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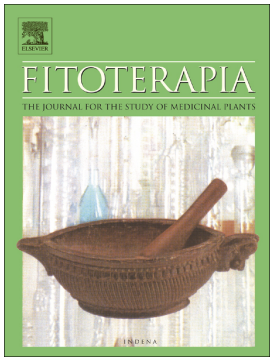

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Epidihydropinidine, the main piperidine alkaloid compound of Norway spruce (*Picea abies*) shows promising antibacterial and anti-*Candida* activity

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ABSTRACT

This study reports for the first time promising antibacterial and antifungal effects of epidihydropinidine, the major piperidine alkaloid in the needles and bark of Norway spruce, *Picea abies* (L.) Karsten. Epidihydropinidine was growth inhibitory against all bacterial and fungal strains used in our investigation, showing the lowest MIC value of 5.37 µg/mL against *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Candida glabrata* and *C. albicans*. Epidihydropinidine was nearly three times more active than tetracycline against *P. aeruginosa* and *E. faecalis.* Promising antibacterial effects were also recorded against *Staphylococcus aureus* and *Bacillus cereus* (MIC 10.75 µg/mL) as well as against *Salmonella enterica* (MIC and MBC 43 µg/mL). Our preliminary results suggest that epidihydropinidine as well related alkaloids of Norway spruce could be powerful candidates for new antibiotics and for preventing food spoilage.

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1. Introduction

Pan-resistant bacterial and fungal strains represent a rising and accelerating threat to global health and therefore new antimicrobial drugs with improved mechanisms of action and safety of use are needed [1,2,3]. Resistance of clinical significance has evolved against virtually all conventional antibiotics [4]. In over 40 years no new classes of clinically relevant antibiotics have been discovered

with the exception of narrow-spectrum daptomycin and linezolid [4]. At the moment twenty new antibiotics are in clinical trials, but most of these do not have new mechanisms of action [5].

Bacteria causing food-borne diseases, such as *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella enterica*, are a major health concern and contribute to significant economic losses globally [6,7]. Food-poisoning caused by *S. aureus* is one of the most common food-borne diseases worldwide [7]. In addition, *S. aureus* causes infections on the skin, endocarditis, pneumonia, osteomyelitis, bacteraemia and central nervous system infections [8]. Methicillin and multi-drug resistant *S. aureus* (MRSA) is a threat to public health in many regions of the world [9,10,11,12]. Increasing consumption of processed refrigerated foods and the increasing percentage of elderly and immunocompromised people contributes to raise the importance of *B. cereus* as a causative agent of food-poisonings [13,14,15,16,17]. To date, *B. cereus* has typically been found to be resistant only to β-lactams [14], but resistance against erythromycin and tetracyclines has been reported in USA and Europe [18]. Non-typhoid and typhoid *Salmonella* infections are of global concern and are complicated by increasing prevalence of acquired multiple drug resistance (MDR) [19,20,21]. Non-typhoid *Salmonella* is one of the most important food-borne pathogens [22]. *Salmonella enterica* is estimated to cause 1.2 million infections yearly in USA and is the leading cause of hospitalizations and death among food-borne diseases [23,24]. Floroquinolones and cephalosporins are used for the standard treatment of Salmonellosis, but during the last decade, increasing resistance to ciprofloxacin has been observed [21,22].

Pseudomonas aeruginosa is an opportunistic pathogen known to cause a range of infections, including infections in wounds, intra-abdominal and urogenital sepsis as well as pneumonia in immunocompromised individuals and in children suffering from cystic fibrosis [25,26]. Moreover, *Pseudomonas* spp. have been associated with food spoilage, such as dairy products [27]. *P. aeruginosa* is known for its high intrinsic resistance against antibiotics [28] and its resistance to many classes of antibiotics, including combination therapies using an aminoglycoside or a fluoroquinolone in combination with a β-lactam is increasing [28,29,30,31].

Enterococcus faecalis is the most common enterococcal species associated with infections in humans [32] and is one of the main nosocomial pathogens [33]. *E. faecalis* inhabits the gastrointestinal and genitourinary tracts and is associated with endodontic diseases [33], bacteraemia [34], infective endocarditis [35], surgical wound infections [36] and urinary tract infections [37]. Enterococci have developed increasing resistance to most conventional antibiotic therapies [38,39]. Antibiotic resistant enterococci have been found in dairy products, meat products and ready-to-eat foods [40,41].

Systemic infections caused by *Candida* species are the fourth leading cause of nosocomial infections with high mortality rates, the rates depending on the species of *Candida* [42]. *Candida albicans* is still the most frequently isolated agent of candidiasis, but non-*albicans Candida* species (NAC), such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. krusei* are increasing [43]. Most of the NAC species exhibit primary resistance or reduced susceptibility against currently used antifungals, such as azoles, polyenes and echinocandins [44].

There is an increasing interest in searching for new antimicrobial compounds from higher plants and other biological sources as synthetic chemical libraries tend to be limited in structural diversity and are therefore poor sources of antimicrobial leads [45]. An important driving force to explore new plants and other organisms for biologically active compounds is also the accelerating pace of habitat destruction and therefore loss of valuable species [46]. Generally higher plants are regarded as a rich source of antimicrobial compounds, among which alkaloids represent a promising class of bioactive compounds [47,48,49,50,51,52]. Antimicrobial activity of alkaloids is dependent on the structural class, so that piperidine, pyrrolidine and pumiliotoxine alkaloids have been found to be the most inhibitory [47,53,54].

Norway spruce (*Picea abies L.* Karsten), one of the most common coniferous species in Northern Europe is a rich source of different compounds [55,56,57,58,59,60]. Norway spruce needles and young stems contain a number of small molecular mass piperidine alkaloids, epidihydropinidine and *cis*-pinidinol being the most abundant ones [55,56,61]. Piperidine alkaloids in conifers are thought to be insect antifeedants but have also been reported to be toxic and teratogenic in a frog embryo test [61,62,63]. Less is known about their possible role as antifungals and antibacterials. However, extracts of spruce cotyledons have been found to effectively inhibit fungal growth, but the active compounds were not characterized [64]. So far *cis*-pinidinol was found to be devoid of antibacterial activity, whereas euphococcinine showed weak activity against gram-negative bacteria [61]. However, structurally related synthetic *trans*-2-methyl-6-n-undecyl (Solenopsine A), -tridecyl (Solenopsine B) and -pentadecyl (Solenopsine C) alkaloids, have been found to give good growth inhibitory effects against gram-positive bacteria [65]. Moreover, piperidine alkaloids in the venom of the fire ant have been proposed to play a protective role against microbial infections of the ant cuticle [66].

Epidihydropinidine (*trans*-2-methyl-6-propylpiperidine) present in Norway spruce needles was chosen as the compound to study since, to the best of our knowledge, this piperidine alkaloid has not been studied before for its growth inhibitory effects against human pathogenic bacteria and yeasts. Volatile and water-soluble epidihydropinidine is known to be present in constant concentrations in both young

and mature needles and twigs of Norway spruce [55,58,59]. In this study we describe the *in vitro* growth inhibitory effects of epidihydropinidine, the major piperidine alkaloid in Norway spruce, against selected human pathogenic bacteria and fungal species. *Bacillus cereus, Salmonella enterica, Staphylococcus aureus* and *Pseudomonas aeruginosa* were selected according to their ability to induce food poisoning or spoilage of food [7,67]. In addition, *Enterococcus faecalis* was chosen as a gram-positive model bacterium commonly causing nosocomial infections [68]. The fungal species, *Candida albicans* and *C. glabrata*, were selected according to their clinical relevance of being the most frequent causative agents of systemic fungal infections as well as of opportunistic infections in mouth [43,69].

2. Experimental

2.1. Extraction and identification of epidihydropinidine from Norway spruce

Biologically relevant concentration of epidihydropinidine was obtained from alkaloid mixture extracted with solid phase partitioning (SPP) method, identified and quantified with GC-MS from the needles and bark of Norway spruce (*Picea abies*) originating from Eastern Finland [55,56,57,58].

2.2. Synthesis of (±)-Epidihydropinidine

Racemic (±)-epidihydropinidine was synthesized according to [70,71,72]. Pure product was identified based on NMR spectra recorded using a Bruker Avance 400 FT NMR spectrometer and EI-MS spectra using an Agilent 6890 gas chromatograph coupled to HP5973 mass selective detector equipped with an HP-1MS column (30 m × 0.25 mm ID, 25-µm film thickness, Agilent Technologies, USA) and G1701DA ChemStation D.00.00.38 software (Agilent Technologies). ^{1}H NMR, ^{13}C NMR, COSY, and HSQC identification was performed according to [71] and GC-MS according to [61].
(±)-Epidihydropinidine (Fig. 1a): Clear volatile oil. Yield: 43%. Purity > 95 %. ^{13}C NMR Spectral data (100.6 MHz, $CDCl_3$): δ 14.2 (*q*, C-9), 19.6 (*m*, C-8 and C-4), 21.3 (*q*, C-10), 30.9 (*t*, C-5), 33.1 (*t*, C-3), 36.4 (*t*, C-7), 45.8 (*d*, C-2), 50.5 (*d*, C-6). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.92 (3H, *t*, *J*=7.2 Hz, H-9), 1.07 (3H, *d*, *J*=6.8 C-10), 1.20 (1H, *m*, H-3a), 1.27 (1H, *m*, H-5a), 1.3 (3H, *m*, H- 4a and H-8), 1.34 (1H, *m*, H-7a), 1.46 (1H, *m*, H-7b), 1.56 (1H, *m*, H-4b), 1.62 (1H, *m*, H-3b), 1.63 (1H, *m*, H-5b), 2.89 (1H, *m*, H-2), 3.05 (1H, *m*, H-6). GC-MS ions 70 eV *m/z* (relative intensity): 141 $[M]^+$ (1), 140 (2), 126 (7), 99 (7), 98 (100), 81 (4), 70 (6).

