World Applied Sciences Journal 20 (2): 182-189, 2012
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2012.20.02.7156

Comparative Antimicrobial Activity of Some Active Constituents of *N. sativa* L.

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Abstract: The comparative antimicrobial activity of tannins, saponins, alkaloids, essential oil, brown and yellow crystals separated from essential oil and thymoquinone, were evaluated, against a yeast (*Saccharomyces cerevisiae*), two molds (*Penicillium notatum* and *Aspergillus niger*), two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis),* five Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium and Shigella flexneri*) and an acid-fast bacterium (*Mycobacterium phlei*). Essential oil, its fractions and thymoquinone were the most active constituents against the tested microorganisms followed by alkaloids and saponins, respectively. On the contrary, tannins of *N. sativa* lacked any activity against all tested microorganisms. The tested Gram-positive bacteria were significantly ($p < 0.0001$) more sensitive than Gram-negative bacteria and *Sac. cerevisiae* was more sensitive than *P. notatum* and *A. niger* ($p < 0.001$) to the tested constituents. Concentrations as low as 4-16 µg/ml of the volatile oil or its fractions killed *S. aureus* and *B. subtilis*. The MICs and MBCs of the tested *N. sativa* constituents against both Gram-positive and Gram-negative bacteria in Muller-Hinton were 2 to 8 times higher than their values in M9 minimal medium suggesting an interaction of the tested constituents with the organic matters.

Key words: *Nigella sativa* · Tannins · Saponins · Alkaloids · Essential Oil · Thymoquinone · Antibacterial Activity · Antifungal Activity

INTRODUCTION

Nigella sativa L. (Family *Ranunculaceae)* has been used for centuries in the Middle East, Eastern Europe, Asia and Africa as a natural remedy for many ailments [1-5]. In Muslim countries *N. sativa* seeds have been used extensively in folk medicine for treatment of different diseases, as Prophet Muhammad of Islam, stated that the black seed can heal all diseases except for death [2, 4].

The multiple use of *N. sativa* in folk medicine encouraged many investigators to isolate possible active components [6-8]. *In vivo* and *in vitro* studies revealed many pharmacological actions for *N. sativa*. These include immune system stimulation [9], anti-inflammatory properties [10], anti-cancer activity [11, 12] antimicrobial activity [7, 10-16], anti-parasitic activity [17], anti-oxidant effect [18], hypoglycemic effect [19, 20], galactagogue, carminative and laxative effects [2].

The antimicrobial activity of *N. sativa* crude extracts against different microorganisms has been studied by several research groups. Toppozad*et al.* [13] was the first to report the anti-bacterial effect of the phenolic fraction of *N. sativa* oil. Diethyl-ether extract of *N. sativa* was found to inhibit Gram-positive and Gram-negative bacteria, as well as pathogenic yeasts [21]. Seed extracts of *N. sativa* inhibit the growth of *Escherichia coli, Bacillus subtilis* and *Streptococcus fecalis* [22]. Morsi [23] reported the antibacterial activity of crude extracts of *N. sativa* against multi-drug-resistant organisms, including Gram-positive bacteria like *Staphylococcus aureus* and Gram-negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli*. *N. sativa* extracts also inhibit the growth of different strains of *H. pylori* [24, 25].

Some purified constituents of *N. sativa* have also been tested for their antibacterial activity. Essential oil of *N. sativa* and some of its components like thymoquinone and hydrothymoquinone, were reported to be lethal to fungi, Gram-positive and Gram-negative bacteria [7, 26-30]. The antibacterial activity of alkaloids and saponins, of *N. sativa,* was recently evaluated qualitatively by agar disc diffusion method [31, 32].

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In most of the above reports, the evaluations of antibacterial activity of *N. sativa* extracts or some of its constituents were done rather qualitatively using agar diffusion method and the constituents were not comparatively evaluated. In this study, tannins, volatile oil, yellow and brown crystals separated from volatile oil, thymoquinone, saponins and alkaloids, of *N. sativa,* were comparatively evaluated for their antibacterial and antifungal activities.

MATERIALS AND METHODS

Extraction of Alkaloids: Powdered seeds of the plant were extracted with 70% methanol. The extract was evaporated under reduced pressure, dissolved into distilled water and acidified with 3% hydrochloric acid. The solution was extracted with petroleum ether. The acidic solution was made alkaline with 25% ammonium hydroxide (pH 9-10) and then extracted with chloroform. The combined chloroform extracts were dried to get the crude *N. sativa* alkaloids [33].

