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

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ABSTRACTING/INDEXING

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Foreword

Welcome to the **Third Issue 2015** of the Journal of Tropical Agricultural Science (JTAS)!

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

This issue contains **eight articles**, out of which **two** are short communications and **six** are regular research papers. The authors of these articles are from **Malaysia, the United Kingdom, Nigeria** and **Iran**.

The prevalence of mouse and rat parasites in resource recovery plants, farms and housing areas of southern Selangor is the subject of discussion in the first short communication (*Priscilla, D., Jambari, H. A.* and *Meenakshii, N.*). The second short communication discusses rice production and climate change as a case study of Malaysian rice (*Tiara Herman, Erik H. Murchie* and *Asgar Ali Warsi*).

The first research paper by researchers from University of Agriculture and Ahmadu Bello University Zaria, Nigeria reports on the effect of planting patterns and age at harvest of two cultivars of *Lablab purpureus* in *Andropogon gayanus* on agronomic characteristics and quality of grass/legume mixtures (*Amole, T. A., Oduguwa, B. O., Onifade, S. O., Jolaosho, A. O., Amodu, J. T.* and *Arigbede M. O.*). The second research paper discusses preservative effects of pineapple and cucumber juices on the viability of refrigerated spermatozoa of West African dwarf bucks (*Daramola, J. O.* and *Adekunle, E. O.*). The next research paper from Fasa University and Payame Noor University, Iran presents the combined effect of soil-applied iron and sulfur fertilisers on monoterpene content and antioxidant activity of *Satureja hortensis* L. extract (*Zahedifar, M.* and *Najafian, S. H.*).

In the next research paper, a group of researchers from Universiti Putra Malaysia, UPM describes the extract potential from the nut of *Areca catechu* to control mango anthracnose (*Aizad Izha Ahmad Rusdan, Jugah Kadir, Mahmud Tengku Muda Mohammed* and *Gwendoline Ee Cheng Lian*), and also from UPM, another group reports on the application of multivariate analysis for detection of crude palm oil adulteration through fatty acid composition and triacylglycerol profile (*Anand Kumar Inthiram, Hamed Mirhosseini, Chin Ping Tan, Rosfarizan Mohamad* and *Oi Ming Lai*). The last research paper in this issue discusses the functional properties of resistant starch type-III from *Metroxylon sagu* as affected by processing conditions (*Zi-Ni, T., Rosma, A., Karim, A. A.* and *Liong, M. T.*).

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

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

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

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Short Communication

Prevalence of Mouse and Rat Parasites in Resource Recovery Plants, Farms and Housing Areas of Southern Selangor: Implication for Public Health

Priscilla, D.*, Jambari, H. A. and Meenakshii, N.

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ABSTRACT

Parasites of small mammals living in human areas cause a tremendous burden of vector borne disease. Small mammals infected with parasites can readily facilitate parasitic transmission to humans and other susceptible animal hosts. The objective of this study was to determine the presence of parasites in small mammals (rats and mice) from the Semenyih Recovery Plant (RESBS) and to compare this with parasites from a plantation farm, Ladang Pertanian Bersepadu, Universiti Putra Malaysia (LPB UPM), and a housing area, Sri Serdang housing area (SSHA), Selangor, Malaysia. The methods employed in this study was to trap small mammals in the resource recovery plant, the plantation farm and the housing area. All the captured small mammals were examined microscopically for the presence of endo and ectoparasites. The brine gravity floatation method was used to determine the presence of parasitic eggs in the feces of the small mammals. The staining method of Semichon's acetic carmine was employed to find the parasites infesting the internal organs of the mammals. Twenty-three small mammals were trapped alive in the study. *Rattus sabanus* (10) and *Suncus murinus* (2) were captured in the Semenyih resource recovery plant; *Rattus argentiventer* (7) in the farm area of Ladang Pertanian Bersepadu UPM and *Mus musculus* (1) and *Suncus murinus* (3) species in the Sri Serdang housing area. *R. sabanus, R. argentiventer* and *M. musculus* were found to be heavily infected with *Echinolaelaps echidinus* (mites). *Polyplax spinulosa* (lice) infected the *R. sabanus* species. The intestinal parasite, cestode *Hymenolepididae* tapeworm, was found to have invaded the small mammals

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from Semenyih, the resource recovery plant and the farm area of Ladang Pertanian Bersepadu UPM. The nematodes were also found in the small mammals from RESBS. The small mammals captured from RESBS were found to be heavily infected with both external and internal parasites including *E. echidinus*, *P. spinulosa*, cestode tapeworms and nematodes compared to the farm and housing areas. Therefore, the existence of small mammals carrying parasites found mainly in waste disposal areas needs to be addressed to prevent serious disease that can cause harm to human health.

Keywords: Parasites, *E. echidinus* (mites), *P. spinulosa* (lice), cestode tapeworms, small mammals, rats, mice

INTRODUCTION

Municipal waste management in Malaysia is managed by the Ministry of Housing and Local Government and with the involvement of the private sector. The quantity of waste is expected to increase to 31,000 tonnes by the year 2020. The Malaysian National Solid Waste Management Policy has aimed to assure the public that public health will not become a concern as a result; however, problems to health are often caused by small mammals carrying parasites that are found mainly in waste disposal areas (Manaf *et al.*, 2009).

The parasites invade small mammals as their hosts and serve as reservoirs for diseases that infect humans. The modes of transmission to host can occur through various parasitic life cycles such as spores, eggs, cysts and juveniles (Roberts & Janovy, 2006). The unhygienic conditions of *Rattus* spp living in an impoverished areas can allow for the transmission of parasites to other susceptible hosts including humans (Claveria *et al.*, 2005). Studies have shown that the ectoparasites (lice and mites) of rats that live on external surface or skin or attached to hair follicles (Roberts & Janovy, 2006) can be transmitted to humans and cause diseases (Hoopman & Baron, 2007; Beck & Folster-Holst, 2009).

Infestations with lice often lead to sub-clinical diseases. The infections may cause anemia, weight loss, dorsum alopecia and even pruritus and cutaneous lesions in severe cases (Hoppmann & Barron, 2007; Beck & Folster-Holst, 2009). The lice species found globally in domestic rats act as vectors to transmit murine hemobartonellosis bacterial disease (Durden, 2002). Mites that live in host bedding usually feed on the host's lachrymal secretions, blood or serous exudates from abraded skin during the night and cause discernible lesions. Moreover, the mite is the vector of *Hepatozoon muris* (blood-protozoan parasites) that infest rats. Small mammals are commonly infected by internal parasites such as cestode tapeworms. The endoparasites that live in the subcutaneous tissue, lymphatics, muscles, trachea, lungs, heart, esophagus, liver, spleen, kidneys, bladder, brain and other internal structures are transmissible to humans (Roberts & Janovy, 2006).

In this study, our intention was to investigate the presence of parasites in mice and rats from Semenyih Recovery Plant (RESBS) and to compare them with

the parasites of mice and rats from a farm, Ladang Pertanian Bersepadu, Universiti Putra Malaysia (LPB UPM) and Sri Serdang housing area (SSHA), Selangor, Malaysia. The researchers also postulated that there is a difference in parasite species in all three entrapment areas and their impact on human populations.

METHODOLOGY

Small mammal sampling was conducted at three locations i.e. (1) the resource recovery plant, Recycle Energy Sdn Bhd Semenyih (RESBS), (2) the plantation farm, Ladang Pertanian Bersepadu, Universiti Putra Malaysia (LPB UPM) and (3) the Sri Serdang housing area (SSHA), Selangor, Malaysia. The sampling was done from May 2007 to May 2008. The RESBS is a resource recovery centre or waste-to-energy plant that processes 1,000 metric tonnes of municipal solid waste per day from Kajang municipality and neighbouring areas. The second site, a vegetation farm, is located in the Ladang Pertanian Bersepadu Universiti Putra Malaysia and the third site, a residential area, is located in Sri Serdang. The ethical approvals were obtained from the Department of Biology, Faculty of Science, Universiti Putra Malaysia and RESBS. Rodents were captured in wire cages that were baited with coconut flesh or cheese and placed in the entrapment areas. Trapped rodents were transported to the Parasitology Laboratory of the Faculty of Science, Universiti Putra Malaysia.

Examination for ectoparasites

Individual hosts were killed with an overdose of chloroform and examined for parasites. Screening for ectoparasites involved a thorough examination of the body of the small mammals including the eyes, nose, mouth, ears, body, anus and vulva or penis. The skin of each rodent was examined grossly for ectoparasites. Those rodents with parasites were identified and recorded. Samples of the observed parasites were removed with a camel hair brush and transferred to a vial containing 70% alcohol. They were fixed on a microscopic slide using a drop of formalin and mounted with DPX or Hoyers medium. The specimens were examined under a bright-field microscope. All the *images were captured* at *100x magnification.*

Examination for endoparasites

Every rodent was dissected, and different internal organs were macroscopically examined for the presence of any parasites. All parts of organ or tissue specimens were placed into separate petri dishes containing normal saline. The hollow organs and duct openings were rinsed with physiological saline and the saline solution was poured through a sieve to retain any parasitic worms. The contents of the esophagus, gall bladder, liver passages, lungs, pancreatic ducts, urinary bladder, brain and other organs were carefully examined under a dissecting microscope. Any clumped parasitic worms were gently teased apart from the lining of these organs with a scalpel and placed in a larger vessel.

The worms were gently rinsed with water to remove any remaining fixative prior to staining. Staining time varied depending on the size of the specimens from 1 to 8 hours. A few drops of 0.5 to 1% hydrochloric acid were added to decolourise the internal structures of the parasites. Next, the specimens were passed through a series of alcohol 70%, 85%, 95% and 100% allowing at least for 30 minutes in each solution. The endoparasites were stained with Semichon's acetic carmine and mounted with DPX or Hoyers medium. The specimens were examined under a bright-field microscope at *100x magnification* and the *images were captured*.

Examination of Feces for Worms, Eggs and Larvae

The feces was mixed with 1 to 2ml of brine solution in a test tube and stirred until the solution took on a pasty consistency. Then the mixture was allowed to stand undisturbed for 30 to 60 minutes. A wire loop or brim of a lipless test tube was used to transfer some of the surface film to a clean slide. All the slides were examined under a low power microscope for the presence of worms, eggs or larvae in the feces.

RESULTS

Twenty-three rodents belonging to four species were live-trapped during the study period. Species of hosts trapped were as follows: *Rattus argentiventer, Mus musculus*, *Suncus murinus* and *Rattus sabanus.* The highest number of rodents was from RESBS (N=12; 52.2%), followed by LPB UPM (N=7; 30.4%) and SSHA (N=4; 17.4%). The species of *R. sabanus* was captured from RESBS, *R. argentiventer* from LPB UPM and *M. musculus* from SSHA. The *S. murinus* species was captured in both RESBS and SSHA.

In the present study, captured rodents were identified to be infested with two ectoparasites, *Echinolaelaps echidinus* (mite) and *Polyplax spinulosa* (louse). Among all *R. sabanus* species, 20% were found to be infested with *E. echidinus* and 40% were infested with *P. spinulosa.* The ectoparasites found in each species are summarised in Table 1 and 3.

About 33.3% of captured rodents from RESBS and 28.6% from LPB UPM were infested with cestode tapeworms and no endoparasites were found in rodents from SSHA. Nematode endoparasites were also found in rodents from RESBS (8.3%). The endoparasites found in each species are presented in Table 2 and 3.

The rodents from the LPB UPM were infected with *E. echidinus* and cestode tapeworms whereas the rodents from SSHA were infected with *E. echidinus* parasites only**.** The images of these parasites are shown in Fig.1 and Fig.2.

DISCUSSION

A total of 23 rodents comprising four species were trapped from the three habitats. The captured species of rodent varied from different localities. The different medium and high density residential areas, social

TABLE 1
Number of Host Species Caught in Each Entrapment Area and the Number of Host Positive for Ectoparasites

Location	(n)	Host	Ectoparasites	No. of host (+) Ectoparasite
RESBS	2	House shrews *S. murinus*	Nil	Nil
	10	Long-tailed giant rat *R. sabanus*	Louse *P. spinulosa*	4
			Mite *E. echidinus*	2
SSHA	1	Mouse *M. musculus*	Mite *E. echidinus*	1
	3	House shrews *S. murinus*	Not found	Nil
LPB UPM	7	Rice field rat *R. argentiventer*	Mite *E. echidinus*	5

Recycle Energy Sdn Bhd Semenyih (RESBS); Ladang Pertanian Bersepadu, Universiti Putra Malaysia (LPB UPM) and Sri Serdang housing area (SSHA), Selangor, Malaysia

TABLE 2
Number of Host Species Caught in Each Entrapment Area and the Number of Host Positive for Endoparasites

Location	(n)	Host	No. of host (+) Endoparasites (Cestode)	No. of host (+) Endoparasites (Nematode)
RESBS	2	House shrews *S. murinus*	2	Nil
	10	Long-tailed giant rat *R. sabanus*	2	1
SSHA	1	Mouse *M. musculus*	Nil	Nil
	3	House shrews *S. murinus*	Nil	Nil
LPB UPM	7	Rice field rat *R. argentiventer*	2	Nil

Recycle Energy Sdn Bhd Semenyih (RESBS); Ladang Pertanian Bersepadu, Universiti Putra Malaysia (LPB UPM) and Sri Serdang housing area (SSHA), Selangor, Malaysia

TABLE 3
Comparison Between Ecto and Endoparasites According to the Species of Host and Locations

Host species	Location	*P. spinulosa*	*E. echidinus*	*Cestode*	*Nematode*
R. sabanus	RESBS	+	+	+	+
R. argentiventer	LPB UPM	-	+	+	-
M. musculus	SSHA	-	+	-	-
S. murinus	1.RESBS	-	-	+	-
	2. SSHA	-	-	-	-

Recycle Energy Sdn Bhd Semenyih (RESBS); Ladang Pertanian Bersepadu, Universiti Putra Malaysia (LPB UPM) and Sri Serdang housing area (SSHA), Selangor, Malaysia

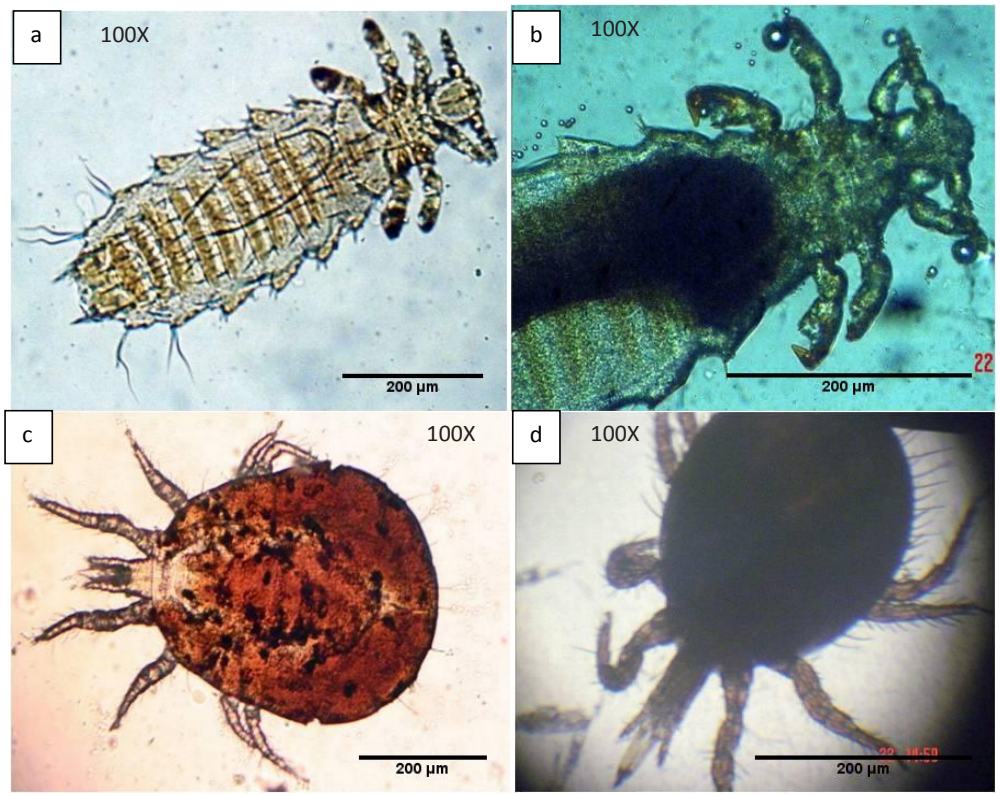

Fig.1: Ectoparasite Anoplura, parasitic (a) *P. spinulosa* morphology mounted with DPX (b) and Hoyers medium. (c) *E. echidinus* (mite) mounted with DPX and (d) Hoyers medium.

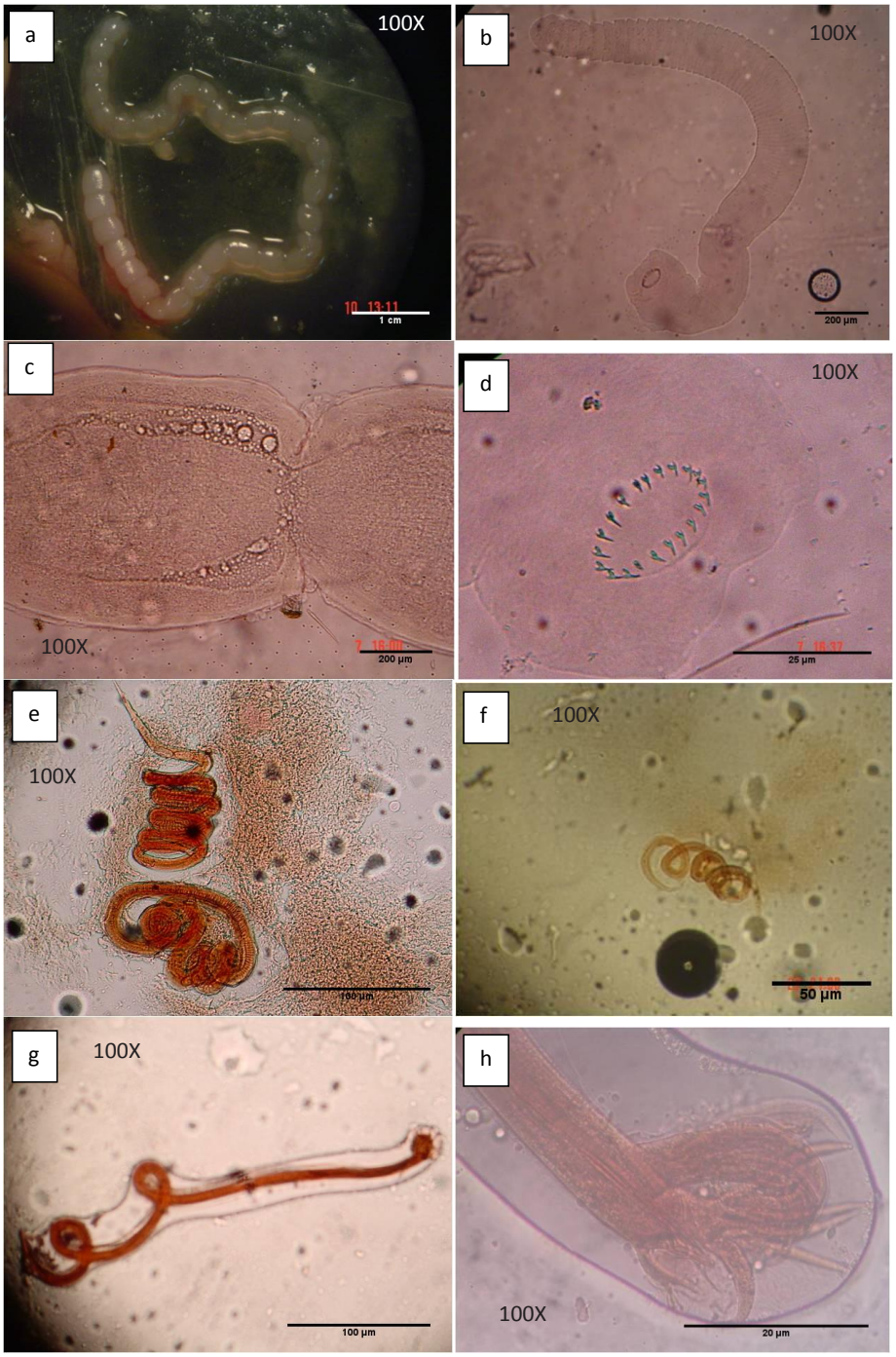

Fig.2: Endoparasites cestode tapeworm from intestine of rodents showing vesicle and proglottids (a, b, c) and scolex with 21 hooks (d), Endoparasites nematode (complete structure) (e, f, g) and bursa of nematode (h).

status and food availability might be the key factors that determined the types of rodents living in different habitats.

Rattus sabanus and *S. murinus* were the two species live rodents trapped from RESBS. *R. sabanus* species are found in Malayan mainland forests (Hoi-Sen, 1970) and it is known as the long-tailed giant rat (Lee, 1965). In the present study *R. sabanus* species were captured only in the RESBS and not in LPB UPM and SSHA. RESBS was only visited by *R. sabanus* that probably came from the bushes surrounding the resource recovery plant because the species mainly live in forested areas or near the bushes. *S. murinus* may have also originated from the same area.

The RESBS is regularly cleaned and the waste disposal does not support the breeding place for rats as the waste is constantly shifted in order to be separated for recovery processes. Even though the *R. sabanus* were found to be heavily infected by ecto and endoparasite that are transmissable to humans, this particular species does not come into human residential areas as it prefers to live in forested areas. Fortunately, there was no captured house rat, *Rattus r. diardi,* in RESBS and this indicated that the house rat from the housing area had not visited the recovery plant.

The long-tailed giant rats (*R. sabanus)* from RESBS were found to be infested with *E. echidinus* (mites), *P. spinulosa* (lice), cestode tapeworms and nematodes. Similarly, Claveria *et al.,* (2005) also reported that rats (*Rattus* spp.) that were trapped from wet markets in Quiapo, Manila and Balayan, Batangas were found to be infested with the same ectoparasites. Infection of the cestodes tapeworms and nematodes in the *R. sabanus* species may be presumably transmitted by consumption of insects from the resource recovery plant.

The rice field rats (*R. argentiventer*) were found in LPB UPM. These rodents prefer to live in a vegetation field and are mainly found in the dikes with a cover of weeds, which provides a good shelter for them to survive (Jakel *et al.*, 2006). In Malaysia they are abundantly present in 55 to 60 organisms per hectare before the rice sowing season (Wood, 1971). Rats from the farm are more vulnerable to ectoparasites infestation. In the present study, 71.4% of rice field rats were infected with *E. echidinus.* The two most commonly reported epidermal parasites of rats were *E. echidinus* and *P. spinulosa* (Soliman *et al.*, 2001). The cestode tapeworms in liver cysts were also found in these rice field rats.

Mus musculus and *S. murinus* were the two species captured in SSHA. The *M. musculus*, known as house mice, was found to be abundantly distributed in eastern Europe and Asia (Pocock *et al*., 2005). The *S. murinus* or house shrews were believed to be transported from India by humans as the species lived in close contact with humans (Meegaskumbura *et al.*, 2010). These house shrews were also captured in RESBS. The *M. musculus* and *S. murinus species* were less infected with the parasites compared to other species.

The *E. echidinus* (mites) classified under the family of Laelaptidae was found in

all three entrapment areas. The adult female mites give birth to live larvae and the larvae usually do not feed until they develop to the protonymphs and deutonymphs stage before becoming adults in three weeks. It has been reported that the ectoparasites, *Laelaps echidninus* (spiny rat mites) belonging to the family Laelaptidae is occasionally found in the *Rattus* species such as Black, Norway rats, house mice and other domestic and wild rodents (Mullen & O'Connor, 2002).

Polyplax spinulosa (spined rat louse) belongs to the family Polyplacidae (Lance & Musser, 1994). *P. spinulosa* is a blood-sucking louse that is easily found by the skin scraping method (Tamura, 2010). Host specific *P. spinulosa* lice are the most common parasites found in rats. The existence of these blood-sucking lice parasites are more in young, neglected and under-nourished rats (Hoppmann & Barron, 2007). The *R. sabanus* from RESBS was the only species infected with *P. spinulosa* compared to the rodents in the two other localities.

The cestode tapeworms found in the study are classified as phylum Platyhelminthes, class Cestoda, known as flatworms (Columbia Encyclopedia, 2007). These cestode tapeworms live in the bodies of vertebrates during their juvenile stage of development and in the digestive tract of the host in the adult stage. The two species of tapeworms found in the study were probably the dwarf cestode tapeworms, *Hymenolepis nana* or rat cestode tapeworms, *Hymenolepis diminuta* (DPDx, 2008). Cestode tapeworms live in the small intestine by anchoring their scolex into the intestinal wall of the host (Williams *et al*., 2011).

A cestode tapeworm requires at least two hosts in order to survive and complete its life cycle. The primary host often carries the larvae that live in the tissue of the host; they are then transmitted to the secondary host, developing to adult stage in the intestine of this final host. Recent studies have shown that *H. nana* and *H. diminuta* are commonly found in rats and mice, respectively (Chai, 2013). Many cestode tapeworms are host-specific and some may only require one host to complete their whole life cycle (Heyneman & Baron, 1996).

The species of roundworm nematodes that was found in the study is probably the *Angiostrongylus sp*. The natural hosts for these roundworms are rodents such as *Rattus norvegicus* and *Rattus rattus*. The intermediate hosts for these roundworms are molluscs. Humans become infected by ingestion of intermediate or paratenic hosts; in humans, infestation can cause eosinophilic meningitis in the brain (Thiengo *et al*., 2013).

Unfed parasites are able to live long without a blood meal. Therefore, careful examination of parasites should be performed not only among the rodents or infected patients but also thorough examination of surrounding areas; this is rarely performed (Beck & Folster-Holst, 2009).

The host animals live in the same general niche and consume similar types of food such as plant materials and insects. They may directly transmit their parasites

to another host. This transmission occurs by burrowing or nesting of parasites in the host and normally happens in conditions of low-host densities in the complex habitat. Similar transmission occurs in the many species of parasites that infest a variety of animals in Malaysia. The process occurs in two stages, firstly through the food and secondly, through the physiological level. All the host species, especially rats, in the previous studies are well known to feed on insects (Hurd, 2003). There are some indications that animal protein is necessary for the survival of some species of rat in Malaysia (Wood & Fee, 2003).