2.3. Antibacterial and antifungal assays

Antimicrobial activity of epidihydropinidine was analyzed with synthetized pure (±)-epidihydropinidine [55,56]. Three gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus cereus* ATCC 10987), two gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Salmonella enterica* ssp. *enterica* ATCC 43845) as well as two species of yeast (*Candida albicans* ATCC 10231 and *C. glabrata* ATCC 2001) were used as test organisms. The strains were obtained from HAMBI culture collection, Department of Food and Environmental Sciences, Division of Microbiology, University of Helsinki, Finland. The antibacterial and antifungal effects of synthesized (±)-epidihydropinidine were explored using a turbidimetric microdilution broth assay according to Clinical and Laboratory Standards Institute [73]. Two-fold serial dilutions of (±)-epidihydropinidine in Mueller Hinton or Saboraud Broth (Becton, Dickinson & Company, USA) were made from 86 to 1.343 µg/mL in sterile Eppendorf tubes. Tetracycline hydrochloride, ampicillin and amphotericin B (Sigma-Aldrich, St. Louis MO, USA) were used as standard antibiotics using two-fold serial dilutions from 1 mg/mL to 0.015 µg/mL. Before the test the bacterial and fungal cultures were grown overnight in Mueller Hinton and Saboraud broth, respectively, at +37 ˚C. Turbidity of 1 mL of this bacterial or fungal suspension was measured at 625 nm. The suspensions were diluted to an absorbance of 0.1 at 625 nm containing approximately 1×10^8 CFU/ mL. The suspensions were diluted further so that the final number of cells in inoculum was 1.0×10^6 CFU/mL. 100 µl of this inoculum was added to the wells of the microplate, whereafter 100 µl of (±)-epidihydropinidine and antibiotic dilutions were added. The final number of cells in the wells at starting point of experiment was therefore 5.0×10^5 CFU/mL. The microplates were incubated for 24 h at +37 ˚C whereafter turbidity at 620 nm was recorded after gently shaking of the plates using a Victor 1420 (Wallac, Finland) spectrophotometer. The percentage of growth and growth inhibition in comparison to the growth control (freely growing bacterial or fungal cells) was calculated. Dose-response curves were generated from the percentage of growth values of a series of two-fold serial dilutions of (±)-epidihydropinidine and antibiotics. The minimum inhibitory concentration (MIC) was estimated as the lowest concentration of (±)-epidihydropinidine or antibiotics resulting in no visible growth as detected by the unaided eye [73]. Spectrophotometrically (A_{620}), these minimum inhibitory concentrations resulting in no visible growth inhibited 90 % or more of the growth observed in the growth control wells. Therefore, the MIC values were estimated as the lowest concentrations showing 90 % or more inhibition of the growth of controls. IC50 was estimated as half-maximal inhibitory

concentration of (±)-epidihydropinidine or antibiotics. For minimum bactericidal and fungicidal concentration estimations the microplate tests were first performed as described above, after which 100 µl from those wells containing MIC and from wells containing the following four higher concentrations above MIC were pipetted and spread out evenly on petri dishes (∅ = 9 cm) containing Isosensitest or Saboraud agar as top layer. The petri dishes were incubated for 24-48 h at +37 ˚C. MBC and MFC were expressed as the lowest concentrations of (±)-epidihydropinidine or antibiotics which totally inhibited the growth of the microorganisms after subculturing, resulting in a clear surface of the petri dishes. All assays were done in duplicate, and repeated at least two times and the results were expressed as mean ± standard deviation (SD).

3. Results

The growth inhibitory effects of (±)-epidihydropinidine, a 2,6-disubstituted piperidine alkaloid, of which the (+)-enantiomer (Fig. 1a) is present as the major piperidine alkaloid in the needles, bark and xylem of Norway spruce, was investigated against bacteria causing food spoilage and food poisonings as well as some other relevant human pathogenic bacteria and two clinically relevant *Candida* species. (±)-Epidihydropinidine showed a broad spectrum of activity, inhibiting the growth of all the tested gram-positive and gram-negative bacteria as well as the *Candida* species (Table 1).

3.1. Antibacterial effects

In this screening, *Pseudomonas aeruginosa* was most susceptible of all the tested bacteria to the effects of (±)-epidihydropinidine, and the lowest IC50 value of 1.34 µg/mL and a MIC of 5.37 µg/mL could be recorded against this bacterial strain (Table 1 and Fig. 2a). It is noteworthy that the MIC of (±)-epidihydropinidine was nearly three times lower than the MIC of tetracycline (15.63 µg/mL) against *P. aeruginosa* (Table 1 and Fig. 2b). The growth inhibition of (±)-epidihydropinidine was dose-dependent and still at 2.68 and 1.34 µg/mL, a 73 % and 52 % growth inhibition could be observed, respectively (Fig. 2a). For comparison, at 1.95 µg/mL, tetracycline inhibited only 4.5 % of the growth of *P. aeruginosa* (Fig. 2b).

Promising growth inhibitory results were also obtained against the gram-positive *Enterococcus faecalis*. (±)-Epidihydropinidine gave a MIC of 5.37 µg/mL and an IC50 of 2.68 µg/mL against this bacterium and was nearly three times more effective than tetracycline which gave a MIC of 15.63 µg/mL (Table 1, Fig. 3a). The growth inhibitory effects of (±)-epidihydropinidine were dose-dependent

and still at 0.67 µg/mL a 22.68 % inhibition of growth could be observed. At 0.33 µg/mL the growth inhibitory effect increased slightly to 27.69 % but decreased again at 0.16 µg/mL to 20.93 % (Fig. 3a). At concentrations below 1.95 µg/ml, however, tetracycline was more effective than (±)-epidihydropinidine. For comparison, at 0.24 µg/mL tetracycline inhibited 46.22 % of the growth of *E. faecalis* (Fig. 3b).

In our study (±)-epidihydropinidine gave a good dose-dependent growth inhibition against *S. aureus*, showing a MIC of 10.75 µg/mL and an IC50 of 5.37 µg/mL (Table 1, Fig. 4a). (±)-Epidihydropinidine was bactericidal at concentrations higher than 21.50 µg/mL. For comparison, MIC and IC50 of tetracycline against *S. aureus* were 0.97 and 0.12 µg/mL, respectively (Fig. 4b).

(±)-Epidihydropinidine gave good dose-dependent growth inhibitory effects against *B. cereus*, showing a MIC of 10.75 µg/mL and an IC50 of 5.37 µg/mL (Table 1, Fig. 5a). In comparison, tetracycline and ampicillin gave MIC values of 0.48 and 250 µg/mL, respectively (Table 1 and Fig. 5b). Thus, the MIC of epidihydropinidine was 23 times lower than the MIC of ampicillin.

S. enterica was most resistant of all the investigated bacteria and fungi against the growth inhibitory effects of (±)-epidihydropinidine. Similarly as for the other bacteria the growth inhibition of (±)-epidihydropinidine was dose dependent and a decrease of inhibitory effects could be seen with decreasing concentrations (Fig. 6a). MIC was 43 µg/mL and IC50 16.50 µg/mL (Table 1). MIC was at the same time also minimum bactericidal concentration (MBC) and this concentration was found to be bactericidal even after two days of incubation. For comparison, tetracycline and ampicillin gave MIC values of 1.95 and 7.81 µg/mL, respectively, against *S. enterica* (Table 1, Fig. 6b).

3.2. Antifungal effects

(±)-Epidihydropinidine gave promising growth inhibitory effects against *C. albicans* and *C. glabrata*, showing MIC and MFC values of 5.37 and 43 µg/mL, respectively, against both fungal species (Table 1). A sharp decline could be observed in the growth inhibitory effects of (±)-epidihydropinidine between MIC and 2.68 µg/mL for both *Candida* species (Fig. 7a). At 2.68 and 1.34 µg/mL, no growth inhibition, but a slight growth stimulation was observed for both *Candida* species. In comparison to (±)-epidihydropinidine the antifungal standard drug, amphotericin B, gave a MIC of 0.24 µg/mL against *C. albicans* (Table 1) and similarly as for (±)-epidihydropinidine there was a sharp decline in inhibitory effects at smaller concentrations such as between 0.24 and 0.12 µg/mL (Fig. 7b). Amphotericin-B was less active against *C. glabrata*, showing a MIC of 15.63 µg/mL and thus (±)-epidihydropinidine was almost three times more effective.

