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TROPICAL AGRICULTURAL SCIENCE

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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

JTAS is a **quarterly** (*February, May, August and November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open to authors around the world regardless of the nationality.

The Journal is available world-wide.

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Pertanika was founded in 1978. A decision was made in 1992 to streamline Pertanika into three journals as Journal of Tropical Agricultural Science, Journal of Science & Technology, and Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

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The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's **Instructions to Authors**.

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Notification of the editorial decision is usually provided within ten to fourteen weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

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3. The chief executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Edito-in-Chief, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.
4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the chief executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).
5. The chief executive editor sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.
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Office of the Deputy Vice Chancellor (R&I)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 Serdang, Selangor Malaysia.
Gen Enq.: +603 8947 1622 | 1616
E-mail: executive_editor.pertanika@upm.my
URL: www.journals-jd.upm.edu.my

PUBLISHER
Kamariah Mohd Saidin
UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia.
Tel: +603 8946 8855, 8946 8854
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Foreword

Welcome to the **Third Issue 2017** 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 **13 articles**, out of which **one** is a review paper, **one** is a short communication, **one** is a clinical case study and **six** are regular papers. This issue also features **four** selected papers from the INTROP Research Colloquium 2015 (IRC 2015). The authors of these articles come from different countries, namely, **Malaysia**, **Nigeria**, **Bangladesh** and **Indonesia**.

Joey Ee Uli, Christina Yong Seok Yien and *Tan Soon Guan* in their review article discuss a glimpse into the genomic outlook of the long-tailed macaque (*Macaca fascicularis*), while the short communication discusses the correlation between sperm parameters in West African dwarf goat bucks during storage (*Daramola, J. O.* and *Adekunle, E. O.*). The clinical case study by *Khairuddin, N. H., Roslim, N., Affandi, S. S., Lau, S. F.* and *Lim, S. Y.* discusses a case of diaphragmatic rupture in a criollo pony.

The 6 regular papers cover a wide range of topics. In the first research paper, the potential use of papaya juice as fermentation medium for bacterial cellulose production by *Acetobacter xylinum* 0416 is studied (*Zahan, K. A., Hedzir, M. S. A.* and *Mustapha, M.*). The next paper discusses the temporal expression of a putative homogentisate solanesyltransferase cDNA in wounded *Aquilaria malaccensis*, an endangered tropical tree (*Azzarina, A. B., Mohamed, R., Siah, C. H.* and *Wong, M. T.*). The other papers are studies on: a genetic analysis of Nigerian 'Egusi' melon

accessions based on sequence-related amplified polymorphism (SRAP) (*Idehen, E. O.*); the response of calves to supplementation of forage legume-based concentrate diets (*Bello, R. A., Fasae, O. A., Oluwatosin, B. O.* and *Olanite, J. A.*); diseases and disease conditions with associated post-mortem lesions diagnosed in cats and dogs at the post-mortem laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia between 2005 and 2015 (*Rathiymaler, M., Zamri-Saad, M.* and *Annas, S.*); and fractionation of anticholesterol bioactive compounds from *Bekasam* (Indonesian fermented fish product) (*Rinto, Nopianti, R., Herpandi* and *Oktaviani, S.*).

I conclude this issue with 4 articles arising from the research colloquium featuring the following: an evaluation of ricinus communis as a phytoremediator of manganese in soil contaminated with sewage sludge (*Nur-Nazirah, P. M., Arifin, A., Shamshuddin, J., Rezaul, K., Muhammad-Nazrin, Mohd-Hadi, A.* and *Aiza-Shaliha*); spectroscopic analysis of epoxidised jatropha oil (EJO) and acrylated epoxidised jatropha oil (AEJO) (*Wong, J. L., Aung, M. M., Lim, H. N.* and *Md. Jamil, Siti Nurul Ain*); characterisation of pulp and paper manufactured from empty fruit bunches and kenaf fibres of oil palm (*Rafidah, D., Ainun, Z. M. A., Hazwani, H. A., Rushdan, I., Luqman, C. A., Sharmiza, A., Paridah, M. T.* and *Jalaluddin, H.*); and finally, the properties of paper manufactured from kenaf as function of alkaline pH medium and retention of precipitated calcium carbonate (*Ainun, Z. M. A., Nurul, I. N., Zaida, S., Hazwani, H. A.* and *Latifah J.*).

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.

Chief Executive Editor
Nayan Deep S. KANWAL, FRSA, ABIM, AMIS, Ph.D.
nayan@upm.my

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Review Article

A Glimpse into the Genomic Outlook of the Long-Tailed Macaque (*Macaca fascicularis*)

Joey Ee Uli[1]*, Christina Yong Seok Yien[2] and Tan Soon Guan[1]

[1]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[2]*Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

ABSTRACT

The long-tailed macaque (*Macaca fascicularis*) is commonly used for biomedical researches. However, genetic variation within a population or among populations can significantly affect phenotypical outcomes of treatments tested on model organisms. As such, it is important for studies involving model organisms originating from different, or even the same geographical locations to have sufficient genomic and transcriptomic background of the model organisms that is used. This paper summarises the utilisation of next-generation sequencing (NGS) technology to sequence genomes and transcriptomes of long-tailed macaques from various geographical populations in general and the Malaysian long-tailed macaque in particular, and its importance in the context of population genetic studies.

Keywords: *Macaca fascicularis*, next-generation sequencing, genomics, genetic variation, transcriptomics, population genetics, biomedical science

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E-mail addresses:
joeyeeuli@gmail.com (Joey Ee Uli),
chrisyong@upm.edu.my (Christina Yong Seok Yien),
sgtan_98@yahoo.com (Tan Soon Guan)
* Corresponding author

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INTRODUCTION

The long-tailed macaque (*Macaca fascicularis*) is currently regarded as the most heavily-utilised non-human primate (NHP) model in biomedical studies (Ogawa & Vallender, 2014). The macaque's close evolutionary relationship with humans enables the macaque to recapitulate human biology, physiology, behaviour, and

symptoms when presented with certain perturbations in the environment. Thus making it useful for researchers studying the various effects of drugs and treatments in a cellular environment. A wide array of studies have utilised the long-tailed macaque as NHP models (Nunamaker et al., 2013; Lee et al., 2014b; Silverstein et al., 2014; Berry et al., 2015; Gardner et al., 2015), and their efficacy in translational studies will continue to attract researchers in the foreseeable future. That being said, thought must be given to the genetic variation of long-tailed macaques originating from different geographical locations. Variations in the genome causes phenotypical changes that is observed through a wider spectrum of the model organism's reactions towards drug treatments. Hence, researchers need to take into consideration the functional variations contained within the genomes and transcriptomes of model NHPs before they embark on biomedical projects.

In recent years, genomics and transcriptomics play a pivotal role in biomedical studies involving NHPs. It is pertinent that genomic and transcriptomic information of model organisms is widely available to facilitate in experiment design. Next-generation sequencing (NGS) provides a platform that can expedite the process of obtaining the genomic and transcriptomic information of NHP organisms. In addition to its low cost and speed of generating sequences, NGS provides a huge volume of genomic and transcriptomic related useful for research. Various attempts have been made to sequence the genomes and transcriptomes of the long-tailed macaque from different geographical locations This paper will attempt to summarise the various genomic and transcriptomic sequencing endeavours carried out on the long-tailed macaques, as well as briefly outline phylogenetic and population genetic studies involving Malaysian long-tailed macaques. We will also discuss the need to sequence genomes and transcriptomes of the Malaysian long-tailed macaque in the context of population management.

GENOMES AND TRANSCRIPTOMES

Genomics and transcriptomics is the mainstay of biomedical studies which require the sequencing of whole genomes and/or transcriptomes, especially where non-model organisms are involved. Since the advent of NGS, whole genomes and transcriptomes can be easily sequenced.

To date, more than 3000 eukaryote genomes of various assembly levels have been deposited to NCBI's Genome database. Without having to rely on reference genomes, genomes can be assembled via de novo assembly to form contigs which subsequently are assembled into scaffolds. Newly assembled genomes are then annotated with gene information and their functions predicted by searching for orthologous regions in closely related species that are already annotated, in which case, established model organisms with their genomes completely sequenced and annotated. Insight into a genome is particularly useful for genomic comparative studies. The focus can either be on

evolutionary relationships between two or more species, or the genomic relatedness of several populations of organisms. Most noteworthy is how researchers no longer need to rely on inferring phylogeny or genetic diversity based merely on several genes, rather in the age of NGS, entire genomes can be used for higher resolution phylogeny inference.

A complementary approach to whole genome sequencing would be to sequence the transcriptome, as transcriptomes provide information pertaining to the quantification of gene expression, the discovery of novel transcripts and their isoforms, intragenic expression, as well as to understand modes of antisense regulation (Tarazona et al., 2011). RNA-seq is a remarkably cost-effective approach to producing massive amounts of data, capable of sequencing complete transcriptomes of almost any tissue, as well as measuring their levels of gene expression in a myriad of conditions (Ozsolak & Milos, 2011). The measurements of gene expression is based on the mapping and quantification of reads generated from the sequencing platform. In addition, RNA-seq provides a higher base pair resolution of gene expression measurement as opposed to conventional microarray techniques (Marioni et al., 2008), and also avoids the need for bacterial cloning of cDNA (Wang et al., 2009) – of which may cause sequences to be misrepresented or incomplete (Mortazavi et al., 2008). Unlike microarrays, which require prior knowledge of the genes of interest, one of RNA-seq's significant utility is the sequencing of novel transcriptomes. Hybridisations of novel transcripts to microarray probes designed for closely-related species may lead to unsuccessful hybridisations due to differences in the reference sequence and transcript in question. This makes it difficult to study non-model organisms which are more likely to not have their entire transcriptomes sequenced and catalogued. With RNA-seq, transcriptomes can be assembled without the need for a reference genome of the organism in question. By using paired-end sequencing reads, researchers can discover exon splice junctions and novel transcript isoforms within a transcriptome, which is not possible with the microarray platform.

WHOLE GENOME SEQUENCING OF THE LONG-TAILED MACAQUES

Following the completion of the human genome project in 2003, concerted efforts have been made to sequence model organisms seen beneficial to the biomedical field. Prior to the onset of next-generation sequencing, researchers have successfully sequenced the genome of an Indian rhesus macaque via whole-genome shotgun sequencing approach (Gibbs et al., 2007). Of major significance to the complete sequencing of the rhesus macaque's genome is its ability to provide insights into the biological pathways and the functionality of an adult rhesus macaque. Using the newly assembled draft rhesus macaque genome (rheMac2) Gibbs et al. (2007) and Osada et al. (2008) compared Philippine and Cambodian-Thailand hybrid *M. fascicularis* via sequencing of complementary DNA (cDNA) clones with

M. mulatta cDNA sequences predicted from the rheMac2 assembly. However, the team relied mostly on aligning the *M. mulatta* and *M. fascicularis* sequences with human genome sequences to identify orthologs, and thus estimate the genetic divergence between *M. fascicularis* and *M. mulatta*. Due to the rheMac2 genome assembly's lack of species-specific transcript annotation information, the presence of gaps, and the discovery of scaffold misassemblies (Norgren 2013), efforts were made to annotate the rheMac2 genome through RNA sequencing studies of the rhesus macaque (Pipes et al., 2013) as well as genome annotation endeavours (Zhang et al., 2013; Peng et al., 2014). A new rhesus macaque genome assembly was produced by Zimin et al. (2014) using NGS technology to correct the assembly errors in the rheMac2 assembly and the rhesus macaque genome assembly done by Yan et al. (2011). This assembly called MacaM, was built independently of the rheMac2 assembly and was also supplemented by RNA-seq data that was carried out simultaneously with the MacaM whole-genome sequencing. The drafting and annotation of the rhesus macaque genome opened doors for biomedical studies which rely y on the model organisms' gene expression patterns towards novel drug treatments.

One of the first whole-genome sequencing endeavour of the long-tailed macaque was accomplished by Ebeling et al. in 2011. They employed the NGS platform to sequence the genome of a Mauritian long-tailed macaque using the whole genome shotgun sequencing approach. The team assembled the long-tailed macaque genome by utilising the rheMac2 genome, as well as the human genome (hg18) based on homology. With the draft genome, the team carried out transcriptome predictions to develop *M. fascicularis*-specific gene expression microarrays. Their ultimate goal was to provide reliable genomic information of the long-tailed macaque for biomedical research and drug testing.

The genomes of a Vietnamese long-tailed macaque and a Chinese rhesus macaque were drafted by Yan et al. in 2011. They compared their genome sequences with that of the rheMac2 genome assembly and their findings suggest that the Vietnamese long-tailed macaque may have undergone introgression after hybridisation with the Chinese rhesus macaque. In addition, they also observed genetic differences in orthologues related to biomedical studies between the three macaque genomes they studied. The researchers postulated that macaque populations from distinct geographical locations are likely to have genetic variation.

In 2012, Higashino et al. endeavoured to construct the genome of the Malaysian long-tailed macaque by employing the resequencing method with reference to the rheMac2 genome assembly. Their rationale was that the Malaysian long-tailed macaque has shown to exhibit higher genetic diversity in contrast with their Vietnamese counterparts (Osada et al., 2010). The team's study supported Yan et al.'s (2011) postulation that the Vietnamese long-tailed

macaque had undergone introgression with the Chinese rhesus macaque.

These findings were further strengthened by Fan et al.'s (2014) assembly of the Tibetan macaque (*Macaca thibetana*) genome by conducting genetic diversity assessments among the genomes of the Tibetan macaque, the Chinese rhesus macaque, the Vietnamese long-tailed macaque, and the Malaysian long-tailed macaque. They found long-tailed macaque populations to display higher genetic diversity than populations of the rhesus macaques. Genome-wide comparisons also showed the Tibetan macaques were more closely related to the rhesus macaques compared to the long-tailed macaques. Subsequently, they also postulated that the Tibetan macaques underwent admixture with the Chinese rhesus macaque resulting from overlapping geographical distribution and mating habits.

The most recent whole-genome sequencing of the long-tailed macaque was undertaken by Osada et al. in 2015. Six Mauritian long-tailed macaque genomes were sequenced and it was determined that the overall nucleotide diversity of the Mauritian macaques was 23% smaller than the Malaysian long-tailed macaques. Further phylogenetic analysis between the newly sequenced Mauritian long-tailed macaque, Malaysian, Vietnamese, and Chinese long-tailed macaque suggested that the Mauritian macaques were genetically closer to the Malaysian macaques. Genome-wide comparisons between two or more closely related species are beneficial for genetic variation discovery studies, annotations of functional genes via orthologue comparisons, phylogenetic inference, and differential gene expression studies.

TRANSCRIPTOMIC SEQUENCING OF THE LONG-TAILED MACAQUE

Prior to NGS technology becoming ubiquitous, cloning and capillary sequencing were essential methodologies for sequencing the transcriptome of an organism. However, this approach involves the time consuming and costly effort of cloning an immense amount of cDNA sequences and sequencing all the cloned sequences. Despite the tedious work flow and cost, this was a method commonly used in sequencing expressed sequence tags (ESTs) to conduct transcript profiling. In 2005, Magness et al. sequenced the transcriptome of the rhesus macaque using this approach. They constructed the rhesus macaque transcriptome by mapping their reads to orthologs in the human messenger RNA (mRNA) RefSeq sequences as well as rhesus macaque mitochondrial sequences found in GenBank. Magness et al. also encountered rhesus macaque cDNA sequences that did not have putative orthologs in the human cDNA or genome, of which they were unable to determine their species specificity due to the then unavailability of a completed rhesus macaque genome. In addition, Magness et al. determined the percentage of sequence divergence between the rhesus macaque and humans in the coding and non-coding sequence levels, as well as in the amino acid level. Osada et al. (2008) also sequenced the transcripts of the long-

tailed and rhesus macaque using a similar approach of sequencing tens of thousands of cDNA clones.

Using RNA-seq technology, Huh et al. (2012) sequenced the transcriptome of long-tailed macaques and identified genes involved in various biological responses of the macaque. A total of 16 tissues originating from two macaque individuals obtained from Vietnam were used and 175 transcripts identified, 81 of which were experimentally validated. In an expansion of Huh et al.'s 2012 study, Park et al. (2013) used Huh et al.'s transcriptome sequencing data to select appropriate reference genes for real-time quantitative PCR (RT-qPCR) normalisation procedures involved in gene expression studies of the long-tailed macaque. This study provides a benchmark for research in gene expression studies on the long-tailed macaque. Subsequently, Lee et al. (2014b) and Park et al. (2015) used transcriptomic data from Huh et al. (2012) in two separate gene expression studies to investigate the quantitative gene expression of insulin/insulin-like growth factor, amyloid precursor protein, and tau-phosphorylation-related genes in Alzheimer's disease with the long-tailed macaque as a model organism.

A collaboration between researchers from many NPRCs, Pipes et al. (2013) set out to profile the transcriptomes of 13 NHP species of biomedical importance. Among the NHPs selected for the large-scale study include long-tailed macaques of Indochinese and Mauritian origin, the pig tailed macaque (*Macaca nemestrina*), rhesus macaques of Chinese and Indian origin, the Japanese macaque (*Macaca fuscata*), and other NHPs. The team employed multiple methods of library preparation to capture the deeper set of transcriptomic data, including coding transcripts, non-coding transcripts, and delineating information for strand-specific and anti-sense transcription detection. Parallel to the RNA-seq studies, the 'NHP Reference Transcriptome Resource' was established to deposit sequencing data as well as to serve as a public database and online community for researchers. Peng et al. (2014) further expanded the NHPRTR project by sequencing the same 15 tissues of 11 NHP species and subspecies – including the Indochinese and Mauritian long-tailed macaque. Their goal was to make tissue-specific RNA-seq data originating from the various NHPs sequenced in this endeavour. They also set out to improve the Indian rhesus macaque and Mauritian long-tailed macaque transcriptome annotations, filling up transcript sequence gaps in the genomes of the respective macaques, and providing novel isoforms for annotated genes and also unannotated intergenic transcripts enriched with noncoding RNA.

Lee et al. (2014a) sequenced the transcriptomes of the long-tailed macaque, African green monkey (*Chlorocebus aethiops*), and rhesus macaque using the Illumina GAIIx platform. They sequenced six tissue samples as well as blood samples harvested from the long-tailed macaque and proceeded with a reference-guided assembly of the long-tailed macaque by mapping the sequencing reads to the long-tailed macaque

genome. The team performed a comparative tissue-specific gene expression analysis of their long-tailed macaque and African green monkey transcriptomes with transcriptomes of other primates, including *Homo sapiens, Pan troglodytes, Gorilla gorilla*, and *Macaca mulatta*. Principal component analysis revealed the consistent gene expression profiles of orthologous protein-coding genes across the primates analysed. Furthermore, the team also identified and validated novel transcripts and splice isoforms obtained from their sequencing data. In addition to the transcriptome assembly, Lee et al. (2014a) also developed a Multi-Species Annotation (MSA) pipeline that enables systematic annotations of transcriptome assemblies by employing the use of BLAST and NCBI's nt database.

GENETIC VARIATIONS IN THE LONG-TAILED MACAQUES AND THE NEED FOR COMPREHENSIVE GENOMIC AND TRANSCRIPTOMIC INFORMATION

Genomic comparisons of the long-tailed macaques are mostly in concordance with genetic data. With genomic datasets, Higashino et al. (2012) and Fan et al. (2014) determined that the Malaysian long-tailed macaque has the highest genetic diversity among the populations of long-tailed macaques, a pattern that is similarly observed in Smith et al. (2007). Osada et al.'s (2015) findings using genomic data to determine the nucleotide diversity of Mauritian long-tailed macaques also mirror the findings of Smith et al. (2007). Genomic data (Yan et al., 2011; Higashino et al., 2012) also tally with findings of introgression between Vietnamese long-tailed macaques and Chinese rhesus macaques based on genetic data (Tosi et al., 2002; Bonhomme et al., 2009; Kanthaswamy et al., 2008). The parallel between genomic and genetic results shows the promise of genomics in population genetic studies. Genomics allows the expansion of the number of genes/ nucleotide characters utilised, providing for a larger sampling size for the construction and inference of phylogenetic trees, and single nucleotide polymorphism analysis. NGS vastly facilitates the acquisition of genomes and transcriptomes, which in turn provides the larger sample size for further downstream analyses at a fraction of the time, cost, and effort.

Due to inter- and intraspecific genetic variations in NHP models, a more robust and complete annotation of reference genomes from various locations and populations is needed in order to select populations with genetic backgrounds suitable for relevant experiments (Haus et al., 2014). Various studies employing macaques have observed different phenotypical reactions involving individuals from different geographical locations, or even among individuals from the same geographical origin. Trichel et al. (2002) observed differences in the progression of acquired immune deficiency syndrome (AIDS)-like viral infection in two different populations of rhesus macaques obtained from India and China, whereby the rhesus macaques from China lived longer than the Indian macaques. A study by Seekatz et al. (2013) into the

efficacy of Shigella vaccines administered to Mauritian and non-Mauritian long-tailed macaques showed that the two populations responded differently, arising from the presence of distinct gut microbiota in the Mauritian macaques. Genotyping efforts of TRIM5α by Zhang et al. (2016) revealed polymorphisms of the gene that suppresses the viral replication of HIV2-ROD in rhesus macaques, and suggests the need to genotype TRIM5α of rhesus macaques before commencing HIV studies that employ these model organisms. While NHP model organisms certainly assist in drug studies, it is essential that reference genomes of NHP models that originate from various locations are sequenced in order to design a solid research framework.

In a decade where Malaysia faces indiscriminate levelling of forests (Hamdan et al., 2016), the population of long-tailed macaques is becoming more fragmentised and threatened. Families of macaques are forced out of their habitats and into human dwellings where they risk of conflict with humans, capture and culling. Though the long-tailed macaques are not yet extinct their numbers are dwindling faster than initially expected (Eudey, 2008). Populations of macaques face the threat of inbreeding due to their fragmented habitats, which will inevitably reduce their genetic diversity overtime (Mona et al., 2014). Researchers can mitigate the possibility of population fragmentation through population management techniques. Following the introduction of high-throughput sequencing technology, whole genomes and transcriptomes can be more easily sequenced, assembled, and analysed. This approach is beneficial for population genetics researchers because large genomic and/or transcriptomic datasets can be generated more cheaply (Shendure & Ji, 2008; Metzker, 2010). From the whole genome or transcriptome dataset, and with appropriate pipelines – reviewed extensively in Ekblom & Galindo, (2011) and Davey et al., (2011) – researchers can easily extract thousands of sequences and design genetic markers for population genetics studies.

Presently, there is a need to sequence the genome and transcriptome of long-tailed macaques of Peninsular Malaysia and Borneo Malaysia. Out of the 50 *M. fascicularis* subspecies classified by Fooden (1995), *Macaca fascicularis* is the most widely distributed *M. fascicularis* subspecies in Peninsular and Borneo Malaysia (Groves 2001; Brandon-Jones et al., 2004). By utilising mitochondrial DNA (Tosi & Coke, 2007; Abdul-Latiff et al., 2014b), mitogenomic phylogeny (Liedigk et al., 2015), and examining Y-chromosome gene flow (Rovie-Ryan et al., 2013), researchers have observed phylogeography separations of Malaysian long-tailed macaque populations into mainland (Peninsular Malaysia) and insular (Borneo Malaysia) populations. Abdul-Latiff et al. (2014a) and Smith et al. (2007) have shown that the Malaysian population of long-tailed macaques are monophyletic with no shared haplotypes with other Southeast

Asian long-tailed macaque populations, with the Malaysian populations having higher levels of nucleotide diversity compared to the other long-tailed macaque populations of Southeast Asia. Higher genetic diversity results in a wider range of genotypic and phenotypic reactions to drug treatments (Osada et al., 2015), which could prove to be a valuable insight in biomedical research. In light of the long-tailed macaque's suitability as NHP models in drug-safety testing, and biomedical and disease-related studies, the importance of obtaining deep-sequencing genomic data of this species is crucial. A genomic and transcriptomic repository of not only long-tailed macaques, but other NHPs from various geographical locations is essential for sustainable and substantial biomedical, disease-related, and drug-safety testing research.

CONCLUSION

With the benefit of cutting costs, saving time, and the usage of relatively small amounts of tissue for the sequencing task, high throughput sequencing of the transcriptome can expedite the acquisition of genetic markers for the population genetic studies of the long-tailed macaques. A faster and more efficient management of the population of long-tailed macaques can hopefully be achieved, although this goes hand-in-hand with the many government and non-government agencies involved. Researchers have a duty to provide information needed for the respective parties to act.

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The Potential Use of Papaya Juice as Fermentation Medium for Bacterial Cellulose Production by *Acetobacter xylinum* 0416

Zahan, K. A.*, Hedzir, M. S. A. and Mustapha, M.

Bioengineering Technology Section, Malaysian Institute of Chemical and Bioengineering Technology, Universiti Kuala Lumpur, 78000 Alor Gajah, Melaka, Malaysia

ABSTRACT

Bacterial cellulose had been proven to be a very versatile natural polymer produced by bacteria. One of the major constraints in producing bacterial cellulose is the high cost of fermentation medium. This study examines the potential use of papaya juice as a low-cost fermentation medium for the production of bacterial cellulose. The fermentation of *Acetobacter xylinum* 0416 was carried out under static fermentation with an initial pH of 5.5, a temperature of 28±1°C and a fermentation period of 5 days using different juice extracted from ripe and unripe papaya. The highest production of bacterial cellulose was detected in ripe papaya pulp juice with a total weight of 35.37 g/l. Juice obtained from various parts of the fruit produced bacterial cellulose between 3.33 g/l and 16.10 g/l. By referring to the standard medium (Hestrin-Schramm (HS) medium), ripe papaya pulp juice shows the highest potential to be used as an alternative for the production of bacterial cellulose. This is due to its high glucose concentration that provide suitable conditions for *A.xylinum* 0416 to grow and produce bacterial cellulose.

Keywords: *Acetobacter xylinum* 0416, bacterial cellulose, fermentation medium, papaya juice

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E-mail addresses:
khairulazly@unikl.edu.my (Zahan, K. A.),
shaifulaizathedzir@gmail.com (Hedzir, M. S. A.),
mahfuzah@unikl.edu.my (Mustapha, M.)
* Corresponding author

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INTRODUCTION

The most abundant natural polymer on Earth is cellulose, which is mostly produced by vascular plants, algae and other species of bacteria. With the increasing need for sustainable biofuel and bioproducts, the demand for plant cellulose has continued to rise (Ernsting, 2012). Typically, plant cellulose can be obtained from lignocellulosic resources or biomass, such as

forestry materials and agricultural residues. Cellulose is embedded in the complex matrix of the lignocellulosic structure and thus, it is difficult to separate it from the biomass. An alternative resource for plant cellulose is bacterial cellulose (BC) (Brown, 2004), which are easily separated compared with its plant cellulose counterparts.

The molecular formula of bacterial cellulose $(C_6H_{10}O_5)n$ is similar with plant cellulose, but its physical and chemical features are different. The BC has unique mechanical and structural properties such as high degree of crystallinity and polymerisation. It is also purer than the fibrous polymer obtained from plant sources in which the cellulose fibrils are embedded with lignin, hemicellulose and waxy aromatic substances (Ross et al., 1991). Additionally, the thickness of cellulose fibrils for bacterial cellulose is between 0.1 µm and 10 µm, which is one hundred times thinner than cellulose fibrils obtained from plant cellulose (Gayathry & Gopalaswamy, 2014).

Due to the numerous potential applications of bacterial cellulose, it must be efficient, cost-effective and produced in high quantities to meet commercial demands. To ensure favourable yields and to lower costs, attention needs to be given to the species and genetic modification of the bacteria used, type and composition of fermentation medium and the type of reactor for the production process (Shi et al., 2014).

Previously, most of the BC was produced from a high cost defined medium such as Hestrin-Schramm (HS) medium containing various types of chemicals such as glucose, yeast extract, ammonium sulphate, peptone and other additional synthetic nutrients (Zahan et al., 2014a). Hungund et al. (2013) revealed that various fruits juice including pineapple, pomegranate, muskmelon, watermelon, tomato, orange, molasses, sugarcane and coconut water were used as alternative carbon sources for BC production. Fruit juice alone as a carbon source was capable of producing a high yield of BC instead of using a high cost defined medium (Zahan et al., 2014b).

Although fruit juices are known to be an efficient fermentation medium to produce BC, the use of papaya juice in particular for this purpose is still limited. Papaya or *Carica papaya* belongs to the genus Carica, found abundantly in Malaysia. Papaya is an excellent source of carbohydrate, vitamins and minerals. Among the carbohydrates, sugars are the principle constituents of papaya with a total content of 48.3% sucrose, 29.8% glucose and 2% fructose (Chan & Kwok, 1975; Gomez et al., 2006). According to Oloyede (2005) and Wall (2006), papaya contains sodium, potassium, magnesium, calcium, iron and other vitamins which encourage growth of bacterium.

Thus, this research aims to study the potential of papaya juice obtained from various parts of the fruit (pulp, peel and seed) to become a low cost medium for the production of bacterial cellulose by fermentation of *Acetobacter xylinum* 0416. Thus, no parts of the perishable fruit are wasted (Rohani et al., 1997).

MATERIALS AND METHODS

Preparation of inoculum *A.xylinum* 0416 using papaya juice

Local ripe papaya was peeled and washed. About 200 g of its pulp was weighed and blended with 200 ml of distilled water. The juice was filtered using a filter cloth to obtain 200 ml of clear juice (Mehtab & Paul, 2014) and transferred to a 250 ml schott bottle. The pH of the juice was adjusted to 5.5 with 2 M sodium hydroxide (NaOH) or 2 M hydrochloric acid (HCl) before it was sterilised at 121°C for 15 minutes. After it was cooled to 28±1°C, 10% (v/v) of *A.xylinum* 0416 (obtained from Biotechnology Research Centre, MARDI, Serdang) was added to the juice aseptically in a laminar flow. The inoculum was mixed by shaking the bottle gently and slowly before it was incubated at 28±1°C for three days (Zahan et al., 2014b).

Preparation of inoculum *A.xylinum* 0416 using standard medium (Hestrin & Schramm (HS) medium)

200 ml of HS medium containing 0.5% (w/v) yeast extract, 0.5% (w/v) peptone water, 2% (w/v) D-glucose, 0.115% (w/v) citric acid ($C_6H_8O_7$) and 0.27% (w/v) disodium hydrogen phosphate (Na_2HPO_4) were mixed homogeneously (Shi et al., 2013). The mixture was transferred to a 250 ml schott bottle, and its pH adjusted to 5.5 with 2 M sodium hydroxide (NaOH) or 2 M hydrochloric acid (HCl). The mixture was sterilised at 121°C for 15 minutes. After it was cooled to 28±1°C, 10% (v/v) of *A.xylinum* 0416 was added to the mixture aseptically in a laminar flow. The inoculum was mixed by manually shaking the bottle gently and slowly before it was incubated at 28±1°C for three days (Zahan et al., 2014b).

Preparation of fermentation medium and synthesis of bacterial cellulose

300 ml of fermentation medium was prepared for HS medium and different parts of papaya (ripe papaya pulp, ripe papaya peel, unripe papaya pulp, unripe papaya peel and papaya seed) as mention in previous section. The juices and HS medium were transferred to a different 500 ml schott bottle, and the pH was adjusted to 5.5 with 2 M sodium hydroxide (NaOH) or 2 M hydrochloric acid (HCl). Then the fermentation medium was sterilised at 121°C for 15 min. After it was cooled to 28±1°C, the fermentation medium was transferred to a sterilised 1 L plastic container aseptically in a laminar flow. Then, 10% (v/v) of *A.xylinum* 0416 inoculum was aseptically poured into the fermentation medium. Finally, the fermentation medium was incubated at 28±1°C for five days. 10 ml of the fermentation medium was sampled every 24 hours. The steps were repeated twice for each type of fermentation medium (Pa'e et al., 2011).

Measurement of bacterial cellulose weight

The BC was later harvested and washed with boiling water at 100°C for 60 minutes. The BC was rinsed again with plenty of water (until pH became 7.0). Excess water on the

surface of the BC was wiped with tissue papers and the total weight were recorded using an electronic balance until a constant value was obtained (Shi et al., 2013).

Determination of *A.xylinum* 0416 growth.

Two dried and clean 1.5 ml microcentrifuge tubes were weighed. Then 10 ml of fermentation medium sample was mixed by manually shaking the bottle gently and slowly. After that, 1.5 ml of fermentation medium sample was pipetted into each tube. The tubes were centrifuged at 3000 rpm for 20 min. Then, the supernatant was decanted, and the tubes were placed in an oven at 90°C for 24 hr. After that, the tubes were weighed to obtain the cell dried weight (Banerjee et al., 1993). The cell dried weight (CDW) was calculated using Eq. 1:

$$\text{CDW (g/l)} = \frac{\text{weight of tube and dried cells, (g) - weight of empty tube, (g)}}{\text{sample volume, (ml)}} \times 1000 \quad [1]$$

Determination of glucose concentration.

A 10 ml of fermentation medium sample was mixed by manually shaking the bottle gently and slowly. After that, 1.5 ml of sample was pipetted into a cuvette. The glucose concentration was detected using a biochemistry analyser (YSI 2700D, USA) (Zahan et al., 2015a).

Analysis of pH

The electrode was rinsed using a distilled water. Then, the electrode was immersed in a universal bottle containing 10 ml of fermentation medium sample obtain at the initial and final day of the fermentation process. The pH values were recorded when the reading was stable.

