British Journal of Pharmacology and Toxicology 1(2): 96-100, 2010
ISSN: 2044-2467

Submitted date: August 30, 2010 Accepted date: September 24, 2010 Published date: November 15, 2010

Antibacterial Activity of *Nigella sativa* L. Seed Extracts

[1]T.V. Suresh Kumar, [2]P.S. Negi and [1]K. Udaya Sankar
[1]Food Engineering Department,
[2]Human Resource Development Department, Central Food Technological Research Institute,
(Council of Scientific and Industrial Research), Mysore-570 020, Karnataka, India

Abstract: Most of the bacterial pathogens are resistant to existing synthetic antibacterial agents demanding an increasing effort to seek effective phytochemicals as antibacterial agents against such pathogens. *Nigella sativa* L. (black cumin) seeds play an important role in folk medicine and some of its major constituents are reported to be pharmacologically active. In this present work, black cumin seed extracts were obtained using supercritical carbon dioxide ($SCCO_2$) and conventional soxtec extraction using various organic solvents. The antibacterial activities of the extracts were investigated by the agar dilution method against Gram-positive bacteria (*Bacillus cereus* F 4810 and *Staphylococcus aureus* FRI 722) and Gram-negative bacteria (*Escherichia coli* MTCC 108 and *Yersinia enterocolitica* MTCC 859). $SCCO_2$-1 (120 bar/40ºC) extract showed effective growth inhibition than conventional solvent extracts against all the tested bacteria. Further the antibacterial principle present in the extract was isolated and characterized found to be thymoquinone.

Key words: Agar dilution method, black cumin, ^1H and ^{13}C NMR, supercritical CO_2 extraction, thymoquinone

INTRODUCTION

The problem of microbial resistance and degenerative diseases are growing and the outlook for the use of synthetic drugs without adverse effects in the future is still uncertain. Synthetic drugs also block receptor sites and hence attempts are being made to control the use of synthetic drugs and develop new drugs from natural resources like medicinal plants. Medicinal plants are important therapeutic aids for various ailments and the use of those that are native to India in various traditional system of medicine are awe inspiring.

Pharmacologically active seeds of *Nigella sativa* L. (*Ranunculaceae* family), is found in southern Europe, northern Africa and Asia Minor. The seeds are small, black and possess aromatic odor and taste. The seeds commonly known as black seed or black cumin or kalunji have been extensively investigated in recent years and used in folk medicine as a natural remedy for a number of diseases such as asthma, hypertension, diabetes, inflammation, cough, eczema, fever and gastrointestinal disturbances. Seed oil also has antipyretic, analgesic and antineoplastic activity (Ali and Blunden, 2003). Thymoquinone, an active constituent of *Nigella sativa* seeds, is a pharmacologically active quinone, which possesses several pharmacological properties including analgesic and anti-inflammatory actions (Abdel-Fattah *et al*., 2000; Randhawa and Al-Ghamdi, 2002).

Selection of extraction technique is important to extract substances or group of constituents of interest. Many of these bioactive constituents possess high volatility, thermo sensitivity and photo reactivity. Hence, the evaluation of the extraction process related to its efficiency to reach target constituents from a solid matrix is of considerable relevance. The development of new extraction technique has gained increasing importance in the pharmaceutical and food industries in recent years.

Supercritical Fluid Extraction (SFE) of bioactive constituents from plant material is a promising field for the industrial application (Reverchon, 1997), since it has certain advantages over steam-distillation and solvent extraction. Steam-distillation can lead to thermal degradation and partial hydrolysis of some essential oil compounds, while SFE can be performed at lower temperatures, thereby preserving the original extract composition and properties. Carbon dioxide is the most used supercritical solvent in extractions of active constituents, especially for application in pharmaceutical, cosmetic and food industries. Carbon dioxide is non toxic and allows SFE at temperatures near room temperature and relatively low pressures (8-10 MPa). To suppress co-extraction of higher molecular weight constituents, Udaya Sankar (1989) and Reverchon (1997) suggested performing SFE of essential oil at temperatures of 40-50ºC and pressures below 10 MPa.

The objective of this study is to investigate the antibacterial activity of *Nigella sativa* seed various extracts obtained by $SCCO_2$ and conventional solvent extraction against different bacterial pathogens and to characterize the antibacterial principle: thymoquinone.

