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Antifungal Activity of Organic and Aqueous Extractions in *Plakobranhus ocellatus* (Gastropoda: Sacoglossa) against *Candida albicans*, *C. parapsilosis* and *C. glabrata*

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ABSTRACT

In the recent years, there has been a growing concern about the effects of marine natural products on human pathogens. It is determined that sacoglossan mollusks have special natural products which attracted more attempts and attentions. For the first time, in the present study antifungal activity of *Plakobranhus ocellatus* collected from the southern waters of Qeshm Island, the Persian Gulf has been investigated. Acetonitrile, ethanol and sodium phosphate buffer as organic and aqueous extracts were prepared from the whole body tissue and tested through seven concentrations against *Candida albicans*, *C. parapsilosis* and *C. glabrata*. The extractions with more responses against fungal strains were identified. Maximum and minimum inhibition zones were estimated for acetonitrile and sodium phosphate buffer extracts against *C. parapsilosis* and *C. albicans*, respectively. The organic extracts were the best solvents for extracting antifungal compounds. Acetonitrile extractions showed the best antifungal activity and *C. glabrata* was the most resistant strain against all *P. ocellatus* antifungal extractions.

Key words: Antifungal activity, *Plakobranhus ocellatus*, sacoglossan opisthobranch, *Candida*, The Persian Gulf

INTRODUCTION

In marine environment, animals have their own specific protection strategy. It varies from countershading in octopuses to swelling in puffer fishes. Some gastropods have hard shell as a main defense cover or are shell-less animals without mechanical defenses but they improve their chemical guards.

It is argued that most of sacoglossans, animals with no hard shell or any physical defense tactics have evolved chemical defense mechanisms by producing bioactive metabolites (Pawlik *et al.*, 1988; Rajaganapathi *et al.*, 2001; Vennila *et al.*, 2010; Bano and Ayub, 2012). There are a lot of studies on effects of bioactive compounds taken from these chemicals against human pathogens such as fungal and bacterial infections, tumors, alzheimer and cancers (Kisugi *et al.*, 1987; Zandi *et al.*, 2007).

As opportunistic commensal, *Candida* is the main factor of serious infections such as vulvovaginal candidiasis (VVC) which can affect 75% of adult women during their life (Sharanappa and Vidyasagar, 2013). It can also increase the chance of cutaneous infections,

vaginitis, intestinal and systemic infections because of immunosuppressive treatments, long-term catheterization, use of broad-spectrum antibiotics and longer survival of immunologically compromised individual infections in last decades (Molero *et al.*, 2010). Opportunistic fungal strains such as *C. albicans* as more frequent species isolated from oral infections (Kantarcioglu and Yucel, 2002) and other *Candida* species with direct effects can cause systemic infections in immunosuppressant patients (such as AIDS, cancer patients and solid-organ transplant recipients). *Candida* strains also are introduced as fourth effective factor in hematological infections for patients hospitalized and responsible for about 40% of death (Anaissie *et al.*, 2003).

Despite many studies have been carried out on the sacoglossans (Evertsen *et al.*, 2007; Handeler *et al.*, 2009; Shenai-Tirodkar *et al.*, 2012), the lack of studies on natural products and bioactive compounds of *Plakobranthus ocellatus* is felt and almost no study has been done on antifungal properties. In the present study the effects of some bioactive extractions in *P. ocellatus* against three *Candida* species (*C. albicans*, *C. parapsilosis* and *C. glabrata*) were investigated for the first time.

MATERIALS AND METHODS

Sampling: Samples of *P. ocellatus* were collected from the southern waters of Qeshm Island in the Persian Gulf (26°90' N, 56°16' E) through diving at 5-15 m depth in 2014. All samples were small in size, about 2-3 centimeters long. The individuals were gently collected by hand picking and were quickly stored in nitrogen liquid tank for transporting to laboratory. They were placed at -70°C in freezer to keep for long time preservation.

Extraction of bioactive compounds: There is no direct way to take the extract out from animals and this is maybe because of differences between chemical structures in different animals. Some later methods were also examined to make extractions (Tsukamoto *et al.*, 2005; Bano and Ayub, 2012) but finally we achieved our specific way.

