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Original Article

An Assessment of the Antibacterial Activities, Phytochemistry and Toxicities of *Nigella damascena* L. Seed Extracts

Jinwoo Kim[1], Enoch John Rusford[1], Gagan Tiwana[1], Sean Robert Alcorn[1], Ian Edwin Cock[2,3], Matthew James Cheesman[1,*]

[1]School of Pharmacy and Medical Sciences, Gold Coast Campus, Griffith University, Gold Coast, AUSTRALIA.
[2]School of Environment and Science, Nathan Campus, Griffith University, Brisbane, AUSTRALIA.

ABSTRACT

Background: *Nigella damascena* L. (ND) seeds have been incorporated into traditional plant-based medicinal systems for millennia. However, there are conflicting reports on the antibacterial activities of the seed extracts, although phytochemicals known to possess antibacterial activity have been found in the seeds of this species. The toxicities of the extracts have not been studied previously. **Aim:** This work assesses the ability for aqueous and methanolic ND seed extracts to inhibit the growth of a panel of skin pathogens on agar and in broth, including methicillin-resistant *Staphylococcus aureus* (MRSA). Qualitative determinations of the phytochemical constituents of the extracts were performed, as well as their toxicity levels. **Materials and Methods:** Bacterial growth inhibition was evaluated using agar disc diffusion and liquid broth microdilution assays, whilst *Artemia* nauplii bioassays were used to screen toxicities. Qualitative phytochemical assays were conducted to assess the relative abundance of several important phytochemical compound classes. **Results:** No inhibition was observed on agar for either the aqueous or methanolic extracts against *S. aureus*, MRSA, *S. epidermidis* or *S. pyogenes*. This was concordant with the liquid microdilution broth assays, with the exception of the methanolic ND extract against *S. pyogenes*, which produced a minimum inhibitory concentration value of 481 µg/mL. Phenols, saponins, tannins and alkaloid were present in both extract types, while flavonoids could be detected in the methanolic extract only. Both extracts were deemed to be nontoxic in the *Artemia* nauplii assay. **Conclusion:** The ND methanolic extract possessed activity against *S. pyogenes* in liquid broth, but not on agar. The presence of flavonoids in this extract may be responsible for this activity. The extracts were inactive against *S. aureus*, MRSA and *S. epidermidis* in agar and broth assays, and were also nontoxic as adjudged by the *Artemia* nauplii brine shrimp assays.

Keywords: *Nigella damascena*, Natural products, Antibiotic resistance, MRSA, Toxicities, Bacterial growth.

Correspondence:
Dr. Matthew James Cheesman
School of Pharmacy and Medical Sciences, Gold Coast Campus, Griffith University, Gold Coast, AUSTRALIA.
Email: m.cheesman@griffith.edu.au

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INTRODUCTION

Antimicrobial resistance (AMR) has become a serious global concern in recent decades. The World Health Organization (WHO) has estimated that the number of worldwide deaths caused by AMR will supersede those caused by cancer by the year 2050.[1] Many cases have arisen due to infections triggered by the multidrug resistant (MDR) *S. aureus* species MRSA (methicillin-resistant *Staphylococcus aureus*), which results in high morbidity and mortality, and novel treatment strategies are urgently needed.[2] Interest in the study of natural products, including those found in traditional plant-based medicines, has gained significant traction, particularly as conventional antimicrobial drugs lose their effectiveness due to the rise of AMR.[3,4]

Nigella damascena L. (ND; commonly known as love-in-a-mist, devil in the bush, and Blue Midget) is a short-lived annual plant of the Nigella genus (the buttercup family, Ranunculaceae). It is native to the Mediterranean region and temperate areas of Europe, although it has been introduced globally and now grows widely in disturbed soils of semi-arid areas globally. It is an annual herb that is cultivated primarily as an ornamental plant, but has also been used as an ingredient in food as a flavouring agent in bread and cheese.[5] Additionally, ND is used in traditional medicine systems in areas in which it grows, with uses for analgesia and antiseptic effects, as well as anti-oedematous and anti-pyretic uses.[6,7] Its

