

## Microbiological and Chemical Analysis of Neem (*Azadirachta indica*) Extracts: New Data on Antimicrobial Activity

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The antimicrobial effects of extracts of neem seed (*Azadirachta indica* A. Juss.) were investigated using microbial growth inhibition assays. A laboratory-prepared neem seed extract along with a commercially available formulated product, were characterized using HPLC, and shown to be effective against a range of bacteria in an agar diffusion assay. The active ingredient, *i.e.*, the unformulated seed extract of the commercial product, also showed activity and this was further investigated in a biochromatogram, using the sensitive bacterium *Bacillus mycooides*. Results showed antibacterial activity as three discrete inhibition zones that did not correspond to the  $R_f$  of the major neem metabolites, azadirachtin, nimbin and salannin. This suggests that these compounds were not antibacterial. The colony radial growth rates of the fungal pathogens that cause 'take-all' and 'snow mould' disease were both significantly affected when the commercial, unformulated, neem seed extract was incorporated into the growth medium. Experiments in liquid culture suggested that the effect was fungistatic. Conidial germination of the commercially important obligate pathogen *Sphaerotheca fuliginea* (powdery mildew) was reduced to 11%. The results show that neem seed extracts possess antimicrobial activity with notable effects on some fungal phytopathogens. This work demonstrates that neem seed extracts have potential for controlling both microbial and insect pests.

KEY WORDS: Neem; antibacterial; antifungal; biochromatogram; phytopathogens; azadirachtin.

### INTRODUCTION

The neem tree, *Azadirachta indica* A. Juss., is a fast growing broadleaved tree, native to the arid regions of the Indian subcontinent (22). It is renowned for its relative paucity of natural pests and pathogens, with over 300 compounds from the tree having been isolated and characterized (17). The most documented compound, azadirachtin, is a potent insect growth regulator, sterilant and antifeedant (20) with over 200 species in seven orders being susceptible to it (26). Coupled with these effects on insects, neem extracts have extremely low mammalian toxicity (16) and are relatively safe to non-target organisms (28). Consequently the use of neem-based products is gaining acceptability as novel, environmentally sound insecticides in both developing and industrialized countries, with products being registered in the USA for use on both non-food and food crops (9).

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In contrast to the wealth of knowledge on the effects of neem extracts – particularly azadirachtin – on insect pests, few studies have investigated their antimicrobial activity. Much of the work that has been undertaken has focused on organisms of medical and veterinary importance (5,13). Many studies are, however, limited by the extracts not having been chemically defined, a problem which is compounded by the fact that the concentration of metabolites in neem varies with factors such as tree age and geographical location (29). There has been some interesting work on antifungal activities of neem extracts and there is evidence that extracts from various parts of the tree have antifungal activity (6,25,33,35). In addition, commercial neem products have been shown to possess varying degrees of activity against a number of fungi (30). More recently, the antifungal fraction of neem oil has been shown to contain a mixture of tetranortriterpenoids (12). Purification of these compounds, however, resulted in decreased activity, suggesting that mixtures, rather than the individual compounds, are required for maximum activity. The aims of this study were to investigate the effects of characterized neem extracts on a number of bacteria and three economically important fungal pathogens of wheat and cucurbits, and to determine whether the major neem metabolites – azadirachtin, nimbin and salannin – possess antibacterial activity. The potential advantage would be that a naturally occurring plant extract could offer dual control of both insect pests and microbial pathogens.