Extracts were taken out by using organic solvents such as acetonitrile 80% (C₂H₃N-Chem Lab) and ethanol 100% (C₂H₅OH-Chem Lab) for non-protein compounds as polar and non-polar compounds and aqueous solvent sodium phosphate buffer (NaH₂PO₄.2H₂O; NaOH-Merk) for antimicrobial peptide and protein compounds. It is important that the sodium phosphate buffer should be kept at 7.2 pH and added to the aqueous extract for taking the neutral protein compounds out.

In the present study the whole body tissue of *P. ocellatus* was investigated. Three grams of samples were well-mixed by homogenizer to break down the cells membrane at room temperature followed by: At first homogenizing in 12700 rpm for 2 min, relaxation for 30 sec and re-homogenizing in 19700 rpm for 2 min. The mixture matter was weighted again and filled by the solvents five times of the weighted amount. Then they were homogenized for 2 min in 5800 rpm. After that, the extracts were shaken at 40°C, 90 rpm for 24 h. Aqueous extractions were taken through the same homogenizing stages but kept in 42°C through all period and the extract was shaken at 4°C, 90 rpm for 2.5 h.

All organic and aqueous extractions then were centrifuged at 4°C in 4000 rpm for 10 min, the supernatant were separated, the remaining matters were weighted and filled by the solvents two times of the weighted amount. The mixture was centrifuged again and all supernatants were freeze-dried for 18 h. The dried crude extractions were kept in -20°C for long time preservation.

Antifungal activity of *Plakobanchus ocellatus* extracts: The antifungal activity of *P. ocellatus* crude extracts was evaluated using Sabouraud Dextrose Agar (SDA) well diffusion methods (Stepanovic *et al.*, 2003; Periyasamy *et al.*, 2012) against *Candida albicans* (Persian Type Culture Collection (PTCC): 5027 ATCC 10231), *C. parapsilosis* (PTCC: 5297 DSM 11226) and *C. glabrata* (Iranian Biological Resource Center (IBRC-M): 30005). All *Candida* strains were unloaded from lyophilized ampoules to SDA media after dissolving in 0.5 mL of distilled water and cultivated by linear cultivation method. All stages were passed with sterile conditions. Colonies of each fungal strain was inoculated on the SDA, separately and incubated at 37°C for 24 h. After two passages, the materials of plates were dissolved in 1 mL of distilled water and well-mixed by pipetting. Then, 100 μ L of each solution was added to the new SDA plates. The plates were rotated to make even distribution of the culture and allowed to solidify. After solidification, numbers of wells were made by using sterile borer of 6 mm diameter (Bano and Ayub, 2012).

To find the effective ranges of crude concentrations, seven different volumes of extracts were categorized (300, 600, 900, 1500, 2000, 2500 and 3000 μ g). Organic crude extracts were dissolved in 30 μ L Dimethyl Sulfoxide (DMSO) and aqueous crude extracts were dissolved in 30 μ L distilled water. All extractions were added to the labeled wells in their respective wells. Then loaded plates were incubated at 37°C for 24 h.

Control treatment: Three antibiotics drugs Ketoconazole, Fluconazole and Nystatin each with 30 μ g mL⁻¹ were prepared to use as positive controls, DMSO and distilled water were also performed as negative controls.

Statistical analysis: Results included the diameters of antifungal activity zone were expressed as Mean \pm SD. One way ANOVA followed by Duncans multiple range test was used to analyze data, using SPSS windows version 16.0 with $p < 0.05$ were considered statistically significant.

RESULTS

Antifungal activity of three extracts was determined using the well diffusion method with three human pathogens *C. albicans*, *C. parapsilosis* and *C. glabrata*. Study on all impressive concentrations of crude extracts of *P. ocellatus* showed that in some concentrations the response of the extracts against *Candida* strains was more than other concentrations. The crude extracts at 1500, 3000, 600 μ g mL⁻¹ for *C. albicans* and *C. parapsilosis* and 900, 1500 and 3000 μ g mL⁻¹ for *C. glabrata* demonstrated more responses against studied fungi (Fig. 1). Mean values were significantly different between concentrations ($p < 0.05$).