Extraction of Saponins: Powdered seeds of the plant were defatted with n-hexane. Further extraction was performed with 70% methanol. After evaporation of the methanol under vacuum, the crude residue was acidified with 5% hydrochloric acid and left overnight in the refrigerator. The precipitate was extracted with chloroform: methanol (75:25), to get crude saponins [34].

Preparation of Volatile Oil and Brown and Yellow Fractions: *N. sativa* powdered seeds were mixed with water and steam distillated. The collected essential oil was kept in a dark glass bottle at -20°C till used. On storage of essential oil at -20°C for several days, yellow and brown crystals were formed, collected separately and stored at -20°C till used.

Extraction of Tannins: Powdered seeds were extracted with 70% methanol and dried under vacuum and dissolved into small amount of distilled water. The extract was applied into a column of XAD-16 resin (Sigma) and washed with copious amounts of water until the elute was clear in color. Adsorbed tannins were eluted several times by methanol and the dark brown solution collected was evaporated at 50 ºC in a rotary vacuum evaporator [35].

Assessment of Antimicrobial Activity by Agar Well Diffusion Method: This was done as previously described by Halawani and Shohayeb [36], with some modifications. Briefly, suspensions (10^7CFU/ml) of each tested microorganism (Table 1) in physiological saline were spread onto the surface of Muller-Hinton agar and Sabouraud's dextrose agar plates for bacteria and fungi respectively. Six mm cork borer was used to punch wells into the plates and 100 µl of each extracted constituent dissolved in DMSO (10 mg/ml) was applied to each well. The plates were incubated for 18 h at 37°C in case of bacteria and for 48 h at 27°C in case of fungi. The diameter of inhibition zones for each extract was measured.

Minimum Inhibitory Concentration (MIC) of Constituents: Each constituent of *N. sativa* was two-fold serially diluted with Muller-Hinton broth or M9 minimum medium to give series of concentrations in sterile 96-well polystyrene micro-titration plates. Each series of dilutions

Table 1: Bacteria and fungi used in the study and their source

Bacteria	Characteristic	Source
Bacillus subtilis	Standard strain (168)	Faculty of Pharmacy, Tanta University, Egypt
Staphylococcus aureus	Ap, Cf, Sm, Gn*	Clinical isolate, TaifUniversity, KSA
Mycobacterium phlei	Standard strain (ATCC- 6841)	Tanta University, Egypt
Pseudomonas aeruginosa	Ap, Pp, Cp, Tc, Cm, Sx, Sm*	Clinical isolate, TaifUniversity, KSA
Escherichia coli	Ap, Tc, Cm, Sx, Sm*	Clinical isolate, Tanta University, Egypt
Klebsiella pneumoniae	Ap, Pp, Cp, Tc, Cm, Sx*	Clinical isolate, Tanta University, Egypt
Shigella flexneri	Ap, Cp, Tc, Cm, Sx*	Clinical isolate, Tanta University, Egypt
Salmonella Typhimurium	Ap, Tc, Sx, Sm*	Clinical isolate, TaifUniversity, KSA
Aspergillus niger	Standard strain (ATCC-13794)	Faculty of Pharmacy, Tanta University, Egypt
Penicillium chrysogenum	Standard strain (ATCC-18226)	Faculty of Pharmacy, Tanta University, Egypt
Saccharomyces cerevisiae	Standard strain (ATCC-9080)	Faculty of Pharmacy, Tanta University, Egypt

* Clinical isolate resistant to: Ap, ampicillin; Cf, cephalexin; Cp, cephoperazone; Pp, piperacillin; Tc, tetracycline; Sm, streptomycin; Cm, chloramphenicol; Sx, sulfamethoxazole; Gn, gentamicin

was inoculated with 10^4 CFU/ml of the tested bacteria (Table 1) and incubated at 37°C for 18 hours before determining the least concentration that inhibited the appearance of visible growth [37].

Minimum Bactericidal Concentration (MBC) of Constituents: The minimum bactericidal activity was determined by sub-culturing inhibitory concentrations of the tested constituents after reading their MICs in micro-titration plates [37].

Statistical Analysis: All determinations were carried out in triplicates and the statistical analyses were carried out using SPSS 13.0 and Microsoft Excel programs.

RESULTS

The antifungal activity of volatile oil, yellow and brown crystals separated from volatile oil, thymoquinone, tannins, alkaloids and saponins of *N. sativa* were assessed against two types of molds (*A. niger* and *P. notatum*) and a yeast (*Sac. cerevisiae*), as shown in Fig. 1 and Table 2. While volatile oil and its fractions were active against all the three tested fungi, saponins and alkaloids were active only against *Sac. cerevisiae* and tannins were inactive against the three tested fungi (Table 2). *Sac. cerevisiae*, was more sensitive to all tested constituents of *N. sativa* than *A. niger* and *P. notatum* and had inhibition zones ranging between 24 and 30 mm compared to 10 and 15 mm for *A. niger* and *P. notatum*, respectively (Table 2).

