Structural and functional characterization of peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*

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Abstract

Tick defensins may serve as templates for the development of multifunctional peptides. The purpose of this study was to evaluate shorter peptides derived from tick defensin isoform 2 (OsDef2) in terms of their antibacterial, antioxidant and cytotoxic activities. We compared the structural and functional properties of a synthetic peptide derived from the carboxy-terminal of the parent peptide (Os) to that of an analogue in which the three cysteine residues were omitted (Os-C). Here we report that both peptides were bactericidal (MBC values ranging from 0.94 – 15 µg/ml) to both Gram-positive and Gram-negative bacteria, while the parent peptide only exhibited Gram-positive antibacterial activity. The Os peptide was found to be two-fold more active than Os-C against three of the four tested bacteria, but equally active against *Staphylococcus aureus*. Os showed rapid killing kinetics against both *Escherichia coli* and *Bacillus subtilis*, whereas Os-C took longer, suggesting different modes of action. Scanning electron microscopy showed that in contrast to melittin for which blebbing of bacterial surfaces was observed, cells exposed to either peptide appeared flattened and empty. Circular dichroism data indicated that in a membrane-mimicking environment, the cysteine-containing peptide has a higher α-helical content. Both peptides were found to be non-cytotoxic to mammalian cells. Moreover, the peptides displayed potent antioxidant activity and were twelve times more active than melittin. Multifunctional peptides hold potential for a wide range of clinical applications and further investigation into their mode of antibacterial and antioxidant properties is therefore warranted.

Keywords: Antimicrobial peptide, Antioxidant, Tick, Defensin, Synthetic peptide, Carboxy-terminal, Multifunctional

List of abbreviations: Os, synthetic peptide derived from the C-terminal of *O. savignyi* defensin isoform 2; Os-C, synthetic analogue of Os without cysteine residues; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; LB, Luria-Bertani; DTT, dithiothreitol; CD, circular dichroism, NaP, sodium phosphate, CFU, colony forming units; SEM, scanning electron microscopy; CV, crystal violet; AUC, area under curve

1. Introduction

Microbial resistance towards commonly used antibiotics is of increasing concern in the medical field and there is thus an urgent need to develop new classes of antibacterial agents [1]. Antimicrobial peptides (AMPs) are an integral part of the innate immune system of all living organisms and are considered to be promising candidates for the development of novel anti-infective agents. These molecules have a broad antimicrobial activity spectrum, various modes of action as well as a decreased incidence of resistance development [2, 3]. One of the major families of AMPs is the defensins found within various organisms including animals, plants and invertebrates [3, 4]. Despite the ability of ticks to harbour and transmit pathogens, they possess an efficient defence system for preventing microbial infection [5]. Defensins are major components of innate immunity in ticks [5, 6].

Tick defensins are constitutively expressed in various tissues at very low levels and their expression is induced either by blood feeding or bacterial challenge [7, 8]. Defensins are cationic with molecular masses of approximately 4 kDa, usually containing six cysteine residues forming three disulfide bonds. Multiple defensin isoforms have been detected in ticks [6] and they are primarily active against Gram-positive bacteria, but some isoforms have also been shown to be effective against Gram-negative bacteria, yeast and protozoa [9, 10]. To date only one mode of action study has been reported for a tick defensin [9]. Synthetic defensin isoform A from *Ornithodoros moubata* showed antibacterial activity against many Gram-positive bacteria, but not against Gram-negative bacteria. The peptide exhibited low hemolytic activity, but was able to disrupt the membrane potential of *Micrococcus luteus* over a period of 30 – 60 min consistent with its relatively slow rate of killing.

Tsuji et al. [10] found that longicin, a defensin-like peptide from the tick *Haemaphysalis longicornis*, displayed bactericidal, fungicidal and parasiticidal activity. Using four overlapping synthetic peptides which spanned the entire sequence of longicin, the authors showed that the peptide's functional region was located in the carboxy-terminal domain. The synthetic peptide (P4), derived from this region displayed similar activities to the parent peptide. In another study, Varkey et al. [11] found that a peptide derived from the C-terminal domain of defensin isoform C from *O. moubata*, but in which the three cysteine residues were omitted, was active against both Gram-positive and Gram-negative bacteria, whereas the parent peptide was only able to inactivate Gram-positive bacteria.

The tick used in this study, *Ornithodoros savignyi*, is a livestock parasite endemic to arid and semi-arid regions in Africa [12]. This tick is a rich source of bioactive molecules and using it

as a model for tick investigations we have described several anti-hemostatic components [13]. More recently, we have identified two defensin isoforms (OsDef1 and OsDef2) from the midgut of this tick [unpublished data]. Ticks are becoming valuable sources of novel proteins and peptides that may serve as templates for the development of anti-infective agents [9, 14]. Multifunctional peptides and fragments thereof hold potential for a wide range of applications [15-18]. The purpose of this study was to investigate whether peptides derived from OsDef2 would display a twofold role. To this end we compared the structural and the functional properties of a synthetic peptide (Os) derived from the carboxy-terminal of the parent OsDef2 peptide to that of an analogue in which the three cysteine residues were omitted (Os-C). The function of the peptides was evaluated in terms of their antibacterial, antioxidant and cytotoxic activities. To our knowledge this is the first report of defensin-derived peptides displaying antioxidant activity.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli (ATCC 700928), *Pseudomonas aeruginosa* (ATCC 10145), *Bacillus subtilis* (13933) and *Staphylococcus aureus* (U3300) were used for the antibacterial assays. Bacteria were grown aerobically in Luria-Bertani (LB) broth at 37 ºC.

2.2. Preparation of synthetic peptides

OsDef2 was obtained from Life tein (US), whereas P4, Os and Os-C were purchased from GenScript (USA). The purity and molecular mass of the peptides were determined by reverse-phase HPLC and mass spectrometry, respectively. Dithiothreitol (DTT, 10 nmol)was added to P4 and Os prior to lyophilisation, the concentration of DTT after resuspension and dilution of the peptides was insufficient to maintain peptides in their reduced states. Peptide concentrations were determined by measuring the absorbance of tyrosine residues at 280 nm and using the equation given below. Stock peptide solutions of 1.2 mg/ml were prepared in dddH_2O.

$$c = \frac{MW \times df \times Abs}{no.of\ Tyr\ or\ Trp \times Extinction\ coefficient}$$

Where c is the peptide concentration in mg/ml; MW the molecular weight of the peptide; df the dilution factor used to determine the absorbance (abs) at 280 nm. The extinction coefficient of tyrosine is1200 and that of tryptophan is 5560 AU/mmole/ml.

