Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

Vol. 32 (1) Feb. 2009

A scientific journal published by Universiti Putra Malaysia Press

About the Journal
Pertanika is an international peer-reviewed journal devoted to the publication of original papers, and it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields. Pertanika Journal of Tropical Agricultural Science which began publication in 1978 is a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped Pertanika Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other Pertanika series include Pertanika Journal of Science and Technology (JST) and Pertanika Journal of Social Sciences and Humanities (JSSH).

JTAS is published in **English** and it is open to authors around the world regardless of the nationality. It is currently published two times a year i.e. in **February** and **August**.

Goal of Pertanika
Our goal is to bring the highest quality research to the widest possible audience.

Quality
We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 12 weeks. The elapsed time from submission to publication for the articles averages 5-6 months. JTAS is an international award-winning journal indexed in SCOPUS (Elsevier) and EBSCO.

Future vision
We are continuously improving access to our journal archives, content, and research services. We have the drive to realise exciting new horizons that will benefit not only the academic community, but society itself.

We also have views on the future of our journals. The emergence of the online medium as the predominant vehicle for the 'consumption' and distribution of much academic research will be the ultimate instrument in the dissemination of the research news to our scientists and readers.

Aims and scope
Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include: *agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.*

Editorial Statement
Pertanika is the official journal of Universiti Putra Malaysia. The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

Editorial Office
Pertanika, Research Management Centre (RMC), 4th Floor, Administration Building
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
Tel: +603 8946 6192, 8946 6185 • Fax: +603 8947 2075
E-mail: *ndeeps@admin.upm.edu.my*

Publisher
The UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia
Tel: +603 8946 8855, 8946 8854 • Fax: +603 8941 6172
penerbit@putra.upm.edu.my
URL : *http://penerbit.upm.edu.my*

Editorial Board

Editorial Office

Pertanika, Research Management Centre (RMC), 4th Floor, Administration Building
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
Tel: +603 8946 6192, 8946 6185 • Fax: +603 8947 2075
E-mail: ndeeps@admin.upm.edu.my

Publisher

The UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia
Tel: +603 8946 8855, 8946 8854 • Fax: +603 8941 6172
penerbit@putra.upm.edu.my
URL : http://penerbit.upm.edu.my

Pertanika Journal of Tropical Agricultural Science
Vol. 32(1) Feb. 2009

Contents

Pertanika J. Trop. Agric. Sci. **32(1)**: 1 - 5 (2009) ISSN: 1511-3701

Nitrate, Ammonia and Phosphate Concentrations in the Surface Water of Kuala Gula Bird Sanctuary, West Coast of Peninsular Malaysia

Lomoljo, R.M.*, Ismail, A. and Yap, C.K.
Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
**E-mail: lomoljo@yahoo.com*

ABSTRACT

This study was undertaken to compare the concentrations of nitrate nitrogen (NO_2-N), total ammonia nitrogen (TAN) and soluble reactive phosphorus (SRP) in the surface intertidal waters of Kuala Gula Bird Sanctuary over a four-month period (June to September, 2007). Three sampling stations were established in the Gula river estuary, labelled as Station 1, Station 2 and Station 3. The highest concentrations of SRP (55.92±7.88 µg/L), nitrate-N (85.68±24.33 µg/L) and TAN (85.91±6.54 µg/L) were recorded in the months of June, July and August, respectively whereas, the lowest concentrations of all the nutrients were recorded in September. The highest concentrations of the nutrients observed for the three months (June, July and August) coincided with the planting season of the nearby paddy fields in Kuala Kurau, Kuala Gula, Salinsing and some parts of Bagan Serai. This might indicate contamination of nitrogen and phosphorus nutrients from fertilizer run-off. Therefore, a continuous monitoring, for the content of nutrient in the surface intertidal waters of the bird sanctuary, is recommended to observe any significant changes which may take place in the area. The results of this study would serve as an important baseline information for future reference.

Keywords: Nitrate (NO_3-N), TAN and SRP concentration, surface intertidal water, west coast, Kuala Gula Bird sanctuary, Peninsular Malaysia

INTRODUCTION

Enhanced availability of phosphorus and nitrogen is a worldwide cause for eutrophication of aquatic ecosystems (Pieterse *et al.,* 2002). Anthropogenic sources of nutrients, coupled with modifications to the environment and climate, are now so pervasive that no aquatic system can be considered as truly pristine. Agricultural activities often provide the dominant input of nitrogen, particularly nitrates (Hunt *et al.,* 2004). In the case of phosphates, there is often a more balanced mix of fluxes from agricultural and various effluent sources. Aquatic ecosystems can eutrophicate when the concentrations of nutrients exceed the critical levels, and this can lead to enhanced primary production (increase of alga biomass), enhanced decay of organic materials, a shortage of dissolved oxygen and species redistribution within aquatic ecosystems (Tyrell, 1999). Moreover, the effects of high nitrate and phosphate can increase severe toxic phytoplankton blooms in many near shore waters worldwide. In Malaysian waters, nevertheless, studies on nitrates and phosphates as chemical pollutants in the coastal waters are still very scarce. Among the studies on the nutrient level backgrounds in the Malaysian coastal waters was carried out by Yap *et al.* (2005). Their study suggested that nitrate contamination, in the coastal waters of the Straits of Malacca, is not serious although further monitoring has to be undertaken.

Kuala Gula is an important bird sanctuary in Malaysia and the Asian region. It serves as a stop-over site for migratory shorebirds during annual migrations and is along one of the major

Received: 13 May 2008
Accepted: 21 November 2008
*Corresponding Author

migratory routes between Asia and Australasia (Pepping *et al.,* 1999; Lane and Mundkur, 1992; Riak *et al.,* 2002; Riak *et al.,* 2003a; Riak *et al.,* 2003b; Parish and Wells, 1984; Edward *et al.,* 1986). Shorebirds are important predators of macrobenthos such as crabs, shrimps and other bivalves in coastal areas, specifically along the coastal mudflat (Hawkins and Howes, 1986; Silvius *et al.,* 1987). According to Williams *et al.* (1986), the elevated levels of nitrates, ammonia and phosphorus in the surface waters, in both marine and fresh water, can affect invertebrate diversity in feeding grounds and migratory birds. Since migratory shorebirds, on the west coast of Peninsular Malaysia, spend most diurnal activity feeding on the mudflats to meet their energy and nutrients requirements to continue migration, maintenance of a healthy ecosystem in the feeding ground is a basic requirement to meet such demand. Therefore, the present study was undertaken with the purpose of monitoring changes in the concentrations of nitrate, ammonia and phosphorus in the surface intertidal waters in the Kuala Gula bird sanctuary.

MATERIALS AND METHODS

The study was carried out monthly for four consecutive months (from June to September, 2007) at Kuala Gula bird sanctuary, which is N 04° 55' 896" and E100° 26' 791", located about 45 kilometers from Taiping in Larut, Matang and Selama districts in Perak (*Fig 1*). For this purpose, three sampling stations were established in the Gula river estuary, namely Station 1 (04° 55.185'N, 100° 27.840'E), Station 2 (04° 55.085'N, 100° 27.960'E) and Station 3 (04° 55.006'N, 100° 27.761'E). All the sampling stations have an average depth of 0.8m ± 0.1m throughout the sampling periods. Due to the shallowness of the waters in the sampling stations, the water samples were collected only at one depth i.e. at 0.5 m the deepest depth which the sampler could be lowered down without disturbing the muddy sediment surface. Triplicate samples of surface water were collected for the nutrient analysis from each station using a 5-L capacity Niskin water sampler, which were then transferred into a 1-L acid washed polyethylene bottles and kept in refrigerated box and brought back to the laboratory for immediate analysis. The water samples were then filtered using a 0.45µm Millipore membrane filter prior to the analysis. The total ammonia nitrogen (TAN) and soluble reactive phosphorus (SRP) were analyzed according to the method suggested by Parsons *et al.* (1984); whereas, nitrate-nitrogen concentration was determined using the hydrazine reduction method introduced by Kitamura *et al.* (1984).

Data were statistically analyzed using the one-way analysis of variance (ANOVA). Significant differences, among the individual treatment effects, were determined using a Post Hoc, Tukey's Test (T-HSD) set at $P<0.05$. In addition, statistical analyses were undertaken using the Statistical Analysis System (SAS Inc. 1992) software program.

RESULTS AND DISCUSSION

The concentrations of nutrients (TAN, SRP and nitrate-N) in the surface waters were found to be different at the three stations. The total ammonia nitrogen was found to be the highest ($p<0.05$) at Station 1 (82.45 ±6.39 µg/L) and lowest at Station 2 (50.37±8.03 µg/L) (Table 1). In contrast, the SRP concentration was highest ($p<0.05$) at Station 3 (at 54.48±7.58 µg/L) and lowest at Station 2 (at 27.72± 6.45 µg/L), as shown in Table 1. Nevertheless, no significant differences ($p>0.05$) were observed in the concentrations of nitrate-N at all the 3 stations (Table 1). During the four-month sampling period, the highest ($p<0.05$) concentrations of SRP, nitrate-N and TAN were recorded in June, July and August, respectively. On the contrary, the lowest concentrations of all nutrients were recorded in September. The highest SRP concentration (55.92±7.88 µg/L) was recorded in June, while the highest nitrate-nitrogen concentration (85.68±24.33 µg/L) was observed in the month of July (Table 2). The total ammonia nitrogen was found to be the highest ($p<0.05$) in the month of August, i.e. at 85.91±6.54 µg/L (Table 2). The lowest ($p<0.05$) concentrations of all nutrients were observed throughout September, with the mean values of 45.10±16.36 µg/L, 36.50±3.77 µg/L and 25.87±1.15 µg/L for TAN, nitrate-N and SRP, respectively. Meanwhile, the highest concentrations of nutrients observed were present during the three months (June, July and August), which coincided with the planting season of the nearby paddy fields located in Kuala Kurau and Kuala Gula (Kerian Irrigation Scheme Malaysia- DOA, 2008). On the other hand, most of the local paddy fields were dry and ready for harvesting in the month of

TABLE 1
The comparison in the concentrations (μg/L) of $N0_2$+$N0_3$ – N, TAN and SRP of the surface water of Kuala Gula bird sanctuary according to stations. The values are mean ± SE

Station			
	$N0_2$ $N0_3$	TAN	SRP
Station 1	48.59^{a} ± 8.72	82.45a ± 6.39	27.72^{b} ± 6.45
Station 2	67.14^{a} ± 17.84	50.37b ± 8.03	31.05^{ab} ± 6.59
Station 3	66.42^{a} ± 16.63	80.91ab ± 11.50	54.48^{a} ± 7.58

Columns with the same superscript are not significantly different at (p>0.05)

TABLE 2
The comparison in the concentrations (μg/L) of $N0_2$+$N0_3$ – N, TAN and SRP of the surface water of Kuala Gula bird sanctuary according to months. The values are mean ± SE

Months	Parameters		
	$N0_2$+$N0_3$	TAN	SRP
June	42.06^{ab} ± 2.68	79.43^{a} ± 3.53	55.92^{a} ± 7.88
July	85.68^{a} ± 24.33	74.52^{a} ± 10.55	33.36^{ab} ± 11.85
August	78.63^{ab} ± 20.80	85.91^{a} ± 6.54	35.87^{ab} ± 8.70
September	36.50^{b} ± 3.77	45.10^{b} ± 16.36	25.87^{b} ± 1.15

Columns with the same superscript are not significantly different at (p>0.05)

September. The changes in the concentrations of N and P were associated with the applications of local fertilizer at the nearby paddy fields. This demonstrated that agricultural use of fertilizers was responsible for the local fluctuations in the organic nutrients of the bird sanctuary water. There are other possible sources of pollutants in the coastal ecosystems which include waste water effluents from municipal and industrial origins, run-off from adjacent pasture ranch and septic tank lecheate (Novonty and Olem, 1994). These sources, however, are available all year round and close to the bird sanctuary therefore, their effects are more likely to be constant throughout the year. The results of the present study showed a trend which correlated with the planting and harvest seasons of the paddy fields in the locality. If this relationship is confirmed as the contamination of N and P nutrients from the fertilizer, it may pose a significant threat to the local waters. According to Neal *et al.* (2005), agriculture is one of the main sources of nitrates and phosphates in the local rivers. Excessive use of readily available conventional chemical fertilizers and livestock manure on agricultural land is widely recognized as the major source of surface waters contamination (Adams *et al.*, 1994; Chang and Entz, 1996; Levallois *et al.,* 1998). The scarcity of the available data on the nutrient concentrations, in the local (Malaysia) surface waters along the rivers estuaries and coastal waters, has made it entirely impossible to compare the present results. However, Yap *et al.* (2005) studied the nitrate profile of waters along the Straits of Malacca and reported the concentrations of nitrate from several coastal waters along the straits. In their study, the concentrations of nitrate in the coastal waters were found to range from 107–330 μg/L, which was significantly higher as compared to the results (36.50 – 85.68 μg/L) of the present study. In addition, the concentrations of all nutrients measured in this study were found to be below the maximum levels of the standard water quality limits approved by the National Water Quality Standards of Malaysia (2005). According to the National Water Quality Standards of Malaysia (2005), for Class IIA water (water appropriate for sensitive aquatic species) phosphorus, ammoniacal nitrogen and nitrate concentrations should not exceed 200μg/L, 300 μg/L and 400 μg/L, respectively.

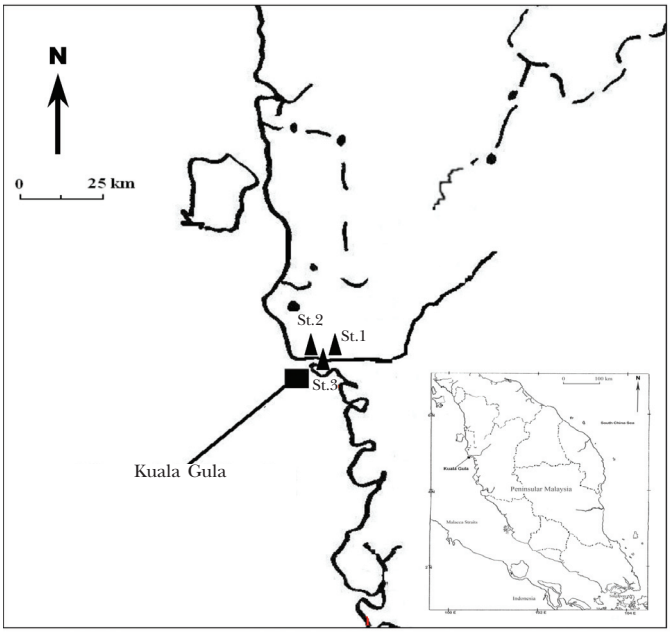


Fig. 1: Map showing the sampling stations along the Kuala Gula, Perak

CONCLUSIONS

The concentrations of nitrite-N, ammonia and phosphate in Kuala Gula bird sanctuary surface intertidal waters fluctuated during the study period, but they never rose above the recognized water quality standards. A positive relationship was also observed; however, this was between the higher levels of nitrate, phosphorus and ammonia and the use of fertilizers for the production of rice at the local paddy fields. Nitrate, phosphorus and ammonia are of great toxicological interest, and they are important macronutrients which can cause eutrophication of waters at raised level. A continuous monitoring of these nutrients in the study area should be conducted more widely. This monitoring data should be able to address the potential around Kuala Gula Sanctuary for the negative impacts on migratory bird habitats in Malaysia.

ACKNOWLEDGEMENTS

The author wishes to thank the Malaysian Wildlife Department for providing the opportunity to conduct research at the Kuala Gula Bird Sanctuary which included free accommodation, water transportation and assistance from department staff during the study period. A special word of thanks also goes to my loving and understanding wife, Evangeline R.M. Lomoljo, for giving me both moral support and encouragement. I would also like to thank Abu Bakar Mat Noon, for his assistance in the field and for the constructive comments given by Hazel Monica Peralta and all my colleagues at the Biology Department, UPM.

REFERENCES

ADAMS, P. L., DANIEL, T.C., EDWARDS, D.R., NICHOLS, D.J., POTE, D.H. and SCOTT, H.D. (1994). Poultry litter and manure contribution to nitrate leaching through the vadose zone. Soil. *Sci.Soc. am. J., 58,* 1206-1211.

CHANG, C. and ENTZ, T. (1996). Nitrate leaching losses under repeated cattle feedlot manure applications in southern Alberta. *J. Environ. Qual., 25,* 145-153.

EDWARDS, P.J., PARISH, D. and NPWO. (1986). Evaluation of Sarawak wetland and their importance to water birds Report no. 2: Western Sarawak Interwader Publication No. 5, Kuala Lumpur.

HAWKINS, A.F. and HOWES, J.R. (1986). Preliminary assessment of coastal wetland and shorebirds in South-west of Peninsular Malaysia. Interwader Publication no. 13, Kuala Lumpur.

Hunt, D.T.E., Dee, A.S. and Oakes, D.B. (2004). Updating an estimate of the sources of nitrogen to UK waters – phase 2. Defra Final Report for Project WT03016. http://www.defra.gov.uk/science/project_data/DocumentLibrary/WT0701CSF/WT03016_1573.FRA.pdf>

Interim National Water Quality Standards for Malaysia. (2005). http://www.did.sarawak.gov.my/wqis/sgsarawak/inwqsm-standards.htm.

Kerian Irrigation Scheme Malaysia-Department of Agriculture. (2008). Schedule of inflow and outflow of water from irrigation system to the rice field for planting and harvesting Compartment D [covering North (Jalan Simpang Lima ke Kuala Kurau), South (Jalan Bagan Serai ke Kuala Kurau), East (Jalan Simpang Lima ke Bagan Serai) and West (Jalan Raya Simpang ke Kuala Kurau)].

Kitamura, H., Ishitani, H., Kuge, Y. and Nakamata, M. (1982). Determination of nitrate freshwater and sea water by a hydrazine reduction method. *Japan J. Wat. Pollut. Res.*, *5,* 35–42.

Lane, B. and Mundkur, T. (1992). Wader study at Malaysian Power Station. *The Stilt, 20,* 42.

Levallois, P., Theriault, M., Rouffignat, J., Tessier, S., Landry, R., Ayotte, P., Girard, M., Gingras, S. S., Gauvin, D. and Chiasson, C. (1998). Groundwater contamination by nitrates associated with intensive potato culture in Quebec. *Sci. Tot. Environ*, *217,* 91-101.

Neal, C., Jarvie, H.P., Neal, M., Love, A.J., Hill, L. and Wickham, H. (2005). Water quality of treated sewage effluent in a rural area of the upper. Themes Basin, southern England, and the impacts of such effluents on riverine phosphorus concentrations. *Journal of Hydrology, 304*, 103-117.

Novonty, V. and Olem, H. (1994). *Water Quality: Prevention, Identification, and Management of Diffuse Pollution* (pp. 1054). New York: Van Nostrand Reinhold.

Parsons, T. R., Maita,Y. and Lalli, C. M. (1984). A manual of chemical and biological method for seawater analysis. *Biological Oceanographic Processes* (3rd edn).

Parish, D. and Wells, D. R. (1984). INTERWADER 83 Report. Kuala Lumpur.

Pepping, M., Piesma, T., Pearson, G. and Lavaleye. (1999). Intertidal sediment and benthic animals of Roebuck Bay, Western Australia (p. 212). NIOZ: Curtin University of Technology, Perth.

Pieterse, N.M., Bleuten, W. and Jorgensen, S.E. (2002). Contribution of point sources and diffuse sources to nitrogen and phosphorus loads in lowland rivers tributaries. *Journal of Hydrology, 271,* 213-225.

Riak, M.K., Ismail, A., Arshad, A. and Ismail, A.R. (2002). The importance of Kapar mudflat and Kuala Selangor Nature Park to the migrant shorebirds. In F.M. Yusoff, M. Shariff, H.M. Ibrahim, S.G. Tan and S.Y. Tai (Eds.), *Tropical marine environment: Charting Strategies for Millennium* (pp. 517-524). Universiti Putra Malaysia, Serdang.

Riak, M.K., Ismail, A., Arshad, A. and Ismail, A. R. (2003a). Intertidal macrobenthic fauna: The food resources for migratory shorebirds in Kapar and Pantai Remis, Selangor, Malaysia. *Appl. Biology, 32*(1), 51-60.

Riak, M.K., Ismail, A., Arshad, A. and Ismail, A. R. (2003b). Species composition and used of mudflat of Kapar, West Coast of Peninsular Malaysia by migratory shorebirds. *The Stilt, 44,* 44-49.

SAS. (1992). Statistical analysis system (SAS Inc. 1992) software program.

Silvius, M. J., Chan, H. J. and Shamsudin, I. (1987). Evaluation of wetlands of the West Coast of Peninsular Malaysia and their importance for natural resources conservation. World Wide Fund for Nature Malaysia.

Tyrrell, T. (1999). The relative influences of nitrogen and phosphorus on oceanic primary production. School of Ocean and Earth Science, Southampton Oceanography Centre, University of Southampton, European Way, Southampton. *Nature, 400,* 525-531.

Williams, K.A., Green, D.W.J. and Pascoe, D. (1986). Studies on the acute toxicity of pollutants to fresh water macroinvertebrates. *Archives fur Hydrobiolgie, 106,* 61-70.

Yap, C.K., Ismail, A., Misri, K. and Tan, S.G. (2005). Nitrate concentration in the surface seawater of the Strait of Malacca. *Asian Journal of Water, Environment and Pollution*, *2*(2), 45-49.

Pertanika J. Trop. Agric. Sci. **32(1)**: 7 - 16 (2009) ISSN: 1511-3701

Freshwater Fish Diversity and Composition in Batang Kerang Floodplain, Balai Ringin, Sarawak

Khairul Adha A. Rahim[1,2*], Siti Khalijah Daud[1], Siti Shapor Siraj[3], Aziz Arshad[3], Yuzine Esa[1,2] and Eza Rena Ibrahim[4]

[1]Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[2]Department of Aquatic Science, Faculty of Resource Science & Technology, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia

[3]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[4] Malaysian Agricultural Research & Development Institute (MARDI) Headquarters, 43400 Serdang, Selangor, Malaysia

[*]E-mail: akhairul@frst.unimas.my

ABSTRACT

The diversity and composition of fish communities in brown and black water habitats at Batang Kerang in Balai Ringin, Sarawak, were evaluated during high and low water seasons. A total of 234 individual fish representing 36 species belonging to 13 families were captured. The fish communities in both the habitats were apparently from 32 species belonging to 12 families in brown water, and only 12 species from 7 families in the black water habitats. The fish fauna in the brown water was dominated by the Cyprinidae (63.8%) family, while the Helostomatidae (59.8%) family dominated the black water habitat. Various water parameters, such as dissolved oxygen, pH values, conductivity and water transparency, total suspended solid (TSS) and ammonium-nitrogen concentrations were significantly different ($p<0.05$) between the black and brown water habitats. The brown water habitat supports more diverse and abundant populations of freshwater fishes than the black water habitat. However, introduced species such as *H. temmincki* and the increase of commercial fishing may also have affected the population of native fish.

Keywords: Black water, brown water, floodplains, *Helostoma temminckii*, fish diversity

INTRODUCTION

Studies of spatial and temporal patterns of diversity, distribution and species composition of freshwater fishes are useful to examine factors influencing the structure of the fish community (Belliard *et al.*, 1997; Galactosa *et al.*, 2004). The distribution and composition of the fish species in each habitat were closely associated with various factors such as the availability of food, breeding sites, water current, depth, topography and physicochemical properties of water (Harris, 1995). There have been a number of studies, conducted within the floodplain of Amazon River, examining the distribution patterns of fish in the white water and black water, poor and rich nutrient (Henderson and Crampton, 1997; Saint-Paul *et al.*, 2000; Hoeinghausa *et al.*, 2003; Cetra and Petrere, 2006). The icthyofauna survey in Amazon has shown that floodplains, including black and white water habitats, support diverse and abundant populations of freshwater fishes. However, there has been limited information documented on the ichthyofauna of the floodplain and freshwater swamp forest in Malaysia (Zakaria *et al.*, 1999).

Received: 4 September 2008
Accepted: 17 December 2008
[*]Corresponding Author

The importance of fish fauna in a particular habitat such as in black water and swampy habitats in Peninsular Malaysia and Borneo has been outlined in several publications (Johnson, 1967; 1968; Mizuno and Furtado, 1982; Davies and Abdullah, 1989; Ng, 1994; Ng *et al.*, 1994; Murtedza *et al.*, 2000; Beamish *et al.*, 2003; Khairul and Yuzine, 2006; Nyanti *et al.*, 2006). The documentation of blackwater fish in Malaysia probably varies from that of the Amazonian rivers (Henderson and Crampton, 1997; Putz, 1997; Francisco *et al.*, 1998; Saint-Paul *et al.*, 2000; Hoeinghausa *et al.*, 2003). Johnson (1968) described that blackwater swamps in Malaysia are generally low in biodiversity and productivity. However, Saint-Paul *et al.* (2000) found that black water fish communities are more diverse in blackwater than white water in the Central Amazonian river.

Batang Kerang floodplain, which is located in Balai Ringin, Sarawak, can be classified on the basis of its water quality into two different types, namely brown water river and black water river. The difference, between the brown water of Batang Kerang and its tributaries i.e. black water is apparent, where the two rivers meet (*Fig. 1*).

Despite of many studies on the ecological characteristics of fish assemblages and species composition in Borneo are available (Watson and Balon, 1984; Roberts, 1989; Abdullah, 1990; Inger and Chin, 1990; Kottelat *et al.*, 1993; Kottelate and Lim, 1995; Nyanti, 1995; Leh, 2000; Khairul Adha *et al.,* 2001; Nyanti *et al.*, 2006), comparative data on the different habitats such as black and brown water floodplain forests within a localized area are still lacking. Flooded forests and floating vegetations of Batang Kerang floodplain are important habitats for fishes. However, the composition of species, as well as the distribution and abundance of fish in these habitats are poorly known. Thus, the aim of the current research was to study the community structure and composition of fish from the brown and black water habitats at the rivers of Batang Kerang floodplain.

MATERIALS AND METHODS

Study Site

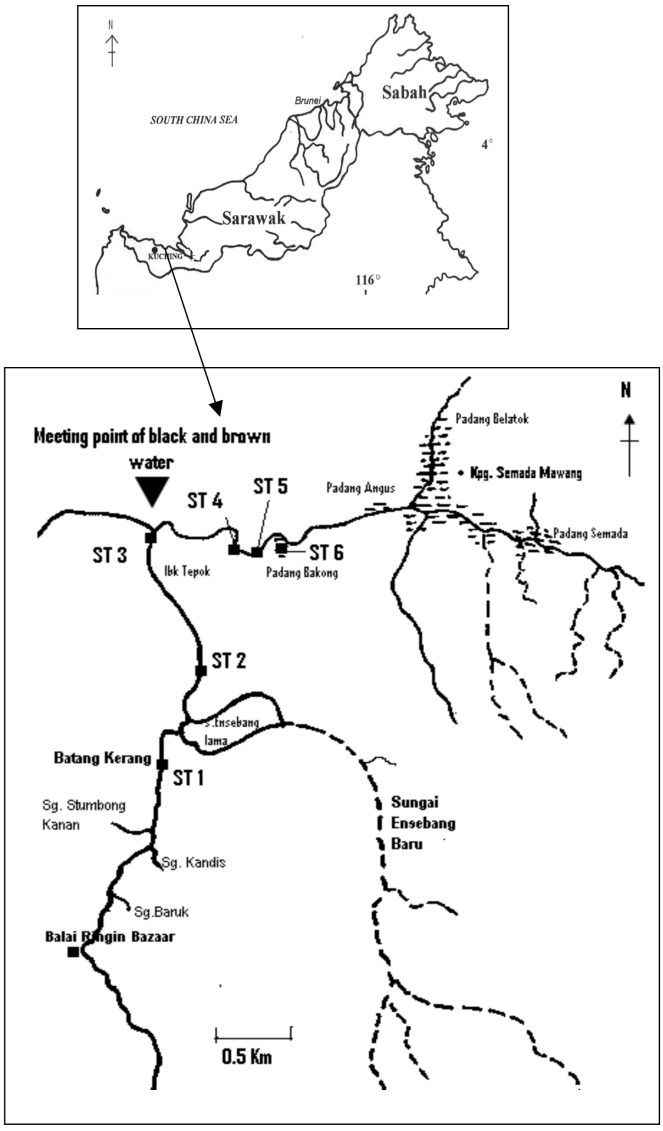
Fishes were sampled from black and brown water habitats in Batang Kerang floodplain (N 01° 14' 00", E 110°41'00") of Balai Ringin, Serian, Sarawak, during high and low water seasons from September 15–18, 2004 and January 27–30, 2005 (*Fig. 1*), respectively. The lower Batang Kerang transverses through a flood plain of largely undisturbed riverine mixed-dipterocarp, swamp forest and marshland. Brown water river is muddy due to its high sediment contents. The mean width and depth of brown water river during sampling were 15.8±1.30 m and 4.2±0.46 m, respectively, with flowing waters at 0.19±0.02 m/s. Some areas of the brown water are characterized with extensive mats of floating vegetation, such as *Hanguana malayana* and *Eichhornia crassipes,* and other submerged aquatic plants. Water draining out of the black water habitat is generally acidic with low pH, inorganic ion and dissolved oxygen level. Black water river has high concentration of humid acids which give the characteristics of dark appearance of the water. Slow flowing water was observed in the black water habitat (0.02±0.01 ms^{-1}) with the mean width of 2.2±0.14 m and depth of 1.4±0.15 m. High and low water level seasons are well defined in this river system. Within the floodplain area, floodplain forests may be inundated from three to eleven months a year.

Sampling

Fish fauna at Batang Kerang floodplain were sampled using monofilament gill nets with different mesh sizes (2.0, 2.5, 3.75, and 5.0 cm) at three stations in brown and black water areas, during low and high water seasons (*Fig. 1*). The gill nets were placed at a suitable depth at the selected stations, and left overnight. Samples were also obtained using a traditional fishing method locally know as 'Selambau'.

Physico-chemical Water Parameters

Water temperature (°C), pH and dissolved oxygen (DO) were measured *in situ* using Hydrolab Water Quality Multiprobe (SVR3, Austin, Texas, U.S.A). Water transparency (Secchi disk), depth and wide of both habitats were measured at the same site. Total suspended solid (TSS) concentrations were estimated using the standard method APHA (1998). The total ammonium-nitrogen concentration was determined using the standard method 8038, based on Nesseler Methods (Hach, 2000) and nitrate was analyzed using the standard methods 8192 based on cadmium reduction method (Hach, 2000).



(ST1, ST2, ST3: Brown water; ST4, ST5, ST6: Black water)

Fig. 1: Maps showing sampling stations at brown water and black water habitats in Batang Kerang floodplains, located in Balai Ringin, Sarawak

Data Analyses

Fish diversity (H') was measured using the Shannon-Weaver (1963) indices. The evenness was determined using the index described by Pielou (1969). Species richness was calculated following Margalef (1958), and modified t-test (Zar, 1996) was used to test for the differences in the fish diversity between the two habitats. Sørensen's index (CC) (Sørensen, 1948) was used to compare the species compositions between the brown and black water habitats. This index considers the number of species common to two sites, and the computed similarity can be between 0% and 100%. The t-test was used to compare the differences in the physicochemical water parameters between the black and brown water habitats.

RESULTS

Physico-chemical Water Parameters

The physico-chemical water parameters for the rivers surveyed are summarized in Table 1. In general, dissolved oxygen, pH, conductivity and water transparency, TSS and total ammonium-nitrogen concentrations were found to be significantly different ($p<0.05$) between the black and brown water habitats. The brown water habitat had higher dissolved oxygen concentrations, pH, conductivity and rich in TSS than those in the black water habitat. However, water transparency and ammonium nitrogen concentration in brown water were significantly lower ($p<0.05$) than those in the black water habitat. Nevertheless, there were no significant differences ($p>0.05$) in terms of water temperature and nitrate concentration between the two habitats.

TABLE 1
Physicochemical water characteristics of black and brown water habitats at Batang Kerang floodplain, Balai Ringin, Serian, Sarawak (Mean ± SD)

Parameters	Brown water	Black water	Significant
pH	5.45 ± 0.10	4.55 ± 0.10	*
Temperature (°C)	25.60 ± 0.60	26.65 ± 0.40	ns
Dissolved oxygen (mg l^{-1})	1.66 ± 0.10	1.15 ± 0.10	*
Secchi transparency (cm)	63.20 ± 4.70	126.30 ± 17	*
TSS (mg/l)	2.20 ± 0.13	0.85 ± 0.20	*
Nitrate (mg/l)	0.05 ± 0.00	0.05 ± 0.01	ns
Conductivity (µS cm^{-1})	31.00 ± 2.30	21.44 ± 0.50	*
Ammonia-nitrogen (mg/l)	0.46 ± 0.10	0.81 ± 0.12	*

Notes: (*) Significant different, (ns) No significant different, n = 18, df =17; (P< 0.05)

Fish Composition

Fish species were identified following Mohsin and Ambak (1983), Robert (1989), Inger and Chin (1990), Kottelat *et al.* (1993) and Kottelat and Lim (1995). In this study, a total of 234 individual fish from 36 species and 13 families were collected from Batang Kerang (Table 2). Black and brown water habitats in Batang Kerang showed different fish species compositions. A total of 152 individual fish from 32 species were caught in brown water, out of which 25 were exclusively found in this habitat. The fish fauna in the brown water habitat were dominated by the Cyprinidae family representing about 63.8% of the total fish caught. *Oxygaster anomalura,* with the mean weight of 24.03±1.63 gm and the standard length of 13.73±0.27 cm, was abundant and presented 25.7% of the fish collection, followed by *Cyclocheilichthys apogon* and *Osteochilus* spp. with 15.8% and 9.9% of fish collection, respectively.

In the black water habitat, 82 individual fish from 12 species were caught. The most abundant black water fish was the *Helostoma temminckii* (Helostomatidae) which represented 59.8% of the total fish caught. This species, with the mean standard length of 13.28±0.44 cm, was found in all stations. *Trichogaster pectoralis, Clarias batrachus, Clarias macrocephalus, Clarias nieuhofi,* and *Rasbora pauciperforata* were found only in the blackwater habitat. Meanwhile, seven species from seven families were found in both habitats. These include *Anabas testudineus, Channa lucius, Clarias teijsmanni, Hemibagrus nemurus, Helostoma temminckii, Oxygaster anomalura*, and *Trichogaster trichopterus.* It is important to highlight that *Helostoma temminckii* and *Oxygaster anomalura* were the most abundant species caught in both habitats. However, about 94.2% of *Helostoma temminckkii* were found in blackwater and 97.5% of *Oxygaster anomalura* in brown water habitats.

The number of individuals and species composition were significantly higher during

TABLE 2
Fish species collected from the black and brown water habitats at Batang Kerang floodplain of Balai Ringin, Serian, Sarawak

Family	Species	Brown	Black
Anabantidae			
	Anabas testudineus	4	7
Bagridae			
	Leiocassis micropogon	4	0
	Hemibagrus baramensis	7	0
	Mystus micracanthus	5	0
	Hemibagrus nemurus	1	1
Belontidae			
	Trichogaster pectoralis	1	5
	Trichogaster trichopterus	1	3
Channidae			
	Channa lucius	2	1
Clariidae			
	Clarias batrachus	0	3
	Clarias macrocephalus	0	1
	Clarias nieuhofi	0	1
	Clarias teijsmanni	4	8
Cobitidae			
	Pangio semicincta	3	0
Cyprinidae			
	Cyclocheilichthys apogon	24	0
	Hampala macrolepidota	4	0
	Osteochilus enneaporos	1	0
	Osteochilus hasseltii	12	0
	Osteochilus kahajanensis	2	0
	Oxygaster anomalura	39	1
	Puntius kuchingensis.	3	0
	Puntius orphoides	4	0
	Puntius lineatus	1	0
	Rasbora caudimaculata	7	0
	Rasbora pauciperforata	0	2
Eleotrididae			
	Bostrychus sinensis	3	0
	Oxyeleotris marmorata	2	0
	Eleotris acanthopomus	1	0
	Prinobutis dasyrhynchus	1	0
Helostomatidae			
	Helostoma temminckii	2	49
Luciocephalidae			
	Luciocephalus pulcher	1	0
Pangasiidae			
	Pangasius sp.	1	0
Siluridae			
	Krytopterus macrochephalus	1	0
	Krytopterus schilbeides	2	0
	Ompok leiacanthus	2	0
	Silurichthys hasseltii	3	0
	Silurichthys phaiosoma	2	0
Tetraodontidae			
	Carinotetraodon salivator	2	0
Total (N)		152	82

the low water level than the high water level (Chi-square, $p< 0.05$). In brown water habitat, only 37 individual fish of 14 species were found during high water level. However, about 115 individual fishes from 24 species were sampled during low water level. In particular, *Carinotetraodon salivator, Hemibagrus nemurus, Puntius lineatus, Bostrychus sinensis, Eleotris acanthopomus, Krytopterus macrocephalus, K. schilbeides and Ompok leiacanthus* were mostly caught during high water level. During low water level, fish species such as *Pangasius* sp., *Anabas testudineus, Mystus baramensis, M. micracanthus, Trichogaster pectoralis, Channa lucius, Clarias teijsmanni, Pangio semicincta, Puntius lineatus, Puntius orphoides, Osteochilus enneaporos, Osteochilus kahajanensis, Rasbora caudimaculata* and *Oxyeleotris marmorata* were collected.

From the black water area, only 10 individual fish belonging to seven species and 72 individual fish from eight species were caught during high and low water seasons, respectively. Fish species such as *Clarias batrachus, C. macrocephalus, C. nieuhofi, and Oxygaster anomalura* were caught during high water level, whilst *Hemibagrus nemurus, Trichogaster pectoralis, T. trichopterus, Channa lucius, and Clarias teijsmanni* were caught during low water level. However, *H. temminckii* was the most abundant during the low water season.

Fish Species Diversity

The fish diversity indices of Batang Kerang floodplain is shown in Table 3. The ecological indices for the two habitats showed that brown water had a significantly higher ($p<0.05$) species diversity, evenness and richness, and the number of individual caught than those in blackwater (t-test). Meanwhile, the diversity indices for brown water were 2.09, and this was 0.65 for the black water. The evenness indices for the two habitats were also different; the indices of 0.60 and 0.26 were detected for brown and black waters, respectively. The richness of the fish species was also higher in the brown water than in the black water habitat. The Sørensen coefficient of community similarity between the brown and black water habitats was 31.8%, in which only 7 species were found in both habitats.

DISCUSSION

The brown water habitat in Batang Kerang floodplains has more species than the adjacent black water habitat, which was 32 and 12 species, respectively. The fish fauna in the brown water habitat were dominated by the Cyprinidae family (63.8%). Robert (1989) stated that about one-third of all the freshwater fishes in Western Borneo were represented by cyprinids. Nyanti (1995) and Leh (2000) reported that approximately 66% and 46% of the fish collections in Sarawak were from the Cyprinidae family.

The unequal fish species composition and distribution in Batang Kerang floodplains could be attributed to many factors. Brown water habitat are characterized by overgrown floating vegetation such as *Eichhornia crassipes* and fringed with tall grasses. The floating meadows are known as the nursery grounds for young fishes which use the submerged roots as refuge from predation and foraging substrate (Putz, 1997). This characteristic could probably create suitable niches for a variety of fish species, and subsequently higher fish abundance and species richness found in that habitat. Brown water has also facilitated wider and deeper habitat than the black water habitat. Zakaria *et al.* (1999) stated that river with wider, deeper and longer channel should be rich in fishes. The presence of macrophytes and larger habitats provide more heterogeneous living spaces, which could support

TABLE 3
The fish diversities of the black and brown water habitats in Batang Kerang, Balai Ringin, Serian, Sarawak

Habitats	s	N	H'	J'	D"
Brown water	32	152	2.09	0.60	14.21
Black water	12	82	0.65	0.26	9.09

Notes: s = number of species, N = number of individuals, H'= species diversity, J'= Pielou eveness index, D"= species richness

a large number of fish species through habitat segregation (Lemly and Dimmick, 1982).

The black water habitats were dominated by *Helostoma temminckii*. This species generally prefers stagnant to slow moving water. This species was abundant and dominant in Baram areas, including Bakong black water river and Logan Bunut, based on the number of individual fish caught (Murtedza *et al.*, 2000; Nyanti *et al.*, 2006). Predator fish such as *Hemibagrus nemurus, Mystus baramensis, Mystus micracanthus, Channa lucius, Clarias batrachus, Clarias teijsmanni, Clarias nieuhofi, Clarias macrocephalus* and *Oxyeleotris marmorata* were commonly found in this habitat.

Most of the specimens caught are native to Borneo and only three fish species (*Clarias macrocephalus, Trichogaster pectoralis* and *Helostoma temminckii*) have been identified as introduced in this river. Local inhabitants reported that the population of *H. temminckii* were abundant during low water level. For instance, *Helostoma temminckii*, which is also known as kissing gouramy, was introduced into Sarawak in 1956 for aquaculture and it was believed that this species escaped from the ponds into the surrounding streams during heavy floods in the Baram area in 1963 (Hans and Morshidi, 2000). Since 1963, the population of *Helostoma temminckii* has increased dramatically and invaded many lakes and rivers of the lower Baram (Hans and Morshidi, 2000; Murtedza *et al.*, 2000). The establishment of non-indigenous species has substantially changed the community structure in some areas, and in some cases, the numbers of introduced fish are greater than the native (Sublette *et al.*, 1990; Vitousek *et al.*, 1996; 1997). The increasing number of the introduced fish (such as *Helostoma temminckii* population) may threaten the biodiversity of indigenous fishes in the areas. However, the effects of the introduced fishes in Batang Kerang floodplain are less understood. Therefore, further studies to examine the degree of habitat-use overlap between native and non-native species in this area must be obtained.

The aquatic system at Batang Kerang floodplains varied considerably between the black and brown habitats. The blackwater habitat of Batang Kerang is a typical Malaysian black water habitat with low pH, dissolved oxygen and conductivity, poor in suspended and dissolved solids, high dark transparent water and low fertility (Ng, 1994; Ng *et al.*, 1994; Murtedza *et al.*, 2000; Beamish *et al.*, 2003; Khairul and Yuzine, 2006). The low TSS reading of 0.85 mg/l and high water transparency suggest that the black water habitat is still in pristine environment. The oxygen concentration level in the brown water was found to be higher than the black water habitats, and this was probably because of the turbulent mixing of the water. The brown water is also rich in TSS reading (2.20mg/l) which may be reflected by the lower light penetration and a high nutrient content, where more aquatic macrophytes plants are found. However, spatial and temporal variations of the water quality, during high and low water seasons, and the correlation with fish species compositions and abundance were not well addressed in this study.

Fish species in the blackwater habitat of Batang Kerang showed adaptation to water with low dissolved oxygen levels and high acidic water including those with accesory respiratory organs and suprabranchial cavities. They were from the families of Anabantidae, Belontiidae, Bagridae, Channidae, Helastomatidae, and Siluridae. The patterns of species composition and abundance indicated that the availability of dissolved oxygen and pH value are the important factors in determining the presence of fish. According to Beamish *et al.* (2003), the abiotic conditions within the swamp, particularly the low pH and dissolved oxygen concentrations, are the indicators of a generally unfavourable environment for fish. The same phenomenon was observed for the fish composition found in Bakong black water rivers, Samarahan peat swamp habitat and Logan Bunut National Park in Sarawak (Murtedza *et al.*, 2000; Khairul and Yuzine, 2006; Nyanti *et al.,* 2006).

Seasonal fluctuations of water level in Batang Kerang floodplains have influenced the fish composition. The greater number of individual fish caught and species composition were observed during the low water season. Different fish communities found during high and low water seasons may be associated with variations in the migratory movements of the fish species (Renato *et al.,* 2000). High water levels increase the size of the aquatic environment and some fish species have migrated from the floodplains to the upper reaches of the river for breeding or expanding their food and habitat resources, then migrated back to downstream after spawning as the water recedes (Lowe-McConnel, 1975; 1987; Welcomme, 1979). Floodwater recession reduces the availability of aquatic habitats and thus,

increases fish densities and biotic interactions (Winemiller, 1989; Rodr'iguez and Lewis, 1994; 1997). This may explain the quantity of fish captured was always lower during raising water than during receding water seasons.

Most of the popular aquarium fishes are from peat swamp forest. Ng *et al.* (1994) noted that 27 species of black water fish have been recognized as the potential valued species for the aquarium industry. Fish species from genera *Rasbora*, *Puntius*, *Trichogaster* and *Sphaeritchys osphromenoides* have been traded as ornamental fish (Khairul and Yuzine, 2006). Pikehead, *Luciocephalus pulcher, Pangio semicincta* and *Carinotetraodon salivator* are known to be popular fish for aquarium found at the study sites. Presently, the market demand for ornamental fish has increased drastically, but the catch from the wild stock is still insufficient. It is important to highlight that sustainable utilization of the fish resources in the black water of Batang Kerang for the aquarium trade may contribute to the economy of the country.

In general, species diversity was considerably higher in brown water than that of black water habitats. Comparing the two sampling sites, the number of species in brown water was found to be 55.6% higher than that of the black water with 31% similarity. The differences in terms of diversity indices between the two habitats may be related to the physicochemical properties of the brown and black waters. Galacatosa *et al.* (2003) found that the diversity indices of fish composition were significantly influenced by both seasonal and habitat differences between the white and black water habitats in the Amazon river. Furthermore, Johnson (1969) and Zakaria *et al.* (1999) found that several environmental factors, such the physicochemistry of the water quality, topographical, hydrological characteristics and habitat destruction, could play major roles in species richness, diversity and species survival in aquatic habitats.

CONCLUSIONS

In this research, the brown water habitat supports diverse and abundant populations of freshwater fishes compared to the black water habitat at Batang Kerang River systems. The present study also provided evidence which showed there were certain distinctive characteristics between the community structure of fish captured in black water and brown water habitats. However, seasonal flooding also seemed to be an important factor which influenced the assemblage and composition of fish. The results of the present study suggested that studies on the spatial and temporal of species compositions, distribution and abundance of floodplain fish should be carried out in Borneo.

ACKNOWLEDGEMENTS

This study was financed by Universiti Malaysia Sarawak through research grant; ASEAN Regional Centre for Biodiversity Conservation (ARCBE, RE-MYS-00) and Shell-MDS-UNIMAS. We thank Zaidi Ibrahim, Zulkefli Ahmad, Harris Norman, Jeman Mardzuki, Mazlan Mardzuki and the local people of Balai Ringin for their field assistance throughout the study.

