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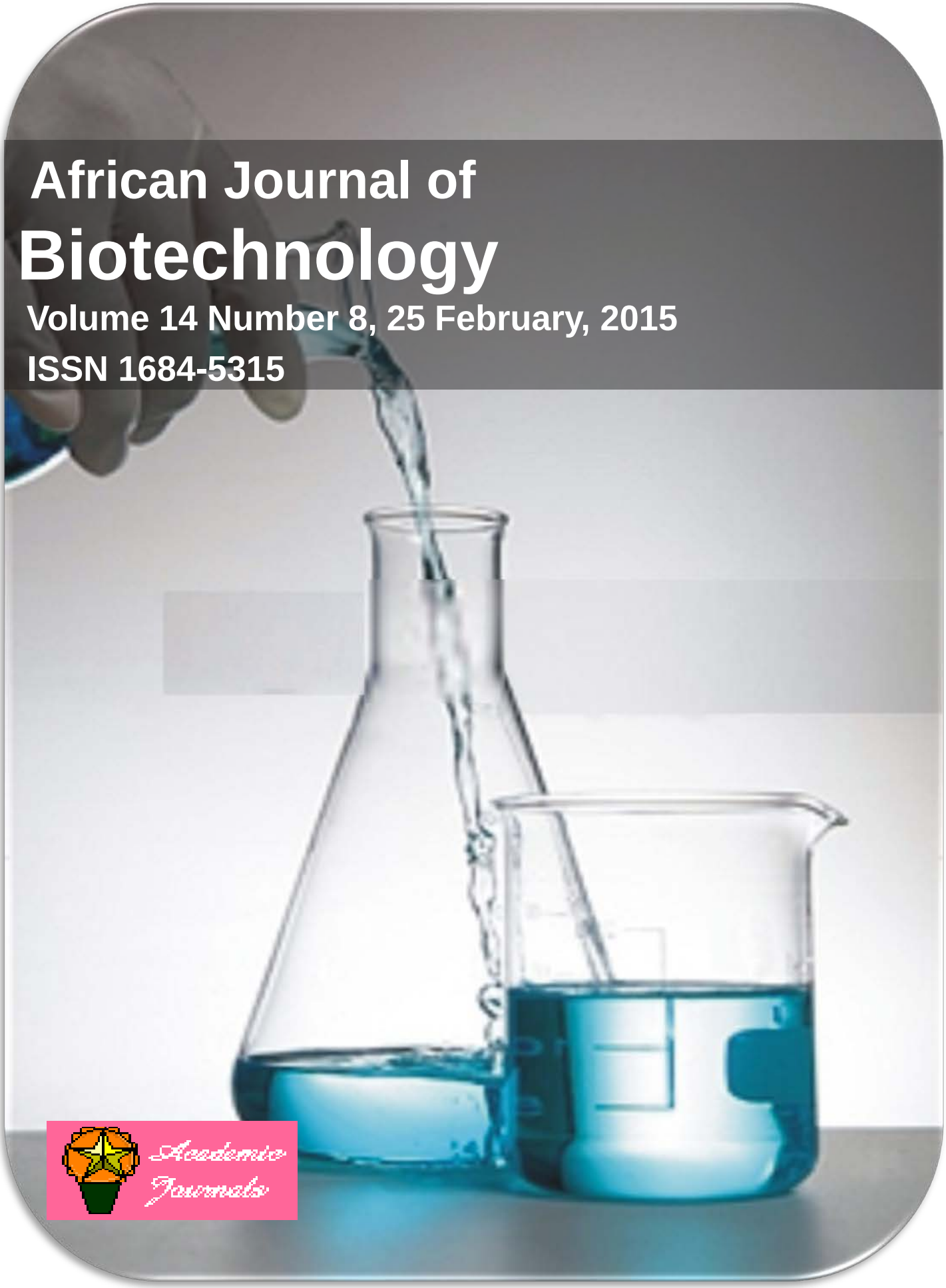

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Review

Properties of Brazil nuts: A review

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Brazil nut is a seed with high nutritional value and of great economic importance to the Northern region of Brazil. In addition to enabling direct consumption, its nutritional potential enables the development of various products. Among its nutrients, emphasis is given to the amino acid-rich proteins, lipid content and selenium, which exhibits antioxidant properties. This review addresses the nutritional value and approaches different technologies applied in the Brazil nut products process.

Key words: *Bertholletia excelsa*, selenium, oleic acid.

INTRODUCTION

Brazil nut (*Bertholletia excelsa*, Bonpl.), a plant in the family of Lecythidaceae, is native to the Amazon rain forest and adjacent areas in Brazil, Bolivia, Peru (Ferreira et al., 2011). It is an extractive product with high ecological, social, economic and nutritional value (Silva et al., 2010). It is considered a good nutritional source for food enrichment and production as an alternative ingredient for some consumer groups (Yang, 2009). The kernels contain about 60 to 70% lipids, 15 to 20% protein, sulfur, vitamin E and antioxidant properties (Martins et al., 2012). The largest part of its production comes from areas of extractive activities, that is, they are collected from the natural forest; the seed is processed in plants by dehydration to obtain the dehydrated kernel (safe humidity below 15%) to be processed in-shell or shelled. Figure 1 shows a flowchart of Brazil nut production. The nuts that do not meet the size standards (small, medium and large) or have undesirable characteristics (color, shape or stains) according to industry standards can be otherwise used in pieces and/or for the production of other products, such as oil, or as ingredients to add to cereal bars and cookies (Cardarelli and Oliveira, 2000). Figure 2 shows the different classifications of Brazil nut used by the processing industry. The Brazilian production has two different purposes in terms of trade: domestic consumption and export. These rates have changed, and today export accounts for 25 to 30% and domestic consumption accounts for 70 to 75% of national production. Some of Brazil nut importing countries are Bolivia, United States (including the processed nuts),

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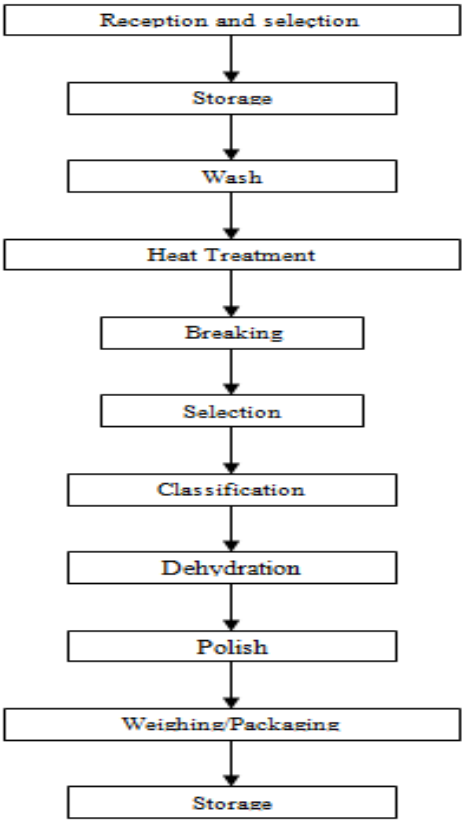

Figure 1. Process flowchart for shelled and unshelled nuts. Source: PACHECO; SCUSSEL, 2006.

Figure 2. Different classifications of Brazil nut used by the processing industry: (a) large, (b) medium and (c) midget.

Hong Kong, Australia and several European countries. The objective of this manuscript was to bring relevant information about the nutritional and technological properties involving Brazil nuts. This way, the study was divided as follows: an overview on nutritional aspects and Brazil nut products, including oil, cake and flour, milk extract and extruded products.

NUTRITIONAL ASPECTS

Brazil nut has high content of proteins, carbohydrates, unsaturated lipids, vitamins and essential minerals. The average content of lipids (60-70%) contributes to its high caloric content, and the ratio of saturated, mono-unsaturated and polyunsaturated fatty acids is 25:41:34 (USDA, 2008). These data show that the content of unsaturated fat in the Brazil nut is higher than that of any other nut. Brazil nuts are a good source of vitamin E (Da Costa et al., 2011) and of essential amino acids (Ampe et al., 1986; Souza and Menezes, 2004; Silva et al., 2010). It is also a source of micronutrients, especially selenium (Se), phytosterols, tocopherols, squalene, and phenolic compounds; all of them are associated with potential health benefits. Furthermore, the FDA has approved the following health claim and disclosure statement: "Scientific evidence suggests", but does not prove, that eating 1.5 ounces per day of most nuts, such as Brazil nuts, as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease (FDA, 2008)." Beta- and gamma-tocopherols are the most abundant tocopherol isomers present in Brazil nuts. The contents of alpha- and gamma-tocopherol in Brazil nuts are 72.55 and 74.35 mg/g of oil, respectively, and the contents of beta-sitosterol, campesterol and stigmasterol are 79.00, 4.00, and 11:33 mg/g of oil, respectively (Costa et al., 2010). Compared to other tree nuts, Brazil nuts are known as the best source of Se (Chunhieng et al., 2004; Pacheco and Scussel, 2007; Chunhieng et al., 2008; Welna et al., 2008). Furthermore, it also contains numerous vital minerals such as calcium, magnesium, phosphorus, and potassium (USDA, 2008; Yang, 2009). Adequate intake of Se is essential for normal activity of numerous selenoenzymes involved in the protection against oxidative stress, maintenance of redox status and regulation of the immune system and thyroid function (Reeves and Hoffmand, 2009). This element can also protect against prostate, lung, and liver cancers due to its high levels of phytonutrients (Yang, 2009). Thomson et al. (2008) demonstrated that 100 µg/day of Se (equivalent to two nuts), for three months increased plasma selenium concentrations and glutathione peroxidase in healthy subjects. Gonzalez and Salas-Salvado (2006) suggested that the risk of developing chronic diseases was reduced with regular consumption of Brazil nuts, and it increased the body's ability to regulate hormone levels. Furthermore, animal experiments, cell culture and observational studies indicate that anti-oxidants can prevent the development of cancer and cardiovascular disease (Kris-Etherton et al., 2001). In another study, Stockler-Pinto et al. (2009) reported that the ingestion of a single serving of Brazil nut (5 g) a day for three months is effective in increasing the concentration of Se and glutathione peroxidase activity thereby improving the antioxidant capacity in patients with disabilities in that capacity. The consumption of only one nut increases the concentration of Se and glutathione peroxidase activity (Cominetti et al., 2010). In addition to the kernel, the seed coat from different geographic origins showed an average content of Se of 6.34 to 20.58 mg/kg (Manfio et al. 2012). Other minerals such as barium (Ba) and radium (Ra) can also be found in Brazil nuts (Martins et al. 2012). The varied levels of Ra in the nuts seem to be influenced by bioaccumulation in the tree, which is transferred to the seeds depending on the concentration of Ra in the soil that is absorbed by the tree. Consequently, the concentrations of Se and Ba in the nuts can vary (Parekh et al., 2008). Tables 1 and 2 shows the major nutrients and minerals present in Brazil nut.

BRAZIL NUT PRODUCTS

Despite of in-shell or shelled Brazil vacuum packaged for sale, the thin brown "skin" covering the seed, is rich in protein, lipids, and selenium. It is obtained as a waste product resulting from processing, but due to the antioxidant potential seems to have the potential to be used as a dietary supplement. In addition, it can be tested in the biotechnology field to obtain enzymes with industrial potential. Among the products obtained from industrial processing of whole nuts or pieces of nuts are oil and cake (partially or fully defatted) obtained by nut pressing and/or by extracting the fatty material (Souza and Menezes, 2004). The oil can be obtained by extraction using n-hexane and ethanol, and it can be used in food or in cosmetic formulations (Freitas et al., 2007). An extract, also called milk, is obtained from the cake by dilution in hot water followed by centrifuging. This milk is intended for culinary use, especially for lactose intolerant individuals. Extrusion is another industrial process used to obtain food. This technique converts a solid material to liquid by combining moisture, heat, compression and shear stress promoting starch gelatinization forcing their passage through a matrix (Borba et al., 2005). Snack foods, animal feed, cereals, etc. are produced by this process. This review describes some Brazil nut products and the processes and technologies used to obtain them.

Oil

Brazil nut oil can be used in cosmetics, foods and

Table 1. Brazil nut proximate composition by different authors.

Proximate composition	Almeida (1963)	Andrade et al. (1999)	Souza and Menezes (2004)	Venkatashalam and Sathe (2006)	Moodley et al. (2007)	USDA (2008)	Felberg et al. (2009)
Calories (Kcal)	666.0	-	676.5	-	-	-	-
Lipids (g%)	65.9	66.8	67.3	66.7	66.8	69.0	70.62
Protein (g%)	14.40	13.60	14.29	19.93	13.60	18.0	14.35
Carbohydrates (g%)	11.00	10.30	3.42	0.69	10.30	13.0	11.61
Fiber (g%)	2.10	-	8.02	-			-

Table 2. Minerals in Brazil nut according different authors.

Component[a]	Furr et al. (1979)[b]	Andrade et al. (1999)	Gonçalves et al. (2002)[c]	Chunhieng et al. (2004)	Ferberg (2004)	Moodley et al. (2007)[b]	Yang (2009)	Naozuka et al. (2010)	Silva et al. (2010)
Calcium	1592	132	206.75	6060	159.04	7432.8	7432.8	-	205
Copper	1.9	1.3	1.17	-	2.22	59.44	59.44	11.0	1.35
Iron	93	3.4	9.67	80	2.82	74.26	74.26	18.3	-
Phosphorus	1.7	674	564.50	23800	721.25	-	-	-	563
Magnesium	3370	160	312.50	13380	381.90	9678.5	9678.5	-	310.10
Manganese	8	0.6	6.85	50	1.34	3.4	3.4	5.02	5.99
Potassium	5405	644	514.75	19690	717.25	-	-	-	512.70
Zinc	41	3.5	7.1	115	4.72	110.31	110.31	92.8	6.90

[a]mg%, [b]retail packaging, [c]origin: trees near Manaus (AM) Brazil.

pharmaceuticals. The oil can be extracted by mechanical or hydraulic press or by using reagents or CO_2 (Neto et al., 2009). Hot or cold pressed extractions are the most commonly used methods for the extraction of oils from most oilseeds at an industrial scale.

In a laboratory scale, solid-liquid extraction is commonly used with solvents such as ethanol and n-hexane (Santos et al., 2012). The difficulties related to oil recovery and the possibility to damage the oil and the cake, due to the temperature increase that occurs during pressing, emphasize the need to develop low temperature extraction methods (Guedes, 2006). Supercritical CO_2 extraction can be used as an alternative method to degrease Brazil nut (Penedo et al., 1997; Rodrigues et al., 2005).

The composition of the Brazil nut crude oil shows high content of unsaturated fatty acids, 36.21 to 51% of monounsaturated oleic fatty acid, and 34 to 38.28% of oleic fatty acid (Silva et al., 2010; Ferreira et al., 2006). According to Silva et al. (2010), the oleic fatty acid is the main component of almond oil, but Brazil nut is also a source of polyunsaturated fatty acids (Silva et al., 2010; Ferreira et al., 2006; Gonçalves et al., 2002). Saraiva et al. (2009) found linoleic acid levels between 30 - 47%. Funasaki et al. (2012) argue that due to the fact that Brazil nut oil is rich in unsaturated fatty acids, which are sensitive to oxidation, the rate of oxidation should be monitored since it is an important quality parameter. The main fatty acids found are shown in Table 3.

"Cake" and flour

The brown residue obtained by extracting the kernel oil, generally called "cake", has aroused great interest among researchers given its high protein content (Gloria and Regitano - D'arce, 2000). According to Ferreira et al., (2006), the cake, besides containing on average 19.17% lipids, 28.34% protein, and 39.63% carbohydrates, it is also an excellent source of Se.

Souza and Menezes (2004) found 0.714 mg Se/100 g of cake, which is 3.56 times higher than the content of Se found in the Brazil nut kernel (0.204 mg /100 g). This difference can be explained by the large number of kernels with their skin that are generally used to obtain the cake and also by its lower lipid content, suggesting that the kernel skin may contain high concentrations of selenium (Berno et al., 2010). The "cake" is considered an excellent source of vegetable protein due to its richness in sulphur amino acids, methionine and cysteine, which are usually missing in other vegetable proteins (Cohen et al., 2007).

Table 3. Major fatty acids present in crude oils from the nuts of Brazil, according to different authors.

Component (acids)	Gonçalves et al. (2002)	Ryan et al. (2006)	Ferreira et al. (2006)	Venkatachalam and Sathe (2006)	Freitas et al. (2007)	Chunhieng (2008)	Yang (2009)	Silva et al. (2010)	Santos et al. (2012)
Palmitic (C16)	13.15	13.5	13	-	13.8	13.0	13.5	13.33	14.24
Stearic (C18)	10.36	11.77		9.51	10.36	11.0	11.77	10.78	11.19
Oleic (C18:1, W-9)	37.42	29.09	51	28.75	31.4	39.3	29.09	36.21	36.26
Linoleic (C18:2,W-6)	37.75	42.80	34	45.43		36.1	42.8	38.28	37.53
Linolenic (C18:3, W-9)	-	0.20	-	0.18	42.5		0.20	-	0.076

Experiments conducted on rats have shown that the flour obtained in the process has higher quality than soy flour and lower quality than casein flour powder. The “cake” has numerous possibilities for application focusing on the enrichment of a wide variety of food groups, such as baked goods, beverages, sausages, flour, milk, cereals, chips and crackers, sweet and savory treats, pastry, ice cream, chocolates, and many others (Souza and Menezes, 2004).

"Milk" extract

The nut "milk" is obtained by shelling fresh nuts, which are then shredded producing a thick white extract (popularly called milk) and are subsequently diluted in water. This product is similar to coconut milk; it is rich in protein and is used as an ingredient in foods (Pacheco and Scussel, 2006). According to Cardarelli and Oliveira (2000), when the cake is diluted in water, the "milk" is produced, which after being pasteurized and refrigerated remains stable for approximately 30 days. These authors found that its additive effect, pasteurization, cooling and the addition of preservatives extended its shelf life to 180 days. Felberg et al. (2002) evaluated the effects of extraction conditions on the yield and quality of dehulled Brazil nut extract. In the disintegration step, four different temperatures (25, 50, 75 and 100°C) were evaluated in one or two extractions. The experimental samples obtained were evaluated for composition, extraction yield and sweetness (sensory analysis). The nut "milk" obtained at 75°C with one and two extractions showed solid, oil and protein yield significantly higher than those obtained under the other temperatures studied. As for the sugar content, different concentrations (2, 3 and 4%) were evaluated and subjected to sensory analysis. The "milk” obtained at 75°C and formulated with 3% sugar was preferred by the majority of the panel (78%). Felberg et al. (2004) formulated a mixed drink of whole soy extract and Brazil nut extract resulting in a drink of high nutritional value. The soy extract drink was formulated with 3% sugar, 0.2% salt, and different concentrations of Brazil nut extract (10, 20, 30, 40, and 50). The whole soy drink formulated with 40% of Brazil nut extract, 3% sugar, and 0.2% salt was accepted by most of the panel.

EXTRUSION

Extrusion technology has become a major process for the development of food products. This process can be done using hot or cold materials and it combines several unit operations including mixing, cooking, kneading, shearing, forming and shaping. Hot extrusion is a high-temperature short-time process, which reduces microbial contamination. On the other hand, in the cold extrusion process, the product is extruded without cooking or distortion of the food (Fellows, 2006). Extrusion promotes starch gelatinization, denaturation and re-orientation of proteins, enzyme inactivation, removal of some toxic substances, and the reduction in microbial counts (Borba et al., 2005; Menegassi et al., 2007). This technology is used to produce instant drinks, modified starches for industrial use, precooked animal feeds, ready-to-eat snacks, precooked cereal flakes, semi-processed sauces, bakery products, and breakfast cereals among others (Souza and Menezes, 2008a). Thermoplastic extrusion stands out in food technology for its versatility, high productivity, low cost, and for not generating waste during or after processing.

According to Souza and Menezes (2008b), this technology is the process by which the mechanical friction is combined with thermal heating to mix, plasticize and gelatinize the starch, leading to its fluidization, in order to obtain products with new textures and shapes. Extrusion confers a beneficial effect on the quality of products since it enables the mixing of different raw materials and other nutrients (Carvalho et al. 2010). Souza and Menezes (2006) evaluated the overall acceptance, flavor, crispness, and purchase intent of sweet, salty, and natural extruded cereals made from Brazil nut cake mixed with cassava after six months of storage at ambient temperature. The results showed that these three types of breakfast cereals obtained higher

scores in all attributes evaluated than those of a similar commercial cereal. The sweet cereal received higher scores than those of the natural and salty cereals. Souza and Menezes (2008a) conducted a study aimed at finding the ideal formula of Brazil nut and cassava flour mixture processed by extrusion in order to obtain a product rich in vegetable protein ready for consumption. They found that the formulations containing higher amounts of nuts were more expanded, had light-color and higher contents of protein, lipid and ash. On the other hand, formulations with lower amounts of nuts did not expand, had grayish color and had higher content of carbohydrates. In a study on the optimization of the processing conditions of thermoplastic extrusion of a mixture of Brazil nut cake and cassava flour as a function of acceptance, Souza and Menezes (2008b) found that by increasing the Brazil nut content, temperature and moisture, global acceptance and purchase intent also increased. Furthermore, Brazil nut cake at very high and/or very low temperature and moisture (extreme levels) can lead to reduce global acceptance scores and purchase intent of the products. The highest global acceptance and purchase intent scores found in that study are in the central points and indicate the validity of the model.

FINAL CONSIDERATIONS

Brazil nut has significant nutritional properties that can help the prevention of certain chronic diseases such as heart disease and cancer. It is a recognized source of selenium, and is rich in unsaturated fatty acids and essential amino acids. The products derived from Brazil nut can be used as raw material for the production of various products increasing their nutritional value.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Rapid analysis for the identification of the seagrass *Halophila ovalis* (Hydrocharitaceae)

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Seagrasses are considered as one of the most important species as they play key ecological roles in various types of ecosystems and also provide a food source for endangered animal species. There are two main characteristics of seagrasses that hinder efforts to correctly identify species based on conventional identification keys alone: i) the variability of morphological characteristics and ii) lack of needed morphological characters especially flowers. A taxonomically unresolved complex such as *Halophila* spp. is reported. Plant DNA barcoding regions (*rbc*L and *trn*H-*psb*A) were used to confirm species of collected seagrasses from the southern coast of Thailand. Small and big-leaved samples of *Halophila* spp. were analysed in this study. The big-leaved samples were identified on the field as *Halophila ovalis* whilst it was uncertain whether the small-leaved samples belonged to *H. ovalis*. DNA analysis revealed that the small-leaved samples could be *H. ovalis*. We also coupled PCR with high resolution melt (HRM) to more cost-effectively identify individuals of *H. ovilis* than using barcoding alone. Using HRM to resolve differences in the sequence of two genes showed that the two unknown seagrasses belonged to the same species as *H. ovalis.* In conclusion, using HRM proved to pose great potential in seagrass identification.

Key words: DNA barcoding, *Halophila ovalis*, *rbc*L, *trn*H-*psb*A, species identification.

INTRODUCTION

Seagrasses are flowering plants that are widely distributed along temperate and tropical coastlines of the world. There are 60 described seagrass species worldwide, with the majority of species found in the Indo-Pacific region (Den Hartog, 1970). Seagrasses play key ecological roles in many shallow, nearshore, marine ecosystems (Short et al., 2007; Orth et al., 2006). These plants provide protective shelter for many animals, including fish and amphipods, and provide a food to endangered manatees, dugongs and green turtles (Heck et al., 2003; Hine et al., 2005; Wongkamhaeng et al., 2009; Wilson et al., 2013). Twelve species of seagrass,

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from two families, have been reported in Thailand, including Andaman Sea and Gulf of Thailand coastlines. Only a few studies on seagrass in Thailand have been reported. Den Hartog (1970) reported five species: *Halophila ovalis* (R.Br.) Hook. f., *Halophila minor*, *Halophila decipiens*, *Halodule uninervis* and *Cymodocea rotundata*. Poovachiranon (1988) provided initial basic information on seagrass beds in Phangnga Bay, Andaman Sea of Thailand. Since 1988, several surveys revealed 12 seagrass species in Thailand (Poovachiranon, 1988; Poovachiranon et al., 1994; Poovachiranon and Adulyanukosol, 1999; Terrados et al., 1999; Hine et al., 2005; Poovachiranon et al., 2006; Sakayaroj et al., 2010). However, closely related species from *Halophila* spp., still form a taxonomically unresolved complex (Den Hartog and Kuo, 2006). The variability of morphological characteristics in seagrasses hinders efforts to correctly identify species based on conventional identification keys alone. Traditionally, biological species were classified according to some morphological features and still are the main basis of taxonomy (Heinrich, 2007).

In addition, accurate classification of individuals requires the expertise of an experienced professional taxonomist. In the case of seagrasses lacking the morphological character needed for identification, correct identification of species can be problematic and difficult. Although, some seagrasses taxa are relatively well known and easily identified, most of them are difficult to identify without specialised training. Two main characteristics of seagrasses contribute to identification difficulties: 1) they predominantly propagate by vegetative growth in units, with sexual reproduction occurring rarely due to irregular and infrequent flowering (Reusch et al., 1999) and 2) within the same species, morphological plasticity is commonly observed, with morphological acclimation to different environmental conditions (Bricker et al., 2011). The past decade has seen increasingly rapid advances in the field of molecular tools, which can be informative at many different levels of analysis. A method to identify plant species has been developed (Hollingsworth et al., 2009; Kress et al., 2005) using a short sequence region of DNA which is referred to as DNA barcoding. Yet the method is relatively expensive and is not suitable for a developing country with limited in-house sequencing capabilities like Thailand. Recently, DNA barcoding coupling with high resolution melting analysis (called Bar-HRM) has been applied to authenticate plant, meat and food products (Ganopoulos et al., 2012; Faria et al., 2013; Sakaridis et al., 2013).

H. ovalis shows morphological variability in the leaves in response to different environmental factors in various habitats. Three *H. ovalis* variants can be distinguished: small, intermediate and big-leaved. *H. ovalis* is a highly polymorphic taxon. Numerous studies pointed out the morphological variability of *H. ovalis* that relates to environmental factors (Duarte, 1991; Longstaff and Dennison, 1999; Ralph, 1999; Annaletchumy et al., 2005). Surveys, such as that conducted by Annaletchumy et al. (2005), concluded that the small-leaved *Halophila* specimen can either be *H. ovalis* or *H. minor*, requiring further study to resolve taxonomic uncertainty.

MATERIALS AND METHODS

Samples collection

Specimens of spoon grass (*H. ovalis*) were collected from mixed seagrass beds (three different locations) in Tungkhen Bay, Phuket Province (7˚48.539'N, 98˚24.692'E), and Laem Hangnak, Krabi Province (8˚01.620'N, 98˚46.420'E) on the southern coast of Thailand (Figure 1).

Molecular analysis

Selecting DNA regions

Several DNA regions were selected for providing molecular data of the species. Previous DNA sequencing analyses of molecular data (Newmaster et al., 2006; 2008; Kress and Erickson, 2007; 2008; Fazekas et al., 2008; Lahaye et al., 2008) suggest that several DNA regions are suitable for barcoding plants. Based on these studies, three regions (*rbc*L, *mat*K and *trn*H-psbA) were chosen for this study.

DNA extractions, PCR conditions, DNA sequencing and accession numbers

Total genomic DNA was isolated from leaf material using DNEasy kits (Qiagen; Venlo, Limburg). Extracted DNA was stored in sterile microcentifuge tubes at -20°C. DNA was amplified in 25 µL reaction mixtures containing 1 U *Taq* Polymerase with 1 x PCR Buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl) and 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 mM of each primer, and 20 ng/µL template DNA. DNA barcodes were amplified by PCR using universal primers of *mat*K, *rbc*L and *trn*H-*psb*A (Supplementary Table 1) through 35 cycles of 94°C for 30 s, 55°C (both *rbc*L and *trn*H-*psb*A) and, 53°C (for *mat*K) for 1 min, and 72˚°C for 1 min. The PCR products were analysed by electrophoresis on 1.5% agarose gels (100 V, 40 mA), stained with ethidium bromide, visualised under UV. The specific DNA fragments were then purified. The amplicons were sequenced (First base, Malaysia) directly in both directions with the primers used for amplification. DNA sequences were then deposited in GenBank.

Sequence analysis

The obtained raw sequence data was analysed using Bioedit 7.0.9. The sequence files obtained were manually assembled to obtain a consensus sequence. The consensus sequence was subsequently analysed to verify the gene fragment and/or taxon. The sequences were blasted against the GenBank database to identify contaminant DNA sequences derived from fungal or other parasitic origins. After verification, the sequence was examined for the appropriate forward and reverse primer sequences. Sequences flanked by the specific primers were maintained. The resulting sequences were subsequently used for further analysis.

HRM analysis

To acquire the characteristic melting temperature (T_m) that was

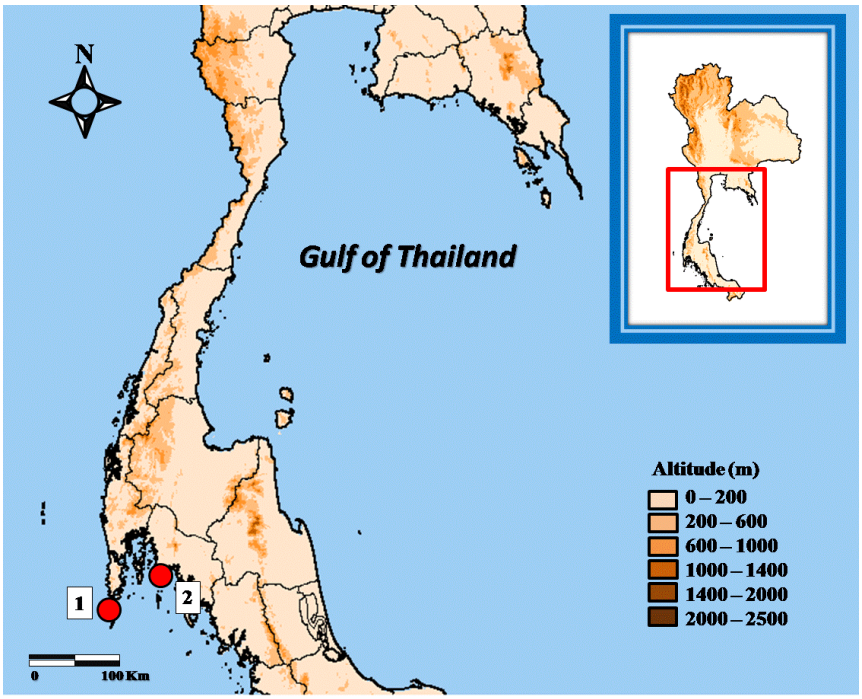

Figure 1. Samples collection map; Andaman sea from three different sites in Tungkhen Bay, Phuket Province (7˚48.539'N, 98˚24.692'E) (1), and Laem Hangnak, Krabi Province (8˚01.620'N, 98˚46.420'E) (2) on the southern coast of Thailand.

capable of distinguishing the different species of seagrass species; *Cymodocea serrulata*, *H. ovalis* and *H. uninervis*, DNA amplification using real-time PCR and DNA were performed using Eco^{TM} Real-Time PCR system (illumina®, San Diego, USA). The reaction mixture for real-time PCR and HRM analysis was done in 10 µl of total volume contained 5 µl of 2x THUNDERBIRD® SYBR qPCR Mix, 0.2 µM forward primer (HRM_rbcL3F: 5'-TAGACCTTTTTGAAGAAGGTTCTGT-3'), 0.2 µM reverse primer (HRM_rbcL3R: 5'-TGAGGCGGRCCTTGGAAAGTT-3') and 1 µl of 25 ng DNA. SYBR fluorescence dye was used to monitor the accumulation of amplified product during PCR and high resolution melting process to derive T_m value. PCR protocol was conducted in 48-well plate Helixis using an initial denaturing step at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 20 s. The fluorescent data were acquired at the end of each extension step during PCR cycles. Before HRM, the products were denatured at 95°C for 15 s, and then annealed at 50°C for 15 s to randomly form DNA duplexes. For HRM experiments, fluorescence data was collected every 0.1°C. The EcoTM software (version 4.0.7.0) was used to analysis the Tm. The negative derivative of fluorescence (F) over temperature (T) (dF/dt) curve primarily displaying the T_m, the normalised raw curve depicting the decreasing fluorescence *vs.* increasing temperature. To generate normalised melt curves and difference melt curves (Wittwer et al., 2003), pre- and post-melt normalisation regions are set to define the temperature boundaries of the normalised and difference plot that were mainly used. *H. ovalis* was set as a reference species.

