Egypt. Acad. J. Biolog. Sci., 4(1): 85-92 (2012) G. Microbiology
Email:egyptianacademic@yahoo.com ISSN: 2090-0872
Received: 20/8/2012 www.eajbs.eg.net

Effect of some natural plant extracts against gram negative bacteria in Njran Area, Saudi Arabia

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ABSTRACT

This study aimed to evaluate the growth inhibitory effect of*Syzygium aromaticum (clove), Nigella sativa* (*black cumin*), *Commiphora molmol* (*myrrh*) and *Allium sativum* (*garlic*) on *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Water, 80% ethanol and n-hexane plant extracts either alone or in combination were tested against 4 gram negative bacteria. Gel diffusion method, minimum inhibitory concentration (MIC) values were used in this investigation. The findings indicated that individual *S. aromaticum, C. molmol*and *A. sativum* extracts had growth inhibitory effect against tested bacteria. Ethanolic extract of *S. aromaticum* exhibited the highest inhibitory effect on all tested microorganisms. Individual water, ethanolic and hexanic extracts of *N. sativa* did not show any growth inhibitory effect against all tested microorganisms. Synergistic inhibitory effects of ethanolic and hexanic extracts combination of the four plants were able to prevent the growth of the tested bacteria. Combination of water extracts of the 4 plants inhibit the growth of *P. aeruginosa* and *A. baumannii*, while *E. coli* and *K. pneumonia* were not inhibited. We are of the opinion that individual ethanolic and hexanic extracts of the clove and combinations of ethanolic and hexanic extracts of the four tested plants could potentially be used for treatment of gram negative bacterial infection especially to the tested microorganisms.

Keywords:Gram negative, Plant extracts,*Syzygium aromaticum , Nigella sativa , Commiphora molmol, Allium sativum.*

INTRODUCTION

Bacterial infectious diseases represent an important cause of morbidity and mortality worldwide. An antibiotic resistant bacterium is a threat which is becoming increasingly common (Lesse 1995). The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs (Gislene *et al.*, 2000; Cos *et al.*, 2006) .Plants are rich source of natural products used for centuries to cure various diseases. The plant-derived medicines are based upon the premise that they contain natural substances that can promote health and alleviate illness. So, a return to natural substances are an absolute need of our time (Swayamjot *et al.,* 2005; Kumar *et al.*, 2007).The inhibitory activity of *clove* (*Syzygium aromaticum*) is due to the presence of several constituents, mainly eugenol, eugenyl acetate, beta-caryophyllene, 2-heptanone (Chaieb *et al.,* 2007), acetyl- eugenol, alpha-humulene, methyl salicylate, iso-eugenol, methyl-eugenol (Yang *et al.,* 2003). Several studies have demonstrated potent antibacterial effects of *clove* (Lopez *et al.*, 2005; Li *et al.,* 2005; Betoni *et al.,* 2006; Fu *et al.,* 2007). *N. sativa* (*black*

cumin) is an herbaceous indigenous plant in the Mediterranean region. Seeds of this plant have been used for centuries as a spice and food preservative, as well as a traditional medicine for the treatment of various diseases (Goreja, 2003). Crude extracts and seed constituents of *N. sativa*, in particular thymoquinone, have been reported to possess a number of pharmacological properties (Ali and Blunden, 2003). *Commiphora molmol* (*myrrh*) is widely distributed in the Kingdom of Saudi Arabia and it is grown in Jizan area on Red Sea coast. It is also found in Somalia and other coast African countries (Mugahid, 1981). It is used in traditional medicine as antiseptic, carminative, anti-inflammatory, (Tarig *et al.,* 1985). The ethanolic extract of *C. molmol* exhibited antimicrobial activity against the Gram negative organisms (Omer *et al.,* 2011). Several studies have proved that garlic has antimicrobial effects (Lawson, 1988; Martin and Ernst, 2003). It inhibits the growth of both gram-negative and gram-positive bacteria (Pai and Platt, 1992; Ross *et al.,* 2001). The development of new antimicrobial agents for the treatment of bacterial infection is of increasing interest. In the last few years a number of studies have been conducted to verify the effectiveness of plant extracts against bacterial infections (Prashanth *et al.,* 2006; Ung *et al.*, 2010). The present study evaluated the individual and in combination growth inhibitory effect of 4 natural plant extracts against 4 Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial Isolates and Culture Media

