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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 four times a year, i.e. in **February**, **May**, **August** and **November**.

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Pertanika Journal of Tropical Agricultural Science
Vol. 36 (4) Nov. 2013

Contents

Foreword
Nayan Deep S. Kanwal i

Editorial
Developing Sustainable Fish Farming in the Western Pacific: A Viewpoint on Potential Reasons for Why Many Attempts Failed iii
Peter B. Mather and Satya Nandlal

Short Communication
Ethnic Differences in the Prevalence, Clinical Outcome and *cag* Pathogenicity Island (*cag*PAI) Virulence Gene Profiles of *Helicobacter pylori* Strains from Malaysia 289
Hamat, R. A., Nor Amalina, E., Malina, O., Zamberi, S., Alfizah, H., Rizal, A. M., Aminuddin, A. and Ramelah, M.

Regular Articles
Variations of Electrical Conductivity between Upstream and Downstream of Langat River, Malaysia: Its Significance as a Single Indicator of Water Quality Deterioration 299
Yap, C. K.

Accumulation of Heavy Metals and Antioxidative Enzymes of *Centella asiatica* in Relation to Metals of the Soils 311
Ong, G. H., Yap, C. K., Maziah, M. and Tan, S. G.

Length-Frequency Distribution, Length-Weight Relationship and Condition Factor of Sompat Grunt *Pomadasys jubelini* (Cuvier, 1830) off Lagos Coast, Nigeria 337
Adebiyi, F. A.

Phylogenetic Tree Construction in Reconfirmation of Parasitoid Species (Braconidae: Opiinae), Reared From Fruit Flies (*Bactrocera papayae*) Infesting Star Fruit (*Averrhoa carambola*) Based on Mitochondrial 16S rRNA Sequences 345
Ibrahim, N. J., Shariff, S., Idris, A. B., Md-Zain, B. M., Suhana, Y., Roff, M. N. and Yaakop, S.

Physico-Chemical Characteristics of Chok Anan Mango Fruit after Hot Water Treatment 359
Phebe Ding and Salumiah Mijin

Freezing Method as a New Non-Destructive Modification of DNA Extraction 373
Yaakop, S., C. van Achterberg, Idris, A. B. and Aman, A. Z.

Antioxidant Activity of Two Mesomeric Heterocyclic Betaines Containing a Pyrimidine Moiety 393
Malki, F., Touati, A. and Moulay, S.

Foreword

Welcome to the **Fourth Issue 2013** of the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for the Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university and run on a non-profit basis for the benefit of the world-wide science community.

This issue contains **eight articles,** out of which **one** is a short communication and **seven** are regular research papers. The authors of these articles are from **Malaysia, Nigeria, the Netherlands** and **Algeria**.

The short communication discusses the prevalence, clinical outcome and *cag* pathogenicity island (*cag*PAI) virulence gene profiles of *Helicobacter pylori* strains in response to ethnic differences in patients in Malaysia (*Hamat, R. A., Nor Amalina, E., Malina, O., Zamberi, S., Alfizah, H., Rizal, A. M., Aminuddin, A. and Ramelah, M.*). The preliminary data made available in this study might explain why many Malaysian patients of Indian show a low prevalence for gastric cancer and peptic ulcer disease despite having had a persistently high prevalence of *H. pylori* infection for many decades ("Indian enigma").

The seven research papers cover a wide range of topics. Researchers from Universiti Putra Malaysia investigate the potential of using a single-chemical parameter, namely electrical conductivity, as an indicator of water quality pollution in relation to dissolved oxygen and suspended solids parameter (*Yap, C. K.*). Another research group from the same university describes in detail the accumulation of heavy metals and antioxidative enzymes of *Centella asiatica, a* medicinal herb in relation to metals of the soil (*Ong, G. H., Yap, C. K., Maziah, M. and Tan, S. G.*). In yet another report, on length-frequency distribution, the length-weight relationship and condition factors of sompat grunt *Pomadasys jubelini,* a fish species found off the coast of Lagos, Nigeria, were investigated in order to study the growth pattern of the species (*Adebiyi, F. A.*).

Collaboration work between Universiti Kebangsaan Malaysia and the Malaysian Agricultural Research and Development Institute (MARDI) has successfully constructed a phylogenetic tree in reconfirmation of the parasitoid species (Braconidae: Opiinae), reared from fruit flies (*Bactrocera papayae*) infesting star fruit (*Averrhoa carambola*) and based on their mitochondrial 16S rRNA sequences (*Ibrahim, N. J., Shariff, S., Idris, A. B., Md-Zain, B. M., Suhana, Y., Roff, M. N. and Yaakop, S.*), while anther group of researchers from UPM has characterised Chok Anan mango fruit after hot water treatment (*Phebe Ding and Salumiah Mijin*).

One more collaborative work between Universiti Kebangsaan Malaysia and Naturalis Biodiversity Center in the Netherlands has proved the usability of the freezing method as a new non-destructive modification method of DNA extraction to obtain a sufficient quantity and a high quality of DNA for molecular work (*Yaakop, S., C. van Achterberg, Idris, A. B. and Aman, A. Z.*).

The last manuscript in this issue is on research conducted by a group of researchers from Algeria. They investigate the antioxidant activity of two mesomeric heterocyclic betaines containing a pyrimidine moiety in *in vitro* assays (*Malki, F., Touati, A. and Moulay, S.)*.

I anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

I would also like to express my gratitude to all the contributors, namely, the authors, reviewers and editors who have made this issue possible. Last but not least, the editorial assistance of the journal division staff is fully appreciated.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

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Editorial[i]

Developing Sustainable Fish Farming in the Western Pacific: A Viewpoint on Potential Reasons for Why Many Attempts Failed

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Capture fisheries and aquaculture have been a major source of food and providers of economic benefits to many communities around the world for a very long time. While the history of aquaculture or fish farming can be traced back for more than 2000 years in some corners of the globe, notably in China, Japan and the Mediterranean, this is not true everywhere, where in general, fish farming is a relatively new industry. Rapid human population growth and increasing urbanisation over the last 20 to 40 years has meant that while fish consumption has doubled globally, returns from capture fisheries have remained static or have declined due to overexploitation and rising pollution levels, with some fisheries either closing or becoming economically unviable. Data from studies suggest that this trend is unlikely to be reversed unless appropriate fisheries management allows depleted wild stocks to rebuild. This has occurred during a time when demand for fish products has grown, in part due to improved purchasing power in some developing countries and changing dietary habits where fish are now considered to have a positive impact on health. Based on the projected population growth over the next two decades, Food and Agricultural Organization (FAO) estimates that at least an additional 40 million tonnes of aquatic food will be required to maintain the current per capita consumption (FAO 2006). Thus, many nations have attempted to address the need to increase fish supply by promoting development of aquaculture as it is perceived as having the greatest potential to meet the growing demand for aquatic foods. For some countries, this change has resulted in development of large, highly competitive aquaculture industries, an outcome driven by market pressure, such as salmonids in Norway, channel catfish in the USA, penaeid prawns and tilapia in Asia and more recently, 'tra' catfish in Vietnam. In contrast, development of productive fish culture industries in other regions has been uneven, notably in Africa and the western Pacific, and has been very slow and experienced many failures after significant effort had been directed at encouraging their growth. While many reasons are likely to have contributed to these failures, the social context, traditional lifestyle and the social systems of indigenous people are not often considered to be the factors that could have contributed to the success or failure of new aquaculture ventures.

Here, we argue that many of the aquaculture failures experienced in the western Pacific may have potentially resulted from a failure to develop fish culture systems that are designed for, and well-adapted to, the traditional lifestyles and social systems present in the region.

The western Pacific is a vast marine ecosystem that contains large numbers of mostly small islands and atolls divided into isolated island chain states (referred to as the Pacific Island Countries and Territories or PICTs). Most island human populations are relatively small and have traditionally relied on sustainable agriculture and fishing to meet their food security needs because terrestrial animal food resources are limited or absent. In reality, fisheries are the principal natural resource available across the region, providing almost 50% of the total animal protein supply on average and in some PICTs, the importance of fish to food security is even greater. According to the Secretariat of the Pacific Community (SPC 2008), up to 50% of daily protein intake in the region, as recommended by World health Organization (WHO) for good nutrition, will need to come from fish. Until recent times, traditional social and cultural systems and practices, while varying among PICTs, essentially controlled access to most resources and by doing so, contributed to their long-term sustainability ensuring ongoing supply of natural foods for local people. Over the last 10 to 15 years, however, many culture systems and associated land/water tenure and resource access traditions have faced increasing challenges from rising human populations, urbanization, coupled with more pollution and increased rates of harvesting of inshore and offshore marine resources (e.g. tuna) by large industrial scale fishing enterprises from outside of the region. Another major concern is that chronic over fishing has occurred in coastal areas to an extent that they may not have the capacity to support for future fish food security requirements (SPC, 2008; Bell *et al.,* 2009a &b). Most PICTs now import a significant amount of food, most noticeably cereals, including flour and rice. In addition, PICTs have experienced increasing food and fuel prices, unstable economic conditions and climate change impacts that together affect the availability of food and also the access to it. In particular, increased local food prices have exacerbated an already high reliance on imported and processed foods. Together, these factors contribute to a general decline in the local food production, and island populations are at increasing risk of malnutrition and related disease impacts. These challenges demand a coordinated response and require an understanding of the factors leading to declining food security and most importantly actions that are acceptable to locals to address the problems.

Following from the above, in recent times, the net result has been that in many PICTs, inshore marine resources have declined to a point where they cannot now meet the animal protein needs of the local indigenous populations. Alternative indigenous animal protein sources are limited in most PICTs because native freshwater aquatic faunas are de-pauperate and terrestrial livestock

are limited in supply and very expensive, so some indigenous populations have been forced to change their diets to include increasing quantities of imported, canned or frozen substitutes (e.g. canned fish, frozen low grade meat) to address this problem. This change, as stated above, is linked to growing human health problems (rapid increases in the rates of Type 1 diabetes and obesity-related diseases) that result from consumption of low quality foods, high in fat content and often low in nutrients because this is essentially all that the poorer sections (particularly urban populations) of the community can afford due to the decline in the access to fresh local food.

Governments across the western Pacific are well aware of this growing problem and have examined ways to address the issue. Fish farming has been identified as one solution because it can potentially address two important social problems at the same time; providing high quality, fresh animal protein at a reasonable cost, while also providing livelihoods and employment to the local people, in particular, to those at the poorer end of the social spectrum. Since fish culture has not been a traditional practice in PICTs, the industry is however new and its development has been slow because skills, infrastructure and experience need to be developed from a very limited starting base. Having said this, it is appropriate to recognise that there has been considerable investment made by some governments (notably in Papua New Guinea and Fiji) and also by some international donor agencies and NGOs) (e.g., Australian Centre for international Agricultural Research-ACIAR, Japan International Cooperation Agency–JICA, and Secretariat of the Pacific Community-SPC) to assist the development of aquaculture there. The problem has been, however, that most aquaculture ventures overtime have either failed or the few that have survived produce products that are either beyond the purchasing power of most local people (marine prawns) or that are exported to wealthy markets overseas or that provide luxury items for local tourist industries (e.g., Pearl oysters). While development of high value export culture industries may appear at first sight to be an indirect way of increasing revenue for local development, the impacts of these industries on the local people can often be marginal because they generally employ only a relatively small number of low paid workers (often casual) and in most cases, the companies are not owned by the local people and profits may be repatriated to home countries. The question then is, why have so many fish farming ventures in the western Pacific that have been directed at assisting local development failed even after considerable financial and technical assistance has been provided?

The answer(s) to this question are likely to be quite complex and in many situations will be case-specific. The authors here have been engaged in research directed at assisting

aquaculture industry development in the western Pacific, both directly in long-term positions in national fisheries agencies and via engagement in Australian research programmes since the early 1990s and we believe that we have developed some knowledge of this issue and can potentially provide some insights that may help to explain this apparent dilemma. In addition, we also believe that we can offer some suggestions that could contribute to better outcomes in the future. As a general observation, we believe that major factors which have contributed to many of the failures of fish farming attempts in the western Pacific often relate to choice of culture species, level of culture intensification attempted and how well the farming system fits into the general life-style and social cultural practices across the region. Our main contention is that fish culture systems that have failed in the region were developed without careful consideration of one, and in many cases, all of the issues identified above. The choice of the species, culture system and intensity of productions practiced have, in our opinion, often been based primarily on observations about what has worked elsewhere (particularly in southeast Asia and some western nations) and the attempt has been to simply transfer similar systems to the Pacific and assume that they will also work there. The following sections will attempt to expand on this idea and provide some evidence where it is available to support the contention.

A diverse array of aquatic species have been trialled, or are currently being trialled in aquaculture in the western Pacific; these include marine prawns, sandfish, milkfish, pearl oysters, marine algae, giant clam, trochus, some carp species, tilapia and giant freshwater prawn. Most have failed or are failing currently. Other species have also been suggested as potential candidates for the region, including some indigenous marine tropical predatory fishes (e.g. groupers and snappers) and non-indigenous marine/ freshwater fish (barramundi), mudcrabs, and even tropical marine lobsters, but the experience has so far clearly shown that the choice of the species for culture by the locals is critical. To date, the only real success story for the local farmers has been the culture of Nile tilapia with freshwater prawns which begin to show some promises as

well, at least in Fiji. The best results have been obtained with pond culture of Nile tilapia, an omnivore that can grow well on a variety of diets, including feeding on plankton in simple fertilised ponds (addition of animal manure or organic fertilisers) and also on various farm by-products and simple artificial feeds. While culture of marine prawns (*L. vannamei*) has been successful in New Caledonia, this is an example of a local industry developed, subsidised and maintained by support directly from the French government with little engagement to date from indigenous farmers. Thus, the success stories for the region are really limited to a single (or perhaps two species). Our view is that the culture system employed in Tilapia farming, intensity of production used and general culture requirements (production cycle, intensity of management required, human and physical resources required to make it work) are such that they are compatible with the local farming practices and engagement in the region, while species that have failed are generally not.

Tilapia produced in ponds, as an economic crop in particular, have adapted well to the local farming systems and practices as well as social and cultural traditions in most PICTs, where they have been trialled. Where tilapia farming was not successful in the region in the past, this has often been attributed to production of 'small-sized' fish due to precocial spawning which results in large numbers of fry swamping ponds and stunted populations. The problem has been addressed in some places, following improvements to husbandry and replacement of poor performing stocks with an improved Nile tilapia strain (Genetically Improved farmed tilapia - GIFT). Tilapia culture in Fiji, PNG, Vanuatu, Samoa etc. are now exclusively based on GIFT. Although early maturation of tilapia in ponds remains a problem in some places, in actual practice scarcity of productive fingerlings has, somewhat strangely also been a major constraint, particularly in small-scale rural farming. Mass production of fingerlings for stocking ponds cannot yet be considered as a well-developed approach that is routinely practiced by rural farmers. Methods of broodstock production, selection and rearing, design and preparation of

spawning ponds and tanks, fry rearing and nursery management will also need additional attention across the region.

Tilapia, while amenable to high input, high resource intensive farming systems, can also be very productive in relatively low input (simple pond fertilisation systems), low resource (feed diversity, ration and quality), low technology systems and can therefore be easily adopted by local farmers with limited land (pond area), culture expertise and experience. Given that the production cycle is also relatively short, i.e. varies from only 3 to 6 months on average, farmers can receive a return from their limited investment rapidly where alternate sources of income are often scarce. Small farms (even consisting of one or a few small ponds) can produce 3 to 4 crops of fish per year and thus provide a regular source of income, while providing fresh animal protein, income and employment for family members. In addition, because tilapia are quite hardy fish, they will survive in limited water volumes at quite high densities in relatively low oxygen levels for a considerable time and hence can be transported and sold live at local markets to maximise prices for 'fresh fish', a practice that may require refrigeration if the product had been sold dead. In contrast, many alternative aquatic species that have failed in culture in PICTs require very different management and culture practices that include much higher resource inputs, longer production cycles, more intense culture stock management, expensive diets and/or large production areas for them to be viable culture options.

While the experience in Asian countries suggests that fish farming can be introduced easily into communities and is successful, where land as well as access to water resources are vested in the individual, in the western Pacific countries in contrast, land ownership, use and leasing systems are controlled by an extended family, clan or communal group that varies according to local custom and to conditions within various indigenous groups. Clans and clan members are bound by traditional obligations that are paramount. Under these systems, land and resources are usually inherited and are commonly based on a founding lineage and its descendants. As such, any community member seeking permission to build a small fishpond would usually need approval from the clan, but use

of large production areas is usually not favoured because all members of the communal group have access rights as well as the rights to a share in any product produced from this land. Therefore, commercial fish farming that requires relatively large land areas for an extended period of time is unlikely to be compatible with community norm practices, while small tilapia ponds are not generally viewed as a business undertaking, but as an activity comparable with taro or cassava farming on which the welfare of a household depends. Some of the 'fish' produced will probably also be shared with other members of the community or clan.

While freshwater prawns (*Macrobrachium rosenbergii*) are not yet widely farmed in the western Pacific and are yet to be identified as a priority species in the development plans of most PICTs, like Tilapia, freshwater prawn farming has been showing a considerable promise where it has been trialled (e.g. in Fiji). This is because recent experimental and commercial trials have identified a better performing strain (from Vietnam) for the local industry that has yielded encouraging results allowing many indigenous tilapia farmers to convert to prawn culture since requirements are similar to those identified above for Tilapia. In general, freshwater prawns can be grown to a marketable size in a relatively short period of time (production cycles are similar to tilapia), and production intensity can be relatively low as are feed costs and required expertise. Prawns also grow well in relatively small ponds and their market demand is very high with a value about 4-5 times higher than for tilapia, so returns to farmers are very favourable. In Fiji, we note that many farmers who have learned their aquaculture skills farming Tilapia often make rapid transition to freshwater prawn culture that produces higher returns. Freshwater prawns are also relatively hardy and can grow well even in comparatively poor pond conditions, giving reasonable yields. This species, like Tilapia, is also omnivorous and is therefore complementary in its feeding habits to Nile tilapia. A potential disadvantage compared with tilapia, however, is the hatchery cycle that is more complex than that for tilapia, because brackishwater and specialised live diets are required for larval metamorphosis to be completed successfully, and this adds a cost and complexity to the system. It has

been demonstrated, however, that PLs can be produced routinely across the year in the tropical/subtropical conditions, even in simple backyard hatchery systems which is an advantage over many alternative species (in particular marine predatory fish and some mollusc species).

Even while scarcity of quality fingerlings and PLs remain major constraints on development of tilapia and freshwater prawn culture in the western Pacific respectively, only standard methods of producing marketable fish and prawns have been developed to a level that matches the simplicity with which locals produce their crops, such as cassava and taro. Once the main constraint on 'seed' supply is overcome, it will be necessary to develop more efficient production systems, including improving pond management measures. Thus, our considered opinion is that the choice of culture species, the production system employed and the intensity of management practiced are all key factors that determine why certain culture species have been farmed successfully by indigenous populations (notably tilapia), while others have been relatively unsuccessful or failed in culture in the western Pacific. We strongly believe that these issues should be considered before new culture species are considered for development in culture across the region in particular, where the primary focus is on improving food security and nutrition for local people while increasing average family livelihoods and employment opportunities for poorer sectors of the community.

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Professor Mather is an academic with expertise in the fields of population genetics and ecology and has research interests that focus on diversity of wild and cultured aquatic species and more recently he has focussed his research in the fields of aquaculture genetics and genomics. He has developed both a national and international research profile in the Asia-Pacific region and engaged in research directed at characterisation of wild and farmed aquatic genetic resources, optimising their productivity in culture, development of improved culture lines and identification of key genes and mutations that affect variation in important production traits. As part of this work, he has developed collaborative research partnerships and networks that engage international governmental agencies and academic institutions working in the fisheries and aquaculture sector in the Asian-Pacific region and south Asia notably in Malaysia, Vietnam, India, and Fiji. This has led to many invitations to present the outcomes of his research group's work at international scientific meetings, to chair national and international sessions at scientific conferences and to provide advice to government agencies, universities and NGOs. He is also a member of the international advisory boards of a number of university science faculties in Malaysia.

Professor Mather has published more than 130 scientific papers, book chapters, and reviews and his work is widely cited. He is a member of the editorial boards of a number of international scientific journals and has been an advisor for over 40 higher degree students from more than 12 countries.

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The views expressed in this article are those of the author and do not necessarily represent the views of, and should not be attributed to, the Pertanika Journal or the Pertanika Editorial Board.

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Short Communication

Ethnic Differences in the Prevalence, Clinical Outcome and *cag* Pathogenicity Island (*cag*PAI) Virulence Gene Profiles of *Helicobacter pylori* Strains from Malaysia

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ABSTRACT

Different *Helicobacter pylori* genes may be well conserved within different ethnic groups and could give rise to different clinical outcomes. In this study, we demonstrated a low prevalence of *H. pylori* infection (19.2%) which is in concordance with the current trend demostrated locally and abroad. The Indians had the highest prevalence of *H. pylori* infection among other ethnic groups (Malays= 8.6 %, Chinese= 24.3 %, Indians= 33.9%). *cagM* and *cagT* were the most predominant genes found (63.4% for each), followed by *cagA* (62.2 %), *cagE* (48.2%), *cag6-7* (46.3%), *cag10* (42.1%), *cag13* (4.9%) and *IS605* (3.7%). No significant association was found between *H. pylori* infection and *H. pylori* genes with ethnic groups or clinical outcomes. Indians who had a combination of *cagA/E/M* genes of *H. pylori* were likely to be associated with 21-time of having non-ulcer dyspepsia (NUD) than peptic ulcer disease (PUD). Therefore, these genes may serve as useful markers in predicting the clinical presentation of a *H. pylori* infection among

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Indians in our studied population. Hence, this preliminary data might explain why Indians have a low prevalence of gastric cancer and peptic ulcer disease despite having persistently high prevalence of *H. pylori* infection for many decades ("Indian enigma") in Malaysian patients.

Keywords: *Helicobacter pylori*, cag pathogenicity island, virulence genes, peptic ulcer diseases, non-peptic ulcer dyspepsia, ethnicity, Malaysia

INTRODUCTION

Helicobacter pylori is present in more than 50% of the world's population (Peterson, 1992). This unique bacterial species is extremely diverse in its genomic structures. This panmicticism is believed to be important in colonization and infection in the human gastro-duodenal system. A variety of diseases including gastritis, gastric ulcers (GU), duodenal ulcers (DU) and gastric cancer have been attributed to *H. pylori*. The prevalence rates of *H. pylori* vary between populations or sub-populations (ethnic groups) within the same geographic locations (Suerbaum & Michetti, 2002). Data from endoscopic surveys and sero-prevalence studies amongst the three Malaysian ethnic groups revealed that Indians have the highest prevalence of *H. pylori* infection (68.9% -75.0%) compared to Chinese (45.0% - 60.6%), while Malays consistently have the lowest prevalence rate (8.0% - 43.3%). Ironically, in terms of the severity of disease caused by *H. pylori*, gastric cancer occurs more frequently in Chinese (68.0%) rather than Indians (16.5%) and Malays (15.5%)(Haron *et al.*, 1994; Kang *et al.*, 1990). In addition, it has been documented that peptic ulcer disease (PUD) is rather low among Indians compared to Chinese (Owen *et al.*, 2001; Goh, 1997). This paradox which is known as the "Indian enigma" (high prevalence of *H. pylori* but low percentage of gastric cancer and PUD) may be explained by differential virulence of the *H. pylori* strains or different susceptibility genotypes in the Indian ethnic group. *cag*PAI, a 40-kb region of first chromosomal DNA is the major genetic determinant of *H. pylori* virulence that consists of 27 genes and it is divided into two segments, *cag* I and *cag* II, by an insertion sequence known as *IS605* (Censini *et al.*, 1996). There is now increasing evidence for the existence of several novel bacterial pathogenicity markers on the *cag* (cytotoxin-associated gene) pathogenicity island (*cag*PAI) that may play important roles in the disease invoking potential of *H. pylori* (Mattar *et al.*, 2007; Van Doorn *et al.*, 1998). Meanwhile, the overall genetic variability of *H. pylori* may also be held responsible for the great diversity of clinical outcomes (Proença-Módena *et al.*, 2007; Maeda *et al.*, 1999). However, this has not been well explored locally in Malaysia and detailed data is lacking. Therefore, we conducted a study to investigate the role of several selected genes in the *cag*PAI (*cagA*, *cagE*, *cagM*, *cagT* and *cag6-7*, *cag10*, *cag13* represented the *cag* I and *cag* II segments, respectively and *IS605*) versus ethnicity and clinical outcomes. To our knowledge, this is the largest set of *cag*PAI genes that has

been studied so far in *H. pylori* from clinical isolates in Malaysia.

MATERIALS AND METHODS

A total of 855 gastric biopsies were obtained from dyspeptic patients who had undergone endoscopy from May 2004 to May 2007 in Universiti Kebangsaan Malaysia Medical Center. These patients were enrolled by a purposive sampling. Selection of patients and study protocols were followed according to a previous local study (Ramelah *et al.*, 2005). The study protocol was approved by the Medical Ethics Committee of Universiti Kebangsaan Malaysia (FF-075-2003). *H . pylori* culture was performed as previously described with modifications (Proença-Módena *et al.*, 2007). Briefly, specimens for culture were immediately transported to the laboratory in Stuart transport medium (Oxoid, UK). Biopsies were sub-cultured onto Columbia agar base medium (Oxoid Ltd., Basingstoke, Hampshire, England) containing Dent's supplement and 7% lysed horse blood. The cultures were incubated under micro-aerophilic conditions (5–6% O_2, 8–10% CO_2, 80–85% N_2, and a relative humidity of at least 95%) at 37°C for a minimum period of 5 days. The bacteria were identified as *H. pylori* based on the typical Gram stain morphology and biochemical tests such as urease, oxidase and catalase reactions. A pool of bacterial colonies obtained from each single plate was used for DNA extraction. Two *H. pylori* reference strains: American type culture collection (ATCC) 700824 (strain J99) and ATCC 43256 were used in this study. Chromosomal DNA of each strain was prepared using the High Pure PCR Template Preparation kit (Roche, Mannheim, Germany) in accordance with the manufacturer's instructions.

PCR analysis for *cag*PAI was performed to amplify *cagA*, *cagE*, *cagM*, *cagT*, *cag6-7*, *cag10*, *cag13* and *IS605* genes. The primers and PCR conditions used for each gene have been reported previously by Maeda *et al*. (1999). Briefly, the PCR was performed in a total reaction volume of 25µl containing 2.5µl 10 X PCR buffer, 1µl of each primer (each at 10pmol/µl), 2 mM $MgCl_2$; 200µM of dNTP, 5 U of super Taq (Super Taq DNA Polymerase Mbiotech) and 1µL (10ng) of genomic DNA from the culture lysates. DNA extracts of *H. pylori* ATCC 700824 and *H. pylori* ATCC 43256 were used as positive controls while negative controls (without DNA) were added to each PCR run. PCR products were visualized by electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and examined under UV illumination. Standard 1-kb DNA ladders (Fermentas Life Science, Hanover, MD) were used as molecular size markers.

Chi-squared and Fisher's exact tests were used to determine the difference in the distribution of *H. pylori* genes and the correlation between genes, clinical disease and ethnicity. p values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In this study, we reported a lower overall prevalence of *H. pylori* amongst our studied population (19.2%; 164 out of 855) compared

to 49% and 22% by Goh *et al*. (1997) and Mahadeva *et al*. (2005), respectively. This is not surprising as high standard of living conditions and improved personal hygiene could contribute to major decline of *H. pylori* infection in Asian populations (Tan & Goh, 2008). Interestingly, our finding corroborates data from a study conducted in a different locality, i.e. the north eastern part of Peninsular Malaysia which was 19.3% (Raj *et al.*, 2001). With regard to ethnic differences, a similar pattern was observed; Indians had the highest prevalence rate of *H. pylori* infection (33.9%; 42/124) compared to Chinese (24.3%; 98/404) and Malays (8.6%; 28/327) (data not shown). This is very much consistent with previous local findings as well (Mahadeva *et al.*, 2005; Goh *et al.*, 1997; Kang *et al.*, 1990) and could be explained by the role of genetic heterogeneity in susceptible hosts or other co-factors that could possibly account for the inter-ethnic variations (Alm & Trust, 1999).

