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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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Full Length Research Paper

In silico characterization and evolution studies of alcohol dehydrogenase gene from *Phoenix dactylifera* L.cv Deglet Nour

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The aim of our study was to isolate the alcohol dehydrogenase (ADH) mRNA from *Phoenix dactifera*, and examine the molecular evolutionary history of this nuclear gene with others ADH genes from palms and other plants species. The *DnADH* gene has been isolated *in silico* by BLAST2GO from a cDNA library of date palm cv *Deglet Nour*. The prediction of candidate's mRNA and protein for *ADH* gene from *khalas* were performed *in silico* from whole genome shotgun sequence (ACYX02009373.1) using FGENESH prediction program. Nucleotide polymorphism using DnaSPv5 was examined in four palm ADH mRNA sequences across the entire 1.098 kb length of ADH mRNA. A primary conclusion of the present study is that nucleotide diversity for ADH between palm species is very low. In order to assess selective pressure, we calculated the ratio of non-synonymous to synonymous substitutions. We conclude that ADH palms genes appear to be under very different selective constraints. Phylogenetic analyses using PHYLIP and Notung 2.8 programs suggest that ADH genes of some plants species resulted from relatively ancient duplication events. In this study, we present for the first time a molecular characterization of ADH protein of *P. dactylifera* L cv Deglet nour and a phylogeny analysis between plants ADH.

Keys word: Alcohol dehydrogenase, palms species, evolution, duplication.

INTRODUCTION

Sequencing of date palm genome and cDNA or expressed sequence tags (EST) using Next generation sequencing provides a rapid method for gene discovery and can be used to identify transcripts associated with specific biological processes (Al-Mssallem et al., 2013). The alcohol dehydrogenase (ADH) genes encode a glycolytic enzyme and have been characterized at the molecular level in a wide range of flowering plants (Clegg et al., 1997; Miyashita et al., 2001), California fan Palm (*Washingtonia robusta*) (Morton et al., 1996) and Oil palm

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Abbreviations: EST, Expressed sequence tags; **ADH,** alcohol dehydrogenase; **CDD,** conserved domains database; **ML,** maximum-likelihood; **SNP,** single nucleotide polymorphisms.

(*Elaeis guineensis*) (Low et al., 2008; Singh et al., 2013). The ADH enzyme is essential for anaerobic metabolism (Dolferus et al., 1994; Dolferus et al., 1997), found in plants and prokaryotes, its activity is required for lignin biosynthesis and apparently also for defense-related functions (Umezawa, 2010). The ADH genes in *Arabidopsis thaliana* (Innan et al., 1996), *Arabidopsis gemmifera* (Miyashita et al., 2013) and Leavenwortia (Charlesworth et al., 1998) in Brassicaceae, cottons (Small et al., 2000), and grasses (Gaut et al., 1996; Gaut et al., 1999) have been subjected to molecular evolutionary studies. However, the broader evolutionary histories of the ADH genes in the angiosperms remain unclear since few studies have investigated the evolution of the ADH genes in a wide range of angiosperms (Strommer, 2011).

Recently, Small and Wendel (Small et al., 2000) suggested that some ADH gene duplications may have predated the origin of each of the flowering plant families. However, the details of the gene duplications and deletions experienced by the ADH genes of most groups of the angiosperms remain unclear. Additional studies are needed to understand the evolutionary history of the ADH genes in various plant groups. A number of studies have been conducted on the evolutionary dynamics of plant gene families, including the gene families coding for the R and MADS-box regulatory proteins (Purugganan et al., 1995), the small heat-shock proteins (Waters et al., 1995), chalcone synthase (Durbin et al., 1995), and the chlorophyll a/b binding proteins (Demmin et al., 1989). Most of these gene families consist of numerous loci and have a great deal of variation in copy number between species. The evolutionary picture emerging for these gene families is one of dynamic fluctuations of copy number through multiple gene duplication/deletion events.

The glycolytic proteins in plants are coded by small multigene families, which provide an interesting contrast to the high copy number gene families studied to date. Isozyme surveys covering an array of dicot and monocot species have revealed that most glycolytic enzymes have two forms in all species (Gottlieb et al., 1982), probably reflecting a small, and stable, number of loci. The apparent stability of these gene families raises important questions regarding evolutionary dynamics. One issue is whether any given gene family emerged once by duplication and then differentiated, as suggested by Gottlieb (Gottlieb et al., 1982). An alternative view posits a continuous, albeit slow, flux of gene duplication and loss that leads to an approximate dynamic equilibrium in copy number. The narrow range of gene family size for glycolytic enzymes suggests that additional constraints may act to determine copy number for this important class of genes.

An analysis of animal and plant ADH genes indicated that the grass ADH1 and ADH2 genes diverged following the divergence of monocots and dicots (Yokoyama et al., 1993). This result provides evidence that the gene family did not emerge from a single duplication event early in angiosperm evolution. Additionally, the isolation of a recent duplication product in barley (Trick et al., 1988), as well as the duplication of ADH I in other species, suggests that the gene family undergoes some copy number fluctuation. Here, we report the isolation of ADH mRNA from *Phoenix dactilifera* L cv Deglet Nour and the prediction of ADH from *P. dactilifera* L cv Khalas. In order to study ADH gene evolution between recalcitrant vs. orthodox palm species, we compared ADH mRNAs and proteins of Khalas, Deglet Nour, *E. guineensis* and *W. robusta* varieties. We also investigated the molecular evolutionary history of the palms species ADH genes with the others species to gain further understanding of the evolutionary dynamics of nuclear gene families.

MATERIALS AND METHODS

cDNA library normalization and *in silico* isolation of DnADH

Fresh leaf tissue from *P. dactylifera* L cv Deglet Nour were processed and flash frozen in liquid nitrogen. Tissues were immediately sent to Bio S&T Inc. (Montreal, QC, Canada) where RNA extraction, cDNA synthesis and normalization were performed. Briefly, RNA was extracted using a modified TRIzol method (Invitrogen, USA). cDNA synthesis was carried out using 18 µg total RNA by a modified SMART™ Cdna synthesis method and then were normalized by a modified normalization method where full-length cDNA was synthesized with two sets of primers for driver and tester cDNA. Single-stranded cDNA was used for hybridization instead of double-stranded cDNA. Excess amounts of sense-stranded cDNA was hybridized with antisense- stranded cDNA. After hybridization, duplex DNA was removed by hydroxyapatite chromatography. Normalized tester cDNA was re-amplified and purified with tester specific primer L4N by PCR, while driver cDNA was unable to amplify using L4N primer. Size fractionation of re-amplified cDNA was done in a 1% agarose gel. Greater than 0.5 kb cDNA fragments were purified by electroelution and after determining the concentrations, purified cDNAs were precipitated and stored in 80% EtOH at −80°C. The normalized cDNA library was prepared for sequencing and approximately 8 µg of purified cDNA was sheared into small fragments via Covaris E210 Acoustic Focusing Instrument and sequenced in three-fourths 454 plate run on a 454 GS-FLX Titanium platform (Roche). To identified DnADH cDNA, assembled contigs were analysed using Blast2GO2.8 bioinformatic (Conesa and Götz, 2008) to provide Gene Ontology, BLAST and domain/Interpro annotation. Evaluation of DnMRE11 predicted protein was done based on the identification of domains in the NCBI Conserved Domains Database (CDD), phytozome of June 2013 (http://www.phytozome.net/) and most recent version of HMMER (HMMERV3.0; Eddy, 2009).

mRNA and protein prediction of KhADH

Candidate's mRNA and protein for ADH gene from *P. dactifera* L cv *khalas* (KhADH) were identified *in silico* from whole genome shotgun sequence (accession number: ACYX02009373.1) using FGENESH prediction program. Our approach to the annotation was based on applying basic gene prediction tools and using the BLASTN and BLASTP programs to improve the accuracy of gene prediction. Sequence motifs related to function were identify through PFAM (Henikoff et al., 1999), and PROSITE (Altschul et al., 1998).