Statistical Analysis

All data were analysed with one way analysis of variance (ANOVA), and mean values were compared at $P<0.05$ significant level test using Microsoft Excel 2010.

RESULTS AND DISCUSSIONS

Production of bacterial cellulose by *A.xylinum* 0416 in different parts of papaya juice and HS medium were evaluated by the total weight of bacterial cellulose produced after 5 days of fermentation. Figure 1 shows that the highest yield of bacterial cellulose was produced in ripe papaya pulp medium (35.37 g/l) and followed by HS medium (35.2 g/l). Result also shown that fermentation of *A.xylinum* 0416 in other parts of papaya juice medium also manages to produce the bacterial cellulose but in different amount; ripe papaya peel (16.1 g/l), unripe papaya pulp (15.93 g/l), unripe papaya peel (5.2 g/l) and papaya seed (3.33 g/l). By comparing with the standard medium (HS medium), ripe papaya pulp medium produced a slightly higher amount of bacterial cellulose which shows a potential to be used as an alternative and low cost fermentation medium for the production of bacterial cellulose. While, the other parts of papaya fruits such as peel and seed also can be utilized for production of bacterial cellulose instead of being disposed.

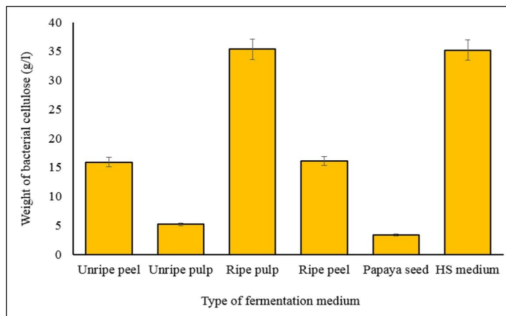

Figure 1. Weight of bacterial cellulose produced in different type of fermentation medium

The analysis of variance (ANOVA) show the calculated F_{value} (184.22) was higher compared with F_{crit} (4.39) and a very low probability value ($P \leq 0.00000177$). Therefore, the juice obtained from different parts of papaya to produce bacterial cellulose by *A.xylinum* 0416 fermentation was statistically proven.

Production of BC is higher when the nutrients in the fermentation medium such as carbon sources is high. The example of carbon sources are glucose, fructose, sucrose and maltose but in this research, the focus is on glucose as primary carbon sources. Glucose was recommended as the ideal carbon source for BC production since most of the bacterial cellulose synthesis are done in glucose culture medium (Santos et al., 2013; Zahan et al., 2014a). Figure 2 shows the highest glucose concentration was 22.4 g/l and 21.3 g/l in ripe papaya pulp medium and HS medium respectively. The lowest glucose concentration was 2.07 g/l in papaya seed medium. The result shows different types of fermentation medium have different levels of glucose concentration. This finding is consistent with that of Chan and Kwok (1975), Gomez et al., (2006) and Duke (1996) which stated that the highest carbon sources present in ripe papaya pulp was glucose.

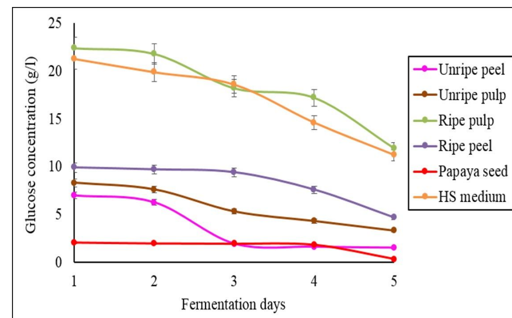

Figure 2. Glucose concentration in different type of fermentation medium

Bungay and Serafica (1999) stated that increasing the glucose concentration above 25 g/l does not correspond to an increase in bacterial cellulose production during the 5 days of the fermentation process, while an inadequate amount of glucose, namely 5 g/l, can inhibit BC production. Therefore, in order to maximise the amount of bacterial cellulose produced, a certain amount of glucose should be maintained during the fermentation process. Based on this research, it is suggested glucose concentration is maintained between 5 g/l and 25 g/l. The results also indicate represent that all types of fermentation medium are suitable for BC production except for papaya seed.

Figure 2 also shows, as fermentation period (days) increases, the glucose concentration (g/l) decreases for all types of fermentation medium because the glucose has been used by *A.xylinum* 0416 for BC formation and its growth. Figure 3 shows

that growth of *A.xylinum* 0416 increases as fermentation period increases where the population growth in a closed system follows the “standard growth curve”. The lag, exponential and stationary phase for *A.xylinum* 0416 in a different type of fermentation medium can be determined. The highest growth of *A.xylinum* 0416 was in ripe papaya pulp medium and HS medium that showed the exponential phase began from the first day until the fifth day of fermentation. While the lowest growth of *A.xylinum* 0416 was recorded in papaya seed medium where the exponential phase occurred from the first day until the third day of fermentation before proceeding to the stationary phase. These results were consistent with the analysis of glucose concentration discussed earlier which proves that when the glucose consumption is high, the growth of *A.xylinum* 0416 is also high.

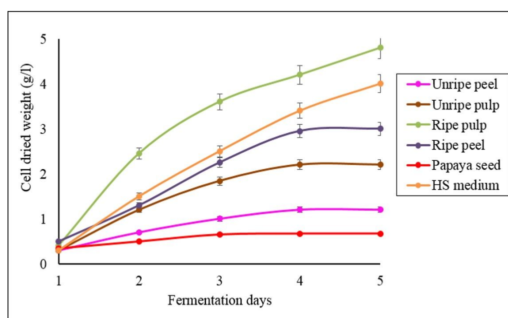

Figure 3. Growth of *A.xylinum* 0416 in different types of fermentation medium

The analysis of variance (ANOVA) showed the calculated F_{value} (3.66) was higher compared with the F_{crit} (2.62) and a very low probability value ($P \leq 0.01323$). Therefore, juice from different parts of the papaya for the growth of *A.xylinum* 0416 was statistically proven.

A.xylinum 0416 reproduction is limited by the availability of nutrients in the fermentation medium. Each *A.xylinum* 0416 cell divided into two, and will continue to do so until it runs out of nutrients. During the exponential phase, a rapid growth of *A.xylinum* 0416 occurs due to suitable conditions. In this phase, the consumption of glucose, and BC production rate is high (Zahan et al., 2015a). The stationary phase shows a linear growth of *A.xylinum* 0416 during fermentation process where the consumption of glucose and bacterial cellulose production is constant. Through this research, there is no death phase for *A.xylinum* 0416 during the 5-day fermentation period. Thus, results indicated that ripe papaya pulp medium can serve as a suitable fermentation medium for the growth of *A.xylinum* 0416 due to the high amount of glucose and supported by other natural elements present.

During the exponential phase of *A.xylinum* 0416 growth, the glucose consumption was high and at the same time, the formation of acetic acid as its by-products is also high (Zahan et al., 2015b). Consequently, there is an increase in acidity levels in the fermentation medium that may suppress the growth of *A.xylinum* 0416 and also the BC production (Vandamme et al., 1998). Figure 4 shows the decreasing trend for the pH for each type of fermentation medium between 3% and 18%. But the pH for each type of fermentation medium is still

in the optimum range. Chawla et al. (2008) reported that the optimum pH of the medium for BC production in the range of 4.0 to 6.0, and the yield decreases when it is below 4.0. Thus, it is important to ensure that the pH is controlled during the fermentation process to reduce the possibility of bacterial inhibition which can affect yield.

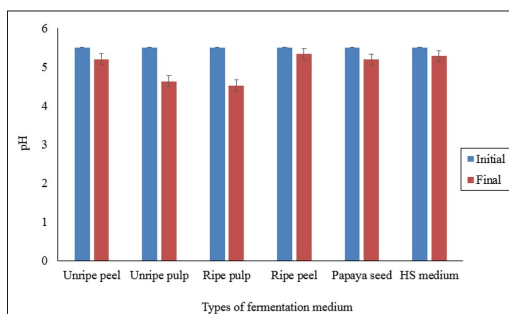

Figure 4. pH for different type of fermentation medium

CONCLUSION

The results show that the production of BC by fermenting *A.xylinum* 0416 in ripe papaya pulp medium was slightly higher than using the HS medium, thus proving that ripe papaya pulp medium can be used as a low-cost medium of fermentation in the production of BC. In addition, the other parts of papaya showed a good potential to produce BC. In conclusion, the growth of *A.xylinum* 0416 is the highest in ripe papaya pulp medium due to its highest glucose concentration and suitable pH .

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Temporal Expression of a Putative Homogentisate Solanesyltransferase cDNA in Wounded *Aquilaria malaccensis*, an Endangered Tropical Tree

Azzarina, A. B., Mohamed, R.*, Siah, C. H. and Wong, M. T.

Forest Biotech Laboratory, Department of Forest Management, Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

ABSTRACT

Homogentisate prenytransferase (HPT) generally catalyses prenylation reactions in tocochromanol and plastoquinone-9 biosynthesis, while homogentisate solanesyltransferase (HST) is specific to reaction leading to plastoquinone, an essential component in the synthesis of carotenoid, a powerful antioxidant and precursor to vitamin A. In *Aquilaria* spp. abiotic stress in the form of wounding is the main trigger for the production of a highly-valued terpene-rich wood known as agarwood. Putative HST cDNA, *AmHST1* was cloned from total RNA of callus tissue of *Aquilaria malaccensis* using reverse transcription approach. Based on a partial HST sequence, specific primers were initially designed to amplify the internal open reading frame region followed by RACE, which successfully amplified the cDNA. The partial length *AmHST1* cDNA measured about 1182 bp nucleotides and encodes a polypeptide of 392 amino acid. Sequence alignment revealed that AmHST1 shares 74% - 77% similarity with HPT from Arabidopsis and *Theobroma cacao*. Gene expression analysis indicated that the *AmHST1* expression was suppressed in wounded tissues. Results suggest that there should be a potential trade-off between genes involved in plastoquinone and terpenoid synthesis as they both share similar upstream genes and precursors. When facing a major abiotic stress such as wounding, the latter is favoured.

Keywords: *AmHST1,* prenyltransferase, terpenoid, Thymelaeaceae, Tocopherol

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E-mail addresses:
mzazzar@gmail.com (Azzarina, A. B.),
rozimohd@upm.edu.my (Mohamed, R.),
pinkspring147@gmail.com (Siah, C. H.),
munthengwong@gmail.com (Wong, M. T.)
* Corresponding author

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INTRODUCTION

Aquilaria malaccensis Lam. is one of the main agarwood-producing species in the world. The genus *Aquilaria* belongs to the family Thymelaeaceae and is found across South East Asia and tropical regions of China and India (Oldfield, 1998). Agarwood, also known as gaharu, eaglewood and aloeswood, is a dark fragrant resin used as medicine to treat asthma, diarrhoea, body ache, and other ailments (Barden et al. 2000), as incense for use in religious rituals, and also as an ingredient in perfumes (Persoon, 2008; Jayachandran et al. 2014). In Asia, huge pieces of agarwood are highly sought for use in sculpturing idols or decorative items. All these make agarwood an extremely prized forest product presently traded internationally. Because of its high demand and prices of agarwood, *Aquilaria* spp. in the wild have been over-exploited resulting in each of its species being listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2013).

Agarwood is a resinous substance produced by the tree in response to 'wound' caused by many natural factors such as insect and microbe attack, lightning strikes and wind. However, the accumulation is rather slow and may take multiple years to yield a substantial amount. The main chemical components of agarwood from various *Aquilaria* species have been identified as sesquiterpenes and phenylethyl chromones (Naef, 2011). Based on plant model systems, the biosynthesis of terpenoids, which are required for cellular growth and survival, involves prenyltransferases (PTs) which catalyse sequential condensation of basic 5-carbon building blocks, isopentenyl diphospate (IPP; C_5) and dimethylallyl diphosphate (DMAPP; C_5) into three intermediate isoprenoid molecules, geranyl diphospate (GPP ; C_{10}), farnesyl diphosphate (FPP ; C_{15}) and geranylgeranyl diphosphate (GGPP ; C_{20}), which are the basis of other longer chain isoprenoid molecules (Aubourg et al. 2002; Dudavera et al. 2013). The terpene synthases (TPSs) then convert the three prenyl diphosphate intermediates into cyclic and acyclic terpenoid skeletons, yielding 10-carbon monoterpenes (monoterpene synthase), 15-carbon sesquiterpenes (sesquiterpene synthase) and 20-carbon diterpene (diterpene synthase) (Lange & Ahkami, 2013). In *Aquilaria*, the PT, also known as farnesyl pyrophosphate synthase (FPP synthase), is a critical enzyme because it catalyses formation of the intermediate FPP isoprenoids to serve as the substrate for subsequent TPS reaction leading to final sesquiterpene product. In previous studies, *Aquilaria* prenyltransferases in the terpenoid pathway were proven to be closely-related to agarwood synthesis (Kenmotsu et al. 2013; Yang et al., 2013).

In its natural state, the FPP is grouped under the general group of enzyme called homogentisate prenyltransferase (HPT) that plays an important role in the biosynthesis of various secondary metabolites such as tocochromanol, tocotrienol, flavonoid, terpenoid and plastoquinone-9. Similar to FPP, the homogentisate solanesyltransferase

(HST), which is specific to plastoquinone (PQ) biosynthesis, is also grouped as HPT. The HST is an important enzyme that catalyses formation of several compounds in the plant, such as tyrosine-derived aromatic compounds, which leads to multiple functions such as biosynthesis in vitamin E, photosystem II (PSII) mobile electron transport co-factor, PQ, and carotenoid (Norris et al., 1995; Sadre et al., 2006; Yang et al., 2011). Many genes encoding HST or its homologs have been isolated and identified from Arabidopsis and other plants (Venkatesh et al., 2006; Soderlund et al., 2009). However, the first HST gene, VTE2, was isolated from *Glycine max* (Venkatesh et al., 2006) and enzyme assay results for cell expression for HST gene in *Escherichia coli* put forward the contribution of HST in catalysing the first step in PQ biosynthesis (Sandre et al., 2006). Unusual expression in HST gene may result in growth abnormality, in which improvement in prenyl lipid, PQ and tocopherol levels was observed in transgenic Arabidopsis when HST gene was overexpressed; disruption of this gene may cause albino phenotype that leads to PQ and tocopherol synthesis deficiency (Norris et al., 1995).

In this study, using mRNA data sequence from *A. malaccensis* transcriptome (Siah et al. 2016), a gene with putative function as homogentisate solanesyltransferase (*AmHST1*) was cloned. Its amino acid sequence shares some similarity with FPP synthase, an important enzyme in agarwood synthesis, but it shares higher homology with HST of other plant origins. *HST* genes are involved in the synthesis of plastoquinone, an antioxidant substance that protects against stress. The temporal expression of the gene was characterised in a time-course wounding experiment and the expression patterns revealed the gene perhaps is not directly involved in agarwood synthesis.

MATERIALS AND METHODS

Plant Materials

In vitro plants were grown from seeds that were sterilised and introduced into half strength MS medium (Murashige & Skoog, 1962) as reported by Daud et al. (2012). Seeds were collected from an *A. malaccensis* mother tree growing at the Sungai Buloh Forest Reserve, Kepong in May 2011. Germinated shoots were cut and transferred into MS medium supplemented with 1.3 μM 6-benzylaminopurine (BAP) as described by He et al. (2005). Plants were sub-cultured onto a fresh medium every 4 weeks until they reach a height of 5cm. Plantlets were grown under long-day conditions (16 hours of light, 8 hours of darkness) with temperature of 25°C.

To initiate callus culture, fresh leaves were collected from four-year-old *A. malaccensis* trees maintained in polybags in the shade house of the Faculty of Forestry, Universiti Putra Malaysia, Serdang. The sterilisation protocol was adopted from Daud et al. (2012). The leaves were washed for 15 minutes under slow running tap water. Then, the leaves were dipped in 70% alcohol and rinsed twice with sterile distilled water. The leaves were surface sterilised in 0.1%

$HgCl_2$ (Sigma Aldrich, USA) for 1 minute; they were later washed four or five times with sterile distilled water. After surface sterilisation, the entire mid rib of the leaf was removed aseptically to produce leaf strip. Leaf explants were prepared by cutting the leaf strips into small squares of 10 mm x 10 mm. Then, they were soaked in 0.5 mg/L ascorbic acid (Sigma Aldrich, USA) for 30 minutes. The explants were dabbed on sterile tissue paper and placed on petri dishes containing MS with 30 g/L sucrose and 2.75 g/L gelrite (Duchefa, Netherlands) without hormone. The Petri dishes were incubated at 25°C in total darkness and observed for occurrence of contamination. After 2 days of observation, the contamination-free explants were transferred onto MS medium supplemented with 2.2 µM BAP and 1.1 µM naphthaleneacetic acid (NAA) following He et al. (2005) and Jayaraman et al. (2014). Calli were sub-cultured every other week in the same fresh media, but in magenta jars, for the next five months. The jars were incubated at 25°C in total darkness.

For gene expression study, wood samples were collected as described in Wong et al. (2013). Briefly, a three-year old *A. malaccensis* tree was drilled with a 3.5 mm diameter drill bit into a depth of 1 to 2 cm. Wounding proceeded in two straight lines with each wound spaced at approximately 10cm. Samples representing 0, 6, 12, 18 and 24 hour post-wounding were collected and stored at -80°C. For callus treatment, using a sharp scalpel, three clumps of calli were cut separately into tiny pieces about 1 – 2 mm and returned to the culturing medium. A portion of the cut callus was collected to represent samples at 0, 6, 12, and 24 hours after wounding treatment. All samples were kept at -80°C for RNA extraction.

RNA Extraction for cDNA Cloning

Stem tissues of *in vitro* plantlets were used for cDNA isolations. Total RNA was extracted from 0.5 g starting material using the RNeasy® Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. DNase treatment was carried out using the DNA-*free*™ Kit (Ambion, USA), according to the manufacturer's instructions. The RNA samples were quantified by measuring the absorbance at 260 nm and 280 nm using nanophotometer (IMPLEN, Germany). The integrity of the RNA samples was measured by 1% agarose gel electrophoresis. First-strand cDNA was synthesised by reverse transcription (RT) from 1µg of DNase-treated total RNA using SuperScript® III First-Strand Synthesis System (Invitrogen, USA) according to the manufacturer's instructions.

Isolation of *AmHST1*

Primers were designed using the Beacon Designer™ 7 software (PREMIER Biosoft, USA) for prenyltransferase gene obtained from our own transcriptome (Siah et al. 2016). All Polymerase Chain Reaction (PCR) experiments were conducted on a SpeedCycler^2 (Analytik Jena, Germany). The specific primers employed were designed to isolate a 760 bp fragment containing the internal sequence (Table

1). The cycle parameters were: 94°C for 5 minutes; 40 cycles at 94°C for 30 seconds, annealing at 60°C for 45 seconds, 72°C for 1 minute; and a final elongation at 72°C for 10 minutes. The PCR product was gel-electrophoresed, the desired fragment cut and cloned into the pGEM®–T Easy Vector (Promega, USA) and sent for sequencing at a commercial lab. Then, the 3'-cDNA end was amplified from RT reaction using the FirstChoice® RLM-RACE (Ambion, USA) according to the manufacturer's protocol. Based on the predicted open reading frame of the partial sequence of *AmHST1*, a Rapid Amplification cDNA Ends (RACE) primer was designed as shown in Table 1. The cycle parameters were: 94°C for 3 minutes; 35 cycles at 94°C for 30 seconds, annealing at the respective temperatures for 30 seconds, 72°C for 1 minute; and a final elongation at 72°C for 7 minutes.

Sequence Verification and Phylogenetic Analysis

A specific pair of primers were designed to verify the gene *AmHST1* using High Fidelity KOD Hot Start Mastermix (Favorgen, USA). The reaction was setup as follows: 10 µl (0.04 U/µl) of KOD Hot Start, both forward and reverse primers at 10 µM each, and 100 ng of cDNA in a final volume of

Table 1
Specific primers used in PCR analysis to obtain AmHST1 cDNA and the primers used in qRT-PCR

Amplification/ Gene	Primer Name	Sequence (5' to 3')	Amplicon size (bp)
Internal	*AmHST1*-F	5'-TCCTCACACCGTCGCCTCTCC-3'	760
	AmHST1-R	5'-CACAGGAGGACTCCACAAGAAAGG-3'	
3' RACE	*AmHST1*-3'O	5'-GGTCATCTCCTTTGCAGTGGCT-3'	749
ORF	*AmHST1*-F-full	5'-ATGGAGCACTCAATCTCTGTTTT-3'	1182
	AmHST1-R-full	5'-CTAAACGAATGGAAATATAGC-3'	
qRT-PCR/	RT-*AmHST1*-F	5'-GCTTCTGAATTATGTTGCTGCCATC-3'	224
AmHST1[1]	RT-*AmHST1*-R	5'-TACCCTAAACGAATGGAAATATAGCG-3'	
qRT-PCR/	TUA-F	5'-GCCAAGTGACACAAGCGTAGGT-3'	183
TUA[2]	TUA-R	5'-TCCTTGCCAGAAATAAGTTGCTC-3'	
qRT-PCR/	PAL-F	5'- GCCTTGCATGGTGGGAACTTTCAG -3'	192
AmPAL[3]	PAL-R	5'- GCCCTTGAAGCCGTAGTCCAG -3'	
qRT-PCR/	PD-F	5'-GAAGTGGCCTTCCTAAGATTTTCACA -3'	216
AmPD[1]	PD-R	5'- ATCGTGACAAATGAAGGTATGCGTC-3'	
qRT-PCR/	GAPD-F	5'-CCG GTC TTT TGG TAT CAG ACG C-3'	251
AmRPL[2]	GAPD-R	5'-CCC GAT AAC CAG GAC GTT CAA G-3'	
qRT-PCR/	WRKY-F	5'-CAACCGACCTAACAACAAC-3'	106
AmWRKY[3]	WRKY-R	5'-TAAATTGTGACCTGGGTTAC-3'	

[1]Primer sequences were designed in this study
[2]Primer sequences derived from Gao et al. (2012)
[3]Primer sequences derived from Wong et al. (2013)

20 µl. The cycling conditions: 95°C for 2 minutes; 40 cycles at 95°C for 20 seconds, annealing at 42°C for 10 seconds, 72°C for 40 seconds; and a final elongation at 72°C for 7 minutes. The PCR product was cloned into the Perfectly Blunt® Cloning Kits (Novagen, USA) and sequenced. The percentage of *AmHST1* nucleotides was predicted using the Bioedit software version 7.2.5 (www.bioedit.software.com). The sequence was searched against the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm to identify similar sequences. The molecular weight and theoretical isoelectric point for the deduced amino acid were calculated using ExPASy online software (http://web.expasy.org/compute_pi/). To identify the conserved domains present in the deduced amino acid, a comparison was made with conserved domain alignments found in the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The prenyltransferase-like sequences from the GenBank were aligned using ClustalW and phylogenetic analysis was performed using MEGA version 6 (Tamura et al. 2013). Bootstrap analysis was carried out with 1000 datasets.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR study, total RNA was isolated from 1g of wood tissue samples derived from the wounded tree using the RNeasy® Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. Similar isolation protocol was conducted for the fresh cut callus. Then, 1µg of total RNA was used to synthesise the first-strand cDNA using the QuantiTech® Reverse Transcription Kit (Qiagen, Germany). The parameters analysed that were used for qRT-PCR (Wong et al., 2013). Briefly, it was conducted in triplicate assays and each assay contained 10µl of 2× Sensifast™ SYBR Lo-ROX Kit (Bioline, UK), 10 µM of forward and reverse primers, and 100 ng of cDNA template in a final volume of 20 µl. The PCR parameters were: 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds, annealing at 60°C for 10 seconds, 72°C for 5 seconds, and the process continued with 95°C for 2 minutes, 60°C for 5 seconds and 95°C for 5 seconds, using a MX3005P™ instrument. It was analysed using MxPro™ QPCR (Software) (Agilent Technologies, USA). The calculation of normalised gene of interest (GOI) expression level was done by dividing the raw GOI quantities for each sample to appropriate normalisation factor. The error propagation rules for independent variables were applied to calculate the standard deviation (SD) on the normalised gene of interest (GO_{Inorm}) expression level.

For expression analysis of *AmHST1* gene, the RT-*AmHST1*-F and RT-*AmHST1*-R primers were utilised. Three other genes were included as comparison and to provide evidence for the function of *AmHST1*: 1) phenylalanine ammonia-lyase (*AmPAL*, GenBank Accession No. KT357522) and *AmWRKY* (GenBank Accession No. KT357521) (Wong et al. 2013), and 2) pyruvate dehydrogenase (PD). The

latter sequence was obtained from our transcriptome (Siah et al. 2016). The *Aquilaria* housekeeping genes, α-tubulin (TUA) and ribosomal gene (RPL), were used as reference genes for data normalisation (Gao et al. 2012). All primers sequences are listed in Table 1.