For instance, *cagA* gene in *cag*PAI region of *H. pylori* strains was responsible for more severe pathological changes and clinical outcomes in Western countries (Crabtree *et al.*, 1993; Xiang *et al.*, 1993). Surprisingly, similar correlations were not found in some Asian countries (Zheng *et al.*, 2000; Maeda *et al*., 1998). *cagA* gene was reported to be insufficient in inducing some disease processes in *H. pylori* infection in China (Hu *et al.*, 1995). Tan *et al*. (2005) reported 84% of their patients who had *cagA* gene were not associated with clinical outcomes. In addition, no significant correlation was observed in the prevalence of this gene among the Malays (76.6%), Chinese (86.4%) and Indians (86.8%). In our previous report, we could not reveal the association between either the *cagA* or 3' end region of *cagA* gene with gastroduodenal diseases. However, Chinese (96%) in whom have been known to have a higher risk of peptic ulcer disease and gastric cancer had the highest prevalence of cagA subtype A strains compared to Malays (72%) and Indians (69%). The difference was statistically significant ($p< 0.0005$) (Ramelah *et al.*, 2005). In a recent work by our group, Eastern CagA strains were significantly predominance among the Chinese (82.9%) compared to Malays (11.4%) and Indians (1.4%). However, no association could be made between Eastern CagA strains and the clinical outcome (Mohamed *et al.*, 2009). It has been known that Eastern CagA strains confer higher tyrosine CagA phosphorylation activity than Western strains, which is related to their pathogenic potentials (Higashi *et al*., 2002a). As previous works were only focussing on cagA and its variants, and could not demonstrate the association with clinical outcome , we hypothesized that other potential virulence genes on *cag*PAI could also be involved in *H. pylori* infection among our multi-ethnic population. Maeda *et al*. (1999) evaluated 15 genes on the *cag*PAI and concluded that *cagA* gene could not be used as a virulence marker in Japanese *H. pylori* strains. Recently, a systematic mutagenesis study of individual genes on *cag*PAI by Fischer *et al.* (2001)

confirmed that 17 and 14 out of 27 genes were involved in *H. pylori* pathogenesis by encoding a type IV secretion system for the translocation of *cag* toxicity protein A (CagA) and for the full induction of interleukin 8 (IL-8). Among the eight genes, *H. pylori cagM* and *cagT* genes were both predominantly found in our study population (63.4% for each) (TABLE 1). To the best of our knowledge, this is the first report on the prevalence of *cagM* and *cagT* genes in Malaysia; nonetheless, we could not reveal any significant association in the prevalence of *cagM* and *cagT* genes as well as other *cag*PAI genes with clinical outcome (TABLE 2). Goh *et al.* (2007) suggested that other virulent genes could account for the paradoxical findings of *H. pylori* infection

TABLE 1
Distribution of the presence of *cag*PAI genes of *Helicobacter pylori* (*cagA*, *cagE*, *cagM*, *cagT*, *cag6-7*, *cag10*, *cag13* and *IS605*) detected in 164 patients in relation to their ethnicity

Gene	Ethnic group			Total
	Malay n = 28 (%)	Chinese n = 94 (%)	Indian n = 42 (%)	
CagA (n=102)				
Positive	16 (57.1)	62 (66.0)	24 (57.1)	102 (62.2)
Negative	12 (42.9)	32 (34.0)	18 (42.9)	62 (37.8)
CagE (n=102)				
Positive	13 (46.4)	44 (46.8)	22 (52.4)	79 (48.2)
Negative	15 (53.6)	50 (53.2)	20 (47.6)	85 (51.8)
CagM (n=102)				
Positive	16 (57.1)	59 (62.8)	29 (69.0)	104 (63.4)
Negative	12 (42.9)	35 (37.2)	13 (31.0)	60 (36.6)
CagT (n=102)				
Positive	16 (57.1)	62 (66.0)	26 (61.9)	104 (63.4)
Negative	12 (42.9)	32 (34.0)	16 (38.1)	60 (36.6)
Cag6-7 (n=102)				
Positive	8 (28.6)	59 (62.8)[a]	9 (21.4)	76 (46.3)
Negative	20 (71.4)	35 (37.2)	33 (78.6)	88 (53.7)
Cag10 (n=102)				
Positive	12 (42.9)	38 (40.4)	19 (45.2)	69 (42.1)
Negative	16 (57.1)	56 (59.6)	23 (54.8)	95 (57.9)
Cag13 (n=102)				
Positive	2 (7.1)	3 (3.2)	3 (7.1)	8 (4.9)
Negative	26 (92.9)	91(96.8)	39 (92.9)	156 (95.1)
IS605 (n=102)				
Positive	0 (0.0)	3 (3.2)	3 (7.1)	6 (3.7)
Negative	28 (100.0)	91 (96.8)	39 (92.9)	158 (96.3)

[a]Chinese vs. Malay (Pearson Chi-square, 2 x 2 table; $\chi 2 = 10.189$, $p = 0.001$)
Chinese vs. Indian (Pearson Chi-square, 2 x 2 table; $\chi 2 = 19.842$, $p = 0.000$)

TABLE 2
Relationship between *Helicobacter pylori* genes (*cagA, cagE, cagM, cagT, cag6-7, cag10, cag13* and *IS605*) and clinical outcomes in 164 patients with *H. pylori* infection

Gene	PUD n=35 (%)	NUD n=129 (%)	Adjusted OR (95 % CI)	p value[a]
cagA (n=102)	19 (54.3)	83 (62.2)	0.658 (0.309-1.402)	0.327
cagE (n=79)	14(40.0)	65 (48.2)	0.656 (0.307-1.403)	0.341
cagM (n=104)	22 (62.9)	82 (63.6)	0.970 (0.447-2.103)	1.000
cagT (n=104)	21 (60.0)	83 (64.3)	0.831 (0.386-1.789)	0.694
cag6-7 (n=76)	17 (48.6)	59 (45.7)	1.121 (0.530-2.367)	0.849
cag10 (n=69)	16 (45.7)	53 (42.1)	1.208 (0.569-2.561)	0.623
cag13 (n=8)	2 (5.7)	6 (4.7)	1.242 (0.240-6.442)	0.679
IS605 (n=6)	1 (2.9)	5 (3.9)	0.157 (0.082-6.455)	1.000

Note: PUD = peptic ulcer disease; NUD = non-ulcer dyspepsia; OR = odds ratio; CI = confidence interval
[a]Fischer's Exact test; p value < 0.05 is considered significant

and clinical outcome in our multi-ethnic groups. They found that only 23.3% of *H. pylori*-positive Indians had gastric cancer compared to 82.3% and 60.5% in Malays and Chinese, respectively. Mohamed *et al*. (2009) reported Western CagA strains were significantly found in Indian patients (43.5%), which could probably explained lower incidence of gastric cancer or PUD in this ethnic group. In our study, when each ethnic group was analyzed, the association was only statistically significant in the proportion of positive *cagA/E/M* genes amongst Indians who had NUD than PUD ($p<0.047$) (TABLE 3). In addition, Indians with the combination of *cagA/E/M* genes were likely (twenty-one times) associated with NUD than PUD. These findings are rather surprising as the presence of these markers has been associated with high risk of developing PUD and bad clinical outcome (Mattar *et al.*, 2007; Censini *et al.*, 1996). *cagA* gene in particular was predominantly found in patients with DU in both European and Polynesian in Auckland (83% and 86%, respectively) (Campbell *et al.*, 1997). Meanwhile, *cagE* gene was proposed to be a more reliable virulence marker than *cagA* gene (Ikenoue *et al.*, 2001; Audibert *et al*., 2001; Maeda *et al*., 1999) but Tan *et al.* (2005) reported that Chinese (39.0%) had significantly lower prevalence rate of *cagE* gene than Malay (70.0%) and Indian (81.6%) patients which appears *cagE* gene is not a good marker. *cagE* and *cagM* genes are responsible for activating the transcription factor NF-κB, which mediates IL-8 secretion (Glocker *et al*., 1998). However , our findings corroborate data with a study conducted in Taiwan which revealed no association between the presence of *cagA*, *cagE* and *cagM* genes with the type of disease and/or the histological findings in their patients (Sheu *et al.*, 2002). Furthermore, the involvement of these genes and others in *H. pylori* pathogenesis has also been disputed (Hsu P-I, *et al*., 2002; Segal *et al*., 1999). In

TABLE 3
Relationship between *Helicobacter pylori* genes (*cagA, cagE, cagM, cagT, cag6-7* and *cag10*) and clinical outcomes in 42 Indian patients with *H. pylori* infection

Gene (s)	PUD n=7 (%)	NUD n=35 (%)	Adjusted OR (95 % CI)	p value
cagA (n=24)	1 (4.2)	23 (95.8)	0.087 (0.009-0.808)	0.031[a]
cagE (n=22)	1 (4.5)	21 (95.5)	0.111 (0.012-1.025)	0.041[b]
cagM (n=29)	2 (6.9)	27 (93.1)	0.119 (0.019-0.731)	0.021[c]
cagT (n=26)	3 (11.5)	23 (88.5)	0.391 (0.075-2.041)	0.397
cag6-7 (n=9)	1 (11.1)	8 (88.9)	0.562 (0.059-5.386)	1.000
cag10 (n=19)	1 (5.3)	18 (94.7)	0.157 (0.051-1.447)	0.105
cagA/E/M (n=21)	1 (4.8)	20 (95.2)	0.125 (0.014-1-151)	0.047[d]

Note: PUD = peptic ulcer disease; NUD = non-ulcer dyspepsia; OR = odds ratio; CI = confidence interval
Statistical analysis was not done for *cag13* and *IS605* genes as number of samples was too small
[a,b,c,d] by Fischer's exact tests; p values <0.05 are considered significant

addition, the prevalence of *cag6-7* gene in this study was significantly higher amongst the Chinese (62.8 %) compared to the Malays (28.6 %) (TABLE 1). The difference in the prevalence of *cag6-7* gene was highly statistically significant between Chinese and Indians (p=0.000). Interestingly, a study in Japan revealed that 93.1% (27/29) of cancer patients had *cag6-7* gene although the significance of this is still uncertain (Deguchi *et al.*, 2004), as this finding was not analyzed statistically.

Our study had several limitations. All *cag*PAI genes could not be chosen for the study due to budget constraints. The number of Indian patients was relatively small despite the significant findings observed in this ethnic group. However, the present study was conducted as a pilot study involving the largest number of *H. pylori* genes in different ethnic groups in Malaysia. Thus, large prospective or multi-centered studies are needed to investigate the pathogenic impact of *H. pylori* virulence genes amongst different ethnic groups in relation to its clinical relevance.

CONCLUSION

The decline in the prevalence of *H. pylori* infection has not only been observed in several developed countries but also in Malaysia. Detection of *cagA/E/M* genes will partially resolve the "Indian enigma": it might be that the presence of different *H. pylori* genes/genotypes that might explain why Indians have a lower risk of developing severe disease outcomes despite having the highest prevalence rate of *H. pylori* infection. This could have clinical implication when initiating anti-*H. pylori* therapy in the multi-ethnic population in Malaysia.

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Variations of Electrical Conductivity between Upstream and Downstream of Langat River, Malaysia: Its Significance as a Single Indicator of Water Quality Deterioration

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ABSTRACT

This paper investigated the potential of using a single chemical parameter namely electrical conductivity (EC) as an indicator of water quality pollution in relation to dissolved oxygen (DO) and suspended solids (SS) which are two of the parameters included in the calculation of Water Quality Index (WQI) in Malaysia based on current scenario. In this study, nine periodic samplings at eight sampling sites along a tropical river which included the polluted downstream and the unpolluted upstream of the Langat River, were conducted between March 1998 and January 1999. The consistent results for the nine month samples (negative and significant correlation between EC vs. DO, and positive and significant correlation between EC vs. SS), indicated that EC could be potentially used as a single chemical parameter to indicate the water quality of tropical rivers such as the Langat River of Malaysia. It is also recommended that EC should be included in the revised WQI in Malaysia in future, in order to better reflect the mineral-related pollution/composition and of the water samples.

Keywords: Electrical conductivity, Langat River, Malaysia

INTRODUCTION

Currently, the Malaysian Water Quality Index (WQI) is based on six water parameters (dissolved oxygen (DO), pH, biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), suspended solids (SS) and ammoniacal nitrogen (AN) (DOE, 2007; WEPA, 2012). This WQI with the chosen six parameters is an opinion-poll formula and this formulation was based on consultations by a panel of experts on the choice of parameters and on the weight to each parameter (DOE, 1985). Although WQI enables large amount of water quality data to be reduced to a single index value

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with its index score (Gupta *et al.*, 2003), there are limitations in the use of WQI and this has been clearly reviewed by Zainudin (2010). According to Zainuddin (2010), indexes by design contain less information than the raw data that they summarize. The major weakness of WQI is that it is not a comprehensive index to quantify all the water quality constituents.

A particular sampling site may be calculated as being a 'good' WQI score but the water quality could be contaminated / deteriorated by other water parameters/ contaminants which are not included in the calculation of the WQI which are only based on six water parameters. For instance, heavy metals such as Cd and Pb , which may be carcinogenic top living organisms (Kromhout *et al.*, 1985), are not included in the calculation of present Malaysian WQI. Subsequently, a good WQI score at a particular sampling site does not necessarily mean that water quality was always in an acceptable level. Hence, further check on the water quality parameters listed in the Interim National Water Quality Standards for Malaysia (INWQS) (WEPA, 2012) should be conducted.

The distribution and abundance of benthic macroinvertebrate of tropical rivers could be potentially influenced by a lot of biotic and abiotic factors. However, owing to limited time and facilities, some researchers limit the measurement of physico-chemicals to only some easy and measurable parameters that are highly dependent on the availability of the sampling devices. If we had only measured a few water quality parameters, can they still be used to indicate the quality of the river water?

The inclusion of electrical conductivity (EC) as one of the parameters in the calculation of WQI is always not found in the literature except for a few cases. Banoeng-Yakubo *et al*. (2009) included nine water parameters in the determination of WQI including EC and assigned three parameters (EC, $NO3^-$, and F^- as assigned the highest weight (value 5) because of their importance in the water quality assessment. According to Dojlido and Best (1993) and Welch *et al.* (1998), EC can be used as a measure of sulfate, bicarbonate, and chlorides of calcium, magnesium, and sodium therefore basically EC can be used as a measurement of the ability of an aqueous solution to carry an electrical current (Das *et al.,* 2006). Only Das *et al*. (2006) proposed the use of electrical conductivity as an indicator of pollution in shallow lakes but not in tropical rivers yet.

Earlier, Azlina *et al.* (2006) documented the anthropogenic impacts on the biodiversity of macrobenthic invertebrates and water quality between the upstream and downstream of the Langat River while Yap *et al.* (2005) reported on the nitrate levels in the Straits of Malacca. However, detailed study on a single parameter such as EC, as an indicator of water quality pollution, has not been discussed widely in the literature. This could be due to the fact that the more chemical parameters are included, the better and more conclusively could the data be interpreted. However, from the present

study, if all the other physico-chemicals are normalized, the use of the single EC could be a potential parameter for water pollution studies.

This is an interesting point if more sampling sites and regular samplings are included to study the above question. Therefore, the objective of this study is to find out the relationships between EC vs. dissolved oxygen (DO) and EC vs. suspended solids (SS) and to discuss the potential use of EC as a single indicator of water quality deterioration.

MATERIALS AND METHODS

Description of the study area

Samplings were conducted over nine months (March, April, May, June, July, August, October and November of 1998 and January 1999) at the Langat River Basin which is located in Selangor, Peninsular Malaysia (Fig.1). The water flows from the upstream area in Hulu Langat until Kuala Selangor at the downstream area where the Langat River becomes the main river in the basin which flows in a southwesterly direction and drains into the Straits of Malacca. The Langat

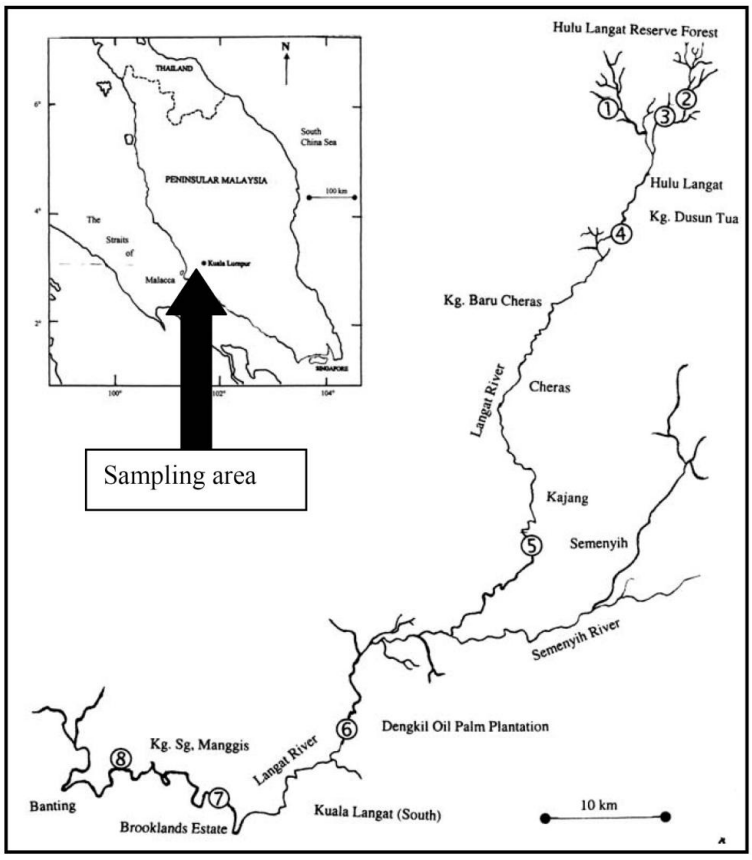

Fig.1: Sampling area along the Langat River, Peninsular Malaysia.

River Basin is located between 101°25' and 101°54' of the Greenwich meridian.

The upstream stations covered Stations 1-4 while the downstream covered Stations 5-8. Azlina *et al.* (2006) reported that that the upstream and downstream of the Langat River were clean and polluted parts of the river, respectively. The first station was located in the Chongkak River . This river is used for camping and picnicking especially during the weekends. The second station was located near the Orang Asli village in Hulu Langat. The third and fourth stations were located at the Lopo River and Kampung Batu 17 in Hulu Langat, respectively . The water was clean and clear and it was used by some villagers for washing and bathing. The common characteristics of stations 1-4 were the presence of cobbles and pebbles and sometimes sand and gravel. There was generally high vegetation coverage at the four upstream sampling stations.

The fifth station was located at Section 4 of the township of Bandar Baru Bangi. The sixth station was located at the Dengkil Oil Palm Plantation and the water could be heavily polluted since colour of the river water was brownish. The seventh station was located at the Brooklands Oil Palm Estate close to a paper factory. The sediment was of a muddy type. The eighth station was located at Telok Dato' near Banting. The river water was milky, smelly and it was likely to contain a lot of suspended matter that could be due to sewage pollution. The substrata of the downstream sampling sites comprised of muddy sediment.

Water Quality Measurement

Three replicates of the EC were obtained by using a YSI meter Model 33 and DO by a YSI oxygen meter Model 57. Suspended solids (SS) was calculated based on the standard method procedures (APHA, 1989).

Data Analysis

All the data were transformed by $\log_{10}(x+1)$. The data transformation was performed to stabilize the variance so that the data frequency distribution was close to the normal distribution (Zar, 1996). T-test and Spearman's correlation analysis (CA) was carried out based on the transformed data by using STATISTICA 99" Edition (version 5.5).

RESULTS AND DISCUSSION

From this study, there are two major points to support the use of EC as a single chemical indicator of water quality. Firstly, from Table 1, the polluted downstream of the Langat River (Azlina *et al*., 2006) had significantly ($P< 0.05$) higher EC values (154-301 µS/cm) and SS (200-1145 mg/L) and significantly ($P< 0.05$) lower DO concentrations (0.97-4.15 mg/L), than those in the clean upstream stations (EC: 29.0-46.0 µS/cm; SS: 4.50-25.0 mg/L and DO: 7.69-10.03 mg/L). Anthropogenic inputs at the downstream of Langat River could be attributed from sources including 1) *wastewater* from sewage treatment plants (**point source** pollutants), 2) *wastewater* from septic systems and drainfield on-site wastewater treatment and disposal systems (**point source** pollutants), 3) *urban runoff*

TABLE 1
The T-test results from comparisons of mean [Suspended solids and dissolved oxygen in mg/L, electrical conductivity in µS/cm] between upstream (stations 1-4) and downstream (stations 5-8) of the Langat River. N= 4.

Parameter	Periods	Upstream (st 1-4)	Downstream (st 5-8)	Significance level
Electrical conductivity	March 1998	36.4	178.8	P< 0.05
	April 1998	43.3	154.9	P < 0.001
	May 1998	41.0	186.5	P < 0.01
	June 1998	39.2	243.3	P < 0.01
	July 1998	42.4	247.8	P< 0.05
	August 1998	46.9	168.0	P> 0.05
	October 1998	37.0	301.3	P< 0.001
	November 1998	29.0	126.8	P> 0.05
	January1999	29.8	210.4	P< 0.05
Suspended solids	March 1998	4.50	231	P < 0.05
	April 1998	8.67	534	P < 0.05
	May 1998	24.9	270	P < 0.05
	June 1998	25.0	209	P < 0.05
	July 1998	8.92	200	P< 0.01
	August 1998	19.3	632	P< 0.001
	October 1998	5.67	132	P< 0.05
	November 1998	13.5	1145	P< 0.05
	January1999	18.0	132	P< 0.05
Dissolved oxygen	March 1998	7.98	3.89	P < 0.05
	April 1998	10.00	3.13	P < 0.0001
	May 1998	7.69	3.02	P < 0.01
	June 1998	10.03	3.42	P < 0.00
	July 1998	9.64	2.12	P< 0.001
	August 1998	8.19	4.15	P< 0.001
	October 1998	8.33	0.97	P< 0.001
	November 1998	8.40	2.54	P< 0.001
	January1999	7.85	3.97	P< 0.001

from roads and 4) *agricultural runoff* of water draining agricultural fields typically has extremely high levels of dissolved salts (Dojlido and Best, 1993). According to INWQS for Malaysia (DOE, 2006; WEPA, 2012), EC is classified as I (Water supply I - Practically no treatment necessary) and IIA (Water supply II – Conventional treatment required) for a value of 1000 µS/cm while 6000 µS/cm for Class IV (Irrigation). Therefore, the present ranges of EC values (29.0-301 µS/cm) are all classified as Class I.

When compared to other reported studies on EC, the present EC values (29.0-301 µS/cm) are lower than those reported by

Banoeng-Yakubo *et al*. (2009) for surface river waters in the Volta region (Ghana), EC as 787-806 µS/cm. However, their values are below the WHO standard for EC (1500 µS/cm) (Banoeng-Yakubo *et al*., 2009), which is considered to be unsuitable for agricultural use (Dojlido and Best, 1993). Zhang *et al*. (2012) reported that EC ranged from 43.9 to 2830 µS/cm and 96.8 to 2680 µS/cm for surface water and groundwater, respectively, in Songnen plain, Northeast China. Positively, they concluded that EC is also a good index to ion concentrations and for the assessment of water quality. The present EC values are also lower than that (875 µS/cm) the groundwaters in the Kathmandu Valley (Nepal) (Pant, 2011).

Secondly, from Table 2, EC values correlated negatively and significantly with DO ($R= -0.739$ to $R= -0.895$, $P< 0.001$) and correlated positively and significantly with SS ($R= 0.594$ to $R= 0.775$, $P< 0.001$). These correlation coefficients indicated that EC is a potential water quality indicator. From Fig.2, the comparison between EC and DO showed a negative pattern with each other. A higher EC would be followed by a decrease in the DO of the river water. The negative relationships between EC and DO were consistent in all the nine sampling periods. Therefore, this supports the use of EC as a single indicator of water quality of the river water.

The use of EC as a single indicator of water quality of the river water is also well supported by some literature. For instance, EC is one of the important water parameters in the determination of surface water quality such as in Jakara River (Nigeria) (Mustapha *et al*., 2012). Mustapha *et al*. (2012) also found that positive correlations between EC and SS. Moreover, Ouyang *et al*. (2006) reported that the lower St. Johns River, located in northeast Florida (USA), with contaminants such as nutrients, hydrocarbons, pesticides, and heavy metals, had significant correlations between EC vs. DO as $R= -0.91$ in spring, $R= -0.80$ in summer, $R= -0.40$ in fall and $R= -0.90$ in winter. They concluded that EC was always one of the most important parameters in contributing to water quality variations for all four seasons. This clearly showed that mineral and nutrient related EC was negatively affected by the physical parameter DO and thus in support of the present findings on the tropical Langat River. The presence of dissolved ionic substances could potentially increase the EC value in water (Pant, 2011). The increased ionic concentrations are practically caused by increments of different ionic substances including organic matters containing ionic charges, metallic ions, nitrate, and ammonium ions (Dzwairo *et al*., 2006).

Eh Rak *et al*. (2010) reported that water discharge at Mengkibol River showed negative relationships with EC and DO. They suggested that a high water discharge will not influence the EC and DO values of the river water. However, they found a positive relationship between river discharges and turbidity, where turbidity levels of the water were influenced by river discharges of the rivers. Therefore, if EC is to be used as a single indicator of water

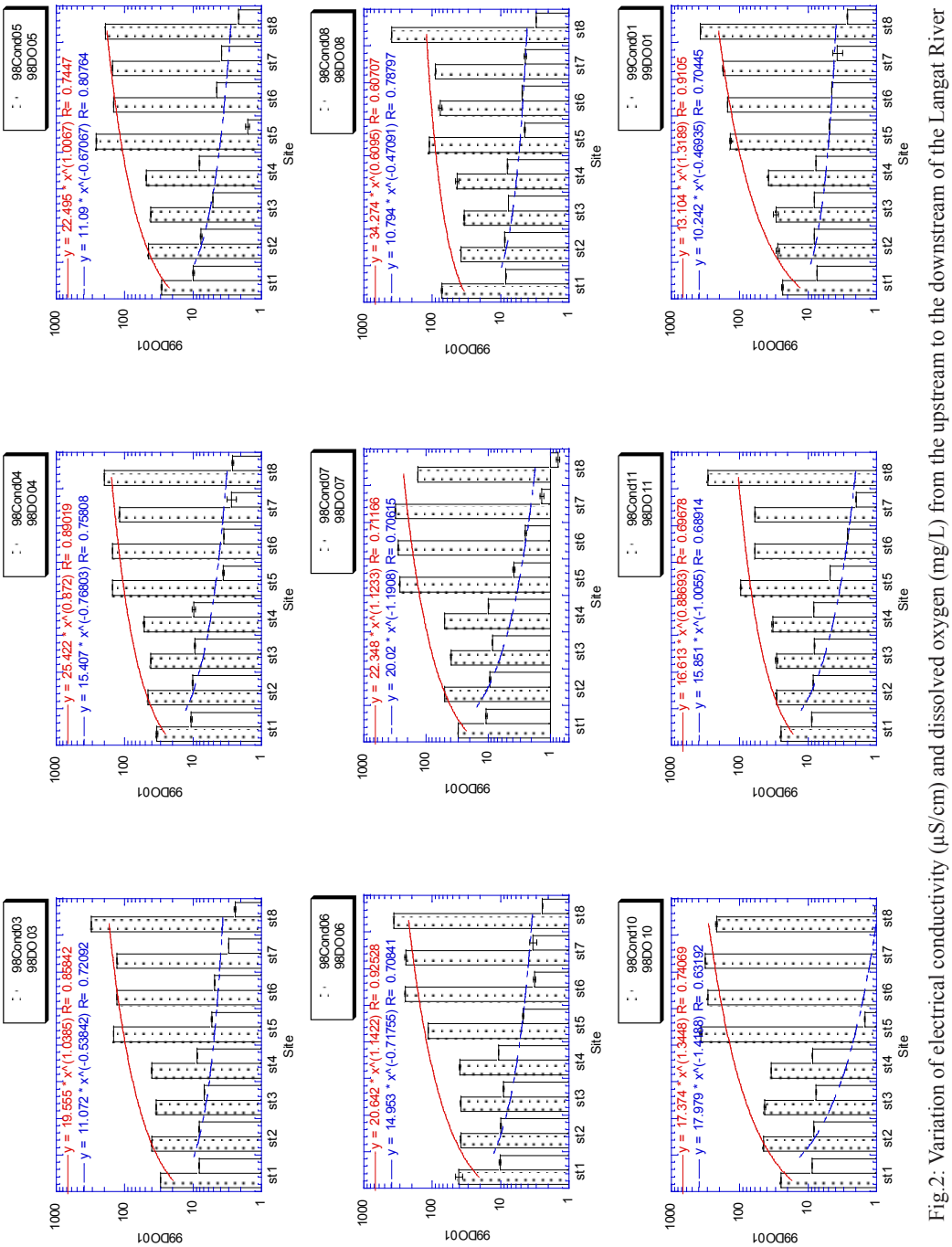

Fig.2: Variation of electrical conductivity (µS/cm) and dissolved oxygen (mg/L) from the upstream to the downstream of the Langat River

TABLE 2
Spearman's correlation coefficients based on log10 (mean +1) of suspended solids (SS), dissolved oxygen (DO) and electrical conductivity (EC) in the water samples collected from March 1998 to January 1999, at the Langat River.

March 1998	N= 24	DO	SS
	EC	-0.739***	0.632***
	DO	1.000	-0.809***
April 1998	N= 23	DO	SS
	EC	-0.810***	0.715***
	DO	1.000	-0.752***
May 1998	N= 22	DO	SS
	EC	-0.895***	0.775***
	DO	1.000	-0.655***
June 1998	N= 24	DO	SS
	EC	-0.817***	0.673***
	NDO	1.000	-0.604***
July 1998	N= 24	DO	SS
	EC	-0.773***	0.772***
	DO	1.000	-0.911***
August 1998	N= 24	DO	SS
	EC	-0.791***	0.594***
	DO	1.000	-0.762***
October 1998	N= 24	DO	SS
	EC	-0.829***	0.728***
	DO	1.000	-0.831***
November 1998	N= 24	DO	SS
	EC	-0.878***	0.730***
	DO	1.000	-0.860***
January 1999	N= 24	DO	SS
	EC	-0.845***	0.693***
	DO	1.000	-0.635***

Note: Level of significance: ***P< 0.001.

quality, the discharge is not contaminated by mineral and nutrient related pollutants.

The use of EC as an indicator of water quality deterioration in general was also reported in the literature. Das *et al*. (2006) reported that water quality is closely linked to many physical and chemical aspects of a lake. They found that EC was a thermophysical property of lake water which has a strong interrelationship with pollution levels. Therefore, measured values of EC indirectly indicate the level of pollution in lake waters. Moreover, measurement of EC is much easier than direct measurement of total dissolved solids (TDS), thus easing for pollution monitoring of river waters.

Owing to SS can be attributed to industrial wastes, wastewater discharges,

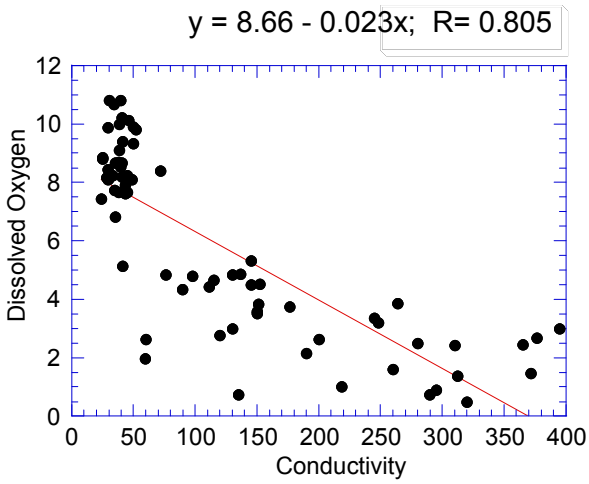

Fig.3: Overall relationship between electrical conductivity (µS/cm) and dissolved oxygen (mg/L) from the upstream to the downstream of the Langat River.

erosion from urban runoff and agricultural land (Liebl, 2009), the positive relationship between EC and SS found from this study could be related to all the above anthropogenic wastes and activities. Since SS is one of the major pollutants in Malaysia with the sources for SS were mostly earthworks and land clearing activities (WEPA, 2012), there is a reason to use EC as a single and effective indicator of mineral-related parameter in Malaysian rivers, because EC is significantly and positively correlated with SS.

CONCLUSION

Since it is much easier and faster to measure EC than to measure all the anions and cations, highly positively correlated with SS and negatively correlated with DO, the use of EC as a single indicator of water pollution in tropical rivers is proposed. The present finding has provided evidence that EC should be included in the revised Water Quality Index in Malaysia in future, in order to better reflect the mineral-related pollution/composition and of the water samples.

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Accumulation of Heavy Metals and Antioxidative Enzymes of *Centella asiatica* in Relation to Metals of the Soils

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ABSTRACT

Antioxidative enzymes have been claimed as being beneficial for enhancing fitness and for preventing disorders in plants due to the production of reactive oxygen species (ROS) caused by heavy metal stresses. *Centella asiatica* plants and soil sediments from nine sampling sites were collected between May and June of 2010. They were tested for their Cd, Cu, Fe, Ni, Pb and Zn contents. The plants were also analyzed for the activities of antioxidative enzymes namely superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX). This study revealed positive and significant ($P<0.05$) correlations between plants (leaves and roots) and soils for Cd, Zn, Ni and Fe content. It also showed that the significant correlations between Cd, Fe and Pb accumulations did not seem to be a factor for the increase in antioxidative enzyme activities due to their low concentrations in the plant; but the accumulated Cu, Zn and Ni levels were significantly ($P<0.05$) correlated with increases in antioxidative activities.