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seeds are also used in Sicilian folk medicine as a galactagogue,[8] and as an anthelminthic agent in Serbian medieval medicine,[9] and to treat trachoma in Italy and Tunisia.[10] There is an abundance of omega-6 fatty acids within the seed, as well as various other phytochemicals including thymoquinone, saponins, alkaloids, and flavonoids.[11]

Several antimicrobial studies have been performed on ND seed extracts. Early studies provided evidence that petroleum ether, dichloromethane and methanol extracts do not inhibit the growth of *B. subtilis, S. aureus, E. faecalis, E. coli* or *P. aeruginosa* on agar,[12] whereas butanol extracts were active against *P. aeruginosa* and *S. aureus*, with minimum inhibitory concentration (MIC) values of 2.25 mg/mL and 1.125 mg/mL, respectively.[13] Later studies reported that successive extraction of ND seeds by *n*-hexane, chloroform, and methanol produced extracts that were inactive, as determined in broth assays against nine different bacterial species.[14] Despite these reports of a lack of antibacterial activity of the various extracts, the potential antimicrobial activities of ND seed extracts should be further explored, particularly against antibiotic-resistant pathogen strains.

The aim of the present study was to prepare aqueous and methanolic ND seed extracts and measure their antibacterial activities against a panel of skin pathogens, including *Staphylococcus aureus*, MRSA, *Staphylococcus epidermidis* and *Streptococcus pyogenes*. Furthermore, a qualitative assessment of their phytochemical constituents was conducted, alongside toxicity assays using a brine shrimp model.

MATERIALS AND METHODS

Bacterial species and antibiotics

Staphylococcus aureus (ATCC 25923), MRSA (ATCC 43300), *Staphylococcus epidermidis* (ATCC 122292) and *Streptococcus pyogenes* (ATCC 12384) were obtained from the American Type Culture Collection (ATCC, USA). All bacteria were maintained in Mueller-Hinton (MH) agar and broth (Oxoid Ltd., Australia), which were supplemented with 2% NaCl for *S. aureus* cultures to preserve their purity.

Antibiotic powders were purchased from Sigma-Aldrich (Australia) and included penicillin-G (potency of 1440-1680 µg/mg), erythromycin (potency ≥850 µg/mg), tetracycline (≥95% purity by HPLC), chloramphenicol (≥98% purity by HPLC), and ciprofloxacin (≥98% purity by HPLC). The powders were used to prepare 1 mg/mL stock solutions for the broth microdilution assays. Preloaded antibiotic discs (Oxoid Ltd., Australia) containing penicillin (10 IU), erythromycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), ciprofloxacin (1 µg) or cefoxitin (30 µg) were used for the agar disc diffusion studies.

Plant source and solvent extractions

Nigella damascena was a gift from Dr. Michael Whitehouse of the School of Medicine, Griffith University, Australia. ND plants were grown from verified seeds in Deepwater, Australia. The plants were grown apart from other species and pesticides and fertilisers were not used in their cultivation. Flowers were harvested from mature plants in the summer of 2020 and the seeds were removed and allowed to air dry in the shade. A voucher specimen (ND-MW_DNSW_Seed2024) is stored at Griffith University.

The dried plant material was ground to a coarse powder using a laboratory grinder, and one-gram quantities were placed into 50 mL tubes. Sterile deionised water or methanol (AR grade, Ajax Fine Chemicals, Australia) was added to 50 mL and the tubes oscillated at 30 rpm for 24 hr at room temperature. The samples were then vacuum filtered through Whatman #1 filter paper, and the filtrates were subjected to evaporation to remove all traces of solvents. The resultant crude extracts were weighed to determine the extraction yields, and then resuspended in 1% dimethyl sulfoxide (DMSO) and sonicated three times with a pulse sonicator for 30 seconds. The solutions were then sterilised by filtration through 0.2 µm filters (Sarstedt, Australia), which also removed any remaining particulate matter. The extracts were stored as 1 mL aliquots at -20°C until required.