RESULTS

Identification of a Putative Homogentisate Solanesyltransferase cDNA

From our transcriptome data (Siah et al. 2016), several sequences similar to prenyltransferases were selected. Using reverse transcriptase-PCR amplification and specific primers designed to amplify the internal region of the transcriptomic sequence, the partial sequence from first-strand cDNA template of *in vitro* plant stems were amplified. The sequence length was 760 bp and matched with the original transcriptome sequence. Using the verified sequence, a near full-length sequence was cloned by 3' RACE using oligo (dT)-primed cDNA. Because the start codon was only 50 bp upstream from the N terminal portion of this sequence, the open reading frame (ORF) was amplified using a forward primer designed from the start codon. The resulting cloned cDNA sequence was 1312 bp and it contained an ORF of 1182 bp, and a 130 bp 3' un-translated region including a poly (A) tail (Figure 1). The sequence has been given a GenBank accession number, KT380852.

```
   1 ATGGAGCACT CAATCTCTGT TTTctcccca tctcgaattt tagctctagc [tcctcacacc
  61 gtcgcctctc c]ttctctact aaagatgggt ttggctccca ataagcccag ttgtagttct
 121 tgtcatttgc tctcgaaatg gtccaatcac ctcccgccaa ccggattctt cagcacgaga
 181 agttgctcga agctcgtttc tgttcgccgg ttcaagctaa attctataac ggcctcttca
 241 caagttggtg ctgctgattc tgatccgata ttgagcaaaa tttcgaattt caaagatgca
 301 tgctggagat ttttgaggcc tcatacaata aggggaacag ctctaggatc tgttgctttg
 361 gttacaagag ctttgattga gaatccacat ctaataaagt ggtctctagt gctcaaggca
 421 ttctctggcc taatagctct catatgcgga aatggttata tagttggcat caatcagatc
 481 tacgatattg ggatagacaa ggtaaacaaa ccttatttac ctatagctgc aggggaccta
 541 tccgttcaat ctgcctggat cttggtcatc tcctttgcag tggctggtct tttaattgtc
 601 ggaaccaact ttggtccatt catcacttcc ctttattctt ttggtctact tctgggcaca
 661 atctattctg ttcctccgct taggatgaag agattccctg ttgcagcatt tcttataatt
 721 gccacggtgc gcggcttcct tcttaatttt ggggtatatt atgccacgag ggctgctctt
 781 ggactt[cctt tcttgtggag tcctcctgtg]gcttttatca caactttcgt gactttgttt
 841 gcgcttgtta ttgccataac taaggatctt ccagatgtag aaggagatcg caagtttcag
 901 atatcaacct ttgcaacaaa gcttggagtt agaaacattg cattccttgg atctggactt
 961 ttgcttctga attatgttgc tgccatcctg gctgcaatat acatgcctca ggcattcagg
1021 cataatgtga tgctacctgc acatttagtc ctggctatat gcttgatctt ccagacatgg
1081 gtgctggaac gagcaaatta cacccaggga gcaatctcag aattctaccg cttcatttgg
1141 aatcttttct atgcggagta cGCTATATTT CCATTCGTTT AGggtaacat ttttgtcttt
1201 tttgcttctc ttttgctttt tatctttgca tggtcattat gaggatcata gaaagttgta
1261 tatgtaaagc atttcatctc taaaatgtat atattcgaga caaaaaaaaa aa
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Figure 1. cDNA sequence of *AmHST1* and primer locations used in PCR, RACE and qRT-PCR. Forward primers are underlined. Primers used in PCR amplication of the 760 bp internal length are in brackets. Primer used in 3'RACE is underlined and in italic. Primers used for amplication of the 1182 bp ORF are indicated in uppercase letters. Primers used in qRT-PCR are boxed

The deduced amino acid sequence of *AmHST1* was used as query to search the GenBank protein databases. The predicted *AmHST1* encodes a protein of 392 amino acids and shares 77% and 74% identities with homogentisate prenyltransferase from *Theobroma cacao* (GenBank accession no. CM001883) and *Arabidopsis thaliana* (GenBank accession no. DQ231060) respectively, indicating it is involved in prenylation catalysis. The *AmHST1* protein had a molecular weight of 43.126 kDa and a theoretical isoelectric point (pI) of 9.79. From the comparison made with conserved domain alignments found in the Conserved Domain Database (CDD), it was predicted that the amino acid contained two D-rich sequence motifs in the active site (Figure 2). The two motifs, NQxxDxxxD and KD(I/L)xDx(E/D), are consistent with other known homogentisate group of prenyltransferases and are responsible for prenyldiphosphate recognition (Venkatesh et al. 2006; Sasaki et al. 2008; Shen et al. 2012). Using the prediction software ChloroP 1.1 indicated that *AmHST1* contained a chloroplast targeting peptide of 77 amino acids in length, while TMHMM 2.0 suggested that the protein has six putative transmembrane domains (http://www.cbs.dtu.dk). These predictions suggested that AmHST1 is a plastidic membrane protein.

To identify similar known HPT proteins from the GenBank, the *AmHST1* was searched against the non-redundant amino acid database using BLAST. A phylogenetic

Figure 2. Multiple sequence alignment of homogentisate prenyltransferases. Sequence comparisons made between *Aquilaria malaccensis AmHST1* and five other homogentisate prenyltransferases from plant origins from the GenBank, *Theobroma cacao* TcHPT1i4 (XP_007029130), *Glycine max* GmVTE2-2 (DQ231061), *Arabidopsis thaliana* AtHPT1 (NP_001154609), *A. thaliana* AtVTE2-2 (DQ231060) and *Artemisia sphaerocephala* ArHPT (ACS34774) using ClustalW version 2.1. Identical and similar residues were shaded in black and in grey, respectively, using the Boxshade 3.3.1 program. Two D-rich motifs are marked with asterisks. The two conserved motifs are boxed

tree was constructed to examine the relatedness between these proteins (Figure 3). The *AmHST1* is grouped together with related prenyltransferases involved in the biosynthesis of plastoquinone-9 (PQ-9) and tocochromanols, also known as vitamin

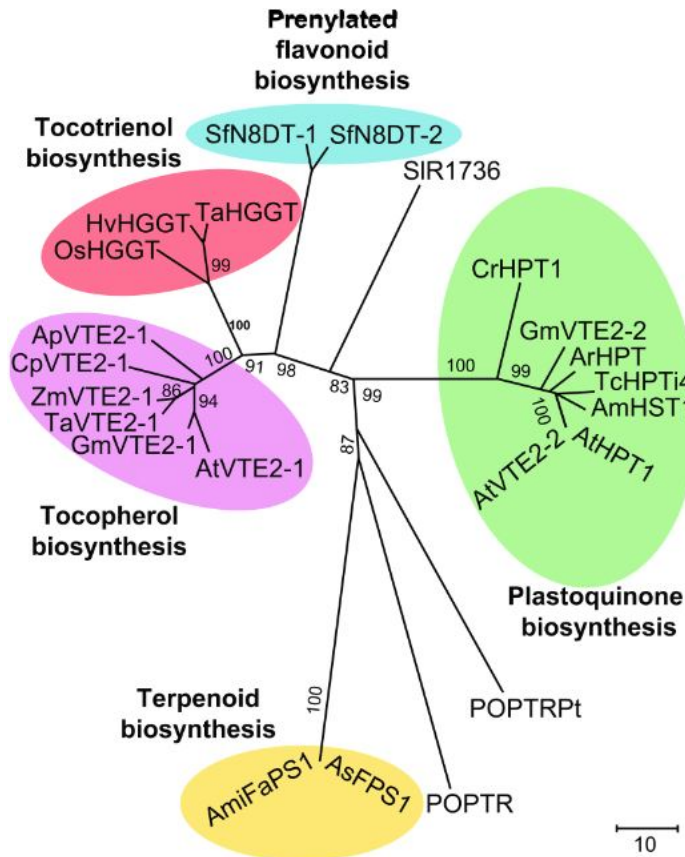

Figure 3. Neighbour-joining phylogenetic relationship of *AmHST1*. An unrooted phylogram was generated using MEGA6 (Tamura et al. 2013). Homogentisate phytyltransferase (VTE2-2) and homogentisate prenyltransferase (HPT) are involved in plastoquinone biosynthesis, putative farnesyl diphosphate synthase (FaPS1) and farnesyl pyrophosphate synthase 1 (AsFPS1) are involved in terpenoid biosynthesis, homogentisate phytyltransferase (VTE2-1) are involved in tocopherol biosynthesis, homogentisic acid geranylgeranyl transferase (HGGT) are involved in tocotrienol biosynthesis and naringenin 8-dimethylallyltransferase (DT-1 and DT-2) are involved in prenylated flavonoid biosynthesis. Accession numbers: *Arabidopsis thaliana* AtHPT1 (NP_001154609), *A. thaliana* AtVTE2-2 (DQ231060), *Artemisia sphaerocephala* ArHPT (ACS34774), *Chlamydomonas reinhardtii* CrHPT1 (CAL01105), *G. max* GmVTE2-2 (DQ231061); Aquilaria microcarpa AmiFaPS1 (ADH95185), Aquilaria sinensis AsFPS1 (AHG54251); *Allium porrum* ApVTE2-1 (DQ231057), *A. thaliana* AtVTE2-1 (AY089963), *Cuphea pulcherrima* CpVTE2-1 (DQ231058), *Glycine max* GmVTE2-1 (DQ231059), *Triticum aestivum* TaVTE2-1 (DQ231056), *Zea mays* ZmVTE2-1(DQ231055); *Hordeum vulgare* HvHGGT (AY222860), *Oryza sativa* OsHGGT (AY222862), *T. aestivum* TaHGGT (AY222861); *Sophora flavescens* SfN8Dt-1 (AB325579), *S. flavescens* SfN8DT-2 (AB370330)

E. It is clearly separated from the clade of *Aquilaria* prenyltransferases (*AmiFaPS1* and *AsFPS1*) in the terpenoid pathway (Yang et al., 2013; Kenmotsu et al., 2013), indicating it has no direct relationship to agarwood synthesis.

qRT-PCR Expression

We investigated expression of this gene to determine if it is wound inducible. In addition, we tested the expression in callus tissue after five months of growing in the dark. The qRT-PCR analysis indicated that *AmHST1* transcripts were expressed in callus tissue, but it was low compared with unwounded wood stem, which was 20-fold higher (Figure 4). Interestingly, when the stem was wounded, the expression levels in 6- to 24-hour post-wounding samples dropped to between 2- to 10-fold lower than unwounded stem (Figure 4). This clearly shows that *AmHST1* is down-regulated by wounding treatment. In a different experiment, so as to avoid compounding effects from natural surroundings such as from microorganisms and herbivores, *in vitro* callus was cut to mimic wounding treatment. The *AmHST1* expression was compared with several other genes from *A. malaccensis*. *AmPAL* and *AmWRKY*, two wound inducible genes had increased expressions of between 9- to 15-folds, respectively, at 24 hours after cutting, when compared with control callus, while *AmPD*,

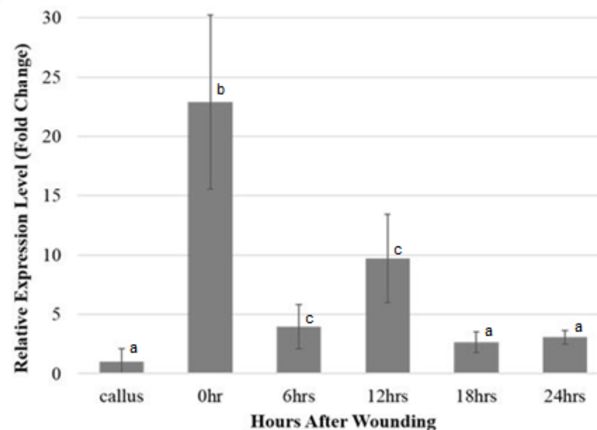

Figure 4. Relative gene expression of *AmHST1* in callus and wounded stem of *Aquilaria malaccensis* at time 0 (untreated control) and 6, 12, 18, and 24 hours after wounding. Callus was used as the calibrator. Bars in the chart represent fold changes in relative expression and the error bars represent standard deviations. Different alphabets indicate significant difference while same alphabets indicate no significant difference between sampling time, using Tukey's test (p<0.05)

a gene involved in basic metabolism and *AmHST1* expressions, was not perturbed (Figure 5). This suggests that *AmHST1* was highly expressed in stem tissue but not in callus.

DISCUSSION

This study reports the first putative homogentisate solanesyltransferase cDNA, *AmHST1*, cloned from *A. malaccensis*, a tropical tree widely known for its agarwood. Sequence and prediction analyses using multiple software suggest that the protein has a putative role in PQ-9 biosynthesis, a pathway closely related to vitamin E biosynthesis. Homogentisate prenyltransferases are enzymes involved in the biosynthesis of vitamin E and quinones (Collakova & DellaPenna, 2001). Neighbour-joining phylogenetic tree (Figure 3) reveals that members of HPT are divided into three main groups (Mène-Saffrané & DellaPenna, 2010): homogentisate phytyltransferases involve in tocopherol biosynthesis, homogentisate geranylgeranyltransferases involve in tocotrienol biosynthesis, and HST responsible for PQ-9 biosynthesis, of which *AmHST1* is most related to. Not much is known about this enzyme, except that it is located in the inner membrane of chloroplast and is hardly active with phytyl diphosphate. However, it catalyses the decarboxylation and prenylation of homogentisate with solanesyl diphosphate, leading to the formation of 2-methyl-6-solanesyl-benzoquinol (MSBQ), the immediate precursor of PQ-9 (Soll et al., 1985). An *AmHST1* homolog in Arabidopsis, when expressed in *E. coli* has been shown to react actively with its substrate, solanesyl diphosphate, consistent with its function in PQ-9 biosynthesis (Sadre et al., 2006). When constitutively over-expressed in *A. thaliana*, the transgenic plants have higher

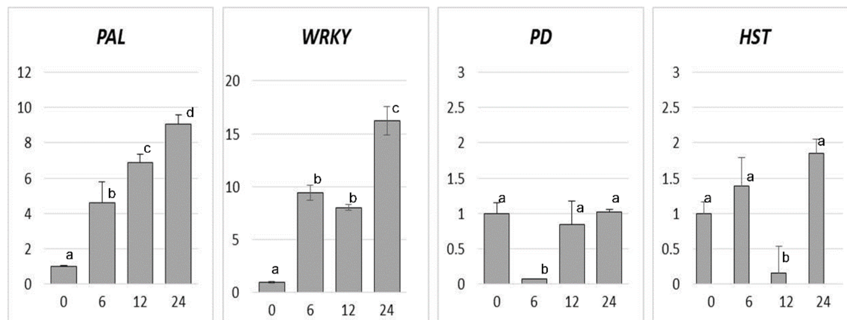

Figure 5. Relative gene expression of several *Aquilaria malaccensis* genes in callus at time 0 (untreated control) and 6, 12, and 24 hours after wounding. Untreated callus was used as the calibrator. Bars in the chart represent fold changes in relative expression and the error bars represent standard deviations. Y-axes are on different scales. (*PAL*=phenylalanine ammonia lyase; *WRKY=WRKY* transcription factor; *PD*=phytyl diphosphate; *HST*=homogentisate solanesyltransferase). Different alphabets indicate significant difference while same alphabets indicate no significant difference between sampling time, using Tukey's test (p<0.05).

PQ-9 level compared with control group. Interestingly there is also evidence that suggest higher levels of tocochromanols in the leaves of the transgenics (Sadre et al., 2006). It has been suggested that HST-mediated prenylation may have as many as two possible alternatives resulting with different intermediate molecules (Sadre et al., 2010). This is not surprising as HST and other homogentisate prenyltransferases share similar substrates in the pathway synthesis of PQ-9 and tocochromanols, therefore, they may over-lap in functions. The latter is a group of vitamin E precursors that are synthesised by photosynthetic organisms and possess antioxidants activity. In plants, vitamin E is believed to protect the cellular components from oxidative stress as significant increase of tocopherol levels are observed in response to various abiotic stresses including exposures to high light, saline, cold and drought conditions (Havaux et al. 2000). High-light stress has been shown to escalate total tocopherol levels in *Arabidopsis* leaves and increase the expression of related genes in tocopherol biosynthesis (Collakova & DellaPenna 2003a). This supports our observations on *AmHST1*, which was suppressed in tissues grown in prolonged darkness such as callus, while being expressed in non-stressed tissue, the 0-hour unwounded tree stem (Figure 4). Wounding induces several genes in defence response, such as the transcription factor *AmWRKY* and *AmPAL* of the phenylpropanoid pathway both in stem (Wong et al., 2013) and callus tissues (Figure 5). However, *AmHST1* did not respond to callus wounding (Figure 5), suggesting the long period in darkness might have suppressed the gene.

In tocopherol biosynthesis (reviewed in Mène-Saffrané & DellaPenna, 2010), HPT activity catalyses the committed step, where homogentisic acid (HGA) and phythyl diphosphate (PDP) are condensed into tocopherol. The PDP is generated from reduced form of GDPP by action of GGDP reductase. In an experiment that applies high-light stress to Arabidopsis, it was shown that genes in the tocopherol pathway have positive relationship with expressions and tocopherol accumulation, while the GGDP showed negative response (Collakova & DellaPenna 2003b). In addition, related downstream genes from GGDP reductase are also downregulated. GGDP is synthesised from IPP and DMAPP. Both are precursors in the biosynthesis pathways of tocochromanols, PQ-9 as well as terpenoids. Many important intermediate isoprenoid molecules including FPP and GGDP, which form the basis of other longer chain isoprenoid molecules originated from IPP and DMAPP (Dudavera et al. 2013).

Wounding is an abiotic stress that plays a major role in terpenoid-rich agarwood induction. The fact that the expression of a gene related to PQ-9 and vitamin E biosynthesis is suppressed when a terpenoid inducing situation emerged suggests that there could be precedence in the activation of controlling genes. It is speculated that genes controlling the committing steps

in PQ-9 and tocochromanol synthesis are down-regulated as to allow the formation of isoprenoids important in defence response against abiotic as well as biotic stresses. The up-regulation of several terpenoid synthesis genes from *Aquilaria* by wounding treatment and addition of biochemical elicitors further support our conclusion.

CONCLUSION

This study cloned the gene *AmHST1*, whose expression is down-regulated in the stems of *A. malaccensis* experiencing wounding and in callus grown in the dark. While reports have shown that abiotic stresses have positive relationships with tocopherol accumulation, the mechanisms that regulate its synthesis pathway and related pathways, such as the isoprenoid pathway that shares identical precursor building blocks in making end products of similar defence functions, remain poorly understood and need further investigation.

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ABBREVIATIONS

BAP	6-benzylaminopurine
DMAPP	dimethylallyl diphosphate
FPP	farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
GOI	gene of interest
GPP	geranyl diphospate
HGA	homogentisic acid
HPT	homogentisate prenyltransferase
HST	homogentisate solanesyltransferase
IPP	isopentenyl diphospate
NAA	naphthaleneacetic acid
PCR	Polymerase Chain Reaction
PDP	phythyl diphosphate
PT	prenyltransferase
RACE	Rapid Amplification of cDNA Ends
TPS	terpene synthase
TUA	tubulin

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Sequence Related Amplified Polymorphism (SRAP) Based Genetic Analysis of Nigerian 'Egusi' Melon Accessions

Idehen, E. O.

Plant Breeding And Seed Technology Department, Federal University Of Agriculture, Abeokuta, P.M.B. 2240 Abeokuta, Ogun State, Nigeria

ABSTRACT

Variability is germane in crop improvement. The ability of molecular markers to reveal polymorphism can assist in identifying the specific loci of a particular trait in crops. Sequence related amplified polymorphism (SRAP) is a useful technique for determining the genetic diversity of plants and was used to discriminate fifty 'egusi' melon accessions from different parts of Nigeria. The fifty accessions were subjected to SRAP analysis and 65.48% of the bands were found to be polymorphic with similarity coefficients of the accessions ranging from 0.51 – 0.96. Accessions DD98/3, NG/AU/MAR/09/012, NG/OE/MAR/09/015, NG/AO/APR/09/032 and A17 were found to be distinct from all other accessions. The high level of polymorphism exhibited by the 'egusi' melon accessions show they would be useful for maintaining genetic diversity in future breeding programs.

Keywords: Dendrogram, 'egusi' melon, genetic diversity, SRAP

INTRODUCTION

Egusi' melon [*Citrulus lanatus* (Thunb.) Matsum. & Nakai] belongs to the family cucurbitacea and it is an annual herb with hairy, trailing and angular stem, and dark green alternate leaves. The mesocarp of the fruit is extremely bitter, but the seeds can be removed, roasted, and eaten, in addition to being made into a soup thickener or flavouring agent (Badifu & Ogunsua, 1991). Conventional methods to characterize melon accessions based on phenotypic observations have been reported (Idehen et al., 2007, Kehinde & Idehen, 2008), but the use of molecular approach has become imperative when considering their diversity. Sequence related amplified polymorphism (SRAP) is a novel Polymerase Chain Reaction (PCR) based marker system as

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E-mail address:
emmaidehen@yahoo.com (Idehen, E. O.)

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described by Li and Quiros, (2001) was to determine genetic diversity in peach and nectarine (Ahmad et al, 2004) and tomato (Ruiz and Garcia-Martinez, 2005). The aim of this study is to determine the relationship among Nigerian 'egusi' melon accessions using the SRAP marker technology.

MATERIALS AND METHODS

Plant Materials

Fifty accessions of 'egusi' melon were collected from Research Institutes and different parts of Nigeria (Table 1).

Genomic DNA Extraction

Genomic DNA was extracted from young leaves according to the modified CTAB method (Liu et al., 2003). DNA was then dissolved in 50 µl of double-distilled water, diluted to a final concentration of 40 ng/µl-1 with 1×TBE buffer and stored at 4°C. Measurement of the DNA concentration and purity was determined using a NanoDrop ND 1000 spectrophotometer.

Primers and PCR Amplification

In this assay 156 primer combinations were screened and twenty-six different combinations which generated amplicons were used (Table 2). The SRAP-PCR reaction mixture totalling 20 µl consisted of 8.2 µl of double distilled water, forward and reverse primers of 1.5 µl each, dNTPs at 1.6 µl, 10x buffer at 2.0 µl, Taq DNA polymerase at 0.2 µl, and 5 µl of genomic DNA (40 ng/ µl). The amplification consisted of a denaturing step of 4 min at 94°C, followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min, followed by cooling to 4°C. The PCR products were electrophoresed on 6% non-denatured polyacrylamide gels in 1× TBE buffer, running at 120 V constant voltage for 3 h, and then silver stained (Bassam et al., 1991; Liu et al., 2007). The band patterns on the gels were photographed over white fluorescent light.

Statistical Analysis

The gel photographs were binary coded, with '1' indicating the presence of bands and '0' absence of bands. The assay efficiency index, also referred to as the polymorphism information content (PIC), was calculated using the algorithm:

$$PIC = 1 - \sum_{i=1} fi^2$$

Where, fi^2 is the frequency of the i^{th} allele.

A dendrogram was constructed using the Unweighted Pair-Group Method of the Arithmetic Mean (UPGMA). Principal component analysis (PCA) was performed to reveal the genetic similarity and diversity between the accessions.

Table 1
Names and sources of 50 'egusi' melon accessions used for the study

S/N	ACCESSION NAME	SOURCE	S/N	ACCESSION NAME	SOURCE
1	DD98/550	NIHORT, IBADAN	26	NG/TO/ APR/09/029	NACGRAB, IBADAN
2	DD99/75	NIHORT, IBADAN	27	NG/AO/ APR/09/032	NACGRAB, IBADAN
3	DD/98/506	NIHORT, IBADAN	28	A1	BENIN, EDO
4	DD/98/4	NIHORT, IBADAN	29	A2	KADUNA
5	DD/98/3	NIHORT, IBADAN	30	A3	ZAMFARA
6	DL/91/71	NIHORT, IBADAN	31	A4	KOGI
7	DD/98/511	NIHORT, IBADAN	32	A5	OYO, NIGERIA
8	NG/SA/ DEC/07/0361	NACGRAB, IBADAN	33	A6	OYO, NIGERIA
9	NG/SA/JAN/09/027	NACGRAB, IBADAN	34	A7	KOGI, NIGERIA
10	NG/SA/JAN/09/028	NACGRAB, IBADAN,	35	A8 (ITO)	ABEOKUTA, OGUN
11	NG/SA/JAN/09/029	NACGRAB, IBADAN,	36	A9 (SEREWE)	OWO, ONDO
12	NG/SA/JAN/09/030	NACGRAB, IBADAN	37	A10	TARABA
13	NG/SA/JAN/09/031	NACGRAB, IBADAN	38	A11	GUSAU, SOKOTO
14	NG/SA/JAN/09/032	NACGRAB, IBADAN	39	A12	ZAMFARA
15	NG/AU/ MAR/09/012	NACGRAB, IBADAN	40	A13	ZAMFARA
16	NG/OE/ MAR/09/015	NACGRAB, IBADAN	41	A14 (EGBIRA)	KOGI,
17	NG/OE/ MAR/09/016	NACGRAB, IBADAN	42	A15	EDO
18	NG/AT/APR/09/001	NACGRAB, IBADAN	43	A16 (PAPA)	OYO
19	NG/AT/APR/09/002	NACGRAB, IBADAN	44	A17 (ITO)	IBADAN, OYO
20	NG/AT/APR/09/003	NACGRAB, IBADAN	45	A18 (SOFIN II)	OYO
21	NG/AT/APR/09/004	NACGRAB, IBADAN	46	A19	ILARO, ABEOKUTA
22	NG/TO/ APR/09/030	NACGRAB, IBADAN	47	A20 (PAPA)	SAKI, OYO
23	NG/OE/ MAR/09/014	NACGRAB, IBADAN	48	A21 (PAPA)	EPE, LAGOS
24	NG/TO/ APR/09/027	NACGRAB, IBADAN	49	A22 (SEREWE)	ABEOKUTA, OGUN
25	NG/TO/ APR/09/028	NACGRAB, IBADAN	50	A23 (IGBA)	ABEOKUTA, OGUN

Table 2
Base sequences of the Sequence Related Amplified Polymorphism (SRAP) primer combinations used for DNA amplification

Forward primer (5' → 3’)	Reverse primer (5' → 3')
Me16: ACTACTTTGATGGACACTTGCCT	Em16: GCTCTGAAGTTGATTAGTCGGTC
Me18: TAGGGTTTAGAGTTAAGGGGTGG	Em18: TCTCCAAGAAAGAAAATGACCAA
Me20: GGGAAGACACTTTGGAGGAGTAC	Em21: GGATAATGTTTATGGTAGCTCGA
Me21: GTTTACGGTTCAGGGTTTAGGGT	Em22: GAAATCCTTTCCATCAGCTTTCT
Me22: ACCTGTCTCCATCTCCACCTTGT	Em23: GCAAGACATTGAGCCTTCTACTT
Me23: TAAAGAGCCAACAAACTCGAAAG	Em24: GTAAAACCGAACCGTACCGAACC
Me24: ATACCAAACCATATCCAAATCCT	Em25: AAACTAGATCTTGACCGCACATC
Me26: ATGGTTAGATATCAAATTGGAAAC	Em26: ACTAGTATTGACCCCATGCTAGG
Me27: TTCCTCCACCACCGCTGACACTA	Em27: GTTTAGCAAAATCTCCAACAACC

RESULTS AND DISCUSSION

Polymorphism in ‘egusi’ melon accessions detected by SRAP primers

A total of 156 different combinations of primers, nine forward and reverse primers, were screened. A total of 26 combinations revealed polymorphism between the ‘egusi’ melon accessions (Table 3), from which one hundred and ninety-seven bands were obtained for analysis.

High number of bands were observed for primer combinations; Me18 - Em18 (15 bands), Me27 - Em27 (13 bands), Me18 - Em24 (12 bands) and Me21 - Em26 (12 bands). While, lower number of bands were recorded for primer combinations; Me26 - Em26, Me22 - Em18, Me22 - Em21), with three bands each. Polymorphic bands ranged between 1 to11 for primer combinations Me22 - Em21 and Me18 - Em18, respectively. The least polymorphism of 27.20% was observed for Me23 – Em26), while the highest polymorphism of 85.70% for Me16 – Em24 (Table 3).

Polymorphism information content (PIC) of the SRAP primers ranged from 0.14 – 0.72 for Me24-Em24 and Me20-Em26, respectively with a mean of 0.51. Representative banding pattern generated using primer Me20-Em26 are shown in Figure 1. Polymorphism information content of the SRAP loci and their ability to detect differences based on genetic diversity showed primer combination Me20 – Em26 is important in discriminating melon accessions. Higher number of polymorphic bands generated per primer pair combinations when compared to the previous study with melon using SSR primers (Idehen et al., 2012), which is an indication of the ability of SRAP primers to effectively identify multiple loci.

Analysis of phylogenetic dendrogram based on SRAP analysis

Dendrogram resulting from SRAP analysis with similarity coefficients for the 50 ‘egusi’

Table 3
Polymorphism and polymorphism information content (PIC) of 26 Sequence Related Amplified Polymorphism (SRAP) primer combinations

Primer Combinations	Total number of bands	Polymorphic bands	Polymorphism (%)	PIC
Me16 - Em16	6	3	50.00	0.58
Me18 - Em18	15	11	73.00	0.52
Me24 - Em24	9	5	55.00	0.14
Me26 - Em26	3	2	66.60	0.66
Me27 - Em27	13	10	76.92	0.22
Me20 - Em18	8	6	75.00	0.51
Me22 - Em18	3	2	66.60	0.39
Me23 - Em18	4	2	50.00	0.55
Me27 - Em18	7	4	57.10	0.43
Me22 - Em21	3	1	33.30	0.49
Me20 - Em22	6	4	66.60	0.54
Me21 - Em22	8	4	50.00	0.47
Me22 - Em23	10	8	80.00	0.66
Me23 - Em22	8	3	37.50	0.45
Me26 -Em22	9	5	55.50	0.36
Me27 - Em22	5	4	80.00	0.52
Me16 - Em24	7	6	85.70	0.60
Me18 - Em24	12	9	75.00	0.48
Me22 - Em24	9	7	77.70	0.49
Me27 - Em24	7	5	71.40	0.43
Me21 - Em25	4	3	75.00	0.54
Me27 - Em25	6	5	83.30	0.68
Me20 - Em26	8	5	62.50	0.72
Me21 - Em26	12	9	75.00	0.51
Me22 - Em26	4	3	75.00	0.68
Me23 - Em26	11	3	27.20	0.67
Mean	7.57	4.96	64.65	0.51
Total	197	129	-	-

melon accessions used in this study (Figure 2), clearly distinguished the accessions. Similarity coefficients for the six groups as revealed by SRAP analysis ranged from 0.51 – 0.96. The 50 'egusi' melon accessions were divided into 6 groups (I, II, III, IV, V and VI) (Figure 2), with group V having the highest number of accessions clustered together (19) and group III, the least (3 accessions). The dendrogram from SRAP analysis was able to distinguish all accessions except two (2) at a similarity coefficient of 0.96, whereas an

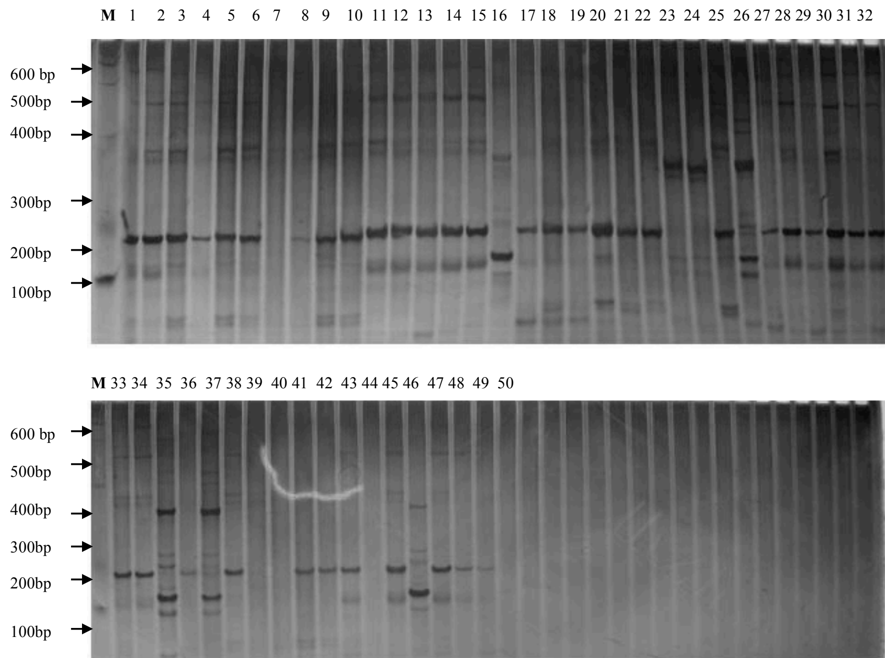

Figure 1. Representative banding pattern generated using SRAP primer combination Me20-Em26

*M- Marker
*Numbers above represents accessions

1=DD98/550	11=NG/SA/JAN/09/029	21=NG/AT/APR/09/004	31=A4	41=A14 (EGBIRA)
2= DD99/75	12=NG/SA/JAN/09/030	22=NG/TO/APR/09/030	32=A5	42=A15
3=DD/98/506	13=NG/SA/JAN/09/031	23=NG/OE/MAR/09/014	33=A6	43=A16 (PAPA)
4=DD/98/4	14=NG/SA/JAN/09/032	24=NG/TO/APR/09/027	34=A7	44=A17 (ITO)
5=DD/98/3	15=NG/AU/MAR/09/012	25=NG/TO/APR/09/028	35=A8 (ITO)	45=A18 (SOFIN II)
6=DL/91/71	16=NG/OE/MAR/09/015	26=NG/TO/APR/09/029	36=A9 (SEREWE)	46=A19
7=DD/98/511	17=NG/OE/MAR/09/016	27=NG/AO/APR/09/032	37=A10	47=A20 (PAPA)
8=NG/SA/DEC/07/0361	18=NG/AT/APR/09/001	28=A1	38=A11	48=A21 (PAPA)
9=NG/SA/JAN/09/027	19=NG/AT/APR/09/002	29=A2	39=A12	49=A22 (SEREWE)
10=NG/SA/JAN/09/028	20=NG/AT/APR/09/003	30=A3	40=A13	50=A23 (IGBA)

earlier study on melon by Idehen et al. 2012, the accessions were not distinguishable even at a similarity coefficient of 1.00 as this was evident in majority of the accessions especially those in group five (5). This might be attributable to an earlier report by Budak et al., 2004 who stated the effectiveness of SRAP analyses in genetic diversity analysis, cultivar identification and phylogenetic studies.

Two-dimension (2D) plots from the principal component analysis (Figure 3) revealed accessions DD98/3, NG/AU/MAR/09/012, NG/OE/MAR/09/015, NG/

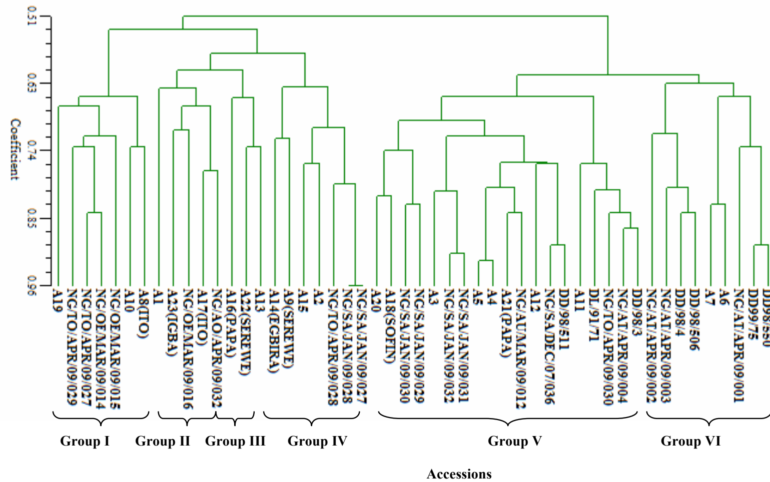

Figure 2. Dendrogram resulting from SRAP analysis showing similarity coefficients for the 50 ‘egusi’ melon accessions used in this study

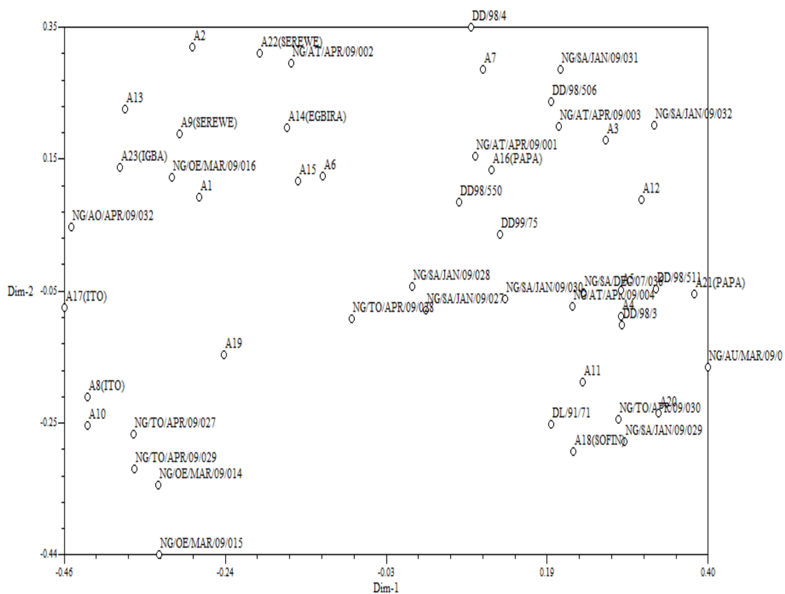

Figure 3. Two-dimension (2D) plot of 50 ‘egusi’ melon accessions based on SRAP markers

AO/APR/09/032 and A19 collected from research Institutes, with the exception of A19 which was sourced locally were distinct from all other accessions. The two (2) dimension plot was also able to clearly discriminate and show the spatial distribution of the accessions.

UPGMA analysis grouped the accessions into six groups, with majority of the accessions clustered in group V. The

clusters observed between the accessions collected from the research Institutes and those from other agro-ecological zones in the country show that they possess one or more character in common and suggest that they may have been from the same origin.

CONCLUSION

This study revealed that the SRAP primers were highly polymorphic and thus able to distinguish the accessions effectively. The highest polymorphism was observed for primer combination Me16 - Em24 and the high PIC of primer combinations Me20 – Em26 shows its ability to effectively discriminate several loci. The highest number of accessions clustered was in group V thus indicating their level of genetic similarity. Hence, selection for hybridization should be made from accessions in distant groups in order to benefit from their genetic variability.

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Response of Calves to Supplementation of Forage Legume - Based Concentrate Diets

Bello, R. A.[1], Fasae, O. A.[1]*, Oluwatosin, B. O.[2] and Olanite, J. A.[3]

[1]*Department of Animal Production and Health, Federal University of Agriculture, P.M.B 2240, Abeokuta, Nigeria*
[2]*Institute of Food Security, Environmental Resources and Agricultural Research, Federal University of Agriculture, P.M.B. 2240, Abeokuta, Nigeria*
[3]*Department of Pasture and Range Management, Federal University of Agriculture, P.M.B 2240, Abeokuta, Nigeria*

ABSTRACT

Sixteen (16) crossbred (White Fulani, Muturu & Keteku) calves aged 7-10 months and with an average weight of 69.78 ± 8.81 kg were sampled in a 12-week experiment to evaluate the response of calves to supplementation of legume - based concentrate diets. These calves graze on natural pastures. The sampled calves were allotted in a completely randomised design into four treatment groups and offered 25% *Leucaena leucocephala, Enterolobium cyclocarpum* and *Glricidia sepium* based concentrate diets and natural pastures (control) for treatments 1 - 4 respectively. Data were collected on feed intake, weight gain, nutrient digestibility, nitrogen balance, blood profile and faecal egg count of calves. The supplementation of forage legume concentrate diets improved ($P < 0.05$) DM intake and weight gain of calves with best results were observed in calves fed *Glricidia sepium* concentrate diets with 450.56 g/day and 188.45 g/day respectively. Nutrient digestibility (%) and nitrogen balance varied ($P < 0.05$) across treatment groups. Blood parameters did not differ ($P > 0.05$) across treatments and falls within the normal range for healthy calves, while the supplementation of forage legume concentrate diets reduced ($P < 0.05$) faecal egg count (egg/g) to ascertain the level of worm burden in calves. The study concluded that supplementation with legume concentrate diets improved the performance of calves with Gliricidia forage supplemented concentrate diet recommended for calves' optimum performance.

