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ENHANCING THE ANTIMICROBIAL ACTIVITY OF *NIGELLA SATIVA* CRUDE OIL BY COMBINATION WITH SOME AROMA COMPOUNDS

Hamdy A. Shaaban[1], Asmaa A. Taha[2] and Amr E. Edris[1]*

[1]Aroma & Flavor Chemistry Department, National Research Center, [2]Department of Food Sciences and Nutrition, National Research Centre, El Behose Street, Dokki, Cairo, Egypt

*Corresponding author: amr_edris@hotmail.com

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ABSTRACT

The antimicrobial activity of *Nigella sativa* crude oil (NCO) was investigated against four pathogenic bacteria namely, *E. coli*, *S. typhimurium*, *L.monocytogenes* and *S. aureus*. The activity was re-assessed after combining NCO at 10.0% of its weight with three individual aroma compounds namely eugenol, carvacrol and cinnamaldehyde. Results showed that NCO has antimicrobial activity against the four tested organisms with pronounced effect against *S. aureus*. Combined NCO/cinnamaldehyde showed significantly higher antimicrobial activity than NCO alone against all microorganisms. NCO/eugenol combination was more antimicrobial active against *L. monocytogenes* only compared with NCO. There was no significant difference between the antimicrobial activity of NCO/carvacrol and NCO alone. The study illustrates the potentials of enhancing the antimicrobial activity of NCO by combining with some aroma compounds especially cinnamaldehyde and eugenol. This combination may be considered as a potential line of protection against food borne pathogenic bacteria when consumed on a daily basis as dietary supplement.

Keyword: *Nigella sativa*, crude oil, dietary supplement, antimicrobial activity, combination with aroma compounds**.**

INTRODUCTION

Nigella sativa (*Black cumin*) is an herbaceous plant belongs to family (Ranunculaceae). *Nigella* crude oil (NCO) extracted from the seeds has been used to promote health and fight disease for centuries especially in the Middle East and Asia. NCO is rich in essential fatty acids especially linoleic (up to 61.0%, Hamrouni-Sellani et al., 2008) beside oleic which represents up to 26.0% (Al-Okbi et al., 2013). NCO was also found to protect the liver from the inflammatory fatty liver disease (Al-Okbi et al., 2013) beside other therapeutic and protective properties (Develi et al., 2014; Ahlatcia et al., 2014; Ahmad et al., 2013; Awadalla, 2012). NCO is also reputed for its antimicrobial activity against different pathogenic bacteria (Islam et al., 2012; Salem et al., 2010; Kumar et al., 2010; Salman et al., 2008; Arici et al., 2007). Most of this antimicrobial activity originated from the volatile oil fraction of NCO (Piras et al., 2013; Harzallah et al., 2011; Bourgou et al., 2010). Therefore, with the increasing awareness of the health promoting potentials of NCO, this oil becomes highly demanded as a dietary supplement. Also due to its antimicrobial activity, daily ingestion of NCO can be a potential line of defense against food borne illness caused by pathogenic bacteria that contaminate minimally processed or unprocessed foods.

It is known that combination of natural plant extracts like essential oils with other antimicrobial agents can improve the antimicrobial activity of that binary antimicrobial system compared with that of a single one. Examples may include conventional antibiotics/essential oils (Fadli et al., 2014), bacteriocins/essential oils (Abdollahzadeh et al., 2014; Turgis et al., 2012), lauric arginate/essential oils (Ma et al., 2013), silver ions/essential oils (Ahmad et al., 2014) and inert dust/essential oil (Campolo et al., 2014). The antimicrobial activity of combinations of different essential oils was assessed (Cherrat et al., 2014) and also reviewed (Bassolé and Juliani, 2012). Three possible biological events may happen due to that combination: including synergism, antagonism or additive action (Bassolé and Juliani, 2012).

Despite the different literatures that evaluated the antimicrobial activity of NCO (as was shown earlier), no attempt was found to enhance this activity by combination with other antimicrobial agents, as in the previous case with essential oils. Therefore, in the current work, the authors aimed to combine NCO with some individual aroma volatile compounds that already characterized with inherent antimicrobial activity. Examples of these aroma compounds will include eugenol, carvacrol, and cinnamaldehyde. The ultimate aim of this work is to report any potential enhancement of the

antimicrobial activity of NCO/aroma compound combination against common food-born pathogenic bacteria. That is expected to add an extra additive value to NCO as daily dietary supplement.