Keywords: *Centella asiatica,* Heavy metals, antioxidative enzymes

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INTRODUCTION

Recently, toxicities caused by heavy metals have become a major issue to the public due to their capabilities to be transferred and accumulated in plants, animals and humans (Garcia-Rico *et al*., 2007). Moreover, there are concerns on the long term persistence of these metals which might cause hazardous health issues to humans (Gisbert *et al*., 2003;

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Stankovic *et al*., 2012). There are several factors influencing the concentrations of these metals in medicinal plants such as the species of the plant, climate, air pollution and other environmental factors (Sovljanski *et al*., 1989).

High concentrations of these metals might cause growth inhibition and even death of the plants (Schutzendubel *et al*., 2001). Heavy metals are involved in many steps in the production of deleterious free radicals/reactive oxygen species (ROS) such as singlet oxygen ($O_{2)}$, superoxide radical ($O^{-}_{2)}$, hydrogen peroxide (H_2O_2), hydroxyl ion ($OH^{-)}$ and free hydroxyl radical (OH) (Halliwell and Gutteridge, 1984). ROS is highly reactive and harmful, but it can also act as a signaling molecule and as an inducer in the antioxidative enzyme system of plants for protecting themselves (Foyer *et al*., 2009; Parra-Lobato *et al*., 2009). The synchronous action of superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), ascorbate peroxidase (APX), dehydroascorbate reductases (GDOR) and glutathione reductase (GR) is part of the system that protects plants against ROS in various compartments of the plant cell (Singh *et al*., 2006).

In this study, we focused on *Centella asiatica* (family: Umbelliferae) which is widely used in folk medicine for hundreds of years to treat a wide range of illnesses (Brinkhaus *et al*., 2000). It is also listed as one of the useful medicinal herbs by WHO (1999). In Malaysia, it is used to rapidly heal small wounds, chaps and scratches, surgical wounds and so on (Ong *et al*., 2011). Of the entire *Centella* genus, only the *asiatica* species is found in commercial drugs today (Zainol *et al*., 2003). Much of the work related to heavy metal biomonitoring featuring plants had been done around the world for example those by Aksoy and Demirezen (2006), Baycu (2003) and Yilmaz *et al.* (2006). Thus , *Centella asiatica* can be chosen as an ideal biomonitor due to it being sedentary, abundant, easy to identify, available for sampling throughout the year, large enough to provide sufficient tissues for (individual) analysis, resistance to handling stress caused by laboratory studies of metal kinetics and/or field transplantations, tolerant to exposure to environmental variations in physico-chemical parameters and most important of all its capability as a net accumulator of the metal with a simple correlation between metal concentration in tissues over a short time period (Rainbow and Phillips, 1993; Wittig, 1993).

In addition, the effects of geochemical fraction of soils on metal concentrations in *C. asiatica* in Malaysia are still unclear. There is very little information available on antioxidative activity in medicinal herbs caused by heavy metals in the recent literature. Therefore, the objective of this study was to determine the relationships between selected metal concentrations and antioxidative activities of *C. asiatica* with the level of these metals in the soil.

MATERIALS AND METHODS

Sample collection

Centella asiatica was collected from nine sampling sites in Peninsular Malaysia

(Fig.1) between May and June, 2010. The plant samples collected from the wild were from Permatang Pauh (PPauh), Karangan, Kluang, Butterworth, Universiti Putra Malaysia (UPM) in Serdang, Kapar, Seremban, Kampung Simpang Renggam (KSR) and Pontian (Table 1). Three replicates were collected per sampling site and around 100 g of fresh weight of samples were collected for each replicate. During collection, surface sediments (top 3-5cm) were also collected to determine the levels of heavy metals. The soil sediment was collected by using a plastic scoop after the litter had been removed and three replicates were collected for each sampling site.

Determination of heavy metal concentrations

Three replicate determinations were done for each sampling site. For determination of metal concentrations, plants were separated into two different parts namely leaves and roots. The separated plant tissues, and the sediments, were then dried in an oven for 72 hours at 60 ºC to constant dry weights. About 0.5g of dried plant tissue parts were placed in a digestion tube and 10 ml of concentrated nitric acid (AnalaR grade, BDH 69%) were added to digest the plant tissues. Then, the digestion tubes were placed in a hot block digester at 40°C for 1 hour and then at 140°C for at least 3 hours

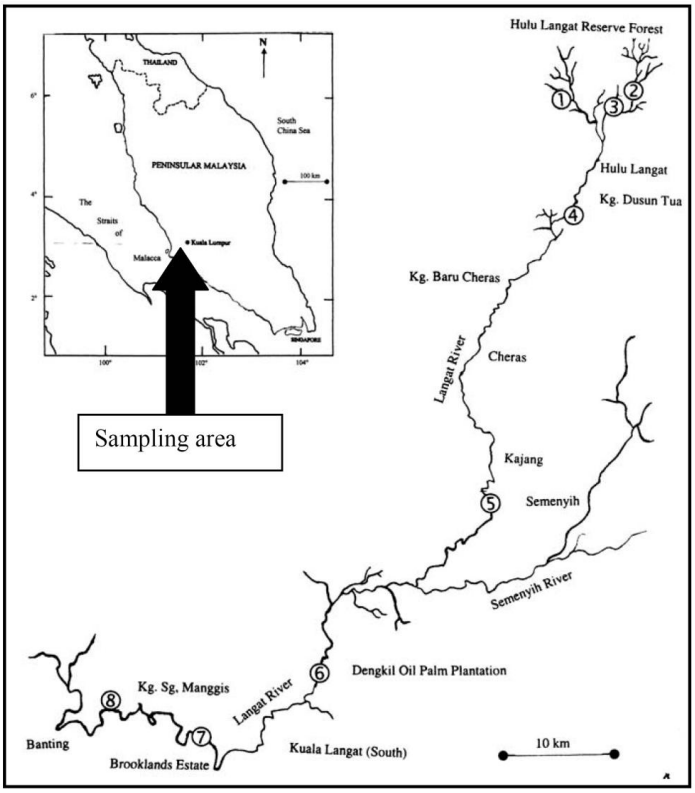

Fig.1: Map showing the sampling sites for *Centella asiatica* in Peninsular Malaysia.

TABLE1
Sampling sites, sampling date and site description of *Centella asiatica*

No	Sampling site	Sampling date	Site description
1.	Pontian, Johore	9 May 10	Near a plant agriculture area.
2.	Kampung Simpang Renggam (KSR), Johore	9 May 10	Near a housing area.
3.	Seremban, Sembilan	4 June 10	Near shop lots and road sides.
4.	Kapar, Selangor	5 June 10	Small scale housing area.
5.	Universiti Putra Malaysia (UPM), Selangor	5 June 10	Near agriculture area.
6.	Butterworth, Penang	12 June 10	Near an industrial area and highway.
7.	Kluang, Johore	19 June 10	Near paddy fields.
8.	Karangan, Kedah	12 June 10	Near oil palm plantations.
9.	Permatang Pauh (PPauh), Penang	12 June 10	Near a housing area and highway.

(Yap *et al*., 2003). After that, the digested samples were left to cool and were topped up (diluted) to 40 ml with double de-ionized water. Lastly, the solution was filtered through a Whatman No. 1 filter paper into an acid-washed (Yap *et al*., 2003) pill box and stored at room temperature until required for metal concentration determinations. Soil sediments were sieved through a 63 µm mesh followed by direct aqua-regia digestion and the sequential extraction technique (SET) described below .

Direct aqua-regia digestion

About 1 g of each dried sample was weighed and placed in an acid washed digestion tube. A combination of concentrated nitric acid (69 %) and perchloric acid (60 %) was prepared in the ratio of 4:1 and added to each digestion tube (Yap *et al*., 2002). The tubes were then placed in a digestion block at 40 ºC for 1 hour and were then fully digested at 140 ºC for 2-3 hours (Yap *et al*., 2002). After they were cooled to room temperature, the digests were topped up (diluted) to 40 ml with double de-ionized water. Each diluted sample was then filtered through a Whatman No. 1 filter paper into an acid-washed pill box. The samples were then stored until used for metal determination .

Sequential Extraction Technique (SET)

Sequential extraction was performed by using a four stage procedure originally recommended by Badri and Aston (1983). Sequential extraction analysis (SET) provides information on heavy metals which are of anthropogenic sources such as easily, freely leachable or exchangeable fraction (EFLE), acid reducible fraction, oxidisable organic fraction and resistant fraction in the sediments.

Extraction of EFLE fraction

For each site, three replicate extractions were done. Ten grams of dried samples were weighed and placed in a 250 ml Erlenmeyer

flask. After that, 50 ml of 1.0 M ammonium acetate at pH 7 was added to the sample and agitated in an orbital shaker model GYROMAX 722 at constant speed (2500 rpm) for 3 hours at room temperature. Then it was filtered through a Whatman No. 1 filter paper into an acid washed pill box. The remainder in the Erlenmeyer flask was washed with 20 ml of double de-ionized water and then filtered through the same filter paper into the same pill box. The residue left on the filter paper was dried in an oven between 40 to 60 ºC until a constant dry weight was obtained. The dried residue was scraped off from the filter paper and placed in the same Erlenmeyer flask. The dry weight was measured and recorded to determine the amount of sediment that dissolved in the extractant.

Extraction of acid reducible fraction

The same procedure as above was followed but 50 ml of 0.25 M hydroxyl ammonium chloride at pH 2 were used instead. The second fraction is known as the 'acid reducible' fraction. The filter paper containing the residue was dried in an oven between 40 to 60 ºC until a constant dry weight was obtained.

Extraction of oxidisable-organic fraction

The dried residue obtained in Section 2.2.2.2 was oxidised with 15 ml 30 % hydrogen peroxide (H_2O_2) in a water bath (90 ºC to 95 ºC). During the experiment, cool water was prepared to slow down the reaction if the reaction was too vigorous. The experiment was carried on until the mixture dried up. After cooling, the metal released from the organic complex was continuously agitated with 50 ml of 1 M ammonium acetate at pH 3.5 at room temperature. The washing, filtration and measurement of dry weight were repeated as described earlier.

Extraction of resistant fraction

The sediments were again dried and scraped off from the filter paper. About 1 g of each sediment sample was weighed from the residue from the extraction of oxidisable–organic fraction and the experiment was carried out based on the direct aqua-regia method which was described previously.

Heavy metal determination

All ~~the~~ stored plant and soil samples were analyzed using an air-acetylene Perkin-Elmer™ flame atomic absorption spectrophotometer model AAnalyst 800. The light used for each metal was different. Each light have its own wavelengths namely 228.8 nm for Cd; 324.8 nm for Cu; 248.3 nm for Fe; 232.0 nm for Ni; 283.3 nm for Pb and 213.9 nm for Zn (Perkin-Emer, 1990). Blank determination was carried out to calibrate the instrument. Standard solutions for Cd, Cu, Fe, Ni, Pb and Zn were prepared from 1000 ppm stock solutions provided by MERCK Titrisol. All data obtained from the ASS w ere presented in µg/g dry weight basis.

Assay of antioxidative enzyme activity

Enzyme extraction

All chemical used for assay of antioxidative activity were freshly prepared to prevent the degradation of its activities. About 0.2g of (leave and root) fresh tissues was homogenized in an ice-cooled mortar with 5 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone (Mishra *et al*., 2006). The pH of the phosphate buffer was adjusted by adding monopotassium phosphate (KH_2PO_4) and dipotassium phosphate (K_2HPO_4). The homogenate was transferred to a 1.5 ml Eppendorf tube and centrifuged at 15000xg for 15 min at 4°C (Mishra *et al*., 2006). The supernatant was used for enzyme activity determination.

Superoxide dismutase (SOD) activity

The activity of SOD was assayed by measuring the inhibition of the photochemical reduction of nitrobluetetrazolium (NBT) (Beauchamp and Fridovich, 1971). The experiments were carried out in test tubes. Two sets of test tubes were prepared: one set of test tubes was under illumination while another set of test tubes was covered with aluminum foil and used as the control.

The assay mixture contained ~~of~~ 1.5 ml of 50 mM phosphate buffer (pH 7.8), 0.3 ml of 130 mM methionine, 0.3 ml of 750 µM NBT, 0.3 ml of 0.1 mM EDTA, 0.3 ml of 20 µM riboflavin, 0.05 ml of enzyme extract and 0.25 ml of deionized H_2O in a total volume of 3.0 ml. Riboflavin was added last, and the tubes were shaken and then illuminated for 15 min under standard florescent light (10 lamp watts per foot of length). The change in absorbance was recorded at 560 nm.

Catalase (CAT) activity

The assay for CAT activity was done based on the method of Aebi (1984). The assay mixture contained 0.2 ml of tissue extract, 1.5 ml of 50 mM phosphate buffer (pH 7.8), 1.0 ml of deionized H_2O and 0.3 ml of 0.1 M H_2O_2. H_2O_2 was added last and the mixture was shaken before the decrease in absorbance was recorded at 240 nm for 3 min.

Guaiacol peroxidase (GPX) activity

GPX activity was determined following the oxidation of guaiacol using the method described by Hemeda and Klein (1990) with some modifications. The assay mixture contained 2.9 ml of 50 mM phosphate buffer with pH 6.0, 1.0 ml of 2% H_2O_2 and 0.1 ml of the enzyme extract. After the addition of 1.0 ml 50 mM guaiacol, the mixture was shaken before the increase in absorbance as guaiacol was oxidized was measured at 470 nm for 3 min.

Ascorbate peroxidase (APX) activity

APX activity was determined by the method of Nakano and Asada (1981). The assay mixture contained 1.8ml of 50 mM phosphate buffer (pH 7, containing 0.2 mM EDTA-Na_2), 0.1ml of 7.5 mM ascorbic acid, 1 ml of 300 mM H_2O_2 and 0.1 ml of enzyme extract. H_2O_2 was added last and the mixture

was shaken before the change in absorbance was recorded at 290 nm.

Total protein determination

Total soluble protein concentration was determined using the method established by Bradford (1976). Standard Bovine Serum Albumin (BSA) was prepared at different concentrations: 0, 40, 80, 120, 160 and 200 µg/ml. Total protein content was expressed as mg of BSA equivalent by using the equation obtained from the standard curve of BSA.

Statistical analysis

The STATISTICA version 8 software was used to determine the correlation coefficient and for hierarchical cluster analysis. The analysis of variance (ANOVA), Student-Newman-Keuls (SNK) and Post hoc test were done using the SPSS software version 17.0 for Windows to find the differences between the means of heavy metal concentrations in the different parts of the plants from different sites (Zar, 1996).

RESULTS

The study was focused on the accumulation of common non-ionised form of metals in the leaves and roots of *C. asitica*, and soil sediments from selected sampling sites in Malaysia. As the metal concentrations in the stems did not show any significant correlations with those in the soils from all sampling sites, they are not further discussed. Based on the concentrations of heavy metals in the leaves of plants from nine sampling sites as listed in Table 2, there were significantly higher ($P<0.05$) Cu concentrations in leaves collected in Butterworth, Seremban and Pontian . For Cd in leaves, only those collected in Butterworth showed significant difference ($P<0.05$) in concentration. The concentrations of Zn in leaves from PPauh and Butterworth were significantly higher ($P<0.05$) than the rest. For Ni, samples from PPauh and Seremban were significantly higher ($P<0.05$) in concentrations. Leaves samples from Butterworth, Kapar, Seremban and Pontian showed significantly higher ($P<0.05$) concentrations of Pb. Fe concentration of plants from Butterworth and Seremban were significantly higher ($P<0.05$) in leaves than in roots .

Table 3 shows the concentrations of heavy metals in roots. Samples collected from Butterworth, UPM , Seremban and Pontian. showed significant differences ($P<0.05$) for Cu concentrations. For the concentrations of Fe and Cd, samples from Butterworth was significantly highest ($P<0.05$). For Zn, samples collected from PPauh and Butterwoth showed significantly higher ($P<0.05$) concentrations. Samples from Seremban and PPauh were observed to be significantly higher ($P<0.05$) for Ni concentrations in roots. Lastly, samples from Butterworth, Kapar and Pontian showed significant ($P<0.05$) difference in Pb concentrations.

For soil samples, the percentages of the resistant and non-resistant fractions are presented in Tables 4, 5, 6, 7, 8, and 9. For Cd, only two sampling sites showed more than 40% in the non-resistant fraction

TABLE 2
Concentrations (mean ± SD, µg/g dry weight) of heavy metals in leaves of *Centella asiatica* collected from 9 sampling sites from Peninsular Malaysia. (N=9)

Sampling sites	Cd				Cu				Fe				Ni				Pb				Zn			
PPauh	1.31	±	0.24	b	11.78	±	0.24	a,b	334.45	±	6.12	c,d	11.86	±	2.42	a	21.07	±	3.04	b	315.6	±	4.02	a
Karangan	1.04	±	0.08	c	6.75	±	1.24	d	291.12	±	13.36	c,d	4.24	±	0.58	d,e	13.44	±	2.27	c	172.24	±	25.01	b,c
Kluang	0.91	±	0.28	c,d	9.33	±	0.38	b,c	67.44	±	3.54	f	6.01	±	0.12	c,d	8.68	±	0.80	d	145.15	±	28.15	d,e,f
Butterworth	2.24	±	0.57	a	13.49	±	0.92	a	1244.8	±	144.95	a	9.33	±	1.45	a,b	46.64	±	7.60	a	336.75	±	24.02	a
UPM	0.88	±	0.42	c,d	11.96	±	2.65	a,b	367.36	±	29.76	c,d	2.59	±	0.84	f	7.46	±	0.76	d	144.59	±	12.53	d,e,f
Kapar	0.16	±	0.08	e	8.93	±	2.41	c	257.2	±	106.64	d	2.91	±	0.47	e,f	50.85	±	11.66	a	121.35	±	0.44	f
Seremban	1.87	±	0.66	a,b	13.28	±	1.25	a	887.12	±	138.2	a	11.87	±	0.48	a	42.45	±	5.19	a	182.19	±	10.11	b
KSR	1.25	±	0.24	b,c	5.36	±	0.64	e	470.8	±	17.12	c	6.13	±	0.49	c,d	26.52	±	9.43	b	160.30	±	2.48	b,c,d
Pontian	1.23	±	0.04	b,c	13.28	±	1.25	a	719.76	±	131.37	b	4.64	±	0.98	d	43.12	±	5.75	a	182.40	±	7.80	b

a,b,c : different alphabet in each column shows the different of significant mean (SNK test, P<0.05)

TABLE 3
Concentrations (mean ± SD, µg/g dry weight) of heavy metals in roots of *Centella asiatica* collected from 9 sampling sites from Peninsular Malaysia. (N=9)

Sampling sites	Cd				Cu				Fe				Ni				Pb				Zn			
PPauh	2.00	±	0.45	b	14.08	±	1.06	a	388.88	±	4.12	b	14.20	±	0.08	a	31.71	±	1.77	b	335.92	±	19.07	a
Karangan	1.36	±	0.28	b,c	7.92	±	0.23	b	311.00	±	12.05	b,c	4.80	±	0.22	b,c,d	15.24	±	0.40	c	207.52	±	11.00	b
Kluang	1.17	±	0.26	a,b	12.32	±	2.27	a,b	220.40	±	23.17	c	7.60	±	0.74	a,b,c,d	9.60	±	4.11	d	184.91	±	2.82	b,c
Butterworth	4.00	±	0.24	a	17.36	±	1.58	a	2008.8	±	13.22	a	10.77	±	2.45	a,b	63.52	±	5.88	d	349.20	±	10.99	a
UPM	1.52	±	0.11	b,c	15.76	±	3.00	a	482.16	±	9.05	b	2.99	±	1.70	d	10.91	±	0.40	d	207.76	±	2.26	b
Kapar	0.20	±	0.06	d	13.36	±	1.13	a,b	400.51	±	25.24	b,c	4.27	±	0.53	c,d	64.56	±	6.00	a	134.32	±	2.91	c,d
Seremban	2.00	±	0.06	b	16.00	±	2.54	a	1182.96	±	10.19	a,b	15.28	±	1.24	a	50.00	±	5.81	a,b	229.80	±	0.06	b
KSR	1.86	±	0.28	b	6.00	±	0.64	b	541.16	±	109.46	b	7.20	±	0.23	a,b,c,d	32.80	±	5.08	b	170.92	±	2.72	b,c
Pontian	1.58	±	0.24	b,c	16.00	±	2.54	a	1379.47	±	169.48	a,b	5.60	±	0.22	b,c,d	59.39	±	5.77	a	213.80	±	35.02	b

a,b,c : different alphabet in each column shows the different of significant mean (SNK test, P<0.05)

TABLE 4
Concentrations (mean ± SD, µg/g dry weight) of Cd in geochemical fractions of sediments collected from 9 sampling sites from Peninsular Malaysia. (N=3)

Sampling sites	EFLE	Acid-reducible	Oxidisable-organic	Resistant	Total (100%)	Non-resistant (%)	Resistant (%)
PPauh	0.074 ± 0.033	0.212 ± 0.018	0.210 ± 0.001	1.160 ± 0.013	1.656	29.95	70.05
Karangan	0.119 ± 0.030	0.088 ± 0.021	0.167 ± 0.026	1.660 ± 0.011	2.034	18.39	81.61
Kluang	0.077 ± 0.040	0.094 ± 0.000	0.038 ± 0.013	1.480 ± 0.020	1.689	12.37	87.63
Butterworth	0.221 ± 0.005	0.697 ± 0.010	0.567 ± 0.031	2.300 ± 0.004	3.885	40.80	59.20
UPM	0.044 ± 0.023	0.090 ± 0.015	0.285 ± 0.027	1.627 ± 0.029	2.046	20.50	79.51
Kapar	0.007 ± 0.005	0.096 ± 0.004	0.035 ± 0.018	1.093 ± 0.032	1.231	11.18	88.82
Seremban	0.116 ± 0.054	0.778 ± 0.025	0.410 ± 0.010	1.720 ± 0.047	3.024	43.12	56.88
Pontian	0.119 ± 0.054	0.225 ± 0.026	0.252 ± 0.016	1.720 ± 0.031	2.316	25.73	74.27
KSR	0.147 ± 0.008	0.300 ± 0.021	0.242 ± 0.022	1.004 ± 0.019	1.693	40.70	59.30

TABLE 5
Concentrations (mean ± SD, µg/g dry weight) of Cu in geochemical fractions of sediments collected from 9 sampling sites from Peninsular Malaysia. (N=3)

Sampling sites	EFLE	Acid-reducible	Oxidisable-organic	Resistant	Total (100%)	Non-resistant (%)	Resistant (%)
PPauh	0.980 ± 0.022	0.154 ± 0.015	34.497 ± 0.165	38.320 ± 0.128	73.951	48.18	51.82
Karangan	1.022 ± 0.010	0.085 ± 0.027	6.254 ± 0.005	10.020 ± 0.057	17.381	42.35	57.65
Kluang	0.721 ± 0.049	0.400 ± 0.005	8.079 ± 1.085	11.740 ± 0.031	20.940	43.94	56.07
Butterworth	7.063 ± 0.084	1.123 ± 0.020	82.343 ± 6.058	100.780 ± 0.208	191.309	47.32	52.68
UPM	0.467 ± 0.015	0.109 ± 0.015	20.046 ± 2.624	33.947 ± 0.229	54.569	37.79	62.21
Kapar	0.686 ± 0.040	0.214 ± 0.023	8.222 ± 0.629	13.400 ± 0.044	22.522	40.50	59.50
Seremban	2.062 ± 0.129	0.961 ± 0.321	61.016 ± 3.144	65.200 ± 0.013	129.239	49.55	50.45
Pontian	3.980 ± 0.084	0.785 ± 0.057	54.779 ± 4.833	90.740 ± 0.406	150.284	39.62	60.38
KSR	0.707 ± 0.084	0.435 ± 0.090	15.162 ± 2.407	27.408 ± 0.190	43.712	37.30	62.70

TABLE 6
Concentrations (mean ± SD, µg/g dry weight) of Fe in geochemical fractions of sediments collected from 9 sampling sites from Peninsular Malaysia. (N=3)

Sampling sites	EFLE	Acid-reducible	Oxidisable-organic	Resistant	Total (100%)	Non-resistant (%)	Resistant (%)
PPauh	259.70 ± 1.91	856.37 ± 81.78	2253.89 ± 10.34	16080.00 ± 1689.38	19449.96	17.33	82.67
Karangan	1405.37 ± 2.47	2675.32 ± 191.47	1475.78 ± 89.94	10038.00 ± 1258.65	15594.46	35.63	64.37
Kluang	569.80 ± 48.51	1939.38 ± 418.03	1008.58 ± 116.01	9662.00 ± 591.14	13179.77	26.69	73.31
Butterworth	518.70 ± 47.52	896.37 ± 86.55	3917.32 ± 223.69	22940.00 ± 3116.93	28272.39	18.86	81.14
UPM	142.80 ± 14.05	416.33 ± 181.44	685.01 ± 50.31	23880.00 ± 3628.11	25124.14	4.95	95.05
Kapar	182.35 ± 15.84	3904.26 ± 525.75	2000.44 ± 29.41	15905.33 ± 3470.10	21992.39	27.68	72.32
Seremban	248.50 ± 22.77	605.73 ± 430.85	3782.67 ± 235.05	24078.00 ± 3193.29	28714.89	16.15	83.85
Pontian	717.15 ± 70.78	2112.72 ± 67.79	2138.61 ± 98.94	15640.00 ± 2319.31	20608.48	24.11	75.89
KSR	480.90 ± 127.76	831.25 ± 0.58	2015.21 ± 5.47	16120.00 ± 2513.26	19447.36	17.11	82.89

TABLE 7
Concentrations (mean ± SD, µg/g dry weight) of Ni in geochemical fractions of sediments collected from 9 sampling sites from Peninsular Malaysia. (N=3)

Sampling sites	EFLE	Acid-reducible	Oxidisable-organic	Resistant	Total (100%)	Non-resistant (%)	Resistant (%)
PPauh	0.700 ± 0.026	0.710 ± 0.046	3.339 ± 0.010	5.800 ± 0.068	10.549	45.02	54.98
Karangan	0.767 ± 0.114	0.417 ± 0.041	1.250 ± 0.099	4.560 ± 0.115	6.994	34.80	65.20
Kluang	0.760 ± 0.045	0.663 ± 0.041	2.325 ± 0.151	7.440 ± 0.088	11.188	33.50	66.50
Butterworth	1.208 ± 0.084	0.662 ± 0.102	4.067 ± 0.325	8.120 ± 0.042	14.057	42.24	57.77
UPM	0.728 ± 0.068	0.315 ± 0.202	1.713 ± 0.143	4.293 ± 0.092	7.049	39.10	60.90
Kapar	0.350 ± 0.129	0.243 ± 0.084	0.354 ± 0.116	3.213 ± 0.015	4.160	22.76	77.24
Seremban	0.795 ± 0.173	0.774 ± 0.385	5.239 ± 0.196	8.600 ± 0.087	15.408	44.19	55.82
Pontian	0.441 ± 0.059	0.254 ± 0.141	2.610 ± 0.141	8.640 ± 0.008	11.945	27.67	72.33
KSR	0.707 ± 0.074	0.315 ± 0.037	3.008 ± 0.295	7.744 ± 0.010	11.774	34.23	65.77

TABLE 8
Concentrations (mean ± SD, µg/g dry weight) of Pb in geochemical fractions of sediments collected from 9 sampling sites from Peninsular Malaysia. (N=3)

Sampling sites	EFLE	Acid-reducible	Oxidisable-organic	Resistant	Total (100%)	Non-resistant (%)	Resistant (%)
PPauh	2.114 ± 0.008	1.442 ± 0.740	17.640 ± 0.165	84.440 ± 0.128	105.636	20.07	79.94
Karangan	2.664 ± 0.342	8.383 ± 0.269	16.345 ± 0.368	52.060 ± 0.179	79.452	34.48	65.52
Kluang	2.282 ± 0.584	1.424 ± 0.617	3.988 ± 0.876	21.000 ± 0.068	28.694	26.81	73.19
Butterworth	3.269 ± 0.178	2.056 ± 0.799	29.310 ± 0.182	125.820 ± 0.547	160.455	21.59	78.42
UPM	2.996 ± 0.303	2.229 ± 0.139	8.844 ± 0.807	55.133 ± 1.463	69.202	20.33	79.67
Kapar	3.617 ± 0.624	2.958 ± 0.159	10.704 ± 0.839	46.533 ± 0.948	63.812	27.08	72.92
Seremban	4.407 ± 0.351	5.785 ± 1.211	34.818 ± 0.869	132.020 ± 0.010	177.030	25.43	74.58
Pontian	4.032 ± 0.752	7.098 ± 0.391	41.857 ± 2.600	147.980 ± 0.267	200.967	26.37	73.63
KSR	2.625 ± 0.467	1.428 ± 0.026	34.453 ± 1.286	92.160 ± 0.177	130.666	29.47	70.53

TABLE 9
Concentrations (mean ± SD, µg/g dry weight) of Zn in geochemical fractions of sediments collected from 9 sampling sites from Peninsular Malaysia. (N=3)

Sampling sites	EFLE	Acid-reducible	Oxidisable-organic	Resistant	Total (100%)	Non-resistant (%)	Resistant (%)
PPauh	8.449 ± 0.018	39.283 ± 0.550	63.752 ± 0.204	112.040 ± 0.188	223.524	49.88	50.12
Karangan	1.995 ± 0.045	3.136 ± 0.398	12.893 ± 0.104	31.860 ± 0.309	49.884	36.13	63.87
Kluang	2.433 ± 0.064	4.919 ± 2.034	10.813 ± 0.790	24.420 ± 1.246	42.585	42.66	57.34
Butterworth	14.732 ± 0.025	52.364 ± 0.295	69.284 ± 1.042	171.220 ± 1.140	307.600	44.34	55.66
UPM	0.443 ± 0.091	5.127 ± 0.375	36.286 ± 2.244	46.613 ± 0.343	88.469	47.31	52.69
Kapar	1.643 ± 0.163	4.358 ± 1.249	5.869 ± 0.825	21.253 ± 0.269	33.123	35.84	64.16
Seremban	3.171 ± 0.079	67.067 ± 0.162	31.624 ± 0.396	107.920 ± 0.448	209.782	48.56	51.44
Pontian	2.149 ± 0.049	25.382 ± 2.138	53.061 ± 1.433	131.260 ± 0.421	211.852	38.04	61.96
KSR	1.932 ± 0.178	22.299 ± 3.446	31.506 ± 0.557	77.076 ± 0.373	132.813	41.97	58.03

TABLE 10
Overall mean for aqua-regia, SET (mean, µg/g dry weight) and the percentage of similarity of soils collected from 9 sampling sites in Peninsular Malaysia. (N=3)

Sampling sites	Cd			Cu			Fe			Ni			Pb			Zn		
	Aqua	SET	%	Aqua	SET	%	Aqua	SET	%	Aqua	SET	%	Aqua	SET	%	Aqua	SET	%
PPauh	1.63	1.66	101.60	62.24	73.95	118.82	21634	19450	89.90	11.72	10.55	90.01	90.97	105.64	116.12	203.43	223.52	109.88
Karangan	1.68	2.03	121.07	24.98	17.38	69.58	13713	15594	113.72	5.37	6.99	130.24	66.18	79.45	120.05	40.50	49.88	123.17
Kluang	1.40	1.69	120.64	25.09	20.94	83.46	12196	13180	108.07	9.03	11.19	123.90	24.72	28.69	116.08	43.68	42.59	97.49
Butterworth	3.72	3.89	104.44	146.99	191.31	130.15	27917	28272	101.27	12.97	14.06	108.38	125.67	160.46	127.68	237.11	307.60	129.73
UPM	1.67	2.05	122.50	50.24	54.57	108.62	25907	25124	96.98	6.95	7.05	101.43	53.73	69.20	128.80	51.92	88.47	170.40
Kapar	1.21	1.23	101.76	22.24	22.52	101.27	23424	21992	93.89	4.01	4.16	103.75	53.64	63.81	118.96	26.12	33.12	126.81
Seremban	2.64	3.02	114.55	102.03	129.24	126.67	27424	28715	104.71	13.49	15.41	114.22	170.43	177.03	103.87	232.10	209.78	90.38
Pontian	1.69	2.32	137.04	120.70	150.28	124.48	24694	20608	83.46	9.27	11.95	128.86	179.03	200.97	112.25	188.79	211.85	112.22
KSR	1.66	1.69	101.99	39.07	43.71	111.88	21832	19447	89.08	10.48	11.77	112.35	117.40	130.67	111.30	150.44	132.81	88.28

namely Butterworth (40.80%) and Seremban (43.12%). Soil samples collected from Seremban, PPauh and Butterworth showed 49.55%, 48.18% and 47.32%, respectively, of Cu in the non-resistant fraction while having 44.19%, 45.02% and 42.24% of Ni there. For Fe and Pb, all soil samples showed less than 40% in the non-resistant fraction. Lastly, soil samples from PPauh and Butterworth showed 49.88% and 44.34% respectively of Zn in the non-resistant fraction.

