ORIGINAL ARTICLE

Evaluation of pharmacognostical, phytochemical and anti-microbial properties of *Porphyra vietnamensis*

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Objective: There is no detailed standardisation work reported for pharmacognostic and phytochemical studies on alga. Thus, present communication attempts to evaluate the pharmacognostical, phytochemical and antimicrobial studies of red algae *Porphyra vietnamensis,* Bangiaceae family. **Material and Methods:** Extraction and isolation were performed to conduct preliminary phytochemical screening and various pharmacognostical studies on *P. vietnamensis.* Fatty acids and minerals were analysed using gas chromatography and atomic absorption spectroscopy. Antimicrobial potential of aqueous and alcoholic fractions was investigated using agar disc diffusion method. **Results:** Preliminary phytochemical screening and pharmacognostical data confirmed the presence of the high amount of dietary fibre, Vitamin C, carbohydrate and lipid content. Fatty acid analysis confirms the presence of the high amount of methyl palmitate. Atomic absorption data revealed the presence of significant amount of Mg, S, Ca, P, Na and K. Aqueous and alcoholic extracts (200–300 µg/ml) was effective against all microbes whereas alcoholic extract was proved to be more effective then aqueous extract. **Conclusion:** This study revealed the specific identities for the commercially important red seaweed, *Porphyra,* which will be useful in identification and prevention of its adulteration. Furthermore anti-microbial elements present in this alga can be further investigated for the development of novel antibiotics.

Key words: Antimicrobial, pharmacognostic, phytochemical analysis, *Porphyra vietnamensis*

INTRODUCTION

Macro-algae (seaweeds) are attached to the bottom in shallow coastal waters and grouped under three divisions namely; *Chlorophyceae* (green algae), *Phaeophyceae* (brown algae) and *Rhodophyceae* (red algae). About 20,000 marine algae species are distributed throughout the world, out of which only 221 species are utilised commercially. These include 145 species for food and 110 species for phycocolloid production (Abdussalam, 1990; Scheuer, 1990). They are considered as source of bioactive compounds and produce a great variety of secondary metabolites characterised by a broad spectrum of biological activities. *Porphyra* (*Bangiales*, *Rhodophyta*) popularly known by "Nori" in Japan, "Kim" in Korea and "Zicai" in China has an annual value of over US$ 1.8 billion. It is an excellent taste traditional Chinese medicine and consumed by local inhabitants as a marine vegetable in Asia. It has been authorised for human consumption by French authorities due to nutritional interests, that is, rich vitamins, oligo elements, minerals and dietary fibres.[1-5]

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Numerous reports related with its anti-oxidant,[6] anti-cancer,[7] anti-aging,[8] antifatigue,[9] anti-coagulants, anti-hyperlipidimic,[10] sunscreen agent,[11] immune-modulation and anti-tumour[12] and anti-viral[13] activities have been found. Various species have been explored so far; however *Porphyra vietnamensis* still needs more attention. Apart from certain potent constituents like Mycosporine like amino acids (MAAs), it contains a large amount of sulphated polysaccharide (porphyran), one of the active principles in *Porphyra* which is having multitude of activities like anticancer, anti-aging, anti-oxidant, etc. Moreover, antimicrobial activity of *Porphyra* is not well-characterised although from a small number of studies it appears that chemical defences may improve its resistance. Despite lacking cell-based immune systems, *Porphyra* suffers remarkably low levels of microbial infection. Atypical millilitre of seawater contains 103 fungal cells, 106 bacteria and 107 viruses, including pathogens that cause widespread mortalities and microbes that initiate fouling of host surfaces. Thus, marine plants and animals are continually exposed to high concentrations of potentially harmful microbes.