The diversity in rat parasites points to their adaptability as well as the enormous capability of the host to support parasites' behavioral, physiological or nutritive and developmental needs. Despite heavy infection with mites, lice and cestode tapeworms, the rodents still appeared healthy and active, reflective of a well-established and presumably successful rat host-parasite interrelationship. The limitations of this study include the disturbance in laying out the grid and food disposal surrounding the entrapment areas; this may have affected the entrapment of the rodents.

CONCLUSION

In summary, the rodents captured from RESBS were heavily infected with parasites including *E. echidinus*, *P. spinulosa* and cestode tapeworms compared to rodents from the farm and housing areas. Therefore, rodents carrying parasites found mainly in RESBS need to be addressed to prevent parasitic infections in humans.

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Short Communication

Rice Production and Climate Change: A Case Study of Malaysian Rice

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ABSTRACT

Rice is the most cultivated and consumed cereal in Malaysia. With the local population rising in number, the yield progress of the crop needs to increase in a sufficient and sustainable manner to meet the increasing demand. However, future productivity is uncertain because of the predicted changes in climate, notably temperature and water availability. Here we highlight the impact of climate change on Malaysian rice production and how it is linked to the current use of nitrogen (N) fertiliser. From literature analysis we propose that the sustainable solution lies in targeting photosynthesis per unit N. Here we show a lower sensitivity of photosynthesis to N deficiency in Malaysian varieties in comparison to other widely grown cultivars, indicating the potential for improvement. This initial study is used to establish baseline measurements for more complex, multi-factor stress analyses.

Keywords: Climate change, Malaysia, nitrogen deficiency, rice

INTRODUCTION

Addressing the issue of global food security in the 21st century is a major challenge. Rice research will play an important role as rice is one of the most important crops in terms of human consumption (as opposed to animal feed) and is produced in 95 countries across the world (Maclean *et al*., 2002). It is the staple food in many countries, accounting for more than 40% of global food production. People in the majority of countries in Asia depend on rice as their main source of nutrition, as well as for income and employment (Maclean *et al*., 2002; Makino, 2011). The Rice Market

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Monitor by the Food and Agriculture Organisation (FAO) in 2012 stated that the current supply of rice outpaces consumption. Nevertheless, an increase in the supply of this cereal crop would be required to meet the future demands of rice in world population. This demands an estimated 50% increase in the yield of rice in order to sustain the predicted world population of 9.3 billion by 2050 (Sheehy & Mitchell, 2013). This is compounded by the uncertainties of the effect of climate change on crop productivity, which will certainly show regional-specific impacts (Parry *et al*., 2005; IPCC, 2013) across the world. In this case study we (1) highlight the localised effect of climate change on the production of rice in Malaysia, and (2) argue that photosynthesis per unit N is a critical sustainable trait in light of climate change and abiotic stress. This is required to tackle the current trend for increased use of N fertiliser in Malaysia.

YIELD LIMITATIONS IN MALAYSIA

Rice makes up a particularly high proportion of the total agricultural area in Southeast Asia (Maclean *et al*., 2002). The climate in this region of the world alternates between the wet and dry seasonal cycle, typical of the tropics. In Malaysia, particularly Peninsular Malaysia, paddy is cultivated as a rainfed or irrigated lowland crop. Rice is currently constrained to eight major granary areas in Peninsular Malaysia. It is mainly grown in states such as Kedah, Perak and Kelantan, which together control more than half of Malaysia's harvested area (FAO, 2002). Dryland cultivation occurs mostly in Sabah and Sarawak, in the islands of Borneo. In comparison to other countries in Asia, Malaysia produces only a small amount of rice. Out of 656.4 million tonnes of rice produced in Asia, only 2.7 million tonnes is from the peninsula and the Borneo islands – approximately 0.4% of Asia's rice production as reported by the FAO in 2011.

The actual average local rice yield was 30-50% lower than achievable potential in 2007, based on MARDI (Malaysian Agricultural Research and Development Institute) local verification trials (Omar, 2008) in granary areas. Due to the inability to currently meet their goal of being 100% self-sufficient in rice, Malaysia still has to import rice from major suppliers – Thailand and Vietnam. The current average yield has been reported as 4.5-5 t/ha, due mostly to farmers applying more than the recommended rate of 170 kg N/ha (Nori *et al*., 2008). FAO reported paddy yields slightly lower, between 3.36 and 3.97 t/ha between 2006 and 2012, in comparison with an average for SE Asia over the same period of 3.90-4.23 t/ha, indicating that Malaysian yields are slightly lower than regional yields (www.faostat.org). Rice yields within the country are seeing a rise due to an increase in usage of N fertilisers despite a stagnant harvested area (Fig.1).

Malaysia is naturally geographically disadvantaged, as most of Peninsular Malaysia is covered in tropical rainforest with a mountainous interior. Fig.2 is a representation of the major environmental and soil constraints affecting the current and future prospects of rice agriculture. In order

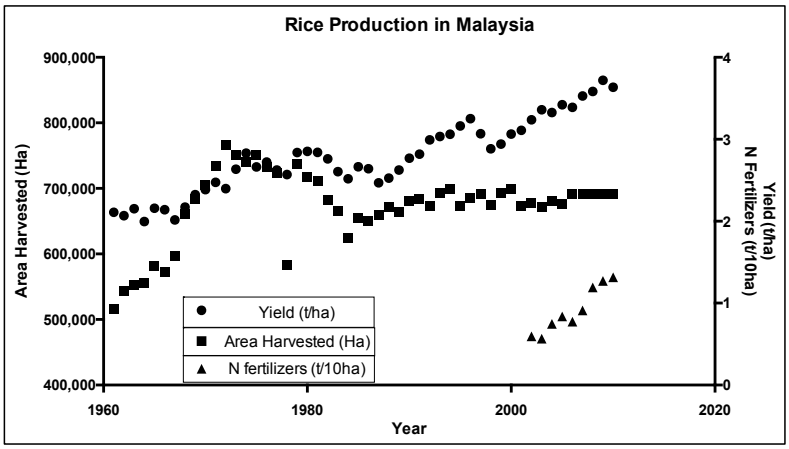

Fig.1: Patterns of rice production in Malaysia. Increases in yield after 2000 are a result of increasing N fertiliser usage. Data source: FAOSTAT.

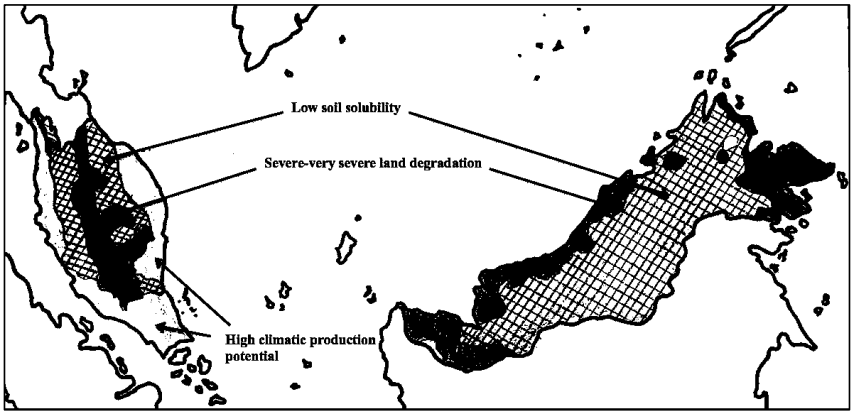

Fig.2: A schematic of the major environmental constraints in Malaysia. Adapted from Major Environmental Constraints for Agricultural Production Project based on FAOCLIM database, ARTEMIS NDVI imagery and soil and terrain data provided by Soil Resources Management and Conservation Service. FAO-GIS. Prepared using Windisp software by Rene Gommes, Environment and Natural Resources Service, for the SOFI 1999 Report.

to fulfil the demand of a rising population, the country has to be able to sustain rice production and at the same time increase productivity of the crop per unit hectare, without having to expand granary areas (Najim *et al*., 2007). This increase in rice production will need to be achieved in the face of a climate that will reduce yield, as we shall describe in the next section.

CO_2 concentrations are forecasted to double by the end of the 21st century, and this anthropogenic rise will result in an increase in global temperatures of between 1.5 and 5.8°C (IPCC, 2013). Although higher CO_2 concentrations have shown field improvements in photosynthesis, biomass and yield across species, largely by reducing photorespiration, this will be mitigated

by the rise in temperature, deterioration of water supply and other factors such as disease and pollution (Ainsworth & Long, 2005; Long *et al*., 2006). Indeed, studies on rice have shown that increased night temperatures are more closely related to yield stagnation across SE Asia than other factors (Peng *et al*., 2004; Welch *et al*., 2010). The mechanism behind this is not yet clear but may be associated with temperature-dependent effects on primary metabolic processes such as growth and maintenance respiration. This is independent of sudden temperature spikes that induce spikelet sterility (Wassman *et al*., 2009). Indeed, temperatures are approaching the upper limit for optimal rice yield and even survival in some regions (Ingram *et al*., 1995). Using existing crop models, rice production in Malaysia is predicted to decline by 0.69t/ha, and giving rise to an economic loss of RM299m per year with a rise of 2°C and a shift in CO_2 from 383 to 574 parts per million (ppm) (Vaghefi *et al*., 2011).

MITIGATING THE EFFECTS OF CLIMATE CHANGE

Climate shifts in the next 50 years are also likely to bring about unpredictability in crop production (Sheehy & Mitchell, 2011). More extreme climatic events such as heavier rainfall and drought in certain areas have been attributed to the rise in temperatures. Other production constraints include increased salinity in coastal granary areas from seawater intrusion as well as pests and diseases such as blast and brown plant hopper. Generally, physiological responses to climate change are complex because of the multiple abiotic factors involved. However, a central process in the response of plants to climate change is photosynthesis. As photosynthesis is intimately linked to climate (temperature and CO_2) and water, this relationship ultimately determines the success of agricultural systems as a whole across multiple environments (Ainsworth & Long, 2005; Murchie *et al*., 2009). Photosynthesis is also the largest sink within the plant for nitrogen fertiliser with 50% of leaf nitrogen in the chloroplast. A factor rarely considered is light: it is essential for photosynthesis, but rice grown in the tropics is especially exposed to high and erratic light intensities. In some cases, very high light intensities cause photoinhibition, a process that damages the photosynthetic apparatus through excessive light absorption and photooxidative stress (Murchie & Niyogi, 2011). High light exacerbates other abiotic stress responses.

Farmers are therefore being driven to confront the inevitable prospect of growing under fluctuating and increasingly unfavourable conditions due to the changing patterns of land use due to climate change. With optimisation of other yield components, rice is increasingly dependent on improvements in biomass production, which require increased nitrogen fertiliser application (Peng *et al*., 2000; Murchie *et al*., 2009), and this is particularly strong in Malaysia (Fig.1). With the increasing cost of fertiliser and unpredictable climate, there is a convincing argument that the improvement

of crop yield should be achieved from the manipulation of photosynthesis at the leaf level, and this is consistent with available information about rice production in Malaysia. Fig.3 compares the responses of photosynthesis to suboptimal nitrogen in widely grown SE Asian rice cultivars and a variety specific to Malaysia. Low N leads to high light sensitivity as shown in rice (Chen *et al*., 2003) and spinach (Verhoeven *et al*., 1997): reduced photosynthetic capacity results in an excess level of absorbed irradiance. Interestingly, MR253 shows higher leaf photosynthesis at suboptimal conditions, demonstrating a potentially improved nitrogen uptake, allocation or remobilisation dynamics (Foulkes & Murchie, 2011) and potentially lower susceptibility to oxidative stress under high light conditions. Additionally, a higher Amax itself is associated with higher canopy photosynthesis and productivity in cereals (Murchie *et al*., 2009: Reynolds *et al*., 2012). The data presented supports a larger study, which will investigate nitrogen–abiotic stress interactions. Table 1 summarises the agronomic traits of the varieties used in this study.

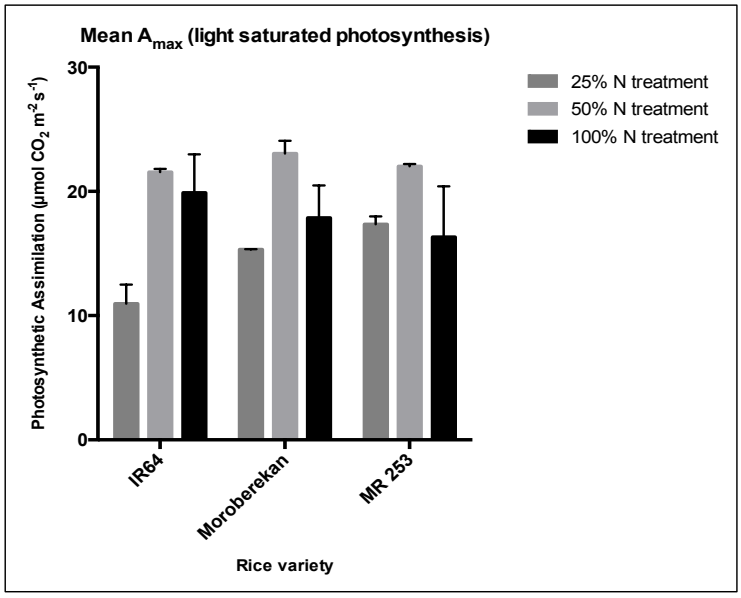

Fig.3: Light saturated rates of CO_2 assimilation in the widely grown IR64, the African cultivar Moroberekan and the Malaysian cultivar MR253. Note the reduced difference in Amax between 50% and 25% for MR253. Means and standard error of the means are shown (n=4). These three varieties were grown in hydroponic solution as in Murchie *et al*., (2005). The experiment was conducted in an environmentally-controlled growth room under three concentrations of NH_4NO_3: 1.4 mM (100% N), 0.7 mM (50% N) and 0.35 mM (25% N). Measurements were made at leaf 11 for the IR64 and MR 253 varieties and leaf 9 for the Moroberekan variety. The photoperiod was 12 hours, temperature 28°C, an irradiance of 600µmol m^{-2} s^{-1} and RH of 40-50%. In-situ light response measurements were made according to Hubbart *et al.* (2012).

TABLE 1
Characteristics of Model and Malaysian Rice Cultivars. Wu *et al.*[1] (2005), Haq *et al.*[2] (2009)

Variety	Origin	Agronomic Characteristics
IR64 (*indica*), released in 1984	IRRI, Philippines	High yielding potential, tolerance to multiple diseases and pests, wide adaptability[1]
Moroberekan (*japonica*)	Africa	Potential drought resistance[2] (deep-rooted), considerable resistance to rice blast
MR 253 (*indica*)	MARDI, Malaysia	Resistant to leaf blast, performs better in marginal soils

CONCLUSION

We have briefly discussed the future problems of increasing rice production in Malaysia in the face of rising demand of rice and unfavourable climatic conditions and economic uncertainties such as the increase in cost of fertiliser. Yield gains in Malaysia are associated with unsustainable increases in N fertiliser applications. We have focused on sustainable approaches for genetic improvements in productivity that include the optimisation of photosynthesis per unit nitrogen and multi-factor stress analyses in the field. Inefficient applications can result in profoundly catastrophic effects on the climate and important ecosystems. Curtailing the use of N fertiliser and promoting better nutrient management are the more immediate cost-effective options, but it is vital that we realise the need for more sustainable and long-term solutions.

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Effect of Planting Patterns and Age at Harvest of Two Cultivars of *Lablab purpureus* in *Andropogon gayanus* on Agronomic Characteristic and Quality of Grass/Legume Mixtures

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ABSTRACT

Research was conducted to investigate the contribution of two legume cultivars of *Lablab purpureus* cv. Highworth (early maturing) and *Lablab purpureus* cv. Rongai (late maturing) to total fodder productivity and nutritive value when intercropped with Gamba grass (*Andropogon gayanus* cv. Kunth). There were nine treatments in all consisting of monocultures of grass, legumes and grass-legume mixtures in the ratios 1:1, 1:2 and 1:3 for each cultivar. The total dry matter yield, Relative yield (RY), land equivalent ratio (LER) and competitive ratio (CR) of the grass and legumes were estimated at 10 and 15 weeks after planting before flowering. Overall total herbage yield of the mixtures was higher than those of grass. In all the mixtures, incorporation of *L. purpureus* cv. Rongai in *A. gayanus* gave higher ($P>0.05$) CP than Highworth. The mixture of *A. gayanus* with double rows of *L. purpureus* cv. Rongai gave the highest ($P<0.05$) mineral contents while the least were recorded in sole *A. gayanus.* It was concluded that double rows of *L. purpureus* were recommended as the most compatible combination to improve the forage quantity and quality of *A. gayanus*.

Keywords: Cultivars, grass-legume, highworth, relative yield, rongai

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INTRODUCTION

In most developing tropical countries, animal production from pastures is low compared to developed countries. Pasture grasses (mainly C4) frequently contain

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crude protein levels of 8% or less, which are inadequate for animal production (Humphreys, 1991). During the low-rainfall season (dry season), there is a drastic decrease in the nutritional quality of tropical grasses, which is reflected by low crude protein (CP) and increased lignin content in the cell wall (Paulino *et al.,* 2008). The limited CP availability provides the critical threshold for adequate microbial growth on the fibrous carbohydrates in basal forage (Lazzarini *et al.,* 2009). This CP deficiency implies poor utilisation of potentially degradable cell wall by microorganisms and results in decreased intake and animal performance (Paulino *et al.,* 2008). Introduction of forage legumes is one of the strategies for improvement of grassland productivity. The benefits of legume-grass association can be expressed, among others, in terms of the enhanced dry matter yield, nitrogen content of herbage, animal intake of forage and animal productivity. Grass-legume mixtures are a means of improving productivity compared to monocultures without any additional investment. Yields are generally higher in mixtures because of more efficient light utilisation and transfer of symbiotically fixed nitrogen to grasses (Ledgard, 1991). Baba *et al*., (2013) reported in an experiment that a Guinea-Arachis mixture gave higher cumulative dry matter yield than pure guinea grass.

Of the species (*Andropogon gayanus, Brachiaria spp* and *Panicum maximum*) that are well adapted to a wide range of relevant environmental conditions, only *Andropogon gayanus* (Gamba grass) grows reasonably well in both the wet season and long into the dry season Phengsavanh, 1997). Gamba grass has soft leaves and grows well on infertile, acid soils in hot climates and in a wide range of climates, but is particularly useful in areas with a long dry season. Gamba grass stays green long into the dry season when most other grasses are already dry, is easy to cut and can tolerate grazing. Gamba provides palatable forage when young but feeding value declines rapidly with age and decreasing leaf/stem ratio. Mineral content is low (0.08 P and 0.27 Ca in DM) (Ajiji *et al*., 2013). Agishi (1985) reported that gamba grass is available in abundance in almost all ecological zones in Nigeria. The problem with gamba, like other tropical grasses, is the rapid decline in crude protein and soluble carbohydrate with age. This is coupled with a progressive increase in crude fibre and lignin (Lambert & Litherland, 2000)

Lablab purpureus combines a great number of qualities that can be used successfully under various conditions. Its first advantage is its adaptability; not only is it drought resistant, it is able to grow in a diverse range of environmental conditions worldwide. Staying green during the dry season, it has been known to provide up to six tonnes of dry matter/ha (Murphy & Colucci, 1999). Being palatable to livestock, it is an adequate source of much-needed protein and can be utilised in several different ways. In several experiments it has been observed to increase livestock weight and milk production during the dry season. *L. purpureus* with its ability to out-

yield conventional crops, especially during the dry season, and its enhanced nutritive value, is a fodder crop of great significance for the tropics.

Though lablab is known in many countries and has the capability of being an outstanding resource for agricultural systems in the tropics, it is not being used to its full potential (Evans, 2002). In many areas where lablab could be beneficial, ability to buy seed is restricted by economic constraints, and producers' willingness to take the risk in trying a new practice is guarded by traditional paradigms. Effort must be devoted to conducting more research to extend both technical and practical knowledge about lablab so that its full potential may be achieved.

The objectives of this study is to evaluate the potential of Andropogon as one of the tropical grasses with rapidly declining crude protein and the solube carbohydrate content in intercrops with different cultivars of lablab, which has a different growth pattern.

MATERIALS AND METHODS

Experimental Sites

The experiment was conducted from May 2011 to May 2012 at the Muturu Paddock of the IFSERAR Farm, Federal University of Agriculture, Abeokuta. The experimental site lies within the savanna agro-ecological zone of South Western Nigeria (latitude: 7°N, longitude 3.5°E, (Google earth, 2011). With an average annual rainfall of 1037mm Abeokuta has a bimodal rainfall pattern that typically peaks in July and September with a break of two to three weeks in August. The relative humidity in the rainy (late March-October) and dry (November-early March) season ranges between 63-96% and 55-84% respectively (Fig.1). The temperature of the soil ranges from 24.5 to 31.0°C (Ogun-

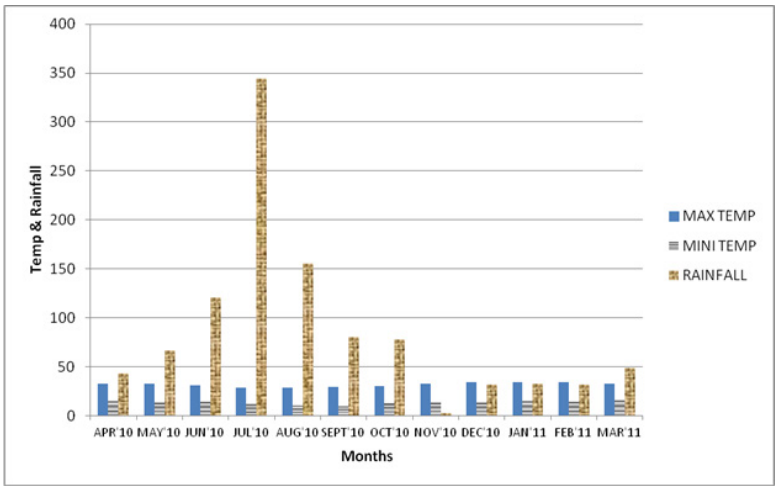

Fig.1: Temperature (°C) and Rainfall (mm) for the experimental period (April 2010 to March 2011)

Source: Ogun-Osun River Basin Development Authority, Abeokuta, Nigeria

Osun River Basin Development Authority, Abeokuta, Nigeria).

Procedure

A land area of 0.15 ha was ploughed, harrowed and levelled before plots layouts. The land was divided into three blocks to cater for the topography of the experiment site and each block represented a replicate. Three core samples of soil (0–15cm) were randomly collected from the plots before planting. These were bulked for each block and analysed for physical (particle size) and chemical properties (pH, total N, organic carbon, C: N ratio, available P, available N, cation exchange capacity and acidity The total nitrogen of the soil was 0.11%, the organic carbon was 1.29 % and the available phosphorus was 53.87mg.kg^{-1}. Exchangeable cations, namely sodium, was 0.80cmol.kg^{-1} and potassium was 0.20cmol. kg^{-1} while calcium and magnesium were 2.77 and2.72cmol.kg^{-1} respectively.

Each block was later divided into nine plots of 7x7m^2 each, representing the number of treatments. The experiment was a randomised complete block design. The trial comprises two cultivars of *L. purpureus*, namely Rongai and Highworth, planted as single, double and triple rows between *A. gayanus* with each legume and grass planted sole to give nine treatments as summarised: single rows of *L. purpureus* cv. Rongai between *A. gayanus* rows, double rows of *L. purpureus* cv. Rongai between *A. gayanus* rows, triple rows of *L. purpureus* cv. Rongai between *A. gayanus* rows, single rows of *L. purpureus* cv. Highworth between *A. gayanus* rows, double rows of *L. purpureus* cv. Highworth between *A. gayanus* rows, triple rows of *L. purpureus* cv. Highworth between *A. gayanus* rows, and sole *A. gayanus*, *L. purpureus* cv. Rongai and sole *L. purpureus* cv. Highworth.

The crown splits of the *A. gayanus* were sourced from previously established plots because the Andropogon seeds were not readily available. The grasses were planted at the spacing of 1m between and within rows on 14^t May 2010, giving 7 rows each with 7 stools and an overall population equivalent to 10,000 stools per ha. The legumes were sowed at 20kg/ha and 6plants/ m^2 giving an equivalent of 60,000 plants/ha. The single rows of legumes were drilled in the middle of the grass rows (50cm from the rows of grasses) while in the double and triple row treatments, the legumes were sown at 30cm from the rows of grasses and 40cm apart and 30cm from the grasses and 20cm apart respectively.

The seed of *L. purpureus* var. Highworth (early maturing with purple flowers and white seeds) and *L. purpureus* var. Rongai (late maturing with white flowers and light brown seeds) were obtained from the National Animal Production Research Institute (NAPRI), Zaria and planted at the recommended rate of 15kg/ha two weeks after planting the grass (28 May) to allow the grass to recover and become established as legume grows faster than grass and so it was critical to avoid competition . The plots were free of weeds throughout the period of study.

Data Collection

Plant growth measurement was taken at two-week intervals throughout the experimental periods while specific measurements were taken six and 10 weeks after planting to estimate the effect of time of harvest. The estimations of tiller density were carried out by counting the number of tillers within two randomly located $1m^2$ quadrats at every harvesting time throughout the experimental period. Estimations of the height of the grass and legumes were carried out by measuring from the base of the plant to where the last leaf on the stem emerged with the aid of a metre rule on 10 randomly selected stands per plot at every harvesting time.

The estimation of total yield, relative yield (RY), land equivalent ratio (LER) and competitive ratio (CR) was carried out by harvesting the herbage materials within the range of two randomly located 1mx1m quadrat at different ages before the flowering of Andropogon at the 10th and 15th weeks after planting. The quadrat was thrown three times per replicate. The dry matter percentage was estimated as dry matter yield (DMY) = dry matter percentage x fresh sample from $1m^2$, which afterwards was extrapolated in tonnes per hectare. Relative yield (RY), land equivalent ratio (LER) and competitive ratio (CR) were determined following the equations of Ghosh *et al.* (2006) as:

$$RY = RYab = DMYab/DMYaa,$$
$$RYba = DMYba/DMYbb$$
$$LER = RYab + RYba$$

In the equation, the following definitions apply: ab refers to performance of *A. gayanus* (a) mixed with either *L. purpureus* cv. Rongai or *L. purpureus* cv. Highworth (b); ba is the performance of either of the legumes (b) mixed with *A. gayanus*; aa is the performance of *A. gayanus* in monoculture and bb is the performance of either of the legumes as a monoculture.