REFERENCES

Abdullah, S. (1990). Taburan dan populasi ikan air tawar di beberapa altitud di Taman Kinabalu Sabah, Malaysia. *Pertanika, 13,* 341-348.

APHA. (1998). *Standard Methods for the Examination of Water and Wastewater.* American Public Health Association (Ed.). New York.

Beamish, F.W.H., Beamish, R.B. and Lim, S.L.H. (2003). Fish assemblages and habitat in a Malaysian blackwater peat swamp. *Environmental Biology of Fishes, 68,*1-13.

Belliard, J., B¨oet, P. and Tales, E. (1997). Regional and longitudinal patterns of fish community structure in the Seine River basin, France. *Environmental Biology of Fishes, 50,* 133–147.

Cetra, M. and Petrere, Jr. (2006). Fish assemblage structure of the Corumbatai River Basin, Sao Paulo State, Brazil: Characterization and anthropogenic disturbance. *Brazilian Journal of Biology, 66,* 431-439.

Davies, J. and Abdullah, A.R. (1989). *Freshwater fish survey of the North Selangor Peat Swamp Forest. Asia* Wetland Bureau Publication No: 46. IPT Asian Wetland Bureau/ WWF Malaysia, Kuala Lumpur: Malaysia.

Francisco, L., Tejerina-Garro, Fortin, R. and Rodriguez, M.A. (1998). Fish community structure in relation to environmental variation in floodplain lakes of the Araguaia River, Amazon Basin. *Environmental Biology of Fishes, 51,* 399–410.

Galactos, K., Barriga-Salazar, R. and Stewart, D. J. (2004). Seasonal and habitat influences on fish communities within the lower Yasuni River basin of the Ecuadorian Amazon. *Environmental Biology of Fishes, 71,* 33–51.

Hach Kit. (2000). *DR/2010 Spectophotometer Procedures Manual.* Copyright of Hach Company. Loveland, USA.

Hans, P.H. and Morsidi, A.K.A. (2000). *National Park of Sarawak.* Malaysian Natural History Publication (Borneo) Sdn. Bhd.

Harris, J.H. (1995). The use of fish in ecological assessments. *Australian Journal of Ecology, 20,* 65-80.

Henderson, P. A. and Crampton, W.G.R. (1997). A comparison of fish diversity and abundance between nutrient-rich and nutrient-poor lakes the Upper Amazon. *Journal of Tropical Ecology, 13,* 175-198.

Hoeinghausa, D.J., Laymana, C.A., Arringtona, D.B.A. and Winnemillera, K.O. (2003). Spatiotemporal variation in fish assemblage structure in tropical floodplain creeks. *Environmental Biology of Fishes, 67,* 379–387.

Inger, R.F. and Chin, P.K. (1990). *The freshwater fish of North Borneo.* Fieldiana: Zoology, Vol. 45 (1962). Reprinted by Sabah Zoological Society Malaysia.

Johnson, D.S. (1967). Distributional patterns of Malayan freshwater fishes. *Ecology, 48,* 722-730.

Johnson, D.S. (1968). Malayan blackwaters. In R. Misra and B. Gopal (Eds.), *Proc. Symp. Recent Adv. Trop. Ecol.*(pp. 303-310). International Society for Tropical Ecology: Varanasi.

Khairul Adha A.R., Shabdin, M.L. and Fatimah, A. (2001). A survey of freshwater fish fauna in the upper rivers of Crocker Range Park, Sabah. In *Scientific Journey Through Borneo, Crocker Range National Park, Sabah* (pp. 119-130). London: ASEAN Academic Press.

Khairul Adha, A.R. and Yuzine, E. (2006). The fish fauna. In F. Abang and I. Das (Eds.), *The Biodiversity of Peat Swamp Forest in Sarawak* (pp. 99-106). Institute of Biodiversity and Environmental Conservation: Universiti Malaysia Sarawak, Kota Samarahan. Sarawak, Malaysia.

Kottelat, M., Whitten, A.J., Kartikasari, S.N. and Wirjoatmodjo, S. (1993). *Freshwater Fishes of Western Indonesia and Sulawesi.* Singapore: Periplus Editions Ltd.

Kottelat, M. and Lim, K.P. (1995). Freshwater fishes of Sarawak and Brunei Darussalam: A preliminary annoted check-list. *Sarawak Museum Journal, 69,* 227-256.

Leh, M.U.C. (2000). Fishes. In E. Soepadomo and P.P.K. Chai (Eds.), *Development of Lanjak-Entimau Wildlife Sanctuary as a Total Protected Area* (pp. 124-136). International Tropical Timber Organization, Japan and Sarawak Forest Department: Sarawak, Malaysia.

Lowe-Mcconnell, R.H. (1975). *Fish Communities in Tropical Freshwater.* New York: Longham Inc.

Lowe-Mcconnell, R.H. (1987). *Ecological Studies in Tropical Fish Communities.* Cambridge: Cambridge University Press.

Lemly, A.D. and Dimmick, J.F. (1982). Structure and dynamics of zooplankton communities in the littoral zone of some North Carolina lakes. *Hydrobiologia, 88,* 299-307.

Margalef, R. (1958). Information theory in ecology. *General System, 3,* 36-71.

Mizuno, N. and Furtado, J.I. (1982). Ecological notes on fishes. In J. I. Furtado and S. Mori, (Eds.), *Tasek Bera: The ecology of freshwater swamp* (pp. 321-354). Hague: Dr.W. Junk Publication.

Mohsin, A.K.M. and Ambak, M.A. (1983). *Freshwater Fishes of Peninsular Malaysia.* Serdang, Selangor: Universiti Pertanian Malaysia Publication.

Murtedza, M., Seng, L., Ling, L.P., Howe, L.Y., Bessaih, N. and Khairul Adha, A.R. (2000). The need and challenges for integrated catchment management-The Bakong catchment in Sarawak. In M. Leigh (Ed.), *Environment conservation and land. Proceedings of the Sixth Binnial Borneo Research Conference Borneo 2000* (pp. 126-140). IEAS, Sarawak Universiti Malaysia Sarawak.

Ng, P.K.L. (1994). Peat swamp fishes of Southeast Asia - Diversity under threat. *Wallaceana, 73,* 1-5.

Ng, P.K.L., Tay, J.B. and Lim K.K.P. (1994). Diversity and conservation of blackwater fishes in Peninsular Malaysia, particularly in the North Selangor peat swamp forest. *Hydrobiologia, 285,* 203-218.

Nyanti, L. (1995). Fish fauna of Sayap-Kinabalu Park, Sabah. In I. Ghazally and D. Laily (Eds.). *A scientific journey through Borneo: Sayap-Kinabalu Park, Sabah* (pp. 189-199). Kuala Lumpur: Pelanduk Publication.

Nyanti, L., Sayok A.K. and Efransjahi, E. (2006). Fish fauna of Loagan Bunut National Park, Sarawak. In A.A. Tuen., A.K. Sayok, A.N. Toh and G. T. Noweg (Eds.), *Scientific journey through Borneo: Loagan Bunut* (pp. 102-129). UNDP/GEF Funded (Mal/99/G31), Sarawak Forest Department, Institute of Biodiversity and Environmental Conservation and Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia.

Pielou, E.C. (1969). *An Introduction to Mathematical Ecology.* New York: Wiley.

Putz, R. (1997). Periphyton communities in Amazonian black- and whitewater habitats: Community structure, biomass and productivity. *Aquatic Science*, *59*, 74–93.

Renato, A.M., Benedito, S., Amaralc, D. and Oyakawad, O. T. (2000). Spatial and temporal patterns of diversity and distribution of the Upper Juru´a River fish community (Brazilian Amazon). *Environmental Biology of Fishes, 57,* 25–35.

Roberts, T.R. (1989). *The Freshwater Fishes of Western Borneo (Kalimantan Barat, Indonesia).* California: Califonia Academy of Science.

Rodr´iguez, M.A. and Lewis, W.M. (1994). Regulation and stability in fish assemblages of Neotropical floodplain lakes. *Oecologia*, *99*, 166–180.

Rodr´iguez, M.A. and Lewis, W.M. (1997). Structure of fish assemblages along environmental gradients in floodplain lakes of the Orinoco River. *Ecology Monographs, 67,* 109–128.

Saint-Paul, U., Zuanon, J., Correa, M.A.V., Garcia, M., Fabre, N.N., Berger, U. and Junk. W. J. (2000). Fish communities in central Amazonian white-and blackwater floodplains. *Environmental Biology of Fishes, 57,* 235-250.

Shannon, C.E. and Weaver, W. (1963). *The Mathematical Theory of Communication.* Urbana: University of Illinois Press.

Sørensen, T. (1948). *A method of establishing groups of equal amplitude in plant sociology based on similarity of species content.* Det. Kong. Danske. Vidensk. Selsk. Biol. Skr: Copenhagen.

Sublette, J.E., Hatch, M.D. and Sublette, M. (1990). *The Fishes of New Mexico.* Albuquerque: University of New Mexico Press.

Vitousek P.M., D'antonio, C.M., Loope, L.L. and Westbrooks, R. (1996). Biological invasions as global environmental change. *American Scientist, 84*, 468–478.

Vitousek P.M., D'antonio, C.M., Loope, L.L., Rejmanek, M. and R. Westbrook, R. (1997). Introduced species: A significant component of human-caused global change. *New Zealand Journal of Ecology, 211,* 1-16.

Watson, D. J. and Balon, E.K. (1984). Structure and production of fish communities in tropical rain forest system of Northern Borneo. *Canadian Journal Zoology, 62,* 927-940.

Welcomme, R.L. (1979). *Fisheries Ecology of Floodplain Rivers.* London: Longman.

Winemiller, K.O. (1989). Ontogenetic diet shifts and resource partitioning among piscivorous fishes in the Venezuelan Llanos. *Environmental Biology of Fish, 26*, 177–199.

Zakaria, R., Mansor, M. and Ali, A.B. (1999). Swamp-riverine tropical fish population: A comparative study of two spatially isolated freshwater ecosystems in Peninsular Malaysia. *Wetlands and Ecology Management, 6,* 261-268.

Zar, J.H. (1996). *Biostatistical Analysis.* N.S. USA: Prentice-Hall.

Pertanika J. Trop. Agric. Sci. **32(1)**: 17 - 23 (2009) ISSN: 1511-3701

Evaluation of Sole and Amended Organic Fertilizers on Soil Fertility and Growth of Kola Seedlings (*Cola acuminate*)

E.I. Moyin-Jesu
Agronomy Department, Federal College of Agriculture, Akure, Ondo State, Nigeria
E-mail: moyinjesu2004@yahoo.com

ABSTRACT

A healthy kola seedling in the nursery is very important for sustainable establishment and high yield of kolanuts in the fields. An investigation was carried out in Akure, in the rainforest zone of Nigeria, to determine the effectiveness of amended forms of wood ash and cocoa husk, turkey, goat and duck manures (sole) as sources of fertilizers, on the growth of kola (*Cola acuminate*) seedlings in the nursery. For this purpose, nine organic fertilizer treatments [duck manure, goat manure, turkey manure (sole), wood ash/duck manure mix, cocoa husk/duck manure mix, goat manure/wood ash mix, goat manure/cocoa husk mix, turkey manure/cocoa husk mix and turkey manure and wood ash mix] were applied at 8t/ha (40g per 10kg soil filled pots), replicated three times with NPK fertilizer and a control (no fertilizer), and arranged in a completely randomized design. The soil, plant and the organic residues were chemically analysed. The findings revealed that the use of organic residues significantly increased plant height, leaf area, stem girth, root length as well as leaf number of kolanut seedlings, soil and leaf N, P, K, Ca, Mg concentrations, soil pH and O.M contents ($p<0.05$), relative to the control treatments. The amended wood ash + duck increased the shoot weight, plant height, root length, leaf area, leaf number and stem girth of kolanut by 6%, 27%, 20%, 35%, 27% and 37% respectively, as compared to using the NPK fertilizer. In addition, it was also found to increase the same parameters by 84%, 80%, 72%, 78%, 56% and 82% respectively, as compared to the control treatment. As for the soil chemical composition, duck manure + wood ash were shown to increase the soil N, P, K, Ca, Mg, pH and O.M by 42%, 26%, 38%, 46%, 59%, 6% and 52% respectively, compared to the duck manure (sole). At the same time, it also increased soil K, Ca, Mg, pH and O.M by 51%, 97%, 93%, 29% and 90% respectively, as compared to using the NPK fertilizer. In particular, the treatment using duck manure + cocoa husk increased the leaf N, P, K, Ca and Mg of kolanut seedlings by 12%, 74%, 56%, 69% and 75%, respectively as compared to merely using duck manure (sole). It also increased the same leaf parameters by 42%, 54%, 92% and 84% respectively, as compared to the control treatment. In this study, the NPK fertilizer was found to decrease soil O.M but it increased soil N and P more than the organic residues. The amended duck manure + wood ash and duck manure + cocoa husk, applied at $8tha^{-1}$ (40g/10kg), were found to be the most effective in improving the performance of kolanut seedlings.

Keywords: *Cola acuminate*, organic fertilizers, kolanut seedlings

INTRODUCTION

Kola belongs to the family of stericuliaceae. The two main species of kola, namely cola nitida and cola acuminate, are of the most important commercial values in the markets. Kolanuts are featured prominently in the religious and social activities of West Africa. They are used particularly during marriages, child naming ceremonies and other cultural activities. Industrially, kola is used for the preparation of drinks such as Coca-cola and Pepsi cola, as well as dyeing purposes and the production of pharmaceuticals (Adeyeye and Ayejuyo, 1994).

Despite the above mentioned importance of kola, its optimum yield has not been attained because of the increasing decline in soil fertility and old age of kola trees in the field. Effort to increase the soil nutrient status, through the

Received: 20 June 2008
Accepted: 11 September 2008

use of chemical fertilizer by farmers, is rather limited due to high cost of fertilizer, and/or its poor availability to farmers locally. Thus, there is need to identify locally available organic fertilizers which can be used to improve the fertility of soils used in raising kola seedlings in the nursery, which takes usually about 8-12 months.

A literature review showed that using different levels of chromelana odorata on kola and coffee, with the exception of the previous research findings of Obatolu (1995). Apart from that, Oladokun (1990) worked on the effects of vegetative propagation on the growth and yield of kolanut, while Abulude (2004) worked on the determination of functional groups in the chemical composition of kola (*Cola acuminate*); nevertheless, there is a scarcity in the research and information on the use of wood ash, cocoa husk amended with goat, duck and turkey manures or the sole application of the manures for raising kola seedlings in the nursery.

The objective of this study was to investigate the effectiveness of various types of organic residues as a source of plant nutrients on the growth, leaf and soil parameters of the kola seedlings in the nursery.

MATERIALS AND METHODS

The experiment was carried out at Akure (7°N, 5° 10° E) in the rainforest zone of Nigeria. The rainfall is between 1100 to 1500mm per annum and the temperature is 24°C.

Soil Sampling and Analysis

30 core soil samples were collected from 0-15cm depth on the site, and mixed thoroughly. The representative samples were taken to the laboratory, air-dried and sieved with 2mm sieve and ready for routine analysis.

The soil pH (1:1 soil/water) was read on the pH meter. Organic matter was determined using wet oxidation method through chromic acid digestion (Walkley and Black, 1934). Soil P was extracted by Bray P_1 extractant and the extract was developed into Murphy blue colouration and determined on a spectronic 20 (Bausch and Lomb Spectronic 20 the Bausch and Lomb France S.A., Boie Postate 3, F-78320 Les Mesnil Saint Denis, France) at 882um (Murphy and Riley, 1962). The soil K, Ca, Mg and Na were extracted with IM NH_4 $0A_c$ pH 7 and the contents of K, Ca and Na were read on the flame photometer (Jenway Clinical PFP7, Designed and manufactured by Jenway Ltd. Felsted Dunmow, Essex CM6 3LB, United Kingdom), while the Mg content was determined on the atomic absorption spectrophotometer (Novaspec II visible spectrophotometer; manufactured by Pharmacia Biotech (Biochron Ltd) Cambridge, England). Meanwhile, the % N was determined using the microkjedahl method (Jackson, 1964).

The Analysis of the Organic Residues Used for the Experiment

The manures from turkey, goat and duck were obtained from their pens at a nearby farm and were air-dried, while wood ash and cocoa husk were obtained from domestic source and cocoa plantation. The cocoa husk was ground using hammer mill for a better utilization by crops, while the wood ash was sieved with 2mm sieve to remove pebbles, wood and charcoals remains. The turkey, goat and duck manures were each stacked under a shade to allow quick mineralization and this was done to reduce the C/N ratio.

The % nitrogen was determined by weighing 2g of each organic material into a digester flask and 5ml of H_2S0_4 with selenium, and copper sulphate tablets were added. After 5ml of NaH was added, the distrillate was collected, and boric acid was added with an indicator before it was titrated with 0.1. M HC1.

Furthermore, two grams of each organic material was weighed into a clean dry tecator digestion tubes to determine the P, K, Ca and Mg contents. 25ml of $HN0_3$ was added down the neck of the flask and swirled to ensure that the organic material was thoroughly wetted. 5ml of $H_2 SO_4$ and 5ml of perchloric acid ($HC1O_4$) were added and the mixture was swirled again. This was then placed on the digestion block and heated carefully by ensuring that the samples did not froth. Digestion was continued until the samples were clear and acids were completely volatized.

The samples were allowed to cool and 10ml of distilled water was added; filtration into 100ml volumetric flask was done and the filtrate was left to cool before it was filled to the mark with distilled water.

As for phosphorus (P), 20ml of phosphor-vanado molybdate solution was added and allowed to stand for at least 2 hours. The colour absorbance was measured on spectronic

20 at 442um. Meanwhile, the % K, Ca and Na contents, an aliquot was measured into 100ml flask and diluted to mark. 1ml of the sample solution was taken, and the flame photometer was adjusted; this was followed by the aspiration of the diluted sample solution. The solution was read and later converted to mg/kg. The Mg content was determined using the atomic absorption spectrophotometer.

Experimental Hypotheses

Three hypotheses were tested using independent variable (X_1) and dependent variables as the Y_1 for kola seedlings. The independent variables (X_1) were defined as organic materials such as turkey manure, duck manure, goat manure, goat manure/cocoa husk mix, turkey manure/cocoa husk mix, wood ash/duck manure mix, cocoa husk/duck manure mix, wood ash/turkey manure mix and cocoa husk/turkey manure mix.

The dependent variables (Y_1) were defined as comprising plant height, leaf area, stem girth, tap root length, shoot weight and leaf number, soil and leaf N, P, K, Ca and Mg, soil pH and O.M.

Each null hypothesis (Ho=U) was tested to determine whether significant statistical relationship existed between each dependent and the observed independent variables.

The three hypotheses tested were as follows:

(i) There is no significant relationship between the organic materials and the plant height, leaf area, stem girth, root length and leaf number of the kolanut seedlings.
(ii) There is no significant relationship between the organic materials and soil N, P, K, Ca, Mg, pH and O.M composition after harvesting.
(iii) There is no significant relationship between the organic materials and leaf N, P, K, Ca and Mg of the kolanut seedlings.

Nursery Experiment

The site was cleared and the debris was removed for laying out polybags on the ground for the purpose of experiment. Each polybag was filled with 10kg soil (0-15cm depth) taken from the site.

The nine organic residue treatments included in the experiment were turkey manure, duck manure, goat manure, goat manure/cocoa husk mix, wood ash/goat manure mix, cocoa husk/duck manure mix, wood ash/duck manure mix, cocoa husk/turkey manure mix and wood ash/turkey manure mix. All these mixes consisted of equal weights (50%) of the two components. The treatments were applied at 8 t/ha (40g residues per 10kg soil).

There was an unamended control treatment (no fertilizer; no manure) and a fertilizer treatment (400kg/ha NPK 15-15-15 fertilizer at 2g per pot). All the treatments were replicated three times and arranged in a completely randomized design (CRD).

The residues were allowed to decay in the soil-filled polybags for one week by watering twice in a day. One pre-germinated kolanut seed was planted in each polybag and watered adequately. After two weeks of planting in the nursery, plant height, leaf area and stem girth of kolanut seedlings were measured using a ruler, graph method and calliper, while the leaf number was done by counts. These growth parameters were measured at every week interval, up to 24 weeks after planting.

Weeding of the site was started at 3 weeks after planting and repeated at 6, 9 and 15 weeks after planting. The kola seedlings were sprayed with karate (i.e. 25g lambda-cyhalotron per litre) at 10ml per 10L of water, every 2 weeks interval to control lead defoliating beetles. Initially, a shade structure was built above the seedlings and this was gradually removed, starting from 12 weeks to thicken the seedlings by receiving more sunlight.

At 10 weeks after planting in the nursery, some leaf samples were taken from the kola seedlings, dried and analysed for the N, P, K, Ca and Mg contents. At 24 weeks after planting (WAP), the seedlings were carefully uprooted, while the shoot weight and tap root length were also measured. The soil samples were taken from each polybag at 25 WAP, air dried and sieved for routine analysis of soil N, P, K, Ca and Mg, soil pH and O.M, as described in the earlier section.

Statistical Analysis

The data collected from the treatment effects of organic residues on the growth parameters, such as plant height, leaf area, stem girth, leaf number, shoot weight and tap root length, were analysed using the ANOVA F test technique and their means were separated and compared using the Duncan Multiple Range Test (DMRT) at 5% level.

RESULTS

Initial Soil Fertility Status

Both the physical and chemical properties of the soils used for rising of kola seedlings in the nursery are presented in Table 1. Using the established critical levels for the soils in South West Nigeria, the soils are acidic, and low in organic matter when compared with the critical level of 3% (Agboola and Corey, 1973). In addition, the total % nitrogen was found to be less than 0.15% N, which is considered as the optimum for crops (Sobulo and Osiname, 1981). The available P was less than 10mg/kg o, which is considered as adequate for the production of crop (Agboola, 1982).

TABLE 1
Soil chemical composition before planting kola seedlings

Soil parameters	Values
Soil pH (1:1) soil/water	5.35
Soil pH (0.01M) $CaCl_2$	5.10
Organic matter (%)	0.36
N%	0.03
Available P (mg/kg)	5.36
Exchangeable K^+ (mmol/kg)	0.09
Exchangeable Ca (mmol/kg)	0.08
Exchangeable Mg (mmol/kg)	0.13
Exchangeable Na (mmol/kg)	0.11
Soil bulk density (g/cm^3)	1.58

The exchangeable K values were very low and crop grown on the soils was expected to respond to K application, with 0.2mmol/kg soil being the critical level. The available Ca, Mg and Na were also found to be low, indicating the soils with poor fertility status. The soil was very sandy and low in clay. The soil bulk density was high (1.58 Mg/m) and would adversely affect the crop in terms of its growth. This soil belongs to the Akure series and is an Alfisol (USDA 7^{th} approximation).

The Analysis of the Organic Materials Used for the Experiment

Among the organic residues used, the manures taken from turkey and duck had the highest N, P and the lowest C/N ratios. In particular, the wood ash had the highest K, Ca and Mg concentrations, and this was followed by cocoa husk. The goat dung was indicated to be fairly high in N, P, K and Ca (Table 2).

The Effect of Organic Fertilizers on the Leaf Chemical Composition of the Kolanut Seedlings

The leaf analysis of the kola seedlings for different organic fertilizer sources is presented in Table 3. Based on the results, there were significant increases ($p<0.05$) detected in the leaf N, P, K, Ca and Mg contents as compared to the control.

The amended and sole forms of the organic residues increases the kola leaf K, Ca and Mg contents compared to the NPK fertilizer; however, the NPK was found to increase the leaf N and P more than the organic residues. Among the organic residues, duck manure and amended duck manure with wood ash and cocoa husk increased the kola leaf N, P, K, Ca and Mg as compared to the others.

The sole forms of the turkey manure, duck manure and goat manure had lowered the kola leaf nutrient contents than the amended forms with wood ash and cocoa husk.

The Effects of Organic Fertilizers on the Soil Chemical Properties after the Experiment on Raising Kola Seedlings

Both organic and inorganic fertilizers were found to increase the soil N, P, K, Ca and Mg significantly ($p<0.05$), relative to the control treatment. On the contrary, the NPK fertilizer decreased soil pH and O.M, as compared to the organic fertilizer treatments (Table 4).

The duck manure, cocoa husk and wood ash amended with duck manure gave the highest values of soil N, P, K, Ca, Mg pH and O.M, as compared to other residues. Meanwhile, the organic fertilizers gave the best values of soil Ca and Mg as compared to the NPK fertilizer.

The Effects of Organic Fertilizers on the Growth Parameters of the Kola Seedlings

The plant height, leaf number, stem girth, leaf area, shoot weight and tap root length of kola seedlings, for the different organic fertilizers, are as presented in Table 5. The organic fertilizers were found to increase the growth parameters of the kola seedlings significantly ($p<0.05$), relative to the control.

TABLE 2
Analysis of the organic materials used for the experiment on raising kola seedlings

Treatment	C/N ratio	N (%)	P mg/kg	K	Ca	Mg mg/kg	Fe	Zn	Cu
Cocoa husk	11.0	1.44	100	20.6	9.3	7.1	50.4	1.69	0.16
Wood ash	11.8	1.53	86	23.0	9.4	8.5	65.5	1.83	0.16
Goat manure	7.9	1.82	168	10.0	2.9	4.5	34.5	1.30	0.16
Duck manure	7.2	2.10	260	6.6	1.9	1.5	21.3	1.13	0.16
Turkey manure	7.10	3.86	346	7.9	2.1	1.8	9.1	1.16	0.14

TABLE 3
The leaf chemical composition of kola seedlings under different organic fertilizers

Treatment	N	P	K	Ca	Mg
			%		
Duck manure (sole)	1.90^{f}	0.32^{d}	1.63^{e}	0.78^{de}	0.33^{c}
Turkey manure (sole)	1.65^{c}	0.28^{c}	1.53^{d}	0.72^{d}	0.36^{d}
Goat manure (sole)	1.48^{b}	0.25^{b}	1.20^{c}	0.63^{c}	0.32^{b}
Goat manure + cocoa husk	1.78^{d}	0.36^{e}	2.10^{f}	1.56^{f}	0.72^{e}
Goat manure + wood ash	1.80^{f}	0.42^{g}	2.43^{g}	1.63^{g}	0.75^{f}
Duck manure + cocoa husk	2.16^{h}	0.43^{h}	3.70^{j}	2.55^{j}	1.26^{i}
Duck manure + wood ash	1.85^{fg}	0.53^{i}	3.90^{k}	2.76^{k}	1.35^{j}
Turkey manure + cocoa husk	1.80^{f}	0.42^{g}	3.20^{h}	2.50^{h}	1.20^{g}
Turkey manure + wood ash	1.79^{de}	0.41^{f}	3.50^{i}	2.52^{hi}	1.23^{h}
NPK 15-15-15	2.23^{i}	0.56^{ij}	0.93^{b}	0.4^{ab}	0.3^{a}
Control	1.25^{a}	0.2^{a}	0.30^{a}	0.2^{a}	0.2^{a}

Treatment means, within each group followed by the same letters, are not significantly different from each other, using DMRT at 5% level.

TABLE 4
The soil chemical composition of kola seedlings under different organic fertilizers

Treatment	N (%)	P mg/kg	K	Ca mg/kg	Mg	Soil pH	O.M %
Duck manure (sole)	0.19^{d}	19.36^{d}	0.83^{e}	0.50^{e}	0.24^{f}	6.80^{e}	1.16^{e}
Turkey manure (sole)	0.18^{c}	17.26^{c}	0.74^{d}	0.48^{d}	0.22^{e}	6.40^{c}	0.98^{da}
Goat manure (sole)	0.15^{b}	15.60^{b}	0.52^{b}	0.36^{b}	0.16^{c}	6.20^{b}	0.70^{c}
Goat manure + cocoa husk	0.20^{e}	19.10^{d}	0.63^{c}	0.42^{c}	0.18^{d}	6.60^{d}	0.99^{d}
Goat manure + wood ash	0.22^{g}	0.94^{f}	0.94^{f}	0.46^{c}	0.22^{e}	6.90^{ef}	1.20^{f}
Duck manure + cocoa husk	0.23^{h}	24.4^{g}	1.24^{i}	0.96^{hi}	0.56^{hi}	7.10^{g}	2.10^{hi}
Duck manure + wood ash	0.33^{j}	26.3^{h}	1.34^{j}	0.92^{h}	0.58^{j}	7.20^{gh}	2.40^{j}
Turkey manure + cocoa husk	0.21^{f}	22.10^{f}	1.05^{fg}	0.85^{fg}	0.52^{g}	7.00^{f}	1.85^{g}
Turkey manure + wood ash	0.27^{i}	23.0^{f}	1.19^{h}	0.81^{f}	0.55^{h}	7.00^{f}	1.96^{h}
NPK 15-15-15	0.36	27.60^{i}	0.66^{c}	0.03^{a}	0.04^{ab}	5.10^{a}	0.25^{a}
Control	0.02^{a}	3.40^{a}	0.04^{a}	0.02^{a}	0.02^{a}	5.10^{a}	0.25^{a}

Treatment means, within each group followed by the same letters, are not significantly different from each other using DMRT at 5% level.

TABLE 5
The growth parameters of kola seedlings under different organic fertilizers treatments between 2 and 24 weeks of planting (WAP)

Treatments	Shoot weight(g)	Plant height(cm)	Tap root length(cm)	Leaf area (cm^2)	Leaf number	Stem girth (cm)
Duck manure (sole)	180.2^{f}	18.2^{f}	8.5^{c}	26.8^{d}	5.0^{d}	0.83^{d}
Turkey manure (sole)	140.1^{c}	15.8^{c}	7.3^{b}	24.5^{c}	4.0^{b}	0.76^{c}
Goat manure (sole)	130.0^{b}	13.4^{b}	7.0^{b}	22.6^{b}	4.0^{b}	0.50^{b}
Goat manure + cocoa husk	163.2^{d}	17.3^{e}	10.4^{d}	27.6^{de}	4.4^{bc}	0.92^{e}
Goat manure + wood ash	175.3^{e}	16.2^{cd}	11.0^{de}	28.2^{f}	5.1^{de}	0.96^{ef}
Duck manure + cocoa husk	193.2^{i}	28.4^{j}	13.3^{h}	32.4^{i}	7.6^{h}	1.16^{h}
Duck manure + wood ash	201.4^{j}	31.6^{k}	14.8^{i}	42.3^{j}	8.2^{i}	1.46^{i}
Turkey manure + cocoa husk	185.1^{g}	26.1^{h}	12.0^{g}	30.5^{g}	7.0^{g}	1.00^{f}
Turkey manure + wood ash	190.0^{h}	27.2^{hi}	13.8^{f}	31.1^{h}	7.4^{gh}	1.10^{fg}
NPK 15-15-15	188.2^{h}	23.2^{g}	11.8^{f}	27.5^{de}	6.0^{f}	0.92^{e}
Control	32.10^{a}	6.3^{a}	4.1^{a}	9.3^{a}	3.6^{a}	0.26^{a}

Treatment means, within each group followed by the same letters, are not significantly different from each other, using DMRT at 5% level.

Among the organic fertilizers, the duck manure (sole), wood ash/duck manure mix and cocoa husk/duck manure mix gave the highest values of plant height as compared to the others. The amended organic fertilizers were found to increase the plant height, leaf area, leaf number, stem girth, tap root length and shoot weight of the kola seedlings much more than the NPK fertilizer.

The amended residues resulted in superior growth compared to the sole forms. The NPK fertilizer, however, increased the growth parameters more than the sole forms of duck, goat and turkey manures.

DISCUSSION

The poor growth of the kola seedlings, in the nursery under the control treatment, was consistent with the low nutrient status of soil K, Ca, Mg, Na, O.M and pH; this fact is supported by Agboola (1982) who had identified poor soil fertility as the main factor in reduced crop yields. The increase in the soil and leaf N, P, K, Ca, Mg soil pH and O.M of the kola seedlings, under the organic fertilizer treatments, was consistent with their chemical composition (Table 3). The view is also corroborated by Swift and Anderson (1993) who reported that organic manures supplied nutrients which NPK fertilizer could not supply to the crops. This showed the potentials of organic fertilizers in increasing the yield of crops.

The increase in the soil pH, through the use of organic fertilizers as compared to the NPK fertilizer, could be responsible for a better growth rate of the kola seedlings because it would favour nutrient release; this view is supported by Raymond (1990) who reported the importance of neutral soil pH in effective nutrient release. In addition, Tisdale and Nelson (1996) also reported that N is important in vegetative growth, protein synthesis and root formation of crops. The best growth performance recorded for the kola seedlings, under the duck manure and turkey manure (sole), wood ash and cocoa husk amended with duck manure, and turkey manure as compared to the others, could be due to the superiority of their nutrients and the low C/N ratio. This is supported by Folorunso (1990) who reported the importance of plant residues (cocoa husk and wood ash) in increasing crop yields.

CONCLUSIONS AND RECOMMENDATIONS

The current study proved that the duck manure and turkey manure (sole) and their amended forms with cocoa husk and wood ash increased the soil, leaf and growth performance of the kola seedlings in the nursery. For this reason, farmers are encouraged to adopt their use at 8 t ha^{-1} for the nursery and field production of kola seedlings.

REFERENCES

Abulude, F.O. (2004). Composition and properties of cola nitida and cola accuminata flour in Nigeria. *Global Journal of Pure and Applied Sciences, 10*(1), 11-16.

Adeyeye, E.I. and Ayejuyo, O.O. (1994). Chemical composition of *Cola acuminate* and Garcinia kola seeds grown in Nigeria. *Int. J. Fd. Sci. and Nutr., 45,* 223-230.

Agboola, A.A. (1982). Soil testing, soil fertility and fertilizer use in Nigeria. *1st National Seminar in Agricultural Land Resources* (pp. 6-8), September 15-18, Kaduna.

Agboola, A.A. and Corey, R.B. (1973). Soil testing N, P, K for maize in the soil derived from metamorphus and igneous rocks of Western State of Nigeria *Jour. West African Sci. Ass.*, *17*(2), 93-100.

Folorunso, O.O. (1999). Use of plant residues for improving soil fertility and the yield of Okra (*Abelmoshuc esculentum* L.) and Amaranthus (*Amaranthus vividis* L.). Ph.D Thesis, Federal University of Technology, Akure.

Jackson, M.L. (1964). *Soil Chemical Analysis.* Englewood Cliffs, New York: Prentice Hall Inc.

Murphy, J. and Riley, J.P. (1962). A modified single solution method for determination of phosphate in natural waters. *Analytical Glumi Acta, 27,* 31-36.

Obatolu, C.R. (1995). Nutrient balance sheet after coffee and maize cropping on an Alfisol supplied with organic fertilizers in Ibadan, Nigeria. *Proc. 3rd all Afric. Soil Sci. Soc.*, (pp. 250), August 20-23, Ibadan.

Oladokun, M.A.O. (1990). Vegetative propagation of kola nut trees. *Ife Jour. Agric. Forestry, 1&2,* 25-30.

Raymon, W.M. (1990). *An Introduction to Soils and Plant Growth.* Englewood Cliff, N.Y., USA: Prentice Hall Inc.

Sobulo, R.A. and Osiname, O.A. (1981). Soils and fertilizer used in Western Nigeria, *Resource Bulletin* (11), I.A. Rot, Univ. of Ile-Ife, 20-26.

Tisdale, S.L. and Nelson, W.L. (1966). *Soil Fertility and Fertilizer.* New York: Macmillan Publisher Ltd.

Walkley, A. and Black, I.A. (1934). An examination of degtagJaroff method for determining soil organic acid filtration method. *Soil Sci., 37,* 29-38.

Swift, M.J. and Anderson, J.M. (1993). Biodiversity and ecosystem function in agricultural systems. In Schultz *et al.* (Eds.), *Biodiversity and ecosystems function* (pp. 201-203). Berlin, Germany: Springer–Verlag.

Pertanika J. Trop. Agric. Sci. **32(1)**: 25 - 27 (2009) ISSN: 1511-3701

Short Communications

Acceptance and Rejection of Peer-reviewed Articles in Environmental Sciences: My Personal Publication Experience

Yap, C.K.
Department of Biology, Faculty of Science,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
E-mail: yapckong@hotmail.com

INTRODUCTION

I am a young lecturer and have so far published 50 peer-reviewed papers from 2002 until March 2007. Still, I think I can contribute more by writing more papers in the future. This article should be somewhat motivating and thought provoking as well as encouraging, if it were to be published in a peer-reviewed journal. The objectives of this article are: (i) to encourage research students and new researchers to write so as to share their knowledge by publishing their findings, and (ii) to share my personal publication experience.

WHY DO WE NEED TO REFER TO PEER-REVIEWED PAPERS?

When I was a student, I was always not confident of what I was doing and regarded international peer-reviewed articles as major references for my research work. When I became a lecturer, I again referred to related peer-reviewed articles for the sake of updating my knowledge and as references. I always tell my students about the importance of peer-reviewed articles for their project work and to improve their future career prospects with a good number of publications. When I was doing my Masters and PhD research projects, I referred to many peer-reviewed scientific journals, either published internationally or locally. Some of them had a relatively high impact factor of more than 2.0. Although some of them had very low impact factor or were not even listed under the International Science Index (ISI) or Science Citation Index (SCI), they were still very useful for my research work. To start as a research student, good peer-reviewed papers are sources of research activities and ideas for doing research work. Besides following methodologies which had been published and done by others on other species, similar ecological and ecotoxicological work, from this country and sometimes this region, had not been reported using the species I had chosen. Although capable of doing a similar type of work, being able to come up with new ideas of presenting the data once again requires appreciable scientific understanding and creativity or innovation, apart from, of course a wide range of knowledge, hard work and lots of perseverance.

A continuation of other people's work or doing a similar type of work which had been done by other prominent scientists using quite a similar methodology, but on other organisms or ecoregion, is acceptable for a start. Since science is about knowing, searching for new pieces of knowledge or to discover interesting phenomena, a research student who is doing ecological research should read a lot and refer to papers related to the ecological species that they are working on.

Received: 28 November 2007
Accepted: 3 September 2008

WHY DO WE NEED TO PUBLISH?

To be confident of what we have learnt through our research activities, our learning process and scientific values need constructive comments, although they are sometimes hard to accept. Peer-reviewed comments are given by the experts in our field of study. The comments given, if constructive, should be regarded as the 'stepping stones' to make our scientific values and understanding better.

Scientists are measured by their publications (Tregenza, 2002) and peer review of journal articles and other technical reports are the key elements in the maintenance of academic integrity (Meier, 1992). Knowing these, writing a scientific journal paper is greatly encouraged by any institutions of higher learning.

'Publish or perish!' has always been a warning given to any researchers or academicians hired in institutions of higher learning, particularly if they are paid to provide scientific articles. Currently, every lecturer in a world class university is required to produce a few peer-reviewed articles with high citation per year. Publication of any scientific work has been an important criterion in the consideration for the promotion of an academician. Hence, the writing of peer-reviewed scientific papers is always focused upon by researchers or academicians.

SOME FACTORS IN DETERMINING THE ACCEPTANCE OR REJECTION OF PEER-REVIEWED PAPERS

Apart from depending upon the merit of the work, there are other factors which could influence the acceptance or rejection of manuscripts including personal biases and preconceptions of the referees and editors involved (Bonnet *et al.*, 2002; Cassey and Blackburn, 2003). According to Tregenza (2002), a manuscript with multiple authors and by native English speakers is more successful. Gosden (2003) explained that this could be due to the fact that non-native speakers of English or novice researchers having to face the challenging task of framing effective replies to referees' criticisms. Based on my personal experience, reviewers usually look at the significant points and findings of my work. I had an experience in which my paper was rejected and this was solely due to the problem of 'bad English language presentation.' According to Bonnet *et al.* (2002), scientists who worked on less 'popular' organisms would usually find difficulty in getting their papers published and they complained that referees were biased against them. Based on this view, my publication experience seems to agree with the finding of Bonnet *et al.* (2002) since I usually work on well-studied and popular species.

MY PUBLICATION EXPERIENCE

The publication experience includes papers being rejected and accepted. However, I think the most important thing is the constructive comments given by the referees, rather than the disappointment of having a paper rejected. It is the good comments by the experts in our field of specialization which will certainly help to shape our understanding and positive scientific values on a particular point of discussion in the manuscripts which are submitted for consideration for publication in an international journal. Although I think that I am still a new and young researcher, the above facts about my journal publication experience should be shared with other people and maintained throughout my academic career.

From my publishing experience, I have learnt a lot by reading the comments given by the referees of international journals. This is because the reviewers whom I had suggested, when I were requested by the journal, are among the best researchers who have published numerous papers in good and highly cited international journals. Almost all of them are professors in the field which is relatively similar to my specialization.
To focus on the work, devotion of time by the authors [sometimes at the sacrifices of time spent for the family] is required and they should stay focus, particularly during the writing process. As for me, when there is no new idea coming into my head, I will keep the draft papers aside until a new 'wave' of ideas comes. In the meantime, I keep searching for as much as information related to the topics which are necessary to explain any ecological and ecotoxicological findings.

This is the reason why some of my papers took me more than 18 months to draft, in addition to the fact that they were still not up to my satisfaction to be submitted for publication. A paper is usually ready to be edited by an English editor after at least 10 drafts, and after that, it is ready to be submitted for consideration for publication in a peer-reviewed journal.

When you have come to a stage whereby your research students have collected a lot of data, you should encourage the students to write or report the data in the form of scientific articles, besides their theses. Writing any articles requires an application of knowledge from many disciplines, scientific understanding and skills, experience or a combination of all of the above. As for me, the 'feeling' and the enthusiasm of presenting an important 'imaginary idea' and facts on an ecotoxicological phenomenon are equally of significant importance.

The preparation of a good journal paper requires a lot of time, devotion and mental concentration, and this is a good way to train research students and any academicians who wish to become up-to-date researchers. Getting your research data accepted and published is an achievement for any researcher and you should be proud of it. An equally important contributing factor is the enthusiasm to do research, which must be maintained even after your research work has been published in a peer-reviewed journal. This is becoming a norm in any academician's life. Finally, it is hoped that this article will be an encouragement to all researchers. Writing a research paper is a matter of knowledge searching [besides of course, reporting the findings] and self discipline, especially in term of time management, while publication of the research data is a contribution to science. Hence, keep on writing and never give up, as 'keep on doing what you have started in the first place and you will finally succeed in completing a task once considered an impossible mission.' To all, I wish you all the best in your publication endeavours.

REFERENCES

Bonnet, X., Shine, R. and Lourdais, O. (2002). Taxonomic chauvinism. *Trends in Ecology and Evolution*, *17,* 1-2.

Cassey, P. and Blackburn, T.M. (2003). Publication rejection among ecologists. *Trends in Ecology and Evolution*, *18,* 375-376.

Gosden, H. (2003). Why not give us full story? Functions of referees' comments in peer reviews of scientific research papers. *Journal of English for Academic Purposes*, *2,* 87-101.

Meier, A. (1992). How to review a technical paper. *Energy and Buildings*, *19,* 75-78.

Tregenza, T. (2002). Gender bias in the refereeing process? *Trends in Ecology and Evolution*, *17,* 349-350.

Pertanika J. Trop. Agric. Sci. **32(1)**: 29 - 33 (2009)

ISSN: 1511-3701

Short Communications

Growth and Phenology of Kenaf (*Hibiscus cannabinus* L.) Varieties

Nor Aini Ab. Shukor[1*], Mohd Basri Hamzah[1], Hazandy Abdul Hamid[2], Ghizan Salleh[3] and Mohd. Fadzhel Mohd Nasir[4]

[1]Laboratory of Sustainable of Bioresource Management, Institute of Tropical Forestry and Forest Products (INTROP), Universiti Putra Malaysia,

[2]Department of Forest Production, Faculty of Forestry, Universiti Putra Malaysia,

[3]Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[4]National Tobacco Board, Ministry of Plantation Industries and Commodities Malaysia, Kubang Kerian, Mail Box 198, 15720 Kota Bharu, Kelantan, Malaysia

[*]E-mail: anishukor@yahoo.com

INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.) holds a promising potential in the Malaysian biocomposite industry, as its long fibres are suitable in the process of making a number of products such as pulp and paper, fibre and particle boards, as well as fibre reinforced plastic components and chemical absorbent.

It can grow to a height of 4 to 6 m in about 4 to 5 months and yield up to 13-24 tonnes/ha total dry matter production (Angelini *et al.,* 1998; Alexopolou *et al.,* 2000; LeMahieau *et al.*, 2003). The late maturity varieties (e.g. Everglades 41 and Tainung 2) are more productive than the early ones (e.g. PI 3234923 and PI 248901) because they have longer vegetative phase (Alexopoulou *et al.*, 2007). Alexopoulou *et al.* (2000) also reported that the early-maturity varieties could grow up to 267 cm tall and produce 6-9 tonnes/ha of dry matter, while the late maturity varieties could go up to 330 cm tall and produce 13-24 tonnes/ha, respectively. In addition, Danalatos and Archontoulis (2004) stated that the final production and quality of fibres were associated with the duration of its growing period.

Early floral initiation and seed production were found to decrease the vegetative rate which resulted in lower stalk and fibre yield (Dempsey, 1975). According to Gray *et al.* (2006), the early maturing varieties (e.g. Line 42) took 72 days, intermediate varieties (e.g. Line 21 and Line 29) between 85 – 86 days, whereas the late varieties (e.g. Tainung 1, Pandora and Endora) took about 121 to 136 days to flower. The mean value of the seed production for the late flowering varieties (Guatemala 4 and Everglades 71) was 12.8 seeds per capsule and the average seed yield at maturity 0.60 and 1.04 tonnes/ha, respectively (Muchow, 1980; Muchow and Wood, 1983). A definite dry season is necessary to achieve high and good quality seeds. The study was conducted to evaluate growth, as well as to study some flowering and seed production characteristics of nine kenaf varieties.

MATERIALS AND METHODS

Nine kenaf varieties were classified into three groups according to their flowering habits, namely early, intermediate and late maturing. These involved two early (i.e. Q-Ping and KK60), five intermediate (i.e. V19, V132, V36, NS and V12) and two late varieties (i.e. V133 and TK). The field trial utilized a complete randomized block design (RCBD) with three replications, established at the Serdang campus of Universiti Putra Malaysia, in Malaysia. The varieties were randomly assigned to five lines, and each

Received: 3 March 2008
Accepted: 15 October 2008
[*]Corresponding Author

consisted of 50 plants, with a spacing of 10 cm between plants and 75 cm between lines per block. Due to different flowering patterns, the planting of these varieties was carried out one month later in different blocks. The growth characteristics and biomass of these varieties were measured and monitored on monthly basis. Ninety samples per variety were selected to measure the height and basal stem diameter, while fifteen samples per variety were assessed for their biomass. Other parameters include flowering characteristics, such as initiation, duration and 50% flowering and seed production (i.e. the number of pods/plant, weight of pods/plant, the number of seeds/pod and the weight of seeds/plant). Data was analyzed for the analysis of variance (ANOVA) and the Duncan's Multiple Range using the Statistical Analytical System (SAS) package, version 6.12.

