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Antifungal Potency of Turtle Eggshell Extract

¹Anggraini Barlian, ²Kusnandar Anggadiredja and ²Indah Prihatin

¹School of Life Science and Technology, ²School of Pharmacy, Institute of Technology Bandung, Indonesia

Corresponding Author: Anggraini Barlian, School of Life Science and Technology, Institute of Technology Bandung, Indonesia

ABSTRACT

Indonesia as tropical country is endemic to various infectious diseases, including that affecting the skin due to fungi infection. In nature, the eggshell, present as hard or soft shell, protects the embryo from microorganism infection. Turtle produces soft shells, which is very unique in that it does not contain albumin. In nature, the turtle eggshell has to cope with high humidity, so it is assumed that turtle soft shell has potent antifungal activity. This study investigated the potency of the eggshell extract from green sea turtle (*Chelonia mydas*) as an antifungal against *Candida albicans*, *Trichophyton mentagrophytes*, *Microsporium gypseum* and *Aspergillus brasiliensis*. Protein extraction was carried out with dialysis followed by lyophilization and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Antifungal activity assay was conducted by Dilution Plating method with nystatin as a standard drug. The possible mechanism of antifungal activity was assessed using Scanning Electron Microscopy (SEM). Fungal growth was inhibited by 8% w/v or 8×10^4 ppm extract, equivalent to 0.53 ppm nystatin against *Candida albicans*, 0.55 ppm against *Microsporium gypseum*, 0.36 ppm against *Trichophyton mentagrophytes* and 0.35 ppm against *Aspergillus brasiliensis*. The SDS-PAGE showed that the turtle eggshell extract had 6 proteins with molecular weight 20; 45, 50, 66, 80 and 116 kDa. This study showed that 8% w/v green turtle eggshell extract has inhibitory activity against *Candida albicans*, *Trichophyton mentagrophytes*, *Microsporium gypseum* and *Aspergillus brasiliensis*, as also proven by SEM results. Taken together, the present results showed that turtle eggshell extract has antifungal potency that could be developed as antifungal drug.

Key words: Antifungal, *Chelonia mydas*, green sea turtle, eggshell, dilution plating, protein, scanning electron microscopy

INTRODUCTION

In a tropical country with high humidity, such as Indonesia, the emergence of skin diseases caused by microorganisms is fairly high. In the long term, skin diseases caused by microorganisms may, indirectly, lead to a decreased immune system. Fungus is one of microorganisms that cause a wide range of skin diseases. Mycosis has been a clinically important disease, with a prevalence of as high as 20-25% of the world's population (Havlickova *et al.*, 2008). Until present, the treatment of mycosis lies on synthetic antifungal agents from the class of azole, polyene and antimetabolite (Di Piro, 2003).

Due to the fact that the eukaryotic fungal cellular structure resembles that of the mammalia, including human, toxic effects resulted from the use of antifungal agents are not uncommon

phenomena in clinical setting. This has, partly, contributed to some sort of 'developmental lag' of antifungal drugs (Pfaller and Yu, 2001). And it gets even worse if particular feature of fungi, such as cellular adaptability to extreme environment (Liu and Xiao, 2005), is taken into consideration.

With the ever-increasing resistance problem against antibiotics, the alternative solution is highly needed. In this regard, peptide-based substance has been shown to be promising. Previous studies indicated that short-chained peptides which have been essential part of body defense machinery of many living organisms (Hancock, 2001; Theis and Stahl, 2004) had potent antifungal activity and were promising candidates for antifungal agents. Indeed, our previous works showed that peptides isolated from the skin gland of *Duttaphrynus melanostictus* frog had the antifungal potency (Barlian *et al.*, 2011).

In nature, eggshell is a protective layer for the embryo to grow and develop. Eggshell protects the embryo from microbial infection and therefore the function of eggshell components is very crucial. There two kinds of eggshells, hard shell and soft shell. Turtle eggshell is more potential and has more advantages to be developed for antimicrobial product compared to others such as avian eggshell, considering its ability to protect the embryo in high humidity condition. Lakshminarayanan *et al.* (2008) isolated pelovaterin, a peptide from turtle *Pelodiscus sinensis* that showed antimicrobial activity against gram-negative bacteria. Wellman-Labadie *et al.* (2010) reported bactericidal activity from avian eggshell against gram-negative and gram-positive bacteria. Until now, there are only limited substances with antifungal activity as compared to those possessing antibacterial action.