The microorganisms which have been used in this study are *E. coli, K. pneumoniae, P. aeruginosa* and *A. baumannii.* The isolates were obtained from the Microbiology laboratory, King Khalid Hospital(K.K.H), Najran region, Saudi Arabia. The organisms were identified by an automated system (MicroScan Walkaway, Siemens) and the results were confirmed (Koneman *et al.,* 1992). The isolates were maintained on agar slant at 4˚C and subcultured on a fresh appropriate agar plates 24 h prior to any antimicrobial test. Mueller Hinton broth (Oxoid, England), Mueller Hinton agar (Oxoid, England) and Nutrient agar (Oxoid, England) were used in this study. All media were prepared according to manufacture recommendations.

Plant materials and extraction

The four natural plant samples used in this study were flowers of *Syzygium aromaticum (clove)*, seeds of *Nigella sativa* (*black cumin*), unorganized part of *Commiphora molmol* (*myrrh*) and bulbs of *Allium sativum* (*garlic*). Samples were purchased from markets distributed in Najran region, King Saudi Arabia, during September 2011, then further dried in an incubator at 37˚C, the samples were ground into fine powders using electric blender and the extracts were prepared by soaking 125 gm. of each sample separately into 500 ml solvents (distilled water, 80% ethanol and n-hexane) using conical flasks plugged with cotton plugs, the mixtures were kept at room temperature for 72 h. under discontinuous shaking. The crude extracts were filtered through sintered glass funnel (500 ml) under vacuum, the filtrates were evaporated to dryness by rota-vapour (Buchi, R-215, Switzerland), the rotary water bath was adjusted to 55˚C, then the extracts were kept overnight under vacuum fume hood to obtain a constant dry weight and the extracts stored in closed amber vessels at 4˚C in refrigerator for further use. The extracts either individual or in combination were weighed and dissolved according to the solvent type (distilled Water, 80% ethanol and n-Hexane) at a concentration of 50 mg/ml.

Antibacterial tests

Independent and in combination water,80% ethanol and n- hexane extracts of 4 plants were tested against *E. coli, K.*

pneumoniae, P. aeruginosa and A. baumanni. The growth inhibitory effect was determined by the agar well diffusion method as previously described by (Berghe and Vlietinck, 1991, Perez *et al.*, 1990 and Collins *et al.*,1995). Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth (Oxoid, England). They were incubated without agitation at 37°C for 24 h. The cultures were diluted with broth to achieve an optical density corresponding to 2.0×10^6 colony forming units per ml (CFU/ml). After agar solidification, Mueller Hinton agar plates were swabbed with a suspension of each bacterial species using sterile cotton swab. The medium was punched with six millimeters diameter wells and filled with 100 µl of the test sample and allowed to diffuse at room temperature for 20 minutes. The final concentration of each individual or in combination extracts was 50 mg/ml. Gentamicin (Oxoid, England) was used as positive control at a concentration of 0.2 mg/ml. The plates were incubated aerobically at 37°C for 24 h and inhibition zone diameters (IZD) formed around the wells were measured (mm) using a ruler. All tests were done in triplicate and the growth inhibitory effect of plant extracts was recorded.

The Minimum Inhibitory Concentration (MIC) of individual and in combination extracts were determined by broth microdilution technique as described by National Committee for Clinical Laboratory Standards (NCCLS, 2000). Extracts were serially diluted with Mueller Hinton broth to give a final concentration ranging from 25 mg/mL to 0.39 mg/mL. Gentamicin was serially diluted to give a final concentration between 2 mg/mL to 0.03 mg/mL. Inoculum size of 1×10^5 CFU/mL of test organism was added to each well. Controls with broth and broth with test bacteria were included in the experiments. Each test strain of bacteria was run in duplicate. Tests were incubated aerobically at 37°C for 24 h. The MIC was considered as the minimum concentration of the dilutions that inhibited the growth of the test microorganism.

Statistical analysis

Data analysis results were expressed as means± S.E.(Standard Error) and differences between means were analyzed statistically using an analysis of variance(ANOVA) according to Tukey's test through SPSS 15.0 software package in Microsoft Windows 7.0 operating system. Differences are considered significant when $P\leq0.05$.