Chemical compositions. Forage samples were harvested at the 10th and 15th weeks after planting, which coincided with 50% and 100% flowering of Andropogon so as to evaluate the effect of legume inclusion when the quality of the grass was declining.

Proximate composition: The contents of dry matter, crude protein, ether extract and ash were determined according to A.O.A.C. (1995). Fibre fraction analysis: Neutral detergent fibre (NDF); acid detergent fibre (ADF); acid detergent lignin (ADL) (Van Soest *et al*., 1991); cellulose was taken as the difference between ADF and ADL while hemicellulose was calculated as the difference between NDF and ADF.

Mineral determination: The samples of the grasses were dried in a forced draught oven at 105°C for 24 hours and were analysed for some macro minerals (Ca, P, K, Na and Mg). The concentration of potassium (K) was estimated with a flame photometer after wet digestion in nitric acid and per chloric acid. Concentration of calcium and phosphorus were determined with atomic absorption spectrophotometry (Fritz & Schenk, 1979).

Statistical Analysis

The data collected was subjected to a two-way analysis of variance (ANOVA), SAS (1999). Duncan's means separation procedure was used to test the level of significance among the means (Duncan, 1955).

RESULTS

Plant growth

The height of the grass in all the mixtures increased as the rows of legume increased to double in each plot (Table 1). Andropogon was tallest ($P<0.05$) when planted with double rows of Highworth but lowest in both varieties when the rows were tripled. Sole grass was comparable in height with mixture except in the triple rows of legumes.

The height of grass increased with age both in cultivars and all planting patterns. Andropogon produced a higher ($P<0.05$) tiller number (15.3) when planted with double rows of *L. purpureus* cv. Highworth than when planted in the sole plot, while the tiller number was similar in the other mixtures

The height of both Rongai and Highworth in the sole plot was higher ($P<0.05$) than in the mixed plots. Within the mixed plots, the height of the Rongai increased from the single to the double rows; however, Highworth reduced ($P<0.05$) in height as its rows in the mixed plots increased. Both cultivars of lablab increased ($P<0.05$) in height with advancing age.

Relative Yield

Planting both legume cultivars in single or double rows had no significant effect on the relative yield of Andropogon in the mixed culture. However, the RY of Andropogon reduced ($P<0.05$) when the rows of Rongai were tripled. The mean RYs of the components of the mixtures throughout the experiment presented in Table 3 shows that the mean RYs of both grasses and legumes were less than unity except for the double rows of Rongai. This indicated that the DM yields of both grasses and legumes in the mixtures were less than those for pure stands; however, all exceeded 0.5 (Table 1). The RY increased in grass as the age at harvest increased but were similar in legume in both harvests. The mean LERs of all grass-legume mixtures in the experimental periods were greater than 1 and ranged between 1.21 and 1.78, demonstrating higher yields in the mixtures than the average of the pure stands.

Dry Matter Yield

Dry matter forage yields of the grass and legumes in the sole and mixed plots were significantly influenced by different planting patterns and age at harvest (Table 1). The dry matter yield of grass increased from the single row to double rows of the legumes and then declined in the triple rows. Planting double rows of legumes with grasses produced higher ($P<0.05$) grass forage. However, these yields were comparable with the yield recorded when grass was planted as a sole plant.

TABLE 1
Plant Growth, Biological Indices and Herbage Yield of Grass-Legume Mixtures as Influenced by Planting Pattern, Cultivars and Age at Harvest

Mixture of *A. gayanus* with	Plant height (cm)		Grass tiller number	Relative yield		LER	Dry matter Yield (t/ha)		Total Dry matter yield (t/ha)
	Grass	Legume		Grass	Legume		Grass	Legume	
Single rows of *L. purpureus* cv. Rongai	103^{b}	49.3^{c}	13.4^{ab}	0.83^{a}	0.87^{b}	1.70^{b}	5.11^{b}	6.25^{ab}	11.4^{c}
Double rows of *L. purpureus* cv. Rongai	122^{ab}	57.4^{b}	9.25^{ab}	0.89^{a}	0.81^{b}	1.70^{b}	6.20^{a}	7.22^{ab}	13.4^{a}
Triple rows of *L. purpureus* cv. Rongai	94.8^{c}	51.4^{b}	10.8^{ab}	0.19^{b}	1.02^{a}	1.21^{d}	5.45^{b}	5.69^{b}	11.1^{c}
Single rows of *L. purpureus* cv. Highworth	115^{ab}	73.2^{ab}	11.9^{ab}	0.80^{a}	0.97^{a}	1.77^{a}	5.21^{b}	7.18^{ab}	12.4^{b}
Double rows of *L. purpureus* cv. Highworth	135^{a}	39.9^{cd}	15.3^{a}	0.81^{a}	0.97^{a}	1.78^{a}	6.59^{a}	7.20^{ab}	13.8^{a}
Triple rows of *L. purpureus* cv. Highworth	83.2^{c}	15.6^{d}	10.2^{b}	0.83^{a}	0.83^{b}	1.66^{c}	4.94^{b}	6.06^{ab}	11.0^{c}
Sole *A. gayanus*	107^{ab}	-	7.63^{b}	-	-	-	6.23^{a}	-	6.20^{e}
Sole *L. purpureus* cv. Rongai	-	88.4^{a}	-	-	-	-	-	7.12^{ab}	7.12^{d}
Sole *L. purpureus* cv. Highworth	-	87.3^{a}	-	-	-	-	-	7.78^{a}	7.48^{d}
SEM	7.63	5.93	0.97	0.01	0.02	0.05	0.13	1.36	1.12
Age									
6 weeks after planting	62.2^{b}	28.3^{b}	6.55^{b}	0.53^{b}	0.73	1.26^{b}	3.28^{b}	5.76^{b}	9.04^{b}
10 weeks after planting	108^{a}	75.8^{a}	10.9^{a}	0.89^{a}	0.85	1.74^{a}	5.55^{a}	6.29^{a}	11.8^{a}
SEM	7.63	5.93	0.97	0.33	0.04	0.23	3.93	3.35	3.04

In columns: values with same letters as superscripts do not differ significantly by DMRT at P<0.05

Sole *L. purpureus* cv. Highworth gave the highest (P<0.05) legume dry matter yield (7.78t/ha) while the least was recorded when triple rows of Rongai were planted in Andropogon plots. The dry matter yield of *L. purpureus* cv. Highworth was similar to that of *L. purpureus* cv. Rongai when both were planted in a single row within Andropogon. Similar results were recorded when both legume cultivars were planted in double and triple rows within Andropogon. Dry matter yield of grass, legumes and their mixtures in the first harvest were generally lower than in the second harvest.

Quality

The proximate composition of the forage mixture and their sole was affected (P<0.05) by legume cultivars, planting pattern and age at harvest (Table 2). The DM content of the mixtures and their sole ranged from the highest value of 950g.kg^{-1} DM in the mixtures *A. gayanus* and double rows of *L. purpureus* cv. Highworth to the least value of 903g.kg^{-1} DM from sole Highworth. The mixture of *A. gayanus* with Highworth gave a higher DM than Rongai, while in both cultivars, an increase in row ratio gave similar dry matter. The DM content of the forages in sole or when mixed at both ages was similar.

The crude protein (CP) of the mixtures of *A. gayanus* and each of the cultivars of *L. purpureus* increased (P< 0.05) as the row ratios of the legume increased from single to double and then declined (P>0.05) as the row ratio tripled. The highest CP content was recorded from the sole plots of both legume cultivars while the least (70.9g. kg^{-1} DM) was recorded from the sole grass plot. The CP content of the forages in the mixture and sole reduced (P<0.05) as the age at harvest advanced.

The EE content trend was similar to that of the CP content with *L. purpureus* cv. Rongai in *A. gayanus* but was reversed with cv. Highworth. Sole Rongai contained the highest EE content of 73.9g.kg^{-1} DM while the EE content was similar in both ages.

The ash content values ranged between 90.2g.kg^{-1} DM in the mixture of *A. gayanus* with single rows of *L. purpureus* cv. Rongai and 124g.kg^{-1} DM in the sole *L. purpureus* cv. Rongai. The ash content of each legume cultivar was similar in mixture to *A. gayanus* in the double and triple row ratios.

Intercropping Rongai and Highworth in a single row with Andropogon had no significant effect on the NDF content of the mixtures, which were comparable (P>0.05) with NDF content of the sole grass, while the NDF content (425g.kg^{-1} DM) was least in the sole plot of *L. purpureus* cv. Highworth.

When intercropped in triple rows with Andropogon, the NDF content of the mixed sward reduced significantly (P<0.05). The NDF content increased in all treatments with advanced age at harvest (Table 2).

The ADF contents of the mixed sward reduced (P<0.05) with increased rows of the legume *L. purpureus* cv. Highworth. ADL content of the mixed sward decreased (P<0.05) as both legumes were intercropped with Andropogon from the single row to double rows and then increased as the

TABLE 2
Proximate and Fibre Composition (g.kg-1 DM) of the Grass-Legume Mixture as Influenced by Planting Rows, Legume Cultivars and Age at Harvest

Mixture of *A.gayanus* with	Dry matter	Crude protein	Ether extract	Ash	Neutral Detergent Fibre	Acid Detergent Fibre	Acid Detergent Lignin
Single rows of *L. purpureus* cv. Rongai	927^{bc}	121^{c}	42.1^{b}	90.2^{b}	606^{a}	384^{a}	135^{b}
Double rows of *L. purpureus* cv. Rongai	930^{bc}	146^{b}	58.9^{ab}	119^{ab}	596^{a}	368^{ab}	123^{c}
Triple rows of *L. purpureus* cv. Rongai	920^{bcd}	129^{bc}	58.8^{ab}	119^{ab}	577^{ab}	362^{ab}	130^{b}
Single rows of *L. purpureus* cv. Highworth	938^{ab}	119^{d}	54.3^{ab}	115^{ab}	597^{a}	340^{b}	126^{bc}
Double rows of *L. purpureus* cv. Highworth	950^{a}	128^{bc}	49.2^{ab}	109^{b}	553^{ab}	313^{c}	116^{c}
Triple rows of *L. purpureus* cv. Highworth	952^{a}	125^{bc}	43.1^{b}	101^{b}	513^{b}	297^{d}	125^{ab}
Sole *A. gayanus*	925^{bcd}	70.9^{d}	54.0^{ab}	104^{b}	637^{a}	473^{a}	160^{a}
Sole *L. purpureus* cv. Rongai	913^{cd}	157^{a}	73.9^{a}	124^{a}	474^{c}	341^{b}	120^{c}
Sole *L. purpureus* cv. Highworth	903^{d}	153^{a}	55.5^{ab}	116^{ab}	425^{d}	317^{c}	128^{bc}
SEM	0.31	0.47	0.38	0.3	2.87	2.1	0.95
Age							
10 weeks after planting	92.89^{a}	129^{a}	58.0	91.5^{a}	729^{b}	501^{a}	182^{a}
15 weeks after planting	92.78^{a}	107^{b}	56.8	86.0^{b}	761^{a}	528^{b}	195^{b}
SEM	0.31	0.89	0.26	0.3	1.9	1.82	0.95

a, b, c, d indicates different superscripts in the same column differ significantly ($P<0.05$)

rows tripled. Both ADF and ADL content increased ($P<0.05$) as the age at harvest increased.

The calcium content of the mixtures showed the same trend in both cultivars as they increased ($P<0.05$) from a single row of legumes to double rows but also decreased as the rows increased from double to triple (Table 3). In all the treatments, the mixture of *A. gayanus* with double rows of *L. purpureus* cv. Rongai gave the highest ($P<0.05$) calcium, sodium, phosphorus and magnesium contents while the least mineral contents were recorded when *A. gayanus* was planted sole.

DISCUSSION

The height of the Andropogon as a sole crop could be attributed to penetration of light, circulation of air and comparatively more nutritional area available to the sole crop under competition in the free environment. However, the reduction noted in the plant height of Andropogon due to intercropping at binary rows with legume was consistent with the report of Rashid and Himayatullah (2003).

In both double and triple rows of lablab, Rongai was taller than Highworth. This was primarily due to differences in growth habit that are genetically controlled. Highworth has a prostrate with a low growth habit of twining while Rongai is an erect bush type of plant (Hector & Smith, 2002). The reduction in the RY of the grass when the rows of Rongai were tripled may be due to a higher population of legumes resulting in higher competition for soil resources than showed by the grass (Baba *et al*., 2011). The mean LERs for the mixtures indicated a yield advantage of 21-78% over that from an average of pure stands of the different species (grass and legumes). This implies that sole crops would require 21-78% more land to achieve the yields obtained by the intercrops. This may indicate N contribution to the grass through nitrogen fixation by the legumes and its transfer from the legume component to the grass. This situation could be attributed to the efficient utilisation of plant growth factors by species in the mixture due to either temporal or spatial differences of their demands.

Relative yield total or LER value well above 1 suggests partial or no competition among species in the mixtures, probably made possible by the contribution of the legumes to the environment of the grass via nitrogen fixation (Tessema & Baar, 2006) or simply the mixtures avoided competition due to a different rooting pattern, which may have prevented the uptake of resources from the same soil strata.

Dry matter production is a function of the nature of competition among the various species in a mixture. The higher dry matter yield recorded when grasses and legumes were planted in 1:2 row ratio might have been due to better utilisation of space, light, nutrients and moisture than other planting patterns. Similar results were also obtained by Singh *et al*. (1983) and Dwivedi (1986). This implies that Andropogon benefited from double rows of legumes by producing high herbage although it was not significantly higher than

TABLE 3
Mineral Composition (g.kg-1 DM) of the Grass-Legume Mixture as Influenced by Planting Rows, Legume Cultivars and Age at Harvest

Mixture of *A.gayanus* with	Calcium	Sodium	Potassium	Phosphorus
Single rows of *L. purpureus* cv. Rongai	2.11^{d}	0.32^{b}	2.10^{c}	0.34^{e}
Double rows of *L. purpureus* cv. Rongai	14.3^{a}	3.26^{a}	24.1^{a}	3.48^{a}
Triple rows of *L. purpureus* cv. Rongai	8.56^{bc}	2.65^{a}	16.7^{ab}	2.72^{ab}
Single rows of *L. purpureus* cv. Highworth	9.69^{b}	3.59^{a}	18.3^{ab}	2.8^{ab}
Double rows of *L. purpureus* cv. Highworth	11.4^{ab}	3.65^{a}	19.2^{ab}	1.72^{c}
Triple rows of *L. purpureus* cv. Highworth	5.77^{c}	2.78^{a}	19.1^{ab}	2.39^{b}
Sole *A. gayanus*	2.01^{d}	0.28^{b}	2.00^{c}	0.25^{f}
Sole *L. purpureus* cv. Rongai	12.8^{ab}	3.27^{a}	11.8^{b}	1.13^{d}
Sole *L. purpureus* cv. Highworth	10.3^{ab}	3.20^{a}	17.1^{ab}	1.19^{d}
SEM	0.83	0.22	1.33	0.18
Age				
10 weeks after planting	11.8a	2.24	6.66b	1.47
15 weeks after planting	16.8b	2.72	9.81a	1.97
SEM	0.83	0.22	1.33	0.18

Values with same letters as superscripts do not differ significantly (P<0.05)

the sole grass. However, the combined total DM yield for grass/legumes intercrops was significantly high. Intercropping has been reported to increase light interception in the intercrops, reduce water evaporation and improve conservation of the soil moisture, resulting in higher DM compared with monocropping (Ghanbari *et al*., 2010). The high dry matter yields of grass in mixtures, which was comparable to the sole plot, could be due to the vigorous nature of grass growth and its ability to rapidly utilise the nitrogen in the soil, which is released following cultivation (Tessema & Baar, 2006). The rapid establishment of the grass may have had a profound effect on the root system that enabled it to extract growth resources from the soil (Kechero, 2008). This result implies that Andropogon can associate well with *L. purpureus*.

Generally, intercrops gave higher forage DM yield than the monocrops. Intercrops with maize, maize/Rongai produced more dry matter than maize pure stands while the yield of maize/Highworth was not significantly different from that of maize in the pure stands (Haque 1989).

The total dry matter yield of forages either in the mixed swards or sole cropping was higher when harvested at 5 weeks after planting than the harvest at 10 weeks after planting. This may be seen in the light of the fact that harvesting at 15 weeks after planting, both grass and legumes were relatively more established and thus able to utilise soil resources better for maximum growth. As both forages advanced to reproductive stage, DM accumulation tends to increased. Njarui *et al*. (2007) reported lower yield when grass-legumes were harvested early because the forages had not developed longer roots to compete for both nutrients and water.

The observed DM yield of grass in the double rows of both legume cultivars was higher than for both single and triple rows and was different from that of Ezenwa and Aken'ova (1996) where grass DM yield was higher with the single than double rows of verano planted between rows of guinea grass. Njarui *et al*. (2007) also recorded no difference in the DM yield of grass either in single or double rows of Seca and Sirato. This could be attributed to the species and their different growth habits and, most importantly, the plant population.

Generally the herbage yield recorded for Lablab in this study was higher than for those reported in Zimbabwe (Jingura *et al*., 2001) but lower than 10.2t/ha, which was obtained at 18 weeks in Zaria Nigeria (Ogedegbe *et al*., 2011). However, the yield recorded in the experiment suggests that both cultivars of lablab have the potential to supply adequate forage quantity for ruminant livestock in sub-Saharan Africa.

The highest CP content recorded when both cultivars were planted sole was lower than what Aganga and Autlwetse (2000) reported (16.4%) for whole plant lablab but was similar to within the range (12.7-14.1%) reported by Evan (2002) for whole plant. Legumes fix atmospheric nitrogen and, therefore, have a higher protein and feed value than associated grasses (Schwenke

& Kerridge, 1990). This contributed to the higher CP content recorded in both legume cultivars when planted sole.

The high CP content of the sole legume was reduced in the intercrop due to the dilution effect of the grass incorporation. However, when two rows of Rongai were incorporated into a single row Andropogon, the CP content was higher than in the other intercrop row ratios. The improvement in quality of forage in the 1:2 row ratios of grass-legume mixed pasture may possibly be the result of the fixation of a higher amount of nitrogen, either by direct excretion from the legume nodule root system or by decomposition of the nodule and root debris. These findings are in conformity with the result obtained by Singh *et al*. (1983).

The CP content of the sole *A. gayanus* obtained from this study was below the recommended CP level (8%) for optimum ruminant performance (Norton, 2003), but was improved by intercropping, which confirmed several reports. The crude protein yield, dry matter yield and ash content of maize forage increased by intercropping with legumes compared with the maize monoculture (Javanmard *et al*., 2009). Increasing the CP content in the diet has been reported to consistently enhance the concentration of NH_3–N in the rumen (Amole *et al*., 2013). It is evident from this study that intercrops of grass with legumes can substantially increase forage quantity and quality and decrease the requirements for protein supplements compared with sole grass.

The NDF content is important in ration formulation because it reflects the amount of forage that can be consumed by animals (Bingol *et al*., 2007). As NDF percentage increases, dry matter intake decreases (Van Soets , 1994). Intercropping Andropogon with three rows of Highworth reduced the NDF concentration. Lauriault *et al*. (2004) noted a similar report that intercropping with peas decreased NDF in all cereals. Thus, addition of lablab to Andropogon reduced the NDF and ADF concentrations, indicating potential for increasing forage intake. Dahmardeh *et al*. (2009) reported that maximum ADF (31.85%) was recorded by sowing maize alone while increasing the proportion of cowpea seeds to 50% in intercropping with maize, resulting in the lowest ADF (25-89%). Both NDF and ADF contents followed generally linear increase with advancing maturity. Similar results were reported by Turgut *et al*. (2008). As plants mature, photosynthetic products are more rapidly converted to structural components, thus having the effect of decreasing protein and soluble carbohydrate and increasing the structural cell wall components (Ammar *et al*., 2004).

Calcium is the most abundant and needed mineral in the animal body and it primarily functions as a component of the skeletal system. It is also involved in vital functions such as blood clotting, transmission of nerve impulse, muscle contractions and cardiac regulation (NRC 2001), *A. gayanus* with double rows of *L. purpureus* cv. Rongai supply the required calcium content without the need of supplementation.

Legumes are said to contain more mineral concentrations including calcium than grass species (Aregheore, 2002.) The mineral concentrations were adequate and sufficiently higher than the requirements of ruminants (NRC, 2001). Legume inclusion increased the sodium content of *A. gayanus*, thus confirming previous reports (Evitayani *et al*., 2004). K content also increased with lablab inclusion, which falls within the recommended range of 1 to 4% of DM in the diet by NRC (2001). The phosphorus content of Andropogon with double rows of *L. purpureus* cv. Rongai is the highest in this study and this can meet the dietary requirements of animals that are fed *A. gayanus* grass because phosphorus is vital for differentiation as a component of RNA and is also responsible for the formation of organic bone matrix in farm animals (NRC, 2001).

CONCLUSION

Gamba grass stays green long into the dry season when most other grasses are already dry, is easy to cut and can tolerate grazing, but there is rapid decline in crude protein and soluble carbohydrate and feeding value with age and low mineral content. It was concluded that *A. gayanus* formed a better association with *L. purpureus* cv. Rongai than with *cv. Highworth*. Double rows of *L. purpureus* are recommended as the most compatible combination to improve the forage quantity and quality of *A. gayanus*. The use of cereal-legume combinations may be attractive to organic meat or milk producers.

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Preservative Effects of Pineapple and Cucumber Juices on Viability of Refrigerated Spermatozoa of West African Dwarf Bucks

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ABSTRACT

This study investigated the preservative effects of pineapple and cucumber juices on the viability of refrigerated spermatozoa of the West African Dwarf (WAD) bucks. Pooled semen from WAD bucks was diluted in Tris-egg yolk extenders containing pineapple and cucumber juices each at 2.5, 5, 7.5 and 10ml/100ml respectively. Microscopic assessments of diluted semen samples were carried out on sperm progressive motility, acrosome and membrane integrities and sperm abnormality after *in vitro* storage at 5^{o}C for 240 hours. The concentration of malondialdehyde (MDA) in the stored semen was measured in thiobarbituric acid reactive substances. The results showed higher ($P<0.05$) sperm progressive motility in extenders supplemented with pineapple and cucumber juices compared to the control. The extenders supplemented with pineapple and cucumber juices had consistent higher ($P<0.05$) acrosome integrity up to 48 hours of post-chilling compared to the control. Higher ($P<0.05$) membrane integrity was obtained in extenders supplemented with fruit juices compared to the control and improved results were obtained in 2.5% pineapple, 2.5% and 5% cucumber fruit juices. The extenders supplemented with 2.5% and 5% cucumber juice had lower ($P<0.05$) abnormality compared to the control The results showed that extenders supplemented with fruit juices had lower ($P<0.05$) MDA concentrations and improved results were obtained at 2.5% and 5% pineapple and 2.5% cucumber fruit juices. The findings indicate preservative potential of pineapple and cucumber juices on sperm viability of chilled semen stored at 5^{o}C.

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INTRODUCTION

Mammalian sperm cells contain a high proportion of polyunsaturated fatty acids, and are susceptible to damage by excessive reactive oxygen species (ROS) causing sperm cells to deteriorate rapidly (Aitken & Fisher, 1994). When semen is stored at 5°C, there is a gradual decline in motility, functional integrity of sperm membranes and fertility (Maxwell & Salamon, 1993; De Lamirande *et al*., 1997). Antioxidants are linked with sperm viability because of their preservative effects against cell damage during preservation (Tai-Wing *et al*., 1991). Goat spermatozoa, like spermatozoa of other mammals, normally contain antioxidants but this endogenous antioxidative capacity may however be insufficient to prevent lipid peroxidation during prolonged storage (Aurich *et al*., 1997). Thus, mammalian spermatozoa lack a significant cytoplasmic component, which contains antioxidants that counteract the damaging effects of reactive oxygen species and lipid peroxidation (Storey, 2007).

Krzyosiak *et al*. (2000) reported that the addition of antioxidants to semen extender improved sperm motility and viability of bovine semen. Natural foods and food-derived antioxidants such as vitamin C and phenolic phytochemicals have received growing attention because they are known to function as chemopreventive agents against oxidative damage (Kiwon *et al*., 2003). Gardner *et al*. (2000) studying the relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices, including orange, grapefruit, pink grapefruit, apple, pineapple and vegetable-juices, found that both vitamin concentrations and total phenolic contents strongly correlated with antioxidant capacity. Improved survival rate of spermatozoa of African Catfish (*Clarias gariepinus*) extended with tomato juice when stored at 5°C (Adeyemo *et al*., 2007) has been observed. In addition, protective effect of orange juice on spermatozoa against the harmful effects of lipid peroxidation of white layer cocks' semen stored for up to 72 hours (Al-Daraji HJ, 2012) has been reported. Information on effects of pineapple and cucumber juices in extender for preserving mammalian spermatozoa is, however, not available in the literature. The objective of this study, therefore, was to determine the preservative effects of these fruit-rich antioxidants on sperm viability of WAD bucks during cold storage.

MATERIALS AND METHODS

Experimental Site and Animals

The experiment was carried out at the Goat Unit of the Directorate of University Farm, Federal University of Agriculture, Abeokuta, Nigeria located at 7^0 10'N and 3^0 2'E. It lies in the south-western part of Nigeria and has a prevailing tropical climate, a mean annual rainfall of 1,037mm and average temperature of 34.7°C. Six intact WAD bucks ranging from 2.5-3 years of age were used for this study. The animals were kept under intensive management and maintained under a uniform nutritional regimen with concentrate feed supplemented with guinea grass (*Panicum maximum*).

Preparation of Fruit Juices

The fruit juice was prepared according to the procedure of Adeyemo *et al*. (2007). Fresh ripe pineapple was washed thoroughly using distilled water. The pineapple was peeled, cut into pieces, blended for 5 minutes and placed in a sieve and pressure was applied manually to squeeze the juice out from the blended pineapple. In the case of cucumber, fully ripe cucumber was washed thoroughly using distilled water, peeled, cut into pieces and seeds were removed. Little pressure was applied to squeeze out the juice. The juices from pineapple and cucumber were separately collected each into plastic test tubes and centrifuged at 3000rpm for 20 minutes. The supernatant fluid was thereafter decanted into a clean beaker and used immediately for the experiment.

