Identification and characterization of antibacterial compound(s) of cockroaches (*Periplaneta americana*)

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Short title: Antibacterial compound(s) of cockroaches

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ABSTRACT

Infectious diseases remain a significant threat to human health, contributing to more than 17 million deaths, annually. With the worsening trends of drug resistance, there is a need for newer and more powerful antimicrobial agents. We hypothesized that animals living in polluted environments are potential source of antimicrobials. Under polluted milieus, organisms such as cockroaches encounter different types of microbes, including superbugs. Such creatures survive the onslaught of superbugs and are able to ward off disease by producing antimicrobial substances. Here, we characterized antibacterial properties in extracts of various body organs of cockroaches (*Periplaneta americana*) and showed potent antibacterial activity in crude brain extract against methicillin-resistant *Staphylococcus aureus* and neuropathogenic *E. coli* K1. The size-exclusion spin columns revealed that the active compound(s) are less than 10 kDa in molecular mass. Using cytotoxicity assays, it was observed that pre-treatment of bacteria with lysates inhibited bacteria-mediated host cell cytotoxicity. Using spectra obtained with LC-MS on Agilent 1290 infinity liquid chromatograph, coupled with an Agilent 6460 triple quadruple mass spectrometer, tissues lysates were analyzed. Among hundreds of compounds, only a few homologous compounds were identified that contained isoquinoline group, chromene derivatives, thiazine groups, imidazoles, pyrrole containing analogs, sulfonamides, furanones, flavanones, and known to possess broad-spectrum antimicrobial properties, and possess anti-inflammatory, anti-tumour, and analgesic properties. Further identification, characterization and functional studies using individual compounds can act as a breakthrough in developing novel therapeutics against various pathogens including superbugs.

Keywords: Cockroach, Antibacterials; Superbugs.

Introduction

Antibiotic resistance is one of the world's most pressing public healthcare problems (WHO 2002). In recent decades, almost every variant of human pathogenic bacteria has become resistant and/or less vulnerable to available antibiotic treatment, threatening new infectious diseases or emergence of super-strains that are difficult to eradicate (Alanis 2005; Nordmann et al. 2007). Moreover, the current decline in the identification of new antibacterial molecules presents a clear and present danger (Alanis 2005; Nordmann et al., 2007; Devasahayam et al. 2010). In particular, the prevalence of multiple drug-resistant bacterial strains in hospital and community settings is a significant challenge to human health (Alanis 2005; Nordmann et al., 2007; Devasahayam et al. 2010). Among a plethora of multiple drug-resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) poses a significant threat to human and animal health (Enright 2003). MRSA is a Gram-positive bacteria, that is resistant to many antibiotics and possesses the ability to produce skin and tissue infections that are often nosocomial in nature (Pantosti et al. 2009). Likewise, neuropathogenic *E. coli* K1 is a leading cause of neonatal meningitis contributing to significant morbidity and mortality, despite advances in antimicrobial chemotherapy (Lee et al. 2011).

We hypothesize that animals living in unsanitary and unhygienic conditions have developed ways of protecting themselves against pathogenic microorganisms (Lee et al. 2011; Sagheer et al. 2014; Bulet et al. 1999; Wilson et al. 1999; Khan et al. 2008). For example, insects such as cockroaches thrive in unhygienic environments. The fact that cockroaches share the environmental niches with humans and animals suggests their routine exposure to infectious agents important to human health. The ability of cockroaches to flourish under such threats and

ward off diseases, indicates their resistance to pathogenic microbes including superbugs as well as toxicants and hazardous materials. Such organisms could be a good source of antibacterials against human pathogens. Insects represent 70% of living fauna and most diverse among the entire animal Kingdom. Insects such as cockroaches have survived millions of years, and withstood catastrophic events (for comparison, humans have been on the planet for 10,000 years), suggesting their ability to adapt and resist environmental threat. These findings support our hypothesis, to search for potential antimicrobials in such creatures. To this end, our studies suggested that insects such as locusts and cockroaches possess antimicrobials. We characterized antibacterial properties of organ extract of cockroaches (*Periplaneta americana*), and tested their effects on human cells, with a view to determine chemical and structural identities as potential therapeutic agents to treat infections.

