Assessment of the antimicrobial potential of the hydro-methanolic extract of Sidr (*Ziziphus spina-christi*) plant against selected pathogens *in vitro*.

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Abstract: *Ziziphus spina-christi* is a native plant growing in the warm and subtropical regions including Middle East. For a long time, its extracts have been used in alternative and complementary medicine with or without a scientific basis. The object of the present study was to evaluate the activity of Sidr plant hydro-methanolic extract against some microbes including Gram-positive, Gram-negative bacteria and *Candida albicans* using agar diffusion assay. The plant aerial parts yielded 5.7% of raw semisolid extract after maceration in hydro-methanol (50:50 v/v) and evaporation. Six concentrations (4, 8, 16, 32, 64 and 128 mg/mL in Muller-Hinton Broth) were used for the assay. The plant extract exhibited concentration-dependent activity against some Gram-positive bacteria, namely, *Bacillus cereus* (15.33 ± 0.52), *Clostridium perfringens* (12.33 ± 0.52), *Listeria monocytogenes* (11.17 ± 0.41) and *Staphyllococcus aureus* (10.17 ± 0.75); and against two Gram-negative bacteria, namely, *Proteus vulgaris* (8.5 ± 0.55) and *Vibrio parahaemolyticus* (8.33 ± 0.52); (the values are in mm after 128 mg/mL extract). The extract was without any visible activity against *Enterococcus faecalis*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri*, *Klebsiella aerogenes* and *Candida albicans*. The minimum inhibitory concentrations (MICs) were 5.16 mg/mL (*Bacillus cereus*), 8.15 mg/mL (*Listeria monocytogenes*), 10.22 mg/mL (*Clostridium perfringens*), 13.71 mg/mL (*Proteus vulgaris*), 13.01 mg/mL (*Vibrio parahaemolyticus*) and 20.37 mg/mL (*Staphyllococcus aureus*). These data may indicate that *Ziziphus* plant extract is active against some pathogenic bacterial strains and thus may be useful in treatment of disease conditions caused by these bacteria.
[Gheith I, El-Mahmoudy A. **Assessment of the antimicrobial potential of the hydro-methanolic extract of Sidr (*Ziziphus spina-christi*) plant against selected pathogens *in vitro*.** *Life Sci J* 2018;15(9):27-34]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 3. doi:10.7537/marslsj150918.03.

Keywords: Antimicrobial, Sidr, Traditional medicine, *Ziziphus*

1. Introduction

Infectious diseases account for approximately one half of all deaths in developing countries. In addition, in advanced ones, clinical problems due to drug resistant microorganisms and the emergence of microbe-related public health concerns are evident despite the progress made in understanding microbes and their pharmacological control (Iwu et al., 1999). For this reason, there is always urgent needs to discover and develop newer antimicrobial remedies with various properties, structures and novel mechanisms of action from various sources to sustain upper therapeutic hand over infectious agents.

Despite emphasis being put in research of synthetic chemical drugs, a special interest in medicinal plants has been focused, in part due to many synthetic drugs are potentially toxic or causing side effects to the patient (Akinpelu and Onakoya, 2006). In addition, plant materials are cheaper and more available contributors to the improvement of human health in terms of cure and prevention of diseases (Okoko and Oruambo, 2008).

Along years, medicinal plants are considered as well-established natural sources for the treatment of various diseases with or without scientific bases. About 20,000 plant species used for medicinal purposes are documented and reported by World Health Organisation (Cos et al., 2006). Particularly in Eastern countries, traditional therapy is more culturally acceptable and is able to meet psychological needs in a way western medicine does not.

Ziziphus spina-christi, also known as Sidr or Nabq, is an evergreen tree and native to the warm-temperate and subtropical regions, including Middle East (Yossef et al., 2011). It belongs to the Rhamnaceae family in the order of Rosales that contains about 60 genera and more than 850 species. The genus *Ziziphus* consists of about 100 species throughout the world (Asgarpanah and Haghighat, 2012). *Ziziphus spina-christi* is a shrub, sometimes a tall tree, with ovate-lanceolate or ellipsoid leaves; subsessile, small-sized flowers and about 1 cm-diameter fruits (Zargari, 1992).