## MATERIALS AND METHODS

**Commercial neem seed formulations and extracts** Commercial neem products (Trifolio-M GmbH, Lahnau, Germany) were used throughout this study. These were all based on the active ingredient, NeemAzal, which consisted of a powdered neem seed kernel extract reported to contain 30% azadirachtin in addition to the limonoids, salannin, nimbin and other compounds. The formulated product NeemAzal-F (containing 5% azadirachtin), prepared commercially using NeemAzal and the formulating agent Azasolv (containing no neem extract), were also used. NeemAzal, provided as a powder, was prepared in 40% ethanol for use in the bioassays described below. To confirm the azadirachtin, nimbin and salannin concentrations, a 1% (w/v) NeemAzal ethanolic solution was analyzed by HPLC. A reverse phase column (Primesphere, 5 $\mu$ , C18-MC 300A, 4.6 mm  $\times$  25 cm, Phenomenex, UK) was used with UV detection at 220 nm and an acetonitrile/water gradient system (15). A water extract of neem seed kernels was prepared (Prof. E.D. Morgan, University of Keele, UK, pers. commun.) and similarly analyzed. Seeds were obtained from a named tree (MI-B) near the village of Maha Illuppallama in the Dry Zone of Sri Lanka (7). Neem kernels (12 g), with shells (endocarp) removed, were ground in a mortar and pestle and the resultant powder was defatted in *n*-hexane under reflux for 2 h (11). The neem seed kernel powder was harvested by vacuum filtration and blended in 80 ml distilled water for approximately 80 sec. The resultant meal–water mixture was centrifuged at 4600 rpm (MSE Chilspin, Fisons, UK) for 10 min. Any fat remaining on the surface after centrifugation was removed and the supernatant decanted. A further 80 ml of distilled water was then used to wash the pellet from the centrifuge tubes and the process of homogenizing and centrifuging was repeated a further three times to give a final combined extract volume of 480 ml. This was filtered (Whatman No. 1) and the extract evaporated to dryness and frozen until use. Prior to HPLC the sample was cleaned (32) using a Pasteur pipette (15 cm  $\times$  0.8 cm internal diameter) that was packed from top to bottom with a small plug of glasswool, 5 cm activated Florisil and 2 cm anhydrous sodium sulphate. The column

was prewashed with 10 ml hexane followed by 10 ml ethyl acetate. The dried sample was reconstituted in 10 ml ethyl acetate and 1 ml put down the Pasteur pipette column. The sample was eluted with 15 ml ethyl acetate without allowing the column to dry. Triplicate samples of both the NeemAzal ethanolic solution and the neem seed water extract were analyzed.

**Detection of antibacterial activity** An agar diffusion assay was used to detect the presence of antibacterial activity in NeemAzal and the formulated product, NeemAzal-F, against *Bacillus cereus*, *B. mycoides*, *B. thuringiensis*, *B. subtilis* NCIMB 9593, *Erwinia carotovora* pv. *carotovora* NCIMB 438, *Pseudomonas syringae* pv. *phaseolicola* NVRS 1281, *Nocardia* sp., *Xanthomonas campestris* NCIMB 11803, *Corynebacterium fascians* and *Agrobacterium tumefaciens* NCIMB 4404. Nutrient Agar (Oxoid, UK) was seeded with late exponential cultures of the test organisms and allowed to set in 9 cm petri dishes. Two 8-mm-diam 'wells' were cut from the medium with a cork borer and filled to capacity with either a 1% solution of NeemAzal or 40% ethanol which served as a control. The plates were incubated for 24 h at 25°C to allow for confluent growth and the diameter of inhibition zones was recorded. A 10% solution of the water-soluble product NeemAzal-F and a sample of the laboratory-prepared seed extract were similarly screened, with sterile distilled water serving as a control. Results are reported as the mean diameter of inhibition zones including the 8 mm diam of the 'well' for duplicate tests. Results were analyzed using a paired t-test. This bioassay was also used to screen higher dilutions of NeemAzal-F and its formulating agent, Azasolv, to determine their critical inhibitory concentrations,  $m_i$  (18).