Table 1. List of accession numbers of mRNA and protein for palms species used in this study.

Nom latin	Abbreviation	mRNA accession number	Protein accession number
Phoenix dactyfera v Deglet Nour	DnADH	KF961040	AHH32692
Phoenix dactyfera v khalas	KhADH		
Elaeis guineensis	EgADH	EU284998.1	ACF06607.1
Washingtonia robusta	WrADH	U65972.1	AAB39597.1

Sequences analysis

The sequences of the ADH genes used in this study were obtained from the GenBank/EMBL/DDBJ database (Table 1). Alignments of four ADH proteins sequences (Table 1) for palms species were performed with ClustalX (Thompson et al., 1997) and ESPript (Robert and Gouet, 2014). The number of segregating sites and levels of nucleotide diversity Pi (π), the average number of nucleotide differences per site between two sequences (Nei et al., 1987) and θ, an estimate of 4Neμ, where Ne is the effective population size and μ is the mutation rate per nucleotide (Watterson et al., 1975), were computed in DnaSP (version 5.10.00). Tajima's D test and Fu and Li's D test were also performed in DnaSP for testing selections deviating from neutrality (Librado and Rozas, 2009). The divergence distance of all mRNA date palm sequence was estimated by using the Kimura two-parameter model (Kimura, 1980) employed by PHYLIP (Felsenstein, 2000) with a transition/transversion ratio of 2.0. Estimation of dN and dS values in order to get information about functional constraints on palm ADH sequences, we also estimated the number of synonymous substitutions per synonymous site (dS), and the number of non-synonymous substitutions per non-synonymous site (dN), using PAML yn00 program with default parameters (Yang, 1979) using Yang and Nielsen (2000) method. A distance matrix based on the aligned amino acid sequences was constructed by using the jones taylor thornton method of the PROTDIST program on PHYLIP.

The phylogenetic relationships between the 38 ADH proteins (Table 2) for different species were analyzed using the maximum-likelihood (ML) method. For the ML analyses, we used the PROTML program of PHYLIP version 3.6 (Felsenstein, 2000). We employed the JTT model of amino acid substitution. All indels were counted as missing. We performed ten random sequence addition searches using the J option and global branch swapping using the G option to isolate the ML tree with the best log-likelihood. In addition, we performed boot- strap analysis with 100 replications. To infer the evolutionary events affecting the ADH genes, an analysis using Notung2.8 (Chen et al., 2000) was performed. The ML tree with the highest log-likelihood was used for the gene tree. Both gene duplications and losses were considered to reconcile the gene tree with the species tree. Evidence of recombination was sought by the program RDP4 (Version 4.16) (Martin et al., 2010).

RESULTS AND DISCUSSION

Sequence analysis of isolated DnADH and predicted KhADH proteins

DnADH (ADH gene from *P. dactifera Deglet Nour)* and KhADH (ADH gene from *P. dactifera* L cv Khalas*)* encode proteins of 380 residues, with the predicted molecular weight of 41.14 and 41.18 kDa, and isoelectric points of 6.16 and 6. 59, respectively. The alignment of DnADH with sequences of different palms ADH proteins shows the presence of a large number of conserved domains (Figure 1), that are typical of this sub-family (Chase, 1999). The identity at the amino acid level between DnADH and other palm species ADHs sub-family is very high and ranges between 78 and 91%. The genetic distance between the four proteins is very low (Table 3). Many very well conserved amino acids that have been implicated in the fixation of zinc are present in DnADH: Cys, His and Cys at the 48, 70 and 178 positions (Figure 1) and four Cys at positions 100, 103, 106, 114 (Figure 1) (Eklund et al., 1976; Yokoyama and Harry, 1993). The Asp in position 237, corresponding to DnADH sequence has been described as implicated in the preference of NAD as cofactor in the dehydrogenase reaction (Eklund et al., 1976; Fan et al., 1991).

Divergence of the palms ADH loci

Pairwise distances based on the Kimura two-parameter model for mRNA sequences of four palm ADH loci are given in Table 4. Two points are apparent from this table. One is that the two KhADH and EgADH mRNA are the most similar. The second is that the ADH gene from palms is moderately diverged and must represent duplication event.

Sequence diversity of ADH gene between palm species

Nucleotide polymorphism was examined in four palm ADH mRNA sequences across the entire length of ADH palms mRNA. The examination yielded 48 single nucleotide polymorphisms (SNP) and 2 insertions or deletions (Indels) in this region (1.098 kb). Nucleotide diversity p of the entire mRNA sequence was 0. 15523 and θ was 0. 10594. Several statistical tests were used to test the hypothesis that ADH sequences have been evolving in accordance with expectations under neutral theory. Several statistical tests were used to test the hypothesis that ADH sequences have been evolving in accordance with expectations under neutral theory. The tests of Tajima (- 0.75403) and Fu and Li (-0.34314) compare different estimates of ɵ (4Ne m) and p; they made assumptions that the four ADH sequences have a

Table 2. List of accession numbers and list of taxa used in this study.

Family	Accession number
Eukaryota	
Aspergillus niger	AnADH : XP001395505.2
Core eudicotyledons	
Dianthus caryophyllus	DcADH :AAP96921.1
Rosids	
Vitis vinifera	VvADH1 :NP001268079.1, VvADH2 :NP001268083.1
Sapindales	
Dimocarpus longan	DlADH :ABF61806.1
Mangifera indica	MiADH1 : ADB43613.1, MiADH2 : ADB43614.1
Brassicaceae	
Arabis hirsuta	AhADH :AAF23543.1
Leavenworthia stylosa	LsADH3 :AAC79418.1
Brassica rapa	BrADH1 :AEC13713.1
Fabids	
Alnus glutinosa	AgADH :CAJ21172.1
Cucumis melo	CmADH1 :ABC02081.1
Papilionoideae	
Lotus corniculatus	LcADH1 : AAO72531.1
Trifolium repens	TrADH1 : P13603.1
Glycine max	GmADHL1 :NP001242142.1
Lamiales	
Salvia miltiorrhiza	SmADH : ACZ48689.1
Solanaceae	
Solanum tuberosum	StADH1 : AAA33806.1
Nicotiana tabacum	NtADH : CAA57446.1
Lactuca sativa	LsADH : AFP72378
Arecaceae	
Coryphoideae	
Phoenix dactylifera	DnADH : AHH32692
Washingtonia robusta	WrADH : AAB39597.1
Elaeis guineensis	EgADH :ACF06607.1
Poaceae	
Oryza sativa	OsADH1 : CAA34363.1, OsADH2 : Q4R1E8.1
Aegilops	
Aegilops tauschii	AtADH3 : ABL74268.1 AtADH2 : ABL74260.1
Aegilops speltoides	AsADH1 : ABL74271
Triticum	
Triticum turgidum	TtADH1 : ABL74262
Triticum aestivum	TaADH1 : ABL74258,TaADH2 : ABL74254, TaADH3 :ABL74253
Hordeum vulgare	HvADH1 :AAK49116
Panicoideae	
Cenchrus americanus	CaADH :CAC37632
Andropogoneae	
Miscanthus sinensis	MsADH1 :ADI24330
Zea mays	ZmADH1 :NP001105409 ZmADH2 : NP001105410
Coix lacryma-jobi	CljADH1 : ABE68381

