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Full Length Article

Phytochemical Analysis of *Nigella sativa* and its Antibacterial Activity against Clinical Isolates Identified by Ribotyping

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Abstract

The study was carried out to find *in vitro* anti-bacterial activity of *Nigella sativa* extracts against seven clinical isolates identified by ribotyping. Crude Extracts of *N. sativa* in eight organic solvents (Aqueous, Methanol, Ethanol, Cloroform, Butanol, Diethyl ether, n-Hexane and Acetone) were evaluated at 5 different concentrations 100 mg/mL, 50 mg/mL, 25 mg/mL, 10 mg/mL and 5 mg/mL by using disk diffusion method against human pathogenic bacterial strains including Gram-positive bacteria (*Enterococcus faecalis* IARS1, *Staphylococuss aureus* IARS4) and Gram-Negative bacteria (*Acinetobacter junii* IARS2, *Escherichia coli* IARS3, *Proteus mirabilis* IARS5, *Serratia marcescens* IARS6, *Enterobacter cloacae* IARS7). All the organic *N. sativa* extracts with minimum concentration of 5 mg/mL showed effective growth inhibition against the tested pathogenic bacterial strains. Standard antibiotics ceftriaxone 100 µg/mL, Amoxycillin 100 µg/mL, Gentamycin 100 µg/mL were used as a positive control. Out of eight extracts Methanolic, Ethanolic, Chloroform, n-Hexane and Acetonic extracts showed maximum antibacterial activity. *N. sativa* extracts were screened for qualitative detection of secondary metabolites and it showed that steroids, tannins, flavanoids, coumarins, cardiac glycosides, saponins and diterpenes were present in methanolic and ethanolic extract of *N. sativa*. The results of this study will provide an insight to characterize bioactive compounds from these extracts, which can act as strong bacterial growth inhibitor against wide range of infectious disease caused by pathogenic bacteria. © 2013 Friends Science Publishers

Keywords: Phytochemical analysis; *Nigella sativa*; Anti-bacterial activity; Disk diffusion; Ribotyping

Introduction

Medicinal plants are the richest bioresource of drugs for traditional systems of medicine, nutraceuticals, food supplements, modern medicines, pharmaceutical intermediates, folk medicines and chemical entities for synthetic drugs. According to World Health Organization (WHO), up to 80% of the people depends on traditional medicinal plants for their medicines (Arunkumar and Muthuselvam, 2009).

Plant products derived from barks, flowers, roots, leaves, seeds, fruits are the part of phytomedicines (Criagg *et al*., 2001). For synthesis of complex chemical compounds knowledge of the chemical constituents of plants is desirable (Mojab *et al.,* 2003; Parekh and Chanda, 2007; 2008). Phytochemical components such as tannins, carbohydrates, alkaloids, terpenoids, phenolic compounds, steroids and flavonoids are responsible for various pharmacological activities of the plants (Abbas *et al*., 2012a, b; Shah *et al.,* 2011; Zaman *et al*., 2012). These phytochemical compounds are synthesized by primary or secondary metabolism of living organisms. Secondary metabolites are taxonomically and chemically diverse compounds with vague function. They are extensively used in agriculture, human therapy, veterinary and related scientific research etc. (Vasu *et al.,* 2009; Mansoor *et al*., 2011).

N. sativa is an indigenous herbaceous plant belongs to the *Ranunculaceae* family that is more commonly known as the fennel flower plant. This plant has finely divided foliage and blue flowers, which produce black seeds and it grows to a maximum height of about 60 cm. The plant is known by many other names e.g. kalonji (Urdu), habba-tu sawda (Arabic), black cumin (English), shonaiz (Persian), kalajira (Bangali) (Khan, 1999). It is cultivated extensively in Pakistan and India, and also grows in the Mediterranean countries. In Islamic medicine, the use of the black seeds is recommended in daily use because it is regarded as one of the greatest forms of healing medicine available. Prophet Muhammad (PBUH) once stated that the black seed can heal every disease-except death-as narrated in the following hadith "Hold onto the use of the black seeds for in it is healing for all diseases except death"(Sahih Bukhari vol. 7 book 71 # 592).

