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ANTIBACTERIAL ACTIVITY OF SIDER (ZIZIPHUS SPINA- CHRISTI), LEAVES EXTRACT AGAINST SELECTED PATHOGENIC BACTERIA.

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ABSTRACT

Background: Increased use of antibiotics, has led to high incidence of resistant bacterial strains infections and associated with increased side effects. Sider (Ziziphus spina- Christi) is a medicinal plant that has many uses in traditional medicine. Ethanol and methanol extracts of the leaves of Sider (Ziziphus spina- Christi) has been tested against selected bacterial strains. **Materials and Methods:** Sider leaves extract was used to evaluate the antimicrobial effects on eight different bacterial cultures, of *Staphylococcus aureus, Streptococcus sp., Escherichia coli, Klebsiella pneumoniae, Salmonella sp., Proteus mirabilis, Pseudomonas aeruginosa, and Enterobacter sp.* Ethanolic and methanolic extracts of the leaves of this plant were prepared. Undiluted neat solution (100mg/ml= 10,000mg/L), followed by concentration of 128, 100, 64, 32 and 16 mg per liter were prepared, from both extracts and antibacterial activities were evaluated by disc diffusion method on selected bacterial strains. **Results:** Ethanol and Methanol extract of sider leaves concentrations was found to be effective antibacterial agent against all the tested bacteria. These extracts had inhibitory effect at various concentration (128, 100, 64 and 32 mg per liter) The ethanolic extract had the highest activity (21 mm zone diameter), against *Salmonella sp* at 128 mg/L, while the lowest activity (9 mm) was demonstrated by the methanolic extract on *Escherichia. coli* at the same concentration. Highest Minimum Inhibitory Concentration (MIC) of the methanolic extract was shown by *Pseudomonas aeruginosa*, at (100 mg/L). Activity Index (A.I) value indicated a significant antibacterial activity, of Sider leaves extracts, against the tested bacterial species, compared to standard antibiotic discs against tested against the same. **Conclusion:** Sider leaves extract, had an inhibitory effect against all tested bacterial species. Also, the antibacterial effect of Sider extract, increased by increasing the concentration. The Activity index (A.I) also showed that the ethanolic extracts were more significant compared to the methanolic extracts. Sider leaves extracts were more significantly effective against all tested bacterial species, specifically *Psuedomonas species*, than the standard antibiotic discs.

KEYWORDS: Sider (Ziziphus spina- Christi), Ethanolic Extract, Methanolic Extract, Disc diffusion method, Minimum inhibitory concentration(MIC).

INTRODUCTION

Infectious diseases accounts for about half of the death in tropical countries. The use of antibiotics to control it has Lead to high incidence of side effects, and emergence of resistant bacterial strains. Herbal remedies used in the traditional folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for chemotherapy which might help to overcome the growing problem of resistance and also the toxicity of the currently available commercial antibiotics (Ali *et al*., 2001). The use of medicinal plants, as traditional health remedies have been most popular for 80% of world population in Asia, Latin America and Africa and is reported to have minimal side effects (Doughari, 2006). The *Ziziphus* species (Rhamnacese) are commonly used in folklore medicine for the curing of various diseases. They are wide-spread in the Mediterranean Region, Africa, Australia and tropical America. *Z. spina-christi* has been used in folk medicine as a demulcent, depurative, anodyne, emollient, Stomach-ache, for toothaches, astringents and as a mouth wash (Shahat et al, 2001).

Many studies indicate that some plants contained peptides, unsaturated long chain aldehydes, alkaloidal constituents, some essential oils, phenols and water, ethanol, chloroform, methanol and butanol soluble compounds (Seyyednejad*et al*., 2008; Alma *et al*., 2003; Klausmeyer*et al*., 2004).*Ziziphus spina-christi* was shown to contain betulic and ceanothic acid, three cyclopeptide alkaloids as well as four saponin glycosides (Mahran*et al*., 1996) and several flavonoids have been isolated from the leaves of *Z. spina-christi* (Amos *et al*., 2001).

The present study aimed to evaluate the antibacterial activity of ethanolic and methanolic extracts of Sider (*Z. spina-christi*) leaves on selected clinically pathogenic bacterial isolates.

MATERIALS AND METHODS

Scientific Name: *Ziziphus spina- Christi*.
Synonyms: *Rhamnusspina-christi*L., *Ziziphusafricana* Mill.
Local Arabic Name(s): Sidr, Nebeq, Jabat, Zejzaj, Zefzoof, Ardeg
Common Name(s): Christ`s thorn, Jujube plant.
Family: Rhamnaceae.