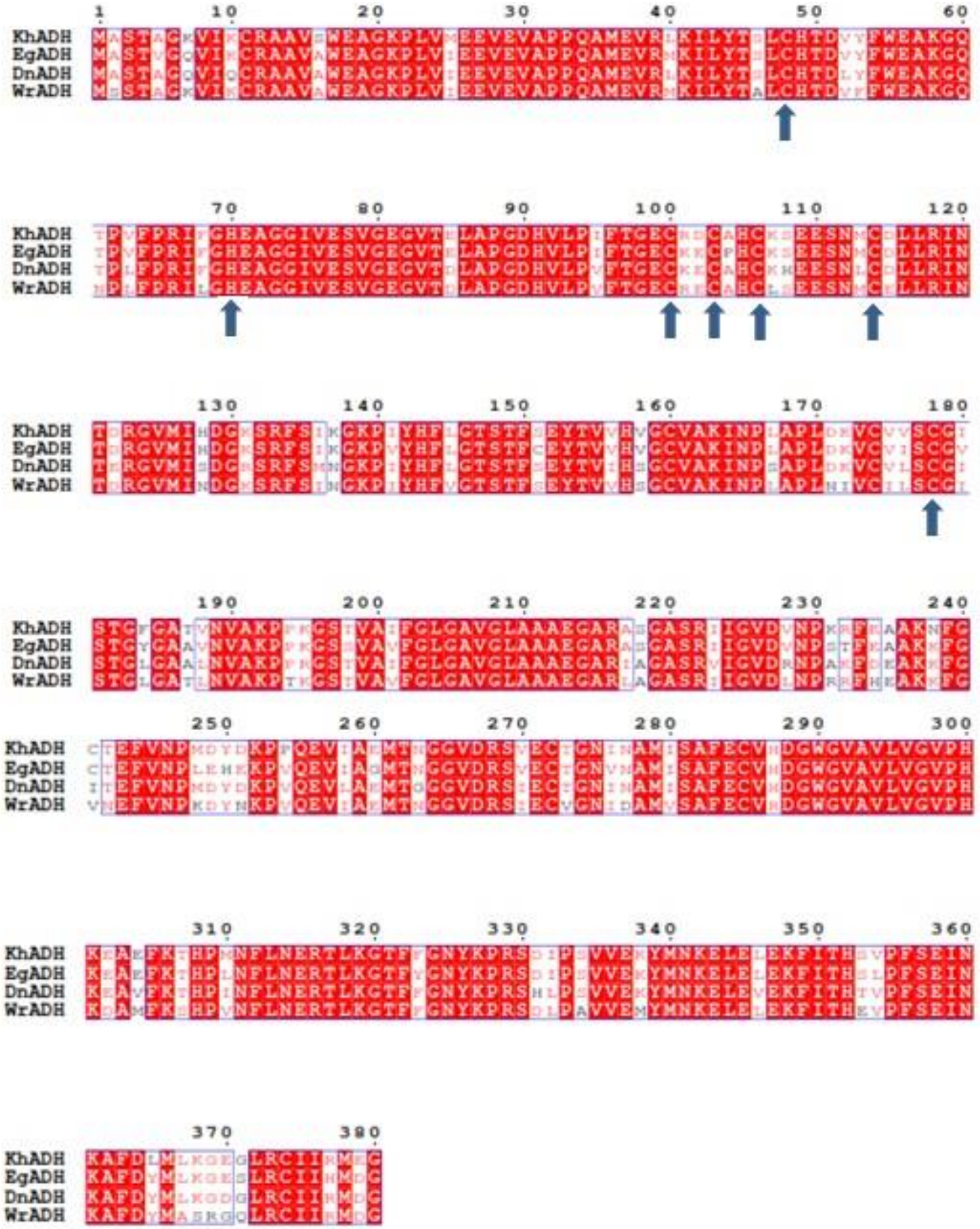

Figure 1. Amino acid sequence alignment of ADH palms sequences using Clustal X and ESPript programs. Conserved residues are shaded in red. The arrows represent the conserved amino acids in short-chain ADHs.

Table 3. Homology percent of amino acid sequence between full length palm ADH protein and protein distance using Jones Taylor Thornton method of PHYLIP program.

Amino acid sequence	KhADH %	EgADH	DnADH	WrADH
KhADH	100	0.090943	0.139001	0.170949
EgADH	91	100%	0.162583	0.197522
DnADH	86	84%	100%	0.168466
WrADH	84	82%	83%	100%

Table 4. Divergences based on Kimura's two-parameter model, between palms ADH mRNA.

	DnADH	KhADH	EgADH	WrADH
DnADH	0.000000			
KhADH	0.202561	0.000000		
EgADH	0.203524	0.086238	0.000000	
WrADH	0.225311	0.221938	0.232504	0.000000

Table 5. Ratio of non-synonymous to synonymous substitutions rate among mRNA palms ADH

Seq	S	N	dN±SE	dS±SE	dN/dS
KhADH-DnADH	285.7	812.3	0.0851±0.0107	0.7850±0.1109	0.202561
EgADH-DnADH	288.2	809.8	0.0969±0.0115	0.6501±0.0823	0.203524
EgADH- KhADH	284.3	813.7	0.0417±0.0073	0.2344±0.0339	0.086238
WrADH-DnADH	288.9	809.1	0.0910±0.0111	0.9349±0.1430	0.225311
WrADH-KhADH	286.2	811.8	0.0914±0.0111	0.8472±0.1205	0.226587
WrADH-EgADH	289.4	808.6	0.1052±0.0121	0.8086±0.1095	0.232504

neutral evolution (Simonsen et al., 1995; Wayne and Simonsen, 1998). None of these tests returned significant *P* values. This is not surprising, given the small number of variable positions and the relatively low statistical power of these tests (Wayne and Simonsen 1998). A primary conclusion of the present study is that nucleotide diversity for ADH between palm species is very low. Estimates reported here are lower than previously reported values not only for plant ADH sequences (Cummings and Clegg, 1998; Liu et al., 1998), but for other plant nuclear genes such as *C1* in maize, (Hanson et al., 1996); *ChiA* in *Arabidopsis* (Kawabe et al., 1997); *ChsA* in *Ipomoea* (Huttley et al., 1997) and *Pgi* in *Dioscorea* (Terachi and Miyashita, 1997). Tests for conversion among the four mRNA palms sequences using RDP v 4.16 with Maxchi program, detected two recombination's: one between DnADH and WrADH (ADH from *Washingtonia robusta*) (KhADH is the recombinant) with a P value of 2.97 10^{-2} and length of 801 nt; one between DnADH and WrADH with EgADH (ADH from *E. guineensis*) is the recombinant (P value of 1.45 10^{-2} and length of 793 nt).

Selection pressure

In order to assess selective pressure, we calculated the ratio of non-synonymous to synonymous substitutions (dN/dS) among mRNA palms ADH. Estimates of dN and dS for the entire coding region between the four palms mRNA are given in Table 5. Comparisons within plant ADH genes show dN/dS < 0.3 (Table 5). For all genes, dS exceeded dN in both comparisons, as would be expected for genes under purifying selection (Nei, 1987). All comparisons with *P* values ≤0.001 remain significant after correcting for multiple tests. The same results were found for the others ADH evolution studies (Yokoyama et al., 1990). By comparing dN/dS ratios of ADH palms genes, we found that the ratio EgADH –KhADH has the lowest value. Therefore, we conclude that ADH genes appear to be under very different selective constraints. This result shows that dN/dS ratios are lower for duplicated genes than for unique genes (Davis et al., 2004; Jordan et al., 2004).