RESULTS AND DISCUSSION

The ANOVA tests showed that there were significant differences between the varieties, in terms of height, basal stem diameter and biomass (Table 1). In more specific, Variety V133 outperformed the others by producing the highest mean total height and basal stem diameter of 286.60 cm and 21.87 mm respectively, in four months. All the varieties (except NS) displayed good growth in the first two months but slowed down in subsequent months. However, growth reduction intermediate and late-flowering varieties was in much less than those for early-flowering varieties (*Fig. 1*).

V133 also took longer time to initiate and achieve 50% flowering at 88 and 146 days respectively and generated more energy used mainly for its prolific vegetative growth (*Fig. 2*). Such postulation is supported by Alexopoulou *et al.* (2000) who found that the late-maturing varieties grew taller (330 cm), exhibited a higher growth rate and developed larger stem diameters (15.36mm) as compared to the early-maturing ones (height-267cm, diameter–13.3mm). The vegetative growth of all varieties continued with time. However, growth was gradually decreased at the onset plants UPM flowering growth deaudelaties with age initiated flowering of the flowering phase. According to Petrini *et al.* (1994), kenaf has an indeterminate type of growth, where grew rapidly in the beginning but gradually decreased. The trend in mean height and basal stem diameter increments of these varieties showed that (*Fig. 1*).

TABLE 1
ANOVA and Duncan's Multiple Range Test of growth of *Hibiscus cannabinus* L. varieties

Variety	Height (cm)	Basal stem diameter (mm)	Leaf dry weight (g)	Branch weight (g)	Stem weight (g)	Root weight (g)	Total dry weight(g)	Yield (t/ha)	CR
Q-Ping	244.4^{abc}	14.23^{c}	21.4^{b}	9.5^{b}	59.6^{bc}	9.9^{b}	100.3^{bc}	13.7	6
KK60	221.11^{cd}	15.30^{c}	22^{b}	8.6^{b}	52.4^{bc}	4.8^{b}	87.7^{c}	12.0	7
V19	252.6^{ab}	20.26^{a}	28^{ab}	11.3^{ab}	68.8^{abc}	13.4^{ab}	121.4^{abc}	16.6	2
V132	231.9^{bcd}	18.56^{ab}	33.9^{a}	10.9^{b}	89.1^{a}	14.3^{ab}	148.1^{a}	20.3	2
V36	239.9^{bcd}	18.89^{ab}	23.5^{b}	10.7^{b}	60.1^{bc}	10.7^{b}	105^{bc}	14.4	5
NS	229.7^{cd}	16.69^{cd}	21.6^{b}	8.8^{b}	44.6^{c}	8.4^{b}	83.4^{c}	11.4	8
V12	197.3^{d}	14.91^{d}	27^{ab}	13^{ab}	43.7^{c}	7.5^{b}	91.2^{c}	12.5	8
V133	286.6^{a}	21.87^{a}	24.7^{b}	12.4^{ab}	78.6^{ab}	11.1^{b}	126.9^{ab}	17.4	1
TK	243.5^{abc}	16.59^{bc}	33.5^{a}	16.3^{a}	56.8^{bc}	23.5^{a}	129.9^{ab}	17.8	4
ANOVA (between varieties)	*	*	*	*	*	*	*		

*significance at $p \leq 0.05$, CR= Composite ranking

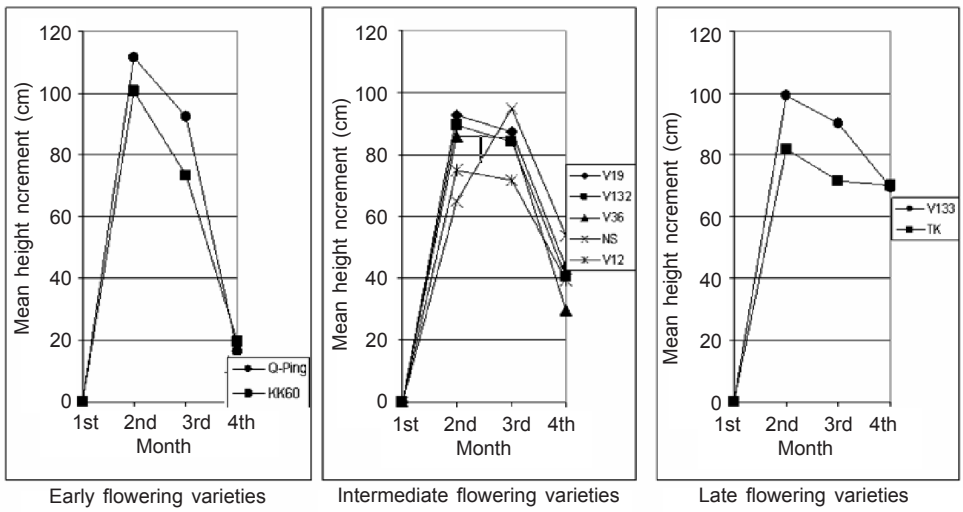


Fig. 1: The mean height increment of Hibiscus cannabinus *L. varieties over 4 months*

Concurrently, all the the early, intermediate and late-flowering varieties on average, initiated flowering within show 43-54, 66-76 and 85-101 days, respectively (Table 2). The days to flowering initiation recorded in this study were earlier than the ones by Gray *et al.* (2006) who reported that the early, intermediate and late maturing varieties took 72, 85–86 and 121–136 days, respectively.

The reduction in growth (height, basal stem diameter and biomass) was particularly marked in early-flowering variety since a subdtantrial growth portion of its energy was allocated for reproductive. In comparison, the intermediate and late-flowering varieties were shown to achieve higher growth due to the fact that they remained vegetative, one and two months longer respectively, than the early ones. This finding was supported by Dryer (1967), Petrini *et al.* (1994) and Alexopolou *et al.* (2000) who stated that flower initiation caused reduction in vegetative growth of kenaf.

Biomass and growth, as given in Table 1, were found to vary considerably according to the maturity type of the kenaf varieties. Biomass (dry matter) for these varieties ranged from 12.0 (KK60-early) to 20.3 tonnes ha^{-1} (V132-intermediate). Biomass of the early varieties gradually declined UPM flowering until the end of the reproductive period. In contrast with the early varieties, the intermediate and late ones exhibited a higher biomass. For example, V132 (an intermediate variety) was found to be the most productive in the third to the fourth month as compared to other varieties (Table 1). This is due to the fact that this variety initiated flowering in the third month. Petrini *et al.* (1994) also reported that there was a positive relationship between the productivity of kenaf and the absence of the flowering phase. Similarly, Alexopoulou *et al.* (2000) also reported that the kenaf variety produced a higher biomass before the beginning of the anthesis stage.

The ANOVA test showed significant differences between the varieties in terms of seed production. The early flowering varieties, especially Q-Ping, were found to produce the highest seed yield in terms of the number of pods/plants, weight of pods/plant, and the number of seeds/pod and weight of seeds/plants. On the contrary, both the late- (e.g. TK) and intermediate-flowering varieties (e.g. V19) produced the lowest yields (*Fig. 3*). Moreover, the production of seeds for the intermediate- and late-flowering varieties coincided with the wet season, i.e. during September to November. These varieties were assumed to be sensitive to relative humidity and moisture, affecting the final development of seeds and reducing its production.

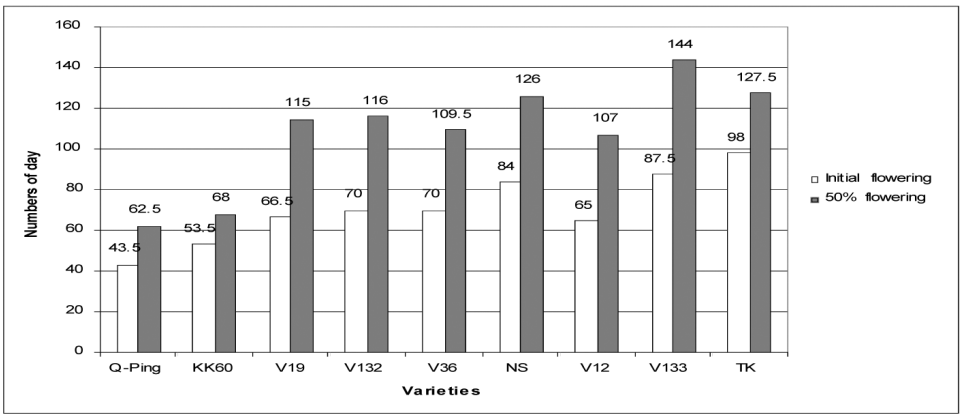


Fig. 2: The average number of days for flower initiation and 50% flowering of Hibiscus cannabinus *L. varieties*

TABLE 2
The number of days taken for flowering initiation, flowering duration and 50% flowering of kenaf varieties

Variety		Range (Days)		
		Flowering initiation	Flowering duration	50% flowering
Q-ping	Early	40-47	40-113	59-66
KK60		46-61	46-119	60-76
V19		64-69	64-154	98-132
V132		64-76	64-143	102-130
36	Intermediate	66-74	66-145	83-136
NS		75-93	75-143	113-139
V12		62-68	62-139	94-120
V133	Late	82-93	82-159	138-150
TK		88-108	105-158	106-149

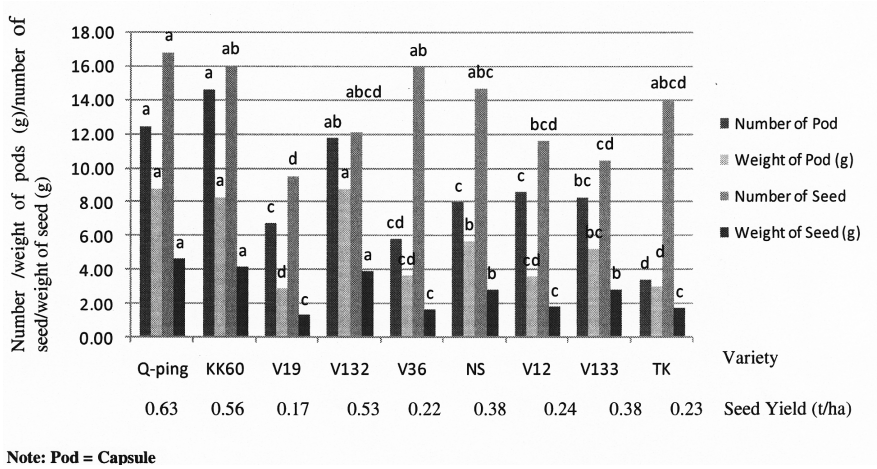


Fig. 3: The mean number of pods, weight of pods, number of seeds per pods and the weight of seeds of Hibiscus cannabinus *L. varieties*

The seed yields varied from 0.17-0.63 tonnes/ha, within the range reported by Muchow (1980) who found that a late flowering variety (Guatemala-4) produced 0.49 tonnes/ha during the wet season. Its yield could however reach three times higher during dry season (1.6 tonnes/ha) under similar planting density. Furthermore, Fehr and Hadley (1980) reported that these varieties differed in their ability to withstand any excess or deficiency in moisture. Despite the confounding influence of rainfall and moisture, both number and weight of seeds varied within the same classification and showed no linear relationship within them, except for the early variety. This suggests that differences in yield could be explained in terms of availability assimilate and/or dry matter partitioning during seed filling (Muchow and Wood, 1983).

REFERENCES

Alexopoulou, E., Christou, M., Mardikis, M. and Chatziathanassiou, A. (2000). Growth and yields of kenaf varieties in central Greece. *Industrial Crop and Products, 11,* 163-172.

Alexopoulou, E., Kipriotis, E., Papatheohari, Y., Moskov, G. and Georgiadis, S. (2007). Cultivation of kenaf in north-east Greece. Part I – Effect of variety, plant density and irrigation on growth and dry yield. *Journal of Food, Agriculture & Environment,* 129-134.

Angelini, G.L., Macchia M., Ceccarini, L. and Bonari, E. (1998). Screening of kenaf (*Hibiscus cannabinus*) genotypes for low temperature and evaluation of feasibility of seed production in Italy. *Field Crop Research,* 73-79.

Danalatos, N.G. and Archontoulis, S.V. (2004). Potential growth and biomass productivity of kenaf under central Greek conditions: II. The influence of variety, sowing time and plant density. In W.P.M. Van Swaaljh, T. Fjalistrom, P. Helm and A. Grassi, (Eds.), *Biomass for energy, industry and climate protection* (pp. 323-326). Proceedings of the *2nd World Biomass Conference*, 10-14 May, Rome, Italy.

Dempsey, J.M.(1975). Fibre crops (pp. 203-204). Gainesville: The University Press of Florida.

Dryer, J.F.(1967). Kenaf seed varieties. Proceedings of the first conference Kenaf for pulp (pp. 44-46). Gainesville, Florida.

Fehr, W. R. and Hadley, H.H. (1980). *Hybridization of crop plants* (pp. vii-viii). Wisconsin: The American Society of Agronomy, Inc.

Gray, L.N., Simon, G.E., Mariotti, J.A. and Collavino, N.G. (2006). Diallelic analysis of genetic effects determining days to flowering in kenaf. *Journal of Industrial Crops and Production,* 194-200.

Lemahieu, P.J., Oplinger, E.S. and Putnam, D.H. (2003). Kenaf. In *Alternative field crops manual.*http://www.corn.agronomy.wisc.edu/FISC/Alternatives/Kenaf.htm.Perusal on 15/3/2007.

Muchow, R.C. (1980). Effect of plant population and season on kenaf (*Hibiscus cannabinus* L.) grown under irrigation in tropical Australia. III. Seed yield and stem yield at maturity. *Journal of Field Crop Research, 3,* 27-32.

Muchow, R.C and Wood, L.M. (1983). Effect of sowing on the growth and yield of kenaf (*Hibiscus cannabinus*) grown under irrigation in tropical Australia. I. Phenology and seed production. *Journal of Field Crop Research, 7,* 81-90.

Petrini, C., Bazzoci, R. and Montali, P. (1994). Yield potetial and adaptation of Kenaf (*Hibiscus cannabinus*) in north-central Italy. *Industrial Crops and Products, 3,* 11-15.

Pertanika J. Trop. Agric. Sci. **32(1)**: 35 - 42 (2009)
ISSN: 1511-3701

Extract of Indian Green Mussel, *Perna viridis* (L.) Shows Inhibition of Blood Capillary Formation *in vitro*

Massoud Mirshahi[1*], Pezhman Mirshahi[1], Sophie Negro[1], Jeannette Soria[1], Sreekumar, P.K.[2], Savita Kotnala[2], Amu Therwath[1] and Anil Chatterji[2]
[1]UMRS 872, E18, Faculté de Médecine Paris VI, 15, rue de l'Ecole de Médecine, 75006 Paris, France
[2]National Institute of Oceanography, Dona Paula, Goa, 403004, India

ABSTRACT

The extract of the Indian green mussel (*Perna viridis* L.) was found to inhibit the formation of endothelial cell capillary tube in a concentration dependent manner *in vitro*. At a concentration of 5 mg/ml of crude extract, there no formation of intercellular junctions and capillary tubes was observed, even after 6 hours. However, at the concentrations below 1 mg/ml, a few cellular junctions and formation of capillaries began to increase at both concentrations of 0.2 and 0.04 mg/ml. The aqueous fraction was also found to inhibit the capillary and junction formations at 5 mg/ml, but it was less effective at lower concentrations, as compared to the total crude extract. The methanol (MeOH) extract was more active than that of the aqueous extract; hence, it was further fractionated into 5 fractions (F1 to F5) and tested for the presence of angiogenic activity. Fraction 3 (F3), which was used at 100 µg/ml concentration, showed a significant inhibitory effect on the formation of intercellular junctions and tube, whereas the other fractions did not show any activity.

Keywords: Indian green mussel, *Perna viridis* (L.), anti angiogenic activity

INTRODUCTION

Substances which promote or inhibit angiogenesis are of clinical importance. Most of the known derivatives have so far reported short half-lives and undesirable side effects, and thus considerably limiting considerably their use in clinical practices. Therefore, it is important to search for and discover novel molecules in controlling angiogenesis without major side effects. A number of compounds from marine organisms possessing anti-bacterial, anti-coagulant, anti-diabetic, anti-inflammatory, anti-fungal, anti-malarial, anti-tuberculosis and anti-viral properties have recently been reported (Mayer and Hamann, 2005). Similarly, some compounds have been found to significantly affect the cardiovascular and nervous systems (Mayer and Hamann, 2004).

The preliminary attempt to identify the presence of angiogenic modulators, in the crude extract from the Indian green mussel *Perna viridis* (L.), revealed that some of the fractions of the crude extract promoted vasculogenesis in chick, while others showed an inhibitory effect (Bichurina *et al.*, 1994). This finding indicated that the crude extract was likely to contain both substances which promote and inhibit vasculo-endothelial development. This interesting observation prompted the researchers to envisage further investigation on the possible role of the active substances contained in these extracts on the formation of blood capillary and angiogenesis at large.

Received: 20 May 2008
Accepted: 8 October 2008
*Corresponding Author

MATERIALS AND METHODS

Cells Cultures

Human microvascular endothelial cells (HMEC) (Golestaneh *et al.*, 2001), were cultured in endothelial basal medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin (PAA Laboratories Inc, Etobicoke, ON, USA), according to the recommendations of the supplier. To ensure endothelial phenotype of the cell lines, expression of some typical markers (vWR, E-selectin, VEGF-R2) was assessed using the PCR and immunocytochemistry.

The Preparation of Extract

The crude extract (GM) from the Indian green mussel (*Perna viridis* L.) was prepared by the process of acid enzyme hydrolysing as described by Chatterji *et al.* (2004). The extract was freeze-dried with the help of a tabletop freeze dryer (Edward Micromodulo, Germany) and stored till further use. The crude extract was sub-fractionated into methanol (MeOH) and aqueous fractions (GM Aqs). When required, all these fractions (crude, aqueous and methanol) were suspended in RPMI-1640 to achieve different final concentrations (5, 1, 0.2 and 0.04 mg/ml). In the present study, the evaluation of the anti-angiogenic activity, in these fractions, was done using the matrigel bi-dimensional models.

Bi-dimensional Angiogenesis Assay

In the bi-dimensional model (matrigel which is a standardized cancer cell extra-cellular matrix), the endothelial cells were seeded on the matrigel, in presence and absence of; a) crude (GM); b) aqueous (GM Aqs); and c) methanol (MeOH) fractions of the mussel extracts. The experiment was conducted using the growth factors-enriched matrigel to find out about the inhibitory effect on angiogenesis. The human microvascular endothelial cells (HMEC) line was continuously propagated in 75 cm culture dishes, containing RPMI 1640 supplemented with glutamine (1 mM), fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and incubated at 37°C, in humidified 5% CO_2 atmosphere.

To assess the formation of tubule, the HMEC were pre-incubated for 18 hrs, and the concentrations of the different fractions to be tested were increased. After the incubation period, the cells were detached by adding 200 µl of accutase, and the culture flask was again kept at 37°C for 10 minutes. 24 well plates were then coated with 100 µl of Matrigel for 2 hrs at 37°C and layered with HMEC ($2x10^5$/well), which was previously incubated for 18 hrs, with the fractions in serum-free RPMI 1640 to be tested. The control test was performed by layering the untreated endothelial cells.

Fractionation of Extract

In this study, the effects of both methanol (MeOH) and aqueous (Aqs) extracts on angiogenesis were compared. As the MeOh extract was found slightly more effective than aqueous extract, it was further fractionated by gel chromatography using Sephadex (G10), and this was followed by the reverse phase HPLC using a C-18 column [mobile phase -water (40%): Acetonitrile (60%)]. This process was found to yield five fractions. Each fraction was then freeze-dried and re-suspended (100 µg/ml) in RPMI-1640 medium with 10% FCS. After that, the five fractions were further tested to determine the most angiogenesis effective fraction using the bi-dimensional model, as described in the earlier section.

RESULTS

Crude Extract of Indian Green Mussel Inhibited the Formation of Endothelial Cell Capillary Tube

In the bi-dimension matrigel assay, the results showed that the crude extract of the Indian green mussel inhibited the formation of the endothelial cell capillary tube. Initially, the endothelial cells (when plated on matrigel) began to organize in several lines with intercellular junctions. At a later stage, the formation of capillary tubes was detected. A dose dependent inhibition of the formation of capillary tube and intercellular junctions were induced by the crude extract of the Indian green mussel (*Figs. 1, 2* and *3*). At a concentration of 5 mg/ml of the crude extract (GM), there was neither formation of capillaries nor intercellular junctions, even after 6 hours of incubation. At the concentration of 1 mg/ml, a few cellular junctions occurred, but no formation of any capillary tube was detected (*Figs. 1, 2* and *3*). Nevertheless, at both concentrations of 0.2 and 0.04 mg/ml of the crude extract, the number of intercellular junctions, as well as the formation of the capillary tube, began to increase.

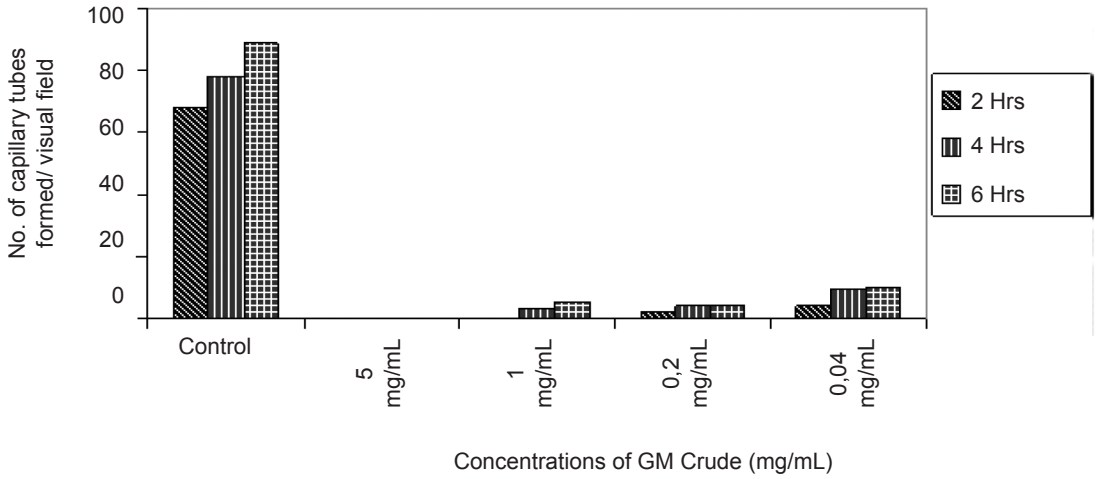


Fig. 1: Dose and time dependant effects of the GM crude on the formation of capillary tubes of human microvascular endothelial cells

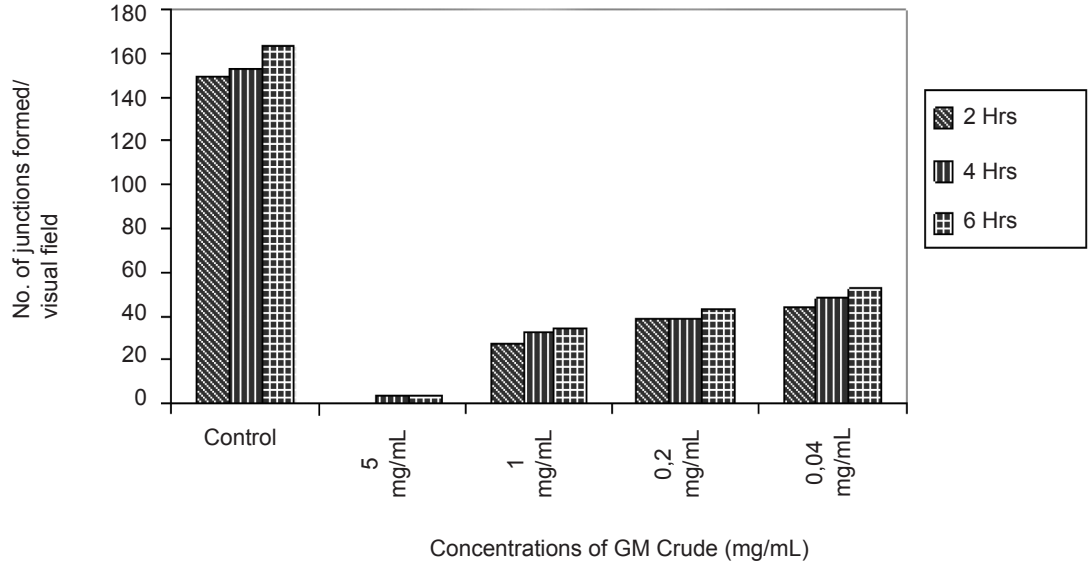


Fig. 2: The dose and time dependant effects of the GM crude on the formation of intercellular junction of human microvascular endothelial cells

Aqueous and Methanol Extracts of Green Mussel Inhibited the Formation of Endothelial Cell Capillary Tube

Although aqueous fractions (GM Aqs) was found to inhibit the formation of capillary and junction at the concentration of 5 mg/ml, it was also indicated as less effective at lower concentrations as compared to the total crude extract (*Figs. 4* and *5*).

The MeOH extract was also shown to inhibit the formation of the capillary tube and junction. At 5 mg/ml of the MeOH extract, neither cell junction nor capillary tube formation occurred (*Figs 6* and *7*). At the concentrations of 1 and 0.2 mg/ml, the MeOH extract was shown to induce a significant decrease in the number of cell junctions and the capillary formation as compared to the control.

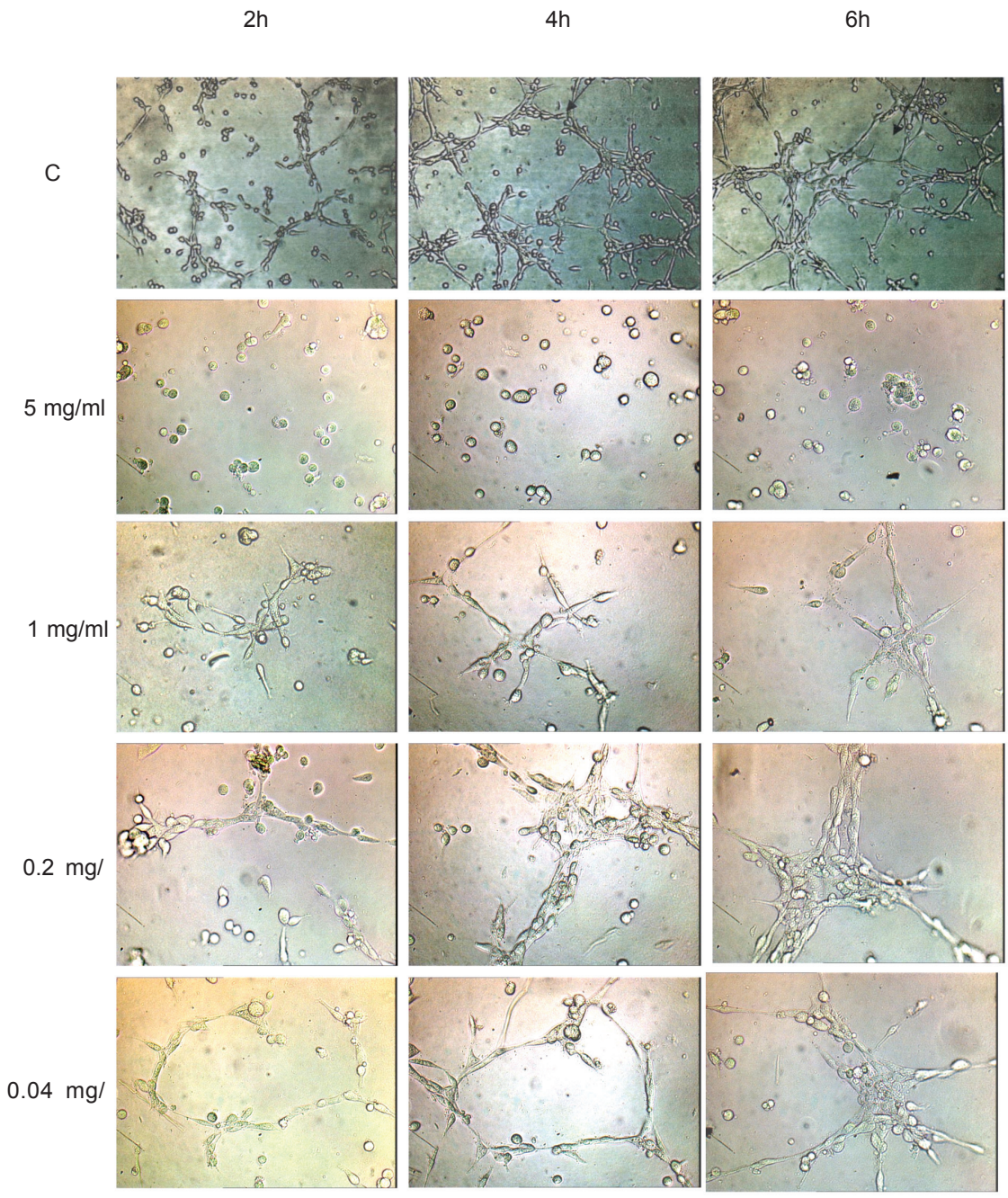


Fig. 3: A dose and time dependent inhibition of the capillary tube formation and intercellular junctions induced by the crude extract of the Indian green mussel

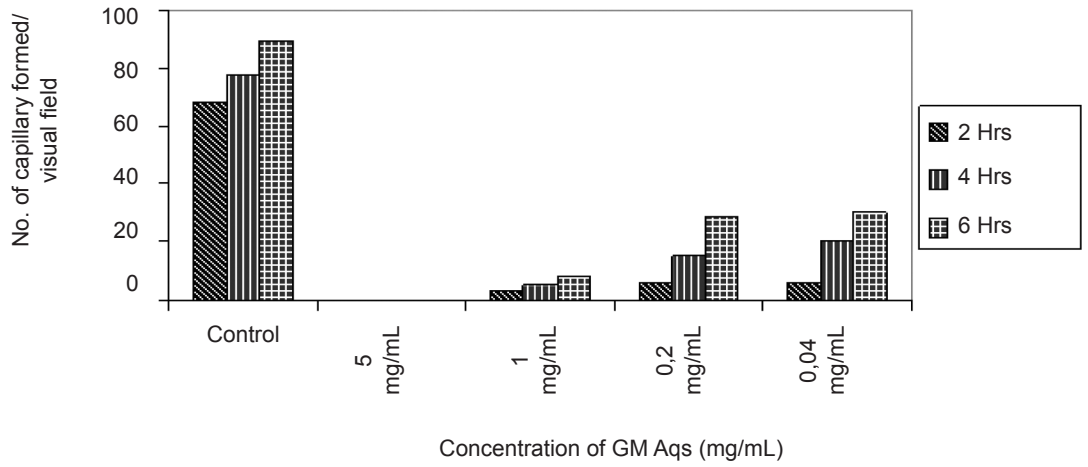


Fig. 4: Dose and time dependant effects of the GM Aqs on the formation of the capillary tubes of human microvascular endothelial cells

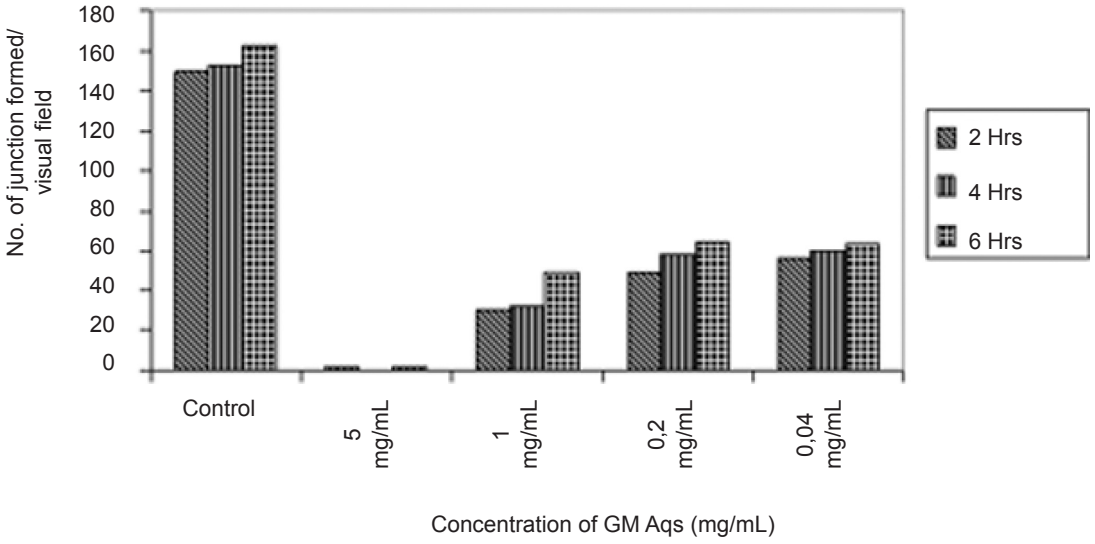


Fig. 5: Dose and time dependant effects of the GM Aqs on the formation of intercellular junction of human microvascular endothelial cells

The results presented in *Figs. 6* and *7* showed that the MeOH extract was found to be more active than that of the aqueous extract. Therefore, only the MeOH extract was used in the subsequent studies. The MeOH extract was further fractionated into 5 fractions (F1 to F5) and tested for the presence of anti-angiogenic activity even up to 18 hrs. As illustrated in *Figs. 8* and *9*, fraction 3 (F3) which was used at 100 µg/ml of the final concentration showed a significant inhibitory effect on the intercellular junctions with respect to the length of capillary (*Fig. 8*) and the number of tube formation (*Fig. 9*), whereas the other fractions did not show any notable activity.

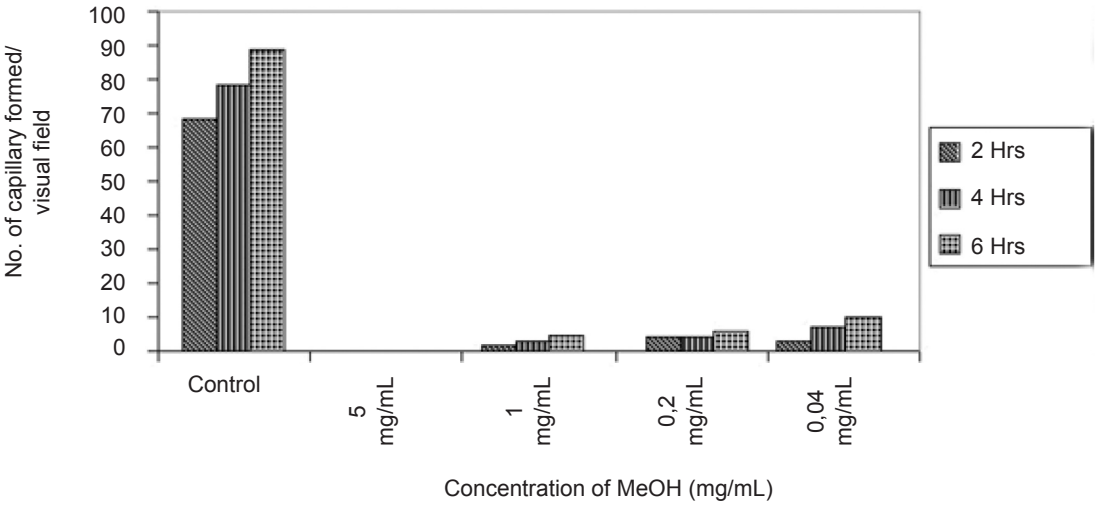


Fig. 6: Dose and time dependant effects of the GM Aqs on the formation of capillary tubes of human microvascular endothelial cells

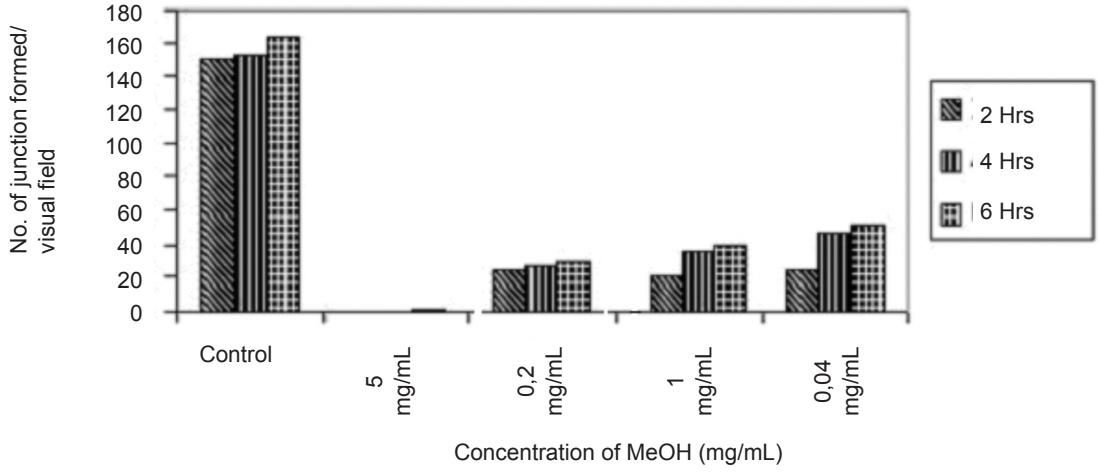


Fig. 7: Dose and time dependant effects of Meoh on the formation of intercellular junction of human microvascular endothelial cells

DISCUSSION

In the course of a screening programme to identify new pharmacologically active compounds from marine organisms, the earlier effort was made by the researchers to search for novel bioactive compounds from the Indian green mussel (Bichurina *et al.*, 1994; Chatterji *et al.*, 2002). Extracts, prepared from the Indian green mussel i.e. *Perna viridis* (L.), showed an inhibition on the formation of osteoclast and based on this finding, it could therefore be used to control osteoporosis (Rao *et al.*, 2003). It also showed the inhibition of HIV virus replication (Mitra and Chatterji, 2004) and the inhibition of replication of *Plasmodium falsiparum* (Malhotra *et al.*, 2003).

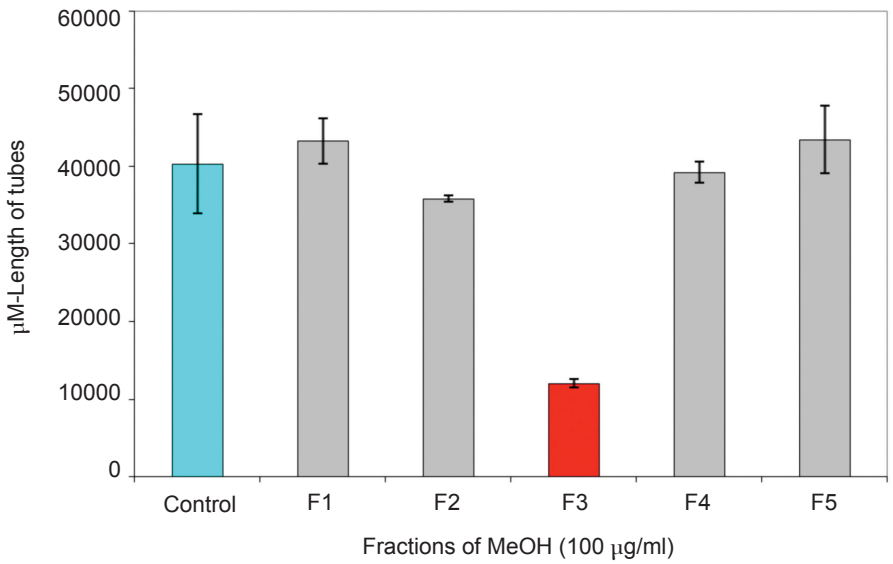


Fig. 8: The MeOH extract was further fractionated into 5 fractions (F1 to F5) and tested for the presence of anti-angiogenic activity, even up to 18 hrs. Fraction No. 3 (F3), used at 100 μg/ml of the final concentration, showed a significant inhibitory effect on the length of capillary

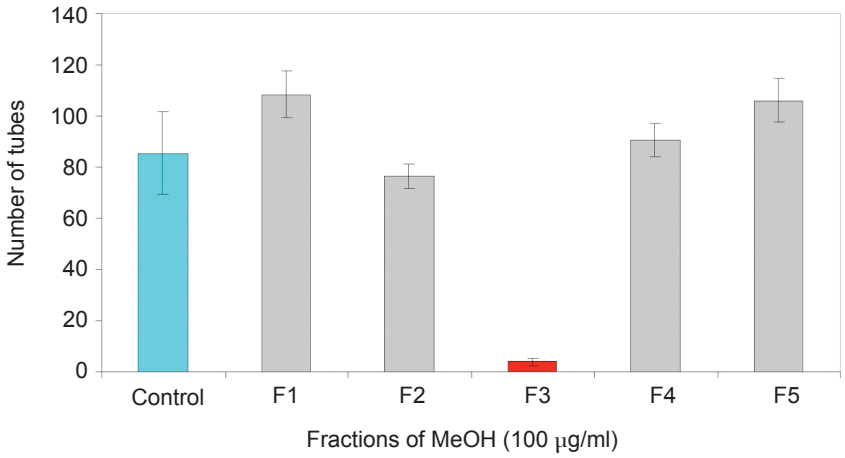


Fig. 9: The MeOH extract was further fractionated into 5 fractions (F1 to F5) and tested for the presence of anti-angiogenic activity, even up to 18 hrs. Fraction No. 3 (F3), used at 100 μg/ml of final concentration, showed a significant inhibitory effect on the intercellular junctions of capillary

Substances or molecules, having anti-angiogenic properties, are useful in cancer therapy. Anti-angiogenic substances can be used to inhibit the growth of tumour mass by preventing neo-vascularization of an early developing tumour. Therefore, such substances are of importance, not only to prevent the development of tumour, but also to get rid of the subsequent metastasis. Thus, pharmaceutical firms, which are aiming at blocking cancer cell proliferation and dissemination, have developed a large number of anti-angiogenic drugs. However, these drugs have adverse effects which include short bench life, high toxicity and high cost.

In this study, it was the first time that the identification, purification and characterization of the compound(s) from the Indian green mussel, *Perna viridis* inhibiting angiogenesis were reported. The anti-angiogenic activity of the green mussel extracts, characterized by a decrease in capillary tube formation, should have interesting applications in several pathologic events such as cancer, inflammation, and age-related macular degeneration (AMD), i.e. a disease associated with ocular neovascularisation.

The present study is therefore of great importance as it has enabled the researchers to identify the anti-angiogenic activity, i.e. a much sought-after property in medical practices, from a marine organism. The purified fraction(s) need to be assayed in the experiments on *in vivo* models. It also has to be subjected to further analysis using the tools of proteomics and gene arrays. At present, various experiments are designed in order to take these observations further so that the researchers could, without long delay, avail of a useful and reliable substance/ molecule in the attempts to contain malignant growth.

ACKNOWLEDGEMENTS

The authors are grateful to the Institute National de la Santé et Researche Médicale (INSERM), Paris, France and the Indian Council of Medical Research (ICMR), New Delhi, India, for their encouragement and support in carrying out the present study.

REFERENCES

Bichurina, M.A., Nikitina, L.E., Sovetora, M.G., Rechina, N.I., Basedina, T.V., Boikov, Y. A. and Noskov, F.S. (1994). Mussels treat humans and animals. *Vopr. Virusol.*, *3*, 134-136.

Boikov, Y., Mukhlenov, A., Bichurina, M. and Dergalyova, I. (1997). Mussels treat humans and animals. *Science in Russia, 5,* 38-41.

Chatterji, A., Ansari, Z.A., Ingole, B.S., Bichurina, M. A., Sovetova, M. and Boikov, Y. A. (2002). Indian marine bivalves: Potential source of antiviral drugs. *Curr. Scie., 82*(10), 1279-1282.

Chatterji, A., Ansari, Z.A., Bichurina, M. and Boikov, Y.A. (2004). An extract obtained from marine animal having antiviral activities and a process for its extraction. Indian Patent # 191701.

Golestaneh, N., Klein, C., Valamanesh, F., Suarez, G., Agarwal, M.K. and Mirshahi, M. (2001). Mineralocorticoid receptor-mediated signaling regulates the ion gated sodium channel in vascular endothelial cells and requires an intact cytoskeleton. *Biochem. Biophys. Res. Commun., 280*, 1300-1306.

Malhotra, P., Dasaradhi, P.V.N., Mohammed, A., Mukherji, S., Manivel, V., Rao, K.V.S., Mishra, G.C., Parameswaran, P.S. and Chatterj, A. (2003). Use of phosphono derivatives of selected aliphatic acids for anti-malarial activity. Indian Patent # 0268NF2003.

Mayer, A.M.S and Hamann, M.T. (2004). Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; affecting the cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action. *Marine Biotechnology, 6,* 37-52.

Mayer, A.S. and Hamann, M.T. (2005). Marine compounds with anthelmintic, antibacterial, anticoagulant, antidiabetic, antifungal, anti-inflammatory, antimalarial, antiplatelet, antiprotozoal, antituberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems and other miscellaneous mechanisms of action. *Comparative Biochemistry and Physiology Toxicology & Pharmacology,* 265-86.

Mitra, D. and Chatterji, A. (2004). Indian green mussel (*Perna viridis*) as a source of anti-HIV activity US Patent #6,770,302 3.

Rao, K.V.S., Wani, M.R., Manivel, V., Parameswaran, P.S., Singh, V.K., Anand, R.V., Desa, E., Mishra, G.C. and Chatterji, A. (2003). Novel molecules to develop drug for the treatment of osteoporosis Indian Patent #0412NF2003, US, 30/12/2003, 10/747,671.