Semen Collection, Dilution and Storage

Semen samples were collected from six WAD bucks with the aid of an artificial vagina. Only ejaculates showing >80% motility were pooled. Pooled semen samples (each pool originating from six bucks) were used. Semen samples were pooled for uniformity and to eliminate individual differences. The extender for the treatment used in this study consisting of Tris-hydroxymethyl-aminomethane (2.42 g), citric acid (1.35 g), glucose (1 g), penicillin (0.028 g), egg yolk (20 ml) and distilled water made up 100ml. The pooled fresh semen was then split into nine equal fractions in different test tubes and diluted with the extenders supplemented with cucumber and pineapple juices at 2.5, 5, 7.5 and 10ml/100 ml of the diluents respectively and the control (no juice) at a final concentration of 235 x 10^6sperm/ml. The pH of the extenders (control: 7.03; pineapple juice: 7.14 and cucumber juice: 6.98) was determined using a digital pH meter. Following dilution, the diluted semen samples were sealed and chilled from 37^{o}C to 5^{o}C at approximately 0.5 ^{o}C/min and maintained at this temperature in a refrigerator for 240 hours and thereafter evaluated for sperm quality characteristics.

Semen Evaluation

Sperm progressive motility. Sperm motility was determined as described by Bearden and Fuquay (1997). Briefly, semen was thawed in Clifton Water bath (Model: 74178 by Nickel Electro Ltd, Weston-S-Mare Somerset, England) at 37^{o}C and accessed for sperm motility using Celestron PentaView compound microscope (LCD-44348 by RoHS, China) at 400 x magnification. A 5µl sample of semen was placed directly on a heated microscope slide and overlaid with a 22 x 22 mm cover slip. For each sample, five microscopic fields were examined for observe progressive sperm motility by three observers and the mean of the five successive evaluations was recorded as the final motility score.

Acrosomes integrity. The percentage of spermatozoa with intact acrosomes was determined according to Ahmad *et al*. (2003) and Ahmad *et al*. (2014). Briefly, 50µl of each semen sample was added to a 500-µl formalin citrate solution (96 ml 2.9% sodium citrate, with 4ml 37% formaldehyde) and mixed carefully. A small drop of the

mixture was placed on a microscope slide and a total of 200 spermatozoa were counted in at least three different microscopic fields for each sample, using a Celestron PentaView LCD compound microscope (400 x magnification). Intactness of acrosome characterised by normal apical ridge of 200 spermatozoa were observed and recorded.

Membrane integrity. Hypo-osmotic swelling test (HOST) was used to determine sperm membrane integrity (Jeyengran *et al*., 1884). This was done by incubating 10µl semen in 100µl Hypo-osmotic solution (7.35g sodium citrate [0.0285M] and 13.5g fructose [0.075M]) at 37^{o}C for 30 minutes; 0.1ml of the mixture was spread over a cover slip warm slide and observed under a Celestron PentaView LCD compound microscope (400 x magnification). Two hundred spermatozoa were counted and the percentage of spermatozoa positive to HOST for their swelling characterised by curled tails, indicating intact plasma membrane was determined and those with no swelling characterised by uncurled tails were classified as spermatozoa with abnormal membrane integrity.

Abnormality. Sperm morphological abnormalities were determined as described by Bearden and Fuquay (1997) with the use of eosin-nigrosin smears. Briefly, a thin smear of mixture of semen and eosin-nigrosin solution was drawn across the slide and dried. The percentage of morphologically abnormal spermatozoa with defects in the head, midpiece and tail were observed under a Celestron PentaView LCD compound microscope (400 x magnification).

MDA concentrations. At the end of every 24 hours, the levels of malondialdehyde (MDA) as indices of lipid peroxidation in the stored semen were measured in a thiobarbituric acid reactive substance (TBARS) according to Buege and Steven (1978), Armstrong and Browne (1994) and Yagi (1998). For this assay, 0.1ml of sperm suspension was incubated with 0.1ml of 150mM Tris-HCl (pH 7.1) for 20 minutes at 37°C. Subsequently, 1ml of 10% trichloroacetic acid (TCA) and 2ml of 0.375% thiobarbituric acid was added followed by incubation in a boiling water bath for 30 minutes. Thereafter, it was centrifuged for 15 minutes at 3000 rpm inside the blank tube and the absorbance was read with a spectrophotometer at 532nm. The concentration of MDA was calculated as follows: The concentration of malondialdehyde MDA (nmol/ml) = AT – AB/1.56 × 10^5; where: AT = the absorbance of the sample serum, AB = the absorbance of the blank, 1.56 × 10^5 molar absorptivity of MDA.

Statistical Analysis

Data obtained were subjected to a two-way analysis of variance (ANOVA) using SPSS version 16 and means separated by the Duncan Multiple Range Test (Duncan, 1955) in the model below:

$$Y_{ijkl} = \mu + A_i + L_j + T_k + (AL)_{ij} + (AT)_{ik} + (LT)_{jk} + (ALT)_{ijk} + \sum_{ijkl}$$

where,

Y_{ijkl}= Dependent variables

μ = Population mean

A_i= effect due to i^{th} fruit juices, i = 1, 2

L_j = effect due to j^{th} level of inclusion, j = 0, 2.5, 5, 7.5, 10

T_k= effect due to k^{th} duration of storage, k = 0, 24, 48, 72, 96 …….240

$(AL)_{ij}$= effect due to ij^{th} interaction between fruit juices and levels of inclusion

$(AT)_{ik}$= effect due to ik^{th} interaction between fruit juices and storage duration

$(LT)_{jk}$= effect due to jk^{th} interaction between levels of inclusion and storage duration

$(ALT)_{ijk}$= effects due to ijk^{th} interaction between fruit juices, levels of inclusion and storage duration

$\sum_{ijkl}$= Experimental error

RESULTS AND DISCUSSION

The results (Table 1) showed higher ($P<0.05$) sperm progressive motility in extenders supplemented with pineapple and cucumber juices compared to the control group. The inclusion of pineapple and cucumber juices in tris-egg yolk extender for *in vitro* cold storage of semen obtained from WAD bucks in this study indicated that these fruit juices have the ability to sustain progressive motility. This could be attributed to the high level of vitamin C, E and other antioxidants present in these fruits (Gebhardt & Thomas, 2002; Djuric & Powell, 2001). Reza *et al*. (2011) showed that vitamin E or C supplementation in stored semen improved motility of spermatozoa. The wholesome effects on sperm viability that accompanied supplementation of semen extender with the fruit juices could be on account of their potential source of vitamin C, a water-soluble antioxidant (Martin *et al*., 2002; Mermeistein, 1999) and vitamin E, both naturally occurring free radical scavengers known to scavenge superoxide anions and singlet oxygen and protect lipoproteins from detectable peroxidative damage (Wainer *et al*., 1986, Donnelly *et al*., 1999). In contrast however, Aurich *et al*. (1997) reported that addition of ascorbic acid did not improve the maintenance of motility of cooled equine spermatozoa during the 96-hour storage period. The antioxidant capacity of the juices might not, however, be due to the vitamin C only but could also arise from some phenolic compounds present in these fruits (Spanos & Wrolstad, 2004). The major phenolic compound in fruits is ferulic acid (Augustin & Williams, 2000), that neutralises free radicals known as superoxide, hydroxyl radical and nitric oxide and in addition acts synergistically with other antioxidants, giving them extra potency to reduce free radical damage to cell membranes (Zuo *et al*., 2002). Progressive motility recorded at various levels of juices could therefore be associated with the concentrations of the vitamins (USDA, 2009) and phenolic compounds present in the juices (Cutler *et al*., 2008). Moreover, in the present study, the concentrations of fruit juices as antioxidants used might have been optimum for preserving buck sperm progressive motility, as effects of pineapple and cucumber juices varied with the level

of supplementation in the extender. Sperm progressive motility was maintained longer at concentrations of 5% of pineapple and 7.5% cucumber juices for 192 and 144 hours respectively.

The extenders supplemented with various concentrations of pineapple and cucumber juices had consistently higher ($P<0.05$) acrosome integrity up to 48 hours of post-chilling compared to the control (Table 2). The results (Table 3) showed consistently higher ($P<0.05$) membrane integrity in extenders supplemented with fruit juices compared to the control and this was more pronounced in extenders supplemented with 2.5% pineapple, 2.5% and 5% cucumber fruit juices after post-chilling. Interestingly, in the present study, the extenders supplemented with fruit juices had not only improved sperm progressive motility, but also enhanced the acrosome integrity and membrane integrity. The beneficial effect of antioxidants from pineapple and cucumber juices on intact acrosome and membrane integrity in the extenders supplemented with fruit juices during cold storage compared to control group observed in this study could be linked to vitamin C and other antioxidative compounds in these juices (Spanos & Wrolstad, 2004; Reza *et al*., 2011). The finding corroborates previous report (Zheng & Zhang, 1997) that ferulic acid was beneficial to sperm viability and reduction of lipid peroxidative damage to sperm membranes. The antioxidant potential of ferulic acid is attributed to its structural characteristics because its phenolic nucleus and unsaturated side chain readily forms a resonance stabilised radical, which accounts for its potent antioxidant activity (Marimuthu *et al*., 2007).

The extenders supplemented with 2.5% and 5% cucumber juice had consistent lower ($P<0.05$) abnormality compared to the control (Table 4). The lower percentage of sperm abnormality observed in the extenders supplemented with these levels of cucumber juice compared to the control after post-chilling suggested that the supplementation had beneficial effects on sperm morphology. It is relevant to mention that semen processing does not necessarily increase the proportion of sperm abnormalities (Revell, 2003). Moreover, the percentage sperm abnormalities observed were within the range for post-thawed goat semen as per the Brazilian College of Animal Reproduction (Henry & Neves, 1998) in extender supplemented with the fruit juice and control.

The results (Table 5) showed that extenders supplemented with fruit juices had lower ($P<0.05$) MDA concentrations compared to the control, and improved results were obtained at 2.5% and 5% pineapple and 2.5% cucumber fruit juices. Flavonoids have antioxidant capacity that is stronger than that of vitamin C and E used to prevent free radical production (Alía *et al*., 2003). The finding indicated that supplementation of tris-egg yolk extender with fruit juices from pineapple and cucumber possibly reduced the suppressive effects of lipid peroxidation on the metabolic activity of buck spermatozoa.

TABLE 1
Progressive Motility (%) of Buck Spermatozoa Chilled with Tris Egg Yolk Extenders Supplemented with Juices

Duration (h)	Control	Pineapple (%)				Cucumber (%)				SEM
		2.5	5	7.5	10	2.5	5	7.5	10	
0	80.00^{c}	90.00^{a}	84.00^{b}	86.00^{b}	84.00^{b}	92.00^{a}	94.00^{a}	92.00^{a}	86.00^{b}	1.171
24	60.00^{d}	88.00^{a}	82.00^{b}	74.00^{c}	50.00^{e}	84.00^{b}	90.00^{a}	88.00^{a}	82.00^{b}	2.242
48	50.00^{e}	80.00^{b}	70.00^{c}	64.00^{d}	46.00^{e}	74.00^{c}	90.00^{a}	84.00^{b}	76.00^{c}	2.557
72	40.00^{d}	78.00^{a}	70.00^{b}	50.00^{c}	44.00^{d}	74.00^{b}	82.00^{a}	80.00^{a}	70.00^{b}	2.714
96	16.00^{d}	76.00^{a}	68.00^{b}	42.00^{c}	12.00^{d}	70.00^{ab}	70.00^{ab}	72.00^{ab}	68.00^{b}	3.571
120	0.00^{f}	60.00^{b}	66.00^{a}	40.00^{d}	10.00^{e}	58.00^{c}	64.00^{b}	70.00^{a}	66.00^{a}	3.685
144	0.00^{f}	52.00^{b}	64.00^{a}	40.00^{c}	10.00^{e}	42.00^{c}	38.00^{d}	68.00^{a}	36.00^{d}	3.571
168	0.00^{f}	32.00^{c}	58.00^{a}	38.00^{b}	4.00^{e}	30.00^{c}	16.00^{d}	36.00^{b}	32.00^{c}	2.857
192	0.00^{e}	30.00^{b}	50.00^{a}	20.00^{c}	0.00^{e}	30.00^{b}	14.00^{d}	34.00^{b}	30.00^{b}	2.457
216	0.00^{d}	22.00^{b}	26.00^{a}	12.00^{c}	0.00^{d}	30.00^{a}	12.00^{c}	30.00^{a}	22.00^{b}	1.900
240	0.00^{e}	6.00^{c}	11.00^{ab}	6.00^{c}	0.00^{e}	8.00^{c}	10.00^{b}	12.00^{a}	4.00^{cd}	1.104

^{abcdef} Values within rows with different superscripts differ significantly ($P<0.05$). SEM: Standard Error of Means

TABLE 2
Acrosome Integrity (%) of Buck Spermatozoa Chilled with Tris Egg Yolk Extenders Supplemented with Juices

Duration (h)	Control	Pineapple (%)				Cucumber (%)				SEM
		2.5	5	7.5	10	2.5	5	7.5	10	
0	92.75^{b}	97.25^{a}	99.00^{a}	98.00^{a}	99.00^{a}	98.00^{a}	97.75^{a}	97.00^{a}	98.50^{a}	0.408
24	92.25^{b}	96.75^{a}	97.50^{a}	98.00^{a}	97.50^{a}	97.25^{a}	97.25^{a}	96.70^{a}	97.00^{a}	0.370
48	88.50^{b}	94.50^{a}	94.00^{a}	94.50^{a}	93.00^{a}	93.25^{a}	94.67^{a}	93.75^{a}	93.75^{a}	0.352
72	84.25^{b}	91.00^{a}	91.50^{a}	84.00^{b}	89.00^{a}	91.00^{a}	91.25^{a}	89.75^{a}	90.75^{a}	0.382
96	80.00^{b}	86.00^{a}	88.50^{a}	82.00^{b}	83.00^{b}	88.00^{a}	87.25^{a}	86.70^{a}	84.00^{a}	0.450
120	75.00^{b}	81.00^{ab}	83.89^{ab}	78.50^{b}	78.50^{b}	82.50^{ab}	82.00^{ab}	81.50^{ab}	79.75^{ab}	0.440
144	71.50^{c}	79.00^{a}	69.50^{c}	71.50^{c}	75.00^{b}	79.00^{a}	77.25^{a}	78.00^{a}	75.50^{b}	0.446

TABLE 2 (*continued*)

Duration (h)	Control	Pineapple (%)				Cucumber (%)				SEM
		2.5	5	7.5	10	2.5	5	7.5	10	
168	65.50^{b}	68.00^{a}	61.50^{c}	60.20^{c}	69.00^{a}	70.00^{a}	68.75^{a}	68.00^{a}	69.50^{a}	0.506
192	58.75^{b}	58.50^{b}	56.00^{b}	58.50^{b}	60.00^{a}	62.50^{a}	62.00^{a}	63.00^{a}	58.75^{b}	0.581
216	53.50^{b}	52.50^{b}	49.00^{c}	55.50^{a}	53.00^{b}	55.75^{a}	55.50^{a}	57.00^{a}	53.00^{b}	0.501
240	45.50^{b}	49.25^{a}	41.00^{c}	47.50^{a}	42.00^{c}	46.75^{a}	47.25^{a}	47.00^{a}	44.75^{b}	0.448

^{abcdef} Values within rows with different superscripts differ significantly (P<0.05). SEM: Standard Error of Means

TABLE 3
Membrane Integrity (%) of Buck Spermatozoa Chilled with Tris Egg Yolk Extenders Supplemented with Juices

Duration (h)	Control	Pineapple (%)				Cucumber (%)				SEM
		2.5	5	7.5	10	2.5	5	7.5	10	
0	89.75^{b}	89.00^{b}	91.00^{a}	92.00^{a}	90.00^{a}	93.00^{a}	93.00^{a}	92.00^{a}	92.50^{a}	0.456
24	88.75^{a}	87.00^{a}	86.00^{a}	86.50^{a}	83.50^{b}	86.50^{a}	87.00^{a}	85.00^{a}	84.00^{b}	0.420
48	81.75^{b}	85.50^{a}	80.00^{b}	82.50^{b}	79.00^{b}	81.00^{b}	83.00^{a}	81.50^{b}	81.50^{b}	0.375
72	74.75^{c}	80.00^{a}	78.00^{a}	77.00^{b}	76.00^{b}	78.00^{a}	79.50^{a}	77.00^{b}	77.50^{b}	0.346
96	71.50^{b}	75.00^{a}	74.00^{a}	75.00^{a}	73.50^{a}	75.50^{a}	74.50^{a}	72.00^{b}	73.00^{a}	0.326
120	65.50^{c}	73.00^{a}	70.00^{a}	73.00^{a}	69.00^{b}	74.00^{a}	73.50^{a}	70.00^{a}	71.00^{a}	0.485
144	59.50^{c}	70.50^{a}	65.00^{b}	69.00^{a}	61.50^{c}	70.00^{a}	70.00^{a}	66.00^{b}	68.50^{a}	0.561
168	55.00^{c}	65.00^{a}	58.50^{b}	63.00^{a}	54.33^{c}	65.50^{a}	64.00^{a}	63.00^{a}	63.50^{a}	0.548
192	49.25^{c}	60.00^{a}	54.00^{b}	50.40^{c}	50.00^{c}	61.50^{a}	59.50^{a}	57.00^{a}	50.00^{c}	0.600
216	43.50^{c}	55.00^{a}	49.00^{b}	48.00^{b}	44.00^{c}	56.50^{a}	54.50^{a}	50.00^{b}	48.00^{b}	0.635
240	38.50^{d}	50.00^{a}	43.50^{bc}	44.00^{b}	33.00^{e}	50.50^{a}	49.50^{a}	46.00^{b}	40.00^{c}	0.620

^{abcdef} Values within rows with different superscripts differ significantly (P<0.05). SEM: Standard Error of Means

TABLE 4
Abnormality (%) of Buck Spermatozoa Chilled with Tris Egg Yolk Extenders Supplemented with Juices

Duration (h)	Control	Pineapple (%)				Cucumber (%)				SEM
		2.5	5	7.5	10	2.5	5	7.5	10	
0	1.50^{a}	1.00^{a}	1.50^{a}	0.25^{b}	0.50^{b}	0.50^{b}	1.50^{a}	0.25^{b}	1.00^{a}	0.276
24	2.00^{a}	2.00^{a}	3.00^{a}	2.70^{a}	3.00^{a}	1.25^{b}	1.50^{b}	1.50^{b}	1.25^{b}	0.273
48	3.50^{a}	3.00^{a}	3.50^{a}	3.50^{a}	3.70^{a}	1.50^{b}	2.70^{b}	2.50^{b}	2.75^{a}	0.241
72	4.70^{a}	3.50^{a}	4.00^{a}	4.50^{a}	4.00^{a}	2.00^{b}	2.80^{b}	3.00^{b}	3.50^{a}	0.233
96	5.50^{a}	4.75^{a}	4.70^{a}	5.50^{a}	4.50^{a}	3.00^{b}	3.20^{b}	3.50^{b}	4.50^{a}	0.233
120	6.00^{a}	5.00^{a}	5.50^{a}	6.00^{a}	5.25^{a}	4.50^{b}	3.70^{b}	4.50^{b}	4.75^{b}	0.236
144	7.00^{a}	5.70^{b}	6.25^{a}	6.50^{a}	5.50^{b}	5.00^{b}	4.50^{c}	6.00^{a}	5.00^{b}	0.196
168	7.75^{a}	6.00^{a}	6.50^{a}	6.50^{a}	6.00^{a}	5.70^{b}	5.70^{b}	7.00^{a}	6.00^{a}	0.160
192	8.00^{a}	7.00^{a}	6.75^{b}	7.40^{a}	6.50^{b}	6.00^{b}	6.70^{b}	7.50^{a}	6.50^{b}	0.151
216	8.50^{a}	7.50^{a}	7.00^{a}	8.00^{a}	6.67^{b}	6.50^{b}	7.00^{b}	8.00^{a}	7.70^{a}	0.143
240	9.50^{a}	8.00^{b}	7.50^{bc}	8.50^{b}	8.00^{b}	7.00^{c}	8.00^{b}	8.75^{b}	8.00^{b}	0.126

^{abcdef} Values within rows with different superscripts differ significantly ($P<0.05$). SEM: Standard Error of Means

TABLE 5
MDA Concentrations (nmol/ml) of Buck Spermatozoa Chilled with Tris Egg Yolk Extenders Supplemented with Juices

Duration (h)	Control	Pineapple (%)				Cucumber (%)				SEM
		2.5	5	7.5	10	2.5	5	7.5	10	
24	0.08^{a}	0.01^{e}	0.02^{c}	0.02^{c}	0.04^{b}	0.01^{e}	0.03^{d}	0.03^{d}	0.04^{b}	0.006
48	0.17^{a}	0.02^{c}	0.03^{b}	0.03^{b}	0.03^{b}	0.01^{d}	0.01^{d}	0.02^{c}	0.03^{b}	0.013
72	0.20^{a}	0.08^{d}	0.10^{c}	0.10^{c}	0.11^{b}	0.05^{e}	0.11^{b}	0.07^{e}	0.09^{d}	0.012
96	0.23^{a}	0.13^{c}	0.13^{c}	0.14^{b}	0.15^{b}	0.11^{d}	0.12^{d}	0.11^{d}	0.13^{c}	0.009
120	0.24^{a}	0.13^{e}	0.16^{c}	0.17^{c}	0.19^{b}	0.13^{e}	0.15^{d}	0.12^{e}	0.15^{d}	0.010
144	0.25^{a}	0.15^{e}	0.17^{d}	0.19^{c}	0.21^{b}	0.13^{f}	0.14^{e}	0.14^{e}	0.16^{d}	0.009
168	0.27^{a}	0.17^{e}	0.19^{d}	0.23^{c}	0.24^{b}	0.16^{e}	0.17^{e}	0.18^{d}	0.19^{d}	0.008

TABLE 5 (*continued*)

Duration (h)	Control	Pineapple (%)				Cucumber (%)				SEM
		2.5	5	7.5	10	2.5	5	7.5	10	
192	0.50^{a}	0.32^{c}	0.32^{c}	0.32^{c}	0.37^{b}	0.26^{e}	0.27^{e}	0.28^{e}	0.30^{d}	0.020
216	0.55^{a}	0.42^{d}	0.43^{d}	0.44^{c}	0.45^{b}	0.37^{e}	0.37^{e}	0.40^{e}	0.40^{e}	0.014
240	0.76^{a}	0.45^{e}	0.46^{e}	0.51^{c}	0.64^{b}	0.46^{e}	0.50^{d}	0.50^{d}	0.52^{c}	0.025

^{abcdef} Values within rows with different superscripts differ significantly ($P<0.05$). SEM: Standard Error of Means

The results of the present study, therefore, suggest that the antioxidants from pineapple and cucumber juices probably work by removing hydrogen peroxide from the medium, thus preventing the generation of hydroxyl radicals, which are powerful oxidants by the Fenton reaction (O'Flaherty *et al*., 2003). This effect may explain the current findings of improved progressive motility, acrosome and membrane integrity, lower abnormalities and reduced MDA when pineapple and cucumber juices were added to the extenders.

CONCLUSION

The improved progressive motility, acrosome and membrane integrities, reduced abnormalities and MDA following supplementation with pineapple and cucumber juices in the extenders indicate that antioxidant properties of these juices may be involved in beneficial effect on the sperm viability and the fruit juices may be used for sperm preservation of chilled West African Dwarf buck semen.

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Combined Effect of Soil Applied Iron and Sulfur Fertilisers on Monoterpene Content and Antioxidant Activity of *Satureja hortensis* L. Extract

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ABSTRACT

Chemical-composition/antioxidant-activity of *Satureja hortensis* L. extract is influenced by many factors including nutrient elements. A factorial completely randomized design greenhouse experiment was conducted to study the effect of three levels of soil-applied iron, Fe (0, 8 and 16mg kg^{-1} soil as ethylene-di-amine-die-hydroxyl-acetic-acid, Fe-EDDHA) and three levels of sulfur, S (0, 50 and 100mg kg^{-1} soil as elemental-S) on monoterpene production and antioxidant activity of *Satureja hortensis* L. The maximum (377.75 mg/L) and minimum (720.406 mg/L) antioxidant activity were obtained with 8mg Fe+100mg S treatment and control, respectively. The main oil constituents in control were γ-terpinene(67%), α-terpinene(11%), myrcene (4%), α-thujene (4%), p-cymene (4%), α-pinene (3%) and carvacrol (2%). The maximum content of α-thujene, α-pinene, myrcene and α-terpinene was obtained with 8mg Fe+50mg S application whereas the control was suitable for obtaining γ-terpinene. Carvacrol was mainly produced with addition of 16mg Fe+100mg S. Furthermore, the α-thujene, α-pinene, myrcene and α-terpinene contents increased with application of 8mg Fe+50mg S. The α-terpinene, myrcene, α-thujene and α-pinene increased by 13, 27, 21 and 43% compared to control, respectively when 8mg Fe+50mg S was applied. The entire component of monoterpenoid fraction with the major constituent of γ-terpinene, α-terpinene, myrcene, α-thujene, p-cymene, α-pinene and carvacrol that constitutes 99.9% of essential oil showed a same trend whereas identified sesquiterpenes and sesquiterpenoid components were relatively low (0.1%). The low molecular weight of γ-terpinene decreased as 8mg Fe+50mg S was applied. In general, it could be concluded that application of 8mg Fe+50mg S kg^{-1} was the most suitable treatment for obtaining

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higher amounts of α-terpinene, myrcene, α-thujene and α-pinene whereas addition of 16mg Fe+100mg S kg^{-1} was preferable for obtaining carvacrol.