2.3. Circular dichroism (CD) secondary structure determination

The effect of two different solvents on peptide secondary structures were determined using far-UV CD spectroscopy, using a Jasco J-810 spectropolarimeter (Jasco Inc., USA), over the 180-250 nm range and a path length of 0.2 cm. The scans were carried out at 20 ºC with a scan speed of 200 nm/min, using a data pitch of 0.1 nm and at a bandwidth of 0.5 nm. Peptides were prepared in either water or 25 mM sodium dodecyl sulfate (SDS) to a final peptide concentration of 50 µM. The results obtained are the average of 10 scans corrected for solvent effects. The signals were converted to mean residue ellipticity, [θ], by using the following equation:

$$[\theta] = \frac{(100 \times \Theta)}{Cnl} \qquad \text{(equation 1)}$$

where *C* is the peptide concentration in millimolar, θ is the measured ellipticity in millidegree, n is the number of residues, and l is the path length (cm). Any data acquired for the CD spectra that was greater than 800 volts was removed, due to the unreliability of data beyond that point.

2.4. Antibacterial assays

The minimum inhibitory concentration (MIC) of OsDef2, Os and Os-C were determined by incubating bacteria with 10 µl of the peptides over a concentration range of 0.06 to 120 µg/ml, to which 10 µl of DTT (1 mM) was added. Briefly, bacteria were grown overnight, diluted 100 times in LB broth and proliferated until OD_{600} of 0.5 was reached. Bacteria were then diluted to OD_{600} of 0.001 in LB broth and incubated (90 µl) with the respective peptides at 37 ºC, whilst shaking at 150 rpm, in a microtitre plate for 20 hrs. The turbidity of the incubated samples was determined at 595 nm. MICs were defined as the concentrations at which a decrease of turbidity (similar to that of only LB broth) was observed.

The minimum bactericidal concentration (MBC) of P4, Os and Os-C were determined by incubating bacteria with the respective peptides over a concentration range of 120-0.06 µg/ml. Bacteria were grown overnight, diluted in LB broth and proliferated until OD_{600} of 0.5 was reached after which the bacterial culture was centrifuged (14500xg for 90 s), washed twice and re-suspended in either 10 mM sodium phosphate (NaP) buffer, pH 7.4, for Gram-negative bacteria, or 1% LB in NaP buffer for Gram-positive bacteria. The washed culture was diluted to approximately 1 x 10^6 CFU / ml in either NaP buffer (Gram-negative) or 1% LB in NaP (Gram-positive) after which it was incubated (90 µl) with the various peptide concentration samples (10 µl) in polypropylene tubes for 2 hours at 37 °C in a shaking incubator. Thereafter incubated samples were diluted 500 times in either NaP buffer (Gram-negative) or LB (Gram-positive), plated out on LB-agar plates and incubated at 37 °C for 16 hrs. The growth control was calculated to contain 180 CFU. MBCs were defined as the concentration at which complete killing (no CFU) was observed.

2.5. Kinetics of bactericidal activity

Bacteria were prepared and diluted as for the MBC determination and incubated with the synthetic peptides, at their respective MBCs, at 37 °C whilst shaking at 150 rpm. At specific time intervals (0, 5, 10, 30, 60 and 120 min), 10 µl of the incubation samples were diluted (500 times) and plated on LB-agar plates. Plates were left over night at 37 °C. The growth control was calculated to contain 180 CFU. Effective killing time was defined as the time (min) after which no more colonies were formed.

2.6. Scanning electron microscopy (SEM)

The sample preparation methods of Mangoni et al. [19] were adapted for this study. Briefly *E. coli* and *B. subtilis* cells were cultured, washed and resuspended as described for antibacterial assays. The cell concentration was adjusted to approximately 1 x 10^6 CFU/ml. The bacterial cells were then exposed to three peptides, melittin (25 µM), Os (0.77 µM) and Os-C (1.74 µM) for 30 min. Control samples were exposed only to NaP buffer. Each sample was incubated at 37 ºC for 30 min on a poly(L-lysine)-coated cover glass to immobilize the cells. The cells attached to cover glasses were fixed with 2.5% gluteraldehyde in NaP buffer, washed with the same buffer and dehydrated with a series of ethanol dilutions. The cells were critical point dried, coated with carbon and viewed with a Zeiss ULTRA Plus FEG (Carl Zeiss NTS GmbH, Oberkochen, Germany) scanning electron microscope.

2.7. Hemolytic assays

To study the effects of the peptides on human red erythrocytes, a modified method of Nakajima et al. [9], was followed. Blood was collected from healthy, consenting donors (Ethical clearance obtained from the Research Ethics Committe, Faculty of Health Sciences, University of Pretoria). Using a sterile needle connected to a 5 ml EDTA vacuum extraction blood tube, 5 ml blood was collected from a vein. The erythrocytes were collected by centrifugation at 2500 x g and the plasma and buffy coat was removed. The erythrocytes were washed with NaP buffer and 10 µl volume of erythrocytes diluted in 80 µl NaP buffer was then exposed to 10 µl of peptide (final concentration range 0.25-100 µM) for 30 min at 37 °C. The samples were centrifuged at 1250 x g and 75 µl supernatants were collected, placed into wells of a 96 well plate and the absorbance measured at 570 nm. Cells incubated with either 2% SDS or NaP buffer, represented the 100% and 0% hemolysis controls, respectively. The results were expressed as % hemolysis, relative to the SDS control.

2.8. Cytotoxicity assays

In order to study the cytotoxicity of the peptides, SC-1 (mouse fibroblasts) and CaCo-2 (human colon cancer) cell lines were used. Cells were incubated with the synthetic peptides, at a concentration range of 1.90 to 120 µg/ml, for 24 hrs at 37 °C and 5% CO_2. Melittin, at the same concentration range, (Sigma Aldrich, South Africa) was used as a control. The cells

were fixed for 20 min at 37 °C after the addition 10 µl of 20% paraformaldehyde. The fixative and medium were removed and the cells blotted dry after which it was stained for 30 min at room temperature using a crystal violet (CV) dye (200 µl) consisting of 0.1% (v/w) CV in 200 mM formic acid, pH 3. Acetic acid (50 µl of 10% solution) was added to the cells to extract the dye and the absorbance of the resulting solution was measured at 630 nm. Results were expressed as % control, no peptide added.