MATERIAL AND METHODS

MATERIALS

Mature dried seeds of *Nigella sativa* were purchased from a specialized herbal retail store located in Cairo, Egypt. Authentic samples of p-cymene (97.0%), thymoquinone (99.0%), carvacrol (98.0%), (99.0%), eugenol (99.0%) and cinnamaldehyde (99.0 %) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

METHODS

EXTRACTION OF *NIGELLA SATIVA* CRUDE OIL (NCO)

The seeds of *Nigella sativa* were pressed twice using commercial scale electric screw expeller pressing machine located in the same herbal retail store from which the seeds were purchased. The oil is received in dark glass bottles that were immersed in cold water to bring back the temperature of NCO to atmospheric temperature (25°C). The bottles were stored in a cooler at 4°C until the beginning of experiments in the same week.

EXTRACTION OF *NIGELLA SATIVA* VOLATILE OIL FROM NCO

The volatile oil fraction of *Nigella sativa* was extracted from NCO using hydro-distillation method for 2.5h according to our previous work (Edris, 2010). The weight of the volatile oil was recorded and the yield percent relative to NCO was calculated. The value of the yield percent was an average of two extractions ± SD.

GAS CHROMATOGRAPHIC ANALYSIS

Nigella sativa volatile oil (20.0 µl) was diluted in 1.0 ml diethyl ether in a glass vial. Then 2.0 µl of this mixture were injected (at a split ratio 10:1) into a Perkin Elmer XL GC equipped with a FID. A fused silica capillary column (60 m x 0.32 mm x 0.25 µm) coated with DB-5 (5% phenyl, 95% methyl polysiloxane) was used to separate the different EO components. The oven temperature was programmed from 50°C to 230°C at a rate of 3°C/min. The injector and detector temperatures were 230°C and 250°C respectively. Helium was used as carrier gas at a flow rate of 1.0 ml/min. Authentic samples were used to identify p-cymene and thymoquinone which are the major constituents of *Nigella sativa* volatile oil by matching their retention times after running on GC under the same conditions. All values of area percent were mean of two injections from two different extractions ± SD.

COMBINATION OF NCO WITH THE PURE AROMA VOLATILE COMPOUNDS

A certain weight of NCO was combined with 10.0% of that weight with carvacrol, eugenol and cinnamaldehyde, separately. Each batch of NCO/aroma compound was stirred using magnetic bar for 15 minute to insure homogeneity then left over night at 4ºC to equilibrate. Therefore the process of combination ends up with three batches of NCO/eugenol, NCO/ carvacrol and NCO/cinnamaldehyde at 10.0 wt% aroma compound per combination. All batches were made in triplicates.

ANTIMICROBIAL EVALUATION

MICROORGANISMS AND CULTURES

The tested microorganisms were provided from the culture collections of the Microbiological Department National Research Center (NRC) Dokki, Giza, Egypt. These include two strains of Gram-positive bacteria *Staphylococcus aureus* (ATCC 43300), *Listeria monocytogenes* (ATCC 35152) and two strains of Gram-negative bacteria *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 27325).

ANTIBACTERIAL ASSAY

Each combined batch of NCO/aroma compound (at 10.0wt%) was diluted with equal amount of isopropanol to decrease their viscosity and facilitate diffusion through the agar gel. The agar well diffusion method (Perez et al.,1990) was employed for the determination of antibacterial activities. In details: 0.1 ml of the diluted inoculums (10^7 CFU/ml) of test organism was spread on tryptone soy agar (TSA) plates. Wells of 5 mm diameter were punched into the agar medium and filled with 40.0 µl of NCO/aroma compound/isopropanol. This volume delivered 20.0 µl of combined NCO/aroma compound/well. The Petri dishes were incubated at 37°C for 18.0h. The antimicrobial activity was evaluated by measuring the zone of inhibition (mm) corresponding to each tested organism. The evaluation was conducted three replicates for each concentration.

STATISTICAL ANALYSIS FOR THE ANTIMICROBIAL EVALUATION

The comparison between the antimicrobial activity of NCO/aroma compound combinations on different bacterial strains was analyzed using one way ANOVA test ($P<0.05$). Statistical analysis was carried out using SPSS software version 16.

RESULTS

Mechanical expression of mature *Nigella sativa* seeds using electric screw expeller machine yielded 20.0±0.9 % (relative to seed's weight) of a typical light brown NCO. Hydrodistillation of NCO yielded 2.6±0.3 wt% (relative to NCO) of a volatile oil fraction. Gas chromatographic analysis indicated that this volatile fraction contained mainly thymoquinone (TQ, 68.1±0.8%) and p-cymene (20.1±0.3%) as the major constituent accounting for 88.2% of the total components. No attempt was made to identify the other compounds in the volatile fraction since full identification is out the scope of this antimicrobial investigation.