RESULTS

The inhibitory effect of water, 80% ethanol and n-hexaneplant extracts (alone or incombination) was evaluated on *E. coli, K. pneumoniae, P. aeruginosa* and *A. baumannii*. The results of IZD and MIC of independent plant extracts against 4 gram negative bacteria are presented in Table 1. Water extract of *S. aromaticum* exhibited inhibitory effect against *P. aeruginosa* with IZD 13.33±0.33mm and MIC 3.12 mg/mL and no inhibitory effect was detected against the remaining bacteria. All the test bacteria were susceptible to ethanolic and hexanic extracts of *S. aromaticum* with IZDs ranged from 11.33±0.33 mm-17.0±0.0mm and MIC from 1.56 mg/mL to 6.25 mg/mL. Water, ethanolic and hexanic extracts of *N. sativa* did not show any inhibitory effect against the tested microorganisms with IZD 0.0±0.0mm and MIC >25 mg/mL. Water extract of *C. molmol*inhibit, show the growth of *P. aeruginosa* and *A. baumannii* with IZD ranged from 10.67±0.33mm to 14.33±0.33 mm and MIC from 3.12mg/mL to 6.25 mg/mL and did not inhibit the growth of *E.coli and K. pneumoniae*. Ethanolic extract of *C. molmol* had inhibitory activity against the tested bacteria except *A. baumannii*.

Hexanic extract of *C. molmol* inhibited the growth of tested gram negative bacteria except *K. pneumoniae*.*P. aeruginosa and A. baumannii* were inhibited by water extract of *A. sativum* with IZD from 12.67±0.33mm to 13.33±0.33 mm and MIC was 3.12 mg/mL, while the other bacteria were not inhibited.All the test bacteria were resistant to ethanolic extract of *A. sativum*except *K. pneumoniae* was inhibited with IZD 10.0±0.58 mm and MIC 12.5 mg/mL.

Table 1: Inhibition zone diameter (IZD in mm) and minimal inhibitory concentration (MIC in mg/mL) of individual water, ethanolic and hexanic plant extracts against 4 gram negative bacteria.

Plant material	Extract	*E. coli*		*K. pneumoniae*		*P. aeruginosa*		*A. baumannii*	
		IZD*	MIC	IZD*	MIC	IZD*	MIC	IZD*	MIC
Syzygium aromaticum	Water	$0.0^{a}\pm0.0$**	>25	$0.0^{a}\pm0.0$	>25	$13.33^{dc}\pm0.33$	3.12	$0.0^{a}\pm0.0$	>25
	80%Ethanol	$14.67^{d}\pm0.33$	3.12	$13.33^{c}\pm0.33$	3.12	$17.0^{e}\pm0.0$	1.56	$13.33^{c}\pm0.33$	3.12
	n-Hexane	$13.67^{d}\pm0.33$	3.12	$11.33^{b}\pm0.33$	6.25	$13.0^{c}\pm0.0$	3.12	$13.0^{c}\pm0.0$	3.12
Nigella sativa	Water	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25
	80%Ethanol	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25
	n-Hexane	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25
Commiphora molmol	Water	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$14.33^{dc}\pm0.33$	3.12	$10.67^{b}\pm0.33$	6.25
	80%Ethanol	$11.0^{b}\pm0.58$	6.25	$11.0^{b}\pm0.58$	6.25	$10.67^{b}\pm0.33$	6.25	$0.0^{a}\pm0.0$	>25
	n-Hexane	$12.33^{c}\pm0.33$	3.12	$0.0^{a}\pm0.0$	>25	$9.67^{b}\pm0.67$	12.5	$10.0^{b}\pm0.58$	12.5
Allium sativum	Water	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$13.33^{dc}\pm0.33$	3.12	$12.67^{c}\pm0.33$	3.12
	80%Ethanol	$0.0^{a}\pm0.0$	>25	$10.0^{a}\pm0.58$	12.5	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25
	n-Hexane	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25	$0.0^{a}\pm0.0$	>25
Gentamicin		$16.67^{e}\pm0.33$	0.06	$14.0^{c}\pm0.00$	0.12	$14.67^{d}\pm0.33$	0.06	$12.33^{c}\pm0.33$	0.12
F- value		826.79		547.67		625.11		660.69	

*Value are the mean of three replicates ± S.E.