2.9. Oxygen radical absorbance capacity (ORAC) antioxidant assay

The method for this assay was based on the method of Ou et al. [20]. A serial dilution of Trolox (an analogue of vitamin E) of 0-800 µM was used as the control standard, with 10 mM NaP buffer as the blank. A range of concentrations between 0.5 and 20 µM of melittin, Os and Os-C peptides were used and the tripeptide, reduced glutathione (GSH), was used as a positive antioxidant control. Fluorescein (0.139 nM) was used as the fluorescent probe and 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) (0.11 µM) was added to generate peroxyl radicals. Fluorescence was measured every 5 min for 4 h on a FLUOstar OPTIMA plate reader (BMG labtechnologies, Offenburg, Germany). Excitation wavelenght was 485 nm and emission was measured at 520 nm. Raw data was exported to an Excel spreadsheet for calculations. The area under the curve (AUC) for each sample was calculated (netACU = $ACU_{antioxidant} - ACU_{blank}$), and the final results were expressed as µMTE (Trolox Equivalent).

3. Results

3.1. CD analysis

In Table 1 the physicochemical properties of the synthetic peptides as well as that of the parent molecule, OsDef2, used in this study, are given. The secondary structures for both Os and Os-C were predicted using PSIPRED (available at: bioinf.cs.ucl.ac.uk/psipred/). As shown in Table 2, there is a considerable difference between the predicted secondary structures of both peptides. For both peptides the % random coil is similar, but part of Os is α-helical, whereas for Os-C it is a β-strand. The secondary structures were determined using CD spectroscopy in water and in SDS. The CD spectra showed that in water (Fig. 1A) both peptides exhibited random coiled structure, whereas in SDS, a membrane-mimicking environment there were significant differences in the secondary structure (Fig. 1B). SDS induced in Os a transition to a predominantly α-helical conformation.

3.2. Antibacterial activities

The parent peptide, OsDef2, was found to be active against *B. subtilis* (MIC of 0.24 µg/ml) and *M. luteus* (MIC of 0.94 µg/ml) butthe MIC for Gram-negative *E. coli* and *P. aeruginosa* could not be determined even at 120 µg/ml (Table 3). On the other hand, peptide derivatives,

Os and Os-C, were inactive against both Gram-positive and Gram-negative bacteria when tested in LB broth. However, potent bactericidal activity was detected for these peptides, with MBC values ranging from 0.94 – 15 µg/ml for Os and 1.90 - 15 µg/ml for Os-C, when tested in NaP buffer (Table 4). The cysteine-containing peptide was found to be two-fold more active than its Os-C counterpart against three of the four tested bacteria, but equally active against *S. aureus*. Both peptides were found to be most active against *P. aeruginosa*, with MBC values of 0.94 µg/ml and 1.90 µg/ml for Os and Os-C, respectively. Os and Os-C were found to be more active than the P4 control for which MBC values ranged from 7.50 – 120 µg/ml.

The kinetics of bactericidal activity of Os and Os-C was evaluated using *E. coli* and *B. subtilis*, as representative Gram-negative and Gram-positive strains. The time-course study revealed that Os inactivated 100% of both Gram-positive and -negative bacteria within the first 5 min, whereas Os-C showed 100% bactericidal activity only after 60 min and 120 min against Gram-negative (Fig. 2A) and Gram-positive bacteria (Fig. 2B), respectively.

3.3. Bacteria morphological changes

The effect of Os and Os-C on the morphology of *E. coli* and *B. subtilis* was investigated with SEM after exposing the bacterial cells to the peptides at the respective MBCs for 30 min. Control bacteria that were exposed only to NaP buffer displayed a rough surface with no evident cellular debris (Fig. 3*A* and *E*). The bacterial cells exposed to melittin showed distinct blebbing on the cell surface (Fig. 3*B* and *F*). Bacterial cells exposed to Os (Fig. 3*C* and *G*) and Os-C (Fig. 3*D* and *H*) showed a collapse of cell structures and flattening in some cells. The *E. coli* and *B. subtilis* cells were considered to have lost part of their cellular contents. No membrane blebbing was observed for bacterial cells exposed to either Os or Os-C.

3.4. Cytotoxic activities

The cytotoxicity of the peptides was firstly evaluated for their ability to cause lysis of human erythrocytes. All peptides were tested at a concentration range of 0.25-100 µM and even at 100 µM (263x the lowest MBC and 14x the highest MBC) Os and Os-C did not cause any significant erythrocyte lysis compared to melittin, used as a positive control (Fig. 4). Melittin caused a dosage related increase in hemolysis from 2.5-100 µM.

Cytotoxicity of the peptides was further evaluated in two dividing cell lines namely SC-1 fibroblasts and CaCo-2 epithelial cells. Both peptides did not cause significant changes in cell number using a concentration range of 1.90-120 µg/ml after 24 h exposure [Fig. 5 (A and 5)]. Even at 120 µg/ml the peptides showed no cytotoxic effects towards SC-1 and CaCo-2 cells, whereas melittin, at the same concentration, was found to reduce the cell number of SC-1 and Caco-2 cells by approximately 60% and 20%, respectively.

3.5. Antioxidant activity

Antibacterial peptides possessing antioxidant activity have increased benefits in terms of clinical applications. For peptides Os and Os-C, a dose-dependent linear increase (R^2-value of 0.998 and 0.983, respectively) in antioxidant activity was seen (Fig. 6). From this data, a molar ratio of 21.63 and 22.87 µmol TE/ µmol peptide for Os and Os-C respectively, compared to melittin which showed minimal activity with a molar ratio of 1.8. Both peptides displayed higher antioxidant activity than GSH even when tested at five times lower concentration.

4. Discussion

Ticks encounter a large number of diverse pathogens and therefore produce numerous antimicrobial factors. With the increasing number of pathogens that are becoming resistant to conventional antibiotics, tick defensins may serve as templates for the development of novel anti-infective agents [9, 14]. Problems associated with the development of AMPs as therapeutic agents include cytotoxicity to mammalian cells, susceptibility to proteases and possibly the biggest limitation being the high cost of manufacturing [15]. One way of addressing the latter is to determine the shortest amino acid sequence of the parent molecule required for antibacterial activity. Another strategy is to use derivatives in which cysteine residues are omitted. Several groups have shown that analogues of invertebrate and vertebrate defensins lacking disulfide bridges retained broad-spectrum activity [11, 21, 22]. As suggested by these authors, such variants would be easier and cheaper to synthesize and thus more attractive in terms of clinical potential.