The antibacterial activity of tested active constituents of *N. sativa* was assessed against two Gram-positive bacteria (*S. aureus* and *B. subtilis*), five Gram-negative bacteria (*E. coli, K. pneumoniae, P. aeruginosa, Sal. typhimurium* and *Sh. flexneri*) and an acid-fast bacterium (*M. phlei*). Six of these strains were clinical isolates resistant to several antibiotics (Table 1).

All the tested constituents exerted inhibitory activities against the tested bacteria except for tannins, which lacked any inhibitory activity (Fig. 1). *S. aureus* and *B. subtilis* were particularly, very sensitive to essential oil, the brown and yellow crystals and thymoquinone. The inhibition zones for *S. aureus* and *B. subtilis* ranged between 45 and 55 mm for essential oil and its fractions compared to inhibition zones ranging between 12 and 14 mm for saponins and alkaloids (Fig. 1).

The inhibition zones produced by the six tested active constituents against *M. phlei* ranged between 10 and 17 mm, while those obtained for the tested Gram-negative bacteria ranged between 6 and 14 mm (Fig. 1). This reflects higher susceptibility of *M. phlei* compared to Gram-negative bacteria.

Table 2: Antifungal activity of the tested constituents of *Nigella sativa* by agar well diffusion method

	Fungus		
Constituent	*Saccharomyces cerevisiae*	*Aspergillus niger*	*Penicillium notatum*
	Zone of inhibition (mm)		
Volatile oil	25± 2.0	10±0.6	10±0.16
Brown oil fraction	24±0.6	12±1.5	14±1.0
Yellow oil fraction	28±1.0	11±0.15	13±0.3
Thymoquinone	30±0.12	13±0.13	15±0.13
Saponins	12±0.16	-	-
Alkaloid	14±0.13	-	-
Tannins	-	-	-

Table 3: Minimum inhibitory and minimum bactericidal concentrations of the tested constituents of *Nigella sativa* seeds

	Bacillus subtilis		*Staphylococcus aureus*		*Mycobacterium phlei*		*Pseudomonas aeruginosa*		*Escherichia coli*		*Klebsiella pneumoniae*		*Shigella flexneri*		*Salmonella typhimurium*	
N. sativa constituent	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	µg/ml															
Volatile oil	8±3.5	8±305	4±3.5	8±7.1	1000±0.0	4000±3.5	2000±0.0	4000±3.5	4000±0.0	8000±0.0	4000±3.5	8000±0.0	4000±3.5	8000±0.0	2000±3.5	4000±3.5
Brown fraction	8±3.5	8±0.0	4±0.0	8±0.0	1000±3.5	2000±0.0	2000±7.1	2000±3.5	4000±0.0	8000±0.0	4000±0.0	8000±0.0	4000±0.0	8000±0.0	2000±3.5	4000±0.0
Yellow fraction	8±0.0	16±7.1	4±7.1	8±7.1	500±7.1	500±7.1	4000±3.5	4000±0.0	4000±3.5	8000±0.0	8000±0.0	8000±0.0	4000±0.0	8000±0.0	4000±0.0	8000±0.0
Thymoquinone	8±7.1	16±3.5	8±3.5	16±3.5	2000±7.1	4000±3.5	4000±0.0	4000±3.5	4000±3.5	8000±0.0	4000±0.0	8000±0.0	2000±3.5	4000±3.5	2000±3.5	4000±3.5
Alkaloids	64±7.1	64±0.0	64±35	128±3.5	4000±3.5	4000±3.5	2000±3.5	4000±0.0	8000±0.0	8000±0.0	8000±0.0	8000±0.0	4000±0.0	8000±0.0	4000±0.0	8000±0.0
Saponins	250±3.5	500±3.5	250±3.5	500±0.0	4000±0.0	4000±0.0	4000±3.5	4000±3.5	2000±3.5	8000±0.0	8000±0.0	8000±0.0	4000±3.5	8000±0.0	4000±0.0	8000±0.0

Table 4: Comparison between MIC and MBC of some *Nigella sativa* constituents in minimum medium and Muller-Hinton medium