Corresponding Author: K. Udaya Sankar, Food Engineering Department, Central Food Technological Research Institute, (Council of Scientific and Industrial Research), Mysore-570 020, Karnataka, India.
Tel: +91 821 2514874; Fax: +91 821 2517233

MATERIALS AND METHODS

This study was conducted during the period of April 2009 to June 2010 in our laboratories at Food Engineering Department and Human Resource Development Department of Central Food Technological Research Institute, (Council of Scientific and Industrial Research), Mysore.

***Nigella sativa* seeds:** The seeds were obtained from Supreem Pharmaceuticals Mysore Pvt. Ltd., India. Cleaned seeds were pulverized into a fine powder by an IKA-10 laboratory mill.

$SCCO_2$ extraction: $SCCO_2$ extraction was carried out in Nova Swiss high pressure extractor as described earlier (Udaya Sankar, 1989). Extractions were carried out on 1 kg of the pulverized black cumin seeds at 40 and 50ºC at 120 and 280 bar pressures. CO_2 flow rate was 1.2 to 2.9 Kg/hr to obtain $SCCO_2$-1 (120 bar/40ºC) and $SCCO_2$-2 (280 bar/50ºC) extracts. Yield of extracts were monitored by weight of extract recovered and then stored at 4ºC in dark until analysis.

Soxtec extraction: 5 g of powdered seed was used in a soxtec apparatus and boiled in respective solvents of hexane, ethyl acetate, methanol, and methanol: Water (70:30 v/v) for 30 min and extracted for 3 h. After extraction, the solvents were removed by rotary vacuum evaporator (40ºC) and dried in a vacuum oven at 30ºC for 2 h.

Preparation of extracts: The known quantities of solvent free extracts and $SCCO_2$ extracts were dissolved in propylene glycol for evaluation of antibacterial activity.

Test bacteria: The microorganisms used were as follows, *Bacillus cereus* F 4810 (Public Health Laboratory, London, UK), *Staphylococcus aureus* FRI 722 (Public Health Laboratory, The Netherlands), *Escherichia coli* MTCC 108 and *Yersinia enterocolitica* MTCC 859 (Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India).

Culture medium: Nutrient agar and brain heart infusion agar media and respective broths were used in the present studies.

Antibacterial activity: The antibacterial activity was determined by agar dilution method (Negi *et al*., 1999) against the test bacteria. *Yersinia enterocolitica* was grown in brain heart infusion agar and the remaining test bacteria were grown in nutrient agar at 37ºC. Each bacterial strain was transferred from stored slants at 4-5ºC to 10 mL of broth and incubated overnight at 37ºC. A preculture was prepared by transferring 1 mL of culture to 9 mL of broth and was cultivated for 48 h. The cells were harvested by centrifugation (1200 g, 5 min), washed and suspended in sterile saline under aseptic conditions.

To flasks containing 20 mL of melted cool agar, different concentrations of extracts in propylene glycol were added. An equivalent amount of propylene glycol was used as controls. One hundred µl (about 10^3 cfu/mL) of each bacterium to be tested were inoculated into the flasks under aseptic conditions. The media were then poured into sterile petri dishes, in triplicate, and incubated at 37ºC for 20-24 h. After the incubation period, the colonies were enumerated and were used to find out the growth inhibition by using the following formula:

$$\text{Inhibition (\%)} = (1 - T/C) \times 100$$

where, T is cfu/ml of extract and C is cfu/mL of control.

The lowest concentrations of the extracts capable of inhibiting the complete growth of the bacteria being tested were expressed as Minimum Inhibitory Concentration (MIC) values.

Purification: 5 mL of $SCCO_2$-1 extract was subjected to purification in a glass column (40 × 450 mm) packed with activated silica gel (60-120 mesh) and eluted with hexane and ethyl acetate at 99:1 ratio. Fractions were collected and continuously monitored by Silica gel-G coated TLC plates with hexane: ethyl acetate (90:10 v/v) as developing solvent. The spots were located by exposing the plates to iodine vapors. Fractions having the similar pattern of the spots with similar Rf values were pooled and concentrated. It was used for spectral characterization studies.

Characterization of thymoquinone:

Spectral characterization: Isolated thymoquinone besides measuring melting point was characterized spectroscopically by UV, IR, Mass and Two-Dimensional Heteronuclear Single Quantum Coherence Transfer (2D HSQCT) NMR spectra. Mass spectra were obtained using a Q-TOF Waters Ultima mass spectrometer. 2D HSQCT were recorded along with ^1H and ^{13}C NMR spectra on a Bruker Avance AQS-500 MHz NMR spectrometer (500.13 MHz for ^1H and 125 MHz for ^{13}C) at 35ºC using 5 mg of the isolated thymoquinone in DMSO-*d6* solvent. In the NMR data, only resolvable signals are shown. Some of the assignments are interchangeable. Certain quaternary carbons and proton signals could not be detected.