Results of extracts antifungal activity tests against *Candida albicans* were revealed in all three extractions but not in all concentrations. *Candida parapsilosis* was shown resistance against almost all concentrations of sodium buffer phosphate except of 3000 μ g mL⁻¹ with the minimum amount of inhibition zone made between all crude extract concentrations against this fungus (Table 1). *Candida glabrata* was more resistant fungi among all three *Candida* strains, totally. Ethanol crude extract was the weakest compound with only one occurrence at 2500 μ g mL⁻¹ and showed less antifungal activity against *C. glabrata* (Table 1). Larger amounts of inhibitory zones were measured between 30.18 and 25.12 mm at five records. Three of these larger zones were made by acetonitrile crude extracts. Acetonitrile extracts showed stronger antifungal activity against *Candida albicans*, *C. parapsilosis* and *C. glabrata*, with inhibition zones of 30.18, 35.12 and 34.12 mm, respectively. Nystatin and fluconazole the two antibiotics

tested as positive controls against *Candida* strains showed stronger antifungal activity against *C. glabrata* and *C. albicans* with inhibition zones of 16.33 mm.

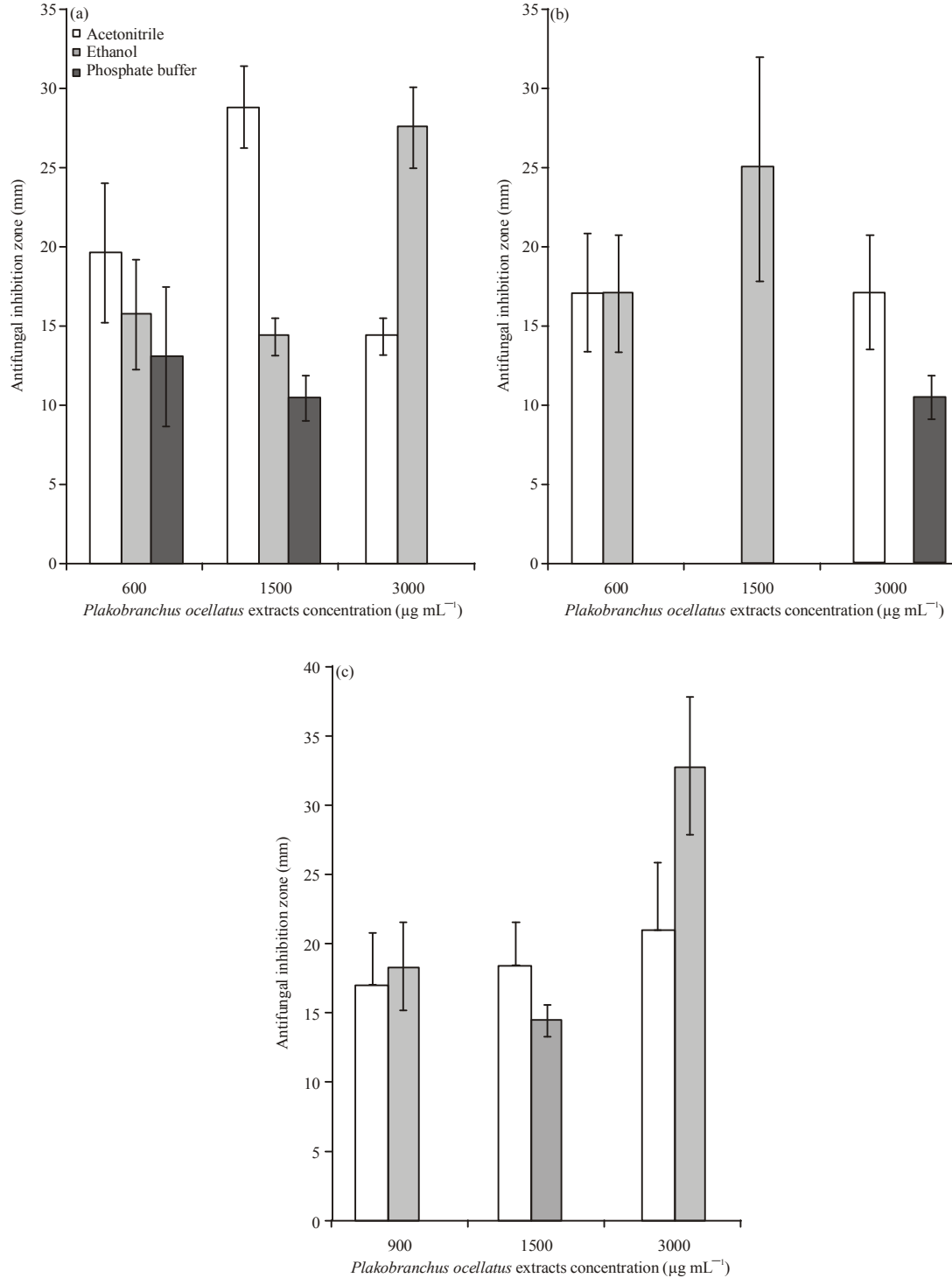


Fig. 1(a-c): Differences between most frequent crude extracts antifungal activity against *Candida* species, (a) *Candida albicans*, (b) *Candida parapsilosis* and (c) *Candida glabrata*