Bacterial growth inhibition assays

The disc diffusion (DD) and liquid dilution (LD) broth assays were conducted as previously described.[15] The zone of inhibition (ZOI) values determined from the DD assays were measured in millimetres and included the 6 mm diameters of the discs used. minimum inhibitory concentration (MIC) values were obtained from the LD microdilution broth assays and were defined as the lowest concentration of extract or antibiotic that caused full inhibition of bacterial growth and was expressed as µg/mL. The extracts were considered to possess low activities if MIC values were 2000-5000 µg/mL, moderate activities at 1000-2000 µg/mL, noteworthy activities at 400-1000 µg/mL, good activities at 100-400 µg/mL and high activities at <100 µg/mL.[16]

Phytochemical and toxicity assays

Plant extracts were assessed for the presence of tannins, flavonoids, water soluble and water insoluble phenols, cardiac glycosides, saponins, triterpenoids, phytosterols, alkaloids and anthraquinones using assays described previously.[17] An *Artemia* nauplii brine shrimp lethality assay[18] was employed to determine the toxicity of the ND leaf extracts. These assays were conducted using *A. franciscana* eggs (Aquabuy, Auburn Australia) in 3.4% sea salt solution (Red Sea Pharm Ltd., Israel) using potassium dichromate as a positive control and artificial sea water as the negative control.

Statistical analyses

The DD assays were conducted in triplicate and one-way analysis of variance (ANOVA) was used to determine statistical differences between the means of test samples and the negative control. Statistical analysis could not be performed on the LD assays, although all assays were repeated on separate days to determine MIC values of the extracts and reference antibiotics.

RESULTS

The concentrations of the aqueous and methanolic extracts and a qualitative assessment of their phytochemical constituents are shown in Table 1. The extraction yields for the aqueous extract was approximately twice that of the methanolic extract, indicating a greater capability for water to extract phytochemicals from the seeds than methanol. Both extracts were rich in saponins. Water soluble and insoluble phenols, as well as tannins, were detected in both extracts, although higher levels were evident in the aqueous extract. Alkaloids were present at moderate levels in both extracts, whilst flavonoids were only be detected at low abundances in the methanolic extract. Cardiac glycosides, triterpenoids, phytosterols and anthraquinones were either absent or below the detection threshold in both extracts.

An initial assessment of the susceptibility of the test bacteria to the ND extracts and the reference antibiotics using agar disc diffusion assays is shown in Figure 1. The majority of the conventional antibiotics inhibited the growth of all four bacterial species tested, with the exception of erythromycin against MRSA, and tetracycline against *S. pyogenes*. Notably, the MRSA strain was far less susceptible to the antibiotics compared to antibiotic-sensitive *S. aureus* strain, verifying the antibiotic-resistant phenotype of this strain. In contrast with most of the antibiotics, the aqueous and methanolic ND extracts failed to produce ZOIs for *S. aureus*, MRSA, *S. pyogenes* or *S. epidermidis*.

Bacterial growth inhibition was also examined in broth using LD assays to determine the MIC values of the reference antibiotics and the ND extracts (Table 2). Due to the low extraction yields, the highest concentrations of the aqueous and methanolic extracts that could be used were 4075 and 1950 µg/mL, respectively. At these concentrations, activity was not observed against any of the bacterial species, except for the methanolic ND extract, which produced noteworthy activity, with an MIC of 481 µg/mL against *S. pyogenes*. The activities of the antibiotics were generally concordant with their activities on agar (Figure 1 and Table 2).

The *Artemia* brine shrimp lethality assay was used to measure the toxicities of the extracts. The brine shrimp larvae were unaffected

Table 1: Concentrations of the ND seed extracts and their qualitative phytochemical analysis.