In agreement with what has been observed for other synthetic tick defensins [9, 14], reduced OsDef2 showed antibacterial activity against only Gram-positive bacteria. The limited number of studies that are available for synthetic tick defensins suggest that these AMPs are active in low µM concentration range whether oxidised (three disulfide bonds) or reduced [9, 14]. Os and Os-C displayed no antibacterial activities when tested in LB broth. The majority of AMPs have been shown to lose antibacterial activity when tested in complex fluids such as broth or serum [15, 23]. We, therefore, tested the MBCs of Os and Os-C in NaP buffer. In our study we used P4, previously investigated by Tsuji *et al*. [10] as a positive control, who determined the MBC for P4 in NaP buffer containg 1% tryptic soy broth. In a similar study, Varkey *et al*. [11] determined the MBC of a peptide derived from a tick defensin from *O. moubata*, in which three cysteine residues were omitted, also in NaP buffer. Both Os and Os-C showed strong antibacterial activities with MBCs ranging from 0.94 to 15 µg/ml and wer both more active than the P4 control. Os was twofold more active than Os-C against three of the four strains tested but equally active against *S. aureus*. Both peptides displayed the greatest activity against *P. aeruginosa*. Although both peptides have the same overall net charge (+6), differences in their antibacterial activity could be as a result of th higher hydrophobicity of Os (<H> = 0.249), compared with the Os-C analogue (<H> =0.045). Another reason for the observed difference in activity could possibly be attributed to the different structures adopted by each peptide in a membrane-environment. Far-UV CD data

showed that in a membrane-mimicking environment, Os has more α-helical properties than Os-C. Although DTT (10 nmol) was added to both cysteine-containing peptides, Os and P4, prior to lyophilisation, the concentration of DTT after re-suspension and dilution of the peptides would be insufficient to maintain them in their reduced state. Tsuji *et al*. [10] did not include DTT in their assays and for comparative purposes, we too excluded DTT.

At their respective MBCs, Os showed rapid killing kinetics against both Gram-positive and Gram-negative strains, whereas Os-C took an hour or longer. The observed differences in the bactericidal activities and killing rates of these peptides suggest a difference in their mechanism of action. The fast rate of killing by Os implies that like for many other AMPs, the primary target of this peptide is the plasma membrane, whereas the slower rate of killing observed for Os-C may indicate additional targets. SEM was used to gain insight into the direct effects of Os and Os-C peptides on the morphology of *E. coli and B. subtilis*, respectively. In contrast to melittin, which showed distinct blebbing of both Gram-negative and Gram-positive bacteria surfaces, cells exposed to both synthetic peptides at their respective MBCs, showed collapse of cell structures, indentation of cell membranes and cells which appear flattened and empty. Most AMPs, including melittin, studied thus far kill cells by causing blebbing on the microbial surfaces, followed by lysis [24]. The effects observed for Os and Os-C are similar to those reported for temporin L and esculentin-1b(1-18) derived from frog skin [19, 25].

Although many AMPs show a preference for prokaryotic cells, several especially at high concentrations are toxic to eukaryotic cells [15]. The erythrocyte is an ideal model system to determine directly the effect of AMPs on the mammalian cell membrane as erythrocytes contain no DNA and are an example of a typical cellular bilayer and hemolysis occurs as a direct result of damage to the cell membrane. Melittin, an effective AMP found to cause significant cellular toxicity [26], in this study caused a dosage related increase in hemolysis. No hemolytic effects were observed for Os and Os-C. In contrast to erythrocytes, cell lines encompass all the above effects as well as metabolism, conjugation and excretion i.e., membrane effects may activate cellular pathways that may cause cellular senescence or death. In both SC-1 and Caco-2 cell lines, Os and Os-C had no effect on cell number even when tested at 120 µg/ml.

Increasingly, research is focussed on bioactive peptides that are multifunctional. Peptides containing both antimicrobial and antioxidant activities do not only efficiently kill microbes, but additional antioxidant activity would promote optimal cellular recovery and functioning by reducing the amount of free radicals [27, 28]. Os and Os-C displayed equally potent antioxidant activities and were found to be more active than both melittin and GSH. Amino acids associated with antioxidant activities include cysteine, methionine, tryptophan, tyrosine, phenylalanine, histidine and proline [29-31]. For a cysteine-rich (six residues) cyanopeptide from *Spirulina fussiformis*, Madhyastha and Vatsala [33] reported using the ORAC assay 9.2 ± 0.2 TE per ng/ml peptide. A similar level of activity of 9.15 and 11.15 TE/ng/ml activity

was calculated for Os and Os-C, respectively. These authors suggested that the antioxidant activity was due to the presence of free cysteine residues where the sulfhydryl group interacts with the radical species by hydrogen donation from the SH-group. Although Os has three cysteine residues, the observed antioxidant activity is most likely not as a result of these residues. Although Os was synthesized in its reduced state, once diluted in the absence of DTT the peptide probably underwent oxidation and these residues were no longer available as free thiols. The fact that the antioxidant activity of Os-C was similar to that of Os further confirms that the cysteines are not responsible for the observed activity. Amino acids that may be contributing to the observed activity are the three tyrosines as well as the single phenylalanine present in both peptides. Various authors have attributed antioxidant activity of peptides to the presence of tyrosine which is a good hydrogen donor due to its phenolic hydroxyl group [33, 34]. In addition to specific amino acids, specific amino acid di- and tripeptide sequences also contribute to activity [35] Using BIOPEP [http://uwn.edu.pl.biochemica/index.php/en/biopep] analysis, a dipeptide sequence, IR, associated with antioxidant activity was found in Os and Os-C close to the N-terminal. However, further investigation into the structure-antioxidant activity relationship of the peptides is required.

In conclusion, the tick defensin-derived peptides Os and Os-C were found to exhibit strong antibacterial activity, especially against Gram-negative bacteria, while causing no damage to mammalian cells. Preliminary findings from mode of action studies suggest that the killing mechanism observed for both peptides is different from that which has been reported for most other AMPs. In addition to their antibacterial activity, the high antioxidant activity observed for both peptides suggests their possible therapeutic application against detrimental effects of oxidative stress associated with infections [26, 27]. We are currently investigating these activities in the presence of physiological ionic strength and serum. Futher studies into their dual mode of action are being pursued.

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References

[1] Hancock, R.E.W. & Patrzykat, A. Clinical Development of Cationic Antimicrobial Peptides: From Natural to Novel Antibiotics. *Curr. Drug Targets Infect. Disord*. 2002; **2:** 79-83

[2] Peters, B.M., Shirtliff, M.E. & Jabra-Rizk, M.A. Antimicrobial Peptides: Primeval Molecules or Future Drugs? *PLoS Pathogens*. 2010; **6:** 1-4.