Table 1: Antifungal inhibition zone of crude extracts concentration against three *Candida* species

Crude concentration ($\mu\text{g mL}^{-1}$)	Antifungal inhibition zone (mm)						
	300	600	900	1500	2000	2500	3000
<i>C. albicans</i>							
Acetonitrile	30.18 \pm 8.49	19.68 \pm 4.42	-	28.87 \pm 2.61	24.93 \pm 6.96	15.75 \pm 3.46	14.43 \pm 1.16
Ethanol	-	15.75 \pm 3.46	-	14.43 \pm 1.16	-	-	27.56 \pm 2.57
SPB	-	13.12 \pm 4.39	-	10.50 \pm 1.4	9.18 \pm 2.24*	-	-
<i>C. parapsilosis</i>							
Acetonitrile	-	17.06 \pm 3.74	-	-	35.12 \pm 5.76**	14.43 \pm 1.16	17.06 \pm 3.74
Ethanol	31.5 \pm 5.43	17.06 \pm 3.74	26.25 \pm 2.72	24.93 \pm 6.96	-	-	-
SPB	-	-	-	-	-	-	10.50 \pm 1.4
<i>C. glabrata</i>							
Acetonitrile	34.12 \pm 4.93	-	17.06 \pm 3.74	18.37 \pm 3.17	-	-	21.00 \pm 4.79
Ethanol	-	-	-	-	-	14.43 \pm 1.16	-
SPB	-	-	18.37 \pm 3.17	14.43 \pm 1.16	-	17.06 \pm 3.74	32.81 \pm 4.93

Antifungal activity inhibition zone Mean \pm SD, - : No antifungal activity, SPB: Sodium phosphate buffer, *Minimum,**Maximum

DISCUSSION

There is a direct relation between increasing in immunosuppressant diseases and fungal infections in last decades. There is also a tangible growing interest in marine natural products or marine secondary metabolites (Periyasamy *et al.*, 2012).

Marine biota has traditional positions in medicine and also referred as a major recourse of pharmacological investigations of marine organisms (Fenical, 1996). Opisthobranch mollusks are more putative and interesting marine animals to extract bioactive compounds (Williams *et al.*, 1986; Sadhasivam *et al.*, 2013; Ramya *et al.*, 2014). They are all almost shell-less benthic animals with inability to escape from predators, competitors fighting for escape with slow moving without any physical defenses but have some effective chemical answers.

Sacoglossan gastropods are herbivorous and associated with green algae. They are often well camouflaged with the help of colors being derived from associated chloroplasts of algae which they feed (Shenai-Tirodkar *et al.*, 2012). *Elysia*, a genus included in family Plakobranhidae reported to accumulates bioactive compounds (Ashour *et al.*, 2006) which sequestered from green algae with strong antifungal effects (Shilabin *et al.*, 2007). *Plakobranhus ocellatus* (van Hasselt, 1824) also stores huge numbers of bright green chloroplasts in ridges hidden from view beneath the parapodial flaps, under a symbiotic relationship. It is estimated that *P. ocellatus* demonstrated long-term retention of chloroplasts as endosymbiosis (Evertsen *et al.*, 2007; Handeler *et al.*, 2009), on the other hand it is expressed *P. ocellatus* is in sitster taxon with all *Elysia*-species (Handeler and Wagele, 2007). The two issues which evaluate the originality of bioactive compounds but it was found that *P. ocellatus* are able to produce their own defensive compounds (Cimino and Gavagnin, 2006).

For the first time, the potential of antifungal activity in extracted compounds of *P. ocellatus* a sacoglossans collected from the Persian Gulf were investigated. Acetonitrile, ethanol and sodium phosphate buffer were used as organic and aqueous solvents, respectively to extract bioactive compounds against three fungal pathogens of *Candida* strains. Antifungal activity was recorded in all crude extract with different values. It was clearly detected; both organic and aqueous extractions of *P. ocellatus* had significant antifungal activity against pathogenic *C. albicans*. In the case of other fungi, ethanolic extracts were showed better antifungal activity against *C. parapsilosis*, whereas it was the weakest against *C. glabrata*. Conversely, the aqueous extracts (compounds extracted by sodium phosphate buffer) were the weakest against *C. parapsilosis* and

showed better conditions against *C. glabrata* (Table 1). Maximum and minimum inhibitory zones were observed for acetonitrile extract against *C. parapsilosis* and phosphate buffer extract against *C. albicans*, both in 2000 $\mu\text{g mL}^{-1}$.