N. sativa seeds, as nutritional and medicinal plant, have traditionally been used for thousands of years as folk

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medicine and some of its active compounds were reported against many ailments (Toncer and Kizil, 2004). Different pharmacological effects such as gastric ulcer healing (Javed *et al.,* 2010), anti-microbial effect (Mariam and Al-Basal, 2009), anti-cancer activity (Shafi *et al.,* 2009), cardiovascular disorders (Sultan *et al.,* 2009), gastroprotective and antioxidant activity (El-Abhar *et al.,* 2003), immunomodulatory, anti-inflammatory and anti-tumor effects (Majdalawieha *et al.,* 2010), antitussive effect (Hosseinzadeh *et al.,* 2008), anti-anxiety effect (Boskabady *et al.,* 2010), anti-asthmatic effect (Navdeep *et al.,* 2009), anti-inflammatory effects in pancreatic cancer cells (Salem, 2010), anti-helicobacter activity (Tingfang *et al.,* 2008), tumor growth suppression (Eugene *et al.,* 2011), anti-viral activity against cytomegalovirus (Salem and Hossain, 2000), hepatoprotective activity (Khan, 1999) have been reported for this medicinal plant.

This study was carried out to find the antibacterial activity of *N. sativa* aqueous and organic extracts against seven human pathogenic bacterial strains which were identified by ribotyping. Ribotyping is a molecular technique, which is used to identify bacteria on the basis of 16S ribosomal RNA gene.

Materials and Methods

Collection of Bacterial Strains

Human pathogenic bacterial strains including Gram-positive bacteria *Enterococcus faecalis* IARS1, *Staphylococuss aureus* IARS4 and Gram-Negative bacteria *Acinetobacter junii* IARS2, *Escherichia coli* IARS3, *Proteus mirabilis* IARS5, *Serratia marcescens* IARS6, *Enterobacter cloacae* IARS7 were isolated from blood samples obtained from microbiology laboratory, Pakistan Institute of Medical Sciences Hospital, Islamabad, Pakistan.

Identification of Bacterial Strains by Ribotyping

Bacterial template DNA was extracted by overnight grown 5 mL bacterial culture. Microcentrifuged 1.5 mL at 14000 rpm for 2 min or until a compact pellet forms. The pellet was resuspended in 567 µL TE buffer. Moreover, 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K, were added to it and mixed thoroughly and the mixture was incubated for 1 hour at 37°C and followed by addition of 100 µL of 5M NaCl and 80 µL of CTAB/NACL and incubation at 65°C. 1 vol (0.7 to 0.8 ml) of 24:1 chloroform/isoamyl alcohol was added into the mixture, mixed thoroughly and microcentrifuged at 14000 rpm for 4 to 5 min. Supernatant was transferred to fresh tube. 0.6 volume of isopropanol was added to supernatant and mixed gently to precipitate DNA. It was then microcentrifuged at 14000 rpm for 2 min at room temperature, supernatant was discarded and 70% ethanol was added to the pellet. Then the mixture was centrifuged for 5 min at room temperature to precipitate the pellet, which was dried in a lypholizer. Finally the pallet was suspended in 100 µL TE buffer.

Molecular identification of the bacterial strains was confirmed by sequencing 16S rRNA gene. Primers RS-1 (5′- AAACTCAAATGAATTGACGG-3′) and RS-3 (5′-ACGGGCGGTGTGTAC-3′) were used to ampilify 16S rRNA gene from bacteria by PCR (Zahoor and Rehman, 2009). Total of 50 µL Reaction mixture was prepared for PCR reaction using Finnzyme kit. Denaturation of DNA at 94°C for 1 min was followed by 30 cycles of amplifications (94°C for 30 sec, 60°C for 30 sec, 72°C for 1:30 min) and final extension at 94°C for 7 min. The PCR product was analyzed on 1% agarose gel. These eluted PCR product was sequenced and Basic Local Alignment Search tool BLAST was then performed for 16S rRNA sequences to identify the sequence by alignment.