Collection of plant materials: The plants used in this study were collected from farms in Medina Province, K.S.A. in 2013.The taxonomic identity of this plant was confirmed by our Voucher specimens, deposited at the Department of Medical Laboratories, Qassim University, Buraidah, K.S.A.

Preparation of extracts: The leaves of sider were shade dried at room temperature for 3 days and crushed into powder using electric blander. Methanol extraction was done by adding, Ten grams (10 gms.) of sider powder, with 100 ml of methanol-concentration 70% solution, and kept on rotary shaker for 24 h. Thereafter, it was filtered and centrifuged at 5000 rpm for 15 min. the supernatant was collected and the solvent was evaporated to make the final volume. It was stored at 4 c in bottles for further studies (Okemo. et al 2001). Ethanol extraction was done by dissolving, Ten gram(10 gm) of sider leaves powder in 100 ml of ethanol –distilled water (8 : 2 w/v), centrifugation 3000 rpm for 15 min and then collecting the supernatants. This process was repeated three times. Finally, the ethanol was removed through evaporation by incubating at room temperature for 48 h. (H. Motamedi, et al 2009).

Test isolates; A total of 8 bacterial species were tested, *Salmonella*, *Proteus*, *E.coli*, *Klebsiella*, *Streptococcus sp.*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter. Sp.* These species were originally isolated from clinical materials collected from patients. They were identified using standard biochemical tests.

Determination of antimicrobial activity: Antibacterial activity of the ethanolic and methanolic extract**s** of the plant sample was evaluated by the paper disc diffusion method. The inhibition zone around each disc was measured in (mm) by ruler. Dilutions prepared from both extracts were (128 -100 – 64 – 32 – 16) mg/L.

The **undiluted neat solution was (100mg/ml= 10,000mg/L).** Five sterile test tubes were taken.
1) First test tube was filled with 9.9 ml of distilled water and 100ul of sider leave extract neat solution. **= 100 mg / L.**
2) The second, third, fourth and fifth tubes were filled with 20 ml of distilled water. Water served to dilute the original solution.

Then, in 2nd tube 256ul from neat solution was added = 128 mg/L, 3rd tube; 128ul from neat, added =64mg/L, 4th tube; 64 ul from neat added= 32 mg/L, and in 5th tube; 32ul from neat added= 16mg/L, as shown in the diagram (Fig.No.1; Serial dilutions). Ethanolic & Methanolic dilutions were made by the mentioned method.

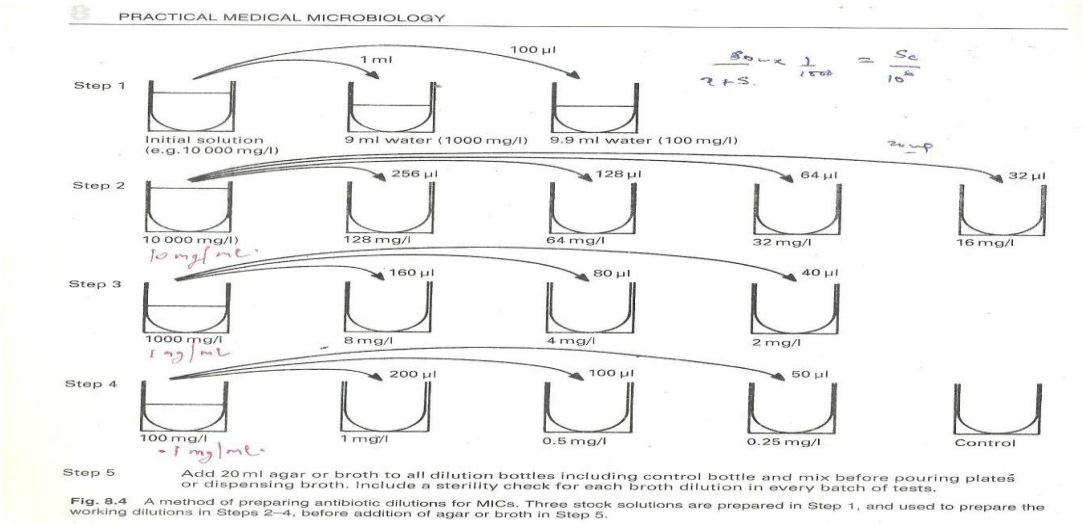

Fig. No. 1 Serial dilutions of Sider extract stock solution protocol.