Extract type	Concentration[a] (mg/mL)	Phytochemical class and abundances									
		Water soluble phenols	Water insoluble phenols	Cardiac glycosides	Saponins	Triterpenoids	Phytosterols	Alkaloids	Flavonoids	Tannins	Anthraquinones
ND-Aq	16.3	++	++	-	+++	-	-	++	-	++	-
ND-MeOH	7.7	+	+	-	+++	-	-	++	+	+	-

[a]Values obtained following resuspension in 1% DMSO. +++ indicates a large response in the assay, ++ indicates a moderate response, + indicates a minor response, - indicates no response. ND=*Nigella damascena* seeds, Aq=aqueous and MeOH=methanol.

Table 2: MIC values of the ND plant extracts and the reference antibiotics against the four bacterial strains tested in this study.

Plant extract or antibiotic		MIC (µg/mL)			
		S. aureus	MRSA	*S. pyogenes*	*S. epidermidis*
ND	Aq	>4075[§]	>4075[§]	>4075[§]	>4075[§]
	MeOH	>1950[§]	>1950[§]	481	>1950[§]
AB	PEN	>2.5[§]	>2.5[§]	0.002	>2.5[§]
	ERY	>2.5[§]	>2.5[§]	0.002	0.313
	TET	0.078	0.078	0.078	>2.5[§]
	CHL	>2.5[§]	>2.5[§]	0.625	2.5
	CIP	0.625	0.625	0.156	0.156

ND=*Nigella damascena* seeds, Aq=Aqueous, MeOH=Methanol. AB=Antibiotics, PEN=Penicillin, ERY=Erythromycin, TET=Tetracycline, CHL=Chloramphenicol and CIP=Ciprofloxacin. [§]Denotes extracts or antibiotics that were inactive at the highest concentrations tested.