** In the same column, means followed by the same letters are not signiffically different(P≤0.05) as analyzed by Tukey's HSD test. F-value is significant at P≤0.001.

None of the tested gram negative bacteria showed any growth inhibition with the hexanic extract of *A. sativum*. The synergistic inhibitory effect of plant extracts combination on the growth of *E.coli, K. pneumoniae, P. aeruginosa* and *A. baumannii* was shown in Table 2. Water extract combination of the four tested plants did not show any inhibitory effect against *E.coli*and *K. pneumonia* while *P. aeruginosa* and *A. baumannii* were inhibited. Ethanolic and hexanic extracts combination exhibited growth inhibitory effect on all tested microorganisms with IZD ranged from 11.33±0.33 – 19.33±0.33 mm and MIC from6.25 mg/mL to1.56 mg/mL. Our results proved that *P. aeruginosa* and *A. baumannii*were more susceptible to all plant extracts combination with IZDs were 14.0±0.0-19.33±0.33 mm and 12.33±0.33–13.33±0.33 mm and MICs were 3.12-1.56 mg/mL and 3.12 mg/mL, respectively.

Table 2: Inhibition zone diameter (IZD in mm) and minimal inhibitory concentration (MIC in mg/mL) of 4 plant extracts combination against4 gram negative bacteria.

Extract combinaion	Plant Materials***	E. coli		K. pneumonia		P. aeruginosa		A. baumannii	
		IZD*	MIC	IZD*	MIC	IZD*	MIC	IZD*	MIC
Water	Sa+Ns+Cm+As	0.0a±0.0**	>25	0.0a±0.0	>25	16.0b±0.58	1.56	12.33a±0.33	3.12
80%ethanol	Sa+Ns+Cm+As	15.33c±0.33	3.12	13.0c±0.58	3.12	19.33c±0.33	1.56	13.0a±0.0	3.12
n-hexane	Sa+Ns+Cm+As	12.67b±0.33	3.12	11.33b±0.33	6.25	14.0a±0.0	3.12	13.33a±0.33	3.12
F-value		698.2		378.3		40.53		3.0	

*Value are the mean of three replicates ± S.E.

** In the same column, means followed by the same letters are not significantly different(P≤0.05) as analyzed by Tukey's HSD test. F-value is significant at P≤0.001.

***(Sa)Syzygium aromaticum, (Ns)Nigella sativa, (Cm) Commiphora molmol and (As)Allium sativum.

DISCUSSION

Plants remain one of the main sources of natural products for new therapies particularly in poor countries, because most of them are cost less, affect a wide range of antibiotic resistant microorganisms, and another reason is there is an erroneous impression that herbal medicines have fewer adverse effects (Ozoula *et al.*, 2010).In this study, extraction was done using water, 80% ethanol and n-hexane. Chemical content of plant extracts differs depending on the nature of the solvent used in the extraction procedure (Jules *et al.*, 2011). In the present investigation, individual ethanolic and hexanic extracts of *clove* showed inhibitory activity against all tested bacteria. *Clove* ethnaolic extract showed the highest growth inhibitory effect against tested microorganisms. Our findings agree with other observations (Sulieman *et al.,* 2007; Ram *et al.*, 2010) who demonstrated that the *clove* ethanolic extract exhibited the maximum zone of inhibition against test bacteria. The data of the in vitro anti-bacterial effect of individual water, ethanolic and hexanic extracts of *N.sativa* seeds revealed that all extracts did not inhibit the growth of gram negative bacteria of the current study, which were fully consistent with other studies on the same plant species. The extract was found to be ineffective on standard and hospital bacterial isolates (Mashhadian *et al.*, 2005). The aqueous, diethyl ether and chloroform extracts of *N. sativa* seeds did not show any inhibitory effect against all the tested gram negative bacteria (Mariam, 2009). However, other studies shown that such extracts of these seeds had an inhibitory effect on the growth of microorganisms (Hanafy and Hatem, 1991). These controversial results can be explained by the different techniques used for extraction. The sensitivity and the accuracy of the anti-microbial test, The concentration and the effectiveness of the constituents in the extracts, The conditions of seed collections, season, storage and the preservation method of the extracts (Mashhadian and Rakhshandeh, 2005; Hanafy and Hatem, 1991; Salman *et al.,* 2008). Variable growth inhibitory effect of individual water, ethanolic and hexanic extracts of *myrrh* on test bacteria was detected with IZD ranged from 0.0±0.0mm-14.33±0.33 mm and MIC from > 25mg/mL to 3.12 mg/mL. This finding was supported with those previously recorded by (Omer *et al.*, 2011). Water extract of *A. sativum* did not inhibit the growth of *E. coli and K. pneumoniae*. This result contradicted with those reported by (Jehan *et al.,* 2011; Meriga *et al.*, 2012) who reported that aqueous extract of *A. sativum* exhibited antibacterial activity against *E. coli* and *K. pneumoniae*. All the tested bacteria were resistant to hexanic *garlic* extract while *P. aeruginosa* and *A. baumannii* were inhibited by water extract and *K. pneumoniae* was susceptible to ethanolic extract. This result was consistent with the result previously cited by (Jehan *et al.*, 2011) who showed that 6 extracts from *garlic* had different ranges of antibacterial activities against the tested microbes. Matthew *et al.*., (2007) observed that the gram-negative diarrheagenic pathogens from the stool samples were highly sensitive to garlic. Combination water extract of the four plants showed growth inhibitory effect on *P. aeruginosa and A. baumannii* and no inhibitory effect was detected against *E. coli and K. pneumoniae.* This result was supported by the results previously reported by (Ncube *et al.,* 2012). Ethanolic and hexanic extract combinations had synergistic inhibitory effect on the four tested microorganisms. Tarek *et al.,* (2010) summarized that the ethanolic extract of different parts of five plants against *E. coli* exerted greater synergistic activity than water extracts. The highest