		Staphylococcus aureus		*Pseudomonas aeruginosa*		*Escherichia coli*		*Klebsiella pneumoniae*		*Shigella flexneri*		*Salmonella typhimurium*	
N. sativa		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
constituent	Medium*	µg/ml											
Thymoquinone	*MH*	8±0.000	16±0.00	4000±7.1	4000±0.0	4000±3.0	8000±7.1	4000±0.0	8000±0.0	2000±0.0	4000±0.0	2000±0.0	4000±3.0
	M9	2±0.000	8±0.000	1000±3.0	1000±3.0	125±7.10	500±3.50	500±7.10	2000±3.5	125±3.00	250±3.50	125±7.10	250±0.00
Alkaloid	MH	64±0.00	128±0.0	2000±0.0	4000±0.0	8000±0.0	8000±0.0	8000±0.0	8000±0.0	4000±3.0	8000±0.0	4000±3.0	8000±0.0
	M9	8±0.000	32±7.10	1000±0.0	1000±7.1	1000±0.0	2000±7.1	4000±0.0	4000±3.0	500±7.10	1000±7.1	1000±7.1	1000±7.1
Saponins	*MH*	250±3.0	500±0.0	4000±3.0	4000±3.0	2000±7.1	8000±0.0	8000±0.0	8000±0.0	4000±3.0	8000±0.0	4000±3.5	8000±0.0
	M9	64±0.00	128±0.0	2000±7.1	2000±0.0	1000±3.5	2000±3.5	2000±0.0	4000±3.5	1000±3.5	2000±3.0	1000±0.0	1000±0.0

*MH, Muller-Hinton; M9, minimal medium

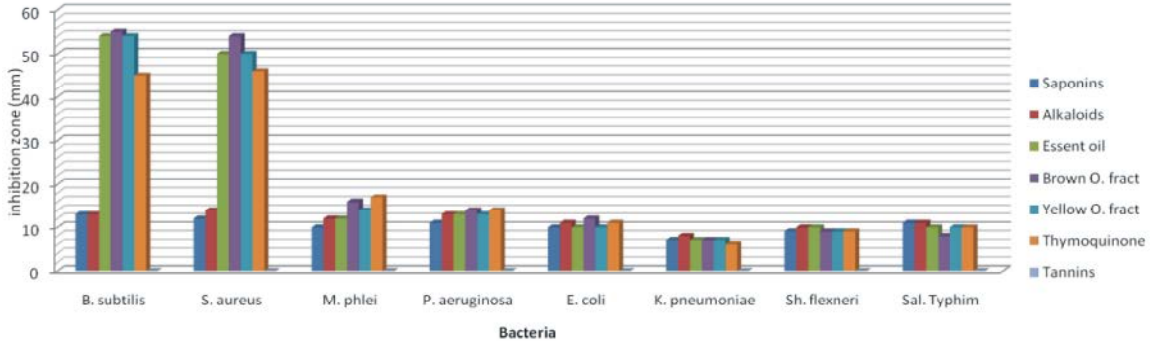

Fig. 1: Zones of inhibition (mm) produced by the tested constituents of *N. sativa* against different types of bacteria

Inhibition zones produced, revealed that, the most sensitive Gram-negative bacterium was *P. aeruginosa* and the most resistant was *K. pneumonia*. The inhibition zones of the former ranged between 11 and 14 mm, while, those of the later ranged between 6 and 8 mm (Fig. 1).

The MICs of *N. sativa* essential oil and its components against *S. aureus* and *B. subtilis* were as low as 4 to 8µg/ml and the MBCs were 8 to 16µg/ml (Table 3). Both *S. aureus* and *B. subtilis* were less susceptible to alkaloids and saponins. The respective MICs and MBCs against *S. aureus* were 64 and 128µg/ml for alkaloids and 250 and 500 µg/ml for saponins (Table 3). The MICs and MBCs for the tested Gram-negative bacteria ranged between, 2000-8000 µg/ml. *P. aeruginosa* was more sensitive than other Gram-negative bacteria (Table 2). The MICs and MBCs for *P. aeruginosa* ranged between 2000 and 4000 while, those other Gram-negative bacteria ranged between 4000 and 8000µg/ml (Table 3).

The MICs of *M. phlei* ranged between 500 and 4000 µg/ml (Table 3), therefore, it is intermediate in its susceptibility for the tested constituents between the tested Gram-positive and Gram-negative bacterias.

The effect of organic matters present in Muller-Hinton on the antibacterial activity of thymoquinone, alkaloids and saponins was checked. The MICs and MBCs were determined in M9 minimal medium and compared with their respective values in Muller-Hinton medium (Table 3). Generally speaking, the MICs and MBCs in Muller-Hinton were 2 to 16 times their values in minimum medium (Table 4). For instance, while the MIC of thymoquinone against *S. aureus* in MH was 8µg/ml, it decreased 4 times in M9 to 2µg/ml. On the other hand, the respective MICs of *Shigella flexneri* in MH and M9 were 2000 and 125µg/ml, indicating 16 times decrease in their value (Table 4).