Thymoquinone: Solid, mp 45.5ºC; UV (alcohol, λ_{max}): 278 nm ($\pi \rightarrow \pi^*$, ε_{278}-2420 M^{-1}); IR (KBr) (stretching

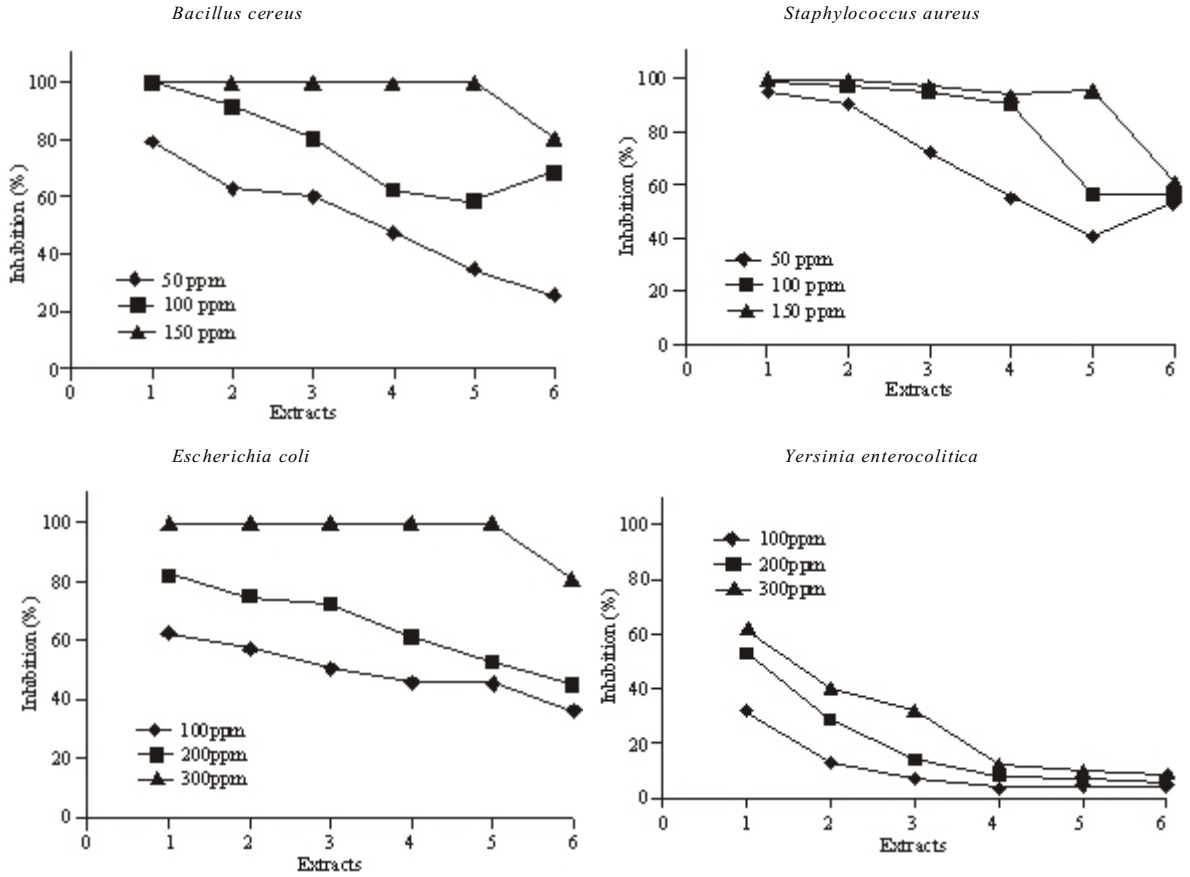

Fig. 1: Inhibition of bacterial growth by black cumin seed extracts: 1. $SCCO_2$-1 (120 bar/40C); 2. $SCCO_2$-2 (280 bar/50C); 3. Ethyl acetate; 4. Methanol; 5. Hexane; 6. Methanol: Water (70:30 v/v) (SD values varied from 0-1.70% for *Bacillus cereus*, 0.39-2.03% for *Staphylococcus aureus*, 0-1.68% for *Escherichia coli*, and 0.10-1.42% for *Yersinia enterocolitica*)