The heavy metal concentrations in the soils based on the aqua-regia method analysis are presented in Table 10. Generally samples from PPauh, Seremban, Butterworth and Pontian showed higher metals concentration. The Cu levels were higher in the samples from Butterworth, Pontian and Seremban. For Cd and Fe, samples from Seremban and Butterworth showed higher concentrations. Samples collected from Butterworth, Seremban and PPauh had higher levels of Zn and Ni. Samples from Seremban and Pontian were higher in Pb in soils.

Fig.5 shows the levels of antioxidative enzymes in leaves; SOD was present at significant levels ($P<0.05$) in the samples from Seremban and Butterworth samples. In the samples collected from Butterworth and PPauh there were observations of significant ($P<0.05$) activities for GPX. CAT and APX did not show any significant value for all metal concentrations in leaves. For antioxidative levels in roots (Fig.6), samples fromSeremban, Butterworth and PPauh showed significant ($P<0.05$) activities for

GPX. APX showed similar results for the samples collected from Butterworth and PPauh. CAT and SOD did not show any significant result for all metal concentrations in roots.

DISCUSSION

Relationships of metals between roots and soils

In general, sampling sites at Butterwoth, Seremban and PPauh showed higher levels of both metals and antioxidative enzymes in plants and soils. Based on the correlations of metals between the soils and different parts of *C. asiatica* (Table 11), the correlations between roots and soils for Cd, Zn, Ni and Fe were high with R=0.855, R=0.827, R=0.888 and R=0.857, respectively. This showed that when the concentrations of Cd, Zn, Ni and Fe were higher in soils, the levels of metal in the roots would also subsequently be higher. This result is supported by Ratko *et al*. (2011) who showed statistically significant correlations between contents of heavy metals (Zn, Cu, Pb and Cd) in soil and plants. Metals in soils were easily taken up by roots and translocated to different parts of the plant such as leaves because ~~only~~ roots are the only organ that is covered in soil all the time when compared to the other parts of the plant. Besides that, root is a good storage area due to the abundance of root hairs present which increased its surface area for adsorption and absorption (Yap *et al*., 2010; Street *et al*., 2009).

Of the metals mentioned above, Fe showed the highest uptake in roots namely 220.40-2008.80 µg/g. This was due to its ability to form octahedral complexes with various ligands and its redox potential in response to different ligand environments (Hell and Stephan, 2003). The phytotoxicities of the trace metals followed the following trend (from the most to the least toxic): Pb >Cu >Cd >≈Ni≈Zn (Kopittke *et al*., 2009). Hence, the results showed higher uptake of Zn (121.35-336.75µg/g) in roots compared to Cd and Cu with each having a total of 0.2-4.0 µg/g and 6.0-17.36 µg/g, respectively, because roots tended to reduce the uptake of heavy metals that were more toxic. González-Miqueo *et al*. (2010) found that *Hypnum cupressiforme* was able to uptake Zn (277 µg/g) Pb (56.6 µg/g), Ni (26.2 µg/g), Cu (21.7 µg/g) and Cd (0.49 µg/g) in Azkoitia, Spain. These results supported our current findings with a similar trend of heavy metal concentrations in *C. asiatica*.

Overall, metal concentrations were highest in roots followed by leaves because plants developed a mechanism which caused immobilization of certain metals when they were bound to their cell walls (Yap *et al*., 2011). This prevents the metal from being further uptaken by the roots and also inhibits the metal translocation to the shoot. The metals that were accumulated in the roots or were unable to enter the plant were kept in the root cells where they would be detoxified by forming complexes with amino acids, organic acids or metal-binding peptides or sequestered into vacuoles (Hall, 2002). This action greatly restricts the translocation of the metals to the above-ground organs. Moreover, it can protect the leaf tissues and the metabolically active photosynthetic cells

from heavy metal damage (Navari-Izzo *et al*., 1998; Sgherri *et al*., 2003).

Relationship between the levels of metals in leaves and soils

For correlations between leaves and soils, all metals showed significant correlations except Pb, with Cu (R=0.720), Cd (R=0.867), Zn (R=0.784), Ni (R=0.903) and Fe (R=0.899). The results showed that when the concentrations of Cu, Cd, Zn, Ni and Fe were higher in soils, the levels of metal concentrations in leaves would subsequently also be higher. There was no further discussion of Fe concentrations in leaves due to the naturally high concentrations of Fe in soils when compared to the other metals and it rarely causes any toxic effects to plants (Ong *et al*., 2011). According to Chojnacka *et al*. (2005), the metal transfer was decreased in the order of Zn > Cu> Ni > Pb and Cd which showed that higher concentrations of Zn ,Cu and Ni were transferred from soils to plants compared to Pb and Cd.

The strategy of uptake of metals in plants depends on some physiological processes which require the cell to conserve the intracellular heavy metal ions in a non toxic form (Cobbet, 2000). Pb is considered toxic to plants; therefore plants will have a series of mechanisms to reduce the entry of Pb. Besides that, Pb is a non-essential metal for plants; thus the uptake of Pb by plant is unfavorable (Mehra and Tripath, 2000). There is a correlation between Cd concentration in the soils and leaves even though Cd is a non-essential metal for plant. This happens when the translocation of other ions from roots to shoots causes some trace Cd to be translocated along. The net amount of Cd translocated was in the range of 0.16-2.24 µg/g in leaves. Furthermore, Cd is a very mobile element in the environment and plants therefore can easily uptake and transfer it to their other organs (Vaněk *et al*., 2004).

Relationship between metal concentrations and antioxidative enzymes

As is shown in Table 12, the correlations between Zn in leaves and the levels of the antioxidative enzymes (CAT, GPX and APX) were significant with R=0.732, R=0.738 and R=0.710, respectively, but SOD did not show any significant correlation. In roots, there were similar significant ($P< 0.05$) correlations between Zn concentration and CAT (R=0.856), GPX (R=0.726) and APX (R=0.794) activities while SOD activity remained insignificant ($P> 0.05$). These results were supported by those of Candan and Tarhan (2003) who reported that GPX and APX activities increased in the presence of Zn^{2+} from roots to leaves. For Ni, the correlations were GPX (R=0.796) and APX (R=0.719) for leaves and CAT (R=0.838), GPX (R=0.734) and APX (R=0.696) for roots. The main response of plants towards increases in the levels of Zn and Ni was to generate SOD to accelerate the dismutation of O_2^- to H_2O_2. For each of these reactions, two molecules of H_2O_2 were produced from one O_2^- causing high concentration of it in the plant. Thus, higher activities of CAT,

TABLE 11
The correlation coefficients between different parts of *Centella asiatica* (Log_{10}mean+1) and aqua-regia concentrations (Cd, Cu, Fe, Ni, Pb and Zn) (N=9).

	Cu	Cd	Zn	Ni	Pb	Fe
Leaves-sediment						
Cu	**0.720**	0.474	0.653	0.528	0.273	0.411
Cd	**0.785**	**0.867**	**0.800**	**0.801**	0.651	0.606
Zn	**0.669**	**0.677**	**0.784**	**0.695**	0.464	0.413
Ni	0.539	0.595	**0.799**	**0.903**	0.397	0.334
Pb	0.443	0.374	0.240	0.242	0.573	0.519
Fe	**0.816**	**0.756**	0.534	0.339	**0.885**	**0.899**
Roots- sediment						
Cu	0.574	0.368	0.495	0.390	0.099	0.295
Cd	**0.767**	**0.855**	**0.779**	**0.726**	0.557	0.616
Zn	**0.712**	**0.704**	**0.827**	**0.710**	0.435	0.405
Ni	0.496	0.548	**0.762**	**0.888**	0.351	0.301
Pb	0.506	0.380	0.305	0.257	0.593	0.577
Fe	**0.886**	**0.794**	0.590	0.488	**0.783**	**0.857**

Note: Bold is significant at the level P< 0.05(two-tailed)

TABLE 12
The correlation coefficients between different parts of *Centella asiatica* (Log_{10}X+1) based on the antioxidant level (SOD, CAT, GPX and APX) (N=9).

	SOD	CAT	GPX	APX
Leave-Antioxidant				
Cu	**0.922**	**0.723**	**0.694**	**0.797**
Cd	0.524	0.451	0.576	0.640
Zn	0.566	**0.732**	**0.738**	**0.710**
Ni	0.554	0.665	**0.796**	**0.719**
Pb	0.425	0.384	0.636	0.543
Fe	0.520	0.279	0.553	0.617
Root-Antioxidant				
Cu	**0.811**	0.561	**0.714**	**0.758**
Cd	0.341	0.640	0.459	0.497
Zn	0.592	**0.856**	**0.726**	**0.794**
Ni	0.616	**0.838**	**0.734**	**0.696**
Pb	0.416	0.399	0.579	0.459
Fe	0.491	0.509	0.594	0.522

Note: Bold is significant at the level P< 0.05(two-tailed)

APX and GPX were required to overcome the mass production of H_2O_2.

Finally, for Cd, Pb and Fe, there were no significant correlations in leaves and roots with the antioxidative enzymes (Table 12). In fact, plants with enhanced activities of antioxidative enzymes had been shown to be tolerant to oxidative stress (Mittler *et al*., 2004). This was due to the uptake and translocation of the metals. Cd and Fe did not show any significant correlation in antioxidative activities because their concentrations in leaves and roots were considered low being 0.16-2.24 µg/g and 0.20-4.0 µg/g in leaves and roots for Cd and only 67.44-1244.95 µg/g (leaves) and 220.40-2008.20 µg/g (roots) for Fe. The levels of both the metals were too low to activate any obvious antioxidative activity. In addition, Cd ions were unable to catalyze the Fenton-Haber-Weiss reaction (Cho and Seo, 2005) which generate ROS. Even though Fe concentration in leaves was high for particular sites, but it did not play a role in causing a high level of SOD. This was supported by the data in Table 12, showed no significant correlation between Fe concentration and SOD. This was due to the soils naturally containing high levels of Fe. For Pb, its translocation in plants was limited and normally it was bounded to leaf surfaces and roots. This was supported by data presented Table 11 where there were no significant correlations between Pb in soils and Pb in leaves or roots. Besides that, data from most experimental studies on Pb toxicity showed that high Pb concentrations in the range of 100 to 1,000 mg/kg soil were needed to cause visible toxic effects on photosynthesis, growth, or other parameters (WHO, 1989; 1995). Thus, Pb can only affect plants in sites with very high environmental concentrations of it.

Usually physiological disorders and metabolic abnormalities in plants were caused by ROS production during normal metabolism when exposed to stresses (Marschner, 1995; Singh, 2007). When free radical production is excessive, or when the antioxidative system is insufficient to overcome ROS, it might damage the plant. Decrease of enzymatic and non-enzymatic free radical scavengers, caused by heavy metal toxicities (De Vos *et al*., 1993), might also contribute to a shift in the balance of free-radical metabolism towards H_2O_2 accumulation.

Hence, an increase in antioxidative enzymes can be expected so as to reduce the oxidative stress caused by heavy metals. Our results showed the differences in antioxidative enzyme activities for different metals in the leaves and roots of *C. asiatica*. Data in Table 12 show the correlations between metals levels in leaves and antioxidative levels. Cu showed significant correlations with all the enzymesnamely SOD (R=0.922), CAT (R=0.723), GPX (R=0.694) and APX (R=0.797). For correlations between metals levels in roots and antioxidative levels, Cu also showed high correlation for SOD (R=0.811), GPX (R=0.714) and APX (R=0.758). This shows that uptake of Cu triggers antioxidative enzyme activities in leaves and roots due to the sensitivity of plants

towards Cu toxicity. These was supported by the results of Candan and Tarhan (2003) who found that all antioxidative enzyme activities correlated positively with increasing Cu^{2+} concentrations in all *M. pulegium* organs**.**

In addition, high levels of Cu in plants might lead to metabolic disturbances and growth inhibition; even in quantities slightly higher than the normal level (Fernandes and Henriques, 1991). These excessive concentrations will cause oxidative stress which in turn increases the reactive oxygen species (ROS) within the subcellular compartments (Mittler *et al*., 2004). As a component of the plant's defense mechanism towards metal uptake, SOD was activated as long as the stress was not too strong for the plant's defense capacity (Siedlecka and Krupa, 2002). SOD is the most effective intracellular enzymatic antioxidative which dismutates O_2^- to H_2O_2 (Sarvajeet and Narendra, 2010). In soil, the usual Cu consists of between 2 and 250ppm whereas healthy plant tissues contain Cu in the range of 20–30µg/g dry weight.

Similarities and differences of metals and antioxidative enzymes

From Fig.2, Cd in all parts of the plant from all the sampling sites except Kapar were shown to be in the same cluster which indicated that all the sampling sites except Kapar accumulated a similar amount of Cd while Kapar accumulated the least amount. For Cu, all the sampling sites were grouped into the same cluster except for Karangan and KSR due to lesser amounts of Cu being accumulated at both these sites. Kluang was grouped in a different cluster compared to the other sampling sites which showed that only Kluang accumulated the least amount of Fe. UPM and Kapar accumulated the least amount of Ni compared to the other sampling sites; therefore they were grouped in the same cluster compared to the others. For Pb, Kluang and UPM were grouped in the same cluster compared to the other sampling sites due to their similarity in Pb concentration in plants. Zn concentrations for all the sampling sites were considered similar except for PPauh and Butterworth.

Fig.3 shows the hierarchical cluster analysis for metals concentrations in soils (aqua-regia and SET). Karangan, Kapar and Kluang which were the least contaminated group were in the same cluster for Cu and Fe when compared to the other sampling sites. For Cd, only Butterworth was grouped in a different cluster due to its high concentration in the soil. UPM was grouped in a different cluster for Fe while Kluang formed its own cluster for Pb. This showed that both sites accumulated less amounts of Fe and Pb respectively in the soils. For Ni, UPM and Kapar were considered as having low concentrations because they were grouped in the same cluster when compared to the other sampling sites.

Based on the geochemical fraction of soils, more than 50% of heavy metals (Cu, Cd, Ni, Fe, Pb and Zn) were accumulated in the 'resistant' fraction. This showed that the mobility of these metals were quite low because the soils from the sampling sites consisted of a higher percentage of

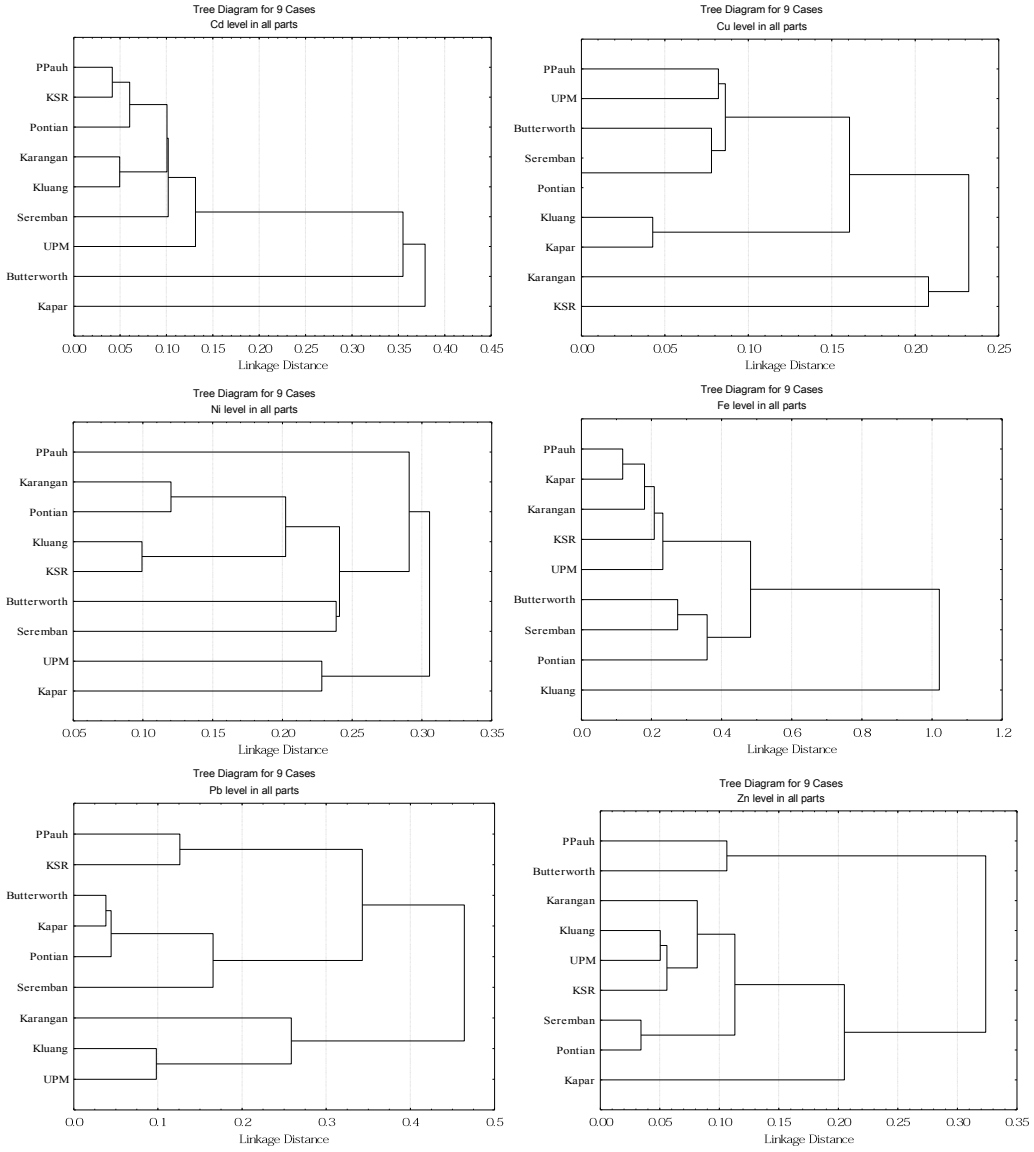

Fig.2: Hierarchical cluster analysis of *Centella asiatica* based on heavy metals (Cd, Cu, Fe, Ni, Pb and Zn) concentrations (Log_{10}mean+1) in all parts (leaves and roots) for all 9 sampling sites.

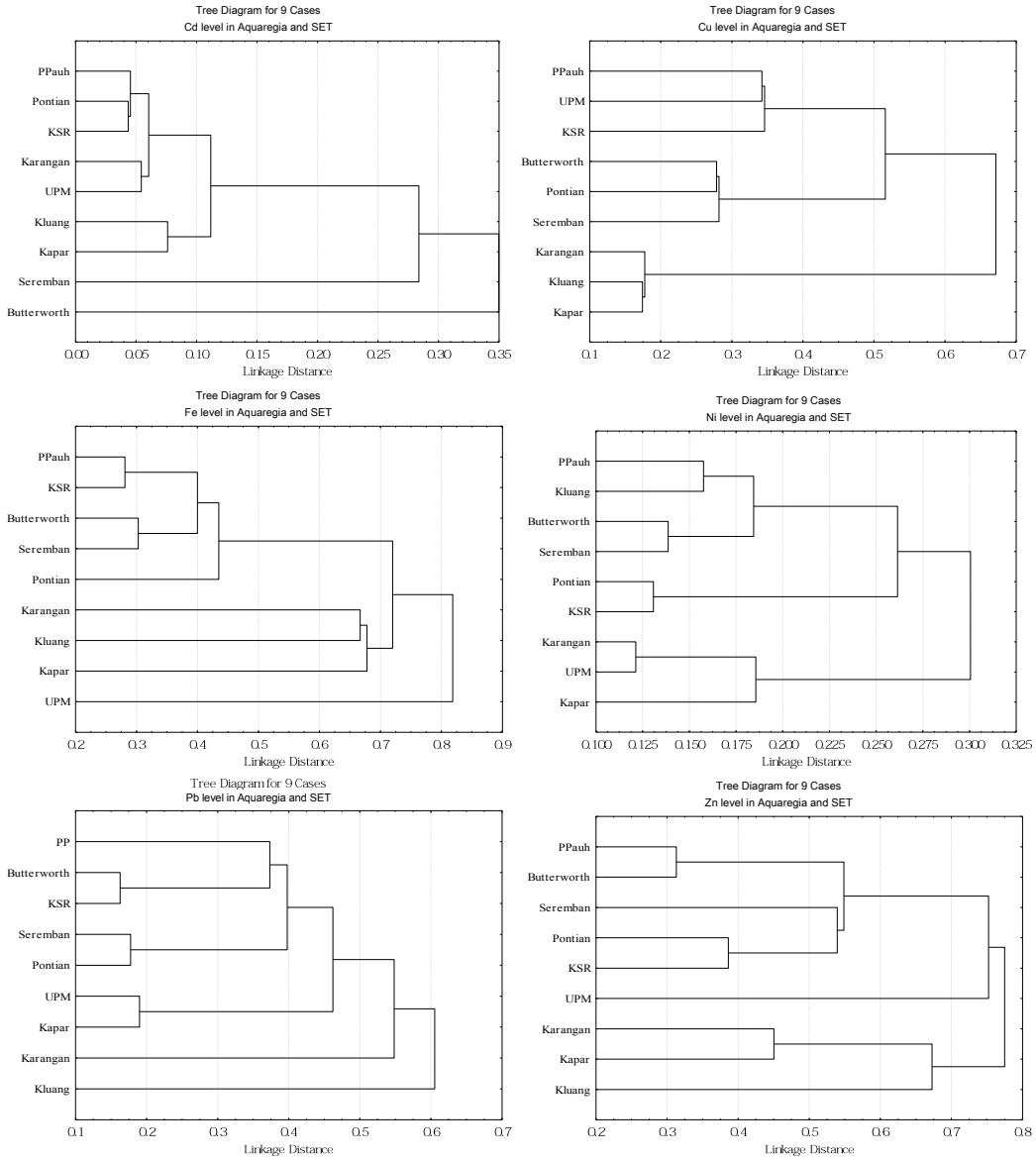

Fig.3: Hierarchical cluster analysis of *Centella asiatica* based on heavy metal concentrations (Log_{10}mean+1) in soils (Aqua-regia and SET) for all 9 sampling sites.

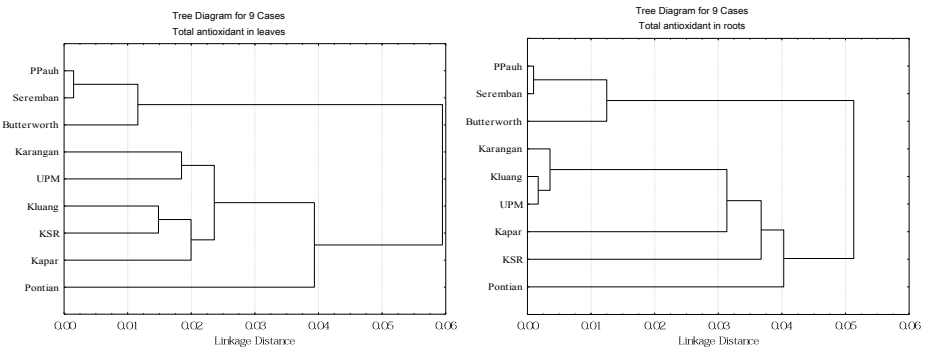

Fig.4: Hierarchical cluster analysis of *Centella asiatica* based on antioxidant enzymes (SOD, CAT, GPX and APX) in leaves and roots (Log_{10}mean+1) for all 9 sampling sites.

non-resistant fraction whereas Butterworth, Seremban, and PPauh were sampling sites suggestive of having more anthropogenic heavy metal inputs other than resistant soils. It was shown that the similarities and differences in the concentrations of metals within plants depended on the concentrations of the metals in the soil when that correlation and cluster analysis were conducted (Miranda *et al*., 1996; Diaz *et al*., 2002; Yongming *et al*., 2006).

It was observed that the uptake of metals into the roots and the translocation of metals to the shoots were highly proportional to the concentration of metals in the soil. In this study Cu did not show any significant correlation between roots and metal concentrations in the soil because generally Cu levels in soils were considered low. The uptake of Cu by osmosis was restricted when competing with other metals. Moreover, it had been postulated (Hill and Matrone, 1970) that elements with similar properties will act antagonistically to one another biologically, as a result of their competition for binding sites on proteins that require metals as cofactors.

For the cluster analysis of the total antioxidative enzymes in leaves and roots (Fig.4), Butterwoth, Seremban and PPauh were shown to be in the same cluster while the other sampling sites were in a different cluster. For the comparison of sites with heavy metal concentrations, Butterworth, Seremban and PPauh were shown to be the sites with the highest metal contamination. As a result of this, a high level of ROS was produced. This would rapidly attack all types of biomolecules such as nucleic acids, proteins, lipids and amino acids (De Vos and Schat, 1991; Mehta *et al*., 1992; Luna *et al*., 1994). Therefore, increased activity of the antioxidative system was required to protect plants from the harmful ROS (Foyer *et al*., 2009; Parra-Lobato *et al*., 2009). The synchronous actions of SOD, CAT, POD and APX were activated to work against ROS in various compartments of the plant cell (Singh *et al*., 2006).

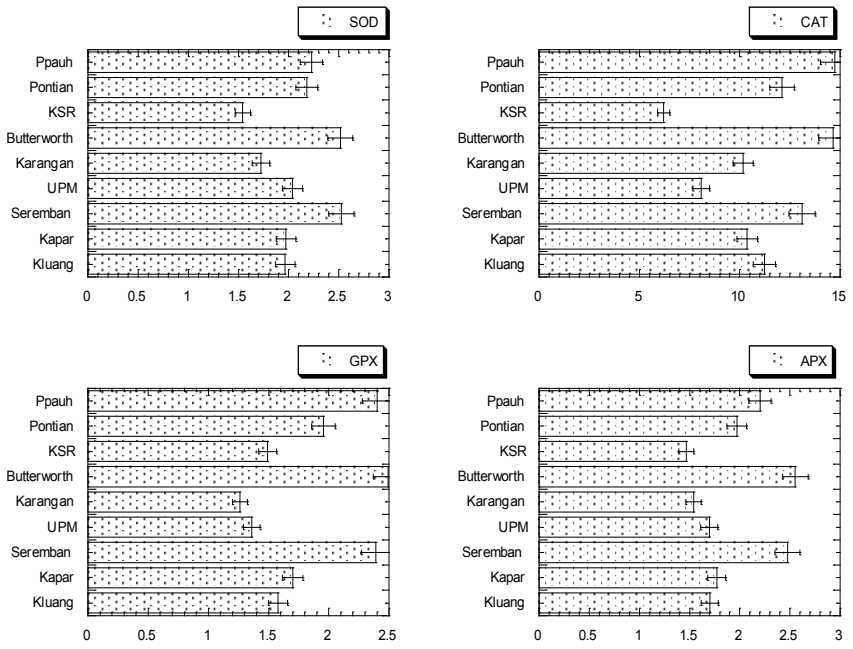

Fig.5: Concentrations (mean ± SD, nmol/mg/g) of antioxidant enzymes (SOD, CAT, GPX and APX) in leaves of *Centella asiatica* collected from nine sampling sites in Peninsular Malaysia. Note: For the activities of SOD, CAT and APX, their actual values are multiplied with 1000.

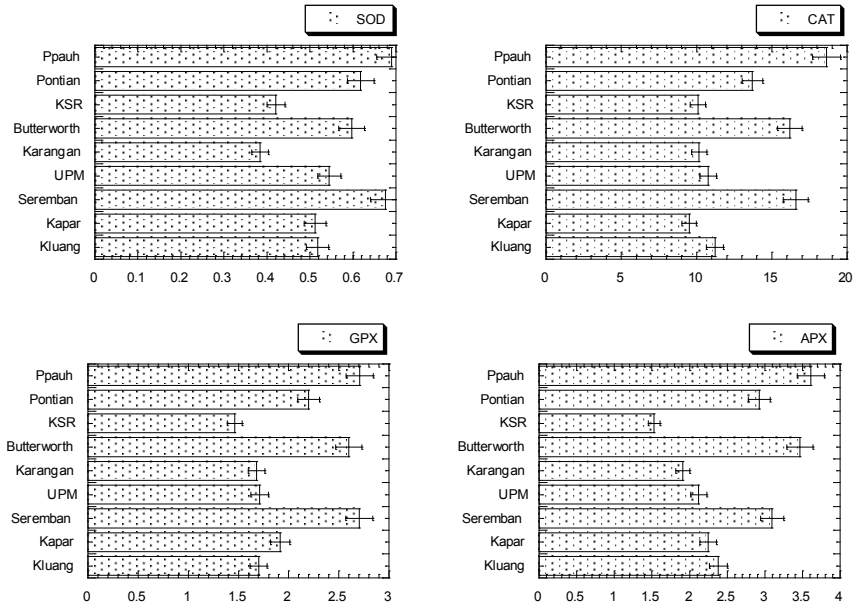

Fig.6: Concentrations (mean ± SD, nmol/mg/g) of antioxidant enzymes (SOD, CAT, GPX and APX) in roots of *Centella asiatica* collected from nine sampling sites in Peninsular Malaysia. Note: For the activities of SOD, CAT and APX, their actual values are multiplied with 1000.