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Microbial pathogens cause green spot rotting disease in the alga *Porphyra*, red spot disease in the kelp *Laminaria* and raisin disease in the brown alga *Sargassum*. The frequency of disease among marine macroorganisms has increased in recent decades and may continue to increase. Because microbes are more widely distributed in seawater than in air, pressures from pathogenic, parasitic, saprophytic and fouling microbes might be greater in marine environments, a circumstance that might be expected to select for potent antimicrobial defences.[14,15] There is no protocol reported available for the standardisation of nutritionally rich *Porphyra* by using pharmacognostic and its antimicrobial studies. Thus, this study was designed to investigate the pharmacognostic, phytochemical, antimicrobial studies of *P. vietnamensis.*

MATERIAL AND METHODS

Plant Material

Porphyra vietnamensis was collected in July 2012 from Ratnagiri coast, Maharashtra, India. Further taxonomic identification was conducted by Professor B. B. Chaugule at the Department of Botany, Pune University, Pune, and Maharashtra, India. A voucher specimen has been deposited in the herbarium at the Laboratory of Ecology under the voucher specimen number *(Bot*/571/12*)*. The sufficient amount of fresh *P. vietnamensis* sample (100 g) was preserved by glutaraldehyde (3%), and sample was entirely washed before its usage.

Extracts Preparation

The coarsely powdered sample drug of *P. vietnamensis* (200 g) was extracted with ethanol by continuous extraction method using soxhlet apparatus. The aqueous extract was filtered and concentrated to a dry mass using the oven till brownish black colour residue was obtained.

Physico-chemical Analysis

The percentage of ash values, extractive values, carbohydrate, protein, Vitamin C, dietary fibre, lipid content, behaviour with different reagents, Rf values of different extracts and fluorescence analysis were determined according to the official methods prescribed.[16-19]

Preliminary Phytochemical Screening

The screening was carried out by standard procedures,[20] using plants as such in their crude form or in the form of extracts, whereby the resulting principle is used as medicinal agent. It is, therefore, obvious that any study in pharmacognosy must embrace a through consideration of both primary and secondary metabolites derived as a result of biosynthetic pathway. Therefore, the plant material was subjected to preliminary phytochemical screening in order to detect plant constituents. As per procedure, the drug was first subjected to extraction with organic solvents in the increasing order of their polarity (Petroleum ether, Benzene, Chloroform, Acetone, Ethanol and Water). Taking the last drop from thimble on a watch glass and observing residue formation ensures complete extraction by each solvent. It is also ensured that powdered material is completely dried and freed from traces of previous solvents. After which the extract were subjected to qualitative chemical test.[21]

Isolation (Polar/Nonpolar Pigments and Fatty Acids)

For pigment isolation, the dried algae (1 g) were taken and dipped it in sufficient quantity of water for polar and acetone for nonpolar pigments isolation. The material was placed in a mortar and triturate (5 min) with the addition of 1 g of sand and triturate till pink colour. After, centrifuge it (1200 rpm), supernatant was collected and left material was discarded. Fatty acid methyl esters (FAME) were extracted by following the method of Garcia *et al*., 2011.[22] For fatty acid extraction, the algae material extract (1.2 g fresh) was taken out in methanol for soxhlet extraction. On evaporation of the organic solvent under vaccum, obtained residue was fractioned with ethyl acetate and water (EtOAc: H_2O; 1:1 v/v) for 3 times. EtOAc layer on evaporation afforded (0.9 g) of the crude extract which was chromatographic on silica gel column and the column was eluted successfully with N-hexane, N-hexane: Diethyl acetate, ether, chloroform, chloroform: Methanol and finally with methanol by increasing order of polarity (with 2% each). The sample is well-closed and kept in the deep freezer to avoid its fatty acid oxidation.

Chromatography

The solutions (prepared by above) were taken and applied over thin layer chromatography (TLC) plates by application of different solvent system. Acetone: water (4:1), toluene: ethyl acetate (5:5), chloroform: methanol (7:3), acetone: pet. ether (5:5), toluene: acetone (5:5), ether: acetone (5:1), ether: acetone: chloroform (7:5:5), cyclohexane: 2-propanol (8:1). Developed chromatogram was visualised in ultraviolet (UV).

Estimation of Pigments and Fatty Acids

For pigment estimation, sample was placed in the large bottles filled with water. Few drops of chloroform were added, and it was placed in a warm place (40–45°C) or under UV. The samples were collected after the regular intervals and colour of the sample was observed. Samples were centrifuged at 1200 rpm the supernatant were collected, and intensity of colour was measured with the UV.

For confirmation of polyunsaturated fatty acid (PUFA) Maxipa (Eicosapentaenoic acid and Docosahexaenoic acid) capsules was purchased from the market and solubility was checked in the following solvents; cyclohexane, DMSO,

toluene, chloroform and methanol. TLC was performed using 7.5 × 2.5 TLC plates.