Materials and Methods

Bacterial cultures

A clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) was used in the present study (Malaysian Type Culture Collection 381123). MRSA strain used in this study was originally derived from blood cultures, obtained from the Luton & Dunstable Hospital NHS Foundation Trust, Luton, England, UK. The sensitivity patterns of MRSA demonstrated its susceptibility to gentamicin, ciprofloxacin, vancomycin and resistance to amoxicillin, augmentin, cephalaxin, cephtazidim, penicillin, flucloxacillin, tetracycline, and erythromycin. In addition, a Gram negative neuropathogenic *Escherichia coli* (a cerebrospinal fluid isolate from a meningitis patient; 018:K1:H7), strain E44 was used as described previously (Malaysian Type Culture

Collection 710859). Bacteria were cultured in Luria-Bertani (LB) broth and grown overnight at 37°C, prior to experiments as previously described (Khan et al. 2008).

Organ lysates of cockroaches

Cockroaches (*Periplaneta americana*) were reared on a diet of dried dog food pellets and were housed in glass fronted metal cages in the dark. The cages were kept in a temperature-regulated insectary at 30°C. For dissections, all instruments were sterilized in 70% ethanol prior to and during each dissection. Cockroaches were immobilized by exposure to 4°C for 15 min. Cockroaches were immobilized by removal of legs and wings and securing to a dissection plate, ventral side up, using a pin on either side of the thorax and one through the distal abdomen. The head and legs were removed, prior to a longitudinal incision made down the midline of the abdomen to expose the fat body tissue, a sample of which was removed aseptically. The thoracic cuticle was opened up with a flap-shaped incision to obtain the haemolymph from the cockroach body cavity using a pipette. The upper hind leg of the cockroach was opened up with a longitudinal incision to expose muscle tissue, a sample of which was aseptically removed. The removed cockroach head was dissected to obtain the intact brain aseptically.

Insect tissue samples were collected in 500 µL of sterile water in batches of samples obtained from 500 cockroaches. The samples were kept on ice during dissection and treated in an identical manner. An estimate was made by eye/weight to ensure that the mass of each tissue was approximately similar. The samples were subjected to four cycles of freeze-thawing in order to cause cellular disruption and lysis. The thawing period was kept as brief as possible and organ extracts were kept cold (4°C) before re-freezing. The samples were then homogenized aseptically with a tissue grinder, prior to centrifugation at 10,000 g for 30 min at 4°C. The

supernatant (crude extract) was then collected, filtered sterilized using 0.2 µm pore size filter, and protein concentration was determined using a Bio-Rad Protein Assay kit. Finally, lysates were stored at -20°C until needed for antibacterial bioassay testing.

Antibacterial assays

To determine the effects of crude extracts of various organ lysates on bacterial viability, antibacterial assays were performed as described previously (Khan et al. 2008). Briefly, the optical density of bacterial broth cultures was adjusted to 0.22 at 595 nm using a spectrophotometer (equivalent to 10^8 colony forming units per mL and confirmed by plating on nutrient agar plates). Approximately 10^6colony forming units (c.f.u.), suspended in 10 µL were incubated with different concentrations of various organ lysates of cockroaches at 37°C for 2 h. Following this, bacteria were serially diluted and enumerated by plating on nutrient agar plates (Khan et al. 2008). For negative controls, bacteria were incubated in PBS alone and for positive controls, bacteria were incubated with 100 µg per mL of gentamicin. Percentage bactericidal effects were determined as the percentage of bacteria surviving relative to the control: 100 - (cfu recovered / original inoculum x 100).

