching assay

The antioxidant activity was determined according to the β-carotene bleaching method of Pratt (1980). A stock solution of β-carotene/linoleic acid mixture was prepared as follows: 0.5 mg of β-carotene was dissolved in 1 ml of chloroform with 25 µl of linoleic acid and 200 mg of Tween-20. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml of distilled water, saturated with oxygen (30 min), was added and the obtained solution was vigorously shaken. Four milliliter (4 ml/ of this reaction mixture was dispensed into test tubes and 200 µl of each sample, prepared at different concentrations was added. The emulsion system was incubated for 2 h at 50°C. The same procedure was repeated with butylated hydroxytoluene (BHT) as a positive control, and a blank as a negative control. After this incubation period, the absorbance of each mixture was measured at 490 nm. Antioxidant

Figure 1. Fruit of *Balanites aegyptiaca* (L.) Del.

activity in β-carotene bleaching model in percentage (A%) was calculated with the following equation: A% = 1- (A0 –At/A'0-A't) × 100, where A0 and A'0 are absorbencies of the sample and the blank, respectively measured at zero time, and At and A't are absorbencies of the sample and the blank, respectively measured after 2 h. All tests were carried out in triplicate.

Test organisms

Ten bacterial pathogens (five Gram-positive and five Gram-negative) and four fungal pathogens were used in this investigation as test microorganisms. Bacterial strains included Gram-positive bacteria: *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6536 and *Enterococcus faecalis* ATCC 29212; and Gram-negative bacteria: *Salmonella enteritidis* (food isolate), *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Klebsiella pneumoniae* ATCC 10031. Fungal strains tested were *Aspergillus niger* CTM 10099, *Fusarium graminearum* (plant isolat), *Fusarium oxysporum* (CTM10402) and *Fusarium culmorum* (ISPAVE 21w). Both bacteria and fungi studied have been obtained from the international culture collections (ATCC) and from the Centre of Biotechnology of Sfax, Tunisia.

Antimicrobial activity assay

The antimicrobial activity of the methanol extract of *B. aegyptiaca* fruit was evaluated using agar-well diffusion method as described by Güven et al. (2006), with minor modifications. To a sterile Petri-dish, 15 ml of molten Mueller-Hinton or potato dextrose agar (PDA) (Oxoid Ltd, UK) was poured and left to solidify. Fresh working cell suspensions were prepared and adjusted to 0.5 McFarland's standard. Then, 100 µl was spread onto the surface of the plates of Mueller-Hinton agar for bacteria or potatoes dextrose agar for fungi. After a while, 6 mm wells were punched into the agar with a sterile cork borer. Methanolic extract of *B. aegyptiaca* fruits was dissolved in methanol/water (1/9 v/v) to a final concentrations of 50 and 100 mg/ml, filtered using 0.22 µm pore-size black polycarbonate filters (Millipore). Afterwards, 80 µl (8000 µg/wells) from each concentration was loaded into the wells of the previously prepared plates and incubated for 24 h at 37°C for bacterial strains and 72 h for fungi at 28°C. Gentamicin 1 mg/ml (10 µg/wells) and Amphotericin B (20 µg/wells) were employed as antibacterial and antifungal positive controls, respectively. Methanol was employed as a negative control. Tests were repeated twice and the mean zone of inhibition was recorded.

Determination of MIC, MBC and MFC

The minimum inhibitory concentration (MIC) of methanolic extract of *B. aegyptiaca* fruits was determined using 96-well microplate dilution assay as reported by Gulluce et al. (2007) with slight modifications. One hundred microliter (100 µl) of Mueller-Hinton broth or potato dextrose broth was loaded from the second to the twelfth test holes. A stock solution of the methanolic extract was prepared by dissolving 100 µl of the extract in methanol and then adjusted to a final concentration of 100 mg/ml using Mueller-Hinton broth. To the first hole, 160 µl of the growth medium and 40 µl of the extract were loaded to reach a final concentration of 10 mg/ml,

Table 1. Preliminary phytochemical analysis for methanol extract of *B. aegyptiaca* fruit.

Active principle	Occurrence
Tannins	-
Saponin	+
Flavonoids	-
Terpenoids	+
Phenolic compounds	+
Alkaloids	±
Anthraquinones	-
Negative control (D.W.)	-

+ = Present, - = absent, ± = weak positive reaction, D.W. = distilled water.

Table 2. Total phenolic and flavonoid contents of *B. aegyptiaca* fruits methanolic extract (100 mg /ml)*.