RESULTS

H. ovalis obtained from Tungkhen Bay, Phuket Province, Thailand showed morphological variability in leaf size and therefore were defined as small-leaved and big-leaved variant. The small-leaved variant has an average leaf length ≤12 mm whilst the big-leaved variant has an average leaf length ≥20 mm. The big-leaved samples were identified by an expert on the field as *H. ovalis* whilst there is uncertain whether the small-leaved samples are belonging to *H. ovalis*. Confusion in identification of *H. ovalis* variants has been long observed especially the small-leaved *H. ovalis*, which is commonly confused with *H. minor.* We only found the small and big-leaved samples although the intermediate *H. ovalis* have been observed in the area. Producing DNA data or short length sequences of chloroplast DNA known as DNA barcodes is seem to be a promising method to confirm *H. ovalis* species identification. In this study, three loci were initially chosen for analysis according to the Consortium for the Barcode of Life (CBOL) recommended primer pairs for the amplification of *mat*K, *rbc*L and *trn*H-*psb*A. However, only *rbc*L *and trn*H-*psb*A primer pairs worked well and led to successful or reproducible amplification. The lengths of the two successful DNA barcodes were 599 bp for *rbc*L and 295 bp for *trn*H-*psb*A. The selected nucleotide sequences obtained from the *rbc*L region (seqRs; sample of small-leaved variant and seqRb; sample of big-leaved variant) and the *trn*H-*psb*A region (seqTs; sample of small-leaved variant and seqTb; sample of big-leaved variant) were uploaded to a database (NCBI: www.ncbi.nlm.nih.gov) with accession numbers JX306023(seqRs),

Table 1. Genetic variation in 599 bp of partial ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene of *Halophila* species. Position based on sequence of GenBank Accession Number JN225349.

Species	GenBank acession numbers	Identity (%)[a]	Nucleotide differences at position									
			78	255	267	336	357	363	429	528	543	582
Halophila ovalis (Thailand: small-leaved)	JX306023	100	C	T	T	C	C	C	C	A	C	T
Halophila ovalis (Thailand: big-leaved)	JX306025	100	●	●	●	●	●	●	●	●	●	●
Halophila ovalis (India)	JN225349	100	●	●	●	●	●	●	●	●	●	●
Halophila decipiens	JN225340	100	●	●	●	●	●	●	●	●	●	C
Halophila minor	JN225347	99.83	●	●	●	●	●	●	●	●	●	C
Halophila stipulacea	JN225356	99.5	●	●	C	●	●	G	T	●	●	●
Halophila beccarii	JN225339	98.5	T	C	C	T	T	A	●	C	T	C

[a]The values are percentage of nucleotides identities for 599 bp calculated from pairwise alignment.

Table 2. Genetic variation in 259 bp of *psbA-trnH* intergenic spacer sequences of *Halophila* species. Position based sequence of GenBank Accession Number GU906229.

Species	GenBank acession numbers	Identity (%)[a]	Nucleotide differences at position					
			59	88-90	154	164-165	169	241
Halophila ovalis (Thailand: small-leaved)	JX306024	100	A	TCC	G	--	T	-
Halophila ovalis (Thailand: big-leaved)	JX306026	100	●	●	●	●	●	●
Halophila ovalis (Australia)	GU906229	99.61	●	●	●	●	●	T
Halophila ovalis (India)	JN225316	98.81	●	GGA	●	●	●	●
Halophila ovata	JN225315	98.81	●	GGA	●	●	●	●
Halophila decipiens	JN225318	98.81	●	GGA	●	●	●	●

[a]The values are percentage of nucleotides identities for 259 bp calculated from pairwise alignment.

JX306025(seqRb), JX306024(seqTs) and JX306026(seqTb), respectively. The nucleotide sequences were then analysed using the rapid identification tool BLAST (Nucleotide BLAST: www.ncbi.nlm.nih.gov) to find regions of local similarity between sequences.

The BLAST results, taking the first three BLAST hits into account, showed that seqRs and seqRb have a similar 100% maximum identity to *rbc*L gene of *H. ovalis* (AB004890, JN225348, JN225349). A close relationship to *H. minor* (JN225347) and *H. decipiens* (JN225340) was observed as shown in Table 1 whereas SeqTs and SeqTb have a similar 99.61% maximum identity to *H. ovalis* (GU906229) (Table 2), confirming that the analysed samples (small-leaved and big-leaved samples) are both, in fact, *H. ovalis*. Based on the searches, both regions offered discrimination at the species level. The DNA barcode results indicated a potential use of both *rbc*L and *trn*H-*psb*A in aiding identification of *H. ovalis* variants. Yet, some limitations of the approach exist. These limitations are included time-consuming and costly method which incurred in the sequencing step. Many laboratories in developing countries like Thailand, commonly do not own sequencing facilities so sequencing works have to be done by outsources and in many cases are done by companies aboard. An expected time to get sequences back is varied from two to five weeks. Thus, we employed a new fast and cost-effective application of DNA barcoding coupled with HRM analysis (Bar-HRM). The use of HRM in confirming the two suspected variants of *H. ovalis* was examined using one universal chloroplast region as marker to amplify polymorphic products from samples. It is appeared that the two variants belonged to the same species according to an analysis of the normalised (Figure 2A) and difference (Figure 2B) HRM curves with the barcode marker *rbc*L. The T_m of each seagrass species derived from HRM curves were shown in Table 3. The curve profiles of *H. ovalis* and both big- and small-leaved variants gaining the T_m value were similar, and could therefore not be visually differentiated, whereas the other tested species (*H. uninervis*, and *C. serrulata*) gave different curve profiles from the two variants. In addition, *H. ovalis* HRM curve was included as reference and the small differences among *H. ovalis* and the two variants melting curves showed in normalised plot. This indicated that the two variants could be *H. ovalis* and was in good agreement with barcodes results (Table 1).

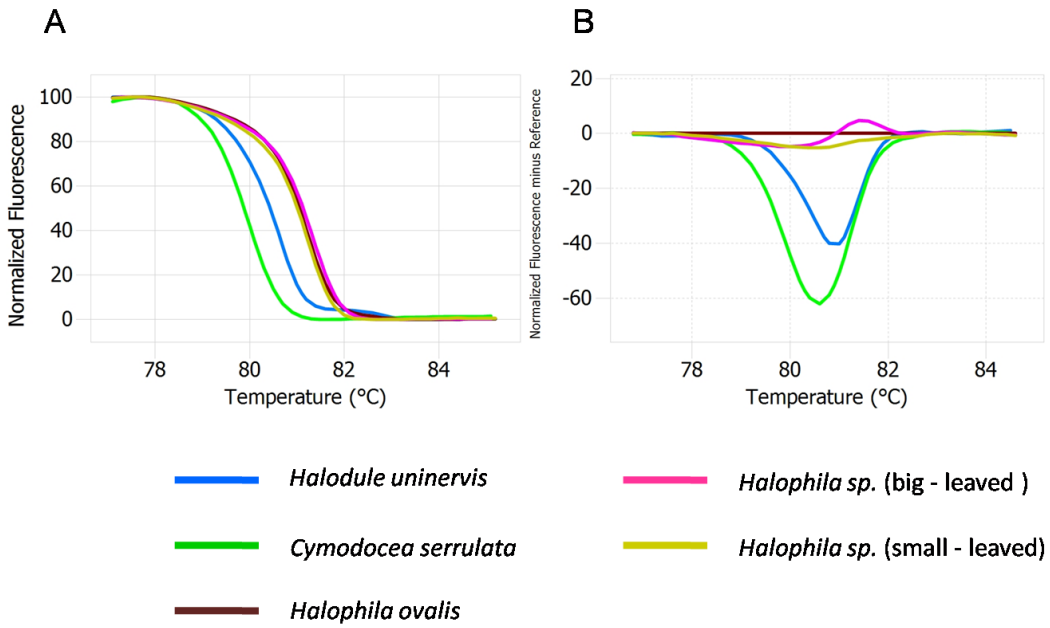

Figure 2. Bar-HRM on three seagrasses species (*Halodule univervis*, *Cymodocea serrulata* and *Halophila ovalis*) and questioned samples of *Halophila* sp. using HRM analysis with *rbc*L chloroplast marker. **(A)** Normalised melting profiles of seagrasses species with the two questioned samples. **(B)** Difference graph of all three species using *Halophila ovalis* as reference genotype. Colour code table with the species used.

Table 3. The melting temperature (T_m) profile of each seagrass species derived from HRM curves of *rbc*L DNA barcoding.

Species	Peak of T_m (°C)
Halodule uninervis	80.75 ± 0.17
Cymodocea rotundata	79.83 ± 0.06
Cymodocea serrulata	79.90 ± 0.00
Halophila ovalis	81.13 ± 0.06
Halophila sp. (big-leaved)	81.10
Halophila sp. (small-leaved)	81.20

DISCUSSION

Many species of seagrasses described by Den Hartog (1970) were identified based on vegetative characteristics, because reproductive structures were often not present. *H. ovalis* was included in a taxonomically unresolved complex given the great variety of different leaf morphologies that is the result of its response to different environmental factors in various habitats. In particular, the small-leaved *H. ovalis* and *H. minor* are frequently confused and difficult to distinguish between, because of several similarities in their morphological characteristics (Annaletchumy et al., 2005). Previous studies mentioned misidentification based on the morphology of seagrasses in the genus *Halophila*, especially *H. ovalis*, which shares considerable morphological plasticity with *H. decipiens* (Short et al., 2010; Lucas et al., 2012). Furthermore, other evidence shows that *H. decipiens* and *H. ovalis* have wide geographic distributions, separated by natural or "jump" dispersal via rafting or floating of vegetative fragments, and that *H. decipiens* is frequently found mixed with *H. ovalis* at 35 m sea depth. With jump dispersal, seagrasses can survive long distance dispersal across a range of different environments. Due to similar lifestyles, seagrass morphology is reduced and shares a number of similarities, even in the same species, but different distributions (Waycott et al., 2006; Short et al., 2010; Lucas et al., 2012). Because of a lack of lignified tissue, the structure of seagrasses is flexible and vulnerable to physical disturbance. Therefore, seagrasses resemble each other when encountered in similar environments, creating cryptic species (Carruthers et al., 2002). In addition, confusion may be caused by the morphology of *H. ovalis* and *H. ovata* not being completely different, as described in Lucas et al. (2012), and thus misidentification may occur between these two species. Thus, finding an accurate, rapid and reliable method for identifying these seagrasses is needed. DNA barcodes seem to be a useful method to support identification of seagrasses in the genus *Halophila* and therefore it used in this study. Here, we collected small and big-leaved variants of *H. ovalis* and then used molecular tools and traditional morphological taxonomic traits to classify

whether these variants belonged to the same or distinct species. They were defined as small or big-leaved variants according to their leaf length (≤12 mm = small-leaved variant and ≥20 mm = big-leaved variant) (Annaletchumy et al., 2005).

DNA barcodes; short DNA sequences, have been applied in the fields of taxonomy and molecular phylogeny in various groups of plants (e.g., Chase et al., 2005; Hajibabaei et al., 2007; Fazekas et al., 2008; Chen et al., 2010; China Plant BOL Group et al., 2011; Goldstein and DeSalle, 2011; Bhargava and Sharma, 2013). DNA barcoding was also reported as having a huge potential in many plants species and so chosen for this study. Several recent works have documented several seagrass DNA barcoding regions of either the nuclear or chloroplast genome (Table 3). However, there is no general agreement on the recommended DNA barcode region for seagrasses. One of the three regions (*mat*K, *rbc*L and *trn*H-*psb*A) chosen for DNA analysis in this study according to CBOL recommendation failed to obtain amplified fragments from the *mat*K; this amplification difficulty using the *mat*K universal primer pair has also been reported in other research (e.g., Hollingsworth et al., 2011). The *rbc*L and *trn*H-*psb*A (two successful markers) meet two of the major criteria of selecting an ideal DNA barcode – the ability of routine amplification using universal primer pair and the generation of unambiguous bidirectional DNA sequences with minimum manual editing (Hollingsworth et al., 2009). Although, several recent work on plant DNA barcoding recommended an informative region in nuclear genome, ITS (Internal transcribed spacer) which found to be effective for species identification of various plant groups (e.g., Li and Dao, 2011; Yang et al., 2012; Pang et al., 2012; Gu et al., 2013). The *rbc*L and *trn*H-*psb*A are effective to categorised the seagrass samples in this study.

The information gained from *rbc*L and *trn*H-*psb*A region analyses indicated that our samples (the two variants) are unequivocally *H. ovalis*. Interestingly, sequences of *rbc*L region from small-leaved variant showed a close-relationship to both *H. ovalis* and *H. decipiens* resulting from BLAST analysis whilst the sequences of *trn*H-*psb*A region confirmed that the samples are indeed *H. ovalis*. Although, we can confirmed now that these two studied variants are *H. ovalis*, the DNA barcodes method itself has some limitations as it is relatively expensive and take some time to produce data, especially when sequencing facilities are not available locally. It is a challenge to develop accurate and reliable methods for more rapid and inexpensive identification of species. Here we proposed, to our knowledge, the first development of HRM analysis coupled with universal chloroplast DNA barcoding region *rbc*L (Bar-HRM) for the rapid identification of seagrasses. Although, *Halophila minor* could not be obtained and used in the analysis, the result of melting curve and *rbc*L sequences were be able to indicate that the tested sampled are *H. ovalis.*

The potential identification power of this method can be observed from melting curves. The shape of the melting curves could be informative when compared difference species and to apply this information to confirm that the two seagrass samples with different average in leave size belong to the same species, other two species were included in the analysis. HRM curve analysis (Fig. 2A and 2B) revealed that the two questioned samples gave the same shape of melting curves as *H. ovalis* whilst other species (*H. uninervis*, and *C. serrulata*) in the analysis could easily be distinguished visually as can be seen from their different melting curves. Although, we could not definitely state that the two variants are not *H. minor* due to lack of the species samples, the results from this study do confirm the two are the same species and could be *H. ovalis*. DNA analysis is not a replacement for morphological identification, but helps to confirm its results and therefore in this case.

Conflict of interests

The authors have not declared any conflict of interest.

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Supplementary Table 1. Oligonucleotide primers used for DNA barcoding in this study.

Primer name	5'-->3'	Ta (˚C)
rbcL F	GTAAAATCAAGTCCACCRCG	
rbcL R	ATGTCACCACAAACAGAGACTAAAGC	55
matK F	CGCGCATGGTGGATTCACAATCC	
matK R	GTTATGCATGAACGTAATGCTC	55
trnH-psbA intergenic spacer F	ATACCCCATTTTATTCATCC	
trnH-psbA intergenic spacer R	GTACTTTTATGTTTACGAGC	55

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Full Length Research Paper

Optimization of explants surface sterilization condition for field grown peach (*Prunus persica* L. Batsch. Cv. Garnem) intended for *in vitro* culture

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The aim of this work was to sterilize nodal explants, so as to mitigate microbial contamination in peach micropropagation. The nodal explants were treated with three concentrations levels (0.15, 0.25 and 0.5% (w/v) active ingredient of chlorine) of locally produced bleach, sodium hypochlorite (NaOCl) for varying exposure times (10, 15 and 20 min). These treatments were carried out in randomized complete design with three replications. Data were recorded on the number of contaminated, dead and survived (clean) cultures after 10 days of culturing. The highest explant contamination (100%) and least explant survival (0%) were recorded when explants were treated with 0.15% active chlorinated local bleach for 10 min. The least culture contamination and minimum tissue death of 9.51 and 4.75%, respectively, and the highest culture survival (85.71%) were recorded when explants were disinfected with 0.25% active chlorinated local bleach for 15 min.

Key words: Explants, local bleach, micropropagation, *Prunus persica,* surface sterilization.

INTRODUCTION

Peach (*Prunus persica* (L). Batsch) belongs to the *Prunoideae,* a sub family of *Rosaceae,* with eight basic and 16 somatic chromosome numbers (2n = 16) (Hesse, 1975). *Prunus* include several species approximately 430 adapted primarily to the temperate regions of the northern hemisphere (http://en.wikipedia.org/wiki/Prunus). China is the native home for peach and was domesticated there 4000-5000 years ago (Aranzana et al., 2010). Peach is grown for its edible fruit consumed as fresh or processed. The total world production of peaches during the year 2012 was 18.1 million tons (Bruke and Change, 2013). The top producer of peaches is typically China, followed by the European Union (EU) and the United States (Daniel et al., 2007). Introduction of temperate fruits especially peach to Ethiopia and North Africa was in sixteenth and seventeenth centuries (Scorza and Sherman, 1996). Because of its early introduction, peach is relatively well established in many highland areas and is introduced much earlier than apple and plum. Recently introduced (2011), Garnem is one of the peach rootstock cultivar introduced from Spain to the country. The production

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Table 1. The treatment combination for sterilization experiment.

Treatment code	NaOCl (Cl %)	Time (min)
ST1	0.15	10
ST2	0.15	15
ST3	0.15	20
ST4	0.25	10
ST5	0.25	15
ST6	0.25	20
ST7	0.5	10
ST8	0.5	15
ST9	0.5	20

potential of peach (McRed is 46 ton/ha) exceeds other temperate fruits production potential, like apple and plum (Endale and Kerssa, 2006).

Recently, breeding practices in *Prunus* have been advanced by the development and application of micropropagation (Martinez-Gomez et al., 2005). Micro-propagation offers the possibility of scale multiplication planting material. Explants surface sterilization is one of the critical steps in micropropagation of plants.

Microbial contamination is one of the most serious problems in micropropagation. Contamination with microorganisms is considered to be the simple most important reason for losses during *in vitro* culture of plants. Such microorganisms include viruses, bacteria, yeast, fungi, etc (Omamor et al., 2007). These microbes compete adversely with plant tissue cultures for nutrients. The presence of these microbes usually result in increased culture mortality but can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003). Determination of effective explant surface sterilization procedure is essential to avoid the problem of contamination during *in vitro* culture. No single sterilization procedure would do for all the species. Even for the same species or the same variety, a single formula may not work at different time. This is due to the fact that load and type of microorganism on explants is dependent on seasons (George and Sherrington, 1984).

Disinfectants such as ethanol, NaOCl, and Tween 20 hamper the growth rate of fungi and bacteria on the growth media (Odutayo et al., 2007). Hypochlorite is known to be a very effective killer of bacteria; even micromolar concentrations are enough to reduce bacterial populations significantly. However, little is known about the exact mechanisms of its bacteriocidal activity. When diluted in water, the hypochlorite salts (NaOCl, $Ca(OCl)_2$) lead to the formation of HOCl whose concentration is correlated with bactericidal activity (Nakagarwara et al., 1998). Sodium hypochlorite is readily available and can be diluted to proper concentrations. A balance between concentration and time must be determined empirically for each type of explants because of phytotoxicity. Therefore, the aim of this study was to assess the effective peach explant-surface sterilization.

MATERIALS AND METHODS

The study was conducted at the Plant Tissue Culture Laboratory, National Agricultural Biotechnology Laboratory, Holeta Agricultural Research Center from October 2013 to January 2014.

Source and choice of plant materials

Young and healthy shoots (4-6 cm long), containing axillary buds (third, fourth and fifth nodes; from shoot apex), were excised and collected from three years old field grown Garnem fruit crop by cutting with sterile scissor and used as explant. Actively growing shoots (juvenile plants) were taken as they are more responsive to shoot regeneration and proliferation than shoot explants from adult forms as reported by (Naghmouchi et al., 2008).

Explant surface sterilization

Before explants were placed on a medium (inoculated), it must be sterilized to make them free of all microorganisms. The leaves were removed from the explants and soaked in tap water and brought to laboratory. The explants were then thoroughly washed with tap water 3-5 times followed by liquid soap for 30 min with agitation to physically remove most microorganisms. Then the explants were treated with 70% ethanol for 30 s under laminar air flow cabinet. After pretreatment with ethanol, the explants were rinsed with distilled water three times, to lower the toxic effect of ethanol. They were then treated with three concentration levels (0.15, 0.25 and 0.5% (w/v) active ingredient of chlorine) of locally produced bleach, sodium hypochlorite (NaOCl) with 5% active ingredient of chlorine for varying exposure times (10, 15 and 20 min) (Table 1). To increase the efficiency of NaOCl, a drop of Tween-20 per 50 ml solution was added as wetting agent. After decanting the sterilizing solutions under safe condition, the explants were washed three times each for 5 min with sterile distilled water to remove traces of NaOCl.

Both ends of the sterilized explants were trimmed under aseptic condition to provide a newly cut surface and to remove any cells damaged by sterilant. Then, the dorsal portion (1 cm long) nodal explants containing a single node were trimmed in a 'V- shape', to expose the xylem and increase the surface area of absorption, under aseptic conditions and cultured on test tubes, containing 10 ml of MS (Murashige and Skoog, 1962) medium fortified with 1 mgL^{-1} 6-benzylaminopurine (BAP), 3% sucrose, and 0.4% agar (agar agar, Type 1). The test tubes with cultured explants were properly sealed with parafilm and labeled. After wards, the cultures were transferred and randomly placed on the growth room shelf with a photoperiod of 16/8h light/dark using cool-white fluorescent lamps (photon flux density, 40 µmol m^{-2} s^{-1} irradiance) at 25 ± 2°C and relative humidity (RH) of 70 to 80%. For each sterilization treatments seven test tubes were line up randomly in completely randomized design (CRD) with three replications. The sterilization experiments data recorded include the number of contaminated, dead and survived (clean) cultures after 10 days of culturing. The data were converted into percentage.

Data collection and Statistical analysis

The sterilization experiments data recorded include the number of contaminated, dead and survived (clean)

Table 2. ANOVA for the effect of local bleach and exposure time on contamination, death and survival of explants.

Source of variation	DF	Mean square		
		Contamination (%)	Mortality (%)	Survival (%)
NaOCl	2	14582.01***	4450.61***	6265.76***
Time	2	1519.09***	1868.03***	211.47***
NaOCl * Time	4	283.62***	643.16***	1742.29***
Error	18	15.06	7.53	7.55
R^2 (%)		0.99	0.99	0.99
CV (%)		12.87	15.28	5.28

***Highly significant (P≤ 0.0001) at α=0.05 significant level; R^2 = coefficient of determination; CV = coefficient of variation; DF, degree of freedom.

Table 3. Effect of different concentrations of local bleach and length of exposure time on contamination, mortality and survival of explants.

NaOCl (local bleach) level (%)	Exposure time (min)	Contamination (%)	Mortality (%)	Clean survived explant (%)
0.15	10	100[a]	0	0
0.15	15	71.43[b]	0	28.57[f]
0.15	20	57.14[c]	0	42.86[e]
0.25	10	28.57[d]	0	71.43[c]
0.25	15	9.51[e]	4.75[e]	85.71[a]
0.25	20	0	28.57[c]	71.43[c]
0.5	10	4.75[ef]	14.29[d]	80.95[b]
0.5	15	0	42.86[b]	57.14[d]
0.5	20	0	71.43[a]	28.57[f]
CV (%)		12.87	15.28	5.3

Means in column with the same letter are not significantly different by DMR test at α=0.05 significant level.

cultures. The data were converted into percentage. The data were subjected to two-way analysis of variance (ANOVA) by SAS computer software (version 9.1). Significant difference between means were assessed by Duncan's multiple range test (DMRT) (*P = 0.05*) (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Analysis of variance (ANOVA) (Table 2) revealed that concentration of local bleach and exposure time, and the interaction effect had highly significant difference (P≤ 0.0001) on the contamination of growth media, death and survival level of explants.

The data (Table 3) revealed that as the exposure time increased from 10 to 20 min for all levels of local bleach, the contamination decreased, and the same was true when concentration of local bleach increased from 0.15 to 0.5% (w/v) for all levels of exposure time.

The highest explant contamination (100%) and least explant survival (0%) were recorded when explants were treated with 0.15% active chlorinated local bleach for 10 min. This might be due to the insufficiency of the concentration of active chlorine in local bleach and short exposure time to take life of microorganisms from cultured explants. The least culture contamination and minimum tissue death of 9.51% and 4.75%, respectively, and the highest culture survival (85.71%) were recorded when explants were disinfected with 0.25% active chlorinated local bleach for 15 min. These findings agree with the result of Ahmad et al. (2003): the application of NaOCl at 0.25% (w/v) for 10 min gave minimum death (5%) and maximum survival (55%).

Surface sterilization with higher concentration (0.5% w/v) of local bleach at and beyond 15 min resulted in no contamination but high rate (71.43%) of explants mortality. This could be due to phytotoxic effect of 0.5% chlorinated local bleach at longer exposure time. Ervin and Wetzel (2002) had also noticed that high concentration of sterilant causing plant tissue death. Surface sterilization should not kill or break off the biological activity of explants, but the contaminants. Explants must be surface sterilized only by treatment with

disinfectant solution at suitable concentrations for a specified period (Oyebanji et al., 2009). Therefore, 0.25% (w/v) local bleach for 15 min exposure time was found to be the most effective one for explants taken from peach shoots.

Conflict of interests

The authors have not declared any conflict of interests.

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Biodegradation of polycyclic aromatic hydrocarbons (PAHs) in spent and fresh cutting fluids contaminated soils by *Pleurotus pulmonarius* (Fries). Quelet and *Pleurotus ostreatus* (Jacq.) Fr. P. Kumm

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The potential of *Pleurotus pulmonarius* and *Pleurotus ostreatus* on the degradation of PAHs in spent and fresh cutting fluids (SCF and FCF) contaminated soils was investigated. Different weights of soil samples were contaminated with varying composition (10, 20 or 30%) of spent and fresh cutting fluids separately then inoculated each sample with *P. pulmonarius* and *P. ostreatus* separately and incubated at 28 ± 2°C for two months. The samples were analyzed in triplicates for 16 priority polycyclic aromatic hydrocarbons (PAHs) by gas chromatography after extracting with hexane and dichloromethane (3:1). The initial PAHs in the cutting fluids were mainly composed of 2 to 6 fused benzene rings. Significant reductions in PAHs concentrations for SCF and FCF were observed after two months of incubation. The percentage total PAHs remaining in FCF soil ranged from 71.7 to 73.6% when inoculated with *P. pulmonarius* and 0.93 to 31.0% when inoculated with *P. ostreatus.* Similarly, the percentage total PAHs remaining in SCF soil ranged from 42.6 to 72.6% when inoculated with *P. pulmonarius* and 54.9 to 62.2% when inoculated with *P. ostreatus.* Overall range of PAHs degradation by *P. pulmonarius* inoculated on FCF contaminated soil was 17.3 to 27.3%, while for *P. ostreatus* inoculated soil was 69.0 to 99.07% at different contamination levels. In contrast, overall PAHs degradation for *P. pulmonarius* and *P. ostreatus* inoculated on SCF ranged from 27.4 to 57.4% and from 37.8 to 45.2%, respectively. Thus, *P. ostreatus* is found more effective as a biodegradation agent for PAHs in contaminated soils when compared with *P. pulmonarius.*

Key words: Biodegradation, polycyclic aromatic hydrocarbons (PAHs), cutting fluids, *Pleurotus ostreatus*, *Pleurotus pulmonarius.*

INTRODUCTION

Soil contamination with various organopollutants is now of great global concern. One major group among the soil contaminants is polycyclic aromatic hydrocarbons (PAHs) which are frequently found in threatening concentrations (Steffen et al., 2007). PAHs members are listed within the list of priority pollutants due to their toxic, mutagenic and carcinogenic effects. The concomitant low water solubility and bioavailability of these contaminants has made them recalcitrant to microbial attack (Petrucciolia et al., 2009). Indiscriminate disposal of lubricating oil by motor mechanics is a common source of soil hydrocarbon contamination in countries like

Nigeria where there is no stringent enforcement of environmental laws (Husaini et al., 2008). Cutting fluid which belongs to the general product family called lubricant is a complex mixture of hydrocarbons, fatty acids, emulsifiers, organo-corrosion inhibitors, amines and glycols (Gannon et al., 1981; Ejoh et al., 2012). However, with the new laws and stricter regula-tions currently in place for cleaning up PAHs contaminated sites, harsh methods such as incineration of soil, bio-augmentation and natural attenuation are employed. Physico-chemical remedial strategies are also adopted in cleaning up sites contaminated by these compounds. These methods have been reported as either not cost effective or adequate enough. The last decade had witnessed the use of fungi in the treatment of a wide variety of solid wastes and wastewaters. Their roles in the bioremediation of various hazardous and toxic compounds in soils had also been established (Leitao, 2009). Fungi have also demonstrated the ability to degrade, in some cases mineralize polycyclic aromatic compounds, polychlorinated biphenyls phenols and halogenated phenolic compounds (Singh, 2006). Mushroom forming fungi (mostly basidiomycetes) are among the nature's most powerful decomposer, secreting strong extracellular enzymes due to their aggressive growth and biomass production (Adenipekun and Lawal, 2012). Therefore, research on decontamina-tion is increasingly focused on biological methods for the degradation and elimination of these pollutants such as PAHs (Jain et al., 2005).

The objective of this study was to assess the ability of *Pleurotus ostreatus*, *Pleurotus pulmonarius* each to biodegrade the 16 priority PAHs in spent and fresh cutting fluids contaminated soils and biodegradation efficiency of the two *Pleurotus* species in spent and fresh cutting fluid contaminated soils were compared.

MATERIALS AND METHODS

Sample location and collection

Soil

The soil used for this experiment was collected from the nursery site of the Department of Botany, University of Ibadan. The physiochemical parameters have been published by Adenipekun et al. (2013). Top soil was dug at 1 to 10 cm deep using stainless hand towel and sieved to 2 mm to remove debris.

Fungi

Pure cultures of *P. pulmonarius* and *P. ostreatus* were collected from Plant Physiology Laboratory of the Department of Botany, University of Ibadan.

Rice straw

Freshly harvested rice straw was collected from International Institute of Tropical Agriculture (IITA) in Ibadan, then taken to the Department of Botany, University of Ibadan and air –dried in a clear open space for seven days to reduce moisture content.

Wheat bran

Wheat bran was collected from the feed mill of Bodija Market, Ibadan.

Cutting fluid

Fresh and spent cutting fluid was obtained from the Physics Department workshop, University of Ibadan.

Substrate preparation

The pure spawn was prepared according to the method of Jonathan and Fasidi (2001). The substrate (rice straw) was cut into 0.50 cm using guillotine then soaked in water for 1 h to moisten the straw and later squeezed using a muslin cloth until no water oozed out. Small quantity of wheat bran (additive) was added to the moist straw which was then put into 350 ml sterile bottles, covered with aluminium foil and autoclaved at 151 lbs and 121°C for 15 min. The bottles were incubated at 28 ± 2°C for three weeks until the substrate was completely ramified to form a spawn.

Experimental set up for culture conditions

The culture condition was carried out according to the method of Adenipekun and Fasidi (2005) and modified as follows: 400 g of sterilized soil was weighed into sterile 350 mL bottles and then mixed thoroughly with varying concentrations of cutting fluid (0, 10, 20 and 30%). A mud balance in Petroleum Engineering Department, University of Ibadan was used to determine the fluid density from which the percent concentration was evaluated. 80 g of moistened rice straw were laid on the contaminated soil in each bottle separated with a wire gauze and covered with an aluminium foil. Three replicates for each experiment were prepared. The bottles were then autoclaved at a temperature of 121°C for 15 min. After cooling, each bottle was inoculated with 10 g of actively grown spawn of *P. pulmonarius* and *P. ostreatus* separately. The bottles were incubated at room temperature (28 ± 2°C) for two months. In the first set of control treatment, cutting fluids was not added to the soil but inoculated with fungi while in the second set different, level of oils were added to the soil but no fungal inoculation. At the end of the incubation period, the mycelia ramified substrate was carefully separated from the soil layer ensuring that soil particles did not mix with it. All contaminated soil samples were extracted and analyzed for PAHs according to the procedure of the USEPA (1996) using AP gas chromatogram model 6890 powered with HP chemstation Rev A. 09.01 (1206) software. The extraction of

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Abbreviations: FCF, Fresh cutting fluid; **SCF**, spent cutting fluid; **PAHs**, polycyclic aromatic hydrocarbons.

Table 1. Initial concentration of PAHs in spent cutting fluid contaminated soil.

PAHs fractions (µg/kg)	Number of benzene rings	Molecular weight (g mol^{-1})	IDL	Initial PAHs concentration (µg/kg)			PAHs fraction of the total PAHs (%)		
				10%	20%	30%	10%	20%	30%
Naphthalene	2	128.17	<0.001	0.575	0.825	1.113	1.34	1.42	1.28
Acenaphthylene	3	154.21	<0.001	0.341	0.468	0.575	0.79	0.81	0.66
Acenaphthene	3	152.2	<0.001	0.363	0.45	0.576	0.84	0.78	0.66
Fluorene	3	178.23	<0.001	0.792	1.035	1.391	1.84	1.79	1.60
Phenanthrene	3	178.23	<0.001	0.504	0.703	5.059	1.17	1.21	5.83
Anthracene	3	166.22	<0.001	3.477	3.658	5.519	8.09	6.31	6.36
Fluoranthene	3	202.26	<0.001	4.361	5.961	6.538	10.1	10.3	7.53
Pyrene	4	228.29	<0.001	2.658	3.442	4.216	6.18	5.94	4.86
Benzo(a)anthracene	4	228.29	<0.001	2.713	3.459	4.141	6.31	5.97	4.77
Chrysene	4	202.26	<0.001	3.828	4.559	5.923	8.90	7.87	6.82
Benzo(b)fluoranthene	5	252.32	<0.001	0.621	0.797	1.975	1.44	1.38	2.28
Benzo(k)Fluoranthene	5	252.32	<0.001	9.215	13.78	23.04	21.4	23.8	26.5
Benzo(a)pyrene	5	252.32	<0.001	11.56	15.71	18.24	26.9	27.1	21.0
Indeno(1,2,3,cd)pyrene	5	278.35	<0.001	0.949	1.127	1.3	2.21	1.94	1.50
Dibenzo(a,h)Anthracene	6	276.34	<0.001	0.537	0.628	0.73	1.25	1.08	0.84
Benzo(g,h,i)perylene	6	276.34	<0.001	0.493	1.356	6.467	1.15	2.34	7.45
Total PAHs remaining				42.99	57.96	86.8	100	100	100

IDL: Instrumental detection limit; PAHs fraction = (Initial PAHs concentration x 100)/Total PAHs.