Based on the previous results and considering the eggshell as a common unused waste, this research was conducted to seek for an alternative source of antifungal peptides. Turtle eggshell was considered potential candidate for developing peptide-based antifungal product.

MATERIALS AND METHODS

Eggshell extraction: Turtle (*Chelonia mydas*) eggshell was obtained from Pangumbahan, Sukabumi, West Java Indonesia. Turtle eggshell was homogenized in 40 mM phosphate buffer at pH 8,0, 25°C for 10 min. The pellets than washed with de-ionized water and settled in room temperature for 24 h until dried to get crude extract. The crude extract is further dialysed in deionized water (1:100) for 24 h at 4°C using cellophane membrane (SIGMA D-9652). Dialyzed extract was then lyophilized.

Lyophilisate of the turtle eggshell was further determined for the total protein concentration using Bradford methods before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Ten percent SDS-PAGE was performed and the gel was stained using Coomassie brilliant blue G-250. Molecular weight marker with molecular range 14.4-116 kDa (Fermentas) was used to estimate the approximate molecular weight.

Antifungal activity test: The test was performed by agar diffusion method, as done in previous studies (Dulger and Gonuz, 2004a, b; Alam and Mostahar, 2005; Musyimi and Ogur, 2008; Ajibesin *et al.*, 2008; Pieme *et al.*, 2008; Sittiwet *et al.*, 2009; Sunilson *et al.*, 2009). Fungi species used in the antifungal activity test were *Candida albicans* (ATCC 10231), *Microsporium gypseum*, *Trichophyton mentagrophytes*, *Aspergillus brasiliensis* (ATCC 16404), obtained from Microbiology

laboratory, School of Pharmacy ITB. The fungi were cultured and maintained on Sabouraud's dextrose agar, SDA (Difco, USA) at 25°C. The lyophilisate was dissolved in sterile distilled water to make a final concentration of 7.5% v/v. Fifteen micro liter of the solution was then dropped onto a paper disc mounted on test fungi-inoculated agar plates in a Petri dish. Three replicates were used in this procedure. Incubation was carried out at 25°C for 72 h for all test fungi. The antifungal activity was determined by measuring the diameter of growth inhibition zone.

Scanning electron microscopic (SEM) assay of antifungal activity: Antifungal effect of the lyophilisate was later confirmed under scanning electron microscope to clarify the antifungal mechanism of action. For electron microscopic examination, two suspensions of each fungus in Sabouraud's dextrose medium were prepared, each with or without the lyophilisate (final concentration of the lyophilisate in the suspension was 7.5% w/v). All suspensions were then incubated in shaking incubator at 25°C for 72 h. After the incubation was completed, the fungal cells were harvested by centrifugation in an initial fixative solution (2% glutaraldehyde in sodium cacodylate buffer). The cells were then washed with phosphate buffer pH 7.4 and added final fixative solution (1% v/v OsO₄ in phosphate buffer pH 7.4). This procedure was continued with dehydration in serial ethanol dilution (50, 75, 88, 94 and 100%). The cells were then suspended in t-butanol. Subsequently, the suspension was dropped onto a cover slip and freeze dried at -50°C. The SEM assay, carried out on analytical SEM machine (JEOL JSM 6360 LA type), required a previous coating with a palladium:gold (80:20) mixture, which was performed in a Fine Coat Sputter (JEOL Ion Sputter JFC 1100) at 1.2 kV of voltage and 7.5 mA of current for 4 min. Observations were done under 5000-20000X magnification.

RESULTS

Our present study tried to reveal the potential antifungal activity of eggshell extract, in search of alternative peptide-based antifungal agents. The results showed that the turtle eggshell extract has antifungal activity. Further analyses performed to uncover the antifungal active ingredients demonstrated that low-molecular weight peptide might be the prime candidate.