Collection of *N. sativa* Seeds

N. sativa seeds were purchased from local herb shop in Jeddah, Saudi Arabia. The plant species was confirmed by taxonomist Dr. Muhammad Qasim Hayat (ASAB, NUST) and it was deposited at Medicinal Plant Laboratory of the Atta-ur-Rahman School of Applied Biological Sciences for future records.

Extract Preparation

N. sativa seeds were first dried at room temperature and then grinded into fine powder by using electric grinder. Eight different solvent extracts of *N. sativa* seeds were prepared to screen the anti-bacterial activity.

Finely grinded *N. sativa* seed powder was subjected to aqueous and seven organic solvents (Methanol, Ethanol, Chloroform, Diethyl ether (DEE), n-Hexane, Acetone, Butanol) separately with ratio 10:100 in a flask and then kept it on shaker at room temperature on continuous shaking for 24-48 h. The solution was then centrifuged for 15 min at 2000 rpm. The supernatant then collected and filtered through Whatmann filter paper 1. The filtrate then dried in a rotary evaporator at 50-60°C until all the solvents gets evaporated and only dry extract left behind. The dry extract then stored at 4°C for further research use.

In-vitro Antibacterial Activity

Antibacterial activities of all eight extracts of *N. sativa* were performed by disk diffusion method (Malika *et al.,* 2004). Mueller Hinton Agar cultures of test microorganism were prepared with standardized inoculums maintaining bacterial culture count at 1×10^8 cells/mL. During antibacterial testing, 40 µL of extracts with different concentrations 100 mg/mL, 50 mg/mL, 25 mg/mL, 10 mg/mL, 5 mg/mL were placed separately on 6 mm filter paper disk with the help of Micropipette. Five filter paper disks with different concentrations of the same extract were placed in a single petri plate. The disk impregnated with standard antibiotics ceftriaxone 100 μg/mL, Amoxycillin 100 μg/mL, Gentamycin 100 μg/mL served as positive control. The disks soaked and evaporated in eight different solvents namely Aqueous, Methanol, Ethanol, Chloroform, DEE, Acetone, Butanol, Hexane and DMSO act as negative control. The plates were then incubated for 8-12 h at 37°C after which zone of inhibition was measured. The experiments were performed in triplicate. The minimum concentrations of extract that inhibited bacterial growth were recorded as minimum inhibitory concentration.

Statistical analysis was carried out using the student´s *t*-test, for the estimation of results as mean ± SD (standard deviation).

Qualitative Phytochemical Secreening for Secondary Metabolites

N. sativa seed extracts (Methanol, Ethanol, Chloroform, Hexane, DEE, Acetone, Butanol and Aqueous) were subjected to phytochemical screening for secondary metabolites. Qualitative phytochemical analysis for steroids, tannins, terpenoids, flavanoids, anthocyanins, leucoanthocyanins, coumarins, cardiac glycosides, saponins and diterpenes was carried out by standard protocols (Harborne, 1973; Trease and Evans, 1989; Sofowra, 1993).

Results

Genomic DNA of seven different human pathogenic bacterial strains were extracted (Fig. 1) and then these Genomic DNA were amplified to 470 bp amplicon, and sequenced (Fig. 2). The resulted sequences were submitted to NCBI genbank JQ863232 (*E. faecalis* IARS1), JQ863233 (*A. junii* IARS2), JQ863234 *(E. coli* IARS3), JQ863235 (*S. aureus* IARS4), JQ863236 (*P. mirabilis* IARS5), JQ863237 (*S. marcescens* IARS6), JQ863238 (*E. cloacae* IARS7) and aligned to search for similar sequences. These Bacterial sequences showed 99% similarity with database sequences.