PAHs was carried out by weighing 5.0 g of soil sample into 250 mL beaker of borosilicate material and 20 mL of double distilled hexane: dichloromethane (3: 1 v/v) was added. The beaker with its contents was placed on sonicator and refluxed for 2 h to extract the hydrocarbons. The organic layer was filtered into 250 mL beaker. The extract was dried with anhydrous sodium sulphate while filtering into a beaker and concentrated with a stream of nitrogen. The clean fractions were analysed for PAHs by a gas chromatography (GC) whose detector was flame ionization detector (FID). The column length, internal diameter and film size were 30 x 0.2 x 0.25 µm. Injection and detector temperatures were 250 and 320°C, respectively, while the detector temperature was 60°C. Mobile phase or carrier Nitrogen column flowed at a pressure of 30 psi. Hydrogen and compressed air pressures were 28 and 32 psi, respectively. Recovery study was carried out by spiking the soil sample, whose PAHs concentrations had previously been determined with an appropriate volume of known concentration of naphthalene standard. The spiked soil sample was homogenized and subjected to extraction procedure for PAHs determination. This spiking was replicated five times with five different soil samples and the average recovery was 94.1±2.3%.

Biodegradation efficiency [BDE]: This was calculated for each concentration of cutting fluid contamination using the ratio of the difference in PAHs concentration after incubation with the fungus to the initial PAHs concentration.

$$BDE\ (\%) = \frac{PAH_i - PAH_f \times 100}{PAH_i}$$

Where; PAHi is the PAHs concentration of PAHs in soil when there was no inoculation. PAH_f is the PAHs concentration of PAH in soil when incubated with white rot fungi *P. pulmonarius* and *P. ostreatus*.

Statistical analysis

The data were subjected to analysis of variance while the treatment means were carried out by Duncan multiple range test at 5% level of probability.

RESULTS AND DISCUSSION

Initial concentrations of PAHs in spent cutting fluid (SCF) and Fresh cutting fluid (FCF) contaminated soils

The initial concentrations of PAHs in SCF and FCF contaminated soil samples are shown in Tables 1 and 2. The PAHs were mainly composed of 2 to 6 fused benzene rings with their molecular-masses ranging from 128.17 g mol^{-1} for naphthalene to 278.35 g mol^{-1} in Indeno(1,2,3,cd) pyrene. The total initial PAHs concentration of SCF contaminated soil samples were 43.0, 58.0 and 86.8 µg/kg for 10, 20 and 30% contamination levels, respectively, (Table 1) while 43.5, 55.2 and 83.8 µg/kg were for 10, 20 and 30% contamination levels, respectively, in FCF contaminated soil samples (Table 2). The most abundant PAH compound in SCF contaminated soil was benzo(a)pyrene constituting 11.56 and 5.71 µg/kg at 10 and 20% contamination levels while at 30% contamination level, benzo (k) fluoranthene constituting 23.04 µg/kg was abundantly present (Table 1). Conversely, acenaphthylene was the least abundant PAH in SCF contaminated

Table 2. Initial concentration of PAHs in Fresh cutting fluid contaminated soil.

PAHs fractions (µg/kg)	Number of benzene rings	Molecular weight (g mol^{-1})	IDL	Initial PAHs concentration (µg/kg)			PAHs fraction of the total PAHs (%)		
				10%	20%	30%	10%	20%	30%
Naphthalene	2	128.17	<0.001	0.633	0.905	1.251	1.46	1.64	1.49
Acenaphthylene	3	154.21	<0.001	0.363	0.394	0.555	0.84	0.71	0.66
Acenaphthene	3	152.2	<0.001	0.383	0.562	0.73	0.88	1.02	0.87
Fluorene	3	178.23	<0.001	0.855	1.188	1.685	1.97	2.15	2.01
Phenanthrene	3	178.23	<0.001	0.692	0.483	0.474	1.59	0.87	0.57
Anthracene	3	166.22	<0.001	3.579	3.806	6.026	8.24	6.89	7.19
Fluoranthene	3	202.26	<0.001	3.900	6.017	10.71	8.98	10.9	12.8
Pyrene	4	228.29	<0.001	2.497	2.694	4.158	5.75	4.88	4.96
Benzo(a)anthracene	4	228.29	<0.001	1.805	2.994	4.744	4.15	5.42	5.66
Chrysene	4	202.26	<0.001	3.109	5.267	7.644	7.16	9.54	9.12
Benzo(b)fluoranthene	5	252.32	<0.001	0.569	0.824	1.142	1.31	1.49	1.36
Benzo(k)Fluoranthene	5	252.32	<0.001	9.598	9.232	16.46	22.1	16.7	19.6
Benzo(a)pyrene	5	252.32	<0.001	12.31	15.82	21.93	28.3	28.7	26.2
Indeno(1,2,3,cd)pyrene	5	278.35	<0.001	0.792	1.013	1.383	1.82	1.83	1.65
Dibenzo(a,h)Anthracene	6	276.34	<0.001	0.439	0.574	0.76	1.01	1.04	0.91
Benzo(g,h,i)perylene	6	276.34	<0.001	1.921	3.438	4.17	4.42	6.23	4.97
Total PAHs remaining				43.45	55.21	83.82	100	100	100

IDL: Instrumental detection limit; PAHs fraction = (Initial PAHs concentration x 100)/Total PAHs

soil with 0.341 and 0.575 µg/kg for 10 and 30% contamination levels while at 20% contamination level, acenaphthene (0.45 µg/kg) was the least abundant PAHs in SCF.

Furthermore, benzo(a)pyrene which ranged from 12.31 to 21.93 µg/kg at different contamination levels was the most abundant PAH compound in FCF soil while acenaphthylene which ranged from 0.363 to 0.555 µg/kg was the least abundant PAH in FCF soil (Table 2).

Reductions in the PAHs fraction residual concentrations by *P. pulmonarius* (*P.p*) and *P. ostreatus* (*P.o*) at two months of incubation period

The extents of degradation of individual PAHs fractions in FCF and SCF contaminated soils by *P. pulmonarius* (*P.p*) and *P. ostreatus* (*P.o*) were shown in Tables 3 and 4. Both *P.p* and *P.o* exhibited some substantial reductions ability after they were able to grow on spent and fresh cutting fluids contaminated soil containing PAHs fractions at varying concentrations. Bishnoi et al. (2008) similarly reported that *Phanerochaete chrysosporium,* a white rot fungus was able to degrade five PAHs fractions in soil from petroleum refinery in both sterile and unsterile conditions. *P.o* significantly reduced the PAHs fractions at all contamination levels compared to *P.p,* which slightly reduced the PAHs fractions in polluted soils except at 10% contamination level with SCF. At this contamination level, *P.p* was able to reduce the PAHs fractions more than *P.o* (Table 3). It is noteworthy that the PAHs fractions at 10 and 20% contamination levels for FCF contaminated soil inoculated with *P.o* were not significantly different (P≤0.05). Also, reduction at 20% contamination level for FCF soil inoculated with *P.o* was significantly high above other levels of contamination (10 and 30%) even when compared with *P.p* inoculated soil at similar levels of contamination. This rapid reduction indicates that 20% contamination level enhances degradation of any PAHs fractions most especially when inoculated with *P.o*. Some PAHs factions might have been used as carbon sources by degradation enzymes during the degradation process. Study by Clemente et al. (2001) explained further that when PAHs fractions such as naphthalene and phenanthrene were used as the carbon source for the growth of strains 837, 870 and 984 of ligninolytic fungi, an enzyme MnP exhibited the highest activity in the degradation of biodegradation of PAHs. The inability of *P.p* to reduced benzo(a)anthracene at 30% contamination level of FCF contaminated soil suggest that PAHs are toxic for the fungus to degrade. Also, an anomalous increase in pyrene from 4.216 to 4.444 µg/kg and benzo(g,h,i)perylene concentration from 6.467 to 7.250 µg/kg (control), respectively, at 30% contamination level for FCF contaminated soil by *P.p* showed that the increase may be due to conversion of one form of aromatics to another as fall-off in the metabolism process. Edema et al. (2011) further explain that magnification of PAHs fraction concentrations in the uncontaminated soil may be due to conversion of one

Table 3. Comparison of the biodegradation of PAHs in spent cutting fluid (SCF) contaminated soil by white rot fungi.

PAHs (µg/kg)	10%			20%			30%		
	Initial PAHs concentration	PAHS Concentration after treatment		Initial PAHs concentration	PAHS Concentration after treatment		Initial PAHs concentration	PAHS Concentration after treatment	
		P.p	*P.o*		*P.p*	*P.o*		*P.p*	*P.o*
Naphthalene	0.575^{d}	0.390^{e}	0.090^{g}	0.825^{c}	0.611^{d}	0.095^{g}	1.113^{a}	0.987^{b}	0.305^{f}
Acenaphthylene	0.341^{d}	0.265^{e}	0.023^{f}	0.468^{c}	0.384^{b}	0.024^{f}	0.575^{a}	0.482^{b}	0.272^{e}
Acenaphthene	0.363^{c}	0.226^{d}	0.030^{e}	0.450^{b}	0.372^{c}	0.031^{e}	0.576^{a}	0.545^{a}	0.287^{d}
Fluorene	0.792^{c}	0.757^{c}	0.021^{d}	1.035^{b}	0.868^{c}	0.021^{d}	1.391^{a}	1.240^{a}	0.189^{d}
Phenanthrene	0.504^{d}	0.313^{e}	0.031^{f}	0.703^{b}	0.587^{c}	0.034^{f}	5.059^{a}	0.681^{b}	0.303^{e}
Anthracene	3.477^{b}	2.892^{b}	0.016^{c}	3.658^{b}	3.656^{b}	0.017^{c}	5.519^{a}	5.443^{a}	1.015^{c}
Fluoranthene	4.361^{b}	4.133^{b}	0.089^{e}	5.961^{a}	3.035^{c}	0.095^{e}	6.538^{a}	5.800^{a}	1.502^{d}
Pyrene	2.658^{c}	2.163^{d}	0.020^{e}	3.442^{b}	2.272^{d}	0.017^{e}	4.216^{a}	4.444^{a}	2.704^{c}
Benzo(a)anthracene	2.713^{c}	2.317^{cd}	0.017^{e}	3.459^{b}	3.195^{b}	0.017^{e}	4.141^{a}	4.141^{a}	1.973^{d}
Chrysene	3.828^{d}	2.645^{e}	0.036^{f}	4.559^{c}	4.051^{cd}	0.038^{f}	5.923^{a}	5.186^{b}	2.438^{e}
Benzo(b)fluoranthene	0.621^{bc}	0.400^{cb}	0.013^{c}	0.797^{cb}	0.577^{cb}	0.014^{c}	1.975^{a}	0.966^{b}	0.444^{cb}
Benzo(k)Fluoranthene	9.215^{e}	6.416^{g}	0.033^{h}	13.78^{c}	11.81^{d}	0.033^{h}	23.04^{a}	20.20^{b}	6.550^{f}
Benzo(a)pyrene	11.56^{c}	6.868^{d}	0.028^{e}	15.71^{b}	12.16^{c}	0.030^{e}	18.24^{a}	12.66^{c}	7.832^{d}
Indeno(1,2,3,cd)pyrene	0.949^{c}	0.708^{d}	0.034^{f}	1.127^{abc}	1.176^{ab}	0.036^{f}	1.300^{a}	1.014^{bc}	0.474^{e}
Dibenzo(a,h)Anthracene	0.537^{cb}	0.435^{c}	0.016^{e}	0.628^{ab}	0.503^{c}	0.016^{e}	0.730^{a}	0.728^{a}	0.306^{d}
Benzo(g,h,i)perylene	0.493^{b}	0.304^{b}	0.019^{b}	1.356^{b}	0.458^{b}	0.023^{b}	6.467^{a}	7.250^{a}	0.310^{b}
Total PAHs remaining	43.0	31.2	0.516	58.0	42.7	0.541	86.8	71.8	26.9
Total PAHs remaining (%)		72.7	1.2		73.6	0.93		82.7	31
BDE (%)		27.3	98.8		26.4	99.1		17.3	69

Each value is a mean of three replicates. Values in the same row with different letters as superscripts are significantly different by Duncan multiple range test (p≤0.05). *P.p: Pleurotus pulmonarius; P.o*: *Pleurotus ostreatus*. BDE: Biodegradation efficiency.

form of aromatics to another as fall-off in the metabolism process.

The overall percentage biodegradation efficiency (BDE) of *P.p* was decreasing with increase in contamination levels for both FCF and SCF soils. BDE of 27.3, 26.4 and 17.3% for 10, 20, and 30% contamination levels for SCF contaminated soil inoculated with *P.p*. In comparison, thehighest BDE of 99.1 at 20% contamination level for *P.o* was obtained (Table 3). For FCF contaminated soil, the highest BDE of 57.4% was obtained at 10% contamination level when the soil was inoculated with *P.p* whereas the highest BDE of 45.2% was obtained at 30% contamination level when the soil was inoculated with *P.o* (Table 4). The reduction in BDE for SCF contaminated soil inoculated with *P.p* could be attributed to high toxicity of contaminants which could have inhibited the fungus degrading enzymes capability retarding the fungus growth and consequently reducing its colonization. The increase in the BDE (%) of *P.o* inoculated on SCF can be attributed to the presence of high organic carbon content of the cutting fluid which is a major factor for sorption of organic pollutants such as PAHs. This is in consonant with the reports of Opuene et al. (2007); Fagbote and Olanipekun (2013) that abundance of total carbon is a significant factor which controls concentration of PAHs in soils.

The comparison of percentage biological degradation efficiency (BDE%) of *Pleurotus pulmonarius* (*P.p*) and *P. ostreatus (P.o*) for PAHs degradation (Table 5)

The range of BDE of *P.o* for FCF contaminated soil were 84.3 to 99.5%, 88.5 to 99.8 % and 35.9 to 94.0% for 10, 20 and 30% contamination levels, respectively, while that of *P.p* were 4.4 to 40.6%, 0.1 to 66.2%, 0 to 86.5%, respectively. Similarly, for SCF contaminated soil, BDE of *P.p* ranged from 25.3 to 85.8%, 5.3 to 88.2% and 10.2 to 88.2% for 10, 20 and 30% contamination levels, respectively, while BDE of *P.o* inoculated soil ranged from 18.4 to 82.8%, 2.2 to 89.6%, 2.7 to 64.5% for corresponding contamination levels. This is similar to the report of Marquez-Rocha et al. (2000) on the degradation of PAHs adsorbed by the white rot fungus, *P ostreatus*, after 21 days. In which case, 50% of pyrene, 68% of anthracene and 63% of phenanthrene were mineralized and their respective biodegradation percentages increased to 75, 80 and 75%, respectively, when 0.18% of Tween 40 was added. This is also similar to the findings of Eggen and Svenm (1999) who observed that the white rot fungus *P. ostreatus* had an overall positive effect on PAH degradation in aged creosote contaminated soil. It is noteworthy that, most PAH higher than naphthalene in molecular weight were almost completely

Table 4. Comparison of the biodegradation of PAHs in fresh cutting fluid (FCF) contaminated soil by white rot fungi.

PAHs (µg/kg)	10%			20%			30%		
	Initial PAHs concentration	PAHS Concentration after treatment		Initial PAHs concentration	PAHS Concentration after treatment		Initial PAHs concentration	PAHS Concentration after treatment	
		P.p	*P.o*		*P.p*	*P.o*		*P.p*	*P.o*
Naphthalene	0.633^{cd}	0.280^{g}	0.365^{f}	0.905^{b}	0.561^{ed}	0.498^{e}	1.251^{a}	0.692^{c}	0.597^{d}
Acenaphthylene	0.363^{b}	0.137^{d}	0.224^{cd}	0.394^{b}	0.217^{cd}	0.356^{b}	0.555^{a}	0.319^{cb}	0.381^{b}
Acenaphthene	0.383^{c}	0.198^{d}	0.211^{d}	0.562^{b}	0.427^{c}	0.341^{c}	0.730^{a}	0.525^{b}	0.334^{c}
Fluorene	0.855^{cd}	0.391^{ef}	0.207^{f}	1.188^{b}	0.662^{ed}	0.218^{f}	1.685^{a}	1.092^{cb}	0.624^{ed}
Phenanthrene	0.692^{a}	0.129^{e}	0.325^{cd}	0.483^{b}	0.268^{d}	0.434^{cb}	0.474^{b}	0.392^{cb}	0.461^{b}
Anthracene	3.579^{cb}	0.685^{e}	2.403^{cd}	3.806^{b}	2.178^{d}	2.099^{d}	6.026^{a}	4.023^{b}	3.542^{cb}
Fluoranthene	3.900^{d}	2.912^{de}	2.090^{e}	6.017^{c}	5.697^{c}	2.152^{e}	10.71^{a}	8.915^{b}	3.804^{d}
Pyrene	2.497^{c}	1.148^{d}	1.887^{cd}	2.694^{cb}	2.046^{c}	2.636^{cb}	4.158^{a}	3.387^{ba}	2.312^{c}
Benzo(a)anthracene	1.805^{ed}	1.125^{e}	1.921^{ed}	2.994^{cb}	2.227^{cd}	2.488^{cd}	4.744^{a}	3.888^{ba}	2.543^{cd}
Chrysene	3.109^{d}	0.643^{f}	2.293^{e}	5.267^{c}	2.991^{d}	3.036^{d}	7.644^{a}	6.865^{b}	2.896^{d}
Benzo(b)fluoranthene	0.569^{cd}	0.372^{ef}	0.341^{f}	0.824^{b}	0.598^{cd}	0.470^{def}	1.142^{a}	0.715^{cb}	0.558^{cde}
Benzo(k)Fluoranthene	9.598^{bc}	3.703^{d}	5.334^{cd}	9.232^{bc}	6.983^{cd}	8.180^{bcd}	16.46^{a}	9.558^{bc}	12.08^{ab}
Benzo(a)pyrene	12.31^{c}	5.864^{e}	8.165^{d}	15.82^{b}	13.71^{c}	9.819^{d}	21.93^{a}	17.30^{b}	12.75^{c}
Indeno(1,2,3,cd)pyrene	0.792^{bcd}	0.393^{e}	0.646^{d}	1.013^{b}	0.686^{cd}	0.637^{d}	1.383^{a}	0.907^{b}	0.715^{cd}
Dibenzo(a,h)Anthracene	0.439^{cb}	0.273^{c}	0.291^{c}	0.574^{ab}	0.445^{cb}	0.415^{cb}	0.760^{a}	0.589^{ab}	0.457^{cb}
Benzo(g,h,i)perylene	1.921^{ab}	0.273^{b}	0.331^{b}	3.438^{a}	0.406^{b}	0.358^{b}	4.170^{a}	0.500^{b}	1.920^{ab}
Total PAHs remaining	43.445	18.526	27.034	55.211	40.102	34.137	83.822	59.667	45.974
Total PAHs remaining (%)		42.64	62.23		72.63	61.83		71.18	54.85
BDE (%)		57.4	37.8		27.4	38.2		28.8	45.2

Each value is a mean of three replicates. Values in the same row with different letters as superscripts are significantly different by Duncan multiple range test ($p \leq 0.05$). *P.p: Pleurotus pulmonarius; P.o*: *Pleurotus ostreatus.* BDE: Biodegradation efficiency.

Table 5. Comparison of percentage biological degradation efficiency of *Pleurotus pulmonarius* (*P.p*) and *P. ostreatus* (*P.o*) for PAHs fractions degradation in fresh and spent cutting fluids contaminated soil.

PAHs fractions (%)	Number of benzene rings	Molecular weight (g mol^{-1})	Fresh cutting fluid contaminated soil						Spent cutting fluid contaminated soil					
			10%		20%		30%		10%		20%		30%	
			P.p	*P.o*	*P.p*	*P.o*	*P.p*	*P.o*	*P.p*	*P.o*	*P.p*	*P.o*	*P.p*	*P.o*
Naphthalene	2	128.17	32.2	84.3	25.9	88.5	11.3	72.6	55.8	42.3	38	45.0	44.7	52.3
Acenaphthylene	3	154.21	22.3	93.3	17.9	94.9	16.2	52.7	62.3	38.3	44.9	9.6	42.5	31.4
Acenaphthene	3	152.2	37.7	91.7	17.3	93.1	5.4	50.2	48.3	44.9	24	39.3	28.1	54.2
Fluorene	3	178.23	4.4	97.3	16.1	98.0	10.9	86.4	54.3	75.8	44.3	81.6	35.2	63.0
Phenanthrene	3	178.23	37.9	93.8	16.5	95.2	86.5	94.0	81.4	53	44.5	10.1	17.3	2.7
Anthracene	3	166.22	16.8	99.5	0.1	99.5	1.4	81.6	80.9	32.9	42.8	44.9	33.2	41.2
Fluoranthene	3	202.26	5.2	98.0	49.1	98.4	11.3	77.0	25.3	46.4	5.3	64.2	16.8	64.5
Pyrene	4	228.29	18.6	99.2	34.0	99.5	*5.4	35.9	54	24.4	24.1	2.2	18.5	44.4
Benzo(a)anthracene	4	228.29	14.6	99.4	7.6	99.5	0.0	52.4	37.7	*6.43	25.6	16.9	18.0	46.4
Chrysene	4	202.26	30.9	99.1	11.1	99.2	12.4	58.8	79.3	26.3	43.2	42.4	10.2	62.1
Benzo(b)fluoranthene	5	252.32	35.6	97.9	27.6	98.2	51.1	77.5	34.6	40.1	27.4	43.0	37.4	51.1
Benzo(k)Fluoranthene	5	252.32	30.4	99.6	14.3	99.8	12.3	71.6	61.4	44.4	24.4	11.4	41.9	26.6
Benzo(a)pyrene	5	252.32	40.6	99.8	22.6	99.8	30.6	57.1	52.4	33.7	13.3	37.9	21.1	41.9
Indeno(1,2,3,cd)pyrene	5	278.35	25.4	96.4	*4.3	96.8	22.0	63.5	50.4	18.4	32.3	37.1	34.4	48.3
Dibenzo(a,h)Anthracene	6	276.34	19.0	97.0	19.9	97.4	0.3	58.1	37.8	33.7	22.5	27.7	22.5	39.9
Benzo(g,h,i)perylene	6	276.34	38.3	96.1	66.2	98.3	*12.1	95.2	85.8	82.8	88.2	89.6	88.0	54.0

*Higher than initial soil PAHs level.

removed by *P.o*. This agrees with the report of Edema et al. (2011) where mushrooms were able to completely removed PAHs higher than naphthalene in molecular mass. Also, there was an anomalous increase in some PAHs concentrations after 2 months of incubation with the fungi. The anomalous increase implies that degradation truly occurred. Similarly, Okparanma et al. (2011) reported an anomalous increase in phenanthrene (3-ring) which was not within the laboratory detection limit but, after the fifty sixth day of incubation with *P. ostreatus*, it increased to 0.01 mg/kg.

Conclusion

In the present study, the effectiveness of *P. pulmonarius* and *P. ostreatus* in biodegradation of PAHs fractions in both spent and fresh cutting fluids contaminated soil at different concentration has being elucidated. In fresh cutting fluid contaminated soil *P. ostreatus* degraded almost all the PAHs fractions more than naphthalene while *P. pulmonarius* degradation was slight one. Such a biodegraded soils can provide a cheap and sustainable means of reverting such soils after damage by cutting fluids and can subsequently be used for agricultural purpose.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Production and antagonistic effect of *Trichoderma* spp. on pathogenic microorganisms (*Botrytis cinerea, Fusarium oxysporium, Macrophomina phasealina* and *Rhizoctonia solani*)

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***Trichoderma* spp. are widely used as bio-fungicides in agriculture. Induction of plant defense and mycoparasitism (killing of one fungus by another) are considered to be the most important mechanisms of *Trichoderma*-mediated biological control. This study is based on the optimized production of *Trichoderma viride*. The media used for the economical production of *T. viride* conidia contain 5% jaggery and 0.5% baker's yeast. It is clear that the growth and sporulation of *Trichoderma* mycelia require different temperatures. Mycelia had significant growth at 37°C and sporulation at 24°C (low temperature). For industrial production of *T. viride* conidia, it is suggested that the culture should be incubated initially at 37°C until the mycelia are formed and then at 24°C to induce sporulation. Formulating *Trichoderma* in talc is better than doing it in oil because the spores are hydrophilic in nature. There is current understanding of the interactions of *Trichoderma* with plant pathogens such as *Botrytis cinerea*, *Fusarium oxysporium*, *Macrophomina phasealina* and *Rhizoctonia solani*, and it is concluded that *Trichoderma* has antagonistic effect against these pathogens.**

Key words: *Trichoderma*, induced resistance, biological control, mycoparasitism.

INTRODUCTION

At present, around 30% of all plant species have been destroyed by plant pathogens. Pesticides and organic compounds are widely used to control plant pathogens in many countries. However, the degradation of such compounds is very difficult and their concentration and accumulation in food chains lead to higher toxicity levels in animals (Chet, 1987; Lynch, 1990). *Trichoderma* spp. (teleomorph Hypocrea) is the most successful bio-fungicide used in today's agriculture; there are more than 60% registered biofungicides world-wide (Verma et al., 2007). *Trichoderma*-based *Trichoderma viride* species have been investigated for over 80 years. In India alone,

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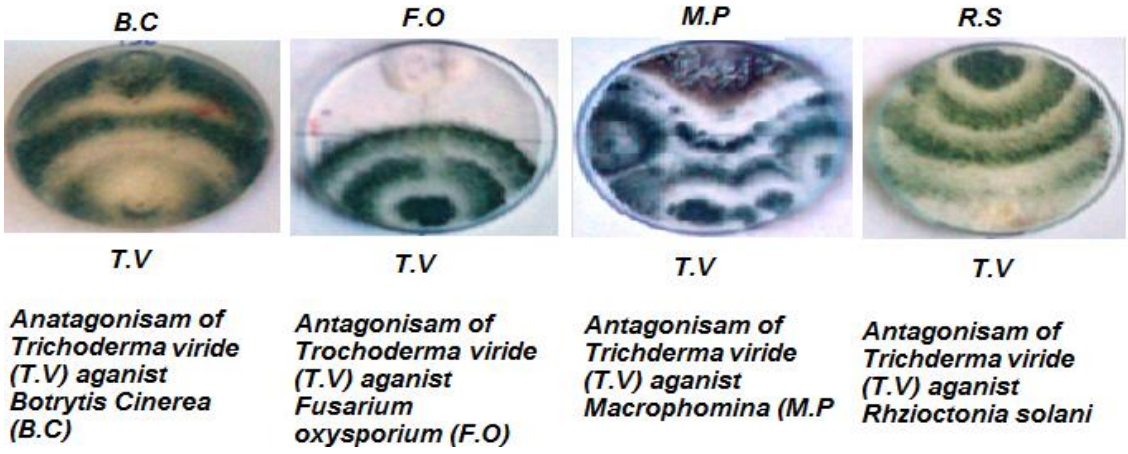

Figure 1. Antagonistic activity of *Trichoderma Viride* to different fungal Strain *Botrytis Cinerea, Fusarium Oxysporium, Macrophomina Phasealina, Rhizoctonia Solani.*

about 250 products are available for field applications (Singh et al., 2009). Recently, they have been used as biological control agents and their isolates have become commercially available (Mukherjee et al., 2012). This development is largely as a result of a change in public attitude towards the use of chemical pesticides and fumigates such as methyl bromide (Elad et al., 1980). In this respect, *T. viride* spp. have been studied as biological control agents against soil-borne plant pathogenic fungi (Küçük, 2000; Chet and Inbar, 1994). Results from different studies showed that several strains of *T. viride* had a significant reducing effect on plant diseases caused by pathogens such as *Rhizoctonia Solani, Sclerotium rolfsii, Phythium aphanidermatium, Fusarium oxysporum, F. culmorum and Gaeumannomyces graminis* var*. tritici* under greenhouse and field conditions (Basim et al., 1999; Sivan and Chet, 1993; Chet and Baker, 1981; Dolatabadi et al., 2011; Ephrem et al., 2011). Isolates of *T. viride* can produce lytic enzymes (Küçük, 2000; Haran et al., 1996) and antifungal antibiotics (Dennis and Webster, 1971; Brewer et al., 1987; Almassi et al., 1991). They can also be competitors of fungal pathogens (Whipps, 1987) and promote plant growth (Inbar et al., 1994). They have ability to grow in a wide range of temperatures, are capable of antagonizing plant pathogens, using lignocellulosic materials for growth. Antibiosis and hyperparasitism make *T. viride* isolates possible bio-control agents (Haran et al., 1996; Rifai, 1969; De La Cruz et al., 1992). For these reasons, *T. viride* has become a successful bio-control agent in agriculture field. Many companies recently started large scale production of *T. viride* conidia for agricultural use like biopesticide. As a beneficial biological agent, *Trichoderma* is a filamentous fungus which has gained attention because of its multi-prolonged action against various plant pathogens (Harman et al., 2004; Shabir-U-Rehman, et al., 2013). One of the most important things considered in the design of a mass production procedure is the compatibility of the product with both formulation and application techniques. For example, the use of oil formulations for application at ultra low volume rates requires the production of lipophilic conidia which suspend easily in oils (Khurana et al., 1993). Submerged conidia are hydrophilic and are not easily formulated in oils. Blastospores are similarly produced in submerged liquid fermentation, but are also hydrophilic and have been found to lose viability relatively quickly during storage. Due to these problems, there is the necessity to develop cost-effective methods to produce conidia of high quality in large-scale. In this study, we attempt to test the bio-efficacy of *T. viride* against fungal pathogens, optimize the culture medium for the production of conidia, and formulate it in a non-lipophilic solid material (talc) instead of using oil. Certain strains of *Trichoderma* species were reported to induce transcriptomic changes in plants and some are known to protect plants from diseases and abiotic stresses (Bailey et al., 2006).

MATERIALS AND METHODS

Fungal strains

The microbial strains used in this study are *Botrytis cinerea, F. oxysporium, Macrophomina phasealina, Rhizoctonia solani* and the antagonistic fungi *T. viride* is obtained from depositary laboratory (Figure 1).

Media optimization for the mass production *of Trichoderma viride*

For the mass production of *T. viride*, different types of media were used (which are cost effective). The media mainly contained Jaggery, yeast extract, glucose and baker's yeast (Figures 2 and 3). 5% baker's yeast and peptone are taken; 3, 4, 5, 10 and 15% of jaggery and 0.5, 1, 1.5, 2, and 2.5% glucose are used to get the optimal composition for the mass production. Acidic condition is

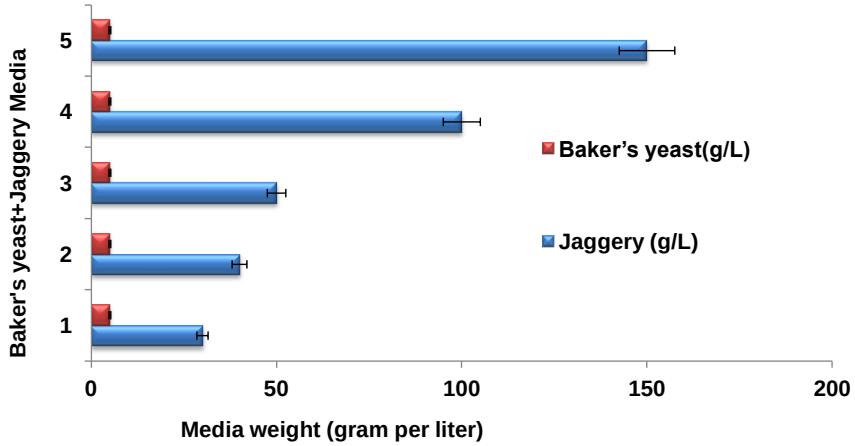

Figure 2. Media composition of baker's yeast and Jaggery.

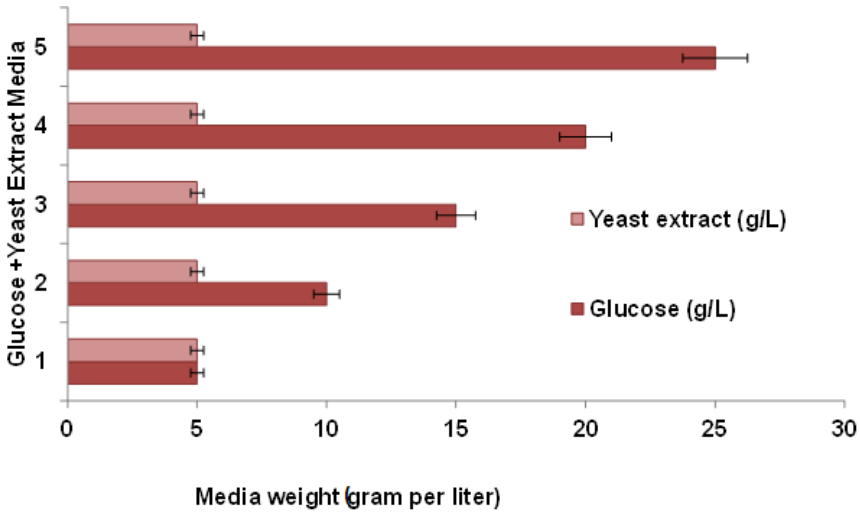

Figure 3. Media composition of yeast extract and glucose.

maintained by adjusting the pH to 5 with 0.1 N HCl. The media are distributed into 20 flasks as each flask contains 50 ml of medium. All the flasks were labeled properly and autoclaved at 121°C and 15 lbs pressure for 30 min. Then the bottles were cooled to room temperature.