Phylogenetic analyses

The palm family emerged -80 million years ago and as such it represents one of the lineages that radiated early in monocot evolution (Wilson et al., 1990; Duvall et al., 1993). The comparative analysis of these four palms monocot families presents an ideal opportunity to

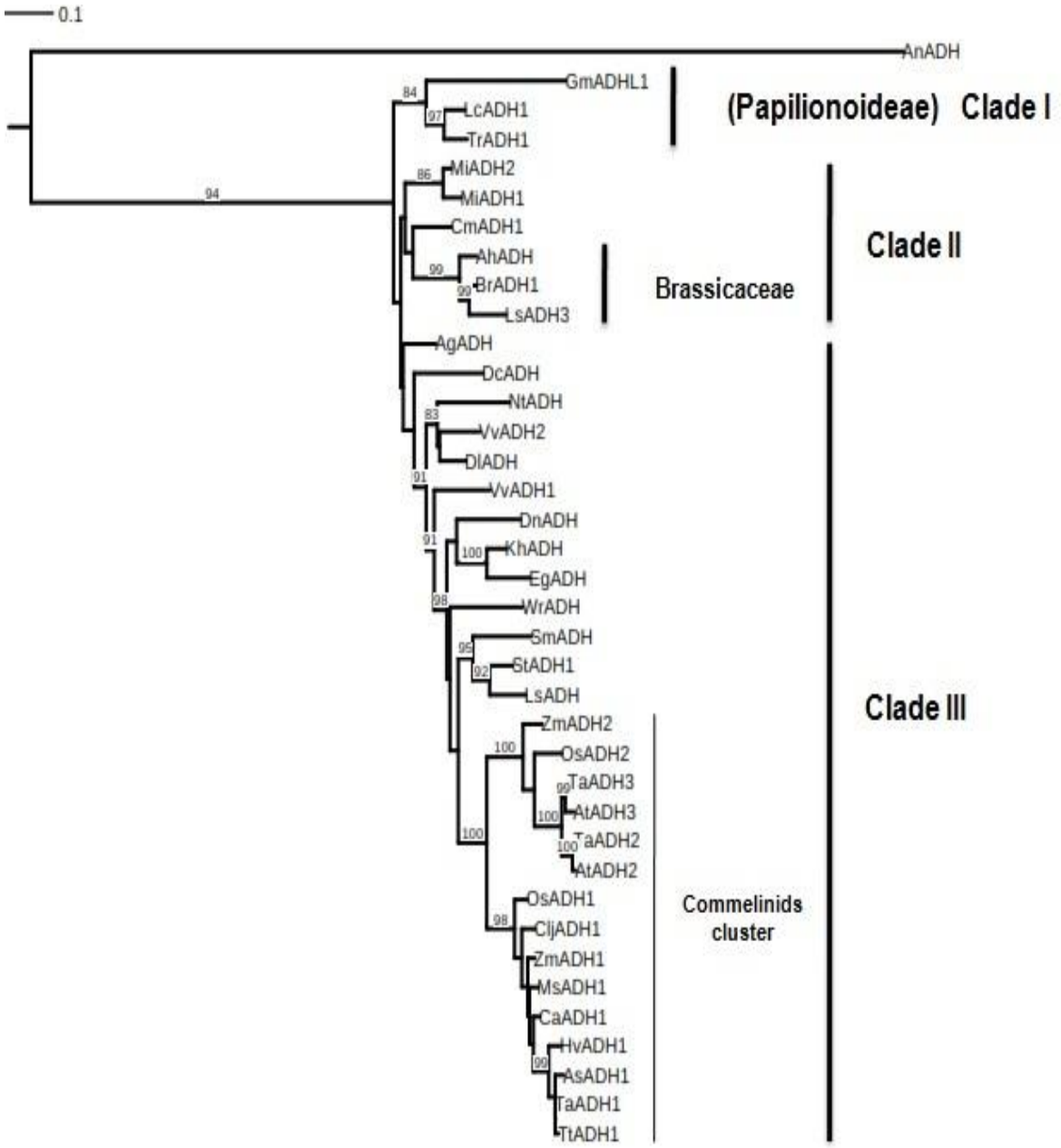

Figure 2. The phylogenetic tree based on ADH gene sequences obtained by the maximum-likelihood method. The log-likelihood of the best ML tree is -3981.05. The numbers below the branches are the bootstrap values of 50% or more support. The ADH genes from plants roughly fall into two clades that we denoted as Clade I and Clade II.

investigate the dynamics of angiosperm gene family evolution, and in particular, to expand our understanding of the evolution of the ADH gene family. A previous analysis of ADH has provided evidence against ADH genes in grasses emerging from a single duplication event early in the evolutionary history of the angiosperms (Yokoyama and Harry, 1993). We conducted phylogenetic analyses of the ADH genes using sequence from *Aspergillus niger* as outgroups. To determine the phylogenetic position of the palms ADH genes isolated and predicted in this study, we subjected their sequences to ML analysis by employing a data set including the previously published ADH gene family sequences from various phylogenetic groups (Clegg et al., 1997; Small et al., 2000). Our resulting ADH gene tree roughly consisted of three monophyletic groups that we denoted "Clade I","Clade II" and Clade III (Figure 2). Clade I contains only ADH genes from Papilionoideae species, while Clade II contains ADH genes from rosids species which contains the Brassicaceae species. The palms ADH proteins appeared with eudicots species in Clade III and not with the Commelinids cluster (monocots) (Figure 2).