All eight extracts with different concentrations showed effective results against seven pathogenic bacterial strains. Antibacterial activity of *N. sativa* extracts against seven pathogenic bacterial strains were summarized in Table 1. *A. juni*, *E. coli* and *S. marcescens* showed sensitivity against aqueous extract of *N. sativa*. Minimum inhibitory concentration of aqueous extract was found to be 5 mg/mL against these bacterial strains.

E. faecalis, *Acinetobater Junii*, *E. coli*, *P. mirabilis*, *S. marcescens* were sensitive to ethanolic extract with MIC value 5 mg/mL, while *S. aureus* isolates were resistant to ethanolic extract. MIC of methanolic extract is 5 mg/mL against all the tested bacterial strains, while it is 25 mg/mL against *S. aureus*. MIC value of chloroform extract against *S. aureus*, *E. cloacae* was 10 mg/mL and 25 mg/mL against *E. coli*. MIC value of Acetonic extract against *E. faecalis* was 100 mg/mL, while it was 50 mg/mL against *S. aureus* and *E. cloacae*. MIC value of n-hexane extract against *E. coli*, *S. aureus* and *P. mirabilis* is 100 mg/mL, 50 mg/mL and 25 mg/mL respectively, while rest of the strains were sensitive to it with MIC value 5 mg/mL. *S. aureus* and *P. mirabilis* were resistant to the diehtyl ether

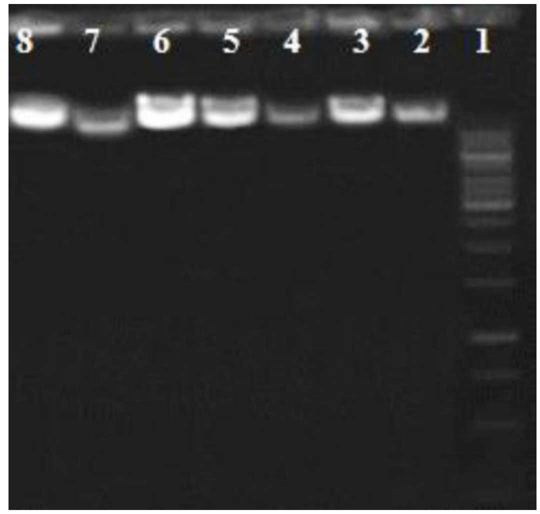

Fig. 1: Lane 1: 1 kb ladder, Lane 2-8 shows genomic DNA of seven bacterial strains. Lane 2= *Enterococcus faecalis*, Lane 3= *Acinetobacter junii*, Lane 4= *Escherichia coli*, Lane 5= *Staphylococuss aureus*, Lane 6= *Proteus mirabilis*, Lane 7= *Serratia marcescens* Lane 8= *Enterobacter cloacae*

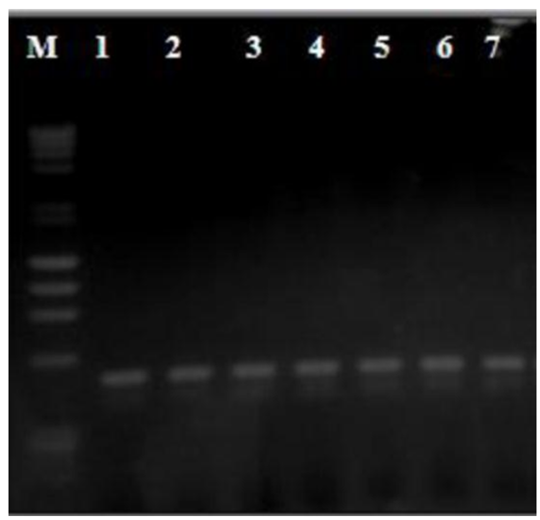

Fig. 2: PCR-based amplification of seven pathogenic bacterial 16S rRNA gene. M: Hindi III ladder, Lane 1-7 shows bands of amplified DNA fragments at approximately 500 bp. Lane 1= *Enterococcus faecalis*, Lane 2= *Acinetobacter junii*, Lane 3= *Escherichia coli*, Lane 4= *Staphylococuss aureus*, Lane 5= *Proteus mirabilis*,Lane 6= *Serratia marcescens* Lane 7= *Enterobacter cloacae*