Keywords: Fertiliser, monoterpene production, lamiaceae *Satureja hortensis*, savory, antioxidant activity

INTRODUCTION

Aromatic plants have been used as natural food additives and spices from a very long time ago due to their antiseptic and aromatic characteristics. Furthermore, these plants have medicinal and industrial applications. The genus *Satureja* L. (savory, saturei) with more than 30 species is one of the most famous aromatic plant species. These species produce essential oils and some secondary metabolites such as antimicrobial agents in their normal growth and development processes or in response to environmental stresses e.g. drought and temperature stresses, nutrient deficiency or toxicity, attack of pathogens etc. *Satureja hortensis* L. species is one of the most well-known species. In addition to having widespread application in cooking and the food industry, it is commonly used in the treatment of some diseases. Therefore, studying the chemical composition of essential oils and extracts of their above-ground parts is very important. The common application of *S. hortensis* L. as a natural compound in the treatment of inflammatory disorders, muscle troubles, spasms and many other disorders as well as in conservation of foods has been justified by some biological and pharmacological investigators due to the presence of anti-spasmodic (Leporatti & Ivancheva, 2003; Hajhashemi *et al*., 2000), anti-oxidant and anti-bacterial (Dorman & Hiltunen, 2003; Adiguzel *et al.,* 2007) and anti-fungal characteristics. Furthermore, high activity of its essential oil against clinical multi-resistant isolates from injuries has been reported by Mihajilov-Krstev *et al*. (2009). Stutte (2006) reported that environmental factors strongly affected the bio-synthesis of secondary metabolites in aromatic or medicinal plants. In this context, the use of organic and chemical fertilisers may increase the amount of essential oil or other major constituents produced by medicinal plants (Khalid *et al*., 2006).

Macro- and micronutrients are important and effective agents on plant yield and composition. High yielding crops need large and regular supply of macro- and micronutrients to develop high photosynthetic capacity and maintain the proper element concentration in the leaves (Lawlor, 1995). The lack of nutrients such as iron and sulfur reduces plant growth (Heidari *et al*., 2011). Sulfur (S) is needed for the optimal production of nutrients; it is also needed in the enzymatic and structural functions of the plant. Sulfur is required for synthesis of protein as a vital constituent of many essential amino acids. It is also needed in synthesis of chlorophyll. It has been reported that the biochemical structure of plant oils mainly depends on the amount of S as one of the major essential nutrients (Mengel & Kirkby, 1978). Sulfur deficiency

could decrease the uptake of nitrogen, yield of crop and plant quality (Marschner, 1995). Ahmad and Sharma (2008) reported that the uptake of S and its assimilation play a key role in determination of yield and quality of seeds in higher plants, and also in resistance to environmental stresses like pests. Higher plants require a continuous supply of sulfur from seed emergence to maturity due to its immobile nature within plants. Khan and Hussain (1999) reported the maximum yield of mustard (*Brassica juncea*) seed and oil was gained with application of 20kg S ha^{-1}. Alizadeh *et al*. (2010) stated that the fresh and dry weight of *S. hortensis* as well as its essential oil yield and efficiency increased in response to applied complete fertiliser. They recorded that 19 components in the essential oil of *S. hortensis* underwent different treatments that represented 97.58-99.24% of its oils, and of these, the main constituents were carvacrol (43.9-59.2%), γ-terpinene (30.7-40.2%), α-terpinene (2.8-4%) and *P*-cymene (1.8-2.2%). They reported that composition of essential oil did not affect significantly in response to different levels of applied fertiliser. They stated that the amount of specific constituents like γ-terpinene, α-terpinene and carvacrol were decreased significantly by fertiliser application while phenolic content and antioxidant activity increased. Zheljazkov *et al*. (2008) showed that application of N and S fertilisers had positive effects on biomass and oil yield of sweet basil as well as on the chemical composition of the plant's oil.

Among the micronutrients, iron plays an important role in the growth and development of plants. Marschner (1995) stated that micronutrients, especially Fe, act either as metal components of various enzymes or as functional, structural or regulatory cofactors; thus, it is associated with saccharide metabolism, photosynthesis and protein synthesis. Iron has important functions in plant metabolism, such as activating catalase enzymes associated with superoxide dismutase as well as in photorespiration, the glycolate pathway and chlorophyll content (Marschner, 1995). Blakrishman (2000) showed that Fe caused an increase in activity of catalase, peroxidase and cytochrome oxidase enzymes. Nasiri *et al*. (2010) reported that foliar applied iron and zinc increased the flower yield of chamomile and the percentage of its essential oil significantly over the control. Yeritsyan and Economakis (2002) determined the growth parameter of oregano and the yield of its essential oil in a hydroponic culture in response to application of three levels of Fe-EDTA (2.5, 5 and 11mg/l). They showed that the content of essential oil decreased when the highest level of Fe (11 mg/L) was applied. Furthermore, they stated that the amount of biomass and essential oil decreased in response to a high level of Fe concentration. Abd El- Wahab (2008) reported that micronutrients such as Fe, Mn and Zn have important roles in plant growth and yield of aromatic and medicinal plants. Furthermore, the role of Fe in biological redox systems (electron transfer chain in photosynthesis and respiration), nitrogen fixation, chloroplast development, enzyme activation, heme proteins (cytochromes,

catalase, peroxidase), Fe-S proteins (e.g. ferredoxin, isoenzymes of superoxide dismutase, aconitase), is well known and documented (Welch, 1995).

Due to the demand for medicinal plants and herbal remedies in the world, the cultivation of these plants has significantly increased in recent years. Meanwhile, the use of chemical fertilisers and nutrients to increase the yield has become very popular (Yazdani *et al*., 2004). However, there has been little research into the effect of different amounts of chemical fertilisers on bioactive components and secondary metabolites in aromatic and medicinal plants. Therefore, this study aimed to evaluate the combined effect of soil applied iron (Fe) and sulfur (S) on essential-oil composition and antioxidant activity of *Satureja hortensis* grown on calcareous soil.

MATERIAL AND METHODS

Soil Analysis

The experiment was carried out on loamy calcareous soil [Typic Calcixerepts] with EC of 0.39mmho cm^{-1}, CCE of 44.8%; organic carbon (OC) of 0.87%; pH of 7.76; available P of 4.47mg kg^{-1}(Olsen *et al*., 1954), DTPA extractable (Lindsay & Norvell, 1978) copper (Cu), manganese (Mn), zinc (Zn), and iron (Fe) content of 1.03, 0.957, 3.73 and 2.34mg kg^{-1} soil, respectively. The mentioned attributes of the studied soil were measured using standard methods.

Statistical Design of Experiment

A completely randomised design experiment with six replications was conducted in greenhouse conditions. Treatments consisted of control (without any Fe or S application), soil application of 8mg Fe and 50mg S kg^{-1} soil (8Fe+50S), 8mg Fe and 100mg S kg^{-1} soil (8Fe+100S), 16mg Fe and 50mg S kg^{-1} soil (16Fe+50S), and 16mg Fe and 100mg S kg^{-1} soil (16Fe+100S). Iron and S were applied as iron-ethylenediamine di-o-hydroxyphenylacetic acid (Fe-EDDHA) and elemental sulfur, respectively.

Soil Preparation and Satureja hortensis planting

Each pot contained 3kg soil. Aforementioned Fe and S treatments were applied before planting. For preventing any probable nutrient deficiency other than Fe and S, 60mg P as $Ca(H_2PO_4)_2.H_2O$, 30, 30 and 15mg Mn, Zn and Cu as in solutions of their sulfates were added uniformly to each pot. Nitrogen (450 mg) was added as $CO(NH_2)_2$ to each pot as well (one half of N was added before planting and the remainder was added as dressed application three weeks after emergence). Twenty seeds of *Satureja hortensis* were planted at a depth of about 10-mm and were reduced to 10 uniform plants two weeks after emergence. Plants were irrigated with distilled water to near FC (field capacity) and maintained at this level of moisture with addition of water to a constant weight. At the 12^{th} week after emergence, the plants were harvested. The plant samples were dried at shade and prepared for analysis.

Extraction of Plant Samples

For extraction of the metabolites in the

plant samples the following steps were carried out: 20g of dry matter was soaked in 0.2L of methanol/water (90/10) solution for 48h (the solvent was changed after 24h). The filtered extract was then concentrated using a rotary evaporator for <10 minutes. The yield was determined by weighting the obtained powder. The powder was preserved at -20°C until used. Just before each analysis, the desired amounts of powder dissolved in methanol and its antioxidant activity and the total content of phenol were determined.

Antioxidant Activity Measurement

The extract of the plant was tested to determine the antioxidant activity and the standard antioxidants on the basis of the radical-scavenging effect of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. gallic acid was used as a standard solution. An improved assay method proposed by of Bruits *et al*. (2001) wherein 0.2 ml of a 0.1M solution of DPPH radical in methanol was mixed with 0.02ml of 0.0125 and 3.200mg ml^{-1} of extracts and gallic acid, respectively was used. The solutions were kept at lab temperature for 0.5h. A micro-plate reader of Biotek ELx 808 model at 515nm was used to measure the DPPH-radical-inhibition. The half maximal inhibitory concentration (IC_{50}) of each extract (concentration in µg/ml required to inhibit DPPH radical formation by 50%) was calculated by MATLAB software package. The methanolic extract solution without DPPH was considered as a blank. Antioxidant activity (AOA) was calculated using the following equation:

$$AOA = 1- [A_{sample}-A_{blank} /A_{control}] \quad [1]$$

where A_{sample} is the absorbance of the samples. $A_{control}$ and A_{blank} correspond to absorbance of DPPH (without plant extract) and methanol, respectively.

The value of IC_{50} (the concentration of the test sample leading to 50-% reduction of the initial DPPH concentration) was determined for each extract sample from the nonlinear regression of the mean value (%) of activity for radical scavenging vs. the log-concentration of the test extract (µg/ml). The IC_{50} value is an appropriate way to measure the oxidation progress in oils and is therefore considered a good indicator for the effectiveness of antioxidants.

Extraction of Headspace Volatiles

Up to 0.003kg of each air-dried *Satureja hortensis* sample was crushed, placed in a 0.02L headspace vial and sealed with silicone-rubber septa and aluminum caps immediately. The vials were then transported to the headspace tray. The headspace continued on the ombiPAL system that consisted of a headspace auto-sampler, agitator and heater. The vial was heated up to 80°C and reserved for 20min while being agitated; the temperature of the sampling needle and transmission lines was 85°C.

Determining of the Oil Components by GC/MS

The GC-MS analysis was conducted using Agilent 7890 operating equipped with a HP-5 MS capillary column (phenyl-methyl-

siloxane, 30mx0.25mm i.dx 25pm) with split ratio of 1:50 and carrier gas of He at 70eV ionisation energy. The retention times of n-alkanes that were injected after the essential oil under the same chromatographic conditions was used to determine the retention indices. The N-alkanes were used as a standard in the determination of retention indices for all the constituents. The identified compounds from the retention indices (RRI, HP-5) were compared with those reported in the literature and by comparison of their mass spectra with the Adams Library, Wiley GC/MS Library, MassFinder 2.1 Library data published mass spectra data (Joulain *et al*., 2001; Adams, 2007; Adams & Yanke, 2007).

Statistical Analysis

The data were statistically analysed using MSTATC (Michigan State University, East Lansing, MI, USA) and Excel (Microsoft, Redmond, WA, USA) software packages and the mean values of the plant responses were compared statistically using Duncan's multiple range test at the probability level of 0.05.

RESULTS AND DISCUSSION

Antioxidant Activity

The combined effect of Fe and S application on the inhibitory effect of *Satureja hortensis* extract is shown in Fig.1. All the treatments possessed antioxidant potential, but variations were observed among them. All the extracts showed significant amounts of inhibitory effects (IC_{50}) from 377.75mg/l in treatment of 8Fe+100S (application of 8mg Fe and 100mg S per kg soil) to 720.406mg/l in control (Fig.1) and decreased in the following order:

8Fe+100S > 8Fe+50S > 16Fe+50S > 16Fe+100S > 0Fe+0S

The results indicated that the best plant was obtained with application of 8mg Fe + 100mg S kg^{-1} soil (IC_{50}=377.75mg/l), whereas the control plants showed the lowest antioxidant activity. Erdemoglu *et al*. (2006) reported that among 60 studied plants of Iran, *S.macrosiphon* (IC_{50} = 2.96 µg) and *S. hypoleuca* (IC_{50}=5.27 µg) failed to show significant antioxidant activity. Another research reported that the *S.macrosiphon* with 404.12mmol of $FeSO_4$ $100g^{-1}$ showed significant antioxidant activity using ferric ion reducing antioxidant power (FRAP) assay (Gohari *et al*., 2011). In the present study all the extracts showed significant amounts of inhibitory effects from 720.4mg/l in control to 377.75mg/l with application of 8 mg Fe+100mg S kg^{-1} by DPPH (Fig.1). Extensive research has been conducted into the antioxidant activity of some species of the Lamiaceae family (Shan *et al*., 2005). Shan *et al*. indicated that this plant family is apowerful antioxidant producer. Some investigators have reported that rosemary had the highest antioxidant effect whereas sage, oregano and basil had the lowest antioxidant activity. Similar to our findings, a linear relationship between the content of total phenolic compounds and their antioxidant

capacity has been demonstrated by some investigators (Djeridane *et al*., 2006; Katsube *et al*., 2004). The maximum antioxidant effect was obtained with application of 8mg Fe + 100mg S kg^{-1} soil. It could be ascribed to an increase in main phenolic content (carvacrol) by about of 1.6 fold because of positive correlation between phenolic contents and antioxidant activity. In other words, our findings revealed that aforementioned levels of applied fertilisers could improve the antioxidant activity of *S. hortensis*. As mentioned (Halliwell & Gutteridge, 1999; Miguel, 2010), plant phenols exhibit in-vitro antioxidant activity, inhibiting lipid peroxidation by acting as chain-breaking peroxyl-radical scavengers. Phenols with two adjacent hydroxyl groups can bind transition metal ions e.g. Fe and Cu. In addition, phenols directly scavenge reactive oxygen species (hydroxyl radicals, peroxynitrite and hypochlorous acid). Sometimes phenols can act as pro-oxidants by reducing transition metal ions.

In contrast, Azaizeh *et al*.(2005) reported that application of chemical fertilisers decreased the antioxidant activity of some other medicinal plants e.g. *E. creticum*. They believed that leaf senescence of studied cultivated plants may account for differences in antioxidant activity in response to different fertilisation regimes.

Chemical Composition of Essential Oil

The identified constituents with their respective RIs and percentages are summarised in Table 1. The main constituents of the oils in control were γ-terpinene (67.4%), α-terpinene (10.8%), myrcene (4.4%), α-thujene (4.2%), p-cymene (3.8%), α-pinene (2.8%) and carvacrol (2.1%). The results showed

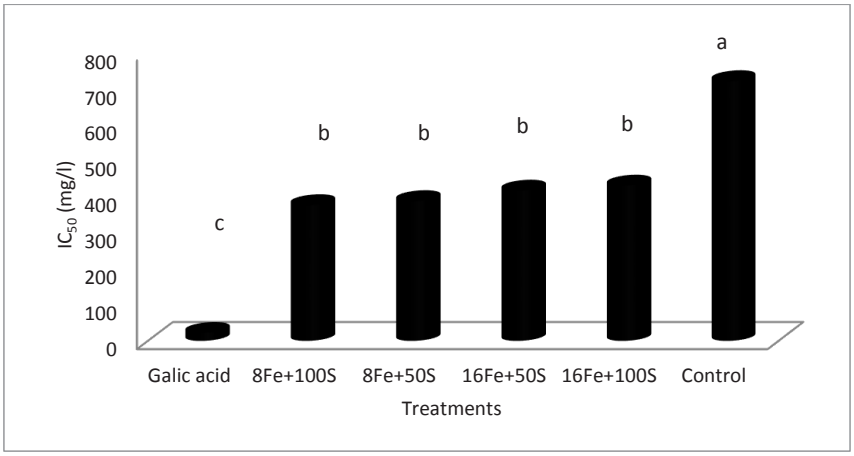

Fig.1: Comparison of antioxidant activity between treatments and gallic acid by DPPH assay. Treatments consisted of 8mg Fe kg^{-1} soil+50mg S kg^{-1} soil, 8mg Fe kg^{-1} soil+100mg S kg^{-1} soil, 16mg Fe kg^{-1} soil+50mg S kg^{-1} soil and 16mg Fe kg^{-1} soil+50mg S kg^{-1} soil (columns with the same statistical letter are not significantly ($P < 0.05$) different by Duncan's multiple range test).

that the maximum α-thujene, α-pinene, myrcene and α-terpinene percentage was obtained with application of 8mg Fe+50mg S kg^{-1} whereas the control was suitable for obtaining special components such as γ-terpinene. On the other hand, carvacrol was mainly produced with the application of 16mg Fe+100mg S kg^{-1}. The most important results of this study were the increasing trend in the quantities of α-thujene, α-pinene, myrcene and α-terpinene with the application of 8mg Fe+50mg S kg^{-1}. The α-terpinene was 10.8% in the control and reached to 12.2% with an application of 8mg Fe+50mg S kg^{-1}, an enhancement equal to 13.0% (Fig.2 and Table 1). The higher percentage of aforementioned constituents in essential oil composition of Fe and S treated plants may correspond to participation of these two studied elements in essential oil constituents or may be due to the improved growth conditions for plants when these two elements were applied. Other researchers like Mengel and Kirkby (1978) also stated that S is one of the major essential nutrients that plays a key role in the biochemical structure of plant oils. Besides, Wierdak (2013) stated that growing conditions, irrigation, cultivation method, fertilisation and date of harvest of plant material can considerably modify both the quantity and quality (composition) of essential oil.

The myrcene also represented a trend similar to that of α-terpinene in response to applied 8mg Fe and 50mg S kg^{-1} soil. This compound showed an increase of about 27.3%. Another important constituent that showed an interesting alteration in trend was α-thujene. As shown in Table 1, the quantity of α-thujene drastically increased by 21.4%. The amount of α-pinene in control harvested plant materials increased over time so that

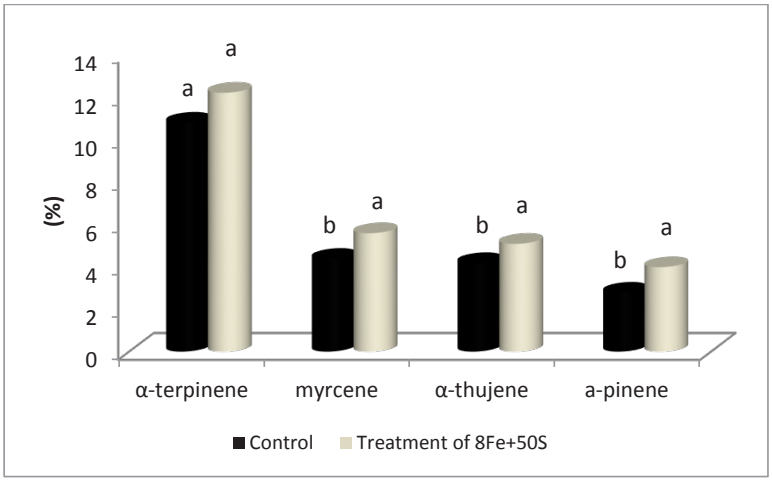

Fig.2: Changes of *S. hortensis* essential oil composition with application of 8mg Fe kg^{-1} and 50mg S kg^{-1} (columns with the same statistical letter are not significantly ($P < 0.05$) different by Duncan's multiple range test).

the amount was 2.8% immediately after oil extraction, gradually increasing to 4.0% and then to 42.85% at the end of the experiment period.

In total, 17 constituents were identified and quantified in the *S. hortensis* essential oil (EO) samples subjected to application of 8mg Fe and 50mg S kg^{-1} soil. The monoterpenoid fraction constituted 99.9% of the oil with the main components γ-terpinene, α-terpinene, myrcene, α-thujene, p-cymene, α-pinene and carvacrol. The percentage of the identified sesquiterpenes and sesquiterpenoid components was relatively low (0.1%). It has been reported that monoterpenes are the primary constituents of plant essential

TABLE 1
Combined Effect of Soil Applied S and Fe on the Chemical Composition (%) of *S. hortensis* Essential Oil

				Applied Fe and S fertilisers (mg kg^{-1} soil)			
No	Compound	RI[a]	Control	8Fe+50S	8Fe+100S	16 Fe+50 S	16Fe+100S
1	α-Thujene	923	4.2[b] b	5.1 a	4.4 b	4.1 b	3.9 b
2	α-Pinene	930	2.8 b	4.0 a	3.3 b	2.9 b	2.8 b
3	Camphene	945	0.2 a	0.3 a	0.2 a	0.2 a	0.2 a
4	Sabinene	969	0.3 bc	0.5 a	0.4 ab	0.3 bc	0.2 c
5	β -pinene	973	1.1 b	2.0 a	1.6 ab	1.4 b	1.3 b
6	Myrcene	987	4.4 b	5.6 a	5.0 a	5.1 a	4.9 ab
7	α-Phellandrene	1003	0.9 a	1.2 a	1.1 a	1.0 a	1.0 a
8	δ-3-Carene	1008	0.1 b	0.2 a	0.2 a	0.2 a	0.2 a
9	α-Terpinene	1014	10.8 a	12.2 a	11.5 a	11.2 a	10.9 b
10	p-Cymene	1021	3.8 b	3.9 b	4.1 b	5.1 a	5.1 a
11	Limonene	1025	0.9 a	0.6 c	0.8 ab	0.7 bc	0.6 c
12	β -Phellandrene	1026	0.2 b	0.8 a	0.5 a	0.6 a	0.7 a
13	(E)- β -Ocimene	1043	0.1 b	0.2 a	0.2 a	0.2 a	0.2 a
14	γ-Terpinene	1059	67.4 a	60.4 b	60.8 b	58.4 b	59.0 b
15	cis-Sabinene hydrate	1064	-	-	-	0.1	0.1
16	Terpinolene	1085	-	-	-	0.1	0.1
17	Thymol methyl ether	1239	-	-	0.1	0.1	0.2
18	p-Cymen-9-ol	1211	0.3	-	-	-	-
19	(Z)-Ocimenone	1224	0.1	-	-	-	-
20	Carvacrol	1299	2.1 b	2.8 b	5.5 ab	7.7 a	8.1 a
21	caryophyllene	1415	-	-	-	-	0.1
22	β -Bisabolene	1504	0.1 b	-	0.2 b	0.4 a	0.5 a
	Total		100%	99.8	99.8	99.8	99.9

[a] RI, retention indices. Treatments consisted of 8mg Fe kg^{-1} soil+50mg S kg^{-1} soil, 8mg Fe kg^{-1} soil+100mg S kg^{-1} soil, 16mg Fe kg^{-1} soil+50mg S kg^{-1} soil and 16mg Fe kg^{-1} soil+50mg S kg^{-1} soil.
[b] Means in each row followed by the same letter are not significantly ($P<0.05$) different by Duncan's multiple range test.

oils and the effects of many medicinal herbs are attributed to the presence of these compounds (Gherlardini *et al*., 2001). In this regard, the findings of our study showed that the concentration of γ-terpinene with a lower molecular weight decreased in response to application of 8mg Fe+50 mg S kg^{-1} (Table 1). This phenomenon could be due to evaporation, oxidation and other unwanted changes in essential-oil components with application of 8mg Fe + 50mg S kg^{-1}. It has been reported that essential-oil biosynthesis depends on a number of factors e.g. the presence of different input substances and enzymes, depending on the metabolic pathway in which a given group of compounds is formed (Woronuk *et al*., 2011). For example, Novak *et al*. (2002) reported that terpenes are produced from a small number of substrates whereas terpene synthases are capable of forming numerous terpene skeletons. Wierdak (2013) stated that the biochemical pathways for synthesis of some volatile compounds, which are essential-oil components, have not yet been fully described. However, some investigators like Lewinsohn *et al*. (2000) showed the combination of the effect of ontogenesis and chemotype on the activity of O-methyltransferase, the enzyme catalysing the transfer of the methyl group from methionine to the acceptor. In addition, these researchers demonstrated the presence of two types of activity of this enzyme in two basil chemotypes; one of them was highly specific for chavicol while the other could accept eugenol as a substrate. In general, as Wierdak (2013) stated, fertilisation and feeding of herbal plants seem to be important factors modifying their aromatic profile and the quantity and quality of their essential oils.

The genus *Satureja* presents great variability in the concentration of the major components of its essential-oil composition due to the presence of different species and subspecies, as well as because of various factors, mostly the environmental and climatic circumstances (Gulluce *et al*., 2003). With reference to previous studies, carvacrol and thymol, in particular, were found to be main components of the oils isolated from numerous Croatian *Satureja* species (Skoibuix & Bezix, 2004). It was interesting to note that different isolates of winter savory from Croatia and Bosnia and Herzegovina had carvacrol (up to 84.19%) as a major component (Kustrak *et al*., 1996). Cazin *et al*. (1985) revealed that the oil composition of winter savory showed large differences in the relative concentration of main constituents: carvacrol (5-69%), linalool (1-62%), γ-terpinene (1-31%) and p-cymene (3-27%), arising from the presence of different chemotypes. The main components of the *S. hortensis* (summer savory) essential oil were the phenols, thymol (29.0%), carvacrol (26.5%), *r*-terpinene (22.6%), p-cymene (9.3%) and other terpenoids (Gulluce *et al*., 2003). Finally, it could be concluded that application of 8mg Fe+50mg S kg^{-1} was the most suitable treatment for obtaining a higher percentage of α-terpinene, myrcene, α-thujene and α-pinene whereas addition of 16mg Fe+100 mg S kg^{-1} was preferable

for obtaining special components such as carvacrol. On the other hand, γ-terpinene was mainly produced in the control. Essential-oil composition in *Satureja* species showed it to be rich in phenolic components like carvacrol, γ-terpinene, thymol, p-cymene, β-aryophyllene, linalool and other terpenoids, but chemical composition and the amount of components varied between the different *Satureja* species oils (Baser *et al*., 2004; Novak *et al*., 2006; Sefidkon and Jamzad, 2006). Some researchers showed that the essential oil and extract of *Satureja* species showed a variety of activities including anti-bacterial and anti-fungal properties and they strongly inhibited the activity of a wide variety of bacteria and fungi in human, food and plant pathogens (Baydar *et al*., 2004; Gulluce *et al*., 2003; Hajhashemi *et al*., 2000). Recent studies showed that some plants from the lamiaceae families were very rich in phenolic compounds such as phenolic-acids, flavonoids and phenolic-diterpenes, and possessed high antioxidant activities (Aaby *et al*., 2004; Wong *et al*., 2006). Flavonoids and phenolic compounds exert multiple biological effects such as anti-oxidant activities, free radical scavenging and anti-inflammatory properties (Miliauskas *et al*., 2004; Shahidi, 2000). Oxidative damage in the human body plays a vital causative role in disease initiation and progression (Jacob & Burri, 1996).

