Membrane Stabilising and Phagocytosis Stimulatory Activities of *Fusarium equiseti* — An Endophytic Fungus of Mangrove Palm (*Nypa fruticans* Wurmb) Fruit

ABSTRACT

Objective: *Nypa fruticans* Wrumb commonly known as mangrove palm thrives well in brackish water; and is an important medicinal plant. It was sampled, surface sterilised, sectioned into tiny chips and plated on sabrose dextrose agar (SDA) media seeded with antibiotics to isolate the endophytic fungi.
Methods: *Fusarium equiseti* was fermented on sterilised solid rice media for 28 days, extracted with ethyl acetate, filtered and concentrated *in vacuo*. A high pressure liquid chromatography (HPLC) assay was performed. While the biological effects, were evaluated by human erythrocyte membrane stabilisation against heat- and hypotonicity-induced haemolysis and stimulation of human polymorphonuclear neutrophilis (PMNs) phagocytosis.
Results: *Fusarium equiseti* (20 – 100 μg/ml) expressed a dose-dependent effect in both bioassays, comparing significantly ($p < 0.05$) with reference drugs: diclofenac (100 μg/ml), indomethacin (50 μg/ml), prednisolone (100 μg/ml), for membrane stabilisation; and ascorbic acid (50 μg/ml) for the later. The HPLC assay identified an isofistularin-3 like-compound as the dominant constituent.
Conclusion: This is first reported on the isolation of endophytic fungi from the Niger Delta mangrove plant species in Nigeria. It also demonstrated the potential of the mangrove palm as a source of novel lead compounds for the development of anti-inflammatory and immune-stimulatory drugs. Future studies will hopefully isolate and characterise the active principles.

Key words: *Fusarium equiseti*, *Nypa fruticans* Wrumb, Erythrocyte membrane stabilisation, Polymorphonuclear neutrophils phagocytosis stimulation, Mangrove ecosystem

INTRODUCTION

Endophytes are microorganisms that leave asymptomatically in and colonise healthy tissues of plants without causing any immediate overt harmful effect. These microbes are known to produce a plethora of bioactive secondary metabolites that usually assist in the host's protection and survival against pathogenic and insect attacks, stress tolerance and disease resistance.[1] Endophytic fungi are important constituents of the endophyte microbial community, and are found in nearly all plant families;[2] including plants living in unique/stressful ecosystems.[3] Researchers have identified fungal endophytes as veritable sources of varied bioactive secondary metabolites with confirmed biological activities like antibiotics, antimycotics, immune-suppressants, anticancer, *et c.*[4] Therefore, endophytic fungi bioprospection will continue to offer great promise in the discovery of novel compounds with therapeutic value. This has attracted increasing attention among microbiologists, ecologists, agronomist and chemists.

Since mangrove plants thrive under an extreme stressful environment, there have been growing interests in them as sources of bioactive compounds. Because such plants have developed special metabolic pathways to produce unique metabolites in order to tolerate harsh environmental conditions. Thus so many reports have indicated the isolation of pharmacological compounds from mangrove plants and mangrove-plants' associated microbiota. These discoveries did highlight the importance of mangrove ecosystems as sources of natural products with interesting medicinal properties, and requires more attention to isolate and confirm their efficiency.

The stabilisation of the cell membranes of human red blood cells (HRBC) by chemical entities, has been indicated to be an important index in the search for anti-inflammatory agents.[5,6] Because it has been shown, that the HRBC membrane is identical to the membranes of lysosomes; and the rupture of the lysosome membranes releases pro-inflammatory mediators that trigger inflammatory response.[7] Therefore, candidate anti-inflammatory entities could exert anti-inflammatory activity by stabilising the membranes of lyposomes. This prevents the release of the pro-inflammatory mediators and inhibits inflammation.

As the mangrove ecosystem and endophytic fungi are important elements in the discovery of novel compounds with diverse bioactivity; the present study focused on the study of the immune-stimulatory and anti-inflammatory effects of the secondary metabolites of *Fusarium equiseti* — an endophytic fungus of *Nypa fruticans* Wurmb fruit. The exercise is the preliminary activities in the isolation and the chemical structure elucidation of the inherent active principles.