The composition of fatty acid was analysed using Bruker 436 GC Gas chromatograph. A 1 µl of aliquot of methylated sample was loaded on a column at 230°C with split ratio 1:30 with nitrogen gas as a carrier gas. Peaks were determined with a flame ionisation detector at 230°C the temperature was programmed to ramp from 50°C for 30 min to 230°C. Fatty acids were identified by comparing the retention time obtained to that of known standard (FAME mixture), Restek.

Mineral Analysis

Mineral content was estimated with the help of Inductively Coupled Plasma Atomic Emission Spectroscopy. Among the minerals of Na, S, Mg, K, C, P, Cu, I, Mn, Zn, Ni, Cr, Mo, As, Cd, Pb, Cr, Ni were estimated. The minerals were identified by comparing with Merck multi element std. that is,-1.11355.0100.

Biological Assay

The agar diffusion test[23] was used to investigate antimicrobial effects of different fractions of *Porphyra* species. In this method, plates containing agar media (10 ml) and Sabouraud's agar medium were overlaid with inoculated stock solution of bacteria/fungi (10 ml). The equidistant holes were made in the agar, then added volume of each sample (1 ml; 200–300 µg/ml) was pipetted into the agar wells and standard compounds (positive control; 100 µg/ml) and ethanol as negative control were used. After 24 h, and 3–5 days no growth (inhibition zones) around the holes in the bacterial lawn were measured. A positive result was defined as an inhibition zone (≥9 mm or more) around the holes indicated the presence of the antibacterial substance in the tested samples.

RESULTS AND DISCUSSION

Pharmacognostic Studies

Porphyra was analysed for moisture content, ash, extractive values, lipid, carbohydrate, protein content, phytochemical test, TLC behaviour, fluorescence behaviour of different extracts with different reagents from *Porphyra* species. Several researchers found that other components of *Porphyra* (e.g. amino acids, soluble fibre, fatty acids and vitamins) play an important role in the human diet; this study focuses on a range of general nutritional components. For all analyses (with the exception of moisture content), algal material was dried in an oven at 100°C for 24 h and ground into a fine powder prior to use. Each analysis was replicated 3 times.

The total Ash values (0.51) gives an idea about the amount of organic and inorganic constituents present in the samples whereas foaming index, swelling index, loss on drying (7.80), pH (6.26) and electrical conductivity (6.90) gives an overview of amount of saponin, moisture content, nature of salts, ions present and extractive values in the sample of *Porphyra* sp. Significant amount of lipid, carbohydrate, dietary fibre, Vitamin C and protein was found as illustrated in [Table 1]. Results showed the maximum extractive value of the aqueous fraction since it contains high molecular weight sulphated polysaccharides and other water soluble components such as polar pigments. Then ethyl acetate, chloroform and methanol have significant extractive value due to unsaturated fatty acids and other soluble compound in the respective solvent. Similarly, other solvents contain certain high molecular weight compounds and more number of compounds which were responsible for their respective extractive values.

Phytochemical screening proven that *Porphyra* fractions has no alkaloid, essential oil, coumarins and steroidal content whereas water soluble fraction has given the positive results against test applied for resins, carbohydrates, pigments, glycosides, tannins, gums and mucilages, amino acids, saponin and phenol. On the other side, alcoholic fractions have also passed the test of amino acids, pigments and tannins as depicted in Table 2.

Thin Layer Chromatography Analysis

The purpose behind performing the TLC of each fraction was to detect the number of components in each solvent extract, and Rf values [Table 3] were determined to fix the position of the components on the plate. The fluorescence of *Porphyra* extracts solvent was determined because

Table 1: Physicochemical observations of *Porphyra* sp.

