Inhibition of *Staphylococcus aureus* by *Nigella sativa* products using different extraction method

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Abstract:

The effect of oil, ethanol and total alkaloids extracted from the seeds of *Nigella sativa* L. (Ranunculaceae) by three different methods (soxhlet, solvent extraction, and reflux techniques) on the growth of *Staphylococcus aureus* was evaluated. Five concentrations (100 µl, 130 µl, 150 µl, 170 µl, 200 µl) of these extracts were utilized. The ethanol and alkaloid extracts performed better antibacterial activities than others, whereas oil extracts showed good activities only in high concentrations. Most of data of extracts showed dose dependent antibacterial effects. This study presents that solvent extraction method was the best method of extraction as it recorded better action on the growth of *Staphylococcus aureus*.

Keywords: Nigella sativa, Ranunculaceae, Soxhlet,, Alkaloid, Staphylococcus aureus.

1. INTRODUCTION:

Seeds of *Nigella sativa* L. (Ranunculaceae) commonly known as black seed or black cumine, are used in folk (herbal) medicine all over the world for the treatment and prevention of a number of diseases and conditions that include asthma, diarrhea and dyslipidaemia [1]. The plant extracts and essential oil showed a broad range of pharmacological effects such as anti diabetic [2,3]. The extracts of the plant also showed anti microbial *in vivo* effect [4,5]. The oil of the plant protects against induced hepato-toxicity and improves serum lipid profile in rats [6]. The proteins fractionated from the plant showed immune modulator effect using non-activated or mitogen activated cells [7], the crude and ethanol extract of the seed showed hepato protective activity [8,9]. *Nigella sativa* seeds possess clinically useful anti-H. pylori activity, comparable to triple therapy clarithromycin, amoxicillin, omeprazole [10].

In the current work, the effect of oil, ethanol and total alkaloid extracts of *Nigella sativa* L. seeds were studied on the growth of pathogenic bacteria *Staphylococcus aureus*, to evaluate the extraction procedure that yields best antibacterial effects bearing in mind that natural products may play a future role by replacing or substituting antibiotics that faces the great threat of over all resistance.

2. MATERIALS AND METHODS:

2.1 Materials

The seeds used in this study were purchased from local market in Hawler\ Kurdistan Region (season 2008). Seeds were cleaned and powdered.

2.2 Methods:

2.2.1 Extraction process: [11].

2.2.1.1 Soxhlet extraction:

Sixty grams of the *Nigella sativa* L. seeds was extracted with (300 ml) petroleum ether (60-80 ℃) using Soxhlet apparatus for 3 hours, after evaporation of the solvent by rotary evaporator, yellow oily extract (yield 20 % w/w) was obtained. The final un-extracted portion was air dried then the same procedure was repeated using ethanol (96%) for 3 hours, the extract was evaporated a yellow-greenish oily extract (18.2%) yielded

2.2.1.2 Solvent extraction [12]:

The powdered seed 60 gm was soaked in 300 ml of petroleum ether (60-80 ℃) at 30 ℃ for 3 hours with stirring. The mixture was filtered using Whatman No.1 filter paper. The final extract was evaporated as previously mentioned to obtain yellow-greenish oily extract (yield 19.5%).

The ethanol extract was obtained by the same method and its colour was greenish (yield 17.8%).

2.2.1.3 Reflux extraction:

Sixty grams of the powdered seed was extracted for 3 hours using petroleum ether (60-80 ℃) by reflux apparatus in a water bath. The mixture was filtered and evaporated as in method 2 the crude extract was yellow-greenish oil (yield 20.9%).

The ethanol extract was obtained by repeating the same procedure after drying the un-extracted portion and greenish oily compound was obtained (yield 19.2%).