In comparison between the extracts concentrations with more responses against fungal strains, results showed that the inhibition zone of acetonitrile and ethanol extracts were measured against all three *Candida* species while sodium phosphate buffer was almost inactive against *C. parapsilosis* and *C. glabrata* (Fig. 1). Antifungal activity in *Elysia grandifolia* was investigated against two plant pathogens and results showed strong inhibition zones about 16-24 mm (Ashour *et al.*, 2006). The stronger antifungal activity were recorded as 30.18, 35.12 and 34.12 mm all belonged to acetonitrile extractions of *P. ocellatus* while the stronger antibiotic activity were recorded as 16.67 mm for fluconazole and ketoconazole against *C. albicans*, *C. parapsilosis* and 16.33 for nystatin, against *C. glabrata*. *Candida glabrata* is a resistant strain to all azoles antifungal drugs and develop resistance during therapy with fluconazole (Silva *et al.*, 2012). According to the present study results, *C. glabrata* was the most resistant fungi against antifungal extractions of *P. ocellatus* and the lowest amounts of antibiotic drugs tests was also recorded for this *Candida* strain.

Based on the solvents used in present study, it supposed that both protein and non-protein compounds were extracted and showed their effects on fungal pathogens. Temperature is the main environmental factor through extractions providing. In aqueous extracts, the probability of the existence of active protein compounds was high because the temperature fixes at 4°C in all stages, whereas, in organic extractions which there is no temperature limitation and it can reach to about 40°C in the process.

Antifungal activity against three studied *Candida* strains showed that the extract (especially organic extractions) of *P. ocellatus* can introduced as potent to product antifungal compounds against fungal infections. This finding is very significant and roles as a base for further studies to determine of new potent drugs against these dangerous pathogens. In the present study, the crude extraction was carried out as antifungal compounds. Results suggest that the purified extracts and specific metabolites of *P. ocellatus* as important sources of antifungal bioactive compounds.

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REFERENCES

- Anaissie, E.J., M.R. McGinnis and M.A. Pfaller, 2003. *Clinical Mycology*. 2nd Edn., Churchill Livingstone, Philadelphia.
- Ashour, M., R. Edrada, R. Ebel, V. Wray and W. Watjen *et al.*, 2006. Kahalalide derivatives from the Indian sacoglossan mollusk *Elysia grandifolia*. *J. Nat. Prod.*, 69: 1547-1553.
- Bano, A. and Z. Ayub, 2012. Antibacterial and antifungal activity in three species of *Siphonaria* (Gastropoda: Pulmonata) collected from rocky ledge of Mubarak Village, Karachi. *Pak. J. Zoo*, 44: 1493-1497.
- Cimino, G. and M. Gavagnin, 2006. *Molluscs: From Chemo-ecological Study to Biotechnological Application (Progress in Molecular and Subcellular Biology/Marine Molecular Biotechnology)*. Springer, USA., ISBN-13: 978-3540308799, Pages: 387.