CONCLUSION

The uptake of heavy metals into plant by roots and their translocations to leaves will induce stress in *Centella asiatica*. The main response towards the uptake of heavy metals and their concentrations was an increase in antioxidative activity to counteract the ROS production. Uptake of Cd, Fe and Pb did not seem to be a factor for the increase of antioxidative enzymes due to their low concentrations in plants. But Cu, Zn and Ni which are essential metals, showed obvious increases in the antioxidative activities of SOD, CAT, APX and GPX due to the higher concentrations of these metals. Thus, monitoring the concentration of these metals was essential to ensure the survival and well being of *C. asiatica*. Overall, antioxidative activities were significant in samples from Seremban, Butterworth and P.Pauh due to higher movement of heavy metals from the soils into *C. asiatica.*

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Length-Frequency Distribution, Length-Weight Relationship and Condition Factor of Sompat Grunt *Pomadasys jubelini* (Cuvier, 1830) off Lagos Coast, Nigeria

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ABSTRACT

Length-frequency distribution, length-weight relationship and condition factor of sompat grunt *Pomadasys jubelini* off the Lagos coast, Nigeria were investigated in order to study the growth pattern of *P. jubelini*. A total of four hundred and fifty specimens of *P. jubelini* were used for this study with body lengths ranging from 13.9-26.6cm (total length) and 11.2-22.1cm (standard length). The body weight ranged from 26.8g to 175.1g. The length-frequency distribution revealed high abundance of *P. jubelini* in the 21.0-21.9cm (total length) size group which accounted for 17.8% of the total specimens examined. The length-frequency distribution showed a poly-modal size distribution. Length-weight relationship was determined by the regression equation Log W = -1.5325 + 2.8177 Log L (n= 450, r^2= 0.69, $p < 0.05$). *P. jubelini* showed negative allometric growth (b = 2.8177) which indicated proportionate growth was more in body length than in body weight. The mean condition factor was significantly higher for males (1.92 ± 0.02) as compared to females (1.75 ± 0.03) ($p < 0.05$). Results of this study are important and relevant for the stock assessment and fishery management of *P. jubelini* in the Lagos coast.

Keywords: Condition factor, growth pattern, Lagos coast, length-frequency distribution, length-weight relationship, *Pomadasys jubelini*, sompat grunts

INTRODUCTION

The Lagos coast supports a natural fishery resource for the Lagos State in Nigeria. There are several economically important fish species in this water body and *Pomadasys jubelini* (Family: Haemulidae) is one of

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the economically important, abundant and widely distributed fish species not only in the Lagos coastal waters but also in the much wider Nigerian coastal waters. It is economically important in both the coastal artisanal fisheries as well as trawl fisheries. *Pomadasys jubelini* belongs to the Sciaenid fish community constituting the demersal resources in the Gulf of Guinea and it inhabits soft, sandy, muddy bottoms at depths between 15-50m (Mensah & Quaatey, 2002). Notwithstanding its economic importance, studies related to growth and size of *P. jubelini* off the Lagos Coast has not been reported.

Information on fish growth is obtained through growth studies where growth parameters like length-frequency distribution, length-weight relationship and condition factor are determined. Length-frequency distribution gives information on specific fish sizes and their corresponding frequency within a given population (Cunha *et al.,* 2007). Information on age groups in a population with modal length range is collected from length-frequency distribution analysis. In temperate regions, scales and otoliths are fish hard parts that are used to determine age and growth of fishes for stock assessment, but this is difficult in tropical regions. Length-frequency distribution however, is a better alternative in tropical regions for fish age determination and for stock assessment (Sparre *et al.,* 1989).

In fisheries assessment, important information is obtained by length-weight relationship of fish species (Haimovici & Velasco, 2000). Fish weight from length, ontogenic allometric changes and condition index are determined from length-weight relationship (Teixeira de Mello *et al.,* 2006). Estimates of length-weight relationship are relevant in stock management and assessment (Koutrakis & Tsikliras, 2003). Variations in length-weight relationship parameters of fish may differ according to season and habitats (Olim & Borges, 2006).

The condition factor expresses the relative degree of robustness or wellbeing of fish and reflects the degree of nourishment and state of sexual maturity. Sex of fish, age of fish, type of fish species, maturity stage and season are some of the factors that influence the condition factor of fish, leading to variation in the condition factor (William, 2000; Anyanwu *et al.,* 2007). The assumption of the condition factor is that fish in better condition are heavier (Bagenal & Tesch, 1978). It also provides information on the physiological state of fish relating to fish welfare from the reproductive and nutritional perspectives (Le Cren, 1951) and also provides useful information that can be used as age, growth and feeding intensity indices (Oni *et al.,* 1983).

There is limited information on the biology of the grunts in Nigeria. Francis and Sikoki (2007) reported the growth coefficient of *P. jubelini* from the Andoni River, Niger Delta, Nigeria. Length-weight relationship of some fresh water and coastal fish species in Nigeria have been reported (King, 1996, Fafioye & Oluajo, 2005; Agboola and Anetekhai, 2008). There is paucity of information on the growth studies of *P. jubelini*. Hence, the objectives

of this study includes determining the length-frequency distribution, length-weight relationship and condition factor of *P. jubelini* from the Lagos coast. It is hoped that this study will enhance knowledge on the biology of *P. jubelini* in the Nigerian waters.

MATERIALS AND METHODS

Study area

The study area was the Lagos coast, Lagos, Nigeria located between longitude 02° 53¹E to 08° 14¹E and latitude 06° 21¹N to 03° 55¹N covering a distance of 85km and lies in between the Gulf of Guinea (Fig. 1). Nigeria's coastline is 853km long bordering the Atlantic Ocean and has a coastal shelf area of approximately 41,000km² (FAO, 1969; Nwankwo & Onyema, 2003).

Fish collection and sampling

Specimens of *P. jubelini* were purchased from fish mongers at the landing centre of trawlers fishing off the Lagos coast at the jetty in Ijora Olopa, Lagos, Nigeria. The specimens were collected from January to

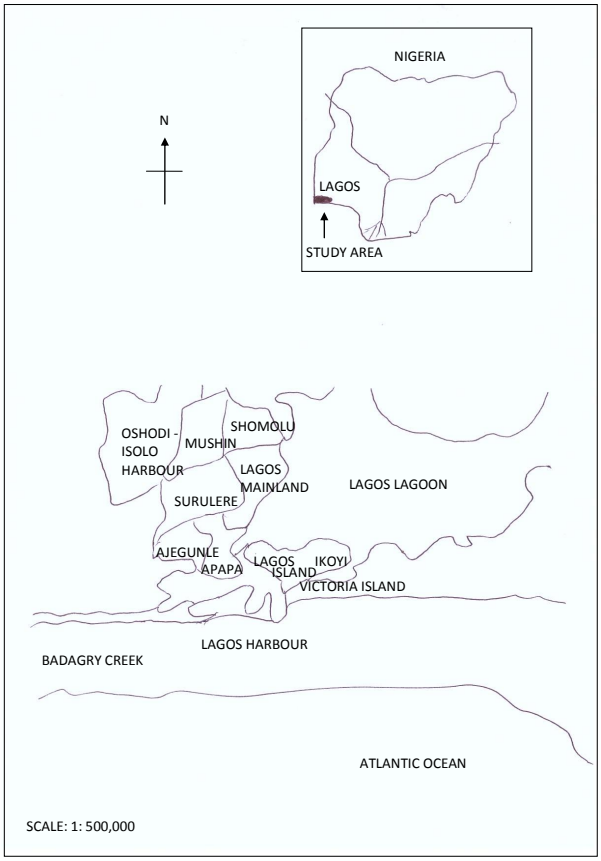

Fig.1: Map of Lagos showing the Lagos coast. Inset: Map of Nigeria showing the study area

September 2005. The fish was identified by the aid of taxonomic keys in Fischer *et al.* (1981). Fifty samples were randomly selected each month, making a total of 450 samples collected during the study period. Specimen selection was done by simple random sampling (Cochran, 2007). Each sample was randomly hand-picked without bias on its size, such that each fish size in the population had an equal chance of being selected. The samples were transported to the research laboratory and preserved in a deep freezer at -20°C. The samples were later allowed to thaw for examination in the laboratory for analysis.

Morphometric measurement

The total and standard lengths were measured using a one-meter measuring board graduated in cm. After wiping off water, the fish body weight was measured using a weighing balance (Sartorius model).

Growth studies

The growth pattern of *P. jubelini* was determined by determining the length-frequency distribution, length-weight relationship and condition factor.

Length-frequency distribution

The fish were grouped into different size classes and the percentage frequency and total lengths were used for the length-frequency distribution.

Length-weight relationship

The standard length and body weight of fish were used for the length-weight relationship.

The length-weight relationship was represented by the regression equation of Dadzie and Wangila (1980):

$$W = a + bL.$$

$$\text{Log } W = a + b \text{ Log } L$$

where W = weight of fish (g), L= standard length of fish (cm), a= regression constant, b= regression coefficient.

Condition factor

The condition factor was calculated according to Bannister (1976). It was calculated for males, females and combines sexes as follows

$$K = \frac{100W}{L^3}$$

where K= condition factor, W= weight of fish (g) and L= standard length of fish (cm).

Statistical analysis

Data were analysed using statistical analysis software (SAS 9.2) and Microsoft Excel 2003 software. Data were expressed as mean ± standard error of the mean. The difference in the condition factor of the male and the female fish was analysed by utilising the student-t test at $\alpha = 0.05$

RESULTS AND DISCUSSION

Length-frequency distribution

The total length of *P. jubelini* ranged from 13.9 cm to 26.6 cm with a mean of 16.75 ± 0.10 cm. The length-frequency distribution

showed polymodal distribution. The modal size class was 21.0-21.9 cm. Fig.2 shows the length-frequency distribution of *P. jubelini*.

Length-weight relationship

Body weight of *P. jubelini* ranged from 26.8 g to 175.1 g (mean = 103.0 ± 5.5g) and the standard length ranged from 11.2cm to 22.1cm (mean = 15.1 ± 0.4cm). The length-weight relationship of *P. jubelini* is shown in Fig.3, and is represented by the regression equation, LogW = -1.5325 + 2.8177 Log L ($r^2 = 0.69$, $r = 0.83$, $p < 0.05$).

Condition factor

The condition factor, K for males (mean = 1.92 ± 0.02; range = 0.69 - 3.15) was significantly higher than that of females (mean = 1.75 ± 0.03; range = 0.71 - 2.79). The condition factor for the combined sexes ranged from 0.69 to 3.15 with a mean value of 1.92 ± 0.02.

The length-frequency distribution suggested that *P. jubelini* belonged to more than one size group due to the poly-modal size distribution observed. This indicated the presence of several size groups in the fish population. The modal class of 21.0 - 21.9 cm which had the highest frequency accounted for 17.8% of the total specimens examined.

The length-weight relationship of *P. jubelini* reflected the expected increase in weight with increasing length irrespective of age or sex. The value of the regression coefficient b showed that the pattern of growth was negative allometric ($b < 3$). It can be inferred that growth was more in body length than in body weight. Similar results of negative allometric growth were also reported for *P. jubelini* from the Badagry Creek (b = 2.91) (Agboola & Anetekhai, 2008) and from the Qua Iboe estuary (b = 2.81) (King, 1996), Nigeria. In contrast, positive allometric

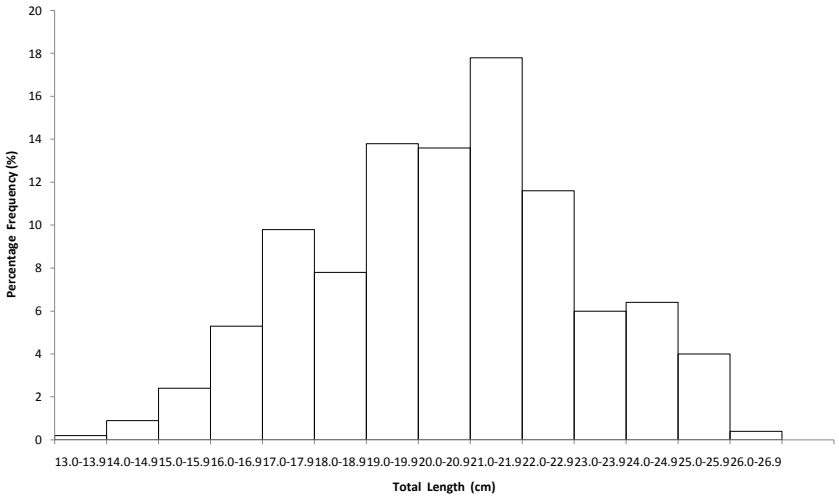

Fig.2: Length-frequency distribution of *Pomadasys jubelini* off Lagos coast

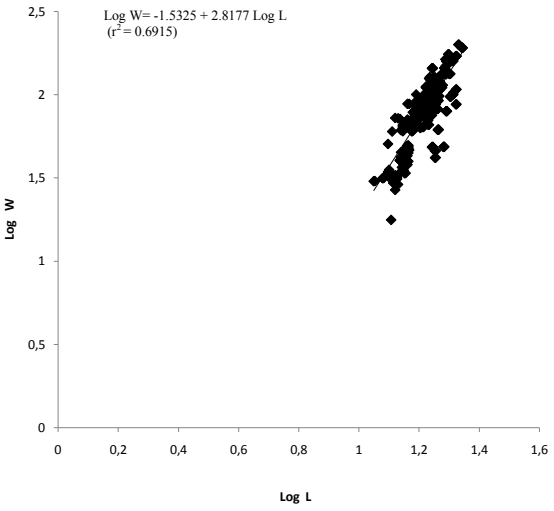

Fig.3: Length-weight relationship of *Pomadasys jubelini* off Lagos coast

growth was reported for *P. jubelini* from the Ivory Coast (b = 3.04) (Konan *et al*., 2007). Conspecifics of *P. jubelini* such as *P. branickii* demonstrated positive allometric growth (b = 3.1) (Rodriguez-Romero *et al*., 2009) while *P. incisus* (bastard grunt) showed negative allometric growth (b = 2.88) (Agboola & Anetekhai, 2008). *Pomadasys incisus* demonstrated similar growth pattern to *P. jubelini* in this study.

P. jubelini were in good condition in the Lagos coast where males had higher condition factor than the females ($p < 0.05$). This implied that male *P. jubelini* were in better condition than the females, and suggested that the males were heavier than the females. This was also demonstrated for *Pomadasys incisus* where the mean value of the male condition factor was 1.98 ± 0.16 and that of the female was 1.40 ± 0.11 (Fehri-Bedoui & Gharbi, 2008).

The information obtained in this study on the length-frequency distribution, length-weight relationship and condition factor of *P. jubelini* has implications on the fishery and fish stock management of Lagos coast. The results are valuable as a guide and would be useful for stock assessment in the development and formulation of fishery management policies in the Lagos coast and sustainability of the stocks available. It is also possible to use the data from this study in estimating the potential yield of *P. jubelini* fish stocks. The information is relevant in determining the future potential for recruitment and useful for monitoring and management of fisheries. This study revealed information on the available fish stocks of *P. jubelini*. Based on the results of the length-frequency distribution 19.0-30.0 cm size classes are in abundance and it is pertinent to monitor and manage these

size groups. Reduction in the population of these size groups (19.0 – 30.0 cm) could have negative effect on the population of *P. jubelini* in the Lagos coast. This is because these size groups (19.0 – 30.0 cm) are susceptible to exploitation and over fishing. The results on length-weight relationship can be used for length-based stock assessment of *P. jubelini.* Generally, this study has contributed to the information on growth parameters of *P. jubelini* in Lagos coast and the information can be utilised for fishery management.

CONCLUSION

It is pertinent to note that the significance of this study in fish biology was that it provided information on the growth parameters (length-frequency distribution, length-weight relationship and condition factor) of *P. jubelini* off the Lagos coast, Nigeria, which is a valuable tool for stock assessment and fishery management of *P. jubelini* in the Lagos coast. The length-frequency distribution suggested that 19.0 – 30.0 cm size class groups are abundant. This data is useful in quantifying the numerical abundance in the by-catch composition of *P. jubelini* in the Lagos coast. Length-based stock assessment can also be done by utilising the information obtained from the length-weight relationship of *P. jubelini* in this study.

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Phylogenetic Tree Construction in Reconfirmation of Parasitoid Species (Braconidae: Opiinae), Reared From Fruit Flies (*Bactrocera papayae*) Infesting Star Fruit (*Averrhoa carambola*) Based on Mitochondrial 16S rRNA Sequences

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ABSTRACT

Opiinae (Hymenoptera: Braconidae) is an important parasitoid of fruit flies (Diptera: Tephritidae). Accurate identification is needed for them to be effectively used as biological control agents. This study demonstrates the use of molecular phylogenetic analyses to reconfirm the species of Opiinae reared from tephritids infesting star fruit species. A total of 14 specimens were isolated and a 401 bp of mitochondrial 16S rRNA gene was amplified and sequenced from each of them. Three individual tephritids hosts (*Bactrocera papayae*) were also identified based on the mitochondrial ND1 gene. Maximum Parsimony (MP) tree was constructed using PAUP 4.0b10. Three species of Opiinae were successfully identified based on the mitochondrial 16S rRNA gene, namely *Diachasmimorpha longicaudata* (Ashmead), *Fopius arisanus* (Sonan), and *Psyttalia incisi* (Silvestri). These molecular-based findings reconfirmed the parasitoid species of *B. papayae* as recorded by previous studies based on morphology. In fact, the identification of Opiinae individuals have been reconfirmed based on topology and branching pattern of the phylogenetic tree as well as based on genetic distance analyses, which matched morphological-based identification.

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INTRODUCTION

Braconidae is a diverse family of Hymenopteran wasps, consisting of 22 genera (Yu *et al*., 2005) and more than 17000

species (Yu *et al*., 2004). Most of them are parasitoids of holometabolous insects, attacking the larval stage (Wang *et al*., 2004). However, braconids consist of subfamilies that are very specific when choosing their host to parasitize (Achterberg, 2002). For example, Microgastrinae preferentially parasitize Lepidopteran larvae, Aphidinae parasitize aphids, while Alysiinae and Opiinae parasitize cyclorraphous Dipteran larvae. These specificities represent the existence of host-parasitoid evolutionary relationships (Whitfield, 1992).

Because Braconidae is the second most important family of parasitoid wasps, a lot of studies were conducted in order to investigate their potential as biological control agents in controlling host pest populations (Greathead, 1986; Duan and Messing, 1997; Vargas *et al*,. 2007; Daane *et al*., 2008). In addition, this interest also arises from significant losses in economical value of commercial crops due to pest species (Clarke *et al*., 2005). The parasitoids include species in the subfamily Opiinae, which are among the important parasitoids of fruit fly species attacking star fruits (Chinajariyawong *et al*., 2000). Star fruit is one of the most preferred crops attacked by fruit fly pests (Tan & Serit, 1994). Aside from economic loss, the pest might also lead to increased costs due to farmers' disinfection and pest control from which might also result environmental pollution. Thus, biological control methods are safer, cheaper and more environmentally-friendly tools in controlling pest populations, with the objective of avoiding economic loss to star fruit crops (Yokomi & Tang, 1996; Daane & Johnson, 2010).

Among the subfamilies within the Braconidae family, Opiinae, the koinobiont endoparasitoid of cyclorraphous Dipterans, constitutes among the largest Braconidae subfamily (Wharton, 1997). All of their developmental stages are spent inside their hosts, where the male Opiinae emerges earlier than the female (Rungrojwanich & Walter, 2000). The genera *Biosteres* (Forester) and *Opius* (Wesmael), commonly used as parasitoids in controlling fruit fly species, belong to this subfamily. The fruit fly species being attacked in Malaysia include *Bactrocera carambolae* Drew & Hancock, *B. latifrons* (Hendel), and *B. papayae* Drew & Hancock (Chinajariyawong *et al*., 2000; Chua & Khoo, 1995). Host preferences are related to host suitabilities (Mohamed *et al*., 2003), which depend on certain factors namely sex ratio, generation interval, and fecundity of the parasitoid's progeny (Vargas *et al*., 2002; Rousse *et al*., 2006). In Malaysia, a total of seven species of fruit flies have been recorded as susceptible to parasitoid attacked, with six of them infesting star fruit (Chinajariyawong *et al*., 2000).

Correct identification is crucial for effective use of Opiinae as a biological control agent against fruit flies (Rugman-Jones *et al*., 2009). However, the classification and identification processes based on morphological characteristics alone are somewhat confusing when identifying Braconidae species (Quicke & Belshaw, 1999; Wharton, 2000; Wharton

& Achterberg, 2000). Identification based on molecular techniques has thus been used to overcome this deficiency (Rugman-Jones *et al*., 2009). Aside from the rapid characteristics of molecular identification, molecular based techniques are also useful in phylogenetic tree construction, which enables individuals of the same species to be grouped together in the same clade. This could also contribute to more accurate species identification based on the grouping of individuals (Rugman-Jones *et al*., 2009). Phylogenetic inferences have also been constructed on Braconidae based on morphological characteristics (Quicke & Achterberg, 1990; Achterberg & Quicke, 1992; Wharton *et al*., 1992). However, some of these studies cannot propose a meaningful reclassification, due to low support levels obtained for most clades in most analyses (Belokobylskij *et al*., 2004), as well as homoplasy brought by the morphological characteristics (Quicke & Belshaw, 1999). There is also incongruence between morphological and molecular data (Zaldivar-Riverón *et al*., 2005). Therefore, many studies have been done in order to construct the phylogeny of Braconidae based on molecular data (Sharanowski *et al*., 2011; Michel-Salzat & Whitfield, 2004) as well as a combination of molecular and morphological data (Yaakop *et al*., 2009; Zaldivar-Riverón *et al*., 2005; Shi *et al*., 2005; Whitfield, 2002; Dowton & Austin, 1998).

To date, a number of studies regarding phylogenetic relationships among Braconids have been successful. The use of mitochondrial genes as molecular genetic markers is very useful in resolving phylogenetic relationships among closely related species (Lunt *et al*., 1996; Xie *et al*., 2006). For example, the mitochondrial ND1 gene was successfully used in the separation of different species due to rapid mutation rate at every third codon of the sequence (Michel-Salzat & Whitfield, 2004; Segura *et al*., 2006; Wan *et al*., 2011; Yaakop, 2009). Where else, the usage of mitochondrial 16S rRNA gene was also successful in molecular identification (Tang *et al*., 2012) including the identification of insects (Li *et al*., 2010; Guo *et al*. 2012). There are also records regarding the usage of the mitochondrial 16S rRNA gene that successfully resolved the relationships among braconid species **(**Dowton & Austin, 1994; Belshaw & Quicke, 1997; Whitfield, 1997; Dowton & Austin, 1998; Dowton *et al*., 1998; Mardulyn & Whitfield, 1999; Whitfield *et al*., 2002). However, these types of studies are still scarce in Malaysia. Thus , the objective of the present study was to investigate the use of phylogenetic tree construction based on mitochondrial 16S rRNA gene in confirming the identification of braconid species reared from fruit flies (Diptera: Tephritidae) infesting star fruits. Aside from that, this study might also add to the current data on the type of species attacking fruit flies infesting star fruits.

MATERIALS AND METHODS

Specimens

A total of 14 taxa originating from Kluang, Johor, Malaysia were selected for this study.

Table 1 represents a list of the analyzed taxa, institutional numbers, locality as well as their accession numbers. The specimens were reared from tephritid larvae collected at some branch campuses of the Malaysian Agricultural Research and Development Institute (MARDI). Slices of star fruits together with the tephritid larvae were placed in transparent plastic containers (24.5 cm x 13.5 cm x 13.0 cm) covered with cloth netting, and the base was lined with a layer of saw dust to provide suitable conditions for the transition process of larval into pupal stages. The insect cultures were maintained at constant room temperature of 23°C and relative humidity of 69% (pers. comm. Mrs . Suhana Yusof). The samples were preserved in 98 % ethanol before being analysed. Species *Aspilota vaga* Belokobylskij (2007) was used as an outgroup in the phylogenetic analyses. *A. vaga* is a parasitoid of the leaf mining Dipteran larvae, instead of the Tephritidae species.

DNA Extraction

DNA samples were extracted from three host Tephritidae by using the Qiagen kit (DNeasy® Blood & Tissue), based on the original guidelines. Individual braconids were also extracted for their DNA using the Qiagen kit (DNeasy® Blood & Tissue), but with modifications of steps one to three. The modified method known as the freezing method, allowed the isolation of DNA from the samples without damaging them so that they were still morphologically identifiable (Yaakop, 2009). 180 µl ATL buffer and 20 µl K proteinase were put into the centrifuge tube along with the sample. The samples were then incubated in a water bath at 55°C for 10 minutes before being mixed. They were then stored in a freezer at -20°C for two days. The DNA extraction then followed the extraction kit's original instructions.

PCR Amplification

Polymerase chain reaction (PCR) amplification was done by amplifying about 600 bp of the NADH1 dehydrogenase (ND1) and 400 bp of mitochondrial 16S rRNA genes from all genomic DNA for tephritids and braconids respectively. The oligonucleotide primers for amplifying tephritids sequences include foward Nad1-F5'-TAGTTGCTTGGTTGTGTATTCC-3' Nardi *et al*. (2005) and reverse Nad1-R5'-AGGTAAAAAACTCTTTCAAGC-3' Nardi *et al*. (2005), while for amplifying braconids sequences were 16S Wb5'-CACCTGTTTATCAAAAACAT-3' Dowton & Austin (1994) 16S outer5'-CTTATTCAACATCGAGGTC-3' Whitfield (1997).

Polymerase Chain Reaction (PCR) amplification for tephritid (ND1 region) was performed in 25 µl reaction volumes: 16.45 µl dd H_20, 2.5 µl PCR buffer 10X (Vivantis), 1.25 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl foward and reverse primers (20 pmol/µl), 0.3 µl *Taq* DNA polymerase (5 U/µl) (Vivantis), and 3 µl DNA template. The PCR's temperature profile consisted of an initial denaturation period of 5 min at 94°C, followed by 35 cycles of 94°C for 30s, 54°C for 1 min, and 72°C for 1 min 30s. A final extension step was added at 72 °C for 10

TABLE 1
List of braconids and tephritids taxa examined with UKM institution numbers, locality and accession numbers.

Label	UKM Institution No.	Species	Locality	Gene	Accession no.
34.1	UKM000087	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233524
34.2.2	UKM000089	*Psyttalia incisi*	Malaysia: Johor, Kluang, MARDI	16S	JX233531
34.3	UKM000090	*Psyttalia incisi*	Malaysia: Johor, Kluang, MARDI	16S	JX233530
34.4	UKM000091	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233525
34.5	UKM000092	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233526
34.6.2	UKM000094	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233527
34.7	UKM000095	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233528
34.8	UKM000096	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233529
34.9.2	UKM000098	*Psyttalia incisi*	Malaysia: Johor, Kluang, MARDI	16S	JX233532
34.9.3	UKM000099	*Psyttalia incisi*	Malaysia: Johor, Kluang, MARDI	16S	JX233533
34.10.2	UKM000101	*Fopius arisanus*	Malaysia: Johor, Kluang, MARDI	16S	JX233534
34.12.2	UKM000103	*Fopius arisanus*	Malaysia: Johor, Kluang, MARDI	16S	JX233535
34.13.2	UKM000105	*Fopius arisanus*	Malaysia: Johor, Kluang, MARDI	16S	JX233536
34.14.2	UKM000107	*Fopius arisanus*	Malaysia: Johor, Kluang, MARDI	16S	JX233537
FF 34.6	UKM000110	*Bactrocera papayae*	Malaysia: Johor, Kluang, MARDI	ND1	JX233538
FF 34.8	UKM000114	*Bactrocera papayae*	Malaysia: Johor, Kluang, MARDI	ND1	JX233539
FF 34.10	UKM000115	*Bactrocera papayae*	Malaysia: Johor, Kluang, MARDI	ND1	JX233540

min. PCR amplification was carried out for braconid (16S region) in 25 µl reaction volumes composed of 15.2 µl dd H_20, 2.5 µl PCR buffer 10X (Vivantis), 1.30 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl foward and reverse primers (20 pmol/µl), 0.5 µl *Taq* DNA polymerase (5 U/µl) (Vivantis), and 4 µl DNA template. The double stranded DNA was amplified in an Eppendorf Mastercycler using initial denaturation period of 3 min at 94°C, followed by 35 cycles of 94°C for 1m, 45°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 5 min was added. Three microlitres of each amplified PCR product was run for electrophoresis on 1.5% agarose gel, stained with ethidium bromide (ETBR), and visualized under ultraviolet light for the detection of the amplified DNA. The PCR product with the correct amplified DNA fragment size was purified using a purification kit (GeneAid) following the manufacturer's instructions. The purified products were sent to First Base Sdn. Bhd. for sequencing.

Sequence Alignment

The sequencing results underwent pairwise alignment using the BioEdit version 7.0.2. software (Hall, 2005) to produce the final sequence. Species names of the samples were then identified by comparing the final sequence with GenBank data through the internet software Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997, Tatusova *et al*., 1999).

Phylogenetic Analysis

The 16S gene sequences of the 14 samples were aligned using Clustal W under default parameter settings, together with the sequence of *Aspilota vaga* (JX233523). The sequences were then adjusted visually by referring to the secondary structures present. All sequences were analyzed using PAUP 4.0b10 for phylogeny reconstruction. The methods of analysis in PAUP included maximum parsimony (MP) with 1000 stepwise addition replicates in a heuristic search (Swofford, 2002). The MP tree was subjected to bootstrap analysis with 1000 replicates to obtain bootstrap value support and topology stability estimates. MEGA 3.1 was used to obtain transition and transversion ratio (Kumar *et al*., 2004), pairwise genetic distances as well as other characteristics of the DNA sequences.

RESULTS AND DISCUSSION

DNA sequences from 14 braconid samples were amplified, producing 401 bp each of 14 sequences. The results from the BLAST search of the tephritid sequences showed that the host species were *Bactrocera papayae* (Table 1). The BLAST results for braconids consisted of three species, namely *Diachasmimorpha longicaudata* (Ashmead), *Fopius arisanus* (Sonan) and *Psyttalia incisi* (Silvestri). Therefore, this present study based on 16S genetic markers and analyses of 14 specimens suggested that *P. incisi*, *F. arisanus*, and *D. longicaudata* appeared to be parasitoids of *B. papayae* infesting star fruits in Kluang Johor. Our molecular results supported the

rearing and morphology based findings of Chinajariyawong *et al*. (2000) but they reported more parasitoids attacking tephritids infesting star fruits namely *F. vandenboschi, P. makii,* and *P. fletcheri* which were not detected in the present study. The fact that these braconids are parasitoids of tephritid fruit flies was also supported by the studies of Stuhl *et al*. (2011), Bautista *et al*. (1999), and Guang-Hong *et al*. (2006). But, these researches done outside of Malaysia found *B. dorsalis* to be the host which was not the case here. In addition, there is a scarcity of records of braconids-tephritid relationships in Malaysia.