Human brain microvascular endothelial cell (HBMEC) cultures

The primary HBMEC were cultured in RPMI-1640 containing 10% heat inactivated fetal bovine serum, 10% Nu-serum, 2 mM glutamine, 1 mM Na-pyruvate, 100 U penicillin per mL, 100 µg streptomycin per mL, non-essential amino acids and vitamins as previously described (Khan and Siddiqui, 2009). For cytotoxicity assays, HBMECs were cultured in 24-well plates by inoculating 5 x 10^5 cells per well per mL and incubating them at 37°C with 5% CO_2, which resulted in the formation of complete monolayers within 48 h.

Cytotoxicity Assay

Cytotoxicity assay were performed as previously described (Khan and Siddiqui, 2009). Briefly, assays were performed in 24 well plates containing confluent HBMEC monolayers. The organ lysates alone, bacteria alone, or bacteria treated with lysates were added to confluent HBMEC layers (final volume of 500 µL RPMI-1640). For treatment, bacteria were incubated with gentamicin (100 µg per mL) or lysates (100 µg per mL) for 2 h at 37°C, followed by incubation with HBMEC monolayers. Plates were incubated at 37°C in a 5% CO_2 incubator for 20 h. After this incubation, the supernatants were collected from each well, centrifuged to remove cellular debris and then cytotoxic effect were determined by estimating the amount of lactate dehydrogenase release from HBMEC using Cytotoxicity Detection kit (Roche Applied Sciences). The percent cytotoxicity was calculated as follows: % cytotoxicity = (sample value – control value) / (total LDH release – control value) X 100. Control values were determined by incubating HBMEC monolayers with RPMI-1640 alone and total LDH release was obtained by completely lysing the HBMEC using 1% Triton X-100. To determine the molecular mass of the active molecule(s), crude brain lysates were filtered through 30 kDa, and 10 kDa molecular weight cut off Spin-X UF columns (Corning). Both the elutate and retentate were used in the aforementioned antibacterial assays.

Liquid Chromatography Mass Spectrometry LC-MS): separation and analysis

All samples were analyzed using a LC-MS on Agilent 1290 infinity liquid chromatograph (Agilent Technologies, Wilmington, DE), coupled with an Agilent 6460 triple quadruple mass spectrometer. Separation of compounds were achieved using reverse-phase HPLC, with a Merck C-18 column of particle size 3 µm (5.5 cm length and i.d. of 4.6 mm) at 25ºC, and equilibrated

with 90% solvent A (0.1% formic acid in MiliQ water) and 10% solvent B (0.1% formic acid in MeOH). A flow rate of 0.6 mL per min with a linear gradient was used as follows: 10% solvent B for 4 min, 80% solvent B over the course of 3.2 min, and 10% solvent B for 2.8 min. The total run time was 14 min, including a 5 min equilibration time.

The compounds were ionized using ESI + jet stream ion mode with the QQQ analyzer. The parameters of ion source were set as follows: capillary voltage at 4500 V, sheath gas flow at 8 L per min, fragmentor voltage 135 V, gas temperature at 350ºC, gas flow at 8 L per min, and nebulizer gas at 40 psi and detector used was MCP Microchannel Plate detector, while blank used after each sample of composition 50% MeOH + 50% MiliQ water.

Identification of compounds through matching with library

Water soluble and methanol-extracted lysates of brain, haemolymph, and muscles of cockroaches were subjected to LC-MS analysis as described above, to obtain the chromatograms and the prospective mass spectra of every separated fraction of the mixture of compounds. The MS spectra for the compounds present in water soluble and methanol-extracted lysates were run against the NIST Mass Spectral Search Program-2009 version 2.0f (National Institutes of Standard and Technology, Gaithersburg, MD) for the identification of homologous compounds through Agilent Mass Hunter software, while keeping in view compensation needed for charges in positive ESI MS as well as electron fragmentations, to ensure searches for the correct parent mass. Reported biological activities of the compounds identified and their novelty were determined with the help of Scifinder software.