Parameters	Values
Total ash	0.551*
Acid insoluble ash	0.377*
Water soluble ash	0.174*
Loss on drying at 110°C	7.80*
Foaming index	2.28*
Swelling index	0.3*
pH (2% w/v)	6.26
Electrical conductivity	6.90
Carbohydrate	60.9±0.26*
Dietary fibre	73.56*
Vitamin C	4.72*
Lipid	1.7±0*
Moisture	21.5±0.08*
Extractive values	
Cyclohexane and CCl_4	0.014*
Petroleum ether and chloroform	0.010*
Acetone and benzene	0.012*
Ethanol and methanol	0.030*
Ethyl acetate and water	0.215*

*Values are expressed in percentage

it contains several photo-protective and UV-absorbing components under visible, short and long UV light [Table 4]. It was observed that besides ether fraction each fraction contains certain compounds which showed fluorescence under UV and visible light. The ether fraction does not show any fluorescence because it contains the maximum amount of fatty acids. Furthermore, the behaviour with different reagents were evaluated to study its reaction and stability with that reagent. Stability studies, e.g. solubility, hydrolysis and degradation were performed to interpret the amount of degradation by noticing some visual characters such as brown colour showed complete degradation of the components [Table 5].

Table 2: Phytochemical analysis of petroleum ether, aqueous and alcohol extract of *Porphyra* sp.

Chemical test	Extract			Reference
	Ether	alcoholic	aqueous	
Alkaloids	Absent	Absent	Absent	[24]
Glycosides	Absent	Absent	Present	[25]
Carbohydrates	Absent	Absent	Present	[26]
Resins	Absent	Absent	Present	[25]
Tannins	Absent	Present	Present	[27]
Steroids	Absent	Absent	Absent	[28]
Saponins	Present	Absent	Present	[25]
Fixed oil	Present	Present	Absent	[24]
Phenols	Absent	Present	Present	[24]
Terpenoids	Absent	Present	Absent	[28]
Essential oils	Absent	Absent	Absent	[25]
Coumarins	Absent	Absent	Absent	[26]
Pigments	Absent	Absent	Present	
Gums and mucilage's	Absent	Absent	Present	[25]
Amino acids	Absent	Present	Present	[28]

Table 3: TLC behaviors of different extracts from *Porphyra* sp.

Extracts	Solvent system	Spots	Rf values
Cyclohexane	Tol: EtoAC: Acetic acid (6.5:3:1)	2	0.67, 0.73
CCl_4		4	0.35, 0.46, 0.68, 0.81
Petroleum ether		2	0.58, 0.84
Chloroform		7	0.19, 0.31, 0.40, 0.51, 0.60, 0.73, 0.83
Acetone		6	0.13, 0.34, 0.42, 0.48, 0.50, 0.58
Ethanol		5	0.31, 0.50, 0.56, 0.68, 0.81
Methanol		8	0.21, 0.31, 0.41, 0.52, 0.63, 0.75, 0.81, 0.94
Ethyl acetate		7	0.14, 0.32, 0.40, 0.50, 0.56, 0.82, 0.93
Water		4	0.12, 0.26, 0.31, 0.67

TLC – Thin layer chromatography

Table 4: Fluorescence behaviors of different extracts *Porphyra* sp.

Extract	Visible light	Short UV light	Long UV light
Cyclohexane	Light buff	Colourless	Colourless
CCl_4	Light buff	Light olivaceous buff	Colourless
Petroleum ether	Colourless	Colourless	Colourless
Chloroform	Honey	Light greenish yellow	Light pale luteous
Acetone	Light buff	Light olivaceous buff	Light pale luteous
Ethanol	Light honey	Colourless	Light pale luteous
Methanol	Light honey	Olivaceous buff	Light greenish yellow
Ethyl acetate	Light buff	Light olivaceous buff	Light pale luteous
Water	Brick	Dark citrine	Dark greenish yellow
Benzene	Light honey	Olivaceous buff	Light pale luteous

Mineral Analysis

Seaweeds are a rich source of minerals, especially macro and micronutrients necessary for human nutrition; however, the nutritional properties of seaweeds are usually determined from their biochemical composition alone viz. proteins, carbohydrates, vitamins, amino acids, etc. The mineral fraction of some seaweed even accounts for up to 40% of dry matter; however, in some cases, the mineral content of the seaweeds is recorded even higher than that of land plants and animal products. The genus *Porphyra*, traditionally known as Nori in Japan, Kim in Korea and Zicai in China, is a popular delicacy, due to its rich content of protein, vitamins, minerals and dietary fibers. This alga is also reported to contain iodine, bioactive substances and anti-fungal compounds of therapeutic value. Evaluation of minerals in any edible seaweed is important from both the nutritional and the toxicological point of view. However, very little is known about the mineral composition of *P. vietnamensis*. In view of the preference of *Porphyra* diet over other seaweeds, mineral composition of *P. vietnamensis* was determined to use as a potential food ingredient. In the present study, the sample was analysed for their macro and micro nutrients. Atomic absorption data revealed the presence of a significant amount of Mg, S, Ca, P, Na and K in *P. vietnamensis* as mentioned in Table 6.