Characteristics of DNA Sequences

A total of 113 out of 385 sites were represented as variable sites. Of these, 91 of them were parsimoniously informative sites (80.5%), while the rest of them were parsimoniously uninformative (19.5%). Only 23% out of 400 characters were informative, indicating that the 16S gene is indeed a conserved mitochondrial gene. Among all of the sequences, 67.6 % of the characters represented conserved sites, indicating that these sequences were highly conserved. The nucleotide composition averages among all the sequences were 38.5% (T), 6.6% (C), 47.1% (A), and 7.8% (G). The highest nucleotide pair average was for AA (155), followed by TT (122), while the lowest was for GA (1). Additionally, no GT and GC pairs existed. Among these pairs, the average values of transitional (ti) pairs and transversional (tv) pairs are 9 and 37 respectively, resulting in a ti/tv ratio of 0.24.

Distance Analysis

Pairwise genetic distance was ascertained based on the Kimura-2-Parameter test (Pevsner, 2009). All positions containing gaps and missing data were eliminated from the dataset. The genetic distance between specimens of the same species was zero, indicating that there was no difference in their genetic compositions. For the outgroup, the genetic distance was the highest between the groups of *D. longicaudata* and *F. arisanus* with a value of 0.202, while the lowest was between *F. arisanus* and *P. incisi* which was 0.174 (Table 2).

The presence of variations among species could be detected even at the smallest genetic distance value. However, the distance analyses conducted showed that no genetic distances existed between individuals of the same species. Thus it was proven that there was no species variation

TABLE 2
Pairwise genetic distance between species; *D. longicaudata, P. incisi,* and *F. arisanus* based on the Kimura two-parameter method.

Distance	*D. longicaudata*	*P. incisi*	*F. arisanus*
D. longicaudata	-	-	-
P. incisi	0.181	-	-
F. arisanus	0.202	0.174	-

among the examined specimens of Opiinae.

Phylogenetic Inferences

A total of 136 most parsimonious trees were constructed by maximum parsimony (MP) analysis based on equally weighted total substitution. The best 136 lengths of MP tree constructed (Fig. 1) had consistency index (CI), homoplasy index (HI), and retention index (RI) values of 0.9044, 0.0956, and 0.9645 respectively. All of the *D. longicaudata* fell into clade A, while the rest were grouped into clade B. The *P. incisi* and *F. arisanus* lineages fell into different subclades. The phylogenetic bootstrap tree produced based on 1000 replicates was identical to the MP tree. The MP tree indicated that *D. longicaudata, P. incisi* and *F. arisanus* formed a monophyletic group. In addition, *P. incisi* and *F. arisanus* were closely related to each other. This statement was also supported by the low genetic distance value between them. They formed sister groups with high 74% bootstrap support. *D. longicaudata* was a sister group to both *P. incisi* and *F. arisanus* with 100% bootstrap value.

Instead of referring only to genetic distance analysis, the absence of species variation of individuals within the same genus emerging from the 16S sequences was further proven based on the constructed

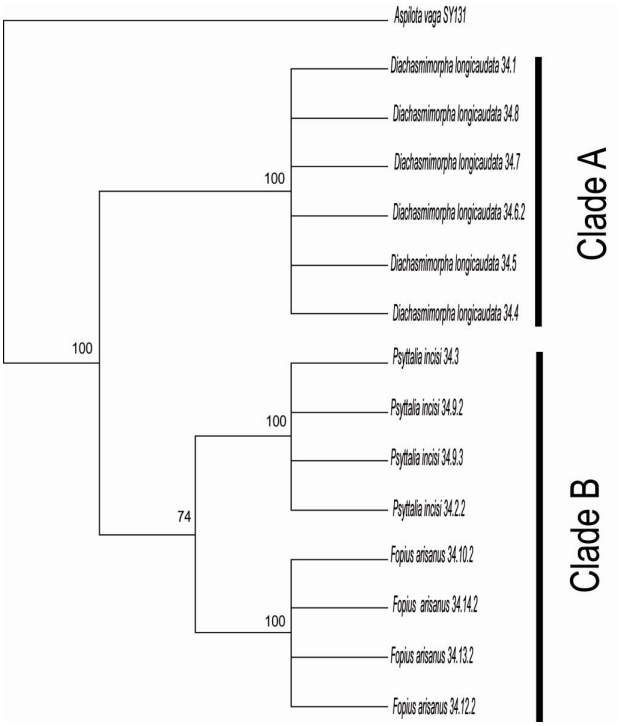

Fig. 1: The Maximum Parsimony tree for 14 species of Opiinae and rooted by outgroup *Aspilota sp.* (Hymenoptera: Braconidae: Alysiinae) based on 16S sequences. Numbers above branches indicate bootstrap values (%).

MP tree. The topology of this tree confirmed that there was no species variation as well as no mistake in the identification of the Opiinae parasitoids based on the molecular methods. The voucher specimens were also re-identified using a stereomicroscope StemiD4 and referring to a morphological genus key (Achterberg, unpublished) to confirm the species taxonomy, and this morphological-based identification matched the identification based on molecular methods using 16S rRNA marker.

CONCLUSIONS

The mitochondrial 16S gene was successfully used to group the Opiinae genus. Molecular approaches are useful for improving the identification of specimens identified initially based on morphological characteristics alone (Chinajariyawong *et al*., 2000; Belokobylskij & Ku, 1998; Aydogyu, 2008; Yilmaz & Beyarsian, 2009). Reconfirmation of the identity of parasitoids was successful, and it was found that *D. longicaudata* (Ashmead), *F. arisanus* (Sonan) and *P. incisi* (Silvestri) appeared to be potential biological control agents of *B. papayae*. Our findings although based on star fruits from a single locality are significant enough to confirm the identities of parasitoids of star fruit pests. However, future studies using star fruits from different geographical origins in Peninsular Malaysia as well as getting more emerging braconids from additional fruit species and using more genes should be done.

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Physico-Chemical Characteristics of Chok Anan Mango Fruit after Hot Water Treatment

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ABSTRACT

Mango fruit is prone to postharvest disease, especially anthracnose and stem end rot. Hot water dip (HWD) treatment has been used to control postharvest disease in fruit but little information about the response of Chok Anan mango fruit towards HWD treatment. This study was conducted to determine optimum duration of HWD in controlling postharvest diseases. Mature green Chok Anan mango fruit were treated at 26 and 55°C for 5, 15 and 25 min and fruits at ripening stage 1, 3 and 5 were analyzed for peel colour, flesh firmness, soluble solids concentration (SSC), ascorbic acid, pH, titratable acidity, disease incidence and heat induced injury. HWD treatment did not affect peel colour, SSC, ascorbic acid, pH and titratable acidity and induced heat injury to the fruit. Disease incidence of ripening stage 5 (fully ripened) fruit reduced significantly after HWD. In addition, the fruit underwent normal ripening as ripening progressed. It is concluded that combination of 55°C hot water for 5 min can be used as postharvest disinfestation treatment for Chok Anan mango fruit while maintaining physico-chemical characteristics of fruit.

Keywords: Anthracnose, disease incidence, heat injury, postharvest quality, peel colour

INTRODUCTION

Mango (*Mangifera indica* L.) belongs to family Anacardiaceae. It is originated from India. It has been cultivated in India since 4,000 years ago. It is one of the popular tropical and subtropical fruit as it contains high antioxidants such as ascorbic acid and carotenoids. Under tropical conditions, the fruits ripen rapidly and then follow by senescence. Mango fruits are susceptible to postharvest fungal pathogen infections with anthracnose and stem end rot as their major problems. The symptoms of infection do not develop until the fruit ripened.

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Colletothrichum spp. is the most important pathogens that cause this type of infection.

Current commercial fungicides such as benomyl and thiabendazole have been widely used to control postharvest pathogens. With the growing consumer awareness about safe food, chemical residues free methods to control postharvest pathogen have been a major focus. One of the safe method used to control postharvest pathogen is by dipping freshly harvested mango fruit in 55°C for 5 to 10 min and this treatment is known as hot water dip (HWD) (Coates *et al*., 1993). HWD treatment is regards as an effective non-chemical method to control postharvest diseases if combinations of suitable temperatures and exposure times being used and at the same time prevent the quality loss of produce (Lurie, 1998). The effectiveness of hot water dips as fungal pathogen control depends on location of fungal spores that are either on the surface or in the first few cell layers under fruit peel.

HWD treatment has been used to control postharvest disease while maintaining fruit quality in 'Kensington' (Jacobi *et al*., 2000), 'Keitt', 'Kent' and 'Tommy Atkins' (Mansour *et al*., 2006) mangoes but little information is available for Chok Anan mango fruit. Chok Anan mango is an important and popular variety in Malaysia. However, there is not much of attention given to this variety especially in postharvest treatment. Furthermore, although heat treatments have been used by many countries as non-chemical disinfection treatments, no single heat treatment has been found to be universally acceptable for all mango cultivars (Jacobi *et al*., 2001). From our literature search, 55°C of hot water for 5 min has been used by Thai to compare efficacy of yeasts antagonists, chitosan or their combination for controlling the severity of anthracnose lesions in Chok Anan mango fruit (Chantrasri *et al*., 2007). Unfortunately, they did not look into physico-chemical characteristics of Chok Anan mango fruit after hot water treatment. The information on fruit quality after hot water treatment is prerequisite in developing technologies. Therefore, the objective of this study was to determine optimum duration of HWD treatment using 55°C as non-chemical disinfection treatment for Chok Anan mango fruit while maintaining fruit quality.

MATERIALS AND METHODS

Plant Material

Chok Anan mango (*Mangifera indica* L.) fruits were obtained from Selangor Wholesale Market, Selangor at mature green stage or ripening stage 1. Fruits were selected for uniformity of shape, colour and size (200–300 g) and any blemishes or diseased fruit were discarded. The fruits were divided into two lots.

For the first lot, fruits were dipped in water at 26°C for 5, 15 and 25 min as control while fruits for the second lot were dipped in heated water of 55°C for same durations. After dipping fruit in 26 and 55°C, fruit were showered with ambient temperature water (26°C) for 20 min and then allowed to air dry before the fruits were packed in a carton and gassed with 0.02 ml acetylene generated from calcium carbide (CaC_2)

at the rate of 20 g CaC_2/kg fruit. Cartons containing the fruit and CaC_2 were placed in a room of 26°C with relative humidity of 75-80% for ripening initiation. After 48 h, the cover of the carton was removed and fruit were allowed to continue ripening until the peel turned full yellow. The fruits of ripening stage 1 (mature green), 3 (50% green – 50% yellow) and 5 (100% yellow) were analyzed. A total of 250 fruits were used for the experiments which repeated four times.

Selected Quality Characteristics

Peel Colour Determination

Peel colour of Chok Anan mango was determined using a chroma meter (Model CR-300 Minolta Corp., Osaka, Japan). The meters were equipped with a measuring head that has an 8-mm-diameter measuring area and calibrated with a standard white tile. Calibration values were L^*=97.95, a^* = -0.07 and b^* = 1.66 using the illumination (CIE 1976). From a fruit, three readings at equatorial region were recorded and mean value was computed. Measurements were expressed in chromaticity values of L^*, C^* and h°.

The L^*, indicates the lightness, with values ranging from 0=black to 100=white. The C^* values refer to the vividness of colour were computed from values of a^* and b^* i.e. $C^* = (a^{*2} + b^{*2})^{1/2}$ which represented the hypotenuse of a right triangle. Hue angle (h°) was calculated as $\tan^{-1}$ b*/a*.

Flesh Firmness Determination

The firmness of the fruit was evaluated using a bishop penetrometer FT 327 (Italy) with an 11-mm-diameter plunger. Forces from constant penetration of the plunger were applied perpendicularly to the 1 cm of peeled mango fruit which was cut from equatorial region of a fruit with a smooth motion in two to three seconds. The readings in kilograms-forces were made at two opposite direction of every slice of the mango fruit and were converted to newton (N).

Titratable Acidity (TA) Determination

The TA of the fruit was determined by slicing out 10 g of the Chok Anan mango fruit. Forty milliliter of distilled water was then added to the 10 g of fruit and blended in a high-speed blender (Model MX V2 National) for one min. The macerate was filtered with cotton into a conical flask. After that, 5 ml of filtrate was titrated with 0.1 mol/ml sodium hydroxide. Three drops of 1% phenolphthalein indicator was added into filtrate. The indicator added filtrate was then titrated until it's turned pink. From the titre, the percentage of citric acid was calculated.

% Citric acid
= [(ml NaOH × 0.1 ml/weight of sample titrated) × 0.64]

Soluble Solids Concentration (SSC) Determination

The SSC of mango fruit were determined using a hand refractometer (Model N1, Atago, Japan). The refractometer was

calibrated with distilled water until the reading reached 0 . A drop of the extracted juice from the remainder of TA determination was then placed on the prism glass of refractometer to obtain the %SSC reading. The readings were corrected to a standard temperature of 20°C by adding 0.28% to obtain % SSC at 27°C.

pH Determination

The remainder of the juice from the TA determination was used to measure pH using glass electrode pH meter (Model Crison Micro pH 2000). The pH meter was calibrated with buffers at pH 4.0 and 7.0 before being used.

Ascorbic Acid (AA) Determination

The AA contained in the Chok Anan mango fruit was determined by using titration method. Ten gram of mango fruit was blended with 3% cold HPO_3. Then five milliliter juice sample was titrated with dye until the juice changed to pink colour. Volume of dye (titre) used was recorded and vitamin C (mg/100 g) was calculated as follows:

Vitamin C (mg/100 g)
= [Titre (ml) × dye factor × volume made up (ml) × 100] / [Aliquot used for estimation (ml) × weight of sample (g)]

To standardize the dye, 5 ml of standard ascorbic solution was taken and 5 ml of 3% cold HPO_3 was added. The mixture was titrated with the dye solution to a pink colour. The dye factor was determined as follows:

Dye factor
= µg acid ascorbic / ml of dye (titre)
= 0.5 / ml dye (titre)

Disease Incidence Assessments

Disease incidence was assessed at ripening stages 3 and 5 during fruit ripening. The severity of disease was assessed after treatment according to the percentage of peel area affected by disease per fruit and then the percentage score was related to a 5-point scale where 0= 0%; 1= 1-5%; 2= 6-15%; 3= 16-30% and 4= 31-100% of peel area affected by disease (Ding & Ong, 2010).

Heat Induced Injury Assessment

The severity of heat induced injury was assessed after treatment according to the percentage of peel area affected by heat per fruit. The symptom of peel injury induced by hot water treatment included translucence, shriveling, dimples, brown discoloration and decay. Mango fruits were sorted into 5-point scale according to their heat injury severity where 0= 0%; 1= 1-5%; 2= 6-15%; 3= 16-30% and 4= 31-100% (Ding & Ong, 2010).

Statistical Analysis

The experimental design was a randomized complete block design with a factorial arrangement of treatments (two water temperatures × three dipping times × three ripening stages) and four replications. Data were analyzed using the analysis of variance (ANOVA) (SAS Institute, Cary, N.C. 1989). When the F values showed significant ($P \leq 0.05$) differences, least significance

difference (LSD) test was used to separate the means. Data for the disease incidence and heat induced injury were transformed into $\log_{10}$ prior to analysis.

RESULTS

The peel colour of Chok Anan mango fruit was not significantly affected by interactions with an exception for hue angle where the three factors were substantially interacted among water temperature x dipping time x ripening stage (Table 1). The insignificant effect of peel colour was also extended to main effect except for the ripening stages. The L^* and C^* values of Chok Anan mango fruit increased significantly as ripening progressed. In contrast, h^o values of Chok Anan mango fruit peel showed an opposite trend to L^* and C^* values with decreasing values as ripening progressed from stage 1 to 5.

There were no significant interactions effects between the factors in flesh firmness of Chok Anan mango fruit (Table 2). However, flesh firmness was affected significantly by water temperature and

TABLE 1
Effects of two water temperatures, three dipping times and three ripening stages on peel colour (L^*, C^* and h°) of Chok Anan mango fruit

Factor	Peel colour		
	L^*	C^*	h°
Dipping temperature (W), ^oC			
26	62.42	40.83	101.00
55	63.13	40.65	100.93
F-test significance	NS	NS	NS
Dipping time (D), min			
5	62.61	40.53	100.72
15	62.87	40.44	101.64
25	62.84	41.25	100.25
F-test significance	NS	NS	NS
Ripening stage (R)			
1	52.00 c[z]	29.85 c	121.44 a
3	65.02 b	42.34 b	94.65 b
5	71.30 a	50.04 a	86.78 c
F-test significance	**	**	**
Interaction			
W x D	NS	NS	NS
W x R	NS	NS	NS
D x R	NS	NS	NS
W x D x R	NS	NS	*

L^* = lightness, C^* = chroma and h^o = hue angle.
[NS], *, ** Non significant or significant or highly significant at $P \leq 0.05$.
[Z]Mean separation within columns and factors followed by the same letter are significantly different by LSD at $P \leq 0.05$.

TABLE 2
Effects of two water temperatures, three dipping times and three ripening stages on firmness (N), soluble solids concentration (SSC), ascorbic acid (AA), pH and titratable acidity (TA) of Chok Anan mango fruit

Factor	Firmness (N)	SSC (%)	AA (mg/100 g)	pH	TA (% citric acid)
Water temperature (W), ˚C					
26	3.31	11.01	24.40	4.46	0.83
55	3.83	10.01	25.26	4.41	0.90
F-test significance	*	NS	NS	NS	NS
Dipping time (D), min					
5	3.65	10.47	25.55	4.45	0.97
15	3.59	10.58	24.68	4.41	0.78
25	3.47	10.48	25.77	4.45	0.84
F-test significance	NS	NS	NS	NS	NS
Ripening stage (R)					
1	9.56 a[z]	3.68 c	29.60 a	3.64 c	1.30 a
3	0.92 b	13.09 b	24.06 b	4.64 b	0.82 b
5	0.22 c	14.77 a	22.34 c	5.02 a	0.47 c
F-test significance	**	**	**	**	**
Interaction					
W x D	NS	NS	NS	NS	NS
W x R	NS	NS	NS	NS	NS
D x R	NS	NS	NS	NS	NS
W x D x R	NS	NS	NS	NS	NS

[z]Mean separation within columns and factors followed by the same letter are significantly different by LSD at P≤ 0.05.
NS, *, ** Non significant, significant or highly significant at P≤0.05, respectively.

ripening stage. Flesh firmness of Chok Anan mango fruit dipped in 55˚C was firmer as compared to the control. As fruit ripened, firmness of fruit encountered significant decrease. SSC for Chok Anan mango fruit was not affected by interactions between water temperature and dipping time (Table 2). However, the SSC was significantly affected by ripening stage with 301% increase when fruit ripened. Similar to SSC, AA, pH and TA of Chok Anan mango fruit was not affected by interactions, water temperature and dipping time (Table 2). However, with the advancement of ripening stage, AA content and TA of fruit decreased significantly while pH increased.

Disease incidence of Chok Anan mango fruit was affected by significant interaction between water temperature and ripening stage (Table 3). Disease incidence occurred in fruit either dipped in 26 or 55˚C (Fig.1). Nonetheless, fruits treated with 55˚C water only started to show disease incidence when at ripening stage five. Control fruit was infected by pathogen as early as ripening stage 3 and became more serious when at

TABLE 3
Effects of two water temperatures, three dipping times and three ripening stages on disease incidence and heat induced injury of Chok Anan mango fruit

Factor	Disease incidence	Heat induced injury
Water temperature (W), °C		
26	0.19	0.01
55	0.01	0.03
F-test significance	**	NS
Dipping time (D), min		
5	0.11	0.00
15	0.10	0.03
25	0.10	0.03
F-test significance	NS	NS
Ripening stage (R)		
1	0.00 c[z]	0.00
3	0.05 b	0.02
5	0.26 a	0.04
F-test significance	**	NS
Interaction		
W x D	NS	NS
W x R	**	NS
D x R	NS	NS
W x D x R	NS	NS

[z]Mean separation within columns and factors followed by the same letter are significantly different by LSD at P≤ 0.05.
NS, ** Non significant or highly significant at P≤0.05, respectively.

stage 5. By ripening stage 5, fruits treated with 55˚C of hot water had successfully inhibited the occurrence of disease by 94% as compared to the control. Although the disease incidence has been suppressed tremendously by hot water, the heat did not cause any injury to Chok Anan mango fruit as found in Table 3.

DISCUSSION

The peel colour, SSC, AA, pH and TA of Chok Anan mango fruit was not affected by water temperatures, dipping times and their interactions (Tables 1 & 2). As ripening stage advanced, the peel colour showed significant increase in lightness and chromaticity with decrease hue values which reflected colour changed from green to yellow. Similar to peel colour, the firmness, SSC, AA, pH and TA of Chok Anan mango fruit also showed significant changes as ripening stage advanced. With the significant changes in these quality characteristics, Chok Anan mango fruit became palatable.

The ability of a fruit to undergo normal ripening is a main concern when using heat

in postharvest chain besides controlling and/or reducing disease incidence (Jacobi *et al*., 2001; Paull & Chen, 2000). Jacobi *et al*. (2001) has reviewed different varieties of mangoes response distinctly to various combination of heat temperature and exposure time. They even ranked different cultivars of mango according to its heat tolerance in their review. 'Irwin', 'Kensington', 'Haden' and 'Strawberry' mangoes are the least heat tolerant cultivars as compared to 'Haden', 'Davis Haden', 'Pahiri' and 'Alphonso' mangoes. Some of the mangoes showed uneven skin development when ripening while some showed fruit softening after heat treatment. However, in the present study, Chok Anan mango fruits did not have these problems after HWD treatment. This indicated that Chok Anan mango could tolerate 55°C hot water for 25 min. It is reported that the influence of heat on postharvest fruit ripening is also dependent on level of field-induced thermotolerance besides differences in cultivar (Paull & Chen, 2000). Chok Anan mango fruit has been exposed to hot and humid field condition throughout its growing season. Therefore, it is expected that this variety of mango could tolerate high postharvest heat treatment compared to other variety of mango planted in subtropical regions. Unfortunately, besides this report, there is no other reports have been found on heat tolerance level of Chok Anan mango fruit whether it could withstand temperature beyond 55°C or not and/or with extended exposure time.

Besides mangoes, there are other species of fruit been treated with hot water which responded differently towards water temperature and exposure time used. Thompson Seedless table grapes dipped in

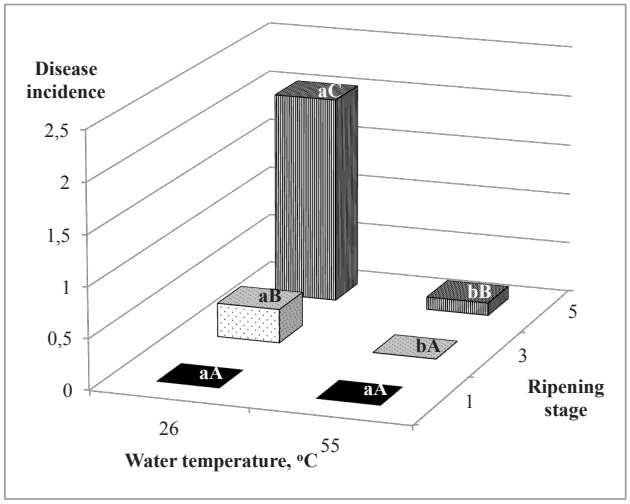

Fig.1: Effects of water temperature and ripening stage on disease incidence of Chok Anan mango fruit. Means followed by the different small and capital letters denote significant differences within water temperatures and among ripening stages, respectively ($P \leq 0.05$, LSD).

30, 40 and 50°C hot water for 3 min and stored for 1 month at 1°C, did not show any differences in peel colour among the temperatures used (Karabulut *et al*., 2004). Litchi dipped in 52°C hot water for 1 min did not show any significant difference from control in terms L^* and C^* values after 49 days of storage at 5°C (Olessen *et al*., 2004). SSC of 'Kent' mango fruit was higher after treatment with hot water at 50°C for 5 min (Mansour *et al*., 2006). For sapote mamey fruit, HWD treatment caused lower SSC than control after 4-day of ripening (Diaz-Perez *et al*., 2001). While in strawberry, SSC was not affected by HWD treatment (Zhang *et al*., 2007). Similar to the finding of this study, AA and TA of strawberry were not affected by hot water treatment. However, HWD treated sapote mamey has higher TA and lower pH as compared to the control (Diaz-Perez *et al*., 2001). 'Hom Thong' banana treated with 50°C hot water for 10 min only showed significant higher AA than control at day 2 during 10 days of storage at 25°C (Ummarat *et al*., 2011). While the rest of storage days did not show any differences in AA as compared to the control.

Heat treatment has inhibited softening of Chok Anan mango fruit (Table 2). This indicated that HWD Chok Anan mango fruit took longer time to soften and thus prolong its shelf life. This characteristic is desired by retailers. A contrary finding was reported in HWD 'Tommy Atkin', 'Keitt', 'Palmer' (McGuire, 1991) and 'Kensington' mangoes (Jacobi and Giles, 1997). These researchers reported that the softening of mangoes fruits were accelerated after heat treatment. For strawberry, HWD did not affect fruit softening even after 3 days of storage at 20°C (Zhang *et al.,* 2007). The firmness of sapote mamey fruit after HWD at 60°C for 60 min followed by 4-day ripening at 25°C was higher than control (Diaz-Perex *et al.,* 2001). Again, the response of fruit towards HWD treatment depended on variety and species of fruit.

Heat treatment gives to fruit during postharvest handling is a kind of stress. The severity of stress is determined by temperature and exposure time given (Lurie, 1998). Difference responses of fruit to temperature and exposure time are related to the level of heat protective proteins at harvest and postharvest production of heat shock protein (Paull & Chen, 2000). Heat shock protein is believed to act as chaperones that are responsible for protein refolding under stress conditions (Wang *et al*., 2004). Chok Anan mango fruit could undergo normal colour changes even though has been treated with 55°C water for 25 min. It has been reported that the change of fruit peel colour is associated with enzymatic degradation of green chlorophyll and synthesis of yellow carotenoid (Ding *et al.*, 2007). Enzymes are well known for its heat sensitive, but in this study the enzymatic reaction involved in colour changes of Chok Anan mango fruit was not affected by heat treatment.

However, this was not true for enzymes involved in Chok Anan mango fruit softening. The firmness of hot water treated Chok Anan mango fruit has been retained. Paull & Chen (2000) have summarized

flesh softening is temperature dependent with slow softening at 38-40°C and faster or disrupted after exposure to 45-50°C of heat. This finding may not apply to all types of fruit. The softening of *Musa* AAA Berangan ripened at 37°C was retained while fruit ripened at 25°C showed faster ripening (Ratule *et al*., 2007). They found out banana ripened at 25°C showed a more advanced dissolution of pectin in the middle lamella than fruit ripened at elevated temperature of 37°C. Most probably the dissolution of pectin in heat treated Chok Anan mango fruit is slower than control fruit. Thus, fruit treated with 55°C hot water has higher firmness than control. Hydrolytic enzymes are needed to dissolve cellulose, hemicellulose and pectin in cell wall which lead to softening. However, the actual enzymes having the central role in softening of heat treated fruit have not been determined yet (Paull & Chen, 2000).

Postharvest pathogen of Chok Anan mango fruits were reduced significantly after treating with 55°C hot water (Table 3). On top of this, the heat used in present study did not cause any injury to fruit. The non-significant effect in heat induced injury to fruit tissue caused by water temperature, dipping time, ripening stage and their interactions, again showed that 55°C can be used to treat Chok Anan mango fruit. Since varying dipping time from 5 to 25 min did not affect the efficacy of heat treatment, it is suggested that the combination of hot water 55°C and 5 min dipping time is sufficient to be used to control postharvest pathogens in Chok Anan mango fruit while retaining fruit quality.

Heat treatments, as one of the disinfestation methods, have been widely used to control disease in mango industry (Jaboci *et al*., 2001). The treatments require mango fruit to be heated to a specific core temperature for a defined period (Paull & McDonald, 1994). Heat is transferred via energy from a heating medium, which is water in this study. After treating Chok Anan mango fruit at 55°C for 5, 15 and 25 min, the fruit core temperatures were 40.08, 48.10 and 51.74°C, respectively (unpublished data). Before heat treatment, the initial core temperature of fruit was 26.55°C. The energy of hot water was transferred from peel (exocarp) then into flesh (mesocarp). Along the energy transfer, fungal infection sites that contain mycelium and spores were reached and killed by the heat. After dipping for 25 min, the fruit core temperature was 51.74°C and yet fruit did not show any symptoms of heat injury. Apparently, Chok Anan mango fruit could tolerate core temperature up to 51.74°C.

Another reason for the success in controlling postharvest pathogen by heat treatment is 'melting' of the cuticular wax which sealed micro-cracks, stomata and/or lenticels that appeared on fruit surface. The peel of a mature mango fruit is composed by a single layer of epidermis with lenticels and a well-defined cuticle with wax deposition (Muhammad & Ding, 2007). Micro-cracks can easily be found among cuticular platelets of mango fruit. These opening structures are important invasion site for pathogens. As reported in 'Oroblanco'

grapefruit, 'Fortune' mandarins, cactus pears and organically grown grapefruit, after heat treatments, the wax platelets melted and eventually lead to covering and sealing of these openings (Schirra *et al*., 2000). This provided mechanical barrier against pathogen. Also, germinated spores, conidia and hyphae appeared covered and mummified by molten wax as occurred in cactus pears that were subjected to curing at 37˚C for 30 h (Schirra *et al*., 1999). However, such beneficial effects may be stalled during prolonged shelf life as cracks tend to reappear and damaged stomata may attract hyphae penetration. The melting point of mango wax is 62˚C (Panhwar, 2005) and thus the 55˚C of hot water used in the present study is able to 'melt' some of the wax. The melted wax could have covered and sealed the lenticels and micro-cracks of Chok Anan mango fruit. Therefore, the disease incidence was not present in ripening stage 3 of hot water treated fruit but not in control fruit (Table 3).

The occurrence of disease incidence in ripening stage 5 of hot water treated Chok Anan mango fruit indicated HWD treatment is not able to inhibit the growth of pathogen completely (Table 3). Most probably more than one type and/or species of pathogen exist in the fruit. It has been proven that the sensitivity of pathogens to temperature and exposure time varied according to pathogen species as found in crown rot of banana cv Bungulan (Alvindia, 2012). Generally, the mycelium growth and spore germination of crown rot-causing pathogens was slower at a combination of higher water temperature and longer exposure time. However, the tolerance of fruit to hot water should take into consideration during using high water temperature and long exposure time. It is advisable to use temperatures less than the lethal temperatures and duration so that short-term disruption of transcription and translation steps in protein synthesis can be reversed (Paull & Chen, 2000).