Keywords: Calves, forage legumes, performance, blood, faecal egg count

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E-mail addresses:
rafbellforall@gmail.com (Bello, R. A.),
animalexp@yahoo.co.uk (Fasae, O. A.),
oduguwabmdl@gmail.com (Oluwatosin, B. O.),
olaniteja@funaab.edu.ng (Olanite, J. A.)
* Corresponding author

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INTRODUCTION

Nigeria is known for its nomadic nature of cattle rearing. The major constraints faced in the production of these animals is the severe drop in body condition during prolonged droughts (Ibrahim & Jayatileka, 2000), due to forage scarcity characterised by low quality, resulting in low digestibility and poor productivity of the animals (Richard et al., 1994). Despite the fact that production of cattle depends solely on natural pastures which have contributed to their dietary needs, there is need to exploit available forage for year-round feeding, which should be of good quality to meet their nutritional requirements so as to attain their genetic potential. With good management, quality forage could be available year-round and where this is lacking, alternative feed can be provided (Karageorge, 2005, Tufarelli et al., 2012).

Forage legumes are important feed resources in ruminant production systems, readily available in Nigeria and are found in natural grasslands, playing a valuable role in providing supplemental nitrogen to ruminant animals. The feeding value of any forage is a function of the characteristics of the species, such as its availability, accessibility and nutrient content as well as the balance between available nutrient and the quantity of the nutrient ingested by the animal (Matlebyane, 2005, Bansi, et al., 2014).

Enterolobium cyclocarpum, Leucaena leucocephala and *Gliricidia sepium* are tropical multipurpose leguminous tree species which are drought resistance, persistent, relatively fast-growing trees, used for forage production. These plants are widely used as a source of nitrogen for ruminants as they are available year-round, and for their rapid growth and re-growth as well as palatability (Adejumo, 1991; Abdulrazak et al., 1997; Reynolds & Atta-Krah, 2006, Fasae et al., 2010). Therefore, the aim of this experiment is to evaluate the supplementation of forage legume based concentrate diets in improving the performance of cross bred calves grazing natural pasture.

MATERIALS AND METHODS

Experimental Animals and Management

Sixteen (16) healthy cross bred calves (White Fulani, Muturu and Keteku) of mixed sex (8 males and 8 females), between 7 and 10 months of age, with weight ranging from 60 kg to 80 kg, managed by the cattle unit of the Directorate of Teaching and Research Farms of the Federal University of Agriculture, Abeokuta were used for this experiment. The calves were tagged for identification and allotted in a completely randomised design into four groups. They were offered 25% *Leucaena leucocephala, Enterolobium cyclocarpum* and *Gliricidia sepium* based concentrate supplements for treatments 1-3, with calves on the fourth treatment grazing natural pasture (NP), serving as the control.

The calves were fed forage legume concentrate supplements at 2-3 % body weight on dry matter basis at 0800 to 1100 hours and released for grazing between

the hours of 1100 -1700 every day for five hours. Refusals of concentrate feeds were recorded in order to ascertain feed intake. Animals were weighed weekly with a weighing bridge. Clean water was provided *ad libitum*.

Experimental Feeds

The leaves of *Leucaena leucocephala, Gliricidia sepium* and *Enterolobium cyclocarpum* were harvested within the University premises, sun dried until the leaves become brittle and incorporated at 25% with other feed ingredients to formulate a concentrate supplement comprising maize (10%), wheat offals (35%), palm kernel cake (27.50%), bone meal (2%) salt and were premix at 0.25% for each treatment and fed to the animals at 5% of the body weight. The calves on the control treatment grazed natural pastures within the experimental site consisting of common forages. namely *Panicum maximum, Cynodon dactylon, Azadirachta indica, Gomphrena celosioides, Aspilia africana, Synedrella nodiflora, Pennisetum purpureum* and *Centrosema pubescens*. These forages were harvested from selected portions of the natural grazing land, sorted out and bulked together for the analysis of their chemical constituents.

Digestibility and Nitrogen Balance Trials

The 16 animals were transferred into metabolic crates at the 84th day of the experiment fitted with facilities for separate collection of faeces and urine. The quantity of feed offered, feed refusal, faecal output and urine were determined for 7 days. Ten percent of the faeces and urine were collected daily over the 7- day period and bulked. Faecal samples were weighed, oven dried at 70°C for 36 hours, milled and stored in air tight bottles for analysis.

Haematology and Biochemical analysis

Ten (10 ml) of blood samples was collected through the jugular vein of each calf using hypodermic needle and syringe at commencement and termination of the experiment. Five ml blood samples were released into sample bottles containing ethyl dimethyl tetra acetic acid as anticoagulant for haematological studies, while the rest were drawn into a clean test tube for he serum tests.

Faecal Egg Count Analysis

Faecal samples from the animals were collected at the onset of the experiment and at 2-week intervals directly from the rectum of the calves and then subjected to the modified McMaster egg-counting technique for nematode counts.

Chemical Analysis

Feed samples were oven-dried at 65°C for 48hrs, and analysed for proximate compositions (AOAC, 2005). The concentration of fibre components and tannin in feeds were determined based on` Van Soest et al., (1991) and Makkar et al., (1993) respectively.

Statistical Analysis

Data collected were subjected to one way analysis of variance in a completely randomized design (SAS, 1999) and significant means separated (Duncan, 1955).

RESULTS AND DISCUSSION

The dry matter content of the forage legumes varied (P<0.05) from 57.74 in *Leucaena leucocephala* to 45.52 % in natural pasture (Table 1). The values for the DM content of leaves for the forage legumes is at variance with that reported for indigenous multipurpose trees in Nigeria (Babayemi, 2006, Anele et al., 2009) which may be attributable to the maturity of the leaves used in this study to feed cattle. The crude protein contents of the forage legumes are within the range for reported values (Carew, 1983; Odeyinka, 2001; Oni et al., 2006), while that obtained for bulked forages from natural pastures is slightly lower than 18.96 % reported by Jolaosho et al. (2011). The forage legumes were found to contain varying percentages of tannin which were within the permissible range of 50 g/kg DM recommended by Frutos et al. (2004). Tannins have been reported to be the most widely occurring anti-nutritional factors found in plants, present in numerous tree and shrub foliages, seeds and agro-industrial by-products (Makkar & Becker, 1999), and have been shown to significantly reduce voluntary feed intakes at high levels.

Table 1
Chemical composition (%) of the forage legumes and natural pasture

Nutrients	LL	EC	GS	NP	SEM
Dry matter	57.74	53.50	50.56	45.52	0.31
Crude protein	20.85	22.07	23.50	17.34	0.21
Neutral detergent fibre	62.43	64.67	52.34	70.57	1.34
Acid detergent fibre	43.45	37.00	31.87	43.23	0.10
Acid detergent lignin	12.54	20.37	10.34	7.35	0.13
Ether extract	3.78	2.50	1.80	5.32	0.17
Ash	7.90	9.03	10.90	12.23	0.37
Tannin	3.72	3.09	3.49	ND	0.15

LL: *Leucaena leucocephala*, EC: *Enterolobium cyclocarpum*, GS: *Gliricidia sepium* NP: natural pastures (bulked forage), ND – Not determined

The chemical composition of forage legume supplemented concentrate (Table 2) compares favourably with forage legume supplemented diets fed to cattle and other ruminant species (Gonzalez et al., 2002, Jokthan, 2013). The CP contents of the diets were above the threshold level of 6% CP required by the microbes in the rumen to support metabolic functions of their host while the NDF contents across the

treatments with exception of the bulked forages from natural pastures were below the permissible limit of 65% guaranteed as optimal intake of tropical feeds by ruminant animals (Van Soest, 1994).

Table 2
Chemical composition (%) of experimental diets fed to calves

Nutrients	LSC	ESC	GSC	NP	SEM
Dry matter	92.93	88.82	92.54	45.52	1.14
Crude protein	22.63	22.82	24.73	17.34	0.31
Neutral detergent fibre	44.67	40.42	45.89	70.57	0.14
Acid detergent fibre	33.55	28.45	35.78	43.23	0.17
Acid detergent lignin	14.67	15.78	13.50	7.35	0.13
Ether extract	7.90	7.72	9.47	5.32	0.15
Ash	12.54	10.45	11.92	12.23	0.23

LSC: *Leucaena leucocephala supplemented concentrate*, ESC: *Enterolobium cyclocarpum supplemented concentrate*, GSC: *Gliricidia sepium supplemented concentrate*, NP: natural pastures (bulked forage)

The effect of forage legume supplement concentrate on the performance of calves is shown in Table 3. The improved ($P<0.05$) weight gain (g/day) of calves supplemented with forage legume concentrate diet may be attributed to the protein content of the forage legumes which would have influenced the available CP for animal performance. The range of 158.92 to 188.45 g/day observed in calves supplemented with ESC and GSC respectively, compares favourably with the findings of González et al., (2002) in Bunaji bulls fed a mixed concentrate containing *Gliricidia sepium*. However, higher values of 214.49 g/day were reported by Jolaosho et al. (2011) in calves fed diets supplemented with *Leucaena leucocephala*.

The improved ($P<0.05$) weight gain (g/day) in calves in the supplementary treatments is an indication of the superiority of the diets in relation to total protein and energy contents; This contributed to better forage digestibility, higher level of gastro intestinal tract fill and longer residence time of particulate and fluid digesta phase in the rumen (Demeyer, 1981) thereby, eventually having an effect on animal performance.

Moreover, the GSC diets appeared to promote faster growth rate and encouraged the best performance compared with the other forage legume supplemented treatments. The best performance in weight gain is justified by the high nutrient digestibility values observed in this study (Wanapat et al., 2009). In addition, the improvement observed in body weight gains of calves on GSC could be partly attributed to better balance and utilisation of absorbed nutrients in the tissue.

The increase in the intake of the supplemented forages could be attributable to the high crude protein content of these forages. Nitrogen supplementation for animals fed low-quality forage have

been reported to favour the growth of fibrolytic bacteria, and increases the ruminal degradation and voluntary intake of fibre, as well as the energy extraction from forage fibre (Detmann et al., 2009).

Table 3
Performance indices of calves fed forage legume supplemented concentrate diets

Parameters	LSC	ESC	GSC	NP	SEM
Initial weight (kg)	70.12	68.80	69.87	69.25	8.81
Final weight (kg)	84.47[ab]	82.15[b]	85.70[a]	70.75[c]	9.36
Weight gain (kg)	14.35[b]	13.35[b]	15.83[a]	1.50[c]	4.31
Daily weight gain (g/day)	170.83[ab]	158.92[b]	188.45[a]	17.85[c]	0.05
Metabolic weight gain (g/dayW0.75)	47.25[b]	44.75[b]	50.86[a]	8.68[c]	3.42
Supplementary feed intake (g/day)	430.54[ab]	350.78[b]	450.56[a]	ND	0.51
Metabolic intake (g/dayW0.75)	94.52[a]	81.05[b]	97.79[a]	ND	8.21

[abc]….. Means along the same rows with different superscript are significant ($P<0.05$).
LSC: *Leucaena leucocephala supplemented concentrate*, ESC: *Enterolobium cyclocarpum supplemented concentrate*, GSC: *Gliricidia sepium supplemented concentrate*, NP: Natural pastures., ND: Not determined

The nutrient digestibility of the supplemented concentrate diets is shown in Table 4. In the present study, the digestibility values obtained for most nutrients were generally high, which could have provided better environment for digestibility to occur. Digestion is dependent on the activity of micro-organisms which require energy, nitrogen, minerals and a suitable medium to enable the microbes perform well (Ranjhan, 2001). The improvement in digestibility of nutrients across forage legume supplemented concentrates, relative to the natural pastures in the present study may be due to the availability of increased nutrient molecule in rumen and small intestine for digestion (Abdel-Ghani et al., 2011).

Further, results showed that nitrogen intake values increased ($P<0.05$) with forage legume supplementation, corroborating Arigbede et al., (2008) that the inclusion of fodder leaves in animal feed improves dietary nitrogen utilisation. The GSC had the highest ($P<0.05$) nitrogen balance, which contradicts the findings of Aye, (2013), in which Leucaena supplemented diet had higher N-value compared with those supplemented with cassava and Gliricidia leaf meals. However, nitrogen balance recorded in all the treatments in this study was positive.

The haematological and serum biochemical parameters of experimental calves were not influenced ($P<0.05$) by the forage legume supplemented concentrate (Table 5). Values observed were in accordance with the recommended values for normal blood parameters in cattle (Merck Veterinary Manual, 2009; RAR,

Table 4
Nutrient digestibility (%) and nitrogen balance of forage legume supplemented concentrate diets fed to calves (n = 16)

Nutrients	LSC	ESC	GSC	NP	SEM
Dry matter	72.56	71.92	73.40	68.56	4.05
Crude protein	76.06[a]	70.48[b]	77.68[a]	61s.01[b]	4.66
Neutral detergent fibre	61.52[b]	64.69[a]	62.32[b]	53.22[c]	5.99
Acid detergent fibre	51.23[ab]	46.70[b]	52.33[a]	40.31[b]	5.77
Acid detergent lignin	58.23[a]	53.34[c]	54.38[b]	55.41[c]	4.33
Ether extract	69.86[a]	57.66[b]	68.71[a]	52.11[b]	6.77
Ash	74.45	67.12	69.33	60.21	4.92
Nitrogen (N) balance					
N-intake(g/calves/day)	42.20[a]	41.10[a]	46.21[a]	30.21[b]	1.26
N-voided in faeces (g/calves/day)	15.76	12.37	16.28	14.11	1.00
N-voided in urine (g/calves/day)	0.60[b]	0.79[a]	0.73[a]	0.69[b]	0.03
N-balance (g/calves/day)	25.84[b]	27.94[b]	29.03[a]	15.41[c]	0.50

[abc]….. Means along the same rows with different superscript are significant (P<0.05).
LSC: *Leucaena leucocephala supplemented concentrate*, ESC: *Enterolobium cyclocarpum supplemented concentrate*, GSC: *Gliricidia sepium supplemented concentrate*, NP: Natural pastures

2009), suggesting that dietary forages are safe and not detrimental to the health of the calves. The knowledge about normal values of biochemical variables in blood serum and other physiological variables is important for assessment of damage of organs and tissues in case of diseases and for general health of the animal (Terosky, 1997; Tufarelli et al., 2015).

Table 5
Hematological and serum biochemical of calves fed forage legume supplemented concentrate diets

Parameters	LSC	ESC	GSC	NP	SEM
Pack cell volume (%)	32.50	32.00	32.25	36.25	1.08
Red blood cells (x 10^{12}/L)	3.03	3.00	2.92	3.41	0.05
Haemoglobin (g/L)	10.70	10.65	10.70	12.10	0.35
White blood cells (x 10^{9}/L)	8.17	7.12	10.60	11.02	0.72
Total protein (g/dl)	8.12	9.02	9.90	8.62	10.47
Globulin (g/dl)	5.01	5.50	6.40	4.80	6.95
Albumin (g/dl)	3.11	3.52	3.50	3.32	3.52
Glucose (Mg/dl)	78.41	76.65	82.22	77.25	79.97

[abc]….. Means along the same rows are significantly different (P<0.05).
LSC: *Leucaena leucocephala supplemented concentrate*, ESC: *Enterolobium cyclocarpum supplemented concentrate*, GSC: *Gliricidia sepium supplemented concentrate*, NP: Natural pastures

The effect of forage legumes supplemented concentrate on the faecal egg count of calves is shown in Table 6. Calves supplemented with forage legume concentrate diets had a reduction (P<0.05) in faecal egg count which could be associated to the presence of tannin in the forage. Studies have confirmed the beneficial effects of dietary tannin in ruminant animals; the presence of tannin in several plant species have been found to reduce faecal egg count as well as increased daily weight gain in animals given protein-rich diets (Alonso-Díaz et al. 2008; Salam, 2015). Tannins have direct anthelmintic properties whereby they lower infections by gastrointestinal nematodes, thereby reducing larval migration and development and directly minimising abomasal and intestinal infections (Kahn & Diaz-Hernandez, 2000, Athanasiadou et al., 2000). Moreover, the high faecal egg parasite observed in calves on natural pastures maybe due to the non - supplementation of forage legume concentrate diets which could be attributed to reduced weight gain of calves on this treatment.

Table 6
Effect of forage legume supplemented concentrates on the faecal egg count of calves

Parameters	LSC	ESC	GSC	NP	SEM
Pre-faecal egg count	802.50	832.00	792.25	836.25	2.08
Post faecal egg count	103.03[b]	117.00[b]	102.92[b]	1003.41[a]	4.05
Reduction (%)	87.16[a]	85.94[a]	87.01[a]	-19.99[b]	0.72

[ab] Means along the same rows with different superscript are significant (P<0.05).
LSC: *Leucaena leucocephala supplemented concentrate*, ESC: *Enterolobium cyclocarpum supplemented concentrate*, GSC: *Gliricidia sepium supplemented concentrate*, NP: Natural pastures

CONCLUSION

In conclusion, the performance of calves was significantly influenced by forage legume supplemented concentrate diets, with *Gliricidia sepium* supplemented concentrate diet promoting higher nutrient intake and weight gain. Forage legume supplemented concentrate diets can therefore be used as an alternative feed supplement source in enhancing the performance of calves grazing natural pastures, as well as controlling nematodes.

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Disease Conditions in Cats and Dogs Diagnosed at the Post-Mortem Laboratory of the Faculty of Veterinary Medicine, Universiti Putra Malaysia between 2005 and 2015

Rathiymaler, M., Zamri-Saad, M. and Annas, S.*

Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

ABSTRACT

Keeping pets is a growing trend in Malaysia. This retrospective study involves data analysis of cases of dogs and cats submitted to the Post-Mortem Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia between 2005 and 2015. A total of 37 disease conditions were diagnosed among dogs with septicaemia (10.2%), mammary gland tumor (8.4%) and canine distemper (7.9%) were significantly ($p<0.05$) more frequently diagnosed. In cats, a total of 27 disease conditions were diagnosed with traumatic injuries (19.5%), feline infectious peritonitis (15.1%) and sporotrichosis (12%) were significantly ($p<0.05$) more frequent. Poor awareness among pet owners towards the health requirements of their pets is a main reason for the above.

Keywords: Post-mortem, common, health problems, diseases, dogs, cats

INTRODUCTION

Keeping pets such as dogs, cats, fish, reptiles and birds has become a growing trend worldwide making the death of pet animals an important issue. One of the ways to identify the cause of death of pet animals is by post-mortem examination (Weber et al., 2008). This method of examination provides an opportunity to undertake a thorough examination of dead animals (Berglund et al., 2003).

There are limited studies on the common health problems and their associated pathological changes in cats and dogs. This study was designed to identify the common health problems

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E-mail addresses:
mrathiymaler@gmail.com (Rathiymaler, M.),
mzamri@upm.edu.my (Zamri-Saad, M.),
annas@upm.edu.my (Annas, S.)
* Corresponding author

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based on pathological examinations on local dogs and cats presented to the Post-Mortem Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). This study also describes the pathological changes associated with the common health problems observed in cats and dogs.

MATERIALS AND METHODS

Data Collection

Case records at the Post-Mortem Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia between 2005 and 2015 were selected. These included the records of necropsy, biopsy and disposal cases involving dogs and cats. Only those cases with a confirmatory diagnosis were included in this study. Details on the identity, age, history, and clinical health history that lead to death, the gross lesions, histological changes and associated laboratory results were analysed.

Data Analysis

The data was analysed using Statistical Packages for the Social Sciences (SPSS) version 22. The data was categorical and non-continuous, thus non-parametric test was used in data analysis. All tests were done at 95% confidence interval level. Subsequently, Pearson's chi-square test was used to determine the occurrence rate of all diseases and to identify the prevalence of the three most common diseases in both dogs and cats.

Histopathology

Following analysis of the records, the three most common diseases in dogs and cats were identified. Ten representative cases for each of the three common diseases of dogs and ten common diseases of cats were randomly selected. Paraffin blocks were collected from the Histopathology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia and were subjected to routine histopathology slide preparation. The blocks were sectioned at 4 µm using a microtome (Reichert-Jung 2045 Multicut Rotary Microtome, Canada) and were then subjected to routine staining using Harris' Haematoxylin and Eosin (H&E).

All tissue sections were viewed with the Olympus CX31 upright microscope which was attached to Olympus U-CAM3 and Hpx2301 monitor. The associated histopathological changes were identified and described for each disease. Gross lesions were noted following analyses of records.

RESULTS

Disease Prevalence among Dogs

A total of 762 cases among dogs were selected and subjected to data analysis. A total of 35 diagnoses were made (Figure 1). The three most common health problems among dogs were septicaemia (10.2%), mammary gland tumor (8.4%) and canine distemper (7.9%). They were diagnosed significantly ($p<0.001$) more frequent than the remaining 32 diseases among dogs (Figure 1).

Gram-negative bacteria were the most common cause of septicaemia (66%), followed by a mixture of Gram-positive and Gram-negative (15%), while Gram-positive bacteria were involved in only 1% of the septicaemic cases of dogs. The involvement of Gram-negative bacteria in canine septicaemia was significantly ($p<0.01$) higher than Gram-positive bacteria.

The common gross and histopathological lesions that were observed in cases of septicemia were multiple organ congestion including the spleen, liver, lungs (Figure 2(a)), kidney and gastrointestinal tract. Haemorrhagic spleen and haemorrhagic suppurative gastroenteritis (Figure 2(b)) were also reported.

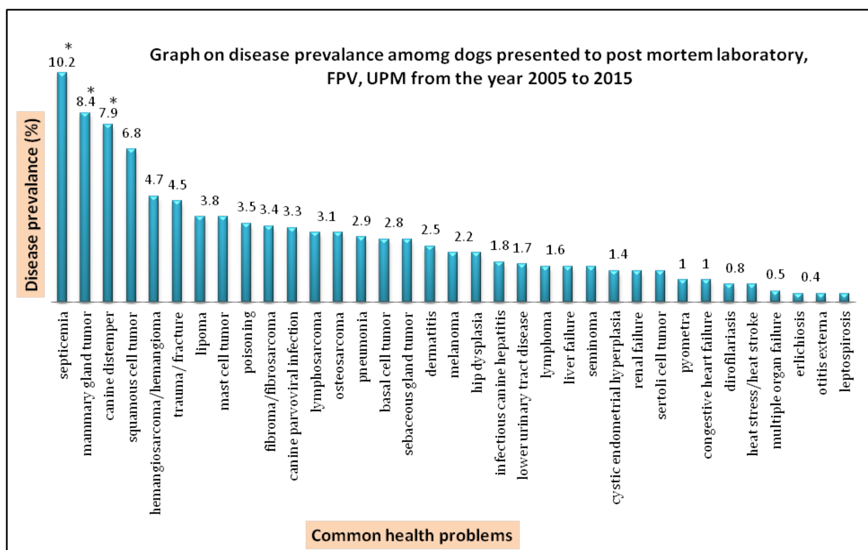

Figure 1. Prevalence of disease and health problems among dogs presented to the Post-mortem Laboratory between 2005 and 2015.Chi-square test, X^2 (34, N=762) = 513.552. *indicates significant difference at $p<0.001$

A total of 64 cases for dogs were diagnosed as mammary gland tumor. The most common type was the mammary gland carcinoma accounting for 37 cases (57.8%), followed by 3 cases (4.7%) of mammary gland adenoma, and a case (1.6%) of mammary gland adenosarcoma. However, a total of 23 cases (35.9%) were unclassified. Thus, the mammary gland carcinoma was significantly more common than other types of canine mammary gland tumours [X^2 (3, N=64) = 73.6, $p<0.05$]. Among the 64 cases of mammary gland tumour, 78.1% (n=50) involved intact dogs and 84% (n=42) of these intact dogs were above 4 years old.

The common gross lesion observed in mammary gland tumour was the presence of masses at the ventral abdomen, which was observed in 58 cases (90.6%) while the remaining 6 cases (9.8%) were from biopsy cases with incomplete reports. On the other hand, histopathological examination

revealed the loss of tubular structure of the mammary gland (Figure 2(c)) and the presence of pleomorphic cells. In mammary gland adenoma, the lesions included loss of myoepithelium of the mammary gland (Figure 2(d)).

There was a total of 60 (7.9%) canine distemper cases. Among these, 91.7% (n=55) involved young dogs of less than 1 year old. The common lesion in canine distemper included cephalic congestion with malacia, oedema and anoxic neurons (Figure 2(e)). Pulmonary congestion and oedema (Figure 2(f)), and mucosal petechiation of the gastrointestinal tract were frequently reported. Hyperkeratosis of the nose and footpads were also noticed. Based on the provided history, many of these dogs experienced central nervous system manifestation such as ataxic gait and depression.

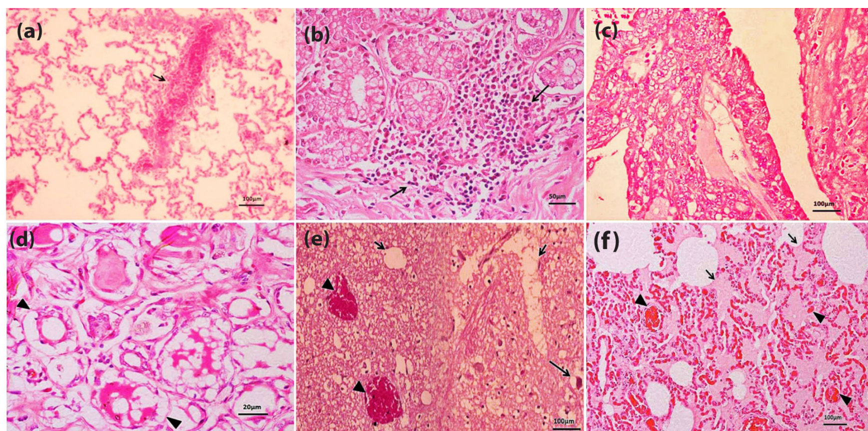

Figure 2. (a) Severe pulmonary congestion of a blood vessel (arrow) and interalveolar capillaries (H&E, 100x magnification, bar = 100 µm). (b) Suppurative gastritis with mixture of macrophages and neutrophils (arrows) in the mucosa of stomach (H&E, 400x magnification, bar = 50 µm). (c) Mammary gland carcinoma showing complete loss of tubular structures (H&E, 100x magnification, bar = 100µm). (d) Loss of myoepithelial surrounding the mammary gland tubules (arrowheads) (H&E, 400x magnification, bar = 20 µm). (e) Severe congestion of the brain (arrowheads), accompanied by generalised mild to moderate spongiosis (short arrow) and anoxic neuron (long arrow) (H&E, 100x magnification, bar = 100 µm). (f) Severe pulmonary oedema with generalised pulmonary congestion (arrowheads) and thickening of interalveolar septa (arrow). (H&E, 100x magnification, bar = 100 µm)

Disease Prevalence among Cats

A total of 866 cases among cats were analysed, resulting in 27 health problems being diagnosed (Figure 3). The three most common health problems among cats were traumatic injury (19.5%), feline infectious peritonitis (FIP) (15.0%) and sporotrichosis (12%). They were diagnosed more frequently ($p<0.001$) than the remaining 24 diseases (Figure 3).

Road traffic accidents appeared to be the most frequent cause of traumatic injuries (96 cases, 56.8%), followed by the high-rise syndrome (39 cases, 23.1%), blunt object

trauma (15 cases, 8.9%) and fighting (10 cases, 5.9%). Traumatic injuries of unknown cause involved 9 cases (5.3%). The road traffic accident was significantly high among the traumatic injury of cats [$X^2(4, N=169) = 200.8, p<0.05$]. The gross lesions observed in traumatic injuries included spinal injury with transected spinal, head trauma with brain haemorrhages, multiple fractures, herniation, haemoabdomen, haemothorax and contusion. Histopathological examination was done in cases of traumatic injury.

The second most common health problem among cats was feline infectious peritonitis (130 cases; 15%). It appeared that effusive FIP was more frequently observed (90 cases, 69.2%) followed by the dry form (28 cases, 21.5%) and the mixed form (13 cases, 10.0%). The effusive form was significantly more frequent compared with the other two forms [X^2 (2, N= 131) = 114.5, p<0.01]. Among the affected cats with effusive form, 75.6% (*n*=68) involved cats between 4 months and 2 years old.

The effusive form of FIP resulted in ascites, hydrothorax and thickening of the wall of small intestine with hemorrhagic mucosa. Histopathology revealed pulmonary vasculitis (Figure 4(a)) and focal pulmonary necrosis (Figure 4(b)). The non-effusive form showed pulmonary and hepatic pyogranuloma. The affected liver was firm with fibrins adhesion. Congestive non-suppurative meningitis (Figure 4(c)) and hydropericardium were also observed. On the other hand, the mixed form involved combination of both effusive and non-effusive forms. The frequently observed lesions were ascites and multifocal diffuse pyogranuloma in multiple organs.

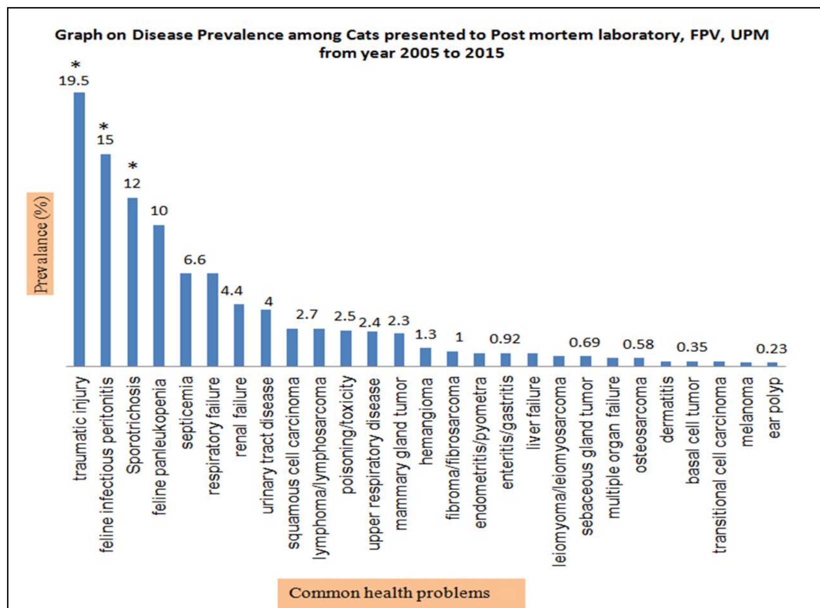

Figure 3. Graph on disease prevalence among cats presented to Post-mortem Laboratory, Faculty of Veterinary Medicine, UPM between 2005-2015. Chi-square test, $X^2(26, N=866) = 1150.1$. * indicates significant difference at p<0.001

Sporotrichosis was the third most frequent health problems among cats with reported 104 cases (12%). The common lesion observed in the affected cats was the non-healing wound. Histology revealed the presence of spores either freely or within the macrophages within the non-healing wounds (Figure 4(d)).

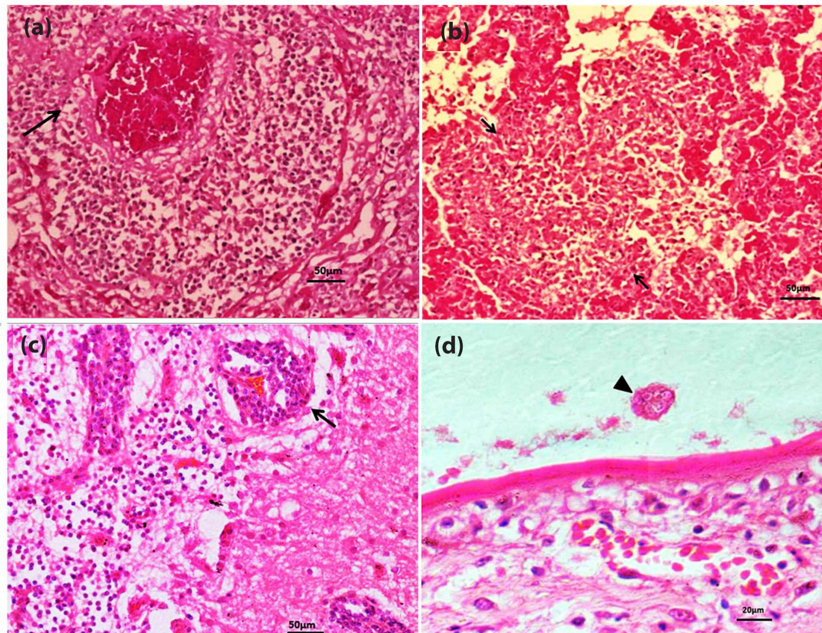

Figure 4. (a) Pulmonary vasculitis (arrows) and pulmonary congestion in effusive FIP (H&E, 400x magnification, bar= 50 µm). (b) Pulmonary focal necrosis (arrows) (H&E, 400x magnification, bar = 50 µm). (c) Histopathological section of the brain showing numerous infiltrations of inflammatory cells mostly neutrophils indicating suppurative meningitis and vasculitis (arrow) (H&E, 400x magnification, bar = 50 µm). (d) Non-healing wound showing spores of *Sporotrix schenkii* (arrowhead) within a macrophage (H&E, 1000x magnification, bar = 20 µm).

DISCUSSION

In this study, septicaemia is the most common health problem among dogs and the fifth most common for cats. This is in agreement with previous reports (Koenig, 2011; Ramachandran 2014). Bacteriological culture revealed frequent involvement of Gram-negative bacteria, as previously report by Svanbom (1979). The Gram-negative bacteria produce potent endotoxins, leading to dilation of blood vessels in various organs and eventually sepsis (Ramachandran, 2014). Similarly, septicaemia is among the important diseases that cause death in humans (Bone et al., 1997).

Most cases of septicaemia reported in this study revealed inflammatory reaction in the mucosa of either stomach