**Isolation of antibacterial components** Thin layer chromatography (TLC) plates (20 × 20 cm, 250  $\mu\text{m}$  thick Silica Gel G, Analtech, UK) were used to separate the components of NeemAzal using a diethyl ether/methanol (49:1) solvent system (34). Azadirachtin, nimbin and salannin (kindly supplied by Dr. A.J. Mordue) were prepared in ethanol to a final concentration of 1 mg ml<sup>-1</sup>, and loaded (15  $\mu\text{l}$ ) at 1.5 cm intervals, 1.5-cm from the bottom of the plate; concentrations of NeemAzal were applied with a 30  $\mu\text{l}$  loading. Plates were visualized by exposing them to iodine vapor in a sealed glass tank for 10 min, treated with 25% H<sub>2</sub>SO<sub>4</sub> using a Dragondorff sprayer, and heated for 2 min at 110°C (24). *B. mycoides* was selected to detect the presence of antibacterial components in the separated neem extract on the basis of its sensitivity and quality of growth in the agar diffusion assay. TLC plates were run, allowed to dry and then overlaid with nutrient agar seeded with *B. mycoides* [1.5 ml overnight culture (*ca* 10<sup>8</sup> cells ml<sup>-1</sup>) in 150 ml nutrient agar]. Plates were incubated at 25°C overnight and then examined for areas of growth inhibition.

**Detection of antifungal activity** (a) *In semi-solid medium.* Various concentrations of NeemAzal prepared in ethanol were incorporated into malt extract agar (MEA, Oxoid, UK) and dispensed into 9 cm petri dishes to give a final ethanol concentration of 1%. Mycelial plugs of *Gaeumannomyces graminis* var. *tritici* (*Ggt*) (supplied by Dr. D. Hall, Cambridge University) and *Microdochium nivale* (supplied by Aberdeen University) were placed in the center of the plates and incubated at 25°C. Colony radial growth rate ( $K_r$ ) was determined by measuring the colony diameter along two intersecting perpendicular lines which passed through the center of the young colony, with a stereo binocular microscope at 24–48-h intervals.  $K_r$  was calculated by linear regression analysis of the mean colony radius as a function of time (1).

(b) *In liquid medium.* The same concentrations of NeemAzal as used in (a) above, were added to 20 ml malt extract broth (MEB, Oxoid, UK) in 50 ml conical flasks and inoculated with a mycelial plug of *Ggt* and *M. nivale*. The flasks were incubated at 25°C and 80 rpm before harvesting the biomass after 7 days. Biomass was harvested by filtering through pre-weighed Whatman Grade 1, 3-cm filter circles under vacuum. The biomass was dried to constant dry weight (70°C for 24 h) and then placed in a desiccator prior to determining the dry weight. *Ggt* was grown in liquid medium in the presence of NeemAzal prepared in both ethanol (final ethanol concentration of 1%) and water to determine the effect on the solubility of any antifungal compounds.

Results were analyzed using one-way analysis of variance with Minitab version 7.2.

**Effect of neem extract on the germination of *S. fuliginea* conidia *in vitro*** This bioassay followed a modified method of Dr. A. Schmitt for *Reynoutria sachalinensis* (A. Schmitt, pers. commun.). NeemAzal was prepared in 1 ml absolute ethanol and added to 25 ml of 0.0125% Tween 20 to give final azadirachtin equivalent concentrations of 0, 62.5, 125, 250 and 500 ppm azadirachtin. Glass slides were flamed and sprayed with the various solutions and allowed to dry. Leaves from a cucumber plant infected with the causative organism of powdery mildew (*Sphaerotheca fuliginea*) were then gently pressed onto the treated surface of the slides to detach the conidia. The slides were placed in a humid chamber, incubated at 22°C, and germination was assessed after 24 h by microscopic examination (Reichert-Jung microscope, Austria).

## RESULTS

HPLC analysis of the laboratory-prepared seed extract confirmed the presence of azadirachtin, nimbin and salannin (Table 1). Similar analysis of the 1% NeemAzal solution confirmed the azadirachtin concentration (*i.e.*, 3000 ppm) specified by the manufacturer (Table 1), which was approximately four times higher than that of the laboratory-prepared seed kernel water extract. The azadirachtin concentration found in the laboratory-prepared seed extract was typical of published reports (14). In addition, salannin and nimbin were detected in NeemAzal but at much lower concentrations compared with azadirachtin (Table 1).