Z. spina-christi is an important plant from both nutritional and medicinal aspects of view. The fruits

are usually eaten fresh or sometimes dehydrated for their high nutritive value. In addition, the flowers are important source for honey production. Pharmacologically, *Ziziphus* has been reported to have potentials of antinociceptive (Adzu et al., 2001), antidiarrhoeal (Adzu et al., 2003), antibilharzial (Ali and Hamed, 2006), anticarcinogenic (Abdel-Wahhab et al., 2007), analgesic (Adzu and Haruna, 2007), hepatoprotective (Shen et al., 2009), antihyperlipidemic (Solati and Soleimani, 2010), central nervous system sedative (Waggas and Al-Hasani, 2010), antioxidant (Abalaka et al., 2011), antidiabetic (Michel et al., 2011), antiplasmodial (Adzu, 2011), molluscicidal (Hassan et al., 2011), and surfactant (Pordel Shahri et al., 2012) activities. In addition, more than one study have been conducted to evaluate the antibacterial and antifungal properties of the Sidr plant different extracts, however they have shown controversial results that might be explained on the basis of different geographical regions, different types of extracts and different types of strains used.

The object of the present study was to investigate the antimicrobial activity of the hydromethanolic extract of *Ziziphus* plant growing in our environment against twelve strains of Gram-positive, Gram-negative and Candida that are pathogenic and hazardous to both human and animal health if remained uncontrolled.

2. Material and Methods

The plant material & extraction procedure

Figure 1: *Ziziphus spina-christi* (the collected plant material)

The aerial plant parts of *Ziziphus spina-christi* (Figure 1) were collected from our local environment and identified.

Plant parts were refluxed in running tap water and then with bi-distilled water, shade dried at room temperature and coarsely crushed using a pestle and mortar. Extracts were prepared by macerating a weighed amount of the crushed plant parts (100 g) in a known volume (1 Litre) of water/organic solvent (bi-distilled water: absolute methanol, 50:50, v/v). Maceration continued for 72 hours in refrigerator with intermittent shaking. The hydro-methanolic extract was then strained through muslin mesh, filtered through Whattman paper #1. The obtained filtrate was then concentrated using a shaking water bath at 56 °C in a wide-mouthed containers and the residue obtained (yield) was then lyophilized (LyoQuest-85, Telstar, Madrid, Spain), weighed and re-constituted by dissolving in measured amount of Muller-Hinton broth (Oxoid, Hampshire, UK). A stock solution of 128 mg/mL was prepared and filter-sterilized using sterile syringe filters (Econofltr PTFE 25 mm 0.45 µm, Agilent Tech., CA, USA). The stock solution was then serially diluted to get 64, 32, 16, 8, 4, 2 & 1 mg/mL which were used for the antibacterial activity testing. The method was modified after (Harborne, 1973).

Culture media and Microbial strains

Maximum Recovery Diluent (MRD, LAB 103, LAB M Ltd., Lancashire, UK) with typical formula (g/L) of Peptone 1.0 and sodium chloride 8.5 was used for diluting the selected reference bacterial strains to obtain the dilution whose turbidity is equivalent to 0.5 McFarland for each bacterial strain.

Muller-Hinton Broth (Oxoid CM0405, Oxoid Ltd., Hampshire, UK) with typical formula (g/L) of Beef dehydrated infusion 300, Casein hydrolysate 17.5 and Starch 1.5 with pH adjusted at 7.3 ± 0.1 was used for serial dilution of *Ziziphus* plant extract.

Muller-Hinton Agar (Biolife Italiana, Milano, Italy) with typical formula (g/L) of Beef extract 2, Acid digest of casein 17.5, Starch 1.5 and Agar 17 was used for antimicrobial assay. The following 12 microbial strains have been used in the present study after preparation of their cultures as described above:

Reference Bacterial Strain	Supplier	NCTC	ATCC
Staphyllococcus aureus	Sigma Trade, Cairo, Egypt	10788	6538
Listeria monocytogenes	Selectrol®, TCS Biosciences Ltd, Buckingham, UK	7973	35152
Bacillus cereus	Selectrol®, TCS Biosciences Ltd, Buckingham, UK	7464	10876
Enterococcus faecalis	Selectrol®, TCS Biosciences Ltd, Buckingham, UK	12697	29212
Clostridium perfringenes	Selectrol®, TCS Biosciences Ltd, Buckingham, UK	8237	13124
Escherichia coli	Sigma Trade, Cairo, Egypt	12241	25922
Salmonella typhimurium	Selectrol®, TCS Biosciences Ltd, Buckingham, UK	12023	14028
Shigella flexneri	KWIK-STIK®, Microbiologics, Inc., Minnesota USA	--	9199
Klebsiella aerogenes	Sigma Trade, Cairo, Egypt	9528	
Proteus vulgaris	Selectrol®, TCS Biosciences Ltd, Buckingham, UK	4175	13315
Vibrio parahaemolyticus	Selectrol®, TCS Biosciences Ltd, Buckingham, UK	10885	--
Candida albicans	LAB M Ltd., Lancashire, UK	--	90028