[3] Lazarev, V.N. & Govorun, V.M. Antimicrobial peptides and their use in medicine. *Appl. Biochem. Microbiol*. 2010; **46**(9): 803-814.

[4] Ganz, T. Defensins: Antimicrobial peptides of innate immunity. *Nat. Rev*. 2003; **3**: 710-720.

[5] Sonenshine, D.E. & Hynes, W.L. Molecular characterization and related aspects of the innate immune response in ticks. *Front. Biosci*. 2008; **2**: 7046-7063.

[6] Chrudimská, T., Chrudimský, T., Golovchenko, M., Rudenko, N. & Grubhoffer, L. New defensins from hard and soft ticks: Similarities, differences and phylogenetic analyses. *Vet. Parasitol*. 2010; **167**: 298-303.

[7] Nakajima, Y., Saido-Sakanaka, H., Taylor, D. & Yamakawa, M. Up-regulated humoral immune response in the soft tick, *Ornithodoros moubata* (Acari: Argasidae). *Parasitol. Res.* 2003; **91**: 476-481.

[8] Nakajima, Y., van der Goes van Naters-Yasui, A., Taylor, D. & Yamakawa, M. Antibacterial peptide defensin is involved in midgut immunity of the soft tick, *Ornithodoros moubata*. *Insect Mol. Biol*. 2002; **11**(6): 611-618.

[9] Nakajima, Y., Ishibashi, J., Yukuhiro, F., Asaoka, A., Taylor, D. & Yamakawa, M. Antibacterial activity and mechanism of action of tick defensin against Gram-positive bacteria. *Biochim. Biophys Acta*, 2003; **1624**: 125-130.

[10] Tsuji, N., Battsetseg, B., Boldbaatar, D., Miyoshi, T., Xuan, X., Oliver Jr, J.H. & Fujisaki, K. Babesial vector tick defensin against *Babesia* sp. Parasites. *Infect. Immun*. 2007; **75**(7): 3633-3640.

[11] Varkey, J., Singh, S. & Nagaraj, R. Antibacterial activity of linear peptides spanning the carboxy-terminal ß-sheet domain of arthropod defensins. *Peptides*, 2006; **27**: 2614-2623.

[12] Hoogstraal, H. Argasid and Nuttalliellid ticks as parasites and vectors. *Adv Parasitol.* 1985; **24**: 135-238.

[13] Maritz-Olivier, C., Stutzer, C., Jongejan, F., Neitz, A.W.H. & Gaspar, A. Review: Tick anti-hemostatics: targets for future vaccines and therapeutics. *Trends Parasitol.* 2007; **23**(9): 397-407.

[14] Chrudimská, T., Slaninová, J., Rudenko, N., Růžek, D. & Grubhoffer, L. Functional characterization of two defensin isoforms of the hard tick *Ixodes ricinus*. *Parasites & Vectors*, 2011; **4**(63): 1-9.

[15] McDermott, A.M. *Bioactive peptides,* (Eds: Howl, J., Jones, S.), CRC Press, Boca Raton, Florida, **2009**, pp. 357-401

[16] Hernández-Ledesma, B., Hsieh, C. & de Lumen, B.O. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochem. Biophys. Res. Commun*. 2009; **390**: 803-808.

[17] Fields, K., Falla, T.J., Rodan, K & Bush, L. Bioactive peptides: signaling the future. *J Cosmet Dermatol*, 2009; **8**: 8-13.

[18] Ramos, R., Silva, J.P., Rodrigues, A.C., Costa, R., Guardão, L., Schmitt, F., Soares, R., Vilanova, M., Domingues, L. & Gama, M. Wound healing activity of the human antimicrobial peptide LL37. *Peptides*, 2011; **32**: 1469-1476.

[19] Mangoni, M.L., Papo, N., Barra, D., Simmaco, M., Bozzi, A., Giulio, A.D. & Rinaldi, A.C. Effects of the antimicrobial peptide temporin L on cell morphology, membrane permeability and viability of *Escherichia coli*. *Biochem J*. 2004; **380**: 859-865.

[20] Ou, B., Huang, D., ,Hampsch-Woodill, M., Flanagan, J.A. & Deemer, E.K. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *J. Agric. Food Chem*. 2002; **50**: 3122-3128.

[21] Varkey, J. & Nagaraj, R. Antibacterial activity of human neutrophils defensin HNP-1 analogs without cysteines. *Antimicrob Agents and Chemother.* 2005; **49**: 4561-4666.

[22] Lundy, F.T., Nelson, J., Lockhart, D., Greer, B., Harriott, P. & Marley, J.J. Antimicrobial activity of truncated α-defensin (human neutrophil peptide (HNP)-1) analogues without disulphide bridges. *Mol. Immunol*. 2007; **45**: 190-193.

[23] Maisetta G, Luca MD, Esin S, Florio W, Brancatisano FL, Bottai D, Campa M, Batoni G. Evaluation of the inhibitory effects of human serum components on bacterial activity of human beta defensin. *Peptides.* 2008; **29**: 1-6.

[24] Henk WG, Todd WJ, Enright FM, Mitchell PS. The morphological effects of two antimicrobial peptides, Hecate-1 and melittin, on *Escherichia coli*. *Scanning Microsc*. 1995: **9**: 501-507.

[25] Marcellini, L., Borro, M., Gentile, G., Rinaldi, A.C., Stella, L., Aimola, P., Barra, D. & Mangoni, M.L. Esculentin-1b(1-18) – a membrane-active antimicrobial peptide that synergizes with antibiotics and modifies the expression level of a limited number of proteins in *Escherichia coli*. *FEBS J.* 2009; **276**: 5647-5664.

[26] Raghuraman, H. & Chattopadhyay, A. Melittin: a membrane-active peptide with diverse functions. *Biosci Rep*. 2007; **27**: 189-223.

[27] Galli, F., Battistoni, A., Gambari, R., Pompella, A., Bragonzi, A., Pilolli, F., Iuliano, L., Piroddi, M., Dechecchi, M.C. & Cabrini, G. Review: Oxidative stress and antioxidant therapy in cystic fibrosis. *Biochim. Biophys. Acta*, 2012; **1822**: 690-713

[28] Morris, D., Khurasany, M., Nguyen, T., Kim, J., Guilford, F., Mehta, R., Grav, D., Saviola, B. & Venketaraman, V. Review: glutathione and infection. *Biochim. Biophys. Acta*. **2012**; DOI: 10.1016/j.bbagen.2012.10.012

[29] Shen, S., Chahal, B., Majumder, K., You, S. & Wu, J. Identification of novel antioxidative peptides derived from a thermolytic hydrolysate of ovotransferrin by LC-MS/MS. *J. Agric. Food Chem.* 2010; **58**: 7664-7672

[30] Yang, H., Wang, X., Liu, X., Wu, J., Liu, C., Gong, W., Zhao, Z., Hong, J., Lin, D., Wang, Y. & Lai, R. Antioxidant peptidomics reveals novel skin antioxidant system. *Mol. Cell. Proteomics.* **2009**; 571-583.