Scanning electron microscopic results showed that the turtle eggs hell layers had thicker inner membrane compared to avian eggshell (Fig. 1). However, turtle eggshell had thinner outer layer

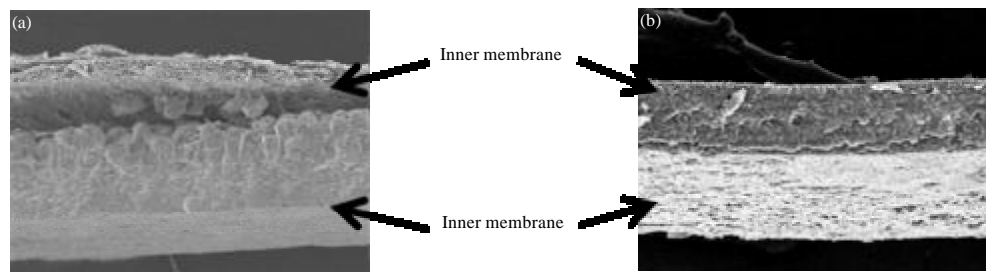


Fig. 1(a-b): Scanning electron microscopic cross section of (a) Avian and (b) Turtle eggshell
Magnification: 200X

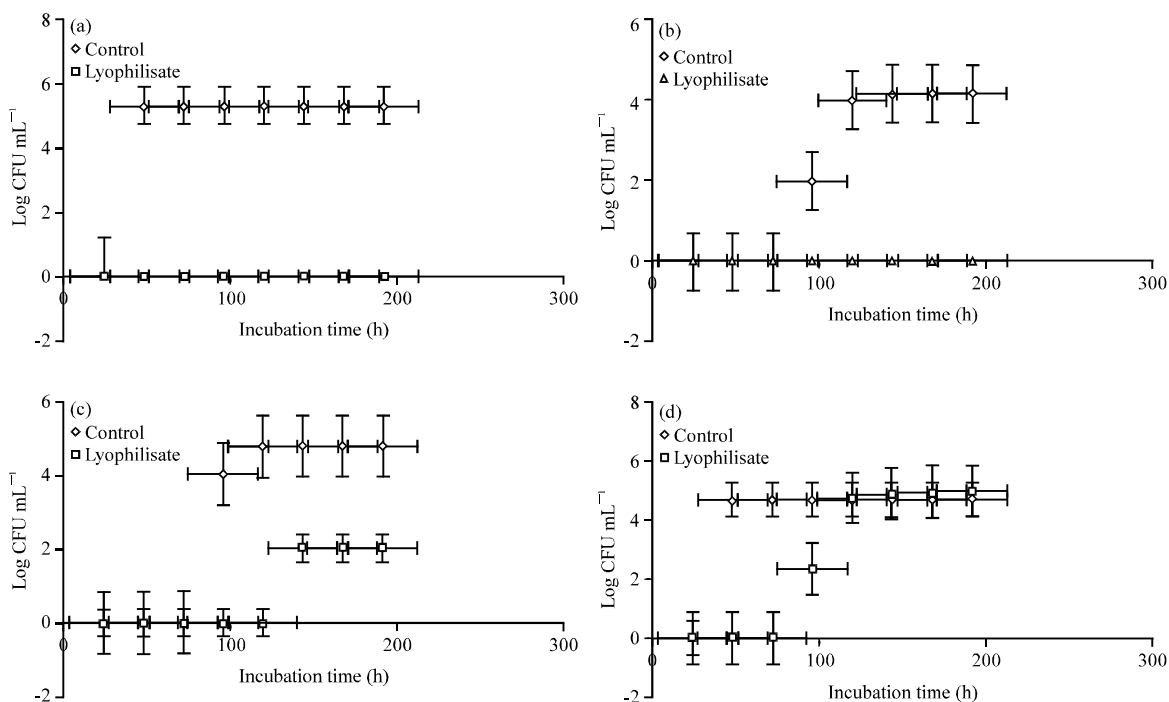


Fig. 2(a-d): Effect of turtle eggshell extract on fungus cultures (a) *Candida albicans*, (b) *Microsporium gypseum*, (c) *Trichophyton mentagrophytes* and (d) *Aspergillus brasiliensis*

than avian eggshell. The eggshell inner membrane may contain proteins with possible antifungal property. Thus, the antifungal activity of turtle eggshell is expected to be derived from the inner membrane of the turtle eggshell.