4. Discussion

Our results on the anti-*Pseudomonas* activity of (±)-epidihydropinidine are promising since gram-negative bacteria in general are more resistant to antibiotic agents than gram-positive bacteria due to the presence of an outer lipopolysaccharide (LPS) rich membrane which acts as a permeability barrier, especially for hydrophilic and negatively charged molecules [74,75,76,77,78]. We suggest that (±)-epidihydropinidine, as a relatively hydrophilic water soluble molecule [79], is exerting its growth inhibitory effects on *P. aeruginosa*, and other gram-negative bacteria, by travelling through porins, water-filled channels in the outer lipopolysaccharide membrane and reaching possible target in periplasmic space or at inner cell membrane [80].

There are only a few other studies on the effects of piperidine alkaloids on the growth of *P. aeruginosa* and some other gram-negative bacterial species; (+)-euphococcinine, another piperidine alkaloid in spruce, was found to give slight growth inhibition against gram-positive bacteria (MIC 100-1000 µg/mL) but no activity against gram-negative bacteria [61]. In another study [81], an alkaloid fraction of *Piper nigrum* fruits was found to be more growth inhibitory against gram-negative than gram-positive bacteria. Moreover, piperine from the fruits of *Piper nigrum* was antibacterial against *P. aeruginosa* giving a MIC of 250 µg/mL [50]. This MIC is, however, 46 times higher than our MIC for (±)-epidihydropinidine against *P. aeruginosa*. Interestingly, in another study piperine, at concentrations of 0.5-10 µg/mL, was found to decrease bacterial swarming and swimming motility in *E. coli* and increased the penetration of ciprofloxacin and azithromycin in *E. coli* biofilms [82]. This warrants further studies on the effects of (±)-epidihydropinidine on the motility of *Pseudomonas aeruginosa*, since motility is important for pathogenesis, i.e. the capacity of an infection to spread. Solenopsine A, a piperidine alkaloid from the venom of the fire ant, *Solenopsis invicta*, has been found to suppress quorum sensing signaling resulting in a decrease in virulence factor production and biofilm formation in *P. aeruginosa* [83] and the authors of this investigation also hypothesized that piperidine alkaloids containing shorter acyl side chains would be even more effective antagonists of quorum sensing inducers than solenopsine A. This would apply for (±)-epidihydropinidine, which has a relatively short acyl chain, and therefore its effects on QS inducers of *P. aeruginosa* should be investigated.

Our results indicate that (±)-epidihydropinidine could be used in very small concentrations to prevent *P. aeruginosa* and other species of *Pseudomonas* to cause spoilage of refrigerated, aerobically stored meat [67,84]. Moreover, (±)-epidihydropinidine might be used for treatment of *P. aeruginosa*

induced bronchial infections in cystic fibrosis patients. However, further studies are needed to clarify possible adjuvant *in vitro* and *in vivo* effects of (±)-epidihydropinidine and its enantiomers, in combination with conventional antibiotics, among them ciprofloxacin, used for cystic fibrosis. Keeping in mind that there are only few novel agents available with anti-pseudomonad activity [5,85], new compounds inhibiting the growth of *Pseudomonas aeruginosa* would be needed urgently. Currently, there is only a limited number of novel drugs in pre-clinical or clinical development for treatment of multi-drug resistant *P. aeruginosa* and with the exception of doripenem, no new anti-pseudomonal drugs have reached the market recently [85].

To the best of our knowledge other plant derived piperidine alkaloids have not been tested for their growth inhibitory effects against *E. faecalis.* Synthetic isomers of phenylpiperidines, originally used as neuroleptics, have been found to inhibit efflux channels in several bacterial strains, among them *E. faecalis*, and in this way to potentiate the effects of conventional antibiotics [86]. It is therefore possible that also (±)-epidihydropinidine might inhibit the function of efflux channels in *E. faecalis* and other bacteria. According to our results (±)-epidihydropinidine could be a new plant derived compound for the treatment of *E. faecalis* induced infections and more research should be performed on its growth inhibitory effects in combination with standard treatment antibiotics such as ampicillin, gentamycin, streptomycin and vancomycin.

Promising growth inhibitory effects against *S. aureus* has been reported for some other piperidine alkaloids: Piperidine alkaloids in general potentiate the effects of antibiotics against *S. aureus* by inhibiting the function of efflux pumps [51,87]. Piperine from the fruits of *Piper nigrum* and other *Piper* spp. was shown to give antibacterial activity against *S. aureus* (MIC 250 µg/mL) [50] and to reduce the MIC of mupirocin fourfold and that of ciprofloxacin eight times against *S. aureus* [51,87,88,89]. The MIC values of hydroxyspectaline and spectaline, two piperidine alkaloids from *Cassia siamaea,* were 12.5-50 µg/mL against *S. aureus* [90], the effects being well comparable to our MIC of 10.75 µg/mL for (±)-epidihydropinidine. In addition to inhibition of efflux pumps, modulation of membrane permeability has been suggested to be one possible antimicrobial mechanism of action of piperidine alkaloids: Karsha et al. [50] demonstrated that treating *S. aureus* with MIC concentrations of black pepper extracts resulted in leakage of proteins and nucleic acid material. In comparison to these reports on the anti-*Staphylococcal* effects of other piperidine alkaloids, with MICs ranging from 12.5-250 µg/mL, our results demonstrate that (±)-epidihydropinidine could be very promising as a scaffold for new antibacterial agents and antibiotic potentiators against *S. aureus*.

Our results are supported by previous reports on good growth inhibitory effects of other piperidine alkaloids and piperidine alkaloid rich extracts against *B. cereus* and other *Bacillus* species: Piperine and piperidine in antimicrobial dichloromethane extracts of *Piper nigrum* were suggested to be the active compounds behind promising growth inhibitory effects against *B. cereus* (MIC 62.5 µg/mL) and in agreement with our results on (±)-epidihydropinidine (MIC 10.75 µg/mL) the extracts were found to be more active than ampicillin [50]. Moreover, a root chloroform extract of *Microcos paniculata* (Tiliaceae), known to contain 2,3,6-trisubstituted piperidine alkaloids such as microcosamines A and B as well as microgrewiapines [91], was shown to give growth inhibitory effects against *B. cereus*, along with some other bacterial species such as *Salmonella typhi* and *Vibrio cholera* [92]. In view of the increasing knowledge on the potential pathogenicity of strains of *B. cereus*, some causing serious central nervous system infections in patients with carcinoma and neutropenia [93,94] as well as other serious infections [15,17], it would be important to find new effective and safe agents for treatment of these infections. Our results demonstrate that (±)-epidihydropinidine could be a candidate.

To the best of our knowledge no other plant derived pure piperidine alkaloids have been investigated for their growth inhibitory effects against *Salmonellae*. Extracts from *Piper nigrum* were found to exert promising growth inhibitory effects against *S. typhimurium* and the good growth inhibitory effects were suggested to be due to the piperidine alkaloid piperine [95]. Our results indicate that (±)-epidihydropinidine could possibly be used as a food preservative for the inhibition of Salmonellosis. In contrast to our results with epidihydropinidine, lignans and stilbenes in polar extracts of Norway spruce knotwood were found to be devoid of growth inhibitory effects against *Salmonella infantis* [96]. It seems therefore that epidihydropinidine would be one potential anti-*Salmonella* compound in Norway spruce.

C. albicans has been reported to be the primary causative agent of candidemia [69] and in view of the increasing resistance of *C. albicans* to the two gold-standard antifungal drugs, amphotericin-B and fluconazole [97,98], our antifungal result for (±)-epidihydropinidine is notable. Multi-drug resistance in *C. albicans* has been reported and is due to expression of ATP binding cassette transporter proteins in the membrane [99]. There is an urgent need for new antifungal compounds against *C. albicans*, and (±)-epidihydropinidine represents a promising candidate.

In our study (±)-epidihydropinidine was nearly three times more active than amphotericin B (MIC 15.63 µg/mL) against *Candida glabrata* (Table 1). This result is especially promising considering that in Europe and the USA, *C. glabrata* has been reported to be the second most important

fungal pathogen causing systemic infections after *C. albicans* [42] and high mortality rates have been described [100]. Amphotericin B is generally applied for primary therapy of severe infections caused by *C. glabrata* [101] but high MIC values of 25 µg/mL against some strains have been demonstrated in agreement with our results [102,103]. Moreover, 5 % of nosocomial isolates of *C. glabrata* have been found to be resistant to caspofungin, a drug belonging to the newest class of antifungals, the echinocandins, which target cell wall synthesis [44].

Some other piperidine alkaloids in spruce have been screened for growth inhibitory effects against yeasts; euphococcinine was found to lack growth inhibitory effects whereas (-)-pinidinol gave slight inhibition [61]. Haloxyline A and B from *Haloxylon salicornicum* (Chenopodiaceae), showed better antifungal effects (MIC 65 and 80 µg/mL, respectively) than the standard antifungal drug miconazol (MIC 110 µg/mL) against *C. glabrata* and *C. albicans* [104]. However, when compared to our results with (±)-epidihydropinidine against both *C. albicans* and *C. glabrata*, the haloxylines were still 10-12 times less effective. Moreover, it was found that mycelial growth of the plant pathogenic fungal species, *Pythium ultimum* was significantly reduced by fire ant venom piperidine alkaloids, which closely resemble epidihydropinidine [105]. Therefore, epidihydropinidine and other structurally closely related piperidine alkaloids seem to have promising potential as scaffolds for new antifungal agents, both against *Candida* species and filamentous fungi.