[31] Elias RJ, Kellerby SS, Decker EA. Antioxidant activity of proteins and peptides. *Crit. Rev. Food Sci*. 2008; **48**(5): 460-441.

[32] Madhyastha, H and Vatsala, T.M. Cysteine rich cyanopeptide β2 from *Spirulina fussiformis* exhibits plasmid DNA pBR322 scission prevention and cellular antioxidant activity. *Indian J. Exp. Biol.* 2010; **48**: 486-493.

[33] Zheng, L., Su, G., Ren, J., Gu, L., You, L. & Zhao, M. Isolation and characterization of an oxygen absorbance activity peptide from defatted peanut meal hydrolysate and its antioxidant properties. *J. Agric. Food Chem.* 2006; **60**: 5431-5437.

[34] Saito, K., Jin, D., Ogawa, T., Muramoto, K., Hatakeyama, E., Yasuhara, T. & Nokihara, K. Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry. *J. Agric. Food Chem.* 2003; **51**: 3668-3674.

[35] Power O, Jakeman P, FitzGerald RJ. Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidation activity and potential applications of milk-derived antioxidative peptides. *Amino Acids*. 2012; DOI: 10.1007/s00726-012-1393-9

Table 1: Physicochemical properties of synthetic peptides

Peptides	Sequence	Length	Mass (Da)	<H>[a]	pI	Net charge
OsDef2[b]	GYGCPFNQYQCHSHCKGIRGYKGGYCKGAFKQTCKCY	37	4185.80	0.371	9.20	+6
Os	KGIRGYKGGYCKGAFKQTCKCY	22	2459.92	0.249	9.67	+6
Os-C	K- Y	19	2150.50	0.045	10.22	+6
P4[c]	SIGRRGGYCAGIIKQTCTCYR	21	2306.70	0.404	9.50	+4
Melittin[d]	GIGAVLKVLTTGLPALISWIKRKRQQ	26	2847.40	0.511	12.02	+5

[a]Hydrophobicity. Data obtained from HeliQuest [heliquest.ipmc.cnrs.fr/]

[b]Parent molecule from which Os and Os-C were derived.

[c]Carboxy-terminal derivative of longicin isolated from the hard tick, *Haemaphysalis logicornis*, used in this study as a control [10]

[d]Peptide isolated from bee venom [26], used as a control

Table 2: Predicted secondary structures of Os and Os-C

Peptide	Secondary structure constituent		
	% Helix	% β-Strand	% Coil
Os	32	0	68
Os-C	0	21	79

Used PSIPRED computer-based program [bioinf.cs.ucl.uk/psipred/]

Table 3: MIC values of OsDef2, Os and Os-C

Bacterial strain	**MIC (µg/ml)**		
	OsDef2	**Os**	**Os-C**
Gram-positive			
B. subtilis	0.24	>120	>120
M. luteus	0.94	nd	nd
Gram-negative			
E. coli	>120	>120	>120
P. aeruginosa	>120	nd*	nd

*not determined

Table 4: Bactericidal activities of synthetic peptides

	MBC (µg/ml)		
	P4	**Os**	**Os-C**
Bacterial strain			
Gram-positive			
B. subtilis	7.5 (3.25 µM)	1.90 (0.77 µM)	3.75 (1.74 µM)
S. aureus	120 (6.50 µM)	15 (6.10 µM)	15 (6.98 µM)
Gram-negative			
E. coli	7.5 (3.25 µM)	1.90 (0.77 µM)	3.75 (1.74 µM)
P. aeruginosa	15 (52 µM)	0.94 (0.38 µM)	1.90 (0.88 µM)

Figure 1

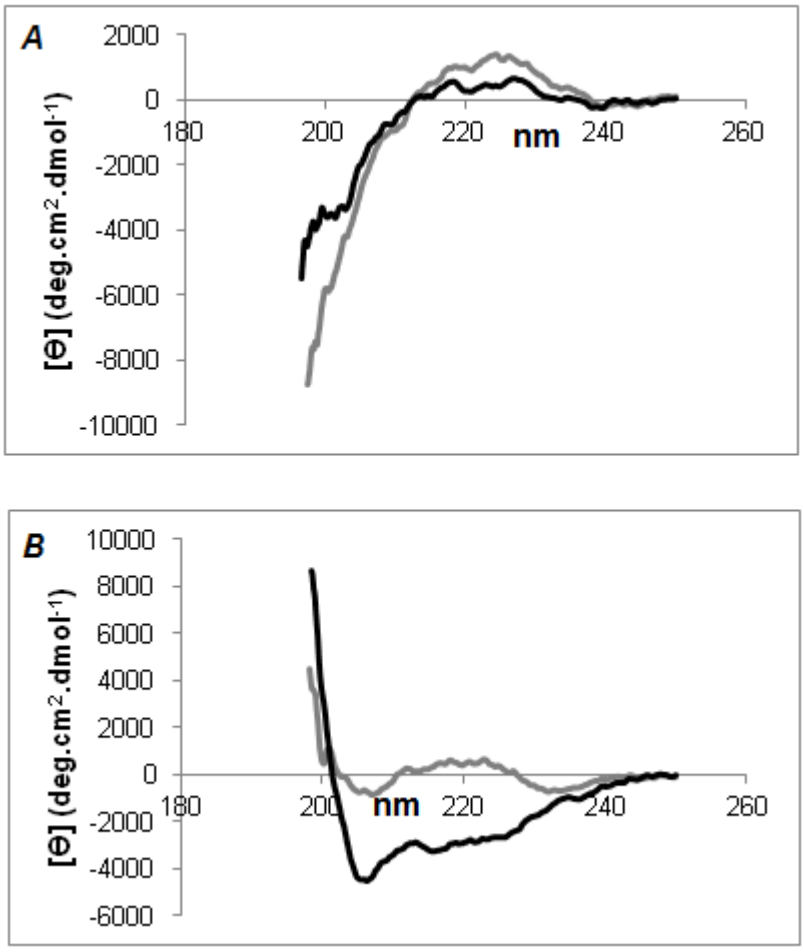

Figure 1: Far-UV CD spectra of Os (black curve) and Os-C (grey curve) in (A) water and (B) SDS.