or small intestinal. This indicates that most septicaemic cases in dogs originated from the gastrointestinal tract (Deitch et al., 1987). In veterinary practice, animals are generally administered with antibiotics to prevent and treat septicaemia, causing lysis of the Gram-negative bacteria and the release of high amount of endotoxin, leading to death caused by endotoxin shock (Remick, 2007; Ramachandran, 2014). Septicemia can also originate from external injuries such as contaminated fight wound or cuts and also pyothorax, peritonitis, pneumonia or ulcerative gastritis (Lefort et al., 2011)

An earlier study reported that mammary gland tumour was the most common tumour in female dogs (Brigitte et al., 2012). This study supported those findings and found the high prevalence of mammary gland tumour among dogs in Malaysia was possibly due to low awareness on the importance of neutering pets.

Canine distemper occurs among dogs that have been not properly vaccinated (Frolich, 2002). Chung et al. (2011) concluded that the main histopathology lesions are lymphoid depletion in the spleen and thymus atrophy, which was not observed in this present study. Greene and Appel (2006) concluded that most cases of canine distemper show acute non-inflammatory lesion in the brain in the form of spongy vacuolation, as observed in this study. Hyperkeratosis of nose and digits is commonly seen in dogs with central nervous signs of delayed response to infection (Greene and Appel, 2006; Martella et al., 2008) was also reported in this study. Vaccination against canine distemper is categorised as vital suggesting the high prevalence of the disease in Malaysia was possibly the result of lack of vaccination of pets.

Traumatic injury is the most common cause of death among cats compared with dogs. Intact and out-door cats are prone to traumatic injuries (Rochlitz et al., 2004) caused by road traffic accident (Rochlitz et al., 2004) and the USA (Kolata,1980).

FIP in many parts of the world (Foley et al, 1997) is due to the absence of effective vaccine (Fehr et al., 1997) and availability of effective treatment regimen (Hartmann & Ritz, 2008).

It is interesting to note that sporotrichosis is one of the most common diseases among cats which leads to systemic infection (Barros et al., 2011) and their prolonged treatment with antifungal drugs (Gremiao et al., 2015). Furthermore, its zoonotic potential leads to most owners opting to euthanise the infected pets.

CONCLUSIONS

The most common health problems among dogs are septicemia, mammary gland tumor and canine distemper. On the other hand, traumatic injuries, FIP and sporotrichosis occurred more frequently in cats, which may be prevented through pet vaccinations.

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Fractionation of Anticholesterol Bioactive Compounds from Bekasam (Indonesian Fermented Fish Product)

Rinto*, Nopianti, R., Herpandi and Oktaviani, S.

Department of Fisheries Product Technology, Faculty of Agricultural, Sriwijaya University, Indralaya, 30862 South Sumatera, Indonesia

ABSTRACT

Bekasam functions as an inhibitor of HMG-CoA reductase. Fractionation was required to determine the bioactive peptide which functions as a HMG-CoA reductase inhibitor. Steps taken for this research were the production of bekasam used salt (15%), rice (15%) and *Lactobacillus acidophilus* as a culture starter, extraction and fractionation of *bekasam* to assay its HMG-CoA reductase inhibition. The results showed that six fractions from *bekasam* extract had different inhibition activity. The fraction of *bekasam* extract without evaporation (F1) contained 3 peptides (peptide of 7.69 kD; 10.71 kD and 20.22 kD). Extract free supernatant fraction (F2) contained 4 peptides (peptide of 7.69 kD; 10.71 kD; 20.22 kD and 35.38 kD). Fractions of *bekasam* extract in the F3 contained 2 peptides (7.69 kD and 10.71 kD). Furthermore, fractionation in the F4 can separate only one peptide band with molecular weight 7.69 kD. Peptides were not discovered in the F3 and F4 fraction while F6 and F4 fractions had the higher inhibition fraction to HMG-CoA reductase activity (92.86%). There was peptide 7.69 kD in F4 fraction and lovastatin (148.30 ppm) in F6 fraction.

Keywords: *Bekasam*, peptide 7.69 kD, anticholesterol, HMG-Coa reductase

INTRODUCTION

The 3-Hidroxy-3-Methylglutaryl-Coenzyme A Reductase (HMG-CoA reductase) is an enzyme which has a limiting factor to regulate cholesterol synthesis, especially in the formation of mevalonic acid from Hidroxy Methylglutaryl-Coenzym A (HMG-CoA). The inhibition to HMG-CoA reductase enzyme can reduce cholesterol in

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E-mail addresses:
rinto.unsri@gmail.com (Rinto),
nopi_81@yahoo.com (Nopianti, R.),
herpandinapis@gmail.com (Herpandi),
serlyoktaviani353@gmail.com (Oktaviani, S.)
* Corresponding author

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the hyperlipidaemia (Lyons & Harbinson, 2009; Rinto, 2016). Statins i.e. compactin, pravastatin, lovastatin, simvastatin (Barrios-Gonzales & Miranda 2010) and some peptides i.e. peptide from herbal *Senna obtusifolia* (Chuhua et al., 2008), potato and soy (Liyanage et al., 2008), milk (Kirana et al., 2005) and fermented fish (Kato et al., 2009) are bioactive compounds that reduce HMG-CoA reductase activity and cholesterol.

Some fermented fish product can block activity of HMG-CoA reductase enzyme. *Narezushi* and *Heshiko* extract are Japanese fermented fish products, containing protein fraction (peptides) and non-protein fraction, which have high inhibition for HMG-CoA reductase (Itou & Akahane, 2009; 2010). *Bekasam* extract (Indonesian fermented fish product) also had high inhibition for this enzyme (Rinto et al., 2015a).

Peptide fractions from bekasam which have activity to inhibit HMG-CoA reductase have not been well studied and documented. This study examined the fractionation of bekasam extract and the content of bioactive peptides that had high inhibition activity to HMG-CoA reductase. In addition to bioactive peptides, peptide profiles were identified and amino acid sequencing was done to discover type of peptide from bekasam that functions as an inhibitor of HMG-CoA reductase.

MATERIALS AND METHODS

Materials

Minnows/carps fish (*Rasbora argyrotenia*) was obtained from Indralaya traditional market, South Sumatera, Indonesia. De Man Rogosa Sharpe (MRS) broth medium were purchased from England. Lovastatin, HMG CoA reductase kit assay, were purchased from Sigma Aldrich (USA). A standard molecular weight protein marker (Low Range Protein Ladder) were purchased from Thermo Scientific (Lithuania). *Lactobacillus acidophilus* was screened and isolated from *bekasam*. All other chemicals were of analytical grade and purchased from the local representative of Sigma and Merck.

The Production of Bekasam with *Lactobacillus acidophilus* as a Culture Starter

Minnows/carps fish (*Rasbora argyrotenia*) (1 kg) was used as main raw material for making *bekasam*. *Minnows/carps* fish was gutted, washed and soaked in the starter culture *Lactobacillus acidophilus* (1 L) for 30 minutes. After that, the fish was separated from bacteria culture. Salt (15%) and rice (15%) was added to the fish and then fermented until seven days to produce *bekasam*.

Extraction of *Bekasam*

Extract of *bekasam* was prepared based on Rinto et al. 2015. Briefly, 10 g *bekasam* was homogenised with 40 mL distilled water. The homogenate was centrifuged at 2000 x g, 4°C for 15 minutes. After separating the first supernatant, 50 mL distilled water was added to the precipitate to obtain the second supernatant in the same manner. These two supernatants were mixed and filtered

through membrane 0.45 μm (Biotechlab, Bulgaria). The filtrate was used in enzyme inhibition assay and its lovastatin content analysed.

Fractionation of *Bekasam* Extract

The purpose of fractionation was to separate bioactive peptides from other compounds in the *bekasam* extract. Fractionation was based on the molecule size using filtration membrane (3 kD and 10 kD MWCO, Thermo-Scientific, UK) and membrane filter 0.02 μm (Whatman, Germany). Six fractions were obtained: non-evaporation fraction (F1) was extracted from *bekasam* using aquabides, free supernatant fraction (F2) was result of evaporation of F1; fraction with molecular weight (MW) of > 10 kD (F3), fraction with MW of 3 – 10 kD (F4), fractions with MW of < 3 kD (F5) and fraction with MW< 1 kD (F6). All fractions (F1-F6) were used for assay of their HMG-CoA reductase inhibitory activity.

Lovastatin Assay

Lovastatin content was detected in the free supernatant fraction (F2). Lovastatin is a bioactive compound with molecular weight < 1 kD and since its content in the *bekasam* was low and thus it didn't need to be fractionated. Lovastatin content was measured using spectrophotometer (UV-Mini-1240, Shimadzu). Five mL of the sample was mixed with 20 mL methanol and the mixture shaken for two hours, before it was filtered through membrane 0.45 μm. The filtrate was centrifuged at 120 x g, 4°C for 15 minutes, and the supernatant was separated. Supernatant (0.5 mL) was mixed with 0.5 mL trifluoracetic acid 1% and incubated for 10 minutes. Homogenate (0.5 mL) was placed in 5 mL volumetric flash and filled up with methanol. The absorbance was measured using spectrophotometer at λ = 238 nm. The concentration of lovastatin was calculated in ppm and corresponded to the calibrate curve. Standard lovastatin at concentrations of 6, 8, 10, 20, 30, 40, 50, and 60 ppm were used (Osman et al., 2011).

HMG-CoA Reductase Inhibition Assay

Supernatant from *bekasam* extract (5 mL) was separated and filtered via 0.45 μm membrane and the filtrate was used in the HMG-CoA reductase inhibition assay using HMG-CoA reductase assay kit containing pravastatin as positive control, HMG-CoA as a substrate, HMG-CoA reductase enzyme, NADPH and assay buffer. The procedure followed manufacturer's instructions. The assay was based on the spectrophotometric measurement of decrease in absorbance at λ = 340 nm, which represented oxidation of NADPH by the catalytic subunit of HMG-CoA reductase in the presence of the substrate HMG-CoA. One unit was defined as 1.0 μmole of NADPH converted to NADP+ per 1 minute. Specific activity was defined as μmol/min/mg-protein (units/mg) (Lachenmeier et al., 2012).

Profile Peptides Assay of *Bekasam* Extract Fractions

The peptide profile was analysed by resolving 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel in the 1 M Tris-HCl pH 6.8. The protein fraction was loaded into the wells of the gel and electrophoresed using 1x running buffer (24.8 mMTris, 192 mM glycine, 0.1% SDS, in the pH 8.3). A standard molecular weight marker (Low Range Protein Ladder Thermo scientific, Lithuania) was loaded onto the gel to compare the molecular weights of the proteins/peptides in different samples. After electrophoresis, the gels were stained with silver staining mechanism (Giri et al., 2012).

RESULTS

HMG-CoA Reductase Inhibitor (Lovastatin Content in the *Bekasam* Extract)

Lovastatin is a mine bioactive compound which inhibit HMG-CoA reductase enzyme activity. The content of lovastatin in the bekasam from *minnows/carps* fish produced with starter *Lactobacillus acidophilus* was 148.30 ppm (Table 1). This is higher compared with Rinto et al. (2015a) that revealed the statins content in the bekasam was between 20.98 and 106.42 ppm. In addition to statin, bioactive peptide in the bekasam extract also inhibits HMG-CoA reductase enzyme activity.

Table 1
The yield of bekasam fraction, lovastatin content, peptides and inhibition of bekasam extract fraction for HMG-CoA reductase enzyme activity

No	Sample	Yield (%)	Lovastatin (ppm)	Peptides (kD)	Inhibition of HMG-CoA R (%)
1	F1 (Non-Evaporation)	NA*	NA	7.69 10.71 20.22	66,67
2	F2 (extract free supernatant)	15	148.30	7.69 10.71 20.22 35.38	85,71
3	F3 (MW > 10 kD)	3.8	NA	7.69 10.71	69,05
4	F4 (WM 3-10 kD)	1.7	NA	7.69	92,86
5	F5 (MW 1- 3 kD)	0.37	NA	-	85,71
6	F6 (MW < 1 kD)	0.7	NA	-	92,86

Note. NA: Not analysed

HMG-CoA Reductase Inhibitor (Peptides in the *bekasam* Extract)

Peptides in the *bekasam* extract are produced by microorganisms and enzymes in the fermentation process; *Lactobacillus acidophilus* as a culture starter in the fermented process helps to produce peptides. It produces bioactive peptide 6.3 kD as an inhibitor of HMG-CoA reductase (Rinto et al. 2015a). Profile peptides assay of fractions in the *bekasam* extract resulted some peptides (Figure 1). In the fraction of bekasam extract without evaporation (F1) contains 3 peptides, i.e. peptide with molecular weight 7.69 kD; 10.71 kD and 20.22 kD. Extract with free supernatant fraction (F2) contain 4 peptides (7.69 kD; 10.71 kD; 20.22 kD and 35.38 kD). Nevertheless, peptide bands in the F2 was thicker than in the F1. This indicated that the concentration process by evaporation in 70°C could increase the intensiveness of bekasam extract. Fractions of *bekasam* extract in the F3 contain 2 peptides band, i.e. peptide with molecular weight 7.69 kD; 10.71 kD. Furthermore, fractionation in the F4 can separate only one peptide band with molecular weight 7.69 kD. In the F3 and F4 fraction there were no peptides band. This showed that there were no peptides with molecular weight less than 3 kD (Table 1 and Figure 1).

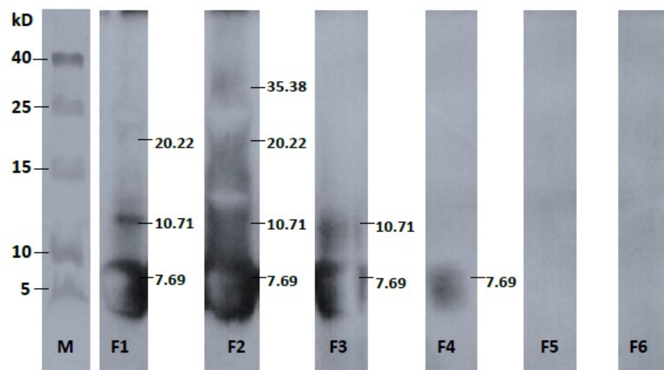

Figure 1. Peptides profile of *bekasam* extract (F1: non-evaporation *bekasam* extract; F2: extract free supernatant; F3: fraction of *bekasam* extract with molecule weight > 10 kD; F4: fraction of *bekasam* extract with molecular weight 3-10 kD; F5: fraction of *bekasam* extract with molecular weight 1-3 kD and F6: fraction of *bekasam* extract with molecular weight < 1 kD)

Inhibition of *Bekasam* Extract

Inhibition assay of each fractions for *bekasam* extract resulted in different inhibition to HMG-CoA reductase enzyme. Overall, inhibition of bekasam extract fractions to HMG-CoA reductase is more than 60%. It indicated that fractions of *bekasam* extract had high level of inhibition to HMG-CoA reductase enzyme. This finding corresponds with a past study

which revealed that the bekasam extract was able to inhibit the activity of HMG-CoA reductase by 64.44% (Rinto et al., 2015a). Crude *bekasam* extract treated without evaporation (F1) had the lowest inhibition. . This caused the concentration of bioactive compounds (peptides) to be lower than the other fractions (Figure 1). The highest inhibition was found inF4 and F5 (92.86%). In F4 fractions contained one band of peptides with a molecular weight of 7.69 kD while there was no band of peptide in F6. This indicated that the F6 fraction of *bekasam* extract involved in inhibition to the enzyme HMG-CoA reductase was lovastatin, while the F4 was a peptide with a molecular weight of 7.69 kD.

DISCUSSION

Fermentation is a chemical process where a substance breaks down into a simpler one. *Bekasam* is one of the fermented fish products. In the fermentation of bekasam, protein is converted to peptides or amino acid by indigenous enzyme and microorganisms. Some microorganisms that are responsible for fermentation is *Lactobacillus plantarum, Lactobacillus mesenteroides, Lactobacillus brevis, Pediococcus*, and *Leuconostoc* (Rhee et al., 2011; Wikandari et al., 2012). Lactobacillus acidophilus are known as lovastatin and peptides producer bacteria which functions as a HMG-CoA reductase inhibitor. Therefore, the use of Lactobacillus acidophilus as a starter culture in the fermentation of bekasam increases the bioactive compounds of HMG-CoA reductase inhibitors (Rinto et al. 2015b).

The extraction and fractionation of *bekasam* produced a peptide fraction (F4) and lovastatin (F6); they displayed high inhibition to HMG-CoA reductase enzyme with a value of 92.86% each . An earlier study showed the inhibitor activity of bekasam extract was 64.44% (Rinto et al., 2015a), it indicates that fractionation process can increase the activity level of inhibition to HMG-CoA reductase enzyme. The evaporation process as are result of *bekasam* extract has an effect on the inhibiting activity causing the peptide to become concentrated. Increasing the concentration and purity of the inhibitor can increase level of inhibition to enzyme activity.

The present of lovastatin in F6 fraction and peptide of 7.69 kD in F4 fraction showed the role of *Lactobacillus acidophilus* as a producer of lovastatin and peptides in the fermentation of *bekasam*. A previous study showed that *Lactobacillus acidophilus* produced lovastatin and peptide as an inhibitor of HMG-CoA reductase. The utilisation of *Lactobacillus acidophilus* as a starter culture in the *bekasam* fermentation improved inhibition of bekasam extract to the activity of HMG-CoA reductase, although the content of lovastatin did not increase. This shows the use of *Lactobacillus acidophilus* as a starter culture in *bekasam* fermentation is important to improve the bioactive peptide as an inhibitor of HMG-CoA reductase enzyme.

CONCLUSION

Fractionation of *Bekasam* extract produces peptide (7.69 kD) and lovastatin (148.30 ppm) which display high inhibition to HMG-CoA reductase enzyme. The use of *Lactobacillus acidophilus* as a starter culture in the fermentation of *bekasam* could increase activity level of peptide (7.69 kD) that functions as HMG-CoA reductase inhibitor.

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Short Communications

Correlation between Sperm Parameters in West African Dwarf Goat Bucks during Storage

Daramola, J. O.* and Adekunle, E. O.

Department of Animal Physiology, Federal University of Agriculture Abeokuta, P.M.B.2240, Abeokuta, Ogun State, Nigeria

ABSTRACT

The relationships among sperm parameters of West African Dwarf (WAD) goat semen were determined. The semen samples were extended in Tris-egg yolk extender and assessed for sperm motility, acrosome and membrane integrities, abnormality and malondialdehyde (MDA) concentrations from 0 to 240 h of storage at 5°C. All the parameters were found to have highly significant correlations (positive/negative) except between motility and MDA concentration as well as abnormality and MDA concentration. This study suggests that a significant correlation exists among the sperm parameters of WAD goat bucks and the semen stored at 5°C up to 48 h is suitable for artificial insemination beyond which deterioration to sperm viability occurred.

Keywords: Bucks, relationships, sperm quality, storage

INTRODUCTION

West African Dwarf (WAD) goats (*Capra hircus*) are found in the South-Western part of Nigeria. The demand for animal protein in the sub region is constantly high; hence, there are prospects for increasing the productivity of this breed of goat Artificial insemination (AI) to improve this breed presents a great potential to alleviate the problem of protein malnutrition in the region. Holt et al. (2007) stated that success of AI depends on the quality of its semen and its potential for fertilisation (as cited in Sharma et al., 2012).

The fertility potential of frozen or chilled semen is made on the basis of fertility rate in

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E-mail addresses:
daramolajames2003@yahoo.com (Daramola, J. O.),
oluwafemiadekunle2010@yahoo.com (Adekunle, E. O.)
* Corresponding author

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females mated, this notwithstanding, semen evaluation provides predictive information on expected performance of the male and insights into the fertilising capacity of the preserved spermatozoa (Januskauskas & Zilinskas, 2002). There is a link between fertility of semen in AI and its measurable parameters (Januskauskas & Zilinskas, 2002). Evaluation tests measuring the physiological and cytological parameters of spermatozoa *in-vitro* such as sperm viability, progressive motility, hypo-osmotic swelling tests, acrosome integrity and morphological abnormalities when carried out reduce the economic and time constraints in field conditions. Knowledge on the value of any one of these parameters could provide a fairly good and adequate prediction of the other. Ho (2006) reported that correlation of two variables is often used to predict the score of one on the other (as cited in Sharma et al., 2012). Adequate base-line information on the evaluation of semen for breeding purposes in WAD goats will be a valuable diagnostic tool to assess the fertility status of these bucks. There is little or no information in literature on the semen evaluation parameters as a valuable diagnostic tool to predict the quality of spermatozoa obtained from WAD goats. This study, therefore, investigated the correlation between sperm progressive motility, acrosome integrity, membrane integrity, percentage abnormality and malondialdehyde concentration of post-chilled spermatozoa obtained from WAD goat bucks.

MATERIALS AND METHODS

Animals and management

The WAD goat bucks used in this experiment were raised at the Goat unit of Teaching and Research Farm of Federal University of Agriculture Abeokuta, Nigeria. The University is located in south-western Nigeria with a prevailing tropical climate (mean annual rainfall of 1,037 mm, mean relative humidity of 82% and average temperature of 34.7 °C, latitude 7° 10' N and longitude 3° 2' E, and altitude 76 m above sea level). The bucks were aged between four and 5 and weighed 18 kg on average. The animals were managed intensively and fed (crude protein = 16 %, fat = 5%) at 300g/ body weight, supplemented with guinea grass (*Panicum maximum*) and fresh water *ad libitum*.

Semen collection, dilution and storage

Semen was collected from the bucks that had earlier responded well to semen collection using artificial vagina (AV). The semen samples collected from six bucks showing >80 % sperm motility were pooled to reduce individual differences. A Tris-egg yolk based extender [Tris [$OHCO_3$] amiozomethane (2.42 g/L), citric acid (1.36 g/L), glucose (1 g/L), penicillin (0.028 g/L), egg yolk (20% v/v) and pH adjusted to 7.03] was used for this study. The semen samples were diluted with the Tris-egg yolk based extender at room temperature. The diluted semen samples were drawn into vials, sealed and then cooled to 5°C, and maintained at

this temperature in a refrigerator for 240 hours. Following storage, evaluation of semen quality characteristics was carried out at interval of 24 hours.

Semen Evaluation

Subjective Microscopic Sperm Motility. Semen was warmed for 2 minutes in Clifton Water bath (Model: 74178 by Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37°C to determine its motility, using Celestron PentaView digital microscope (LCD-44348 by RoHS, China) at 400x magnification. Semen sample (5 µL) was placed directly on a warmed microscope slide overlaid with a cover slip. Each semen sample was examined for sperm motility rate from different slides. Ten microscopic fields were examined by three observers simultaneously to observe progressively motile spermatozoa that moved forward in basically a straight line. The mean (each from the three observers) of the 10 successive evaluations was recorded as the final motility score.

Acrosome Integrity. Percentage of spermatozoa with intact acrosome was determined by adding 50 µL of each semen sample to a 500 µL formalin citrate solution (96 mL of 2.9% sodium citrate with 4 mL of 37 % formaldehyde) and mixed carefully. A small drop of the mixture was placed on a glass slide and a total of 200 spermatozoa were counted in different microscopic fields for each sample. Normal apical ridge of spermatozoa was assessed as intact acrosome using Celestron PentaView LCD digital microscope (400x magnification).

Sperm Membrane Integrity. Hypo-osmotic swelling test (HOST) assay was carried out by incubating chilled semen (10 µL) in 100 µL of a 100mOsm hypo-osmotic solution (9 g fructose plus 4.9 g tri-sodium citrate dihydrate mixed with 1000 mL of distilled water) at 37°C for 30 min. Thereafter, 0.1 mL of the mixture was dispersed on a warmed slide overlaid with a with a cover slip and observed under Celestron PentaView LCD digital microscope (400x magnification). A total of 200 spermatozoa were counted on each slide. Spermatozoon that swelled in response to the test is indicative of having an intact plasma membrane and was counted.

Sperm Abnormality. Sperm abnormality was carried out using eosin-nigrosine stain. Semen sample (3 *u*L) was placed on a microscopic slide and 2 *u*L of eosin-nigrosin was dropped on it and dried. The percentage of morphologically abnormal spermatozoa with defects in the head, midpiece and tail were observed under Celestron PentaView LCD digital microscope (400x magnification).

Malondialdehyde concentrations. Malondialdehyde concentration in the stored semen was measured in a thiobarbituric acid reactive substances (TBARS) according to Pipan et al. (2014, pp. 132-133). This was carried out by incubating sperm suspension

(0.1 mL) in 0.1 mL of 150 mM Tris-HCl (pH 7.1) for 20 min at 37°C. Subsequently, 1 mL of 10 % trichloroacetic acid (TCA) and 2 mL of 0.375 % thiobarbituric acid was added and incubated in boiling water for 30 minutes. Thereafter, it was centrifuged for 15 minutes at 3000 g. The absorbance was read using UV spectrophotometer (SW7504 model by Surgifriend Medicals, England) at 532 nm. The concentration of malondialdehyde was calculated as follows: Malondialdehyde (nmol/mL) = AT – AB/1.56 × 10^5; where AT = the absorbance of the semen sample, AB = absorbance of the blank, 1.56 × 10^5 is the molar absorptivity of malondialdehyde.

Statistical analysis. Data were subjected to analysis of variance using general linear model of SAS package. Duncan Multiple Range was used to separate means while Pearson's correlation coefficients were used to determine the relationship between the semen quality characteristics.

RESULTS

The means of sperm parameters for the different hours of cold storage are presented in Table 1. The results of this study showed that period of storage had significant effect on motility, acrosome integrity, membrane integrity and percentage abnormality. The percentage motility, acrosome integrity and membrane integrity decreased with increased storage periods while the percentage of sperm abnormality and malondialdehyde concentration increased in tandem with increased storage periods. The relationships between the various sperm parameters along with their regression equations are shown in tables 2 and 3. All the parameters had significant correlations (positive/ negative) except correlation between motility and malondialdehyde concentration as well as percentage abnormality and malondialdehyde concentration. Motility showed significant positive correlation

Table 1
Sperm parameters evaluated for the different hours of storage at 5°C (Mean ± SD)

Duration (h)	MOT (%)	ACI (%)	MI (%)	ABN (%)	MDA (μmol/L)
0	88.00 ± 6.00^{a}	93.58 ± 0.87^{a}	90.54 ± 1.26^{a}	1.02 ± 0.63^{g}	0.00^{h}
24	69.33 ± 8.08^{b}	92.37 ± 0.12^{a}	84.37 ± 4.37^{b}	2.48 ± 1.40^{f}	0.13 ± 0.11^{g}
48	58.00 ± 6.92^{c}	87.25 ± 1.25^{b}	76.37 ± 5.37^{c}	3.36 ± 1.15^{e}	0.22 ± 0.16^{fg}
72	49.33 ± 8.08^{d}	80.12 ± 4.12^{bc}	71.37 ± 3.96^{cd}	4.10 ± 1.10^{de}	0.28 ± 0.18^{f}
96	41.33 +21.93^{e}	76.50 ± 3.50^{c}	66.25 ± 5.25^{d}	4.63 ± 1.25^{d}	0.31 ± 0.16^{e}
120	36.00 ±31.17^{f}	69.50 ± 5.50^{d}	58.75 ± 6.75^{e}	5.36 ± 1.73^{c}	0.32 ± 0.16^{e}
144	34.66 ± 30.02^{fg}	64.50 ± 7.00^{e}	54.50 ± 5.00^{ef}	5.44 ± 1.69^{c}	0.34 ± 0.17^{de}
168	28.00 ± 24.24^{g}	58.75 ± 6.75^{f}	49.50 ± 5.50^{f}	5.96 ± 1.78^{bc}	0.35 ± 0.18^{d}
192	26.66 ± 23.09^{h}	49.37 ± 9.37^{g}	41.75 ± 6.49^{g}	6.49 ± 1.51^{b}	0.56 ± 0.36^{c}
216	26.67 ± 23.09^{h}	42.75 ± 10.75^{h}	39.87 ± 6.38^{g}	6.86 ± 1.73^{b}	0.62 ± 0.36^{b}
240	10.66 ± 9.23^{i}	36.75 ± 8.75^{i}	33.00 ± 5.50^{h}	8.13 ± 1.29^{a}	0.78 ± 0.53^{a}

^{a,b,c,} ….i Mean values with different superscripts within columns are significantly different at $p<0.05$.
MOT: Motility, ACI: Acrosome Integrity, MI: Membrane Integrity, ABN: Abnormality, MDA: Malondialdehyde

Table 2
Correlation between the evaluated sperm parameters

	MOT (%)	ACI (%)	MI (%)	ABN (%)	MDA (μmol/L)
MOT (%)	1.00000	0.47332**	0.49986**	-0.81133***	-0.03150^{NS}
AI (%)		1.00000	0.98193***	-0.60388***	-0.80019***
MI (%)			1.00000	-0.62657***	-0.75699***
ABN (%)				1.00000	0.12981^{NS}
MDA (μmol/L)					1.00000

** (P<0.01), *** (P<0.001), ^{NS} (not significant), MOT: Motility, ACI: Acrosome Integrity, MI: Membrane Integrity, ABN: Abnormality, MDA: Malondialdehyde

Table 3
Inter-relationship between semen evaluation parameters in WAD bucks

S/No	Relationship between parameters	Correlation coefficient	Regression estimate	Regression equation
1	MOT with ACI	0.47332**	0.3198	y = - 11.758 + 0.796x
2	MOT with MI	0.49986**	0.3865	y = -12.787 +0.914x
3	MOT with ABN	-0.81133***	0.7373	y = 91.816 - 10.049x
4	MOT with MDA	-0.03150^{NS}	0.0215	y = 49.851 – 20.245x
5	ACI with MI	0.98193***	0.9577	y = 5.822 + 1.032x
6	ACI with ABN	-0.60388***	0.4503	y = 96.910 – 5.839x
7	ACI with MDA	-0.8001***	0.6798	y = 87.661 – 54.059x
8	MI with ABN	-0.62657***	0.5153	y = 89.479 – 5.903x
9	MI with MDA	-0.75699***	0.6206	y = 78.154 – 49.13x
10	ABN with MDA	0.12981^{NS}	0.0628	y = 4.069 + 2.313x

** (P<0.01), *** (P<0.001), ^{NS} (Not significant), MOT: Motility, ACI: Acrosome Integrity, MI: Membrane Integrity, ABN: Abnormality, MDA: Malondialdehyde

with acrosome integrity (r=0.47332) and membrane integrity (r=0.49986). Acrosome integrity also showed significant positive correlation with membrane integrity (r=0.98193). However, a negative correlation (P<0.001) was recorded between percentage abnormalities with the following sperm parameters: motility (r=-0.81133), acrosome integrity (r=-0.60388), membrane integrity (r=-0.62657) as well as MDA with acrosome integrity (r=-0.80019) and membrane integrity (r=-0.75699).

DISCUSSION

The assessed viability parameters (motility, acrosome integrity and membrane integrity) were found to deteriorate with time while sperm abnormality and malondialdehyde concentrations increased with time. The survival of ejaculated sperm in seminal plasma alone is limited to few hours (Kheradmand et al., 2006, p. 40). The present study therefore indicated the importance of storage period for the refrigeration of buck sperm. Storage of semen at low temperature

decreases the functional integrity of spermatozoa (Rasul et al., 2001).