Antimicrobial activity using agar diffusion assay (ADA)

Sensitivities of the above mentioned microbial strains to *Ziziphus* plant extract have been measured in terms of zone of inhibition using agar diffusion assay (ADA) (Bauer et al., 1966). The Mueller-Hinton agar was prepared (31 g of agar powder were mixed with 1080 mL of bi-distilled water in a suitable flask and then autoclaved. The amount of agar solution was divided in 4 smaller flasks, 270 ml each. Evaporation occurs during autoclaving and 250 out of 270 mL remains in every flask. Flasks were kept on water bath at 56 °C to maintain their liquid state. Each flask was then inoculated with 0.5 ml of the microbial inoculum to match a 0.5 McFarland turbidity standard, which is equivalent to 1.5×108 cells per mL. After inoculation, the infected agar was spread into sterile Petri dishes (15 ml of agar solution into 10 mm diameter Petri dish to form a uniform layer of about 2.7 mm deep). The plates were left to cool and solidify and then wells (7 mm diameter) have been cut out from the agar plates using a sterilized stainless steel pore maker. Each well was then filled with 0.1 ml of *Ziziphus* extract at a particular concentration (1~128 mg/mL in Muller-Hinton Broth). The wells of a set of plates were filled only with Muller-Hinton Broth, the solvent of the plant extract, and kept as negative control. All of the prepared plate sets were then incubated at 37°C for 24 h either under anaerobic (Clostridium) or aerobic (other bacteria) condition and the diameter of any resultant zone of inhibition was measured. Zone sizes are measured from the edge of the well to the end of the clear zone. For each combination of a particular extract concentration and a bacterial strain, the experiment was performed in triplicate.

Minimum inhibitory concentration (MIC)

MIC of the *Ziziphus* plant extract was calculated from Agar diffusion assay results; where, the value of MIC is determined as the zero intercept of a linear regression of the squared size of the inhibition zones (x^2), plotted against the natural logarithm of the antibiotic concentration (lnC) as follows:

$$\ln (MIC) = \ln (C) - x2/4Dt$$

where, D is the diffusion coefficient, presumed to be independent of concentration, and t the time of antibiotic diffusion (Bonev et al., 2008). Ln (C) was calculated from the linear equation by setting (Y) value as zero; then MIC was calculated as (C) at Y-intercept.

Statistical analysis

All values were expressed as mean ± standard deviation of the mean of the performed triplicates. Linear regression was prepared using Excel software, 2013.

3. Results

The yield % from the extracted 100 grams of the chopped areal *Ziziphus* plant parts was 5.7%. This was calculated according to the equation of (Yield % = 100 × Extracted residue / Original plant).

Table 1: Inhibition zones of different concentrations of *Ziziphus* plant extract on selected Gram-positive bacterial strains and Candida (Mean ± SD; triplicates):

Ziziphus extract conc. (mg/mL)	*Staphyllococcus aureus*	*Listeria monocytogenes*	*Bacillus cereus*	*Enterococcus faecalis*	*Clostridium perfringens*	*Candida albicans*
128	10.17 ± 0.75	11.17 ± 0.41	15.33 ± 0.52	0.0 ± 0.0	12.33 ± 0.52	0.0 ± 0.0
64	7.33 ± 0.52	8.17 ± 0.41	13.50 ± 0.55	0.0 ± 0.0	10.33 ± 0.52	0.0 ± 0.0
32	5.33 ± 0.52	7.00 ± 0.63	10.33 ± 0.52	0.0 ± 0.0	8.33 ± 0.52	0.0 ± 0.0
16	0.0 ± 0.0	5.17± 0.41	7.50 ± 0.63	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
8	0.0 ± 0.0	2.5 ± 0.55	5.17 ± 0.52	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4	0.0 ± 0.0	0.0 ± 0.0	2.17 ± 0.41	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 2: Inhibition zones of different concentrations of *Ziziphus* plant extract on selected Gram-negative bacterial strains (Mean ± SE; triplicates):