Preparation of inoculums

The media used to inoculate *T. viride* for spore production were yeast extract (2%), peptone (0.5%), glucose (3%), and pH was maintained to 5 with 0.1 N HCl. The media were autoclaved at 121°C and 15 lbs pressure for 20 min. *Trichderma viride* spores were taken from PDA slant. The spores were added as the concentration of spores reached $5x10^4$ per ml; 0.01% of sodium lauryl sulphate was added to the spore suspension. The flask was kept on the shaker for two hours to achieve uniform distribution of spores. Sodium lauryl sulphate helps to separate the spore clumps. 1 ml of spore suspension was added to each mass production medium flask in a sterile condition and mixed properly. Two flasks from each type of medium were not inoculated with spores and used as a control. Among 20 flasks of each type, 9 inoculated flasks and 1 control flask were incubated at 24°C. Nine (9) inoculated flasks and 1 control flask were incubated at 37°C ; the flasks were incubated for seven days (Figure 4).

Measurement of wet weight

The inoculated media were centrifuged at 4000 rpm for 20 min and the pellet was separated. The supernatant was stained with lactophenol cotton blue and observed under 40x magnification of microscope. If spores were found in supernatant, it was centrifuged again at 5000 rpm for 20 min. Then the weight of the pellet was measured. 1 g of pellet was taken and mixed with 1 kg of sterile talc and 0.5 g of carboxy methyl cellulose (CMC); and sieved carefully. Then 1 g of sample was taken and serially diluted with sterile distilled water. Few dilutions were taken to count the colony forming units (CFU) on potato dextrose agar plates. The medium

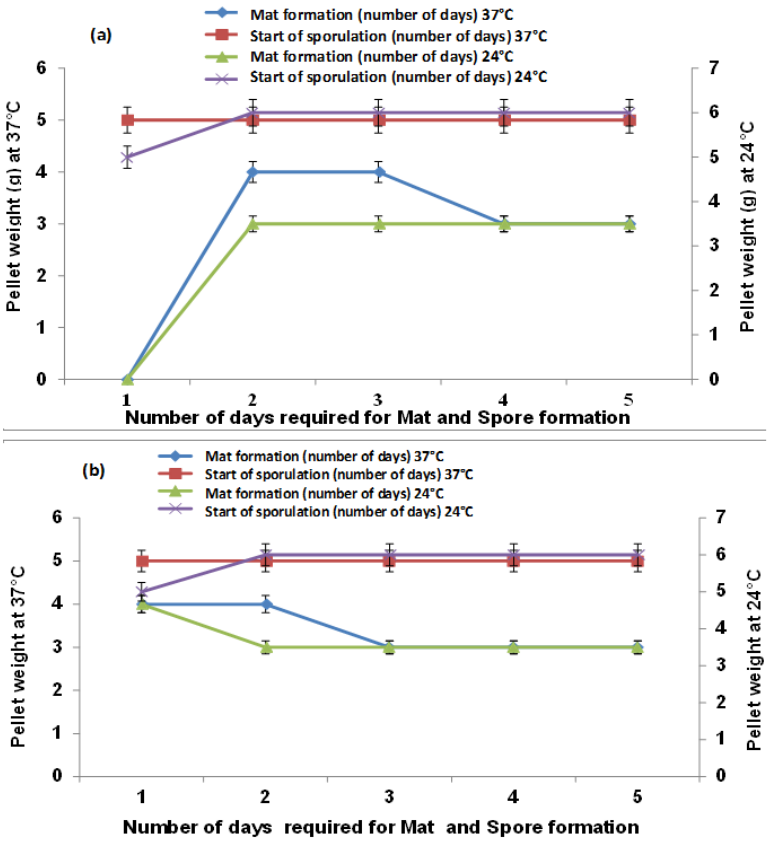

Figure 4. Effect of medium concentration (weight) on mat and spore formation at different temperature: (a) low concentration (b) high concentration.

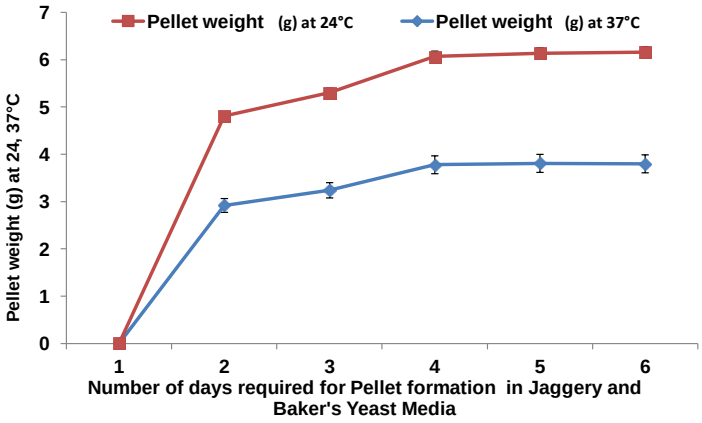

Figure 5. Pellet formation at different temperature of Jaggery and baker's yeast.

with the maximum spore count was selected for submerged fermentation in 25 L fermenter (Figures 5 and 6).

Submerged fermentation in 25 L fermenter

The optimized medium was used for submerged fermentation in a 25 L fermenter. 25 L of medium was prepared based on the composition and poured into the fermenter. Impellers were set properly and fermenter was closed and autoclaved at 121°C and 15 lbs pressure for 20 min. After autoclaving, the fermenter was cooled for two days.

T. viride spore suspension was prepared in spore suspension medium and spore concentration was adjusted to 2.5×10^6/ml. The spores were suspended evenly by adding sodium lauryl sulphate and keeping on shaker for 2 h. 100 ml of spore suspension was

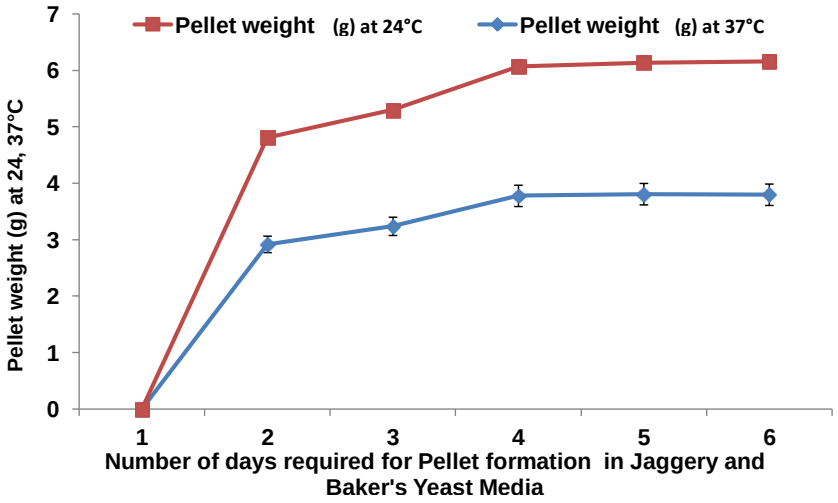

Figure 6. Pellet formation at different temperature of glucose and yeast extract Media.

inoculated into fermenter using vacuum pump in a laminar air flow. Temperature and aeration were continuously monitored for seven days. After every 24 h, samples were collected to observe the spores. After seven days of incubation, 10 ml of sample was collected and centrifuged at 4000 rpm for 20 min, of which the pellet was separated. The supernatant was stained with lactophenol cotton blue and observed under 40x magnification of microscope. If spores were found in supernatant, it was centrifuged again at 5000 rpm for 20 min. Then the weight of the pellet was measured. The pellet was formulated in talc, and colony forming unit (CFU) was counted on potato dextrose agar plates.

Solid state fermentation

For mass production of *T. viride spores* in solid state fermentation, substrates such as sorghum seeds, rice and wheat flour were used.

Solid state fermentation with sorghum seeds

Sorghum seeds were washed for 20 min under running tap water. Then they were washed with distilled water for two times and allowed to dry. The seed coats were ruptured carefully in mixer. Then the seeds were soaked in distilled water for 20 min; excess water was removed. 150 g of wet seeds was weighed and autoclaved at 121°C and 15 lbs pressure for 30 min.

Solid state fermentation with rice and wheat flour

For solid state fermentation with rice and wheat flour, same protocol was followed. 100 g of flour was weighed, 30 ml of water was added and autoclaved at 121°C and 15 lbs pressure for 30 min. After autoclaving, seeds (150 g) or flour (100 g) were spread in each tub. Each tub was inoculated with 5 ml of spore suspension containing 10^6 spores/ml. Then the tubs were covered with plastic sheets and incubated at 27°C for a week. After a week, the seeds were sieved on a mesh to obtain the spores. The weight of the spores formed per 100 g of the seed was measured. 1 g of spore was taken and formulated in talc; and CFU count was calculated on potato dextrose agar plates.

Determination of antagonistic efficacy of *Trichoderma viride*

Antagonistic efficacy of *T. viride* was tested on potato dextrose agar plates. Antagonistic efficacy was observed by plating both the organisms on same Petri plate. Presence of inhibition zone indicates that *T. viride* is antagonistic to given pathogen. The four fungal pathogens such as *Botrytis cinerea, F. oxysporium, M. phasealina, R. solani* and *T. viride* were separately inoculated and labeled as control; on the other plates a line was drawn in the middle. On one side, *T. viride* was inoculated; on the other side, *B. cinerea* was inoculated. Likewise, *F. oxysporium, M. phasealina, and R. solani* were inoculated opposite to *T. viride.* Plates were incubated at 27°C for a week; then the plates were observed for zone of inhibition.

Statistical analysis

Data were analyzed by Descriptive Statistics in MS-EXCELL-2007 Software.

RESULTS AND DISCUSSION

T. viride was cultured on different media for seven days at different temperatures. Mycelial mat formation and sporulation started at different intervals in different media (Figure 4). As the results suggest, at low concentrations of jaggery and glucose, mycelial mat formation was not satisfactory. As their concentration increased, mat formed completely and within less time. At low concentrations of jaggery and glucose, due to low nutrition, sporulation was induced at early stage and hence it was observed on the 5^{th} day. As the medium became nutrient rich, induction of sporulation was delayed. Therefore, it indicates that sporulation can be induced by nutrient starvation. Leo et al. (2010) show that at a concentration of 4 g Kg^{-1} under field conditions, an abiotic stress tolerant *T. viride* was effective against root rot disease and enhanced yield of

Table 1. The intervals at which mycelial mat formation, sporulation occurred.

Medium composition (g/L)		Mat formation (No. of days) 37°C	Start of sporulation (No. of days) 37°C	Mat formation (No. of days) 24°C	Start of sporulation (No. of days) 24°C
Jaggery	**Baker's yeast**				
30	5	4±0.02	5±0.03	4±0.02	5±0.03
40	5	4±0.02	5±0.03	3±0.01	6±0.04
50	5	3±0.01	5±0.03	3±0.01	6±0.04
100	5	3±0.01	5±0.03	3±0.01	6±0.04
150	5	3±0.01	5±0.03	3±0.01	6±0.04
Glucose	**Yeast extract**				
5	5	-	5±0.03	-	5±0.03
10	5	4±0.02	5±0.03	3±0.01	6±0.04
15	5	4±0.02	5±0.03	3±0.01	6±0.04
20	5	3±0.01	5±0.03	3±0.01	6±0.04
25	5	3±0.01	5±0.03	3±0.01	6±0.04

Table 2. Spore formation at different medium composition, at 24 and 37°C temperature.

Medium composition (g/L)		Pellet weight(g) 37°C	Pellet weight g) 24°C
Jaggery	**Baker's yeast**		
30	5	2.92±0.001	1.89±0.00
40	5	3.24±0.03	2.06±0.001
50	5	3.78±0.03	2.29±0.01
100	5	3.81±0.03	2.33±0.01
150	5	3.80±0.03	2.36±0.01
Glucose	**Yeast extract**		
5	5	1.69±0.00	1.14±0.00
10	5	2.31±0.01	1.38±0.00
15	5	2.68±0.01	1.44±0.00
20	5	2.81±0.02	1.66±0.00
25	5	3.04±0.03	1.84±0.00

Vigna mungo when applied as seed dresser. With respect to incubation temperature, sporulation and mycelial vegetative growth were favored by different temperatures. When the cultures were grown at 37°C (Tables 1 and 2), mycelia grew rapidly; but initiation of sporulation was delayed. At low temperatures, mycelia growth was not satisfactory and complete mat formation was delayed; but at this temperature, mycelia were converted into spore within less duration. Therefore, it is suggested that mycelial vegetative growth is favored by room temperature (37°C), and sporulation is favored by low temperature. The pellets were collected upon centrifugation, and the weight of the pellet was measured to compare the growth on different media.

These values indicate that the most efficient growth of *T. viride was* found when jaggery concentration exceeded 50 g/L. After this, there is no significant increase in the pellet weight. It is better to use jaggery than glucose (Figure 5 and 6). Pellet weight was measured at high room temperature instead of low temperature (Figure 5). This might be due to the less vegetative growth at low temperature. As the growth of *T. viride* is found far better in jaggery and baker's yeast medium, at 37°C, these samples were formulated in sterile talc to find out the number of colony forming units in one gram of pellet obtained. The Colony Forming Units count decreased gradually with increased jaggery concentration. This might be due to incomplete sporulation as the jaggery concentration increased. As the jaggery concentration increased, medium became rich in nutrient and sporulation was delayed. So such cultures require more time to be completely converted into spores. In these cultures, vegetative cells are still present at the time of harvest and centrifugation. On the basis of these results, the medium containing 50 g of Jaggery and 5 g of baker's yeast is selected as the best medium for submerged fermentation

Table 3. Colony forming units count at different Jaggery concentration and 0.5% Baker's yeast.

Medium composition (g/L)		No. of CFU/g of pellet
Jaggery	Baker's yeast	
30	5	3.12×10^9
40	5	2.98×10^9
50	5	2.88×10^9
100	5	2.82×10^9
150	5	2.8×10^9

(Table 3). When these samples are formulated, it was concluded that 3×10^{12} spores were present in 10 ml of medium.

In vitro antagonistic tests

The isolate *T. veride* (Tr 8) showed 70, 68.2, 70, 73.3, 69.3 and 70.1% growth inhibition against *R. solani, S. rolfsii, M. phaseolina, A. alternata, F. solani and C. capsici*, respectively (Mishra et al., 2011). The antagonistic activity of *T.viride* against *B. cinerea, F. oxysporium* (Farkhondeh et al., 2013; Shi et al., 2012), *M. phasealina and R. solani* (Rahman et al., 2014) inhibited growth, after the 4th day of dual plate culture. *Trichoderma* started sporulation on day 9th and 10th in *B. cinerea, M. Phasealina* and *Rhizoctonia Solani* respectively. But in *F. Oxysporium* dual plate *Trichoderma* sporulation was inhibited by *F. Oxysporium*. *T. viride* was more effective against *Botrytis Cinerea* compared to other strains (Figure 1). But *F. Oxysporium* was resistant to *Trichoderma* antagonistic activity, hence *Trichoderma* was unable to overgrow and sporulate the strain in dual plate.

Conclusion

Being biotechnologically important, mycoparasitic *Trichoderma* spp. are broadly researched for both field applications as well as basic biology .This is a simple and cost effective medium for dual plate culture. *T. viride* showed antagonistic activity against all the pathogens such as *B. cinerea, F. oxysporium, M. phasealina* and *R. solani;* therefore, *T. viride* can be used as a biopesticide against all the phytopathogens.

Conflict of interests

The authors did not declare any conflict of interest.

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Nutritional composition, phytochemicals and microbiological quality of the legume, *Mucuna pruriens*

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The aim of this study was to evaluate the nutritional and phytochemical compositions and microbiological quality of seeds of the legume *Mucuna pruriens* (MP) grown in northeastern Brazil. MP flour and extract were produced and evaluated for proximate, mineral, and phytochemical compositions, fatty acid profile, and microbiological quality. MP flour and seed extract showed 43.12 and 43.4% of protein, 7 and 7.6% lipid matter, 37.19 and 33.33% starch, and 5.64 and 2.36% fiber ($p<0.05$), respectively. Abundant minerals found were in both the extract as flour, such as potassium (635 and 679 mg/g), iron (79 and 158 mg/g), and phosphorus (83 and 93 mg/g). Flavonoids, steroids, and saponins were detected. The main fatty acids found were myristic, palmitic, oleic and linoleic acids. Microbiological evaluation did not indicate the presence of pathogenic or spoilage microorganisms. MP produced in Northeastern Brazil is an alternative source of carbohydrate, fiber, protein, essential fatty acids, minerals, saponins and flavonoids, which may encourage its potential consumption and marketing.

Key words: Mineral composition, microbiological quality, phytochemicals, legume.

INTRODUCTION

Mucuna pruriens (MP) is a member of the *Fabaceae* family, composed of approximately 650 genera and 2,000 species. This leguminous plant grows pods about 12 cm long that contain about 7 seeds of varied coloration from beige to brown and black, and also striped ones. This legume is also known as “velvet bean, lion bean, nescafe, and cowage”, among others. It is a leguminous plant originating in India and cultivated in Sri Lanka, Malaysia, southeastern Asia, and in tropical regions of Central and South America (Hammerton, 2003). MP is largely grown in northeastern Brazil, where it is used as “green manure” due to its capacity to fix nitrogen for the soil or as a supplement for animal feed and in a minor volume used as food source or in the traditional medicine (Raina et al., 2012), being an important source of income for many farmers and consequently for the economy.

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Several studies have highlighted some medicinal properties of this plant and its use has been proposed especially in the treatment of Parkinson's disease (Tharakan et al., 2007). Studies have shown hypoglycemic (Majekodunmi et al., 2011), anti-inflammatory and diuretic (Bala et al., 2011), hypolipidemic (Eze et al., 2012), and aphrodisiac properties (Suresh et al., 2009), and other studies have shown its androgenic (Muthu and Krishnamoorty, 2011; Ahmad et al., 2012) and estrogenic activities (Shahaji and Parnu, 2011).

Studies analyzing the composition of MP grown in India have shown high percentages of protein (28.23%) and carbohydrate (60.03%) and a low content of lipids (2.69%), and a mineral composition of good nutritional quality (Siddhuraju and Becker, 2003). Regarding the phytochemical composition, Manalisha and Chandra (2012) detected the presence of proteins, carbohydrates, resins, flavonoids, alkaloids, sterols, phenols, and glycosides in MP seeds after washing and drying in the shade. antinutritional components such as tannins, phytic acid, enzyme inhibitors, trypsin, saponins, lectins, as well as hemagglutinin activity and high levels of L-dopa (Vadivel and Pugalenthi, 2010; Nwaoguikpe et al., 2011; Ahmad et al., 2012), which require treatments such as peeling, cooking, irradiation, and others to be inactivated (Betancur-Ancona et al., 2008). Studies on the nutritional composition of MP have characterized this leguminous plant grown mainly in India; however, it is known that the nutritional composition of foods is influenced by climatic variations, soil composition, temperature, surrounding vegetation, and rainfall volume (Luizão, 2007). Accordingly, the aim of this study was to evaluate the nutritional and phytochemical composition and microbiological quality of *M. pruriens* grown in northeastern Brazil, with the purpose of encouraging the human consumption of this legume.

MATERIALS AND METHODS

Preparation of *M. pruriens* flour and seed extract

MP seeds (4 kg) were obtained in the local market of the city of João Pessoa - PB. To prepare the flour, seeds were washed twice in tap water, subsequently dried with absorbent paper at room temperature (26°C), crushed in grinder, and sieved to obtain a fine powder, which was kept in a drying oven at 50°C for 100 h. MP extract was obtained by hydroalcoholic extraction by adding 200 mL distilled water and 200 mL alcohol in 100 g of dry flour. This mixture was homogenized for 72 h and sieved to remove solid residues, being then submitted to water bath at 45°C for 24 h to form a creamy material of beige-brown coloration. The samples were frozen and then lyophilized at -48°C with pressure of 130 mmHg in lyophilizer model Liotop, L101 (São Carlos, Sao Paulo, Brazil) for 24 h (Muthu and Krishnamoorthy, 2011; Suresh et al., 2009).

Nutritional composition of *M. pruriens* flour and extract

Proximate composition

All chemical composition analyses were performed in triplicate. Moisture analysis was performed by drying in oven at 105°C, and ash by incineration in muffle furnace at 550°C; the protein percentage was obtained using the Kjedhal method, the Soxhlet methodology was used for the extraction and determination of total lipids and the amount of carbohydrate was obtained by determining the starch and fiber contents (AOAC, 2002).

Fatty acid profile

The lipid extract was initially obtained by the method of Folch et al. (1957) and this extract was used to obtain methyl esters by esterification (Hartman and Lago, 1973). The identification and quantification of methyl esters were performed on gas chromategraph model GC-Master (Ciola & Gregori Ltda, São Paulo, Brazil) with flame ionization detector. The chromatographic conditions were: fused silica polyethylene glycol column (Carbowax 20 M) with 30 m in length, 0.53 mm in diameter and 0.25 µm of film thickness in the stationary phase. The temperatures used were: vaporizer: 150°C, detector 200°C and oven programming: 80°C for 3 min, 10°C/min up to 120°C, remaining at 200°C for 6 min and later decreasing 3°C/min up to 180°C. The mobile phase was hydrogen at flow rate of 5 mL/min. The injected volume was 1 µl, with a split ratio of 1:25. The characterization of fatty acids was performed by comparison of mass spectra obtained with standards that were also injected into the chromatograph.

Mineral composition

The analysis of minerals present in MP was performed by energy-dispersive X-ray fluorescence spectrometry (Teixeira et al., 2012). Initially, the samples were dried at 75°C for 1 h in watch glasses. Then, the samples were placed in appropriate sample holders of the Energy Dispersive X-ray Spectrometer device (model EDX-720, Japan), being sealed on both ends with thin polypropylene film and at one end a hole was opened to prevent extrusion of samples when activating the vacuum to be then analyzed by EDX.

Phytochemical screening

Phytochemical screening of MP flour and extract to detect the presence of flavonoids, saponins, tannins, sterols, and alkaloids was performed according to methodology proposed by Matos (2009). For the detection of flavonoids, chloroform was added for the separation of layers. Then, methanol was added and evaporation was conducted in rotary evaporator. The dissolved material was distributed into two test tubes, where 10% HCl solution and magnesium tape were added, leaving to react until the tape disappeared and observing the appearance of pink color (positive test). In the second tube, acetone, oxalic acid and boric acid were added. The mixture was dried in water bath, ethyl ether was added and the fluorescence was observed under UV spectrophotometer at wavelength of 510 nm (Biospectro model SP-220/Brazil).

Qualitative analysis of saponin was performed using the foam test, which consisted of dissolving the sample in water in a test tube, stirring for 1 min and leaving to rest for 10 min. After the rest, the foam disappeared, which characterizes negative test for this phytochemical. The test for tannins was carried out with evaporation of samples to complete drying, and filtering with cotton funnel. The filtrate was distributed in six test tubes, the first three were tested with 0.5% gelatin and the others with 2% iron chloride at different concentrations (0.5, 1.0 and 2.0 mL), observing the formation of precipitates, which indicates the presence of tannins. For the analysis of steroids, samples were submitted to evaporation until complete drying, adding chloroform for dissolution. Then, the samples were divided into three test tubes with 0.12, 0.25 and 0.5

Table 1. Proximate composition of *Mucuna pruriens* flour and seed extract.

Proximate composition	Flour	Extract
Protein (%)	43.12±0.19	43.40±0.07
Starch (%)	37.19±0.4	33.33±1.6
Crude fiber (%)	5.64±0.3	2.36±0.06
Lipids (%)	7.00±0.5	7.60±1.4
Moisture (%)	8.20±0.04	9.90±0.02
Ash (%)	3.10±0.04	2.90±0.02
Total energy (Kcal)	384.24±2.46	375.32±7.06

Means ± standard error with different letters in the same row differ by the Student t test ($p<0.05$).

mL. Chloroform, acetic anhydride and concentrated sulfuric acid were added to each tube. The results were observed according to the color standard, where the color blue indicated the presence of steroids.

Qualitative analysis of alkaloids was performed by evaporation of the alcoholic extract to complete drying, and alkalinizing the medium with 1% sodium hydroxide. Distilled water with chloroform was added; the system was filtered with cotton filter and the extract was separated from the chloroform layer. Then, 1% hydrochloric acid was added to the chloroform layer, stirred and allowed to settle until it becomes clear. Subsequently, it was distributed into four test tubes of 1 mL, followed by testing with the following reagents: Bouchardat, Mayer, Dragendorff and silicotungstic acid, observing the formation of precipitate if positive.

Microbiological evaluation

The prior preparation of MP extract and flour samples for microbiological analysis consisted of homogenizing the products with 0.1% of buffered peptone water (BPW), where 10^{-1}, 10^{-2} and 10^{-3} dilutions were prepared. The microbiological quality of MP flour and extract was assessed by counts of coliforms at 35°C, standard plate count of mesophilic aerobic bacteria, yeasts and molds, *Staphylococcus aureus* and *Salmonella* spp. (Vanderzant and Splittstoesser, 1992). All analyses were performed in triplicate.

Statistical analysis

The results regarding the proximate composition were submitted to the Student's t-test at 5% significance level ($p <0.05$) using the GraphPad InStat software version 3.0.1 (GarphPad InStat, San Diego, CA, USA).

RESULTS AND DISCUSSION

Proximate composition

Difference ($p<0.05$) in the percentages of moisture, ash, starch and fiber was observed between MP flour and extract, which is justified by the different processing techniques that the samples were submitted (Table 1). Both MP flour and extract have high protein content (from 43.12 to 43.40%) and carbohydrates as starch (from 37.19 to 33.33%) and significant ash content (from 2.90 to 3.10%). By comparing with results obtained by Josephine and Janardhanan (1992), the present study obtained higher protein percentage (34.4%), similar lipid percentage (7.7%) and lower carbohydrate percentage (45.9%). In relation to results obtained by Siddhuraju et al. (1996), results obtained in this research were higher for lipids and protein (6.7 and 31.5%, respectively) and lower for carbohydrates (52.5%). Nwaoguipe et al. (2011) analyzed raw MP flour and found 28.2% proteins, 2.7% lipids and 60% carbohydrates and when raw MP flour was submitted to water logging followed by cooking, only reduction in the protein content was observed. The fiber results of MP flour and extract are lower than the fiber percentage of cowpea (19.4 ± 1.07) determined by Frota et al. (2008); however, MP flour can be considered as a source of fibers, since it exceeded the minimum 3 g fibers/100 g of food (Brasil, 1998). The grinding and sifting processes that the MP grain was submitted to may be responsible for this decrease, since some amount of bark was removed during processing. Moreover, the extraction process that the flour was submitted to may have removed additional amounts of bark, so there was difference between fiber values obtained between flour and extract. Variations in proximate composition reported in literature and compared with the present study may be justified by different varieties and cultivation conditions such as soil characteristics, temperature, humidity and rainfall volume (Freire Filho, 2011).

Fatty acids profile

The main fatty acids detected in MP flour were myristic, palmitic and linoleic acids. MP extract showed higher percentages of myristic, palmitic, oleic and linoleic acids (Table 2). The present study detected higher concentrations of saturated fatty acids in MP flour and similar proportion of saturated and polyunsaturated fatty acids in the MP extract; similar results were found by Frota et al. (2008), who analyzed cowpea and found higher concentrations of linoleic acid followed by palmitic and stearic acids, so that the highest percentage obtained was of saturated fatty acids. Among the fatty acids of higher

Table 2. Fatty acids profile of *Mucuna pruriens* flour and seed extract.

Fatty acids	Flour	Extract
Myristic acid - C 14:0	52.05 ± 3.3	23.8 ± 3.3
Palmitic acid - C 16:0	14.15 ± 1.0	12.61 ± 0.4
Stearic acid - C18:0	4.82 ± 0.53	4.94 ± 0.2
Saturated fatty acids (%)	71.02	41.35
Oleic acid - C18:1	8.02 ± 0.6	17.07 ±0.88
Monounsaturated fatty acids (%)	8.02	17.07
Linoleic acid - C 18:2	18.51 ± 1.5	37.46 ±1.71
Linolenic acid - C 18:3	2.44 ± 0.17	4.11 ± 0.13
Polyunsaturated fatty acids (%)	20.95	41.57

Means ± standard error with different letters in the same row differ by the Student t test ($p<0.05$).

Table 3. Mineral composition of *Mucuna pruriens* flour and seed extract.

Mineral	Flour (mg/g)	Extract (mg/g)	*DRI (adults)
Potassium	635	679	4.700 mg
Iron	158	79	14 mg
Phosphorus	83	93	700 mg
Calcium	70	68	1.000 mg
Sulfur	38	48	N/A
Zinc	6	34	7 mg
Copper	3	34	900 µg
Magnesium	**nd	20	260 mg

*Daily recommended intake (Brazil, 2005); ** nd = not detected; N/A = no recommended daily intake.

concentration in MP flour and extract, saturated fatty acids myristic and palmitic acids can raise total cholesterol and all its fractions, participating in the development of atherosclerosis in humans. Monounsaturated and polyunsaturated oleic and linoleic acids in MP extract have antiatherogenic action, since they reduce total and LDL-C cholesterol without reducing HDL-C. Furthermore, they increase vasodilation and reduce platelet aggregation (Nicod et al., 2015). Polyunsaturated fatty acids present in higher concentration in the MP extract are considered essential in humans because there is no endogenous biosynthesis of these nutrients. They are important for maintenance of cell membranes, brain function and nerve impulse transmission, and take part in the transfer of atmospheric oxygen to the blood plasma, hemoglobin synthesis and cell division (Martin et al., 2006). The American Heart Association (Stone et al., 2013) limits the intake of saturated fatty acids to 7% the total calories ingested per day, 20% for monounsaturated fatty acids and 10% for polyunsaturated fatty acids. Both samples have a high content of saturated fatty acids; however, the MP extract shows fatty acids profile more beneficial to health than MP flour, since it presents greater proportion of monounsaturated and polyunsaturated fatty acids.

Mineral composition

Mineral composition of MP showed amounts of potassium and iron in the flour, and potassium and phosphorus in the extract, and magnesium was only detected in the extract. The presence of toxic minerals under the Brazilian law was not detected (Brasil, 2005) (Table 3). By comparing the concentration of minerals present in MP flour or extract with values proposed by the DRI for adults (FAO/WHO/UNU, 2001), it was observed that MP flour or extract have concentrations higher than recommendation for the following minerals: iron, copper and zinc. Both samples had values greater than 10% the DRI values for minerals potassium and phosphorus. Magnesium was found only in the MP extract, representing

7.7% of the recommended daily intake. Only calcium showed concentration lower than 10% the DRI values for flour (7%) and extract (6.8%). Vegetables are natural sources of magnesium, iron, zinc, and copper (Mesquita et al., 2007). Vadivel and Janardhanan (2005) found higher potassium concentrations (83.51%), followed by calcium (30.45%) and magnesium (20.88%) and Bhat et al. (2008) found 24.5% of phosphorus, 6.65% of calcium and 1.94% of selenium in *M. pruriens*. Other vegetables such as soybeans, widely consumed by the Brazilian population, presents varying concentration of minerals such as calcium (275 mg/g), phosphorus (674 mg/g) and iron (53 mg/g) (Penha et al., 2007). Bean, a major Brazilian food source, shows 0.72 g potassium/100 g, 1.51 g calcium/100 g, 0.45 g phosphorus/100 g and 126.9 mg iron/kg (Mesquita et al., 2007).

Minerals play important and specific roles in the body. Potassium is important for cardiac patients who wish to reduce blood pressure through diet (Terker et al., 2015). Iron stands out for participating in oxidation and reduction reactions, respiratory and blood transport of oxygen and carbon dioxide and enzyme activation (Moura and Canniatti-Brazaca, 2006). Phosphorus participates in essential functions of the organism, since DNA and RNA are based on phosphate, a source of energy in the form of ATP, cell membrane component, formation of hydroxyapatite (a component of teeth and bones) and others (Rostami et al., 2014). The main function of calcium is to gain and maintain bone mass and density, transmembrane transport, nerve transmission and muscle contraction (Pedrosa and Castro, 2005).