Contrary to the positive correlation between sperm motility and membrane integrity observed in this study, Ollero et al. (1998) reported that many sperm cells that exhibit damaged membranes are motile after thawing. Ollero et al. (1998) attributed the inclusion of non-viable sperm cells into the population of motile sperm cells as a possible reason. Similar to other small ruminants, the plasma membrane of buck sperm is rich in polyunsaturated fatty acids, and this makes it susceptible to peroxidative damage (Jones & Mann, 1976). Lipid peroxidation is known to induce membrane damage (Aitken, 1995), and this is evidenced by the positive correlation between sperm motility and membrane integrity observed in this study contrary to negative correlation with sperm motility earlier reported in (Kasimanickam et al., 2006).

The significant correlation among the parameters assessed in this study is consistent with Jeyendran et al. (1984). The results obtained in this study could probably be due to the dependence of sperm motility on compounds transported across membrane of spermatozoa (Jeyendran et al., 1984,). For this reason and in line with Kordan and Strze_ zek (1997), plasma membrane damage due to death or anisosmotic conditions causes a rapid leakage of intracellular adenosine triphosphate (ATP), which is required to maintain sperm motility. The content of ATP has been reported to be highly correlated with progressive sperm motility of fresh and cryopreserved bull semen (Januskauskas & Rodriguez-Martinez, 1995). Moreover, as the production of mitochondrial ATP is a membrane-dependent process, any negative change to the cell membrane could reduce mitochondrial ATP production that could possibly lead to mitochondrial membrane potential (Januskauskas & Rodriguez-Martinez, 1995). In the absence of sufficient energy, spermatozoa are not progressively motile. Therefore, the correlation between the membrane integrity and sperm motility reflected this observation.

When the relationship between motility and percentage of sperm with intact acrosome was evaluated in semen from yearling Hereford or Angus bulls, Bemdtson et al. (1981) reported that post-thawed motility and acrosome integrity constituted distinct features of sperm integrity that varied independently of each other. The results obtained in this study were in agreement with previous work on cattle (Lodhi et al., 2008) and human (Jeyendran et al., 1984), semen. The correlation that was recorded among motility, acrosome integrity and membrane integrity was in line with Brito et al. (2003) and this was expected since they are all related to plasma membrane integrity and possibly because the sperm plasma membrane is a continuous structure covering the head, mid-piece and tail (Karp, 2009). Membrane integrity and the stability of its semi-permeable features are required for viable spermatozoon and this is because if the plasmalemma is intact but functionally unstable, the spermatozoon is not capable of interacting with its environment and thus,

is unable to fertilise (Rodriguez-Martinez, 2007).

Furthermore, the correlation between the membrane integrity and motility may be attributed to the fact that motility is a function of intracellular ATP content (Januskauskas & Rodriguez-Martinez, 1995). Higher correlation between motility and membrane integrity was probably due to the fact that both are determinants of the integrity of the tail membrane. Similarly, a correlation has earlier been established between acrosome intact sperms with percentage of motile sperms (Kirk et al., 2005). Negative correlations of these parameters with morphological abnormalities at various periods of storage have also been reported (Vyas et al., 1992). The significant relationship of the morphological abnormalities established with acrosome integrity in this study could be attributed to the principle of classification of the abnormalities. The relationship with acrosome integrity may be ascribed to the association of acrosome abnormalities with head abnormalities.

Kobayashi et al. (1991) demonstrated that malondialdehyde concentration in spermatozoa was significantly related to the number of non-motile spermatozoa. In the present study, the negative correlation between the malondialdehyde activity and membrane integrity was consistent with the finding of Kobayashi et al. (1991). Increased malondialdehyde activity could represent the pathologic lipid peroxidation of sperm membrane and inhibition of sperm motility.

CONCLUSION

The results indicated positive correlations between motility, acrosome integrity and membrane integrity, while abnormality was negatively correlated with motility, acrosome integrity and membrane integrity. The MDA concentration was negatively correlated with acrosome integrity. The parameters provide fair estimate of sperm quality of WAD goat bucks and the semen stored at 5°C up to 48 hours is suitable for artificial insemination beyond which deterioration of sperm viability is witnessed.

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Clinical Case Study

A Case of Diaphragmatic Rupture in a Criollo Pony

Khairuddin, N. H.[1]*, Roslim, N.[1], Affandi, S. S.[1], Lau, S. F.[2] and Lim, S. Y.[2]

[1]*Department of Farm and Exotic Animal Medicine and Surgery, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[2]*Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

ABSTRACT

This report describes a case of diaphragmatic rupture with migration of small and large intestines into the thoracic cavity of an 18-year-old Criollo pony. The pony was initially presented to the University Veterinary Hospital (UVH), Universiti Putra Malaysia (UPM), with mild colic and increased respiratory effort. A diagnosis of diaphragmatic rupture was made based on thoracic auscultation, radiographic and ultrasonographic findings. Due to financial constraints, surgical management was not an option and so the pony was managed medically. The pony was diagnosed with diaphragmatic rupture and concurrent bronchopneumonia and was observed closely. Mild colic was treated with analgesics while dyspnoea was managed symptomatically with bronchodilators and antibiotics. The pony remained clinically stable for more than eight (8) weeks. However, another episode of colic lead to a deterioration in its clinical condition and subsequent death. Post-mortem findings revealed migration of loops of small intestines and part of the large colon into the thoracic cavity. There was a linear tear (estimated to be about 15cm in length) in the right dorsal segment of the diaphragm at the border of the muscular and fibrous portion, which confirmed our clinical and diagnostic imaging findings of diaphragmatic rupture that had caused the pony to suffer from colic and dyspnoea.

Keywords: Diaphragmatic rupture, dyspnoea, colic, radiography, ultrasonography, pony

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E-mail addresses:
nurul.leman82@gmail.com
nurulhayah@upm.edu.my (Khairuddin, N. H.),
noorashimah@upm.edu.my (Roslim, N.),
syaaffandi@upm.edu.my (Affandi, S. S.),
lausengfong@upm.edu.my (Lau, S. F.),
sueyee@upm.edu.my (Lim, S. Y.)
* Corresponding author

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INTRODUCTION

Diaphragmatic rupture is uncommon in horses (Speirs & Reynolds, 1976; Santschi, et al., 1997; Dabareiner & White, 1999). In adult horses, traumatic diaphragmatic injury has been recorded following parturition or excessive strenuous activities (Bristol, 1986). Following a diaphragmatic rent, various abdominal organs may pass through the defect although migration of small intestine into the thoracic cavity is most commonly recorded (Wimberly et al., 1977). Repair of diaphragmatic hernia is possible in horses but the prognosis following surgery is generally poor (Hart & Brown, 2009; Romero & Rodgerson, 2010; Efraim & Kelmer, 2015). In this report, we describe the diagnosis and conservative management of a case of diaphragmatic rupture in a Criollo pony that was presented with recurrent low-grade colic and dyspnoea. Diagnosis of rupture of the diaphragm was made based on findings from clinical, radiographic and ultrasonographic examinations.

CASE HISTORY

An 18-year old Criollo pony, gelding , with 3/5 body condition was presented to UVH, UPM, with signs of mild colic that had lasted for four hours. The pony was reported to have rolled repeatedly in the stable when the colic was first noticed. According to the caretaker, there was no history of trauma or changes in its routine. Initially, administration of flunixin meglumine (1.1 mg/kg, IV) and intravenous fluids (0.9% NaCl, replacement rate at 20 mg/kg/hour, IV) provided relief as the clinical signs subsided, and all clinical parameters such as heart and respiratory rates returned to their normal limits. However, 12 hours after the initial episode, the horse showed signs of recurrent mild colic, and another dose of flunixin meglumine (1.1 mg/kg, IV) was administered. At this stage, the horse showed increased breathing effort and was sweating profusely.

CLINICAL FINDINGS AND DIAGNOSTICS

Physical examination revealed a heart rate of 44 beats per minute and a respiratory rate of 36 breaths per minute. The pony displayed considerable respiratory effort and obvious abdominal breathing with a notable heave line. Rectal temperature, capillary refill time and the colour and hydration of mucous membranes were all within normal limits. Internal examination by rectal palpation was unremarkable. Normal borborygmi, which included gaseous components, were detected on auscultation of the abdomen. Auscultation of the left and right thoracic regions revealed sounds typical of intestinal activity. In addition, enhanced respiratory sounds, including wheezes and crackles, were evident. A radiograph of the thoracic region was taken from the left side and tubular, gas-filled structures, highly suggestive of distended loops of bowel, were seen in the cranio-ventral, caudo-ventral and caudal dorsal regions of the thorax (Figure 1). The diaphragmatic outline at the caudo-dorsal region of the thorax was not well defined, suggesting a disruption of the diaphragm in this location.

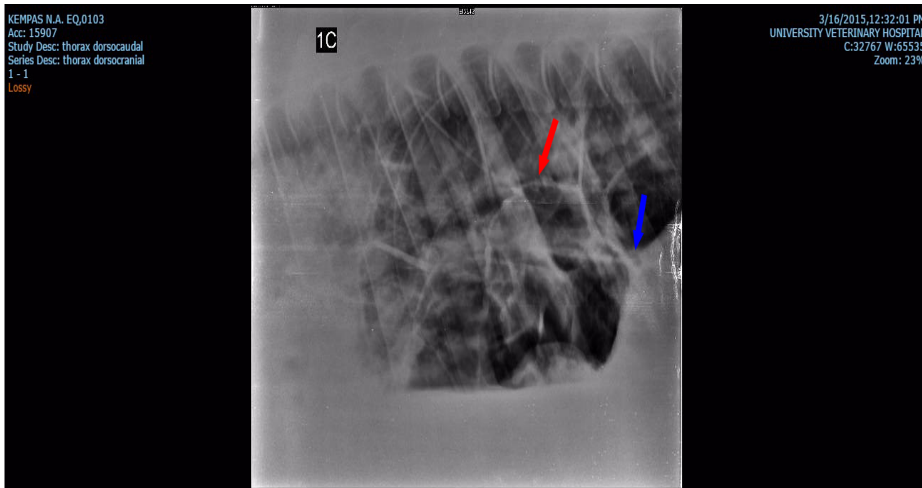

Figure 1. Left lateral thoracic radiograph revealed the presence of gas-filled organs highly suggestive of distended loops of bowel at the caudo-dorsal of the thoracic field (red arrow) and the irregularity of diaphragmatic outline at the caudo-dorsal region of thorax (blue arrow)

Transcutaneous ultrasonographic examination of the abdomen and thorax was performed using an ultrasound scanner (MyLab™ 40, Esaote) with a 3-9 MHz curvilinear probe (CA 123VET). With the horse in standing position, the ultrasound probe was placed longitudinally between two ribs and advanced sequentially cranially from the 13th intercostal space. A large amount of intra-thoracic free fluid was observed within the thoracic cavity at the level of the 11th intercostal space of the left side. Moving the probe further cranially, loops of intestines and free fluid were observed. The intestines were recognised by the reverberation artifact caused by intestinal gas. Marked pleural effusions were observed in the lung field (Figure 2). It was not possible to visualise any areas of discontinuity in the diaphragm based on ultrasonographic examination. Video of the ultrasound examination of the thorax is available as supplementary material. Abdominal ultrasound revealed that the spleen and liver were displaced ventrally and cranially. The motility of observable intestines appeared normal.

A diagnosis of diaphragmatic rupture with passage of intestines into the pleural cavity was made based on thoracic auscultation, radiographic and ultrasonographic findings.

TREATMENT

Mild colic was treated with flunixin meglumine. Dyspnoea and tachypnea were managed with a bronchodilator (Clenbutarol, 5 g/100kg BW, BID for 10 - 14 days, PO) and a course of antibiotics (gentamicin, 6.6mg/kg BW, IV, every 24 hours and penicillin streptomycin, 22,000 IU/kg BW, IM, every 24 hours) were

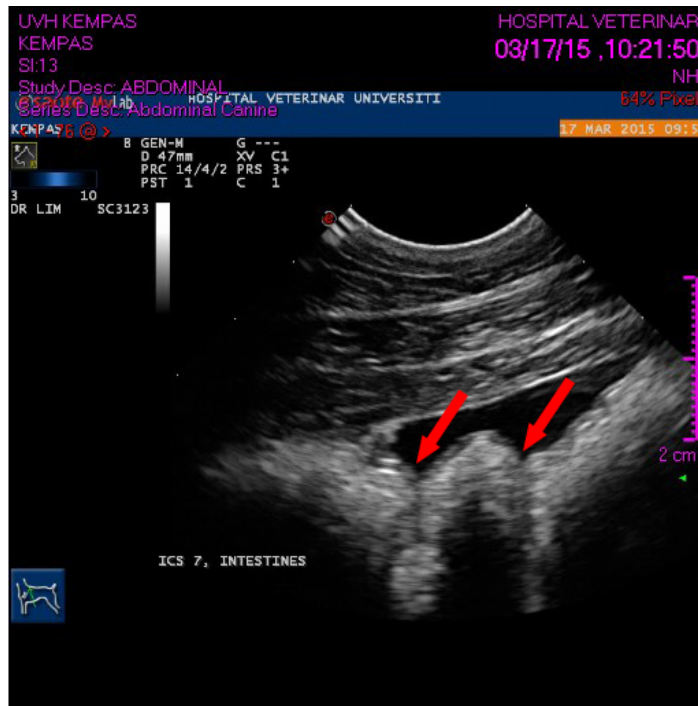

Figure 2. Transcutaneous ultrasonographic image of the thorax at the 7th intercostal space (ventral to the left, dorsal to the right, medial to the bottom). Loops of intestines (arrows) and intra-thoracic free fluid were observed. This image was obtained using a 3-9 MHz curvilinear probe

administered. Signs of colic resolved, and the pony regained its normal appetite and demeanour although it showed increased respiratory effort, with a respiratory rate of 36-40 breaths per minute.

All medications were discontinued after day five of hospitalisation at which stage the pony was discharged. It remained clinically stable for more than eight weeks. However, it then suffered another episode of colic, leading to clinical deterioration and its eventual death.

POST-MORTEM FINDINGS

Post-mortem examination revealed presence of few litres of green-coloured fluid within the thoracic cavity and multiple loops of small intestine and part of the left large colon within the pleural space. The lungs were displaced cranially and were consolidated over more than 50% in all lobes. There was a large, linear tear (estimated to be about 15cm in length) in the right dorsal segment of the diaphragm at the border of the muscular and fibrous portions (Figure 3). The edge of the tear was smooth and was free of adhesions to any other structures. The walls of the intestine displaced in the thoracic cavity were thick and appeared congested.

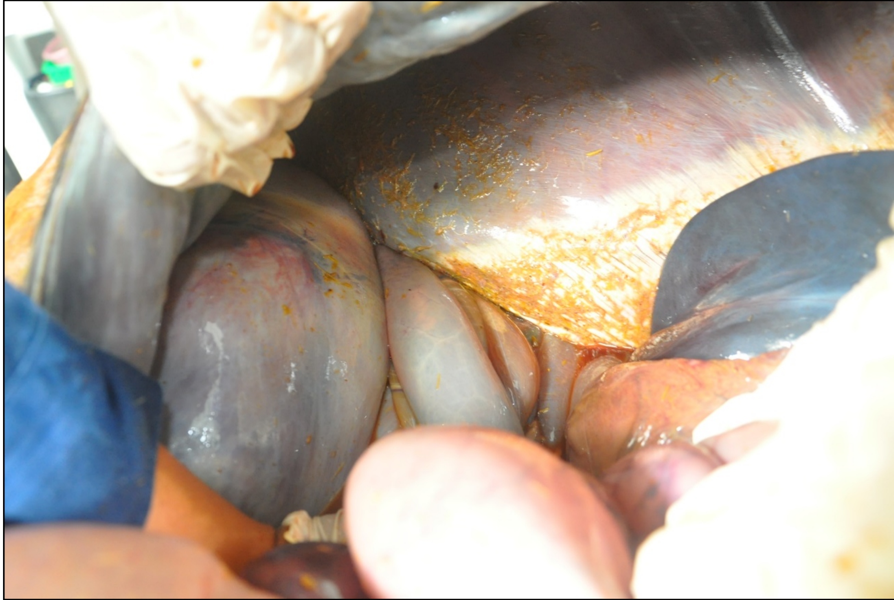

Figure 3. Post-mortem image of the thoracic cavity where small and large intestines pushing through the large linear tear at the right dorsal border of the diaphragm at the border of the muscular and fibrous portion. The edge of the tear was smooth, and no adhesion was evident. The tear was estimated to be about 15 cm in length

DISCUSSION

Diagnosis of diaphragmatic hernia in a horse can be challenging. In this case, the pony was presented with signs of acute colic, dyspnoea and detection of borborygmi that appeared to emanate from the thoracic cavity. This alerted us to the possibility of this rare condition.

In horses, the most common presenting sign of diaphragmatic rupture and passage of abdominal viscera into the pleural space is abdominal pain (Romero & Rodgerson, 2010). In cases where the vascular supply to bowel is compromised, signs are acute, severe and unresponsive to medical therapy. However, where bowel is not strangulated or obstructed, colic may be low grade, chronic and recurrent in nature. Findings from rectal examination, abdominocentesis and clinical pathology may all be within normal limits. Most commonly, horses suffering from this condition are presented with severe signs of colic that requires urgent surgical intervention. The condition is often not definitively diagnosed until the abdomen is systematically explored at celiotomy. Occasionally, the diaphragmatic rent is identified incidentally at necropsy (Bristol, 1986).

Identification of diaphragmatic rupture before induction of general anaesthesia is beneficial as it alerts the anaesthetist to the respiratory compromise that is likely to be present and will facilitate more effective surgical planning. In this case, both radiography and ultrasonography revealed evidence of gas filled loops of intestines within the thoracic cavity that proved to be

helpful in achieving an accurate diagnosis. In addition, radiography showed loss of a distinct diaphragmatic line at the level of the rupture, seen as radiopacity in the caudo-dorsal part of the chest, with the presence of gas filled within.

Rupture of the diaphragm and subsequent migration of intestine into the thoracic cavity resulted in the loss of pleural seal, leading to atelectatic lungs. Surprisingly, in this pony, despite evidence of pleural effusion on radiography and ultrasonography, and a very large tear on the diaphragm seen at post-mortem, the respiratory distress was not very severe. Indeed, the pony survived comfortably for more than two months from the time of presumed diaphragmatic rupture. The final abdominal crisis that resulted in severe deterioration in the pony's condition may be explained by the migration of portions of the gastrointestinal tract into the thoracic cavity including the large colon, which may have also caused further and more severe respiratory distress. Due to financial restraints and a poor prognosis for long-term survival, surgical repair of the diaphragmatic hernia was not an option. The pony was left with an acknowledged problem of diaphragmatic hernia. It has been reported that an unrepaired diaphragmatic rent can be partly sealed by adjacent stomach and liver (Stick, 2006). However, this may be unlikely or impossible if the tear is large and abdominal organs are displaced ventrally and cranially, as was observed in this case.

It was difficult to deduce if the diaphragmatic defect was congenital or acquired in nature. Congenital diaphragmatic hernia can be a result of failed fusion of many of the four embryonic components of the diaphragm (Malone et al., 2001). It can also be associated with diaphragmatic tear due to rib fractures in young foals (Stick, 2006). Congenital defects are reported to be small (only 2.5 cm in diameter) with smooth round edges (Schambourg, et al., 2003).

Diaphragmatic tears following trauma are more common in adults, following road traffic accidents, recent parturition (Stick, 2006) or even breeding in stallions (Riggs, personal communication, August 20, 2015). The tear is usually large and originates from the dorsal body wall as a result of increased intra-abdominal pressure. In this case, the pony survived for two months as the bowel was only mildly compromised and did not become incarcerated thus, displaying signs of low grade colic.

CONCLUSION

In conclusion, the presenting clinical sign of low-grade colic, dyspnoea, and borborygmi within the thoracic cavity, were suggestive of diaphragmatic rupture and migration of abdominal contents into the thoracic cavity. Radiographic and ultrasonographic examinations confirmed the diagnosis. Survival of a horse with unrepaired diaphragmatic hernia depends on the abdominal organs eviscerated, whether the organs have incarcerated, and also the severity of the respiratory distress.

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Evaluation of *Ricinus communis* as a Phytoremediator of Manganese in Soil Contaminated with Sewage Sludge

Nur-Nazirah, P. M.[1], Arifin, A.[1,2]*, Shamshuddin, J.[3], Rezaul, K.[1,2,4], Muhammad-Nazrin[1], Mohd-Hadi, A.[1] and Aiza-Shaliha[1]

[1]*Department of Forest Management, Faculty of Forestry, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[2]*Laboratory of Sustainable Bioresource Management, Institute of Tropical Forestry and Forest Product, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[3]*Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*
[4]*Department of Soil Science, EXIM Bank Agricultural University, Bangladesh, Chapainawabganj 6300, Bangladesh*

ABSTRACT

The cost to dispose of sludge is very high, and Malaysia usually treats it by composting. However, composting is not a feasible method because it is time consuming. The application of sewage sludge on cropland is economical method because it provides the plant nutrients. However, it has a serious impact on the plants, animal and environment due to high concentration of heavy metals. Therefore, phytoremediation is a green technology to remove pollutants especially heavy metals from contaminated soil. This study was conducted to determine the potential of *Ricinus communis* to remove heavy metal in soil. The seedlings were planted on six different planting media T_0 (100% soil-Control), T_2 (80% soil + 20% sewage sludge), T_3 (60% soil + 40 % sewage sludge), T_4 (40% soil + 60% sewage sludge), T_5 (20% soil + 80% sewage sludge) and T_6 (100% sewage sludge). The highest accumulation of Mn was observed in leaves. *Ricinus communis* were found to be suitable as phytoremediators to clean heavy metals especially Mn as its TF value was higher than BCF.

Keywords: Heavy metals, manganese, phytoremediation, sewage sludge

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E-mail addresses:
naz_irah89@yahoo.com (Nur-Nazirah, P. M.),
arifin_soil@yahoo.com (Arifin, A.),
shamshud@upm.edu.my (Shamshuddin, J.),
reza2007_karim@yahoo.com (Rezaul, K.),
deadman17_039@yahoo.com (Muhammad-Nazrin),
mohdhadiakbar@gmail.com (Mohd-Hadi, A.),
aizajamaluddin@gmail.com (Aiza-Shaliha)
* Corresponding author

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INTRODUCTION

Population growth in Malaysia have led to accumulation of sewage sludge, an industrial and domestic by-product. According to Kadir and Mohd (1998), about 3 million metric tonnes of sewage sludge is produced yearly while Indah Water Konsortium (2003) estimated about 7 million cubic meters of sewage sludge yearly by 2022. The current method to dispose of sewage sludge is through incineration (Ødegaard, et al., 2002). Previous studies have shown that sewage sludge has its benefits as soil amendment, soil fertiliser, soil conditioner and improves soil aggregation (Sommers, et al., 1980; Mininni, & Santori, 1987; Xu et al., 2013; Xu et al., 2013). The reason is it contains a high amount of nutrient (N, P, K, Mg and Ca) and organic matter that acts as a natural fertiliser (Majid, et al., 2011). The application of sewage sludge on cropland provides the plants with nutrients and is much more economical compared with incineration, excavating soil, pumping and treating contaminated groundwater, electro kinetic systems, chemical treatment, physical barriers, soil vapour extraction, in-situ oxidation and *in-situ* vitrification.

Sewage sludge can be described as solid waste or biosolids that contain numerous heavy metals from wastewater treatments, mining residues and agricultural waste. The sewage sludge is a source of surface and ground pollution which produces bad odours (Zaini et al. 2014). Heavy metals from sewage sludge would contaminate cultivated crops and natural vegetation as they exceeds the maximum permissible limit set by the local authorities (Ahmad et al., 2015; Nasira, et al., 2015). It is concentrated in a food chain that has an adverse effect on human health and wildlife (Ahmadpour et al. 2010). According to Ahmad et al. (2015), heavy metals can harm the natural environment at low concentration through their tendency to accumulate in the food chain or because of their inherent toxicity. Accidental digestion of heavy metals may cause health problems such as skin allergies and anaemia. Thus, improper disposal of solid waste (sewage sludge) has serious repercussions to the environment. Most countries use sewage sludge sparingly as a soil amendment due to its high heavy metal concentrations.

There have been efforts to remove heavy metals from the soil by using physical and chemical processes to treat it. These have proven to be expensive (Majid et al. 2011). Phytoremediation is a low cost, low impact and environmentally sound green technology to remove heavy metals from contaminated soil using selected plants, The plant is selected based on its characteristics such as fast growing, have high biomass and natural tolerance to toxic substances (Majid et al. 2011). The objective of scientists in applying phytoremediation is to treat contamination by using the most rational and natural process.

Ricinus communis (Family Euphorbiaceae) known as castor bean was selected as a phytoremediator for this study. Previous studies have shown that this plant is a fast growing species and

able to grow in polluted soil that contains heavy metals (Vara Prasad & de Oliveira Freitas, 2003; Rockwood et al., 2004; Giordani, et al., 2005; Vanaja et al. 2011). Limited information is available regarding the evaluation of *Ricinus communis* as phytoremediator of manganese. This study was initiated with the following objectives: (i) to quantify heavy metal accumulation in different plant parts such as roots, stems and leaves; and (ii) to quantify Mn concentration in growth medium before planting and after harvesting period.

MATERIALS AND METHODS

The study was conducted at the greenhouse of Universiti Putra Malaysia, Serdang, Malaysia. The duration of the experiment was six months (February 2014 to July 2014). Healthy seedlings of Ricinus communis were collected from Seri Subuh Agrofarm, Negeri Sembilan. The growing medium for Ricinus communis was soil, organic matter, river sand, in a 3:2:1 ratio. The seedlings were transplanted into suitable pots (32.0 cm height, 106.0 cm upper diameter and 69.0 lower diameters) that were filled up with the mixture of soil and sewage sludge.

The soil used for this study was from the Munchong Series. while sewage sludge was taken from STP Bandar Tun Razak. The soil samples were used as growth media and the soils were mixed with sewage sludge. Physical and chemical properties of soil and sewage sludge were analysed. There were six different treatments with four replicates for each treatment; T_0 - Control (100% soil), T_1 (80% soil + 20% sewage sludge), T_2 (60% soil + 40% sewage sludge), T_3 (40% soil + 60% sewage sludge), T_4 (20% soil+ 80% sewage sludge) and T_5 (100% sewage sludge). The pots were arranged in a Randomized Complete Block Design (RCBD) in a greenhouse. Soil samples were collected from each pot before planting and after harvesting. All soils and sewage sludge were air dried and sieved to <2 mm.

The aqua regia method was used to determine the concentration of Mn in soil. The aqua regia solution contained a 3:1 mixture of concentrated hydrochloric acid (HCl) and nitric acid (HNO_3). Five grammes of soil were put into 50 ml of Aqua Regia solution in a PTFE (polytetrafluoroethylene) beaker and left to react overnight. Then, the samples were heated on the hot plate to approximately 200°C for two hours. When cooled and after making up to 250 ml, the samples were filtered by Atomic absorption spectrophotometry (AAS) (Justin, et al., 2011).

The dry ashing method was used to determine the concentration of heavy metals in the plant. The plants were harvested and washed gently with distilled water to remove soil particles and dust. The leaves stems and roots were cut and the fresh samples were weighed and dried in the oven at 60°C for 24 hours (Heryati et al. 2011). After the samples had cooled, 1 g of ashed tissue was dissolved in a 10 ml dilute nitric acid- hydrochloric acid digestion in 50 millilitres beaker. Finally, the samples were filtered to remove the suspended solids .The heavy metals were determined using atomic absorption spectrophotometry (AAS) (Justin et al. 2011).

The plant height and a number of leaves were measured every month. Plant biomass was measured separately according to its leaves, stems and roots. Two indicators are used to detect the accumulation of heavy metals: a) Bioconcentration Factors (BCF) which indicate the ability of a plant species that can accumulate metals into its tissue from the soils or water; b)Translocation Factors (TF) which indicate the competence of the plant species in translocating metals from roots to shoots. The plant's ability to accumulate metals from soils and translocate metals was estimated using the TF in equation 1 and the BCF in equation 2 (Yoon, et al., 2006):

$$TF = \frac{Metal\ concentration\ aerial\ parts}{Metal\ concentration\ in\ roots} \quad [Eqn. 1]$$

$$BCF = \frac{Metal\ concentration\ in\ roots}{Metal\ concentration\ in\ soil} \quad [Eqn. 2]$$

The analysis of variance (ANOVA) was used for growth parameters, heavy metals in the soil and plant parts and the mean separation test were carried out using Tukey's test ($p \leq 0.05$). The computation and preparation of graph were done using SPSS 16.00 and Microsoft EXCEL 2003 software program.

RESULTS AND DISCUSSION

Physical and Chemical Properties of Soil and Sewage Sludge

The physical and chemical analysis of the control media are shown in Table 1.1. In this experiment, the texture in the control was sandy clay (because the topsoil contained some sand) at a depth of 0-25 cm. The proportion of sand, silt and clay was 41.24%, 7.72%, 51.04% respectively. A high percentage of clay helps to store enough water. Due to the negative charge that belongs to clay, we can determine the fertility of the soil from clay minerals. The percentages of total N and C were 0.11% and 1.62%, while the C/N ratio was 1.62%. The soil was acidic, with a pH of 5.51 (normally, Malaysian soils are very acidic). The available phosphorus was 0.92 mg kg^{-1} and available CEC was 7.38 $cmolckg^{-1}$. The concentrations of exchangeable bases, such as K, Mg, Ca and Na, were 0.40 $cmolckg^{-1}$,

Table 1
Selected physical and chemical analysis of soil and sewage sludge

Analysis	Soil	Sludge
Texture	Sandy clay	-
Sand (%)	41.24	-
Silt (%)	7.72	-
Clay (%)	51.04	-
Total N (%)	0.11	1.39
Total C (%)	1.62	63.65
C/N ratio	14.73	45.79
pH (1:2.5 soil: water)	5.51	6.19
Available P (mg kg^{-1})	0.92	80.68
CEC ($cmolckg^{-1)}$	7.38	27.88
Exchangeable cations (cmolckg-1)		
K ($cmolckg^{-1}$)	0.40	2.50
Mg ($cmolckg^{-1}$)	0.15	1.80
Ca ($cmolckg^{-1}$)	0.62	5.44
Na ($cmolckg^{-1}$)	0.11	0.73
Total heavy metal (mg kg^{-1})		
Cu (mg kg^{-1})	0.008	4.93
Fe (mg kg^{-1})	30.875	1602.13
Mn (mg kg^{-1})	0.003	9.73
Pb (mg kg^{-1})	0.087	10.35
Zn (mg kg^{-1})	0.135	68.38

0.15 cmolckg^{-1}, 0.62 cmolckg^{-1} and 0.11 cmolckg^{-1} respectively. The concentrations of Cu, Fe, Mn, Pb and Zn were 0.008 mg kg^{-1}, 30.875 mg kg^{-1}, 0.003 mg kg^{-1}, 0.087 mg kg^{-1} and 0.135 mg kg^{-1} respectively.

Plant Height, Number of Leaves and Heavy Metal Accumulation in Plant Parts

Figure 1 shows the significance difference ($P \leq 0.05$) in plant height based on treatments and age. In these experiments, plant height ranged from 8 cm to 114 cm at 6 months of age. On average, application of sewage sludge increased plant height of *Ricinus communis*. The number of leaves increased with increasing time except for T_5 (Figure 2). It was proven that, *Ricinus communis* are not tolerant in highly contaminated soil with sewage sludge.