Paper disc method: The discs, prepared from filter paper (whatman No.3) with 6 mm diameter, were sterilized in autoclave and allowed to dry. 50 ul each, of sider leave dilutions ethanolic and methanolic solution of variable concentrations (128mg /l – 100mg/l – 64mg/l - 32mg/l – 16mg/l) were applied to the filter paper discs, allowed to dry. A bacterial suspension in sterile normal saline was prepared with reference to the 0.5 McFarland standard. Turbidity of the bacterial suspension was compared with 0.5 McFarland standard solution, followed by lawn culture of bacterial suspension on Mueller-Hinton agar plates by sterile cotton swab and kept in incubator at 37^{o}C for 15 minutes. Prepared methanolic and ethanolic discs of above mentioned concentrations were placed on the lawn cultures from mentioned bacterial strains and the plates incubated at 37^{o}C for 24 h. A separate set of mentioned lawn cultures was tested with standard antibiotic discs, as positive controls. The inhibition zone around each disc was measured in (mm) and the assay was carried out three times for each extract dilutions.

Sensitivity tests of Sandard antibiotics Disc.

A set of seven antibiotics were used to compare with sider activity, Amikacin (Ak 30), Vancomycin(VA30), Clarimazole (CLT 10), Doxycycline (DO 30), Ceftazidime (CAZ 30), Neomycin (N 30) Novobiocin (NV 5). Sensitivity of antibiotics against test strains was assessed by agar disc diffusion method. Sensitivity was predicted by degree of clear zone surrounding the disc after 24 hrs. measured as (mm diameter of zone of inhibition). The results of sensitivity tests were expressed as (0) for no sensitivity, + for (below 12) for low sensitivity, ++ (12-29) for moderate sensitivity and +++ (30-45) for high sensitivity.

STATISTICAL ANALYSIS

Each experiment was repeated in triplicate sets and **the means** from the absolute data has been mentioned. The comparison of antibacterial activity of the medicinal extracts, with standard antibiotics was evaluated by activity index (AI) (Sekhawat and Vijayvergia 2010).

RESULTS

Inhibitory effect of selected antibiotics, as shown in Fig.no.2 & Table No. 1.

Abbreviations	Used for
(Ak 30)	Amikacin
(VA30)	Vancomycin
(CLT 10)	Clarimazole
(DO 30)	Doxycycline
(CAZ 30)	Ceftazidime
(N 30)	Neomycin
(NV 5)	Novobiocin

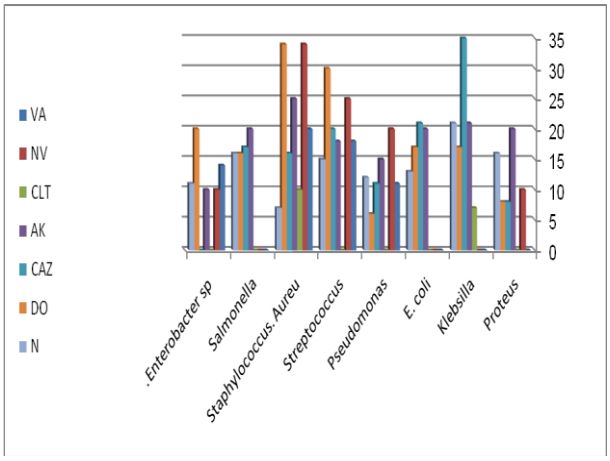

Fig 2. Inhibition zones in (mm) diameter of all tested bacterial species, with known antibiotic discs.

Sensitivity tests revealed that *Enterobacter sp.* was highly senstitive to Doxycycline with zone of inhibition of (20mm), moderately sensitive to Vancomycin, Neomycin, Amikacin and Novobiocin showing zone of inhibition as (14mm, 11mm, 10mm & 10mmrespectively). on the other hand *Enterobacter* was resistant against Ceftazidime and Clarimazole. *K. pneumoniae*, was highly sensitive to Ceftazidime, zone of inhibition(35mm), moderately sensitive to Amikacin, Neomycin, & Doxycycline with zone of inhibition(21mm, 21mm, & 17mmrespectively), while Clarimazole had lowest effect of (7mm). *S.aureus* was highly sensitive to Novobiocin, Doxycycline, Amikacin, and Vancomycin (34mm, 34mm, 25mm, & 20mm respectively). Similarly *Streptococcus* also was highly sensitive to Doxycycline, Novobiocin, Ceftazidime and Amikacin, &Vancomycin zone of inhibition (30mm, 25mm, 20mm, 18mm & 18mm respectively). On the other hand Neomycin had least effect on both *Streptococcus and S.aureus*(15mm & 7mm).