It has been reported that the response of fruit to heat varies with species, genotypes within species, physiological stage or fruit maturity, fruit size and morphological characteristics, exposure to different environmental and/or preharvest factors (such as rainfall, soil type and production practices), the type of heat treatment applied, heat transfer rate and energy balance (thermal difference, heat capacity and relative humidity), final temperature and the duration of exposure at different temperatures, and whether postharvest conditioning treatments have been given before and after a heat treatment (Jacobi *et al*., 2001; Paull & Chen, 2000). In short, there is no single heat disinfestation treatment has been found to be applied for all mango cultivars including other fruits as a lot of factors involved in determining the success of the treatment.

From the findings of the present study, it can be concluded that postharvest pathogen of Chok Anan mango fruit can be controlled using 55°C hot water for 5 min without having to compromise the postharvest qualities of the fruits with further improvement of firmness. This report provided basic understandings on Chok Anan mango

fruits response to 55°C hot water dipped for 5, 15 and 25 min. Since there are so many factors affecting the success HWD treatment, knowledge of basic principles of physiological and biochemical responses of mango fruit to temperature stress is essential. In future, a more comprehensive work using different hot water temperatures, type of heat treatment, duration of heat exposure and even different physiological state of fruit should be carried out to provide a solid foundation of information to possibly predict Chok Anan mango fruit response to heat treatment. In order to market this fruit internationally, this information is needed to enable the development of effective strategies for heat treatment since the number of health conscious consumers is growing every year.

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Freezing Method as a New Non-Destructive Modification of DNA Extraction

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ABSTRACT

A modification of a DNA extraction method by freezing specimens is recognized as one of new non-destructive techniques. In this study, the freezing method has been applied on dried and fresh, tiny and economically important insect samples, i.e. on adults and larvae of wasps, fruit flies and thrips. The modification entails freezing instead of a lengthy incubation of the sample. Most importantly, the sample is not cut into small pieces, but is soaked in a lysis buffer and then frozen in -22°C for a minimum of 20 minutes. After that, the remaining protocols from the manual of DNeasy Blood and Tissue Kit are followed. Several other non-destructive methods also require incubation for at least 20 minutes in a lysis buffer at 55°C. However, the duration of that incubation process is not standard for all insect and arthropod species. This is because the optimization process is based on species size and the thickness of the insect cuticle. With the freezing method, samples are not damaged, and remain available for morphological re-examination. Hence, the sample can also be re-used for taxonomic work with no distortion of samples, no loss of coloration and no phenotypic changes on the external morphology. The complete protocol for the freezing method is described in this paper. With this freezing method, DNA concentration of 0.2-5.61 ng/µl was recovered on various tiny insect species. Furthermore. several specimens of *Bactrocera* and *Heratemis* species were selected as control specimens in analyzing a variety of extraction methods. The freezing method was proven as a new technique to obtain sufficient quantity and a high quality of DNA for molecular work.

Keywords: Freezing method, molecular DNA, non-destructive samples, sufficient DNA, phylogenetic analysis.

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INTRODUCTION

Describing a new species based on a single individual is a valid procedure in taxonomy, but should be avoided if possible. For tropical taxa, there is often no other option or opportunity to obtain more specimens. There are several published taxonomic descriptions based on a single specimen or small series of type specimens (Nitz *et al*., 2009; Schmidt-Rhaesa, 2001; Targino & Wild, 2009). This situation becomes more complicated and difficult if that single type specimen is required for molecular studies. From the point of view of a taxonomist who needs to study shape and form, molecular studies are a nightmare as destruction of the whole body or the sacrifice of some parts of a unique specimen for DNA extraction is required. Morphological characters have traditionally been used in defining species; more recently, the use of molecular data has become regular practise (Johnson *et al.*, 2009; Salvo *et al*., 2011; Smith *et al.*, 2003). Both methods seem useful in phylogenetic studies to show similarity or to resolve conflict between two different data (Friedrich *et al*., 2006; Hillis 1987; Whiting *et al*., 1997), and provide an additional approach to resolving conflicts arising from external morphology. Molecular tools are now widely used in species identification (Göker *et al*., 2009) particularly because morphological characters present often limited data for phylogeny, so molecular data is especially helpful and informative in resolving species relationships.

In the molecular process, DNA extraction is the most important technical step to obtain because the procedure affects the quality of DNA. For this reason, the appropriate extraction method and techniques specific to the intended purposes must be identified. One of the main aims of choosing the right extraction technique is to maintain the voucher specimen for taxonomy and to make sure the specimen remains externally as complete as before the extraction process. Although the samples may not be classified as ancient museum samples, the right techniques have to be applied to maintain as complete a structure as possible so as to preserve it for further use. Thus, some modification of the usual extraction method should be considered to maximize the use of the voucher specimen after extraction; this modification will be presented in this study.

Several molecular procedures are available that enable DNA to be obtained from samples without causing morphological damage (Gillbert *et al.*, 2007; Hofreiter, 2012; Rohland, 2012). Usually, the whole insect body or entire body part (e.g hind leg, antenna etc.) are extracted to obtain DNA. Even if a small portion of an insect body is used for extraction, that method is not the best option since a complete voucher specimen is very important and highly necessary for description or re-examination (Yaakop *et al*., 2009, 2010). Furthermore, small and fragile body parts of minute insect samples e.g. thrips, small braconid species (alysiines, opiines) may accidentally be destroyed during the process of removing appendages for DNA extraction.

There are several published papers on non-destructive DNA extraction for insects and other arthropods without any obvious alteration on the morphological characteristics (Castalanelli *et al*., 2010; Dittrich-Schröderet *et al*., 2012; Favret, 2005; Gilbert *et al.*, 2007; Hunter *et al.*, 2008; Pons 2006; Rowley *et al.*, 2007; Thomsen *et al.*, 2009). However, each paper has provided a non-destructive DNA extraction method, but do not seem as efficient as the novel proposed freezing method because the methods provided may require a longer incubation process, invite contamination and require maceration of samples. The freezing method use the commercial kit with some modifications and has proven to be successful for extraction and analysis on several insect samples.

Currently, only limited insect samples deposited in museums are used for extraction. This might be due to the limited number of samples available for similar taxa, or concern regarding damage to the samples and loss of body parts. Very small insect specimens can be damaged during processing or if parts must be removed for molecular work. Therefore, some modification of the typical extraction process is really needed in order to improve and obtain DNA for molecular work. By applying the freezing method, tiny insect samples will remain intact and complete in structure. The extracted voucher specimens can also be kept in the museum repository as a holotype, while the DNA sequences are stored in GenBank. In addition, the voucher specimens can be used again after the extraction process for future studies. The main goal of this study is to document a new modification of the DNA extraction designated as the freezing method, on several minute insects.

MATERIALS AND METHODS

Insect specimens

Fresh insect samples which were preserved in 90% alcohol and dried museum specimens (collected since 1986) were tested with the freezing method . These samples consisted of several insect Orders e.g. adults and larvae of Hymenoptera (braconids), Diptera (tephritids) and Thysanoptera (thrips). A total of 52 individuals of various sizes (0.5-3.0 mm) were used in this study. Three *Bactrocera* larvae which were more or less similar in size were selected as controls. Each specimen was extracted using 1) a destructive method followed by incubation at 55°C until the specimen is completely lysed; 2) a non-destructive method, without freezing and with incubation of the sample at 55°C overnight; and 3) a non-destructive method, with freezing and without incubation at 55°C of the samples. In addition, two individuals of *Heratemis* sp. were selected as control specimens using the first method of DNA extraction.

DNA extraction

The insect samples were completely immersed and soaked in microtubes with distilled water for 3 days in order to wash them (except for thrips samples). [Fresh thrip samples used in this study were washed

several times by rinsing them in absolute alcohol before being soaked it in proteinase K]. Specimens were then dried and DNA extraction was carried out using the DNA isolation Kit, DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, U.S.A.). The manufacturer's steps one and two were modified. According to blood and tissue extraction protocol, samples should be cut into small pieces and placed in 180 ml of buffer ATL + 20 ml of proteinase K, then the sample has to be incubated at 55°C, followed by the remaining general protocol. However, with the freezing method, the sample was soaked with 180 ml of buffer ATL + 20 µl of proteinase K without destroying it (it was not cut into pieces) and then kept in a freezer at -22°C until totally frozen (for a minimum time of 20 min). After that, the remaining general protocol was carried out; with 200 µl Buffer AL and vortex for 15s added to it. 200 µl ethanol (96-100%) was then thoroughly mixed into it again. The mixture was pipetted into a DNeasy Mini spin column in a 2 ml collection tube and centrifuged at ≥ 6000 *x g* (8000 rpm) for 1 min. The flow-through and collection tube were then discarded. The spin column was placed in a new 2 ml collection tube and 500 ul Buffer AW1 was added and centrifuged for 1 min at ≥ 6000 *x g*. The flow-through and collection tube were again discarded. The previous step was repeated, but this time 500 µl Buffer AW2 was added and centrifuged for 3 mins at 20,000 *x g* (14 000 rpm). The flow-through and collection tube were discarded again. Finally, the spin column was carefully removed to ensure that DNA did not come into contact with the flow-through. A new 1.5 ml or 2 ml microcentrifuge tube was transferred to the spin column by adding 200 µl Buffer AE for elution. The sample was incubated for 1 min at room temperature and centrifuged for 1 min at ≥ 6000 *x g*.

DNA concentration measurement and PCR analysis

The DNA concentration was measured using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer after DNA extraction and purification. The extracted samples were then analyzed with PCR. The conditions for PCR analysis varies between species. A total of 25 µl of PCR used contains 0.5 µl of 0.2 mM DNTPs, 10 pmol of each primer, 1.25U of Taq polymerase, and 1µl of 15 mM $MgCl_2$ from Vivantis. The PCR was performed using MyGene MG96G Thermalcycler or Thermocycler Perkin Elmer 240 under different conditions for each primer combination, starting with denaturation for 3 mins at 94˚C, followed by 39 cycles of denaturation for 1 min at 92˚C, annealing for 15 sec-1 min at 45-62˚C, extension for 1 min at 72˚C and final extension for 5 mins at 72˚C. Several sets of primer combinations of 28S, COI and ND1 markers are used in this study. The list of primers, the anneal temperature and duration for PCR analysis are presented in Table 2-3.

Sequencing and BLAST analysis

PCR products for each species were then sent to Macrogen Inc., Korea and First Base Company, Selangor, Malaysia for

sequencing. The status of the species was confirmed using BLAST search and then they were used in the phylogenetic analyses. Prior to that, the sequences were edited using Sequencher 4.8 and aligned using MacClade 4.08.

Quality and Efficiency of the Extraction Process

The quality and efficiency of the freezing method were measured based on comparison of eight DNA sequences of *Bactrocera* (adults and larvae). The phylogenetic analyses used were based on earlier references on constructing phylogeny (Yaakop *et al*., 2009, 2010). In this study, the phylogeny of *Bactrocera* is presented (Fig.4). The DNA used was obtained from the larvae and adult of *Bactroceracarambolae* Drew and Hancock by using the freezing method (MARDI-sample 0E, F, 0I, FF) (Table 1).

For phylogenetic analysis, the maximum parsimony (MP) tree(s) PAUP* 4.0- test version 4.0d63 (Swofford 1998) was used to get the most parsimonious tree(s). A heuristic parsimony search (Hillis *et al.* 1996) was performed using 100 replicates of random addition sequences, including the TBR (tree bisection reconnection) option for branch swapping. Each base was treated as an unordered character with equal weight, with gaps treated as missing data. Statistical support was obtained by bootstrap analysis with 100 replications (Felsenstein, 1985).

Photograph specimens

Extracted specimens of braconids, thrip and larvae of tephritids (Fig.1 to Fig.3) using the freezing method were photographed with a Stereomicroscope Stemi-D4 (braconids and tephritid's larvae) and Olympus BX41 Universal Transmitted DIC microscope (thrips) attached to a Canon camera digital EOS 1000D DSLR.). Photographing is essential to compare the specimens prior to and after DNA extraction. It is also important to have a control in case specimens are lost or misslabelled.

RESULTS AND DISCUSSION

The PCR amplification based on the COI marker of three of the *Bactrocera* samples (controls) that were extracted using the freezing and non-freezing method was successful, but each contained different concentrations of DNA. Of the three methods in the control experiment, we found that the destructive method showed the highest concentration of DNA, 22 ng/µl. However, there were no specimen remains left as vouchers. The second highest concentration obtained was from the specimen that used the non-destructive method and was then incubated at 55°C overnight. That method successfully collected 14.5 ng/µl of DNA. However, it required a long incubation process and was more time consuming. The lowest concentration of DNA (0.07 ng/µl) was obtained with the non-destructive method without incubation at 55°C and without freezing. However, the DNA concentration extract edusing the freezing method was between 1.54-5.61 ng/µl for the *Bactrocera* larvae and 0.2-5.61 ng/µl for the other insect species used in this study (Table 1), and required a minimum

TABLE 1
List of sample used with institute code, locality, accession numbers and DNA concentration after extraction.

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
1.	New genus near *Coelalaysia* SY24*	RMNH100044 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534336	EF535636	EF534349	EF535624	1.3
2.	*Phaenocarpa* sp. 2104*	RMNH100045 (Dried)	Malaysia: Hutan Kuala Lompat	EF534337	EF535637	EF534350	EF535625	0.9
3.	*Cratospila* sp.SY21*	RMNH100046 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534326	EF535626	EF534339	EF535615	0.76
4.	*Heratemis pahangensis* 2147*	RMNH100047 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534334	EF535634	EF535347	EF535622	2.4
5.	*Heratemis pahangensis* 2144*	RMNH100048 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534333	EF535633	EF534346	EF535621	1.6
6.	*Heratemis devriesi*SY7 *	RMNH100049 (Fresh)	Malaysia: Hutan Kuala Lompat	EF534335	EF535635	EF534348	EF535623	2.5
7.	*Heratemis filosa* SY50*	RMNH100050 (Fresh)	Malaysia: Penang, Telok Bahang	EF534338	EF535628	EF534341	EF535617	0.84
8.	*Heratemis filosa*SY6*	RMNH100051 (Fresh)	Malaysia: Pahang, Cameron Highlands	EF534328	-	-	EF535618	0.77
9.	*Heratemis malayensis* SY44*	RMNH100052 (Fresh)	Malaysia: Perak, Bukit Larut	EF534332	EF535630	EF534345	EF535620	0.65
10.	*Heratemis malayensis* SY33*	RMNH100053 (Fresh)	Malaysia: Pahang, Taman Negara Merapoh	EF534331	EF535629	EF534344	EF535619	1.32
11.	*Heratemis malayensis* 2005* [control] (a destructive method followed by incubation at 55°C until the specimen is completely lysed)	RMNH100054 (Fresh)	Malaysia: Johor, Endau Rompin, Selai	EF534329	EF535631	EF534342	-	12.7
12.	*Heratemis malayensis* 2007* [control] (a destructive method followed by incubation at 55°C until the specimen is completely lysed)	RMNH100055 (Fresh)	Malaysia: Pahang, Taman Negara Endau Rompin	EF534330	EF535632	EF534343	-	10.9

TABLE 1 (*continue*)

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
13.	*Heratemis cubicep* SY46*	RMNH100056 (Fresh)	Malaysia: Pahang, Hutan Kuala Lompat	EF534327	EF535627	EF534340	EF535616	0.55
14.	*Apodesmia irregularis* * SY160	RMNH100187 (Fresh)	The Netherlands: Waarder	HQ416433			HQ416441	0.94
15.	*Aloeides* sp. * SY257	RMNH100188 (Fresh)	Malaysia: Johor, Selai	HQ416431			HQ416436	1.7
16.	*Bracon* sp. * SY318	RMNH100189 (Fresh)	Malaysia: Sarawak, Betong	HQ416427			HQ416435	1.1
17.	*Colastes braconius* * SY152	RMNH100073 (Fresh)	United Kingdom: nr. Bristol organic farm 2005	HQ416430			HQ416439	0.86
18.	*Colastes braconius* * SY280	RMNH100074 (Dried) '?'	Belgium: Prov. Liege, Eben-Emael	HQ416428			HQ416438	0.2
19.	*Gnamptodon* sp. * SY81	RMMH100186 (Fresh)	Malaysia: Pahang, Kuala Lompat	HQ416428			HQ416437	0.45
20.	*Tanycarpa* sp.* SY320	RMNH100126 (Fresh)	Malaysia: Pahang, Cameron Highlands	HQ416432			HQ416440	1.23
21.	*Utetes* sp. * SY74	RMNH100174 (Fresh)	Malaysia: Selangor, Hutan Simpan Bangi	HQ416434			HQ416442	0.64
22.	*Bactrocera carambolae* * FF	UKM0000070 (Fresh)	Malaysia: Selangor, Bangi		JN833638		JN833639	1.54
23.	*Fopius arisanus* * 117	UKM000059 (Fresh)	Malaysia: Selangor, Bangi	JN833636			JN833637	0.96
24.	*Bactrocera carambolae*+ MARDI-0E	UKM000006 (Fresh)	Malaysia: Pahang, Sg. Tekam		X			5.61
25.	*Bactrocera carambolae*+ MARDI-0F	UKM000005 (Fresh)	Malaysia: Selangor, Serdang, DOA		X			2.53
26.	*Bactrocera carambolae*+ MARDI-0G	UKM000004 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			1.75

TABLE 1 (*continue*)

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
27.	*Bactrocera carambolae*+ MARDO-0I	UKM000002 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			2.39
28.	*Scirtothrips dorsalis* * SN13	UKM000019 (Fresh)	No locality		X			0.78
29.	*Scirtothrips* sp. 1 * SN11	UKM000020 (Fresh)	No locality		X			0.90
30.	*Scirtothrips* sp. 2* SN04	UKM000021 (Fresh)	No locality	X				2.70
31.	*Scirtothrips* sp. 3 * SD03	UKM000022 (Fresh)	No locality	X				2.00
32.	*Scirtothrips* sp. 4 * SD02	UKM000028 (Fresh)	No locality	X				1.56
33.	*Scirtothrips* sp. 5 * SD01	UKM000030 (Fresh)	No locality	X				4.07
34.	*Alysia* sp. * SY94	RMNH000180 (Dried) '1987'	Malaysia: S.W. Sabah, nr. Long Pa Sia (West), c. 1050 m	X				3.5
35.	*Bobekoides* sp. * SY95	RMNH000181 (Dried) '1987'	Malaysia: S.W. Sabah, nr. Long Pa Sia (West), c. 1050	X				2.8
36.	*Aphaereta* sp. *SY94	RMNH000182 (Dried) '1988'	Malaysia: S.E. Sabah, nr. Danum Valle, Field C, c. 150 m	X				3.5
37.	*Psyttalia* sp. * SY101	UKM000069 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	1.53
38.	*Diachasmimorpha* sp. * SY105	UKM000030 (Fresh)	Malaysia: Melaka, D-Paradise (Jambu batu)	X	X			0.5
39.	*Biosteres* sp. * SY109	UKM000066 (Fresh)	Malaysia: Selangor, Serdang, MARDI	X			X	0.38

TABLE 1 (*continue*)

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
40.	*Heratemis* sp. * SY111	UKM000065 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X	X		X	0.12
41.	*Heratemis* sp. * SY112	UKM000064 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X	X		X	3.29
42.	*Dinotrema* sp. * SY115	UKM000061 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	0.97
43.	*Diachasmimorpha* sp. * SY116	UKM000060 (Fresh)	Malaysia: Melaka, D-Paradise (Jambu Batu)	X	X			4.52
44.	*Bitomus* sp. SY119	UKM000057 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	0.2
45	*Orientopius* sp. SY121	UKM000055 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	5.17
46.	*Asobara* sp. 1 SY122	UKM000054 (Fresh)	Malaysia: Pahang, Raub, Hutan Lipur Lata Jarum	X			X	2.35
47.	*Asobara* sp. 2 SY143	UKM000036 (Fresh)	Selangor, Bangi, Hutan Simpan Bangi	X			X	2.03
48.	*Asobara* sp. 3 SY144	UKM000035 (Fresh)	Selangor, Bangi, Hutan Simpan Bangi	X			X	2.03
49.	*Aspilota* sp. SY145	UKM000034 (Fresh)	Selangor, Bangi, Hutan Simpan Bangi	X			X	0.27

TABLE 1 (*continue*)

No.	Species (Family)	Institute Code (Type of specimen)	Locality	Accession No.				DNA concentration (ng/µl) after DNA extraction
				28S	**COI**	**16S**	**ND1**	
50.	*Cratospila* sp. 146	UKM000033 (Fresh)	Selangor, Bangi, Hutan Simpan Bangi	X			X	1.89
51.	*Bactrocera* sp. + [control] (a destructive method followed by incubation at 55°C until the specimen is completely lysed)	UKM000119 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			22.0
52.	*Bactrocera* sp.+ [control] (a non-destructive method, without freezing and with incubation of the sample at 55°C overnight)	UKM000120 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			14.5
53.	*Bactrocera* sp.+ [control] (a non-destructive method, without freezing and without incubation of the sample)	UKM000121 (Fresh)	Malaysia: Selangor, Serdang, MARDI		X			0.07

Notes:
(*)= adult; (+)= larva; (H)= Braconidae; (D)= Tephritidae; (T)= Thripidae.
(-)= not successful with PCR
No PCR attempted for the blank boxes.

TABLE 2
List of primers sequences.

Gene	Sequences 5'-3'
28S	28S 3665 (5' AGA GAG AGT TCA AGA GTA CGT G 3') (Forward) (Belshaw & Quicke, 1997)
	28S 4047 (5' TTGGTCCGTGTTTCAAGACGGG 3') (Reverse) (Campbell *et al.*, 1993)
	28S SYR (5' CCGAATAGCCAGTCAGGAAA 3' (Reverse) (Yaakop, 2011)
COI	Ron (5' GGA TCA CCT CAT ATA GCA TTC CC 3') (Forward) (Monteiro & Pierre, 2000; Simon *et al*., 1994)
	Nancy (5' CCC GGT AAA AAT TAA AAT ATA AAC TTC 3') (Reversed) (Monteiro & Pierre, 2000; Simon *et al*., 1994)
	COI SY F (5' CATGGGGGAATTTCTGTTGA 3') (Forward) (Yaakop, 2011)
	D23 (5' TACAATTTATCGCCTAAACTTCAG 3') (Forward) (Han & Ro, 2005)
	D25 (5' CATTTCAAGTTGTGTAAGCATC 3') (Reverse) (Han & Ro, 2005)
16S	16SWb (5'CACCTGTTTATCAAAAACAT 3') (Forward) (Dowton & Austin 1994)
	16S outer (5' CTTATTCAAATCGAGGTC 3') (Reversed) (Whitfield, 1997)
ND1	ND1F (5' ACT AAT TCAG ATT CTC CTT CT 3') (Forward) (Crozier & Crozier, 1993; Smith *et al*. 1999; Smith & Kambhampati, 1999)
	ND1R (5' CAA CCT TTT AGT GAT GC 3') (Reversed) (Crozier & Crozier 1993; Smith *et al.* 1999; Smith & Kambhampati, 1999)
	ND1 SY F (5' GAGCAATTGAGCGGATTGAT 3' (Forward) (Yaakop, 2011)

TABLE 3
PCR procedure (anneal duration and temperature) for each primer combination.

Gene	Anneal duration (sec)	Anneal temperature (˚C)
28S 3665/28S 4047	15	45
28S 3665/ 28S SYR	15	55
Ron/ Nancy	15	45
COI SY F/ Nancy	15	56
D23/D25	60	56
16S SWb/ 16S outer	60	62
ND1 F/ ND1 R	60	50
ND1 SYF/ ND1 R	60	51

of 20 minutes for the freezing procedure. We also compared the DNA concentrations extracted for similar-sized insect bodies after extracting several samples of *Heratemis* sp.by freezing and by the usual method of grinding the entire insect's body. We found that DNA concentrations were relatively higher if we used the whole body (RMNH100054-RM100055, 10.7-12.7 ng/l) than the freezing method (0.55-3.29) ng/µl (Table 1).

However, the 'freezing method' allows the voucher specimen to remain intact. Therefore, if necessary, the specimen can be re-examined and sequenced again. The samples were confirmed and did not show any changes in their morphological feature as proven from the visible morphology noted prior to and after the extraction process. The non-destructive method did not damage the insect cuticle (e.g. scutellum and pronotum), there was no loss of setae (e.g. on the tibia) and did not change the shape and size of the wing, in spite of the shrivelling process. Photographs of the specimens after the extraction process are shown in Fig.1 to Fig.3. In addition, the freezing method was also tested on samples of thrips. Specimens preserved after being mounted on slides are clear, fully macerated the tissue samples and retained their color, which is necessary for identification. Dr. Ng Yong Foo (pers. comm., 2011) confirmed that the freezing method allowed fat tissues of the thrips samples to be taken out during the freezing step without the need to squeeze the body content (Fig.3).

Interestingly, fragile, dried museum specimens were also successfully tested with the freezing method and DNA extraction (Table 1). However, shorter fragments of the DNA are amplified by applying a different set of primer combinations. The short DNA amplification band is assumed to be due to degradation occurring on the dried samples. The dried samples were collected since 1985. Furthermore, the samples were preserved with chemicals using the AXA Alcohol-Xylene-Amylacetate method (van Achterberg, 2009) and probably with empty body tissue. The concentration of extracted DNA was measured before the amplification process and showed lower and sometimes higher concentrations when compared to fresh specimens that are more or less similar in size (0.9-3.5 ng/µl) (Table 1).

The freezing method was successfully scored on 1.5% gel with TAE 1X buffer for 40 min (80 volt) after completion of the PCR process. A minimum amount of DNA, 5 µl was used as template for PCR. The PCR results showed very clear amplification bands. The targeted band sizes are between 300-1300 bp and vary with insect size and type of samples (fresh or dried).

After the extraction process, PCR products are purified before the samples were sent for sequencing. Technically, 5 µl was loaded on the 1.5% TAE 1X buffer for 40 min (80 volt) gel to confirm that there is enough DNA for sequencing. The DNA concentration is measured and between 35-95 ng/µl was obtained. Generally, the DNA band was clearly visible on the gel after purification. This provides an indication of the samples that can proceed for sequencing.

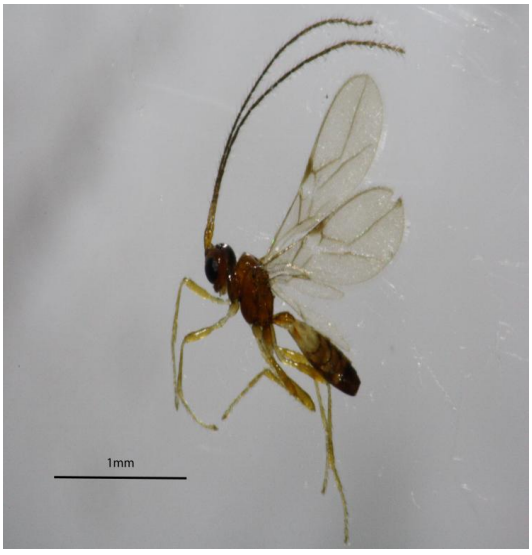

Fig.1: Photograph of extracted specimen of braconid using freezing method

Fig.2: Photograph of extracted specimen of tephritid's larvae using freezing method.

The results from the sequencing process did not show any difference between those that were extracted from body parts or from the entire body. The results showed very nice chromatograms and were very convenient for editing. The edited sequences were aligned and then implemented in BLAST and phylogenetic analyses.

In this study, MP analysis was carried out and implemented on the sequences of the larvae and adults of the *Bactrocera* samples to measure the quality and efficiency of the freezing method by implementing the phylogenetic analysis. The results showed that the freezing method still provides high quality of DNA in a short

Fig.3: Photograph of extracted specimen of thrips using freezing method.

duration. The samples those were were not extracted by freezing method namely *B. occipitalis* and *B. latifrons*+ *B. umbrosa* were successfully separated from the *B. carambolae*, supported by100% and 93% bootstrap values. Besides that, higher length of DNA fragment (approximately 760-1300 bp) obtained from the *Bactrocera* larvae specimens (2-39-5.61 ng/ul) compared to the adult specimen (1.54 ng/ul) and showing DNA obtained from the freezing method still provided enough data for producing a robust phylogeny of *Bactrocera* species (Fig. 4).

In this study, a modification of a commercial manual extraction kit DNeasy Blood & Tissue Kit was carried out. The freezing method does not require a long incubation process, unlike the blood and tissue extraction procedure, which requires a very long process of incubation (Ball & Armstrong, 2008; Thomsen *et al.*, 2009). This is because the freezing method is believed to lead the DNA fragmented and only requires a minimum of 20 minutes to freeze the lysis buffer (ATL buffer and proteinase K) with the whole insect specimen. According to Castalanelli *et al.* (2010), Qiagen Dneasy is often used for the non-destructive method; however no evaluation has been done on that method.

PCR amplification of all insects with selected markers using both the freezing and non-freezing methods (as control) was successful; concentrations were found to be very low to moderately low in the freezing method. In the non-freezing method (method 1-2 in the control experiment), the DNA obtained was high, but there were no remains of the insect body left after the extraction and the process was very time consuming. The DNA amount was found to be very low if there was no incubation after soaking the samples in a lysis buffer and without freezing the samples. After having compared

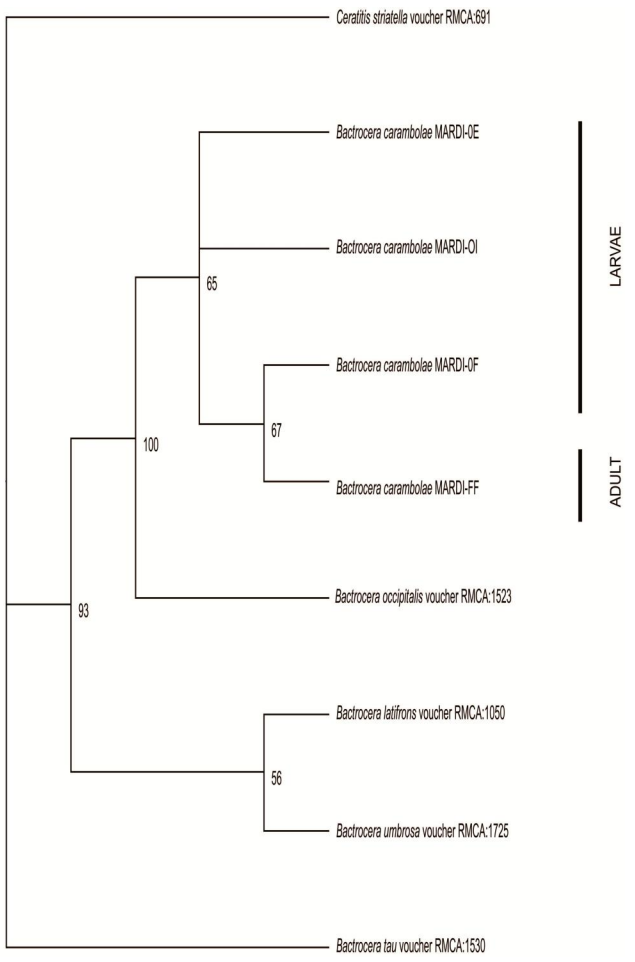

Fig.4: Maximum Parsimony tree of larvae and adults of *Bactrocera* samples using COI markers.

all the methods, we strongly believe that the freezing method provides more benefits and has shown to have a high significance when applied. As a result, we have come up with minor modifications of the normal procedure to ensure that there is no damage of the samples, reduce the duration of the process and avoid contamination.