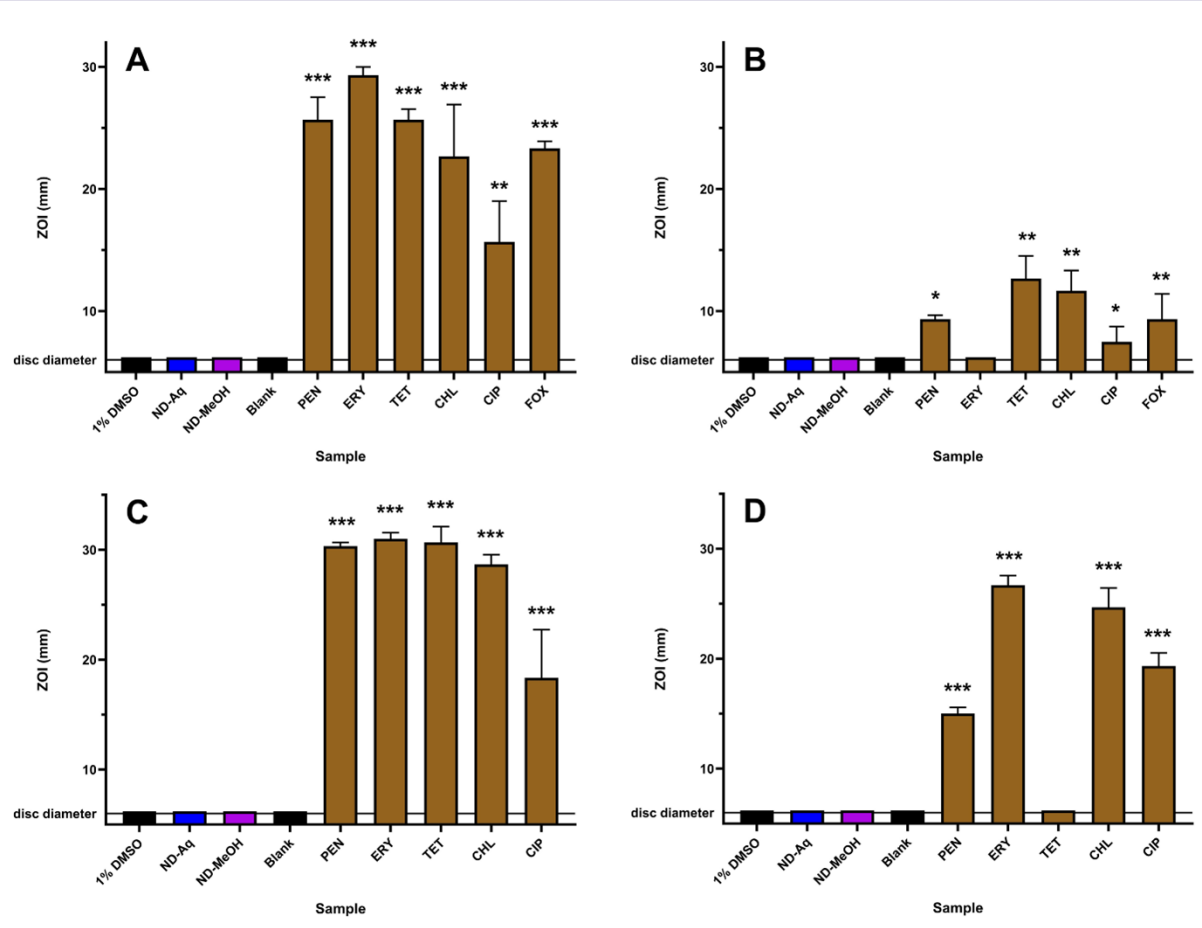

Figure 1: Agar disc diffusion assays for plant extracts and reference antibiotics against (A) *S. aureus*, (B) MRSA, (C) *S. pyogenes* and (D) *S. epidermidis*. The ZOI values were measured in mm and the disc sizes were 6 mm, as indicated on the y-axis. Negative control discs contained 1% DMSO (for extracts) or blank discs (for antibiotics) and are shown by the black bars. Aqueous and methanolic extracts are indicated in blue and purple bars, respectively, while antibiotics are shown as brown bars. Each value is expressed as mean ± SEM of triplicate assays of each extract and antibiotic. Results are significantly different to the negative control if $p<0.05$ (*), highly statistically significant if $p<0.01$ (**) and very highly statistically significant if $p<0.001$ (***). ND = *Nigella damascena* seeds, Aq = aqueous, MeOH = methanol; PEN = penicillin, ERY = erythromycin, TET = tetracycline, CHL = chloramphenicol, CIP = ciprofloxacin and FOX = cefoxitin.

by the aqueous and methanolic ND extracts, and were similar to the results observed in the seawater negative control assays. In contrast, all *Artemia* nauplii had died following incubation with the positive control potassium dichromate. Thus, both ND extracts were deemed to be nontoxic in the brine shrimp assay.

DISCUSSION

The purpose of the present study was to investigate the antibacterial properties of ND seeds extracted with either water or methanol, against four selected bacterial species. Extract toxicities and qualitative measures of their phytochemical constituents were also assessed. This was done in an attempt to find evidence that would support the presence of antibacterial agents within the extracts and that they may be safe to use therapeutically.

Initial antibacterial experiments focused on growth inhibition on agar. The ND extracts failed to inhibit the growth of any of the four bacterial species in these assays. These findings were generally concordant with the lack of inhibition in LD broth assays. Interestingly, the methanolic ND extract did yield a noteworthy MIC value of 481 µg/mL in the LD assays, despite the extract being unable to produce ZOIs in the agar DD assays. This finding suggests that components in the ND methanolic extract are only capable of *S. pyogenes* growth in liquid media. It is possible that two or more compounds, such as an antibiotic molecule and a potentiator, must be free to work in combination, and that this is only possible in liquid media. Contrastingly, the two (or more) phytochemical molecules may diffuse at different rates in the agar media due to their differing polarities, solubilities, or molecular sizes, and thus their separation leads to loss of extract activity.[19,20] Volatile phytochemicals may also evaporate from the agar surface, with their resultant loss leading to loss of activity.[20] It is also intriguing that this effect was observed in *S. pyogenes* only, suggesting that it is specific to *Streptococcus* and did not occur with the *Staphylococcus* species at the highest concentrations

tested (1950-4075 µg/mL). Further work in required in order to investigate this species-specific result.