Pertanika J. Trop. Agric. Sci. **32(1)**: 43 - 50 (2009) ISSN: 1511-3701

Antibacterial and DPPH Free Radical-Scavenging Activities of Methanolic Extracts of *Aaptos* sp. (Marine Sponges)

Habsah Mohamad[1*], Zalilawati Mat Rashid[1], Khozirah Shaari[2], Jalifah Latip[3], Md. Nordin Hj. Lajis[3] and Abd. Manaf Ali[4]

[1]Department of Chemical Sciences, Faculty of Sciences and Technology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia
[2]Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[3]Department of Chemical Science, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM, Bangi, Selangor, Malaysia
[4]Faculty of Agrobiotechnology, Universiti Darul Iman Malaysia, 20400 Kuala Terengganu, Terengganu, Malaysia
[*]E-mail: habsah@umt.edu.my

ABSTRACT

This study reports on the evaluation of the antioxidant and antibacterial activities of twelve methanolic extracts (A-L) of *Aaptos* sp., collected from various locations of Terengganu Islands, namely Pulau Bidong, Pulau Kapas, Pulau Perhentian and Pulau Redang. The antioxidant activity of the twelve specimens was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method. The antibacterial bioassay against 5 bacteria, i.e. *Bacillus subtilis* (*gram-positive*), *Escherichia coli* (*gram-negative*), *Bacillus proteus, Streptococus agalatea* and *Streptococus fecalis,* was carried out using the disc-diffusion method. In the DPPH method, all extracts exhibited moderate and strong radical scavenging activity when compared to the standards used, i.e. quercetin and butylated hydroxyanisole (BHA) with the inhibition percentage in the range of 55–89%. In particular, specimen H exhibited the strongest radical scavenging activity with IC_{50} value of 0.1mg/ml. On the contrary, all the specimens showed antibacterial activity at least against one test organism. Interestingly, specimens C, G and L, which were collected from Perhentian, Bidong and Kapas Islands respectively, exhibited weak to strong activity against all bacterial strains. Beside that, specimen F (collected off Redang Island) was weakly bactericidal only against *Bacillus proteus*. Meanwhile, specimen G (collected off Bidong island) was primarily selected for further isolation to yield cholestan-3β-ol and aaptamine.

Keywords: DPPH free radical scavenging, antibacterial, *Aaptos* sp., cholestan-3β-ol, aaptamine

INTRODUCTION

The ocean provides a huge resource bank to the discovery of novel compounds. Marine sponges, as one of the most interesting phyla with respect to pharmacological active marine compounds, were investigated widely in the last decade (Blunt *et al.*, 2005). More than 5000 different compounds have been isolated from about 500 species of sponges (Rifai *et al.*, 2005). An extensive study has also been done on the isolation of bioactive compounds from marine sponges worldwide. However, only a few studies have reported on the isolation of chemical compounds from the Malaysian sponges, and these include *Pseudaxinyssa* sp (Fernandez *et al.*, 1992) and *Leucoploeus fenestrata* (Siraj *et al.*, 1988). A few reports have also revealed cytotoxicity and liver metabolizing enzyme activity of the Malaysian sponge extracts (Abas *et al.*, 1999; Habsah *et al.*, 2005a, 2005b). Recently, the isolation of bioactive compound, from marine sponge-derived fungi, has gained a great attention, which resulted in

Received: 20 May 2008
Accepted: 8 October 2008
[*]Corresponding Author

the isolation of brefeldin A. mycophenolic acid and cladosporin (Nor Ainy *et al.*, 2005).

Aaptos sp., a marine sponge from the family Suberitidae, has been found as a rich source of 1*H*-benzo[*d*,*e*][1,6]-naphthyridine alkaloid, aaptamine which comprises of α-adrenoceptor blocking activity (Nakamura *et al.*, 1982) and other pharmacological activities including anti-tumour, anti-viral, anti-microbial, and PKC or GFAT enzyme inhibitor (Bobzin *et al.*, 2000; Coutinho *et al.*, 2002). To date, a few aptaminoid analogues, including aaptamine, 9-demethylaaptamine, bisdemethylaaptamine, bisdemethylaaptamine-9-*O*-sulfate, isoaaptamine, aaptosamine, aaptosine, 9-demethyloxyaaptamine and 4-methyloxyaaptamine, have been isolated from this species (Nakamura *et al.*, 1982; Rudi and Kashman, 1993; Herlt *et al.*, 2004). Besides, isoagelaxanthin A, 3-[(13-methylhexadecyl)oxyl-1,2-propanediol and 3-[(15-methyloctadecyl)oxyl-1,2-propanediol were also successfully isolated from this species (DNP on CD-ROM, 1982-2001). Considering the importance of *Aaptos* sp, the researchers also made an attempt to isolate anti-bacterial compounds.

MATERIALS AND METHODS

Specimen Preparation

The marine sponges, *Aaptos* sp., were collected via SCUBA at a depth of 8 to 15 meters from Bidong, Kapas, Redang and Perhentian Island, Terengganu. Voucher specimens were deposited at the Museum Biodiversity, Institute of Oceanography, Universiti Malaysia Terengganu. Sponges were cleaned, chopped and dried in air-grafted oven (45°C), prior to extraction with methanol. The extracts were filtered and dried under reduced pressure using a rotary evaporator. The dried extracts were de-salted prior to the analysis. The Methanol extract of 12 specimens were subjected to thin layer chromatography (*Fig. 1*).

Thin Layer Chromatography (TLC)

TLC was perform using TLC sheets (Merck 1.05735.0001), which were pre-coated with silica gel GF_{254} of 0.25 mm thickness, with a mobile phase of chloroform-methanol (8:2). Silica gel plates were visualized under UV 365 nm and UV 254 nm without treatment.

Bacteria

For the purpose of antibacterial evaluation, five bacterial strains, i.e. *Bacillus subtilis* (Gram-positive), *Bacillus proteus*, *Escherichia coli* (Gram-negative), *Streptococus agalatea* and *Streptococus fecalis* were cultured in appropriate broths at 30°C for overnight, and their concentrations adjusted to 10^5-10^6 colony forming units (CFU) per ml, using a spectrophotometer (λ 600nm).

Antibacterial Disc Diffusion Method

The agar cultures of the tested micro-organisms were prepared as described by Mackeen *et al.* (1997). 10 mg of extract was loaded onto each Whatman No. 1 filter paper disc (ϕ 6 mm) and placed on inoculated agar for initial screening. The plates were inverted and incubated for 24 h at 30°C. The presence of antimicrobial activity was confirmed by the occurrence of clear inhibition zones around the disc. The assay was carried out in triplicates. The strength of the activity was classified as 'strong' for the inhibition zone having diameters of ≥15.0 mm, 'moderate' (good) for the diameters ranging from 10.0 to 14.5 mm, and weak for the one with diameters < 10 mm.

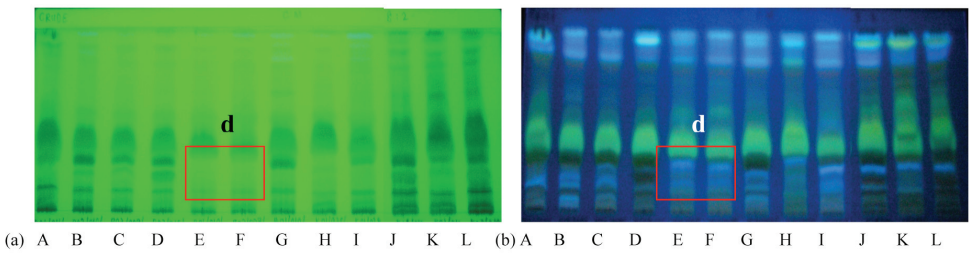


Fig. 1: TLC chromatogram of 12 specimens (band A-L) under (a) 254 nm and (b) UV 365 nm, respectively

DPPH Free Radical Scavenging Assay

The stock solutions of the specimens were prepared at 10 mg/ml in DMSO. The reaction mixture of 50 µl sample was added to 1.95 ml 0.1 mM DPPH solution in a disposable cuvette (Plastibrand® Kartell, 1940). After that, the reaction mixture was shaken and incubated for 30 min at room temperature and the absorbance was read at 517 nm against a blank. The standards used in this assay were butylated hydroxyanisole (BHA) and quercetin. The specimens showing strong activity (D, G, H, J and K) were subjected for further identification to evaluate the IC_{50} values of the different concentrations, using the 96-well micro plate method proposed by Lee *et al.* (1998) with a slight modification. A solution of DPPH was prepared by dissolving 5 mg DPPH in 2 ml of methanol. The stock solutions of the specimens (1 mg/ml) were diluted (two fold dilution) in 96-well micro plates to varying concentrations, topping from 500 ug/ml down to the lowest of 7.81 µg/ml. Then, 5 µl of methanolic DPPH solution was added. Each well was shaken before incubation in a dark place at room temperature. After 30 minutes, the absorbance was read at 517 nm. The assay was carried out in triplicates and calculated using the following formula:

$$\text{Scavenging effect (\%)} = [A_{\text{Blank (517nm)}} - A_{\text{Sample (517nm)}} / A_{\text{Blank(517nm)}}] \times 100$$

A=Absorbance

*Isolation of **1** and **2***

Methanolic extract of B01/010/04 was primarily selected for further isolation because of its significant radical scavenging and antibacterial activities. The extract (150 g) was fractionated by silica gel 60 (0.063-0.200 mm) (70-230 mesh ASTM Merck) gravity chromatography, employing a gradient (0-100% of hexane in chloroform and then from 0-100% chloroform in methanol). Based on their TLC profiles, the fractions were combined to yield 14 fractions. The active fraction 3 (0.5 g) was further purified, using silica gel 230-400 mesh ASTM Merck (0 to 100% hexane in chloroform), to yield **1** (12.9 mg). Fraction 11 (5 g) which was then further purified by silica gel 230-400 mesh ASTM Merck (0 to 100% chloroform in methanol) and to give **2** (250 mg).

*Cholestan-3β-ol (**1**)*

White powder ($CHCl_3$), 12.9 mg: m.p. 128-129°C; $C_{27}H_{48}O$; EIMS (+) ion mode *m/z*: 388 $[M]^+$, 373 $[M + CH_3]^+$, 264, 233, 215, 201, 147; IR (KBr) V_{max}: 3400, 2930, 2850, 1657, 1467, 1375, 1331, 1170, 1137, 1078, 1039 cm^{-1} (for ^1H and ^{13}C NMR data, *see* Table 1) (Gauvin *et al.*, 1998; Dzeha *et al.*, 2002; Santalova *et al.*, 2004).

Aaptamine (***2***)

Greenish yellow crystal ($CHCl_3$), 250 mg: m.p. 111-112°C; $C_{13}H_{12}N_2O_2$; EIMS (+) ion mode *m/z*: 228 $[M]^+$, 213, 183, 170, 142; IR (KBr) *v*max: 3450, 1633, 1325, 1248, 1111, 1026, 777 cm^{-1} (for ^1H and ^{13}C NMR data, *see* Table 1) (Nakamura *et al.*, 1982; Herlt *et al.*, 2004).

RESULTS AND DISCUSSIONS

It is a known fact that sponges contain bioactive compounds which are of potential medical importance (Thakur and Muller, 2004). In this research, the results of the preliminary studies on *Aaptos* sp. were reported for the presence of antibacterial and DPPH free radical scavenging activities. *In vitro,* the antibacterial screening of twelve methanolic extracts of *Aaptos* sp.(A-L) demonstrated activity against one or more bacteria, tested with less activity than standards (gentamycin, streptomycin and penicillin), as shown in Table 2. Specimens J, K and L showed an equally strong activity against *Streptococus fecalis,* whereas specimens H and B exhibited a strong activity against *Bacillus subtilis* and *Streptococus agalatea,* respectively. Considerable antibacterial activity was also shown by few samples against certain bacteria; specimens A, B, D and L against *Bacillus subtilis*; specimens B, C, D, E, H and I against *Streptococus fecalis*; A, D, E, I, K and L against *Streptococus agalatea.* Specimen C displayed a strong activity against *Bacillus proteus*, while other extracts showed only weak activity. Meanwhile, specimens C, G and L showed a weak activity whereas all the remaining extracts were inactive against *Escherichia coli.* In conclusion, specimens C, G and L (collected off Perhentian, Bidong and Kapas islands) showed mostly strong activity against all bacteria and in contrary to this, specimen F (collected off Redang island) was weakly bactericidal against only one bacterium, i.e. *Bacillus proteus.*

TABLE 1
^{1}H and ^{13}C NMR assignments for cholestan-3β-ol (**1**) and aaptamine (**2**)

Position	A1			A2		
C#	δH (J_{HH})	δC	HMBC	δH (J_{HH})	δC	HMBC
1		37.22	C1/H19			
				7.15 *d*	142.49	H2/C4,C12,C13
2		35.7	C2/H19	(6.0)		
				6.27 *d*	99.08	H3/C2,C13
3	3.59 m	71.6		(6.0)		
4		45.07			151.52	
5		54.57	C5/H19			
				7.69 *d*	130.21	H6/C3,C4,C8
6		21.47		(6.0)		
				6.77 *d*	102.25	H7/C6,C13
7		24.43		(6.0)		
8		31.75			135.10	
9		56.49		6.92 *s*	117.90	H9/C10,C11,C13,
10		32.3			158.77	
10'				3.97 *s*	57.06	H10'/C10,C11'
11		19.82			133.27	
11'				3.85 *s*	61.22	H11'/C11,C10'
			C12/H18		134.16	
12		40.26	C17,C14			
13		36.38			114.37	C13/H7,H9
14		42.81	C14/C12,C17,H18			
15		24.05				
16		28.47				
17		56.71	C17/H21			
18	0.65 *s*	12.29	H18/C12,C14,C17			
19	0.81 *s*	12.54	H19/C1,C5,C9			
20		38.44				
	0.90 *d*					
21	(6.6)	18.88				
22		36.01	C22/H21			
23		28.23	C23/H26			
24		39.73	C24/H26			
25		28.95	C25/H26			
	0.87 *d*					
26	(6.6)	22.78	H26/C27			
	0.86 *d*					
27	(6.6)	23.04	H27/C26			

All spectra in $CDCl_3$, ^{1}H at 400 MHz, ^{13}C at 400 MHz; assignments by ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, and HMBC experiments acquired on a Varian-Unity INOVA spectrometer.

TABLE 2
Antibacterial activity of methanol extracts of *Aaptos* sp. collected from various locations

Code	Specimens	Bacteria species*				
		B.sub	B.Pro	S.fea	S.aga	E.coli
P01/011/04	A	+	+	-	+	-
P02/010/04	B	+	+	+	++	-
P02/009/04	C	++	++	+	++	+
P03/015/04	D	+	+	+	+	-
R01/010/04	E	-	+	+	+	-
R03/007/04	F	-	+	-	-	-
B01/010/04	G	++	+	++	++	+
K01/025/04	H	++	+	+	-	-
K01/028/04	I	-	+	+	+	-
K01/010/05	J	-	+	++	++	-
K02/011/05	K	-	+	++	+	-
K03/010/05	L	+	+	++	+	+
	Gentamycin	39	15	19	21	19
Control	Penicillin	-	-	18	-	-
	Streptomycin	20	20	20	19	20

Note: B - Bidong Island; R - Redang Island; P - Perhentian Island; K - Kapas Island
* B.sub: *Bacillus subtilis*; B.pro: *Bacillus proteus*; S.aga: *Streptococus agalatea*; S.fea: *Streptococus* sp.; E.coli: *Escherichia coli* * (–) No activity, (+) weak activity (7–10-mm halo), (++) good activity (10–15-mm halo)

Twelve crude extracts of *Aaptos* sp. (A-L), from different localities, were assayed for antioxidant activity using DPPH free radical scavenging assay (Table 3). Five specimens (D, G, H, J and K) exhibited a strong free radical scavenging although they were less active as compared to butylated hydroxyanisole (BHA) and quercetin with the inhibition percentages in the range of 79-89%. The remaining extracts showed only moderate to weak activity, with the inhibition percentages in the range between 55-78%, with the weakest activity detected for the specimens collected from Redang Island; E and F (inhibition percentage of 55.37% and 58.89%, respectively). The five specimens, which showed strong activity, were further analyzed to determine the concentration values for their 50% inhibition of DPPH free radical scavenging activity (IC_{50}), using different concentrations (2-fold dilution) topping from 7.81 to 500 µg/ml. The IC_{50} value of specimens D, G, H, J and K ranged from 0.1 to 0.12 mg/ml. *In vitro* screening of antioxidant and antibacterial activity of 12 methanolic extract of *Aaptos* sp. showed that the specimen collected off Bidong Island (G) displayed potential significant activity. Unlike other specimens, which somehow exhibited moderate activity in both assays, specimens E and F had weak activities. According to the TLC profiling of all 12 specimens (*Fig. 1*), the bands in area d were absent in the TLC profile of E and F. These could justify the low DPPH free radical scavenging activity of specimens E and F.

Cholestan-3β-ol (**1**) was purified from the hexane fraction and its structure was confirmed by comparing the spectral data with the literature values. The EIMS spectrum showed the molecular ion peak at m/z 388.2, indicating the molecular formula as $C_{27}H_{48}O$ (Gauvin *et al.*, 1998). The other fragment ions were at *m/z* 373 [M-CH_3] (28), 233 ($C_{16}H_{25}O^+$) (63), 215 ($C_{15}H_{19}O^+$) (100), 147($C_{11}H_{15}^{\bullet}$) (30) and ($C_5H_7^+$) (32). The interpretation of the ^1H and ^{13}C NMR (Table 1) is in agreement with the data in the literature (Dzeha *et al.*, 2002; Santalova *et al.*, 2004).

TABLE 3
Free radical scavenging activity (%) of methanol extract of *Aaptos* sp. collected from various locations

Code	Specimen	Free radical scavenging activity (%)	IC_{50} (mg/ml)
P01/011/04	A	73.84 ± 1.0	NT
P02/010/04	B	76.32 ± 0.3	NT
P02/009/04	C	78.69 ± 1.9	NT
P03/015/04	D	80.51 ± 0.5	0.13
R01/010/04	E	55.37 ± 0.1	NT
R03/007/04	F	58.89 ± 1.3	NT
B01/010/04	G	78.80 ± 0.5	0.12
K01/025/04	H	89.28 ± 0.7	0.11
K01/028/04	I	78.57 ± 0.8	NT
K01/010/05	J	81.57 ± 0.7	0.26
K02/011/05	K	81.05 ± 2.3	0.12
K03/010/05	L	72.21 ± 0.9	NT
Standard	BHA	94.38 ± 0.6	0.04
Standard	Quercetin	94.15 ± 0.6	0.04

NT-not tested

The structure of aaptamine (**2**) was determined by interpreting the data of 1D and 2D-NMR, and it was also in agreement with the literature value (Nakamura *et al.*, 1982; Herlt, 2004). The EIMS spectrum of **2** showed that the molecular formula as $C_{13}H_{12}N_2O_2$ with molecular weight 228 $[M]^+$ (46). Other fragments ion are 213 (100), 183 (23), 170 (36), 142 (13). The ^{1}HNMR spectrum showed the presence of two methoxy group at δ 3.97 (3H, s, 8-OCH_3) and 3.85 (3H, s, 9-OCH_3). In the aromatic region δ 6 – 8 ppm, 5 peaks integrated a proton each was observed, which were at δ 6.27 (J = 6.81 *Hz*, H-3) and δ 7.68 (J = 6.87 Hz, H-2), δ 7.15 (J = 7.56 Hz, H-5) and 6.77 (J = 6.78 Hz, H-6). The assignment of carbons and protons of **2** and the HMBC correlation is given in Table 1. These biological activities, in all the samples, might be contributed by aaptamine and demethyloxyaaptamine (DNP on CD-ROM, 1982-2001).

CONCLUSIONS

In conclusion, to the best of the researchers' knowledge, Bidong Island is a suitable location for the collection of sample in search of bioactive constituent from *Aaptos sp.* From the results gathered from the *in vitro* screening, specimen G was found to yield a cholestanol compound known as cholestan-3β-ol (**1**) and an alkaloid called aaptamine (**2**). The isolation of the other bioactive compounds is in progress.

ACKNOWLEDGEMENTS

The authors acknowledge the support from the Ministry of Science, Technology and Innovation, for the fund provided (IRPA 01-02-12-0068-EA 10707) and FRGS (vote: 59004). The authors are also grateful to the co-operation given by the Department of National Marine Parks for the permit on sampling.

REFERENCES

Abas, H.H., Zulfigar, Y. and Chan, K.L. (1999). Cytotoxicity and drug metabolism screening of several marine sponges from Pulau Pasir, Kedah and Pulau Aur, Johor. *Asean Review of Biodiversity and Environmental Conservation, (ARBEC),* 1-5.

Blunt, J., Copp, B.R., Munro, M.H.G., Northcote, P. T. and Prinsep, M.R. (2005). Marine natural product. *Natural Product Report, 22*, 15-61.

Bobzin, S.C., Yang, S. and Kasten, P. (2000). Application of liquid chromatography-nuclear magnetic resonance spectroscopy to the identification of natural products. *Journal of Chromatography B*, 748, 259-267.

Coutinho, A.F., Chanas, B., E. Souza, T.M.L., Frugrulhetti, I.C.P.P. and Epifanio, R.D.A. (2002). Anti HSV-1 alkaloids from a feeding deterrent marine sponge of the genus *Aaptos*. *Heterocycles, 57*, 1265-1272.

Dictionary of Natural Product (DNP) on CD-ROM, Version 10:1. (1982-2001). Chapman& Hall/ CRC.

Dzeha, T., Jaspars, M. and Tabadravu, J. (2003). Chionasterol, a triterpenoid from the Kenyan marine green macroalga *Halimeda macroloba. Western Indian Ocean J. Mar. Sci., 2*, 157-161.

Fernandez, R., Siraj, O., Feliz, M., Quinoa, E. and Riguera, R. (1992). Malaysiatin, the first cyclic heptapeptide from a marine sponge. *Tetrahedron Letters, 33*, 6017-6020.

Gauvin, A., Smadja, J., Aknin, M. and Gaydou, E. M. (1998). Cylopropane-containing sterols in the marine sponge *Petrosia spheroida. Comparative Biochemistry and Physiology, Part B 121,* 451-456.

Habsah, M., Zalilawati, M.R., Nurhana, F., Khamsah S.M., Jasnizat, S., Ali, A.M., Lajis M.N., Faizah S-Harisson and Mohamad H. Z. (2005a). Cytotoxic activity of extracts of *Aaptos* spp. (marine sponges) against HL60 and MCF-7 cell lines (pp. 313-316). In Ibrahim Jantan, Jamia Azdina Jamal, Juriyati Jalil, Nor Fadillah Rajab, Hidayatul Fathi Othman & Jacintha Santhanam (Eds.), 21st *Annual Seminar of Malaysian Natural Product Society.*

Habsah, M., Wan Ainur, N.W.A.J., Khamsah S.M., Nurhana, F., Ali, A.M., Lajis M.N. and Mohamad H.Z. (2005b). Cytotoxic activity of the Methanol extracts of some marine sponges (*Halichondria* sp.) (p.289-294). In Ibrahim Jantan, Jamia Azdina Jamal, Juriyati Jalil, Nor Fadillah Rajab, Hidayatul Fathi Othman and Jacintha Santhanam (Eds.), 21st *Annual Seminar of Malaysian Natural Product Society.*

Herlt, A., Mander, L., Rombang, W., Rumampuk, R., Soemitro, S., Steglich, W., Tarigan, P. and Nussbaum, F.V. (2004). Alkaloids from marine organisms. Part 8: Isolation of bisdemethylaaptamine and bisdemethylaaptamine-9-O-sulfate from an Indonesian *Aaptos* sp. marine sponge. *Tetrahedron, 60*, 6101–6104.

Lee, S.K., Mbwambo, Z.H., Chung, H.S., Luyengi, L., Gamez, E.J.C., Mehta, R.G., Kinghorn, A.D. and Pezzuto J.M. (1998). Evaluation of the antioxidant potential of natural products. *Combinatorial Chemistry & High Throughput Screening, 1,* 35-46.

Mackeen, M.M., Ali, A.M., El-Sharkawy, S.H., Salleh, K.M., Lajis, N.H. and Kawazu, K., (1997). Antimicrobial and cytotoxic properties of some Malaysian traditional vegetables. *International Journal of Pharmacognosy, 35*, 174–178.

Nakamura, H., Kobayashi, J. and Ohizumi, Y. (1982). Isolation and structure of aaptamine a novel heteroaromatic substance possessing α-blocking activity from the sea sponge *Aaptos aaptos. Tetrahedron Letters, 23*, 5555-5558.

Nor Ainy, M., Cole, A., Munro, M., Blunt, J., Cummings, N., Lang, G. and Mitova, M. (2005). Marine-invertebrate-derived fungi and their bioactive compounds (pp.35-47). In Ibrahim Jantan, Jamia Azdina Jamal, Juriyati Jalil, Nor Fadillah Rajab, Hidayatul Fathi Othman and Jacintha Santhanam (Eds.), 21st *Annual Seminar of Malaysian Natural Product Society.*

Santalova, E.A., Makarieva, T.N., Gorshkova, I.A., Dmitrenok, A.S., Krasokhin, V.B. and Stonik, V.A. (2004). Sterols from six marine sponges. *Biochemical Systematics and Ecology, 32*, 153-167.

Siraj, O., Tenenbaum, L.V., Manes, L. and Crews, P. (1988). Novel marine sponge derived amino acids 7. The fenestins. *Tetrahedron Letters, 29,* 5489-5492.

Rudy, A. and Kashman, Y. (1993). Aaptosine-a new cytotoxic 5,8-diazabenz[cd]azulene alkaloid from the Red Sea sponge *Aaptos aaptos*. *Tetrahedron Letters, 34*, 4683-4684.

Rifai S.A., Fassouane, A., El-Abbouyi, A. Wardani, A. Kijjoa and Van Soest, R. (2004). Screening of antimicrobial activity of marine sponges extracts. *Medical Mycology, 15*, 33-38.

Thakur, N.L. and Muller, W.E.G. (2004). Biotechnological potential of marine sponges. *Review Article Current Science, 86*, 1506-1512.

Pertanika J. Trop. Agric. Sci. **32(1)**: 51 - 55 (2009)

ISSN: 1511-3701

Octacosanoic Acid, Long Chains Saturated Fatty Acid from the Marine Sponges *Xestospongia* sp.

Habsah Mohamad[1*], Wan Ainur Najmiah Wan Abdul Jamil[1], Faridah Abas[2], Khamsah Suryati Mohamad[1] and Abdul Manaf Ali[3]

[1]Department of Chemistry, Faculty of Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

[2]Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia,

[3]Faculty of Agrobiotechnology, Universiti Darul Iman Malaysia, 20400 Kuala Terengganu, Terengganu, Malaysia

[*]E-mail: habsah@umt.edu.my

ABSTRACT

Octacosanoic acid has been isolated from the marine sponges *Xestospongia* sp. collected from Bidong Island, Terengganu. The structure was elucidated using various spectroscopic techniques (IR, MS, ^{1}H NMR and ^{13}C NMR). The structure and bioactivities of this long chains saturated fatty acid compound were reviewed.

Keywords: Marine sponges, *Xestospongia* sp., Bidong Islands, bioactivities, long chains saturated fatty acid

INTRODUCTION

Fatty acids are widely distributed in nature and are important as nutritional substances and metabolites in living organisms. In marine organisms, particularly sponges, fatty acids have provided some of the most interesting structural varieties. Many of these marine fatty acids originate from unusual biosynthetic pathways. In recent years, excellent reviews have appeared on the fatty acid structural types which are present in these organisms, their possible roles in membranes, and their biogenesis. However, little is known about or has been reviewed on the biomedical potential of these unusual sponge fatty acids, specifically about the differences which exist in their bioactivity, as compared to what have been reported for more common fatty acids (Carballeira, 2008).

In addition to their wide range of natural product contents, sponges turn out to be a rich source of unusual lipids which play a primary structural and functional role in their plasma membranes. Since the early studies by Litchfield *et al.* (1976), several investigations have proven the presence of long-chain, unsaturated carboxylic acids in a variety of sponges from different marine environments, all of which are members of the Demospongiae (Thiel *et al.*, 1999). These compounds display the characteristic of unsaturated patterns and may in some cases, exhibit terminal as well as mid-chain branching. They may occur as mono-, di- and trienoic compounds (tetraenoic and pentaenoic exceptionally) and cover a relatively broad carbon-number range, typically between C_{24} and C_{30}. Other organisms are apparently lacking these characteristic compounds which have thus been introduced as "demospongic" acids into the literature (Thiel *et al.*, 1999).

The total FA content of a sponge is the sum of FA of the own sponge cells and symbiont bacteria, cynobacteria and/or algal species. As a result, the total mixture of sponge FA is very complex, while the number of components

Received: 20 May 2008
Accepted: 8 October 2008
[*]Corresponding Author

exceeds 100. A lot of sponge FA is specific for the symbiont types in the sponge tissue. Some lipids isolated from marine sponges showed a cytotoxic and anti-cancer activity; therefore, these sponges may be a good source of bioactive compounds with a unique chemical structure (Imbs and Rodkina, 2004). This study reports on the isolation and structure elucidation of the new fatty acid metabolites and its bioassays.

MATERIALS AND METHODS

Sample Preparation and Extraction

Samples of *Xestospongia* sp. were collected *via* SCUBA diving at the depth of 5-10 meters off Kapas Island, in Terengganu. The sponges were directly frozen after the collection and transferred into a –20°C freezer for storage. One part of the fresh sample was preserved in methanol as a taxonomic voucher. Specimens were also deposited at the Biodiversity Museum, Oceanography Institute, Universiti Malaysia Terengganu (Voucer speciman no: B01/002/04). The remaining sponges were cleaned, chopped and dried in an air grafied oven (40°C) prior to extraction. The dried sponges were macerated in methanol 99.7%. The extracts were filtered and dried under reduced pressure using a rotary evaporator. After that these dried extracts were de-salted and kept under –20°C prior to analysis.

Isolation

The concentrated hexane extract was chromatographed over silica gel with hexane/$CHCl_3$/EtOAc to give the oily cream fractions. Further purification of the fractions by repeated colum chromatography and recrystalized from methanol yielded compound **1** (30mg, fraction 12-17 from B01/002/04).

Free Radical-Scavenging Activity (1, 1-diphenyl-2-picrylhydrazyl)

The scavenging activity of DPPH free radical of 1 was done according to the method reported by Oktay *et al.* (2003) with some modifications. The compound (50 µl) was mixed with 1.95 ml of 0.1 mM DPPH-methanol solution. Methanol was used as a blank sample in this experiment. After 30 min. of incubation at room temperature in the dark, the reduction of the DPPH free radical was measured by reading the UV absorbance at 517 nm. Butylated hydroxyanisole (BHA) and Quercetin were used as positive controls. In this study, the scavenging activity (%) was calculated using the following equation:

Free radical scavenging activity (%) = $\{(abs_{control} - abs_{sample})/abs_{control}\} \times 100$.

The scavenging activity (SA) of each test sample was classified into four ranks as follows: strongly active (SA≥70%); moderately active (70%>SA≥50%); weakly active (50%>SA≥30%) and inactive (SA<30%).

Micro-organisms and Media

Antibacterial activity was determined against certified strains of three micro-organisms, namely *Bacillus cereuss* (Gram-positive), *Escherichia coli* and *Klebsiella pneumoniae* (Gram-negative), which were cultured in the appropriated broths at 30°C for overnight. They were obtained from the Pathology Centre, Sultanah Nur Zahirah Hospital, Kuala Terengganu, Terengganu. The bacterial stock cultures were maintained on the nutrient agar (NA) respectively, which were stored at 28°C.

Antibacterial Activity Assay

The agar cultures of the test micro-organisms were prepared as described previously by Mackeen *et al.* (1997). For an initial screening, 1 mg of compound was loaded onto each Whatman No.1 filter paper disc (∅ 6 mm) and placed on the previously inoculated agar. The plates were inverted and incubated for 24 hour at 30°C. For this purpose, streptomycin and gentamycin were used as positive controls. A stock solution of compound was made in DMSO. The presence of antibacterial activity would be indicated by the occurrence of clear inhibition zones around the disc. The assay was carried out in triplicates. The strength of the activity was classified as strong for inhibition zone diameters (i.d.) >16.0 mm, moderate (good) for diameters ranging from 11 to 16 mm, weak for diameters 7 to 11 mm and no activity for diameters < 7 mm. The diameters (mm) of the growth inhibition halos were measured using a ruler.

Cytotoxic Assay

Cytotoxic assay was carried out against Human Caucassian Promyelocytic leukaemia cells (HL-60 cell line) obtained from the National Cancer Institute, USA. The cells were maintained in

RMPI-1640 medium supplemented with 10% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cytotoxic effect of the compound was assessed using the MTT Assay. Briefly, 100µl of exponentially growing HL-60 cells, at the concentration of $1x10^5$ cells/ml, were seeded into 96-flat bottom microwell plate in the presence of various concentrations of compound and incubated for 3 days. A volume of 20µl MTT solution (5mg/ml in PBS) was added to each well. After 4 hours of incubation, the medium was replaced with 100µl of DMSO. The MTT-formazan product, dissolved in DMSO, was estimated by measuring the absorbance at 570nm in the ELISA plate reader (BIO-TECH INSTRUMENT, INC). The cytotoxicity was expressed as inhibition concentration fifty percent (IC_{50}), i.e. the concentration to reduce the absorbance of the treated cells by 50% with reference to the control (untreated cells).

RESULTS AND DISCUSSIONS

Homogenized *Xestospongia* sp. (8 kg, dry weight) was extracted with methanol. The extract (394 g) was then subjected to solvent partitioning, i.e., aq. MeOH against hexane, ethyl acetate and butanol. The extracted hexane (76.34 g) was chromatographed using silica gel Merck Kieselgel 60 PF_{254} Art no. 7749, eluted with hexane/$CHCl_3$/EtOAc. Sequential CC on silica gel led to the isolation of fatty acid compound, octacosanoic acid (*Fig. 1*).

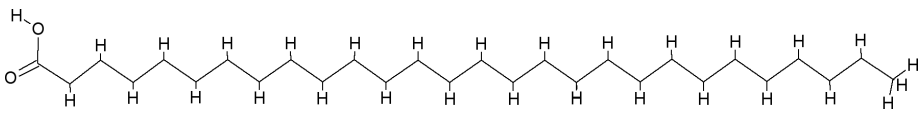


Fig. 1: The octacosanoic acid

Octacosanoic acid (*Fig. 1*) was isolated as a colourless amorphous solid and showed a molecular ion peak at m/z 424 $(M)^+$ in the EIMS. The presence of 28 carbon signals in the ^{13}C NMR spectrum was consistent with the molecular formula of $C_{28}H_{52}O_2$. The data of ^1H NMR and ^{13}C NMR are summarized in Table 1. The characterization of 1 was as follows:

Octacosanoic acid (1). IR (CCl_4) 3649, 2918, 2848, 1702, 1464, 1432, 1410, 1297 cm^{-1}; ^1H-NMR ($CDCl_3$) 0.864 (brt, J =, 3H, -CH_3), 1.265 (brs, 26H, -CH2-), 1.644 (m, 2H), 2.358 (t, 2H); MS, m/e (relative intensity) 424 (M^+), 410 (19), 354 (7), 326 (17), 311 (24), 297 (36), 283 (31), 269 (21), 255 (21), 241 (33), 227 (29), 213 (24), 199 (32), 185 (51), 171 (43), 157 (49), 143 (44), 129 (71), 87 (100).

TABLE 1
^1H-(400MHz) and ^{13}C-(100 MHz) NMR chemical shifts ($CDCl_3$) of octacosanoic acid

Position C	Octacosanoic acid	
	δH	δC
1		178.572
2	2.358 (2H, t)	33.965
3	1.644 (2H, m)	24.929
4-21	1.265 (26H, br, s)	29.926-30.412
22		32.194
23		22.929
24		27.323
25		37.336
26		32.988
27		19.959
28	0.864 (3H, t)	14.351

Three new brominated fatty acids, (5*E*, 11*E*, 15*E*, 19*E*)-20-bromoeicosa-5,11,15,19-tetraene-9,17-diynoic acid, (5Z, 11*E*, 15*E*, 19E)-6,20-dibromoeicosa-5,11,15,19-tetraene-9,17-diynoic acid and (Z *E*)-14,14-dibromo-4,6,13-tetradecatrienoate (characterized as the methyl ester) were isolated from *Xestospongia* sp. from the Indian Ocean (Brantley *et al.*, 1995).

The bioassay results for the isolated compound are shown in Table 2. The octacosanoic acid was inactive in free radical-scavenging activity exhibited only 10.03% inhibition. The growth-inhibition, induced in cancer cells after exposure to the compound, was expressed as the percentage of the cell viability observed in the treated cells versus untreated HL-60. The results are summarized in Table 2. When tested *in vitro* against Human Caucassian Promyelocytic Leukaemia (HL-60) cell line, octacosanoic acid demonstrated cytotoxicity at IC_{50} 2.2 µg/ml, respectively.

In this study, octacosanoic acid was found to give a weak activity against *Bacillus cereuss* and *Escherichia coli* when tested for antimicrobial activity. Only *Klebsiella pneumoniae* inhibited significantly moderate activity by this isolated compound. These results are also presented in Table 2.

TABLE 2
Activities of pure compound isolated from *Xestospongia* sp.

Specimen		[a]AO (%)	[b]Cytotoxic	[c*] Antibacterial		
				B. cereus	*E. coli*	*K. pneumoniae*
Compound	Octacosanoic acid	10.03	2.2	+	+	++
Control	Gentamycin			+++	+++	+++
	Streptomycin			+++	+++	+++

** B.cereus: Bacillus cereuss*; E.coli: *Escherichia coli; K. pneumoniae: Klebsiella pneumoniae*
(-) No activity, (+) weak activity (7-11 mm halo), (++) moderate activity (11-16 mm halo), (+++) high activity (>16 mm halo).
The experiments were run in triplicates. [a]The reduction of the DPPH free radical was measured by reading the UV absorbance at 517 nm. [b]The cytotoxic effect of compound human caucassian promyelocytic leukaemia cells (HL-60 cell line) was assessed by MTT Assay. [c]Antibacterial activity of Octacosanoic acid as measured by the disc diffusion method. The inhibition zones were measured in mm.

CONCLUSIONS

The isolation of hexane extract, from *Xestospongia* sp., yielded a long chain saturated fatty acid compound, i.e. octacosanoic acid, which might not have been reported previously in *Xestospongia*. For its bioactivities, the compound was found to be inactive against antioxidant activity (DPPH), moderate activity against *Klebsiella pneumoniae* in antibacterial activity and strong cytotoxicity against Human Caucassian Promyelocytic Leukaemia (HL-60) cell line. This showed that the Malaysian water is a potential source of sponge secondary metabolites worthy as a lead compound for developing a therapeutic agent for various kind of diseases.

ACKNOWLEDGEMENTS

The authors acknowledge the support from the Ministry of Science, Technology and Innovation for the fund provided (IRPA 01-02-12-0068-EA 10707) and FRGS (vote: 59004). The authors also thank the Department of National Marine Parks for the sampling permit.

REFERENCES

Biavatti, M.W., Vieira, P.C., Silva, M.F.G.F., Fernandes, J.B., Albuquerque, S., Magalhães, C.M.I. and Pagnocca, F.C. (2001). Chemistry and bioactivity of *Raulinoa echinata* Cowan, an endemic Brazilian Rutaceae species. *Phytomedicine*, *8*, 121-124.

Brantley, S.E., Molinski, T.F., Preston, C.M. and DeLong, E.F. (1995). Brominated acetylenic fatty acids from *Xestospongia* sp., a marine spongebacteria association. *Tetrahedron*, *51*, 7667-7672.

Carballeira, N.M. (2008). New advances in fatty acids as antimalarial, antimycobacterial and antifungal agents. *Progress in Lipid Research*, *40,* 50-61.

Imbs A.B. and Rodkina S.A. (2004). Isolation of 2-methyl branched unsaturated very long fatty acids from marine sponge *Halichondria panicea* and identification of them by GC–MS and NMR. *Chemistry and Physics of Lipids*, *129*, 173-181.

Litchfield, C., Greenberg, A.J., Noto, G. and Morales, R.W. (1976). Unusually high levels of C_{24}–C_{30} fatty acids in sponges of the class *Demospongiae*. *Lipids, 11,* 567–570.

Mackeen, M.M., Ali, A.M., El-Sharkawy, S.H., Manap, M.Y., Salleh, K.M., Lajis, N.H. and Kawazu, K. (1997). Antimicrobial and Cytotoxic properties of some Malaysian traditional vegetables. *International Journal of Pharmacognosiy*, *35,* 174-178.

Munro, M.H.G., Dumdei, J.W., Hickford, E.J., Lill, S.J., Shangxiao, R.E., Battershill, C.N. and Duckworth, A.R. (1999). The discovery and development of marine compounds with pharmaceutical potential, *J. Biotech.*, *70,* 15-25.

Oktay, M., Gulcin, I. and Kufrevioglu, O. (2003). Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seeds extract. *Lebensmittel-Wissenschaft and Technology*, *36,* 263-271.

Thiel, V., Jenisch, A., Wörheide, G., Löwenberg, A., Reitner, J. and Michaelis, W. (1999). Mid-chain branched alkanoic acids from "living fossil" demosponges: a link to ancient sedimentary lipids? *Organic Geochemistry*, *30*, 1-14.

Pertanika J. Trop. Agric. Sci. **32(1)**: 57 - 61 (2009) ISSN: 1511-3701

Osteoconductive Nanocomposite Coating of Apatite-Wollastonite and Chitosan

S. Sharma[1], V.P. Soni[1] and Jayesh Bellare[1,2,3*]
[1]School of Biosciences and Bioengineering, and
[2]Department of Chemical Engineering I.I.T. Bombay, Powai, Mumbai – 400 076, India
[3]Piercy Visiting Chair Professor, Department of Chemical Engineering,
University of Minnesota, Minneapolis, MN 55455, USA
[*]E-mail: jb@ iitb.ac.in

ABSTRACT

Here, we show the importance of the marine biomaterial chitin and its derivative, chitosan, in bioimplant applications. Titanium and titanium alloys have shown high potential for load bearing in bioimplant applications. However, after their application, a bond with living bone often does not develop or the integration of the implants with bone tissue takes several months. Moreover, the surface of any material implanted in living body changes over time. Titanium ions may be released from the implant surface, following corrosion and wear. Therefore, the metallic implants coated with bioactive materials are able to induce a biological bonding with both soft and hard tissues. Chitin, an important marine biomaterial, and its derivative, chitosan, is an excellent bioactive material compatible with human biological environment and has good flexural strength and osteoconductive properties. Apatite-wollastonite (AW) has been regarded as a promising biomaterial due to its high biocompatibility, good bioaffinity and osteoconductivity and its crystallographic and chemical similarity with human bone and teeth. However, coating them at ambient temperatures onto titanium implants have proven difficult. We will discuss a new technique that we have developed using electrophoresis to coat a chitosan-AW composite onto a titanium substrate and demonstrated its useful mechanical and biochemical properties and its bioactivity.

Keywords: Electrophoretic deposition, chitosan, apatite-wollastonite, titanium implants

INTRODUCTION

Titanium and titanium alloys are proven to be potentially very suitable materials for load bearing in bioimplant applications due to their biocompatibility, high corrosion resistance and reliable mechanical properties. Nevertheless, from the biochemical point of view, they are considered nearly inert materials (Hench, 1991; Stoch *et al.*, 1994; Flavio *et al.*, 1999) because they do not bond with the bone through chemical or biological interaction, but simply by a morphological connection to the bone. This insufficient adhesion to the bone, due to the lack of a specific biological response from the living tissues, can progressively form a non-adherent fibrous capsule around the implant and this can lead to clinical failure. However, metallic implants, coated with bioactive materials, are able to induce a biological bonding with both soft and hard tissues.

Glass-ceramics containing apatite and wollastonite crystals (AW) have been found to have the highest mechanical strength among bioceramics and they have also been shown to display the ability to directly bond to living bone by forming an apatite layer on their surface in the body environment (Osborn and Newesely, 1980). Although ceramic coatings have been shown to improve bone attachment and integration of implants, long-term stability of coating is still a challenging issue. Thus, attention has been directed towards the potential use of ceramic-

Received: 20 May 2008
Accepted: 8 October 2008
[*]Corresponding Author

polymer composites in the recent years so as to improve the biocompatibility and mechanical strength of implant materials.

Chitin, an important marine biomaterial and its derivative, chitosan are excellent bioactive materials found to be compatible with human biological environment. Chitosan is a principal constituent in the shells of crabs, lobsters and other crustacean species. It has been used in a number of biomedical applications such as drug encapsulation, fat absorbers, and wound dressing materials. Chitosan/Wollastonite composite scaffolds have earlier been used for tissue engineering (Zhao *et al.*, 2004).

Different coating techniques have been used to produce coating on metallic substrates including plasma spray, sol-gel, enamelling, slurry dipping, electrophoretic deposition and sputter coating. In particular, the sputtering and plasma spray techniques could present some problems related to difficulty in coating complex shapes and the eventual compositional modifications due to the high temperature (Liu and Ding, 2002). Electrophoretic deposition has been known as an efficient technique used in assembling fine particles because of its simplicity, low equipment cost, the possibility of deposition on substrates of complex shape, high purity and micro-structural homogeneity of deposits.

In this work, the researchers developed nano-composite coatings which could help in surface modification of titanium implants, thereby improving its performance. The approach used in achieving the optimal nano-composite coating makes use of a simple, cost-effective electrophoretic deposition technique. The various parameters of operation were optimized and these included pH, surface of titanium and approach of coating. The coated titanium implants, using electrophoretic deposition and characterized by different techniques and its biocompatibility, were also studied.

Electrophoretic Deposition and pH Optimization

The prepared coating had lamellar structure with alternating layers of ceramic and polymer. The advantage of using polymer, as the innermost layer, is to achieve stronger interaction of the coat with the metal substrate.

pH optimization was performed over a range of 1-6.5 pH value, using a fixed current density and the final pH was set as 1.6.pH. At this pH, a uniform, crack free coat was obtained.