Figure 2

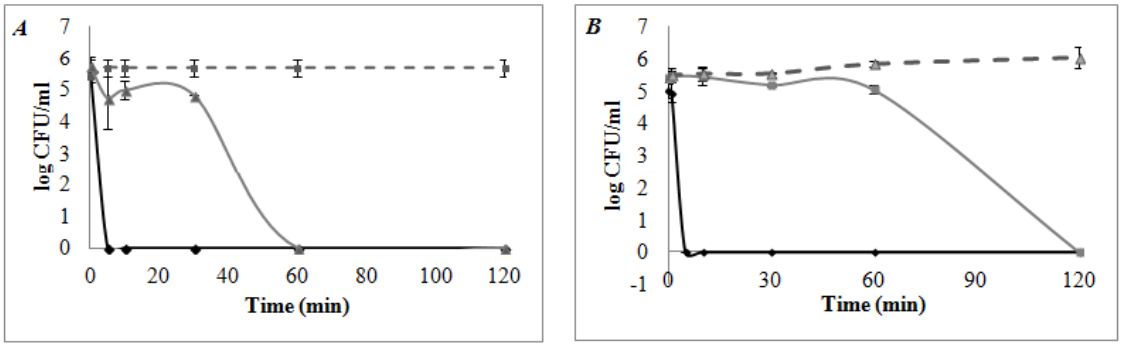

Figure 2: Kinetics of bactericidal activities of Os (black curve) and Os-C (grey curve) when targeted against (A)*E. coli* and (B) *B. subtilis*. The log CFU/ml is given in comparison with the growth control (gray dotted curve) in which no peptide was added. Error bars represent the standard error of the mean of two independent experiments. Detection limit of 30 to 300 CFU was used.

Figure 3

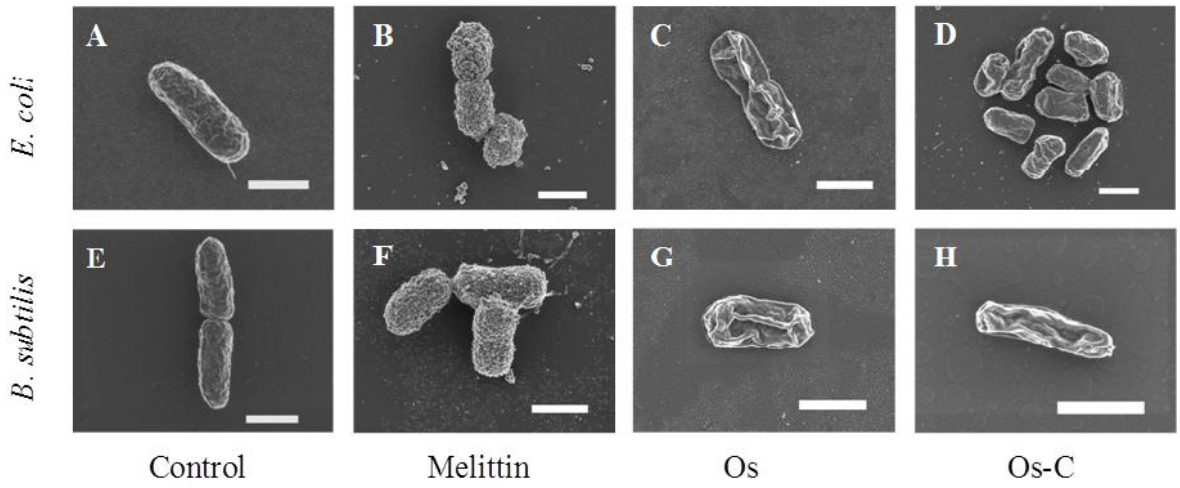

Figure 3: Scanning electron microscope images of *E. coli* and *B. subtilis* treated with melittin (B, F), Os (C, G) and Os-C (D, H) at the respective minimum bactericidal concentrations. Scale bars = 1 µm.

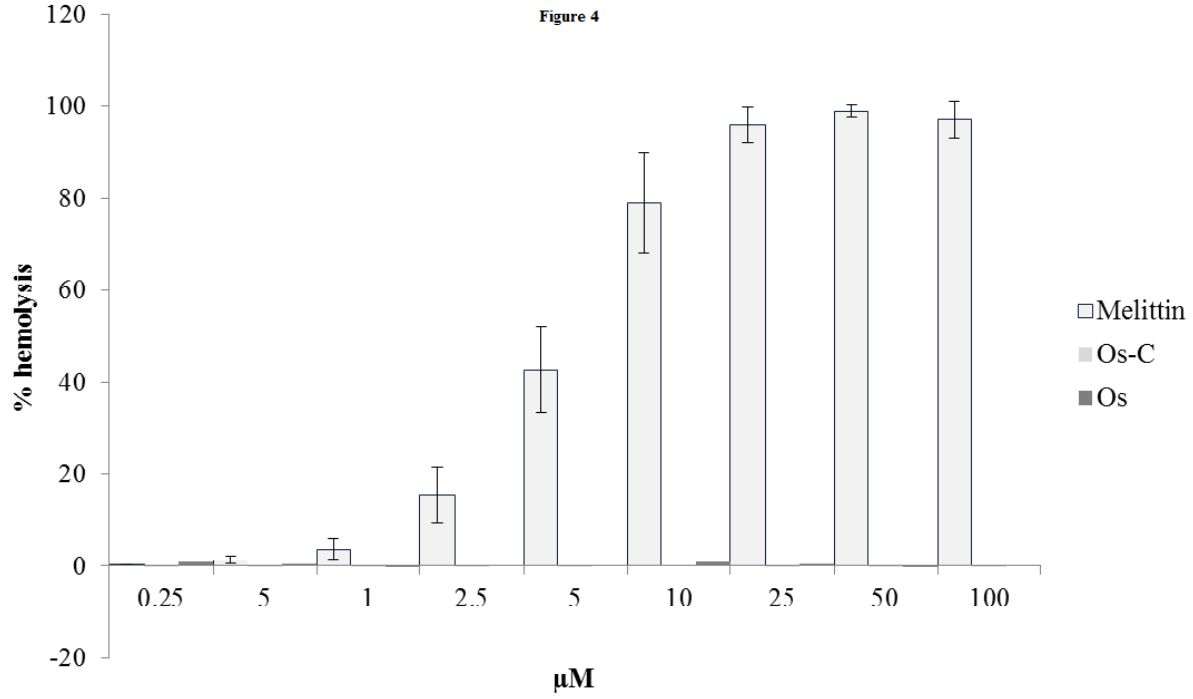

Figure 4: Hemolysis of human erythrocytes caused by synthetic peptides. Erythrocytes were incubated with peptides spanning a concentration range of 0.25 – 100 µM. Data expressed as mean ± standard error of the mean obtained from three independent triplicate experiments.