Extract	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)
Methanol	212 ± 2.6	11.5 ± 1.3

*Each value represents the mean ± S.D. of three experiments. (mg GAE /g): mg of gallic acid equivalent per g of dry plant extract. (mg QE/g): mg of quercitin equivalent per g of dry plant extract.

and then 100 µl of serial dilutions was made from the second to the ninth hole. Thereafter and from each well, 10 µl of the suspension was removed and replaced by the bacterial or fungal suspensions to final inoculum concentrations of 10^6 colony forming unit (CFU)/ml for bacteria and 10^5 spores/ml for fungi. Though, the final concentrations of the extracts ranged from 0.039 to 10 mg/ml. The 10^{th} well contained Mueller-Hinton media for bacterial strains or potato dextrose broth for fungi and was considered as a positive growth control. Twenty microliter (25 µl) of microorganism growth indicator of 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to each hole, where microbial growth was inhibited. The solution in the well remained clear after incubation with MTT (Eloff, 1998). Finally, the microplate was covered with the sterile plate cover and incubated for 24 h at 37°C for bacteria or 72 h at 28°C for fungi. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extract to inhibit the growth of microorganisms. The minimum bacterial concentration (MBC) or minimum fungal concentration (MFC) was defined as the concentration of extract that did not show any microbial growth.

RESULTS AND DISCUSSION

The dramatic spread of multi-drug resistant microorganisms all over the world has put the search for new antimicrobial drugs from other sources such as plants on top priority (Abdallah, 2011). However, integrated study on the nominated plant of antimicrobial properties is important in order to investigate its biological activities and possible side effects. As shown in Tables 1 and 2 and Figures 1 and 2, the phytochemical analysis for methanol extract of *Balanites aegyptiaca* fruit revealed the presence of saponin, terpenoids, phenolic compounds and alkaloids, with considerable quantities of total phenolics and total flavonoids. It was documented that the major classes of antimicrobial compounds from plants are phenolics, terpenoids, alkaloids, lectins, poly-peptides and poly-acetylenes (Cowan, 1999). Also, saponin is a phytochemical compound of antimicrobial activity (Abdallah, 2011). Therefore, our phytochemical results confirm the presence of antimicrobial compounds in the fruit of *B. aegyptiaca*. Moreover, a similar study about phytochemical investigation on leaves of *B. aegyptiaca* methanolic extract showed the presence of saponins, tanins, phenols and anthraquinones (Doughari et al., 2007).

The effect of *B. aegyptiaca* extracts on DPPH-radical-scavenging showed a dose-dependent activity that can be evaluated by the determination of the IC_{50} values corresponding to the amount of the fraction required to scavenge 50% of DPPH radicals present in the reaction mixture. As shown in Figure 2, the radical scavenger extract showed an IC_{50} of 3 µg/ml. Therefore, the authors can conclude that this extract was able to reduce the stable free radical DPPH to the yellow colored diphenylpicryl hydrazine. In the β-carotene bleaching method, the degree of linoleic acid oxidation is determined by measuring oxidation products (lipid hydroperoxides conjugated dienes, and volatile by-products) of linoleic acid which simultaneously attack β-carotene, resulting in the bleaching of its characteristic yellow color in an aqueous solution. The antioxidant activity of methanolic extract of *B. aegyptiaca* was evaluated using different concentrations of extracts and it was compared with BHT and used as a reference (Figure 3). The addition of the *B. aegyptiaca* and the BHT at a concentration of 20 and 50 µg/ml prevented the bleaching of β-carotene at different degrees. Therefore, in the light