Interestingly, the mounting process after applying the freezing method on the thrips specimen was easier and more efficient. It is because no maceration was needed after the DNA was extracted from the thrips' body. The method used by Castalanelli *et al*. (2010) without freezing on Eriophyid mites also showed the possibility of sample fragmentation during heating. The process also required short term storage prior to the DNA extraction and mounting to prevent further sample fragmentation. However, this is not necessary when using the freezing method. Furthermore, the method was not standard for several insect species such as mites and beetles, in terms of the duration of

the incubation in order to heat the samples at 99˚C. This is because the duration varies depending on the thickness of the cuticle. On the other hand, when using the freezing method, the process of incubation, in term of the freezing procedure is standard for several species of insects and only requires a minimum of 20 minutes.

DNA concentration was found to be very low when using freezing method compared to the non-freezing methods. In addition, the DNA concentration of dried museum specimens was unstable and sometimes slightly lower or higher compared to the fresh specimens. This might be because the DNA contains too many proteins, phenol and other contaminants; this can be evaluated by measuring the 260/230 ratio. This might also be due to the unpurified DNA samples being measured at that stage (Wilfinger *et al.*, 1997).

We have also proven the quality of DNA obtained in spite of the short time duration required to process the dried and fresh specimens, especially when using the freezing method without a long incubation process (Thomsen *et al.*, 2009). Most importantly, the specimens are are not destroyed. Basically, the ATL Buffer from the isolation kit works similar to the lysis buffer, which functions to dissolve and neutralize cellular components. The ATL buffer functions as a lysis or extraction buffer with the purpose of lysing cells to prepare them for molecular biology experiments. DNA is freed from cellular membranes and becomes soluble using the lysis buffer. Proteinase K is then applied to break down cellular proteins or to digest protein and remove contamination from the nucleic acids. Proteinase K makes nucleases that might degrade the DNA during the purification process inactive. The freezing method is then continued and the remaining protocol is followed through. Through this procedure, it is very clear that it is not necessary for the specimen to be to cut into pieces or to be grinded using liquid nitrogen for DNA collection.

Although several papers have been published on a variety of extraction methods, the freezing method deserves consideration. In other methods, samples may need to be cleaned after the DNA extraction process using ANDE solution, creating the risk of contamination as they have to be pierced with micro pins for the larval specimen (Castalanelli *et al.*, 2010; Rowley *et al.*, 2007). Yet other methods require a long incubation process (Dittrich-Schröderet *et al.*, 2012), which is not necessary for the freezing method. According to Dittrich-Schröder *et al.* (2012), DNA extraction of minute sized insect specimens always results in very low amounts of DNA. It is also very difficult and challenging to obtaining results from the PCR of these specimens. This might be due to the very low amount of DNA templates used. In this method, low amounts of DNA were measured using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the insect's intact body remained as a voucher specimen. DNA extraction was successful for all the fresh adult samples and higher DNA amounts were retrieved compared to the dried

samples, but this also depends on the insect's body size. The DNA concentration also was not affected only by the specimen size, but was due to the detection of contaminants in the DNA sample. In spite of this, the small amount of DNA could be used as a template and the concept of PCR has been shown by amplifying and duplicating a billion copies of DNA from the available small DNA pieces. This modification technique has been approved after many studies on insect extraction and published in several journal papers (Yaakop et al 2009, 2010).

CONCLUSION

The freezing method is absolutely useful and important in cases where there is a conflict of taxonomical status and only a small sample is available. This method has also been proven to provide very informative data for phylogenetic analyses. This new method would allow tiny insects to be kept intact and available for other purposes even after undergoing the DNA extraction process. This method is also recommended for museum loan specimens, as extraction can also be carried out without the removal of any portion of the sample's body. We would also like to stress that the freezing method provides sufficient quantity or high-quality DNA for molecular work. Besides that the freezing method is highly significant because DNA can be obtained rapidly, it can minimize DNA contamination, does not require a long incubation process and maceration process.

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Antioxidant Activity of Two Mesomeric Heterocyclic Betaines Containing a Pyrimidine Moiety

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ABSTRACT

Two mesomeric heterocyclic betaines, phenyl pyrimidinium betaine (BT_1) and undecyl pyrimidinium betaine (BT_2), were investigated for their antioxidant activity using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The antioxidant propriety was assessed by the effective concentration of the betaine (EC_{50}), the time required to reach the steady state of DPPH discoloration at EC_{50} concentration (TEC_{50}), the antiradical efficiency (AE), and the reduction kinetics . Vitamin E (α-tocopherol) and di-*tert*-butylhydroxytoluene (BHT) were used as the standard antioxidants in *in vitro* assays. Kinetic studies showed that BT_1 was more effective than vitamin E and BT_2.

Keywords: Betaine, pyrimidine, antioxidant, DPPH assay, reduction kinetic

INTRODUCTION

Antioxidant substances play an important role in scavenging the deleterious oxygenated radical species, thus providing protection to humans against infectious and degenerative diseases (Thirunavukarasu *et al*., 2010); under an aerobic medium, oxidation of natural substances such as lipids may occur via the reaction of formed radicals ($R^{\cdot}$) with oxygen, resulting in hydroperoxides (ROOH) which may degrade and causes damages. Therefore, consumption of food rich in a natural or synthetic antioxidant, would annihilate the endogenous oxidative deterioration. In the last few years, much attention has been focused on the synthesis of compounds and the extraction of natural ones that can potentially act as antioxidants (Pereira *et al*., 1999; Lebeau *et al*., 2000; Du Toit *et al*., 2001). In addition, new methods

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of estimating the antioxidant efficiency were developed (Sanchez-Moreno, 2002; Schwartz *et al*., 2001). Among these, the 2,2-diphenylpicrylhydrazyl (DPPH) trapping technique has been the most common one.

DPPH is a stable free radical and becomes a stable diamagnetic molecule by pulling out an electron or a hydrogen atom. In the DPPH radical-scavenging assay, antioxidants react with DPPH, and convert it to yellow- colored diphenylpicrylhydrazine. The color fading extent proves indirectly the radical-scavenging capacity of the antioxidant (Blois, 1958). The DPPH tests provided in the literature are based on the same principle as described by Brand-Williams *et al*. (1995), but the analytical protocols differ in several parameters.

The term "betaine" was originally coined for *N*, *N*, *N*-trimethylglycine. Being a polar amphoteric compound, betaine acts as an osmoprotectant in plants and as a protective agent for the liver, the heart and the vessels in humans. It is present in several plants (beet, wheat, spinach), and animals micro-organisms (*Propionibacterium shermanii*, *Denitrifying Pseudomonas*) (Huang et al., 2008). Today, the chemistry of betaines has become a subject of particular interest due to their applications in biological research, especially with regard to their metabolic roles in the living organism (Myers & Jibril, 1957). Indeed, alkylbetaines nowadays have a variety of uses in medicine, pharmacy, biology and other scientific fields (Domingo, 1996; Gonzaleza & Mesab, 2009). Some glycine betaine derivatives are employed as surfactants in cosmetics (Nsimba *et al*., 2010).

In the aim at investigating the antioxidant property of betaines, the previously synthesized mesomeric heterocyclic betaines (Malki *et al.*, 2011): phenyl pyrimidinium betaine (BT_1)* and undecyl pyrimidinium betaine (BT_2)**; the latter one having a fatty alkyl chain as a typical example of alkyl pyrimidinium betaines used as amphoteric surfactants (Fig.1). However, reports on biological activities of pyrimidinium betaines are very limited (Lindner *et al*., 2009; Gonzalez *et al*., 2009). To our knowledge, there was only one work unraveling the antioxidant potency of betaines, namely $(CH_3)_3N^+(CH_2)_nCOO^-$ with n =1-5, using hydroxyl radical scavenging (Kalvinsh *et al*., 1999;. M.M. Islam *et al*. 2009).

The antioxidant activity was ascertained by the radical scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH), a radical widely used in the reactivity studies of phenolic antioxidants (Blois, 1958; Molyneux *et al*., 2004).

Aside from its biological activity (Naik & Chikhalia, 2007), pyrimidine ring in the herein chosen betaines would favor the formation of a nitrogen-containing conjugated system ($>N^+=C-^{\cdot\cdot}N<$) that stabilizes free radicals (Wentrup, 1984).

*4-*H*-4-oxo-1,2,3,5 -tetraphenyl-1-pyrimidinium-6-olate

**4-*H*-4-oxo-1,3, 5-triphenyl-2-undecyl-1-pyrimidinium-6-olate

MATERIALS AND METHODS

Synthesis of pyriminium betaines

Betaines BT_1 and BT_2 were prepared as described previously (Malki *et al*., 2011) and as illustrated by the reaction shown in Fig.1 (2).

A typical synthetic protocol of BT_1 is as follows: Into a 10 mL round-bottomed flask, 5 mL of acetone was added and sequential addition of the following reactants was made under stirring at room temperature : 10^{-3} mole of dipentachlorophenyl phenylmalonate, and 10^{-3} mole of *N, N'*-diphenylbenzamidine. To the milky suspension obtained , 2×10^{-3} mole of triethylamine was added. Within one minute after the latter addition, a yellow solid precipitated at the bottom of the flask, leaving a yellow solution. The whole system was then stirred for 30 min at room temperature. Afterwards, the yellow precipitate was filtered off and recrystallized from chlorobenzene, yielding betaine BT_1 as yellow bright crystals.

By using the same procedure, undecylpyrimidinium betaine was obtained as white solid by the reaction of *N, N'*-diphenylundecamidine and *bis*pentachlorophenyl ester of phenyl malonic acid in diethyl ether for 1 h.

The betaines BT_1 and BT_2 were characterized by spectroscopic analyses, including UV-visible, IR, ^{1}H ^{13}C NMR and MS (see Malki et al., 2011). Their structures, along with those of vitamin E (*α*-tocopherol) and di-*tert*-butylhydroxytoluene (BHT), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH), are illustrated in Fig.2 (1). And, their physical characteristics are gathered in Table 1.

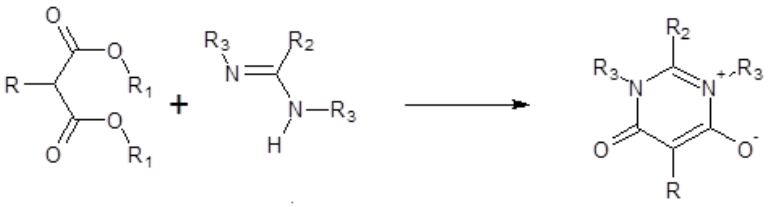
Protocol of DPPH radical scavenging assay

Methanolic solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was prepared and was added to the sample in ethanol at different concentrations (50, 100, 300, 500, 1000 µg/ml).

The first step was to determine the stabilization time for the DPPH methanolic solution discoloration. Then, the inhibition kinetics was studied for the two pyrimidinium betaines at each concentration. The reduction kinetics of DPPH at different concentrations in the tested antioxidants was followed until a plateau was reached, corresponding to the stabilization time (T_{eq}). At this stage, the UV-visible analysis was taken, and the percentage of unreacted DPPH, $(DPPH)_{unr}$ was estimated for each betaine concentration and at different times (T), from absorbance

TABLE 1
Physical characteristics of BT_1, BT_2, vitamin E (α-tocopherol), BHT, and DPPH.

Compound	Appearance	Molecular weight (g/mol)	Melting point °C
BT_1	Yellow crystals	416	317-319
BT_2	White needles	494	134-138
Vitamin E	Oily colorless liquid or yellow-brown liquid	430	2-3
BHT	White powder	220	70-73
DPPH	Black to green powder (purple in solution)	394	135



BT_1: $R = R_2 = R_3 = C_6H_5$
BT_2: $R = R_3 = C_6H_5$; $R_2 = C_{11}H_{23}$

Fig.1: Synthetic pathway of BT_1 and BT_2

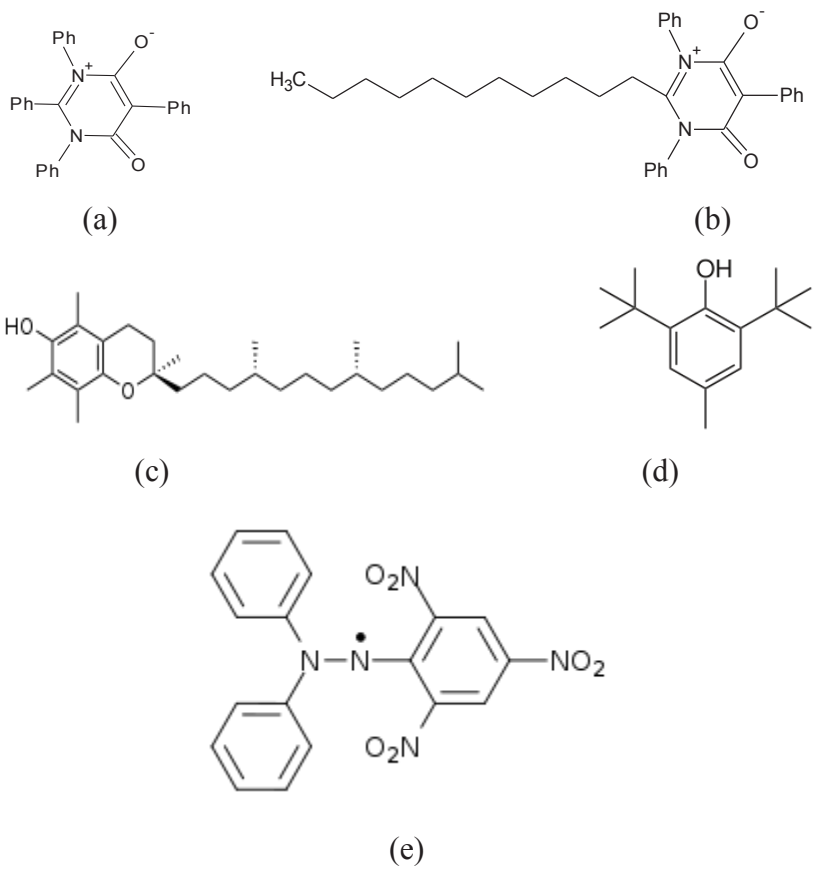


Fig.2: Structures of phenyl pyrimidinium betaine BT_1 (a), undecyl pyrimidinium betaine BT_2 (b), vitamin E (α-tocopherol) (c), di-*tert*-butylhydroxytoluene (BHT) (d), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (e).

at λ_{max} = 517 nm via equation Eq.1.

$$\% (DPPH)_{unr} = 100 \times (DPPH)_{T = Teq}/(DPPH)_{T = o} \quad [Eq.1]$$

where $(DPPH)_{T = Teq}$ and $(DPPH)_{T = 0}$ are the absorbances of DPPH solution at the start of reaction with sample (T = 0) and at T = T_{eq}, respectively.

The well-known antioxidants, vitamin E (α-tocopherol) and di-*tert*-butylhydroxytoluene (BHT), were employed as positive controls.

Effective concentration of sample required to scavenge 50% of DPPH radicals (EC_{50}) was computed from the plot of the percentage of unreacted DPPH curve versus sample concentrations. In order to easily characterize the behavior of a substance as an antioxidant, the antiradical efficiency parameter (AE) was also calculated. The latter parameter combines the two parameters, EC_{50} and T_{EC50}. Antiradical efficiency (AE) was then determined according to equation Eq.2:

$$AE = 1 / (EC_{50} \times T_{EC50}) \quad [Eq.2]$$

Where EC_{50} is the concentration required to reduce the DPPH discoloration by 50%, and T_{EC50} is the time required by each compound to reach the steady state of DPPH discoloration at EC_{50} concentration (Sanchez-Moreno *et al*., 1998).

RESULTS AND DISCUSSION

DPPH has been used extensively as a free radical to evaluate reducing substances (Motlhanka *et al*., 2008) and a reagent for investigating the free radical scavenging activities of compounds (Duan *et al*., 2006). As the electrons become paired off, the solution color faded stoichiometrically depending on the number of electron taken up (Blois, 1958). Hence, this assay provides an insight into the reactivity of the tested samples towards a stable free radical (Senthilkumar *et al*., 2010).

The kinetic effects of scavenging BT_1 and BT_2 by DPPH at their different concentrations are illustrated in Fig.3, showing the extent of DPPH inhibition obtained at each concentration for each betaine at the steady-state time (Teq). From this figure, the DPPH radical scavenging activity towards pyrimidinium betaines is clearly demonstrated, and the DPPH drop was concentration-dependent. The decrease in the UV-visible absorbances of the DPPH radical engendered by test samples was due to the radical scavenging by electron donation.

The kinetic effects of DPPH scavenging of vitamin E and BHT used as standards are presented in Fig.4.

The effective concentrations (EC_{50}) of BT_1 and BT_2 were obtained from the curve plotting the percentage of unreacted DPPH and its concentration at T = T_{eq}, using graphic interpolation.

The different antioxidant parameters EC_{50}, T_{EC50} and AE obtained are gathered in Table 2. It can be easily noticed that the synthesized betaines BT_1 and BT_2 revealed a radical scavenging activity. The kinetic studies showed that the obtained results for the betaines were closer to those of vitamin E and drastically lower when compared

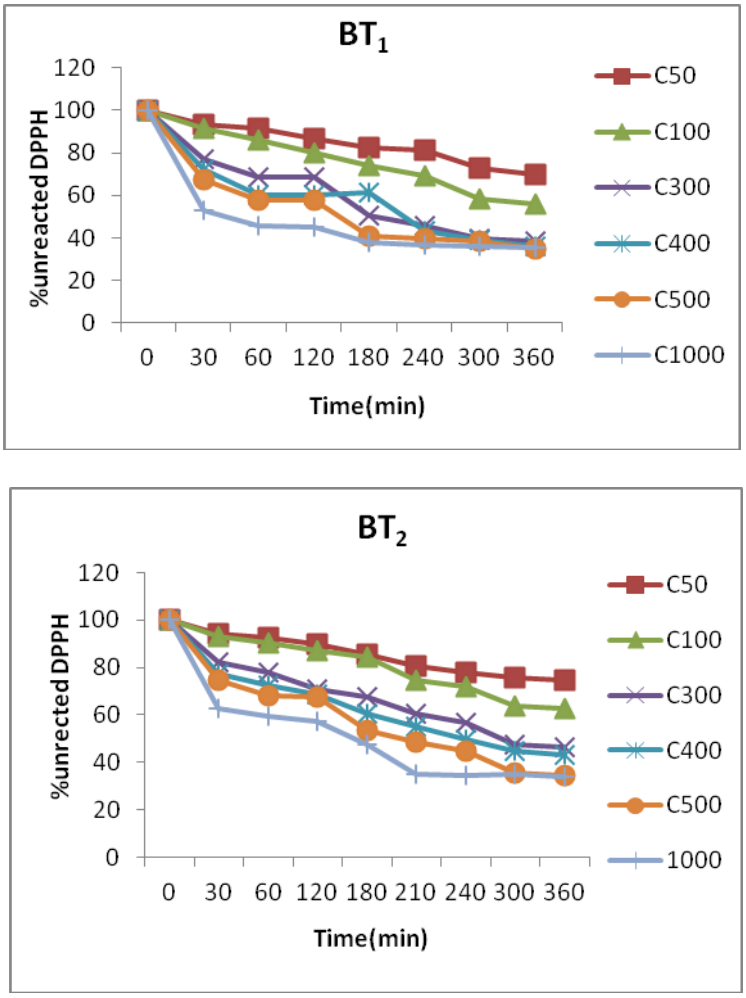


Fig.3: Kinetics of DPPH scavenging effects of BT_1 and BT_2

TABLE 2
Results of the kinetics of reduction of DPPH.

Compound	EC_{50}(μg/mL)	T_{EC50}(min)	AE(μg/mL.min)$^{-1}$
BT_1	150	180	3.70×10^{-5}
BT_2	250	240	1.66×10^{-5}
Vitamin E	152	210	3.13×10^{-5}
BHT	1.5	150	4.44×10^{-3}

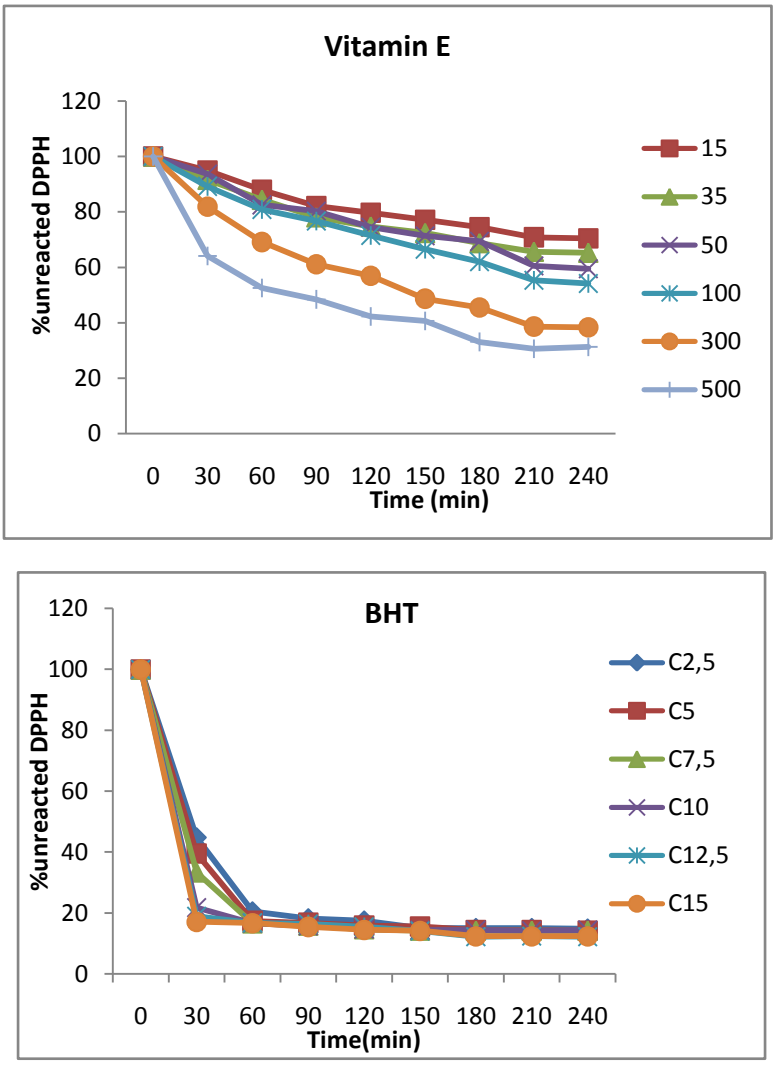
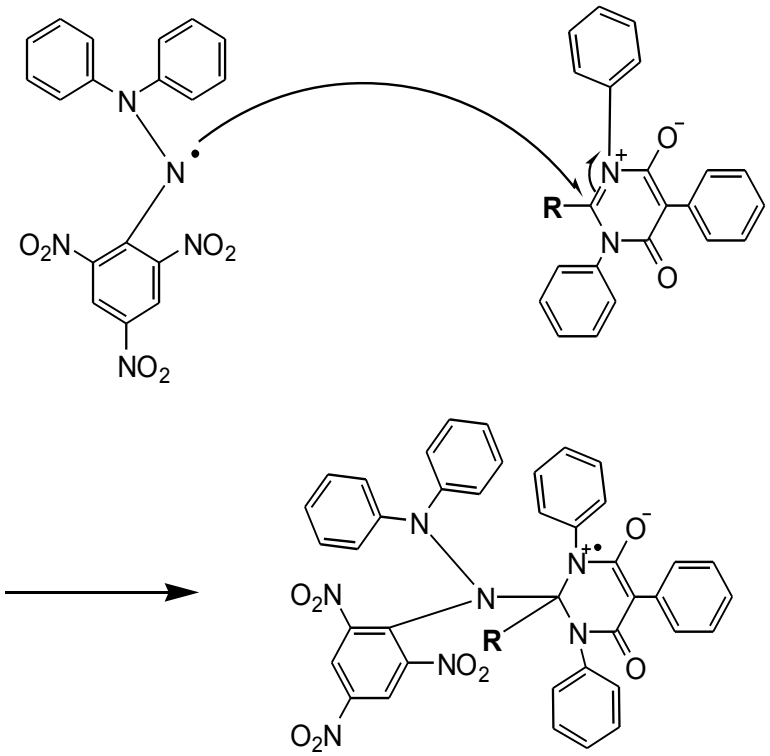


Fig.4: Kinetics of DPPH scavenging effects of vitamin E and BHT

with those of BHT. Considering the two pyrimidinium betaines and vitamin E, it is interesting to note that BT_1 was the most potent antiradical reactivity, because it has higher AE than BT_2 and vitamin E, and took a short time to reach the plateau. Whereas a lower antioxidant capacity was obtained for the BT_2. Steric effects might play a fundamental role in the reaction with DPPH. Hence, the antioxidant efficacy is probably related not only to its reducing properties, but also to steric factors that might influence its ability to approach the electron deficient reactive sites. Indeed, the low reactivity of BT_2 towards the DPPH radical can be ascribed to the presence of the fatty alkyl chain, being a long one, hence providing a steric hindrance; with a shorter alkyl chain



R = C_6H_5; $C_{11}H_{23}$

Fig.5: Proposed mechanism of the attack of betaine by DPPH

or a small group, such steric would be greatly insignificant. The higher reactivity of BT_1 could be associated with the size of the phenyl group. Fig.5 depicts the plausible mechanism of the reaction of DPPH with pyrimidinium betaines. As can be seen, the hindrance factor definitely aroused, not only from the betaines but also from the DPPH molecule. With regard to the mechanism of the action of DDPH towards vitamin E and BHT, as known, it takes place by pulling out the hydrogen atom of the phenolic hydroxyl group, being very labile (Patt & Hudson, 1990; Bondet *et al*., 1997). However, that for the action towards pyrimidinium betaines occurred differently; DPPH may attack the betaine and forms a covalent bond as shown in Fig.5; the radical on the nitrogen atom is greatly stabilized by the two phenyl and carbonyl groups of the pyrimidinium betaine.

By comparing the values of the antiradical efficiency (AE), the scavenging effect on the DPPH radical decreased in the order of: BHT >> BT_1 > Vitamin E > BT_2.

Although BHT has an antiradical efficiency higher than that of vitamin E, the lower antioxidant activity of the latter

could be attributed to steric effect due to the presence of the long alkyl chain in this molecule. On the other hand, the antioxidant activity of pyrimidinium betaines could be attributed to the conjugated systems with nitrogen atoms which are known to stabilize free radicals (Wentrup, 1984). In the case of $BT_{2,}$ the low antioxidant activity could be also imputed to the steric effect of fatty alkyl chain.

CONCLUSION

DPPH radical scavenging method has ascertained the oxidant potency of pyrimidinium betaines. The above results suggested the effects of the nature of the substituent and the steric hindrance of length of the alkyl chain affixed on the hydrophilic head of the betaine. The kinetic studies showed that phenyl pyrimidinium betaine BT_1 is more active than undecyl pyrimidinium betaine BT_2 and standard vitamin E and less active than BHT. Further investigation will decipher the exact mode of action of these compounds at molecular level.

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      [2]Office of the Deputy Vice Chancellor (R&I), Universiti Putra Malaysia, Serdang, Malaysia.

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   The results have been interpreted (Kanwal *et al.,* 2009).

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Pertanika began publication in 1978 as the Journal of Tropical Agricultural Science (JTAS).

In 1992, a decision was made to streamline *Pertanika* into **3 journals**. i.e.,

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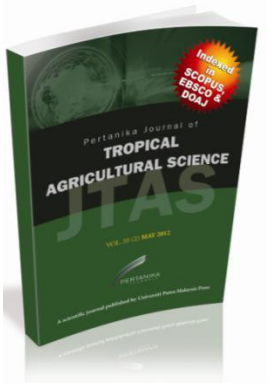

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Freezing Method as a New Non-Destructive Modification of DNA Extraction 373
Yaakop, S., C. van Achterberg, Idris, A. B. and Aman, A. Z.

Antioxidant Activity of Two Mesomeric Heterocyclic Betaines Containing a Pyrimidine Moiety 393
Malki, F., Touati, A. and Moulay, S.

Pertanika Journal of Tropical Agricultural Science
Vol. 36 (4) Nov. 2013

Contents

Foreword
Nayan Deep S. Kanwal i

Editorial
Developing Sustainable Fish Farming in the Western Pacific: A Viewpoint on Potential Reasons for Why Many Attempts Failed iii
Peter B. Mather and Satya Nandlal

Short Communication
Ethnic Differences in the Prevalence, Clinical Outcome and *cag* Pathogenicity Island (*cag*PAI) Virulence Gene Profiles of *Helicobacter pylori* Strains from Malaysia 289
Hamat, R. A., Nor Amalina, E., Malina, O., Zamberi, S., Alfizah, H., Rizal, A. M., Aminuddin, A. and Ramelah, M.

Regular Articles
Variations of Electrical Conductivity between Upstream and Downstream of Langat River, Malaysia: Its Significance as a Single Indicator of Water Quality Deterioration 299
Yap, C. K.

Accumulation of Heavy Metals and Antioxidative Enzymes of *Centella asiatica* in Relation to Metals of the Soils 311
Ong, G. H., Yap, C. K., Maziah, M. and Tan, S. G.

Length-Frequency Distribution, Length-Weight Relationship and Condition Factor of Sompat Grunt *Pomadasys jubelini* (Cuvier, 1830) off Lagos Coast, Nigeria 337
Adebiyi, F. A.

Phylogenetic Tree Construction in Reconfirmation of Parasitoid Species (Braconidae: Opiinae), Reared From Fruit Flies (*Bactrocera papayae*) Infesting Star Fruit (*Averrhoa carambola*) Based on Mitochondrial 16S rRNA Sequences 345
Ibrahim, N. J., Shariff, S., Idris, A. B., Md-Zain, B. M., Suhana, Y., Roff, M. N. and Yaakop, S.

Physico-Chemical Characteristics of Chok Anan Mango Fruit after Hot Water Treatment 359
Phebe Ding and Salumiah Mijin

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