Results of antifungal tests against test fungi are presented in Fig. 2. The potency of the test substance, lyophilisate of turtle eggshell at 8% w/v (obtained from preliminary experiment) against all test fungi were comparable. Figure 2a shows that the formation of *Candida albicans* colony was inhibited through all the observation period. The colony of *Microsporium gypseum* was only formed after 100 h of incubation, as shown in Fig. 2b. It took more than 100 h for *Trichophyton mentagrophytes* colony to appear (Fig. 2c), while the colony of *Aspergillus brasiliensis*, as seen in Fig. 2d was observed after 100 h of incubation.

Morphological changes induced by exposure to lyophilisate of the eggshell extract are presented in Fig. 3. As shown in Fig. 3a and b, it is clear that deformation in the form of hyphal shrinkage (arrow) was observed in eggshell extract-incubated *Aspergillus brasiliensis*. *Microsporium gypseum* incubated with eggshell extract was shown to undergo structural deformation in the form of conidial wasting (arrow, Fig. 3d), as compared to the structurally intact fungus (Fig. 3c). The extract induced cellular shrinkage when exposed to *Candida albicans* (arrow, Fig. 3f) which could be clearly distinguished from normal cell (Fig. 3e). When the eggshell extract was contacted with *Trichophyton mentagrophytes*, marked hyphal shrinkage was observed (arrow, Fig. 3h) which was absent in the normal fungus (Fig. 3g).

SDS-PAGE results (Fig. 4) showed that there were 3 bands around 116, 45 and 20 kDa found in turtle eggshell extract.