MATERIALS AND METHODS

Materials and Reagents

Reagents

Sabrose dextrose agar (SDA), malt extract agar (MEA) and 96 % ethanol were procured from local chemical shops and used as supplied. Also, chloramphenicol sulphate BP obtained from a pharmacy shop was used to inhibit the growth of bacteria at the endophytic fungi isolation stage.

Equipment
Autoclave, laminar air flow hood, pH meter, BOD incubator, vacuum rotary evaporator, analytical balance, centrifuge, microscope, ultraviolet-visible light spectrophotometer, and analytical high performance liquid chromatography (HPLC), Dionex UltiMate-3400SD with an LPG-3400SD pump, coupled with photodiode array detector (DAD 300RS) were used.

Collection of *Nypa fruticans* Wurmb specimens
Anatomical segments and whole-plant of *Nypa fruticans* Wurmb, mangrove palm, were collected from the mangrove forest at Degema, Rivers State, Nigeria at 4.00 – 5.30 pm on August 16, 2015. The whole-plant specimen was identified and authenticated by Dr Chimezie Ekeke, Taxonomist/Curator, Herbarium Unit, Department of Plant Science and Biotechnology, University of Port Harcourt, Port Harcourt, Nigeria. Voucher specimen of the sampled mangrove plant was deposited at the University of Port Harcourt Reference Herbarium for Research and Germplasm Conservation (UPH/RHRGC) with the herbarium number UPH/V/78.

Isolation of endophytic fungi
Fungal isolation was performed under sterilised conditions from the inner tissue of *N. fruticans* fruits following an isolation protocol described previously.[8] The fungus was cultured on solid rice medium and identified based on the morphology of the fungal culture, the mechanism of spore production and characteristics of the spore. A voucher specimen of the fungus was deposited at our mycology laboratory.

Extraction of endophytic fungi secondary metabolites
The fermentation was performed on solid rice for four (4) weeks. The culture was diced and extracted with ethyl acetate, and the crude extract dried *in vacuo* using a rotating evaporator. The dried extract was weighed and stored in a refrigerator at less than 4 ºC, before used for bioassays.

Membrane stabilising effect assay
a. Heat-induced hemolysis: Fresh whole human blood (5 ml) was collected and transferred to an ethylene diamine tetraacetate (EDTA) centrifuge tube. The tube was centrifuged at 2000 rpm for 5 min, and washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as a 40 % v/v suspension with isotonic phosphate buffer solution (pH 7.4). The composition of the buffer solution (g/l) was NaCl (4.4 g), NaH_2PO_4 (1.6 g), and Na_2HPO_4 (7.6 g). The isotonic buffer solutions (5 ml) containing 20, 50, or 100 μg/ml of the secondary metabolites were each put in sets of four (4) centrifuge tubes per concentration. Control tubes contained 5 ml of vehicle, 5 ml of 50 μg/ml indomethacin, or 5 ml of 100 μg/ml prednisolone, or 5 ml of 100 μg/ml diclofenac. Erythrocyte suspension (0.005 ml) was added to each tube and gently mixed. A pair of the tubes from each set was incubated at 54 °C for 20 min in a regulated water bath. The other pair was maintained at 0 – 4 °C in a freezer for 20 min. At the end of the incubation, the reaction mixture was centrifuged at 1000 rpm for 3 min and the absorbance (OD) of the supernatant measured spectrophotometrically at 540 nm. The percent inhibition of haemolysis,[9] was calculated using the following formula:

$$\text{Inhibition of haemolysis (\%)} = \{1 - [(OD2 - OD3)/(OD3 - OD1)]\} * 100$$

Where OD1 = absorbance of test sample unheated, OD2 = absorbance of test sample heated, and OD3 = absorbance of control sample heated.