Results

Cockroach brain crude lysates exhibit potent bactericidal activity against MRSA and neuropathogenic E. coli K1

The crude extracts of cockroach fat body, muscle, and brain were prepared and tested along with aspirated haemolymph in antibacterial bioassays against MRSA. Fat body and muscle lysates showed no bactericidal activity against MRSA and *E. coli* K1 at 100 μg per mL, while haemolymph exhibited 35% ± 5.1 and 20% ± 3.5 bactericidal effects, against MRSA and *E. coli* K1 respectively. In contrast, brain extracts exhibited more than 90% bactericidal effects against both MRSA and neuropathogenic *E. coli* K1 (Fig. 1).

Partial characterization of cockroach brain crude lysates and their effects on human cells

When crude brain lysates were filtered through 30 kDa, and 10 kDa molecular weight cut off Spin-X UF columns, the antibacterial activity was observed in elutate, suggesting that antibacterial compound(s) are less than 10 kDa in molecular mass. To assess potential cytotoxicity to human cells, lysates were added to HBMEC monolayers and lactate dehydrogenase, a marker for cellular lysis, was measured using a Roche cytotoxicity detection kit. When incubated with MRSA or neuropathogenic *E. coli* K1 alone, more than 70% HBMEC cytotoxicity was observed (Fig. 2). In contrast, MRSA or neuropathogenic *E. coli* K1 pretreated with gentamicin or lysates followed by incubation with HBMEC produced minimal host cell damage. Notably, lysates alone showed minimal HBMEC cytotoxicity, indicating selective toxicity against bacteria tested.

Identification of compound(s) present in cockroach lysates using liquid chromatography mass spectrometry

The brain, haemolymph, and muscles lysates of cockroaches were processed for water and methanol extractions and subjected to LC-MS (Agilent Technologies 6460 Triple Quadrupole LC/MS) for qualitative analyses. Figure 3 shows spectra from respective extractions. These compounds were separated based on m/z ratio and retention time in the column. The data obtained from the LC-MS for brain lysates contained over 168 peaks for water soluble and over 193 peaks for methanol-extracted brain lysates (Fig. 3). Similarly, the data obtained from LC-MS for haemolymph lysates contained over 182 peaks for water soluble and over 172 peaks for methanol-extracted haemolymph lysates (Fig. 3). Similarly, the data obtained for muscles extract contained over 180 peaks for water soluble and 190 peaks for methanol soluble lysates of muscles (Fig. 3).

For brain lysates, among hundreds of compounds, 5 were identified from water extracted lysates, and 15 generated from LC-MS were identified from methanol-extracted compounds (Table 1). Similarly, for haemolymph lysates, among hundreds of compounds, 19 were identified from water-extracted lysates, and 18 from methanol-extracted compounds (Table 2). For muscles lysates, among hundreds of compounds, 12 were identified from water soluble lysates, and 18 from methanol-extracted compounds were identified using LC-MS (Table 3).

The compounds were searched through Scifinder software to determine any reported biological activity. Among various homologous compounds from brain lysates, many of them were shown to possess biologically active molecules (Table 1). For example, compounds 1, 2, 3, 6, 10,13, 15, 16, 17, 18, and 19 possess antimicrobial activities against various Gram positive

and Gram negative bacteria including, *Listeria monocytogenes, Bacillus subtilis, Salmonella typhimurium, Staphyloccus aureus, Pseudomonas aeruginosa, E. coli, Klebsiella pneumonia, Bacillus mycoides, Proteus vulgaris, Proteus mirabilis, Enterobacter cloacae, Enterococcus faecalis, Morganella morganii, Providencia stuartii*, fungi including *Candida albicans, Curvularia lunata, Aspergillus flavus, Aspergillus niger, Verticillium alboatrum, Colletrichurm lagenarium, Alternaria alternate, Phytophora infestans, Dermatophytes* etc., viruses including Parainfluenza virus, Influenza A virus, Respiratory syncytial virus , Herpes virus etc. Additionally, compounds homologous to 1, 2, 6, 11, 13, 16, and 17 are shown to possess anti-tumour properties against various types of cancers, including oral squamous cell carcinoma, breast cancer, hepatic carcinoma, nasopharyngeal carcinoma, prostate cancer, lung cancer, intestinal tumour etc. while some homologous compounds are shown to exhibit biological activities such as anti-diabetic, anti-inflammatory, anti-platelet aggregation, antioxidant, and analgesic activities (Table 1).