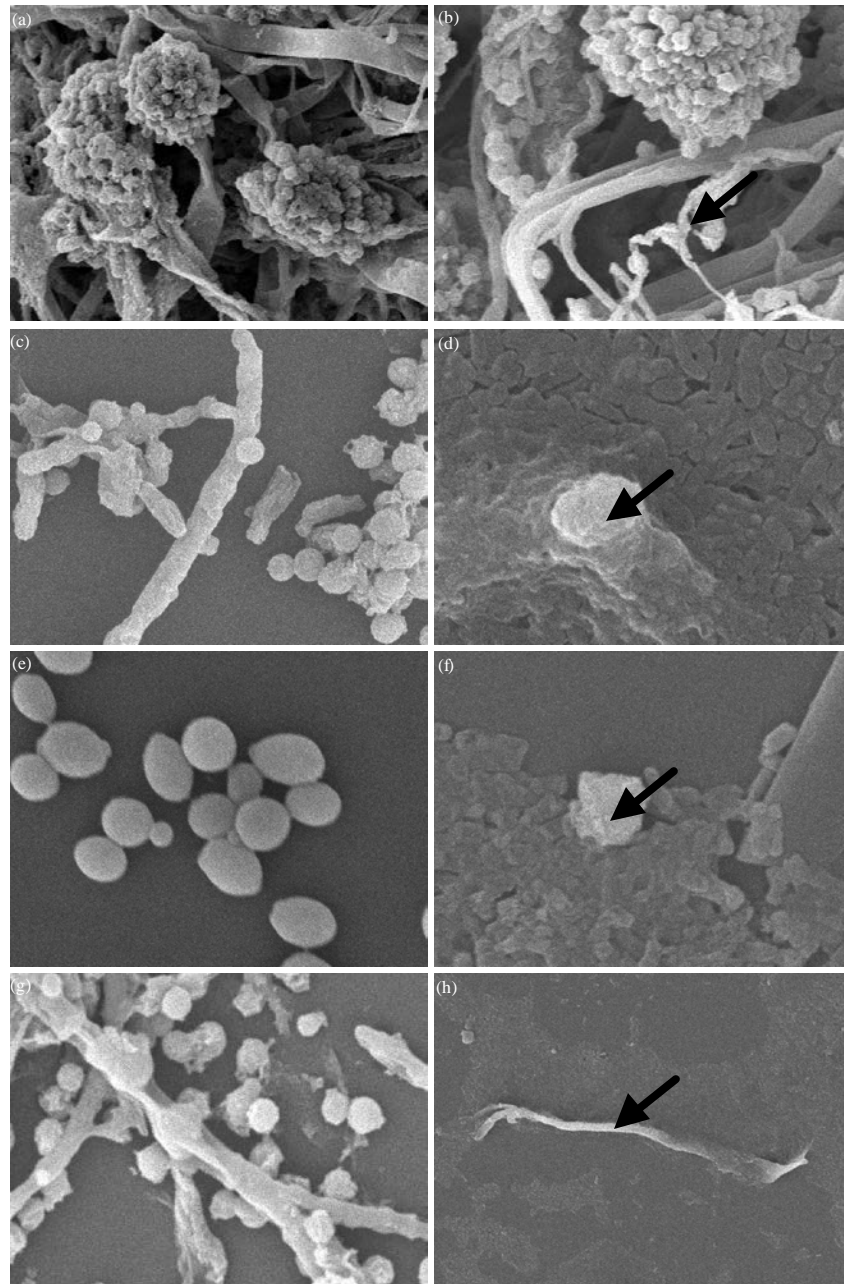


Fig. 3(a-h): Scanning electron microscopic presentation of (a) control *Aspergillus brasiliensis* (1000X), (b) Turtle eggshell extract-incubated *Aspergillus brasiliensis* (1500X), (c) Control *Microsporium gypseum* (3500X), (d) Turtle eggshell extract-incubated *Microsporium gypseum* (7500X), (e) Control *Candida albicans* (5000X), (f) Turtle eggshell extract-incubated *Candida albicans* (7500X), (g) Control *Trichophyton mentagrophytes* (3500X) and (h) Turtle eggshell extract-incubated *Trichophyton mentagrophytes* (1000X). Arrows: Structural damage following exposure to eggshell extract