CONCLUSION

S. hortensis L. is a medicinal and aromatic plant of the Lamiaceae family used in Iranian folk medicine for various purposes. Our results showed that chemical fertilisers increased the essential oil constituents. The amount of some components such as α-terpinene, myrcene, α-thujene, p-cymene, α-pinene and carvacrol could be changed in response to S and Fe fertiliser application. Our findings indicated that savoury oils, in addition to other properties, had potential in topical antioxidant activity and its antioxidant activity increased when plants received S and Fe fertilisers.

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Potential of the Extract from the Nut of *Areca catechu* to Control Mango Anthracnose

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ABSTRACT

Anthracnose is a common disease that attacks mangoes in many regions, including Malaysia. In this study, extracts from the nuts of *Areca catechu* were tested for their antifungal activities in controlling the disease. Antifungal screening tests were done using six extracts i.e. hexane, chloroform and methanol from ripe and unripe nuts of *A. catechu* to determine their ability to inhibit mycelium growth and spore germination of *Colletotrichum gloeosporioides* isolated from mango. Of the six extracts, the chloroform extract from unripe nuts at a concentration of 10 mg/mL showed the best antifungal activity, inhibiting about 52% of mycelium growth and 100% of spore germination. Thus, this particular extract was selected to treat the fruit against anthracnose in two different ways, namely, by dipping them in the extract solution at 27°C for one hour (normal dip) and also at 52°C for five minutes (hot dip). Meanwhile, control and benomyl solutions (each applied in both dipping methods) were used as comparisons. The test proved that the treatment using the extract reduced 34% of disease infection and 27% of disease rate from the control. However, the treatment using benomyl was slightly effective compared to using the extract, reducing around 47% of disease infection and 38% of disease rate from the control. Hence, results from test also proved that the treatment applied at 52°C reducing 51% of disease infection and 35% of disease rate than those conducted at 27°C. Compound screening tests on the chloroform extract of the unripe nuts revealed that the extract contained alkaloids and phenolics. Many previous studies

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have proven that alkaloids and phenolics from various plants could cause antifungal activities and these substances might be responsible for controlling anthracnose development in the study done.

Keywords: Mango, anthracnose, betel nut extract

INTRODUCTION

Mangoes grow in the tropic and sub-tropic regions (Sangeetha & Rawal, 2008). One of the known varieties grown in Malaysia is the Chok Anan. Anthracnose is one of the common diseases that infect mango during the post-harvest period. This common disease is caused by fungal pathogen, *Colletotrichum gloeosporioides*. Several methods and techniques have been used in controlling mango anthracnose including using resistant varieties of the plant, doing the sanitation process by pruning and removing debris to reduce inoculums and applying fungicide sprays in the field on a regular basis (Nishijima, 1993). After harvesting, suitable treatments have to be applied to reduce potential disease development. The common methods applied to treat the disease are hot water dip and fungicide dip (Ploetz, 2003). Furthermore, fungicides can be added to the hot water to increase the effectiveness of the method. This method, which is also known as fungicide dip, is applied by dipping the mango in hot water of 52^{o}C added with 500 to 1000 ppm benomyl for a duration of 3 to 5 minutes (Lim & Khoo, 1985). However, the usage of chemical fungicides may cause many negative implications, such as bad effects to health and environmental pollution and increasing the resistance mechanisms in pathogens due to frequent application. Many studies have discovered the potential of plant extracts in controlling plant diseases or growth of plant pathogens. One of the potential is the nut of *Areca catechu*, commonly known as the betel nut. The plant can be found in many regions including East Africa, the Arabian Peninsula, the tropical regions of Asia and Indonesia, as well as the central Pacific and New Guinea (Staples & Bevacqua, 2006). The potential of the *A. catechu* nut as an alternative antifungal agent can be related to the presence of its important substituents, alkaloids and phenolics. According to Wang and Lee (1996), there are various kinds of phenolic compound found in the nut including tannin. Meanwhile, the medicine and stimulant brought by consuming the nut can be related to the alkaloid content, which produces euphoria and can treat pain (Petterson *et al*., 1991). Many alkaloids and phenolics extracted from various plants are potential antifungal substances, as has been proved (Deng *et al*., 2011; Veloz-Garcia *et al*., 2010; Hussin *et al*., 2009; Nissanka *et al*., 2001; Baumgatner *et al*., 1990). In Malaysia, however, there has been no research done to determine the potential of the *A. catechu* nut as a biopesticide for post-harvest diseases. The objective of this study was to determine the potential of *A.catechu* nut extract to control mango anthracnose by applying the normal dip method and integrated with the hot water dip technique.

MATERIALS AND METHODS

Isolation of Colletotrichum gloeosporioides

The mango variety, Chok Anan, with common symptoms of anthracnose (black spots and necrotic lesion on the skin) (Fig.1), were bought from a wet market, Pasar Borong Selangor. The fruits were dipped into 10% of Clorox solution for 15 minutes before being dried inside a running laminar flow. The half infected and half visibly healthy fruit skins were cut with a sterile blade knife and placed inside the prepared potato dextrose agar (PDA) medium in a petri dish. All the cultures were placed in a culture chamber at 27°C. After two to three days, potential cultures of *C. gloeosporioides* were transferred into a new PDA medium. The pure cultures were observed each day and identified as *C. gloeosporioides* (Fig.2). The process of culture identification was conducted based on morphological characteristics. The isolated mature culture was observed and its morphological characteristics were compared with common *C. gloeosporioides*. Spore identification was also conducted by placing the slight portion of mycelium from the culture on a drop of lactophenol cotton blue (LCB) on a glass slide and the spores were observed using a light microscope (Fig.3). The spores were compared with the common shape of the *C. gloeosporioides* spores.

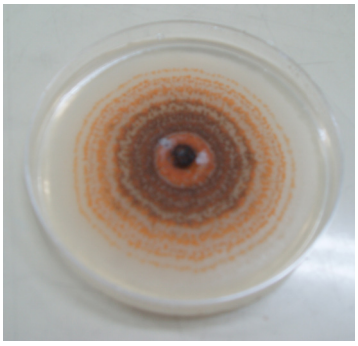

Fig.2: The pure cultures of *C. gloeosporioides*

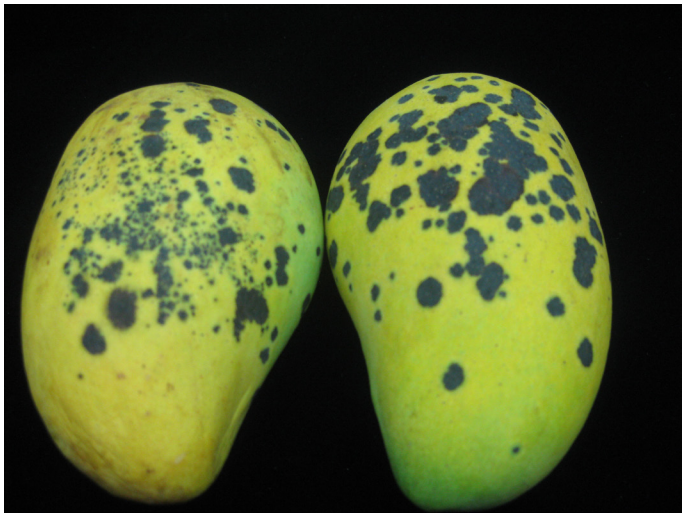

Fig.1: Chok Anan, with common symptoms of anthracnose (black spots and necrotic lesion on the skin)

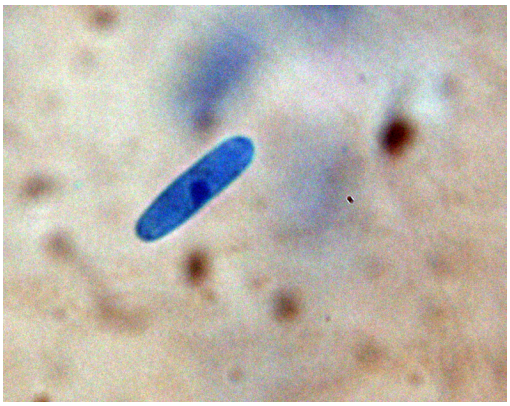

Fig.3: Spore of *C. gloeosporioides*

The Process of Sample Extraction

The ripe and unripe fruits of *A.catechu* were harvested in Kuala Kangsar, Perak, in Peninsular Malaysia. The skin of the ripe fruits was red-orange in colour, while the unripe fruits were dark green. The peel and husk of the fruits were separated from the nuts. The nuts were cut into pieces and dried at normal room temperature for approximately one month to eliminate all the water content. Pieces of the nuts were ground using a blender. Three different solvents were used to extract both the ripe and unripe nuts, which were hexane, chloroform and methanol.

Each nut sample comprising 1kg in weight was extracted continuously starting with hexane followed by chloroform and, finally, methanol. Six extracts comprising hexane, chloroform and methanol were produced from both nut samples. The extracts were then dried using a rotary evaporator. The concentrated extract suspensions were kept at 24 ± 2°C to let the remaining solvents evaporate, leaving only the crude dry extract.

Preparation of Extract Suspensions

Extract stock solutions with concentration of 80mg/mL were prepared by dissolving the solid crude extracts into hot dimetylsulfoxide (DMSO) heated using a hot plate. A 200-µL (0.2mL) extract solution with final concentrations of 20, 40, 60 and 80 mg/mL was obtained by diluting 50, 100, 150 and 200µL stock solutions with 150, 100, 50 and 0µL of DMSO, respectively.

Antifungal Screening Tests

All 1.6mL extract solutions were prepared by diluting each of the 0.2mL already prepared extract solutions, each with the concentration of 20, 40, 60 and 80mg/ml, prepared as described in preparation of extract suspensions, with 1.4mL of sterile distilled water to make four concentrations of extract solutions, which were 2.5, 5.0, 7.5, 10.0mg/mL (all with 12.5% DMSO concentrations), respectively. This method was done to prepare all the extract solutions. A control solution was prepared by dissolving 0.2mL DMSO into 1.4mL sterile distilled water to make 1.6mL of 12.5% DMSO solution. A mycelium growth test was done based on procedure detailed by Rahman (2008) with some modifications. The fungal plug measuring 0.5cm diameter from a seven-day culture of *C. gloeosporioides* was dipped into the extract and control solutions in a sterile glass tube for four hours at 25 ± 2°C. After that, the plugs were placed at the centre of the PDA medium plate in a 5.5-mm diameter Petri dish. Four replications were done for each treatment. The average diameter of the fungal growth was measured

and recorded from day two until day five. The inhibition percentage (%) of mycelium growth was calculated using the following formula:

$$\frac{\text{Mycelium diameter of control culture-Mycelium diameter of treated culture}}{\text{Mycelium diameter of control culture}} \times 100$$

In the spores germination test, spore suspensions were prepared by a flood of a seven-day-old culture of *C. gloeosporioides* with sterile distilled water before being streaked using a sterile L-bent glass rod. The suspension was filtered using double layers of sterile muslin cloth into a sterile flask. Later, 1.4mL of the suspension with concentration of 2.4 x 10^5 conidia mL^{-1} was transferred into glass tubes containing 0.2mL extract solutions, each with a concentration of 20, 40, 60 and 80mg/ml, prepared as described in the earlier section, to make four concentrations of extract mixed with the spore suspension i.e. 2.5, 5.0, 7.5, 10.0mg/mL respectively, all with 12.5% DMSO concentrations,. A control suspension was prepared by dissolving 0.2mL DMSO into 1.4mL spore suspensions. All the mixed suspensions were incubated for 30 minutes at 25 ± 2°C. After that, 0.1mL was transferred from the suspensions and spread over prepared PDA medium plates. All the plates were incubated for 12 hours at 24 ± 2°C. A drop of LCB was used to inhibit any germination after the completion of the incubation period. Spore was considered as being germinated if the germ tube was half the length of the spore (Sariah, 1994). Light microscope was used to observe the germination. A total of 250 spores were counted randomly on each plate. Meanwhile, four replications of the plate were used for each treatment in the test. The inhibition percentages (%) of spore germinations were calculated using the following formula:

$$\frac{\text{Spore germination of control-Spore germination of extract treatment}}{\text{Spore germination of control}} \times 100$$

Screening for Potential Compounds in the Extracts

Two screening tests were carried out to detect the presence of alkaloids and phenolic compounds in the extracts. The screening for alkaloids was based on Touchstone and Dobbins (1983). A stock solution of Dragendorf reagent was prepared by mixing a solution of 0.85g bismuth sub-nitrate in 10mL acetic acid and 40mL water with a solution of 8g potassium iodide in 20mL water. The spray solution was prepared by mixing 1mL of the stock solution with 2mL acetic acid and 10mL water before use. The crude extracts were spotted on a thin layer chromatography (TLC) plate. The solvent systems used for hexane, chloroform and methanol extracts for both ripe and unripe nuts were hexane:chloroform (5:5), chloroform:methanol (7:3) and 100% methanol, respectively. The plate was developed and sprayed with the Dragendorf reagent. Orange coloured spots indicated the presence of alkaloids. For phenolics screening, an iron (III) chloride solution was prepared by dissolving 1.0g iron (III) chloride in 100mL methanol. The

crude extracts were similarly spotted on a developed TLC plate end. The solvent systems used were the same as that applied in the screening of the alkaloids. The developed plate was stained with an iron (III) chloride solution. Blue greyish coloured spots indicated the presence of phenols.

In vivo Test on Mango Using the Selected Extract

The extract causing the best antifungal reaction against *C. gloeosporioides* in both the mycelium growth and spore germination tests was applied in controlling the anthracnose infection on the Chok Anan mangoes. The test was done on the artificially inoculated fruit. Two different treatments using the extract were done on the mangoes, and this was carried out by dipping the fruit in the extract solutions at 27°C for one hour and dipping them in the extract solutions at 52°C for 5 minutes. In this study, unripe green mangoes were used. The three treatments selected were: i) Extract solution; ii) Benomyl solution; and iii) Control solution. The extract solution was prepared by dissolving 250mL extract stock solution with concentration of 80mg/mL (prepared by dissolving 20g extract in 250mL DMSO) in 1750mL sterile distilled water. A benomyl solution was prepared according to suggestion (according to manufacturer recommendations) by dissolving 100mg benomyl powder into 2L sterile distilled water to make a 0.05g/L benomyl solution. A control solution was prepared by dissolving 250mL DMSO in 1750mL sterile distilled water.

The mangoes were cleaned and surface sterilised by dipping them in 10% Clorox solution for 15 minutes; this was followed by drying in a running laminar flow. Spore suspensions, in the concentration of 6.7 x 10^7 conidia mL^{-1}, which had been prepared following the procedure described in the earlier section, were sprayed on the whole fruit. After three hours, the mangoes were treated using two different dipping methods. The first method (normal dipping) was done by dipping the fruits into the prepared extract solution at 27°C for one hour, while the other method (hot dip) was carried out by dipping the fruits in the extract solution at 52°C for 5 minutes using a hot-water bath. For each method, a 12.5% DMSO solution and a 0.05mg/mL benomyl solution were used as the negative control and the positive control, respectively. The mangoes were then dried under a running laminar flow after being treated before being incubated in a chamber. The fruits were sprayed with sterile distilled water. Conditions in the chamber were maintained with relative humidity at 88 ± 2% RH in a temperature of 24 ± 2°C. Five replicates of the fruit were used for each treatment. The experiment was done twice. The spots that appeared on the fruit skin were counted every day for seven days. Disease severity was scored on a 1–5 scale based on Koomen and Jeffries (1993) and Pordesimo (1979) with some modifications, where 1=1-5 spots, 2=6-10 spots, 3=11-20 spots, 4=21-30 spots, and 5=>30 spots. Disease severity percentages (%) were calculated based on the following formula:

$$\frac{\Sigma\ (\text{Disease scale} \times \text{Total fruits in the scale})}{\text{Total fruits in the experiment} \times \text{Highest scale}} \times 100$$

The graphs of the disease severity on the mangoes during the seven days of treatments were plotted. The area under the disease progress curve (AUDPC) was determined from the graphs based on the formula as described by Madden *et al*. (2007).

$$\text{AUDPC} = \sum_{i=1}^{N_i-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_i + t_{i+1})$$

y = data of disease severity or disease incidence collected

t = time of data collected

The disease rate was determined by using a regression of transformed diseased severity values using logistic model ln (y/(1-y)) (Berger, 1981). Percentage data were first transformed using an arc sine transformation before analysis.

Statistical Analysis

All the experiments were carried out in a Completely Randomized Design (CRD). The analysis of variance (ANOVA) using SAS statistical software was utilised to analyse the data. The results showing significant differences were subjected to the mean separation of the Duncan multiple range test (DMRT) at $P \leq 0.05$.

RESULTS

Antifungal Screening Tests

The chloroform extract from the unripe nut had the best antifungal activities against *C. gloeosporioides*. In the spore germination test (Table 1), both chloroform extracts from the ripe and unripe nuts inhibited 100% of spore germination when applied at the concentrations of 7.5 and 10.0mg/mL. Both hexane extracts from the unripe nut inhibited 65.2% and 44.5% of germinations respectively when applied at the highest concentration of 10mg/mL. Methanol extracts from both nuts had the least inhibition among others. In the mycelium growth test (Table 2), the chloroform extract from the unripe nut showed the best result, which inhibited the mycelium growth at 52.2% when applied at the highest concentration of 10 mg/mL. This was followed by the chloroform extract from the ripe nut (44.5%), the hexane extract from the ripe nut (36.7%), the hexane extract from the unripe nut (36.0%), the methanol extract from the ripe nut (12.4%) and the methanol extract from the unripe nut (12.4%).

Screening for Potential Compounds in the Extracts

From both screening tests, positive results indicated the presence of phenolics and alkaloids in the crude extracts (Table 3). Both compounds were present in the two chloroform and the two methanol extracts. However, both compounds were not detected in the two hexane extracts.

TABLE 1
Percentage Inhibition (%) of Spore Germination of *C. gloeosporioides* Treated with Extracts from the *A. catechu* Nuts

Extracts	Concentrations (mg/mL)			
	2.5	5.0	7.5	10.0
Hexane ripe	19.2 Ca[z]	26.8 Cb	45.0 Cc	65.2 Cd
Chloroform ripe	73.7 Da	94.0 Db	100.0 Dc	100.0 Dc
Methanol ripe	5.8 Aa	10.3 Ab	19.2 Ac	26.1 Ac
Hexane unripe	11.0 Ba	19.2 Bb	33.9 Bc	44.5 Bd
Chloroform unripe	82.2 Ea	94.7 Db	100.0 Dc	100.0 Dc
Methanol unripe	7.6 Aa	8.2 Aa	18.5 Ab	25.2 Ac

[z]Means followed by the same uppercase or lowercase letter, for each fungus, within each column or row did not differ significantly at DMRT $P \leq 0.05$.

TABLE 2
Percentage Inhibition (%) of Mycelium Growth of *C. gloeosporioides* Treated with Extracts from the *A. catechu* Nuts

Extracts	Concentrations (mg/mL)			
	2.5	5.0	7.5	10.0
Hexane ripe	2.9 Ba[z]	18.7 Bb	20.5 Cc	26.5 Bd
Chloroform ripe	15.7 Ca	28.9 Cb	36.9 Dc	44.5 Cd
Methanol ripe	2.2 Ba	4.8 Aa	8.9 Bb	12.4 Ac
Hexane unripe	1.0 Ba	5.4 Ab	9.2 Bc	13.0 Ad
Chloroform unripe	18.3 Da	28.6 Cb	43.4 Ec	52.2 Dd
Methanol unripe	0.0 Aa	5.1 Ab	6.7 Ab	12.4 Ac

[z]Means followed by the same uppercase or lowercase letter, for each fungus, within each column or row did not differ significantly at DMRT $P \leq 0.05$.

TABLE 3
Results on Alkaloids and Phenolics Presence in Crude Extracts of Ripe and Unripe Nuts of *A. catechu*

Crude extracts	Alkaloids present	Phenolics present
Hexane ripe nut	-	-
Chloroform ripe nut	+	+
Methanol ripe nut	+	+
Hexane unripe nut	-	-
Chloroform unripe nut	+	+
Methanol unripe nut	+	+

The positive sign (+) indicates the presence of the compounds in the extracts and the negative sign (-) indicates absence of the compounds in the extracts.

In vivo Test on Mango Using the Selected Extract

Interaction between treatments solutions and dipping methods is significant (Table 5 and Table 6). In both hot and normal dip methods, the fruits that had been treated with the control solution were found to be the most infected by anthracnose; this was followed by the fruits treated with the extract and benomyl solutions (see Fig.4). The control fruits also had the fastest disease development, followed by the extract, and benomyl, with the least disease rate (Table 4). The fruits treated with the extract had 34% less disease infection and 27% less disease rate, as compared with the control. However, benomyl-treated fruits had a slightly better result with 47% disease infection reduction and a 38% disease rate from the controlled fruits.

The fruits treated with the normal dip were found to be severely infected by anthracnose during the experiment whereas those treated with the hot water dip had the least disease development. Fruits treated with normal dips also had a higher disease rate compared to fruits treated with the hot dip method (Fig.5). Thus, the hot dip method was proven to have effectively reduced 51% of the anthracnose infections and to have slowed down more than 35% of the disease rates as from the normal dip method (Table 4).

TABLE 4
Effects of the Treatments Applied on Anthracnose Development on Artificially Inoculated Mangoes for Seven Days after Inoculation

	AUDPC	Disease rate
Solution		
Control	226.0 a[z]	0.85 a
Extract	148.5 b	0.62 b
Benomyl	119.0 c	0.53 c
Temperature		
Normal temperature dip	221.0 a	0.81 a
Hot dip	108.0 b	0.52 b

[z]For each treatment, the means within a column followed by the same letter are not significantly different by DMRT at P≤0.05.

TABLE 5
ANOVA Table on Effects of the Treatments Applied on AUDPC

Source of Variation	df	SS	MS	F
Treatment	5	64639		
Solution	2	24434	12217	268**
Temperature	1	38307	38307	839**
Solution x Temperature	2	1898	949	21**
Error	6	274	46	
Total	11	64913		

** are significant at P≤0.01.

TABLE 6
ANOVA Table on Effects of the Treatments Applied on Disease Rate

Source of Variation	df	SS	MS	F
Treatment	5	0.5442		
Solution	2	0.2135	0.1067	143.90**
Temperature	1	0.2611	0.2611	352.01**
Solution x Temperature	2	0.0695	0.0348	46.96**
Error	6	0.0045	0.0007	
Total	11	0.5486		

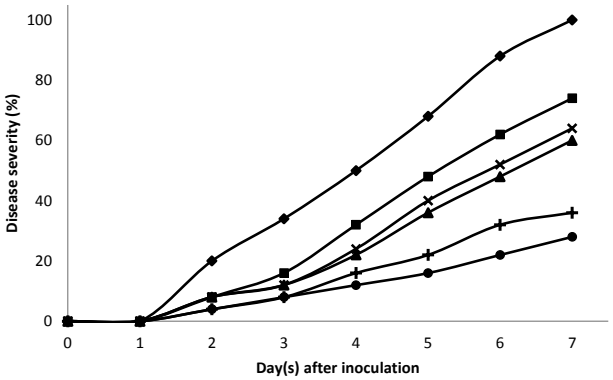

Fig.4: Disease progress curve of anthracnose by *C. gloeosporioides* on mango treated by dipping into control (♦), extract (■) and benomyl solutions (×) at 27°C temperature for 1 hour and dipping into control (▲), extract (+) and benomyl solutions (•) at 52°C temperature for 5 minutes

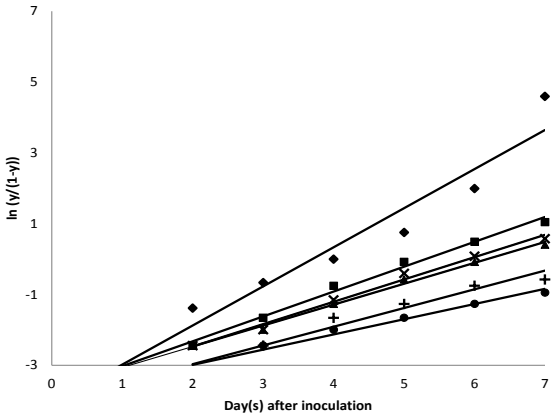

Fig.5: Regression of transformed diseased severity values of disease progress curve of anthracnose by *C. gloeosporioides* on mango treated by dipping into control (♦), extract (■) and benomyl solutions (×) at 27°C temperature for 1 hour and dipping into control (▲), extract (+) and benomyl solutions (•) at 52°C temperature for 5 minutes using logistic model ln (y/(1-y), the equation for the line being Y=1.10x-4.08, R^2=0.91, Y=0.70x-3.72, R^2=0.99, Y=0.63x-3.73, R2=0.99, Y=0.57x-3.61, R^2=0.99, Y=0.53x-4.02, R^2=0.96 and Y=0.43x-3.85, R^2=0.98, respectively

DISCUSSION

Many studies including by Oxenham *et al*. (2002), Holdsworth *et al*. (1998), Wang *et al*. (1997), Wang and Lee (1996) and Huang and McLeish (1989) proved both alkaloids and phenolics are the two substances in *A.catechu* nut most well studied and discovered. As these two groups of compounds are important in the nut, it is possible to relate the antimicrobial effects brought by both alkaloids and phenolics in the extracts. During both mycelium growth and the spore germination tests, both hexane extracts from ripe and unripe nuts were seen to be less effective; this might be due to the absence of alkaloids and phenolics in both extracts. The chloroform extracts of both the ripe and unripe nuts of *A.catechu* gave the best antifungal activities against *C. gloeosporioides*. Between the two, the extracts from the unripe nut proved to be better in inhibiting the growth of the pathogen. A study by Wang *et al*. (1997) found that the unripe nuts contained higher concentrations of alkaloids as compared to the ripe nuts. Since the chloroform extract of the unripe nut had better antifungal activity compared to the chloroform extract of the ripe nuts, the higher concentration of alkaloids it contained might be one of the reasons. Many kinds of alkaloids are able to cause fungitoxic and bacteriostatic actions (Petterson *et al*., 1991).

During the screening test, phenolics were detected in both the ripe and unripe nuts of the chloroform extracts. A study by Wang *et al*. (1997) revealed that the contents of total phenolics and condensed tannins in the nuts increased upon maturity. Due to a better inhibition by the chloroform extract from the unripe nuts compared to the chloroform extract of the ripe nuts, phenolic compound concentrations might have little effect on the antifungal activity of the nuts. Based on the spots on the TLC plates, both methanol extracts from the ripe and unripe nuts contained both phenolics and alkaloids. However, both were found to be ineffective in controlling the growth of *C. gloeosporioides*. Among all the solvents used in the extraction, methanol, being a polar solvent extract, revealed the highest quantity of compounds as compared to the chloroform and the hexane extracts, which can be said to be ineffective. The abundance of compounds in both the methanol extracts might cause each compound in the extracts to act against each other or interfere with each other's mechanisms (Dellavalle *et al*., 2011).