Escherichia coli and *Salmonella* revealed a similar pattern of antibiotic sensitivity. Both of them were highly sensitive to Amikacin with zone of inhibition of (20mm each). *E.coli* was also highly sensitive to Ceftazidime (21mm), while Doxycycline and Neomycin had moderate effect on both *E.coli and Salmonella*, as (17mm, 13mm & 16mm, 16mm respectively. Both were resistant against, Vancomycin, Novobiocin & Clarimazole. *Proteus* was highly sensitive to Amikacin (20mm), moderately sensitive to Neomycin and Novobiocin(16mm & 10mm), while Doxycycline and Ceftazidime had lowest effect as (8mm each). On the other hand *Proteus sp.* was resistant against Vancomycin and Clarimazole. On the contrary *P.aeruginosa* was highly sensitive to Novobiocin as (20mm) zone of inhibition, moderately sensitive to most other antibiotics, least sensitive to Doxycycline (6mm) and resistant against Clarimazole.

Table No.1; Comparison of Antibacterial Effect of Sider Leaves Extract (Ethanolic & Methanolic) with known Antibiotic discs (Zone of inhibition in mm).

Metha 16 mg/L.	Metha 32 mg/L.	Methan. 64 mg/L	Metha 100 mg/L.	Metha 128 m/L.	Metha Un. D.C	Etha 16 mg/L.	Ethan 32 mg/L.	Ethan 64 mg/L.	Ethan. 100 mg/L.	Ethan 128 mg/L.	Ethan. Un. D.C	Antibi NV.	Antib VA.	Antibi AZ.	Antibi . Ak..	Antib i DO.	Anti. CLT.	Anti. N	Bacteria l species.
R (0)	R (0)	7 (+)	9 (+)	10 (+)	20 (++)	R (0)	R (0)	6 (+)	10 (+)	12 (++)	26 (++)	10 (+)	14 (++)	R (0)	10 (+)	20 (++)	R (0)	11 (+)	Enterobacter Sp.
R (0)	7 (+)	9 (+)	10 (+)	13 (++)	20 (++)	R (0	8 (+)	9 (+)	10 (+)	12 (++)	24 (++)	R (0)	R (0)	35 (+++)	21 (++)	17 (++)	7 (+)	21 (++)	K. pneumoniea
R (0)	6 (+)	7 (+)	9 (+)	10 (+)	25 (++)	R (0)	7 (+)	8 (+)	10 (+)	10 (+)	30 (+++)	34 (+++)	20 (++)	16 (++)	25 (++)	34 (+++)	10 (+)	7 (+)	S.aureus
R (0)	R (0)	7 (+)	10 (+)	11 (+)	30 (+++)	R (0)	R (0)	8 (+)	11 (+)	13 (++)	30 (+++)	25 (++)	18 (++)	20 (++)	18 (++)	30 (+++)	R (0)	15 (++)	Streptococcus
R (0)	R (0)	9 (+)	12 (++)	15 (++)	26 (++)	R (0)	R (0)	10 (+)	15 (++)	17 (++)	30 (+++)	10 (+)	R (0)	8 (+)	20 (++)	8 (+)	R (0)	16 (++)	P. mirabilis
R (0)	R (0)	R (0)	8 (+)	10 (+)	25 (++)	R (0)	R (0)	R (0)	10 (+)	11 (+)	32 (+++)	20 (++)	11 (+)	11 (+)	15 (++)	6 (+)	R (0)	12 (++)	Pseudomonas
R (0)	8 (+)	10 (+)	17 (++)	20 (++)	25 (++)	R (0)	8 (+)	10 (+)	18 (++)	21 (++)	33 (+++)	R (0)	R (0)	17 (++)	20 (++)	16 (++)	R (0)	16 (++)	Salmonella
R (0)	6 (+)	7 (+)	8 (+)	9 (+)	18 (++)	R (0)	7 (+)	7 (+)	9 (+)	10 (+)	21 (++)	R (0)	R (0)	21 (++)	20 (++)	17 (++)	R (0)	13 (++)	E. coli

Inhibitory effect of Sider Leaves extract (Ethanol & Methanol extraction) as shown in Table no.1.