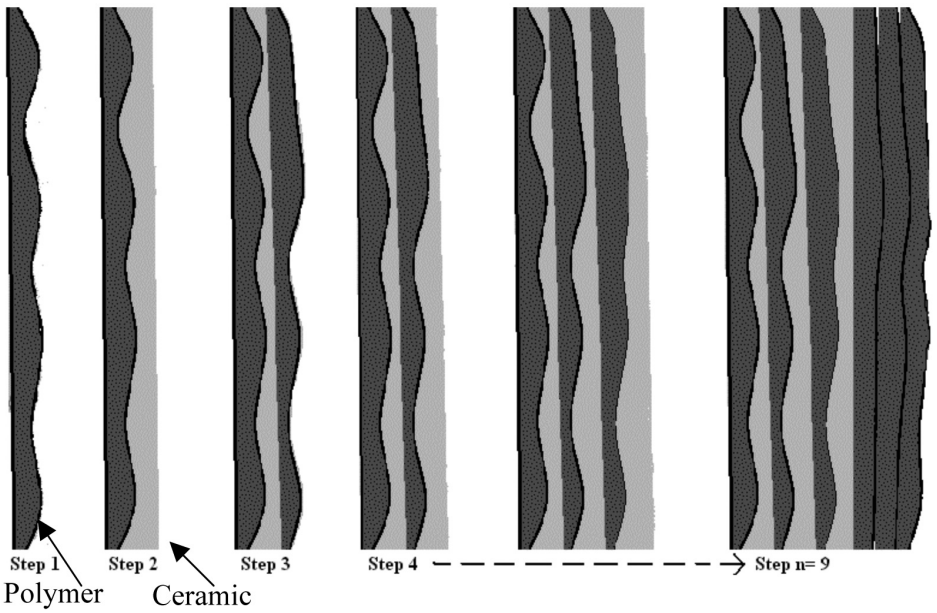


Fig. 1: Coating pattern used to coat titanium substrate

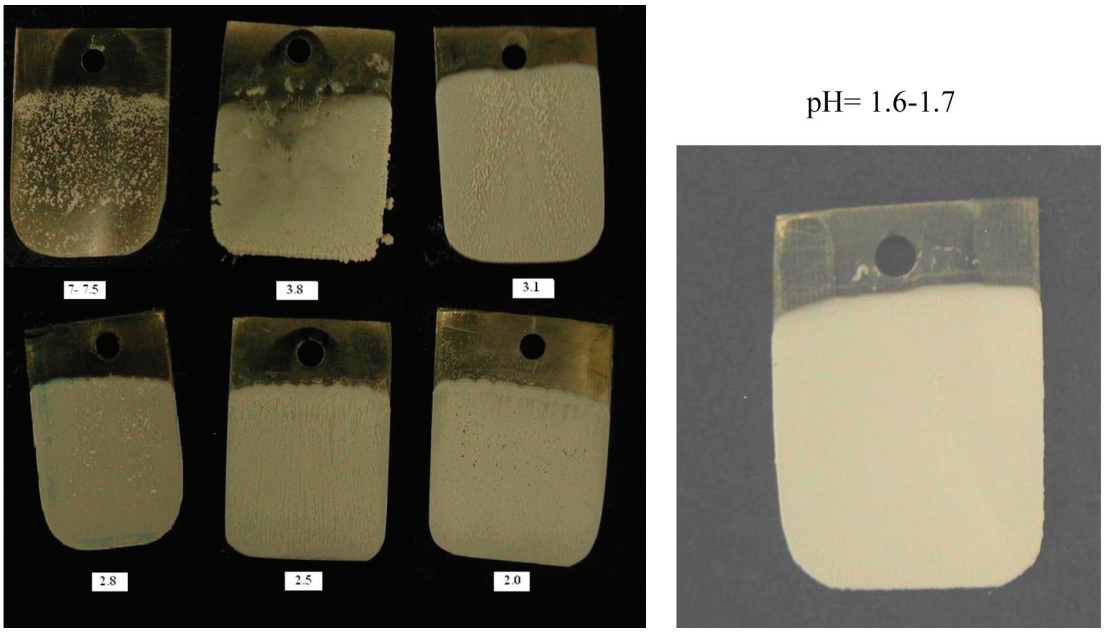


Fig. 2: Coats shown at various pH levels

Particles do not have a definite morphology and are irregularly shaped. The coat contains interstices and pores; the thickness of the coat is found to be approximately 40 µm. The XRD pattern indicates the presence of Wollastonite phase with retention of substantial crystallinity of 65%.

The samples show significantly increased apatite gwowth on their surface till the 21st day.

Cell culturing experiments were carried out and the result showed that the coating has osteoconductive potential and promises to improve the performance of metal implants, apart from its good bioactivity.

Characterization Studies

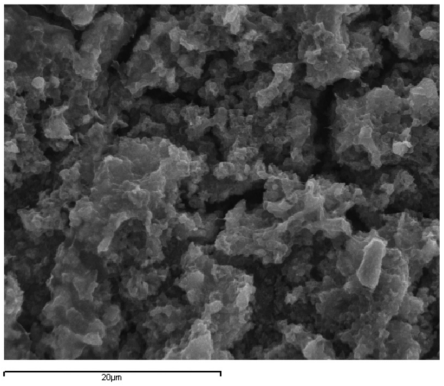


Fig. 3: ESEM micrograph showing particle morphology

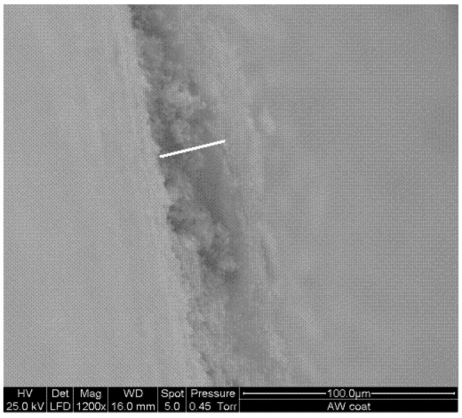


Fig. 4: ESEM micrograph showing the thickness of coating

Bioactivity Studies

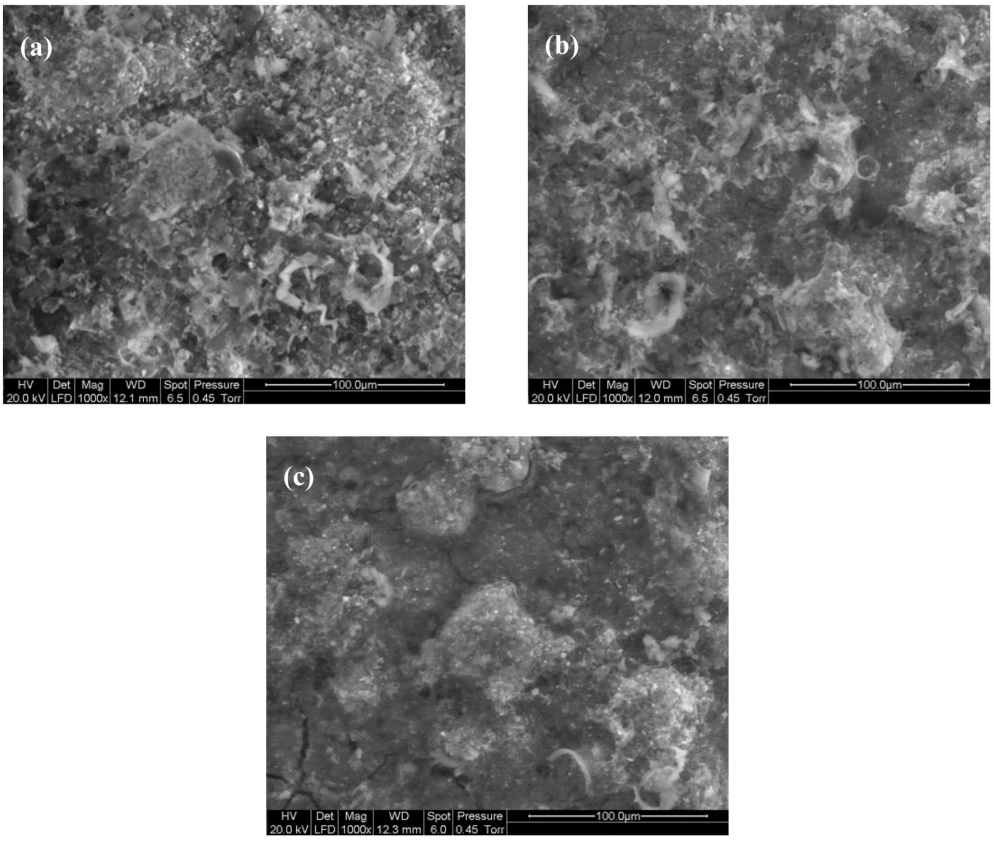


Fig. 5: The ESEM images of the coated samples immersed in SBF at 1000 X (a) 7 days, (b) 14 days and (c) 21 days

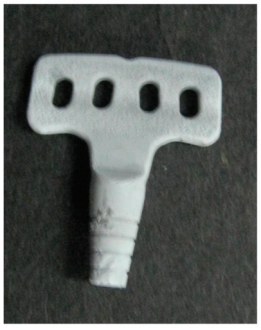


Fig. 6(a): Coated implant using EPD

Fig. 6(b): Implant before coating

REFERENCES

Stoch, A., Stoch, J. and Rakewska, A. (1994). Adhesion an XPS and SEMS study of silica sol-gel/metal substrate interaction. *Surf. Interface Anal., 32,* 242.

Flavio, L.S., Carvalho, Christiano, S., Borges, R.J., Branco, T. and Pereira, M. (1999). Structural analysis of hydroxyapatite/ bioactive glass composite coatings obtained by plasma spray processing. *J. Non-Crystalline Solids, 247,* 64-68.

Hench, L.L. (1991). Bioceramics:from concept to clinic. *J. Amer. Ceramic Soc., 74*, 1487-1510.

Osborn, J.F. and Newesely, H. (1980). The material science of calcium phosphate ceramics. *Biomaterials, 1,* 108.

Zhao, L. and Chang, J. (2004). Preparation and characterisation of macroporous chitosan/wollastonite composite scaffolds for tissue engineering. *J. Mat. Sci.:Mat. Med., 15,* 625-629

Liu, X. and Ding, C. (2002). Plasma sprayed wollastonite/TiO_2 composite coatings on titanium alloys. *Biomaterials, 23*, 4065-4077.

Pertanika J. Trop. Agric. Sci. **32(1)**: 63 - 67 (2009)

ISSN: 1511-3701

Molecular – Bioassay Methods: Complementary Approaches for Development and Evaluation of Anti Infective Marine Product

Mariana Nor Shamsudin[1,3*], Norfarrah Mohamed Alipiah[1,3], Fatimah Md Yusoff[2,3] and Aziz Arshad[2,3]

[1]Faculty of Medicine and Health Sciences, [2]Faculty of Science,
[3]Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: mariana@medic.upm.edu.my

ABSTRACT

The current trends in drug development employ biotechnological approach to expedite effective drugs discovery program. Molecular biotechnological approach, in combination with bioassay, is practical in attaining effective drugs since the two platform methods complement each other by target identification, as well as compound screening, profiling and validation. The research on antimicrobial properties of marine products, targeting membrane function through membrane permeabilizing ability, has been carried out using molecular- and cellular-based approaches. The molecular approach for the screening of membrane permeabilizing peptide gene in local marine organism was found to successfully amplify a conserved gene sequence of the antibacterial peptide gene. Bacterial membrane permeabilizing ability of the methanolic extract was indicated through alteration of mRNA nucleotides, genes coding for membrane development in *Staphylococcus aureus* (MRSA) and the non-methicillin resistant strains. The alteration of nucleotides affected the transportation of lysine to the phospholipid bilayer of bacterial membranes, resulting in incomplete membrane structure, eventual lysis and cell death. Through cellular approaches, the methanolic extract of marine organisms affecting membranes of *S. aureus,* were confirmed. In specific, the extract showed a good inhibitory activity against *S. aureus* through plate and tube methods, and the cellular assay illustrated the penetration of fluorescence dye in treated bacterial pathogens, similar to the pathogens treated with positive antibiotic controls. The research constitutes a scientific advancement in the field of medical treatment of drug resistant bacteria and a forefront study of drugs discovery program focusing on drugs target genes.

Keywords: Membrane permeabilizing, drugs target, antibacterial peptide gene

INTRODUCTION

The evolution of antibiotic resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from various alternative sources. The efforts can be futile if the rate of alternative agent discovery is not fast enough since pathogens keep improving on developing resistance mechanisms. New drug discovery approaches have to be amplified by searching the best infective targets in resistant pathogens, coupled with complementary anti-infective agent discovery, from diverse sources including natural marine products. Methicillin–resistant *Staphylococcus aureus* (MRSA) is a virulent organism which causes substantial infection-related morbidity and mortality in hospitalized patients. MRSA is now one of the most common causes of bacterial nosocomial infections, accounting for 40–70% of *S. aureus* infections in intensive care units (Zetola *et al.,* 2005). The emergence of high levels of penicillin resistance, followed by the development and spread of strain resistant to the semi-synthetic penicillin (methicillin, nafcillin, and oxacilin), macrolides, tetracyclines, and aminoglycoside, has made therapy of staphylococcal diseases a

Received: 20 May 2008
Accepted: 8 October 2008
[*]Corresponding Author

global challenge. The rate of MRSA infection is increasing and its treatment is not only expensive, but available drug of choice is toxic to human host. The aim of the current study was to validate the inhibitory activity of the MRSA and non-MRSA strains by methanolic marine extract from local marine resource. The validation tests, based on two approaches namely bioassay and molecular assay, were attempted. The validation of an active extract for antibacterial agent screening purpose generally involved an experimental demonstration that inactivation of a gene product leaves the bacterial cell non-viable.

MATERIALS AND METHODS

Methanolic Extract Inhibitory Determination

The antibacterial activity of methanolic extract, on MRSA and non-MRSA strains, was determined according to the methods outlined by Kirby-Bauer (1966) for antibacterial disc diffusion assay and minimum inhibitory concentration (MIC) assay using tube dilution method, followed by colonial growth determination of treated bacterial cells on Mueller Hinton Agar. The treatment controls were Luria Bertani broth with no extract, broth and discs with positive and negative antibiotics, respectively. Following the MIC dosages determination, test bacterial strains were grown in broth containing extract and antibiotics diluted in two folds.

Molecular Assay

Studies on active compound from marine resource were conducted to perform prediction of genes affected by these compounds. Primers for RT-PCR were manually designed based on the interest region from gene coding for the target genes obtained from the public domain MRSA database. Genes targeted are genes related to membrane development of MRSA.

Partial expression profiles of bacterial target genes after the treatment with extract and controls were determined using the RT-PCR analysis. Methods and reagents used were according to the manufacturer's instructions stated on the commercial kit (Master Pure, Alleights) with slight modifications. Total RNA were extracted from the treated and untreated MRSA on MIC's plate, using the RNA Extraction kit. The total RNA was then converted into cDNA using the Monster Script (Alleights), which was also according to the manufacturer's instruction for the first and second strands, before projecting it to the Reverse Transcriptase PCR (RT-PCR) using prior designed primers.

The PCR was carried out in a total volume of 25 µl based on the methods introduced by Maniatis (1989) with some modifications. Standard PCR amplification steps were based on Sambrook *et al.* (1989) with some modifications for thirty cycles. The RT-PCR product was run on 1.2% agarose gels, stained in 0.2 µg/ml of ethidium bromide and viewed under UV light (Alpha Imager™ 2200, Alpha Innotech). The bands of interest were extracted with QIAquick gel extraction kit (Qiagen) and sent for commercial sequencing.

Profiling

The sequence analyses were carried out using the DNAsis and Biology Workbench alignment to align the sequencing product for the treated, untreated MRSA and membrane genes from the database. The alignments were done to determine the nucleotide changes after the treatment of marine extract.

Bioassay Validation

Bacterial cells previously treated in two folds, diluted extract and antibiotic controls were subjected to fluorescence dye assay to determine the cellular penetration of the extract and antibiotics. Bacterial cells (0.5 Mc Farland), antibiotics or marine organism extract and SYTOX green dye (5 µM) were combined and dispensed into black micro plate at 100 µl/well. The black micro plate was incubated at 37°C and the fluorescence intensities were measured with the fluorescence multi-well plate reader (SpectraMAX GeminiXS), at an excitation wavelength 485 nm and emission wavelength of 530 nm for every 30 minutes up to 6 hours of treatment.

RESULTS

Methanolic Extract Inhibitory Determination

The minimum inhibitory concentration (MIC) of the marine methanolic extract was indicated as 10.2 mg/ml for *S. aureus* ATCC 29247 and 12.8 mg/ml for *S. aureus* ATCC 700698 (*Fig. 1*).

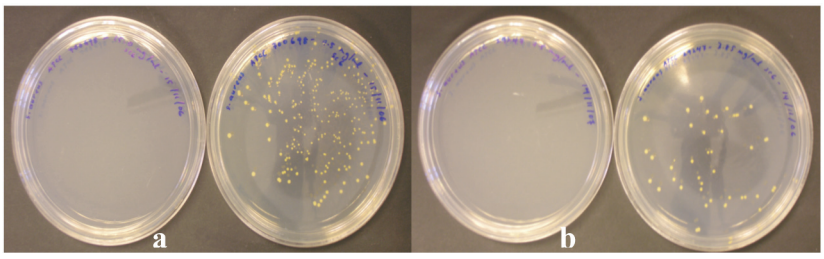


Fig. 1: Minimal inhibitory concentration of MRSA treated with marine organism extract (a) ATCC 700698; (b) ATCC 29247

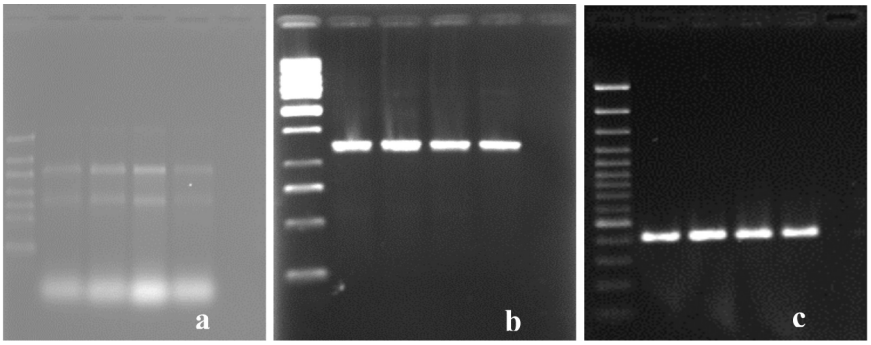


Fig. 2: a) RNA band of treated and untreated MRSA and non MRSA reference strain, ATCC 700698 and ATCC 29247; b) Amplification of a band from extracted RNAs at a size of 1241bp; c) Amplification of a band from extracted RNAs at a size of 406 bp

Molecular Assay

RT - PCR resulted in a single amplification band at 1241 bp and 406 bp respectively, for both strains of ATCC 29247 and 700698 which were treated with marine extract and controls (*Fig. 2*).

Target Identification

From the gene bank database, the predicted genes were encoded for proteins related to membrane development in *S. aureus.*

Profiling

The bands upon purification and commercial sequencing showed changes in the nucleotide sequences of the treated bacterial strains, but no changes in the untreated strains (*Fig. 3*).

Validation

Bacterial cell membrane permeabilizing assay is indicated by the changes in the fluorescence intensities of the treated bacterial strains, as shown in *Fig. 4.* The results showed obvious

```
29247U_membF_complete          TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
membF_[921_-_2180]_AF145699    TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
29247T_mprF_complete           TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
                               **************************************************

29247U_membF_complete          ATTATTTTTGTTCTGCTTATTGTAGCTTTCCGTAGAGCACGTAGGTTGA-
membF_[921_-_2180]_AF145699    ATTATTTTTGTTCTGCTTATTGTAGCTTTCCGTAGAGCACGTAGGTTGAA
29247T_membF_complete          ATTATTTTGTGGCGACATTCTTCACTTACGCTTCATATATTTTAAT-AAC
                               ********    * * * * * * * *   * *  * * * *
```

Fig. 3: Alignment of sequencing results from the treated, untreated S. aureus *and gene bank sequence of a putative membrane synthesis gene*

increase in the intensities of fluorescence in *S. aureus*, which was treated with marine extract and positive control, using membrane inhibitor antibiotic at MIC and 2x MIC for both strains ATCC 29247 and ATCC 700698 in the first 2 hours. After 2 hours of treatment, the intensities were found to slowly increase until 5 hours of post-treatment, and after which, there was no increase detected in the fluorescence intensity. As for *S. aureus* treated with negative control of membrane non-inhibitor antibiotic, the intensities were generally constant with no significant fluctuation throughout the experimental duration of 6 hours.

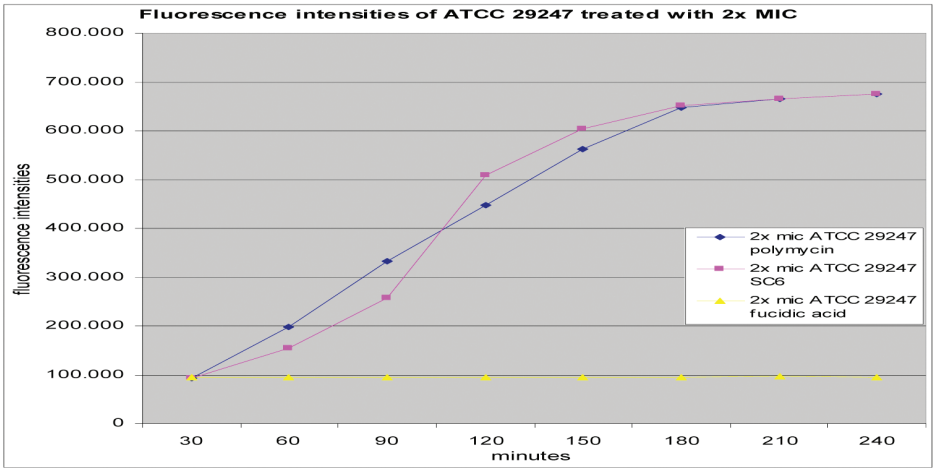


Fig. 4: Fluorescence intensities of ATCC 29247 treated with positive antibiotic control, marine methanolic extract, and negative antibiotic controls

DISCUSSION

Marine methanolic extract is a polar extract which comprises of compound with potential bacterial membrane permeabilizing ability. The extract at a minimum concentration of 10.2 mg/ml was found to inhibit the growth of non MRSA, strain ATCC 29247, and at a higher minimum inhibitory concentration of 12.8mg/ml for MRSA, strain ATCC 700698. When investigated at the molecular level, the inhibitory activity of the extract exhibited bands at two different positions for the MRSA and non-MRSA strains of the treated and untreated controls after the RT-PCR analysis, respectively. The amplification of these bands, visualized on gel after electrophoresis, suggested that the respective genes (with and without treatment) did not affect the partial expression, based on the size of the bands, respectively. However, upon purification, and the sequencing analysis by aligning the sequences of treated, untreated, and sequence of putative membrane development gene from the GenBank, showed there were nucleotide changes only in the treated samples. These findings indicate that the extract has the ability to alter the membrane of *S. aureus*.

In determining the effect of the extract on bacterial membrane integrity, the intensities of SYTOX green fluorescence dye in *S. aureus* cells (treated with marine methanolic extract at the MIC values) increases with time. SYTOX green is an organic compound which fluoresces upon interaction with nucleic acid. When the extract disrupts the membrane integrity of *S. aureus*, the dye enters the cell and easily colours the nucleic acid. In this study the increase in the intensities of fluorescence was also seen in the positive antibiotic control. The fluorescence intensities were found to increase drastically during the first 120 minutes of treatment, and this indicated that the membrane integrity was affected and the dye coloured the nucleic acid. Accordingly, the negative antibiotic control did not affect the bacterial cell membrane integrity and thus

showed a constant fluorescence intensity even after 6 hour of incubation in the antibiotic.

The present study validated the bacterial inhibiting activity of the methanolic marine extract of a local invertebrate species. The significance of the present study is that the two approaches in combination could be used to confirm the activity of the extract and elucidate the activity at the molecular level. The fluorescence dye bioassay approach clearly illustrated the mechanism of the methanolic extract and the positive antibiotic control in destructing the viability of the test bacterial strains via the integrity of bacterial membrane. As membrane plays an important role for cell integrity, the disruption of bacterial cell membrane thus provokes cell leakage and exposes the inner part to antibacterial compounds which serve as an effective alternative to cause the mortality of bacterial cells. An added advantage of the membrane, as an infective target, is the conservative nature of the structure, whereby the cell membrane is found and formed in all cells and the possibility of mutation is very low, making the membrane a readily available target.

ACKNOWLEDGMENTS

The authors are grateful to University Putra Malaysia for providing the facilities and MOSTI for the grant awarded to one of the authors (AC) to complete this manuscript.

REFERENCES

Isnansetyo, A. and Kamei. Y. (2002). MC21-A, a bactericidal antibiotic produced by a new marine bacterium, *Pseudoalteromonas phenolica* sp. Nov O-BC30 against Methicillin- resistant *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy,* 480-488.

Juliet, M.R., June, R.S.C., Graham, D.K. and Valerie, J.S. (1999). Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from granular haemocytes of the shoe crab, *Carcinus maenas. Eur. J. Biochem., 264,* 350-357.

Lee, I.H., Zhao, C. and Nguyen, T. (2001). Clavaspirin, an antibacterial and haemolytic peptide from *Styela clva. J. Peptide Res., 58,* 445–456.

Mariana, N.S., Norfarrah, M.A., Yusoff, F.M. and Arshad, A. (2009). Selective *in vitro* activity of marine extract on genes encoding membrane synthesis of methicillin resistance *Staphylococcus aureus. Journal of Biotechnology, 8,* 180–183.

Oku, Y., Kurokawa, K., Ichihashi, N. and Sekimazu, K., (2004). Characterization of the *Staphylococcus aureus* mprF gene, involved in lysinylation of phosphatidyglycerol. *Journal of Microbiology, 150,* 45–51.

Ridzwan, B.H., Kaswandi, M.A., Azman, Y. and Fuad, M. (1995). Screening for antibacterial agents in three species of sea cucumber from coastal areas of Sabah. *Gen. Pharmac., 26,* 1539-1543.

Saint, N., Cadiou, H. and Bessin, Y. (2002). Antibacterial peptide pleurocidin forms ion channels in planar lipid bilayers. *Biochimica et Biophysica Acta,* 359–364.

Tor, H., Kjuul, A.K. and Styrvold, O.B. (2002). Antibacterial activity in *Strongylocentrotus droebachiensis* (Echinoidea), *Cucumaria frondosa* (Holothuroidea), and *Asterias rubens* (Asteroidea). *J. Invertebrate Pathology, 81,* 94–102.

Thierry, J., Ursula, E.L., Christophe, M. and Sanjeev, K. (2003). Validation of the hexose transporter of *Plasmodium falciparum* as a novel drug target. *Journal of Biochemistry, 100,* 7476–7479.

Sambrook, J., Fritsch, E.F. and Maniatis T. (1989). *Molecular Cloning: A Laboratory Manual* (2nd Ed). Cold Spring Harbor: Cold Spring Harbor Press.

Pertanika J. Trop. Agric. Sci. **32(1)**: 69 - 75 (2009)
ISSN: 1511-3701

Screening for the Presence of Antimicrobial Activity in Few Indian Seaweeds

Savita Kotnala[1], Aakriti Garg[1] and Anil Chatterji[2*]
[1]National Institute of Oceanography, Dona Paula, Goa, India
[2]Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia
[*]E-mail: anilch_18@yahoo.co.in

ABSTRACT

Methanolic extracts of 17 commonly found seaweeds in the west coast of India were screened for the presence of antimicrobial activity against *Bacillus subtilis*, *Escherichia coli, Pseudomonas* sp., *Streptococcus pyrogenes, Staphylococcus aureus, Proteus vulgaris*, *Klebsiella pneumoneae, Serrratia marganii* and *Candida albicans*. The results of this study indicated that the extracts of *Padina tetrastomatica* and *Jania rubens*, brown and red alga respectively, were promising. Further purification of the active extracts followed by bioassay indicated that fraction of Ethyl Acetate (EtOAc) was more active as compared to other fractions. Phytochemical investigation revealed the presence of phenols, tannins, saponinis, cardiac glycosides, terpenoides, alkaloids, anthraquinones and flavonoides.

Keywords: Antimicrobial activity, phytochemical analysis, Indian seaweeds

INTRODUCTION

The marine world offers an extremely rich resource for important compounds of structurally novel and biologically active metabolites. It also represents a great challenge which requires inputs from various scientific areas to bring the marine chemical diversity up to its therapeutic potential. So far, many chemically unique compounds of marine origin, with different biological activities, have been isolated and a number of them are under investigation or are being developed as new pharmaceuticals (Faulkner, 2000a,b; Da Rocha *et al.*, 2001; Schwartsmann *et al.*, 2001).

Seaweeds are marine plants divided into three categories, based on their colours, such as red (4,500 species), green (900 species) and brown (1,000 species). Seaweeds have been used as food, fertilizer and for medicinal purposes for a long time. It has been reported that seaweeds contain high levels of minerals, vitamins, essential amino acids, indigestible carbohydrates and dietary fibre (Jiménez-Escrig and Goni, 1999). In food manufacturing, seaweeds have been developed as raw or semi-processed food products (Mabeau and Fleurence, 1993). In the present study, antimicrobial activity of the seaweeds and their phytochemical analyses were carried out. The methanolic extracts of different seaweeds were screened for the presence of potential antimicrobial activity against some medically important bacterial strains.

MATERIALS AND METHODS

In the present study, 17 species of seaweed were collected from the beaches of Goa and Maharashtra (India) on low tide from intertidal regions (Table 1). Seaweeds were collected and washed carefully for about 3 to 4 times with fresh water and kept for drying in shade. Methanol extracts were prepared from all the seaweeds. After this preliminary step, the same extracts were partially purified into hexane, ethyl acetate and aqueous fractions.

Received: 20 May 2008
Accepted: 8 October 2008
[*]Corresponding Author

TABLE 1
List of seaweed species and collection points

Sr. N	Seaweeds	Place	Sr. N.	Seaweeds	Place
	Phaeophyceae			**Rhodophyceae**	
1	*S. marginatum*	Marvel beach, Goa	9	*Asparagopsis taxiformis*	Anjuna beach, Goa
2	*St. marginatum*	Marvel beach, Goa	10	*Amphiroa fragilissima*	Anjuna beach, Goa
3	*P. tetrastromatica*	Baga beach, Goa	11	*Jania reubens*	Malvan, Maharashtra
4	*P. gymnospora*	Marvel beach, Goa		**Chlorophyceae**	
5	*C. implexa*	Malvan, Maharashtra	12	*Caulerpa racemosa*	Malvan, Maharashtra
6	*D. australis*	Malvan, Maharashtra	13	*Caulerpa peltata*	Marvel beach, Goa
7	*D. bartayresiana*	Malvan, Maharashtra	14	*Caulerpa taxifolia*	Malvan, Maharashtra
8	*S. aspermum*	Malvan, Maharashtra	15	*Codium fragile*	Anjuna beach, Goa
			16	*Chlorodesmis fastigiata*	Anjuna beach, Goa

The methanolic extracts of 17 seaweeds were subjected for preliminary phytochemical testing for the detection of major chemical groups. The details of the tests are as follows: Braemer's test for tannins, phosphomolybdic acid test for phenols, borntrigers test for anthraquinones and alkaloids, as described by Singleton and Rossi (1965). Forthing test for saponins, Keller - kiliani's test for cardiac glycosides of Harborne (1973). Salkowski test for terpenoides and shinoda test for flavonoids of Sofowara (1993) and Harborne (1973).

The pathogenic strains, such as 5 Gram-negative bacteria (*Klebsiella pneumonia*, *Pseudomonas sp., Escherichia coli, Proteus vulgaris* and *Serrratia marganii*) 3 Gram-positive bacteria (*Bacillus subtilis, Streptococcus pyrogenes, Staphylococcus aureus)* and one fungal strain (*Candida albicans),* were obtained from the Goa Medical College (India) from which, the strains were isolated from the patients admitted at the hospital. These isolated strains were maintained in the laboratory at NIO, Goa (India) and used for microbial assay for the present study.

The antimicrobial activity of the 17 seaweed extracts was screened using a standard disc diffusion method. Sterilized filter paper discs (5 mm diameter), loaded with 10 µl of seaweed extract (10 mg/100µl) along with one disc loaded with 10 µl (0.01 mg/ml) of Gentamycin (positive control) and another with 10 µl of sterilized DW/ methanol (negative control), were also placed on the nutrient agar. These plates were incubated at 32±1°C for 18 hrs. Zone of inhibition (measured in mm) was considered as an indicator for the presence of antimicrobial activity of a particular extract against the set of pathogenic micro-organisms. During the bioassay, zones of inhibition appearing above 8 mm diameter were considered as excellent, between 7–8 mm diameter as good, between 6 and 7 mm as moderate and less than that as poor indicators of the anti-microbial activity.

Column with Sephadex LH-20 (70 cm X 5 cm) was prepared and Ethyle acetate fraction of *P. tetrastromatica* (PTOH.2) was loaded onto this column. The fractions were eluted with methanol-chloroform (1:1) solvent. This yielded

180 fractions of 5 ml each and every fraction was analysed for the presence of antimicrobial activity.

RESULTS

The results of the assay, on the methanolic extracts of 17 seaweeds, confirmed the presence of phenols, cardiac glycosides, tannins, alkaloids, anthraqu-inones, saponins and flavonoids in all the seaweed species. Cardiac glycosides were absent in *S. marginatum*. Steroids and Terpenoids were *absent* in *S. marginatum, A. taxiformis, A. fragilissima, J. reubens* and *Cd. fastigiata* (Table 2).

In the present study, the methanolic extracts of 17 seaweeds were assayed for the presence of antimicrobial activity. They were mainly active against Gram negative bacteria as compared to Gram positive bacteria (Table 3). Seaweed (*P. tetrastromatica*) also showed a very good antimicrobial activity against the Gram positive bacteria (*S. pyrogens, B. subtilis and S. aureus*). On the basis of the preliminary results, the crude extract *of P. tetrastromatica* was further fractionated with hexane (PTOH.1), ethyl acetate (PTOH.2) and the remaining fraction was the aqueous part (PTOH.3). These fractions were concentrated and lyophilized. After that, the anti-microbial activity of these fractions was evaluated (Table 4). The ethyl acetate fractions of *P. tetrastromatica* (PTOH.2) were found to be active against *P. vulgaris*, *S. pyrogens* and *Pseud.* sp.

PTOH.2 were further loaded onto LH –20 column, with methanol-chloroform (1:1) solvent and fractions were merged into six fractions, based on the TLC results (PTOH.2.1, PTOH.2.2, PTOH.2.3, PTOH.2.4, PTOH.2.5, PTOH.2.6) and bacterial activities were checked. The findings indicated that the fraction of PTOH.2.2 was active against *S. pyrogens* and *P. vulgaris*, while the fraction of PTOH.2.6 was also active against *S. pyrogens* (Table 5).

TABLE 2
Preliminary phytochemical screening of methanolic extracts of seaweeds

Sr. No.	Phytochemical analysis								
	Seaweeds	Phenols	Tannins	Alkaloids	Anthraqu-inones	Flavonoids	Cardiac glycosides	Saponins	Steroids and terpenoids
1	*S. marginatum*	++	++	++	+++	++	-	++	-
2	*St. marginatum*	+++	+++	+++	+++	++	++	+++	+++
3	*P. tetrastromatica*	+++	+++	+++	+++	+++	++	+++	++
4	*P. gymnospora*	+++	+++	+++	+++	+++	+++	++	+++
5	*C .Implexa*	+++	+++	+++	+++	+++	+++	+++	+++
6	*D. australis*	+++	+++	+++	+++	++	++	+++	++
7	*D. bartayresiana*	++	+++	+++	+++	++	++	+++	++
8	*S. aspermum*	+++	+++	+++	+++	+++	++	+++	++
9	*A. taxiformis*	++	++	+++	+++	++	++	+++	-
10	*A. fragilissima*	++	++	+++	+++	++	++	++	-
11	*J. reubens*	++	++	+++	+++	++	++	+++	-
12	*C. racemosa*	+++	+++	++	+++	+++	+++	++	+++
13	*C. peltata*	+++	+++	+++	+++	+++	+++	+++	++
14	*C. taxifolia*	++	++	+++	+++	++	++	+++	++
15	*C. fragile*	++	++	+++	+++	+++	++	+++	++
16	*Cd. fastigiata*	++	++	+++	+++	+++	++	++	-

(- absent, + traces, ++ moderate, +++ abundance)

TABLE 3
Antimicrobial activity (zone of inhibition in mm) of the methanolic extracts of seaweeds extracts

S. No.	Seaweeds	Micro-organisms								
		S. aureus	*S. pyrogenes*	*B. subtilis*	*E. coli*	*Pseud.* sp	*S. marganii*	*P. vulgaris*	*K. pneumon*	*C. albicans*
1.	*S. marginatum*	6	-	6	-	-	-	-	7	-
2.	*St. marginatum*	-	6	-	-	-	6	6	7	-
3.	*P. tetrastromatica*	7	8	9	-	9	7	7	9	-
4.	*P. gymnospora*	8	-	-	-	-	-	-	9	6
5.	*C. implexa*	-	-	-	-	-	-	-	6	-
6.	*D. australis*	6	-	-	-	7	6	6	6	6
7.	*D. bartayresiana*	-	-	-	-	-	-	-	6	6
8.	*S. aspermum*	6	-	-	-	-	-	-	6	-
9.	*A. taxiformis*	-	-	-	-	-	-	-	6	6
10.	*A. fragilissima*	-	-	-	-	-	7	-	-	-
11.	*J. reubens*	-	9	6	8	-	8	6	6	9
12.	*C. racemosa*	-	4	-	-	6	6	-	-	6
13.	*C. peltata*	-	-	-	-	6	6	6	6	-
14.	*C. taxifolia*	6	6	6	-	-	6	-	6	6
15.	*C. fragile*	-	-	6	-	6	6	6	6	6
16.	*Cd. fastigiata*	-	-	6	-	-	6	-	7	6

TABLE 4
Antimicrobial activity (zone of inhibition in mm) of fractions of *P. tetrastromatica*

S. No.	Microorganisms	*P. tetrastromatica* (Fractions)		
		PTOH.1	PTOH.2	PTOH.3
1.	*S. pyrogenes*	8	9	-
2.	*B. subtilis*	-	-	9
3.	*P .vulgaris*	8	8	-
4.	*Pseud.* sp	-	8	-

The active fraction was again fractionated on Sephadex LH-20 column [methanol-chloroform (1:1) solvent]. For this, a total of 21 fractions were collected and after TLC, they were pooled in 8 fractions. Antimicrobial activity was checked against the active pathogens for all 8 fractions. The results indicated that fractions 1-5 (PTOH.2.2.1, PTOH.2.2.2, PTOH.2.2.3, PTOH.2.2.4 and PTOH.2.2.5) were active against *P. vulgaris*. Meanwhile, PTOH.2.2.5 was also found to be active against *S. pyrogens* (Table 6).

The preliminary NMR of the fraction PTOH.2.2.2 was carried out to find out the nature of the compounds responsible for the antimicrobial activity. The compounds appeared to be fatty acids. The major fatty acid appeared to be similar to stearic acid (*Fig. 1*). On the basis of the NMR, the antimicrobial activity of pure stearic acid (0.01 µg/ml) was checked against *S. pyrogens*, *B. subtilis*, *P. vulgaris* and *Pseud* sp. However, no activity was observed against these bacteria.

TABLE 5
Antimicrobial activity (zone of inhibition in mm) of gel fractions *P. tetrastromatica*

S. No.	Microorganisms	*P.tetrastromatica* (Fractions)					
		PTOH.2.1	PTOH.2.2	PTOH.2.3	PTOH.2.4	PTOH.2.5	PTOH.2.6
1.	*S. pyrogenes*	-	9	-	-	6	-
2.	*B. subtilis*	-	-	-	-	-	-
3.	*P .vulgaris*	-	8	-	-	-	-
4.	*Pseud.* sp	-	-	-	-	-	-

TABLE 6
Antimicrobial activity (zone of inhibition in mm) of gel fractions *P. tetrastromatica*

S. No.	Microorganisms	*P. tetrastromatica* (Fractions)					
		PTOH.2.2.1	PTOH.2.2.2	PTOH.2.2.3	PTOH.2.2.4	PTOH.2.2.5	PTOH.2.2.6
1.	*S. pyrogenes*	-	-	-	-	9	-
2.	*B. subtilis*	-	-	-	-	-	-
3.	*P .vulgaris*	9	10	9	9	9	9
4.	*Pseud.* sp	-	-	-	-	-	-

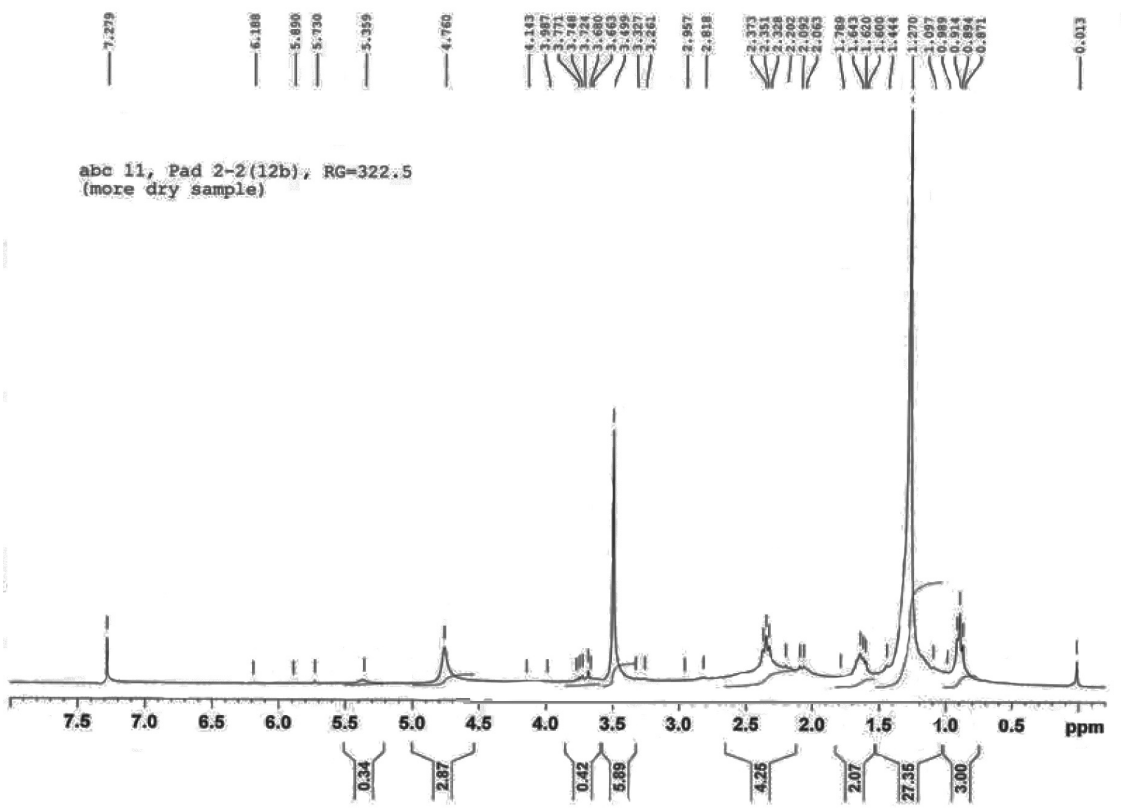


Fig. 1: NMR of the fraction PTOH.2.2.2

DISCUSSION

Recently, much attention has been directed towards extracts and biologically active compounds isolated from seaweeds. Marine algae have received comparatively less bioassay attention. On the contrary, there are a number of seaweeds with economic potential (Critchley *et al.*, 1998). Alternatively, findings from academic laboratories could result in new cultivation initiatives. It is important to highlight that the red alga *Sphaerococcus coronopifolius* has shown to possess antibacterial activity (Donia *et al.*, 2003) and the green alga (*Ulva lactuca)* has been indicated as an anti-inflammatory compound. An anti-tumour compound has been isolated from *Portieria hornemannii* (Faulkner *et al.*, 2002). *Ulva fasciata* produces a novel sphingosine derivative which has been found to have antiviral activity *in vivo* (Garg *et al.*, 1992). A cytotoxic metabolite, stypoldione, inhibiting microtubule polymerization and thereby preventing the formation of mitotic spindle, has been isolated from tropical brown alga - *Stypodium zonale* (Gerwick *et al.*, 1985). *P. hornemannii* has been found to be a novel source of cytotoxic; whereas penta, halogenated monoterpene, halomon, exhibit one of the most extreme examples of differential cytotoxicity in the screening conducted by the National Cancer Institute (NCI), USA. In specific, halomon has been selected for preclinical drug development since this compound shows toxicity to brain, renal and colon tumour cell-lines. The preliminary *in vivo* evaluations have been extremely encouraging (Carte *et al.*, 1996).

The review of literature in some studies shows that methanol extraction appears more effective, particularly in terms of antimicrobial activity than *n*-hexane and ethyl acetate (Febles *et al.,* 1995), whereas in others, chloroform has been shown to be better than methanol and benzene (Sastry and Rao, 1994). In the present study, the crude extract with methanol was prepared as it was evident from the experience of the previous study and the fact that the use of organic solvents always provides a higher efficiency in extracting antimicrobial activities, as compared to water extraction (Rosell and Srivastava, 1987). Methanol extract was further fractionated using *n*-hexane and ethyl acetate to separate compounds in increasing polarity.

The result from the antimicrobial assay of the methanolic extracts of 17 seaweeds in the present study showed the presence of biologically active compounds. The antimicrobial assay of the methanolic extracts of all the 17 seaweeds tested showed varying degrees of antibacterial activity, indicating that most of the active compounds are polar in nature. *P. tetrastromatica* and *J. reubens* showed activity against the maximum number of bacteria, whereas *A. fragilissima* and *C. implexa* showed activity against only one bacterium. Different bacterial species were found to differ in susceptibility to the methanol extract of different seaweeds, with the Gram positive organisms being generally more susceptible than the Gram negative bacteria (Rao and Parekh, 1981; Pesando and Caram, 1984; Reichelt and Borowitzka, 1984). Ethyl acetate extract (PTOH.2) exhibited a higher degree of antimicrobial activity as compared to water and hexane extract fractions.

The production of antimicrobial activities is considered to be an indicator of the capability of the seaweeds to synthesize bio-active secondary metabolites. The complexity of antimicrobial properties in seaweeds is due to their multiple inhibitory properties. In several cases, different substances have been found in the same seaweed (Olesen *et al.*, 1963). The pytochemical properties (phenols, tannins, alkaloids, anthraquinones, flavonoids, cardiac glycosides, saponins, steroids and terpenoids) of the screened seaweeds were observed to differ from traces to abundance. Therefore, it is not surprising that there are differences in the antimicrobial activity of different seaweeds.

The preliminary NMR study of fraction PTOH.2 indicated the presence of a number of fatty acids and one of the major compounds is similar to stearic acid. However, the antimicrobial assay of pure stearic acid on the tested pathogens did not show any activity.

The data presented in this study constitutes the initial results of a screening programme. The seaweed appears as a potential source of antibacterial drugs, and as about 720 species are common in Indian waters (Sajid and Satam, 2003), many more seaweeds could be investigated for the presence of potential bioactivities.