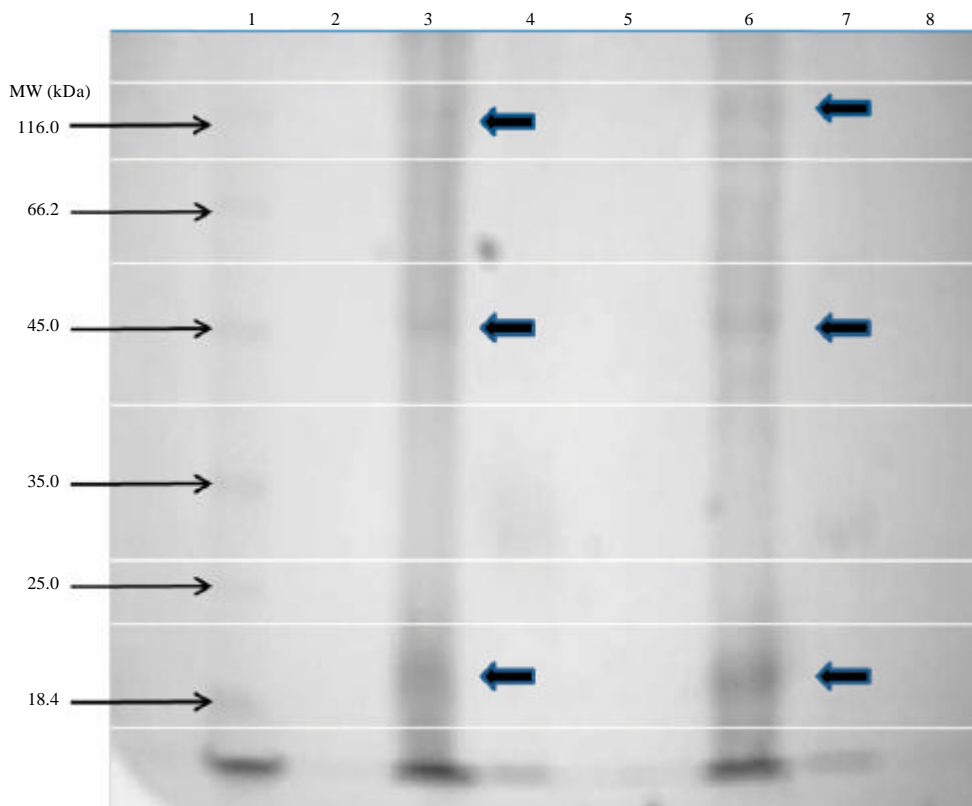


Fig. 4: SDS-PAGE from turtle eggshell extract, Lane 1: Molecular weight marker, numbers show the molecular weight (kDa), range in from 18.4-116 kDa, Lane 3: 15 $\mu\text{g } \mu\text{L}^{-1}$ turtle eggshell extract, Lane 6: 20 $\mu\text{g } \mu\text{L}^{-1}$ turtle eggshell extract. Arrows from top to the bottom show three proteins bands from turtle eggshell extract around 116, 45 and 20 kDa. Lane 2, 4, 5, 7 and 8: No. of samples

DISCUSSION

Results of the antifungal testing showed that the lyophilisate of the green turtle eggshell exerted growth inhibition activity against all test fungi to almost the same degree. It might be, thus, assumed that the lyophilisate contained ingredients which are potent against *T. mentagrophytes*, *M. gypseum* and *C. albicans* and *Aspergillus brasiliensis* infections. This finding might have positive clinical impact because candidal infection, with its ever-changing epidemiology, has been contributing significantly to the morbidity and mortality in health care setting (Horn *et al.*, 2009). Meanwhile, *T. mentagrophytes* and *M. gypseum* have been indicated as the major cause of dermatophytes infections (Adefemi *et al.*, 2010).

Detailed examination on the antifungal activity of the lyophilisate through SEM assay showed that the exposure to the lyophilisate led to deteriorating structural changes in test fungi. In general, the cellular surface became shrunken and pores were formed throughout the cell. These cellular deformations have been implicated to lead to the loss of water, electrolytes and other vital intracellular components essential for the cell survival. This mode of action has been demonstrated to be the cellular mechanism by which cell wall integrity-interfering antifungal agents work. Thus,

nystatin was shown to increase ionic, while amphotericin B was revealed to increase nonelectrolyte permeability (Andreoli and Monahan, 1968; Andreoli *et al.*, 1969). Our present findings suggest that the fungal active ingredients in the lyophilisate also affected the integrity of fungal cell wall or membrane and the damages thus produced would eventually lead to a striking degree of detrimental changes in the trans-membrane transport of essential electrolytes and water.

Earlier works have unveiled the presence of peptide antifungal agents extracted from particular frog species (such those reported by Simmaco *et al.* (1993, 1996), Conlon *et al.* (2009, 2010), Subasinghage *et al.* (2010) and Conlon *et al.* (2010)). These peptides are of short-chained type and they are body's natural protection components with potent fungicidal activities (Theis and Stahl, 2004). Thirty seven amino acids was known to constitute cecropin A, a peptide which was capable of complexing lipopolysaccharide (Boman *et al.*, 1985; De Lucca *et al.*, 1995). This peptide was later found to target ergosterol as well as cholesterol in *Aspergillus* and *Fusarium* species (De Lucca *et al.*, 1997). Further investigation has shown that a synthetic 17-amino acid-long proteolytic-resistant peptide bound cholesterol in the same fungal isolates (De Lucca *et al.*, 1998a). De Lucca *et al.* (1998b) further found physicochemical evidence showing that cecropin B and dermaseptin bound to ergosterol and cholesterol, the constituents of conidial wall, but not to chitin or beta-1,3-glucan.

As with the active component present in the lyophilisate of the green turtle eggshell, it might be expected that the occurring peptides exerted the antifungal effect due to its amphipathic characteristic that could induce pores formation in membrane plasma of fungi and the ensuing ionic leakage and damage to cell wall integrity. The antifungal peptides might also interfere with plasma membrane and other essential cell components syntheses (De Lucca, 2000). C-type lysozyme, ovotransferrin and ovocalyxin-32 are examples of peptides isolated from avian eggshell (*Gallus gallus*), ducks eggshell (*Anas platyrhynchos*) and goose eggshell (*Anser anser*), respectively that has antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* (Wellman-Labadie *et al.*, 2008).