The 5 compounds identified in water extracted brain lysates, i.e., Cpd1: 6,7-dimethoxy-1-methoxymethyl-3,4-dihydroisoquinoline, Cpd2: (3S,6S,6aS,8S,10S,11aR,12R,12aS,13S)-1-ethyl-8,10,13-trimethoxy-3-(methoxymethyl)tetradecahydro-1H-3,6a,12-(epiethane[1,1,2]triyl)-7,9-methanonaphtho[2,3-b]azocine-6,11a,12-triol, and Cpd3: 3,6-Dimethylbenzofuran-4(5H)-ol, Cpd4: 3-((6-methoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)methyl)phenol, and Cpd5: 4-benzyl-4,5-dihydroisoxazole, have reported biological activities mentioned above. Among the 15 compounds identified from brain methanol extracts, 3 of them (Cpd11: 2-Amino-3,6-dimethylimidazo[4,5-b]pyridine, Cpd18: 2-isopropoxy-5-phenyl-1,3,4-oxadiazole, and Cpd20: 4,4'-(iminomethylene)bis(N,N-dimethylaniline) have reported biological activities. However, Cpd8: 1,4-dihydro-2,3-benzoxathin-3-oxide, and Cpd9: 2,2,4,7a-tetramethyl-6-(phenylsulfonyl)-

3a,4,5,7a-tetrahydrobenzo[d][1,3] dioxole do not have any reported biological activities. The remaining 10 compounds have homologous structures, reported in the literature with various biological activities (Table 1).

For haemolymph lysates, several homologous compounds were shown to possess biologically active molecules. For example, compounds 1, 2, 3, 4, 6, 10, 12, 14, 15, 16, 19, 20, 23, 24, 26, 28, 29, 30, 31, 32, 33, and 37 were shown to possess antimicrobial activities against broad range of microorganisms including bacteria such as *Bacillus subtilis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus cereus*, *Enterococcus feacalis*, *Staphylococcus aureus*, *Salmonella typhi*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pasturella multicoda*, *E. coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes* etc., fungi such as *Aspergillus niger*, *Fusarium proliferatum*, *Aspergillus parasiticus*, *Trichoderma reesei*, *Candida albicans*, *Curvularia lunata*, *Geotricum candidum*, *Pyricularia oryzae*, *Helminthosporium oryzae*, *Rhizoctonia bataticola*, *Alternata alternate*, *Pythium aphanidermatum*, *Fusarium solani*, and *Sclerotium folfsi* , *Saccharomyces cerevisiae*, parasites such as *Leishmania chagasi*, parasitic worm embryos, *Plasmodium*, *Toxoplasma*, and *Theileria*, phytoparasitic nematodes, viruses such as HIV-1 , HSV-1 and HSV-2, Parainfluenza virus, Tobacco mosaic virus, Norovirus, Flavivirus, Avian influenza virus (H5N1). Moreover, compounds 1, 3, 4, 5, 6, 9, 10, 12, 14, 15, 16, 19, 23, 24, 25, 27, 28, 29, 31, and 35 were shown to possess anti-tumour activities against ovarian, hepatic, lung, prostate, nasopharyngeal, colon, skin, and vincristine resistant nasopharyngeal, breast cancer, as well as in the treatment of metabolic disorders, inflammation, diabetes, neurodegenerative diseases, convulsing disease, platelet-related disease conditions.