ACKNOWLEDGEMENTS

I wish to thank Department of Science and Technology, New Delhi, India, for the financial support rendered to finance this study. The authors are also grateful to the Director of the National Institute of Oceanography, Goa, India,

for providing the necessary laboratory facilities. One of the authors (AC) is also grateful to University Malaysia Terengganu for the award of a research fellowship.

REFERENCES

Carte, B.K. (1996). Biomedical potentials of marine natural products. *Bioscience, 46,* 271–286.

Critchley, A.T., Gillespie, R.D. and Rotmann, K. W.G. (1998). Seaweed resources of South Africa. In A.T. Critchley and M. Ohno (Eds.), *Seaweed resources of the world* (pp. 413-425). Jap. Interntl. Coop. Agen., Japan.

Donia, M. and Hamann, M.T. (2003). Marine natural products and their potential applications as anti-infective agents. *The Lancet, 3,* 338–348.

Faulkner, D.J. (2000a). Highlights of marine natural products chemistry (1972–1999). *Nat. Prod. Rep., 17,* 1–6.

Faulkner, D.J. (2000b). Marine Pharmacology. *Antonie Van Leeuwenhoek,* 77, 135–145. Da Rocha, A.B., Lopes, R.M. and Schwartsmann, G., 2001. Natural products in anticancer therapy. *Curr. Opin. Pharmacol., 1,* 364–369.

Faulkner, D.J. (2002). Marine natural products. *Nat. Prod. Rep, 19,* 1–48.

Febles, C.I., Arias, A., Gil-Rodriguez, M.C., Hardission, A. and Sierra Lopez, A. (1995). *In vitro* study of antimicrobial activity I algae (Chlorophyta, phaeophyta and Rhodophyta) collected from the coast of Tenerife (in Spanish). *Annuario del Instituto de Estudios Canarios, 34,* 181-192.

Garg, H.S., Sharma, M., Bhakunibrenda, D.S., Pramanikajay, N. and Bose, K. (1992). An antiviral sphingosine derivative from the green alga, *Ulva fasciata. Tetrahedron Lett., 33,* 1641–1644.

Gerwick, W.H., Fenical, W. and Norris, J.N. (1985). Chemical variation in the tropical seaweed *Stypopodium zonale* (Dictyotaceae). *Phytochem., 24,* 1279-1283.

Jimenez-Escrig, Antonio and Goni, Isabel Cambrodón.(1999). A., Evaluación nutricional y efectos fisiológicos de macroalgas marinas comestibles. Archivos *Latinoamericanos de Nutrición, 49:* 114–120 Goñi.

Mabeau, S. and Fleurence J. (1993). Seaweed in food products: biochemical and nutritional aspects. *Trends in Food Science and Technology, 4,* 103–107.

Olesen, P.E., Maretaki A. and Almodovar L.R. (1963). An investigation of antimicrobial substances from marine algae. *Bot. Mar., 6,* 224-232.

Pesando, D. and Caram, B. (1984). Screening of marine algae from the French southeastern Mediterranean coast for antibacterial and antifungal activities. *Bot. Mar., 27,* 381-386.

Rao, P. S. and Parekh K.S. (1981). Antibacterial activity of Indian seaweed extracts. *Bot. Mar., 24,* 577-582.

Reichelt, J.L. and Borowitzka, M.A. (1984). Antimicrobial activity from marine algae: Results of a large-scale screening programme. *Proc. Int. Seaweed Symp., 11,* 158-167.

Rosell, K.G. and Srivastva, L.M. (1987). Faty acids as antimicrobial substances in brown algae. *Hydrobiologia, 151/152,* 471-475.

Sastry, V.M.V.S. and Rao, G.R.K. (1994). Antibacterial substances from marine alsgae; successive extraction using benzene, chloroform and methanol. *Bot. Mar., 37,* 357-360.

Sajid, I.K. and Satam, S.B. (2003). Seaweed Mari culture: Scope and Potential in India. *Aquaculture Asia* (VIII), *4,* 26-29.

Schwartsmann, G., Rocha, D.A., Berlinck, A.B. and Jimeno, J. (2001). Marine organisms as a source of new anticancer agents. *Lancet Oncol., 2,* 221–225.

Singleton, V. L. and Rossi, J.R. (1965). Colorimetry of total phenolic with phosphomolybdic acid-phosphomngstic acid reagents. *Ame. J. Enol. Viticult., 16*(3), 144-158.

Pertanika J. Trop. Agric. Sci. **32(1)**: 77 - 83 (2009)

ISSN: 1511-3701

Effect of Different Extraction Procedures on Antimicrobial Activity of Marine Bivalves: A Comparison

Sumita Sharma[1*], Anil Chatterji[2] and Partha Das[1]

[1]National Institute of Oceanography, Dona Paula, Goa-403 001, India

[2]Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

ABSTRACT

Anti-bacterial activity was evaluated in different crude extracts of five commercially important edible marine bivalves, namely *Meretrix casta* (Chemnitz), *Polymesoda* (Geloina) *P. erosa* (Solander), *Perna viridis* (Linnaeus), *Crassostrea gryphoides* (Schlothim) and *Villorita cyprinoides* (Grey), collected from the coast of Goa (India). Three different procedures, *viz.* methanol (MeOH), PBS and acid-enzyme hydrolysis (AEH), were used to prepare the extracts. The efficacy of the extraction procedure was assessed on the antimicrobial activity. Antimicrobial assay was carried out against 8 bacterial strains (3 Gram positive and 5 Gram negative bacteria) and 1 species of fungi. The AEH extracts showed higher activity against the tested organisms as compared to MeOH and PBS extracts. The findings of the present study confirmed that the antimicrobial activity in bivalves appeared to be dependent on the extraction process. Considerable interspecies variation was also observed.

Keywords: Extraction procedures, methanol and PBS extracts, acid enzyme hydrolysate, antimicrobial peptides

INTRODUCTION

Due to an alarming rise in the occurrence of antibiotic resistant bacterial strains, the identification of new antimicrobial compounds has become one of the frontier areas in biomedical research. Marine invertebrates are known to rely on innate immune mechanisms which include both interacting cellular and humoral components to protect against potential pathogen (Tincu and Taylor, 2004). Innate immune mechanism in marine invertebrates is known to protect these organisms against potential pathogens. Moreover, it has been well known that the innate immunity is triggered immediately after microbial infection to produce antimicrobial compounds including small antimicrobial peptides (AMP). In recent years, it has widely been recognized that AMPs are strong defensive weapons against bacteria and/or fungi, viruses, or parasites in multicellular organisms (Zasloff, 2002). Furthermore, AMPs are also known as major components of innate immune defence system in invertebrates (Seo *et al.*, 2005).

Considering the fact that the marine animals can survive in a hostile environment where they are surrounded by various pathogenic organisms, including human pathogens (Bouchriti and Goyal, 1992) and that they are potential sources for bioactive compounds, an attempt was done in the present study to evaluate the antimicrobial activity in five commonly occurring edible bivalves, such as *Meretrix casta* (Chemnitz), *Polymesoda* (Geloina) *P. erosa* (Solander), *Perna viridis* (Linnaeus), *Crassostrea gryphoides* (Schlothim) and *Villorita cyprinoides* (Grey). In

Received: 20 May 2008
Accepted: 8 October 2008
*Corresponding Author

continuation with the same effort, an attempt was also made to assess and compare the efficacy of the extracts prepared using three different extraction procedures.

MATERIALS AND METHODS

Live bivalves used in the present study were collected from various beaches of Goa, Maharashtra and Karnataka in India. These bivalves were not collected during the summer months to avoid stress related to disease, elevated water temperature, hypoxia or gametogenesis.

Bivalves were brought to the laboratory in seawater, washed, and de-shelled; tissue and mantle fluids were also collected. Material collected from each animal was divided into three equal parts for the preparation of Methanol (MeOH), phosphate buffer saline (PBS) extracts with protease inhibitors and acid enzyme hydrolysate (Chatterji *et al.*, 2000). These procedures yielded four different types of extract from each animal. They were designated as MeOH, PBS, AEH -MeOH and AEH- Aq extracts.

All the extracts were quantitatively analyzed using the standard methods for the estimation of total protein (Lowry *et al.*, 1951), carbohydrates (Dubois *et al.*, 1956) and lipids (Parsons *et al.*, 1984).

The antimicrobial activity of the bivalve extracts prepared was evaluated against a set of 9 pathogenic micro-organisms (*Bacillus subtilis, Escherichia coli, Pseudomonas* sp, *Streptococcus pyrogenes, Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumoneae, Serrratia marganii* and *Candida albicans*) using standard disc diffusion assay (qualitative) and liquid growth inhibition assay (quantitative). The liquid growth inhibition assay was carried out at 32±1°C for 30 hrs. Three sets of sterilized glass vials, with 2.5 ml nutrient broth (NB), were prepared for each pathogenic micro-organism. These sets were inoculated with one bacterial suspension (18 hr old), and out of which, one set each was inoculated with 10 µl of Gentamycin (1 mg/ml). This was treated as the positive control. In another set, all the micro-organisms were inoculated with 10 µl of animal extracts (50 mg/ml). One set was maintained as the negative control to see the normal growth of the tested organisms. After 2 hrs, an aliquot was taken in a micro titer plate and optical density was measured at 570 nm using an ELISA reader (BioRad, Microplate Reader, Model No. 680). The percentage of the inhibition growth was calculated with absorbance values using the following equation, where CI is the percentage Inhibition index, A = A_{570} of bacteria with NB (control) and B = A_{570} of bacteria with extract or Gentamycin (experimental).

$$C\,I = \left[\frac{A - B}{A}\right] \times 100$$

RESULTS

The total protein content was the highest as compared to the total contents of carbohydrate and lipids in all the extracts. The total content of lipid in all the extracts was very less as compared to the total protein and carbohydrate (Table 1).

Bioassay of MeOH extracts, using standard disc diffusion assay, showed that *P. erosa* appeared to be the most active one against seven tested organisms. In case of the PBS extracts, *M. casta* showed the highest antimicrobial activity against all pathogens. Meanwhile, in the case of AEH-MeOH extract, significant antimicrobial activity was shown by *C. gryphoides* against all the pathogens. On the other hand, the AEH-Aq extracts of *P. erosa* showed a very good activity against all the tested organisms (Table 2).

As compared to Gentamycin, all the MeOH extracts showed more than 30 to 50% antimicrobial activity against *E. coli* when liquid growth inhibition assay was performed. The MeOH extract of *P. erosa* appeared to be the most promising extract showing antimicrobial activity against both Gram positive and Gram negative tested organisms for 18-24 hrs (*Fig. 1*). The activity of the extract of *M. casta* PBS was better than Gentamycin for 10 hrs. It was interesting to note that Gentamycin did not have very good antimicrobial activity against *S. marganii*, but all the PBS extracts (except *V. cyprinoides*) were able to inhibit the growth (>50%) of Gram negative bacterium for more than 6 hrs (*Fig. 1*). In the case of AEH–MeOH extracts, it was observed that *C. gryphoides, V. cyprinoides, M. casta* and *P. erosa* appeared to be better than or at par with Gentamycin in successfully inhibiting the growth of some pathogens (*Fig. 1*). In the group of AEH–Aq extracts, different extracts (except *V. cyprinoides*) inhibited the growth of *E. coli, K. pneumoneae, S. marganii* and *S. aureus* better than Gentamycin for 4–10 hrs (*Fig. 1*).

TABLE 1
Biochemical analysis of different bivalves

Extracts		Biochemical analysis		
		Protein (µg/ml/mg)	Carbohydrate (µg/ml/mg)	Lipid (µg/ml/mg)
P. erosa	MeOH	420	24.48	0.6
	PBS	640	2.31	0.07
	AEH-MeOH	528	7.08	0.19
	AEH-Aq	154	6.54	0.02
P. viridis	MeOH	280	24.72	0.09
	PBS	830	32.05	0.04
	AEH-MeOH	440	2.04	0.05
	AEH-Aq	170	1.05	0.01
M. casta	MeOH	190	5.76	0.15
	PBS	960	24	0.08
	AEH-MeOH	356	7.2	0.03
	AEH-Aq	194	32.77	0.01
C. gryphoides	MeOH	270	27.61	0.2
	PBS	370	15	0.07
	AEH-MeOH	744	6.48	0.03
	AEH-Aq	126	16.68	0.02
V. cyprinoides	MeOH	246	34.28	0.15
	PBS	415	19.58	0.05
	AEH-MeOH	720	22.08	0.06
	AEH-Aq	176	5.25	0.02

All the experiments were carried out in triplicates and the results are expressed as mean values. The results were then compared using the two-way ANOVA test (StatSoft, 1999). p values < 0.05 were considered as significant. In this study, it was observed that when the antimicrobial activity of one extract was compared to all the tested organisms, the p value < 0.05 indicated that the presence of antimicrobial compound/ peptide in the extract could have different degrees of activity against various organisms.

DISCUSSION

Most of the work carried out on antimicrobial compounds from marine bivalves deal with *M. edulis, M. galloprovicialis, G. demissa, C. verginica* and *C.* gigas (Haug *et al.*, 2004; Tincu and Taylor, 2004; Seo *et al.*, 2005). In this study, an attempt was done to screen a few more bivalves, especially the commonly occurring edible ones. The source for majority of the AMPs reported has been from the hemocytes (Charlet *et al.*, 1996; Mitta *et al.,* 1999a,b), epithelial tissues (Marshall and Arenas, 2003; Noriaki *et al., 2003*) and the tissues of gut and respiratory organs (Tincu and Taylor, 2004). Considering this an important aspect, the extracts were prepared using both mantle tissue and mantle fluid of the bivalves.

Presuming that the antimicrobial compounds were either protein or peptide in nature, a combination of 'soft techniques' was selected for the preparation of crude Methanol (homogenization with chilled Methanol + filtration) and PBS extracts (homogenization with PBS + protease inhibitor + centrifugation) to ensure that the functionality and/or the biological activity of the analytes remained intact (Visser *et al.,* 2005). For this, methanol

TABLE 2
Antimicrobial activity (zone of inhibition in mm) induced by different extracts of five bivalves

Test Organism	*P. erosa*				*P. viridis*				*M. casta*				*C. gryphoides*				*V. cyprinoides*			
	Me OH	PBS	AEH MeOH	AEH Aq	Me OH	PBS	AEH MeOH	AEH Aq	Me OH	PBS	AEH MeOH	AEH Aq	Me OH	PBS	AEH MeOH	AEH Aq	Me OH	PBS	AEH MeOH	AEH Aq
S. aureus	11	9	8	6	-	6	-	6	11	8	8	9	10	-	6	12	-	-	8	7
S. pyrogenes	12	8	7	11	11	9	-	7	7	8	6	7	11	9	12	-	-	-	9	-
B. subtilis	13	7	12	12	-	8	-	-	-	12	7	-	9	-	6	-	-	-	-	11
E. coli	8	-	11	12	9	-	-	10	6	11	13	6	9	-	7	12	-	-	7	9
Pseud. sp	12	-	10	11	10	6	6	-	-	7	11	-	-	9	8	-	-	-	9	11
S. marganii	11	8	-	6	-	6	6	6	12	7	-	6	-	9	13	-	-	-	-	11
P. vulgaris	-	-	10	12	-	-	-	7	6	6	-	7	-	-	12	7	-	-	-	13
K. pneumon.	-	6	9	7	-	6	-	6	6	12	7	6	-	7	9	7	-	9	9	12
C. albicans	10	6	6	12	-	10	7	7	-	11	9	10	-	12	8	10	-	-	9	13

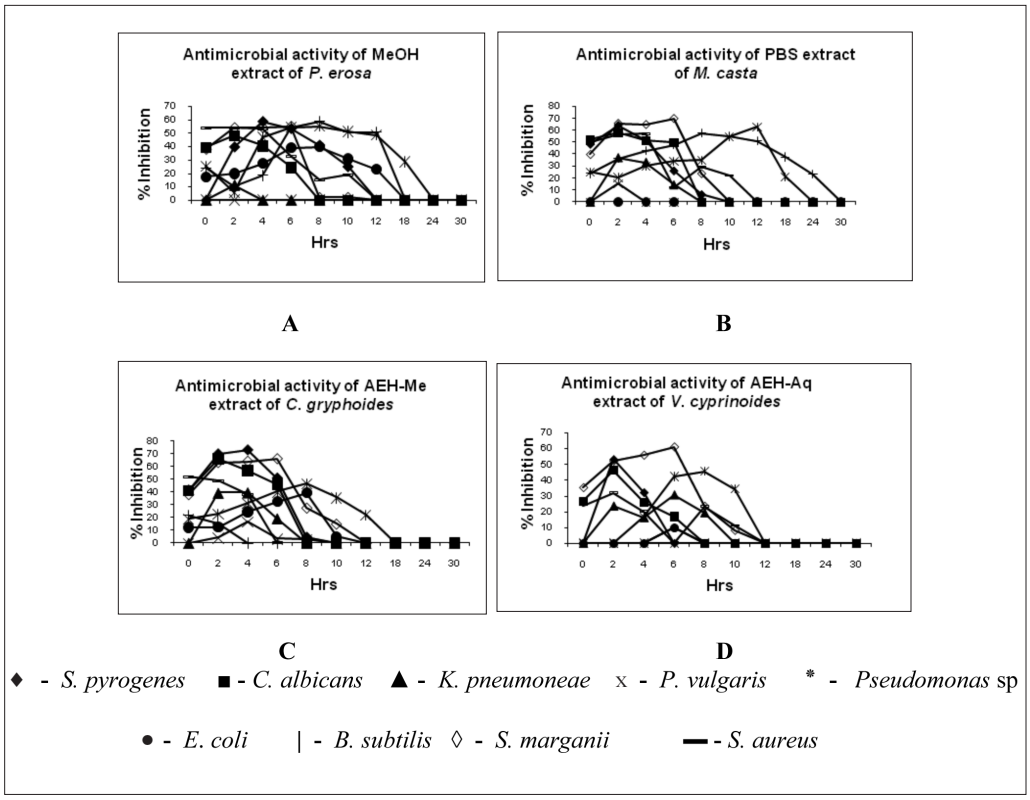


Fig. 1: Antimicrobial activity induced by different extracts against pathogenic organisms [Best activity of *Gentamycin against* - S. pyrogenes - 73% (10 h), C. albicans - 74.5% (10 h), K. pneumoneae - 28% (10 h), P. vulgaris - 48% (2 h), Pseudomonas *sp* - 63.5% (10 h), E. coli - 8.5% (0 h), B. subtilis - 78% (30 h), S. marganii - 39% (30 h), S. aureus - 69% (10 h)]

was selected as a suitable solvent as it gave good extraction efficiency. Most of the low molecular weight proteins/peptides (stable at room temperature) were extracted from it and had an added advantage of allowing rapid sample concentration through evaporation. The other solvent/homogenization medium is PBS as most of the active high molecular weight proteins and peptides are known to get extracted in it. Protease inhibitor simultaneously deactivated proteolytic enzymes in the tissue which would otherwise cause rapid degradation of the proteins/peptides (Conlon, 2007). The results of antimicrobial assay in the present study indicated that these extracts showed high antimicrobial activity against the tested pathogens, indicating that these procedures are capable of extracting the antimicrobial compound(s), with relatively higher activity, without degrading the nature of the compound.

Extraction from a biological matrix can also be achieved using the 'harsh' techniques in which extraction conditions deviate from physiological conditions and result in completely different physico-chemical properties of the analytes and interfering molecules. The AEH extraction procedure employed in the present study was very harsh as all the conditions (e.g. highly acidic pH, 100°C temperature) were totally different from the physiological conditions of the biological matrices. Enzyme protosubtilin hydrolyzed high-molecular animal proteins to short peptides and a mixture of free amino acids. The crude extract has been reported to possess short peptides, free amino acids (conjugated with metals like Cu, Zn etc) and minerals. They were also reported to possess low fat and salt contents (Chatterji *et al.*, 2000). The antimicrobial activity of the AEH extract might be either due to short peptides, amino acids conjugated with metal ions

or both or it could also be due to the generation of artefacts during the extraction process.

The standard disc diffusion method is a sensitive and highly accepted method used for the detection of antimicrobial activity, but many feel that it is a qualitative method and should not be used to quantify the activity (Rios *et al.*, 1988). Therefore, the results were quantified using the liquid growth inhibition assay. These results largely confirmed the findings of disc diffusion assay and helped in calculating the inhibition percentage of culture growth caused by a particular extract towards the pathogenic organism(s). This assay was carried out for 30 hrs to compare the time when the extracts started inhibiting the growth of the organism and when it lost the activity. It was observed that most of the crude extracts were able to induce inhibition for ±10 hrs and also showed antimicrobial activity at par (or better at some places) with a purified antibiotic like Gentamycin.

The results presented in this paper are the first stage of a bioassay-based baseline survey to achieve the isolation, purification, structure elucidation and biological testing of the active compound(s) from the potent marine bivalves. This comparative data suggests that *P. erosa, M. casta,* C. *gryphoides* and *V. cyprinoides* are the potential candidates for the isolation and purification of potent antibiotics. Moreover, the extraction of the antimicrobial compound(s) appeared to be dependent on the extraction procedure and the nature of the solvents used for extraction.

ACKNOWLEDGEMENTS

I wish to thank the Department of Science and Technology, New Delhi, India, for the financial support given to finance this study. The authors are also grateful to the Director of the National Institute of Oceanography, Goa, India, for providing the necessary laboratory facilities. One of the authors (AC) is also grateful to University Malaysia Terengganu for the award of a research fellowship.

REFERENCES

Bouchriti, N. and Goyal, S.M. (1992). Evaluation of three methods for the concentration of poliovirus from oysters. *Microbiologica*, *15*, 403-408.

Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J.A. and Bulet, P. (1996). Innate Immunity. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis. J. Biol. Chem.*, *271*, 21808-21813.

Chatterji, A., Ansari, Z.A., Bichurina, M.A., Sovetova, M. and Boikov, Y.A. (2000). An extract obtained from marine animal having antiviral activities and process for its extraction. *Indian Patent, 2000, Application No. NF 159/00.*

Conlon, J.M. (2007). Purification of naturally occurring peptides by reversed-phase HPLC. *Nat. Protocols.*, *2,* 191– 97.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, *28*, 350.

Haug, T., Stensvag, K., Olsen, M., Orjan, M., Sandsdalen, E. and Styrvold, O.B. (2004). Antibacterial activities in various tissues of the horse mussel, *Modiolus modiolus. J. Invertebr. Pathol., 85*(2), 112-119.

Lowry, O., Rosebrough, N.J., Farr, A.L. and Randall, H.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, *193*, 265–275.

Marshall, S.H. and Arenas, G. (2003) Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology. *Electro. J. Biotech., 6*, 1-14.

Mitta, G., Hubert, F., Noel, T. and Roch, P. (1999a). Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel *Mytilus galloprovincialis. Eur. J. Biochem.*, *265*, 71-78.

Mitta, G., Vandenbulcke, F., Hubert, F. and Roch, P. (1999b). Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. *J. Cell. Sci.*, *112*, 4233-4242.

Noriaki, I., Norio, T., Yohko, E., Yohko, M., Kazumasa, U., Tomoya, M. and Toshihiro, N. (2003). Purification and characterization of three isoforms of chrysophsin, a novel

antimicrobial peptide in the gills of the red sea bream, *Chrysophrys major. Eur. J. Biochem.*, *270*, 675-686.

Parsons, T.R., Maita, Y. and Lalli, C.M. (1984). *A Manual of Chemical and Biological Methods for Seawater Analysis*. Oxford: Pergamon Press.

Rios, J.L., Recio, M.C. and Viller, A. (1988). Screening as methods for natural products with antimicrobial activity: Review of the literature. *J. Ethnopharmaco.*, *23*, 127–149.

Seo, J.K., Crawford, J.M., Stone, K.L. and Noga, E. J. (2005). Purification of a novel arthropod defencin from the American oyster, *Crassostrea virginica. Biochem and Biophys Res Commun., 338*, 1998-2004.

Statsoft, Statistica FOR Windows [Computer program manual]. (1999). StatSoft Inc Tulsa, OK. 1999.

Tincu, A.J. and Taylor, S.W. (2004).Antimicrobial peptides from marine invertebrates. *Antimicrob Agents Chemother.*, *48*, 3645-3654.

Visser, N.F.C., Lingeman, H. and Irth, H. (2005). Sample preparation for peptides and proteins in biological matrices prior to liquid chromatography and capillary zone lectrophoresis. *Anal. Bioanal. Chem.*, *382*, 535–558.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, *24*, 389-395.

Pertanika J. Trop. Agric. Sci. **32(1)**: 85 - 90 (2009) ISSN: 1511-3701

Identification of Lysozyme Activity from Two Edible Bivalves - *Perna viridis* (Linnaeus) and *Meretrix casta* (Chemnitz)

Sumita Sharma[1*], Tanu[2] and Anil Chatterji[3]
[1]National Institute of Oceanography, Dona Paula, Goa, India
[2]Banasthali Universiti, Rajasthan
[3]Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

ABSTRACT

Lysozyme activity of two commercially important edible bivalves, namely *Perna viridis* (Linnaeus) and *Meretrix casta* (Chemnitz), was analyzed against *Micrococcus luteus*. The results of this study indicated that the extract, prepared by homogenizing the mantle fluid and meat, had more lysozyme activity as compared to the acetic acid extract. The extract of *P. viridis* showed a better activity than *M. casta*. Partial purification of lysozyme was carried out using gel filtration chromatography and initial ion (IE) exchange chromatography.

Keywords: **Lysozyme activity, marine bivalves, specific activity, protein concentration**

INTRODUCTION

Lysozymes are enzymes which are widely distributed in organisms, from bacteriophages to human. The major functions of lysozymes are lysing bacterial cells by hydrolyzing the beta-1-4-linked glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of peptidoglycan, a major component of bacterial cell walls (Salton, 1957; Chipman and Sharon, 1969; Jolles, 1996). In addition, they are believed to play an important role in host defence and digestion (Allam *et al.,* 2000; Cronin *et al.*, 2001; Xue *et al.,* 2004). Therefore, they are considered as one of the important antimicrobial agents of natural origin. In contrast to the many examples of the commercial use of chicken egg white lysozyme, there is little information available on the potential use of lysozymes from other sources. It has been hypothesized that lysozymes from aquatic species may have inhibitory activity against both Gram negative and Gram positive bacteria.

Several types of lysozyme viz. *c*, *g*, *i* have been identified from a wide range of organisms (Xue *et al.,* 2004). There has been an increasing interest in the distribution and characterization of invertebrate *i*-type lysozymes (including lysozymes of bivalve molluscs) in the recent years (Bachali *et al.,* 2002; Olsen *et al.,* 2003; Takeshita *et al.*, 2003; Zavalova *et al.*, 2003; Bachali *et al.,* 2004). Lysozyme activity has been detected in the body fluids and tissues of many bivalves' molluscs. Since marine bivalve molluscs are osmoconformers and poikilotherms, they are exposed to a wide range of environmental conditions and thus lysozymes from these bivalves have been evolved to be active under different environmental conditions (Bachali *et al.,* 2004). Lysozyme of marine bivalves are active at higher salt concentrations, lower temperatures and have higher activities than the specific activities of lysozyme of egg white; this makes them better suited for food and pharmaceutical industries (Zavalova *et al.*, 2003).

The present work is an attempt to identify the lysozyme activity and partially purify the lysozyme from the whole body extracts of two edible and commercially important marine

Received: 20 May 2008
Accepted: 8 October 2008
*Corresponding Author

bivalves, i.e. *Perna viridis* (Linnaeus) and *Meretrix casta* (Chemnitz). To the best of our knowledge, no lysozyme has been purified from these marine bivalves so far.

MATERIALS AND METHODS

Bivalves

Live specimens of *M. casta* were collected from St. Josinto Island from the intertidal zone during low tide, whereas *P. viridis,* being a sessile animal, was collected with the help of the divers from the deeper zone of the Dona Paula estuary in Goa (India) during the month of December, 2007 and March, 2008.

Extract Preparation

Animals were brought to the laboratory to be washed and de-shelled. Meat and mantle fluid was collected and divided into two equal parts: i) Acetic acid extract - One part was homogenized with chilled 2% acetic acid using a blender. The extract was centrifuged at 5000 rpm for 15 min and the supernatant containing lysozyme was collected. The second extraction was performed to further release lysozyme activity from the pellet using more than 2% acetic acid. The supernatants were collected, pooled, lyophilized and used as crude extract to check for lysozyme activity against *Micrococcus luteus.*

ii) Liquor extract - The other part was homogenized, centrifuged and supernatant was lyophilized and used as crude extract for lysozyme activity against *Micrococcus luteus.*

Determination of Protein Concentration

Protein assay (Lowry *et al.*, 1951) was carried out in triplicates to assess the concentration of protein for both the extracts of *M. casta* and *P. viridis* with bovine serum albumin as a standard protein.

Determination of Lysozyme Activity

20 µl of extract was mixed with 180 µl of bacterial suspension of *Micrococcus luteus* suspended in 0.2 M acetate buffer at pH 5.8 in a 96-well microplate at room temperature. The absorbance of the mixture was immediately measured at 450 nm with an ELISA plate reader (BioRad, Microplate Reader, Model No. 680). Absorbance was measured again 5 min after the initial reading and the decrease in the absorbance at 450 nm per min was calculated. All measurements were done in triplicates. One unit of lysozyme was defined as thet quantity which caused a decrease in the absorbance of 0.001 per min of bacterial suspension suspended in 0.2 M acetate buffer at pH 5.2 (Xue *et al.*, 2004).

Determination of Specific Activity

Specific activity was determined using the protein and lysozyme activity values:

$$\text{Specific activity} = \frac{\text{Lysozyme activity}}{\text{Protein Concentration}}$$

Partial Purification

Gel filtration chromatography - A Sephadex G-50 column was prepared and equilibrated with 0.02 M sodium acetate buffer (pH 5.0). Lyophilized extract was dissolved in sodium acetate buffer and loaded on the column. Fractions of 1 ml each were collected. The absorbance of these fractions was also monitored at 280 nm. The lysozyme activity was checked against *Micrococcus luteus.*

Ion exchange chromatography - The lyophilized active samples (McL2 and PvL2) were loaded on a SP-Sepharose FF column, equilibrated with 0.02 M sodium acetate buffer, at pH 5. The column was successively washed with 0, 0.1, 0.3 and 0.6 M of NaCl in 0.02M sodium acetate buffer pH 5.0 (Datta, 2005). The absorbance of the eluted fractions was monitored at 280 nm and lysozyme activity was checked.

SDS-PAGE – The approximate comparative molecular mass of the compounds, present in the crude lysozyme extracts, were estimated using the SDS-PAGE with a 12.0% running gel and a 4% stacking gel. The low-range (14–100 kDa) protein molecular markers, from Genei Pvt. Ltd., India, were used as the standards to calculate the molecular mass.

RESULTS AND DISCUSSION

Two different extracts, acetic acid and liquor extracts, were prepared from each animal. The total protein content was found to be more in the acetic acid extract of both the bivalves (*Fig. 1*). Surprisingly, the results of the lysozyme assay showed that the liquor extract of *P. viridis* had more lysozyme activity as compared to the acetic acid extract. On the other hand, both the

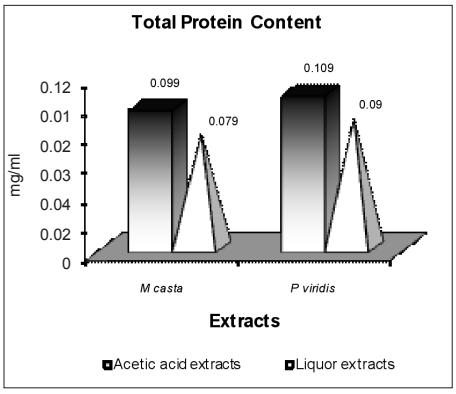


Fig. 1: Total protein content of different extracts

extracts of *M. casta* showed the same lysozyme activity. The determination of specific activities was shown to be better in the liquor extracts of both *M. casta* and *P. viridis* (Table 1). It may be possible that the acetic acid extract shows higher concentration of protein due to the fact that acetic acid also extracts proteins other than lysozymes from the animal tissues. On the other hand, liquor extract contains only those proteins which are either freely present in the mantle fluid, hemolymph or extracted due to homogenization and centrifugation (*Fig. 1*). Extracts were prepared in winter and once again at the onset of summer to see whether the different seasons have any effect on the lysozyme activity of these bivalves. It was observed that the extracts prepared in the month of March showed marked decrease in the activity and protein concentration as compared to the extracts prepared in December. Datta (2005) reported that a wide variation in the protein concentration and lysozyme activity took place in *Crassostrea virginica* due to seasonal variations. In addition, plasma lysozyme activity has been reported to be higher in winter (Chu and La Peyre, 1989).

The first step of purification using gel filtration (Sephadex G – 50) showed very encouraging results in the present study. When compared to the crude extracts, both the protein content and lysozyme activity were found to increase by ten times. The TLC carried out on these fractions confirmed the presence of proteins. The fractions were also read at 280 nm and fraction 7 of *M. casta* was observed to show the highest concentration (*Fig. 2*). On the contrary, the concentration of fraction 5 of *P. viridis* was found to be quite less (*Fig. 3*). Protein assay also showed the same pattern. Since both these fractions showed the same lysozyme activity against *M. luteus,* this might indicate the possibility that either the lysozyme present in the *P. viridis* is more potent than the one present in *M. casta* or the *M. casta* fraction has more proteins other than the lysozymes (Table 2).

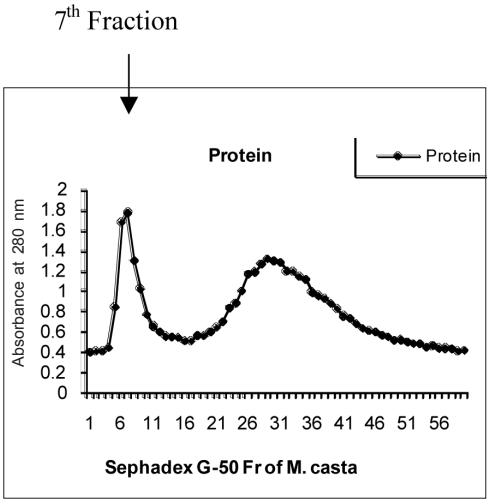


Fig. 2: Absorbance of Sephadex fractions of M. casta *at 280 nm*

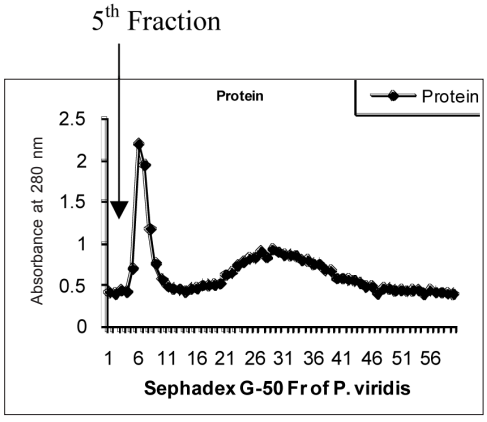


Fig. 3: Absorbance of Sephadex fractions of P. viridis *(crude and G-50 fractions)*

On the basis of the absorbance at 280 nm, the results of bioassay fractions were merged and reduced to 6 fractions in the case of *M. casta* – McL1, **McL2 (fr 7)**, McL3, McL4, McL5 and McL6, and 7 in the case of *P. viridis* - PvL1, **PvL2 (fr 5)**, PvL3, PvL4, PvL5, PvL6, PvL 7.

TABLE 1
The total protein concentration, lysozyme and specific activity of *M. casta* and *P. viridis* crude extracts

S. No	Extracts	Lysozyme activity (U/min/mg)	Total protein (mg/ml)	Specific activity (U/mg)
1.	Acetic acid extract of *M. casta*	12.8	0.099	1.29 x 10^2
2.	Liquor extract of *M. casta*	12.8	0.079	1.62 x10^2
3.	Acetic acid extract of *P. viridis*	14.0	0.109	1.28 x10^2
4.	Liquor extract of *P. viridis*	16.8	0.09	1.86 x10^2

TABLE 2
The total protein concentration, lysozyme and specific activity of merged G-50 fractions of *M. casta* and *P. viridis*

Merged G-50 Frs of *M. casta*	Lysozyme activity (U/min/mg)	Total protein (mg/ml)	Specific activity (U)	Merged G-50 Frs of *P.viridis*	Lysozyme activity (U/min/mg)	Total protein (mg/ml	Specific activity (U)
McL1 (Fr 1 - 4)	75	0.107	7.0 X10^2	PvL1 (Fr 1 - 2)	60	0.155	3.87x10^2
McL2 (Fr 7)	220	0.215	10.23X10^2	PvL2 (Fr 5)	220	0.180	12.22X10^2
McL3 (Fr 11 -12)	110	0.160	6.87X10^2	PvL3 (Fr 6 - 7)	140	0.280	5.0X10^2
McL4 (Fr 15 -19)	100	0.134	7.46X10^2	PvL4 (Fr 13 - 21)	90	0.176	5.11X10^2
McL5 (Fr 21 - 30)	150	0.180	8.33X10^2	PvL5 (25 - 28)	125	0.196	6.37X10^2
McL6 (Fr 45 - 60)	125	0.141	8.86X10^2	PvL6 (Fr 34 - 37)	80	0.198	4.04X10^2
-	-	-	-	PvL7 (Fr 47 - 55)	75	0.196	3.82X10^2

A comparative SDS PAGE of both the crude liquor extracts, McL2 and PvL2 were carried out, along with the molecular marker of 14–100 KDa. As a result, unclear bands were observed in the crude extracts of *M. casta* and *P. viridis*. However, a clear band was observed in McL2, while two were observed in PvL2, i.e. between 14 KDa and 29 KDa, indicating the possible presence of lysozymes (*Fig. 4*).

McL2 and PvL2 were further taken on a strong cation exchange column (SP Sepharose) for the next step of purification. Most of the fractions collected from McL2 did not show any activity against *M. luteus*. The fractions of PvL2 showed a better activity as compared to McL2 fractions, but these were still found to be less than the G-50 fractions (Table 3). This could be due to the fact that the solvent used (0.02 M sodium acetate buffer of pH 5 with increasing concentration of NaCl -0.00, 0.1, 0.3, and 0.6M) was not adequate to elute the lysozyme present in these samples (Xue *et al.*, 2004). In another work by Olsen *et al.* (2003), lysozyme was successfully eluted by employing a 0–0.5 M NaCl gradient in 0.05 M sodium acetate buffer (pH 5) from blue mussel (*Mytilus edulis*).

TABLE 3
Total protein concentration, lysozyme and specific activity of SP Sepharose fractions of McL2 and PvL2

SP Sepharose Fractions of McL2	Lysozyme activity (U/min/mg)	Total protein (mg/ml)	Specific activity (U)	SP Sepharose Fractions of PvL2	Lysozyme activity (U/min/mg)	Total protein (mg/ml)	Specific activity (U)
1	-	0.125		1	-	0.120	
2	-	0.123		2	-	0.121	
3	-	0.140		3	-	0.120	
4	-	0.170		4	-	0.152	
5	-	0.160		5	-	0.125	
6	-	0.165		6	-	0.135	
7	-	0.140		7	-	0.138	
8	1	0.155		8	-	0.125	
9	-	0.105		9	-	0.145	
10	-	0.140		10	-	0.145	
11	-	0.140		11	-	0.150	
12	-	0.185		12	-	0.145	
13	-	0.145		13	9	0.148	
14	-	0.145		14	-	0.140	
15	-	0.146		15	-	0.130	
16	-	0.145		16	-	0.185	
17	-	0.145		17	-	0.140	
18	-	0.130		18	-	0.140	
19	-	0.130		19	-	0.120	
20	-	0.135		20	5	0.110	
21	-	0.155		21	-	0.125	
22	-	0.160		22	-	0.128	
23	70	0.145	$4.82X10^2$	23	-	0.134	
24	80	0.145	$5.51X10^2$	24	-	0.156	
25	-	-		25	-	0.155	
26	-	0.165		26	-	0.140	
27	-	-		27	-	0.139	
28	-	0.125		28	98	0.135	$7.25X10^2$
29	-	0.120		29	-	0.140	
30	-	0.140		30	-	0.125	
31	-	0.130		31	150	0.135	$11.11X10^2$
32	-	0.185		32	155	0.135	$11.48X10^2$
33	5	0.196		33	163	0.145	$11.24X10^2$
34	100	0.180	$5.55X10^2$	34	-	0.135	
35	-	-		35	-	0.135	
36	-	-		36	-	0.120	

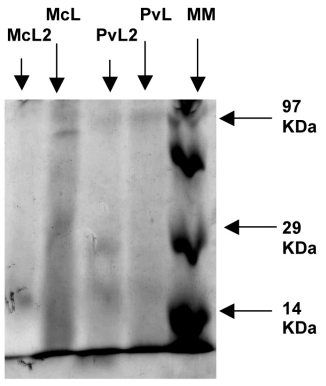


Fig. 4: Comparative SDS-PAGE of M. casta *and* P. viridis *at 280 nm*

ACKNOWLEDGEMENTS

I wish to thank Department of Science and Technology, New Delhi, India, for the financial support given to carry out this study. The authors are also grateful to the Director of the National Institute of Oceanography, Goa, India for providing the necessary laboratory facilities. One of the authors (AC) is also grateful to University Malaysia Terengganu for the award of a research fellowship.

REFERENCES

Allam, B., Paillard, C. and Auffret, M. (2000). Alterations in hemolymph and extrapallial fluid parameters in the Manila clam, *Ruditapes philippinarum*, challenged with the pathogen *Vibrio* tapetis. *J. Invertebr. Pathol., 76,* 63–69.

Bachali, S., Jager, M., Hassanin, A., Schoentgen, F., Jolles, P., Fiala-Medioni, A. and Deutsch, J.S. (2002). Phylogenetic analysis of invertebrate lysozymes and the evolution of lysozyme function. *J. Mol. Evol., 54,* 652–664.

Bachali, S., Bailly, X., Jolles, J., Jolles, P. and Deutsch, J.S. (2004). The lysozyme of the starfish Asterias rubens. A paradygmatic type i lysozyme. *Eur. J. Biochem., 271,* 237-242.

Chipman, D.M. and Sharon, N. (1969). Mechanism of lysozyme action. *Science, 165*, 454-65.

Chu Fu-Lin, E. and La. Peyre, J. F. (1989). Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*), *J. Invertebr. Pathol., 54*, 224–232.

Cronin, M.A., Culloty, S.C. and Mulcahy, M.F. (2001). Lysozyme activity and protein concentration in the haemolymph of the flat oyster *Ostrea edulis* (L.). *Fish Shellfish Immunol., 11,* 611–622.

Data, S. (2005). Purification of lysozyme from shell liquor of eastern oysters (*crassostrea virginica*) and its use in antimicrobial films to Preserve smoked fish. Thesis Louisiana State University and Agricultural and Mechanical College, Baton Rouge, LA 70803.

Jolles, P. (1996). From the discovery of lysozyme to the characterization of several lysozyme families. *EXS., 75,* 3-5.

Lowry, O., Rosebrough, N.J., Farr, A.L. and Randall, H.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem., 193*, 265–275.

Olsen, M., Nilsen, I.W., Sletten, K. and Myrnes, B. (2003). Multiple invertebrate lysozymes in blue mussel (*Mytilus edulis*). *Comp. Biochem. Physiol., B136*, 107–115.

Salton, M.R.J. (1957). The properties of lysozyme and its action on micro-organisms. *Bacteriol. Rev., 21*, 82–99.

Takeshita, K., Hashimoto, Y., Ueda, T. and Imoto, T. (2003). A small chimerically bifunctional monomeric protein: *Tapes japonica* lysozyme. *Cell. Mol. Life Sci., 60,* 1944–1951.

Xue Qing-Gang, Schey, K.L., Volety, A. K., Fu-Lin Chu, E. and La Peyre., J.F. (2004). A novel slow-tight binding seyine serine protease inhibitor from eastern oyster (*Crassostrea virginica*) plasma inhibits perkinsin, the major extracellular protease of the oyster protozoan parasite *Perkinsus marinus.* Comparative Biocemistry and Physiology Part B: *Comp. Biochem. Physiol., 139*, 11-25.

Zavalova, L.L., Artamonova, I.I., Berezhnoy, S.N., Tagaev, A. A. Baskova, I. P., Andersen, J., Roepstorff, P. and Egorov, T.A. (2003). Multiple forms of medicinal leech destabilase-lysozyme. *Biochem. Biophys. Res. Commun., 306,* 318–323.

Pertanika J. Trop. Agric. Sci. **32(1)**: 91 - 98 (2009) ISSN: 1511-3701

Identification of Putative Pre-B Cell Leukaemia Transcription Factor 1 Gene by Differential Display: A Novel Fish mRNA Expressed Upon Cadmium Exposure

K.G. Vijayendran[1*], A.W.M. Effendy[1], T.S. Cha[2] and T. Mariam[2]
[1]Institute of Marine Biotechnology, Universiti Malaysia Terengganu,
[2]Department of Biological Science, Faculty of Science and Technology,
Universiti Malaysia Terengganu, Mengabang Telipot,
21030 Kuala Terengganu, Terengganu, Malaysia
[*]E-mail: vijay0701@yahoo.com

ABSTRACT

Pre-B cell leukaemia transcription factor (Pbx1) is a member of a ubiquitous class of homeodomain (HOX) proteins. Subsequent studies have shown that Pbx1 is widely expressed and acts as a co-factor for a variety of HOX proteins. This also means that Pbx1 is a one of the markedly and specifically suppressed gene in Promyelocytic leukaemia zinc finger (PLZF) which has been characterized as one of the regulated genes in cancer. In addition, PLZF is also a transcription repressor which suppresses the transcription of genes such as c-myc, cyclin A2 and HoxD11. In this study, the use of differential display reverse transcriptase polymerase chain reaction was reported to identify a novel hybrid tilapia mRNA sequence which is highly homologous to Pbx1 gene. For this purpose, hybrid tilapia Pbx1 was cloned. Then, a specific primer for the hybrid tilapia was designed for the Pbx1 mRNA measurements using the real-time PCR. The hybrid tilapia was exposed to 0.469, 0.938, 1.875 and 2.813 mg/l cadmium (Cd) to determine the relationship between Pbx1 mRNA expression levels. The cloned Pbx1, consisting of 343 bp encoding a protein of 53 amino acids, showed higher than 60% identity with the deduced amino acid sequence. Pbx1 mRNA expression and Cd accumulation appeared to be dose-responsive following cadmium treatment. Based on these results, the Pbx1 mRNA expression levels could be used as a bio-indicator to monitor the carcinogenic level of Cd in biological samples. The study is currently in progress to obtain the full gene sequence of Pbx1 using RACE-PCR.