DISCUSSION

Medicinal plants are considered a rich source of antimicrobial agents [38-40]. *N. sativa* has gained a special interest as a medicinal plant and there has been several reports dealing with the antimicrobial activity of its crude extracts and some of its constituents [21, 22, 24, 25, 27].

Although, in this study, all tested *N. sativa* constituents except for tannins showed inhibitory activity against *Sac. cerevisiae,* only volatile oil and its constituents were active against the tested molds. This confirms a previous study on the antifungal activity of *N. sativa,* volatile oils [29].

Several constituents of *N. sativa* like essential oil, thymoquinone and hydrothymoquinone which are components of the essential oil, alkaloids and saponins were qualitatively reported to possess antibacterial activity [7, 26-28, 30-32]. In this study, we qualitatively and quantitatively investigated the antibacterial activity of saponins, alkaloids and essential oil against different types of bacteria. All the three active constituents possessed antibacterial activity as previously reported [30-32]. We also investigated the antibacterial activity of tannins and brown and yellow crystals separated from essential oil. While, tannins of *N. sativa* had no antibacterial activity, the brown and yellow crystals separated from essential oil, were active against the tested bacteria. Although tannins lacked antimicrobial activity against both bacteria and fungi, it should be mentioned, however, that tannins of some other plants have been reported to possess both bacteriostatic and bactericidal activities [42, 43].

While Gram-positive bacteria, *S. aureus* and *B. subtilis*, were highly sensitive to the tested constituents, acid-fast and Gram-negative bacteria were moderately sensitive. The higher susceptibility of Gram-positive bacteria compared to Gram-negative bacteria ($p < 0.0001$) has been previously reported for other natural products [43-47]. This is likely because Gram-positive bacteria lack the outer membrane of Gram-negative bacteria, which acts as a barrier for penetration of numerous molecules [48].

It is rather interesting that although, both *P. aeruginosa* and *M. phlei* are usually less susceptible to antimicrobials including antibiotics, preservatives, antiseptics and disinfectants [49-50], they were relatively more sensitive to the tested constituents than the tested Gram-negative bacteria.

The high susceptibility of *S. aureus* and *B. subtilis* to volatile oil and its fractions, demonstrated in this study, has been previously reported [28-31] and the values obtained for the MIC of thymoquinone in this study are comparable to those obtained byChaieb, *et al.* [51].

The increasing incidence of multi-drug resistant bacteria is a major concern in different countries [52-56] and therefore, inrecent years, there has been an emphasis on the need to find alternative antibacterial agents [56]. In this study, multi-drug resistant clinical Gram-positive and Gram-negative were inhibited by the tested active constituents of *N. sativa*. *S. aureus*in particular was inhibited by volatile oil and its fractions at concentrations as low as 4 to 8 µg/ml. Therefore, the essential oil of *N. sativa* or its fractions might be considered as possible alternatives for treatment of resistant *S. aureus* infections.

It should be mentioned that the antibacterial activity of the tested active constituents of *N. sativa* was not affected by multi-drug resistance of both clinical Gram-positive and Gram-negative bacterial strains. This phenomenon which was previously reported for crude extracts of *N. sativa* [23, 41], suggests that the mechanisms of action of active constituents of *N. sativa* are different to those of antibiotics.

The MICs and MBCs of the tested *N. sativa* constituents against Gram-negative bacteria in Muller-Hinton were 2 to 16 times their values in minimum medium. This reflects the interaction of the tested constituents with organic matters. Phytoconstituents of some medicinal plants were reported to suffer from reduction in their antibacterial activities in presence of organic matters [57].

In conclusion, volatile oil and its yellow and brown fractions, thymoquinone, saponins and alkaloids of *N. sativa* were all inhibitory to different types of bacteria and fungi. All constituents except tannins were significantly more active against Gram-positive than Gram-negative bacteria ($p < 0.0001$) and more active against *Sac. cerevisiae* yeast than *A. niger* and *P. notatum* molds ($p < 0.001$). Volatile oil and its tested fractions, as well as thymoquinone, were exceptionally highly lethal to Gram-positive bacteria at low concentrations and therefore, they might be considered as possible alternatives to antibiotics for treatment of *S. aureus* infections.

ACKNOWLEDGEMENT

The authors are thankful to TaifUniversity for their financial support and for providing research facilities for this research work.

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