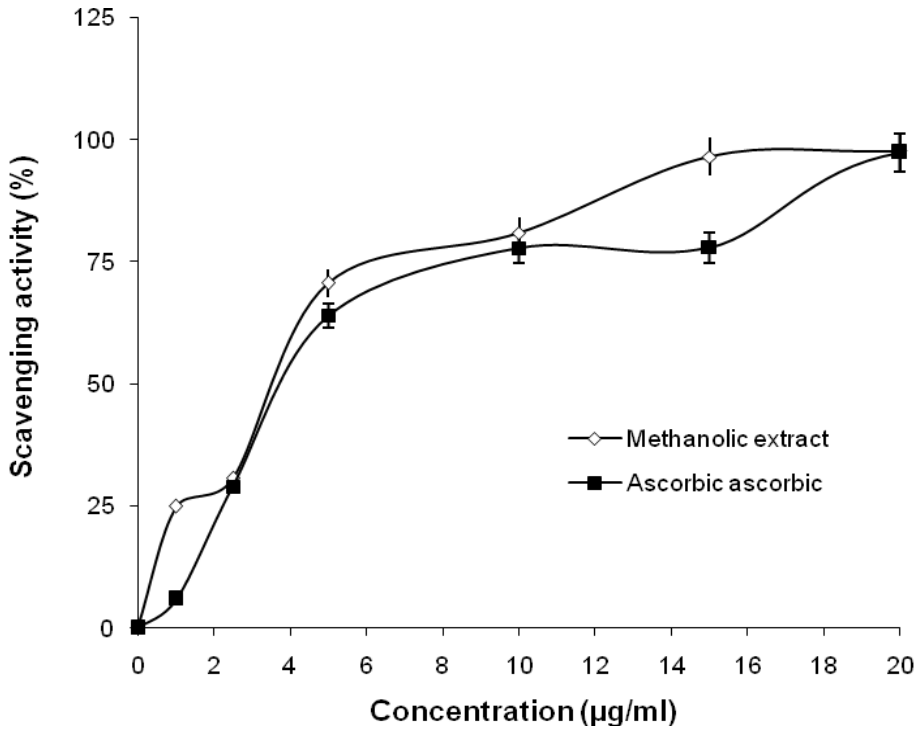

Figure 2. The DPPH free radical-scavenging activity of methanolic fractions of *B. aegyptiaca* at different concentrations (µg/ml).

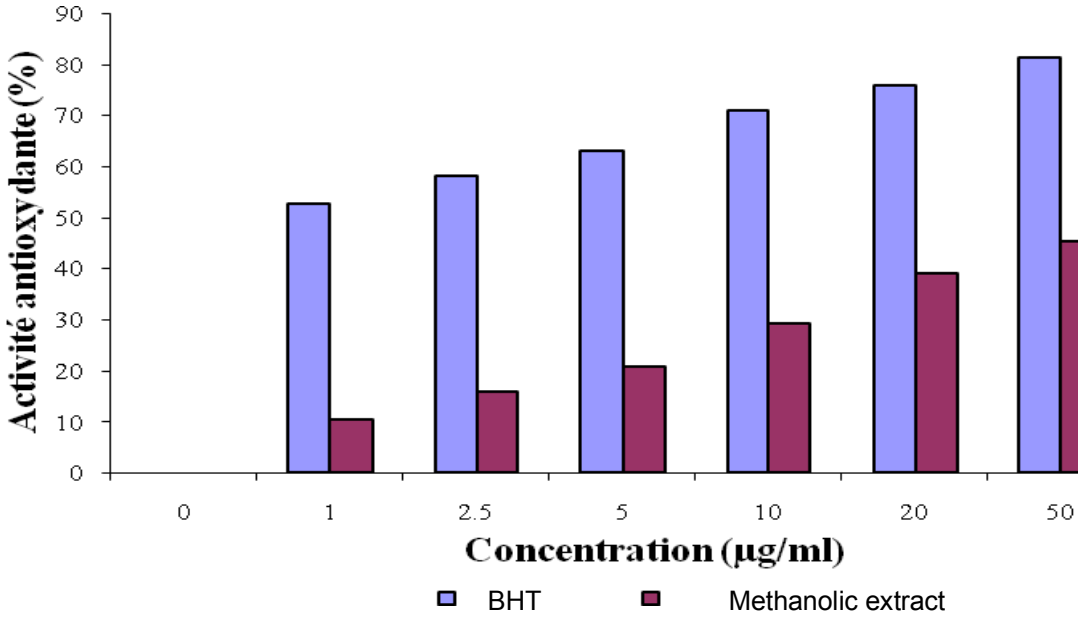

Figure 3. Antioxidant activities of methanolic fraction from fruits of *B. aegyptiaca* and BHT at different concentrations (µg/ml). The values represent the percent of inhibition of autoxidation of the linoleic acid/b-carotene emulsion. Measurements were carried out in triplicates.

of these results *B. aegyptiaca* showed antioxidant activity. These results also revealed that the methanolic extract of *B. aegyptiaca* fruit was free radical scavengers, acting possibly as primary antioxidants. The inhibition of LPO by the addition of OE can be used to improve the quality and stability of food products.

Table 3. Antibacterial activity of methanolic extract of *B. aegyptiaca* fruits*.