Keywords: **Hybrid tilapia, cadmium, cancer, bioindicator, mRNA differential display**

INTRODUCTION

Over the past decades, the contamination of cadmium (Cd) has increased drastically. By the early 1990s, the world- wide annual release of Cd reached 22000 tons, which was largely found in water and soil (Liu *et al.*, 2007). Cadmium is a member of the IIb group in the periodic table of elements and one of the most toxic heavy metals able to produce genotoxic and mutagenic events at high concentrations (Waalkes, 2003). Moreover, Cd has been classified as a human carcinogen by International Agency for Research on Cancer. When Cd enters into the aquatic environment, it poses a serious threat to the living organisms as it has been found to be carcinogenic and mutagenic (Carginale *et al.*, 2002). In fish, Cd can damage gills because chloride cells are the primary targets of waterborne Cd and this metal decreases the activity of gills Ca2+-ATPase, which leads to fish hypocalcemia (Wong and Wong, 2000). In addition, Cd has adverse effects on growth, reproduction, respiratory functions and osmoregulation (Wu *et al.*, 2007). The first response to the carcinogenic

Received: 20 May 2008
Accepted: 8 October 2008
[*]Corresponding Author

of Cd is the regulation of genes which includes immediate early response genes (IEGs), stress response genes (metallothionein genes, heat-shock genes, genes controlling glutathione and related proteins), transcription factors, translation factors and miscellaneous genes (Waisberg *et al.*, 2003). Various approaches have been employed to identify such genes, including mutant screening (Price *et al.*, 1998), micro arrays (Akhtar *et al.*, 2002; Panda *et al.*, 2002) and differential display (Carginale *et al.*, 2002; Basile *et al.*, 2005). Differential display is a sensitive mRNA screening technique which enables the comparison of reverse transcribed and arbitrarily amplified cDNAs from two or more cell or tissue types. The isolated cDNAs can be identified by sequencing and interrogation of databases; subsequently, they can be cloned and used as probes for further analysis.

This study reports on the use of differential display, in the identification of a novel hybrid tilapia mRNA sequence which is highly homologous to Pre-B cell leukaemia transcription factor (Pbx1) and its expression level using real time PCR analysis. In particular, Pbx1 has been reported as IEG and belongs to the group of non-Hoxhomeodomain transcription factors which significantly contribute to embryonic differentiation and organogenesis. During this process, Pbx1 allows homeodomain proteins such as Hox (1), engrailed (2), MyoD (3), Meis (4), or Pad (5) to bind to DNA with higher affinity and specificity. Complexes of Pbx1/Hox can act as transcriptional activators or as repressors, depending on the recruitment of other transcription factors and modulation through extra cellular signals. Pbx1 is one of the markedly and specifically suppressed genes in Promyelocytic leukaemia zinc finger (PLZF) which has been characterized as one of the regulated genes in cancer. Similarly, PLZF is also a transcription repressor which suppresses the transcription of genes such as c-myc, cyclin A2, and HoxD11.

MATERIALS AND METHODS

Fish and Experiment Design

Immature hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) (15 g) were purchased from a local supplier. Fish were then transferred to flow through the system, and 30 days prior exposure for acclimatization to laboratory conditions. Acute bioassay procedure was based on the standard methods (APHA-AWWA-WPCF, 1998). The experiment was run for 96 hours and repeated for three times. Acute toxicity effect of Cd on hybrid tilapia was determined using the Finney's Probit Analysis LC_{50} Determination Method (Finney, 1971). The computer analysis was also carried with LC_{50} 1.00 software developed by EPA (1999). Based on this, the test result of the LC_{50} of Cd 96 h was indicated as 4.832 mg/l. Tilapia fingerlings were tested against lower concentrations of cadmium based on the data obtained from the acute toxicity test. Fish were exposed to 10, 20, 40 and 60% of the 96 hours LC_{50} for 7 days.

Total RNA Extraction, Removal of Genomic DNA Contamination

Gills of hybrid tilapia were ground into fine powder, under liquid nitrogen (5 ml), using pestle and mortar. The total RNA was isolated from 100 mg of this material using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Genomic DNA contamination was removed from the total RNA by a treatment of 10 µg RNA with 5U Dnase I from Promega. A quality control of the total RNA was done by the standard PCR amplification using differential display primer.

Differential Display

Differential display was performed using the Gene Fishing ™PCR Kit (Seegene Inc., Korea), according to the supplier's protocol. The DNA free total RNA (3µg) from gills, were reversely transcribed (RT) with oligo-DT primer (dT-ACP1: 5`-CTGTGAATGCTGCGACTACGATX XXXX$(T)_{18}$-3`). The reaction contained 5 x RT buffer (25 mM Tris-Cl pH 8, 35 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT), 2 mM dNTPs, 40 U RNase Inhibitor and 200 U of MMLV reverse transcriptase. The RT reactions were incubated in 42°C for 90 min, 94°C for 2 min and chilled on ice for 2 min. As for the PCR amplification, each reaction mixture of 20 µl contained 5 µl of first-strand cDNA (50 ng), 10 µl of 2 x See Amp™ ACP Master Mix, 1 µl of 10 µM dT-ACP2 (5`-CTGTGAATGCTGCGA CTACGATXXXXX$(T)_{15}$-3`) and 2 µl of 5 µM arbitrary primer. The PCR program consisted of 1 cycle of 94°C for 5 min, 50°C for 3 min, 72°C for 1 min; 40 cycles of 94°C for 40 s, 65°C for 40 s. 72°C for 40 s and followed by a final elongation step at 72°C for 5 min.

Re–amplification, Purification and Cloning of PCR Product

Differentially expressed bands were excised. The DNA was recovered and re-amplified into a 40 µl reaction. All the reaction conditions were identical to the ones described above for the PCR amplification. The re-amplification cDNA was purified using Gel Purification Mini Kit (Favorgen, Biotech Corp, Taiwan) and cloned into pGEM-T vector (Promega). Specific primers were designed from this clone for total quantification using real time PCR.

Quantification Real Time PCR

The differential expressed gene (DEG) transcripts were quantified by real-time quantitative PCR using Mini Opticon Real Time PCR machine (BioRad) and SYBR Green Fluorescein mix per manufacturer's specification (Qiagen). The quantification was based on a 210-bp amplicon generated using the specified primer set (forward primer: 5`-CGACGATGATGAAGTGGATG-3` and reverse primer: 5`-TCAGCCTTGGTGGTAGTGGT-3`) of the DEG. The reaction mixture of 25 µl was set using the following concentrations: 100 nM each forward and reverse primers, 1x SYBR Green Fluorescein mix and 50 ng of cDNA. The reaction conditions were 94°C for 5 min for 1 cycle; 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; and melting at 60-90°C for 15 s. The number of cDNA transcripts was determined using the standard curves generated with references plasmid of DEG.

Statistical Analysis

Data are presented as the mean ± SD. Results were analyzed using the one way ANOVA with Tukey`s multiple comparison. The statistical significance was accepted at a level of $p< 0.05$.

RESULTS

Identification of the Cd Induced Gene by Differential Display

Messenger RNA (mRNA) expression patterns of the control and cadmium-treated hybrid tilapia were compared in order to identify the genes whose transcription were up-regulated or down regulated by cadmium. To prevent isolation of 'false positive', all amplification experiments were performed on two different dilutions of each cDNA sample. Only cDNA bands, whose levels of expression were affected by cadmium in both dilutions, were selected for further analysis. *Fig. 1* shows a representative differential display gel. The cDNA band (a33.9) was excised and cloned. The cloned size of the cDNA band was 343 bp and renamed as pA33.9 for further analysis.

Nucleotide Sequencing, Homology Searching and Classification of the Isolated Gene

The DEG (pA33.9) was completely sequenced and registered at genbank (Genbank accession no: EU717966). The nucleotide sequence was then analyzed, by searching for protein homologies against the GenBank database, using the BLASTX programme and the results are summarized in *Fig. 2.* The clone showed

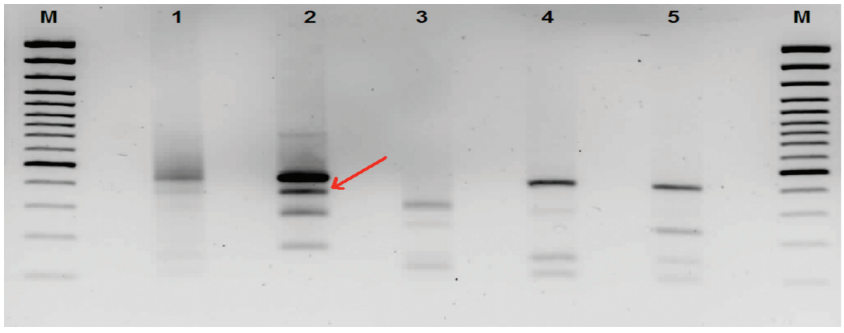


Fig. 1: Differential display of mRNAs from normal and exposed hybrid tilapia to Cd. N = O mg/l; 1 = 0.469 mg/l; 2 = 0.938 mg/l; 3 = 1.875 mg/l and 4 = 2.813 mg/l. Arrow indicates cDNA band which was only present, specifically at 0.938 mg/l excised and renamed pA33.9 for further analysis

significant amino acid homologies to the known protein (Pre-B cell leukaemia transcription factor interacting protein (Pbx1) in the database. The clone product encodes a 53 amino acids (nt. 3-158) partial sequence, with 93.6% homology to the *Mus muscles*, 80.5% homology to the *Rattus norvegius* and 68% to *Homo sapiens*.

Real-time PCR Analysis

The quantification of transcripts, amplified by real-time RT-PCR in this study, was based on the widely used standard curve method. This method assumed that the efficiency in the amplification of the samples was the same (Livak, 1997). The references plasmid, used to generate the standard curves for quantification of pA33.9 gene transcript, was pGEMT*pA33.9*. The real-time PCR amplification of this reference plasmid was performed together with the samples in the same run and reaction conditions. The Ct values were plotted versus the log of the initial amount of plasmid DNA to give the standard curves, as shown in *Fig. 3*. The correlation coefficient for the standard curves was 0.998 with the PCR reaction efficiency of 1.88. The transcript levels were determined based on the amount of DNA in femtogram (fg) of reference plasmid. *Fig. 4* shows the transcript levels for gene a33.9 at various cadmium exposures. It can be seen that the level of gene a33.9 was approximately 500-fold higher at the 0.938 mg/l as compared to the control. However, the expression level started to decrease at 1.875 mg/l and 2.813 mg/l.

```
pA33.9          EVDDFEDFVFGHFFGDKALKKRSRKKEKHSWNPRVVGPREEHSRHPHHYHQG 52
Mus             EVDDFEDFVFGHFFGDKALKKRSRKKEKHSWNPRVVGPREEHSRHPHHYHQG 52
Rattus          EVDDFEDFIFSHFFGDKALKRRSKKKEKQPWNHRAVGPREEHSRHPHHYHQG 52
Homo            EVDDFEDFIFSHFFGDKALKKRSGKKDKHSQSPRAAGPREGHSHSHHHHHRG 52
                ********:*.*********:** **:*:. . *..**** **:  **:*:*
```

Fig. 2: Alignment of the partial amino acid translated from pA33.9 (from this study) to a Pre-B cell leukaemia transcription factor interacting protein (Pbx1) from Mus muscles *(GenBank Acc. No: EDL15193),* Rattus norvegius *(GenBank Acc. No: EDM00624) and* Homo sapiens *(Genbank Acc. No: CAI13238). The alignment was performed with Clustal* W program

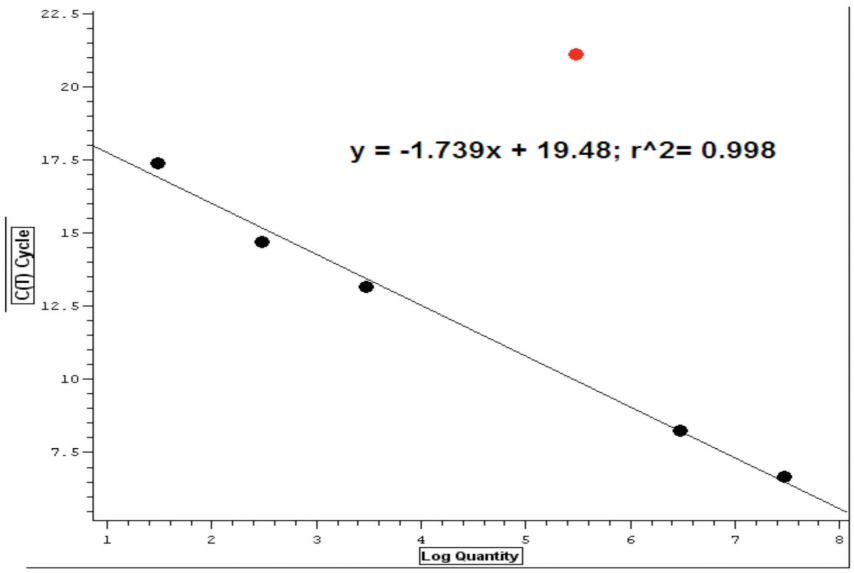


Fig. 3: Standard curves for pGEMTpA33.9. The graph shows the threshold cycle (Ct) plotted against log_2 starting quantity of plasmid defined by copy number. The efficiency of the reaction was 1.88

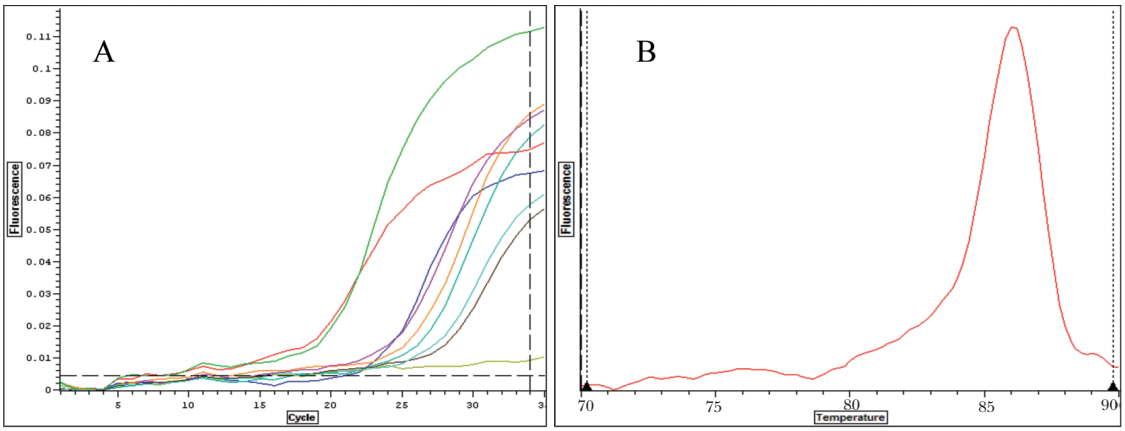


Fig. 4: (A) Fluorescence profiles of the cDNA of a33.9 gene amplicons; (B) Melting curve of the reaction for a33.9 gene

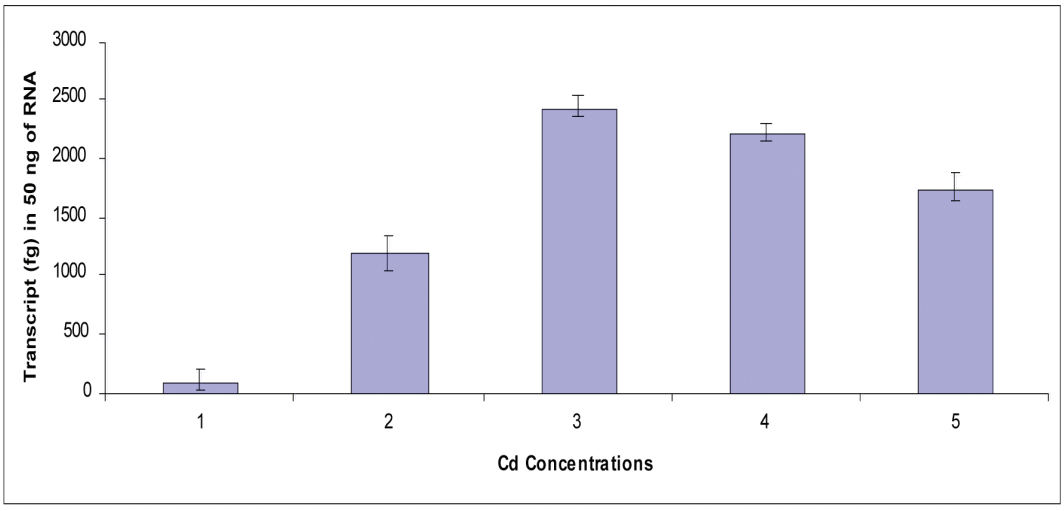


Fig. 4: Expression profiles for gene a33.9 at various cadmium exposure. 1 = O mg/l 2= 0.469 mg/l; 3 = 0.938 mg/l; 4 = 1.875 mg/l and 5 = 2.813 mg/l. All concentrations showed significant difference as compared to control (p<0.05)

DISCUSSION

Finney's Probit Analysis gave 96-h LC_{50} value for the hybrid tilapia exposed to different Cd concentrations as 4.832 mg/l. The results of the present study are within the range of the results reported by the following researchers. For instance, the LC_{50} values of cadmium on rainbow trout (*Oncorhynchus mykiss)* for 24, 48, 72 and 96 h were found to be 7.76, 1.95, 0.5, and 0.45mg/l, respectively (Oryan and Nejatkhah, 1997). In addition, Chambers (1995) investigated the effect of acute cadmium toxicity on marron, *Cherax tenuimanus* (Smith, 1912); the author found 96-h LC_{50} value as 17.9 (13.4–23.9) mg/l. Furthermore Muley *et al.* (2000) reported the 96-h LC_{50} value of cadmium on *C. carpio* as 121.8 ppm. The 96-h LC_{50} values of cadmium on *Salmo gairdneri* and *Xenopus laevis* larvae were reported to be between 80 and 100mg/l (Woodal *et al.*, 1988). According to Wright (2001) there are many factors involve in determining the 96-h LC_{50} and these include temperature, pH, alkalinity, water hardness, dissolved oxygen, total organic carbon, etc.

Differential display is a useful method used to identify genes; the expression of which may be up- or down-regulated in one sample relative to another. Following its introduction by Liang and Pardee (1992), the methodology has been widely used and improved. In this study, a significant expression of Pbx1 was observed from 0.938 mg/l of fish exposed to Cd toxicity. The over expression of PBx1 is a result of carcinogenic effect of Cd. However, the expression of Pbx1 started to decrease at 1.875 mg/l of Cd exposure onwards. This might due to the presence of detoxification genes such as heat shock proteins, metallothioneins and oxidative stress response gene. For example, metallothioneins sequester cadmium, with high affinity, resulted in decreased availability of Cd^{2+} capable of interacting with cellular targets to elicit toxicity, including carcinogenicity (Waalkes, 2003).

Generally, Pbx1 has widely been expressed during embryonic differentiation and organogenesis. However, Kikugawa *et al.* (2006) revealed that Pbx1 was one of the markedly and specifically suppressed genes in PLZF over-expressed DU145 cells. It was analyzed by Western blotting and RT-PCR that Pbx1 was endogenously higher expressed in DU145 cells than in LNCaP cells. Forced expression of androgen receptor in DU145 cells recovered the response of androgen-dependent PLZF expression and subsequent repression of Pbx1 expression. In particular, Pbx1 was found to achieve high-affinity DNA binding when Hox proteins became a partner of hetero-dimerization and the formed Hox-Pbx heterocomplex acted as an activator of transcription. This means, cadmium has the potential to influence the activity of several transcriptional factors leading to deregulation of gene expression. The proto-oncogenes *c-fos* and *c-jun* code for proteins which are the members of the AP-1 element that functions as a transcriptional factor regulation, i.e. the expression of a large number of genes controlling the growth and division of cell (Angel and Karin, 1991). This shows that Cadmium is a powerful inducer of *c-fos* and c-jun and this has been considered as a major mechanism for cadmium-induced cell transformation and tumorigenesis. Several other transcription factors (for example, metal regulatory transcription factor 1 or MTF1, upstream stimulator factor or USF, nuclear factor kB or NF-kB and NF-E2-related factor or NRF2) were activated by exposing them to Cd, resulting in modulation of gene expression (Li *et al.*, 1998; Alam *et al.*, 2000; Smirnova *et al.*, 2000). On the other hand, the exposure to Cd also resulted in the suppression of the DNA binding activities of the transcription factors hypoxia-inducible factor-1 (HIF-1) (Obera *et al.*, 2003) and Sp1 (Watkin *et al.*, 2003). HIF-1 is involved in controlling the expression of the erythropoietin gene, whereas Sp1 plays a key role in cell proliferation and its inactivation leads to cell death.

Cadmium is a complete carcinogen in experimental animals. Among other, Cd stimulates the expression of many genes and changes in these genes may be the precursors of some of the numerous effects reported at higher levels of biological organization. Furthermore, some can be used as a potential biomarker for hazard identification in the risk assessment of eco-toxicological studies as they are very specific to Cd toxicity. The results from this study indicated that the Pbx1 gene could be considered as a biomarker to monitor the carcinogenic level of Cd in the biological samples, especially fish.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Higher Education, Malaysia, and University Malaysia Terengganu, with the Grant Number: 59009. K.G. Vijayendran is the recipient of PASCA Scholarship, from the Ministry of Science, Technology and Innovation, Malaysia. The authors wish to thank the staff at the Institute of Oceanography and Marine Biotechnology for their kind supports.

REFERENCES

APHA, AWWA and WPCF. (1998). *Standard Methods for the Examination of Water and Wastewater* (20th ed.). New York: American Publication of Health Association.

Akthar, R.A., Reddy, A.B, Maywood, E.D., Clayton, J.D., King, V.M., Smith, A.G., Gant, T.W., Hastings, M.H. and Kyriacou, C.P. (2002). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Current Biology, 12*, 540– 550.

Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbeins, S., Choi, A.M., Burow, M.E. and Tou, J. (2000). Mechanism

ofheme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. *Journal of Biological Chemistry, 275,* 27694-27702.

Angel, P. and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica et Biophysica Acta, 1072*, 129-157.

Basile, A., Nuzzo, R.A.D., Capasso, C., Sorbo, S., Capasso, A. and Carginale, V. (2005). Effects of cadmium on gene expression in the liverwort Lunularia cruciata. *Gene, 356*, 153-159.

Carginale, V., Capasso, C., Scudiero, R. and Parisi, E. (2002). Identification of cadmium sensitive genes in the Antarctic fish Chionodraco hamatus by messenger RNA differential display. *Gene, 431,* 117-124.

Chambers, M.G. (1995). The effect of acute cadmium on marron, Cherax tenuimanus (Smith 1912; Parastacidae), Freshwater Crayfish 10. In *Tenth International Symposium of Astacology,* 10-15 (pp. 209-220).

Finney, D.J. (1971). *Probit Analysis*. New York: Cambridge University Press.

Kikugawa, T., Higashiyama, S., Tanji N. and Yokoyama, M. (2006). Role of promy elotic leukaemia zinc finger (PLZF) and pre-B cell leukaemia transcription factor (PBX1) androgem-independent prostate cancer cell growth. *European Urology Supplement, 5(2),* 123.

Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science, 257,* 967-971.

Liu, W., Yang, Y.S., Zhou, Q., Xie, L., Li, P. and Sun, T. (2007). Impact assessment of cadmium contamination on rice (*Oryza sativa* L.) seedlings at molecular and population levels using multiple biomarkers. *Chemosphere, 67*, 1153-1163.

Livak, K. J. (1997). Comparative Ct method. ABI Prism 7700 Sequence Detection System. User Bulletin no.2. PE Applied Bioystems.

Muley, D.V., Kamble, G.B. and Bhilave, M. P. (2000). Effect of heavy metals on nucleic acids in *Crprinus carpio. Journal of Environmental Biology, 21*, 367-370.

Obara, N., Imagawa, S., Nakano, M., Suzuki, N., Yamamoto, M. and Nagasawa, T. (2003). Suppression of erythropoietin gene expression by cadmium depends on inhibition of HIF-1, not stimulation of GATA-2. *Archive Toxicology, 77*, 267-273.

Oryan, S. and Nejatkhah, P. (1997). Effects of cadmium on plasma cortisol and prolactin levels in the rainbow trout (*Oncorhynchus mykiss*). *J. Fac. Vet. Med. Univ.Tehran, 51*, 39–53.

Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S. and Hogenesch, J.B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell, 109,* 307–320.

Price, J.L., Blau, J., Rothenfluh, M., Abodeely, B., Kloss and Young, M.W. (1998). Double-time is a new Drosophila clock gene that regulates PERIOD protein accumulation. *Cell, 94,* 83-95.

Smirnova, I.V., Bittel, D.C., Ravindra, R., Jiang, H. and Andrews, G.K. (2000). Zinc and cadmium can promote rapid nuclear translocation of metal response element-binding transcription factor-1. *Journal of Biological Chemistry, 275,* 9377-9384.

Waalkes, M.P. (2003).Cadmium carcinogenesis. *Mutation Research, 533*, 107-120.

Waisberg, M., Pius, J., Beverly, H. and Detmar, B. (2003). Molecular and cellular mechanisms for cadmium carcinogenic. *Toxicology, 192*, 95-117.

Watkin, R.D., Nawrot, T., Potts, R.J. and Hart, B. A. (2003). Mechanisms regulating the Cadmium-mediated suppression of Sp1 transcription factor activity in alveolar epithelial cells. *Toxicology, 184,* 157–178.

Woodal, C., Maclean, N. and Crossley, F. (1988). Responses of trout fry (*Salmo gairdneri*) and Xenopus laevis tadpoles to cadmium and

zinc. *Comparative Biochemistry and Physiology, 89C*, 96-99.

WONG, C.K.C. and WONG, M.H. (2000). Morphological and biochemical changes in the gills of tilapia (*Oreochromis mossambicus*) to ambient cadmium exposure. *Aquatic Toxicology, 48*, 517-527.

WRIGHT, D.A. (2001). *Environmental Toxicology*. Cambridge: Cambridge University Press.

WU, S.M., SHIH, M.J. and HO, Y.C. (2007). Toxicological stress response and cadmium distribution in hybrid tilapia (*Oreochromis* sp.) upon cadmium exposure. *Comparative Biochemistry and Physiology, C 145*, 218-226.

Pertanika J. Trop. Agric. Sci. **32(1)**: 99 - 110 (2009)
ISSN: 1511-3701

From Biological Control to Bioactive Metabolites: Prospects with *Trichoderma* for Safe Human Food

H.B. Singh* **and D.P. Singh**
Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University,Varanasi 221 005, India
**E-mail: hbs1@rediffmail.com*

ABSTRACT

Trichoderma, a commonly known mycoparasite inhabiting nearly all agricultural soils, has shown outstanding biological properties in controlling growth of other less desirable or more harmful (pathogenic) types of fungi. What makes *Trichoderma* very interesting is that it uses local materials (decaying products) for proliferation, is non-toxic and biodegradable, produces numerous useful metabolites with complex chemistry and performs diverse biological activities. What is more intriguing, however, is its ability to target a specific mechanism rather than killing or repelling organisms indiscriminately. Although the biological control ability of *Trichoderma* has been studied and proven for many years, the ability of these fungi to increase the rate of plant growth and development, particularly to enhance the production of more robust roots, is now being documented. While working on more than 260 strains of *Trichoderma* collected from different habitats, the researchers have documented the bio-control ability of these organisms, not only at laboratory level but also at the field level, as well as up to the extent of commercialization. Based on the study, the researchers also discovered that several strains increased the size and numbers of deep roots which were quite below the soil surface. These deep roots cause crops, such as corn, fruit crops and ornamental plants, to become more resistant to drought. Besides such potentialities, certain *Trichoderma* species are highly efficient producers of many extracellular enzymes and are used commercially for the production of cellulases and other enzymes which degrade complex polysaccharides. They are frequently used in the food and textile industries. In particular, *Trichoderma* protease appears to exhibit excellent mechanisms of action in controlling grey mold on the surface of bean leaf by preventing germination of mold spore and deactivating harmful mold enzymes. The researchers' recent interests warrant the use of secondary metabolites as potential biopesticidal and biofungicidal agents. From the local free-living *Trichoderma* isolates, different chemical fractions are extracted and separated in search of novel bioactive metabolites for their *in vitro* testing against phytopathogenic fungi, bacteria and pests. In addition to this, the researches are now planning to extend such studies to the marine-derived *Trichoderma* species, such as *Trichoderma reesie,* with an aim to evaluate their biological activity and ability to be successfully used in field trials to control many crop pathogens.

Keywords: *Trichoderma*, biological control, bioagent, bioactive metabolites

INTRODUCTION

Access to food remains a central issue to every civilization from the time immortal, and agriculture, as a whole, is the only basic activity performed by the humans to feed all civilizations. However, with the increasing need and greed for acquiring more and more and global commercialization of the agricultural products to gain maximum economic returns, even at the cost of deviation from the natural way of growing food crops, the griming scenario of agriculture all over the world has started. Huge industrialization, chemicalization in farm inputs, unscientific farming practices and vastly expanding population have worsened the situation even more. Therefore, the concept of sustainability in agriculture is being advocated in farming communities to re-establish the losing

Received: 20 May 2008
Accepted: 8 October 2008
*Corresponding Author

potential of farms, natural quality of foods and feeds, as well as to regain the decline in human health, due to the consumption of contaminated food. All these motives can only be achieved with the dependency of low-input agriculture practices, by recycling agriculture wastes into the farms, minimizing the use of chemical farm inputs, promoting bio-inputs such as microbes, beneficial fungi and nematodes, bioagents, worms, etc., to keep the soil and plant in good health, and over and above, harmonizing the natural ways of producing crops.

Pesticide consumption in India amounts to 288 g/ha, and crops produced for agricultural exports worth US$ 25 million are being rejected yearly due to the presence of high pesticide residues. There is a growing movement in many countries to mitigate the amount of chemicals being released into the environment or stop its use altogether. The persistence and accumulation of these chemicals in the food chain and environment (especially in the soil and aquatic ecosystem), over a period of time, are major causes for concern due to their inherent hazards to plants and human life. It is a fact that world food production needs to be increased to feed the ever-increasing population. However, it would be better if such practices, which enhance the quality of food in terms of less residual or phytotoxic effects of chemicals, are adapted. It is for this reason that the natural ways of pest and disease control, using natural enemies of the pest or disease-causing agents, have been emerged as a potential alternative to hazardous farm chemicals. For such an approach, the use of biofertilizers and biopesticides is being recommended, along with the use of other practices of organic agriculture. When human civilization started cultivation of crops approximately 10,000 years ago, it encountered the losses in crop yields due to plant diseases, decreased soil fertility, unsuitable soil, and moisture conditions. The early farmers observed that when the same land was used continuously for the cultivation of the same crop, the yields decreased in subsequent years and that by shifting to new fields for cultivation, the yield could be increased in many folds. The management of plant pathogens by the use of crude chemicals, like Bordeaux mixture, was started in the nineteenth century and their use kept on increasing in the ensuing years. Nevertheless, to maintain sustainability, the use of microbes as fertilizers and pesticides has become instrumental in keeping plant healthy and enhancing the yield qualitatively and quantitatively.

Trichoderma *as Biocontrol Agent*

Trichoderma, a genus of asexually reproducing fungi, is present in nearly all temperate and tropical soils. The strains of *Trichoderma* spp. are strong opportunistic invaders, fast growing, prolific producers of spores and powerful antibiotic producers. These properties make these fungi ecologically very successful and are the reasons for their ubiquitousness (Kubicek *et al.,* 2002). They show a high level of genetic diversity and can be used to produce a wide range of products of commercial and ecological interest. Much of the known biology and many of the uses of these fungi have been documented recently (Buhariwalla, 2005; Druzhinina, 2006).

Trichoderma *as a Source of Potential Bioactive Metabolites*

With the growing interest in the biological properties of *Trichoderma* species, several bioactive metabolites have been isolated and identified from different strains. These compounds possess diverse structures which belong to different classes of molecule and accordingly possess a range of bioactivities (Table 1).

Trichoderma species produce different volatile and non-volatile compounds which inhibit the growth of fungal phytopathogens. The mechanism of antibiosis constitutes a much more complex system, leading to the phenomenon of biological control. Among these antibiotics, the production of gliovirin, gliotoxin, viridin, pyrones, peptabiols and others have been described extensively (Vey *et al.*, 2001). Besides, the production of a large variety of volatile secondary metabolites by *Trichoderma* (e.g. ethylene, hydrogen cyanide, alcohols, aldehydes and ketones up to C4 chain-length) also plays an important role in biocontrol. Another antibiotic compound, i.e. peptabiols, which are non-ribosomally synthesized antimicrobial peptides and exhibit antibacterial and antifungal properties, represented by alamethicin (Reusser, 1967; Landreau *et al.*, 2002), do play an important role in antagonism. To date, 309 peptabiols have been sequenced, among them more than 180 are synthesized by *Trichoderma.* Xiao-Yan *et al.* (2006) studied antimicrobial metabolites, produced by *Trichoderma koningii* SMF2, and

TABLE 1
Bioactive metabolites from *Trichoderma* species

Principles constituents/ metabolites	Isolated from	Biological activity	References
Trichodermin, Trichodermol Sesquiterpenoids	*Trichoderma polysporum and T. sporulosum*	Growth inhibitors	Adams and Hanson. Sesquiterpenoid metabolites of *Trichoderma polysporum* and *T. sporulosum. Phytochemistry,* 1 *92,* 423.
Trichoviridin and isocyanides	*T. hamatum*	Inhibited growth of *micrococcus luteus*	Brewer, D., Taylor, A., Keeping, J. W., Taha, A .A. and Thaller, V. (1982). Production of experimental quantities of isocyanide metabolites of *Trichoderma hamatum. Can. J. Microbiol., 28,* 1252-60.
1,3,6,8-tetrahydroxyanthraquinone and 1-acetyl-2,4,5,7-tetrahydroxy-9, 10-anthracenedione Anthroquinone	*T. viride*	Pigments	Betina, V., Sedmera, P., Vokoun, J. and Podojil, M. (1986). Anthraquinone pigments from a conidiating mutant of Trichoderma viride. *Experientia, 42,* 196-7.
T-2 toxin	*Trichoderma lignorum*	Toxin	Bamburg, J. R. and Strong, F. M. (1986). Mycotoxins of the trichothecane family produced by *Fusarium tricinctum* and *Trichoderma lignorum. Phytochemistry, 8,* 2405-2410.
Lignoren sesquiterpenoid	*Trichoderma lignorum* HKI 0257		Berg, A., Wangun, H. Kemami, V., Nkengfack, A. E. Schlegel, B. (2004). Lignoren, a new sesquiterpenoid metabolite from *Trichoderma lignorum* HKI 0257. *Journal of Basic Microbiology, 44,* 317-319.
	antibacterial and antifungal activities.		
Trichogin A IV			
Lipopeptaibol	*Trichoderma longibrachiatum*		Auvin-Guette, C., Rebuffat, S., Prigent, Y., Bodo, B. and Trichogin, A IV. (1992). An 11-residue lipopeptaibol from *Trichoderma longibrachiatum. Journal of the American Chemical Society, 114,* 2170-4 .
2',4'-dihydroxy-3'-methoxymethyl-5'-methylacetophenone and 2',4 '-dihydroxy-3',5'-dimethylacetophenone (clavatol)	*Trichoderma pseudokoningii*		Astudillo, L., Schmeda-Hirschmann, G., Soto, R., Sandoval, C., Afonso, C., Gonzalez, M. J. and Kijjoa, A. (2000). Acetophenone derivatives from Chilean isolate of *Trichoderma pseudokoningii* Rifai. *World Journal of Microbiology & Biotechnology, 16,* 585-587.

TABLE 1 *Cont.*

octaketide-derived acetal diol	*Trichoderma koningii and T. harzianum*	Antagonistic activity against fungus	Almassi, F., Ghisalberti, E. L., Narbey, M. J. and Sivasithamparam, K. (1991). New antibiotics from strains of *Trichoderma harzianum. Journal of Natural Products, 54*, 396-402.
Trichodermin	*Trichoderma polysporum and T. sporulosum*	Antibiotic	Abrahamsson, S. and Nilsson, B. (1966). Molecular structure of trichodermin, *Acta Chemica Scandinavica* (1947-1973).
Bisorbicillinoid	*Trichoderma* sp. *USF-2690*	–	Abe, N., Arakawa, T., Yamamoto, K., and Hirota, A. (2002). Biosynthesis of bisorbicillinoid in *Trichoderma* sp. USF-2690; evidence for the biosynthetic pathway, via sorbicillinol, of sorbicillin, bisorbicillinol, bisorbibutenolide, and bisorbicillinolide. *Bioscience, Biotechnology, and Biochemistry, 66*, 2090-2099.
Demethylsorbicillin and oxosorbicillinol	*Trichoderma* sp. *USF-2690*	Free radical scavenging activity	Abe, N., Yamamoto, K. and Hirota, A. (2000). Novel fungal metabolites, demethylsorbicillin and oxosorbicillinol, isolated from *Trichoderma* sp. USF-2690. *Bioscience, Biotechnology, and Biochemistry, 64*, 620-622.
Bisorbicillinoid	*Trichoderma* sp. *USF-2690*	free radical scavenger	Abe, N., Sugimoto, O., Arakawa. T., Tanji, K., Hirota, A. Sorbicillinol, a key intermediate of bisorbicillinoid biosynthesis in *Trichoderma* sp. USF-2690, School of Food and Nutritional Sciences, University of Shizuoka, Japan. abe@fns1.u-shizuoka-ken.ac.jp
Crude extracts	*T. sect. Trichoderma, T. sect. Pachybasium and T. sect. longibrachiatum*	Antimicrobial activity	Juan A. VIZCAÍNO a1, Luis SANZ a1, Angela BASILIO a2, Francisca VICENTE a2, Santiago GUTIÉRREZ a3, M. Rosa HERMOSA a1 and Enrique MONTE. (2004). Screening of antimicrobial activities in Trichoderma isolates representing three Trichoderma sections. *International Journal of Biology and Biotechnology, 1*, 355-363
Harzianopyridone	*Trichoderma harzianum*	antifungal	Dickinson, J. M., Hanson, J.R., Hitchcock, P.B. and Claydon, N. (1989). Structure and biosynthesis of harzianopyridone, an antifungal metabolite of *Trichoderma harzianum. J. Chem. Soc., Perkin Trans. 1, 1*885 - 1887.

showed them to be effective against a wide range of fungal phytopathogens. sAlthough production of antibiotics by *Trichoderma* involved in biocontrol is a well-documented phenomenon, clear identification and understanding of the role of antibiotics in disease control lags far behind than that in bacteria and therefore needs to be addressed (Whipps, 2001; Singh *et al.*, 2002). Further research is needed to study the toxicity of antibiotic compounds and the mechanisms of their biocontrol activity against phytopathogens.

Trichoderma - *Selection of Potential Strains for Biocontrol*

The discovery of fungal antagonists has led to new challenges in research, development, and registration of biocontrol products in a market where chemical pesticides dominate. Bringing a biocontrol product into market is a multilayered process including discovery, efficacy trials, toxicological testing, mass production, formulation, and registration (Singh *et al.*, 2001, 2002, 2004). Four steps mark the process of strain selection viz., the selection of effective strains in relation to plant pathogens; screening of isolates with high biotechnological indices, analysis of properties specific for plants in pathogens, useful insects, animals and humans, as well as search for economically viable substrates, convenience for mass multiplication of fungus which maintain high colony forming units (cfu) for longer periods (Singh *et al.,* 2002, 2003, 2007). The first step in developing a fungal biocontrol agent is the discovery, through empirical or targeted screening. Isolation and screening of potential fungal biocontrol agents have been identified in numerous cropping systems or natural ecosystems (Baker and Cook, 1982). Following isolation of potential strain of *Trichoderma* spp. for a particular or broad range of diseases, screening of the fungal isolates for biocontrol activity is generally performed *in vitro*, along with certain tests to establish the identity and relatedness of the newly found strains. This is most often accomplished using various available standard techniques. The relative ease of finding the right antagonist strain of *Trichoderma* and other biocontrol micro-organisms under laboratory and field conditions is an added advantage, but it does ensure that the agent will work consistently and effectively in the field.

The development of a highly successful strain, T-22 of *T. harzianum*, by Harman and his colleagues took more than a decade and it was only in later part of the last century that the sale of *Trichoderma*-based products started to pick up from the scratch (Mathre *et al.*, 1999; Harman, 2000;). Similarly, the researcher also had gone through a similar period of time in their endeavour to isolate the potential strain of *Trichoderma*. In the study, several strains of *Trichoderma* of different species (such as *T. harzianum*, *T. viride, T. atroviride, T. koningii)* were isolated. They have been found effective against a large number of fungal phytopathogens affecting several economically important crops (Singh *et al.*, 2006). Kalra *et al.* (2002) developed a potential strain of *Trichoderma harzianum* (ATCC-PTA3701) which is useful as a nematode inhibitor, biofungicide and plant growth promoter (US Patent No. 6,475,772). The application of this strain, as a biocontrol agent of soilborne fungal pathogens, had additional advantages in improving the growth of plants and economic yields of crop plants, contribution to the reduction of deleterious nematode population in the host tissue and rhizosphere, and thereby, reducing the severity of root knot disease. Moreover, its use as soil amendment in reducing the application of hazardous chemical fungicides and nematicides has been found to disturb the natural beneficial soil micro-flora and pollute the soil and soil water. The application of this strain in nursery has also been found to reduce the input of chemical fungicides, which sometimes inhibit the rooting of cuttings. Similarly, Singh and Singh (2004) screened two strains, viz, *T. harzianum* (IMI No. 359869) and *T. virens* (ITCC No. 1066.95) having the potential to control the collar rot disease of *Mentha* species caused by *Sclerotium rolfsii* and also increasing the oil yield significantly, which was otherwise drastically reduced in diseased plants. It would be boon to the industries associated with the development of biocontrol products, if such potential strains could be produced successfully at large scale and marketed for use by the farmers.

Once the identity of the fungus had been established, the researchers screened different isolates to assess the diversity within the species. This further added in selecting the most efficient and antagonistic strains for biocontrol ability *in vivo*. Overall, ecological fitness is a fundamental

requirement for bio-control agents (BCAs) because of the relatively narrow window of parameters, particularly relative humidity, type of soil, osmotic potential, and temperature. The adaptability to these factors is a major requisite for *Trichoderma* strains to grow efficiently in the natural environment. Due to this reason, it is therefore essential to select fungal isolates under a range of conditions.

Biological Control: How Does Trichoderma *Offer Disease Protection?*

The antagonistic nature of *Trichoderma* had been demonstrated more than seven decades ago (Weindling, 1934), although the first report on the biological control experiment using *Trichoderma* under natural field conditions came much later (Chet *et al.*, 1997). Furthermore, progress has been made towards the improvement of *Trichoderma,* as biological control agents, in the last 15 years than in the previous 60 years. Since then, many potential strains of *Trichoderma* have been isolated and characterized from different natural habitats and used in biocontrol experiments against several plant pathogenic fungi.

The prevention of infection or suppression of disease by *Trichoderma* is based on hyperparasitism, antibiosis, reduction of the saprophytic ability, induced resistance in the host plant, competition for nutrients and space, as well as reducing spore dissemination and/or restraining of pathogenicity factors of the pathogens, which may act co-ordinately and whose importance in the antagonistic process depends on several parameters (Elad and Freeman, 2002; Howell, 2003; Harman *et al.,* 2004).

Its mycoparasitism involves a complementary action of antibiosis, nutrient competition and cell wall degrading enzymes, such as chitinases, β-1, 3-glucanases and proteases. Since chitin is the major component of most fungal cell walls, a primary role has been attributed to chitinases in the biocontrol activity of *Trichoderma* (Harman, 2000). Therefore, studies on the molecular structure and characteristics of genes encoding enzymes of the chitinase complex in *Trichoderma* will contribute to a better understanding of the relationships between the different enzymes involved in the biocontrol mechanisms.

Competition and Rhizosphere Competence

For a *Trichoderma* species to be rhizosphere-competent, it must colonize the rhizosphere beyond 2 cm depth from the seed (Chao *et al.,* 1986; Ahmad and Baker, 1987) or proliferate to a concentration which exceeds the initial population coated on the seed (Beagle-Ristaino and Papavizas, 1985). Rhizosphere competence of a particular isolate of *Trichoderma* makes it a successful bio-control agent. *Trichoderma* species, either added to the soil or applied as seed treatments, grow readily along with the developing root system of the treated plant (Zhang *et al.*, 1996; Harman, 2000; Howell *et al.*, 2000). Although competition through rhizosphere competence may not be among the principal mechanisms which drive biological control, it is certainly a valuable adjunct to those that do. One concept associated with competition and rhizosphere competencies is the replacement of indogenous fungi on the root surface (Harman, 2001). Possibly, the ability of *Trichoderma* to colonize rhizosphere leads to increased levels of defence-related plant enzymes viz., peroxidases, chitinases and β-1,3-glucanases. In addition, root colonization by these beneficial fungi also induces significant changes in the plant metabolic machinery. Biochemical analysis of sunflower, soybean, mustard and maize plants treated with *Trichoderma* formulations at different intervals of time after sowing, shows substantial increase in the level of proteins, phenols, antioxidants, total chlorophyll and vitamins.