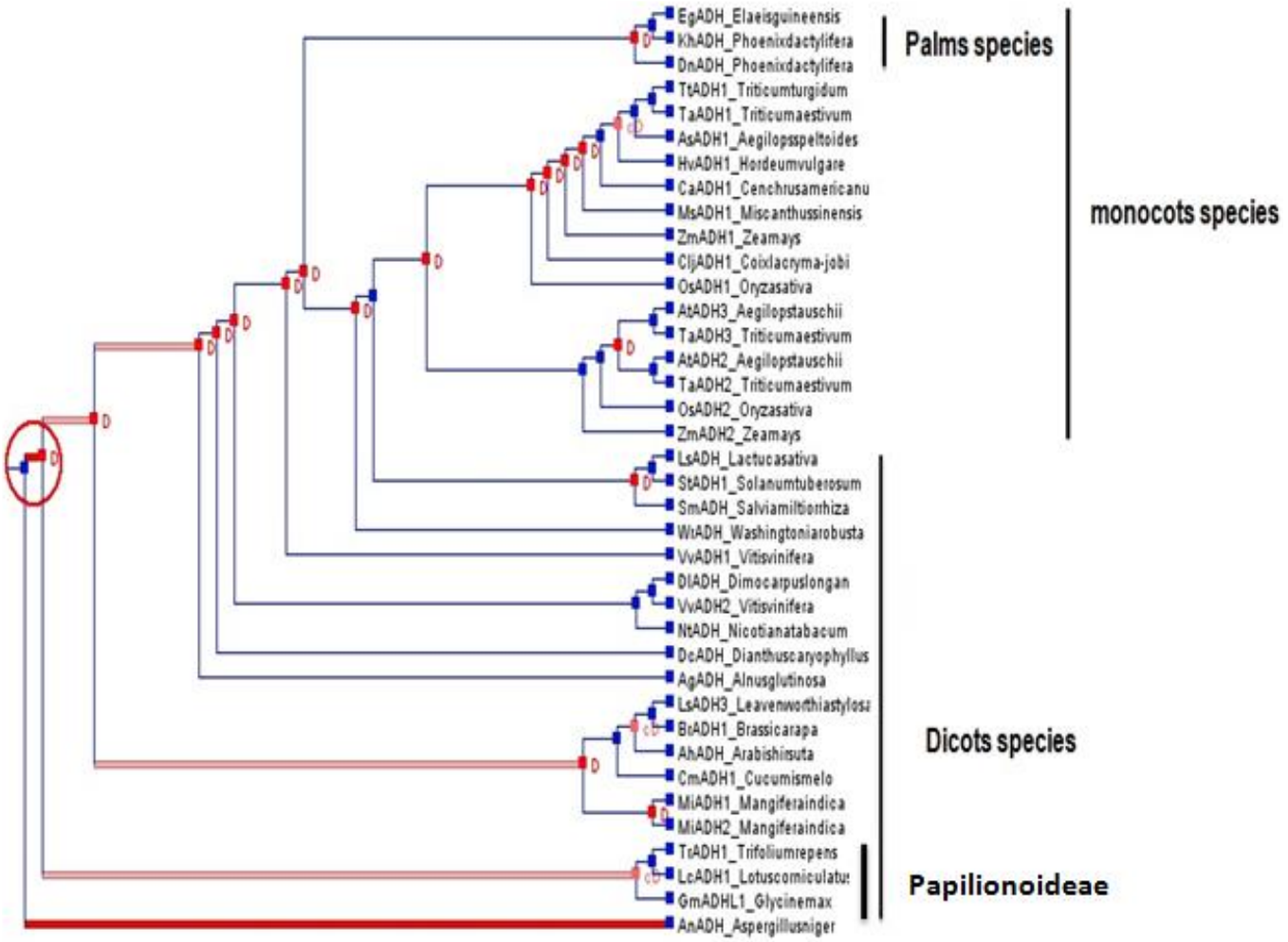

Figure 3. Reconciled tree for the ADH plants family**.** The ML tree of ADH proteins (figure 2) was reconciled using Notung 2.8 with a species tree complied from a phylogeny of model organisms. The reconciled tree involves 18 gene duplications (D) and 3 gene co-duplication (cD).The solid red and pink boxes indicate gene duplications that were inferred on the basis of mismatches between the gene tree and the species tree.

Notung2.8 analysis using the ADH gene sequences suggested that the first ADH gene divergence event shown in Figure 3 by a circle separate monocot and dicots species from Papilionoideae species. The palms ADH proteins diverged within the monocots species (Figure 3) except WrADH and before the others monocots species (Figure 3) (Strommer, 2011). Our analysis revealed that in palms, the divergence of WrADH genes occurred after the others palms species diverged. This study revealed the complicated evolution of the ADH gene family that occurred during the course of plant diversification.

In our study, the phylogenic tree resulting from Notung 2.8 analysis showed that some ADH genes in flowering plants evolved in complex manner that included several duplication events (Figure 3). Duplication events in ADH genes have also been detected in other plant groups at various evolutionary levels. For example, we revealed duplication events in *A. thaliana* ADH copies (AtADH2 and AtADH3) and *Triticum astivum* ADH copies (TaADH2 and TaADH3) (Figure 3). Sang et al. (1997) showed that diploid species of *Paeonia* (Paeoniaceae) had two or three ADH sequences and that repeated duplication or deletion events occurred after the diversification of this genus. Small and Wendel analyzed ADH genes in *Gossypium* (Malvaceae) in great detail and found that these ADH sequences had experienced duplication events both before and after the divergence in *Gossypium*. Duplicated genes arise frequently in eukaryotic genomes through local events that generate tandem duplications, large-scale events that duplicate chromosomal regions or entire chromosomes, or genome-wide events that result in complete genome duplication (Dujon et al., 2004). Indeed, the existence of multigene families is evidence of the repeated gene duplication that has occurred over the history of life. One

of the examples of the comprehensive analysis of gene duplication events in plants is the study of the MADS-box gene family. This gene family, which plays a central role in the morphogenesis of plant reproductive organs such as ovules and flowers, had experienced duplication events before the origin of angiosperms (Theissen et al., 2000). Moreover, some specific functions were gained through duplication events that took place after the diversification of flowering plants (Theissen et al., 2000). Thus, gene duplication has long been recognized as an important mechanism for the creation of new gene functions (Wagner, 1998; Wagner, 2001). It is likely that each of the ADH genes in the palms that were identified in the present study would have been subjected to different selective pressures over a long period. To determine whether this resulted in new functions, functional analysis of the palms ADH genes in each clade will have to be performed in the future.

Conclusion

The *Adh* genes in the date palm that were identified and analysed in the present study would have been subjected to different selective pressures over a long period. This is the first report revealing that palms species have a ADH genes loci belonging to the same clade. Phylogenetic analyses suggest that these genes resulted from relatively ancient divergence and duplication events.

Conflict of interests

The authors did not declare any conflict of interest.

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Responses of potatoes plants inoculated with arbuscular mycorrhizal fungi and litter in greenhouse

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A pot experiment was set to examine the impact of the foliar litter (*Hardwickia binata* and *Azadirachta indica)* and an *arbuscular mycorrhizal* (AM) fungus on the development of two varieties of potato plants (Aida, Atlas). Three litter doses (0, 25 and 50 g) were applied to the pots after bedding plantlets. The plants were inoculated with AM, *Glomus aggregatum*. Mycorrhizal colonization, shoot dry weight, size and number of minitubers were evaluated after 12 weeks on the potato growth. Results show that shoot dry weight of plants was improved by litter of the *H. binata* at 25 and 50 g. Thus, *A. indica* litter increased size of plants Aida at 50 g and the minitubers numbers Atlas at 25 g. On the other hand, root colonization decreased with increase in the dose of litter with both varieties of potato.

Key words: Arbuscular mycorrhizal fungi, potato, litter, micropropagation.

INTRODUCTION

Plant residues are an important source of nutrients (Musvoto et al., 2000). Leaf litter makes it possible to restore soils by a vertical transfer of minerals (Feller, 1995). The use of litter also stimulates the activity and development of soil microorganisms by a direct effect with the addition of carbon substrate in soil-vegetation systems (Vance and Chapin, 2001). The capacity of high fertilization of the litter is related to the type of organic matter use (Larkin and Tavantzis, 2013). Many tropical trees are used for agricultural purposes. The leaves of trees of *Hardwickia binata* Roxb, *Azadirachta indica* A. Juss, *Faidherbia albida* are used as green manure in Senegal. Some leaf litters used (*Andropogon gayanus*, Kunth and *Eragrostis tremula*, Steud) do not provide significant organic reserves whereas those of *F. albida* and *A. indica* show a high potential to improve the growth of the plants (Diallo et al., 2008).

For the plants like potato (*Solanum tuberosum*), which have a low root density and a strong potential of growth, the arbuscular mycorrhizal symbiosis may be of particular significance in coping with phosphorus and water deficiency stress in tropical soils. Arbuscular mycorrhizal

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Abbreviations: MS, Murashige and Skoog; **AM,** arbuscular mycorrhizal; **LBC,** laboratory of fungal Biotechnologies; **MC,** mycorrhizal colonization; **SDW,** shoot dry weight; **NM,** number of minitubers; **SM,** size of minitubers.

Table 1. Characteristics of the soil used.

Component	Contents (%)
Clay	3.6
Silt	1.6
Fine silt	2.9
Fine sand	51
Coarse sand	40.9
Organic matter	1.06
Total carbon	2.5
Total nitrogen	0.3
Available phosphorus	3.1
pH (sol/water ratio 1:2)	6.7
pH (sol/KCl ratio 1:2)	4.5

(AM) fungi is known to increase yield and mineral nutrition. Their association makes it possible to supplement the nutrition of the plant in limiting elements (Diallo et al., 2010). Mycorhization of the potato vitroplants is compatible with P fertilization (Ndiaye et al., 2003). AM fungi can be ranked as *Glomus aggregatum*, *Glomus mosseae, Glomus versiforme* for improving yield as well as nitrogen, phosphorus, and potassium acquisition of *Solanum* cultivar (Diop et al., 2003). However, little information is available on the interactions between AM fungi with organic fertilization based on litter. Understanding this compatible is a trump to potatoes production.