5. Conclusions

(±)-Epidihydropinidine gives promising broad-spectrum antibacterial and antifungal activities showing growth inhibitory effects against all test organisms in this investigation with the smallest MIC values ranging from 5.37-10.75 µg/mL. Of special significance is the low MIC of 5.37 µg/mL against *Pseudomonas aeruginosa*, being comparable to the MIC of Tomopenem (4 µg/mL), a new carbapenem compound in clinical trial [106]. Bactericidal and fungicidal effects at concentrations of 43 µg/mL were observed against *Salmonella enterica* as well as *Candida albicans* and *C. glabrata*.

Our results indicate that (±)-epidihydropinidine could have special potential as a new active compound or drug adjuvant to be developed against bacterial and fungal infections. It must be noted here, that since the biological activity usually is highly enantiomer specific, and in coniferous trees, such as Norway spruce, epidihydropinidine appears in the (+)-enantiomeric form [61,62], further antimicrobial studies are needed using pure (+)-epidihydropinidine and (-)-epidihydropinidine. In the racemic mixture of epidihydropinidine, which we have used in this investigation, the activity could be

due to synergistic effects between the two isomers. Studies on the potential of (±)-epidihydropinidine and its isomers as adjuvants in combination with antibiotics are also warranted.

Further studies are needed to investigate the toxicity of (±)-epidihydropinidine and its enantiomers since piperidine alkaloids in general have been found to be both teratogenic and toxic [61,107]. The LD50 value for epidihydropinidine has not been determined, but we have found that field voles (*Microtus agrestis*) seem to be resistant to Norway spruce alkaloids in general [55].

Mechanistic studies should be carried out to elucidate the action of (±)-epidihydropinidine and its enantiomers on drug efflux pumps, cell division, cell wall synthesis and integrity, as well as on biofilm formation. In addition, other piperidine alkaloids in spruce, which have not been studied for their antimicrobial effects should be explored, alone and in combinations with (±)-epidihydropinidine. Synergistic effects of piperidine alkaloids in alkaloid enriched extracts from spruce are possible.

The results on the antimicrobial potential of epidihydropinidine are especially promising in view of the increasing global needs for new and effective broad-spectrum antibiotics, useful for the treatment of antibiotic resistant microbes [108].

Table 1
Antibacterial and antifungal effects of (±)-epidihydropinidine and reference antibiotics. MIC, MBC/MFC and IC_{50} in µg/mL. For the positive controls MIC values of ≥250 µg/mL indicate that the microorganism is resistant to these agents.

Test compound and antibiotics	*Staphylococcus aureus* ATCC 25923	*Bacillus cereus* ATCC 10987	*Salmonella enterica* ATCC 43845	*Enterococcus faecalis* ATCC 29212	*Pseudomonas aeruginosa* ATCC 27853	*Candida albicans* ATCC 10231	*Candida glabrata* ATCC 2001
(±)-Epidihydropinidine							
MIC	10.75 (IC_{92})	10.75 (IC_{100})	43.00 (IC_{98})	5.37 (IC_{94})	5.37 (IC_{99})	5.37 (IC_{100})	5.37 (IC_{100})
MBC/MFC	> 21.50	> 21.50	43.00	NT	> 10.75	43.00	43.00
IC_{50}	5.37	5.37	16.50	2.68	1.34	NE	NE
Tetracycline							
MIC	0.97 (IC_{99})	0.48 (IC_{98})	1.95 (IC_{94})	15.63 (IC_{99})	15.63 (IC_{94})	NT	NT
MBC/MFC	NT	1.95	> 3.91	NT	NT	NT	NT
IC_{50}	0.12	< 0.24	< 0.48	0.48	7.80	NT	NT
Ampicillin							
MIC	NT	250 (IC_{100})	7.81(IC_{98})	NT	NT	NT	NT
MBC/MFC	NT	> 250	> 15.63	NT	NT	NT	NT
IC_{50}	NT	125	3.91	NT	NT	NT	NT
Amphotericin B							
MIC	NT	NT	NT	NT	NT	0.24 (IC_{99})	15.63*
MBC/MFC	NT	NT	NT	NT	NT	NT	NT
IC_{50}	NT	NT	NT	NT	NT	NT	NT

NT, Not tested; NE, Not estimated; MIC, minimum inhibitory concentration; *MIC obtained using agar diffusion method; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; IC50 (half-maximal inhibitory concentration),

concentration leading to 50 % reduction of growth compared to control. Results which are not exact MBC/MFC and IC50 values but estimates close to these values are marked with < or >. IC, inhibitory concentration. In brackets the percentage of the bacterial/fungal cell growth inhibited by the indicated concentration.

References

[1] D.M. Livermore, Discovery research: the scientific challenge of finding new antibiotics. J. Antimicrob. Chemoth. (2011) 1–4.

[2] T.R. Walsh, M.A. Toleman, The emergence of pan-resistant gram-negative pathogens merits a rapid global political response, J. Antimicrob. Chemoth. 67 (2012) 1–3.

[3] U. Theuretzbacher, Accelerating resistance, inadequate antibacterial drug pipelines and international responses. Int. J. Antimicrob. Ag. 39 (2012) 295–299.

[4] A.E. Clatworthy, E. Pierson, D.T. Hung, Targeting virulence: a new paradigm for antimicrobial therapy, Nature Chem. Biol. 3 (99) (2007) 541–548.

[5] M. Vaara, Novel derivatives of Polymyxins, J. Antimicrob. Chemoth. (2013) 1213–1219.

[6] Y. Le Loir, F. Baron, M. Gautier, *Staphylococcus aureus* and food poisoning, Genetics and Molecular Research 2 (1) (2003) 63–76.

[7] J. Kadariya, T.C. Smith, D. Thapaliya, *Staphylococcus aureus* and Staphylococcal Food-Borne Disease: An Ongoing Challenge in Public Health, BioMed Research International Article ID 827965 (2014) 1–9.

[8] C. Liu, A. Bayer, S.E. Cosgrove, R.S. Daum, S.K. Fridkin, R.J. Gorwitz, S.E. Kaplan, A.W. Karchmer, D.P. Levine, B.E. Murray, M.J. Rybak, A.D. Talan, H.F. Chambers, Clinical Practice Guidelines by the Infectious Diseases Society of America for the Treatment of Methicillin-Resistant

Staphylococcus Aureus Infections in Adults and Children, Clinical Infectious Diseases Advance Access 2 (2011) 1–38.

[9] H. Grundmann, M. Aires-de-Sousa, J. Boyce, E. Tiemersma, Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat, Lancet 368 (2006) 874–885.

[10] B.A. Diep, A.M. Palazzolo-Ballance, P. Tattevin, L. Basuino, K.R. Braughton, A.R. Whitney, L. Chen, B.N. Kreiswirth, M. Otto, F.R. Deleo, H.F. Chambers, Contribution of Panton-Valentine leukocidin in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis, PLOS ONE 3 (9) (2008) 1–8.

[11] S.C. de Oliveira, V. dos Santos Falcão-Silvia, J. Pinto Siqueira-Júnior, D.P. Harding, F.B. Lira, F.G.F. Lorenzo, J.M. Barbosa-Filho, P.F. de Athayde-Filho, Drug Resistance Modulation in *Staphylococcus aureus*, A New Biological Activity for Mesoionic Hydrochloride Compounds, Molecules 16 (2011) 2023–2031.

[12] B.P. Howden, C.R.E. McEvoy, D.L. Allen, K. Chua, W. Gao, P.F. Harrison, J. Bell, G. Coombs, V. Bennett-Wood, J.L. Porter, R. Robins-Browne, J.K. Davies, T. Seemann, T.P. Stinear, Evolution of Multidrug Resistance during *Staphylococcus aureus* infection involves Mutation of the Essential Two Component Regulator WalKR, PLoS pathogens 2011; 7 (11): 1–15.

[13] M. Ehling-Schulz, M. Fricker, S. Scherer, *Bacillus cereus*, the causative agent of an emetic type of food-borne illness, Mol. Nutr. Food Res. 48 (2004) 479–487.

[14] V.A. Luna, D.S. King, J. Gulledge, A.C. Cannons, P.T. Amuso, J. Cattani, Susceptibility of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre® automated microbroth dilution and Etest® agar gradient diffusion methods, J. Antimicrob. Chemoth. 60 (2007) 555–567.

[15] A.S. Ginsburg, L.G. Salazar, L.D. True, M.L. Disis, Fatal *Bacillus cereus* sepsis following resolving neutropenic enterocolitis during the treatment of acute leukemia, Am. J. Hematol. 72 (2003) 204–209.

[16] T. Lund, M.I. De Buyser, P.E. Granum, A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis, Mol. Microbiol. 38 (2005) 254–261.

[17] J.M. Miller, J.G. Hair, M. Hebert, L. Hebert, F.J. Roberts Jr, R.S. Weyant, Fulminating bacteremia and pneumonia due to *Bacillus cereus*. J. Clin. Microbiol. 35 (1997) 534–537.

[18] D.M. Citron, M.D. Appleman, In vitro activities of daptomycin, ciprofloxacin and other antimicrobial agents against the cells and spores of clinical isolates of *Bacillus* species, J. Clin. Microbiol. 44 (2006) 3814–3818.