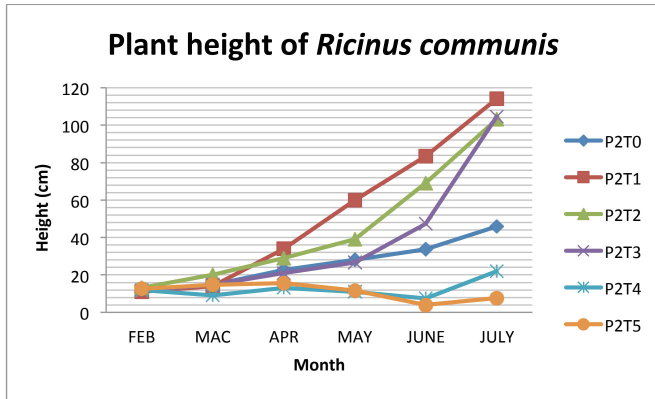

Figure 1. Plant height of *Ricinus communis* from February 2014 to July 2014

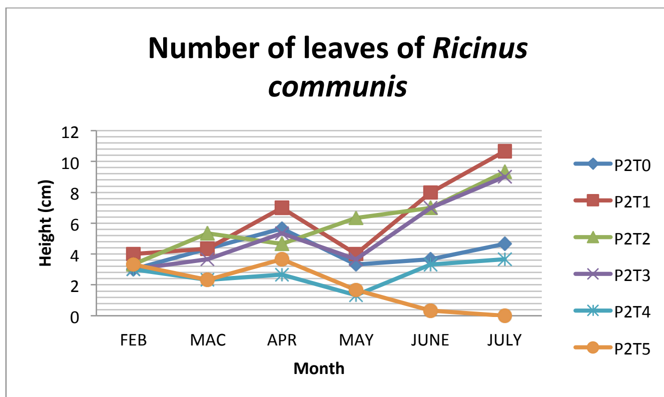

Figure 2. Number of leaves of *Ricinus communis* from February 2014 to July 2014

Mn concentration had a significant effect (P ≤0.05) on different plant parts at different levels of treatments (Figure 3). The range of Mn concentration in leaves, stems and roots were between 0.04 mg/kg and 0.60 mg/kg (T_5). The graph showed that the concentration of Mn was in the order of leaves>roots>stems. According to Sánchez,et al., (2005) and Zakir, et al., (2008), Mn concentration was high in the soil and plant parts after sewage sludge was applied in the growth medium. Besides, the increased concentration of Mn are related to the excess of Zn in plant shoots (Abdu et al. 2011). Hyperaccumulator plant species can accumulate Mn element up to 100 or 1000 times followed by non-accumulator plants (Tangahu et al. 2011).

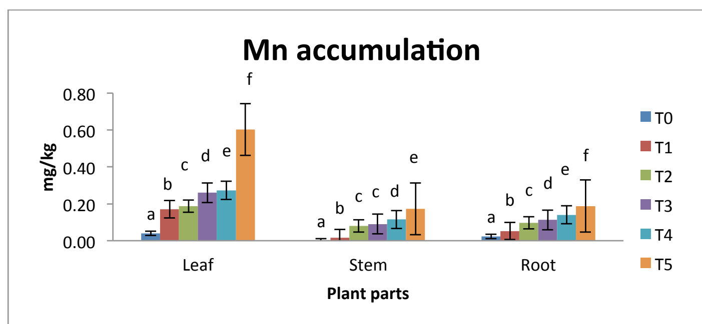

Figure 3. Concentrations of Mn in plant parts after harvesting of *Ricinus communis*. Different letters indicate difference between means at each treatment before planting and after harvesting. T_0 = Control, T_1 = 80% soil + 20% sewage sludge, T_2 = 60% soil + 40% sewage sludge. T_3 = 40% soil + 60% sewage sludge, T_4 = 20% soil + 80% sewage sludge, T_5 = 100% sludge

Concentration of Mn before planting and After Harvesting in the Growth Medium

There is a stark difference (P≤0.05) between Mn concentrations and treatments before planting and after harvesting (Figure 4). The results showed that Mn concentration was high due to the concentration of sewage sludge. However, the Mn concentration decreased after harvesting. The ranged of Mn concentration in growth medium with *Ricinus communis* was between 1.10 mg/kg (T_1) and 9.73 mg/kg (T_5). Parisa et al. (2010) stated that Mn concentration in the soil and plant tissue increased after applying the sewage sludge in the growth medium. In these experiments, Ricinus communis contained high Mn concentration in growth medium with a value of 9.73 mg/kg (T_5). High concentration of Mn in the soil restricted germination of plants as well as reduced root and shoot growth. According to Schulte and Kelling (1972), the availability of Mn was influenced by soil pH, organic matter content, moisture and soil aeration. Soil pH increased when Mn concentration in growth medium is increase due to the high content of sewage sludge. Soil pH affects

plants' heavy metal uptake. The minimum soil pH in *Ricinus communis* was found in T_0 (the control), with a value of 5.26, while the maximum soil pH in *Ricinus communis* was found in T_5, with a value 6.36. Commonly, acidic soils are likely to contain high Mn and cause toxicity to the plants. However, this sewage sludge contains high calcium due to lime treated sludge. There are many symptoms of Mn toxicities to the plants such as distorted leaves, dark specks on leaves and leaf tissue dying at the leaf margins.

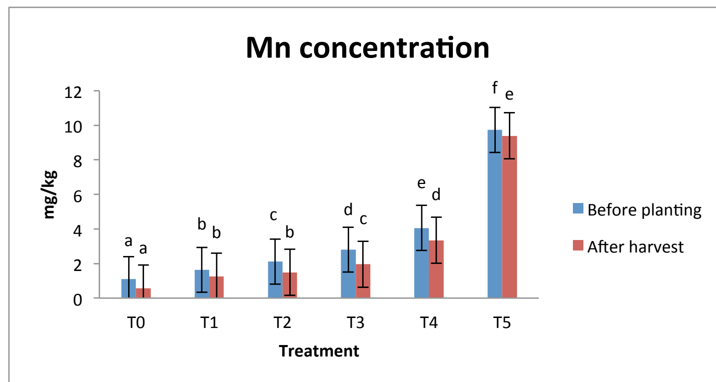

Figure 4. Concentrations of Mn in growth medium of *Ricinus communis* before planting and after harvesting. Different letters indicate difference between means at each treatment before planting and after harvesting. T_0 = Control, T_1 = 80% soil + 20% sewage sludge, T_2 = 60% soil + 40% sewage sludge. T_3 = 40% soil + 60% sewage sludge, T_4 = 20% soil + 80% sewage sludge, T_5 = 100%

Bioconcentration Factor (BCF) and Translocation Factor (TF) in *Ricinus communis*

Based on Table 1, the values of Mn BCF's was below 1 (0.02 to 0.07), while the TF values were above 1 (1.93 to 4.16). The results showed that *Ricinus communis* could be a good phytoextractor because its TF value was above 1 while BCF was low.

Table 2
Bioconcentration factor and translocation factor of Mn in different plant species

Species	BCF						TF					
	T_0	T_1	T_2	T_3	T_4	T_5	T_0	T_1	T_2	T_3	T_4	T_5
Ricinus communis	0.04^b	0.04^b	0.07^c	0.06^c	0.04^b	0.02^a	1.93^a	3.62^d	2.83^b	3.10^c	2.72^b	4.16^e

BCF = Metal concentration ratio of plant roots to soil and TF = Metal concentration ratio of plant shoot to roots

CONCLUSIONS

The present study confirmed that the *Ricinus communis* are suitable as phytoremediators to treat soil contaminated with sewage sludge with a high concentration of Mn. The plant was able absorb heavy metals and stored them in the stem based on the results that showed that the TF value was higher compared than BCF. However, the duration of the study needs to be extended. These experiments need to be conducted in the field in order to verify if this species can be used as a good accumulator for heavy metals in large areas.

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Spectroscopic Analysis of Epoxidised Jatropha Oil (EJO) and Acrylated Epoxidised Jatropha Oil (AEJO)

Wong, J. L.[1]*, Aung, M. M.[1,2], Lim, H. N.[2] and Md. Jamil, Siti Nurul Ain[2]

[1]*Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

[2]*Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

ABSTRACT

The non-edible seed oil of the Jatropha plant is a renewable and sustainable material to produce vegetable oil-based epoxy and epoxy acrylate as raw polymeric material. The objective of this study is to synthesis Jatropha seed oil-based epoxy and acrylate epoxy resins through conventional methods. An epoxy ring of Epoxidised Jatropha Oil (EJO) was formed through an *in-situ* epoxidation process using hydrogen peroxide and formic acid as an oxygen donor and oxygen carrier respectively. Acrylated Epoxidised Jatropha Oil (AEJO) was produced by reacting EJO with acrylic acid with the existence of triethylamine and 4-methoxyphenol as a catalyst and inhibitor respectively. The measured oxirane-oxygen content (OOC) of EJO was 4.99%. The acid value of the AEJO was determined at 4.42 mg KOH/g. Both the EJO and AEJO were characterised by FTIR and ^{1}H NMR spectroscopies.

Keywords: Acid value, acrylated epoxidised jatropha oil, epoxidised jatropha oil, FTIR, NMR, oxirane-oxygen content

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E-mail addresses:
wjialig829@gmail.com (Wong, J. L.),
minmin_aung@upm.edu.my (Aung, M. M.),
hongngee@upm.edu.my (Lim, H. N.),
ctnurulain@upm.edu.my (Md. Jamil, Siti Nurul Ain)
* Corresponding author

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INTRODUCTION

Natural vegetable oil consists of 93-98 wt.% triglyceride and minor components of diglycerides, monoglycerides and phosphoglycerides (Sharmin,et al., 2015). Triglycerides can be defined as ester where three molecules of one or more different types of fatty acids are linked to the alcohol glycerol. Fatty acids are normally made up of 14 to 18 carbon atoms linearly. It

consists of saturated, monounsaturated and polyunsaturated fatty acids. Belgacem and Gandini (2011, p. 52) found that some of the fatty acids consist of hydroxyl and epoxy groups such as ricinoleic and vernolic acids respectively, and the presence of these functional groups is more reactive but it is rare in naturally produced oil. Therefore, active sites of vegetable oil such as double bonds, allylic carbons, oxiranes and ester groups can be chemically and enzymatically transformed into functionalised polymeric material to increase the reactivity of vegetable oil (Xia & Larock, 2010).

Jatropha Oil (JO) can be used in polymer material synthesis compared with edible oils such as palm oils, soybean oils and sunflower oils because it is abundant and classified as non-edible oil due to the toxic phorbol ester in the seed (Hazmi,et al., 2013; Islam, et al., 2014). *Jatropha curcas* is a flowering plant of family Euphorbiaceae (Martinez-Herrera, et al., 2006; Kumar & Sharma, 2008), genus Jatropha and this family contains 175 of the known species (Singh et al., 2008; Karaj & Müller, 2010). It originates from Central America, Africa, South and South East Asia (Martinez-Herrera et al., 2006; Vyas & Singh, 2007; Singh et al., 2008; Zippel, et al., 2010) and can be grown in tropical and sub-tropical zones. Its seed is blackish, thin-shelled and oblong in shape (Kabir, et al., 2009). Jatropha seed is a good source of oil where the decorticated Jatropha seed consists of 40-60% oil (Makkar,et al., 1998; Openshaw, 2000; Sharma, et al., 1994), 75% unsaturated fatty acid, mainly oleic acid (18:1) and linoleic acid (18:2) at percentages of 42.8% and 32.8% respectively (Akbar, et al., 2009) and thus it is known as an oleic-linoleic oil.

Highly unsaturated non-edible JO is suitable to be transformed from its less reactive double bond into various Jatropha-based derivatives which are more reactive than virgin oil. Due to the low iodine number and high cetane concentration of mature Jatropha seed oil, much research had been focused on the potential of Jatropha seed oil as a renewable energy resource for biodiesel (Openshaw, 2000; Contran et al., 2013; Sinha, et al., 2015). The performance of trans-esterified Jatropha seed oil by using methanol in the presence of an alkali catalyst was achieved and which meets the standard requirements of American and European countries (Gübitz, et al., 1999) and is within the limits of the American Society for Testing and Materials (ASTM) specification for diesel, D 6751 (Kywe & Oo, 2009). In addition, the high content of unsaturated fatty acids makes Jatropha-based polyurethane adhesives exhibit better shear strength and good resistance towards water, acid and alkali solvents compared with palm oil based adhesives (Aung, et al., 2014). The transformed JO has been projected as a potent base stock for bio-lubricants at high temperature due to its high flash point property (Sammaiah, et al., 2014).

Epoxidised vegetable oil acts as a vital raw material in the synthesis of polymer derivatives such as acrylates, polyols, polyesters, polyamides and other derivatives via the ring opening process.

Acrylate epoxy resin is usually applied in UV-cured coatings due to its high reactivity of acrylate unsaturated functional groups. Acrylate epoxy is known to be applied in graphic arts, automotive coatings, and floor coatings (Ronda, et al., 2013). In the past, acrylate epoxy resin was produced commercially with petroleum as the raw materials. Utilisation of petroleum-based products over the long term has raised environmental and health hazard concerns, not to mention the depletion of petroleum resources which has attracted the attention of many researchers in the utilisation of plant oil derived polymeric materials (Alam, et al., 2014). For instance, the use of palm oil (Cheong, et al, 2009), soybean oil (Habib & Bajpai, 2011) and tung oil (Thanamongkollit, et al., 2012) in several applications such as adhesives, lubricants, biodiesels, coatings etc. has been studied in recent years. The objective of this study is to synthesis Jatropha-based acrylate epoxy resin by reacting Epoxidised Jatropha Oil (EJO) with acrylic acid in the presence of 4-methoxyphenol and triethylamine.

MATERIALS AND METHOD

Materials

Jatropha oil with a composition of oleic acid (41.3%), linoleic acid (34.4%), palmitic acid (12.8%), stearic acid (7.3%) and unsaturated fatty acid (2.7%) was obtained from Bionas Malaysia Sdn. Bhd. Hydrogen peroxide (30%), sulphuric acid (95%) and hydrated magnesium sulphate (99.5%) were purchased from Merck, Germany. Triethylamine was supplied by Fluka, Spain. Formic acid (98%), acrylic acid (99.5%) and 4-methoxyphenol (99 %) were obtained from Across Organic, New York.

Epoxidation of Jatropha Oil

The *in-situ* epoxidation of JO was carried out in a three-necked round bottom flask fitted with a mechanical stirrer, a thermometer and a dropping funnel. The JO (100 g) was charged into the flask followed by formic acid (11.66 g) and the flask was continuously stirred in a water bath at 45-55°C. The molar ratio of the double bond of oil to formic acid and hydrogen peroxide was 1: 0.6: 1.7. A corresponding quantity of hydrogen peroxide (73.25 g) was added into the reaction mixture at such a rate as to reach a dropping time of 30 minutes. The dropping rate of hydrogen peroxide should be slow enough to prevent a sudden increase in reaction temperature. Precaution was taken to avoid overheating due to exothermic reaction. After that, the mixture was heated to 60°C and further stirred for 4 hours. The cooled EJO was washed three times with distilled water in order to remove the remaining acid. The aqueous layer was removed and a little bit of anhydrous $MgSO_4$ was added and then left overnight.

Acrylation of Epoxidised Jatropha Oil

Previously prepared EJO (100 g) was charged in a three-necked flask equipped with a mechanical stirrer, a thermometer and a dropping funnel. The flask was placed in a silicone oil-bath at room temperature and stirring at 500 rpm. After that, triethylamine

(2 wt.% of EJO and acrylic acid) and 4 methoxyphenol (1wt.% of EJO and acrylic acid) were added to the mixture at 110°C and stirred for 15 minutes. Under continuous stirring, a calculated amount of acrylic acid (3.18 g) was added drop by drop to the mixture for about 30 minutes. The molar ratio of EJO to acrylic acid (EJO: CH_2=CHCOOH) was 1:1. After the addition of acrylic acid, the reaction was stopped until a constant acid value (less than 5 mg KOH/g) was achieved. Cooled Acrylated Epoxidised Jatropha Oil (AEJO) was washed three times with distilled water in order to remove the remaining acid. The aqueous layer was removed and the AEJO resin underwent rotary evaporation to remove excessive acrylic acid until a clear and viscous product was obtained. A little bit of anhydrous $MgSO_4$ was added and then left overnight. The reactions of epoxidation and acrylation are shown in Figure 1.

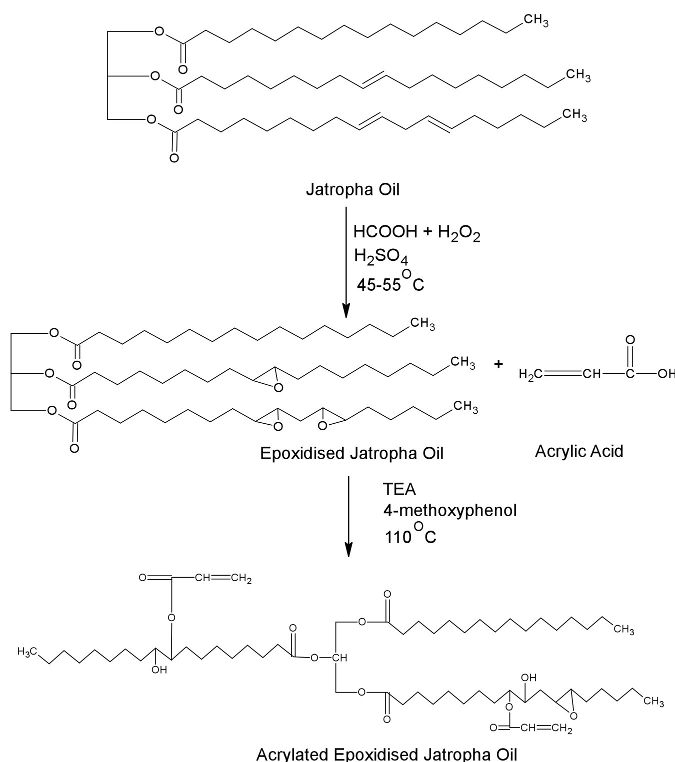

Figure 1. Epoxidation of JO and acrylation of EJO reactions

Fourier Transform-Infrared Spectroscopy (FT-IR) Analysis

The Fourier Transform-Infrared Spectroscopic (FT-IR) spectra of the received JO, EJO and AEJO were determined by means of a Perkin Elmer Model 1650 FTIR spectrometer using KBr pellets.

[1]H Nuclear Magnetic Resonance ([1]H NMR) Analysis

The received JO, EJO and AEJO were sent for [1]H NMR analysis by utilising the model Varian NMR System.[1]H Nuclear Magnetic Resonance ([1]H NMR) spectra were obtained with a Varian Unity INOVA Spectrometer. Chemical shifts (δ) were recorded in ppm relative to tetramethylsilane (TMS). Deuterated chloroform ($CDCl_3$) was used as a solvent.

RESULTS AND DISCUSSION

The composition of unsaturated fatty acid in JO and other plant oils is shown in Table 1. The total unsaturated fatty acid of JO is higher than the commercial palm oil at 78.9%. Its high composition of oleic acid (43.4%) and linoleic acid (34.4%) are comparable to sunflower oil. JO is known as an oleic-linoleic oil.

Table 1
Unsaturated fatty acids of plant oils

Fatty Acid	Percentage (%)			
	Jatropha Oil	Palm Oil	Soybean Oil	Sunflower Oil
Palmitoleic (C16/1)	1.4	-	0.1	0.8
Oleic (C18/1)	43.1	43.4	23.4	20.6
Linoleic (C18/2)	34.4	13.2	53.2	66.2
Linolenic (C18/3)	-	-	7.8	0.8
Total Unsaturated	78.9	56.6	84.5	88.4

Adapted from (Meyer et al., 2008)

One of the important properties in the characterisation of epoxy vegetable oil is the determination of the oxirane-oxygen content (OOC), in order to ensure the epoxy groups are present in the products. Epoxy resins with a high OOC are desired in the production of polymer. EJO was synthesised through *in-situ* reaction. Peroxyformic acid was formed by formic acid accepting oxygen atoms from an oxygen donor, hydrogen peroxide as shown in Figure 2. Unstable peroxyformic acid then attacked the double bonds of the JO, causing the ring formation, and resulting in EJO as shown in Figure 3. An OOC test was conducted by the direct method with hydrobromic acid (HBr) solution in acetic acid according to the ASTM D1652-97 Test Method A Standard to form the following expression:

$$\text{OOC Percentage} = (V-B) \times N \times 1.60 / W \quad [1]$$

where V is the volume of HBr solution used for titration of the sample in ml, B is the volume of HBr solution used for titration of the blank in ml, N is the normality of HBr solution in acetic acid, and W is the weight of sample (in gram).

Figure 2. Formation of peroxyformic acid

Figure 3. Formation of epoxy ring

The OOC was determined every half an hour and the reaction was stopped when the OOC dropped after achieving a maximum value. The epoxy content of the synthesised EJO was 4.99% which is higher than the EJO that was synthesised from chemo-enzymatic treatment with an OOC percentage of 4.60% (Togi, 2010). According to the recorded OOC values, the reaction achieved the highest OOC value within 4h as shown in Figure 4. Along with the reaction, the oxirane content was increased due to the formation of epoxy rings (Hazmi et al., 2013).

Figure 4. A graph of OOC percentage versus reaction time

In the acrylation process, triethylamine and 4-methoxyphenol acted as catalyst and gel inhibitor respectively. The lone pair electrons on the nitrogen atom of triethylamine will attack the unstable epoxy group and thus a complex intermediate will be formed. The nucleophile of acrylic acid substituted triethylamine and formed a stable ester bond through a nucleophilic substitution reaction. As a result, AEJO was produced. Figure 5 shows the mechanism of the acrylation reaction. An acid value (AV) was measured along with the reaction to indicate the mass of potassium hydroxide (KOH) in milligrams that was required to neutralise one gram of product. An AV test was carried out according to the ASTM D4662-98 Test Method A Standard by measuring the acidic constituents in polyols and other materials of high acidity

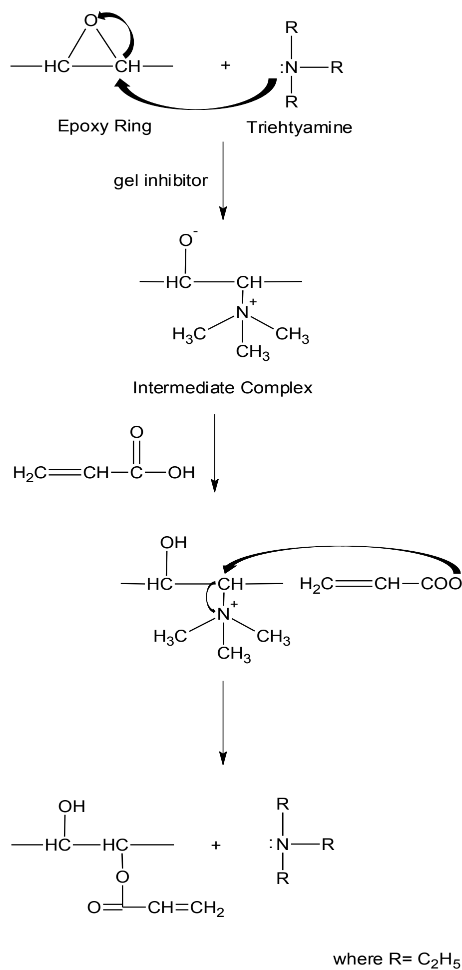

Figure 5. Mechanism of acrylation reaction

that are soluble in mixtures of toluene and ethyl alcohol. The AV was calculated by the following expression:

$$AV = (V-B) \times N \times 56.1/ W \quad [2]$$

where V is the volume of KOH solution used for titration of the sample in ml, B is the volume of KOH used for titration of the blank in ml, N is the normality of KOH solution in ethanol, and W is the weight of sample used in gram.

The rate of addition of acrylic acid and the temperature of the reaction must not exceed 110°C in order to prevent the formation of gel during the reaction. The rate of stirring should be increased when the viscosity of the mixture increases. The acid value of the synthesised AEJO was 4.42 mg KOH/g. Table 2 shows the OOC percentage and AV of the samples. The AV decreased over time for each sample due to the process of acrylation.

Table 2
OOC percentage and acid value of samples

Sample	Oxirane-Oxygen Content Percentage (%)	Acid Value (mg KOH/g)
EJO	4.99	23.86
AEJO	1.56	4.42

Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Fourier Transform Infrared (FTIR) spectroscopy is a fast, non-destructive technique that is used widely in the characterisation of lipids because of the absorption bands of the functional groups within the lipids at the infrared region in the electromagnetic spectrum. The FTIR spectra of JO, EJO and AEJO are shown in Figure 6. The spectrum of JO showed a CH stretching of saturated fatty acid backbone at 2929 cm^{-1} and 2858 cm^{-1} which represent a stretching band of terminal methyl group (CH_3) and methylene proton

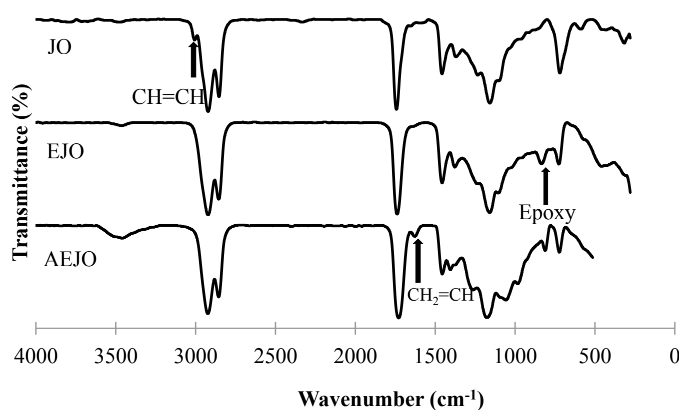

Figure 6. FTIR spectra of received JO, EJO and AEJO

(CH_2) of triglyceride molecule respectively. The peak around 1749 cm^{-1} is attributed to the carbonyl group (C=O) of triglyceride backbone. A significant functional group of unsaturated fatty acid was displayed at 3006 cm^{-1} which indicated the vinyl proton of unsaturation double bond (CH=CH). For EJO FTIR analysis, the alkene double bond of JO at 3006 cm^{-1} disappeared due to the formation of epoxy rings. This is supported by many researches done on epoxidation of vegetables oil (Adhvaryu & Erhan, 2002; Dinda, et al., 2008; Hazmi et al., 2013). A C-O-C oxirane group was observed at 830-850 cm^{-1} and the small intensity at 830 cm^{-1} represented the most significant peak indicating the presence of the oxirane group. These findings are consistent with the study by (Habib & Bajpai, 2011). In the FTIR spectrum of the AEJO, the diminishing small peak at 830 cm^{-1} represented the open ring reaction and indicated that not all epoxy groups have been consumed during the acrylation reaction. The OH bond showed a broad peak at 3476 cm^{-1} and a CH_2=CH stretch at an intensity of 1637 cm^{-1} indicated the formation of AEJO. The results are similar with those of (Rengasamy & Mannari, 2013). Table 3 represents the FTIR absorption peaks of JO, EJO and AEJO.

Table 3
FTIR absorption peaks of JO, EJO and AEJO

Functional Group	Absorption Peak (cm^{-1})		
	JO	EJO	AEJO
CH=CH	3006	-	-
CH stretch	2929, 2858	2923, 2857	2925, 2858
C=O	1749	1738	1737
CH scissoring band	1465	1453	1453
CH symmetric band	1381	1373	1371
C-C-O	1174	1163	1160
C-O-C oxirane group	-	830-850	830
OH (strong)	-	3473	3473
CH_2=CH	-	-	1636

^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy Analysis

^{1}H NMR spectroscopy was utilised to verity the structures of the received JO, EJO and AEJO. The spectra of the samples are shown in Figure 7. In ^{1}H NMR spectrum of JO, a triplet peak at chemical shift, δ of 0.80-0.88 ppm is represented by the terminal methyl protons ($-CH_3$) of fatty acid. Chemical shift at 1.19- 1.25 ppm, 1.23-1.26 ppm, 2.21-2.28 ppm and 1.53-1.69 ppm are revealed as the methylene protons adjacent to terminal proton ($-CH_2CH_2CH_3$), methylene protons in saturated fatty acid chain ($-CH_2CH_2CH_2-$), methylene protons adjacent to carbonyl

group (α,β-proton) (-OC=OCH$_2$CH$_2$-) respectively. Methylene protons of unsaturated double bonds (-CH=CH-) were assigned to the δ at 5.24-5.32 ppm and the δ at 5.19-5.24 ppm and 4.06-4.29 ppm represented the protons in the glyceride backbone of JO (CH(CH$_2$~)$_2$OCO~ and CH$_2$(CHOCO~)OCO~). For the ^{1}H NMR spectrum of EJO, the disappearance or diminishing of unsaturation at 5.24-5.32 ppm and the presence of epoxide group with a δ at 3.06-3.07 ppm is determined. This confirmed the formation of epoxy ring in EJO. This finding is similar with those of study worked on the epoxidation of soybean oil where a new peak was found at 3.0 ppm and diminishing of peaks at 5.2 ppm (Habib & Bajpai, 2011). A small signal at chemical shift of 1.71-1.99 ppm is revealed as the methylene proton adjacent

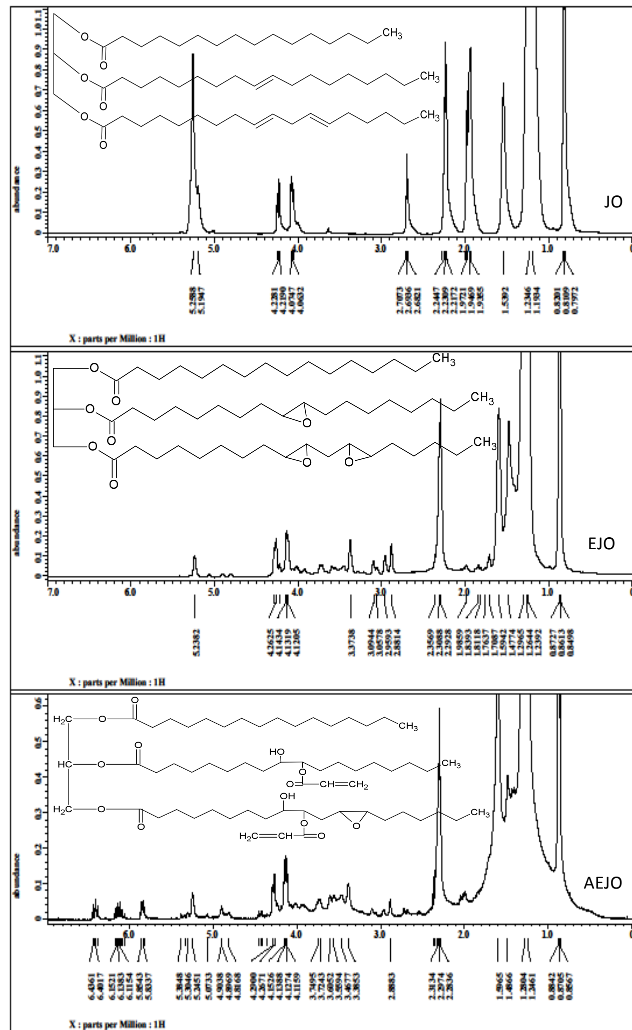

Figure 7. ^{1}H NMR spectra of received JO, EJO and AEJO

to epoxy group (Epoxy-CH_2-) whereas 2.88-2.96 ppm is represented as the methylene proton adjacent to two epoxy groups (Epoxy-CH_2-Epoxy). On the other hand, the formation of an acrylate group in the AEJO was proved by the new peaks at 5.83-6.43 ppm assigned to protons of acrylate ester (-OCO-CH=CH_2). The formation of hydroxyl proton (-OH) due to the opening of epoxy ring is attributed to chemical shift of 2.88 ppm, similar to findings of earlier studies (Téllez, et al., 2009). Methylene proton adjacent to hydroxyl proton (-CH-OH) appears at 3.37-3.61 ppm which is a more de-shielded region compared with methylene proton which is adjacent to the epoxy group. This is because these methylene protons are adjacent to oxygen atom of hydroxyl group which experiences stronger anisotropy effect compared with methylene proton adjacent to the epoxy group (Salih et al., 2015).

CONCLUSION

The AEJO with an acid value of 4.42 mg KOH/g was successfully synthesised from JO through two significant reactions, epoxidation and acrylation. From ^{1}H NMR spectra, the disappearance of methylene proton in unsaturated double bonds of JO at chemical shift of 5.24-5.32 ppm and the presence of epoxide group at 2.7-2.9 ppm confirmed the conversion of EJO from JO. On the other hand, the formation of an acrylate group and hydroxyl group at 5.83-6.43 ppm and 2.88 ppm respectively represent epoxy ring opening and grafting of acrylate groups.

RECOMMENDATION

The green acrylate epoxy resin can be used in the coating and adhesive industry which is able to reduce environmental impacts. The application of AEJO as anticorrosion coating could form the basis of further study in order to increase the corrosion performance of the coating on a mild steel substrate.

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Characterisation of Pulp and Paper Manufactured from Oil Palm Empty Fruit Bunches and Kenaf Fibres

Rafidah, D.[1], Ainun, Z. M. A.[1]*, Hazwani, H. A.[3], Rushdan, I.[2], Luqman, C. A.[1], Sharmiza, A.[2], Paridah, M. T.[1] and Jalaluddin, H.[3]

[1]Programme of Pulp and Paper and Pollution Control, Laboratory of Biopolymer and Derivatives, Institute of Tropical & Forest Products (INTROP), Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[2]Pulp and Paper Programme, Forest Products Division, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia

[3]Malaysian Timber Industry Board, Level 13-17, Menara PGRM, No.8, Jalan Pudu Ulu, 56100 Cheras, Kuala Lumpur, Malaysia

ABSTRACT

In papermaking, blending or mixture of fibres is one of the ways to enhance mechanical properties of paper. The objective of this study was to evaluate the properties of paper manufactured from mixture of oil palm empty fruit bunch (EFB) and kenaf fibres. The papers were prepared according to 10, 30, 50 and 70 percentages of kenaf whole stem blended into oil palm empty fruit bunch fibres. The preparation and testing of papers were carried out based on TAPPI Test Methods. Results showed that using kenaf whole stem fibres improved the mechanical properties of the blended papers and complied with the standard requirement for writing and printing grade paper.

Keywords: Kenaf whole stem, papermaking, paper properties, pulp properties, oil palm empty fruit bunch

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E-mail addresses:
epot77@yahoo.com (Rafidah, D.),
ainunzuriyati@upm.edu.my (Ainun, Z. M. A.),
husnabdullah@gmail.com (Hazwani, H. A.),
rushdan@frim.gov.my (Rushdan, I.),
chuah@upm.edu.my (Luqman, C. A.),
sharmiza@frim.gov.my (Sharmiza, A.),
parida.introp@gmail.com (Paridah, M. T.),
dr.jalaluddin@mtib.gov.my (Jalaluddin, H.)
* Corresponding author

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INTRODUCTION

Malaysia is the largest producer for oil palm which accounts for 67% (4.85 million ha) of its plantation area. In 2011, the land under oil palm cultivation reached 5 million hectares. The expansion of palm oil industry worldwide led to an increase in the amount oil palm biomass residues. One of the residues is empty fruit bunches which accounted for about 95.3 million

tonnes of dry lignocellulosic biomass in 2009 (Basiron, & Simeh, 2005; Wan Rosli & Law, 2011). The enormous amount of lignocellulosic biomass includes fresh fruit bunches which can be transformed into many resources such as pulp, paper, paper board and other composites (Faizi et al., 2017). It was reported that in 2010 up to 7 million tonnes (dry weight) empty fruit bunches (EFB) were generated in Malaysia. Every 5 tonnes of EFB could produce up to a tonne of pulp (Shuit, et al., 2009). Earlier works proved that EFB is a suitable raw material for pulp and paper production (Rushdan, 2003). Availability in large quantity, continuous supply, low lignin content, high cellulose content and high strength placed EFB has a potentially high commercial value as a non-wood fibre to substitute recycled fibre. The products may be produced from the fibre alone or may be mixed with other Fibres. A study showed that blending EFB with recycled pulps enhanced the structural, mechanical and optical properties of paper (Rushdan, 2003). Recycled paper is enhanced by adding EFB pulps (Wan Rosli, et al., 2005).

There is a great potential for kenaf to be used as a national commodity by the paper industry. Kenaf is an herbaceous plant which can be harvested after 4-5 months of planting, allowing two cycles of harvest in a year (Kaldor, et al., 1990). Kenaf is readily considered as an alternate fibre, derived from its stem (Kaldor, 1992; Mossello et al., 2010). The long fibres contribute to strength while the core provides better smoothness and formation. Thus, the quality of paper could be improved by using both fibres (Villar, et al., 2009). Soda-anthraquinone (NaOH-AQ pulping) was selected based on its better pulp quality (Akamatsu, et al., 1987; Law & Jiang, 2001; Rushdan, 2002) in which no sulphur was incorporated during the pulping process. Blending is a common practice in papermaking to attain certain physical and mechanical properties to meet the demand for high strength papers to be used for packaging, furniture, paperboard and even as building materials (Fagbemi et al., 2017).

In most applications, two types of fibres are used, hardwood or softwood blended with non-wood fibres. The interaction of oil palm EFB fibres with a hardwood kraft pulp of Acacia mangium had been studied (Wan Rosli, et al., 2012). They also investigated the effect of blending EFB Fibres with softwood pulps and found that the product had great potential as liners and medium. Rushdan, et al. (2007) studied the blending of EFB with old corrugated container (OCC) to produce medium paper. The findings showed that by mixing EFB Fibres to OCC the product was similar to commercial hardwood species, *Acacia mangium* and *Eucalyptus globulus*.

Due to the increasing demand for fibre, the sources of raw material for it has become important. The reality is that paper production is dependent on the availability of fibre. Substituting the lignocellulosic material can reduce the burden on forest while supporting the natural biodiversity and ameliorate its waste management problem. This research is anticipated to reduce the

dependency on wood fibres by taking into consideration the abundant raw materials in the form of EFB and by blending it with the fast-growth kenaf whole stem fibres. Therefore, the focus of this study is to determine the suitability of adding two types of non-wood fibres in papermaking. To date, no study has been carried out on blending of non-wood to make paper and specifically, on the use of kenaf and EFB in papermaking.