The aim of this study was to investigate the effect of dual inoculation with fungi AM and litter on two varieties of micropropagated potatoes in greenhouse.

MATERIALS AND METHODS

Soil

Soil used in this study was collected at 5 to 20 cm depth from Sangalkam, (50 km from Dakar, Senegal). Soil was sterilized by autoclaving at 12°C for 1 h. Soil characteristics are given in Table 1.

The plant material

The plant material consisted of potato tubers of two varieties (*S. tuberosum*), Atlas and Aida imported from GERMICOPA SA. (Quimper, France). These varieties are well adapted to agroclimatic conditions of Senegal. Dormancy was removed by chemical treatment (Bryan, 1989). Tubers were removed from the solution, dried and placed in a sealed chamber, dark and airy at 25°C until germination. After sprouting, the germs of 1 to 2 cm in height were gently lifted tubers using a sterile scalpel and closed at their ends by dipping in a bath of liquid paraffin at 40°C. Disinfection of germs was carried out in a host of laminar airflow. First, they were immersed in distilled water with 20 drops of Tween 80 for 10 min water and then pre-soaked for 10 s in alcohol at 70°C before putting them in a solution of mercuric chloride ($HgCl_2$) to 0.1% for 10 min. After disinfection, germs wiped and recovered, were sterilized on Whatman paper. Briefly, germs were placed in sterile culture glass tubes (22 × 150 mm) filled with 15 ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) while respecting the apical-basal polarity original. This medium solidified with agar (8 $g.l^{-1}$) was devoid of growth regulators and adjusted to pH 5.9 before sterilization (120°C for 30 min). The culture tubes were placed in a growth chamber (temperature 28°C, 16 h day photoperiod, light intensity 101.4 $\mu moles.m^{-2}.s^{-1}$, relative humidity 55%). After 4 weeks of culture, the explants formed, were cut into as many nodal cutting (3 to 7) and transplanted in jars with a capacity of 660 ml. Each jar contained 50 ml of MS medium. Older plantlets (10 days) and measuring approximately 7 cm were used as plant material (Figure 1).

Mycorrhizal inoculum

Mycorrhizal inoculum containing indigenous species of *G. aggregatum* was obtained from Laboratory of Fungal Biotechnologies (LBC) of the Plant Biology Department (University Cheikh Anta Diop / Senegal) was multiplied by using maize as host plant. Mycorrhizal inoculum consisted of rhizospheric soil mixture from pure culture containing spores, hyphae and mycorrhizal root fragments (an average of 40 spores per gram and 85% of roots infected) were used for the experiment.

Litter

Two trees litter were selected: *A. indica* A Juss (*Meliaceae*) and *H. binata* Roxb (*Fabaceae*). This tree species were chosen based on their availability and their leaves have good anti-microbial activity. Also, both trees are considered to be adaptable to diverse habitats and climatic conditions. Their leaves were taken when they fall between April and June. The leaves were immersed in 15% hydrogen peroxide solution for 90 s to reduce bacteria and fungi without phytotoxicity and dried in the host open air. Two weeks after, they were crushed to 0.75 mm fraction to accelerate decomposition upon contact with the ground and then stored in bags (Recous et al., 1995). Plant residues (carbon, nitrogen, hemicellulose, cellulose and lignin) were determined by the method of Van Soest (1963). The physical-chemical characteristics of litter used are given in the Table 2.

Experimental procedure

This study was conducted in a greenhouse of the department of Plant Biology (Cheikh Anta Diop University/Senegal) during 3 months. Soil was sterilized by autoclaving at 120°C for 1 h. All underwent incubation for seven months to accelerate the process of mineralization. Potato plant of two varieties, Aida and Atlas, were transplanted to the plastic pot with the same soil (5 kg). Each pot contained one plant. During this process, plants were inoculated with AM fungi *G. aggreegatum* by adding 20 g of inoculum directly in contact with the roots. Pot was laid in randomized block with ten replicates. Three factors were studied: (i) inoculation; (ii) litter and (iii) variety. Plants were grown in greenhouse with the following conditions: day/night cycle of 12/12 h, 32/25°C and 40 to 50% air humidity. Plants were irrigated with tap water.

Measured parameters

After sowing, roots and shoots were harvested separately. Fresh roots were taken to evaluate root colonization. Roots were first cleared with 10% KOH at 50°C for 60 min and then stained with 0.05% Trypan Blue at 50°C (Philips and Hayman, 1970). Mycorrhizal colonization (MC) root infection was evaluated under a

Figure 1. Older plantlets of potatoes Atlas on MS (Murashige and Skoog) medium 10 days in culture chamber (temperature of 28°C; 16 h day photoperiod, light intensity of 101.4 µmoles.m^{-2}.$s^{-1}$and relative humidity of 55%).

Table 2. Physical-chemical characteristics of leaves litter *Azadirachta indica* and *Hardwickia binata* used in the study.

Litter	Lignin (%)	Cellulose (%)	Hemicellulose (%)	C (mg/g)	N (mg/g)	C/N
Azadirachta indica	22	20.1	11	440.6	14.5	30.3
Hardwickia binata	18.3	17.6	11.3	424.1	21.0	20.2

binocular microscope by grid-line intersect method according to Giovannetti and Mosse (1980). The shoot dry weight (SDW) was measured after oven-drying at 75°C for 72 h. The minitubers were counted and means of number of minitubers per plant (NM) were calculated. The average size of minitubers (SM) per plant was measured using a caliper. Analysis of variance was carried out with the software XLSTAT (version 13.2). The comparisons between the averages were done using the software by Fischer LSD test at 5%.

RESULTS

Litter effect on plant development

A statistical analysis performed shows that the litter has a significant effect on biomass plants potato mycorrhizal (R^2 = 0.96, p <0.001). For both varieties, the contribution of litter (*H. binata* or *A. indica*) increased significant shoot dry weight. The application of litter *H. binata* at a dose of 50 g, provides the greatest biomass (1654.00 mg) in the mycorrhiza Atlas range (Table 2).

Litter effect on yield

The litter input causes a significant increase in the number of minitubers products by mycorrhized potato seedlings. Litter *H. binata* gave the largest number of minitubers (3.83 to 25 g) (Table 3). In plants both

Table 3. Shoot dry weight (SDW), Number of minitubers of plant (NM) and Size minitubers (SM) of plant potato (Atlas, Aida) during three months under different treatment of litter (*Azadirachta indica, Hardwickia binata*) inoculed or not with *Glomus aggregatum*.