With the exception of the ND methanolic extract and its effect on *S. pyogenes* in liquid media, all other antibacterial assays revealed a lack of activity in the DD and LD assays. This suggests that, against the species tested, ND seeds extracted with water or methanol are inactive. It is also generally in agreement with previous findings where the seeds extracted with other solvents were inactive, or showed low activities, against Gram-negative and Gram-positive bacteria.[12-14] However, the ND seeds contain a myriad of different phytochemicals.[7] Our findings showed that the extracts contained a number of different phytochemical classes as measured by qualitative assays. These have been shown to possess antibacterial activities, such as phenolics,[21] saponins,[22] triterpenoids,[23] flavonoids,[24] tannins[25] and alkaloids.[26] It is possible that specific combinations and concentrations of these phytochemicals in extracts are required for antibacterial activity, and that the ND extracts lack these conditions and are thus inactive. However, it is important to note that the one instance of activity observed, the ND methanolic extract against *S. pyogenes* showed activity in LD media and was the only extract that contained detectable levels of flavonoids. Therefore, the presence of flavonoids may be essential for activity towards *S. pyogenes*, and this may only be possible in liquid media so that the flavonoids can act in conjunction with other phytochemicals in the liquid media. The flavonoid(s) may also evaporate on agar, or their mobility through the agar may be altered such that it cannot produce antibacterial activity on agar. Further work using liquid chromatography-mass spectrometry should be used to determine the metabolites contained in the extracts, particularly the flavonoids in the ND methanolic extract, in order to ascertain the phytochemicals that may be responsible for the activity profiles of the extracts in this study.

All extracts used in this study were nontoxic in *Artemia* nauplii brine shrimp assays. This indicates that they may be safe to use therapeutically, which is particularly important as they may be applied to skin to treat *Staphylococcus* and *Streptococcus* pathogens included in our study. It should also be noted that methanolic ND seed extracts have been reported to be nontoxic in mice and human erythrocytes in both acute and sub-chronic studies,[27,28] further indicating their safety.

CONCLUSION

Aqueous and methanolic extracts were prepared from ND seeds and their antibacterial activities assessed on agar and in liquid media against four different skin bacterial pathogens. Activity was not observed in most instances, although the ND methanolic extract showed noteworthy activity in liquid media against *S. pyogenes*. The presence of low levels of flavonoids may be responsible for this activity, although future work should be directed at identifying the specific phytochemicals involved. Otherwise, the lack of antibacterial activity observed with the extracts was generally in agreement with previous work using ND seed extracts, although different solvents were used for extraction in those studies. The extracts were nontoxic in the *Artemia* nauplii assay, indicating that they may be safe to use therapeutically. Mammalian cell cultures using skin-cell derived cell lines should be undertaken to confirm their safety of the extracts on epidermal surfaces.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AMR: Antimicrobial resistance; **ANOVA:** One way analysis of variance; **Aq:** Aqueous; **CHL:** Chloramphenicol; **CIP:** Ciprofloxacin; **DD:** Disc diffusion; **DMSO:** Dimethyl sulfoxide; **ERY:** Erythromycin; **FOX:** Cefoxitin; **LD:** Liquid dilution; **MDR:** Multi-drug resistant; **MeOH:** Methanol; **MH:** Mueller Hinton; **MIC:** Minimum inhibitory concentration; **MRSA:** Methicillin-resistant *Staphylococcus aureus*; **ND:** *Nigella damascena*; **PEN:** Penicillin; **SEM:** Standard error of the mean; **TET:** Tetracycline; **ZOI:** Zone of inhibition.

SUMMARY

- *Nigella damascena* (ND) seed extracts were examined for their antibacterial activities on agar and liquid media
- Only the ND methanolic extract showed activity, which was towards *S. pyogenes* and only in liquid broth assays
- The extracts contain saponins, phenolics, alkaloids and tannins, but only the ND methanolic extract contained flavonoids
- All extracts were nontoxic in *Artemia* nauplii lethality bioassays.

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