Out of 19 compounds identified in haemolymph water lysates, 5 were shown to possess biological activities including, Cpd2: 3,6-Dimethylbenzofuran-4-ol , Cpd3: 6,7-dimethoxy-1-

methoxymethyl-3,4-dihydroisoquinoline, Cpd5: 3-Phthalimidopropionaldehyde , Cpd17: 3-ethyl-5-phenylimidazolidine-2,4-dione, and Cpd19: Cholestane,3,5-dichloro-6-nitro-(3beta,5alpha,6beta) (Table 2). For homologous searches, all compounds except Cpd7: N-Benzyl (4-Phenyl-3-propyl-1-oxaspiri[4,5]dec-3-enylidene) amine, possess reported biological activities (Table 2). For methanol-extracted haemolymph lysates, 18 compounds were identified and 5 of them were shown to possess biological activities including, Cpd21: 2-Ethyl-2-methoxypentanoic acid, Cpd23: 7-(Isopropoxy)-2,2,5-trimethylchromene, Cpd29: 5-methyl-2-(thiophen-2-yl)pyridine, Cpd34: 2-Hydroxy-2-methylhexanoic acid, Cpd36: 2,7,12,17-tetraethyl-3,8,13,18-tetramethyl-21H,23H-Porphine Copper (Table 2).

When compared with compounds extracted from muscles lysates with no antibacterial activity, out of a total of 20 compounds identified from cockroach brain lysates, 5 of them were found to be common in muscles lysates and brain lysates, indicating that possibly these were the compounds which are not responsible for the tested biological activities. However, the remaining 15 compounds were absent in the brain lysates and these included, i.e. Cpd 2: (3S,6S,6aS,8S,10S,11aR,12R,12aS,13S)-1-ethyl-8,10,13-trimethoxy-3-(methoxymethyl)tetradecahydro-1H-3,6a,12-(epiethane[1,1,2]triyl)-7,9-methanonaphtho[2,3-b]azocine-6,11a,12-triol, Cpd 3: 3,6-Dimethylbenzofuran-4(5H)-ol, Cpd 4: 3-((6-methoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)methyl)phenol, Cpd 5: 4-benzyl-4,5-dihydroisoxazole, Cpd6: 6a-hydroxy-4-methoxy-6a,6b,7,8,9,10,10a,10b-octahydro-6H-benzo[3,4]cyclobuta[1,2-c]chromen-6-one, Cpd 9: 2,2,4,7a-tetramethyl-6-(phenylsulfonyl)-3a,4,5,7a-tetrahydrobenzo[d][1,3]dioxole, Cpd 10: 3-(2'-Phenylethyl)-2,3-dihydro-6-phenyl-2,4-dioxo-4H-1,3-thiazine, Cpd 11: 2-Amino-3,6-dimethylimidazo[4,5-b]pyridine, Cpd 12: 3,9-Dimethoxy-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-6-benzyl, Cpd 13: (S,S)-1,1-

Bis(ethoxycarbonyl)-2,2-bis-p-tolylsulfinyl-1-ethanol, Cpd 14: 1-methyl-[14-(13)C]-9,12-methanobenzo[h]pyridazino[1,2-b]phthalazine-7,14-dione, Cpd 15: allyl(2-nitro-1-phenylethyl)sulfane, Cpd 17: KARAKOLIDINE, Cpd 18: 2-isopropoxy-5-phenyl-1,3,4-oxadiazole and Cpd 19: 1-(4-hydroxybenzyl)-3,4-dihydroisoquinoline-6,7-diol. However, compounds 1, 7, 8, 16 and 20 are common in muscles indicated that might be these does not possess potent biological activities against MRSA and neuropathogenic *E.coli* K1.