Ziziphus extract conc. (mg/mL)	*Escherichia coli*	*Salmonella typhimurium*	*Shigella flexneri*	*Klebsiella aerogenes*	*Proteus vulgaris*	*Vibrio parahaemolyticus*
128	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.5 ± 0.55	8.33 ± 0.52
64	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.33 ± 0.52	6.33 ± 0.52
32	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.33 ± 0.52	4.67 ± 0.55
16	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.33 ± 0.52	3.17 ± 0.41
8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 3: MICs of *Ziziphus* plant extract against the studied susceptible bacterial strains.

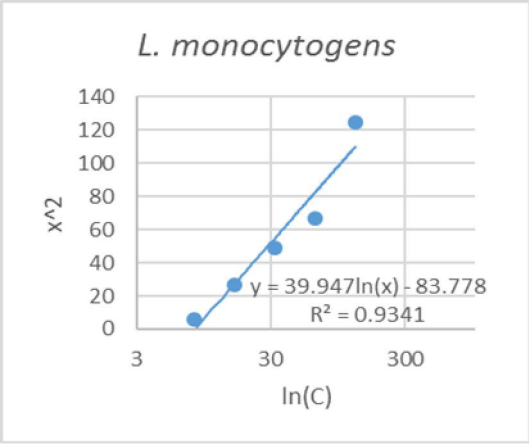
Ziziphus extract	*Bacillus cereus*	*Listeria monocytogenes*	*Clostridium perfringens*	*Vibrio parahaemolyticus*	*Proteus vulgaris*	*Staphyllococcus aureus*
MIC (mg/mL)	5.16	8.15	10.22	13.01	13.71	20.37

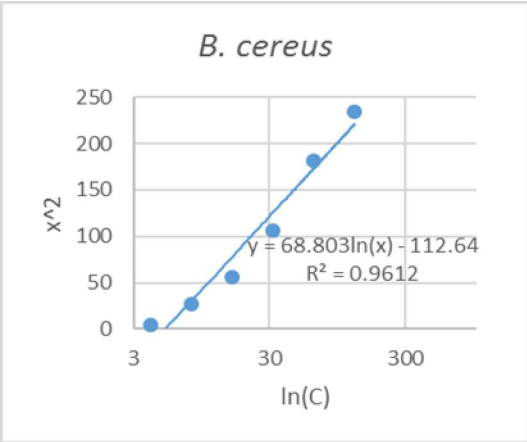
Ziziphus plant extract showed significant inhibitory effects against most Gram-positive microbial strains, namely, *Staphyllococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus* and *Clostridium perfringens*; and against only two of Gram-negative ones which are *Proteus vulgaris* and *Vibrio parahaemolyticus*.

Meanwhile, the extract was without effect against most of Gram-negative strains, namely, *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri* and *Klebsiella aerogenes*; as well as without effect against the Gram-positive strain *Enterococcus faecalis* and the mycotic strain *Candida albicans*.

Inhibition zones and the corresponding *Ziziphus* plant extract concentrations are tabulated in tables 1 and 2. MICs are depicted from Figure 2 and shown in Table 3.