Bacteria	Inhibition zones diameter (mm) ofmethanol extract**		Gentamicin (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
	50 (mg/ml)	100 (mg/ml)			
Bacillus subtilis ATCC 6633	16.3 ± 0.3	22.2 ± 0.2	20.1 ± 0.1	0.312	0.625
Bacillus cereus ATCC 14579	15.4 ± 0.2	22.3 ± 0.4	20.4 ± 0.2	1.25	0.312
Staphylococcus aureus ATCC 25923	13.3 ± 0.3	17.0 ± 0.5	25.2 ± 0.2	0.625	1.25
Staphylococcus aureus ATCC 6536	15.1 ± 0.6	20.5 ± 0.5	16.2 ± 0.7	0.312	1.25
Enterococcus faecalis ATCC 29212	17.4 ± 0.1	20.1 ± 0.3	12.2 ± 0.2	0.312	0.625
Salmonella enteridis (Food isolate)	10.1 ± 0.1	15.2 ± 0.5	18.6 ± 0.1	0.625	1.25
Escherichia coli ATCC 25922	14.4 ± 0.4	20.0 ± 0.5	21.1 ± 0.3	0.625	1.25
Escherichia Coli ATCC 8739	15.4 ± 0.3	19.1 ± 0.4	20.2 ± 0.2	1.25	1.25
Pseudomonas aeruginosa ATCC 9027	15.2 ± 0.2	18.2 ± 0.1	18.5 ± 0.5	1.25	1.25
Klebsiella pneumoniae ATCC 10031	12.4 ± 0.2	15.0 ± 0.5	12.3 ± 0.3	2.5	1.25

*, Mean of 2 replicates ± S.E.M; **, diameter of inhibition zones are including diameter of well 6 mm.

Interestingly, results of the antimicrobial activity tests came highly positive. For antibacterial testing, as shown in Table 3 and Figure 4, the methanol extract of *B. aegyptiaca* fruit exhibited a considerable antibacterial activity compared with the standard antibiotic (Gentamicin 1 mg/ml). Generally, the antimicrobial activity correlates with the extract concentration (Tables 3 and 4, Figures 4 and 5); this could be related to the phytochemical constituents of this fruit. At concentration of 100 mg/ml, the highest susceptible bacterium was *Bacillus cereus* ATCC 14579 (22.3 mm), followed by *Bacillus subtilis* ATCC 6633 (22.2 mm), *Staphylococcus aureus* ATCC 6536 (20.5 mm), *Enterococcus faecalis* ATCC 29212 (20.1 mm), *Escherichia coli* ATCC 25922 (20 mm), *Escherichia Coli* ATCC 8739 (19.1 mm), *Pseudomonas aeruginosa* ATCC 9027 (18.2 mm), *Staphylococcus aureus* ATCC 25923 (17.0 mm), *Salmonella enteridis* (15.2 mm) and *Klebsiella pneumoniae* ATCC 10031 (15 mm). The inhibition zone equal or above 14 mm is considered as a high antibacterial activity (Philip et al., 2009). Obviously, inhibition zones from all strains were higher than 14 mm, revealing the potency of the extract as a promising effective antibacterial agent against different bacterial pathogens.

Interestingly, most strains tested were much susceptible to the tested extract rather than the standard antibiotic (Gentamicin). High level resistance to Gentamicin is well documented possessing a serious healthcare problem (Simonsen et al., 2003). The MIC values ranged from 0.312 to 2.5 mg/ml, while the MBC values ranged from 0.312 to 1.25 mg/ml (Table 4). Fluctuations and variations in MIC and MBC were observed (Tables 3 and 4). It is known that low MIC and MBC indicate a high antibacterial activity (Salvat et al., 2004). However, high MIC and MBC values of susceptible bacteria/or fungi could be due to the presence of active ingredients in low concentrations in the extract depending on the method of extraction itself (Abdallah et al., 2009). Here, intensive studies on fractions of the extract and the mode of action are a must.