Phytochemical screening

There was no (-) alkaloids and tannins in MP, but a weak presence (+) of steroids and saponins and moderate presence (++) of flavonoids in MP. MP is widely used as aphrodisiac and stimulant of testosterone biosynthesis (Suresh et al., 2009; Muthu and Krishnamoorty, 2011). It may be due to the high concentration of this steroid in the grain, since steroids stimulate the production of anabolic androgenic hormones; however, in this study, it was observed that steroids are weakly present in MP grown in northeastern Brazil. Still, Manalisha and Chandra (2012) evaluated the phytochemical composition of MP and observed absence of saponins, weak presence of resins, tannins, flavonoids, alkaloids, steroids, phenols and glycosides. Phytochemical analysis showed moderate presence of flavonoids in the MP extract. Flavonoids comprise a large group of polyphenolic compounds responsible for a variety of pharmacological activities related to its antioxidant activity, inducing protective enzyme systems in humans against infectious, degenerative, cardiovascular and age-related diseases. These phytochemicals are widely distributed in foods of plant origin and cannot be synthesized by humans or animals; therefore, foods that are sources of these nutrients should be consumed (Kumar and Pandey, 2013). There is a great demand for the consumption of food or food supplements rich in flavonoids; however, it is difficult to identify an average dietary intake of this phytochemical, since there is a wide distribution in plant food sources (Izzi et al., 2012). Accordingly, there has been an increasing interest in the production of foods with therapeutic potential attributed to the presence of flavonoids (Kumar and Pandey, 2012), which can boost MP consumption after the conduction of *in vivo* studies evaluating the antioxidant capacity of the grain.

Saponins also play an important role due to their activities beneficial to health such as significant anticholesterolemic, antidiabetic and anticancer activity, as well as the ability to reduce plasma cholesterol concentration (Park et al., 2001). Despite many beneficial effects attributed to saponins, these substances are also considered anti-nutritional factors. These compounds are very common in foods of plant origin and are considered anti-nutritional substances because they reduce the nutritional value of foods, decreasing the digestibility and absorption of nutrients. When in excess, saponins can be toxic and lead to undesirable side effects (Santos, 2006). Antinutritional factors have led the scientific community to find ways to reduce their concentration or inactivate them, especially through the application of methods such as cooking, roasting or drying, considering that the majority of anti-nutritional factors are sensitive to high temperatures. In addition to these techniques, fermentation, alkaline solubilization, or isoelectric precipitation can also be used to reduce antinutritional factors that are insensitive to high temperatures (Betancur-Ancona et al., 2008).

Microbiological evaluation

The tested samples showed no contamination by coliforms at 35°C and no *S. aureus* or *Salmonella* spp. were isolated. The MP extract showed mesophilic aerobic bacteria count of 1.0×10^3 CFU/g, and this value is within standards established by the National Health Surveillance Agency (ANVISA) from 1.0×10^3 to 1.0×10^5 CFU/g (Brasil, 2001). Yeast and mold counts were 2.34×10^6 CFU/g in the extract and 3×10^2 CFU/g in the flour. The MP extract and flour obtained showed no contamination by pathogenic and spoilage microorganisms and can be considered suitable for human consumption.

Conclusions

M. pruriens flour and extract are sources of carbohydrates, fiber and protein and exhibit considerable amounts of iron, potassium and phosphorus for humans.

Moreover, they present essential fatty acids, and the presence of flavonoids in this vegetable represents possible antioxidant activity. The microbiological evaluation indicated satisfactory sanitary quality of the product. These results may encourage the consumption and marketing of this vegetable in the domestic and international market as food source or ingredient in industrial formulations.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Nutritional, antinutritional and phytochemical status of okra leaves (*Abelmoschus esculentus*) subjected to different processes

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The aim of this study was to analyze the nutritional quality and antinutritional factors of okra leaves subjected to different process. Proximate composition, calcium, magnesium, potassium were determined, as well as lectin, tannin, saponin and total phenolic compounds. The okra leaves showed a predominance of carbohydrates, fibers and proteins that were not significantly affected by process, being also considered source of calcium, magnesium and potassium. Extracts obtained from bleached, cooked, lyophilized leaves and from 30% fraction, buffer showed the presence of lectin. The tannin contents found were 3.39% in lyophilized leaves, 0.45% in fresh leaves, 0.44% in bleached leaves and 0.27% in cooked leaves. The presence of saponin was not detected. The extract showed content of phenolic compounds of 19.27 mg of GA/g. The okra leaves can be included in human diet as a nutritionally suitable food.

Key words: *Abelmoschus esculentus*, antinutritional factors, minerals, nutritional quality.

INTRODUCTION

The world population growth has led to an increase in nutritional deficiencies and diseases related to the lack of essential nutrients in human diet, particularly affecting vulnerable populations. One of the world's greatest challenges is to secure sufficient and healthy food for all, and to do so in an environmentally sustainable manner.

In order to reduce these conditions, the attention has been increasingly focused on exploring non-conventional food sources that provide nutritional and pharmaceutical benefits, highlighting dark-green leafy vegetables, good sources of minerals and vitamins (Raju et al., 2007; Burchi et al., 2011). Vegetables have gained prominence

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because they are sources of bioactive compounds, and are important sources of ingredients for use as functional foods (Nithiyanantham et al., 2012). The use of okra leaves (*Abelmoschus esculentus*) as vegetable popularly known as okra, belonging to the family Malvaceae, widely distributed in Africa, Asia and America is among the possible alternatives (Doreddula et al., 2014). World production of okra as fresh vegetable is estimated at 6 million t/year (Sergius and Esther, 2014); are commonly used both as food as salad fresh or cooked and for curative purposes, showing low calories, a good source of edible fiber,contains important bioactive compounds such as carotene, folic acid, thiamine, riboflavin, niacin, vitamin C, oxalic acid and amino acids (Roy et al., 2014). Besides, the nutritional properties mentioned above, some authors have reported a variety of functional activities attributed to this fruit such as anti-diabetic, anti-hyperlipemic, anti-inflammatory, anti-fungal and antioxidant (Khomsug et al., 2010; Doreddula et al., 2014). The antioxidant activity of the fruit is due to its content of phenolic compounds, which are effective antioxidants and can be used in the prevention of degenerative processes such as cancer, cardiovascular diseases and diabetes (Doreddula et al., 2014).

Moreover, vegetal sources may contain substances harmful for human health, affecting the bioavailability of nutrients. Among these substances, lectins, tannins and saponins stand out. However, to use plant leaves as an alternative source of nutrients, it is necessary to study them for the presence of antinutritional factors and how to inactivate them for subsequent safe consumption. Studies on the nutritional characteristics and phytochemical compounds of okra leaves are scarce. In this sense, in order to introduce the market a new plant product, a study on the nutritional quality and presence of phytochemical compounds in okra leaves *(Abelmoschus esculentus)* subjected to different treatments was carried out, aiming its use for human consumption.

MATERIALS AND METHODS

Samples

Okra leaves (*A. esculentus*) were grown in the municipality of Sapé, state of Paraiba, Brazil. A total of 6 kg of okra leaves were rinsed thoroughly in running water and distilled water and dried at room temperature. Samples of fresh leaves were separated for the following thermal treatments: blanching for 2 min in boiling water and cooking for 20 min in boiling water. Cooked, bleached or fresh leaves were dried at 25°C and stored. Another portion of the leaves were separated for lyophilization at temperature of -36°C and pressure of 300 mmHg, and ground in a Willey-type electric mill, obtaining fine flour.

Proximate composition and minerals

Moisture, ash, proteins and lipid determinations were performed according to AOAC (2006). The magnesium (Mg), potassium (K) and calcium (Ca) concentrations were determined by atomic absorption spectroscopy from the ash solution (AOAC, 2006). All analyzes were performed in triplicate.

Phytochemical analysis

Fresh, bleached, cooked and lyophilized leaf samples were crushed and submitted to extraction in Tris-HCl 0.1 M pH 7.4 with 0.15 M NaCl under stirring for 3 h at 25°C. The suspension obtained was centrifuged at 5000 rpm at 4°C for 20 min and the precipitate was discarded. The supernatant, called total extract, was filtered and submitted to hemagglutinating activity assay. The filtrate of the lyophilized sample was submitted to precipitation with ammonium sulfate $(NH_4)_2SO_4$ using the saturation range: 30%. After saturation with ammonium sulfate, the solution was left to rest for 8 h and centrifuged at 5000 rpm at 4°C for 20 min. The fraction obtained was dialyzed and lyophilized, and then submitted to hemagglutination activity, in which human erythrocytes types A, B and O were used, provided by the Blood Center of Paraíba and rabbit erythrocytes, obtained from the Experimental Laboratory of the Department of Molecular Biology, UFPB. The hemagglutinating activity of the different treatments was determined by double serial dilutions and the presence of HA were macroscopically determined overnight. The lectin specificity was determined by inhibition with sugar, and was macroscopically viewed (Soares et al., 2012). Lectin of fraction 30% (2 mg/mL) was initially diluted in 50 ml of 0.1 M Tris-HCl pH 7.4; 0.15 M NaCl buffer, separate into two equal aliquots and submitted to pH from 2.0 to 13.0. Then, the mixtures were incubated in 37°C for 30 min and the hemagglutinating activity was performed with rabbit erythrocyte at 3% by double-serial dilution. The resistance of lectin from okra leaves (*A. esculentus)* was assessed against trypsin-like proteolytic enzyme. The effect of the β-mercaptoethanol reducing agent on the hemagglutinating activity and the denaturing agent urea was assessed (Soares et al., 2012). To assess the effect of temperature on the lectin present in 30% fraction of okra leaves (*A. esculentus*), 2 mg/mL was used. The solution was aliquoted and submitted to heating in a thermal cycler with temperatures ranging from 40 to 100°C. Every 10 min corresponding to a variation of 10°C, an aliquot of 100 µL was collected and the hemagglutinating activity was determined as previously described. The sample was tested in duplicate and in serial dilution.

Tannin levels were determined in bleached, cooked and lyophilized okra leaves (*A. esculentus*) according to procedure recommended by F to D method (Bubba et al., 2009). Saponin levels were determined using an agar solution buffered with 25 mM bibasic phosphate pH 7.4. About 5 mL of agar solution were added to 0.5 ml of rabbit erythrocytes at 3%, homogenized and placed in a Petri dish to solidify. After solidification, 20 and 40 µL of samples were added in wells. The result was observed within 30 min and overnight by the presence of a hemolytic halo. As positive control, the fraction of *Luffa operculata* seeds presenting hemolytic halo was used.

The total phenolic compounds were determined using the Folin-Ciocalteau reagent with gallic acid as stock solution and the reading was performed in UV / VIS at 765 nm (Vallverdú-Queralt et al., 2011).

Statistical analyses

The results were submitted to statistical tests, using the Assistat software version 7.6 beta. In order to meet the methodological assumptions of parametric tests and obtain consistent results, the sample homogeneity and the Kolmogorov - Smirnov (KS) normal distribution tests were applied. Analysis of variance (ANOVA) for multiple comparisons was also applied where $p \leq 0.05$ was set to indicate statistical significance with the Tukey's t test .

Table 1. Proximate composition and Levels of minerals of okra leaves (*Abelmoschus esculentus)*, wet basis.

Parameters evaluated	Lyophilized leaf	Fresh leaf	Bleached leaf	Cooked leaf
Moisture (%)	9.13±0.19[c]	74.83±1.17[b]	73.31±0.38[b]	81.53±0.11[a]
Lipids (%)	2.00 ±0[a]	0.93±0.18[b]	1.00±0.16[b]	1.07±0.18[b]
Protein (%)	23.62±0.23[a]	6.10±0.61[b]	6.22±0.23[b]	6.06±0.39[b]
Ashes (%)	15.39±0.08[a]	2.75±0.44[b]	2.84±0.84[b]	2.49±0.40[b]
Calcium (mg/100 g)	691±0.22[a]	382.50±0.71[b]	357±0.43[c]	366.50±0.42[d]
Magnesium (mg/100 g)	438±0.64[a]	232.50±0.31[b]	237.50±0.18[c]	138.50±0.66[d]
Potassium(mg/100 g)	670.50±0.55[a]	167.50±0.68[b]	110.5±0.86[c]	63±0.91[d]

Means followed by different letters between columns indicate significant differences with an error probability of p ≤ 5%, according to the Tukey's t test.

RESULTS AND DISCUSSION

Proximate composition and minerals

The proximate composition of lyophilized, fresh, bleached and cooked okra leaves are shown in Table 1. The cooking process was able to significantly increase the moisture content by 6% compared to the fresh leaf due to the swelling of plant cells. There was no significant difference in the protein, lipids and ash contents between fresh, bleached and cooked leaves. The moisture content in the fresh leaf (74.83%) found in this study corroborates value found in literature (Effiongh et al., 2009), of 78.83%, assessing fresh okra leaves. In fresh leaves of *Petroselinum crispum*, *Anethum graveolens*, *Lactuca sativa* and *Brassica oleracea*, Caunii et al. (2010) found levels higher than those found in this work; 87.71, 85.95, 94.91 and 92.52 %, respectively. The leaves of okra had lower lipid content and similar ash content compared to okra seeds, which showed lipid content 28 to 31% and ash 3.42% (Adelakun et al., 2012), indicating that the chemical composition varies according to the examined part of the plant. Mensah et al. (2008) found low proteins in fresh leaves *Amaranthus cruentus* (4.6%). Caunii et al. (2010) analyzed fresh *L. sativa* and found values of 1.62% protein. Singh et al. (2001) found higher protein values in coriander and spinach leaves; 22.2 and 26.5%, respectively. Effiong et al. (2009) studied fresh okra leaves and found values twice greater than those found in this study, 13.75%; variations in macronutrients within the same species are a reflection of soil, climate and irrigation conditions of vegetables during cultivation. The calcium, magnesium and potassium contents were also higher in lyophilized leaves due to the concentration of nutrients found in dehydrated products, while the cooking process reduced these levels due to the solubilization of minerals in the cooking water, and it is important to use the cooking water for other culinary preparations. The calcium and magnesium contents in fresh, bleached, cooked and lyophilized okra leaves account for more than 15% of the recommended daily intake (RDA) for these minerals, which are 800 and 300 mg, respectively, being then considered food sources of these minerals (IOM, 2000).

The seeds of okra have a lower content of calcium, magnesium and potassium (Adelakun et al., 2012) in comparison to the leaves of okra. The magnesium contents were higher when compared to other leafy vegetables such as coriander, spinach, amaranth and carrot leaves, which values are 3.7 ± 0.04, 10.2 ± 0.05, 3.1 ±0.04 and 1.8 ± 0.01 mg/100 g, respectively (Singh et al., 2001). Caunii et al. (2010) found for lettuce, calcium, potassium and magnesium contents of 36, 45 and 6 mg/100 g, respectively. The calcium and magnesium contents in fresh leaves are very close to those found in a similar study (Effiongh et al., 2009), of 321.00 ± 0.88 for calcium, 180.00 ± 2.30 for magnesium; however, for potassium, there is value well above that found in this study and in other studies with leaves, of 1128.1 ± 2.19 mg/100 g.

Phytochemical analysis

The lectin present in the extracts of bleached, cooked, lyophilized leaves and in the fraction 30% has specificity for sugars present in the membrane of rabbit erythrocytes, not recognizing membrane carbohydrates present in ABO human blood system. The analyses showed that the lectin present in extracts was inhibited by mucin (625 mM). The presence of lectin was not detected in fresh leaves due to the high mucilage content in which this protein is complexed, because the lectins are proteins that are capable of specifically and reversibly binding to carbohydrates in their mono- or oligosaccharide forms, thereby agglutinating cells and precipitating the oligosaccharides and glycoproteins (Soares et al., 2012), however, with application of the thermal treatment, it was observed that, lyophilized okra leaves had a higher specific hemagglutination activity, of 11.14 UH / mgP, followed by fraction 30%, with 4.77 UH / mgP, bleached leaves of 2.57 UH / mgP and cooked leaves of 2.10 UH /

mgP. Similar results obtained by Seena et al. (2006) and Leite et al. (2009) in cooking causes the reduction of specific hemaglutination activity in plants. Lectin of fraction 30% of okra leaves was inactivated at 100°C in a time of 30 min. As for the stability against different pH, the lectin found in okra leaves was stable in acidic pH (1 to 6), with maximum activity at pH 7 and inactivation at pH 12 and by the action of agents such as beta mercaptoethanol in concentration of 20 mM and by denaturing agent urea. Similar results for this study were reported by Cheung et al. (2010), that evaluated the lectin from *Musa acuminata* remains functional within the pH range from 1 to 13 and rated by Yao et al. (2010), that evaluated the lectin from *Setcreasea purpurea* as capable of promoting clustering at extreme pH values, with 100% hemagglutination within pH range from 5 to 9. Lectins from *Hypnea cervicornis*, *Craniela australiensis* and *Aspergillus nidulans* are specific to mucin glycoprotein (Shing et al., 2011). Leite et al. (2009) studied the presence of lectin in fresh, bleached and cooked *Amaranthus* leaves and detected the presence of lectin only in fresh leaves. The presence of saponins in fresh, bleached, cooked and lyophilized leaves was not detected because there was no hemolysis of erythrocytes. Ee and Yates (2013) in experiments with wattle *Acacia saligna* seeds observed the presence of saponin in raw seeds (3.0 $g.100^{-1}$) and after cooking for 10 min (1.06 $g.100^{-1}$) and that heat processing is usually applied to the seeds before consumption to eliminate antinutritional factors, such as protease inhibitors, lectins, alkaloids, saponins and oxalates, which can interfere with the digestion and absorption of nutrients.

The analysis of the presence of tannin in fresh okra leaves found values of 0.44 ± 0.02% tannic acid equivalent and there was no significant difference when leaves were bleached (0.45 ± 0.05%); however, the cooking process reduced the tannin content (0.27 ± 0.06%) to acceptable levels. Astringent and antinutritional functions are assigned to tannins; the latter, if ingested in large amounts. The analysis of the presence of tannin in fresh okra leaves found values of 0.44 ± 0.02% tannic acid equivalent, lower than that reported in literature, of 1.2% in fresh okra leaves (Singh et al., 2001). Leite et al. (2009) investigating this component in *Amaranthus* leaves observed significant increase from fresh (1.07%) to bleached leaves (2.79 %). Ferreira et al. (2008) found higher concentrations of tannins and saponins in *Moringa oleifera* leaves compared to those determined in the present study. The content of total phenolics in *A. esculentus* extract was 19.27 ± 0.9 mg GAE/g. The content of total phenolics in *A. esculentu* was lower than that found in other leaves; *T. brasiliensis* (38.53 ± 0.63 GAE/g)), *C. macrophyllum* (66.14 ± 3.56 GAE/g) (Sousa et al., 2007); *B. crassifolia* (35.93 mg GAE/g), *I. edulis* (24.50 mg GAE/g) (Pompeu et al., 2012), but higher than values found in fresh okra leaves (less than 0.15 mg GAE/g), seeds (2.13 µg/g) and root (0.55 µg/g) (Leiote et al., 2009), in *Cosmos caudatus* (18.83 mg GAE/g), *Centella asiatica* (7.79 mg GAE/g) and *Oenanthe javanica* leaves (7.41 mg GAE/g) (Huda-Faujan et al., 2009). For some derivatives of phenolic acids, antioxidant activity has been reported, except for those of high molecular weight, such as tannins.

Conclusion

The heat treatment showed the absence of saponins, inactivation of lectins, reduced levels of tannins and maintained the protein, lipids and minerals similar to fresh leaves. The lyophilization was also an appropriate processing for conservation of nutrients in okra leaves. Fresh, lyophilized and heat-treated leaves of okra showed a source of calcium and magnesium, and furthermore antioxidant activity can be included in the human diet as a nutritionally adequate food.

Conflict of interests

The authors did not declare any conflict of interest.

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Effects of ethanolic extract of garlic, roselle and ginger on quality attributes of chicken patties

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Efficiency of ethanolic extracts of garlic, ginger and roselle on quality attributes of chicken patties was investigated. Sensorial qualities were evaluated using a 9-point hedonic scale. Lipid oxidation was assessed by monitoring malondialdehyde formation with 2-thiobarbituric acid (TBARS) assay. Total plate count (log_{10}CFU/g) and Warmed Over Flavour (WOF) were determined on days 0, 7 and 14 of refrigerated storage while proximate composition was determined on freshly prepared patties using a standard procedure. Sensory evaluation revealed high scores for Overall Acceptability (OA) of patties containing the plant extracts while the highest score of aroma was recorded in products with ginger extract. The nutrient composition of the products were not affected (P>0.05) by the plant extracts. The cost of production reduced by 1.81, 0.49, 2.75 and 0.53% with the addition of 0.05% of garlic, ginger, roselle extracts and α-tocopherol respectively in comparison to the negative control. The WOF formation reduced from 17.65 to 39.29% by the inclusion of the extracts. The microbial load also reduced in comparison to the negative control. In conclusion, the plant extracts used in this study provided antioxidant and antimicrobial benefits to raw chicken patties during cold storage (4°C). As herbs/spices, they could be used to extend the shelf-life of chicken patties and provide the consumer with food containing natural additives, which might be more healthful.

Key words: Chicken patties, plant extracts, sensory attributes, lipid oxidation.

INTRODUCTION

Lipids oxidation is a major problem in Sub-Saharan Africa especially as the ambient temperature is very high and food preservation becomes a challenge. In other to maintain food product quality especially during cold storage, the application of suitable substance that has both antioxidant and antimicrobial activities may be useful to extend their shelf life and prevent economic loss (Yin and Cheng, 2003). This intervention becomes necessary because the products of lipid oxidation such as malondiadehyde (MDA) have been implicated to cause pathological changes in the mucous membranes of the alimentary tract and to increase the cholesterol and peroxides in blood serum. Apart from all these serious implications on human health, lipid oxidation is responsible for reduction in food nutritional quality (Aguirrezabal et al., 2000), while microbial contamination can cause major public health hazards and economic loss in terms of food poisoning and meat spoilage.

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Table 1. Formulation of experimental chicken patties (% Weights).

Ingredient (%)	Treatment				
	P_1	P_2	P_3	P_4	P_5
Breast Muscle	70.00	70.00	70.00	70.00	70.00
Fat	7.00	6.95	6.95	6.95	6.95
Binder	5.00	5.00	5.00	5.00	5.00
Water/ice	10.00	10.00	10.00	10.00	10.00
Sugar	1.00	1.00	1.00	1.00	1.00
Salt	2.00	2.00	2.00	2.00	2.00
[a]Spices	3.50	3.50	3.50	3.50	3.50
Monosodium glutamate	1.50	1.50	1.50	1.50	1.50
Garlic extract	-	0.05	-	-	-
Ginger extract	-	-	0.05	-	-
Roselle extract	-	-	-	0.05	-
α-Tocopherol	-	-	-	-	0.05
Total	100.00	100.00	100.00	100.00	100.00

[a]White pepper (30%), hot pepper (40%) and powdered nutmeg (30%). P_1, No additive (Negative control); P_2, 0.05% of garlic extract; P_3, 0.05% of ginger extract; P_4, 0.05% roselle extract; P_5, 0.05% α-tocopherol (positive control).

Fortunately, many researchers have indicated that lipid oxidation and microbial growth in meat products can be controlled or minimized by using either synthetic or natural food additives (Gray et al., 1996; Mielnik et al., 2008). However, the use of synthetic antioxidants, such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) have been related to humans health risks resulting in strict regulations over their use in foods (Hettiarachchy et al., 1996). On the other hand, natural substances possessing antioxidant and antimicrobial properties have the advantage of being readily accepted by consumers who are becoming more health conscious.

This concern has aroused a great interest in natural additives (Pokorny, 1991) and their utilization in meat and meat products is increasingly important because consumers are daily demanding additive-free or natural products. Research for new bio-efficient antioxidants has particularly focused on natural antioxidants to respect consumer's concern over safety and toxicity. Fortunately, the Sub Saharan Africa is endowed with many herbs and spices that can function as both antioxidant and antimicrobial. Garlic (*Allium sativum*), ginger (*Zingiber officinale*), and roselle (*Hibiscus sabdariffa)* are among some tropical plants commonly found in the study area and have been reported to have antioxidant and antimicrobial properties (Agarwal, 1996). The objective of this study was therefore, to investigate the effect of ethanolic extracts of garlic, ginger and roselle on cost of production, sensory, physical, and keeping qualities of chicken patties.

MATERIALS AND METHODS

Dried ginger, garlic and roselle were obtained from the Crop Research Institute of Nigeria, Ibadan. Meat curing salt and pork fat (lard) were obtained from the Meat Processing Unit of the Department of Animal Science, University of Ibadan, Nigeria. Food grade sodium tripolyphosphate was obtained from Germany (GmbH andCo.KG, Adalbert-Raps-str.1–D95326). All other additives were purchased from the Bodija Central Market in Ibadan, Nigeria.

Extraction of plant materials

The extraction was carried out according to the method described by Fatope et al. (1993). 20 g each of the powdered plant samples were percolated at room temperature (25°C) with 400 ml 97% ethanol for ginger, garlic and roselle, respectively in 400 ml beakers (thus achieving 1:20 ratio). These were prepared in multiples to ensure enough extractions for the study. The beakers were covered with foil paper, shaken and left to stand for two weeks with regular shaking. After two weeks, the suspensions were filtered and the filtrates were concentrated using Rotatory Evaporating Machine at 40°C. The extracts were labelled accordingly and stored in the refrigerator (4°C) until used.

Meat patties preparation/ experimental design

The experimental product formulation recipe is shown in Table 1. Pork fat and breast muscle meat from eight week old freshly slaughtered Arbor Acre broiler chicks, manually deboned were ground using a Super Wolf (MADOMEW 513, Maschinferbrik Domhan, GmbH, Germany) grinder through 4 and 3 mm sieve plates, respectively. Ground chicken breast muscle, lard and spices were chopped using a table top MTK 561 meat cutter (MA$^{(R)}$ Grant, Germany). The chopping temperature was maintained at 15°C for 20 min to obtain a meat emulsion of desirable consistency. A total of 10 kg emulsion was prepared and used for the chicken patties preparation. 2 kg of the emulsion was assigned to each treatment while each treatment was replicated four times in a completely randomized design.

The antioxidant extracts were added to each of the emulsion portions as follows; treatment 1 (P_1), no additive (negative control); treatment 2 (P_2), 0.05% of garlic extract; treatment 3 (P_3), 0.05% of

ginger extract; treatment 4 (P_4), 0.05% roselle extract; treatment 5 (P_5), 0.05% vitamin E (positive control) (Table 1). The extracts were thoroughly mixed with the respective emulsion. Thereafter, 100 g of the thoroughly mixed emulsion was shaped using a patty cutter and cooked in an electric oven at 180°C to an internal temperature of 72°C. The oven was preheated for 10 min to ensure uniform temperature was achieved inside before the actual cooking process commenced.

The core temperature of each patty was measured using a meat piercing thermometer (Troy, USA). All cooked patties were conditioned at room temperature (27°C) after which they were chilled at 2°C overnight. The chilled patties were weighed and vacuum packed separately and store at -4°C for further analysis. The chilled samples were coded P_1, P_2, P_3, P_4 and P_5 for treatments 1, 2, 3, 4 and 5, respectively (Table 1).

Proximate composition, pH and physical properties of chicken patties

The proximate composition of chicken patties was determined using procedures described by AOAC (1990) for moisture, protein, fat and ash determinations. The analyses were made in triplicates for all the treatments. A pH meter fitted with glass electrode (FC200, H19024C, Hanna Instruments, Singapore) was used to measure the pH of the cooked patties after cooling to room temperature (27°C). The weight of samples in each treatment was taken before cooking and after to determine the cooking loss.

Water Holding Capacity (WHC) was determined following the method of Suzuki et al. (1991). In the process, cooked patties (10 x 10 x 5 mm) from each treatment were weighed individually onto two filter papers and pressed between two plexi glasses for a minute using a vice. The samples were then oven dried at 65^{o}C for 48 h to determine the moisture content. The amount of water released from the samples was measure indirectly by measuring the area of the filter paper wetted relative to the area of pressed sample.

The WHC was calculated as follows:

$$WHC = \frac{100 - [(Ar - Am) \times 9.47)]}{Wm \times Mo} \times 100$$

Where, Ar = Area of water released form meat (cm^2); Am = Area of meat sample (cm^2); Wm = Weight of meat in mg; Mo = Moisture content of meat (%); 9.47 is a constant factor.

Yield of the product was calculated using the following formula:

$$Yield = \frac{\text{Weight of product}}{\text{Initial weight of sample}} \times 100$$

Lipid oxidation

The 2-thiobarbituric acid (TBARS) assay was carried out according to the procedure of Schmedes and Holmer (1989). Patty sample (10 g) was mixed with 25 ml of trichloroacetic acid solution (200 g/l of TCA in 135 ml/l phosphoric acid solution) and homogenized in a blender for 30 s. After filtration, 2 ml of the filtrate were added to 2 ml TBA solution (3 g/l) in a test tube. The test tubes were incubated at room temperature in the dark for 20 h; then the absorbance was measured at 532 nm by using UV–VIS spectrophotometer (model UV-1200, Shimadzu, Japan). TBA value was expressed as mg malonaldehyde per kg of patty. The analyses were made in duplicates for all the treatments.

Microbial load evaluation

Patty sample (10 g) was homogenized with 90 ml of sterile peptone water (1 g/l) in a laboratory homogenizer (AM-5 Ace homogenizer, Nihonseiki, Japan) and serial dilutions were prepared, then 0.1 ml of each dilution was spread with a bent sterile glass rod on duplicate plates of pre-poured and dried standard plate count agar (Nissui Pharmaceutical, Japan). After 48-h incubation at 25°C, colonies were counted and results were expressed as $\log_{10}$ CFU/g of patty sample.

Sensory attributes and Warmed Over Flavour (WOF) evaluation

Twenty-five (25) consumer panellists made up of staff and students of the University of Ibadan evaluated the flavour, juiciness, tenderness, appearance, taste and overall acceptability of the product using a 9-point Hedonic scale (9 = like extremely, 5 = neither like nor dislike and 1 = dislike extremely). The chicken patties were sliced to approximately equal bite size of 2 cm^2, wrapped in kitchen foil and warmed in an oven at 180°C for 5 min before serving. Similar methodology was applied to samples for WOF determination except that WOF was determined at days 1, 7 and 14 after storage at - 4°C. All products were blind coded with 3-digital random numbers and the orders of serving samples were randomised. Water was offered to rinse the mouth in-between tasting. Panellists sat in such a manner that ensured independence throughout the entire duration of product evaluation. The evaluation room was well illuminated with white fluorescent lights (Poste et al., 1991) and there were nothing such as noise and unpleasant odours to detract the attention of the panellists.

Statistical analysis

The data generated from the study were subjected to one-way analysis of variance (ANOVA) and significant differences ($P<0.05$) between means were determined by Scheffe multiple comparison test using SPSS (2006) 16.0.1 for Windows.

RESULTS AND DISCUSSION

The percentage compositions for moisture, protein, fat and ash of chicken patties are reported in Table 2. These parameters were not affected by the inclusion of the plant extracts as there were no significant differences between their means and those of the two controls. The protein values were 28.12, 28.41, 28.01, 28.78 and 28.92% for P_1, P_2, P3, P4 and P_5 samples, respectively. The pH values were not affected significantly ($P>0.05$) with the use of the plant extracts. Addition of the different extracts did not cause any significant change in the nutritional content of the products. The finding was in agreement with the report of Sallam et al. (2004) that the addition of different garlic forms did not cause any significant change in pH value, protein, ash and fat contents of chicken sausage.

The pH values of patties from both the negative and positive control were 5.91 respectively, while the treated patties (P_2, P_3 and P_4) had similar pH values of 5.92 each. Although, there were no significant differences in the pH values obtained in all the products however, it is worthy

Table 2. Proximate composition (%) and pH of cooked chicken patties with or without plant extracts.

Parameter	Treatment					SEM
	P_1	P_2	P_3	P_4	P_5	
Moisture	32.50	34.50	33.47	33.00	32.52	1.21
Protein	28.12	28.41	28.01	28.78	28.92	1.16
Fat	14.42	14.19	14.16	14.58	14.49	0.66
Ash	2.76	2.93	2.95	3.01	2.68	0.76
pH	5.91	5.92	5.92	5.92	5.91	0.01

Means in same row with similar superscripts are not significantly different (P>0.05). P_1, no additive (negative control); P_2, 0.05% of garlic extract; P_3, 0.05% of ginger extract; P_4, 0.05% roselle extract; P_5, 0.05% α-tocopherol (positive control); SEM, standard error of the means.

Table 3. Yield and water holding capacity (WHC) of chicken patties as influenced by ethanolic extracts of ginger, garlic and roselle.

Parameter	Treatment					SEM
	P_1	P_2	P_3	P_4	P_5	
Yield (%)	84.39	86.02	84.88	86.85	85.45	1.54
WHC (%)	50.96^a	44.36^b	46.25^b	47.19^b	52.20^a	2.55
Production cost (#/kg)	700.32	687.63	696.87	681.06	696.61	-

Means in same row with similar superscripts are not significantly different (P>0.05). P_1, no additive (negative control); P_2, 0.05% of garlic extract; P_3, 0.05% of ginger extract; P_4, 0.05% roselle extract; P_5, 0.05% α-tocopherol (positive control); SEM, standard error of the means.

of note that at the acidic pH the condition becomes hostile for microbial growth. The range of pH values obtained in this study is lower than the pH range of 6.65 (in control samples) to 6.78 (in fresh garlic-formulated sausage) obtained by Sallam et al. (2004). The difference in the pH values could be due to the differences in product and product formulations as pointed out by Akwetey et al. (2014).