Table 1: Zone of inhibition of eight extracts against seven pathogenic bacterial strains

			Zone of Inhibition (mm)					
			Identified by Ribotyping					
Extract		*E. faecalis*	*A. junii*	*E. coli*	*S. aureus*	*P. mirabilis*	*S. marcescens*	*E. cloacae*
	100 mg/ml	N.Z	15±1.0	14.0±1.0	N.Z	N.Z	12.0±1.0	N.Z
Aqueous	50 mg/ml	N.Z	13.3±0.57	12.33±1.0	N.Z	N.Z	12.3±0.5	N.Z
	25 mg/ml	N.Z	12.6±0.57	10.66±1.1	N.Z	N.Z	13.5±1.3	N.Z
	10 mg/ml	N.Z	11.5±0.8	9.1±1.2	N.Z	N.Z	13.6±1.5	N.Z
	5 mg/ml	N.Z	9.5±0.8	8.83±0.7	N.Z	N.Z	14.0±1.0	N.Z
	100 mg/ml	15.33±1.5	15.16±1.0	13.5±1.3	N.Z	12.0±0.0	17.33±1.1	14.4±0.5
Ethanolic	50 mg/ml	13.33±0.5	14.0±1.0	12.0±1.0	N.Z	11.0±1.0	14.7±1.5	13.4±1.1
	25 mg/ml	12.66±0.7	12.67±0.5	11.33±1.2	N.Z	9.6±1.0	14.0±1.0	12.3±0.5
	10 mg/ml	11.33±0.5	11.5±0.5	10.0±1.3	N.Z	10.0±0.0	12.33±0.5	11.0±1.0
	5 mg/ml	8.67±0.5	10.5±0.5	9.5±1.0	N.Z	8.6±0.5	10.33±0.5	N.Z
	100 mg/ml	16.33±0.5	15.33±0.5	15.0±1.0	14.6±1.5	13.33±0.5	15.33±0.5	15.0±1.0
Methanolic	50 mg/ml	14.0±0	14.33±1.5	12.6±0.5	12.0±1.0	11.66±0.5	13.66±.5	14.4±1.1
	25 mg/ml	13.33±1.1	14.33±1.5	12.33±0.51	9.3±1.5	10.16±0.7	12.0±1.0	12.7±0.5
	10 mg/ml	11.33±1.1	12.33±0.7	11.33±1.2	N.Z	9.3±0.7	11.33±0.5	10.7±1.1
	5 mg/ml	11.33±1.1	11.0±0.1	10.8±1.4	N.Z	8.83±1.0	10.0±1.0	9.0±0.0
	100 mg/ml	12.83±1.0	13.0±1.0	12.0±1.5	15.33±1.1	14.0±1.0	11.66±0.5	13.7±1.5
	50 mg/ml	13.83±0.2	13.0±1.0	11.66±1.5	14.0±1.0	13.33±0.5	13.33±1.5	13.$+1.2
Chloroform	25 mg/ml	14.83±0.2	14.67±0.7	11.0±1.3	12.0±2.0	10.5±0.5	13.6±1.5	11.0±1.7