Also, the antifungal activities of methanol extract of *B. aegyptiaca* fruit were detected against all fungal strains tested particularly at the concentration of 100 mg/ml (Table 4 and Figure 3), recording inhibition zones higher than the reference antibiotic (Amphotericin B, 2 mg/ml). The highest susceptible fungi was *Aspergillus niger* CTM 10099 (24.8 mm) followed by *Fusarium graminearum* ISPANVE 271 (20.2 mm), *Fusarium oxysporum* CTM 10402 (18.0 mm) and *Fusarium culmorum* ISPAVE 21W (14.5 mm), respectively. MIC and MFC results confirmed the antifungal properties of this extract (Table 4). In literature, studies on the antimicrobial activities of the fruit of *B. aegyptiaca*, particularly the Sudanese varieties are scanty. However, some studies have been carried out on other parts of *B. aegyptiaca* and revealed a considerable antimicrobial activity, such as roots (Henna et al., 2010), leaves (Doughari et al., 2007) and stem park (Maregesi et al., 2008).

Conclusion

The current study revealed that the methanolic extract of the fruit of *B. aegyptiaca* (desert dates) is a promising source of potential antimicrobial activity against wide-spectrum microorganisms, as many phytochemical investigation. Accordingly, the fruit of this ancient medicinal plant could play significant role in the search compounds of antimicrobial activity have been detected, in addition to the positive results of the antimicrobial for new antimicrobial drugs.

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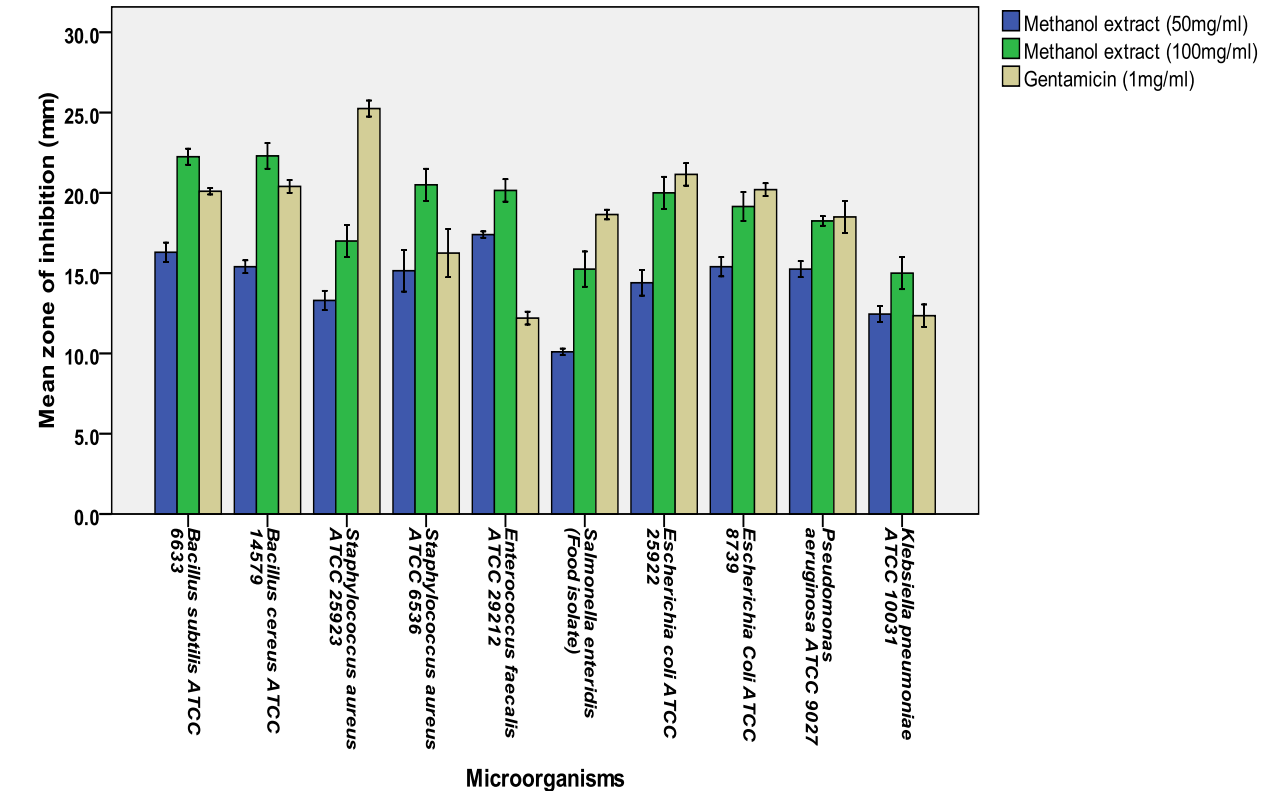

Figure 4. Antibacterial activity of methanolic extract of *B. aegyptiaca* fruit.