4. Discussions

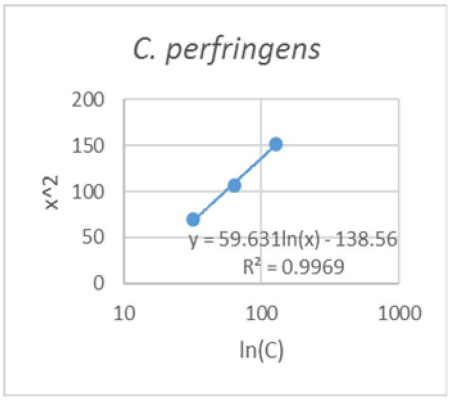


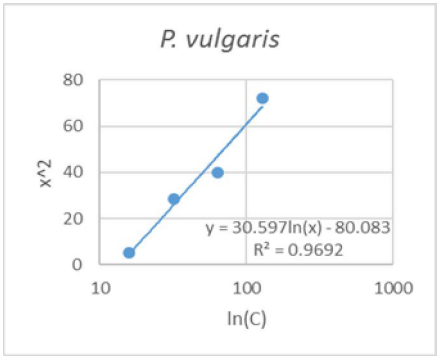


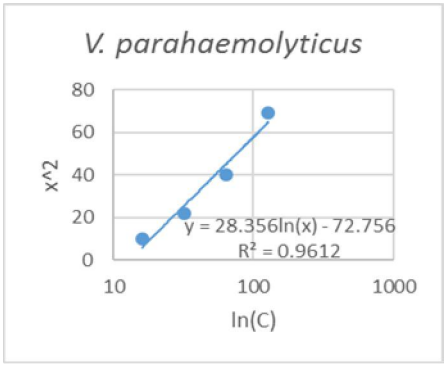
Arising from their microorganism eliminating features and disease treatment, antimicrobials are considered indispensable drugs. Among the available great variety of antimicrobial agents, a particular antimicrobial agent should be ideally selected to employ against a disease-causing particular microorganism, to eliminate it effectively without harming the host organism. However, uncontrolled and/or unsupervised use of antimicrobials, either by patients themselves or from prescriptions written without culture analysis, resulted in emergence of resistant bacterial strains. Increased rate of resistance as well as some other problems associated with safety necessitated more demand and interest in antimicrobials from natural sources including plant extracts (Friedman, 2007).

For centuries, medicinal plants have been used as remedies for various human and animal disease conditions depending on the basis that they contain bioactive principles of therapeutic value (Nostro et al., 2000). Antimicrobials of plant origin are not associated with side effects, cheap, available worldwide and have good therapeutic potential to heal many infectious diseases (Iwu et al., 1999). For these reasons, antimicrobial assays are continuing to screen plants for their antimicrobial activity with the hope of discovery of very effective, yet safer, pharmacological antimicrobial tools.

In the present study, we investigated the possible antimicrobial activity of *Ziziphus spina-christi* extract against twelve microbial pathogens that are potentially hazardous in both human and veterinary fields if they are not controlled. Agar gel diffusion assay was applied for fulfilling this purpose.







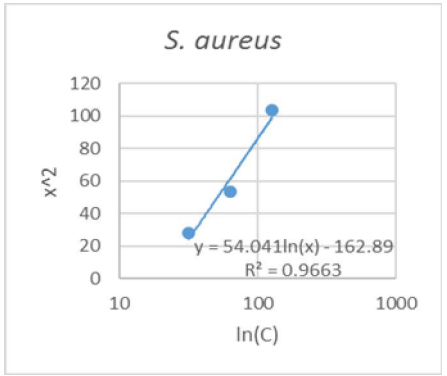


Figure 2: Plotting of natural logarithmic concentrations (X-scale) against squares of inhibition zones (Y-scale) to calculate MICs of *Ziziphus* extract against susceptible bacteria.

One of the most important tasks in the clinical microbiology laboratory as well as clinical pharmacology laboratory is the performance of antimicrobial susceptibility tests on significant bacterial isolates and strains. Susceptibility testing is thus useful to predict the possible outcome of treating a patient's infection with a particular antimicrobial agent (Jorgensen and Ferraro, 1998).

Disk Diffusion Test (Bauer-Kirby Procedure) is one of the simplest and most reliable susceptibility testing methods (Woods, 1995; Bauer et al., 1966). This method has been widely studied and well standardized over a number of years. The test is performed by applying a standardized inoculum of up to 1.5 × 108 cfu/mL to the surface of a large (150-mm diameter) Mueller-Hinton agar plates. Up to 12 commercially prepared, fixed-concentration filter-paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–18 hours in ambient air at 35°C before the results are determined. The diameters of the zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter by viewing the plate with reflected light when it is held a few inches above a black, nonreflecting background. The diameter of the zone of inhibition is related to the susceptibility of the isolate and to the rate of diffusion of the drug through the agar medium. The zone diameter correlates inversely with the approximate MIC for that antibiotic, i.e., linear regression analysis of zone diameters plotted against natural log of MIC values demonstrate a consistent relationship. However, in practice, the results of a disk diffusion test are interpreted by comparing the measured zone diameter with the interpretive criteria published by the National Committee on Clinical Laboratory Standards, NCCLS, (Cockerill et al., 2012) and included in the disk's FDA-approved product insert. The results of the disk diffusion test are ''qualitative'' (Susceptible, Intermediate, or Resistant) is derived from the test in addition to an MIC, which is the endpoint of the test or the lowest concentration of antibiotic that prevents visible growth of microbes. In general, the results of a susceptibility test are interpreted as drugs with the lowest MICs for a given bacterial isolate are the best for treatment of an infection due to that isolate.