- Evertsen, J., I. Burghardt, G. Johnsen and H. Wagele, 2007. Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean. *Mar. Biol.*, 151: 2159-2166.
- Fenical, W., 1996. Marine biodiversity and the medicine cabinet the status of new drugs from marine organisms. *Oceanography*, 8: 23-27.
- Handeler, K. and H. Wagele, 2007. Preliminary study on molecular phylogeny of *Sacoglossa* and a compilation of their food organisms. *Bonn. Zool. Beitr.*, 55: 231-254.
- Handeler, K., Y.P. Grzybowski, P.J. Krug and H. Wagele, 2009. Functional chloroplasts in metazoan cells: A unique evolutionary strategy in animal life. *Front Zool.*, Vol. 6
- Kantarcioğlu, A.S. and A. Yücel, 2002. The presence of fluconazole-resistant *Candida dubliniensis* strains among *Candida albicans* isolates from immunocompromised or otherwise debilitated HIV-negative Turkish patients. *Revista Iberoamericana de Micología*, 19: 44-48.
- Kisugi, J., H. Kamiya and M. Yamazaki, 1987. Purification and characterization of aplysinin E, an antitumor factor from sea hare eggs. *Cancer Res.*, 47: 5649-5653.
- Molero, G., R. Diez-Orejas, F. Navarro-García, L. Monteoliva and J. Pla *et al.*, 2010. *Candida albicans*: Genetics, dimorphism and pathogenicity. *Int. Microbiol.*, 1: 95-106.
- Pawlik, J.R., M.R. Kernan, T.F. Molinski, M.K. Harper and D.J. Faulkner, 1988. Defensive chemicals of the Spanish dancer nudibranch *Hexabranhus sanguineus* and its egg ribbons: Macrolides derived from a sponge diet. *J. Exp. Mar. Biol. Ecol.*, 119: 99-109.
- Periyasamy, N., M. Srinivasan and S. Balakrishnan, 2012. Antimicrobial activities of the tissue extracts of *Babylonia spirata* Linnaeus, 1758 (Mollusca: Gastropoda) from Thazhanguda, Southeast coast of India. *Asian Pac. J. Trop. Biomed.*, 2: 36-40.
- Rajaganapathi, J., K. Rajagopal and J.P. Edward, 2001. Antifungal and cytotoxic effects of methanol extracts of three marine molluscs. *Indian J. Exp. Biol.*, 39: 85-86.
- Ramya, M.S., S. Ravichandran and R. Anbuhezian, 2014. *In vitro* antioxidant properties of marine Sea Slug *Armina babai* from Pazhayaar, Southeast coast of India. *Int. J. Nat. Prod. Res.*, 4: 105-109.
- Sadhasivam, G., A. Muthuvel, W.M. Vitthal, A. Pachaiyappan, M. Kumar and B. Thangavel, 2013. *In vitro* antibacterial, alpha-amylase inhibition potential of three nudibranchs extracts from South East coast of India. *J. Coastal Life Med.*, 1: 186-192.
- Sharanappa, R. and G.M. Vidyasagar, 2013. Anti-Candida activity of medicinal plants: A review. *Int. J. Pharm. Pharm. Sci.*, 5: 9-16.
- Shenai-Tirodkar, P., N.M. Desai and T.G. Jagtar, 2012. Ecological observations and GC-MS analysis of methanolic extract of sacoglossan *Elysia bangtawaensis* (Swennen). *Int. Quart. J. Life Sci.*, 7: 457-461.
- Shilabin, A.G., N. Kasanah, D.E. Wedge and M.T. Hamann, 2007. Lysosome and HER3 (ErbB3) selective anticancer agent kahalalide F: Semisynthetic modifications and antifungal lead-exploration studies. *J. Med. Chem.*, 50: 4340-4350.
- Silva, S., M. Negri, M. Henriques, R. Oliveira, D.W. Williams and J. Azeredo, 2012. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: Biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.*, 36: 288-305.
- Stepanovic, S., N. Antic, I. Dakic and M. Svabic-Vlahovic, 2003. *In vitro* antimicrobial activity of propolis and synergism between propolis and antimicrobial drugs. *Microbiol. Res.*, 158: 353-357.
- Tsukamoto, S., Y. Yamashita and T. Otha, 2005. New cytotoxic and antibacterial compounds isolated from the Sea Hare, *Aplysia krodai*. *Marine Drugs*, 3: 22-28.

- Vennila, R., S. Kanchana, M. Arumugam and T. Balasubramanian, 2010. Investigation of antimicrobial and plasma coagulation property of some molluscan ink extracts: Gastropods and cephalopods. *Afr. J. Biochem. Res.*, 5: 14-21.
- Williams, D.E., S.W. Ayer and R.J. Andersen, 1986. Diaulsterols A and B from the skin extracts of the dorid nudibranch *Diaulula sandiegensis*. *Canadian J. Chem.*, 64: 1527-1529.
- Zandi, K., M.H. Farsangi, I. Nabipour, M. Soleimani, K.H. Khajeh, R. Hassan Sajedi and S.M. Jafari, 2007. Isolation of a 60 kDa protein with in vitro anticancer activity against human cancer cell lines from the purple fluid of the Persian Gulf sea hare, *Aplysia dactylomela*. *Afr. J. Biotechnol.*, 6: 1280-1283.