b. Hypotonicity-induced hemolysis: This was done by a slight modification of the method reported by Mounnissamy *et al*.[10] Two per cent (2 % w/v) dextrose, 0.8 % w/v, sodium citrate, 0.05 % w/v citric acid and 0.42 % w/v sodium chloride was dissolved in distilled water and sterilised to obtain sterilised Alsever solution. Blood was collected from median cubital vein of healthy volunteers, placed in ethylene diamine tetraacetic acid (EDTA) centrifuge tubes and swirled for 30 seconds. A portion of the collected blood was mixed with equal volume of sterilized Alsever solution, centrifuged at 3000 rpm for 10 minutes and the packed cells were washed trice with sterilised isosaline (0.9 % NaCl) solution in distilled water), and a suspension of compact packed blood cells in 10 % (v/v) sterile isosaline was made. A 1 ml aliquot of filtered solutions of varying concentrations (20 – 100 μg/ml) of the *F. equiseti* secondary metabolites in phosphate buffer (pH 7.4) was mixed with 2 ml hyposaline (0.3 % NaCl solution in distilled water) in a centrifuge tube, followed by addition of 0.5 ml of the human red blood cell (HRBC) suspension. The assay mixture was incubated at 37 ºC for 30 minutes and centrifuged at 3000 rpm for 10 minutes. Haemoglobin content in the supernatant solution was estimated using ultraviolet/visible light spectrophotometry at 560 nm. Diclofenac sodium (100 μg/ml) was used as the reference drug and positive control. For the negative control, the 2 ml of hyposaline was replaced with an equivalent volume of sterile distilled water. The per cent inhibition of haemolysis was calculated by assuming the haemolysis inhibition produced in the presence of distilled water was 0 %. The percentage of HRBC membrane stabilization or protection was calculated using this equation:

$$\text{Percentage inhibition of Haemolysis} = [(OD1 - OD2)/OD1] * 100$$

Where OD1and OD2, are the optical absorbance of the negative control and test sample respectively.

Phagocytises stimulatory assay

a. *Candida albicans* culture was incubated in Sabouraud dextrose broth (SDB) overnight and centrifuged to form a cell button at the bottom of the test tube. The supernatant was discarded and the cell button washed four (4) times with sterile phosphate buffer saline (PBS) and centrifuged. The washed cell button was re-suspended in equivolume of a mixture of PBS and serum in the ratio 4:1.

b. Slides were appropriately labelled for the various concentrations of the secondary metabolites, ascorbic acid, and control (water). One drop of human blood was smeared on each slide and the slides were incubated at 37 ^{o}C for 20 minutes to allow for clotting. The slides were drained slowly with sterile normal saline, taking care not to wash off the adhered neutrophils. The slides consisting of polymophornuclear neutrophils (PMNs) were flooded with 5 ml of solution of predetermined concentrations of the various test samples, respectively, and incubated at 37 ^{o}C for 15 minutes. After which, the PMNs on the slide was covered with 1 ml of the *Candida albicans* suspension and incubated at 37 ^{o}C for 1 hour. The slides were drained with normal saline, fixed with methanol and stained with Giemsa stain.

c. The slides were viewed under the microscope and the number of *Candida albicans* cells phagocytosed by PMNs on the slides was determined by a count of the dark blue spots on gray background. This number was regarded as the phagocytic index (PI) and was compared with the PI of the control treatment as described in the equation below.[11]

$$\text{Stimulation of phagocytosis} = \frac{\text{PI (test)} - \text{PI (control)}}{\text{PI control}} \times \frac{100}{1}$$

Analytical high-pressure liquid chromatography

Analytical HPLC was used to identify the distribution of compounds in the crude extract of *F. equiseti* secondary metabolites. This assay was performed as previously described by Okonkwo *et al*.[12]

Statistical analysis

Where applicable, results were expressed as mean ± SEM. Means were compared for statistical significant difference by one-tailed *t*-Test and one-tailed analysis of variance (ANOVA) using Duncan post-hoc test, as programmed in Graphical Prism® statistical software version 7.0. Effects were considered significant at p = 0.05 in all cases.