The cost (#/kg) of producing chicken patties with ethanolic extracts of garlic, ginger and roselle inclusion varies from N700.32 (negative Control, P_1) to N681.06/kg in patties containing 0.05% roselle extract (Table 3). These resulted in 1.81, 0.49, 2.75 and 0.53% reduction in production cost respectively for using 0.05% of garlic extract, ginger extract, roselle extract and α-tocopherol. Chicken patty processors who would adopt and utilize roselle extract stand to benefit more by way of higher savings (2.75 %) on production cost. While, the use of garlic and ginger extract resulted in 1.81 and 0.49% reduction in cost of production respectively. This reduction in cost of production is an added advantage since these extracts were used essentially as antioxidant and antimicrobial. Such savings on the cost of production could lead to reduction in the price /kg of the product and also encourage increased consumption which might lead to increment in the volumes of the product sold.

Water holding capacity (WHC) varies significantly (P<0.05) in chicken patties containing the plant extracts as compared to the two controls. At high WHC the product yield was expected to increase correspondently however, the product yield obtained in this study contradicted that assertion.

The product yield was 84.39% for the negative control (P_1) and 86.02, 84.88 and 86.85% for patties containing garlic, ginger and roselle extracts respectively while the patties with α-tocopherol gave product yield of 85.45%.

Results of the warmed-over flavour of chicken patties as assessed by the consumer panellists are reported in Table 4. There were significant differences in the values obtained in each of the storage days with the negative control (P_1) having the highest values in each storage day. The use garlic extract resulted in 34.62% reduction in warmed-over flavour after 24 h storage at 4°C while the use of ginger and roselle led to 26.92 and 23.08% reduction, respectively. The result obtained on the 7^{th} day of storage showed no significant difference (P>0.05) in the values obtained for samples treated with garlic extract and α-tocopherol while those of ginger and roselle were also similar (P>0.05). The efficacy of garlic and roselle extract seemed to have reached its peak on day seven of storage as there were noticeable reduction in the rate at which warmed-over flavour was reduced beyond day seven whereas; in the case of ginger extract, there was an improvement from 25.00 to 29.41%. For a short term

Table 4. Warmed over flavour (WOF) of chicken patties with or without plant extract.

Days of storage	Treatment					SEM
	P_1	P_2	P_3	P_4	P_5	
1(%)	2.60^{a} NA	1.70^{c} (34.62)	1.90^{b} (26.92)	2.00^{b} (23.08)	1.50^{d} (42.31)	0.01
7(%)	2.80^{a} NA	1.70^{c} (39.29)	2.10^{b} (25.00)	2.10^{b} (25.00)	1.70^{c} (39.29	0.02
14(%)	3.40^{a} NA	2.80^{b} (17.65)	2.40^{c} (29.41)	2.50^{c} (26.47)	2.20^{d} (35.29)	0.01

Means in same row with similar superscripts are significantly different (P>0.05). Numbers in parenthesis indicate percent change (reduction) relative to negative control (P_1). NA, Not applicable; P_1, no additive (negative control); P_2, 0.05% of garlic extract; P3, 0.05% of ginger extract; P_4, 0.05% roselle extract; P_5, 0.05% α-tocopherol (positive control).

Table 5. Lipid oxidation / thiobarbituric acid values (mg/Kg) of chicken patties with or without plant extract.

Days of storage	Treatment					SEM
	P_1	P_2	P_3	P_4	P_5	
1(%)	0.78^{a} NA	0.68^{b} (12.82)	0.56^{c} (28.21)	0.72^{b} (7.69)	0.56^{c} (28.21)	0.01
7(%)	1.05^{a} NA	0.95^{c} (9.52)	0.93^{c} (11.43)	0.98^{b} (6.67)	1.01^{b} (3.81)	0.02
14(%)	1.90^{a} NA	1.52^{b} (20.00)	1.10^{d} (42.11)	1.22^{c} (35.79)	1.07^{d} (43.68)	0.01

Means in same row with similar superscripts are significantly different (P>0.05). Numbers in parenthesis indicate percent change (reduction) relative to negative control (P_1). NA, Not applicable; P_1, no additive (negative control); P_2, 0.05% of garlic extract; P3, 0.05% of ginger extract; P_4, 0.05% roselle extract; P_5, 0.05% α-tocopherol (positive control).

intervention (1-7 days storage) in chicken patties, any of the studied extract could be used while, for storage beyond 7 days the use of ginger extract is preferred.

Each of the ethanolic plant extract (Table 5) lowered the thiobarbitutic acid level of their respective patties in comparison with the negative control (P_1). Ginger extract consistently gave the highest reduction in oxidation with values of 28.21, 11.43 and 42.11% for days 1, 7 and 14 of storage respectively as against values of 12.82, 9.52 and 20.00 for roselle and 7.69, 6.67 and 35.79 % for garlic treated samples for similar storage days. The percent reduction in oxidative value obtained for ginger were comparable to those of α- tocopherol with the exception of day 7 where the ginger extract had a higher value (11.43%) than that of α- tocopherol (3.81%).

In an earlier study, Formanek et al. (2009); Ibrahim et al. (2011) and Abu-almaaly (2011) reported that ginger extract as antioxidant was effective against TBA formation when incorporated into meat during frozen storage. Moreover, polyphenolic extracts are excellent electron and proton donors, and their intermediate radicals are quite stable due to electronic delocalization phenomena as well as the lack of position attackable by oxygen (Djenane et al., 2005). As the day of storage lengthened especially from 7 to 14 days, the oxidative effect of the extracts became more pronounced. Sallam et al. (2004) determined the antioxidant activity of garlic in chicken sausages and reported a reduction in values in garlic treated samples compared with the control. In a similar way, the efficacy of garlic as antioxidant was reported by Park et al. (2008), in their study with garlic and onion in fresh pork belly and loin during refrigerated storage. Garlic also has high total phenolic content showing high antioxidant activity. These properties make garlic good free radical scavengers (Kikuzaki and Nakatani, 1993; Schulick, 1993; Thippeswamy and Naidu, 2005). The calyx of the roselle plant on its own part has long been recognized as a source of antioxidants (Mohd-Esa et al., 2010).

Roselle calyces were reported to contain higher antioxidant properties compared to BHA and vitamin E most probably due to its high polyphenol components (Rhee et al., 2001). In the current study, since the natural extracts used in preparing chicken patties contained phenolic compounds, these substances could cause an inhibition of the chain reactions during lipid oxidation (El-Diwani et al., 2009).

Antimicrobials agents are used in food for two main reasons: to control natural spoilage processes (Naidu, 2000) and to prevent/control growth of micro-organisms for food safety. This study demonstrated that the use of ehanolic extract of garlic, ginger and roselle reduced the microbial load of the patties across the treatments in each of the storage period compared to the control (Table 6). Ginger extract conferred the highest antimicrobial activity on the product in each of the storage day since products containing ginger consistently has the least microbial load in each of the days. Apart from its noticeable antioxidant activity ginger has been reported to be effective as antimicrobial (Shamsuddeen et al., 2009; Ibrahim et al., 2011). Ginger has been shown to be effective against the growth of both Gram-negative and

Table 6. Microbial load of chicken patties as influenced by ethanolic extract of ginger, garlic and roselle (X 10^2 CFU/gm).

Days of storage	Treatment					SEM
	P_1	P_2	P_3	P_4	P_5	
0	3.46^a	3.29^a	2.95^b	3.32^a	3.38^a	0.11
7	4.51^a	3.94^b	3.02^c	4.13^b	4.27^a	0.08
14	4.98^a	4.12^b	4.07^c	4.86^b	4.93^a	0.17

Means in same row with similar superscripts are significantly different (P>0.05). P_1, No additive (Negative control); P_2, 0.05% of garlic extract; P3, 0.05% of ginger extract; P_4, 0.05% roselle extract; P_5, 0.05% α-tocopherol (positive control).

Table 7. Sensory attributes of cooked chicken patties as influenced by antioxidant extracts.

Parameter	Treatment					SEM
	P_1	P_2	P_3	P_4	P_5	
Aroma	3.60^c	4.20^b	4.70^a	4.20^b	4.40^b	0.01
Tenderness	5.30^c	5.60^a	5.70^a	5.50^b	5.60^a	0.02
Juiciness	5.60	5.80	5.70	5.60	5.70	0.01
Colour	6.20	6.30	6.40	6.50	6.20	0.13
O. Acceptability	6.00^d	6.50^b	6.20^c	6.60^b	7.10^a	0.06

Means along the same row with the same superscripts are not significantly different (P< 0.05). 9, Like extremely; 5, neither like or dislike; 1, dislike extremely; P_1, no additive (negative control); P_2, 0.05% of garlic extract; P_3, 0.05% of ginger extract; P_4, 0.05% roselle extract; P_5, 0.05% α-tocopherol (positive control).

positive bacteria including *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Streptococccus viridans* (Thompson et al., 1973). Sulphur and polyphenols present in garlic is responsible for its antibacterial, antifungal and antioxidant activity (Benkeblia, 2004).

The sensory evaluation of food products to any food processing technology is very important in determining the consumer acceptability (Mohamed et al., 2011). Results of the sensory attributes of chicken patties as assessed by the consumer panellists are reported in Table 7.

There were no significant differences (P>0.05) in juiciness and colour score in both control (negative and positive) and the ethanolic extract treated products. The extracts significantly increased the aroma and tenderness score (Table 7) in comparison with the negative control products (P_1). However, the patties containing ginger extract has the highest score for aroma while there was no significant difference in score for products containing garlic, roselle and α- tocopherol.

Garlic and ginger extracts gave comparable result to that of α- tocopherol in terms of tenderness while that of roselle treated samples was lower. The order of the overall acceptability of the chicken patties was $P_5 > P_4 > P_2 > P_3 > P_1$.

Conclusion

This study concluded that the ethanolic extract of garlic, ginger and roselle provided antioxidant and antimicrobial benefits to raw chicken patties during cold storage (4°C). However, ginger seemed to have longer antioxidant potency than garlic and roselle. The result shows that the ethanolic extracts had salutary effects on the sensory profile of chicken patties especially by reducing the development of Warmed-Over Flavour (WOF). Therefore, it is suggested that ginger, garlic or roselle extract as a natural herb, could be used to extend the shelf-life of chicken patties and provide the consumer with food containing natural additives, which might be more healthful.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of methotrexate combined with ginger, silymarin or propolis on the mRNA expression levels of cytochrome P450 oxidoreductase (POR), caspase 3 (CASP-3) and interlukin 6 (IL-6)

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The present study was performed to evaluate the effect of three natural antioxidants on the adverse effect of methotrexate (MTX) in normal liver cells. TaqMan RT-PCR technology was used to estimate the mRNA expression levels for three genes after rats injection with a single dose of 20 mg/kg b.w MTX or the same MTX dose combined with ginger, silymarin or propolis oral administration. The doses of ginger, silymarin or propolis were similar (200 mg/kg b.w) and were daily administrated to rats for 21 days before MTX injection and four days after MTX injection. The three genes were: cytochrome P450 oxidoreductase (POR) that encodes POR enzyme, caspase 3 (CASP-3) that encodes CASP-3 enzyme and interlukin 6 (IL-6) that encodes IL-6 pro- inflammatory cytokine. Results indicate that the used MTX single dose of 20 mg/kg b.w did not significantly affect POR mRNA expression level in rat liver. Moreover the administration of ginger, silymarin or propolis with MTX did not significantly increase or decrease POR mRNA expression level. Results also reveal insignificant increase in CASP-3 mRNA expression level after MTX injection and also after administration of ginger or propolis with MTX. The administration of silymarin with MTX significantly increased the CASP-3 mRNA expression level. IL-6 mRNA expression level was insignificantly upregulated after injection with MTX and also after administration of silymarin with MTX whereas a significantly upregulation in the IL-6 mRNA expression level was reported after administration of ginger or propolis with MTX.

Key words: Methotrexate, ginger, silymarin, propolis, mRNA expression.

INTRODUCTION

Methotrexate (MTX) is a cytotoxic chemotherapeutic agent that is widely preferred in the treatment of malignancies and some autoimmune diseases (Cetin et al., 2011). MTX is a structural analogue of folic acid. It works as a dihydrofolate reductase (DHFR) inhibitor that blocks DNA synthesis through the depletion of the

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intracellular-reduced folate pools required for the purines and thymidine biosynthesis, and leads to cell cycle arrest and apoptosis in different cell types. MTX promotes adenosine release and inhibits pro-inflammatory cytokines production. It also suppresses the lymphocytes proliferation and reduces serum immunoglobulin through the folic acid metabolism inhibition (Kobayashi et al., 2002).

While the cytotoxic effect of MTX is not selective for cancer cells, it also affects the normal tissues specially that have a high rate of proliferation, including the hematopoetic cells in the bone marrow and actively dividing cells of the intestinal mucosa (Jahovic et al., 2004). It was also reported that MTX induced damage to the normal liver cells that was well characterized by fatty changes in hepatocytes and sinusoidal lining cells, mild necrosis and inflammation (Hemeida and Mohafez, 2008).

Numerous studies showed that different antioxidants such as flavonoids reduce the adverse effects of chemo-therapeutic agents on normal cells (Choi et al., 1999; Borek, 2004). In addition to that, other studies have suggested that antioxidants can directly induce apoptosis in tumor cells (Nomura et al., 2001).

Propolis (bee glue) is a natural antioxidant produced by the honeybee (*Apis mellifera ligustica*). It has been suggested that the therapeutic activities of propolis depend mainly on the presence of flavonoids which have been reported to induce the immune system. Many of the physiological actions of these flavonoids have been attributed to their antioxidant properties (Bhadauria et al., 2008).

Silymarin is an active extract from milk thistle. It is a powerful antioxidant said to protect liver cells (and other cells in the body and brain) from toxins. Sylimarin apparently promotes liver cell protein synthesis and decreases the oxidation of glutathione. Silymarin may potentially be beneficial in a number of diseases involving liver disease, if in the early stages. Early research indicates that silymarin may also have anti-cancer and pro-apoptotic properties (Yassin et al., 2010).

Ginger or ginger root is the rhizome of the plant *Zingiber officinale*. Ginger is known to possess antioxidant properties. 6-Gingerol, a natural product of ginger, has been known to possess anti-tumorigenic and pro-apoptotic activities (Oyagbemi et al., 2010).

Previous studies were performed to investigate the beneficial effect of propolis and ginger extracts against MTX-induced hepatotoxicity using histopathological and biochemical approaches (Cetin et al., 2011; Badr et al., 2011; Mansour et al., 2012). However, to the best of our knowledge, no studies were performed to estimate the mRNA expression levels of genes encoding for the liver enzymes or other genes that are involved in MTX mode of action after treatment with MTX combined with propolis, ginger or silymarin. In the present study three of these genes were chosen for investigation after MTX injection combined with propolis, silymarin and ginger.

The first gene is cytochrome P450 oxidoreductase (POR) that encodes the P450 oxidoreductase enzyme which is required for the normal functioning of more than 50 enzymes in the cytochrome P450 family. Cytochrome P450 enzymes are involved in the formation (synthesis) and breakdown (metabolism) of various molecules and chemicals within cells (Rhee and Galivan, 1986). The two other genes caspase 3 (CASP-3) and interlukin 6 (IL-6) are involved in the processes of apoptosis and inflammation. *CASP-3* gene encodes for the enzyme caspase 3 which is required for DNA fragmentation and the morphological changes associated with apoptosis (Yang et al., 2004). Where, IL-6 gene encodes interleukin 6 cytokine that functions in inflammation and the maturation of B cells. The IL-6 pro-inflammatory cytokine was reported to have an important role in MTX uptake inside the cell (Yoshida et al., 2005; Hashizume et al., 2012).

The aim of this work was to study the effect of MTX alone and MTX in combination with propolis, silymarin or ginger on the mRNA expression levels of the three genes: POR, CASP-3, and IL-6 in rat liver cells using RT-PCR technology.

MATERIALS AND METHODS

This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy, Cairo University, Cairo, Egypt, following the 18th World Medical Association (WMA) General Assembly, Helsinki, June 1964 and updated by the 59th WMA General Assembly, Seoul, October 2008.

Methotrexate (MTX) was purchased from Ebewe Pharma, Austria. Silymarin and Gingerol were kind gift from Kahira Pharm. and Chem. IND. Co., Egypt and Mepaco Co., Egypt, respectively. Propolis was purchased from TWIN Laboratories Inc.,N.Y.(USA).

Experiment strategy and treatments

Sixty adult male albino rats (*Rattus norvegicus*) were used in this study. The animals were allocated into five groups. Group 1 (G1) and group 2 (G2) were administered with saline; group 3 (G3) received a daily dose of 200 mg/kg b.w ginger extract; group 4 (G4) received a daily dose of 200 mg/kg b.w silymarin and group 5 (G5) received a daily dose of 200 mg/kg b.w propolis. The daily dose of 200 mg/kg b.w of ginger, silymarin and propolis was chosen in the present work according to the previous studies which reported that the daily administration of the above antioxidants is not toxic at this dose (El-Abhara et al., 2008; Eminzade et al., 2008; Bhadauria et al., 2007). All treatments were orally administered for 21 days. On day 21, animals in G2, G3, G4 and G5 were injected intraperitoneally with a single dose of 20 mg/kg b.w MTX. This dose of MTX was reported to cause injury in liver cells (Gulgun et al., 2010). The oral administration of saline, ginger, silymarin and propolis were continued for 4 consecutive days in all groups. All animals were sacrificed on day 25.

Extraction of RNA and complementary DNA (cDNA) synthesis

Liver tissue samples from all animal groups were obtained for RNA

Table 1. The mean relative quantity values (mean RQ values) for each target gene in the five rat groups.

Groups	Parameter		
	POR mRNA mean RQ values ± standard deviation	CASP-3 mRNA mean RQ values ± standard deviation	IL-6 mRNA mean RQ values ± standard deviation
Control	1.00±0.00	1.00±0.00	1..00±0.00
MTX	0.95±0.01	2.48±0.69	2.78±0.03
Ginger + MTX	0.77±0.04	2.15±0.67	71.28±9.12[ab]
Silymarin + MTX	0.97±0.18	3.06±1.77[a]	27.66±8.36
Propolis + MTX	1.07±0.30	1.89±0.83	113.75±10.03[ab]

Significance level: at P<0.05; a: significant increase compared to control group; b: significant increase compared to MTX group.

extraction. Total RNA was extracted from each sample using PeqGold TriFast™ (PEQLAB Biotechnologie GmbH) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on the extracted total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences) according to the manufacturer's instructions. The resulting cDNA from each sample was subjected to real time PCR amplification (RT-PCR) using TaqMan technology in order to quantify mRNA expression levels of three target genes; POR , CASP-3 and IL-6 in the different groups of the experiment.

Primers and fluorogenic probes design

Sequences from the cDNA of the three target genes in rat; POR, CASP-3, IL-6 and from the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) which was used as an internal control were amplified using TaqMan RT-PCR technology. The design and synthesis of the primers and probes of the three tested genes cDNA were performed using TaqMan® Gene Expression Assays-Applied Biosystem. TaqMan probe/primer sequences were designed to be specific for rat cDNA of POR (Assay ID: RN00580820), CASP-3 (Assay ID: RN00563902), IL-6 (Assay ID: RN01410330). The probe/primer set designed for GAPDH cDNA, although amplifying GAPDH cDNA in the rat was also capable of amplifying mouse and human GAPDH because of its conserved nature across species.

Rt-PCR amplification conditions and profile

Real time-PCR reaction of each target gene cDNA was performed with the housekeeping gene cDNA in the same tube (multiplex RCR) where the probe of the housekeeping gene cDNA was labeled by VIC fluorescent dye and the three target gene cDNA probes were labeled by FAM fluorescent dye. RT-PCR reactions were conducted in 25 µL final volume. Reaction mixture consisted of 12.5 L of 2x master mix (TaqMan Universal PCR Master Mix, ABI), 1.25 µL of the target gene primer/probe mix (20x), 1.25 µL of the housekeeping gene primer/probe mix (20x), 5 µL of cDNA (typically 25 g of total RNA) and 5 µL of DEBC-treated water. The quantitative real-time PCR reactions were carried out in an RT-PCR Cycler-Rotor-Gene Q 2 Plex- with 2 channels (QIAGEN). RT-PCR amplification profile started by a hold step for 15 min at 95°C followed by 45 repeats of 15 sec at 94°C and 60 sec at 50°C.

Calculation of mRNA expression levels

Data were analyzed by the software version Rotor-Gene 2.0.2.4 as following: The delta ct (threshold cycle) was calculated for the target gene in each sample of the different groups by subtracting the ct value of the housekeeping gene from the ct value of the target gene, where the ct value is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold. The delta delta ct (ΔΔCt) of the target gene in each sample in all groups was calculated by subtracting the delta ct value of the target gene in the calibrator (The calibrator: is one of untreated control animals) from the delta ct value of the same gene in each sample. Finally, the mRNA expression level of the target gene in a given sample was calculated as a relative quantity (RQ) compared to its expression in the calibrator; where, $RQ= 2^{-\Delta\Delta Ct}$. RQ value < 1 means that the mRNA expression is down regulated whereas RQ value > 1 means an up regulation in the expression.

Statistical analysis

For the determination of significant inter-groups differences in the mRNA expression of the three genes under investigation, statistical analysis for the obtained RQ data was performed using one-way analysis of variance (ANOVA) included in SAS version 11 for windows. The ct values of the housekeeping gene in the different groups of each multiplex PCR were also analyzed by the ANOVA test to investigate the effect of the different treatments of the present study on its mRNA expression level. Significance values were determined at P<0.05.

RESULTS

In the present study, quantitative gene expression data of POR, CASP-3 and IL-6 genes were normalized to the expression level of the housekeeping gene GAPDH. Aanalysis of the ct values of the GAPDH gene in the different tested groups of each multiplex PCR indicated that there were no significant differences in the expression levels of GAPDH mRNA due to the different treatments compared to the control and the methotrexate treated groups.

The mRNA expression levels of POR, CASP-3 and IL-6 genes are presented as the mean values of RQ ± standard deviation for each target gene in five animal groups: control (G1), MTX (G2), ginger + MTX (G3), silymarin + MTX (G4) and propolis + MTX (G5) (Table 1). The mean RQ values of each gene in the different groups were statistically compared with the same gene mean RQ values of the control and MTX groups (at P<0.05) and the

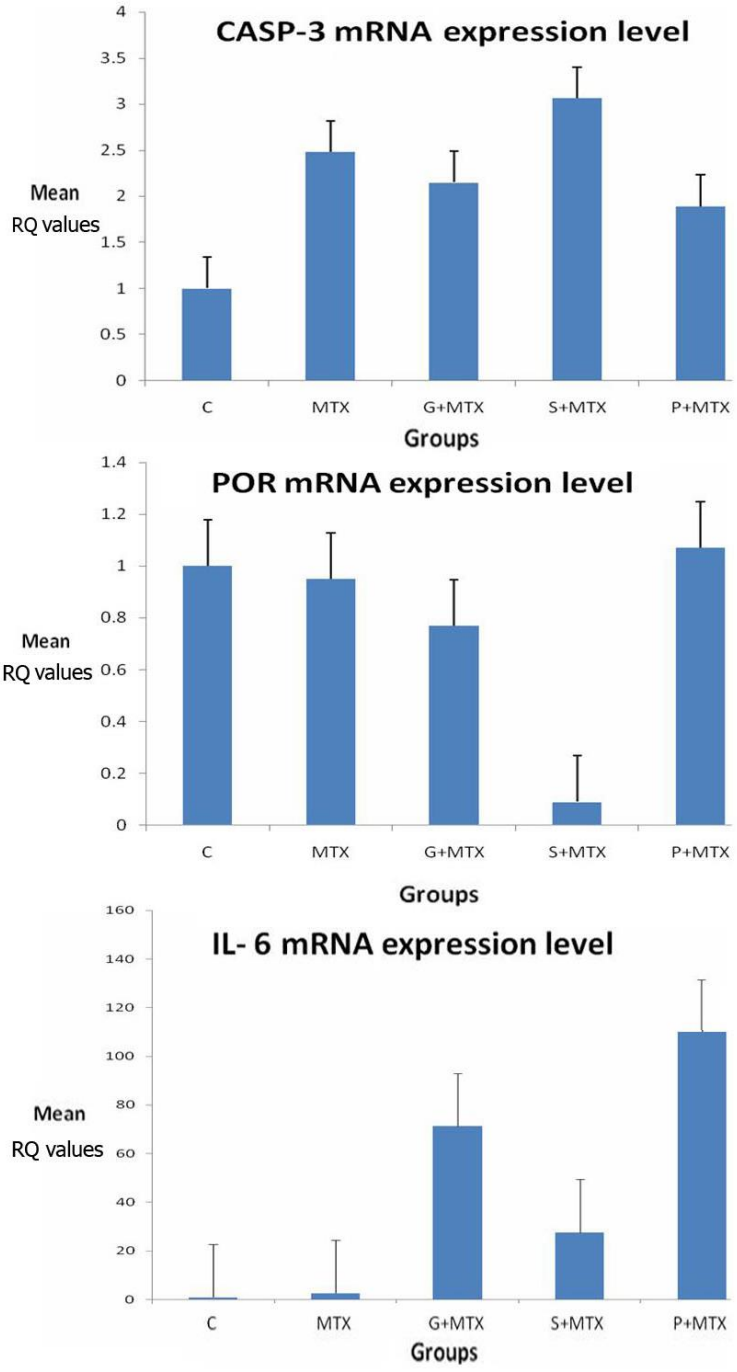

Figure 1. mRNA expression levels of each of the tested genes in the five rat groups. C= Control; MTX= Methotrexate; G = Ginger; S = Silymarin; P = Propolis.

significant differences in mRNA expression levels are illustrated (Table 1). Results of mRNA expression levels are also represented in a histogram (Figure 1) that was done using the mean RQ values of each gene in each of the five animal groups.

Results reveal that there was no statistical difference in the mRNA expression level of POR gene in the different groups compared to control and to the MTX groups.

The mRNA expression level of CAPS-3 gene was elevated in the MTX group (mean RQ=2.48±0.69) compared to its expression level in the control group (mean RQ=1.00±0.00). It was also elevated in ginger + MTX, silymarin + MTX and propolis + MTX but the increase in the expression level was significant (at the

level 0.05) only in the silymarin + MTX group (mean RQ=3.06±1.77) compared to control group (mean RQ=1.00±0.00).

Interleukin 6 mRNA expression level was also elevated in all the treated groups compared to the control group. The increase in the mean RQ values was statistically significant (at the level 0.05) only in the ginger + MTX (mean RQ=71.28±9.12) and propolis + MTX (mean RQ=113.75±10.03) groups compared to both control (mean RQ=1.00±0.00) and MTX (mean RQ=2.78±0.03) groups.

DISCUSSION

In the present study TaqMan RT-PCR technology was used for the estimation of mRNA expression levels of POR, CASP-3 and IL-6 genes in rat liver cells after injection of methotrexate (MTX) alone or MTX with ginger, silymarin or propolis oral administration. TaqMan technology has a number of advantages over conventional methods for assessing the potential of a drug to cause a target gene induction. The key advantages of this method that makes it both precise and reproducible (Bustin, 2000; Ginzinger, 2002) are features, such as it is a completely homogenous assay with a specific target gene being detected. The TaqMan method is also exquisitely sensitive, being able to amplify small amounts of mRNA in contrast to commonly used methodologies, which typically require relatively large amounts of total RNA and are unsuitable for high throughput and usually only semi-quantitative in nature (Baldwin et al., 2006).

To evaluate the effect of MTX alone and MTX in combination with ginger, silymarin or propolis on the liver enzymatic system, the POR gene mRNA expression level was chosen for investigation. It is known that POR enzyme is required for the normal functioning of more than 50 enzymes in the cytochrome P450 family. Cytochrome P450 enzymes are involved in the synthesis and metabolism of various molecules and chemicals within cells (Ding et al., 2001; Rhee and Galivan, 1986). Results of the present study revealed that no significant difference were detected in the POR mRNA expression level after injection of MTX neither alone nor in combination with ginger, silymarin or propolis. These results indicate that the used MTX single dose of 20 mg/kg did not significantly affect POR mRNA expression level in rat liver. Moreover the administration of ginger, silymarin or propolis with MTX did not significantly increase or decrease POR mRNA expression level.

Results of the present study also indicate that the mRNA expression level of CAPS-3 gene was elevated in the group treated with MTX (mean RQ=2.48±0.69) compared to its expression in the control group (mean RQ=1.00±0.00) but the increase in the expression level was not significant. It is well known that apoptosis is the main mechanism of action of most cancer chemotherapeutic agents including methotrexate (Kobayashi et al., 2002). The increase of the mRNA expression level of CAPS-3 gene due to MTX treatment was expected because caspase3 enzyme is required for DNA fragmentation and the morphological changes associated with apoptosis (Yang et al., 2004). Previous studies indicated that the response to methotrexate in tumor cells depends on caspase 3 function (Hattangadi et al., 2004). The administration of ginger, silymarin or propolis with MTX also elevated mRNA expression level of CASP-3 where the elevation was significant only in the silymarin + MTX group (mean RQ=3.06±1.77) compared to control group (mean RQ=1±0.00). This result indicate that administration of silymarin with MTX significantly increase the CASP-3 mRNA expression level. This may consequently leads to the increase of the CASP-3 enzyme activity and apoptosis tendency in liver cells. It was reported before that silymarin has pro-apoptotic activities and it has been shown to inhibit skin carcinogenesis in mice by the induction of apoptosis through caspase3 enzyme activation (Katiyar et al., 2005).

Interleukin 6 is an interleukin that acts as a pro-inflammatory cytokine. It is encoded by the IL-6 gene. Interleukin 6 secretion during infection leads to inflammation and stimulation of the immune response (Nishimoto, 2006). Although MTX is known to have anti-inflammatory actions and it was reported to inhibit pro-inflammatory cytokines production in a dose-dependent manner (Yoshida et al., 2005), results of the present study revealed that the single dose of MTX (20 mg/kg) insignificantly upregulated the IL-6 mRNA expression level (mean RQ=2.78±0.03) compared to its expression in the control group (mean RQ=1.00±0.00). It was reported that IL-6 cytokine has a role in the uptake of MTX inside the cell (Hashizume et al., 2012) as it will be explained in the following. Methotrexate enters cells via the reduced folate carrier SLC19A1, suggesting that SLC19A1 is associated with the efficacy of MTX. IL-6 cytokine is responsible for the regulation of SLC19A1 expression and it is able to reduce the efficacy of MTX by decreasing the expression of SLC19A1 (Hashizume et al., 2012).

The slight upregulation of the IL-6 mRNA expression level which reported in the present study against the single dose of 20 mg/kg MTX treatment may support the finding of Hemeida and Mohafez (2008) that the same single dose of MTX had caused mild inflammation in normal liver cells. The administration of ginger + MTX, silymarin + MTX or propolis + MTX over increased the mRNA expression of IL-6 compared to its expression in the control and MTX groups.

The increases were considered statistically significant only in the ginger + MTX (mean RQ=71.28±9.12) and propolis + MTX (mean RQ=113.75±10.03) groups compared to control (mean RQ=1.00±0.00) and methotrexate (mean RQ=2.78±0.03) groups. It was reported in a previous

study that the oral administration of squeezed ginger augmented the production of IL-6 in mouse leukemic monocytes (Ueda et al., 2010). In another study, it was reported that IL-6 expression increased by a dose-dependent manner due to silymarin administration (Johnson et al., 2003). Also, propolis oral dose of 200 mg/kg, for three consecutive days, was reported to upregulat IL-6 cytokine production in mice spleen cells (Orsatti et al., 2010). The increase of IL-6 mRNA expression level that reported in the present study after administration of silymarin, ginger or propolis with MTX perhaps will lead to the increase in the production of IL-6 pro-inflammatory cytokine, reduction of SLC19A1 expression and consequently reduction of the efficacy of MTX in rat liver cells.

Conclusion

Results of the present study indicated that: the upregulation of mRNA expression level of casp-3 gene after the administration of silymarin with MTX may add to the adverse effect of methotrexate in the normal rat liver cells.

Also, the upregulation of mRNA expression level of IL-6 gene after the administration of silymarin, ginger or propolis with MTX perhaps may decrease the efficacy of MTX in the normal rat liver cells.