Isolation and Detection of Pigments

Porphyra contains high amount of polar and nonpolar pigments which were detected by several UV and TLC methods. Several solvent systems were firstly used in which different bands were separated and clearly detected. Among all the solvent system toluene and acetone (5:5) show best results for nonpolar pigments Rf values [Table 7]. This solvent system possesses rose colour of phyco-erytherin with a strong orange fluorescence by absorbing blue-green light. As shown in the above observations the release of phyco-erytherin was more influenced by UV in comparison to sunlight [Table 8]. The observed range of observed readings was somewhat similar to the reported range which indicates that these pigments were present in *Porphyra* with varying amount.

Fatty Acids Determination

Porphyra sp. contains high amount of fatty acids especially PUFA. So here attempt was made to detect PUFA by TLC.

For this, a standard was purchased from the market which contains docosahexaenoic acid, eicosapentaenoic acid, linolenic acid. It was observed that Maxipa was soluble in cyclohexane, DMSO and Toluene [Table 9]. The Rf value of the sample is 0.50, and 0.57 (two brown bands were appeared in chromatogram as like the standard marker Rf values (0.48 and 0.56). Fatty acid analysis proven that *Porphyra* sp. contains a significant amount of methyl palmitate [Table 10].

Table 5: Behavior of *Porphyra* sp. with different reagents

Reagent	Observations
Concentrated H_2SO_4	Particles float on surface, brown in colour
Concentrated HCl	Particles float on surface, on shaking particles remains suspended, brown in colour
Concentrated HNO_3	Particles float on surface, on shaking particles remains suspended, rust in colour, thick consistency
Acetic acid	Particles float on surface, slowly settle, on shaking particles remains suspended, brown in colour
NaOH and KOH	Particles float on surface, slowly settle, on shaking particles remains suspended, amber in colour
$FeCl_2$	Particles float on surface, amber in colour, on shaking particles remains suspended
Iodine	Particles float on surface, slowly settle, on shaking particles remains suspended, yellowish brown in colour
Organoleptic	Colour: dark green, odourless, Taste: tasteless, Touch: soft

Table 6: Mineral analysis of *Porphyra vietnamensis*

Minerals	Quantity (mg/100 g)
N	1.05±0.01
P_2O_5	266.77±0.95
K_2O	207.78±2.67
Ca	740.13±7.83
Zn	76.67±0.96
Cu	0.97±0.02
Fe	9.52±0.11
Mn	81.1±0.26
Na	382.76±2.4
S	1750.7±28.21
Mg	629.89±9.49
I	57.98±2.66
Mo	0.09±0.01
As	0.18±0.015
Hg	ND
Cd	0.39±0.01
Pb	0.26±0.01
Cr	0.25±0.01
Ni	0.27±0.01

All values are expressed in mg/100 g

Table 7: Rf values of different pigments

Pigments	Theoretical Rf	Observed Rf
Chlorophyll a	0.68	0.61
Chlorophyll b	0.54	NA
Chlorophyll b	0.03	NA
β-carotene	0.94	0.86
Fucoxanthine	0.51	0.49
Lutein	0.43	0.38
Neoxanthine	0.22	NA
Violaxanthine	0.08	NA

NA – Not appeared

Antimicrobial Study

Marine organism's antimicrobial defences are largely uncharacterised, although from a small number of studies it appears that chemical defences may improve host resistance. Atypical millilitre of seawater contains 103 fungal cells, 106 bacteria and 107 viruses, including pathogens that cause widespread mortalities and microbes that initiate fouling of host surfaces. *Porphyra* is a macroalgae which contains several unknown compounds that protect the macroalga from microbial attack different those usually occurs in the sea. Several compounds such as porphyran (sulphated polysaccharide), is now a day's gaining more researches

Table 8: Pigments UV analyses

Types of pigments	λ_{max}	
	Reported (nm)	Observed (nm)
Polar pigments		
Phycoerythrin	650	610-650
Phycocyanin	615	
Allophycocyanin	650	
Non-polar		
Chlorophyll	557	538