RESULTS

The membrane stabilising effect of *F. equiseti* secondary metabolites against hypotonicity induced haemolysis is presented in Figure 1. While Table 1 and Figure 2, respectively indicated the inhibitory activity against heat induced haemolysis and phagocytosis stimulatory effect on PMNs. Figure 3 indicated the ultraviolet/visible light absorption spectrum of the principal constituent of *F. equiseti* secondary metabolites.

Table 1: Mean ± SEM per cent inhibitory activity of *F. equiseti* secondary metabolites and standard anti-inflammatory drugs against heat induced haemolysis

Drug concentration (µg/ml)	*F. equiseti* secondary metabolites	Diclofenac	Indomethacin	Prednisolone
100	111.78 ± 6.86	70.09 ± 1.34	—	39.21 ± 2.18
50	70.02 ± 2.86	—	27.76 ± 3.62	—
20	57.62 ± 6.27	—	—	—

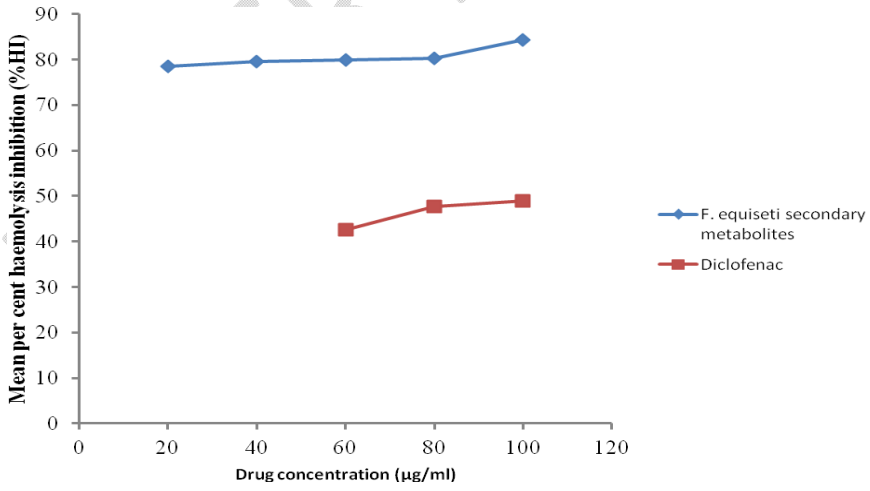

Figure 1: Mean per cent inhibitory activity of *F. equiseti* secondary metabolites and diclofenac against hypotonicity induced haemolysis

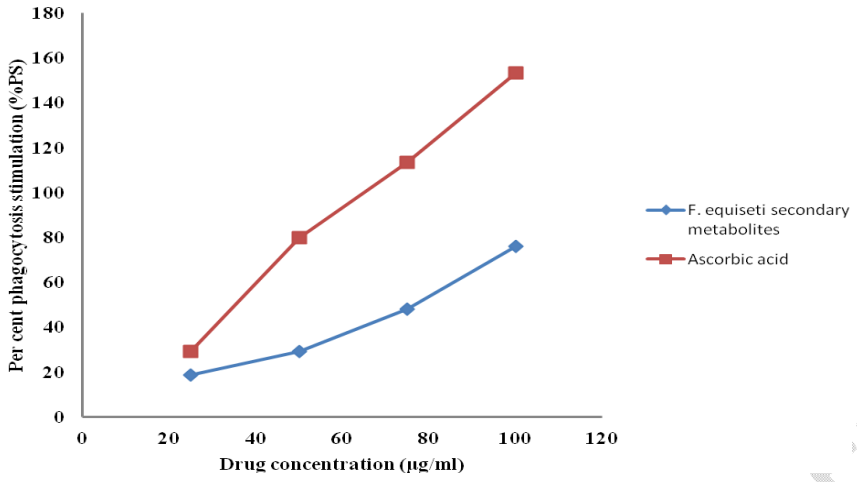

Figure 2: Mean per cent phagocytosis stimulatory activity of *F. equiseti* secondary metabolites and ascorbic acid