2.2.2.1 Separation of alkaloids:

Three grams of the ethanol extract from each of the three methods were added slowly, with stirring into 100 ml of 2% hydrochloric acid. The acidic extract is then cooled, filtered and placed in a separator funnel, where it is made alkaline by adding ammonium hydroxide solution. The alkaline solution was extracted, three times with 100 ml of chloroform. The chloroform layer is then evaporated by rotar evaporator under reduced pressure to obtain total brownish alkaloid (yield 8.5, 8.3 and 8.7%) respectively [13].

The total alkaloids were identified by thin layer chromatography, that a small amount of the extract to be analyzed is spotted near the bottom of a sheet of glass coated with a thin layer of a solid (Al2O3) as adsorbent. The plate is then placed in a shallow pool of a solvent in a developing chamber which is the mobile phase, solid iodide was used for visualization. Retention factor R_f was measured using a ruler.

$R_{f1\ (\text{for the total alkaloids using method 1})}$ = 0.5 0.75 0.86 (Benzene 5: chloroform 5)

$R_{f2\ (\text{for the total alkaloids using method 20})}$ = 0.5 0.8 (Benzene 5: chloroform 5)

$R_{f3\ (\text{for the total alkaloids using method 3})}$ = 0.54 0.67 0.77 0.89 (Benzene 5: chloroform 5)

2.2.3. Antibacterial activity:

Thirty clinical strains of the bacterial cultures were obtained from Rizgary teaching Hospital and the College of Science, Department of Biology, Microbiology laboratory. Bacterial cultures were activated by transferring a loop full of bacterial culture to a test tube containing 10 ml of sterilized nutrient broth, incubated at 37 ˚C for 4 hours. Appropriate dilutions were made by using sterilized peptone water 0.1% (10^{-1}-10^{-5}).

Nutrient agar prepared and poured into sterile Petri dishes to a depth of 4 mm. In each plate, five holes were made each of 7 mm in diameter using a sterile cork. The plates were streaked by sterile cotton wood swab which dipped into diluted bacterial suspension (10^{-5}) and 100, 130,

150, 170 and 200 µl of the petroleum ether, ethanol and alkaloid extracts of the three methods were added to these holes with sterile micro pipettes, the plates were incubated at 37 ˚C for 18-24 hours, then the diameter of inhibition zone were measured in millimeter using a ruler [11].

2.2.4. Statistical analysis:

All data are expressed as means of standard error of the mean (M ±SE) and statistical analysis was carried out using statistically available software (SPSS Version 15). Data analysis was made using two-way analysis of variance (ANOVA). The comparison between methods and extracts were done using Duncan-test..(P<0.05)was considered as statistical significant.

3. RESULTS:

The effect of different concentrations of oil, ethanol, and total alkaloids of the seeds of *Nigella sativa* L., extracted by three different methods soxhlet (1), solvent extraction (2), and reflux (3) techniques for 3 hours, were tested against gram-positive bacteria *Staphylococcus aureus* using diffusion method. Table (1) shows the antibacterial effect of the oil, ethanol and total alkaloids using soxhlet apparatus.

Table (1): In vitro effect of different amounts of the extracts of *N. sativa* L seed by using soxhlet apparatus against *Staphylococcus aureus.*

Amounts of extracts µl	Mean diameter of inhibition zone (mm) ± standard error			
	Oil	Ethanol	Alkaloid	Mean
100	0.000± 0.000^{a}	0.000± 0.000^{a}	0.000± 0.000^{a}	0.000± 0.104a
130	0.000± 0.000^{a}	0.000± 0.000^{a}	0.000± 0.000^{a}	0.000 ± 0.104^{a}
150	0.000± 0.000^{a}	10.633± 0.31^{b}	0.000± 0.000^{a}	3.544 ± 0.104^{b}
170	13.966± 0.548^{c}	11.000± 0.000^{b}	15.000± 0.00^{d}	13.322 ± 0.104^{c}
200	16.00± 0.115^{e}	14.000± 0.251^{c}	16.90± 0.057^{f}	15.633 ± 0.104^{d}
Mean	5.993± 0.080^{a}	7.126± 0.080^{c}	6.380± 0.080^{b}	P= 0.000

The same letters mean no significant difference. The different letters mean significant difference at P < 0.05.