The results from the *in vivo* tests had proven that dipping the fruits in the extract solution did reduce the disease severity of anthracnose. First, the extract solution treatment might induce the resistance level of the fruits. Other than affecting the defence mechanism of the host plant, the plant extracts might cause action mechanisms on fungal pathogens. The mechanisms of crude plant extracts might be due to several different actions on pathogens (Niño *et al*., 2012). The results from the *in vivo* test showed that the treatment using fungicides of the benomyl solution had proven to be effective in slowing down the anthracnose development on the fruits. However, it did

not stop the infection from deteriorating the fruits.

The results from the above study also showed that hot water dipping significantly reduced anthracnose infection on mangoes compared to normal dipping. Antifungal mechanisms by hot water treatment might directly damage the pathogen cells and indirectly increase the resistance level of the host (Karabulut *et al*., 2010). Many plant materials have been successfully controlled using a hot water dip but not many studies have been carried out on integrating the extract application and hot water dipping. Using integration methods in controlling plant diseases causes a difficulty in the pathogen defence mechanism due to the different kinds of antifungal mechanisms caused by each of the treatment (Sharma & Tripathi, 2008).

CONCLUSION

The chloroform extract of the unripe nut reduced disease severity of anthracnose on the mangoes. Both alkaloids and phenolics were found to be present in the extract. These compounds might be involved in the antifungal properties of the extract. However, there have been no reports done on the antifungal activities of these compounds in the study. In the future, a study has to be done on the use of specific compounds from the nut in controlling plant diseases. Dipping fruits in the extracts at 52^{o}C increases the effectiveness of controlling the infections on fruits. More studies have to be carried out in the future to maximise the potential of the extracts by integrating the treatment using the extracts with other available methods.

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Application of Multivariate Analysis for Detection of Crude Palm Oil Adulteration through Fatty Acid Composition and Triacylglycerol Profile

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ABSTRACT

This study focused on developing a reliable procedure for the identification of the adulteration of crude palm oil (CPO) by blending sludge oils (SO) and used vegetable oils (UVO) ranging from 1 to 20% (v/v). Fatty acids methyl esters (FAME) and Triacylglycerol composition consisting of all single and blended CPO were analysed using a gas chromatography (GC)-flame ionisation detector (GC-FID) and high performance liquid chromatography evaporative light scattering detector (HPLC-ELSD), respectively. The results were processed using the multivariate analysis i.e. principal component analysis (PCA) and cluster observation (CO) to discriminate the most applicable factors useful for detecting this adulteration. The results revealed that the combination of chemical properties and multivariate analysis resulted in a strong differentiation between the blends according to the amount of adulterant in the CPO. PCA and CO provided good results, allowing detection of the adulteration of the CPO with the SO and UVO as low as 5% and 2% respectively for each multivariate analysis.

Keywords: Adulteration, crude palm oil, fatty acid methyl esters, GC-FID, HPLC-ELSD, principal component analysis, triacylglycerol

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INTRODUCTION

The oil palm is believed to be originated from Africa, but at present, production is mainly based in the tropical areas of

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America, Africa and Asia, with Malaysia and Indonesia being the most productive countries (Corley, 2003; Sundram, 2005). Oil palm has the highest yield per hectare compared to any other oil crops. In the year 2012, Malaysia accounted for 39% of world oil palm production and 44% of world exports. Generally, oil palm produces two types of oil from its fruit: mesocarp oil or crude palm oil (CPO) and palm kernel oil (PKO). CPO consists of 50% saturated and 50% unsaturated fatty acids. Its major triglycerides are tri-saturated (10.2%), di-saturated (48%), mono-saturated (34.6%), and triunsaturated (6.8%) fatty acids (Corley, 2003; Sundram, 2005; Soh, 2008).

Palm oil is one of the most commonly used vegetable oils in the world today, which accounts for 33% of all oils consumed globally (Kumar, 2013). This situation demands for a high quality of palm oil to be produced for domestic and industrial applications. However, the quality of palm oil is affected by various factors from harvesting, handling, processing fresh fruit bunches and the storing methods of crude palm oil. Recently, there has been speculation that crude palm oil is being adulterated in order to increase profit margin. Countries like Nigeria and India have been found guilty with adulterated cases that have an impact on consumer's health (Soh, 2008; Okonkwa, 2012). Since there is variability within the fatty acid and triacylglycerol composition of crude palm oil, mixing crude palm oil with other oils with similar composition while examining the oil on the physical, chemical and nutritional properties is an analytical challenge.

Principal component analysis (PCA) is widely used for the evaluation of olive oil and virgin olive oil (Pizarro, 2011; Rohman, 2012; Salces, 2010). The use of PCA treated data enables the detection of adulterants in olive oil and virgin olive at different levels. On the other hand, through cluster analysis, samples are clustered together based on the similarity of their chemical and physical properties and the results are displayed as a connection dendrogram. Previous researchers (Fragaki, 2005; Obeidat, 2009) reported that a clear and independent cluster was obtained for further prediction of the adulterant in the virgin olive oil blends.

Therefore, the aim of this study was to verify the effectiveness of fatty acid composition determined by GC-FID and triglyceride composition identified by HPLC-ELSD. This is followed by chemometric tools, principal component analysis and cluster observation to detect the adulteration of crude palm oil with sludge oil and recycled oil in the level of 1 to 20% (v/v). To our best knowledge, there is no published report investigating the adulteration of crude palm oil using multivariate analysis. Most of the studies on the authentication and quality of oil that were profoundly investigated and thereafter published focused on olive oil or virgin olive oil. (Christopoulou, 2002; Gamazo-Vazquez, 2003; Diza, 2005; Jafari, 2009; Bucci, 2002; Capote, 2007; Zabaras, 2004; Gurdeniz, 2009).

MATERIAL AND METHODS

Materials

All reagents used were analytical grade. The analysed samples were crude palm oil (CPO), sludge oil (SO) and used vegetable oil (UVO) and admixtures of CPO. CPO samples were obtained from Genting Ayeh Item Oil Mill (GAIOM, Ayeh Hitam, Johor, Malaysia) and used vegetable oil was obtained from oils used in the cooking of french fries and chicken nuggets in the laboratory. To avoid oxidation and chemical composition changes, all samples were kept at -80^{o}C in the freezer immediately after arrival in the laboratory. Mixtures of the CPO sample with each one of the adulterant oils were prepared. For each adulterant oil, five mixtures with double replicate (Treatment=7, replicate=2) were prepared with percentages 1, 2, 5, 10 and 20% of the respective oil in the genuine CPO. Altogether, n=28 samples of genuine oil and admixtures were prepared. These admixtures together with the genuine oil were analysed after the preparation.

Analysis of Fatty Acid Composition (FAC)

Fatty acid composition of oil sample was determined by taking 1g oil sample and refluxing with 40mL 0.01 M of sodium methoxide solution and 1M methanolic hydrochloric acid solution for 30 minutes (Ainie *et al.,* 2005). Hexane was added in the solution and shaken vigorously. The upper layer of the solution was then analysed using a gas chromatograph (Model: 2014, Shimadzu, Fisher Scientific, Kyoto, Japan). The analysis was performed on a 30mx0.2 capillary column using a gas chromatograph (GC) connected to a flame ionisation detector (FID). The GC conditions used were as follows: injection volume 1 uL, split injection 50:1 at 240^{o}C; oven temperature set at 90^{o}C, then ramped to 165^{o}C at 5^{o}C min^{-1}, then ramped again to 205^{o}C at 2^{o}C min^{-1} and final ramping to 220^{o}C at 15^{o}C min^{-1}(hold 4min). The total run time was approximately 40 minutes. The helium gas carrier was held at a constant flow rate of 1mL min^{-1}, whilst the detector was set at a temperature of 300^{o}C. Individual peaks of fatty acid methyl esters were determined using Supelco F.A.M.E. Mix, C8 –C24 fatty acids standards.

Analysis of Triacylglycerol (TAG) Profile

Triacylglycerol composition was determined by a high performance liquid chromatograph-evaporative light scattering detector (HPLC-ELSD, Model 2695, Waters, Massachusetts, USA). The HPLC system used was equipped with an autoinjector and *evaporative light scattering* detector. The mobile phase was a mixture of acetone/acetonitrile (63.5:36.5 v/v) and the flow rate was 1mL/min. The injection volume was 10µL of 5% (w/v) oil in acetone. Triglyceride peaks were identified based on the retention time of supelco lipid standards of triglyceride mixtures.

Experimental Design and Multivariate Data Analysis

Fatty acid and triglycerides compositions were the main components applied in

this experiment to detect the adulteration of the CPO with the SO and UVO. Five treatments with double replicate were assigned based on the applied combination levels. The experimental design, data analysis, optimisation procedure and method validation were performed using the Minitab v. 14.0 statistical package (Minitab Inc., State College, PA, USA).

Principal component analysis (PCA) and cluster observation (CO) were applied to obtain an overview of correlation between the samples. The correlation matrix was applied with multivariate analysis using Minitab 14.0. Multivariate analysis for processing chromatographic data is an efficient tool for classification and searches for similarities of oil samples, and this provides good quality control.

RESULTS AND DISCUSSION

Detection of Adulterants in Crude Palm Oil Based on FAC and TAG

Table 1 and 2 present the results of the analysis of the crude palm oil (CPO), genuine adulterant oil (Sludge Oil (SO) and used vegetable oil (UVO)) and their admixtures with CPO. The values of fatty acid and triglycerides in the CPO were similar as that reported by Sundram, 2005. However, there were no official values of fatty acid and triglycerides for SO and UVO that have been reported. Comparison between the CPO with SO and UVO showed that there were similarities with regards to triglycerides with ECN of 46, 48 and 50 and fatty acid compositions (C12-C120). Sludge oil (SO) or palm oil mill effluent is the voluminous liquid waste that comes from the sterilisation and clarification sections of the oil palm milling process whereas UVO is produced from used cooking oil derived from refined CPO. Since both SO and UVO are derivatives from the CPO milling process and refined, their triglycerides and fatty acid compositions are similar to that of the native CPO.

In the present study, detection of adulteration of CPO up to the concentration of 20% was investigated. The results for the fatty acid and triglyceride composition of admixtures of CPO with SO and UVO are presented in Table 1 and 2. Based on the results, parameters for detecting adulteration were examined. From the results presented in Table 1 and 2, it could be concluded that the analysis of fatty acid and triglyceride composition does not provide satisfactory results and could not be used as a basis for detecting the adulteration. As the level of adulteration increased gradually from 1 to 20 % there were no major changes in the compositions observed. Changes were very minor in the admixtures of CPO. This is mainly due to the dominant effect of the CPO, which suppressed the adulterants' chemical profiles even at 20% concentration level.

Additionally, a one-way ANOVA was performed on the whole data set in Tables 1 and 2 in order to compare for each variable and the variance within any category. The results showed that there were no differences among the treatments. Hence, a one-sample T-test (df=6 and CI=90%) was applied. The results from the T-test revealed that

TABLE 1
Fatty Acid Composition (%) and Triacylglycerol ECN (%) of Crude Palm Oil (CPO) and Admixtures with Sludge Oil (SO)

Sample	Fatty Acid Composition					Triglycerides (ECN)		
	Carbon 12*	Carbon 14	Carbon 16	Carbon 18*	Carbon 20	46	48*	50*
Genuine Crude Palm Oil (CPO)	0.42	1.09	44.47	53.67	0.35	17.28	69.70	4.00
99 CPO : 1 SO	0.42	1.09	44.44	53.70	0.35	17.28	69.69	3.99
98 CPO : 2 SO	0.43	1.09	44.41	53.72	0.35	17.28	69.68	3.99
95 CPO : 5 SO	0.44	1.09	44.31	53.80	0.35	17.29	69.66	3.97
90 CPO : 10 SO	0.47	1.10	44.15	53.93	0.35	17.30	69.62	3.95
80 CPO : 20 SO	0.53	1.11	43.82	54.19	0.35	17.32	69.54	3.89
Genuine Sludge Oil (SO)	0.96	1.21	41.19	56.29	0.35	17.48	68.92	3.46

Each value in the table represents the mean of fatty acids (n=2)
Carbon 12: Methly Laurate; Carbon 14:Methyl Myristate; Carbon 16:Combination of Methyl Palmitate, Palmitoleic Acid Methyl Ester; Carbon 18:combination of Cis-9-oleic methyl acid, Methyl Linoleate, Methyl Linolenate; Carbon 20:Methyl Eicosenoate. ECN Equivalent Carbon Number; ECN 46:Combination of MPL , PLO, PPL; ECN 48:Combination of MMP, OOO, OOP, PPO,PPP; ECN 50:Combination of OOS, POS (P:Palmitic, M:Myristic, O:Oleic, L:Linoleic)

TABLE 2
Fatty Acid Composition (%) and Triacylglycerol ECN (%) of Crude Palm Oil (CPO) and Admixtures with Used Vegetable Oil (UVO)

Sample	Fatty Acid Composition					Triglycerides (ECN)		
	Carbon 12*	Carbon 14	Carbon 16	Carbon 18*	Carbon 20	46	48*	50*
Genuine Crude Palm Oil (CPO)	0.42	1.09	44.47	53.67	0.35	17.28	69.70	4 .00
99 CPO : 1 UVO	0.41	1.09	44.43	53.72	0.35	17.27	69.72	4.00
98 CPO : 2 UVO	0.41	1.09	44.39	53.76	0.35	17.25	69.73	3.99
95 CPO : 5 UVO	0.41	1.09	44.26	53.90	0.35	17.21	69.78	3.99
90 CPO : 10 UVO	0.40	1.09	44.04	54.12	0.36	17.15	69.85	3.97
80 CPO : 20 UVO	0.39	1.08	43.61	54.57	0.37	17.01	69.99	3.94
Used Vegetable Oil (UVO)	0.30	1.03	40.14	58.14	0.40	15.92	71.16	3.68

Each value in the table represents the mean of fatty acids (n=2)
Carbon 12: Methly Laurate; Carbon 14:Methyl Myristate; Carbon 16:Combination of Methyl Palmitate, Palmitoleic Acid Methyl Ester; Carbon 18:combination of Cis-9-oleic methyl acid, Methyl Linoleate, Methyl Linolenate; Carbon 20:Methyl Eicosenoate. ECN Equivalent Carbon Number; ECN 46: Combination of MPL , PLO, PPL; ECN 48: Combination of MMP, OOO, OOP, PPO,PPP; ECN 50:Combination of OOS, POS (P:Palmitic , M:Myristic, O:Oleic, L:Linoleic)

there were significant differences between CPO and admixtures with adulterant on fatty acids and triacylglycerols at different concentrations of adulterant. The parameters that were identified as discriminatory markers based on the T-test for CPO adulteration are Carbon 12, 18 and ECN 48 and 50 for fatty acid and triacylglycerols composition, respectively.

Detection of Adulterants in Crude Palm Oil by Principal Component Analysis (PCA)

Based on the shortlisted chemical properties of CPO and admixtures with SO and UVO, PCA modelling was carried out and the calculation was tabulated. The PCA score plots were used to determine segregation between CPO, SO and UVO (Fig.1). The results indicated that the first two principal components explained 55.4% and 44.6% of the total variability, respectively. Despite the first two principal components that showed 100% total variation, the remaining principal components did not account for any variability and was not important.

Variables with positive loading on PC1 (Fig.2) were Carbon 12 and 18 whereas variables with negative loadings towards PC1 were ECN 48 and 50. Such distribution was strongly related towards the fatty acid and triacylglycerol (TAG) profiles of each oil. A combination of chemical profile markers and PCA was able to segregate CPO and admixtures (Fig.1) into three main clusters.

Each point across the cluster represents the concentration of adulterant in the crude palm oil ranging from 1 to 20% (v/v). As the concentration of adulterant was reduced to 1% (v/v), clusters were formed closer to the crude palm oil. This explains that those blends containing a lower concentration of adulterant exhibited similar profiles with genuine CPO. A similar observation was reported by Monfreda *et al*. (2012) in vegetable oils and by Kim *et al*. (2014) for discriminating cheeses. Blends with 5% adulterant concentration and higher was able to be identified and were distinguishable. With this proposed method it is possible to distinguish pure oils from mixtures. It also should be possible to predict the type and the percentage of an oil used to adulterate pure CPO. This has been demonstrated by Monfreda *et al*. (2014), whose results were obtained from PCA on adulteration of olive olive with seed oils (peanut, corn, rice and grape seeds) dividing based on type of seed oil and percentage of adulterant.

Detection of Adulterants in Crude Palm Oil by Dendrogram

Dendrograms were constructed using the single linkage of the individual adulterant (SO and UVO) with CPO (Fig.2). The dendrograms were fragmented into five main clusters; cluster one comprised genuine CPO and admixtures of adulterant less than 2% (v/v). Clusters 2, 3 and 4 comprised CPO adulterated with 5, 10 and 20% adulterant concentration, respectively and the final cluster was the genuine adulterant (Kostadinović, 2010; Mirhosseini, 2010).

The segregation level was able to be observed according to the concentration of

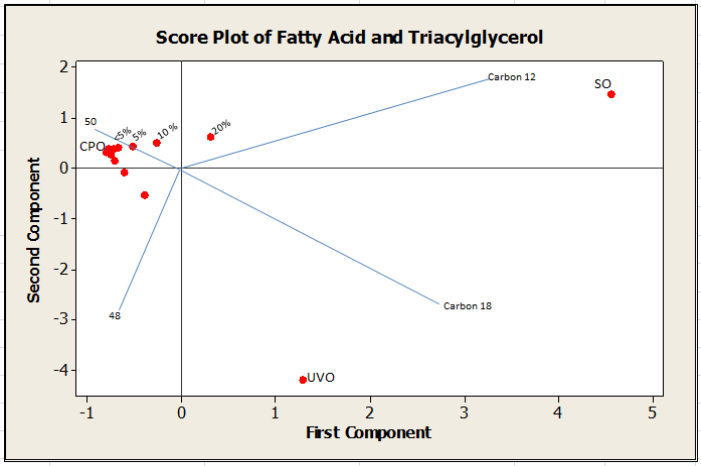

Fig.1: Principal Component Analysis (PCA) scores of crude palm oil (CPO) and blends; PCA of CPO, sludge oil (SO), used vegetable oil (UVO) and PCA loading distribution of fatty acid composition and triacylglycerol

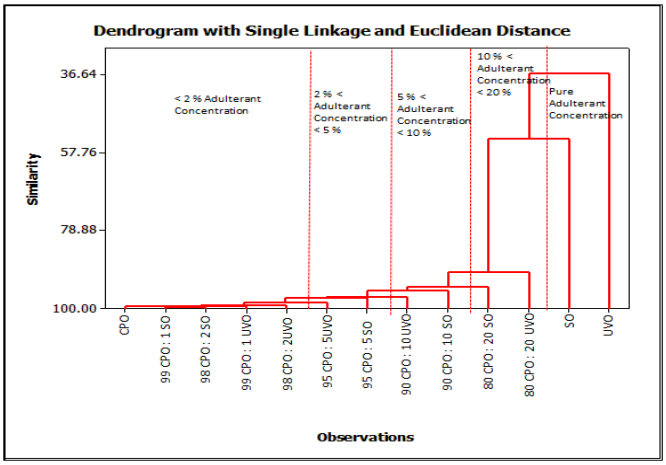

Fig.2: Cluster dendrogram of crude palm oil and adulterant admixtures.

adulterant added to the CPO. Lower level (<2% v/v) adulteration exhibited a similar chemical profile with the genuine crude palm oil that resulted in a single cluster due to the dominant effect of crude palm oil. Such groups are, in fact, well separated despite the existence of adulterant in CPO with less than 2% that was not able to be separated. Thus, detection of adulterant lower than 2% was difficult to be identified.

CONCLUSION

Gas chromatography (GC) equipped with a flame ionisation detector (FID) and high performance liquid chromatograph (HPLC) was employed to analyse the

chemical properties of adulterated crude palm oil (CPO) with sludge oil (SO) and used vegetable oil (UVO). PCA allowed the segregation between CPO and adulterant (SO and UVO) and gave variable loadings for each separated group. Apart from that, the dendrogram cluster allowed the construction of a linkage between crude palm oil and the adulterant that was possible to be used for the identification of blends. Overall, the results of both analyses showed a good indication. The adulterant level as low as 5% and 2% (v/v) was able to be detected by PCA and dendrogram correspondingly. Although this method showed a great potential of CPO quality assessment, the level of detection is still unfavourable. This was due to the dominant effect and similarity of the chemical properties of the adulterant with CPO at the lower level of the adulterant content. The findings of this study can be made more accurate if precision of detection is improved by increasing the number of replications and treatments used as well as by using alternative analytical instruments such as thermal and infrared equipment.

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Functional Properties of Resistant Starch Type-III from *Metroxylon sagu* as Affected by Processing Conditions

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ABSTRACT

Type III resistant starch (RS_3) was produced from native sago starch using different processing conditions. Native sago starch contained 93.5% total starch, of which was 25.8% amylose and 67.7% amylopectin. A sample with the highest RS_3 content (35.7%) was produced when the native sago starch was suspended in distilled water, gelatinised by autoclaving at 121°C for 1 h, followed by debranching with 20U pullulanase per g starch at 60°C for 24 h, autoclaved again at 121°C for 1 h before storage at 4°C for 24 h. The sago RS_3 sample contained 54.0% amylose and 38.8% amylopectin. The powder had solubility, swelling power, water-holding and oil-holding capacity of 27.4%, 2.8g/g, 1.7g/g and 1.1g/g, respectively. Treatment of the sago RS_3 with 0.5M HCl acid at 60°C for 24 h produced HCl-sago RS_3 with 68.30% RS_3 content. The solubility and swelling power of HCl-sago RS_3 was 14.9% and 1.9g/g, respectively. Different processing conditions had significantly influenced the amount and properties of RS_3 produced from sago starch.

Keywords: Resistant starch, *Metroxylon sagu*, sago, amylose, amylopectin, starch property

INTRODUCTION

Consumption of resistant starch in daily meals has captured increasing worldwide attention owing to its health-promoting benefits. Resistant starch refers to the nondigestible starch fraction that resists absorption and digestion along the gastrointestinal tract and may be completely or partially fermented in the colon (Englyst *et al*., 1992). The beneficial physiological effects of resistant starch have been extensively reviewed, and these include prevention of colonic cancer, hypoglycaemic effects, hypercholesterolemia effects, prebiotic function, inhibition of fat accumulation, reduction of gall stone formation, and

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increased absorption of minerals (Fuentes-Zaragoza *et al*., 2011).

Adequate intake of resistant starch is necessary to exert its health benefit effects. The Joint Food and Agricultural Organisation of the United Nations or World Health Organisation Expert Consultation on Human Nutrition have not yet recommended levels for resistant starch consumption. However, the recommended daily consumption of resistant starch by Australia's Commonwealth Scientific and Industrial Research Organisation is approximately 20g (Baghurst *et al*., 2001). This intake level can only be achieved by consuming foods with added resistant starches as food ingredient because foods generally contain low resistant starch (per 100g food): breakfast cereals, 0-3.6g (Alsaffar, 2011); white bread, 0.9g (Brown, 2004); cooked white rice, 7.1g (Vatanasuchart *et al*., 2009) and starchy foods, 0.2-10g (Liljeberg, 2002). Hence, it is suggested that resistant starch be added to foods to increase its amount.

Resistant starch is described as a linear molecule of α-1,4-D-glucan derived from the retrograded amylose fraction of starch that has a relatively low molecular weight of 1.2×10^5a (Fuentes-Zaragoza *et al*., 2011). There are five different types of resistant starch: RS_1, RS_2, RS_3, RS_4 and RS_5. A description and the food sources of each type of resistant starches may be found in Fuentes-Zaragoza *et al*. (2011). RS_3, which was produced in this research, is preferred from among the resistant starches as a functional food ingredient due to its thermal stability high melting temperature at the range of 140°C to 160°C (Shamaia *et al*., 2003). On the other hand, RS_1 and RS_2 are thermally instable, causing them to lose their functional benefits after food processing (Zhao & Lin, 2009), while the legality of RS_4 being used in food production is a major concern (Lunn & Buttriss, 2007). RS_5 is an amylose-lipid complex starch formed from high amylose starches that require a high gelatinisation temperature (Jiang *et al*., 2010). The thermal stability characteristic allows food with added RS_3 to retain its functional benefits even after cooking. Research has also shown that RS_3 can be incorporated into batter without compromising consumer acceptability (Sanz *et al*., 2008).

Production of RS_3 involves four sequential processing steps: disruption of starch granules, enzymatic debranching of starch polymer, starch retrogradation and drying. Every processing step has its own influencing factors in addition to the starch botanical sources, ratio of amylose and amylopectin content and the presence of other components in the starch (Sajilata *et al*., 2006). Previous research has focused on the production of RS_3 from readily accessible starch sources such as maize (Zhao & Lin, 2009), wheat, rice and potato (Garcia-Alonso *et al*., 1998). Less research has been reported on the production of RS_3 from sago (*Metroxylon sagu*) except for our two previous research studies (Leong *et al*., 2007; Siew-Wai *et al*., 2012).

Sago starch is one of the major export commodities for Malaysia, with an increased

output from 44,448.84 metric tonnes in the year 2010 to 50,965.39 metric tonnes in 2011, with an increase in revenue from USD19.1 million to USD27.8 million (Department of Agriculture Sarawak, 2014). Moreover, sago palm produces a relatively higher starch yield, 3 to 4 times more than rice, corn and wheat and 17 times more than cassava in per unit plantation area (Karim *et al*., 2008). Therefore, sago starch was chosen as the raw material to produce RS_3 in this work.

Our previous research produced sago RS_3 with resistant starch content of 11.5% (Leong *et al*., 2007) and 12.2% (Siew-Wai *et al*., 2010). This research was aimed to investigate different processing conditions in increasing the resistant starch content and to investigate the effects of these conditions on functional properties of the resistant starch produced from sago.