UV – Ultraviolet

Table 9: Solubility of Maxipa

Solvents used	Solubility
Cyclohexane	Soluble
DMSO	Soluble
Toluene	Soluble
Chloroform	Soluble
Methanol	Insoluble
Water	Insoluble

Table 10: Fatty acid composition of *Porphyra vietnamensis*

Fatty acids	Quantity in percentage
Methyl palmitate	71.647
Methyl palmitoleate	-
Stearate	3.189
Oleate	5.667
Linoleate	1.893
Linolenate	-
Arachidate	1.335
11-eicosenoate	0.789
11,14-eicosadianoate	1.37
Behenate	18.087
Erucate	1.501
Docosadienoate	-
Lignocerate	-
Nervonate	-

in both applied and basic sciences. Methanolic extract of *Porphyra* contains *Porphyra*-334, several fatty acids (saturated and unsaturated) and several other unknown substances that are responsible for antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as fungi.[28-30] The polysaccharide fraction consists of several macromolecules, having broad-spectrum antimicrobial activity against bacteria and fungi. In conclusion, the two potent samples of *Porphyra* species presented a wide spectrum of antimicrobial activity. It constitutes a potential source of novel antimicrobial agents because of a resurgence of interest in aromatherapy,[31] particularly for the local population, which need cheap drug.

In the present study, the concentration of *Porphyra* sp. (200–300 µg/ml) was effective against all microbes but at low concentration (100 µg/ml); it is ineffective against *E. coli, Flavum oxysporum, Pencillium* and *A. fumigatus.* The samples showed better inhibition against *S. aureus, B. subtilis, Penicillium, A. niger, A. flavus* and *P. aeruginosa*. Whereas, *Porphyra* extract was not effective against *Penicillium* in comparison to the porphyran. The extract is effective against *E. coli* in comparison to the porphyran whereas porphyran show better activity *B. subtilis* in comparison to the *Porphyra* extract at 300 µl/ml. While studying the effectiveness against fungus *Porphyra* extract is effective against *Flavum oxysporum* and *A. fumigatus* in comparison to the porphyran while porphyran is more effective against *A. niger, A. flavus* and *Penicillium in* comparison to *Porphyra* extract at 300 µl/ml [Table 11].

CONCLUSION

Seaweeds are potential renewable resources of the marine environment which could be termed as the future promising drug. Seaweed has been a source of food and medicine in the orient as well as in the west, since ancient times. *Porphyra* (Nori) widely distributed in India, Pakistan, Yemen, China, Taiwan, Vietnam and Hawaiian Islands. The whole compositional analysis was performed to explore the therapeutic potential of *P. vietnamensis*. From the above pharmacognostical results, it has been concluded that Indian *Porphyra* contains various active components. Experimental data have been also proven that *Porphyra* is rich in several fatty acids, fibres, amino acids, pigments and carbohydrates as well. Antimicrobial potential (against bacterial and fungal strains) was also analysed by simultaneous evaluation of two fractions, *Porphyra* (alcoholic) and porphyran, sulphated polysaccharide (water-soluble fraction) of *P. vietnamensis*. It has been proven that the alcoholic extract is much active then aqueous extract. Furthermore, water soluble fraction has shown much better resistance against different fungal strains in comparison to bacterial strains.

Table 11: *Porphyra* alcoholic and aqueous extract antimicrobial activity*

Microorganism	PE (300 µg/ml)	Porphyran (300 µg/ml)
Bacteria		
Escherichia coli	1.5±0.22	0.9±0.11
Staphylococcus aureus	2.0±0.22	1.0±0.21
Bacillus subtilis	1.7±0.33	0.6±0.16
Pseudomonas aeruginosa	3.5±0.74	1.3±0.41
Fungi		
Flavum oxysporum	0.8±0.25	0.5±0.17
Pencillium	0.8±0.19	1.4±0.19
Aspergillus niger	1.6±0.21	1.8±0.28
Aspergillus flavus	1.5±0.57	1.8±0.31
Aspergillus fumigatus	1.5±0.43	0.9±0.66

*Data are mean value of triplicate determinations±SD. SD – Standard deviation

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