IZD was recorded for ethanolic extract combination against *P. aeruginosa* (19.33±0.33 mm) and MIC (1.56 mg/mL). This result consistent with those reported by (Kuete *et al.,* 2011).

CONCLUSION

The present study revealed that *Syzygium aromaticum* (*clove*), *Commiphora molmol* (*myrrh)* and *Allium sativum* (*garlic)* extracts had growth inhibitory effects against tested gram negative bacteria. *Clove* ethanolic extract showed the highest growth inhibitory effect against tested microorganisms. *N. sativa* seeds extracts did not show any inhibitory effect against all tested bacteria. Synergistic inhibitory effects of ethanolic and hexanic extracts combination of the four plants were able to prevent the growth of the tested bacteria. Combination of water plant extracts inhibit the growth of *P. aeruginosa* and *A. baumannii*, while *E. coli, K. pneumoniae* were not inhibited.

ACKNOWLEDGMENTS

We would like to thank Najran University for its financial support for this investigation (grant NO.NU64/11). We grateful to Dr.Mohamed Alshehry Dean of scientific research and finally we would like to thank Microbiology laboratory, King Khalid Hospital for providing test organisms.

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ARABIC SUMMARY

تأثير بعض المستخلصات النباتية الطبيعية على البكتريا سالبة الجرام بمنطقة نجران - المملكة العربية السعودية

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هدفت هذه الدراسة إلى تقييم التأثير المثبط لمستخلصات نباتات القرنفل،الحبة السوداء،المر والثوم على بكتريا الايشرشياكولاي ،والكلبسيلا نيموني ،السيدوموناساريجينوزا والأسينتوباكتربوماني. أظهرت هذه الدراسة أن مستخلصات القرنفل والمر والثوم لها أثر مثبط على نمو البكتريا سالبة الجرام بينما جميع مستخلصات الحبة السوداء ليس لها أي أثر مثبط على البكتريا محل الدراسة.استطاع مستخلصي القرنفل الايثانولي والهكساني أن يثبطا نمو جميع البكتريا وكان لمستخلص القرنفل الايثانولي الأثر الأكبر كمثبط لنمو البكتريا مقارنة بمستخلصي الماء والهكسان.بينت الدراسة أن مستخلص الايثانول والهكسان لنبات القرنفل منفردا ومخلوط مستخلصات النباتات الأربعة مجتمعة لها أثر مثبط على نمو جميع البكتريا تحت الدراسة .ولذا فإننا نرى امكانية استخدام مستخلصي الايثانول والهكسان لنبات القرنفل بمفردة أو مخلوط من الأربعة نباتات في معالجة العدوى بالبكتريا سالبة الجرام ولا سيما البكتريا محل الدراسة.