Table (1) showed that NCO had antimicrobial activity against the four tested pathogenic bacteria;

however the activity was more profound in the case of *S. aureus*. Combination of NCO/eugenol had no significant antimicrobial activity against *E. coli, S. typhimurium* and *S. aureus* compared with the NCO. However, *L. monocytogenes* was more affected by NCO/eugenol combination compared with NCO alone. On the other hand, the combination of NCO/carvacrol showed no significant antimicrobial activity against all tested pathogens compared with NCO along. Interestingly NCO/cinnamaldehyde combination showed significantly enhanced antimicrobial activity against all tested bacterial species comported with NCO. Moreover, NCO/cinnamaldehyde had significant higher antimicrobial activity than NCO/carvacrol against all tested microorganisms. There was no significant difference in antimicrobial activity between NCO/cinnamaldehyde and NCO/Eugenol against *S. typhimurium* and *L. monocytogenes*.

DISCUSSION

The yield of NCO (20.0 wt% relative to the seed's weight) is considered to be parallel (22.5±3.0 wt%) to other studies (Al-Okbi *et al.*, 2013) that used the same screw expeller pressing technique for the extraction of NCO. In fact the performing efficiency of the pressing machine and the genetic quality of the seeds can affect greatly the yield of NCO obtained by screw expeller pressing technique. It is worth indicating that solvent extraction using Soxhelt apparatus can yield much more NCO than the pressing machines (~ 33.0% based on seed's weight, data not published). However, this technique was avoided in the current study due to the potentials of contamination of NCO with solvent residue.

Hydrodistillation of NCO yielded 2.6±0.3 wt% (relative to NCO) of a volatile oil fraction. It is well known that the percentage of volatile fraction in NCO is a crucial factor that can affect on the general biological activity of that oil. For instance, NCO containing 1.8 wt% volatile fraction was found to be 10-fold more resistant to oxidation than NCO containing only 0.1% volatile oil (Edris, 2011). That is due to the high antioxidant activity of thymoquinone (Rifaioglu et al., 2013) which is the major constituent of *Nigella sativa* volatile fraction. Axiomatically, the same trend can be potentially expected regarding the antimicrobial activity of NCO due to the high antimicrobial activity of thymoquinon (Chaieb, et al., 2011; Kouidhi et al., 2011; Halawani, 2009).

The antimicrobial activity of NCO against the four tested bacterial species (Table 1) is obviously due to the high content of thymoquinone (68.1% by GC) and p-cymene (20.1% by GC) in the volatile oil fraction which represents 2.6 wt% of NCO. It was reported that thymoquinone kills *S. aureus* at 6.0µg/ml in broth dilution assay (Halawani, 2009). The vapors of thymoquinone were also found completely inhibiting to the growth of *S. aureus* in broth microdilution assay (Novy et al., 2014).

The second major constituent of the volatile fraction of NCO namely p- cymene (20.1% by GC) showed an affinity for microbial membranes however, it does not affect the membrane permeability but it may decrease its enthalpy and melting temperature (Cristani et al., 2007). In addition, this compound can perturb the microbial membranes, causing them to expand thus affecting the membrane potential of the intact cells (Ultee et al., 2002)

From Table (1) it is evident that there is an exceptional profound activity of NCO against *S. aureus* than the other microorganisms. This observation came in agreement with previous investigation which indicated that Gram +ve bacteria are more susceptible toward thymoquinone (the major constituent in the volatile fraction of NCO) than Gram –ve (Halawani, 2009). On the other hand, *L. monocytogenes* is considered to be an exception due to its well known antimicrobial resistance genes (Morvan et al., 2010) which make this organism relatively resistant to NCO despite its Gram +ve identity. From the above mentioned, NCO investigated in the current study can potentially be used as natural anti- *S. aureus* agent.

Table (1) indicated that NCO/eugenol combination had higher anti- Listerial activity compared with NCO alone. This compound performs its anti-Listerial action by affecting bacteria cell membrane. That was concluded from the observed accumulation of external K^+ above 300 µg mL^{-1} of eugenol (Filgueiras and Vanetti, 2006).

Combination of NCO/carvacrol showed no enhancement of the antimicrobial activity compared with NCO. This data was unexpected due to the well known and wide spectral antimicrobial activity of this compound (Higueras et al., 2014; Juliane et al., 2014; Dharmalingam and Nazni (2013), Nostro and Papalia, 2012). That may indicate neither antagonism nor synergistic can happen between carvacrol and NCO constituents.