TABLE 1. Mean concentration (ppm) of azadirachtin, nimbin and salannin in a 1% (w/v) solution of the unformulated commercial extract NeemAzal and a laboratory-prepared seed kernel extract as determined by HPLC (Concentrations calculated from the mean area under the appropriate HPLC peaks of triplicate samples)

Compound	NeemAzal	Seed kernel extract
Azadirachtin	3000	752
Nimbin	80	30
Salannin	92	122

**Detection of antibacterial activity** Most of the organisms tested showed sensitivity to the laboratory-prepared neem extract and the commercial products (Table 2). *B. mycooides*, *B. thuringiensis*, *B. subtilis*, *Nocardia* sp. and *C. fascians* were inhibited by the laboratory-prepared neem seed kernel extract. In some cases this extract showed better inhibition than 1% NeemAzal *e.g.* *B. thuringiensis* had an inhibition zone of 19.5 mm compared with

TABLE 2. Diameter of inhibition zones formed with the laboratory-prepared neem seed extract, the active ingredient NeemAzal, the commercial product NeemAzal-F, and its formulation Azasolv, against different bacteria after 24 h incubation at 25° C

Organism	Diameter of inhibition zones (mm) <sup>z</sup>			
	Seed extract	1% NeemAzal	10% NeemAzal-F	10% Azasolv
<i>B. cereus</i>	nz	10.0	20.0	24.0
<i>B. mycoides</i>	14.5	11.0	22.0	22.0
<i>B. thuringiensis</i>	19.5	11.0	21.0	23.0
<i>B. subtilis</i>	11.0	9.5	19.0	24.0
<i>E.c. ssp. carotovora</i>	nz	9.7	24.0	25.0
<i>P.s. pv. phaseolicola</i>	nz	10.0	12.0	12.0
<i>Nocardia</i> sp.	22.0	13.5	27.5	25.5
<i>X. campestris</i>	nz	–	31.0	29.0
<i>C. fascians</i>	16.0	11.5	23.5	23.5
<i>A. tumefaciens</i>	nz	nz	nz	12.0
	$P < 0.05^y$	$P < 0.05$		$P > 0.05$

<sup>z</sup>Mean diameter for duplicate tests.

nz, no zone; – not tested.

<sup>y</sup>Statistical analysis using the paired t-test.

TABLE 3. Colony radial growth rates (mm day<sup>-1</sup>) of *Gaeumannomyces graminis tritici* (*Ggt*) and *Microdochium nivale* on malt extract agar plates containing different concentrations of NeemAzal (prepared as ppm azadirachtin)

NeemAzal (ppm azadirachtin)	<i>Ggt</i>	<i>M. nivale</i>
0	9.0 ± 0.13 <sup>z</sup>	14.6 ± 0.29
62.5	7.2 ± 0.03	12.9 ± 0.57
125	6.6 ± 0.03	7.2 ± 0.58
250	5.4 ± 0.09	5.4 ± 0.19
500	4.3 ± 0.19	4.6 ± 0.08
1000	3.5 ± 0.11	3.9 ± 0.01
LSD	1.20	3.86

<sup>z</sup>Values are the mean of three replicates ±SE;  $P < 0.05$ .

only 11 mm for the commercial extract and *Nocardia* had an inhibition zone of 22 mm vs 13.5 mm for the commercial extract. Most of the organisms screened were sensitive to NeemAzal, its formulated product NeemAzal-F and also the formulating agent itself (Table 2). The organisms showed varying degrees of sensitivity, with the *Nocardia* isolate being most sensitive, and the phytopathogen *A. tumefaciens* being least sensitive. Proof that the formulating agent alone, i.e., Azasolv, was also biologically active was established by determining its  $m_i$ , which was not significantly different ( $P > 0.05$ ) from the formulated product NeemAzal-F for any of the organisms tested (data not shown). Eight of the organisms tested, including some of the plant pathogens, were, however, still inhibited by the active ingredient, NeemAzal at 1%, which did not contain any formulating agent, indicating that the neem seed extract *per se* had antibacterial activity (Table 2).