Discussion

Infectious diseases remain one of the leading causes of death worldwide (WHO 2002). Antibiotic-resistant bacteria continue to rise at an alarming rate (Nordmann et al. 2007; Devasahayam et al. 2010). Most currently available antibacterial drugs were discovered between 1940 and 1980 using traditional approaches, which have now become saturated. Most of the newer antibacterial drugs have arisen from chemical modification of existing antibiotic structures. Efforts to create new drugs using existing antibiotic scaffolds are challenging because these semi-synthetic derivatives are often not able to penetrate the bacterial cell wall adequately. More innovative, non-traditional strategies are therefore required in order to provide the urgently needed next generation of antimicrobial drugs. Insects represent a plentiful and untapped potential source of new antimicrobial drugs prompting us to investigate the antibacterial activity of their various tissues. The tissues of living multicellular organisms are potentially a rich source of nutrients for microbes and effective strategies have to be developed by the host organism to prevent microbial digestion. Cockroach lysates prepared from fat body and muscle tissue showed no antibacterial activity against bacteria tested in this study. This is in contrast to previous

studies, which showed that insect such as *Drosophila* fat body produce seven distinct antimicrobial peptides, which are then secreted into the haemolymph to participate in a systemic response to septic injuries (Clynen and Schoots 2009; Metz-Boutigue et al. 2003; Amiche et al. 1999; Salzet 2001). However, this response is mounted as a result of microbial challenge. Since cockroaches used in our study were not immune-stimulated, prior to dissection, this could explain why no antibacterial activity was seen in fat and muscle tissues, while limited activity was observed in the haemolymph. Future work will involve immune stimulation of the cockroach, prior to dissection, by injecting heat-killed bacteria into the haemocoel to investigate if an inducible antibacterial effect can be detected. In contrast, cockroach brain lysates exhibited high antibacterial activity against bacteria tested in the present study. Furthermore, high antibacterial activity was observed at micro-level, showing high potency of the active component(s), which is presumably present at even lower amounts. Moreover, antibacterial molecule(s) were less than 10 kDa in molecular mass, and non-toxic to human cells.

Using LC-MS, spectra was acquired from brain, haemolymph and muscles lysates. Out of hundreds of compounds, only few homologous compounds were identified (Table 1, 2 and 3). The identified compounds possess characteristics in their structure like arrangement of specific functional groups and presence of active components, which makes them biologically significant for their potential therapeutic value against various infectious and non-infectious diseases. For example, several compounds in the brain lysates and haemolymph possess isoquinoline group, chromene derivatives, thiazine groups, imidazoles, pyrrole containing analogs, and are biologically active against broad-spectrum microorganisms, such as bacteria, fungi, viruses, parasites, and known to exhibit anti-tumour and anti-diabetic properties (Ali and El-Kazak 2010; Iwasa et al. 2001; Khafagy et al. 2002; Vennerstrom et al. 1995; Ozkay et al. 2010; Facchinetti et

al. 2012; Bhardwaj et al. 2015). Similarly, sulfonamides and furanones are found to possess active components to treat many tumours, possess broad-spectrum antimicrobial activities, anti-inflammatory and analgesic properties (Ozbek et al. 2007; He et al. 2015; Rappai et al. 2009). Other compounds contained flavanones. Flavanones are naturally occurring compounds present in fruits and vegetables and have considerable therapeutic potential in platelet inhibition, antibacterial, anti-tumour, antioxidant properties (Cushnie and Lamb 2005).

Notably, the majority of compounds in this study remain unidentified and/or whose biological activities have not yet been reported. It is hoped that chemical identities of all compounds and *in vitro* testing for their antibacterial, anti-tumour, antioxidant, anti-diabetic will determine their value for potential clinical applications. Although a detailed characterization of all compounds is needed, the present study suggest the potential significance of these naturally occurring compounds derived from novel sources, i.e., species living in challenging environments, can be a useful resource. To this end, insects such as cockroaches are potentially an important source of therapeutic molecules, as they share several environmental niches with humans and animals. This is evident from our findings that cockroach brain lysates possess potent antibacterial properties. Compared to vertebrates, the insects' nervous system is far more de-centralized. Behaviour such as feeding, movement and mating is controlled by segmental ganglia running the length of the insects' body, instead of the brain. In some cases, the brain may stimulate or inhibit activity in the segmental ganglia. The neuronal cells of the brain synthesize neurotransmitter and neuropeptide chemical messengers to communicate with each other and with peripheral organs (Clynen and Schoots 2009; Metz-Boutigue et al. 2003; Amiche et al. 1999; Salzet 2001). These chemical messengers represent the highest physiological hierarchy in animals controlling many crucial biological processes. It is therefore essential to the survival of