Inoculation Treatment	Litter treatment (g)	Atlas			Aida		
		SDW (mg)	NM	SM (cm)	SDW (mg)	NM	SM (cm)
M+	Control	661^{e}	2.10^{d}	2.14^{c}	220^{g}	2.06^{d}	1.53^{def}
	Hb25	698^{d}	2.80^{a}	3.39^{a}	610^{b}	3.83^{a}	1.54^{de}
	Hb50	1654^{a}	2.50^{b}	3.41^{a}	960^{a}	2.87^{b}	1.82^{cd}
	Ai25	758^{c}	2.75^{a}	2.45^{b}	412^{d}	2.50^{c}	2.80^{b}
	Ai50	1584^{b}	2.30^{c}	2.40^{b}	541^{c}	2.87^{b}	3.17^{a}
M-	Control	418^{h}	1.30^{f}	1.20^{g}	194^{h}	1.13^{e}	1.04^{g}
	Hb25	470^{g}	1.41^{f}	1.33^{e}	204^{h}	1.41^{e}	1.18^{fg}
	Hb50	475^{g}	1.45^{f}	1.35^{de}	195^{h}	1.41^{e}	1.45^{fef}
	Ai25	513^{f}	1.73^{e}	1.27^{f}	256^{f}	1.36^{ef}	1.67^{de}
	Ai50	515^{f}	1.65^{e}	1.37^{d}	277^{e}	1.53^{e}	2.02^{c}

In column, values followed by the same letters are not significantly different (Fischer's protected LSD P < 0.05). M+ = Inoculated with *Glomus aggregatu;* M- = non inoculated with *Glomus aggregatum.* Hb25=*Hardwickia binata* 25 g litter; Hb50=*Hardwickia binata* 50 g; Ai25= *Azadirachta indica* 25 g litter; Ai50=*Azadirachta indica* 50 g l.

Table 4. Root colonization mycorrhizal rate of potato plants (Aida, Atlas) during three months at different litter addition levels of *Azadirachta indica* and *Hardwickia binata*.

Litter treatment	Inoculation treatment	Mycorrhizal colonization (%)	
		Aida	Atlas
Control	M-	0	0
Hb0	M+	21.66^{a}	23.33^{b}
Hb25	M+	13.33^{c}	14.66^{c}
Hb50	M+	08.00^{d}	08.00^{f}
Ai0	M+	16.27^{b}	24.16^{a}
Ai25	M+	09.33^{d}	13.50^{d}
Ai50	M+	07.33^{d}	08.83^{e}

In column, values followed by the same letters are not significantly different (Fischer's protected LSD P < 0.05). M+ = Inoculated with *Glomus aggregatum;* M- = non inoculated with *Glomus aggregatum.* Hb25=*Hardwickia binata* 25 g litter; Hb50=*Hardwickia binata* 50 g; Ai25= *Azadirachta indica* 25 g litter; Ai50=*Azadirachta indica* 50 g litter.

varieties, the average size of minitubers increased when the amount of litter *H. binata* increased. With the addition of litter A. *indica*, only the size of the Atlas minitubers variety improved with the increase of litter (Table 3).

Litter effect on mycorrhizal colonization

A mycorrhizal root colonization was influenced by the amount of litter made. It gradually decreased with increasing amount of litter. The litter input inhibits root colonization of potato by AM fungi *G. aggregatum* (Table 4). Control plants have obviously not been colonized by *G. aggregatum.* Analysis of variance showed a significant interaction between inoculation litter and variety (Table 5).

DISCUSSION

The litter has a stimulatory effect on the biomass, the number and size of minitubers. This stimulation of growth can be explained by a greater availability of minerals. Indeed, litter stimulates the activity and diversity of soil microorganisms (Shiralipour et al., 1992; Carpenter-Boggs et al., 2000). In turn, this microbial community degrades organic matter and release mineral elements to promote the development of plants (Samba, 2001; Diallo et al., 2005). Although, both bacteria and fungi contribute to litter decomposition, fungi are thought to use available C substrates more efficiently than bacteria. In our experimentation, this is the *G. aggregatum* that ensure this role, AM fungi may be involved both in decomposition processes and in the capture of the less mobile amino-

Table 5. Effects of different factors interactions on variables based on analysis of variance; root mycorrhizal colonization (MC), shoot dry weight (SDW), size minitubers (SM) and number of minitubers of plant (NM).

Factor	MC	SDW	SM	NM
R^2	0.967	0.999	0.935	0.941
F	152.465	6111.385	75.008	83.381
Pr > F	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Variety*G. *aggregatum**litter	152.465 < 0.0001	6111.385 < 0.0001	75.008 < 0.0001	83.381 < 0.0001

acids or ammonium ions. AM fungi hyphal were shown to facilitate enhancement of N capture from the litter, the N gain in the plants being linearly related to hyphal density in the organic matter (Read and Moreno, 2003). Both litters used also have a positive impact on the performance of potato mycorrhiza earth. However, that stimulatory effect of litter *H. binata* is more significant than *A. indica*. The same trend was obtained by Diallo et al. (2008) on the growth of maize and millet. This can be explained by the higher C/N ratio in *A. indica* than *H. binata.* In fact, when the nitrogen in the soil is low, microorganisms assimilation of soil mineral nitrogen reduces and crop yields decline. So, the nature of the litter determines its efficiency, due to the variable biochemical characteristics of plant residues (Williams, 1974) which interesting correlates to the anti-microbial activity of both the trees with the arbuscular mycorrhizal fungi.

Micropropagated potato plants can benefit from inoculation with AM fungi (Vosátka et al., 2000). These finding are also supported by our recent observations. Our results have showed that, the litter inhibits mycorrhizal roots of potato at 25 g. This inhibition increases with the amount of litter made. Impact is more significant at Atlas with litter *A. indica*. Several authors demonstrated that the mycorrhizal colonization can decrease in fertile medium (Duke et al., 1994). The release of soluble sugars in the decomposition of lignin litter is another nutrient for the fungus. This can happen for carbon plant and directly meet the needs of sugars from the litter. Thus, the fungi increasing decomposition of litter and the total P uptake by the plant is as important as when the mycorrhizal contribution was supplied with P in organic form (Read and Moreno, 2003).

We found that the decline of mycorrhiza is a concomitant expression of good performance metrics of the potato. This once again confirms that the benefits of mycorrhizae are not always related to a more intense roots colonization inside (Plenchette et al., 1982). These benefits may be explained by a better decomposition of organic matter or a good viability inoculation of *G. aggregatum* (Schädler et al., 2010). Inoculation of micropropagation potato plants with AM fungi during the transfer from *in vitro* conditions may improve the viability of potato and their physiological state (McArthur and Knowles, 1993; Ndiaye et al., 2005). This viability may be increased by activity of antimicrobial of the litter of both trees. The addition of litter is compatible with potato mycorrhizae. However, to increase the potential of the inoculation in practical production of potato, it is necessary to consider the growth response of different potatoes varieties, as well as appropriate combination of litter and AM fungi. Also, biochemical tests will be determined to see nutritional contents of *S. tuberosum*.

Conclusion

Our experiment shows that the contribution of organic fertilizers based on litter *H. binata* or *A. indica* is compatible with a good expression of the mycorrhizal roots of potato obtained by micropropagation. This beneficial effect is a function of the dose and the nature of the litter made. *H. binata* litter has a significant positive impact on the biomass and size of minitubercules. The results of this study contribute to the understanding of the biological processes involved in litter decomposition with AM fungi.

Conflict of interests

The authors did not declare any conflict of interest.

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Induced polygenic variability using combination treatment of gamma rays and ethyl methane sulphonate in blackgram (*Vigna mungo* (L.) Hepper)

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Induced mutation in plant improvement has been proven to be one of the alternative ways to generate new sources of genetic variation in blackgram. In this study, dry seeds of VBN 4 blackgram were treated with combination treatment of both gamma rays (400, 500 and 600 Gy) and ethyl methane sulphonate (EMS) (50, 60 and 70 mM) to study the polygenic characters in M_2 generation. The mean values for plant height, number of primary branches, number of clusters per plant, number of pods per plant, number of seeds per pod and single plant yield decreased below the control in most of the treatments. The mean single plant yield was more than for the control at 400 Gy+60 mM and 600 Gy+50 mM. Moderate and high phenotypic coefficients of variation (PCV) and genotypic coefficients of variation (GCV) were recorded in yield component characters such as plant height, number of primary branches, number of clusters per plant and number of pods per plant. A high amount of heritability and GA as per cent of mean was noted for plant height, number of clusters per plant, number of primary branches per plant, number of pods per plant, pod length, number of seeds, 100 seed weight and single plant yield. This denoted that these characters are governed largely by additive gene effect, which may be favorably exploited for improvement through simple selection in M_2 generation.