[19] X.-Z. Li, H. Nikaido, Efflux-Mediated Drug Resistance in Bacteria: An Update, Drugs 2009; 69 (12): 1555–1623.

[20] T. Kruger, D. Szabo, K.H. Keddy, K. Deeley, J.W. Marsh, A.M. Hujer, R.A. Bonomo, D.L. Paterson, Infections with Nontyphoidal *Salmonella* Species Producing TEM-63 or a Novel TEM Enzyme, TEM-131, in South Africa, Antimicrob. Agents Ch. 48 (2004) 4263–4270.

[21] M.A. Bisi-Johnson, C.L. Obi, J. Eloff, B.B. Samuel, K. Baba, S. Vasaikar, M.A. Adefisoye, Can herbal remedies be the answer to multidrug resistance? Profile of drug resistance in *Salmonella* species in Eastern Cape, South Africa, J. Exp. Integr. Med. 2 (2) (2012) 147–153.

[22] R.M. Humphries, F.C. Fang, F.M Aarestrup, J.A. Hindler, In Vitro Susceptibility Testing of Fluoroquinoline Activity Against *Salmonella*: Recent Changes to CLSI Standards, Clin. Infect. Dis. 55 (8) (2012) 1107–1113.

[23] B.R. Jackson, P.M. Griffin, D. Cole, K.A. Walsh, S.J. Chai, Outbreak-associated *Salmonella enterica* Serotypes and Food Commodities, United States, 1998-2008, Emerg. Infect. Dis. 19 (8) (2013) 1239–1244.

[24] E. Scallan, R.M. Hoekstra, F.J. Angulo, R.V. Tauxe, M.-A. Widdowson, S.L. Roy, J.L. Jones, P.M. Griffin, Foodborne illness acquired in the United States – major pathogens, Emerg. Infect. Dis. 17 (2011) 7–15.

[25] F. Arancibia, T.T. Bauer, S. Ewig, J. Mensa, J. Gonzales, M.S. Niederman, A. Torres, Community-acquired pneumonia due to gram-negative bacteria and *Pseudomonas aeruginosa*: incidence, risk and prognosis, Arch. Intern. Med. 162 (16) (2002) 1849–1858.

[26] S. Fujitani, H.-Y. Sun, V.L. Yu, J.A. Weingarten, Pneumonia due to *Pseudomonas aeruginosa*. Part I. Epidemiology, Clinical diagnosis, and Source, Chest 139 (4) (2011) 909–919.

[27] A.A. El-Leboudy, A.A. Amer, M.E. Nasief, S.M. Eltony, Occurrence and Behaviour of *Pseudomonas* Organisms in White Soft Cheese. Alexandria J. Vet. Sci. 44 (2015) 74–79.

[28] K. Poole, *Pseudomonas aeruginosa*: Resistance to the max, Front. Microbiol. 2, Article 65 (2011) 1–13.

[29] M.G. Page, J. Heim, Prospects for the next anti-*Pseudomonas* drug, Curr. Opin. Pharmacol. 9 (2009) 558–565.

[30] P.D. Lister, D.J. Wolter, N.D. Hanson, Antibacterial-Resistant *Pseudomonas aeruginosa*: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. Clin. Microbiol. Rev. 22 (4) (2009) 582–610.

[31] T. Rasamiravaka, Q. Labtani, P. Duez, M. El Jaziri, The Formation of Biofilms by *Pseudomonas aeruginosa*: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms, BioMed Res. Int., Article ID 759348 (2014) 1–17.

[32] B.D. Shepard, M.S. Gilmore, Differential expression of virulence-related genes in *Enterococcus faecalis*. Response to biological cues in serum and urine, Infect. Immun. 70 (2002) 4344–4352.

[33] A.L. De Castilho, J.P.C. Da Silva, C.H.C. Saraceni, I.E.C. Diaz, M.L.B. Paciencia, A.D. Varella, I.B. Suffredini, *In vitro* activity of Amazon Plant extracts against *Enterococcus faecalis*, Braz. J. Microbiol. 45 (3) (2014) 769–779.

[34] D.R. Murdoch, S. Mirrett, L.J. Harrell, J.S. Monahan, L.B. Reller, Sequential emergence of antibiotic resistance in enterococcal bloodstream isolates over 25 years, Antimicrob. Agents Ch. 46 (2002) 3676–3678.

[35] J.C. Graham, F.K. Gould, Role of aminoglycosides in the treatment of bacterial endocarditis, J. Antimicrob. Chemoth. 49 (2002) 437–444.

[36] M.B. Pasticci, A. Mencacci, A. Moretti, N. Palladino, L.M. Lapalorcia, F. Bistoni, F. Baldelli, In vitro Antimicrobial Activity of Ampicillin-Ceftriaxone and Ampicillin-Ertapenem Combinations against Clinical Isolates of *Enterococcus faecalis* with High Levels of Aminoglycoside Resistance, The Open Microbiol. Journal 2 (2008) 79–84.

[37] D. Morrison, N. Woodford, B. Cookson, Enterococci as emerging pathogens of humans, J. Appl. Microbiol. Symp. Suppl. 83 (1997) 89–99.

[38] P.N. Malani, L.A. Thal, S.M. Donabedian, B. Robinson-Dunn, C.A. Kaufmann, J.W. Chow, E. Herschberger, M.J. Zervos, Molecular analysis of vancomycin-resistant *Enterococcus faecalis* from Michigan hospitals during a 10 year period. J. Antimicrob. Chemoth. 49 (2002) 841–843.

[39] E.E. Udo, N. Al-Sweih, P. John, T.D. Chugh, Antibiotic resistance of enterococci isolated at a teaching hospital in Kuwait, Diagn. Micr. Infec. Dis. 43 (2002) 233–238.

[40] A. Koluman, L.S. Akanb, F.P. Çakiroğlu, Occurrence and antimicrobial resistance of enterococci in retail foods, Food Control 20 (2009) 281–283.

[41] J. Barbosa, V. Ferreira, P. Teixeira, Antibiotic susceptibility of enterococci isolated from traditional fermented meat products, Food Microbiol. 26 (2009) 527–532.

[42] M.A. Pfaller, D.J. Diekema, Epidemiology of invasive candidiasis: a persistent public health problem, Clin. Microbiol. Rev. 20 (2007) 133–163.

[43] M.A. Pfaller, D.J. Diekema, D.L. Gibbs, V.A. Newell, D. Ellis, V. Tullio, A. Rodloff, W. Fu, T.A. Ling and the Global Antifungal Surveillance Group, Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* Species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion, J. Clin. Microbiol. 48 (2010) 1366–1377, doi:10.1128/JCM.02117-09.

[44] L.A. Walker, N.A. Gow, C.A. Munro, Elevated chitin content reduces the susceptibility of *Candida* species to caspofungin, Antimicrob. Agents. Ch. 57 (2013) 146–154.

[45] I. Chopra, The 2012 Garrod Lecture: Discovery of antibacterial drugs in the 21st century, J. Antimicrob. Chemoth. 68 (2013) 496–505.

[46] W.H. Lewis, M.P. Elvin-Lewis, Medicinal plants as sources of new therapeutics. Ann. Mo. Bot. Gard. 82 (1995)16–24.

[47] T.T.P. Cushnie, B. Cushnie, A.J. Lamb, Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities, Int. J. Antimicrob. Ag. 44 (2014) 377–386.

[48] A. Chakraborty, B.K. Chowdhury, P. Bhattacharyya, Clausenol and clausenine–two carbazole alkaloids from *Clausena anisata*, Phytochemistry 40 (1995) 295–298.

[49] M. Cowan, Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev. 12 (4) (1999) 564–582.

[50] P.V. Karsha, O.B. Lakshmi, Antibacterial activity of black pepper (*Piper nigrum* Linn.) with special reference to its mode of action on bacteria. Ind. J. Nat. Prod. Res. 1 (2) (2010) 213–215.

[51] M.Z. Mirza, A. Kumar, N.P. Kalia, A. Zargar, I.A. Khan, Piperine as an inhibitor of the MdeA efflux pump of *Staphylococcus aureus*, J. Med. Microbiol. 60 (2011) 1472–1478.

[52] W. Maneerat, W. Phakhodee, T. Ritthiwigrom, S. Cheenpracha, T. Promgool, K. Yossathera, S. Deachathai, S. Laphookhieo, Antibacterial carbazole alkaloids from *Clausena harmandiana* twigs, Fitoterapia 83 (2012) 1110–1114.

[53] C. Macfoy, D. Danosus, R. Sandit, T.H. Jones, H.M. Garraffo, T.F. Spande, J.W. Daly, Alkaloids of anuran skin: antimicrobial function? Z. Naturforsch. C 60 (2005) 932–937.

[54] A.E. Mina, A.K. Ponti, N.L. Woodcraft, E.E. Johnson, R.A. Saporito, Variation in alkaloid-based microbial defenses of the dendrobatid poison frog *Oophaga pumilio*, Chemoecology (2015) 1–9.