the organism that this system is constantly protected against challenge from pathogens or noxious agents. Constitutive expression of antibacterial factors could provide this protection as a first line of defense before an inducible response has had time to manifest. In addition to metabolites that may possess antibacterial activity and/or inflammatory mediators, large neuropeptide precursor molecules such as proenkephalin and prodermaseptin, are known to be processed into smaller peptides, some of which have antibacterial activity (Clynen and Schoots 2009; Metz-Boutigue et al. 2003). The antimicrobial peptides have diverse amino acid sequences and structures but most are small amphipathic, cationic peptides that exert their antimicrobial effect by disrupting the structural integrity of cell membranes.

In conclusion, these findings suggest that compounds present in cockroach brains are of potential therapeutic value. Further identification, characterization and functional studies *in vitro* and *in vivo* using individual compounds can act as a breakthrough in developing novel therapeutics against various pathogens including superbugs. It is hoped that these molecules could eventually be developed into treatments for bacterial infections that are increasingly resistant to presently available drugs, however, this will require intensive research over the next few years.

Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure legends

Figure 1. Crude extract from cockroach brains were prepared and tested (100 μg) for antibacterial activity, as described in Materials and Methods. The results revealed that cockroach brain lysates exhibited more than 90% bactericidal activity against MRSA and neuropathogenic *E. coli* K1. For positive control, gentamicin (100 μg per mL) exhibited more than 99% killed rate. The data is presented as the mean ± standard mean of three independent experiments performed in duplicate. **(B)** Representative effects of crude extracts from cockroach brains on MRSA and neuropathogenic *E. coli* K1. Both MRSA and neuropathogenic *E. coli* K1 were treated with lysates for 2 h at 37°C for then plated on nutrient agar plates. Note that pretreatment of MRSA and neuropathogenic *E. coli* K1 exhibited bactericidal effects.

Figure 2. Cockroach brain lysates inhibited methicillin-resistant *Staphylococcus aureus* (MRSA)-mediated human brain microvascular endothelial cells (HBMEC) cytotoxicity. (A) Cockroach brain lysates were prepared and tested for their protective effects against MRSA-mediated cytotoxicity on HBMEC as described in Materials and Methods. Note that MRSA alone exhibited >70% cell death. In contrast, MRSA pretreated with gentamicin or lysates for 2 h at 37°C and then incubated with HBMEC monolayers for 20 h, exhibited minimal cell death. Likewise, 100 μg of brain lysates alone had minimal cytotoxic effects on HBMEC. The data is presented as the mean ± standard mean of three independent experiments performed in duplicate. **(B)** Representative effects of MRSA, lysates, gentamicin-treated MRSA, and lysates-treated MRSA on HBMEC monolayers for 20 h. Pretreatment of MRSA with gentamicin did not produce visual disruption of HBMEC monolayers. Similar results were observed with lysates

alone and MRSA pretreated with lysates. In contrast, HBMEC monolayers treated with MRSA exhibited complete monolayer disruptions. X200.

Figure 3. Cockroach brain and haemolymph lysates (water and methanol extractions) were subjected to LC-MS (Agilent Technologies 6460 Triple Quadrupole LC/MS) for qualitative analyses. The compounds were separated based on m/z ratio and retention time in the column. The data obtained from the LC-MS for brain extract contained over 168 peaks from water extracted compounds **(A)**, over 193 peaks from methanol-extracted compounds **(B),** and the data obtained from haemolymph extract contained over 182 peaks from water extracted compounds **(C)** and over 172 peaks from methanol-extracted compounds **(D)**, and the data obtained from muscles extract contained over 180 peaks from water-extracted compounds **(E)** and over 190 peaks from methanol-extracted compounds **(F)**.