Induced Resistance

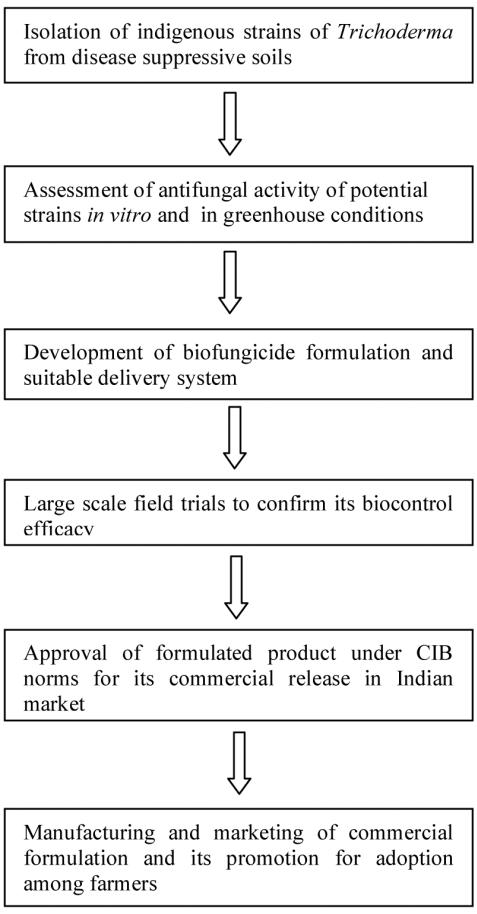
The treatment of plants with various agents (e.g., virulent or avirulent pathogens, non-pathogens, cell wall fragments, plant extracts, and synthetic chemicals) is now well-documented and this leads to the induction of resistance to subsequent pathogen attack, both locally and systemically (Walters *et al.*, 2005). The ability of *Trichoderma* spp. to induce local and systemic resistance has been shown with *T. harzianum* in agricultural crops such as bean, cotton, tobacco, lettuce, tomato and maize (De Meyer *et al.*, 1998; Ahmed *et al.*, 2000; Hanson and Howell, 2004; Harman *et al.,* 2004), with *T. asperellum* in cucumber (Yedidia *et al.*, 2000, 2003), and with *H. virens* (*T. virens*) in cotton (Howell *et al.*, 2000). According to Harman *et al.* (2004), on the contrary to the previously held opinions on biocontrol mechanisms, direct effects on plant pathogens are only one mechanism of biocontrol, and are perhaps less important than induced resistance. With rhizosphere-competent strains which grow

continuously with the plant, long-term systemic resistance is a possibility. The molecular basis of resistance has been partially elucidated with the fact that *Trichoderma* strains produce a set of ATP-binding cassette (ABC) transporters (Lanzuise *et al.*, 2004). These include resistance to environmental toxicants which are produced by soil micro-flora or introduced by human activity and secretion antibiotics and cell-wall-degrading enzymes necessary for the establishment of a compatible interaction with a host fungus.

According to Harman *et al.* (2004) and Viterbo *et al.* (2005), at least three classes of substances which elicit plant defense responses have been identified (e.g. peptides, proteins and low-molecular weight compounds). The systemic response in plants occurs through the JA/ethylene, signaling a pathway in a manner similar to the rhizobacteria-induced systemic resistance (Sarma *et al.*, 2002). These are proteins with enzymatic or other functions, homologues of proteins encoded by the avirulence (*Avr*) genes, oligosaccharides and other low-molecular-weight compounds which are released from fungal or plant cell walls by the activity of *Trichoderma* enzymes (Zeilinger *et al.*, 1999).

Commercialization of Trichoderma *Based Biopesticide*

In India, the majority of farmers continue to express interest in biologically-based pest management strategies of all types as the central components of an IPM approach. Such market realities promote the development of biocontrol products. However, the path to developing and applying effective biocontrol methods is still a long one, fraught with many difficulties. In addition to this, scientific, regulatory, business



Scheme 1: Sequence of events in the development of commercial *Trichoderma* formulation

management and marketing issues, all must be handled effectively for a biocontrol product to be successful in the marketplace.

For successful commercial production of *Trichoderma* based biopesticide, the following properties are essential:

1. Abundant and cost effective production of microbial propagules
2. Optimization of culture conditions to produce high yield and high quality conidial biomass
3. Development of low cost production, storage, and distribution systems
4. Ability to survive downstream processing, particularly drying, which is required to avoid contamination
5. Stability and adequate shelf-life of the final product upon storage, preferably without refrigeration
6. Ability to withstand environmental variations in temperature, desiccation, relative humidity in order to survive and establish active populations in soil
7. Consistent efficacy under varying agro-climatic conditions at commercial feasible states
8. Integration of biocontrol into current agronomic practices.

Biopesticides - Where Do We Stand?

Despite slight reluctance to promote the use of biofertilizers and biopesticides till recently, its use seems to be emerging as an alternative to the use of chemicals; this is rather slowly but surely. This is evident from the recent trend among an increasing number of farmers in the Green Revolution belts of the country to voluntarily switch over to organic agriculture using various ecofriendly means as well as their willingness to gain knowledge about more and more products based on microbes and plants. Organic agriculture requires less financial input and places more reliance on the available natural and human resources. It has the potential to become a viable alternative for the resource-poor farmers of the country. In fact, a switch-over to organic farming can go a long way in improving the economic well-being of these impoverished cultivators if they can take advantage of the rapidly growing global markets for organic products which offer handsome premiums. It will expand more if home grown products could be developed using the available resources, and for this to happen, knowledge dissemination is needed on the benefits of shift to the use of biofertilizers and biopesticides.

The government has to play a very important and proactive role, if scientists, industrialists and farmers have to come together to change the way the agriculture is being practiced. It is the most appropriate time that the Government of India and different states make a structural shift in their current policy stance of promoting agriculture based on the natural resources and the industrial products. The Ministry of Agriculture will have to devise a full-fledged long-term policy framework to create an environment conducive enough for organic agriculture to flourish. Firstly, grants for the development of technologies based on microbial- or plant-based products should be given to the specialist institutions and rigorously monitored. Moreover, efforts must be made for knowledge dissemination through print and audio visual media. Organic certification processes must be made less tardy and faster, while maintaining their integrity. Market development needs to be hastened with the help of the governments for various products such as biofertilizers, biopesticides or bio-control agents. They should be made available in adequate quantities and at reasonable prices. For this purpose, production units may be established for large-scale. For this, Cuba can be a model, i.e. a country where organic conversion has been successfully undertaken on a nation-wide scale, as a consequence of a conscious policy decision on the part of the Fidel Castro government.

Conclusions and Future Prospects

For industrialization of any product, all the requisite parameters should be such that the chances of failure at any stage are non-existent or minimal. The solid state fermentation technology, for the production of *Trichoderma*-based biopesticides, is one such technology. The researchers have successfully standardized the technology and several industries have successfully marketed their products in India. This particular research group is very much interested in developing new methods of biological control which are more suitable to agriculture as what has been practiced in our country due to small landholdings and which may be industrialized profitably. In particular, the researchers are interested in developing consortium of the different species or isolates

of *Trichoderma* itself and *Trichoderma* as well as other microbes which can be effectively integrated in different integrated management modules currently used by farmers. In addition, we would also like to focus genes encoding on specific proteins in *Trichoderma* species. These proteins degrade chitin, a structural component of most pathogenic fungi and herbivorous insects. Furthermore, there is also a need for tolerant strains of *Trichoderma,* which could withstand high temperature encountered during summer in the country. Moreover, cooperative efforts from the researchers at the Banaras Hindu University and elsewhere should lead to a series of next generation of biocontrol products for commercialization.

REFERENCES

Ahmad, J.S. and Baker, R. (1987). Competitive saprophytic ability and cellulolytic activity of rhizosphere-competent mutants of *Trichoderma harzianum. Phytopathology, 77,* 358–362.

Ahmed, S.A., Sanchez, C.P. and Candely, M.E. (2000). Evaluation of induction of systemic resistance in pepper plants (*Capsicum annum*) to *Phytophhtora capsici* using *Trichoderma harzianum* and its relation with capsidiol accumulation. *Eur. J. Plant Pathol., 106,* 817–824.

Altomare, C., Norvell, W.A., Björkman, T. and Harman, G.E. (1999). Solubilization of phosphates and micronutrients by the plant growth promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Appl. Environ. Microbiol., 65,* 2926-2933.

Anonymous. (2006). *Annual Report,* National Botanical Research Institute, Lucknow, pp. 61-74.

Baker, K.F. and Cook, R.J. (1982). *Biological Control of Plant Pathogens.* St. Paul, Minn: APS Press.

Beagle-Ristaino, J.E. and Papavizas, G.C. (1985). Biological control of *Rhizoctonia* stem canker and black scurf of potato. *Phytopathology, 75,* 560-564.

Buhariwalla, H.K., Srilakshmi P., Kannan, S., Kanchi, R.S., Chandra, S., Satyaprasad, K., Waliyar, F., Thakur, R.P. and Crouch, J.H. (2005). AFLP analysis of *Trichoderma* spp. from India compared with sequence and morphological-based diagnostics. *P Zeit., 153,* 389–400.

Chao, W.L., Nelson, E.B., Harman, G.E. and Hoch, H.C. (1986). Colonization of the rhizosphere by biological control agents applied to seeds. *Phytopathology, 76,* 60–65.

Chérif, M. and Benhamou, N. (1990). Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp. on *Fusarium oxysporum* f. sp. *radicis lycopersici. Phytopathology, 80,* 1406-1414.

Chet, I., Inbar, J. and Hadar, I. (1997). Fungal antagonists and mycoparasites. In D.T. Wicklow and B. Soderstorm (Eds.), *The Mycota IV: Environmental and Microbial Relationships* (pp. 165-72). Berlin Heidelberg, New York: Springer.

Das, A., Gokhale, D.V. and Peberdy, J.F. (1988). Protoplast fusion and genetic recombination in intra- and interstrain crossing in *Aspergillus niger. Enzyme Microb. Technol.*, *11,* 2–5.

De Meyer. G., Bigirimana. J., Elad, Y. and Hofte, M. (1998). Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea. Eur. J. Plant Pathol.*, *104,* 279–286.

Druzhinina, I.S., Schmoll, M., Seiboth, B. and Kubicek, C.P. (2006). Global carbon utilization profiles of wild-type strains, mutants and transformants of *Hypocrea jecorina. Appl. Environ. Microbiol.*, *72,* 2126–2133.

Elad, Y. and Freeman, S. (2002). Biological control of fungal plant pathogens. In K. Esser and J.W. Benett (Eds.), *The Mycota A comprehensive Treatise on fungi as experimental systems for basic and applied research (*Vol. XI, pp. 92-109). Germany: Springer Press.

Elad, Y. and Kirshner, B. (1993). Survival in the phylloplane of an introduced biocontrol agent (*Trichoderma harzianum*) and populations of the plant pathogen *Botrytis cinerea* as modified by abiotic conditions. *Phytoparasitica*, *21*, 303-313.

Elad, Y., Barak, R. and Henis, Y. (1983). Ultrastructural studies of the interaction between *Trichoderma* spp. and plant pathogenic fungi. *Phytopathol Z, 107,*168-175.

Elad, Y., Zimand, G., Zaqs, Y., Zurial, S. and Chet, I. (1993). Use of *Trichoderma harzianum* in combination or alternation with fungicides to control cucumber grey mould (*Botrytis cinerea*) under commercial greenhouse conditions. *Pl. Pathol., 42,* 324-332.

Hanson, L.E. and Howell, C.R. (2004). Elicitors of plant defense responses from biocontrol strains of *Trichoderma virens. Phytopathology, 94,* 171–176.

Harman, G.E. (2000). Myths and dogmas of biocontrol: Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Pl Dis., 84,* 377-391.

Harman, G.E. (2001). Microbial tools to improve crop performance and profitability and to control plant diseases. In D.D.S. Tzeng (Eds.), Proceedings of International Symposium on Biological Control of Plant Diseases for the New Century-mode of Action and Application Technology (pp. 71-84). Taichung City, Taiwan: National Chung Hsing University. //4-1–4-14 in: Int Symp Biol Contl. Pl Dis New Century-Mode Action Application Technol. Incomplete///

Harman, G.E., Hayes, C.K. and Ondik, K.L. (1998). Asexual genetics in *Trichoderma* and *Gliocladium*: Mechanisms and implications. In C.P. Kubicek and G.E. Harman (Eds.) *Trichoderma and Gliocladium*, Vol.1 (pp. 243-270). London: Taylor & Francis.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. and Lorito, M. (2004). *Trichoderma* species - opportunistic, avirulent plant symbionts. *Nat Rev Microbiol., 2,* 43-56.

Howell, C.R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. *Pl Dis., 87,* 4-10.

Howell, C.R., Hanson, L.E., Stipanovic, R.D. and Puckhaber, L.S. (2000). Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens. Phytopathology, 90,* 248–252.

Jin X, Harman, G.E. and Taylor, A.G. (1991). Conidial biomass and desiccation tolerance of Trichoderma harzianum produced at different medium water potentials. *Biol Contl, 1,* 237-243.

Kalra, A., Singh, H.B., Pandey, R., Patra, N.K., Katiyar, N., Gupta, M.L., Dhawan, O.P. and Kumar, S. (2002). Strain of *Trichoderma harzianum* useful as nematode inhibitor, fungicide and plant growth promoter and a process for the isolation thereof. (US Patent No. 6,475,772).

Krishna, C. (2005). Solid state fermentation systems: an overview. *Critical Reviews in Biotechnology, 25,* 1-30.

Kubicek, C.P., Bissett, J., Druzhinina, I., Kullnig-Gradinger, C. and Szakacs, G. (2002). Genetic and metabolic diversity of *Trichoderma*: a case study on Southeast Asian isolates. F*ungal Genet Biol, 38,* 310–319.

Kullnig-Gradinger, C. M., Szakacs, G. and Kubicek, C.P. (2002). Phylogeny and evolution of the fungal genus *Trichoderma*—a multigene approach. *Mycol Res., 106,* 757–767.

Lalithakumari, D. (2000). *Fungal Protoplasts—A Biotechnological Tool.* New Delhi, India: Oxford & IBH Publishing Company Private Limited.

Landreau, A., Pouchus, Y.F., Sallenave-Namont, C., Biard, J.F., Boumard, M.C., Robiou du, P.T., Mondeguer, F., Goulard, C. and Verbist, J. F. (2002). Combined use of LC/MS and a biological test for rapid identification of marine mycotoxins produced by *Trichoderma koningii. J Microbiol Meth, 48,* 181–194.

Lanzuise, S., Ruocco, M., Scala, V., Catapano, L., Woo, S., Ciliento, R., Ferraioli, S., Soriente, I., Vinale, F., Scala, F., Del Sorbo, G. and Lorito, M. (2004). Cloning of first ABC transporter encoding gene from *Trichoderma* spp. and its expression during stress and mycoparasitism. 11th SIPAV Annu Meet, Milan, Sep 29 – Oct 1 2004, abs. *J. Pl. Pathol., 86*(4, Special issue), 322.

Lavaania, M., Chauhan, P.S., Chauhan, S.V.S., Singh, H.B. and Nautiyal, C.S. (2006). Induction of plant defense enzymes and phenolics by treatment with plant growth promoting rhizobacteria *Serratia marcescens* NBRI1213. *Current Microbiology, 52,* 363-368.

Lewis, J.A. and Papavizas, G.C. (1983). Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. *Soi Biol Biochem, 15,* 351–357.

Mathre, D.E., Cook, R.J. and Callan, N.W. (1999). From Discovery to Use: Traversing the world of commercializing biocontrol agents for Plant Disease Control. *Plant Disease, 83*(11), 972-983.

Mishra, R.C., Singh, R., Shahi, S.K., Singh, H.B. and Dixit, A. (2000). Biological management of patchouli (*Pogostemon cablin*) wilt caused by *Rhizoctonia solani. Current Science, 78*(3), 230-232.

Mrinalini, C. and Lalithakumari, D. (1998). Integration of enhanced biocontrol efficacy and fungicide tolerance in *Trichoderma* spp. by electrofusion. *J Pl Dis Prot, 105,* 34–40.

Nautiyal, C.S., Mehta, S. and Singh, H.B. (2006). Biological control and plant growth promotion by *Bacillus* strains from milk. *J Microbiol Biotechnol, 16*(2), 184-192.

Nautiyal, C.S., Mehta, S., Singh, H.B. and Pushpangadan, P. (2006). Synergistic bioinicoualnt composition comprising bacterial strains of Accession Nos. NRRL B-30486, NRRL B-30487 and NRRL B-30488 and a method of producing said composition thereof. US Patent No. 71097,830 B2

Ogawa, K., Ohara, H., Koide, T. and Toyama, N. (1989). Interspecific hybridization of *T. reesei* by protoplast fusion. *J. Ferment. Bioeng. 67,* 207–209

Papavizas, G.C. (1981). Survival of *Trichoderma harzianum* in soil and in pea and bean rhizospheres. *Phytopathology, 71,* 121–5.

Papavizas, G.C. (1985). *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. *Annu. Rev. Phytopathol. 23,* 23–54.

Peberdy, J.F. (1980). Protoplast fusion-a tool for genetic manipulation and breeding in industrial microorganisms. *Enzyme Microb. Technol., 2,* 23-29.

Prabavathy, V.R., Mathivanan, N.E., Sagadevan, K., Murugesan and Lalithakumari, D. (2006). Self-fusion of protoplasts enhances chitinase production and biocontrol activity in *Trichoderma harzianum. Biores Technol., 97,* 2330-2334.

Reusser, F. (1967). Biosynthesis of antibiotic U-22,324, a cyclic polypeptide. *J Biol Chem., 242,* 243–247

Sarma B.K., Singh D.P., Mehta S. Singh H.B. and Singh U.P. 2002. Plant growth promoting rhizobacteria-mediated alternation in phenolic profile of chickpea (*Cicer arietinum*) infected by *Sclerotium rolfsii. Journal of Phytopathology, 150*(4&5), 277-282.

Singh, A. 2002. Studies on the collar rot of betelvine (*Piper betle* L.) Lucknow University, Lucknow. pp. 187.

Singh, A. and Singh, H.B. (2004). Control of collar rot of mints (*Mentha* spp.) caused by *Sclerotium rolfsii* by using biological means. *Current Science, 87,* 362-366.

Singh, A., Srivastava, S. and Singh, H.B. (2007). Effect of substrates on growth and shelf life of *Trichoderma harzianum* and its use in biocontrol of diseases. *Bioresource Technology, 98,* 470–473.

Singh, H.B. (2006). Achievements in biological control of diseases with antagonistic organisms at National Botanical Research Institute, Lucknow. In B. Ramanujan and R. J. Rabindra (Eds.), *Current Status of Biological Control of Plant Diseases using Antagonistic Organisms in India (*pp. 329-340). Technical Document, Project Directorate of Biological Control, Bangalore.

Singh, H.B., Kalra, A. and Patra, N.K. (2001). Potential for biological control of wilt and rot in mints by *Trichoderma harzianum* and *Gliocladium virens. Kavaka, 28 & 29,* 45-48.

Singh, H.B., Kalra, A., Patra, N.K., Kumar, S., Pandey, R., Khanuja, S.P.S. and Shasany, A.K. (2003). Process for the preparation of novel growth media from distillation and other plant wastes for mass multiplication of bio-control fungi. (US Patent No: 6,511-821)

Singh, H.B., Rehman, A., Srivastava, S. and Nautiyal, C.S. (2006). A synergistic composition useful as bioinoculant (US Patent Filed NF No. 0020NF2006/IN).

Singh, H.B., Singh, A. and Nautiyal, C.S. (2002). Commercialization of biocontrol agents: problems and prospects. In G.P. Rao *et al.* (Eds.), *Frontiers of Fungal Diversity in Indian Subcontinent (Prof. Kamal Fetschrift)(pp.847-861)*. Lucknow, India: International Book Distributing Company.

Singh, H.B., Singh, A., Singh, S.P. and Nautiyal, C.S. (2004). Commercialization of biocontrol agents: the necessity and its impact on agriculture. In S.P. Singh and H.B. Singh (Eds.), *Eco-agriculture with Bioaugmentation: An emerging concept* (pp.1-20). Lucknow, India: Rohitashwa Printers.

Singh, H.B., Tripathi, A., Srivastava, S., Vikas, Singh, P.P. and Singh, A. (2006). Validation of IPM technologies for the management of corm rot and wilt of gladiolus. *Journal of Eco-friendly Agriculture, 1,* 49-53.

Sivan, A. and Harman, G.E. (1991). Improved rhizosphere competence in a protoplast fusion progeny of *Trichoderma harzianum. J. Gen. Microbiol., 137,* 23–29.

Vey, A., Hoagland, R.E. and Butt, T.M. (2001). Toxic metabolites of fungal biocontrol agents. In T.M. Butt, C. Jackson and Magan, N. (Eds.), *Fungi as Biocontrol Agents: Progress, Problems and Potential* (pp.311–346). Bristol: CAB International.

Viterbo, A., Harel, M., Horwitz, B.A., Chet, I. and Mukherjee, P.K. (2005). *Trichoderma* MAP-kinase signaling is involved in induction of plant systemic resistance. *Appl. Environ Microbiol., 71,* 6241-6246.

Walters, D.R., Newton, A.C. and Lyon, G.D. (2005). Induced resistance: helping plants to help themselves. *Biologist, 52,* 28-33.

Weindling, R. (1934). Studies on a lethal principle effective in the parasitic action of *Trichoderma lignorum* on *Rhizoctonia solani* and other soil fungi. *Phytopathology, 24,* 1153-1179.

Whipps, J.M. (2001). Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot., 52,* 487–511.

Xiao-Yan, S., Qing-Tao, S., Shu-Tao, X., Xiu-Lan, C., Cai-Yun, S. and Yu-Zhong, Z. (2006). Broad-spectrum antimicrobial activity and high stability of Trichokonins from *Trichoderma koningii* SMF2 against plant pathogens *FEMS Microbiol Lett., 260,* 119-125.

Yedidia, I., Benhamou, N., Kapulnik, Y. and Chet, I. (2000). Induction and accumulation of pathogenesis related protein activity during the early stage of root colonization by *Trichoderma harzianum. Pl. Physiol. Biochem., 38,* 863–873.

Yedidia, I., Shoresh, M., Kerem, Z., Benhamou, N., Kapulnik, Y. and Chet, I. (2003). Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. *Appl. Environ. Microbiol., 69,* 7343–7353.

Yedidia, I., Srivastva, A.K., Kapulnik, Y. and Chet, I. (2001). Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Pl. Soil, 235,* 235–242.

Zeilinger, S., Galhaup, C., Payer, K., Woo, S. L., Mach, R.L., Fekete. C., Lorito, M. and Kubicek, C.P. (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genet. Biol., 26,* 131–140.

Zhang, J., Howell, C.R. and Starr, J.L. (1996). Suppression of *Fusarium* colonization of cotton roots and *Fusarium* wilt by seed treatments with *Gliocladium virens* and *Bacillus subtilis. Bioconl. Sci. Technol., 6,* 175-187.

Pertanika J. Trop. Agric. Sci. **31(1)**: ? - ? (2009) ISSN: 1511-3701

Erratum for Yap, C.K. *et al.*, *Pertanika J. Trop. Agric. Sci.* 31(1) 79-90

Journal of Tropical Agricultural Science, Vol. 31(1)
Feb. 2008: pp. 79-90.© 2008 UPM Press.

The February 2008 issue of Vol. 31(1), article entitled "Distribution of Ni and Zn in the surface sediments collected from drainages and intertidal area in Selangor", authors: Yap, C. K., Fairuz, M. S., Cheng, W. H. and Tan, S. G., pp. 79-90, **Tables 6** and **7 contained errors**.

The corrected tables 6 and 7 are reprinted below in its entirety.

TABLE 6
Spearman's correlation coefficients among the geochemical fractions of Ni in the sediments from some places in Selangor. N = 11. All are significantly (P< 0.05) correlated except for those indicated by ns= not significant (P> 0.05).

	F1	F2	F3	F4	Sum	Total
F1	1.00	0.17^{ns}	0.39^{ns}	0.62	0.66	0.66
F2		1.00	0.05^{ns}	0.42^{ns}	0.46^{ns}	0.68
F3			1.00	0.70	0.74	0.61
F4				1.00	0.98	0.86
Sum					1.00	0.92
Total						1.00

Note: F1= EFLE, F2= acid-reducible, F3= oxidisable-organic and F4= resistant. Total= based direct aqua-regia method. SUM= summation of all the 4 fractions based on sequential extraction technique.

TABLE 7
Spearman's correlation coefficients among the geochemical fractions of Zn in the sediments from some places in Selangor. N = 11. All are significantly (P< 0.05) correlated.

	F1	F2	F3	F4	Sum	Total
F1	1.00	0.63	0.77	0.68	0.86	0.88
F2		1.00	0.90	0.68	0.86	0.88
F3			1.00	0.83	0.97	0.96
F4				1.00	0.86	0.84
Sum					1.00	0.99
Total						1.00

Note: F1= EFLE, F2= acid-reducible, F3= oxidisable-organic and F4= resistant. Total= based direct aqua-regia method. SUM= summation of all the 4 fractions based on sequential extraction technique.

The main author apologises for the above errors.

Yap, C. K., Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia. yapckong1973@yahoo.com.sg and yapckong@hotmail.com

Pertanika

Our goal is to bring high quality research to the widest possible audience

Journal of Tropical Agricultural Science

INSTRUCTIONS TO AUTHORS

(Manuscript Preparation & Submission Guidelines)

Revised January 2009

We aim for excellence, sustained by a responsible and professional approach to journal publishing. We value and support our authors in the research community.

Please read the guidelines and follow these instructions carefully; doing so will ensure that the publication of your manuscript is as rapid and efficient as possible. The Editorial Board reserves the right to return manuscripts that are not prepared in accordance with these guidelines.

About the Journal

Pertanika is an international peer-reviewed journal devoted to the publication of original papers, and it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields. Pertanika Journal of Tropical Agricultural Science which began publication in 1978 is a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped Pertanika Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other Pertanika series include Pertanika Journal of Science and Technology (JST) and Pertanika Journal of Social Sciences and Humanities (JSSH).

JTAS is published in **English** and it is open to authors around the world regardless of the nationality. It is currently published two times a year, i.e. in **February** and **August**.

Goal of Pertanika

Our goal is to bring the highest quality research to the widest possible audience.

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. JTAS is an international award winning journal indexed in SCOPUS (Elsevier) and EBSCO.

Future vision

We are continuously improving access to our journal archives, content, and research services. We have the drive to realise exciting new horizons that will benefit not only the academic community, but society itself.

We also have views on the future of our journals. The emergence of the online medium as the predominant vehicle for the 'consumption' and distribution of much academic research will be the ultimate instrument in the dissemination of research news to our scientists and readers.

Aims and scope

Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include: *agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance,* and *veterinary medicine.*

Editorial Statement

Pertanika is the official journal of Universiti Putra Malaysia. The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

Guidelines for Authors

Publication policies

Pertanika policy prohibits an author from submitting the same manuscript for concurrent consideration by two or more publications. It prohibits as well publication of any manuscript that has already been published either in whole or substantial part elsewhere.

Editorial process

Authors are notified on receipt of a manuscript and upon the editorial decision regarding publication.

Manuscript review: Manuscripts deemed suitable for publication are sent to the Editorial Advisory Board members and/or other reviewers. We encourage authors to suggest the names of possible reviewers. Notification of the editorial decision is usually provided within to eight to ten weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

Author approval: Authors are responsible for all statements in articles, including changes made by editors. The liaison author must be available for consultation with an editor of *The Journal* to answer questions during the editorial process and to approve the edited copy. Authors receive edited typescript (not galley proofs) for final approval. Changes **cannot** be made to the copy after the edited version has been approved.

Please direct all inquiries, manuscripts, and related correspondence to:

The Executive Editor
Pertanika Journals
Research Management Centre (RMC)
4th Floor, Administration Building
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor
Malaysia
Phone: + (603) 8946 6192
Fax: + (603) 8947 2075
ndeeps@admin.upm.edu.my

or visit our website at http://rmc.upm.edu.my/pertanika for further information.

Manuscript preparation

Pertanika accepts submission of mainly four types of manuscripts. Each manuscript is classified as **regular** or **original** articles, **short communications**, **reviews**, and proposals for **special issues**. Articles must be in **English** and they must be competently written and argued in clear and concise grammatical English. Acceptable English usage and syntax are expected. Do not use slang, jargon, or obscure abbreviations or phrasing. Metric measurement is preferred; equivalent English measurement may be included in parentheses. Always provide the complete form of an acronym/abbreviation the first time it is presented in the text. Contributors are strongly recommended to have the manuscript checked by a colleague with ample experience in writing English manuscripts or an English language editor.

Linguistically hopeless manuscripts will be rejected straightaway (e.g., when the language is so poor that one cannot be sure of what the authors really mean). This process, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

The instructions for authors must be followed. Manuscripts not adhering to the instructions will be returned for revision without review. Authors should prepare manuscripts according to the guidelines of Pertanika.

1. Regular article
Definition: Full-length original empirical investigations, consisting of introduction, materials and methods, results and discussion, conclusions. Original work must provide references and an explanation on research findings that contain new and significant findings.

Size: Should not exceed 5000 words or 8-10 printed pages (excluding the abstract, references, tables and/or figures). One printed page is roughly equivalent to 3 type-written pages.

2. Short communications
Definition: Significant new information to readers of the Journal in a short but complete form. It is suitable for the publication of technical advance, bioinformatics or insightful findings of plant and animal development and function.

Size: Should not exceed 2000 words or 4 printed pages, is intended for rapid publication. They are not intended for publishing preliminary results or to be a reduced version of Regular Papers or Rapid Papers.

3. Review article
Definition: Critical evaluation of materials about current research that had already been published by organizing, integrating, and evaluating previously published materials. Re-analyses as meta-analysis and systemic reviews are encouraged. Review articles should aim to provide systemic overviews, evaluations and interpretations of research in a given field.

Size: Should not exceed 4000 words or 7-8 printed pages.

4. Special issues
Definition: Usually papers from research presented at a conference, seminar, congress or a symposium.

Size: Should not exceed 5000 words or 8-10 printed pages.

5. Others
Definition: Brief reports, case studies, comments, Letters to the Editor, and replies on previously published articles may be considered.

Size: Should not exceed 2000 words or up to 4 printed pages.

With few exceptions, original manuscripts should not exceed the recommended length of 6 printed pages (about 18 typed pages, double-spaced and in 12-point font, tables and figures included). Printing is expensive, and, for the Journal, postage doubles when an issue exceeds 80 pages. You can understand then that there is little room for flexibility.

Long articles reduce the Journal's possibility to accept other high-quality contributions because of its 80-page restriction. We would like to publish as many good studies as possible, not only a few lengthy ones. (And, who reads overly long articles anyway?) Therefore, in our competition, short and concise manuscripts have a definite advantage.

Format

The paper should be formatted in one column format with the figures at the end. A maximum of eight keywords should be indicated below the abstract to describe the contents of the manuscript. Leave a blank line between each paragraph and between each entry in the list of bibliographic references. Tables should preferably be placed in the same electronic file as the text. Authors should consult a recent issue of the Journal for table layout.

There is no need to spend time formatting your article so that the printout is visually attractive (e.g. by making headings bold or creating a page layout with figures), as most formatting instructions will be removed upon processing.

Manuscripts should be typewritten, typed on one side of the ISO A4 paper with at least 4cm margins and double spacing throughout. Every page of the manuscript, including the title page, references, tables, etc. should be numbered. However, no reference should be made to page numbers in the text; if necessary, one may refer to sections. Underline words that should be in italics, and do not underline any other words.

Authors are advised to use Times New Roman 12-point font. Be especially careful when you are inserting special characters, as those inserted in different fonts may be replaced by different characters when converted to PDF files. It is well known that 'μ' will be replaced by other characters when fonts such as 'Symbol' or 'Mincho' are used.

We recommend that authors prepare the text as a **Microsoft Word** file.

1. Manuscripts in general should be organised in the following order:

   - **Page 1: Running title.** (Not to exceed 60 characters, counting letters and spaces). This page should **only** contain your running title/ full title of your paper. In addition, the **Subject areas** most relevant to the study must be indicated on this page. Select one or two subject areas (refer to the *Scope Form).*
     A **list** of number of **black and white / colour figures and tables** should also be indicated on this page. Figures submitted in color will be printed in colour. See "*5. Figures & Photographs*" for details.

   - **Page 2: Author(s) and Corresponding author information.** This page should **repeat** the title of your paper with name(s) of all the authors, institutions and corresponding author's name, institution and full address (Street address, telephone number (including extension), hand phone number, fax number and e-mail address) for editorial correspondence.

     Authors' addresses. Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers:

     George Swan[1] and Nayan Kanwal[2]
     [1]Department of Biology, Faculty of Science, Duke University, Durham, North Carolina, USA.
     [2]Research Management Centre, Universiti Putra Malaysia, Serdang, Malaysia.

   - **Page 3**: This page should **repeat** the title of your paper with only the **Abstract** (the abstract should be less than 250 words for a Regular Paper and up to 100 words for a Short Communication). **Keywords** must also be provided on this page (Not more than eight keywords in alphabetical order).

   - **Page 4 and subsequent pages**: This page should begin with the **Introduction** of your article and the rest of your paper should follow from page 5 onwards.

   Abbreviations. Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the introduction and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

   Footnotes. Current addresses of authors if different from heading.

2. **Text.** Regular Papers should be prepared with the headings **Introduction, Materials and Methods, Results and Discussion, Conclusions** in this order. Short Communications should be prepared according to "*8. Short Communications.*" below.
3. **Tables.** All tables should be prepared in a form consistent with recent issues of Pertanika and should be numbered consecutively with Arabic numerals. Explanatory material should be given in the table legends and footnotes. Each table should be prepared on a separate page. (Note that when a manuscript is accepted for publication, tables must be submitted as data - .doc, .rtf, Excel or PowerPoint file- because tables submitted as image data cannot be edited for publication.)

4. **Equations and Formulae.** These must be set up clearly and should be typed triple spaced. Numbers identifying equations should be in square brackets and placed on the right margin of the text.

5. **Figures & Photographs.** Submit an original figure or photograph. Line drawings must be clear, with high black and white contrast. Each figure or photograph should be prepared on a separate sheet and numbered consecutively with Arabic numerals. Appropriate sized numbers, letters and symbols should be used, no smaller than 2 mm in size after reduction to single column width (85 mm), 1.5-column width (120 mm) or full 2-column width (175 mm). Failure to comply with these specifications will require new figures and delay in publication. For electronic figures, create your figures using applications that are capable of preparing high resolution TIFF files acceptable for publication. In general, we require **300 dpi or higher resolution for coloured and half-tone artwork** and **1200 dpi or higher for line drawings**. For review, you may attach low-resolution figures, which are still clear enough for reviewing, to keep the file of the manuscript under 5 MB. Illustrations may be produced at extra cost in colour at the discretion of the Publisher; the author could be charged Malaysian Ringgit 50 for each colour page.

6. **References.** Literature citations in the text should be made by name(s) of author(s) and year. For references with more than two authors, the name of the first author followed by 'et al.' should be used.

   Swan and Kanwal (2007) reported that ...
   The results have been interpreted (Kanwal et al. 2009).

   - References should be listed in alphabetical order, by the authors' last names. For the same author, or for the same set of authors, references should be arranged chronologically. If there is more than one publication in the same year for the same author(s), the letters 'a', 'b', etc., should be added to the year.
   - When the authors are more than 11, list 5 authors and then et al.
   - Do not use indentations in typing References. Use one line of space to separate each reference. For example:
     - Jalaludin, S. (1997a). Metabolizable energy of some local feeding stuff. *Tumbuh, 1*, 21-24.
     - Jalaludin, S. (1997b). The use of different vegetable oil in chicken ration. *Mal. Agriculturist, 11*, 29-31.
     - Tan, S.G., Omar, M.Y., Mahani, K.W., Rahani, M., Selvaraj, O.S. (1994). Biochemical genetic studies on wild populations of three species of green leafhoppers *Nephotettix* from Peninsular Malaysia. *Biochemical Genetics, 32*, 415 - 422.
   - In case of citing an author(s) who has published more than one paper in the same year, the papers should be distinguished by addition of a small letter as shown above, e.g. Jalaludin (1997a); Jalaludin (1997b).
   - Unpublished data and personal communications should not be cited as literature citations, but given in the text in parentheses. 'In press' articles that have been accepted for publication may be cited in References. Include in the citation the journal in which the 'in press' article will appear and the publication date, if a date is available.

7. **Examples of other reference citations**:
   Monographs: Turner, H.N. and Yong, S.S.Y. (2006). *Quantitative Genetics in Sheep Breeding*. Ithaca: Cornell University Press.

   Chapter in Book: Kanwal, N.D.S. (1992). Role of plantation crops in Papua New Guinea economy. In Angela R. McLean (Eds.), *Introduction of livestock in the Enga province PNG* (p. 221-250). United Kingdom: Oxford Press.

   Proceedings: Kanwal, N.D.S. (2001). Assessing the visual impact of degraded land management with landscape design software. In N.D.S. Kanwal and P. Lecoustre (Eds.), *International forum for Urban Landscape Technologies* (p. 117-127). Lullier, Geneva, Switzerland: CIRAD Press.

8. **Short Communications** should include **Introduction, Materials and Methods, Results and Discussion, Conclusions** in this order. Headings should only be inserted for Materials and Methods. The abstract should be up to 100 words, as stated above. Short Communications must be 5 printed pages or less, including all references, figures and tables. References should be less than 30. A 5 page paper is usually approximately 3000 words plus four figures or tables (if each figure or table is less than 1/4 page).

   *Authors should state the total number of words (including the Abstract) in the cover letter. Manuscripts that do not fulfill these criteria will be rejected as Short Communications without review.

STYLE OF THE MANUSCRIPT

Manuscripts should follow the style of the latest version of the Publication Manual of the American Psychological Association (APA). The journal uses British spelling and authors should therefore follow the latest edition of the Oxford Advanced Learner's Dictionary.

SUBMISSION OF MANUSCRIPTS

All articles submitted to the journal **must comply** with these instructions. Failure to do so will result in return of the manuscript and possible delay in publication.

The **four copies** of your original manuscript, four sets of photographic figures, as well as a CD with the **electronic copy in MS Word** (including text and figures) together with a **cover letter, declaration form, referral form A, scope form** need to be enclosed. They are available from the Pertanika's home page at http://rmc.upm.edu.my/pertanika or from the Executive Editor's office upon request.

Please do **not** submit manuscripts directly to the editor-in-chief or to the UPM Press. All manuscripts must be **submitted through the executive editor's office** to be properly acknowledged and rapidly processed:

Dr. Nayan KANWAL
Executive Editor
Research Management Centre (RMC)

4th Floor, Administration Building
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia
email: ndeeps@admin.upm.edu.my; tel: + 603-8946 6192
fax: + 603 8947 2075

Authors should retain copies of submitted manuscripts and correspondence, as materials can not be returned.

Cover letter

All submissions must be accompanied by a cover letter detailing what you are submitting. Papers are accepted for publication in the journal on the understanding that the article is original and the content has not been published or submitted for publication elsewhere. This must be stated in the cover letter.

The cover letter must also contain an acknowledgement that all authors have contributed significantly, and that all authors are in agreement with the content of the manuscript.

The cover letter of the paper should contain (i) the title; (ii) the full names of the authors; (iii) the addresses of the institutions at which the work was carried out together with (iv) the full postal and email address, plus facsimile and telephone numbers of the author to whom correspondence about the manuscript should be sent. The present address of any author, if different from that where the work was carried out, should be supplied in a footnote.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed on a cover sheet.

Note When your manuscript is received at Pertanika, it is considered to be in its final form. Therefore, you need to check your manuscript carefully before submitting it to the executive editor (see also **English language editing** below).

Electronic copy

Preparation of manuscripts on a CD or DVD is preferable and articles should be prepared using MS Word. File name(s), the title of your article and authors of the article must be indicated on the CD. The CD must always be accompanied by four hard-copies of the article, and the content of the two must be identical. The CD text must be the same as that of the final refereed, revised manuscript. CDs formatted for IBM PC compatibles are preferred, as those formatted for Apple Macintosh are not acceptable. Please do not send ASCII files, as relevant data may be lost. Leave a blank line between each paragraph and between each entry in the list of bibliographic references. Tables should be placed in the same electronic file as the text. Authors should consult a recent issue of the Journal for table layout.

Peer review

In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts. The Journal uses a double-blind peer-review system. Authors are encouraged to indicate in **referral form A** the names of three potential reviewers, but the editors will make the final choice. The editors are not, however, bound by these suggestions.

Manuscripts should be written so that they are intelligible to the professional reader who is not a specialist in the particular field. They should be written in a clear, concise, direct style. Where contributions are judged as acceptable for publication on the basis of content, the Editor or the Publisher reserves the right to modify the typescripts to eliminate ambiguity and repetition and improve communication between author and reader. If extensive alterations are required, the manuscript will be returned to the author for revision.

The editorial review process

What happens to a manuscript once it is submitted to Pertanika? Typically, there are seven steps to the editorial review process:

1. The executive editor and the editorial board examine the paper to determine whether it is appropriate for the journal and should be reviewed. If not appropriate, the manuscript is rejected outright and the author is informed.
2. The executive editor sends the article-identifying information having been removed, to three reviewers. Typically, one of these is from the Journal's editorial board. Others are specialists in the subject matter represented by the article. The executive editor asks them to complete the review in three weeks and encloses two forms: (a) referral form B and (b) reviewer's comment form along with reviewer's guidelines. Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the literature.
3. The executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Editorial Board, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.
4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors submit a revised version of the paper to the executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor.
5. The executive editor sends the revised paper out for review. Typically, at least one of the original reviewers will be asked to examine the article.
6. When the reviewers have completed their work, the executive editor in consultation with the editorial board and the editor-in-chief examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.
7. If the decision is to accept, the paper is sent to that Press and the article should appear in print in approximately two to three months. The Publisher ensures that the paper adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any queries by the Publisher. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, only essential changes are accepted. Finally, the article appears in the pages of the Journal and is posted on-line.

English language editing
Authors are responsible for the linguistic accuracy of their manuscripts. Authors not fully conversant with the English language should seek advice from subject specialists with a sound knowledge of English. The cost will be borne by the author, and a copy of the certificate issued by the service should be attached to the cover letter.

Author material archive policy
Authors who require the return of any submitted material that is rejected for publication in the journal should indicate on the cover letter. If no indication is given, that author's material should be returned, the Editorial Office will dispose of all hardcopy and electronic material.

Copyright
Authors publishing the Journal will be asked to sign a declaration form. In signing the form, it is assumed that authors have obtained permission to use any copyrighted or previously published material. All authors must read and agree to the conditions outlined in the form, and must sign the form or agree that the corresponding author can sign on their behalf. Articles cannot be published until a signed form has been received.

Lag time
The elapsed time from submission to publication for the articles averages 5-6 months. A decision of acceptance of a manuscript is reached in 2 to 3 months (average 9 weeks).

Back issues
Single issues from current and recent volumes are available at the current single issue price from UPM Press. Earlier issues may also be obtained from UPM Press at a special discounted price. Please contact UPM Press at penerbit@putra.upm.edu.my or you may write for further details at the following address:

UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang
Selangor Darul Ehsan
Malaysia.

Pertanika

Our goal is to bring high quality research to the widest possible audience

Pertanika is Indexed in SCOPUS & EBSCO

Pertanika is an international peer-reviewed leading journal in Malaysia which began publication in 1978. The journal publishes in three different areas — Journal of Tropical Agricultural Science (JTAS); Journal of Science and Technology (JST); and Journal of Social Sciences and Humanities (JSSH).

JTAS is devoted to the publication of original papers that serves as a forum for practical approaches to improving quality in issues pertaining to tropical agricultural research or related fields of study. It is published twice a year in **February** and **August**.

JST caters for science and engineering research or related fields of study. It is published twice a year in **January** and **July**.

JSSH deals in research or theories in social sciences and humanities research with a focus on emerging issues pertaining to the social and behavioural sciences as well as the humanities, particularly in the Asia Pacific region. It is published twice a year in **March** and **September**.

Call for Papers

Pertanika invites you to explore frontiers from all fields of science and technology to social sciences and humanities. You may contribute your scientific work for publishing in UPM's hallmark journals either as a ***regular article, short communication,*** or ***a review article*** in our forthcoming issues. Papers submitted to this journal must contain original results and must not be submitted elsewhere while being evaluated for the Pertanika Journals.

Submissions in **English** should be accompanied by an abstract not exceeding 300 words. Your manuscript should be no more than 6,000 words or 10-12 printed pages, including notes and abstract. Submissions should conform to the Pertanika style, which is available at www.rmc.upm.edu.my/pertanika/index.htm or by mail or email upon request.

Papers should be double-spaced 12 point type (Times New Roman fonts preferred). The first page should include the title of the article but no author information. Page 2 should repeat the title of the article together with the names and contact information of the corresponding author as well as all the other authors. Page 3 should contain the title of the paper and abstract only. Page 4 and subsequent pages to have the text - Acknowledgments - References - Tables - Legends to figures – Figures, etc.

Questions regarding submissions should only be directed to the Executive Editor, *Pertanika* Journals.

Remember, ***Pertanika is the resource to support you in strengthening research and research management capacity.***

Why should you publish in Pertanika Journals?

Benefits to Authors

PROFILE: our journals are circulated in large numbers all over Malaysia, and beyond in Southeast Asia. Recently, we have widened our circulation to other overseas countries as well. We will ensure that your work reaches the widest possible audience in print and online, through our wide publicity campaigns held frequently, and through our constantly developing electronic initiatives via Pertanika Online and e-pertanika.

QUALITY: our journals' reputation for quality is unsurpassed ensuring that the originality, authority and accuracy of your work will be fully recognised. Our double-blind peer refereeing procedures are fair and open, and we aim to help authors develop and improve their work. Pertanika JTAS is now over 30 years old; this accumulated knowledge has resulted in Pertanika being indexed in SCOPUS (Elsevier) and EBSCO.

AUTHOR SERVICES: we provide a rapid response service to all our authors, with dedicated support staff for each journal, and a point of contact throughout the refereeing and production processes. Our aim is to ensure that the production process is as smooth as possible, a borne out by the high number of authors who publish with us again and again.

LAG TIME: Submissions are guaranteed to receive a decision within 14 weeks. The elapsed time from submission to publication for the articles averages 5-6 months. A decision of acceptance of a manuscript is reached in 3 to 4 months (average 14 weeks).

An Award Winning International-Malaysian Journal

FEB 2008

Mail your submissions to:

The Executive Editor
Pertanika Journals
Research Management Centre (RMC)
Publication Division
4th Floor, Administration Building
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia

Tel: +603-8946 6192
ndeeps@admin.upm.edu.my
www.rmc.upm.edu.my/pertanika

Pertanika Journal of Tropical Agricultural Science
Vol. 32(1) Feb. 2009

Contents

Regular Articles

Short Communications

Selected Articles from the International Conference on Marine Organisms and their Biomedical Potentials 2008

Guest Editorial Board: Amu Therwath, Anil Chatterji, Prameswaran P.S., Sreepada R.A. and Massoud Mirshahi

Research Management Centre (RMC)
4th Floor, Administration Building
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

http://www.rmc.upm.edu.my
E-mail : pertanika@rmc.upm.edu.my
Tel : +603 8946 6185/ 6192

UPM Press
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

http://penerbit.upm.edu.my
E-mail : penerbit@putra.upm.edu.my
Tel : +603 8946 8855/8854

ISSN 1511-3701
02
9 771511 370234