The results showed that these extracts were effective against all of the tested bacterial isolates. The highest activity was demonstrated by the ethanolic extract of sider leaves. Among the Gram negative bacteria, *Salmonella sp* (inhibition zone diameter of 21 mm) was observed at a concentration of 128mg/L and 13 mm against Gram positive bacteria *Streptococcus*). The lowest activity by the ethanolic extract, (inhibition zone diameter about 6 mm) was demonstrated by the *Enterobacter sp*. at a concentration of 64mg/L. All tested bacterial isolates, were resistant at concentration of 16mg/L. The highest activity demonstrated by the methanolic extract was at 128mg/L, against Gram negative bacteria, *Salmonella* (20mm) and (11mm) against Gram positive *Streptococcus sp*. However, the methanolic extract showed the lowest activity (inhibition zone diameter about 6 mm for *E. coli*, and *Staph. aureus*, at 32mg/L. These results suggest that antibacterial activity of sider ethanolic and methanolic extracts against tested bacteria were increased when used in higher concentrations. Also the methanolic extract generally showed lower activity against the test organisms compared to the ethanolic extract**.** Highest Minimum Inhibitory Concentration (MIC) of both ethanolic & methanolic extract was shown by *Pseudomonas aeruginosa*, as MIC (100 mg/L).

Analysis Through Activity Index as shown in Table no. 2;

Table No. 2; Comparison of Activity index of Ethanolic and Methanolic Extracts of Sider

Activity index= Zone of inhibition of extracts / Zone of inhibition of antibiotics. A.I = 0 - (could not be divided)								
	Novobiocin.	**Vancomycin**	**Ceftazidime.**	**Amikacin**	**Doxycycline.**	**Clarimazole.**	**Noemycin.**	**Bacteria l species.**
Ethanolic Sider Leave Extract 10 gm / 100ml. (Undiluted Concentration).	A.I= 2.6	A.I= 1.85.	A.I= 0	A.I= 2.6	A.I=1.3	A.I= 0	A.I= 2.36.	Enterobacter Sp.
	A.I= 0	A.I= 0	A.I= 0.68	A.I= 1.14	A.I= 1.41	A.I= 3.43.	A.I=1.14	K. pneumoniea
	A.I= 0.88	A.I= 1.5	A.I= 1.88	A.I= 1.2	A.I= 0.88	A.I= 3.0	A.I= 4.28	S. aureus
	A.I= 1.2	A.I= 1.66	A.I= 1.5	A.I= 1.66	A.I= 1.0	A.I= 0	A.I= 2.0	Streptococcus.
	A.I= 3.0	A.I= 0	A.I= 3.75	A.I= 1.5	A.I= 3.75	A.I= 0	A.I= 1.88	P. mirabilis
	A.I= 1.6	A.I= 2.90	A.I= 2.90	A.I= 2.13	A.I= 5.33	A.I= 0	A.I= 2.66	Pseudomonas
	A.I= 0	A.I= 0	A.I= 1.94	A.I= 1.65	A.I= 2.06	A.I= 0	A.I= 2.06	Salmonella
	A.I= 0	A.I= 0	A.I= 1.0	A.I= 1.05	A.I= 1.23	A.I= 0	A.I= 1.61.	E. coli
Methanolic Sider Leave Extract . 10 gm / 100ml. (Undiluted Concentration).	A.I=2.0	A.I= 1.42	A.I= 0	A.I= 2.0	A.I= 1.0	A.I= 0	A.I=1.81.	Enterobacter Sp.
	A.I= 0	A.I= 0	A.I= 0.57	A.I= 0.95	A.I= 1.76	A.I= 2.85	A.I= 0.95	K. pneumoniea
	A.I= 0.73	A.I= 1.25	A.I= 1.56	A.I= 1.0	A.I= 0.73	A.I= 2.5	A.I= 3.57	S. aureus
	A.I= 1.2	A.I= 1.66	A.I= 1.5	A.I= 1.66	A.I= 1.0	A.I= 0	A.I= 2.0	Streptococcus
	A.I= 2.6	A.I= 0	A.I= 3.25	A.I= 1.3	A.I= 3.25	A.I= 0	A.I= 1.63	P. mirabilis
	A.I= 1.25	A.I= 2.27	A.I= 2.27	A.I= 1.66	A.I= 4.16	A.I= 0	A.I= 2.08	Pseudomonas
	A.I= 0	A.I= 0	A.I= 1.47	A.I= 1.25	A.I= 1.56	A.I= 0	A.I= 1.56	Salmonella
	A.I= 0	A.I= 0	A.I= 0.85	A.I= 0.9	A.I= 1.0	A.I= 0	A.I= 1.38	E. coli

Leaves.