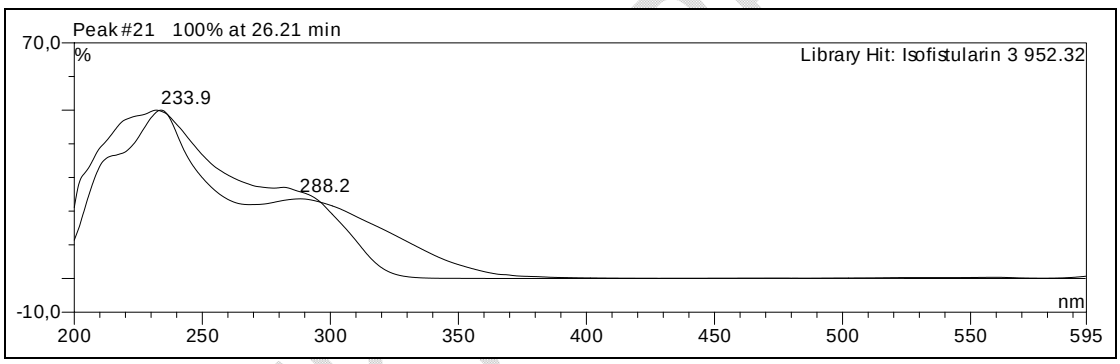

Figure 3: Ultraviolet-visible light (Uv/visible) absorption spectrum of the major constituent (8.01 % relative abundance) of *F. equiseti* secondary metabolites (NB: the curve above, starting from top left, is the Uv/visible light absorption curve of isofistularin-3)

DISCUSSION

F. eqiseti secondary metabolites at 20 – 100 µg/ml significantly ($p < 0.05$) inhibited both heat-induced and hypotonicity-induced lyses of human erythrocytes in a dose-dependent manner. In both models, the inhibitory activities of *F. equiseti* (50 – 100 µg/ml) were significantly ($p < 0.05$) higher than the corresponding effects of diclofenac (100 µg/ml) against the lyses of HRBCs. The observed effect against the heat-induced lyses of HRBCs was generally much higher than the effect of indomethacin (50 µg/ml) and prednisolone (100 µg/ml); which exerted 27.76 ± 3.62 and 39.21 ± 2.18 per cent inhibitions respectively. These findings possibly suggested that *F. equiseti* expressed natural products with anti-inflammatory activity. The chemical structure(s) of the compounds, on isolation and elucidation, may be exploited as lead template(s) in the development of new anti-inflammatory drug(s).

Ascorbic acid concentrates in polymorphonuclear neutrophils (PMNs) and other leucocytic cells,[13,14] via an active transport involving insulin.[15] Thus there is a concentration gradient between the plasma level and the PMNs level of the vitamin. Existing reports indicated a rapid expenditure of vitamin C within PMNs during infection and phagocytosis,[16] thus suggesting a role for ascorbic acid in the immune process. Biochemical studies did reveal that Vitamin C stimulates the hexoes monophosphate (HMP) shunt,[17] which produces nucleotides (ribose nucleic acid, RNA, and dexoyribose nucleic acid, DNA) and nicotinamide diphosphate hydrogen (NADPH) that are very essential in the immune function of leucocytes. Further evidence confirmed that vitamin C is involved in the migration, chemotaxis and phagocytosis by marcrophages and leucocytes.[18] A clinical trial on the effect of dietary supplementation with ascorbic acid in patients on steroid therapy with the resulting steroid-induced depletion of leucocyte ascorbate and diminished phagocytic activity of human neutrophilis, revealed an increased phagocytic activity of neutrophilis in the patients.[19] An earlier report,[20] indicated that treatment of normal human blood leucocytes *in vitro* with ascorbic acid increased erythrophagocytosis.