MATERIALS AND METHODS

Materials

Native sago starch (Soon Huat Moh Trading Co.) was purchased from a local grocery in Kuching, Sarawak, Malaysia. Pullulanase, a debranching enzyme (Promozyme D2), was purchased from Novozymes (Bagsvaerd, Denmark) and used upon arrival. The enzyme was a technical grade enzyme with a specific activity of 1350 PUN/g (one Pullulanase Unit Novo) and a density of 1.20g/mL. All other chemicals used were of analytical grade and purchased from Sigma Chemicals Ltd. (St. Louis, Missouri, USA).

Production of Sago RS_3

In every processing step, factors that were thought to influence the amount of RS_3 produced were individually assessed. The experiments were conducted in triplicate. Fig.1 summarises the flow of the processing conditions. There were four main processing steps involved in the production of sago RS_3:

Step 1: disruption of starch granules. Two disruption methods were investigated in this step as follows:

Gelatinization by heat treatment. Twenty gram of native sago starch (20%, w/v) was suspended in 100mL of 0.1M acetate buffer, pH5 or distilled water and subjected to heat treatment; boiling for 10 min and/or autoclaving at 121°C for 1 h. The starch gel was cooled to 60°C prior to the enzymatic debranching step (Step 2).

Partial acid hydrolysis. Native sago starch was suspended with continuous stirring at ambient temperature (25°C) in 1M hydrochloric acid (HCl) at a ratio of 1 g starch to 3.5mL acid for 24 h. The pH of starch-acid suspension was then adjusted to pH7.0 with 2M of NaOH and centrifuged (2330×*g*, 15 min). The pellet was washed three times with distilled water. It was then oven-dried at 40°C until its moisture content was less than 13% and ground to fine powder with particles of less than 180µm. This acid-treated powder (20%, w/v) was then suspended in 0.1M acetate buffer (pH5.0), and subjected to enzymatic debranching and further processing steps.

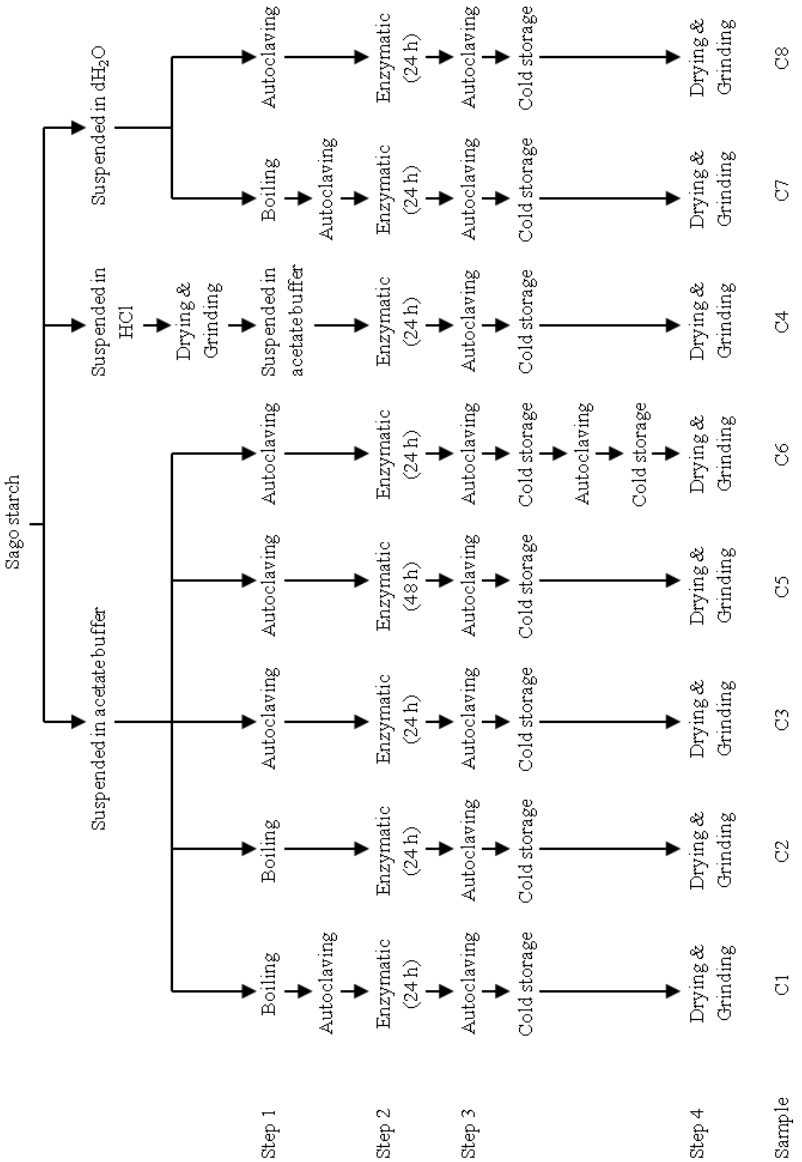

Fig.1: Different processing conditions in the production of sago RS_3.

Step 2: Enzymatic debranching. Pullulanase enzyme was added at 20 PUN/g starch, and the starch-enzyme suspension was incubated in an orbital incubator shaker (Certomart *SII*, Sartorius, Melsungen, Germany) at 60°C for 24 h or 48 h. The reaction was stopped by heating the starch-enzyme suspension in a water bath (80°C) for 15 min.

Step 3: Thermal processing and cold storage. Starch-enzyme suspensions from Step 2 was autoclaved at 121°C for 1 h and cooled to ambient temperature (25°C) before storing in a refrigerator at 4°C for 24 h. Some sample suspensions were subjected to few cycles of thermal processing and cold storage.

Step 4: Drying and grinding. The resulting starch was oven-dried at 40°C until its moisture content was less than 13%, and finally ground to fine powder with particles of less than 180µm. The ground powder was used as sago RS_3 samples.

Acid hydrolysis of sago RS_3 to produce HCl-sago RS_3

Sago resistant starch sample (sago RS_3 from Step 4) with the highest resistant starch content was further subjected to hydrolysis with 0.5M HCl at a ratio of 1:3.5 (sago RS_3:HCl) at 60°C for 24 h with continuous shaking (150rpm) to produce HCl-sago RS_3. The starch slurry was centrifuged at 2330×*g* for 15 min. The starch pellet was washed with distilled water several times and dried in an oven at 40°C until its moisture content was less than 13%. Dried starch was then ground to fine particles of less than 180µm.

Chemical Analyses

Native sago starch was analysed for its moisture, ash, crude protein, crude fat and crude fibre contents (AACC, 2000). Native sago starch was also analysed for total starch (Goni *et al*., 1997) and amylose contents (Hoover & Ratnayake, 2001). Amylopectin content was determined by the difference of total starch and amylose contents.

Resistant starch content was determined according to Goni *et al*., (1996) and calculated as follows:

$$\text{Resistant starch (\%)} = \frac{\text{mg glucose} \times \text{dilution factor} \times 0.9}{\text{sample weight (mg,dry basis)}} \times 100\% \quad [1]$$

The method involved the removal of protein from samples with pepsin (HiMedia RM084, 400U/mg sample, 40°C, pH 1.5, 60 min), hydrolysis of digestible starch with pancreatic α-amylase (Sigma A-3176, 4 U/mg sample, 37°C, pH6.9, 16 h), solubilisation of precipitates with 4M KOH and hydrolysis of samples with amyloglucosidase (Sigma 10115, 0.12U/mg sample, 60°C, 45 min, pH4.8). Finally, the liberated glucose in the sample was determined by using the glucose oxidase assay.

Physical characterisation of sago RS_3 and HCl-sago RS_3

Swelling power and solubility of samples were determined (Chan *et al*., 2010) whereby 100mg of sample was accurately weighed in a pre-weighed 50mL centrifuge tube and 10mL of distilled water was added. The tube was placed in a water bath at 90°C for 30 min and centrifuged at 2330×*g* for 15 min. Then, 5mL of the supernatant was carefully pipetted to a pre-weighed moisture dish and dried in an oven at 110°C overnight. The moisture dish was then cooled in a dessicator and weighed. The wet sediment in the centrifuge tube was also weighed. The swelling power and solubility were calculated as follows:

$$\text{Swelling power (g/g)} = \frac{\text{weight of wet sendiment (g)}}{\text{weight of sample used (g)}} \times 100\% \quad [2]$$

$$\text{Solubility (\%)} = \frac{\text{weight of wet supernatant (g)}}{\text{weight of sample used (g)}} \times 100\% \quad [3]$$

Water-holding capacity (WHC) of samples was determined (Chau *et al*., 1997) whereby 1g of sample was vortexed in 10mL of distilled water at an ambient temperature (25°C) for 1 min and followed by centrifugation at 2200×*g* for 30 min. The supernatant was removed and the wet sediment was weighed. Oil-holding capacity (OHC) was determined by replacing the distilled water with corn oil (Yee Lee Edible Oils Pvt. Ltd., Malaysia). The water-holding and oil-holding capacities were expressed as weight of water or oil held per gram of sample, and was calculated as follows:

$$\text{WHC or OHC (g/g)} = \frac{\text{Weight of wet sediment - weight of sample used (g)}}{\text{weight of sample used (g)}} \times 100\% \quad [4]$$

Statistical Analysis

All the data from triplicate experiments with triplicate analyses were subjected to one-way ANOVA using a computer software, SPSS version 14.0 (Illinois, USA) and the significance of difference between means was determined by the Duncan test at 5% probability level. Pearson's correlation coefficients between the amount of RS_3 produced with amylose content, solubility, water-holding capacity and oil-holding capacity were analysed and considered significantly different at a 1% probability level.

RESULTS AND DISCUSSION

Chemical Analysis of Native Sago Starch

Table 1 shows the chemical composition of native sago starch. Ash, crude protein, crude fat and crude fibre content of the native sago starch in dry basis were 0.17%, 0.18%, 0.13% and 0.64%, respectively. The native sago starch contained 93.45% total starch of which was 25.77% amylose and 67.68% amylopectin. The crude fibre content in the sago starch was higher than that of

sago starch from various manufacturers as determined by Ahmad *et al*. (1999) whereas other values were similar. The native sago starch contained 34.38% of resistant starch. Fuentes-Zaragoza *et al*. (2011) had reported that the resistant starch of various food sources including native starches from corn, wheat rice and potato ranged from 1.6 to 11.0%. However, a comparison of resistant starch content of native starch in this study could not be made with other native starches because of the difference in analysis protocol. Furthermore, the flours were subjected to processing stages of grinding, fine milling, sieving and steam processing (Fuentes-Zaragoza *et al*., 2011), and this processing method had converted the native starches into RS_3. Considering all the factors influencing the resistant starch content determination, Leong *et al*. (2007) had reported that native sago starch contained 41.8% RS, which is higher than what was obtained from this research.

Resistant Starch, Amylose and Amylopectin Content of Samples

Table 2 shows the resistant starch, amylose and amylopectin contents in samples obtained from eight different processing conditions. A decreasing order of resistant starch content from 35.71% to 12.34% was obtained: C8 > C5 ≥ C3 > C7 > C1 > C6 ≥ C2 > C4. A similar decreasing order of amylose content in the samples from 54.05% to 3.84% was also observed.

Production of RS_3 is dependent on amylose content of the starting starchy materials. This was demonstrated by Sievert and Pomeranz (1989) whereby seven different types of starch with different amylose contents ranging from less than 1% to 70% were used to produce RS_3. It was found that the highest RS_3 yield (21.3%) was from amylomaize VII, which contained initial amylose content of 70%. Resistant starch content produced from sago starch in this research was higher (35.71%) with a lower initial amylose content of 24.08%

TABLE 1
Chemical Composition of Native Sago Starch

Analysis	Content (%, dry basis)
Ash	0.17±0.06
Crude fat	0.13±0.02
Crude protein	0.18±0.02
Crude fibre	0.64 ± 0.02
Total starch	93.45±1.19
Resistant starch	34.38 ± 0.22
Digestible starch (by difference)*	59.07 ± 0.22
Amylose	25.77±0.20
Amylopectin (by difference)#	67.68 ±0.20

Results are expressed as means ± standard deviation (N=3).
* Digestible starch = Total starch – Resistant starch; # Amylopectin = Total starch – Amylose.

than amylomaize. This indicated that the production of RS_3 was influenced not only by the initial amylose content of the starchy materials but also by the processing conditions.

Apparently, suspending the sago starch in 0.1M acetate buffer, pH 5 did not significantly improve the production of sago RS_3 as compared to suspending the starch in distilled water (comparing samples C3 and C8 and samples C1 and C7). Debranching α-1,6-glucosidic linkages in sago starch polymer with pullulanase enzyme for a longer time (48 h) also did not significantly improve the RS_3 content (comparing samples C3 and C5). A longer enzymatic reaction time probably produced debranched samples with an abundance of short chain amylose chains that were unable to form RS structure. Consequently, the yield of RS, which varied with average chain length of amylose would be low. It is generally recognised that RS is a 20-25 glucose residue long, retrograded or recrystallised and hydrogen-bonded, polydisperse linear oligosaccharide (Eerlingen *et al*., 1993).

The most apparent reason for high production of sago RS_3 was the gelatinisation step. Gelatinisation of sago starch by autoclaving for 1 h had significantly produced higher RS_3 content than by boiling (comparing samples C2 and C3, and samples C7 and C8). Samples C1 and C7 that were boiled and subsequently autoclaved also contained lower RS_3 content than that of samples C3 and C8, respectively. This is because during boiling, formation of starch gel in samples was observed. The

TABLE 2
Resistant Starch, Amylose and Amylopectin Contents, and Swelling Power and Solubility of Samples Obtained from different processing Conditions

Sample	Resistant Starch (%)	Amylose (%)	Amylopectin (%)	Swelling at 90°C (g/g, dry basis)	Solubility at 90°C (%, dry basis)
C8	35.71 ± 0.59^{a}	54.05 ± 0.53^{a}	38.79 ± 0.53^{g}	2.82 ± 0.05^{f}	27.36 ± 0.80^{a}
C5	33.00 ± 0.09^{b}	49.97 ± 0.60^{b}	42.87 ± 0.60^{f}	4.01 ± 0.22^{d}	21.64 ± 0.03^{b}
C3	32.99 ± 0.41^{b}	46.88 ± 0.83^{c}	45.96 ± 0.83^{e}	3.49 ± 0.06^{e}	19.15 ± 0.42^{c}
C7	31.00 ± 0.36^{c}	43.32 ± 0.38^{d}	49.52 ± 0.38^{d}	3.52 ± 0.08^{e}	21.45 ± 1.05^{b}
C1	28.42 ± 0.57^{d}	37.11 ± 0.23^{e}	55.73 ± 0.23^{c}	4.37 ± 0.11^{c}	17.00 ± 0.03^{d}
C6	26.47 ± 0.34^{e}	25.42 ± 0.39^{f}	67.42 ± 0.39^{b}	5.04 ± 0.07^{ab}	16.56 ± 0.36^{d}
C2	26.31 ± 0.84^{e}	24.47 ± 1.17^{f}	68.37 ± 1.17^{b}	4.81 ± 0.05^{b}	16.74 ± 0.38^{d}
C4	12.34 ± 1.63^{f}	3.84 ± 0.19^{g}	89.00 ± 0.19^{a}	5.15 ± 0.18^{a}	9.93 ± 0.38^{e}

Results are expressed as means ± standard deviation (N=3).
Mean values in the same column followed by different superscript lower case letters^{abcdefg} are significantly different at 5% probability level.

subsequent autoclaving process did not affect sample gelatinisation as much as during boiling because the starch was in gel form with less available water. It was shown that gelatinisation of wheat and corn starches at 120°C had increased RS_3 yield more than gelatinisation at 100°C (Garcia-Alonso *et al*., 1998). One research study had also proved that increasing the heating temperature of locust bean starch from 50ºC to 110°C increased the yield of RS_3 from 39% to 45% (Sankhon *et al*., 2012). However, the effect of gelatinisation temperature on RS_3 yield was also dependent on the botanical source of starches. It was reported that autoclaving had decreased the RS_3 content of rice and potato starches (Garcia-Alonso *et al*., 1998).

RS_3 content in this research was very much improved than in our two previous research studies (Leong *et al*., 2007 and Siew-Wai *et al*., 2010). It was noted that in those two previous studies, sago starch was subgelatinised at 60°C prior to enzymatic debranching. The extent of starch gelatinisation affects the degree of solubilisation of amylose chains and the available amount of amylopectin for the subsequent enzymatic debranching process. A higher temperature applied ensures that starch granules are fully gelatinised (Yao *et al*., 2010) while pressure enhances the diffusion of water molecules into starch granules (Liu *et al*., 2009). During the gelatinisation process, the heating of starch suspension in excessive water raises its temperature progressively, allowing starch molecules to absorb heat energy and increasing the vibration causing the breakage of hydrogen bonds among the starch molecules (Bryksa & Yada, 2009). Meanwhile, hydrogen bonds are formed between water molecules and starch molecules, allowing water to penetrate into the starch granules to such an extent that the irreversible swelling of starch granules occurs (Vaclavik & Christian, 2014). Swelling causes starch granules to lose their birefringence and their ordered crystalline structure. Eventually, they are disrupted, allowing polymer chains to leach out from the starch granules (Vaclavik & Christian, 2014).

Pullulanase enzyme hydrolyses α-1,6-glucosidic linkages of amylopectin in sago starch polymer, releasing short and long linear chains of amylose molecules (Leong *et al*., 2007). Therefore, together with the starch gelatinisation steps, availability of amylose molecules increased and recrystallisation of amylose polymers formed resistant starch easily (Zhao & Lin, 2009). It was shown that the RS_3 content of samples C1 to C8 were positively correlated ($r=0.97$) with amylose content ($p<0.01$).-

Two cycles of heat treatment and cooling (comparing samples C3 and C6) did not improve the RS_3 content in this present research. The effect of the cycle was pronounced with a higher number of cycles (up to 20 cycles), which could raise the RS_3 yield of corn starch from 21.3% to over 40% (Sievert & Pomeranz, 1989).

We also hydrolysed the sago starch polymer with 1M HCl for 24 h to replace starch gelatinisation by heat treatment

while maintaining the other processing steps, producing sample C4 with 12.34% RS_3 content. Although this was an energy-saving approach that utilised only one cycle of autoclaving, the condition had produced the lowest RS_3 content among the samples. It was also noted that sample C4 contained low amounts of amylose (only 3.84%) and 89% amylopectin. It was thought that the hydroxonium ion (H_3O^+) from acid easily attacks the glycosidic linkages of branched polymers in the amorphous region to allow increased polymer mobility for molecular rearrangement and produce short linear chains of amylose (Thompson, 2000). The short linear chains appeared to participate in the rearrangement and recrystallisation of starch during autoclaving and the cooling treatment for the formation of RS_3. However, without starch gelatinisation by heat treatment, a high amount of amylopectin still remained in the crystalline region of the starch granules, and this limited the access of the pullulanase enzyme.

Swelling Power and Solubility

The amount of amylose and amylopectin content in a starchy sample is important, as it does not only influence the amount of RS_3 content of the samples, but also influences the physical characteristics of the samples. Table 2 shows the swelling power and solubility of the RS_3 samples from different processing conditions. One research study showed that swelling power of a product was positively correlated with amylopectin content (Tester & Morrison, 1990). In this research, sample C4 contained the highest amylopectin content ($p<0.05$). Consequently, sample C4 had the highest swelling power than other samples because amylopectin can swell freely without restriction by amylose. In contrast, sample C8 with the lowest amylopectin content had the lowest swelling power ($p<0.05$).

Generally, samples with a higher amount of resistant starch, regardless of the type of resistant starch, have lower solubility. This is demonstrated in research by Shin *et al*. (2003) and Ozturk *et al*. (2009). Similarly, gelatinised and retrograded banana starch with higher RS_3 content also exhibited lower solubility of the sample at 90°C (Aparicio-Saguilan *et al*., 2005). However, processing conditions to produce resistant starch had influenced the solubility of the samples. As noted in this research, the solubility of samples C1 to C8 was positively correlated with the RS_3 content (r=0.91). A similar trend was also observed in the research by Ozturk *et al*. (2009) whereby the sample with the highest RS_3 content from corn starch demonstrated the highest solubility. The main factor that is thought to have contributed to this observation was the use of enzymatic debranching in the processing steps. RS_3 samples that were produced without going through the enzymatic debranching process, as in the case of banana RS_3 (Aparicio-Saguilan *et al*., 2005), had negative correlation with their solubility. As mentioned earlier, pullulanase enzyme cleaves α-1,6 glycosidic linkages of amylopectin to release branching chains and generates a mixture of long and short units of amylose for the production of

resistant starch (Leong *et al*., 2007). These linear chains of amylose unit cause the RS_3 samples to solubilise more. Significant increases in solubility were observed in samples hydrolysed longer with pullulanase (Ozturk *et al*., 2009).

Water-Holding and Oil-Holding Capacity

Water-holding capacity (WHC) measures the interaction magnitude of samples with water molecules. It was noted that WHC of sample was negatively correlated with RS_3 content (r=0.97). Table 3 shows that the sample with the highest RS_3 content (35.71%) had the lowest WHC (1.66 g/g) while the sample with the lowest RS_3 content (12.34%) had the highest WHC (2.45 g/g).

RS_3 mainly comprises retrograded amylose that is joined tightly by a hydrogen bond. Due to the tight bonding within the insoluble crystalline structure, RS_3 is not able to form a hydrogen bond with water molecules. Hence, increasing the RS_3 content in the formulation of processed food can lower the food's WHC. In the baking industry, resistant starch-containing bread with a lower WHC provides a better texture and is more easily handled during processing (Sajilata *et al*., 2006). It also decreases the loaf volume (Sajilata *et al*., 2006) and maintains the structure of the crumb (Ranhotra *et al*., 1999). Hence, RS_3 in food can exert both nutritional benefit and improve the property of the food.

Table 3 shows the oil-holding capacity (OHC) property of the sago RS_3 samples produced from different conditions. The OHC was used to measure the ability of starches to hold the oil. Sample C8 with the highest resistant starch content (35.71%) had the highest capacity in holding oil (1.04 g/g) while sample C4 and C1 had the lowest OHC although RS_3 content of sample C1 was double that of sample C4. Statistical analysis showed that RS_3 content of sample C1 to C8 was not significantly (p>0.01) correlated with the OHC. Previous research

TABLE 3
Water-holding (WHC) and Oil-holding Capacity (OHC) of Samples Obtained from Different Processing Conditions

Condition	Resistant Starch (%)	WHC (g/g)	OHC (g/g)
C4	12.34 ± 1.63^{f}	2.45 ± 0.01^{a}	0.85 ± 0.02^{e}
C2	26.31 ± 0.84^{e}	1.85 ± 0.06^{b}	0.88 ± 0.03^{cde}
C1	28.42 ± 0.57^{d}	1.85 ± 0.05^{b}	0.82 ± 0.03^{e}
C6	26.47 ± 0.34^{e}	1.79 ± 0.05^{bc}	0.87 ± 0.04^{de}
C7	31.00 ± 0.36^{c}	1.78 ± 0.01^{bc}	0.94 ± 0.03^{bc}
C3	32.99 ± 0.41^{b}	1.71 ± 0.09^{c}	0.93 ± 0.01^{bcd}
C5	33.00 ± 0.09^{b}	1.69 ± 0.01^{c}	0.96 ± 0.03^{b}
C8	35.71 ± 0.59^{a}	1.66 ± 0.07^{c}	1.04 ± 0.04^{a}

Results are expressed as means ± standard deviation (N=3).
Mean values in the same column followed by different superscript lower case letters ^{abcde} are significantly different at 5% probability level.

has shown that the OHC levels of lentil and chickpea flours were not dependent on the amount of RS_3 (Aguilera *et al*., 2009). Further research is anticipated to study the functionality and suitability of this sago RS_3 to be incorporated in food formulation especially in fried foods.

RS_3 content was further increased with the treatment of sago RS (sample C8) with 0.5M HCl (sample designated HCl-sago RS3) to 63.80%. It was thought that the hydroxonium ion (H_3O^+) of acid hydrolysed the digestible portion of the sago RS_3 sample, contributing to the enhanced level and purity of the RS_3 content in the HCl-sago RS_3 sample. Native sago starch, sago RS and HCl-sago RS samples were subjected to further analyses. It was found that the solubility and the swelling power of HCl-sago RS was 14.90% and 1.94 g/g, respectively, which was lower than sago RS due to higher RS_3 content.

CONCLUSION

Processing conditions influenced the amount of sago RS_3 and its functional properties. Processing condition with gelatinisation of starch suspension in distilled water by autoclaving at 121°C for 1 h, followed by pullulanase debranching of the starch polymers at 60°C for 24 h prior to autoclaving and cold storage at 4°C for 24 h produced a sago RS sample with 35.71% RS_3 content. Among the sago RS samples, sample C8 had the highest amylose content, highest solubility and oil-holding capacity while it was the lowest in amylopectin content, swelling power and water-holding capacity. Hydrolysis of this sago RS with 0.5M HCl acid at 60°C for 24 h produced HCl-sago RS with 68.30% RS_3 content. The solubility and swelling power of HCl-sago RS were lower than those of sago RS.

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     - Jalaludin, S. (1997b). The use of different vegetable oil in chicken ration. *Malayan Agriculturist, 11,* 29-31.
     - Tan, S. G., Omar, M. Y., Mahani, K. W., Rahani, M., & Selvaraj, O. S. (1994). Biochemical genetic studies on wild populations of three species of green leafhoppers *Nephotettix* from Peninsular Malaysia. *Biochemical Genetics*, *32,* 415 - 422.

   - In case of citing an author(s) who has published more than one paper in the same year, the papers should be distinguished by addition of a small letter as shown above, e.g. Jalaludin (1997a); Jalaludin (1997b).

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7. **Examples of other reference citations**:

   Monographs: Turner, H. N., & Yong, S. S. Y. (2006). *Quantitative Genetics in Sheep Breeding*. Ithaca: Cornell University Press.

   Chapter in Book: Kanwal, N .D. S. (1992). Role of plantation crops in Papua New Guinea economy. In Angela R. McLean (Ed.), *Introduction of livestock in the Enga province PNG* (p. 221-250). United Kingdom: Oxford Press.

8. ***Proceedings***: Kanwal, N. D. S. (2001). Assessing the visual impact of degraded land management with landscape design software. In Kanwal, N. D. S., & Lecoustre, P. (Eds.), *International forum for Urban Landscape Technologies* (p. 117-127). Lullier, Geneva, Switzerland: CIRAD Press.

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