CONCLUSION

Results of the current study demonstrated detrimental effects of *Chelonia mydas* turtle eggshell extract against pathogenic fungi. The results are expected to, further, contribute to the isolation of new fungi-active peptides.

REFERENCES

- Adefemi, S.A., M.A. Abayomi and J.M. Abu, 2010. Superficial fungal infections seen at a tertiary health centre: Clinical and mycological studies. *West Afr. J. Med.*, 29: 267-270.
- Ajibesin, K.K., N. Rene, D.N. Bala and U.A. Essiett, 2008. Antimicrobial activities of the extracts and fractions of *Allanblackia floribunda*. *Biotechnology*, 7: 129-133.
- Alam, S. and S. Mostahar, 2005. Studies of antimicrobial activity of two synthetic 2',4',6'-trioxygenated flavones. *J. Applied Sci.*, 5: 327-333.
- Andreoli, T.E. and M. Monahan, 1968. The interaction of polyene antibiotics with thin lipid membranes. *J. Gen. Physiol.*, 52: 300-325.
- Andreoli, T.E., V.W. Dennis and A.M. Weigl, 1969. The effect of amphotericin B on the water and nonelectrolyte permeability of thin lipid membranes. *J. Gen. Physiol.*, 53: 133-156.
- Barlian, A., K. Anggadiredja, A. Kusumorini and U. Ekawati, 2011. Structure of *Duttaphrynus melanostictus* frog skin and antifungal potency of the skin extract. *J. Biol. Sci.*, 11: 196-202.

- Boman, H.G., I. Faye, V.P. Hofsten, K. Kockum, J.Y. Lee and K.G. Xanthopoulos, 1985. On the primary structures of lysozyme cecropins and attacins from *Hyalophora cecropina*. *Dev. Comp. Immunol.*, 9: 551-558.
- Conlon, J.M., E. Ahmed, T. Pal and A. Sonnevend, 2010. Potent and rapid bactericidal action of alyteserin-1c and its [E4K] analog against multidrug-resistant strains of *Acinetobacter baumannii*. *Peptides*, 31: 1806-1810.
- Conlon, J.M., E. Akhmed and E. Condamine, 2009. Antimicrobial properties of brevinin-2-related peptide and its analogs: Efficacy against multidrug-resistant *Acinetobacter baumannii*. *Chem. Biol. Drug Des.*, 74: 488-493.
- De Lucca, A.J., 2000. Antifungal peptides: Potential candidates for the treatment of fungal infections. *Expert Opin. Investig. Drugs*, 9: 273-299.
- De Lucca, A.J., T.J. Jacks and K.A. Brogden, 1995. Binding between lipopolysaccharide and cecropin A. *Mol. Cell. Biochem.*, 151: 141-148
- De Lucca, A.J., J.M. Bland, T.J. Jacks, C. Grimm, T.E. Cleveland and T.J. Walsh, 1997. Fungicidal activity of cecropin A. *Antimicrob. Agent Chemother.*, 41: 481-483.
- De Lucca, A.J., J.M. Bland, C. Grimm, T.J. Jacks and J.W. Cary *et al.*, 1998a. Fungicidal properties, sterol binding, and proteolytic resistance of the synthetic peptide D4E1. *Can. J. Microbiol.*, 44: 514-520.
- De Lucca, A.J., J.M. Bland, T.J. Jacks, C. Grimm and T.J. Walsh, 1998b. Fungicidal and binding properties of the natural peptides cecropin B and dermaseptin. *Med. Mycol.*, 36: 291-298.
- Di Piro, J.T., 2003. Antimicrobial Regimen Selection. In: *Pharmacotherapy Handbook*, 5th Edn., Wells, B.J., J.T. Di Piro, T.L. Schwinghammer and C.W. Hamilton (Eds.). McGraw-Hill, New York, pp: 358-370.
- Dulger, B. and A. Gonuz, 2004a. Antimicrobial activity of certain plants used in Turkish traditional medicine. *Asian J. Plant Sci.*, 3: 104-107.
- Dulger, B. and A. Gonuz, 2004b. Antimicrobial activity of some Turkish medicinal plants. *Pak. J. Biol. Sci.*, 7: 1559-1562.
- Hancock, R.E., 2001. Cationic peptides: Effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.*, 1: 156-164.
- Havlickova, B., V.A. Czaika and M. Friedrich, 2008. Epidemiological trends in skin mycoses worldwide. *Mycoses*, 51: 2-15.
- Horn, D.L., D. Neofytos, E.J. Anaissie, J.A. Fishman and W.J. Steinbach *et al.*, 2009. Epidemiology and outcomes of candidemia in 2019 patients: Data from the prospective antifungal therapy alliance registry. *Clin. Infect. Dis.*, 48: 1695-1703.
- Lakshminarayanan, R., S. Vivekanandan, R.P. Samy, Y. Banerjee and E.O. Chi-Jin *et al.*, 2008. Structure, self-assembly and dual role of a beta-defensin-like peptide from the Chinese soft-shelled turtle eggshell matrix. *J. Am. Chem. Soc.*, 130: 4660-4668.
- Liu, Q. and C.L. Xiao, 2005. Influence of nutrient and environmental factors on conidial germination of *Potrebniomyces pyri*. *Phytopathology*, 95: 572-580.
- Musyimi, D.M. and J.A. Ogur, 2008. Comparative assessment of antifungal activity of extracts from *Eucalyptus globules* and *Eucalyptus citriodora*. *Res. J. Phytochem.*, 2: 35-43.
- Pfaller, M.A. and W.L. Yu, 2001. Antifungal susceptibility testing. New technology and clinical applications. *Infect. Dis. Clin. North Am.*, 15: 1227-1261.
- Pieme, C.A., J.P. Dzoyem, F.A. Kechia, F.X. Etoa and V. Penlap, 2008. *In vitro* antimicrobial activity of extracts from some Cameroonian medicinal plants. *J. Biol. Sci.*, 8: 902-907.