Thus the per cent human PMNs phagocytosis stimulation (%PS) observed for ascorbic acid (29.30 ± 4.80 – 153.30 ± 7.05), in this report, confirmed the established effect of the vitamin on phagocytosis by neutrophilis and its boosting effects on the immune function of leucocytes. Bio-effects across the standard drug concentrations were both dose-dependent and linear, with a linearity index (R^2) of 0.993 ± 0.003. Thus the 50 % stimulatory concentration (SC_{50}) was evaluated as 35.38 ± 1.27 μg/ml. The test drug, also, expressed a dose-dependent and a linear phagocytic stimulatory effect on human PMNs at 25 – 100 μg/ml. Its effect (76.00 ± 8.33 %PS) at the maximum concentration was significant ($p < 0.05$) to the %PS of ascorbic acid (80.00 ± 4.62 %PS) at 50 μg/ml. The R^2 was 0.960 ± 0.001, while the IC_{50} was deduced as 71.68 ± 1.89 μg/ml. Our findings indicated that *F. equiseti* secondary metabolites possess immune-stimulatory activity, but more *in vivo* studies are required to confirm this effect; as well as the mechanism of action.

Analytical HPLC assay identified about 50 – 52 distinct chemical compounds in the *F. equiseti* crude extract. Their retention time ranged from 7.99 – 49.57 minutes, while an isofistularin-3 like-compound (based on a similarity index, SI, of 95.232 % in Figure 3) had the highest per cent relative abundance of 8.01 %. Aplysinamisin-1 related-compound (95.618 %SI) constituted 1.94 % relative abundance. Both isofistularin-3 and aplysinamisin-1 are brominated alkaliods found commonly in marine sponges.[21] Structurally, isofistularin-3 is a spiroisoxazoline; while aplysinamisin-1 is a bromotyramine drevative.[21] The occurrence of these and similar compounds in *F. equiseti* is expected, since *F. equiseti* is a marine organism possessing the ability to *de novo* syntheses marine compounds.

Other researchers have reported some other biological effects of the *Fusarium* genus of endophytic fungi, and the isolation of active principles from some of its species.[22] This and other studies on endophytic fungi,[23] have confirmed the potential of fungal endophytes as productive sources for the discovery of useful drug leads for innovative and improved pharmaceuticals. Therefore, the present study holds a promise in the discovery of novel drug lead compounds.

CONCLUSION

This is the first study that described the biological activity of *Fusarium* endophytic fungi from the mangrove palm found in the Niger Delta, Nigeria. It revealed that *Nypa fruticans*

Wrumb harbours some endophytic fungi which are capable of producing bioactive natural products. The endophytic *Fusarium equiseti* exhibited promising anti-inflammatory and immune-stimulatory effects. These findings also suggested that endophytes from harsh and stressful environments have great potential to be a viable source for the discovery and development of drug leads.

REFERENCES

1. Pamoda BR, de Silva1 ED, Williams DE, Andersen RJ. Antimicrobial Activities of Endophytic Fungi obtained from the Arid Zone Invasive Plant *Opuntia dillenii* and the Isolation of Equisetin, from Endophytic *Fusarium sp*. *BMC Complem & Altern Med* 2015; **15**: 220–226.

2. Musavi SF, Balakrishnan RM. Biodiversity, Antimicrobial Potential, and Phylogenetic Placement of an Endophytic *Fusarium oxysporum* NFX 06 Isolated from *Nothapodytes foetida*. *J Mycolo* 2013; http://dx.doi.org/10.1155/2013/172056

3. Strobel G, Daisy B, Castillo U, Harper J. Natural Products from Endophytic Microorganisms. *J Nat Prod* 2004; **67**(2): 257–268.

4. Zhang HW, Song YC, Tan RX. Biology and Chemistry of Endophytes. *Nat Prod Rep* 2006; **23**(5): 753–771.

5. Okoye FBC, Osadebe PO, Proksch P, Edrada-Ebel RA, Nworu CS, Esimone CO. Anti-inflammatory and Membrane-stabilizing Stigmastane Steroids from *Alchornea floribunda* Leaves. *Planta Med* 2010; **76**: 172–177.

6. Okoye FBC, Osadebe PO. A New Anti-inflammatory Flavonol Glycoside from *Alchornea floribunda* Leaves. *Nat Prod Res* 2010; **24**(3): 266–273.