The amount of (100, 130 and 150) µl of the oil extract had no significant (p< 0.05) inhibition effect while (170) µl of this oil significantly (p< 0.005) inhibited bacterial growth. However, (200µl) of the total alkaloid had the highest significant inhibitory effects. When solvent extraction (Table2) is used, a significant (p<0.05) difference between type and the amount of the extracts was observed. When comparing alkaloid extract to oil and ethanol extracts, alkaloids statistically produced a large zone of inhibition (Table2).

Table (2): In vitro effect of different amounts of the extracts of *N. sativa* L. seed by using solvent extraction method against *Staphylococcus aureus.*

Amounts of extracts µl	Mean diameter of inhibition zone (mm) ± standard error			
	Oil	Ethanol	Alkaloid	Mean
100	9.966± 0.120[a]	11.066± 0.03[b]	12.033± 0.06c	11.000 ± 0.09[a]
130	11.066± 0.03[b]	14.03± 0.120[e]	14.033± 0.03e	13.044 ± 0.09[b]
150	13.033± 0.03[d]	15.00± 0.152[f]	20.000± 0.10[g]	16.011 ± 0.09[c]
170	11.000± 0.09[a]	25.03± 0.466[h]	20.000± 0. 00[g]	22.51 ± 0.11[d]
200			25.033± 0.06[h]	25.033 ± 0.066[e]
Mean	11.355± 0.090[a]	16.26± 0.078[b]	18.220± 0.07[c]	P= 0.000

The same letters mean no significant difference. The different letters mean significant difference at P<0.05.

Table (3): In vitro effect of different amounts of the extracts of *N.sativa* L seed by using reflux apparatus against *Staphylococcus aureus.*

Amounts of extracts µl	Mean diameter of inhibition zone (mm) ± standard error			
	Oil	Ethanol	Alkaloid	Mean
100	0.000± 0.000[a]	0.000± 0.000[a]	11.03± 0.03[b]	3.677± 0.22[a]
130	0.000± 0.000[a]	14.033± 0.066[e]	12.00± 0.000c	8.677± 0.22[b]
150	0.000± 0.000[a]	15.000± 0.152[f]	12.00± 0.00c	9.000± 0.27[c]
170	0.000± 0.000[a]	25.0± 0.466[h]	12.93± 0.8d	12.666± 0.038[d]
200			12.93± 0.3[d]	12.933± 0.038[d]
Mean	0.000± 0.022[a]	13.33± 0.027[c]	12.18± 0.017[b]	P= 0.000

The same letters mean no significant difference. The different letters mean significant difference at P < 0.05.

Table (4): In vitro effect of different amounts of three types of oil of *N.sativa* L seed against *Staphylococcus aureus.*

Amounts of extracts µl	Mean diameter of inhibition zone (mm) ± standard error			
	Oil(1)	Oil(2)	Oil(3)	Mean
100	0.000± 0.000[a]	9.966± 0.120[b]	0.000± 0.000[a]	3.322± 0.100[a]
130	0.000± 0.000[a]	13.06± 20[e]	0.000± 0.000[a]	3.688± 0.100[a]
150	0.000± 0.000[a]	15.0± 0.152[f]	0.000± 0.000[a]	4.344± 0.100[b]
170	13.96± 0.48[e]		0.000± 0.000[a]	13.966± 0.173[c]
200	16.0± 0.115[f]			16.000± 0.173[d]
Mean	5.93± 0.078[b]	11.35± 0.100[c]	0.000± 0.000[a]	P= 0.000

The same letters mean no significant difference. The different letters mean significant difference at P < 0.05.

Table (3) summarize the in vitro effect of the extracts using reflux method (3), as it shown from the Table, the oil extract showed no significant inhibition, while ethanol and alkaloid extracts resulted in most significant ($p<0.05$) inhibition. The effects of different amounts of oils extracted using three different methods (1, 2, and 3) in Table (4), showed a significant difference between the oils and the amount of the oils observed, oil (2) exhibited better inhibitory effect at (100µl) and oil (3) showed no significant ($p< 0.005$) inhibitory effects.