- Simmaco, M., G. Mignogna, D. Barra and F. Bossa, 1993. Novel antimicrobial peptides from skin secretion of the European frog *Rana esculenta*. *FEBS Lett.*, 324: 159-161.
- Simmaco, M., G. Mignogna, S. Canofeni, R. Miele, M.L. Mangoni and D. Barra, 1996. Temporins, antimicrobial peptides from the European red frog *Rana temporaria*. *Eur. J. Biochem.*, 242: 788-792.
- Sittiwet, C., D. Puangpronpitag and N. Niamsa, 2009. *In vitro* antimicrobial activity of *Schefflera leucantha*: The potential of respiratory tract and urinary tract infection treatment. *Int. J. Pharmacol.*, 5: 240-243.
- Subasinghage, A.P., J.M. Conlon and C.M. Hewage, 2010. Development of potent anti-infective agents from *Silurana tropicalis*: Conformational analysis of the amphipathic, alpha-helical antimicrobial peptide XT-7 and its non-haemolytic analogue [G4K]XT-7. *Biochim. Biophys. Acta. Proteins Proteomics*, 1804: 1020-1028.
- Sunilson, J.A.J., R. Suraj, G. Rejitha, K. Anandarajagopal, A.V.A.G. Kumari and P. Promwichit, 2009. *In vitro* antimicrobial evaluation of *Zingiber officinale*, *Curcuma longa* and *Alpinia galangal* extracts as natural food preservatives. *Am. J. Food Technol.*, 4: 192-200.
- Theis, T. and U. Stahl, 2004. Antifungal proteins: Targets, mechanisms and prospective applications. *Cell Mol. Life Sci.*, 61: 437-455.
- Wellman-Labadie O., S. Lemaire, K. Mann, J. Picman and M.T. Hincke, 2010. Antimicrobial activity of lipophilic avian eggshell surface extracts. *J. Agric. Food Chem.*, 58: 10156-10161.
- Wellman-Labadie, O., J. Picman and M.T. Hincke, 2008. Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. *Br. Poult. Sci.*, 49: 133-143.