**Isolation of antibacterial components** The TLC methodology gave very good separation of the major neem metabolites with defined spots being presented for azadirachtin, nimbin and salannin (respectively black, dark brown and gray/black in color), with  $R_f$  values of 0.43, 0.84 and 0.61, respectively. All three metabolites, in addition to other unidentified components, were clearly observed in NeemAzal. When biochromatograms

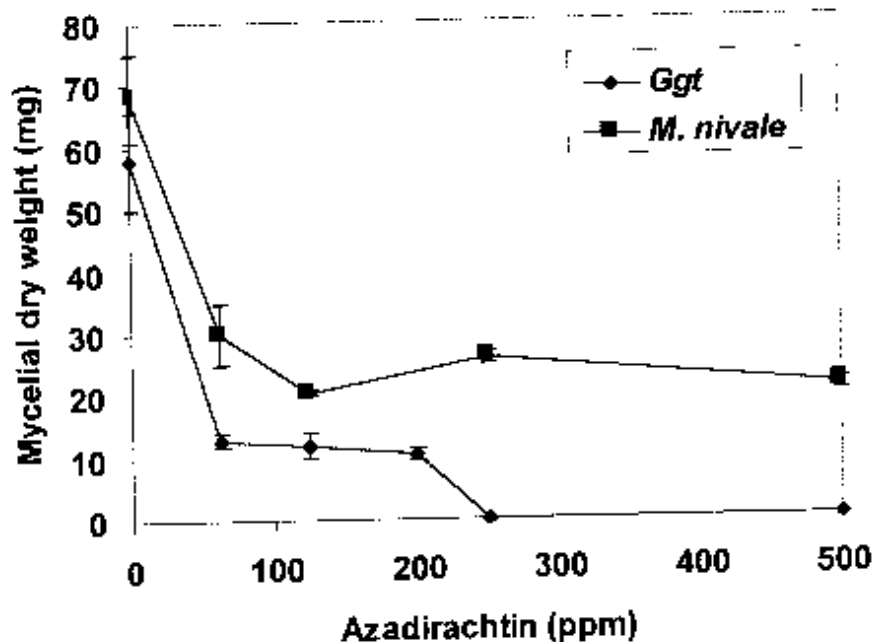


Fig. 1. Growth of *Gaeumannomyces graminis tritici* (*Ggt*) and *Microdochium nivale* in malt extract broth containing NeemAzal (ppm azadirachtin) prepared in ethanol (final ethanol concentration = 1%). Values are the mean of three replicates  $\pm$  SE;  $P < 0.05$ .

were prepared by overlaying TLC plates with bacteriological growth medium seeded with *B. mycooides*, no zones of inhibition were observed for the three major neem metabolites in any of the samples. However, separate discrete zones of inhibition were observed with NeemAzal that had  $R_f$  values of 0.15, 0.29 and 0.57. The sizes of these inhibition zones increased with increasing concentration of NeemAzal. Quantification of the concentration of the bioactive compounds was not attempted, as it was felt that this would have been inaccurate due to the diffusion of the compounds during the period required for bacterial growth and inhibition.

**Detection of antifungal activity** NeemAzal reduced the  $K_r$  of both *Ggt* and *M. nivale* significantly compared with the control at concentrations  $\geq 62.5$  and 125 ppm azadirachtin, respectively (Table 3). In liquid medium NeemAzal prepared in ethanol to concentrations of 250 ppm azadirachtin and above completely inhibited growth of *Ggt* (Fig. 1). When the inoculum was transferred to fresh medium the fungus grew, indicating the effect of the treatments was fungistatic. In contrast, when NeemAzal was prepared in water, *Ggt* grew in concentrations previously found to be inhibitory. The growth of *M. nivale* was significantly reduced ( $P < 0.05$ ) in liquid medium by the presence of NeemAzal containing 62.5 ppm azadirachtin and above but not completely inhibited even with concentrations of 500 ppm azadirachtin (Fig. 1).