The antibacterial activity of ethanol extracts (1, 2, and 3) against *Staphylococcus aureus* is shown in Table (5) a significant difference were observed between the ethanol extracts (1, 2, and 3). Ethanol (2) had significantly ($p<0.05$) the most inhibitory effect and ethanol (1) showed the least significant inhibition. The *in vitro* effect of total alkaloids (1, 2,

and 3) is shown in Table (6), it is clear that alkaloid (2) resulted in a most significant inhibition at all amounts, and only the amounts (170µl and 200µl) of the alkaloid extract (1) showed inhibitory effects.

Table (5): In vitro effect of different amounts of the ethanol extracts of *Nigella sativa* L seed against *Staphylococcus aureus.*

Amount of extracts µl	Mean diameter of inhibition zone (mm) ± standard error			
	Ethanol 1	Ethanol 2	Ethanol 3	Mean
100	0.00± 0.00^{a}	9.966± 0.120^{b}	0.000± 0.000^{a}	3.688± 0.113^{a}
130	0.00± 0.00^{a}	13.06± 0.120^{e}	0.00± 0.00^{a}	9.333± 0.113^{b}
150	0.00± 0.00^{a}	15.00± 0.152^{f}	0.00± 0.00^{a}	13.33± 0.138^{c}
170	13.96± 0.58^{e}		0.00± 0.00^{a}	20.022± 0.138^{e}
200	16.0± 0.115^{f}			14.000± 0.195^{d}
Mean	5.93± 0.078^{b}	11.35± 0.100^{c}	0.00± 0.00^{a}	P =0.000

The same letters mean no significant difference. The different letters mean significant difference at $P < 0.05$

Table (6): In vitro effect of different amounts of the alkaloid extracts of *N. sativa* L seed against *Staphylococcus aureus*

Amounts of extracts µl	Mean diameter of inhibition zone (mm) ± standard error			
	Alkaloid (1)	Alkaloid (2)	Alkaloid (3)	Mean
100	0.000± .000^{a}	12.033± .033^{c}	11.03± 0.033^{b}	7.689± 0.027^{a}
130	0.00± 0.000^{a}	14.033± .033^{e}	12.00± 0.000^{c}	8.678± 0.027^{b}
150	0.000± .000^{a}	20.00± 0.100^{h}	12.00± 0.000^{c}	10.67± 0.027$^{c\,c}$
170	15.000 0.000^{e}	20.00± 0.000^{h}	12.933± 0.88^{d}	15.978± 0.027^{d}
200	16.900± .050^{g}	25.033± 0.06^{i}	12.93± 0.033^{d}	18.289± 0.027^{e}
Mean	6.380± 0.021^{a}	18.22± 0.021^{b}	12.180± 0.020^{c}	P=0.000

The same letters mean no significant difference. The different letters mean significant difference at $P < 0.05$

4. DISCUSSION:

The results of this study, indicates that crud extracts of *Nigella sativa* L. have antibacterial activity towards gram–positive bacteria *Staphylococcus aureus*. The results are in agreement with previous reports that crude extracts of *Nigella sativa* have antimicrobial effects and cytotoxic actions [14, 15, 16, 17, 18]. *Nigella sativa* oil is active against sensitive as well multi drug resistant strains of staphylococcus aureus and may be used therapeutically in susceptible cases [19].

This study was an attempt to evaluate the extraction method which yields the best antibacterial action on *Staphylococcus aureus* using agar diffusion method.