	10 mg/ml	15.5±0.5	14.83±0.7	N.Z	9.1±0.7	10.0±0.0	15.0±1.7	11.0±1.4
	5 mg/ml	16.33±0.5	16.16±0.2	N.Z	N.Z	8.6±0.5	15.0±1.7	N.Z
	100 mg/ml	11.5±0.8	14.67±0.5	12.66±1.1	13.66±1.5	12.66±1.1	15.0±1.0	15.4±1.1
Acetonic	50 mg/ml	N.Z	14.0±0.0	11.83±1.4	10.33±2.5	11.33±2.3	13.6±0.5	14.0±1.1
	25 mg/ml	N.Z	12.83±0.7	10.1±0.2	N.Z	10.0±2.6	12.3±0.5	N.Z
	10 mg/ml	N.Z	12.1±1.0	9.5±1.0	N.Z	9.16±0.7	12.4±0.5	N.Z
	5 mg/ml	N.Z	11.3±1.5	N.Z	N.Z	8.83±0.76	12.0±0.0	N.Z
	100 mg/ml	14.86±0.8	16.0±1.0	7.75±0.3	15.8±1.0	15.0±1.0	14.0±1.0	17.4±1.1
Hexane	50 mg/ml	12.83±2.0	14.67±0.5	N.Z	13.0±1.0	12.6±0.5	12.5±0.5	17.0±1.0
	25 mg/ml	11.33±1.5	13.16±1.2	N.Z	N.Z	9.7±0.5	10.8±0.7	14.7±1.1
	10 mg/ml	11.0+1.7	10.8±0.28	N.Z	N.Z	N.Z	10.0±0.0	12.6±0.5
	5 mg/ml	10.33±2.5	10.0±0.0	N.Z	N.Z	N.Z	9.3±0.7	10.83±0.7
	100 mg/ml	N.Z	13.33±0.5	13.1±1.0	N.Z	N.Z	16.7±0.5	13.66±0.5
Diethyl Ether	50 mg/ml	N.Z	12.67±0.5	11.3±1.1	N.Z	N.Z	14.4±0.5	12.0±0.0
	25 mg/ml	N.Z	12.16±0.7	10.2±1.2	N.Z	N.Z	13.7±0.5	11.33±1.1
	10 mg/ml	15.83±1.0	10.8±0.28	N.Z	N.Z	N.Z	12.0±1.0	10.0±1.0
	5 mg/ml	11.67±1.5	10.0±0.0	N.Z	N.Z	N.Z	11.0±1.0	N.Z
	100 mg/ml	N.Z	N.Z	N.Z	N.Z	N.Z	N.Z	N.Z
Butanol	50 mg/ml	N.Z	N.Z	N.Z	N.Z	N.Z	N.Z	N.Z
	25 mg/ml	N.Z	N.Z	N.Z	N.Z	N.Z	N.Z	13.7±1.5
	10 mg/ml	12.0± 0	N.Z	N.Z	N.Z	N.Z	N.Z	11.7±1.5
	5 mg/ml	8.0±0	N.Z	N.Z	N.Z	N.Z	N.Z	10.33±1.5
Gentamycin	100 µg/ml	22±0.5	25±0.1	24±0.2	20±0.2	28±0	20±0.1	24±.1
Amoxycillin	100 µg/ml	20±0	N.Z	N.Z	18±0	18±0.2	N.Z	10±0
Ceftriaxone	100 µg/ml	25±0	18±0.2	15±0.3	32±0.1	N.Z	17±0	N.Z
Negative control	DMSO	N.Z	N.Z	N.Z	N.Z	N.Z	N.Z	N.Z