MATERIALS AND METHOD

Oil palm empty fruit bunch fibres (EFB) were obtained from Sabutek Sdn. Bhd.. The Fibres were packed in a bale where cleaning and cutting were done prior to pulping. The cut Fibres of about 2 cm length were kept in sealed bags. The EFB Fibres were pulped using soda-anthraquinone (NaOH-AQ pulping). Kenaf was acquired from National Kenaf and Tobacco Board (NKTB) and was harvested at the age of 4-5 months. Stalks without flowers and leaves were chipped at about 2 cm in diameter using chipper machine from Germany. These whole stem chips were screened at about 2 cm of width to allow better penetration of chemicals during pulping and air-dried until 10% of moisture content for storage. The kenaf whole stem was chosen due to their properties (physical and mechanical properties). Kraft pulping technique was used to cook the kenaf chips. Pulping conditions for both kenaf and oil palm EFB chips are summarised in Table 1.

Table 1
Pulping conditions for EFB and kenaf whole stem chips

Pulping condition	Kraft pulping	NaOH-AQ pulping
Type of fibres	300 g of kenaf chips	300 g of EFB fibres
Sulphidity	25.0%	27.3%
Active alkali	17%	-
AQ	-	0.1%
Fibre: liquor	1:7	1:8
Temperature during cooking	170°C	170°C
Time to maximum temperature	60 min	60 min
Time at maximum temperature	120 min	90 min

After pulping, the pulps were washed to eliminate the pulping chemicals and screened using fibre Somerville fractionator. Therefore, cleaned and unbleached pulps were obtained. The next process was using elemental chlorine-free bleaching to bleach both EFB and kenaf, namely D-Ep-D; D refers to chlorine dioxide and Ep is a peroxide-enhanced extraction. The bleaching condition is summarised as in Table 2. The amount of bleaching solutions was calculated based on oven-dried weight of pulp. These solutions were added with unbleached pulp in high temperature

resistant container. The container was immersed in hot water bath for certain duration to allow the reaction to occur. After treatment, the bleached pulp was washed using distilled water, dried using spin dryer and kept in an opaque container to protect against direct contact with light.

Table 2
Sequences and conditions used in ECF bleaching for both EFB and kenaf unbleached pulps

Parameters	Sequences		
	D1	Ep	D2
Chemical charge, %	2	E = 1.5, p = 1	1.25
Consistency, %	10	10	10
Treatment temperature, °C	70	70	70
Reaction time, min	180	90	90

Both kenaf and EFB bleached pulps were beaten according to TAPPI Standard T 248 sp-00. The beating degrees applied was 4000 revolutions. Before beating, the bleached pulp was disintegrated in a pulp disintegrator for 20 minutes. The pulp was made to 10% consistency for beating in the PFI mill machine for 4000 revolutions. Subsequent to beating, the pulp was re-disintegrated prior to blending. The bleached and beaten pulps were obtained, ready to be blended. The blending process involved proportion, based on pulp weights of kenaf to EFB at 10, 30, 50 and 70 percentages. These blended pulps were then measured their freeness according to TAPPI Standard T 227om-99 Canadian Standard Method.

Finally, papermaking was conducted based on TAPPI Standard T 205 sp-02 producing 60 g/m^2 piece of paper. These handsheets were conditioned at temperature of 23°C ± 1°C and 50% ± 2% relative humidity for 24 hours prior to any testing. Optical, physical and mechanical characteristics namely as opacity, smoothness, tensile and tear were tested based on TAPPI Standard T 425 om-01, T 479, T 494om-01 and T 414om-98. Besides, in order to understand the morphology of the produced pulp and handsheets, observation under Scanning Electron Microscope (SEM) was done.

RESULTS AND DISCUSSION

The screened yields for both soda and kraft pulping of EFB and kenaf were 41% and 40% respectively. Both EFB and kenaf was produced at about the same screened yields. These unbleached pulps were tested for their Kappa number. The Kappa number for unbleached EFB pulp was 5 while kenaf pulp was 15. The results for EFB and kenaf are sound due to the percentage concentration of soda used in the pulping processes. The lignin content for both EFB and kenaf are considered low which may ease the bleaching process. The chosen pulping condition seemed to contribute

to the elimination of lignin constituents. By using higher soda concentration of pulping condition, the Kappa number of produced EFB pulp was increased which is confirmed by Tay, et al. (2009). Udohitinah and Oluwadare (2011) found out that unbleached kenaf pulp prepared via kraft process exhibited Kappa number 12.04 to 20.50. After the D-Ep-D bleaching, the bleached pulps were tested and their Kappa number was 0 and 1.5 for EFB and kenaf bleached pulps respectively. The pulping condition was found to aid the bleaching process.

The Canadian Standard Freeness (CSF) is the rate of drainage via diluted pulp slurry. It is correlated to the efficiency of fibres in holding or releasing water either before or after beating (refining). In this study, a decision was made to beat both fibres at same level, 4000 revolutions. The freeness readings for both bleached and beaten fibres were 286.5 and 216.0 for EFB and kenaf respectively. Based on the blending of kenaf into EFB pulp slurry, the values of CSF dropped simultaneously as shown in Table 3. Such finding suggests that the fibres in the mixture held more and released less water which is most probably due to the presence of kenaf bast fibres. This finding is supported by the results of a past study (Villar et al., 2009). There was substantial difference between freeness value of kenaf bast and core. The kenaf bast fibres held more water compared with its core, which allowed less water to be released during the drainage test. Increasing percentage of kenaf fibres seemed not to exhibit extreme differences where the freeness values are at static range of 180 to 200.

Table 3
Canadian Standard Freeness, opacity and brightness values of kenaf, EFB and their blended Fibres (amount of kenaf pulps added into EFB pulp slurry).

Property	Kenaf, 100%	EFB, 100%	10%	30%	50%	70%
CSF, ml	216	287	206	204	181	206
Opacity, %	75.69	80.24	99.63	99.58	99.55	99.36
Smoothness, ml/mm	165.63	82.00	111.30	115.00	119.00	133.80

In terms of opacity and smoothness, the product is better compared with that of unblended paper as shown in Table 3. Both EFB and kenaf paper had 80.24% and 75.69% of opacity readings. The readings for smoothness are 82.00 ml/mm and 165.63 ml/mm for EFB and kenaf accordingly. The EFB paper is found to have better opacity which may be due to the arrangement of short fibres by leaving least voids between the fibre-to-fibre bonding. This arrangement will cause the surface of the paper to become smoother. These results are supported by the morphological observation of the EFB, kenaf and blended papers which can be seen in Figure 3. The EFB fibres are smaller in size compared with kenaf fibres and hence, it produces more compact paper. Higher

compactness provides better paper surface smoothness.

Tensile index is a measurement of resistance of paper to direct tension divide by paper grammage (Smook, 2000). It is observed that the blending has dropped the papers earlier property of tensile strength. Referring to Figure 1, the EFB paper had the highest tensile index value compared with that of blended samples. These results may be due to the presence of kenaf core fibres in the blended samples which expand the amount of short fibres. The effect of pulp blending influences the pulp blending which in turn depends on the quality of preliminary pulps (Fagbemi et al., 2017). Therefore, the addition of 70% of kenaf pulps has the ability to increase the tensile strength better than the existing EFB paper. It is believed that the use of kenaf bast would increase the strength performance as low as blending 10% kenaf into EFB mixture instead of applying kenaf whole stem. Earlier study (Latifah, et al., 2009) proved that the incorporation of only 10% kenaf bast pulps increased the tensile index of old corrugated container pulps. Kenaf pulp contributes tremendously to the improvement of paper strength which is a better choice than softwood (Fagbemi et al., 2017)

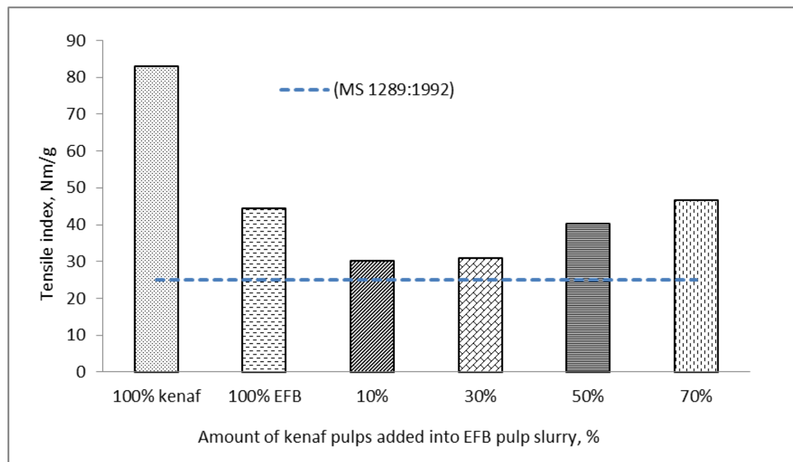

Figure 1. Tensile index versus percentage of kenaf pulps added into the EFB pulp slurry

The blending of kenaf whole stem fibres with EFB decreased the tear strength at earlier addition of 10% as shown in Figure 2. Later, the blending which began with 30% and more of kenaf whole stem fibres began to increase the tear strength until a drastic tear strength improvement of almost 70% increment. Tearing resistance is dependent on the amount of fibres in the sheet rupture, fibre length, fibre numbers, and strength of fibre-to-fibre bonds. The presence of kenaf fibres is believed to cause better fibre-to-fibre bonding. The presence of kenaf whole stem fibres produced better

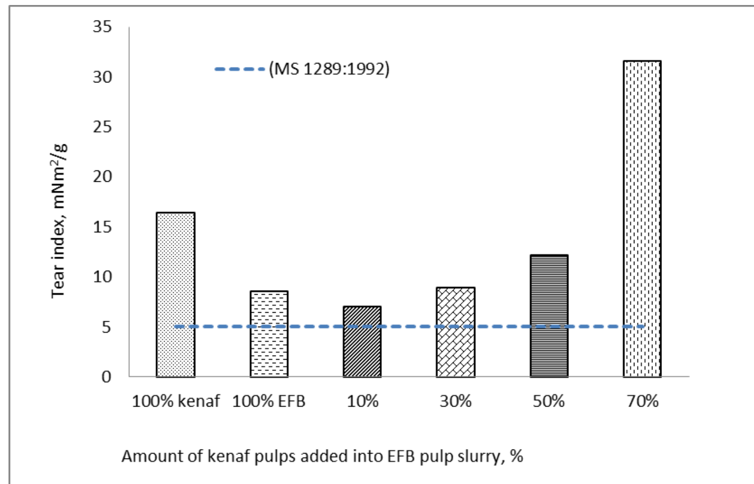

Figure 2. Tear index versus percentage of kenaf pulps added into the EFB pulp slurry

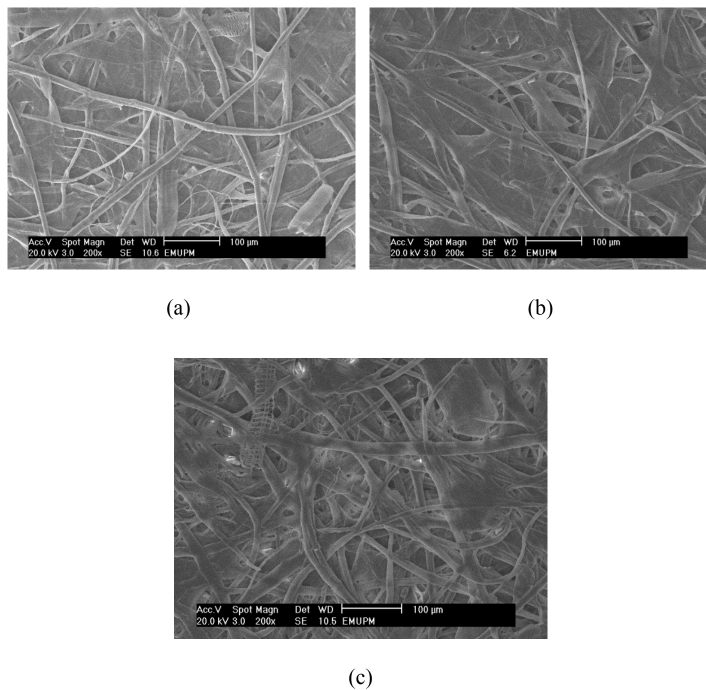

Figure 3. (a) Paper contains bleached EFB fibres; (b) Paper contains bleached kenaf fibres; (c) Paper contains blended fibres of EFB and kenaf whole stem

distribution which led to better formation of paper. Therefore, the tear index of the blended papers became more resistant. This finding is consistent with that of Gulsoy and Erenturk (2016) who used softwood fibres to enhance the strength of recycled fibres.

Results obtained from the tensile and tear index achieved higher than the minimum requirement for offset paper, which were 25 Nm/g and 5 mNm^2/g respectively. Incorporating other natural fibres may enhance mechanical properties of the paper such as its tensile strength (Sanjay, et al., 2016). The addition of at least 10% of kenaf whole stem is enough to obtain this properties which is confirmed by Latifah et al., (2009).

CONCLUSION

Results of this study showed the blending enhanced the properties of EFB paper by adding kenaf fibres. It is recommended that the blending of at least 10% kenaf whole stem fibres into EFB fibres is sufficient to fulfil the requirement for tensile, tear, opacity and smoothness paper properties as well.

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Properties of Paper Manufactured from Kenaf as Function of Alkaline pH Medium and Retention of Precipitated Calcium Carbonate

Ainun, Z. M. A.[1]*, Nurul, I. N.[1], Zaida, S.[1], Hazwani, H. A.[1] and Latifah J.[2]

[1]Program of Pulp and Paper and Pollution Control, Laboratory of Biopolymer and Derivatives, Institute of Tropical Forestry and Forest Products (INTROP), Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]Pulp and Paper Program, Forest Products Division, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia

ABSTRACT

The objective of this study was to have a better understanding of the effect of alkaline pH medium in the retention of filler in papermaking using kenaf bleached pulp. Three stages of experiments were carried out involving papermaking at alkaline pH medium 8 to 13, usage of precipitated calcium carbonate (PCC), Albacar (ABC) of needle-shaped and Albafil (ABF) of circular-shaped, and the application of low and high molecular weight of polyacrylamides (PAM LM and PAM HM). Paper samples were manufactured based on TAPPI Test Method T295 om-88. Characterisation of specimens in terms of filler content, tensile, tear and burst strength were carried out. The results showed that pH medium influenced the inter-fibre bonding of the fibres during papermaking whereby pH 8-9 is found as the best medium in producing stronger paper. The findings are significant in order to suit the pH according to certain shape and size of such fillers.

Keywords: Alkaline papermaking, kenaf, paper strength, precipitated calcium carbonate, retention

INTRODUCTION

Alkaline papermaking was initiated in Scotland using ground chalk to gain whiter products. This was also widely used in Europe since 1950s. In 1970s, due to the introduction of inexpensive filler namely ground calcium carbonate and improvements in alkaline sizing agents have increased the application of alkaline

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E-mail addresses:
ainunzuriyati@upm.edu.my (Ainun, Z. M. A.),
izzatinordin@gmail.com (Nurul, I. N.),
zaida_suod@yahoo.com (Zaida, S.),
husnabdullah@gmail.com (Hazwani, H. A.),
latifah@frim.gov.my (Latifah J.)
* Corresponding author

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papermaking (Gill & Scott, 1987). In 2002, about 70% of paper mills converted to alkaline papermaking due to availability of good alkaline sizing agents and precipitated calcium carbonate (PCC). The percentage rose where the conversion to alkaline papermaking reached 80% in North America and 85% in Western Europe (Chapnerkar, 2004). The most important advantage using alkaline medium in producing paper is alkaline papers could last for hundreds of years whereas acid paper disintegrates within 30 to 40 years. This is due to the use of calcium carbonate that neutralizes acid and prevents acid hydrolysis, which causes the disintegration of cellulose (Casey, 1981).

Since the 8th century, papermakers began substituting virgin pulp during papermaking with mineral fillers. There are many types of fillers such as clay, titanium dioxide, calcium carbonate, and PCC. Precipitated calcium carbonate is produced synthetically and used extensively in alkaline papermaking due to its contribution to paper brightness, opacity, maintaining bulk, sheet porosity and reducing fibre usage. It is also the most common mineral used in fine papers due to its high purity and light scattering (Kocman & Bruno, 1996). These benefit the papermakers in reducing manufacturing cost and improving printability (Kamiti & van de Ven, 1994). Filler decreases the energy demand in papermaking process because of the slighter amount of fibrous mass per unit weight of paper (Chauhan, et al., 2012). Precipitated calcium carbonate can be found at different crystalline forms such as rhombohedral (barrel-shaped), prismatic, spherical, symmetrical, scalenohedral rosette-shaped and aragonite needle-like (Passaretti, et al., 1993). Such modified fillers can be a good approach to improve function and reduce drawbacks (Shen, et al., 2009).

Another important additive in alkaline papermaking is the retention aid. The demand for retention aid increased due to the incorporation of fillers in alkaline papermaking. Polyacrylamide (PAM) is a well-known retention aid and is widely used in the papermaking industry. It can be applied as anion, cation and neutral charge polymer. Based on Reynolds & Wasser (1981) PAM is usually used as cationic polymer. Additionally, the molar mass of PAM is an important factor in obtaining effective retention aid of filler. Polyacrylamide also functions as a dry strength agent in papermaking. The advantage of using PAM is its effectiveness at a wide range of pH, good performance without alum and its suitability in many types of pulp either virgin pulp or secondary pulp. It can increase the strength of paper such as its tensile, burst, internal bonding and fold. It also provides better retention for fine and fillers, easier drying process and increase paper size.

Based on a previous study, the pH equilibrium of PCC is reported to be around 9.4 where no additional chemicals are needed to adjust the pH because the PCC buffers the system (Chapnekar, 2004). Therefore, a study was carried out to investigate the influence of alkaline pH medium in the retention and precipitation performance

of PCC on kenaf whole stem fibres and in order to reduce undesired precipitation of calcium carbonate. The selected range of alkaline pH medium is 8-13. According to Berger, et al. (2008) kaolins, a kind of fillers with different morphologies, help to influence and prognosticate the properties of supercalendered paper. Therefore, PCC of different morphologies were selected and tested for their ability to retain fibres as the controlled alkaline medium. Besides, the polymers of high and low molecular weight polyacrylamide were used in order to enhance mechanical strength characteristics of the paper.

MATERIALS AND METHODS

Raw Material

The whole stem of kenaf chips, 2 cm in diameter, was pulped and bleached. The unbleached kenaf pulp was obtained from kraft pulping with 17% alkali active and 25% sulphidity. Bleaching was carried out according to D_1EpD_2 sequences (D_1: bleaching using chlorine dioxide at first stage, Ep: extraction with NaOH, D_2: bleaching using chlorine dioxide at second stage). The whole stem was used throughout the experiment. Filler employed in the experiment was PCC of two types supplied by Specialty Minerals Incorporation, USA, labelled as Albacar (ABC) and Albafil (ABF). Both fillers were received in the dry state and are white in colour. Table 1 shows the properties of the fillers. Two cationic polyelectrolytes – a higher (HM) and lower (LM) molecular mass PAM were used as retention aids. The PAMs were obtained from Malaysian Adhesives Chemical Sdn. Bhd., Shah Alam, Selangor, Malaysia. Table 2 displays the properties of PAMs used for the experiments.

Table 1
The properties of two types of precipitated calcium carbonate used throughout the study

Typical properties of raw material	PCC ABC	PCC-ABF
Particle shape	rosette	symmetrical
Median particle size, µm (Sedigraph 5100)	1.3	0.7
Dry brightness (Hunter Y, Rd value)	98	98

Table 2
The properties of PAMs used throughout the study

Typical properties	Viscosity (poise)	Polimerisation rate (min)	Molecular weight (g/mol)
PAM LM	21.5	60	300,000
PAM HM	45.0	180	3,000,000

Manufacturing Paper Samples

The papermaking was carried out in three stages (a) the preparation of control paper at a range of pH medium from 8 to 13, labelled as P-8, P-10 and P-12, (b) the preparation of filled-paper at a range of pH medium from 8 to 13 with the addition of PCC and (c) the preparation of selected filled-papers with the addition of PAM. These samples were labelled as ABC-126, ABC-123, ABF-106, ABF-103, ABC-8, ABC-10, ABC-12, ABF-8, ABF-10 and ABF-12. In the second stages of experiment, the mixture of kenaf bleached fibres and PCC fillers were carried out at a ratio, 1:2. The polymer at 1% (g/g pulp) was added to the mixture and stirred in a British disintegrator, subjected to 3,000 rpm for ~25 mins. The amount of polymer was selected at 1% due to its best performance of filler loading and paper strength as reported in a previous study (Ainun, 2010). The mixture was adjusted to a required pH such as pH 8-9 or pH 10-11 or pH 12-13 throughout the papermaking process. The papermaking was carried out according to TAPPI T 295 om-88 producing basis weight of 60 g/m^2 paper. Table 3 shows the content of each sample.

Table 3
Content of paper produced in the experiment

Sample	pH medium	Type of PCC	Type of PAM
P-8	8-9	-	-
P-10	10-11	-	-
P-12	12-13	-	-
ABF-8	8-9	ABF	-
ABF-10	10-11	ABF	-
ABF-12	12-13	ABF	-
ABC-8	8-9	ABC	-
ABC-10	10-11	ABC	-
ABC-12	12-13	ABC	-
ABC-126	12-13	ABC	PAM HM
ABC-123	12-13	ABC	PAM LM
ABF-106	10-11	ABF	PAM HM
ABF-103	10-11	ABF	PAM LM

Morphological Observation of the PCC and Handsheets

A model of Leica Scanning Electron Microscope was used to observe the morphology of PCC ABC and ABF. The aggregates of PCC located on the surface and cross-cut of filled-papers were also observed. Prior to scanning, the PCC and papers were coated with gold for a sharper view of micrograph.

Testing of the Samples

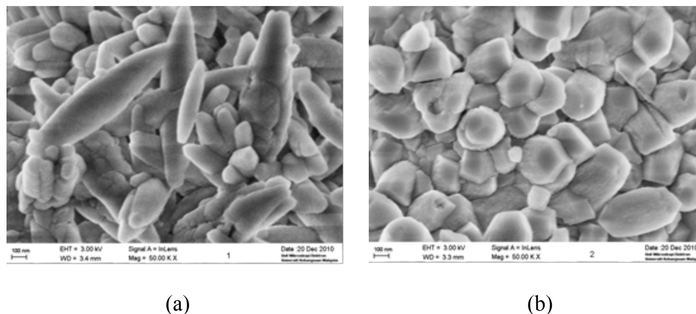
Filler content was determined by ashing the paper according to TAPPI Standard T413

but the ignition temperature is modified to 600°C. Ashing at 600°C is reported as the optimum temperature for isolating PCC in paper and at the same time protecting its' structure (Ferreira, et al., 2005). Tensile, tear and burst strength of the paper were measured according to TAPPI Test Methods T494, T414 and T403 om-02 for 'Tensile Properties of Paper and Paperboard', 'Internal Resistance of Paper' and 'Bursting Strength of Paper' respectively.

RESULTS AND DISCUSSION

Morphological Observation of Filler Particle Shape, Control Paper and Filled-paper

Micrographs of the PCCs are shown in Figure 1a and b. Both fillers are different in shape and size. The ABC is observed as rosette, needle-shaped while the ABF is symmetrical, circular-shaped. The diameter of PCC ABC is in the range of 100-600 nm with 450-900 nm length while the PCC-ABF has 300-500 nm of diameter. Micrographs of kenaf whole stem control-papers prepared at alkaline medium of pH 8-9 are shown in Figure 2. Figure 3 illustrates the micrographs of the kenaf whole stem filled-papers using PCC ABC and ABF. The selected samples were ABC-12 and ABF-10 which performed high level of filler content of 20.69% and 29.04% respectively (Figure 5). Morphologically, the paper surface displayed that the distribution of fillers is better in sample ABF-10 rather than in ABC-12. Almost all fibre surfaces were covered with the flocs of fillers. The thickness of filler deposition on the fibre surfaces and in between the fibre linkages can be seen in the micrographs. These results indicated that shape and size of fillers influence the efficiency of filler retention. Circular-shaped fillers are proven to be better in terms of retention compared with needle-shaped ones.



(a) (b)

Figure 1. SEM micrographs of (a) PCC ABC and (b) PCC-ABF

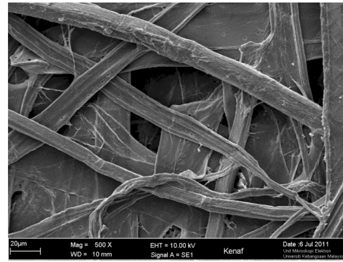
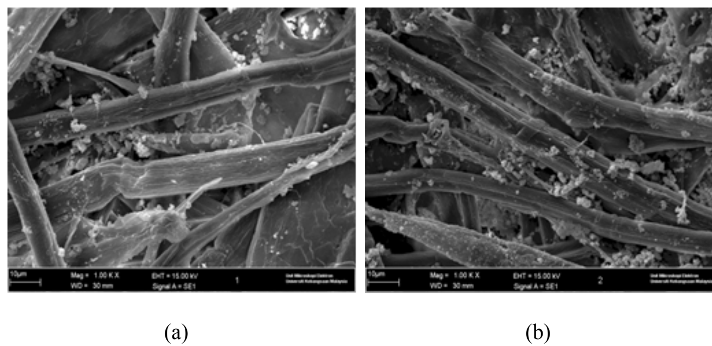


Figure 2. SEM micrograph of kenaf whole stem control papers prepared at alkaline medium of pH 8-9



(a) (b)

Figure 3. SEM micrographs of paper samples containing (a) PCC ABC and (b) PCC-ABF

The Properties of Control-paper Specimens Prepared at a Range of Alkaline Medium

Paper produced at pH 8-9 as shown in Figure 4 has better strength of tensile and burst indices compared with that produced at pH 10-11 and pH 12-13. It is believed that, inter fibre bonding is excellent at pH range of 8-9. The medium is believed to have achieved the stabilization level which means the electrostatic diffuse layer overlap forces is far from the isoelectric point (Borkovec & Papastavrou, 2008). The neutralisation produces optimum negative-positive attraction between fibres in the slurry during papermaking. However, tear strength for P-10 is higher than P-8 which correlates with fibre length and strength of fibres, and energy of fibre-to-fibre interactions (Przybysz, et al., 2016). The number of fibres in the paper sheet and surface area of fibre per unit mass of paper may be more in P-10 rather than in P-8. Chauhan et al. (2012) showed that less fibre in a paper sheet may decrease the tear strength of paper.

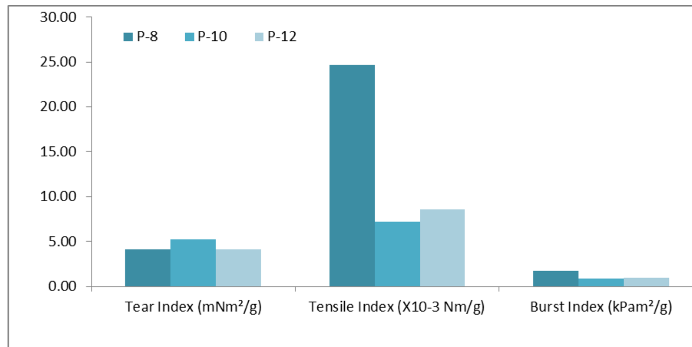


Figure 4. Mechanical properties of produced paper at pH 8-9, pH 10-11 and pH 12-13

The Properties of Filled-paper Prepared at a Range of Alkaline Medium with the Addition of PCC and PAM

The first objective of this experiment is to determine the influence of alkaline medium in the attachment of PCC. Six samples were prepared which involved ABC and ABF in making papers at 3 ranges of alkaline medium (see Table 4). Previously, the alkaline medium of pH 8-9 produced the best mechanical strength of control-paper. However, the addition of PCC ABC and ABF, has substantially changed the trend. The filler content for both series of paper containing ABC and ABF have shown the best retention at different alkaline medium, pH 12-13 and pH 10-11 respectively. These results can be seen from samples ABC-12 and ABF-10 having filler content of 20.69% and 29.04% correspondingly. In filler loading technique, 20-30% of filler content is categorised as having high filler content in paper. Due to the presence of high content of fillers, the tear, tensile and burst strength for these samples has dropped to the lowest among the series of ABCs and ABFs.

Table 4
Mechanical properties and filler content of produced paper using ABC and ABF at pH 8-9, pH 10-11 and pH 12-13

Samples	Tear Index (mNm²/g)	Tensile Index (X10-3 Nm/g)	Burst Index (kPam²/g)	Filler content (%)
ABC-8	4.15	12.10	1.054	15.58
ABC-10	3.62	12.60	0.615	14.81
ABC-12	3.27	7.50	0.972	20.69
ABF-8	4.44	13.90	1.105	12.55
ABF-10	4.49	10.20	0.543	29.04
ABF-12	4.75	10.60	0.599	19.00

The performance of ABF as filler retention was better compared with ABC. This may be due to its filler size and shape. The circular-shaped of filler is believed to floc and attach easier onto the fibre surfaces and in between fibre linkages. This is in contrast to needle-shaped of ABC which has higher surface area and less stable during the filler flocculation and filler-fibre attachment.

The second objective of this experiment was to determine the effect of adding PAM to improve the mechanical properties of filled-papers. There are four samples prepared using ABC and ABF at pH 12-13 and pH 10-11 accordingly, added with PAM LM and HM. The PAM is a bridging polymer which may act in enhancing filler content or paper strength. Regardless of the polymer involved, the results were not promising as shown in Figure 5. The expected results were not obtained which could be due to the congeniality among fillers-fibres-retention aids that affected the amount of filler in attaching the fibre surfaces. Ainun (2010) reported that the presence of retention aid disturbed and influenced the location and distribution of the magnetic fillers in their papermaking. As explained by Alince, et al. (2001), opposite charge of fibres (negative charges) and fillers (positive charges) produced electrostatic attractions which induced the PCC to attach on the fibres. The presence of such polymers may influence the electrostatic attraction as well.

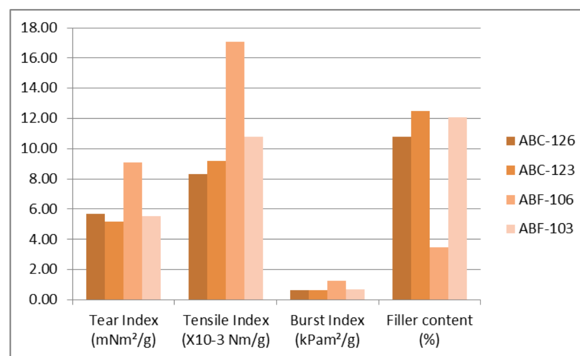


Figure 5. Mechanical properties and filler content of produced paper, using ABC and ABF at pH 12-13 and pH 10-11 respectively, with and without PAM

Based on Figure 5, samples which utilised lower molecular weight of PAM, ABC-123 and ABF-103 were found to load better than these of ABC-126 and ABF-106. No study was carried out at pH 8-9 because retention of both fillers was found better at solution of pH 10-11 and pH 12-13 as discussed earlier. This showed that PAM HM did not aid better filler loading. It is believed that the mutual attraction between PCC-ABC or ABF with the fibres is better with the presence of low molecular weight of PAM. This is confirmed by Alince (1988) who found that cationic polyethyleneimine

affected the mutual attraction between fibres and fillers (clays) particles, thus decreasing the amount of filler attachments in his papermaking. In addition, higher molecular weight of PAM tends to attract and form bigger and heavier flocs of fillers which then is subjected to filler dislocation and prone to be washed away (Ainun, 2010). Studies have shown the presence of such polymer in the suspension leads to higher flocculation (Gaudreault, et al., 2009). Therefore, the PCC are disabled to disperse properly onto the fibre surfaces. Ek, et al. (2009) also highlighted over saturation at fibre surfaces results in desorption process.

According to Alince (1988), the rate of polymer adsorption on filler is faster than on fibres. Appropriate usage of retention aid chemical may greatly influence the retention and drainage of the filler (Cadotte et al., 2007).

In this study, the addition of PAM LM is believed to be optimally adsorbed on the PCC which produces flocs of fillers and attaches onto the fibres. However, there is a decrease in value compared with samples without polymer addition which can be seen from ABC-12 and ABF-10 as illustrated in Figure 6. As explained earlier, a phenomenon that may occur during the pulp preparation is the forming of filler aggregation and flocculation after adding PAM. These flocs may become bigger and at certain level will be rejected from the papermaking system. The choice of retention aid for specific filler is crucial in papermaking (Chauhan, et al., 2012). Based on Gaudreault et al. (2009), higher molecular weight polymers can also function to pre-flocculate the filler into coarser aggregates to secure enhanced first-pass retention. However, the characteristics of the filler suspension, such as pH, temperature, consistency and ionic composition, can dramatically affect the efficiency of these additives (Pelton, Allen, & Nugent, 1980) and make them more difficult to use. It is generally accepted that fillers often form aggregates before sheet formation which could include fines (Li, et al., 2002). Furthermore, both deposition and filtration retention mechanisms predicted that filler retention is particle size dependent.

High filler content was achieved by sample ABC-123 and ABF-103 of 12.49% and 12.09% filler content respectively (see Figure 5). Due to the content of fillers, the strength properties decreased compared with those of ABC-126 and ABF-106. If comparison is made between sample ABC-12 and ABC-123, the percentage of filler decrement is 60.37%. The percentage of filler decrement between ABF-10 and ABF-103 was 41.63%.

CONCLUSION

Alkaline medium of pH is important to provide good inter-fibre bonding which leads to high mechanical strength of paper. The pH acts as a medium provider for the filler to respond whereby different filler acts differently in dissimilar pH medium.

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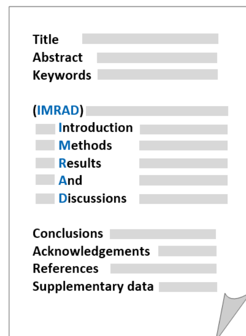

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Pertanika Editorial Office, Journal Division
Office of the Deputy Vice Chancellor (R&I)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

http://www.pertanika.upm.edu.my/
E-mail: executive_editor.pertanika@upm.my
Tel: +603 8947 1622 / 1619

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http://penerbit.upm.edu.my
E-mail : penerbit@putra.upm.edu.my
Tel : +603 8946 8855 / 8854
Fax : +603 8941 6172