Interestingly, NCO/cinnamaldehyde showed significantly higher antimicrobial activity against all tested microorganisms compared with NCO alone. The potential mechanism of action of cinnamaldehyde is by inhibition of cell wall synthesis (Kwon *et al.*, 2003) or inhibition of biosynthetic enzymes (Wendakoon, and Sakaguchi, 1995).

From the above mentioned results illustrated in Table (1) one can notice the profound antibacterial activity of two combinations that includes NCO/cinnamaldehyde followed by NCO/eugenol. Previous investigation shown that both cinnamaldehyde and eugenol exhibited strong antimicrobial properties on different pathogenic bacteria like *E. Coli* O157:H7 and *Salmonella* (Yossa *et al.*, 2012; Nazni and Dharmaligam, 2013). These compounds showed high antimicrobial activity against beta lactamase producing entero-bacteriaceae with cinnamaldehyde being more active than eugenol (Dhara, and Tripathi, 2013). Both compounds also showed antimicrobial activity against human gastric pathogen like *Helicobacter pylori* (Ali et al., 2005).

Applying the results of the current study in actuality, i.e. by combining NCO with cinnamaldehyde or eugenol or both in one dosage form for application as daily supplement may seem to be attractive. However there is a noticeable drawback of this combination manifested by the restrictions that regulate the consumption of cinnamaldehyde and eugenol on a daily basis.

In more details, NCO is usually used as a daily supplement in capsules at an average recommended daily intake dose that varies depending on the manufacturer. For instance, one of the manufacturers of NCO capsules in Egypt recommends that NCO be taken as two capsules (450 mg each), three times a day, and accounting for a total of 2700 mg NCO/person/day. Combining NCO with 10.0% of its weight of cinnamaldehyde or eugenol (as described in the current study) will dictate the decrease of that daily intake dose to only 875 mg and 1750 mg /person /day, for cinnamaldehyde and eugenol fortified NCO respectively. That is obviously due to the legislations that regulate the acceptable daily intake (ADI) of consuming these two aroma chemicals on a daily basis.

Table 1 Antimicrobial activity of *Nigella* crude oil and its combination with individual aroma compounds.

Pathogenic bacteria	Tested formula (20.0 µl/well) dissolved in 20 µl isopropanol			
	NCO	NCO/Eugenol*	NCO/Carvacrol*	NCO/cinnamaldehyde*
Control (isopropanol)	-ve	-ve	-ve	-ve
Escherichia. coli	11.0^{b}±0.8	11.0^{b} ±2.6	12.0^{b}±2.2	16.0^{a} ± 1.3
Salmonella typhimurium	11.0^{b}± 0.5	11.0^{ab}±0.5	11.0^{b}±1.1	13.0^{a}±0.5
Listeria monocytogenes	11.0^{c}±1.3	13.0^{ab}±0.8	11.0^{bc}±2.1	15.0^{a}±2.1
Staphylococcus aureus	18.0^{b}±0.5	18.0^{b}±0.5	19.0^{b}±0.5	21.0^{a}±1.0

NCO: *Nigella* crude oil
*Each aroma compound namely eugenol, carvacrol and cinnamaldehyde represented 10.0% of the weight of the tested formula (without isopropanol) .
Within the same row, means (inhibition zone mm) ±SD, with different letters are significantly different ($p < 0.05$)
-ve: no antimicrobial activity

For instance, the ADI of cinnamaldehyde and eugenol is 1.25mg and 2.5 mg/kg body weight/day (Joint FAO/WHO, 2000; Joint FAO/WHO, 1982), respectively. Therefore, for a 70.0 Kg average person, the ADI of cinnamaldehyde and eugenol will be 87.5mg/day and 175.0 mg/day, respectively. Considering 10.0 wt% of these aroma chemicals in NCO, that will restrict the ingestion of NCO to only 875 mg and 1750 mg /day instead of the recommended 2700 mg/day. Therefore, one should consider the reduction in the amount consumed of that nutritive NCO which bears up to 87.0 wt% of total essential fatty acids versus the potentially high antimicrobial protective benefits of a daily dose of NCO/aroma compounds.

This problem can easily be overcome by including capsules containing the regular pure NCO in the same package beside the NCO/aroma capsules, to finally deliver the recommended dose of NCO/day.

CONCLUSION

Combination of NCO with 10.0% of its weight of eugenol or more preferably cinnamaldehyde can increase its protecting effect against some food transmitted pathogenic bacteria. However, that will be on the expense of reducing the usual dose of daily ingested NCO which is a manageable problem that can be managed as indicated previously in the study.

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