**Effect of neem extract on the germination of *Sphaerotheca fuliginea* conidia in vitro**  
A concentration of NeemAzal containing 62.5 ppm azadirachtin was found to reduce

conidial germination of *S. fuliginea* significantly compared with the control. Thus, germination for the control was 100%, whereas with 62.5 ppm azadirachtin germination was  $11\% \pm 6.0$  (mean of 3 replicates  $\pm 1$  SE). The reduction in germination in this assay was reproducible and consistent, with no germination observed with concentrations of NeemAzal containing 125 ppm azadirachtin and above, up to 500 ppm azadirachtin.

## DISCUSSION

The antimicrobial activity of commercial and laboratory-prepared neem seed extracts, which have been defined in terms of their azadirachtin, nimbin and salannin contents, was investigated. Analysis of the active ingredient, NeemAzal, and the laboratory-prepared seed extract by HPLC revealed their high azadirachtin concentration compared with nimbin and salannin (Table 1). These results are similar to those found in other studies (7), although the concentration of azadirachtin in NeemAzal was particularly high ( $300 \text{ mg azadirachtin g}^{-1}$ , as opposed to  $10 \text{ mg g}^{-1}$  as reported for seed kernels harvested from natural trees, ref. 14). The concentrations of nimbin and salannin in NeemAzal, however, were not proportionally higher (Table 1). The exact method of extracting NeemAzal is not known but its high azadirachtin content is presumably a result of its concentration for use in commercial products developed for insect control.

The agar diffusion assay clearly showed that neem extracts have antibacterial activity (Table 2). However, the formulating agent for NeemAzal-F, *i.e.*, Azasolv, was also inhibitory to the bacteria tested. The fact that *A. tumefaciens* was not affected by any of the samples containing neem extracts but was sensitive to the formulation, suggests that the formulation has additional inhibitory compounds. This is confirmed in Table 2, which shows that the inhibition zones obtained for five of the test organisms were larger with 10% Azasolv than for 10% NeemAzal-F. This work, which has developed the idea of using neem extracts for microbial control, illustrates the importance of testing both the formulating agents and final formulated products on all potential target organisms. The non-formulated active ingredient and the laboratory-prepared seed extract also inhibited some of the bacteria tested, so work progressed into establishing whether the major neem metabolites, in terms of insect control, affect microbes. The fact that the laboratory-prepared extract showed better inhibition of the sensitive organisms than 1% NeemAzal suggested that the main neem limonoids, especially azadirachtin, which is highly concentrated in NeemAzal, may not be responsible for the observed inhibition.

*Bacillus mycoides* was chosen for further studies due to its sensitivity to NeemAzal in the agar diffusion assay, its extremely low  $m_i$  ( $3.01 \times 10^{-4} \%$ ), and its dense and confluent growth – which facilitated observation of inhibition zones in the TLC bioassay. It was used successfully in the biochromatograms to detect antibacterial components in the neem extracts. Discrete zones of inhibition were observed with NeemAzal containing 2000–5000 ppm azadirachtin, indicating the presence of at least three distinct antibacterial compounds. The  $R_f$  values of these zones did not correspond to azadirachtin, nimbin or salannin and thus it is clear that the antibacterial activity did not arise from these compounds. Indeed, no inhibition zones were found with these limonoids when tested at a concentration of  $1 \text{ mg ml}^{-1}$ . The high cost and low availability of these pure compounds prevented their testing at higher concentrations but no inhibition was observed at the anticipated sites in the TLC assay with NeemAzal containing 2000–5000 ppm azadirachtin. These results show clearly that azadirachtin, nimbin and salannin possess no activity against *B. mycoides*. This