E. faecalis=Enterococcus faecalis, A. junii=Acinetobacter junii, E. coli= Escherichia coli, S. aureus= Staphylococcus aureus, P. mirabilis=Proteus mirabilis, S. marcescens=Serratia marcescens, E. cloacae=Enterobacter cloacae. Values are expressed as mean± SD (n = 3). N.Z= No zone

Table 2: Phytochemical Analysis of *Nigella Sativa* linn. Seed Extracts

EXTRACTS	Methanol	Ethanol	Chloroform	DEE	Acetone	Aqueous	Butanol	Hexane
Steroids	+	+	++	++	+++	-	-	+
Tannins	++	++	-	-	-	++	+	-
Terpenoids	-	-	+++	-	-	-	+++	-
Flavanoids	++	++	+	-	-	+++	-	-
Anthocyanins	-	-	-	-	-	-	-	-
Leucoanthocyanins	-	-	-	-	-	-	-	-
Coumarins	+++	++	+	+	+	+++	-	+
Cardiac Glycosides	++	+	+	++	++	-	+	+
Saponins	++	++	-	+	-	-	+	-
Diterpens	++	++	-	-	-	+	+	-

+ = weakly present, ++ = Moderately present, +++= Strongly Present, **-** = Not present extract, while it is sensitive to *E. faecalis, A. junii, E. coli, S. marcescens E. cloacae*. Butanolic extract is only sensitive to *E. faecalis* and *E. cloacae*, while it is resistant to rest of bacterial strains. Results on the phytochemical screening of aqueous and seven organic extracts of *N. sativa* seeds are summarized in Table 2.

Discussion

In the Current study, antibacterial activity of organic and aqueous extracts of *N. sativa* seeds in comparison with standard drugs Ceftriaxone, Amoxycillin, Gentamycin was determined and found to proceed in dose dependent manner against major clinical isolates. Methanolic extract of *N. sativa* seeds shows antibacterial activity against all bacterial strains under investigation. Thymol is present in the methanol extract of *N. sativa* (Enomoto *et al.*, 2001) that has been reported to possess antibacterial activity (Karapinar and Aktug, 1987). It was also investigated by (Mason *et al.*, 1987) that thymol is responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through nonspecific interactions with the proteins.

In present study, phytochemical screening for secondary metabolites shows that steroids, tannins, flavanoids, coumarins, cardiac glycosides, saponins and diterpenes were present in methanolic extract. Previously it was reported that *N. sativa* seeds contain tannins, which is extracted in methanolic extract (Eloff *et al.*, 1998). Tannins forms complexes with proteins through forces such as hydrophobic effects, hydrogen bonding and covalent bond formation, thus, tannins act as antibacterial agent by inactivating microbial adhesins, enzymes, cell envelope transport proteins (Hashem and El-Kiey, 1982).

The use of *N. sativa* oil in preserving food has shown the oil to be a potent inhibitor of food hazardous and spoilage bacteria (El-Sayed *et al.*, 1996; Zuridah *et al.*, 2008). Current study also proves that oil extracted by different *N. sativa* seed extracts show antibacterial activity against food spoilage bacteria including *E. coli* and *S. aureus* with MIC value 5 mg/mL.

Present study shows that chloroform extract possess antibacterial activity against all human pathogenic bacterial strains under study. This antibacterial activity is due to the presence of steroids, terpenoids, flavonoids, coumarins and cardiac glycosides in the chloroform extract. Aqueous extract of *N. sativa* seed showed less antibacterial activity. This may be due to lower accessibility of the aqueous extract to the micro-organism or low extraction of antibacterial agents into this extract (Morsi, 2000).

All the extracts except aqueous extract contain oily content due to which extract appears oily. It was investigated three decades ago that volatile oil of *N. sativa* had antimicrobial activity, which is responsible for growth inhibition of both gram positive and gram negative bacteria (Toppozada *et al.*, 1965). This is proved by current study that oil extracted by methanolic, ethanolic, diethyl ether, acetonic and *n*-hexane shows effective antibacterial activity against both gram positive and gram negative bacteria.

In conclusion, bacterial resistance against antibiotics is a great challenge. The results of this study shows that *N. sativa* seeds extracts have antibacterial activity against most common bacterial strains involved in human pathogenesis. In addition, phytochemicals evaluation of *N. sativa* seed provides information about number of medicinally important secondary metabolites, which impart antibacterial characteristics. Further studies are recommended to find the antiviral activity of *N. sativa* against life threatening viral diseases e.g. HCV, HBV and Dengue.

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