The significant use of the sider leaves extract, with standard antibiotic discs, was calculated through Activity Index (table no.2). More than 1 Activity Index (A.I) value indicated a significant role of Sider leaves extracts, while a value below zero, showed a stronger effect of standard antibiotic discs against tested pathogens. More (A.I) values showed more significant results by Sider leaves extracts.

DISCUSSION

The antimicrobial activity of antibiotics can be administered through various ways to treat human diseases. The sensitivity of antibiotics were consistent with the reported literature (J.Gerald et al). The overuse of antibiotics leads to produce multidrug resistant microorganism. In the current scenario herbal products are considered as safe alternatives of synthetic drugs. Results from the present investigation showed that the growth of bacterial pathogens was inhibited with the crude extracts of medicinal plants.

Ethanolic and methanolic extracts of sider leaves extract were screened for their antibacterial activity against eight bacterial organism. Phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and herbivores (Doughari, 2006).

The results of this study showed that ethanolic and methanolic extracts from the sider leaves, inhibited the growth of various species of Gram-positive and negative bacteria. The ethanolic extract showed slightly better killing action than the methanolic extract which means that the ethanolic extract could be used more. Minimum inhibitory effect of the extracts was observed at concentration 100 mg/l for *Pseudomonas* and concentration 64mg/l for *Enterobacter & streptococcus*. Other bacterial species, demonstrated minimum inhibitory effect at concentration 32 mg/l.(For both Ethanolic & Methanolic extracts). The Activity index (A.I) also showed that the ethanolic extracts were more

significant compared to the methanolic extracts. Sider leaves extracts were more significantly effective against *Psuedomonas species*, than the standard antibiotic discs.

The result by (Shahat, A.A, Nazeif, N.S et al 2001) and Awadh Ali (2001) show that the *E. coli* was resistant to methanolic and ethanolic extract**s** of Sider leaves**,** while in this study, *E.coli* was effected by sider leave extracts. This difference could probably be due to cell membrane permeability, demographic & genetic factors. Study done by Ali-Shtayeh et al. (1998) found that ethanolic extract of the *Z.* spina-christi was active against *E.coli* and *P. aeruginosa.* Our study also showed that, the ethanolic and methanolic extracts of sider leaves was active against *E.coli*, but mostly resistant against *P.aeruginosa.*

In study by Khalid, K. Al-Bayatti, et al. (2011) it was found that the inhibitory effect of the Sider leaves extracts was observed upto the concentration of 50 mg/l, while in our study the activity of sider stops at concentration of 32mg/l. In contrast, a study done by Maleki et al, 2008, supports our result. Based on earlier studies on active constituents of sider, unsaturated fatty acids represent the major components (83.5%). These unsaturated fatty acids maybe responsible for the broad spectrum antimicrobial activity of this plant (Shahat 2001). The high mucilage content about 7.5%, makes it a promising demulcent and emollient in folk medicine (Duke, 1985). In other studies, it has been reported that several bioactive flavonoids such as furocoumarins and furanocoumarins (Manderfield et al., 1997) and also phenolic compounds have been isolated from parsley leaf and are known to exhibit antibacterial activity (Wong and Kitts, 2006;Maleki et al., 2008). Furocoumarins can inhibit bacterial growth by reacting with DNA and disrupting DNA replication (Seyyednejad et al., 2008).On the other hand the hydrophobic character of phenolic compounds can potentially impair cellular function and membrane integrity (Raccach, 1984). The capacity of **phenolic compounds** to chelate transition metals also lowers the reactivity of metal ion by forming an inert metal-ligand complex. Chelation of transition metals, such as iron and copper, reduces bioavailability for bacterial growth (Seyyednejad et al., 2008).

CONCLUSION

The result of this study indicated that, the leaves of sider plant contain some major bioactive compound that inhibits the growth of microorganism thereby proving very effective as alternative source of antibiotics**.** Results from our study suggested that, Sider leaves extract, had an inhibitory effect against all tested bacterial species. Also, the antibacterial effect of Sider leaves extract increased by increasing the concentration. The Activity index (A.I) also showed that the ethanolic extracts were more significant compared to the methanolic extracts. Sider leaves extracts were more significantly effective against *Psuedomonas species*, than the standard antibiotic discs.

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