confirmed results of another study which suggested that the concentration of azadirachtin was not a useful marker for the antimicrobial properties of neem products (30). It would be of great interest to identify the three antibacterial compounds found in this study. Once identified and isolated, future work could establish their effectiveness against a range of microorganisms. This would be of interest with respect to research that showed that antifungal activity was best when mixtures of neem compounds were used, in comparison with single purified compounds (12). The identity, effectiveness and environmental toxicity of the antibacterial compounds found in this study are obviously important for commercial considerations which could influence decisions concerning the use of neem mixtures or single compounds for antibacterial use.

The plant pathogen *Ggt* is responsible for the economically significant root disease 'take-all' of wheat and barley which can dramatically reduce yields (23). It is of interest therefore that this pathogen showed great sensitivity to NeemAzal *in vitro*. Mycelial growth was reduced significantly compared with the control in the presence of concentrations of 62.5 ppm azadirachtin and above in both solidified (Table 3) and liquid culture (Fig. 1). Concentrations of 250 ppm azadirachtin and above in the liquid medium completely inhibited growth, with a fungistatic effect. These different inhibitory concentrations presumably reflect the different growth characteristics in solid and liquid medium. Interestingly, the effectiveness of NeemAzal against *Ggt in vitro* was found to be dependent on the solvent used for its preparation. Thus, growth was inhibited in a fungistatic manner when NeemAzal was prepared in ethanol whereas no inhibition was observed when it was prepared in water. Solubility of the product is an obvious concern for formulation of any antimicrobial agent and is an obvious concern here. The laboratory-prepared seed extract actually enhanced the growth of *Ggt* (data not shown), presumably by acting as a source of nutrients in a situation where the concentrations of the inhibitory compound(s) were lower than their effective dose. This therefore points to a limitation on the use of crude extracts, since nutrients supplied in such extracts may stimulate the growth of phytopathogens and indeed other residential microflora. The nature of the mycelial growth of *Ggt* made it unsuitable for the biochromatographic work but clearly inhibitory metabolites need to be identified so that these can be considered for control of such phytopathogens.

These investigations have also shown some promising results with *M. nivale*, a seedborne pathogen of wheat (4) and the obligate pathogen *S. fuliginea*, one of the most important fungi causing powdery mildew in greenhouse cucurbits (8). Interestingly, W.R. Grace and Co. have developed a neem oil preparation as a fungicidal / acaricidal product named 'Neemgard' (2). The product has been taken over by Thermo Trilogy Corp. under the name of 'Trilogy' or 'Triact' and is recommended, *inter alia*, for the control of different types of mildews, rusts and anthracnose on both vegetables and ornamentals (3,10,19). Obviously *in planta* studies are now required for this work to progress. Indeed, formulated neem oils have been shown to reduce powdery mildew disease in cucumber, with two different modes of action being suggested (31). Thus, the oil, *i.e.*, the crude neem extract, provided a physical barrier to conidial germination while its limonoids and other compounds provided fungicidal activity. It should be noted that these neem oils, although containing different quantities of limonoids, all contained the major neem compounds azadirachtin, nimbin and salannin.

The extracts tested in this study exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria, and the fungal phytopathogens *Ggt*, *M. nivale* and *S. fuliginea*. In terms of insect control, it is currently recommended that commercial neem preparations be applied at 50–100 ppm azadirachtin (21) although even lower concentrations would be advantageous in order to reduce possible harmful effects on beneficial species (27). These azadirachtin concentrations are much lower than those required to inhibit the phytopathogens tested in this study. The TLC bioassay results, however, indicate that the effectiveness of the neem extract in protecting against bacteria is not dependent on azadirachtin content but on other minor neem components, which must be identified and studied further. The use of neem extracts for control of both insects and microbial pathogens may therefore be possible, if the concentration of the unidentified antimicrobial components could be increased without adversely affecting the final product.

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