Figure 5

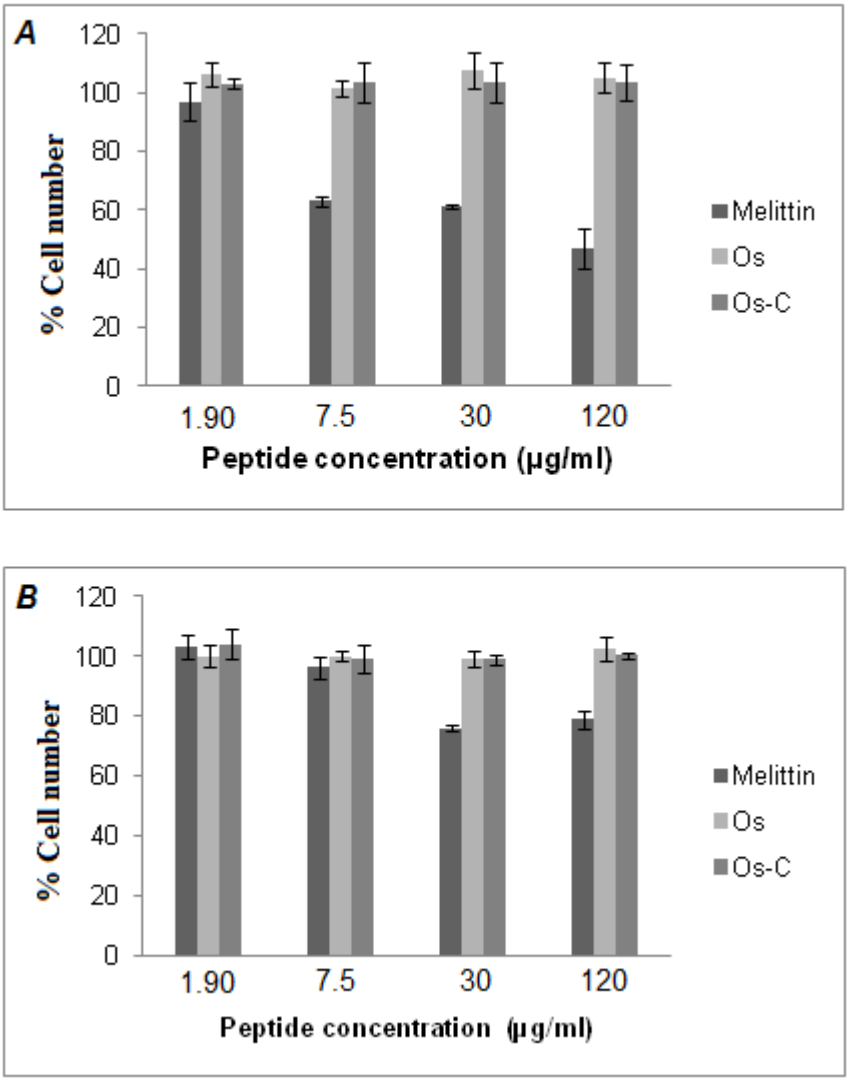

Figure 5: Cytotoxicity, measured as cell number, of melittin (control), Os and Os-C synthetic peptides, 0 – 120 µg/ ml tested on (A) SC-1 and (B)Caco-2 cells following exposure for 24 hours. A total of 120 µg/ml of melittin, Os and Os-C corresponds to 42 µM, 49 µM and 55 µM, respectively. Data expressed as mean ± standard error of the mean obtained from two independent triplicate experiments.

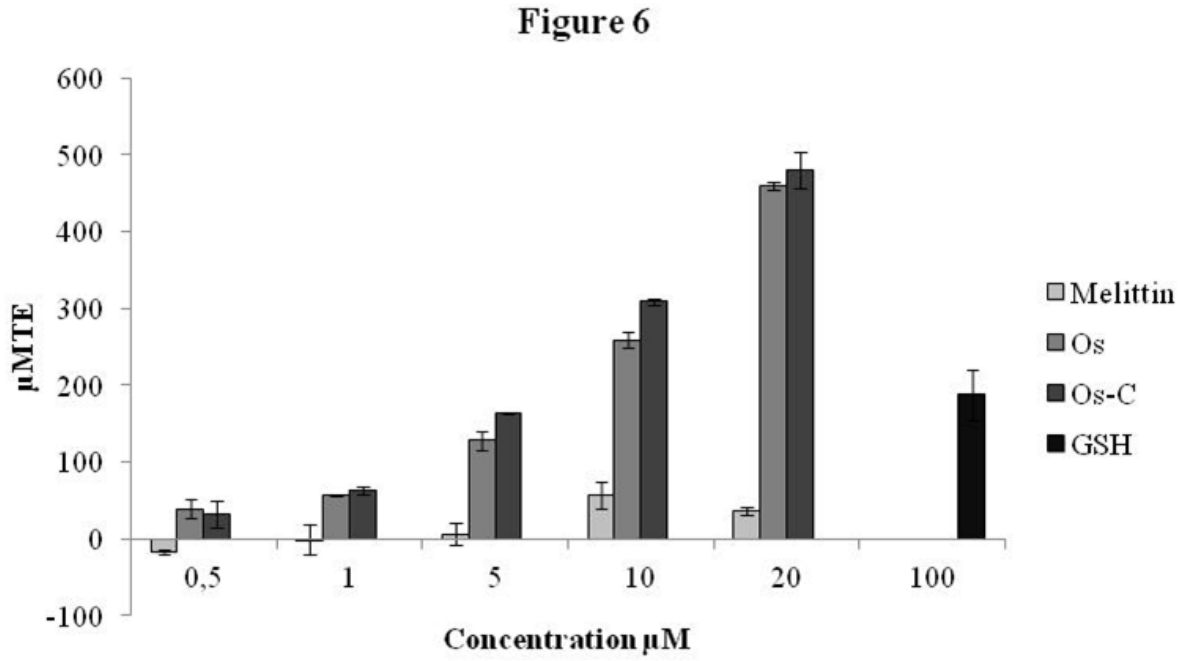

Figure 6: Antioxidant activity measured with the ORAC assay of Os and Os-C compared to melittin and GSH. Data expressed as mean ± standard error of the mean obtained from three triplicate experiments.