The administration of silymarin, ginger or propolis with MTX may protect normal cells from MTX damage but it may also have an adverse effect by decreasing the efficacy of MTX in the diseased cells.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Characterisation of botanical starches as potential substitutes of agar in tissue culture media

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Seven botanical starches; cassava, sweet potato, Irish potato, maize, rice, wheat and sorghum were characterized to determine physicochemical properties influencing gel formation for preparation of tissue culture media. Total starch, protein, fat, amylose content, swelling power and pH were determined using acid hydrolysis, enzymic and spectrophotmetric methods. Each type of starch was tested for ability to support *in vitro* plant growth and 0.8% agar (w/v) was used as standard. Nodal explants cultures were initiated in Murashige and Skoog medium supplemented with 3% sucrose and 0.5 mg/l benzylaminopurine (BAP). After 21 days, number of leaves and nodes, plant height and fresh weight were determined for each treatment. Significant (0.05) differences were observed between starch types in total starch, protein content and fats. Cassava had the highest starch content (81.5%) and irish potato had the lowest (29.3%). Highest protein content (12%) was observed in maize starch and the lowest (3.4%) in cassava. Fat content was highest (6.2%) in wheat and lowest in cassava (0.2%). Starch extract from rice had the highest amylose content (31.12%) while the cassava starch extracts had the lowest (20.75%). The starch extracted from wheat had the highest swelling power (105.1%) while irish potato starch had the lowest (52.1%). Sorghum starch had the lowest pH of 4.57 while the highest (6.92) was recorded in Irish potato starch. The growth of shoots *in vitro* on agar gelled media outperformed those of starch gelled media except for the number of leaves per shoot which cassava starch media recorded significantly the highest response (P = 0.05). Of all the starch gelled media, the best growth response was observed with cassava. Good performance was observed in starches with high starch content, low amylose content and high swelling powers.

Key words: Starch, agar, gelling agent, *in vitro* plant growth.

INTRODUCTION

Agar, an extract of red algae sea weeds is a natural polysaccharide made up of agarose and agaropectin (Villanueva et al., 2010). It is widely used in food and non food industries including biotechnologies like tissue culture (TC). Its wide application is due to its gelling ability and gel characteristics. The gel characteristics

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include ability to form a gel at around 30°C that melts at about 100°C and re-solidifies at about 45°C (Gangopadhyay et al., 2009; Marinho-Soriano and Bourret, 2005). It is highly preferred in preparation of solid TC medium because the gel has high clarity and stable gel strength. Also, it does not react with components of the medium and it is not digested by plant enzymes (Vaz-Pires et al., 2008). In addition, agar has high purity levels and high resistance to alteration during culture (Gangopadhyay et al., 2009). Due to these favorable physical-chemical attributes, agar has been a gelling agent of choice of majority TC systems as it assures adequate support and translocation of water and mineral nutrients to explants. However, although several reports show that agar is non toxic with highest purity level, others have reported presence of impurities and differences in type and quality between brands which have been associated with adverse effects like inhibition of growth, hyperhydricity, necrosis and cytotoxic effects (George et al., 2008; Ivanova and Van Staden, 2011; Ozel et al., 2008). For many decades, tissue culture was confined only in research laboratories until it started to be used in commercial mass propagation. From that point the concept of cost effectiveness in TC became important. Reports show that the total cost of micropropagation is influenced by a number of factors including chemical media. However there are some differences in opinion whether media costs really contribute a significant portion of the total cost (Goel et al., 2007) and others are convinced that the refined agar powder generally used in plant tissue culture media is a costly commodity (Escobar et al., 2006; George et al., 2007). This is supported by a reports that 30% of the total cost of production is due to chemical media of which more than 70% is the cost of gelling agent (Prakash, 1993). Apart from being expensive, also overreliance on agar could result to over-exploitation from the natural ecosystem. Therefore research to investigate the potential substitute has been a justifiable agenda for many years. Several botanical alternatives have been proposed since 1980s. These include those of Henderson and Kinnersley (1988) who first demonstrated that growth and differentiation of plant cell cultures was increased when media were gelled with corn starch instead of agar. Results of other workers show that starch based media promoted callusing although growth of plants *in vitro* was improved when starch was mixed with agar (Babbar et al., 2005; Babbar and Jain, 2006).

Several workers who have reported the use of starch as gelling agents, show that, the *in vitro* plant growth was higher in starch gelled medium than the agar gelled medium (Jain et al., 2005). However others record growth lower than the agar based media and some improvement is observed when starch is mixed with agar at certain ratios (Kuria et al., 2008; Mohamed et al., 2010). It has been established that the different results could be due to differences in physical and chemical nature of the used starch (George et al., 2008). Starch, is made up of two major polymers: amylose and amylopectin which are structurally different. The former is linear while the later is highly branched and each structure plays a critical role in the ultimate functionality of starch. The amylose/ amylopectin ratio which is both environmentally and genetically manipulated determines the functional properties such as swelling power, water biding capacity, gel strength, shear resistance, gelatinization, textures, solubility, tackiness, gel stability, cold swelling, viscosity and retro gradation (Mweta et al., 2008). Colour and clarity are also important properties of starch gels in regeneration and management of tissue cultures. From the review, it is clearly noted that although, the physical-chemical characteristics of starchy gelling agents are important, reports on relationship of these properties with performance of plants *in vitro* are lacking. This paper therefore reports the physical and chemical characteristics of seven botanical starches and their ability to support growth of *in vitro* plant.

MATERIALS AND METHODS

Starch was extracted from maize (*Zea Mays* L; var Stuka) and wheat (*Triticum aestivum* L; Var Riziki) which were obtained from Agricultural Research Institute (ARI)-Seliani, Arusha, Tanzania. Sorghum (*Sorghum bicolour* L; Var Macia) and rice (*Oriza sativa* L;Var TXD 305) were obtained from ARI-Ilonga and Cholima research station respectively both in Morogoro region, Tanzania. Fresh tuberous roots of cassava (*Manihot esculanta* Crants; Var Kiroba), Irish Potato (*Solanum tuberosome* L; Var CAP) and sweet potato (*Ipomoea batatas* L) were bought from local market. Sweet potato (var. Ukerewe) which was used as test plant was obtained from germplasm collection at ARI-Kibaha, Coast region Tanzania.

Starch isolation

Standard alkali methods described in the literature were used for the extraction of starch from sorghum and maize grains (Beta and Corke, 2001; Ji et al., 2004). One kilogram of both maize and sorghum (var Macia), were air screened and washed using tape water to remove contaminants before the maize sample was taken to a grinding mill for course grinding to remove the pericarp and germ. The samples were then rewashed and maize steeped in 1% NaOH and sorghum in 0.25% NaOH (w/v) solution at 45°C water bath for 24 h to loosen the endosperm. The steeped grains were washed using distilled water and wet ground (1:1 w/v) in commercial blender. The slurry was filtered through triple cheese muslin cloth and the remaining materials were re-washed, re-filtered and the remaining grains subjected to repeated grinding and filtration. The filtrate was left to sediment at room temperature for 8 h before the supernatant was drained off. The remaining sediments were oven dried at 30°C for 24 h. The dried starch in form of pellets was subjected to a laboratory hummer mill (3000 rpm) to obtain a fine powder.

Rice and wheat starches were extracted using methods described in the literature (Lumdubwong and Seib, 2000) with some modification. One kilogram of rice and wheat grains were air screened and washed to remove foreign materials before they were air dried. The samples were ground into flour using hummer mill (3500 rpm) before the flour was manually mixed with water in doses

to a 1:1 (w/v) flour to water ratio forming coherent dough. The dough was then incubated at room temperature for 4 h before the dough was knead under distilled water to release the starch into water. Ten (10) washings were done to ensure starch is removed from the dough matrix remaining with gluten protein. The slurry was washed through filtering through a series of sieves (500, 200, 120 and 75 µm). All other operations regarding the filtrate to a final starch powder are as described in the method for maize and sorghum.

Extraction from cassava, sweet potato and potato was carried out using a modified method based on standard procedures (Benesi, 2005; Riley et al., 2006). Two kilograms of each of the fresh tubers and roots were washed, peeled and grated into a pulp. The pulp (mixture) was stirred for 2 min and filtered using a triple cheese (muslin) cloth. The filtrate was allowed to stand to facilitate starch sedimentation for 8 h before the supernatant was decanted and discarded while the remaining sediments were air dried to obtain the starch powder.

Determination of physicochemical properties of starch extracts

Starch purity

Starch content of triplicate samples of each of the seven extracts was determined according to Bhagya et al. (2006). A total of 100 mg of starch extract was defatted by boiling in 30 ml of 80% ethanol for 10 min and the residue collected was dried at 70°C for 4 h. To the residue, 10 ml of 52% $HClO_4$ was added to digest the starch for 15 min at 28°C of the water bath and made up to 25 ml with distilled water after which the mixture was filtered through Whatman No.1. Dubois method (Dubois et al., 1956) was employed to determine the total sugars.

About 20 µl of the filtrate was made up to 1 ml with distilled water then 1 ml of 5% phenol followed by 5 ml 36N H_2SO_4 were added. Pure glucose (Sigma) (20-100 µm) was taken as standard from which a standard curve was established. Absorbance of the solution was read at 490 nm using a spectrophotometer (Altrospec 2000 by Pharmacia biotech). The amount of sugar contained in each sample of starch extract was calculated from the standard curve and the respective starch content was calculated as total sugar multiplied by 0.9.

Amylose content

A triplicate sample which weighed 100 mg of starch was defatted according to the standard AOAC methods (Riley et al., 2006) with modification. About 1 ml of absolute (99.9%) ethanol was added to the sample followed by votexing for 1 min before leaving the mixture to settle for 30 min after which ethanol was decanted off. To this mixture, 9.0 ml of 1 M NaCl was added and the volume was made up to 100 ml with distilled water. From this mixture, 5.0 ml of an aliquot was transferred to a volumetric flask containing 25.0 ml of water. To this volume 19.0 ml of 1 M acetic acid and 1.0 ml of 0.2% Iodine solution were added making the volume up to 50 ml with distilled water.

Absorbance of the resulting solution at 620 nm for amylose was read using a spectrophotometer (Altrospec 2000 by Pharmacia biotech). Standard amylose in varying concentrations was used to construct a standard curve from which the concentrations of amylose in different samples of starch were calculated based on Lambert and Beer's equation represented as; A = ebc, where A is absorbance, e is the molar absorptivity with units of L mol^{-1} $cm^{-1,}$ b is the path length of the sample and c is the concentration of the compound in solution, expressed in mol L-1 (Nelson et al., 2008).

Swelling powers

The swelling power of the starch granules was determined using previously described methods (Li and Yeh, 2001; Leach et al., 1959) with little modification. Firstly the centrifuge tubes were weighed (W1). Secondly, a triplicate sample weighing 0.1 g of starch (W2) from the seven sources were put in pre-weighed tubes and incubated in water bath at 60°C and another same amount at 30°C both for 30 min. After incubation, the samples were centrifuged at 7000 rpm for 20 min followed by decanting the supernatant before weighing the wet sample (W3). Swelling power was determined as the percentage change in the weight of samples of starch calculated using the formula:

$$Swelling\ power = \frac{W3 - W1 + W2}{W2}\ X\ 100 \quad (1)$$

Protein content

Protein was estimated using the Bradford method (Kruger, 2009). The standard procedures were used to prepare a Bradford reagent and kept was in brown glass bottle. Starch samples each weighing 5 g in triplicate were mixed with 10 ml of distilled water from which 0.5 ml was drawn to be mixed with 0.5 ml of de-Ionized water followed by adding 5 ml of Bradford reagent. This mixture was well mixed by vortexing for 1 min and thereafter left to settle for 5 min. After settling, absorbance at 595 nm was read from spectrophotometer (Ultraspec 2000 by Pharmacia biotech). The protein concentration was then estimated from a standard curve, which was prepared using a standard protein (Bovine serum albumin).

Crude fat content

Crude fat content of the samples was estimated using Soxhlet apparatus (Kruger, 2009). In this method, 2 g of starch (W2) in triplicate samples were put in a pre-weighed (W1) filter paper (Whatman No 1) after which they were taken into the soxhlet system for lipid extraction using chloroform and hexane mixed in 1:1 ratio. The remaining fat free sample was dried and re-weighed (W3) then fat content was calculated as a percentage change from the formula:

$$Crude\ Fat = \frac{W1 + W2 - W3}{W2}\ X\ 100 \quad (2)$$

Starch pH

Starch pH was determined based on method described in (Agudelo et al., 2014). A triplicate sample weighing 5.0 g was homogenised using 25.0 ml distilled water for 60 s. The starch was then allowed to settle for 15 min. pH of the water phase was measured using pH meter glass electrode.

Gel strength, colour and clarity

A total of 63 formulations of tissue culture (TC) media gelled by seven types of starch each with six concentrations: 6, 9, 12, 15, 18 and 21% (w/v) were prepared using standard procedures for media preparations. Three people experienced in preparing TC medium were assigned to score each treatment for the degree of gel

Table 1. Starch characteristics showing the influence of different botanical sources.

Starch source	Starch (%)	Protein (%)	Fat (%)	Amylose (%)	Swelling power		pH
					at 30°C	at 60°C	
Cassava	81.49±0.79^{d}	3.60±0.67^{a}	0.26±0.16^{a}	20.75±0.23^{a}	43.08±5.38^{bcd}	118.11±5.01^{d}	5.92±0.04^{d}
Sweet potato	77.51±1.85^{cd}	9.90±0.14^{cd}	1.94±0.05^{b}	21.82±0.47^{b}	37.50±1.90^{bc}	83.95±3.99^{c}	6.22±0.07^{e}
Irish potato	29.32±3.89^{a}	8.23±0.31^{bc}	4.78±0.49^{c}	23.42±0.33^{c}	27.59±1.66^{a}	73.26±7.92^{bc}	6.90±0.06^{f}
Rice	75.63±3.58^{cd}	22.86±1.60^{e}	0.65±0.03^{a}	31.12±0.42^{d}	50.59±0.77^{d}	74.13±2.29^{c}	4.72±0.04^{ab}
Wheat	72.35±1.07^{bcd}	10.75±1.47^{cd}	6.54±0.22^{d}	24.09±0.28^{c}	34.76±1.24^{ab}	50.48±4.92^{a}	5.42±0.05^{c}
Sorghum	67.35±2.21^{bc}	6.36±0.45^{b}	1.83±0.09^{b}	30.10±0.40^{d}	44.85±1.55^{cd}	59.96±2.13^{ab}	4.57±0.20^{a}
Maize	62.81±7.50^{b}	12.08±0.06^{d}	0.46±0.18^{a}	23.73±0.04^{c}	29.69±4.47^{e}	52.53±0.84^{a}	4.93±.01^{b}

Means followed by the same letter within the column are not significantly different at P ≤ 0.05.

strength based on modified 7 point scale (0-1=liquid, 2-3=semi liquid, 4-5= semi solid, 6-7 = solid) (Lee and Chung, 1989). A colour of starch powder was determined using a colour checker (Park et al., 2006) in a range scores of 1-3 = light medium gray, 4-8 = medium gray, 9-12 = light dark gray and 13 -16 = dark gray. The gel clarity was scored using a four point scale (1=Transparent, 2=Semi transparent, 3 =Semi opaque and 4 =Opaque)(Novelo-Cen and Betancur-Ancona, 2005).

Assessment of the ability of starch based media to support in vitro plant growth

Sweet potato cultures were established in tissue culture media which were solidified by starches extracted from wheat, rice, sorghum, maize, cassava, sweet potato, Irish potato and 0.8% agar (w/v) was used as standard. Surface sterilized nodal explants cultures were initiated in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.5 mg/l benzylaminopurine (BAP). After 21 days, number of leaves and nodes, internode length, plant height and fresh weight were determined for each explant.

Data collection and analysis

Variance analysis was calculated based on the statistical models $Y_{ij} = \mu + A_i + \varepsilon_{ij}$; where, Y_{ij} is the j^{th} observation in treatment i., μ is the overall mean, A_i is the i^{th} treatment effect, (type of starch), and ε_{ij} is random error component using a software; GENSTAT discovery edition 3 (Payne, 2009); mean separation tests were performed using least square differences (LSD).

RESULTS

Chemical characteristics of starch extracts

Analysis of Variance in Table 1 shows significantly ($p \leq 0.05$) different properties of starch extracts from different botanical sources. The extracts contained none starch biomolecules like protein and fat in different ($p = 0.05$) proportions. Starch purity (81.49%) of cassava was almost three times higher than the extracts from Irish potato. Highest protein (22.86%) was observed in rice starch while wheat had the highest (6.54%) fat content (Table 1).

The seven starch extracts had significantly different ($P \leq 0.05$) amounts of amylase content. The starch extract from rice was found to have the highest (31.12%) amylose content while the cassava starch extracts had the lowest (20.75%) (Table 1).

The swelling power of starch varied ($p \leq 0.05$) with its botanical source (Table 1). Also the swelling power of each starched type increased with increasing incubation temperature except the starch from maize. Significantly ($p = 0.05$) highest increase was observed in wheat starch where a temperature change from 30 to 60°C increased the swelling by 140.72% while in the same change of temperature, the swelling power of sorghum increased by only 15.31%.

The pH of different starch extracts varied ($p \leq 0.05$) with the botanical sources (Table 1). Sorghum starch had the lowest pH of 4.57 while the highest (6.92) was recorded in Irish potato starch. Generally the pH of cereal starch extracts was lower than those of root and tuber starches.

Gel characteristics of starch based tissue culture media in different concentrations

Gel strength

The summary of results for gel strength scores are presented in Figure 1. The gel strength of TC media which were solidified by all starch types increased with increasing concentration. Maize starch formed a semi-solid gel at the lowest (9% w/v) concentration while rice starch attained the same score at the highest (18% w/v).

Gel colour

The media which were solidified using the different botanical starches consistently changed colours from semi transparent to opaque. Root and tuber starch gels attained an dark gray from a concentration of 18% while the cereal starch gels were medium gray except sorghum

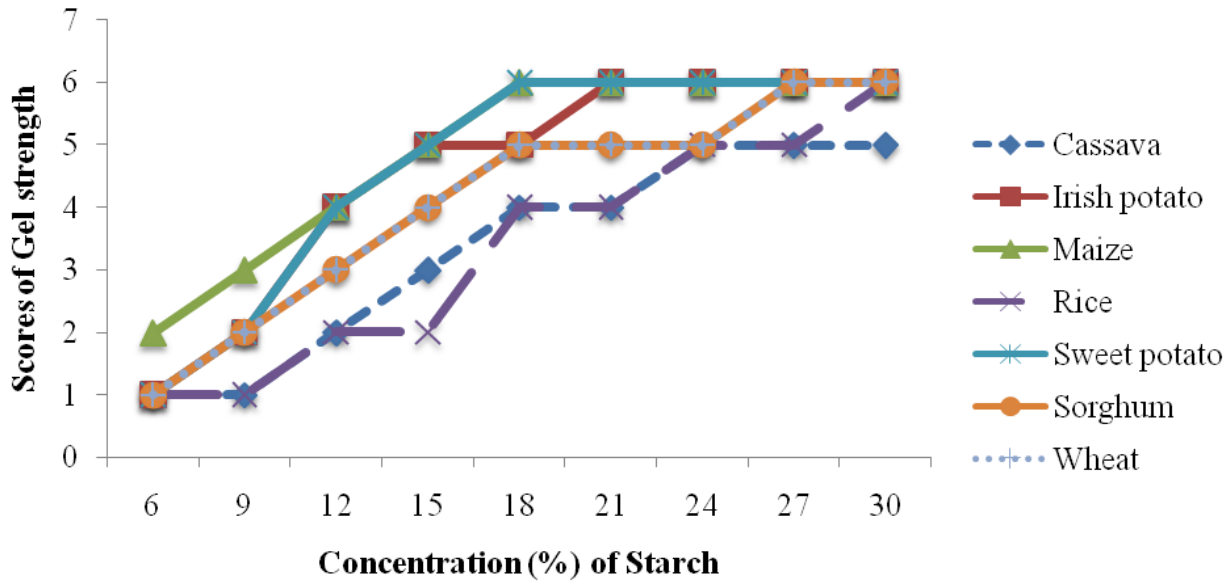

Figure 1. Effect of botanical source on gel strength of starch based tissue culture (TC) medium.

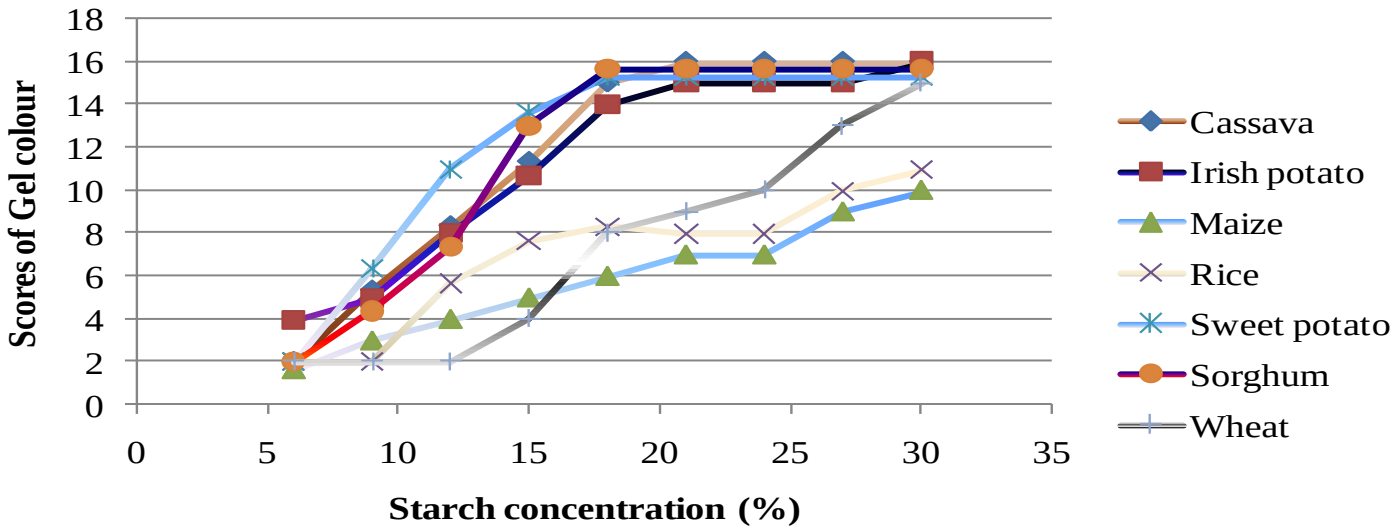

Figure 2. Effect of botanical source and concentration on colour of gels for starch based tissue culture (TC) medium.

starch gels which had results similar to those of root and tubers (Figure 2). Of the three root and tuber starch based medium, cassava produced medium with lowest scores of both gel colour.

Gel clarity

Figure 2 shows lost clarity with increasing concentration after 15 min autoclaving at 121°C. Clarity and colour scores of TC medium based on roots and tuber starches were lower than those from cereal sources (Figure 3).

Influence of types of botanical sources of starches on *in vitro* plant development

The results presented in Figure 4 show that the influence of starch based TC medium solidified starches of cassava, sweet potato and Irish potato on foliar (Figure 4A) and nodal (Figure 4B) development were not significantly different ($p = 0.05$) from the control. Nodal and foliar development of explants grown in TC medium solidified by starches from cereals (maize, rice, wheat and sorghum) were significantly ($P \leq 0.05$) lower than for the control (Agar based media) as well as TC media

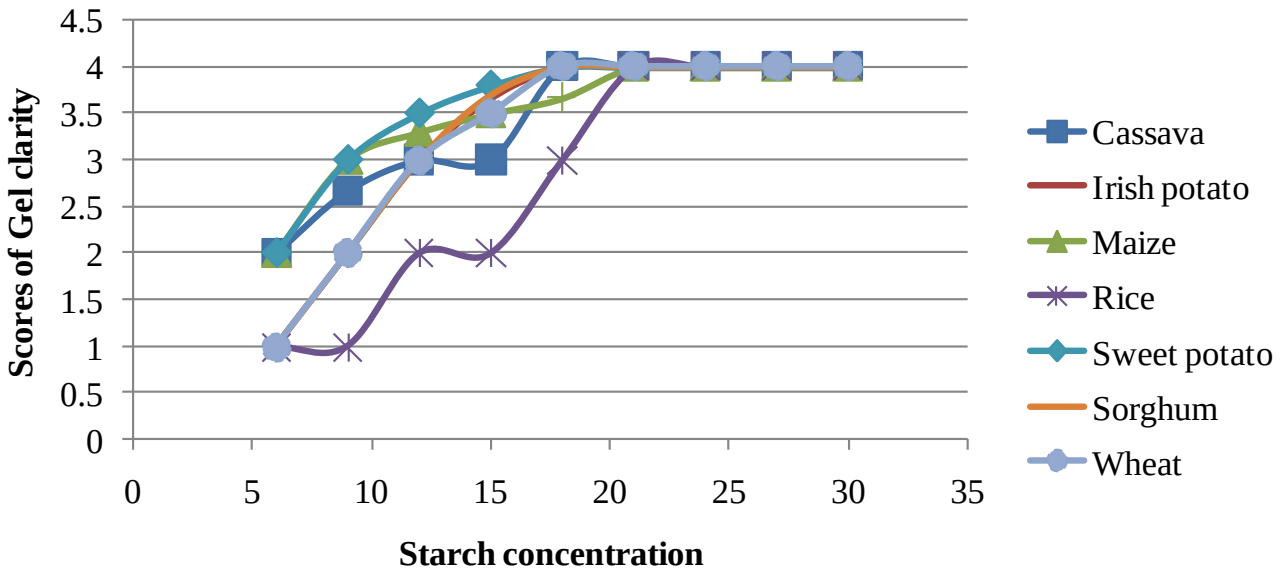

Figure 3. Influence of botanical sources of starch on clarity of gels for starch based tissue culture (TC) media.

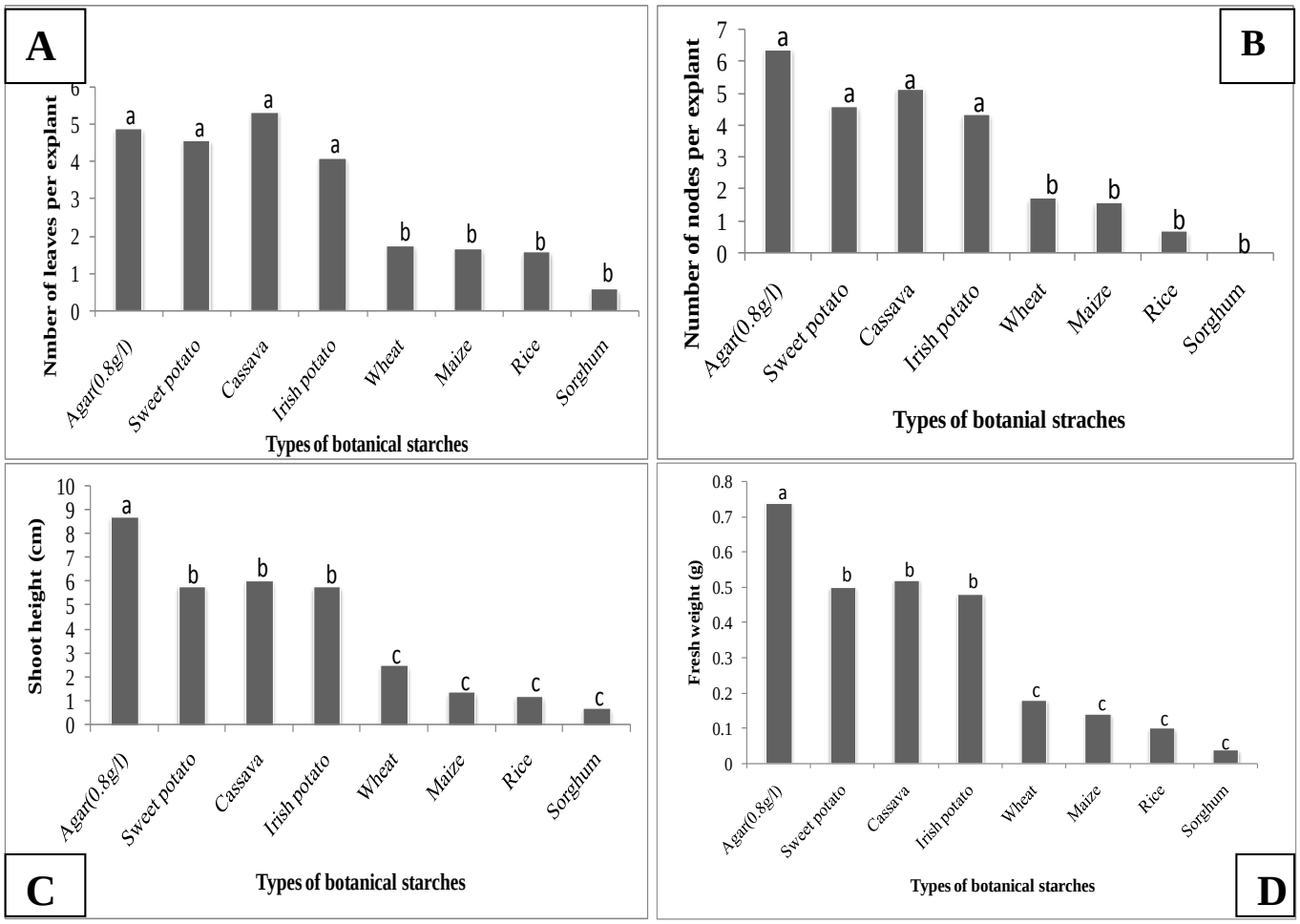

Figure 4. The potential of different botanical sources as alternative gelling agent of tissue culture (TC) media for plant micro-propagation (means which are represented by bars headed with same letters are not significantly different (p = 0.05).

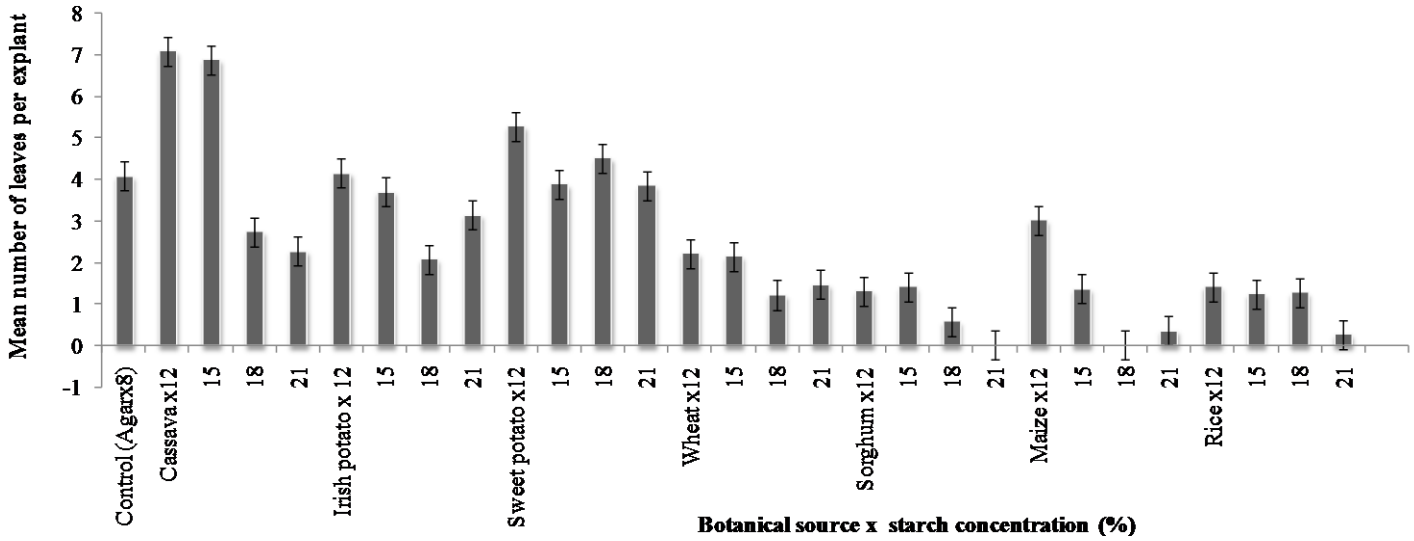

Figure 5. the influence of botanical source of starch and concentration (%) on *in vitro* development of leaves *in vitro*; Means represented with none overlapping standard error bars are significantly different (p = 0.05).

solidified by starch extracts of cassava, sweet potato and Irish potato.

Significant differences (p = 0.05) between types of TC medium were observed in terms of height (Figure 4C) and fresh weight (Figure 4D). Height (Figure 4C) and fresh weight (Figure 4D) of plantlets growing in the control (Agar based TC medium) were significantly (p = 0.05) higher than all starches.

Height and fresh weight parameters of explants growth in TC medium gelled by cassava, sweet potato and Irish potato were not statistically different (p = 0.05) but they significantly outperformed those grown in cereal based starches.

The influence of botanical source and concentration of starch on *in vitro* growth of sweet potato

Development of photosynthetic apparatus

The result in Figure 5 shows significantly different (p = 0.05) numbers of leaves produced per explants in TC media solidified by starch of different botanical sources and concentration (Figure 5). Cassava starch at concentrations of 12, 15% and sweet potato at 12% outperformed the control by more than a unit (Figure 5). The number of leaves per shoot recorded in TC media gelled by 12% (w/v) Irish potato starch (4.58) was not statistically different (p = 0.05) from the performance of the control on the same. Exceptionally, the number of leaves per shoot in maize starch based TC medium at 12% concentration was significantly higher than the rest of cereal starches though lower than root and tuber starches as well as the agar based TC medium (Figure 5).

Development of propagation units

Number of axillury buds produced by explants in TC medium solidified by starch of different botanical sources and concentrations were significantly different (p = 0.05) (Figure 6). The TC media gelled by cassava starch at the concentration of 15% produced shoots whose number of axillary buds was higher (8.14) than those of the control (5.87) but also was the highest of all starch based media (Figure 6).