7. Weissmann G. The Role of Lysosomes in Inflammation and Disease. *Ann Rev Med* 1967; **18**: 97–112.

8. Kjer J, Debbab A, Aly AH, Proksch P. Methods for Isolation of Marine-derived Endophytic Fungi and their Bioactive Secondary Products. *Nature Protocols* 2010; **5**: 479–490.

9. Shinde UA, Phadke AS, Nari AM, Mungantiwar AA, Dikshit VJ, Saraf MN. Membrane Stabilization Activity – A Possible Mechanism of Action for the Anti-inflammatory Activity of *Cedrus deodora* Wood Oil. *Fitoterapia* 1999; 70: 251–257.

10. Mounnissamy VM, Kavimani S, Balu V, Quine SD. Evaluation of Anti-inflammatory and Membrane Stabilizing Properties of Ethanol Extract of *Cansjera rheedii* J. Gmelin (*Opiliaceae*). *Iranian J Pharmacol & Therapeutics* 2007; **6**: 235-237.

11. Ganachari MS, Kumar S, Bhat KG. Effect of *Ziziphus jujuba* Leaves Extract on Phagocytosis by Neutrophils. *J Nat Remedies* 2004; **4**(1): 47–51.

12. Okonkwo TJN, Osadebe PO, Proksch P.. Bioactive Phenylpropanoids, Phenolic Acid and Phytosterol from Landolphia owariensis P. Beauv Stringy Seed Pulp. *Phytother Res* 2016; **30**:78–83.

13. Ströhle A, Hahn A. Vitamin C and Immune Function. *Med Monatsschr Pharm* 2009; **32**(2):49–54.

14. Moser U. Uptake of Ascorbic Acid by Leukocytes. *Ann NY Acad Sci* 1987; **498**: 200–215.

15. Ottoboni F, Ottoboni A. Ascorbic Acid and the Immune System. http://www.orthomolecular.org/library/jom/2005/pdf/2005-v20n03-p179.pdf

16. Thomas WR, Holt PG. Vitamin C and Immunity: An Assessment of the Evidence. *Clin Exp Immunol* 1978; **32**: 370–379.

17. DeChatelet LM, Cooper MR, McCall CE. Stimulation of the Hexose Monophosphate Shunt in Human Neutrophils by Ascorbic Acid: Mechanism of Action. *Antimicrob Agents Chemother* 1972; **1**: 12–16.

18. Levy R, Shriker O, Porath A, Riesenberg K, Schlaeffer F: Vitamin C for the Treatment of Recurrent Furunculosis in Patients with Imparied Neutrophil Functions. *J Infect Dis* 1996; **173**(6): 1502–1505.

19. Olson GE, Polk HK. *In vitro* Effect of Ascorbic Acid on Corticosterone-caused Neutrophil Dysfunction. *J Surg Res* 1977; **22**: 109.

20. Greendyke RM, Brierty RE, Swisher SN. *In vitro* Studies on Erythrophagocytosis. II. Effects of Incubating Leukocytes with selected Cell Metabolites. *Lab Clin Med* 1964; **63**: 1016.

21. Lira NS, Montes RC, Tavares JF, da Silva MS, da Cunha EVL, de Athayde-Filho PF, Rodrigues LC, Dias C da S, Barbosa-Filho JM. Brominated Compounds from Marine Sponges of the Genus *Aplysina* and a Compilation of Their 13C NMR Spectral Data. *Mar Drugs* 2011; **9**: 2316–2368.

22. Ratnaweera PB, Dilip de SE, Williams DE, Andersen RJ. Antimicrobial Activities of Endophytic Fungi obtained from the Arid Zone Invasive Plant *Opuntia dillenii* and the Isolation of Equisetin, from Endophytic *Fusarium* sp. BMC *Compl & Alter Med* 2015; **15**: 220–226.

23. Chen H, Daletos G, Okoye F, Lai D, Dai H, Proksch P. A New Cytotoxic Cytochalasin from the Endophytic Fungus *Trichoderma harzianum. Nat Prod Commun* 2015; **10**(0): 1–3.