It appears that there are significant differences ($p<0.05$) between the effects of oil, ethanol, and total alkaloid extracts, which extracted by using the same method,on growth of *Staphylococcus aureus*, because each extract may contain different active compounds. The significant differences observed between the effects of same extracts by using different methods, could be due to the differences in ethanol 3 and alkaloids 3 which extracted by the same method, the reasons for this could be due to the evaporation and / or destruction of the active compounds such as aromatic and saturated organic compounds during refluxing, while alkaloids have high melting points will remain.It has has been found that soxhlet apparatus is a good method for extracting essential oils and organic compounds without alkaloids which may be due to the time of extraction, because the traditional soxhlet extraction is time and energy consuming.

However, the findings that oil 2, ethanol 2 and alkaloid 2 extracted by using solvent extraction method were more active against *Staphylococcus aureus*, could be due to the type of extraction, because this method is rapid, more efficient, consumes less solvent, it dose not need heating and has been the most common method in fruit preparation [20].

According to our results we can conclude that, significant differences were found between the three types of extraction methods, and solvent extraction is the selected method for the extraction of natural products, since it has the greatest impact on the growth of *Staphylococcus aureus.*

The procedures of extraction. Also significant difference ($p<0.05$) were observed in antibacterial effects by increasing the amounts of extracts from (100 to 200µl), because the later contains more active compounds.

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ڕاگرتنی گەشەی بەکتریای *Staphylococcus aureus* لەلایەن بەرهەمی ڕەشکە بەبەکارهێنانی ڕێگەی دەرهێنانی جیاواز

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پوختــە

پێکهاتەی کیمیاویی و لە دەرەوە چالاکی دژە بەکتریای *Staphylococcus aureus* ***ی هەندێک لە وەرگیراوەکانی دەنکی ڕەشکە*** لەم لێکۆڵینەوەیەدا سێ ڕێگای جیاواز (سۆکسلێت، دەرهێنان بە تووێنەر، ڕێگای خەستکەرەوە) بۆ دەرهێنانی سێ وەرگیراوی دەنکی ڕەشکەی کوردستانی عێراق، هەولێر، ساڵی 2008 هەڵبژێردران. وەرگیراوەکان بریتی بوون لە: وەرگیراوی ئیثەری پترۆڵی (ڕۆن) و، وەرگیراوی کهولی و ماددە لە تفت چووەکان. هەر ڕێگایەک بۆ ماوەی سێ کاتژمێر بەکارهێنرا. وە هەموو وەرگیراوەکان بە چینی تەنکی کرۆماتۆگرافی دا بردران و بە پێنج پەیتی جیاواز دژی گەشەی بەکتریای (*Staphylococcus aureus*) بەکارهێنران. بەگشتی جیاوازییەکی مەعنەوی ($p < 0.05$) لە نێوان ڕێگاکانی دەرهێنان بەدی کرا. هەروەها لەم تووێژینەوەیەدا تێبینی ئەوە کرا کە وەرگیراوەکانی ڕێگای دەرهێنان بە تووێنەر چالاکتر بوون لە وەرگیراوەکانی ڕێگاکانی تر.

تثبيط نمو البكتريا *Staphylococcus aureus* من قبل نواتج الحبة السوداء باستعمال طرق استخلاص مختلفة

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الخلاصــة

تم في هذا البحث اختيار ثلاث طرق مختلفة وهي: طريقة سوكسليت، طريقة النقع وطريقة الرفليكس. لإستخلاص ثلاث مستخلصات من بذور حبة السوداء المجني في كوردستان العراق في سنة 2008، كل طريقة لمدة ثلاث ساعات. المستخلصات كانت عبارة عن: مستخلص الإيثر البترولي، المستخلص الكحولي والقلويدات. وتم تمرير جميع المستخلصات عبر كروماتوطرافيا الطبقة الرقيقة. واختبر كل مستخلص بخمس تراكيز مختلفة ضد نمو بكتريا *Staphylococcus aureus*. بينت النتائج أن هناك فرق معنوي ($p < 0.05$) بين الطرق المستخدمة. ووجد أيضاً أن المستخلصات المستخلصة بطريقة الإستخلاص بالمذيب أكثر فعالية من المستخلصات الأخرى.