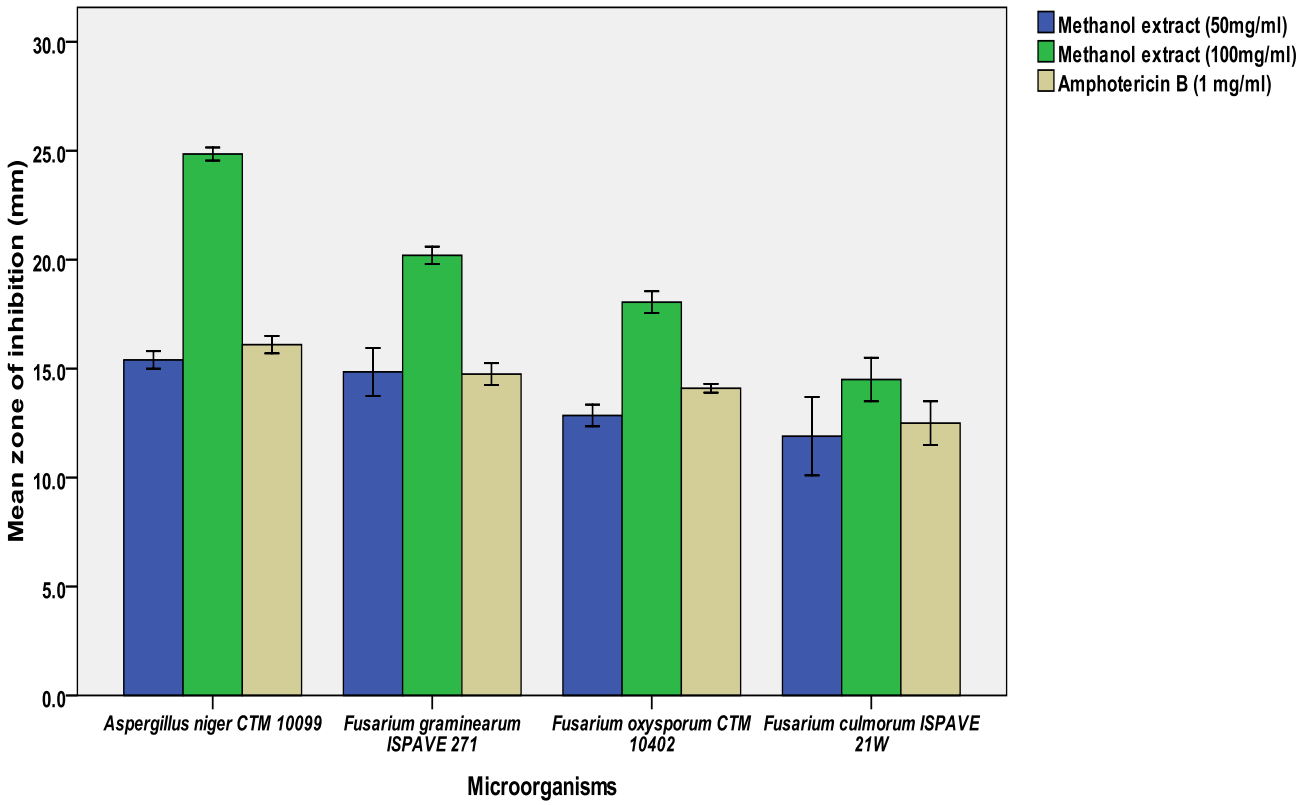

Figure 5. Antifungal activity of methanolic extract of *B. aegyptiaca* fruit.

Table 4. Antifungal activity of methanolic extract of *B. aegyptiaca* fruits*.

Fungi	Inhibition zones diameter (mm) ofmethanol extract**		Amphotericin B2 (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
	50 (mg/ml)	100 (mg/ml)			
Aspergillus niger CTM 10099	15.4 ± 0.2	24.8 ± 0.1	16.1 ± 0.2	1.25	0.312
Fusarium oxysporum CTM 10402	12.8 ± 0.2	18.0 ± 0.2	14.1 ± 0.1	2.5	1.25
Fusarium culmorum ISPAVE 21W	11.9 ± 0.9	14.5 ± 0.5	12.5 ± 0.5	1.25	1.25
Fusarium graminearum ISPANVE 271	14.8 ± 0.5	20.2 ± 0.2	14.7 ± 0.2	2.5	0.625

*Mean of 2 replicates ± S.E.M.** diameter of inhibition zones are including diameter of well 6 mm.

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