[55] V. Virjamo, R. Julkunen-Tiitto, H. Henttonen, E. Hiltunen, R. Karjalainen, J. Korhonen, O. Huitu, Differences in Vole Preference, Secondary Chemistry and Nutrient Levels Between Naturally Regenerated and Planted Norway Spruce Seedlings, J. Chem. Ecol. 39 (2013) 1322-1334.

[56] V. Virjamo, Piperidine alkaloids of Norway spruce (*Picea abies* L. Karsten). Relations with genotypes, season, environment and phenolics, Doctoral thesis 2013, University of Eastern Finland, Joensuu, ISBN 978‐952‐61‐1324‐1 (2013) 61 pp.

[57] V. Virjamo, S. Sutinen, R. Julkunen-Tiitto, Combined effect of elevated UVB, elevated temperature and fertilization on growth, needle structure and phytochemistry of young Norway spruce (*Picea abies*) seedlings, Glob. Change Biol. 20 (2014) 2252–2260.

[58] V. Virjamo, R. Julkunen-Tiitto, Shoot development of Norway spruce (*Picea abies*) involves changes in piperidine alkaloids and condensed tannins, Trees 28 (2) (2014) 427–437.

[59] V. Virjamo, R. Julkunen-Tiitto, Variation in piperidine alkaloid chemistry of Norway spruce (*Picea abies*) foliage in diverse geographical origins grown at the same area, Can. J. Forest Res. 46 (4) (2016) 456–460.

[60] S. Metsämuuronen, H. Siren, Antibacterial Compounds in Predominant Trees in Finland: Review, J. Bioproc. Biotechniq. 4 (5) (2014) 1–13.

[61] J.N. Tawara, A. Blokhin, T.A. Foderaro, F.R. Stermitz, H. Hope, Toxic piperidine alkaloids from pine (*Pinus*) and spruce (*Picea*) trees. New structures and a biosynthetic hypothesis, J. Org. Chem. 58 (1993) 4813–4818.

[62] M.J. Schneider, J.A. Montali, D. Hazen, C.E. Stanton, Alkaloids of *Picea*, J. Nat. Prod. 54 (1991) 905–909.

[63] L. Shtykova, M. Masuda, C. Eriksson, K. Sjödin, E. Marling, F. Schlyter, M. Nydén, Latex coatings containing antifeedants: Formulation, characterization, and application for protection of conifer seedlings against pine weevil feeding, Prog. Org. Coat. 63 (2008) 160–166.

[64] G. Kozlowski, J.-P. Métraux, Antifungal properties of Norway spruce (*Picea abies* (L.) Karst.) seedling homogenates, Acta Soc. Bot. Pol. 68 (1999) 191–195.

[65] D.P. Jouvenaz, M.S. Blum, J.G. MacConnell, Antibacterial activity of venom alkaloids from the imported fire ant, *Solenopsis invicta* Buren, Antimicrob. Agents Ch. 2 (1972) 291–293.

[66] M.S. Obin, R.K. Vander Meer, Gaster Flagging by Fire Ants (Solenopsis spp.). Functional significance of venom dispersal behavior, J. Chem. Ecol. 11 (1985) 1757–1768.

[67] J. Petrova, A. Pavelkova, L. Hleba, J. Pochop, K. Rovná, Microbiological Quality of Fresh Chicken Breast Meat after Rosemary Essential Oil Treatment and Vacuum Packaging, Animal Sci. Biotechnol. 46 (1) (2013) 140–144.

[68] A.L. De Castilho, C.H.C. Saraceni, I.E.C. Diaz, M.L.B. Paciencia, I.B. Suffredini, New trends in dentistry; Plant extracts against *Enterococcus faecalis*. The efficacy compared to chlorhexidine. Braz. Oral Res. 27 (2) (2013) 109–115.

[69] M.A. Pfaller, D.J. Diekema, R.N. Jones, H.S. Sader, A.C. Fluit, R.J. Hollis, S.A. Messer, International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and in vitro susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from

1997 through 1999 in the SENTRY antimicrobial surveillance program, J. Clin. Microbiol. 39 (2001) 3254–3259.

[70] H.C. Brown, U.S. Racherla, Organoboranes. 43. A convenient, highly efficient synthesis of triorganylboranes via a modified organometallic route, J. Org. Chem. 51(1986) 427–432.

[71] Y.N. Bubnov, E.V. Klimkina, A.V. Ignatenko, Preparation of trans and cis-2-allyl-6-alkyl(aryl)-1,2,3,6-tetrahydropyridines based on the reductive trans-2,6-dialkylation of pyridine. Synthesis of (±)-epidihydropinidine and (±)-dihydropinidine, Russ. Chem. B+ 47 (1998) 451–458.

[72] W. Chen, Y. Liu, Z. Chen, A highly efficient and practical new allylboronate tartramide for the asymmetric allylboration of achiral aldehydes, Eur. J. Org. Chem. 8 (2005) 1665–1668.

[73] Clinical and Laboratory Standards Institute, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard- Ninth Edition, 2012, M07-A9 32 (2), 1–67.

[74] I.M. Helander, H.L. Alakomi, K. Latva-Kala, Characterization of the action of selected essential oil components on gram-negative bacteria, J. Agr. Food Chem. 46 (9) (1998) 3590–3595.

[75] S. Mullin, N. Mani, T.H. Grossman, Inhibition of Antibiotic Efflux in Bacteria by the Novel Multidrug Resistance Inhibitors Biricodar (VX-710) and Timcodar (VX 853), Antimicrob. Agents Ch. 48 (11) (2004) 4171–4176.

[76] T. Takahashi, R. Kokubo, M. Sakaino, Antimicrobial activities of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculate*, Lett. Appl. Microbiol. 39 (2004) 60–64.

[77] A.K. Tyagi, D. Bukvicki , D. Gottardi, M. Veljic, M.E. Guerzoni, A. Malik, P.D. Marin, Antimicrobial Potential and Chemical Characterization of Serbian Liverwort (*Porella arboris-vitae*): SEM and TEM observations, Evidence-Based Compl. and Alt. Med. vol. 2013, Article ID 382927 (2012) 1– 8.

[78] B. Jamil, Enterobacteriaceae: At the verge of treatment, Int. J. Innov. Appl. Studies 9 (4) (2014) 1736–1745.

[79] E.A. Gerson, R.G. Kelsey, Piperidine alkaloids in Sitka spruce with varying levels of resistance to white pine weevil (Coleoptera: Curculionideae), J. Econ. Entomol. 95 (2002) 608–613.

[80] J.-M. Bolla, S. Alibert-Franco, J. Handzlik, J. Chevalier, A. Mahamoud, G. Boyer, K. Kieć-Kononowicz, J.-M. Pagés, Strategies for bypassing the membrane barrier in multidrug-resistant Gram-negative bacteria. FEBS Lett. 585 (201) 1682–1690.

[81] A.W. Al-Shahwany, Alkaloids and Phenolic Compound Activity of *Piper nigrum* against some Human Pathogenic Bacteria, Biomed. & Biotechnol. 2 (1) (2014) 20–28.

[82] D.H. Dusane, Z. Hosseinidoust, B. Asadishad, N. Tufenkji, Alkaloids Modulate Motility, Biofilm Formation and Antibiotic Susceptibility of Uropathogenic *Escherichia coli*, PLOS ONE 9 (11) (2014) 1–9.

[83] J. Park, G.F. Kaufmann, J.P. Bowen, J.L. Arbiser, K.D. Janda, Solenopsin A, a venom alkaloid from the fire ant *Solenopsis invicta*, inhibits quorum-sensing signaling in *Pseudomonas aeruginosa*, J. Infect. Dis. 198 (2008) 1198–1201.

[84] F. De Filippis, A. La Storia, F. Villani, D. Ercolini, Exploring the Sources of Bacterial Spoilers in Beefsteaks by Culture-Independent High-Troughput sequencing, PLOS ONE 8 (7) (2013) 1–10.

[85] M.G. Page, J. Heim, Prospects for the next anti-*Pseudomonas* drug, Curr. Opin. Pharmacol. 9 (2009) 558–565.

[86] J.E. Kristiansen, O. Hendricks, T. Delvin, T.S. Butterworth, L. Aagaard, J.B. Christensen, V.C. Flores, H. Keyzer, Reversal in resistance of microorganisms by help of non-antibiotics, J. Antimicrob. Chemoth. 59 (2007) 1271–1279.

[87] M. Stavri, L.J.V. Piddock, S. Gibbons, Bacterial efflux pump inhibitors from natural sources, J. Antimicrob. Chemoth. 59 (2007) 1247–1260.

[88] I.A. Khan, Z.H. Mirza, A. Kumar, V. Verma, G.N. Quazi, Piperine, a phytochemical potentiator of ciprofloxacin against *Staphylococcus aureus*, Antimicrob. Agents Ch. 50 (2006) 810–812.

[89] A. Kumar, I.A. Khan, S. Koul, J.L. Koul, S.C. Taneja, I. Ali, F. Ali, S. Sharma, Z.M. Mirza, M. Kumar, P.L. Sangwan, P. Gupta, N. Thota, G.M. Qazi, Novel structural analogues of piperine as inhibitors of the NorA efflux pump of *Staphylococcus aureus*, J. Antimicrob. Chemoth. 61 (2008) 1270–1276.