The number of axillury buds per shoot recorded in the control media was statistically the same as those produced in TC media which were solidified by Irish potato and sweet potato starches both at the concentration of 12% (w/v). The response observed in all TC media which were solidified by starch from cereal extracts ranged from 0 to 4.93 which were significantly (p = 0.05) lower than the control (Figure 6). The number of axillury buds was negatively affected by increasing starch concentration beyond 18%

Growth performance of in vitro plantlets

Figure 7 shows significant different (p = 0.05) heights of *in vitro* plantlets in TC media which were solidified by starches from different botanical sources and applied at different concentrations. Plant height in cassava starch gelled media (15% cassava) did not vary (p = 0.05) from the control but outperformed all other treatments. Within each treatment, lowest plant height was observed at concentration higher than 18% except sweet potato starch gelled media (Figure 7).

Different types of gelling agents applied at different concentrations had different effect on fresh weight of the

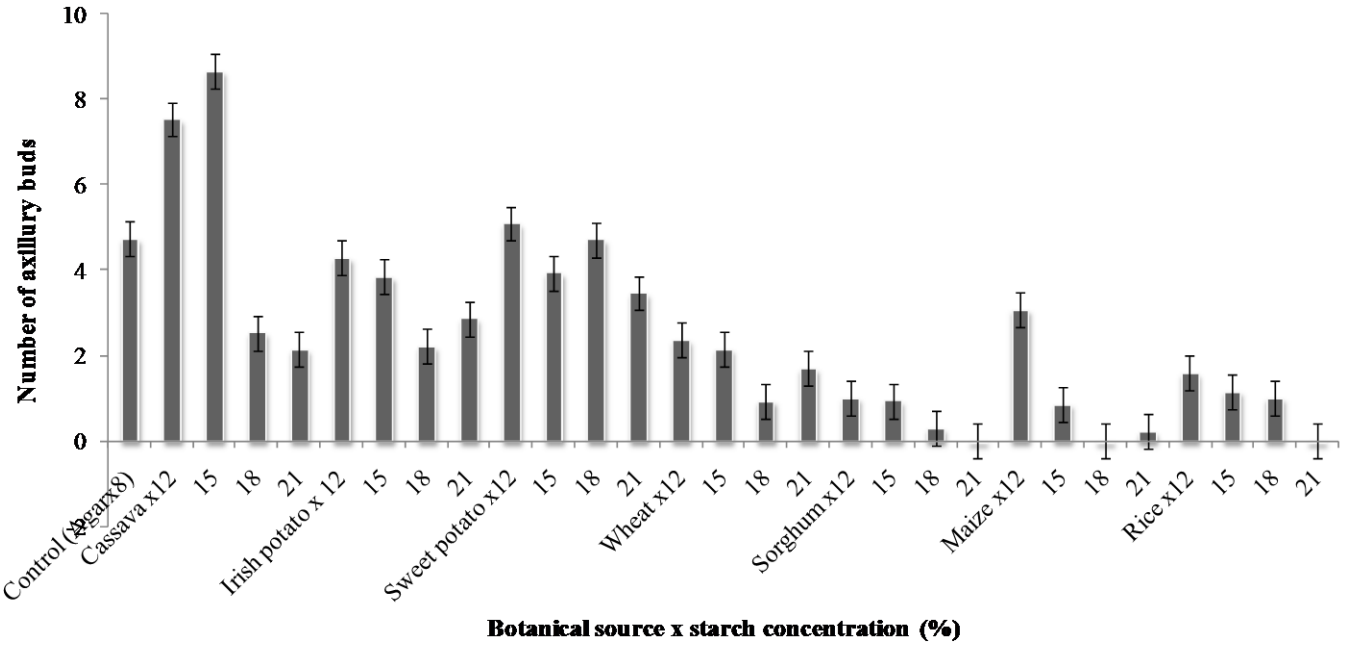

Figure 6. the influence of botanical source of starch and concentration on *in vitro* production of budded propagules; Means represented with none overlapping standard error bars are significantly different (p = 0.05).

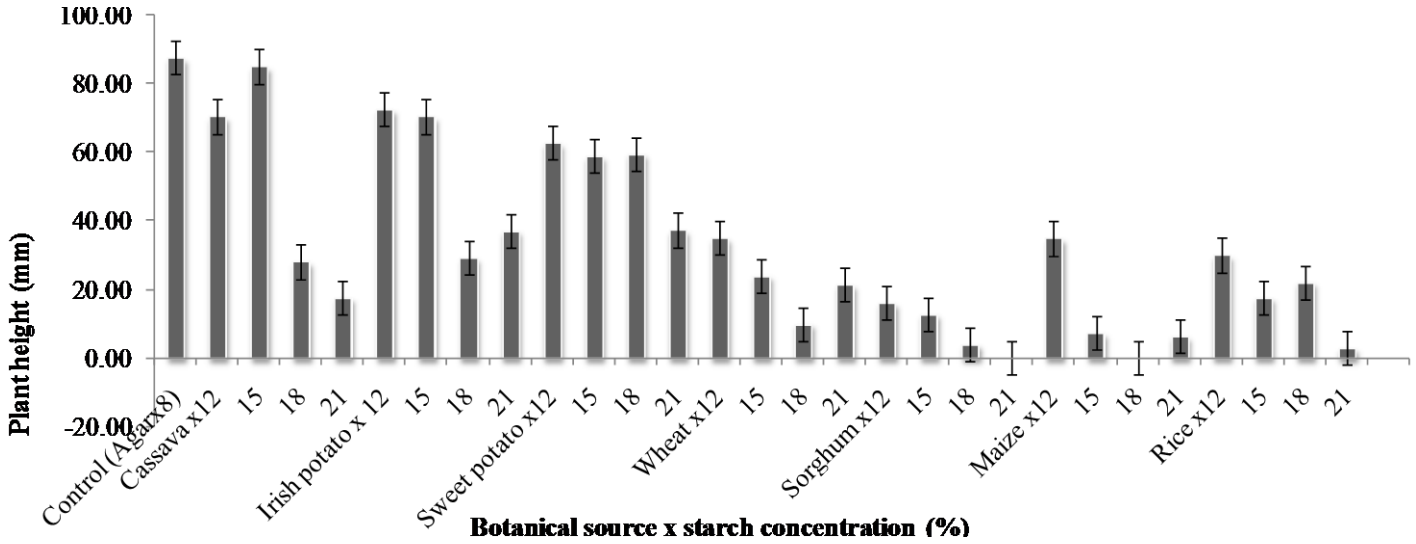

Figure 7. Influence of botanical source of starch and concentration on height of *in vitro* plantlets; Means represented with none overlapping standard error bars are significantly different (p = 0.05).

in vitro shoots (P = 0.05). Plantlets grown in 12% cassava starch accumulated the highest biomass followed by 12% Irish potato and sweet potato starches. Fresh weight attained in 15% cassava, Irish potato and sweet potato did not vary (p = 0.05) from the control. The fresh weight of shoots grown on TC media solidified by root/tuber starches ranged from 0.16 to 0.8 g which was higher than the range of 0 - 0.35 for shoots produced on cereal starch (Figure 8).

DISCUSSION

The results indicate that, starch extracts from cassava and sweet potato contained the highest amount of pure starch. This result is attributable to the characteristic

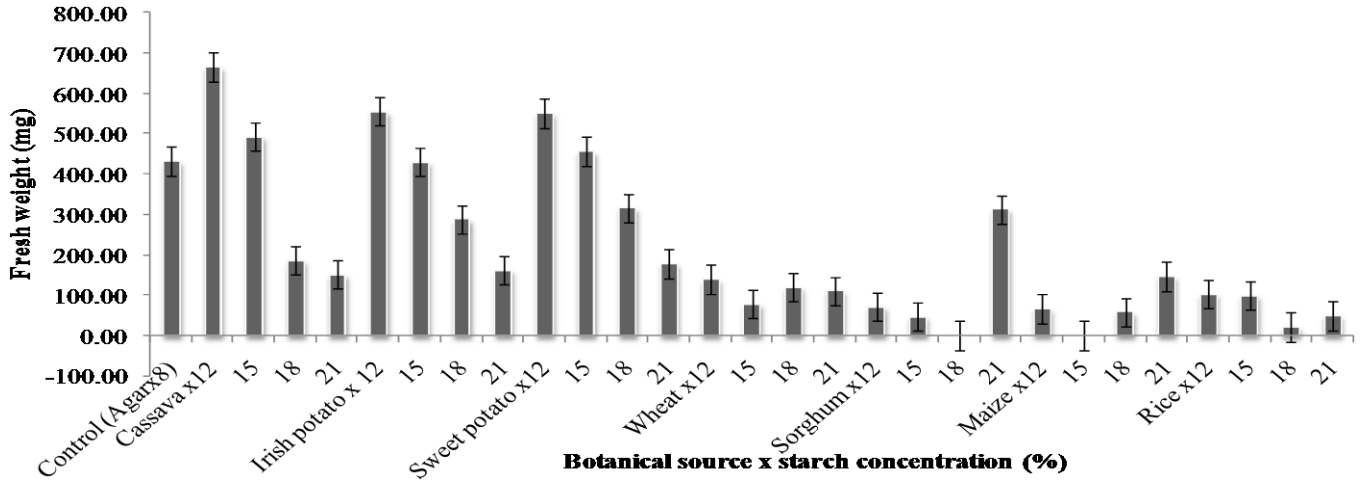

Figure 8. Influence of botanical source of starch and concentration on fresh weight of *in vitro* plantlets; Means represented with none overlapping standard error bars are significantly different (p = 0.05).

behavior of root crops of accumulating large amount of starch in roots and small amounts of other components as was also reported by many authors (Charles et al., 2005; Zaidul et al., 2007). The findings of cassava and sweet potato are consistent with other researchers (Chen et al., 2003; Hoover, 2001; Jangchud et al., 2003). Although the amount of pure starch in extracts from cereals was lower than those in root crops, these results are within established ranges (Kouakou et al., 2008) for the same cereals but different genotypes. These observations in cereal extracts can be associated the genetic tendency of many cereals to accumulate larger amounts of non starch biopolymers such as protein, fats and other organic acids which are usually tightly entangled in grain endosperms than it is in root crops (Kouakou et al., 2008) observed in maize, rice, millet and wheat. Large amount of protein and fat of cereal grains is accumulated in the germ parts as food for the growing embryo during seed germination.

The observed differences in amylose contents between starch extracts may be due to genetic differences between botanical types. This is because the variation between and within species in terms of amylose content is genetically controlled and is a trait with high heritability (Mishra and Rai, 2006). High amylose contents in cereal starch extracts and low levels in root and tuber crops is reported for the same species but different cultivars (Nuwamanya et al., 2010; Oludare and Macdonald, 2010). Due to the direct influence of amylose content on properties of starch gel and that it has a negative correlation with gel strength (Sumnu, 2001), it can be predicted that, starch extracts with low amylose content may form stable semisolid TC media at a lower concentration than those with high amylose content. In this case therefore high amylose starch extracts like those from rice and sorghum will require large amount of starch powder to form a standard semisolid TC media.

Differences in swelling power between starch extracts may be attributed to amylose content, granular structure and amounts of non starch biopolymers of each starch extract. The low swelling power observed on some of the cereal starches may be a result of the extensive and strongly bond micellular structure which makes them relatively resistant to swelling (Nuwamanya, et al., 2010). High swelling power in starch extracts with low amylose content such as cassava, sweet potato and wheat is expected because, as the starch granules absorb water the amylose reinforces the internal network within the granules thus restricting swelling of starch with high amylose content compared to those with small amounts of amylase (Riley et al., 2006).

Furthermore, the low swelling power of starch extracts of Irish potato, rice, maize and sorghum can be associated to low starch purity as was observed in Table 1. This is because starch extracts with large amounts of non starch biopolymers like lipids seem to inhibit swelling of starch granules (Riley et al., 2006). This observation is strongly supported in the previous reports which established that swelling power of many starches are negatively correlated to amylose and lipid contents with correlation coefficients of about -0.84 and -0.68 respectively (Tester et al., 2006; Tester and Karkalas, 1996; Tester et al., 2004). Another reason could be high protein content (Table 1) since the protein molecules have a tendency of interacting with amylose molecules due to opposite charges which form protein-amylose complex hence it restricts swelling (Shimelis et al., 2006).

Swelling power of starches is of great significance in TC media formulations as it enhances movement and availability of water, growth regulators and nutrient solutes to the *in vitro* plants. The results therefore suggest that the TC media gelled by starches with higher

swelling power would be expected to release the nutritional ingredient from its matrices at a faster rate than those with low swelling power. Therefore, these results imply that the TC media gelled with wheat, cassava and Sweet potato starches would support *in vitro* plant growth better than those solidified by rice, maize, sorghum and irish potato.

The results show that, swelling power increased with increasing temperature from 30 to 60°C. This result can be explained as due to the insoluble starch granules which start to swell during hydration as they begin to integrate with water a molecule which is probably enhanced by increment in incubation temperatures. However within the same change in incubation temperature, wheat, sweet potato and cassava showed higher rate of increasing swelling power than the rest. The low amylose content in cassava and sweet potato is probably due to the longer chains in amylopectin structure which is water loving (Riley et al., 2006). These findings agree with those reported for sweet potato starch (Chen et al., 2003), yam (*Diascorea alata* L) starch (Riley et al., 2006), common bean (*Phaseolus vulgaris* L) starch (Shimelis et al., 2006) and cassava and Irish potato starches (Yuan et al., 2007). However the decrease in swelling power of maize starch disagrees with other findings (Fasasi et al., 2007) that the swelling power of both starch and flour from maize increased with increasing temperature.

Compared to root crop (cassava and sweet potato) starches, the low pH of cereal starches might be due to the presence of high amounts of non starch impurities which are acidic in nature like amino and fatty acids from protein and fat biopolymers as was noted in Table 1. The normal pH of TC media for many plant species including sweet potato is usually around 5.7. Therefore the TC media which is solidified by gelling agents like starch from maize, rice, wheat and sorghum with lower pH than the normal values would need more of base/alkali to adjust the pH to normal. Similarly, gelling agents like cassava, sweet potato and irish potato which have pH that is higher than the normal values would need a lot of acid to neutralize it to normal. Excessive application of acid or base to bring the pH of TC media to normal has implication on the production cost. Therefore, gelling agents with extremely low or high pH values may be expensive in terms of acid-base requirements.

The differences between treatments in gel strength are associated with starch purity and amylose contents and this observation is in agreement with many other authors (Blazek and Copeland, 2008; Campo and Tovar, 2008). It has been noted that at the same concentration, starches with high amylose content formed weaker solid gel than those with low amylose content. Due to similar reasons, the results show that at the same concentration of starch, the gel strength of TC medium varied with the botanical sources. As a result therefore, the minimum concentration of starch extract that was required to form a stable semisolid TC medium with gel strength enough to support *in vitro* plant growth also varied with the source of starch. The stable TC medium was characterized of being semi-solid, which adheres well to the bottom of the container (for example, culture bottle) such that it does not become sloppy and flow when the container is tilted. The increase in the amount of any gelling agent in TC media increases the cost of micropropagation, hence low concentration of starchy gelling agent which form TC media with stable gel strength are preferred for cost effectiveness.

Since starch granules are usually white, occurrence of different colours of the starch extracts may a result of presence of non starch impurities such as polyphenols, ascorbic acid and carotene which may have negative effects on the starch quality and the final functionality of starch as reported elsewhere (Shimelis et al., 2006). Reports show that the colour might be affected by the irreversible structural change of the molecular order of the starch granules that occurs after a gelatinization temperature is reached during heat treatment (Sasaki, 2005; Spigno and De Faveri, 2004).

Lack of transparency or clarity of starch gels is attributable to presence of non starch components like protein, lipids and phosphorous which the available findings show that they influence the transparency and clarity of starch gels (Shimelis et al., 2006; Spigno and De Faveri, 2004). Both gel colour and clarity of TC media are important properties in management of *in vitro* culture especially the identification of contaminated cultures as well as the type of contaminant. This is because exudates of some microbial contaminants have colours similar to the colour of the starch based TC media. In such a situation it may be difficult to note the growth and identify the type of contaminant as early as possible.

The growth of shoots *in vitro* on agar gelled media outperformed those of starch gelled media except for the number of leaves per shoot which cassava starch media recorded the highest response. Of all the starch gelled media, the best growth response was observed in cassava. In comparison with other starch based media, high numbers of nodes and leaves per shoot, height and fresh weight on cassava starch media may be due to its characteristic low gel strength which probably enhanced the availability of water and nutrients due to low resistance to diffusion and closer contact between the explant and the medium.

Previous reports establish that the better response on cassava starch gelled media could be due to the absence of inhibitors which have been reported to be present in agar (Kuria et al., 2008). The results in this study are in agreement with previous report for cassava flour (Maliro and Lameck, 2004), corn starch (Van et al., 2012) and Irish potato starch (Ibrahim et al., 2005). Other reports show that the good growth responses on root starch (cassava and sweet potato) based media may be due to their tendencies of acting as an additional source of carbon (Onuweme, 1982). Also, they could be source of

the beneficial nutrients present which acts as ionic supplements (35% carbohydrates, 1% mineral matter) to the medium, which may have resulted into improved cell growth and morphogenesis. Although the results was a declining plant performance with increasing concentration of all botanical starches, the improved performance at 15% cassava starch, 12% sweetptato and irish potation can be associated with optimal conditions created by the TC media such as good anchorage and free movement of nutritional factors within the media matrix. Low performance of cassava starch in 12% concentration could be associated with low gel strength leading to inadequate anchorage of explants, keeping explants submerged and photo-chemically inactive. Poor plant growth in concentrations above 18% can be explained by limited movement of nutrients within media matrix which is also reported elsewhere (Kuria et al., 2008)

Conclusions

In terms of supporting *in vitro* plant growth, agar based media are superior to starch based media. However, it was observed that good performance was observed in starch extracts with large amount of total starch, low amylose content and high swelling powers. From these results therefore, starches extracted from cassava, sweet potato and Irish potato have high potential of being alternative gelling agents upon some improvement. Concentration required to create optimal gel strength is high and provide room for improvement. The colour and clarity of starch gels is not favourable in management of cultures.

Conflict of interests

The authors have not declared any conflict of interests.

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Polymorphism of glucagon-like peptide-1 receptor gene (*rs1042044*) is associated with bone mineral density in Chinese Han postmenopausal women

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Previous investigations indicated that glucagon-like peptide-1 (GLP-1) played important roles in bone turnover via GLP-1 receptors (GLP1Rs) in postmenopausal state. Furthermore, polymorphisms in *GLP1R* gene were suggested to affect the function of GLP1Rs and be associated with many diseases. However, the relationships between *GLP1R* polymorphisms and osteoporosis susceptibility and bone strength remain unexplored. To address this issue, a total of 458 Chinese Han postmenopausal women were included in this study. The bone mineral density (BMD) in the lumbar spine (L_2-L_4) and femoral neck was measured by dual-energy X-ray absorptiometry (DEXA). A missense mutation (rs1042044) in *GLP1R* was genotyped using allele specific TaqMan probes. Our data showed that genetic variants of rs1042044 were significantly associated with osteoporosis (*P* = 0.003) and that the C allele of rs1042044 was a protective factor against osteoporosis compared to the A allele with gene dosage-dependent manner (OR, 0.579; 95% CI, 0.366 to 0.916 for AC genotype and OR, 0.404; 95% CI, 0.238 to 0.688 for CC genotype). These findings indicate that polymorphisms in *GLP1R* gene may affect BMD and development of osteoporosis in Chinese Han postmenopausal women, which provide novel insight into the mechanisms of osteoporosis development and target for personal prevention and treatment of osteoporosis.

Key words: Glucagon-like peptide-1 receptor, single nucleotide polymorphism, osteoporosis, bone mineral density.

INTRODUCTION

Osteoporosis is a prevalent metabolic bone disease and an important and complex health problem in postmenopausal women (Riggs and Melton, 1986; NIH, 2001). This kind of skeletal disease is characterized by low bone mineral density (BMD) and deterioration of bone tissue, and generally believed to be a multifactorial

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disorder with genetic influence accounting for up to 70% of individual variance in BMD (Jouanny et al., 1995). It was reported that, in postmenopausal state, the BMD was affected by a series of bone mass modulating factors, such as vitamin D receptor, estrogen receptor, and osteoprotegerin etc., and that polymorphisms in genes encoding these factors were significantly associated with osteoporosis risk (Horst-Sikorska et al., 2013; Gennari et al., 2005; Arko et al., 2005; Wang et al., 2013). Therefore, to define certain candidate genes responsible for regulation of bone mass and susceptibility to osteoporosis may be a beneficial approach for better understanding of the etiology and mechanisms of osteoporosis.

Recently, it was suggested that glucagon-like peptide-1 (GLP-1), a gastrointestinal hormone, plays important roles in bone modulation via its receptors (Yamada et al., 2008). The GLP-1 receptor (GLP1R) is a cognate G-protein-coupled receptor and its activation by exogenous GLP-1 analogue, exendin-4, enhances bone strength and prevents the deterioration of trabecular microarchitecture (Ma et al., 2013). These findings suggest that GLP1R is an important bone mass modulating factor. Furthermore, some single nucleotide polymorphisms (SNPs) of *GLP1R* gene were found to lead to changes of expression and function of *GLP1R* and to be associated with development of certain diseases (Sheikh et al., 2010; Beinborn et al., 2005; Sathananthan et al., 2010). Therefore, it is rational to hypothesize that *GLP1R* gene might also be a candidate gene participating in bone mass turnover and development of osteoporosis.

However, to our best knowledge, no published studies regarding this issue have been performed so far. In order to prove this hypothesis, in the present study, we investigated the relationship between a common tag-SNP (rs1042044) of *GLP1R* and BMD in Chinese Han postmenopausal women.

MATERIALS AND METHODS

Subjects

A case-control study was performed with a total of 458 Chinese Han postmenopausal women, including 223 randomly selected primary postmenopausal osteoporosis patients (age from 48 to 79), who were enrolled from Kunming General Hospital of Chengdu Military Command between 2009 and 2013, and 235 health controls (age from 49 to 82). Menopausal status was defined as the date of last menses followed by 12 months of no menses. Individuals with current hepatic disease, renal disease, diabetes mellitus, surgical removal of both ovaries, and/or taking any drugs known to affect bone metabolism were excluded. The study protocol was designed in compliance with the principles of the Helsinki Accord, and was reviewed and approved by the local Ethical Committees. Informed consent statement was obtained from all participants after full explanation of the procedure.

Measurement of BMD

BMD was measured at the lumbar spine (L_2-L_4) and femoral neck by dual-energy X-ray absorptiometry (DEXA) (Lunar Expert 1313, Lunar, Madison, WI). The value of BMD was automatically calculated from bone mineral content (g) and bone area (cm^2) and expressed in g/cm^2.

Genotyping assay

Peripheral venous blood samples (3 ml) were collected into tubes containing ethylenediaminetetraacetic acid and stored at -80°C for analysis. Genomic DNA extraction was performed using the QIAGEN DNA MicroKit® (Valencia, CA) according to the manufacturer's instructions. The genotyping of the non-synonymous tag-SNP (rs1042044) was performed according to previous report (Sheikh et al. 2010) by TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA). Briefly, TaqMan PCR was performed in a 10 µL system containing 1× ABI TaqMan Genotyping Master Mix, TaqMan 40× probe mix, and 25 ng of DNA on an ABI StepOne™ Real-Time PCR System (Applied Biosystems) and analyzed using SDS v3.0 software (Applied Biosystems). The PCR conditions were: DNA denaturing at 95°C for 10 min, 50 cycles of replication at 95°C for 15 s, and then annealing and extension at 60.0°C for 1 min followed with final extension for 3 min. The primers used were as follows: forward 5'CCTAGCCAGAGATGTGAGCT3' and reverse 5' CAGCAGTGCGTTCCAAGTAC 3' (chr6:39041252+39041636).

Statistical analysis

Data were analysed with SPSS 18.0 for Windows (PASW Statistics, SPSS Inc., Chicago, IL). The genotypic frequency for rs1042044 was tested for departure from Hardy–Weinberg Equilibrium (HWE). Chi-square (χ^2) test was used to determine the differences of genotypic and allelic distributions between osteoporosis case and control group, and also the odds ratios (OR) with 95% confidential intervals (CIs) of osteoporosis risk. Student *t* test was used to determine the differences in anthropometric characteristics between case and control group. Differences in anthropometric characteristics according to genotypes were tested by one-way analysis of variance. Differences in BMD according to genotypes were analyzed by using analysis of covariance adjusting for potential confounders, that is, age, body mass index (BMI) and years since menopause.

RESULTS

Population characteristics

Descriptive statistics of both the case and control groups are shown in Table 1. There were no significant differences regarding the age (P = 0.979), BMI (P = 0.662), and years since menopause (P = 0.834) between the 2 populations. Meanwhile, the BMD values in osteoporosis case group were significantly lower compared to health controls at both lumbar spine and femoral neck (P < 0.001 for both positions).

Distributions of genotype and allele frequencies of rs1042044 between osteoporosis patients and health controls

The distributions of genotype and allele frequencies of

Table 1. Characteristics of osteoporotic and control postmenopausal women.

Parameter	Case (*n* = 223)	Control (*n* = 235)	*P*[a]
Age (years)	59.9 ± 7.2	59.9 ± 7.3	0.979
BMI (kg/m^2)	23.4 ± 2.8	23.3 ± 2.8	0.662
Years since menopause (years)	10.1 ± 6.9	10.0 ± 7.0	0.834
BMD (g/cm^2)			
Lumbar spine	0.785 ± 0.039	0.973 ± 0.073	**<0.001**
Femoral neck	0.653 ± 0.071	0.825 ± 0.058	**<0.001**

Table 2. Frequency distribution of *GLP1R* rs1042044 polymorphisms and their associations with the risk of osteoporosis in postmenopausal women.

rs1042044	Case	Control	OR (95% CI)	*P*[a]
	n (%)	*n* (%)		
Genotype				
AA	69 (30.9)	44 (18.7)	1.000 [Ref]	
AC	109 (48.9)	120 (51.1)	0.579 (0.366 - 0.916)	**0.003**
CC	45 (20.2)	71 (30.2)	0.404 (0.238 - 0.688)	
Allele				
A	247 (55.4)	208 (44.3)	1.000 [Ref]	**0.001**
C	199 (44.6)	262 (55.7)	0.640 (0.493 - 0.830)	

OR odd ratio, *CI* confidence interval, *Ref* reference category. [a] *P* values were calculated from two-sided chi-square tests for genotype or allele distribution.

GLP1R rs1042044 in the two groups are listed in Table 2. The χ^2 test revealed that the genotypic frequencies of both groups did not deviate from HWE (χ^2 = 0.027, *P* = 0.870 for case group; and χ^2 = 0.287, *P* = 0.592 for control group). In the following association analysis, we found that the genotypic frequencies in osteoporosis case group were significantly different from those of healthy control group (*P* = 0.003). Furthermore, osteoporosis was much less frequently observed in the AC (OR, 0.579; 95% CI, 0.366 to 0.916) and CC (OR, 0.404; 95% CI, 0.238 to 0.688) genotypes than in the AA genotype, which exhibited an obvious gene dosage effect. Accordingly, data about the distribution of allele frequency between the two groups also showed that the C allele was significantly frequent in control group and may be a protective factor against osteoporosis (*P* = 0.001; OR, 0.640; 95% CI, 0.493 to 0.830).

Association of GLP1R rs1042044 genetic polymorphisms with BMD

We then combined the two groups and analyzed the differences of anthropometric characteristics according to genotypes. As shown in Table 3, no significant differences in age (*P* = 0.204), BMI (*P* = 0.181), or years since menopause (*P* = 0.117) according to genotypes of rs1042044 were observed in the present study. However, the rs1042044 polymorphism was found to be still significantly associated with BMD at both lumbar spine and femoral neck (*P* < 0.001) even after adjustment for the possible confounding factors (age, BMI, and years since menopause). Interestingly, these data also exhibited a gene dosage effect that the number of the C allele in the genotypes was positively correlated with the BMD values (Table 3).

DISCUSSION

GLP-1, which induces a variety of physiological effects by activating GLP1R, is mainly produced by L-cells primarily localized in the ileal/colonic mucosa and is hence considered as an important gastrointestinal hormones (Drucker, 1998; Kieffer and Habener, 1999). However, the role of *GLP1R* gene in the pathology of osteoporosis is unclear and need to be investigated. It was recently reported that the entero-bone endocrine axis was proposed as a mediator of bone turnover (Isales and Hamrick, 2008). Therefore, the association between *GLP1R* and BMD and osteoporosis seems to be quite plausible with regard that growing evidence indicated that osteoporosis is a multifactorial and polygenic disease (Zhao et al., 2005; Lee et al., 2014). In the present study, we assessed the effects of rs1042044 polymorphism in *GLP1R* gene on BMD values and osteoporosis risk in

Table 3. The characteristics of *GLP1R* rs1042044 polymorphisms in the total group of subjects in the total group of postmenopausal women.

Genotype	AA	AC	CC	*P*[a]
n (%)	113 (24.7)	229 (50.0)	116 (25.3)	-
Age (years)	59.2 ± 7.1	59.7 ± 7.0	60.9 ± 7.8	0.204[a]
BMI (kg/m^2)	23.8 ± 2.8	23.2 ± 2.7	23.3 ± 2.9	0.181[a]
Years since menopause (years)	9.2 ± 6.8	9.9 ± 6.6	11.1 ± 7.5	0.117[a]
BMD (g/cm^2)				
Lumbar spine	0.838 ± 0.099	0.887 ± 0.110	0.912 ± 0.112	**<0.001**[b]
Femoral neck	0.700 ± 0.108	0.741 ± 0.106	0.780 ± 0.098	**<0.001**[b]

BMI body mass index, *BMD* Bone mineral density. [a] *P* values were calculated by one-way analysis of variance (ANOVA). [b] *P* values were calculated with analysis of covariance adjusted for age, body mass index and years since menopause.

Chinese Han postmenopausal women. Our findings revealed for the first time that genetic variants of rs1042044 in *GLP1R* were significantly associated with osteoporosis and that the C allele of rs1042044 was a protective factor against osteoporosis, which gene dosage-dependently increased BMD values.

The rs1042044 of *GLP1R* is a tag-SNP located in exon 7 of the *GLP1R* gene. This SNP is a nonsynonymous variant resulting in a cytosine (C) to adenine (A) change at the sequence level and phenylalanine (Phe) to leucine (Leu) at position 260 of the GLP1R protein (Phe260Leu). Furthermore, the minor allele frequency of this SNP is much greater than 5% in the Chinese Han population according to Data from HapMap (http://www.hapmap.org). Therefore, we chose this SNP in this study trying to assess its association with osteoporosis. According to our data, the minor allele (A) frequencies of rs1042044 in total participants was 0.497, which was close to the allele frequency data of Chinese Han population from HapMap (http://www.hapmap.org), indicating that the participants consist of a representative population sample for this case-control study.

The functionality of rs1042044 has previously been studied by Fortin et al. (2010) who demonstrate, using a cAMP reporter gene assay, that this polymorphism did not alternate the pharmacological properties of GLP1R (Fortin et al., 2010). However, given the location of the amino acid substitution, it is possible that other aspects of the receptor function may be affected (desensitization or internalization), which need to be further clarified. Since the nature of the present study is exploratory, we did not preformed further mechanism investigations, which is an intrinsic limitation of this study. However, our findings are in accordance with previous investigation which also indicated that variants of this SNP altered the protein function of GLP1R (Sheikh et al., 2010). Some characters of rs1042044 may explain the mechanisms. The location of this SNP is within the second intracellular loop of the mature translated GLP1R protein, whose variation may possibly affect the interaction between GLP1R and G-proteins and therefore alter downstream GLP1R signaling (Bavec et al., 2003). It is also possible that this tag-SNP variant may capture other nearby functional SNPs that influence either ligand affinity or downstream signaling of GLP1R (Sheikh et al., 2010; Bavec et al., 2003; Al-Sabah and Donnelly, 2003; Lopez de Maturana and Donnelly, 2002; Lopez de Maturana et al., 2004; Xiao et al., 2000). Further research is needed to elucidate this issue in the future. Another limitation of our study is the relatively small sample size (223 cases and 235 controls). Therefore, larger sample size as well as meta-analysis is warranted to validate the results of our work. Meanwhile, the race of the studied participants is Han Chinese. Whether our present conclusions can be appropriately drawn in other human races or not still needs to be clarified.

In summary, our present study finds novel evidence that variants of rs1042044 in *GLP1R* gene is associated with BMD values and osteoporosis risk in Chinese Han postmenopausal women, which provides new insight into the mechanisms of osteoporosis development and target for personal prevention and treatment of osteoporosis.

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Conflict of interest

The authors have not declared any conflict of interest.

Abbreviations: BMI, Body mass index; **BMD,** bone mineral density; **CI,** confidence interval; **GLP-1,** glucagon-

like peptide-1; **GLP1R,** glucagon-like peptide-1 receptor; **HWE,** Hardy–Weinberg Equilibrium; **ORs,** odds ratios; **SNP,** single nucleotide polymorphism.

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