The antimicrobial potential of *Ziziphus* extract was evaluated using the same pattern. In the present study, the extract exhibited positive result against Staphylococcus aureus whose infections are of a great threat to both humans and animals; it spreads pneumonia at slow rates (Simor et al., 2002). An inhibition zone of about 10 mm was recorded by extract concentration of 128 mg/mL and MIC of 20.37 mg/mL. This result is inconsistent with that of Al-Saimary (2006) who found that *Ziziphus* leaf extract (up to 250 mg/ml) has no effect on the growth of Staphylococcus aureus, while higher concentrations (500 & 750 mg/mL) inhibited that growth by zones of about 9 & 12 mm, respectively. This difference in antibacterial efficiencies of extracts might be due to the better extraction efficiency or to variation in the quantitative compositions of the same plant species in different geographical regions or to differences in the environmental conditions. The data is also inconsistent with that of Abalaka et al. (2010) who reported that *Ziziphus* extract at 50 mg/mL showed average inhibition zone of 12 mm.

Listeria monocytogenes is a Gram-positive, facultative anaerobic, motile bacterium responsible for listeriosis infections in man and animals. In man it is responsible for 10% of gastroenteritis and meningoencephalitis in animals. In the present study, *Ziziphus* extract exhibited average growth inhibition zone of 11 mm at concentration of 128 mg/mL with MIC of 8.15 mg/mL. The result may be parallel with that of Al-Reza et al. (2010) who reported that *Ziziphus* jujube extract was effective against some strains of Listeria.

The highest activity of the present extract was against *Bacillus cereus* that was 15 mm at concentration of 128 mg/mL with MIC of 5.16 mg/mL. *Bacillus cereus* is a Gram-positive, rod-shaped, motile bacterium that is harmful to humans causing foodborne illness. However, some strains are beneficial as probiotics (Ryan, 2004).

Ziziphus extract showed 12 mm growth inhibition zone with MIC of 10.22 mg/mL against Clostridium perfringenes. The bacterium that was formerly known as *C. welchii* is a Gram-positive, rod-shaped, anaerobic, spore-forming bacterium. It is one of the most common causes of food poisoning.

Among the selected Gram-negative bacteria in the present study, *Ziziphus* extract was only effective against *Proteus vulgaris* and *Vibrio parahaemolyticus* with growth inhibition zone of about 8 mm at concentration of 128 mg/mL with MIC of 13.01 & 13.71 mg/mL, respectively. *Proteus vulgaris* is a rod-shaped bacterium that inhabits the intestinal tracts of humans and animals. It is an opportunistic pathogen and is known to cause urinary tract infections and wound infections. Our result may be parallel with that of (El-Kamali and Mahjoub, 2009) who reported that different types of extracts from different parts of *Ziziphus* caused inhibition of growth of *Proteus vulgaris* at zones ranging between 12-24 mm but at much lesser MICs i.e. micromolar levels. *Vibrio parahaemolyticus* is a curved, rod-shaped motile bacterium found in brackish saltwater, which, when ingested, causes gastrointestinal illness. The present result of *Ziziphus* extract against Vibrio may be partially parallel with (Banerjee et al., 2010).

Other studied Gram-negative bacteria, namely, *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri* and *Klebsiella aerogenes* were found resistant to *Ziziphus* extract at all of its studied concentrations. These results are inconsistent with (Banerjee et al., 2010) and (El-Kamali and Mahjoub, 2009) who recorded inhibition zones of these strain growths in presence of *Ziziphus* extract.

In the present study, *Ziziphus* extract showed no activity against *Candida albicans*. This result is consistent with that of (Abalaka et al., 2010) who stated that extracts of two types *Ziziphus* extracts showed no activity against the fungal isolates, namely Aspergillus niger and *Candida albicans*. However, our and Abalaka's results are inconsistent with some other studies as that of (Tom et al., 2009) who recorded MIC of 6.25 mg/mL against *Candida albicans*.

In conclusion, the findings of the present study have shown clearly that *Ziziphus* plant extract is active against some pathogenic bacterial strains and thus may be useful in treating disease conditions caused by them.

No-Conflict-Interest

The authors hereby declare that there is no conflict of interest related to the present study.

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9/17/2018