frequency): 2970 cm^{-1} (CH), 1690 cm^{-1}(C=O); MS (*m/z*) 164.12 $[M]^+$; 2D HSQCT (DMSO-d_6) ^1H NMR δ_{ppm} (500.13): 6.59 (H-2), 6.72 (H-5), 2.12 (H-8, 5.3 Hz), 1.93 (H-9, 5.3 Hz), 1.94 (H-10, 5.3 Hz); ^{13}C NMR δ_{ppm} (125 MHz): 145.1 (C1), 133.5 (C2), 188.3 (C3), 187.3 (C4), 133.3 (C5), 156.4 (C6), 14.9 (C7), 31.0 (C8), 26.7 (C9), 26.7 (C10).

Quantification of thymoquinone: Quantification was carried out by HPLC on a C18 reversed-phase µ Bondapak analytical column (300 x 3.9 mm, 10 µm particle size), using an isocratic mobile phase of water: methanol: 2-propanol (50:45:5% v/v) at a flow rate of 1 ml/min. The elution was monitored by diode array detector at 254 nm.

RESULTS AND DISCUSSION

The present investigation reveals that the $SCCO_2$ extracts showed an effective antibacterial activity against the tested bacteria than the conventional solvent extracts. The $SCCO_2$-1 (120 bar/40ºC) extract was more effective to inhibit the growth of tested bacteria which was followed by $SCCO_2$-2 and then by ethyl acetate extracts (Fig. 1). *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli* showed maximum inhibition to both $SCCO_2$ and solvent extracts except methanol: water (70:30 v/v) extract. These observations were reflected in the MIC values of these extracts for different bacteria. MIC value of the $SCCO_2$-1 extract for different bacteria was the lowest at 100-350 ppm followed by $SCCO_2$-2 and ethyl acetate extracts. *Yersinia enterocolitica* was the most resistant bacterium to all the tested extracts as inferred from higher MIC values observed for it (Table 1).

All the evaluated extracts were found to be active against Gram-positive than Gram-negative bacteria. The higher resistance of Gram-negative bacteria to external agents has been earlier documented, and it is attributed to the presence of lipopolysaccharides in their outer membranes, which make them inherently resistant to antibiotics, detergents and hydrophilic dyes (Nikaido and Vaara, 1985). Results of the present study are consistent with the literature data reported by Agarwal *et al.* (1979) and Alhaj *et al.* (2008).

Thymoquinone was characterized spectroscopically by UV, IR, Mass and 2D HSQCT NMR. ^1H and ^{13}C 2-D

Table 1: Minimum inhibitory concentration (MIC) of black cumin seed extracts

Extracts	MIC[a] (ppm)			
	Bacillus cereus	*Staphylococcus aureus*	*Escherichia coli*	*Yersinia enterocolitica*
$SCCO_2$-1 (120bar/40ºC)	100	175	300	350
$SCCO_2$-2 (280 bar/50ºC)	150	175	300	450
Ethyl acetate	150	200	300	500
Methanol	150	200	300	500
Hexane	150	225	300	550
Methanol: water (70:30 v/v)	200	250	350	550

[a]: MIC is defined as the lowest concentration of the extract capable of inhibiting the complete growth of the bacterium

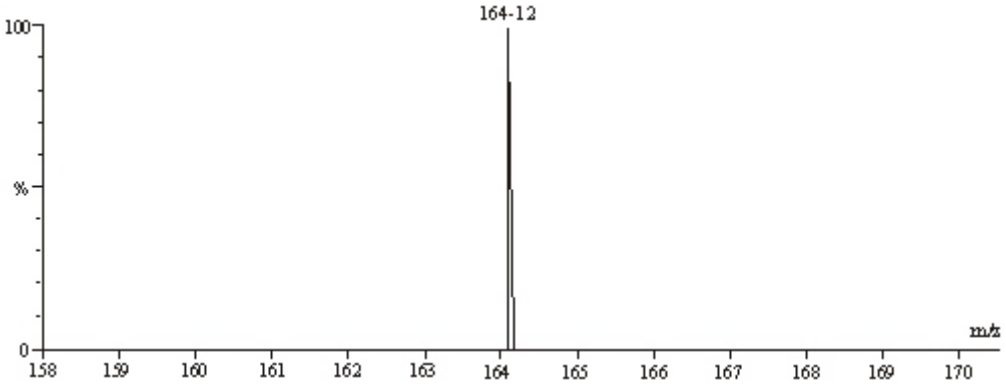

Fig. 2: ESI (-ve) MS finger print of purified $SCCO_2$-1 (120 bar/40ºC) extract of black cumin seeds