[90] X. Yang, P. Zhao, X. Liu, Z. Mei, Antibacterial evaluation of piperidine alkaloids from *Cassia siamea* Lam, Asian J. Chem. 24 (11) (2012) 5222–5224.

[91] P.C. Still, B. Yi, T.F. Gonzáles-Cestari, L. Pan, R.E. Pavloviz, H.-B. Chai, T.N. Ninh, C. Li, D.D. Soejarto, D.B. McKay, A.D. Kinghorn, Alkaloids from *Microcos paniculata* with Cytotoxic and Nicotinic Receptor Antagonistic Activities, J. Nat. Prod. 76 (2013) 246–249.

[92] A. Aziz, M.F.A.K. Shawn, S. Rahman, T. Islam, M. Mita, A. Faruque, S. Rana, Secondary metabolites, antimicrobial, brine shrimp lethality & 4th instar *Culex quinquefasciatus* mosquito larvicidal screening of organic and inorganic root extracts of *Microcos paniculata*, IOSR J. Pharm. & Biol. Sci. (IOSR-JPBS) 8 (5) (2013) 58–65.

[93] J. Schlegelowa, J. Brychta, E. Klimova, E. Napravnikova, V. Babak, The prevalence of and resistance to antimicrobial agents of *Bacillus cereus* isolates from foodstuffs, Vet. Med. –Czech 48 (11) (2003) 331–338.

[94] E.J. Bottone, 2010, *Bacillus cereus*, a Volatile Human Pathogen. Clin. Microbiol. Rev. 23 (2) 382–398.

[95] S.K. Reshmi, E. Sathya, P. Suganya Devi, Isolation of piperidine from *Piper nigrum* and its antiproliferative activity, Afr. J. Pharm. & Pharmacol. 4 (8) (2010) 562–573.

[96] A.-L. Välimaa, U. Honkalampi-Hämäläinen, S. Pietarinen, S. Willför, B. Holmbom, A. von Wright, Antimicrobial and cytotoxic knotwood extracts and related pure compounds and their effects on food-associated microorganisms, Int. J. Food Microbiol. 115 (2007) 235–243.

[97] Z.A. Kanafami, J.R. Perfect, Resistance to Antifungal Agents: Mechanisms and Clinical Impact. Clin. Infect. Dis. 46 (2008) 120–128.

[98] J.H. Rex, M.G. Rinaldi, M.A. Pfaller, Resistance of *Candida* species to Fluconazole, Antimicrob. Agents Ch. 39 (1) (1995) 1–8.

[99] K. Gulshan, W.S. Moye-Rowley, Multidrug Resistance in Fungi, Eukaryotic cell 6-11 (2007) 1933–1942.

[100] M.A. Pfaller, D.J. Diekema, Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. J. Clin. Microbiol. 42 (2004) 4419–4431.

[101] D.A.N. Mario, L.B. Denardi, L.A. Bandeira, M.S. Antunes, J.M. Santurio, L.C. Severo, S.H. Alves, The activity of echinocandins, amphotericin B and voriconazole against fluconazole-susceptible and fluconazole-resistant Brazilian *Candida glabrata* isolates, Mem. Inst. Oswaldo Cruz, Rio de Janeiro 107 (3) (2012) 433–436.

[102] V. Krcmery, A.J. Barnes, Non-*albicans Candida* spp. causing fungemia: pathogenicity and antifungal resistance, J. Hosp. Infect. 50 (2002) 243–260.

[103] N. Kiraz, I. Dağ, M. Yamac, A. Kiremitci, N. Kaşifoğlu, Y. Akgun, Antifungal Activity of Caspofungin in Combination with Amphotericin B against *Candida glabrata*. Comparison of Disc Diffusion, Etest and Time-Kill Methods, Antimicrob. Agents Ch. 53 (2009) 788–790.

[104] S. Ferheen, E. Ahmed, N. Afza, A. Malik, M.R. Shah, S.A. Nawas, M.Y. Choudhary, Haloxylines A and B, Antifungal and Cholinesterase Inhibiting Piperidine Alkaloids from *Haloxylon salicornicum*, Chem. Pharm. Bull. 53 (5) (2005) 570–572.

[105] S.Z. Li, X. Jin, J. Chen, Effects of piperidine and piperideine alkaloids from the venom of red imported fire ants, *Solenopsis invicta* Buren, on *Pythium ultimum* Trow growth in vitro and the application of piperideine alkaloids to control cucumber damping-off in the greenhouse, Pest. Manag. Sci. 68 (2012)1546–1552.

[106] H.W. Boucher, G.H. Talbot, J.S. Bradley, J.E. Edwards Jr., D. Gilbert, L.B. Rice, M. Scheld, B. Spellberg, J. Bartlett, Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America, Clin. Infect. Dis. 48 (2009) 1–12.

[107] B.T. Green, S.T. Lee, K.E. Panter, D.R. Brown, Piperidine alkaloids: Human and food animal teratogens, Food Chem. Toxicol. 50 (2012) 2049–2055.

[108] S.A. Muhammad, A. Ali, A. Naz, A. Hassan, N. Riaz, S. Saeed-ul-Hassan, S. Andleeb, D. Barh, A New Broad-Spectrum Peptide Antibiotic Produced by *Bacillus brevis* strain MH9 Isolated from Margalla Hills of Islamabad, Pakistan, Int. J. Pept. Res. Ther. (2016) 271–279.

Acknowledgements

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Figure captions:

Fig. 1. Molecular structure of a) (+)- and b) (-)-epidihydropinidine found in *Picea abies* needles and bark.

Fig. 2. Dose-response of inhibition of growth for a) (±)-epidihydropinidine and b) tetracycline against *Pseudomonas aeruginosa*.

Fig. 3. Dose-response of inhibition of growth for a) (±)-epidihydropinidine and b) tetracycline against *Enterococcus faecalis*.

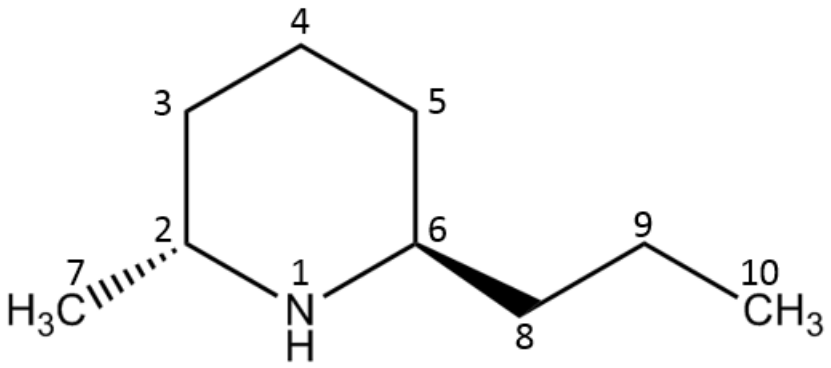
Fig. 4. Dose-response of a) (±)-epidihydropinidine and b) tetracycline against *Staphylococcus aureus*.

Fig. 5. Dose-response of a) (±)-epidihydropinidine and b) tetracycline against *Bacillus cereus*.

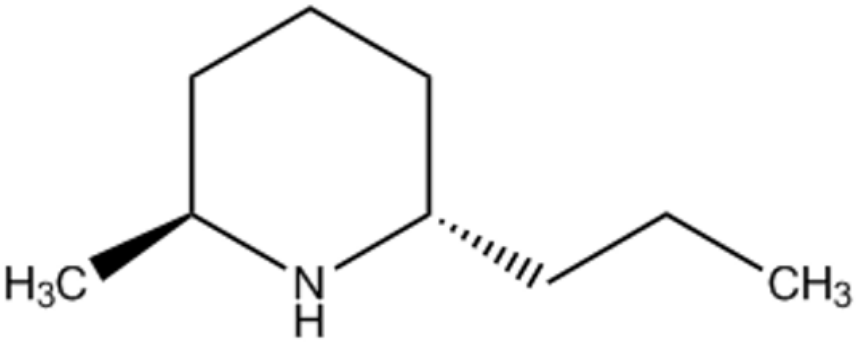
Fig. 6. Dose-response of a) (±)-epidihydropinidine and b) tetracycline against *Salmonella enterica*.

Fig. 7. Dose-response of a) (±)-epidihydropinidine and b) amphotericin-B against *Candida albicans*.

Figure 1



a)



b)

Figure 2

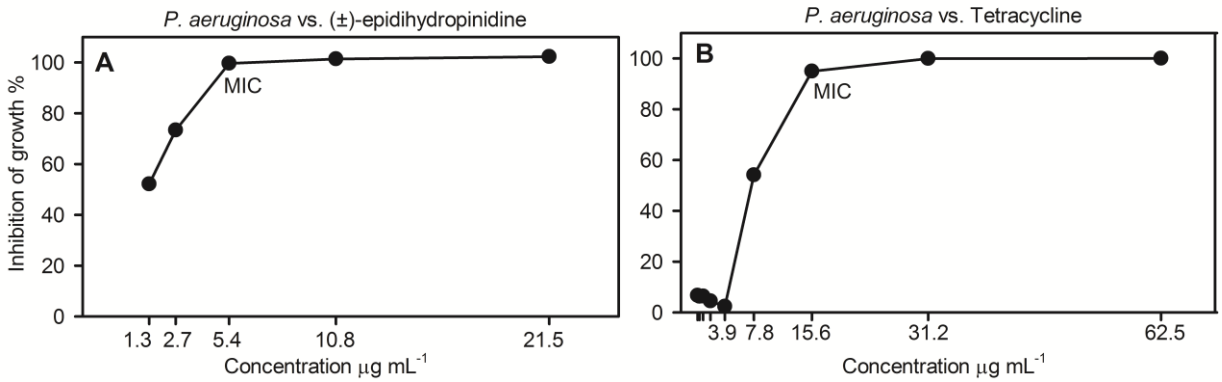
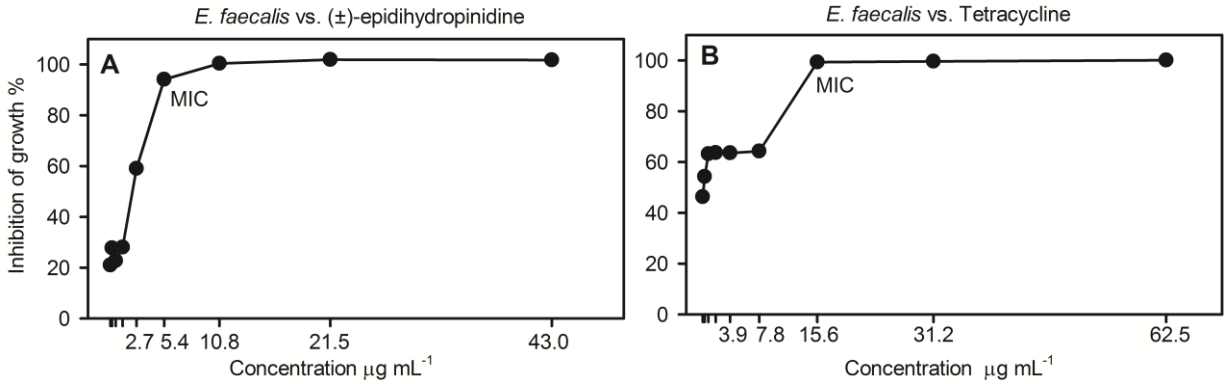


Figure 3



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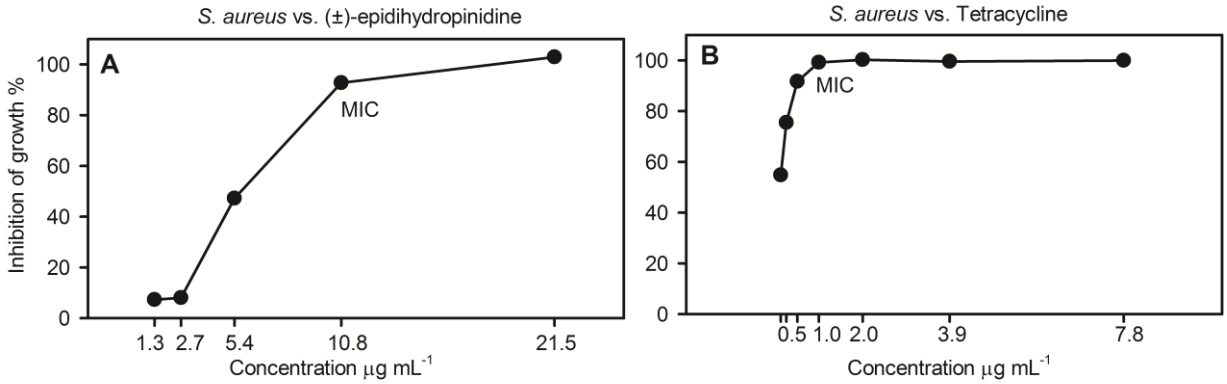

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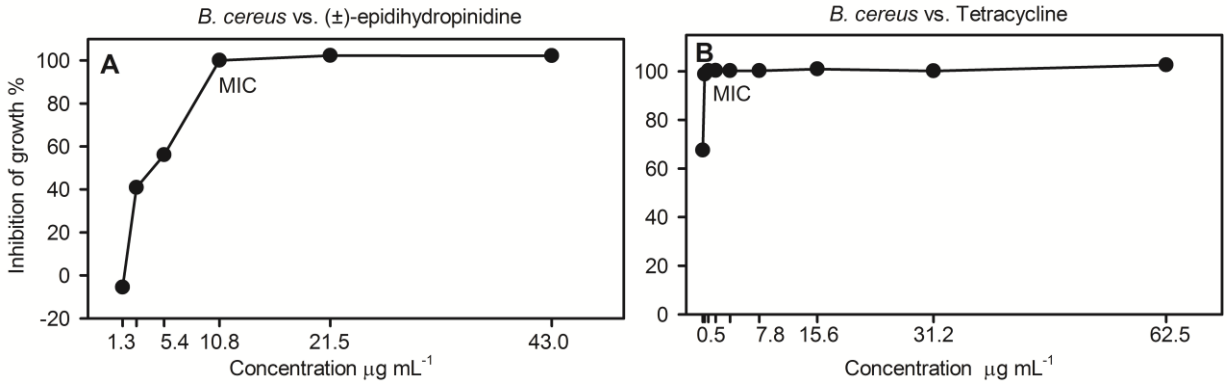

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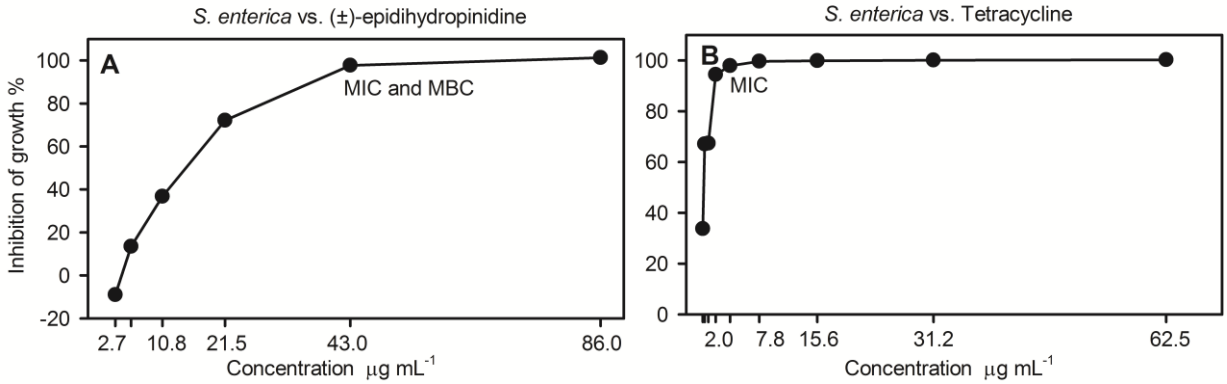
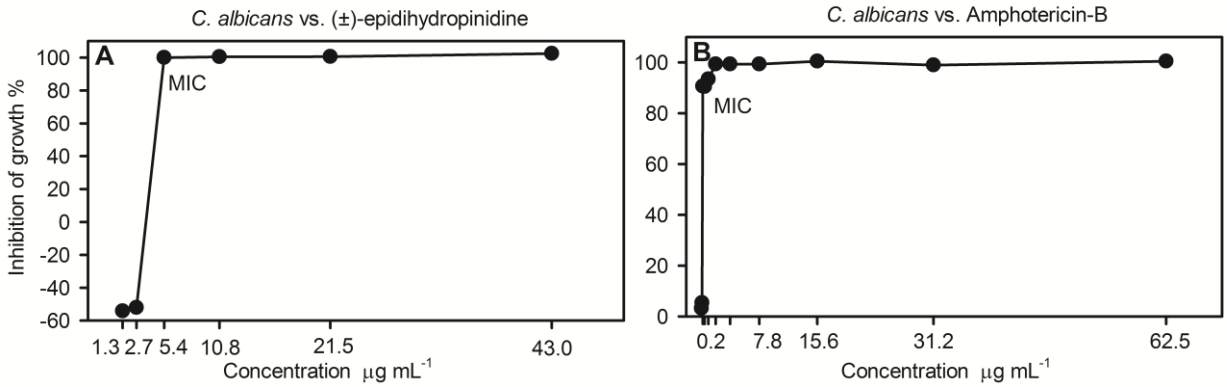


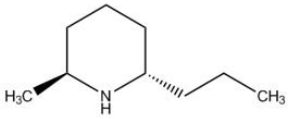
Figure 7



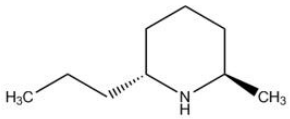
Graphical abstract

Picea abies

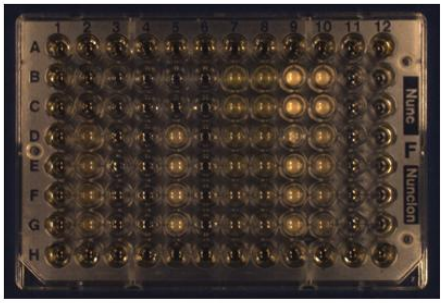
(±)-epidihydropinidine



(+)-epidihydropinidine



(–)-epidihydropinidine



MIC 5.37 µg/ml:

Pseudomonas aeruginosa
Enterococcus faecalis
Candida albicans
Candida glabrata