Table 2: Thymoquinone content and extract yield for different extracts of black cumin seed

Extracts	Thymoquinone content (%)	Extract yield[a] (%)
$SCCO_2$-1 (120bar/40ºC)	12.3±0.24	07.8±0.32
$SCCO_2$-2 (280 bar/50ºC)	07.1±0.32	26.0±1.21
Ethyl acetate	03.2±0.05	42.7±1.21
Methanol	02.7±0.16	37.3±1.30
Hexane	01.2±0.11	32.0±1.01
Methanol: water (70:30 v/v)	00.6±0.03	10.6±0.48

[a]: Extracts yield were expressed as % on dry weight basis of seeds

HSQCT spectra showed the presence of two carbonyl groups at 187.3 ppm and 188.3 ppm. Further, two aromatic protons were detected at 6.59 ppm and 6.72 ppm. A CH_3 group attached to an aromatic ring was detected at 14.9 ppm in the carbon spectrum. The presence of isopropyl group was detected by observing signals at 1.93 ppm and 1.94 ppm (doublet) and 2.12 ppm, all with the coupling constant of 5.3 Hz. A typical ESI (- ve) MS finger print of purified $SCCO_2$-1 extract was depicted in Fig. 2 showed good correspondence for an *m/z* value of 164. All these confirmed that it could be the antibacterial principle: thymoquinone.

Using the calibration curve, the quantification of thymoquinone was achieved at 254 nm ranging from 0.6 to 12.3% (Table 2). $SCCO_2$-1 extract had higher thymoquinone content of 12.3% which is in agreement with similar values reported by Erkan *et al.* (2008). The extracts yield was calculated based on dry weight of the seed material and it ranged from 7.8 to 42.7% dw (Table 2).

CONCLUSION

The present study shows that supercritical CO_2 extraction at 120 bar/40ºC is effective to obtain *Nigella sativa* extracts with antibacterial activity and better recovery of thymoquinone. It was also found that, the maximum recovery of thymoquinone without degradation is possible only in $SCCO_2$ extraction, which was higher than to conventional solvent extracts besides exhibiting a direct correlation to antibacterial activity.

ACKNOWLEDGMENT

The first author acknowledges the management of RVS College of Arts and Science, Coimbatore, Tamil Nadu, India for sponsoring as a Teacher Research Fellow at CFTRI, Mysore and also the Director of CFTRI, Mysore for providing the necessary facilities.

REFERENCES

Abdel-Fattah, A.F.M., K. Matsumoto and H. Watanabe, 2000. Antinociceptive effects of *Nigella sativa* oil and its major component, thymoquinone, in mice. Eur. J. Pharmacol., 400: 89-97.

Agarwal, R., M.D. Kharya and R. Shrivastava, 1979. Antimicrobial and anthelmintic activities of the essential oil of *Nigella sativa* Linn. Ind. J. Exp. Biol., 17: 1264-1265.

Alhaj, N.A., M.N. Shamsudin, H.F. Zamri and R. Abdullah, 2008. Extraction of essential oil from *Nigella sativa* using supercritical carbon dioxide: Study of antibacterial activity. Am. J. Pharmacol. Toxicol., 3: 225-228.

Ali, B.H. and G. Blunden, 2003. Pharmacological and toxicological properties of *Nigella sativa*. Phytother. Res., 17: 299-305.

Erkan, N., G. Ayranci and E. Ayranci, 2008. Antioxidant activities of rosemary (*Rosmarinus officinalis* L.) extract, black seed (*Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. Food Chem., 110: 76-82.

Negi, P.S., G.K. Jayaprakasha, L.J.M. Rao and K.K. Sakariah, 1999. Antibacterial activity of turmeric oil - A byproduct from curcumin manufacture. J. Agric. Food Chem., 47: 4297-4300.

Nikaido, H. and M. Vaara, 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev., 49: 1-32.

Randhawa, M.A. and M.S. Al-Ghamdi, 2002. A review of the pharmacotherapeutic effects of *Nigella sativa*. Pak. J. Med. Res., 41: 77-83.

Reverchon, E., 1997. Supercritical fluid extraction and fractionation of essential oils and related products. J. Supercrit. Fluids., 10: 1-37.

Udaya Sankar, K., 1989. Studies on physico-chemical characteristic of essential oil of pepper (*Piper nigrum* L.) obtained by supercritical carbon dioxide. J. Sci. Food. Agric., 48: 105-112.