Key words: Gamma rays, ethyl methane sulphonate (EMS), blackgram, polygenic variability, M_2 generation.

INTRODUCTION

Pulses constitute an important role in human dietary. They are important source of protein and are essential adjuncts to a predominantly cereal based diet and enhance the biological value of protein consumed. Pulses are often attributed as “poor man’s diet,” which are really important in Indian diet as a source of protein. Though pulses contributed significant role in human consumption but they have not yet reached a comfortable level of production. Blackgram [*Vigna mungo* (L.) Hepper], one of the important proteinaceous pulse crop grown largely in

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Abbreviations: EMS, Ethyl methane sulphonate; **RBD,** randomized block design; **GA,** genetic advance; **PCV,** phenotypic coefficients of variation; **GCV,** genotypic coefficients of variation.

India, needs crop improvement to a considerable extent. It contains about 26% protein, which is almost three times more than cereals. It belongs to the family leguminosae and subfamily papilionaceae. The chromosome number of this crop is 2n=2x=22 (Bhatnagar et al., 1974). It is a highly self-pollinated crop. In India, it covers an area of about 3.24 million hectares and produces 1.46million tonnes. Its productivity is only 526 kg per ha. In Tamil Nadu, blackgram covers an area of about 3.41 lakh hectares with production of 1.21 lakh tonnes and 355 kg per ha. For any successful breeding programme, the variability is the basic tool on which the selection is exercised for genetic improvement. Creation of variability through pollination and artificial hybridization is very difficult in this crop as the flowers are cleistogamous and delicate to handle. Even if hybridization is carried out the seed set is less than 5%. Also, this crop lacks proper male sterility system commercially to be utilized for hybridization (Anbu Selvam et al., 2010).

Research on blackgram is lagging behind than that of cereals and other legumes (Arulbalachandran and Mullainathan, 2009). In order to improve yield and other polygenic characters, mutation breeding can be effectively utilized (Deepalakshmi and Ananda Kumar, 2004). Induction of mutation forms an important part of breeding programme as it widens the gene pool through creation of genetic variability. According to Raje and Rao (2000), genetic variability is essential in order to realize response to selection pressure as the estimates of genetic parameters of variation are specific for a particular population and the phenotypic expression of the quantitative character may be altered by environmental stress that affect plant growth and development. Therefore, an attempt has been made to study the magnitude of variability through induction of mutation using both gamma rays and ethyl methane sulphonate (EMS) and their combinations.

MATERIALS AND METHODS

Blackgram variety Vamban-4 (VBN 4) was selected to induce mutation by combination treatment of gamma rays and EMS to analyze polygenic variability in M_2 generation. VBN 4 is resistant to Recent Developments on yellow mosaic virus (YMV), late senescence suitable for all seasons (June to July, September to October, and February to March). This study was carried out in Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Madurai during the year 2009 to 2011.

Induction of mutation: fixing LD_{50} value under laboratory

Physical mutagen (gamma rays)

For fixing LD_{50} value of physical mutagen, nine sets containing 75 well filled seeds were treated with gamma rays (100 Gy to 900 Gy with an interval of 100 Gy) in the gamma chamber installed at the Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore where cobalt-60 serves as source of gamma rays. Non-irradiated dry seeds were also taken as control.

Chemical mutagen (EMS)

Five different concentrations of EMS ranging from 30 to 70 mM with 10 mM interval were used initially to fix LD_{50} value. Five sets containing 75 well filled seeds were presoaked for 16 h in distilled water initially (Malarkodi, 2008). Then soaked seeds were treated with EMS for 6 h. A sample of 150 seeds was soaked in distilled water for the respective duration to utilize it as control. After soaking the seeds in the mutagen, they were thoroughly washed in running tap water for half an hour. The seeds were then subjected to germination test. Based on the effect of physical and chemical mutagen on germination, LD_{50} value was obtained.

Combination treatment (gamma rays + EMS)

Based on the LD_{50} value of both the mutagens, initially the seeds were exposed to gamma irradiation and then same seeds were treated with EMS following the same procedure as mentioned above. Non-irradiated wet seeds are also taken to utilize it as control for combination treatment.

Raising M_1 generation

The LD_{50} values for the mutagens were worked out based on observations recorded on seed germination under laboratory conditions. 50% reduction in germination was obtained at 500 Gy for gamma rays and at 60 mM for EMS treatments. Based on this, the mutagenic doses studied under field condition for combination treatments *viz.*, 400 Gy + 50 mM, 400 Gy + 60 mM, 400 Gy + 70 mM, 500 Gy + 50 mM, 500 Gy + 60 mM, 500 Gy + 70 mM, 600 Gy + 50 mM, 600 Gy + 60 mM and 600 Gy + 70 mM. For M_1 generation, in each treatment a total of 150 seeds were sown in the field along with control in a Randomized Block Design (RBD) with three replications by adopting a spacing of 30 cm between rows and 15 cm between plants. Recommended agronomic practices and plant protection measures were followed as per crop production manual (TNAU and Department of Agriculture).

Raising M_2 generation

The M_2 generation was raised on M_1 plant basis following plant to progeny method in a RBD with three replications. Thirty plants per treatment were forwarded from the M_1 to the M_2 generation. Data on various quantitative traits *viz.,* seed yield (g), plant height (cm), days to 50% flowering (days), primary branches number, cluster number, pod number, number of seeds per pod, pod length (cm) and 100 seed weight (g) were taken and analyzed statistically to estimate the PCV, GCV (Burton, 1952), broad sense heritability (h^2) and genetic advance (GA) as per cent of mean (Johnson et al., 1955).

RESULTS AND DISCUSSION

Effect of population mean

Aastveit (1968) and Scossiroli (1977) suggested that an estimation of the extent of induced genetic variability in quantitative traits in M_2 itself would provide valuable information for designing selection programme. It is therefore, considered worthwhile to study the shift of mean values and gather information on induced genotypic variance, heritability and GA as per cent of mean for different quantitative traits in the M_2 families of different

Table 1. Mean performance of blackgram in relation to different concentration of gamma rays + EMS in M_2 generation.

Gamma rays (Gy) + EMS (mM)	Plant height (cm)	Days to 50% flowering	Number of primary branches	Number of clusters per plant	Number of pods per plant	Number of seeds per pod	Pod length (cm)	100 seed weight (g)	Yield / plant (g)
Control	25.40	39.13	2.00	7.36	15.80	5.82	5.20	4.57	4.75
400 Gy + 50 mM	26.71	38.95	2.20	7.98	12.15	5.60	4.73	3.54	4.15
400 Gy + 60 mM	22.41	40.00	2.15	6.80	9.92	6.35	4.99	4.25	4.88
400 Gy + 70 mM	20.84	41.99	1.40	9.89	15.35	6.40	4.49	3.23	4.18
500 Gy + 50 mM	27.14	38.80	2.40	7.65	8.98	5.50	3.93	4.14	3.39
500 Gy + 60 mM	22.80	41.35	1.75	7.58	19.85	5.35	4.94	4.65	4.68
500 Gy + 70 mM	24.59	39.90	1.33	6.90	12.64	5.20	5.04	3.99	3.04
600 Gy + 50 mM	26.70	42.69	2.00	8.25	18.65	4.60	4.58	2.77	5.01
600 Gy + 60 mM	23.30	38.87	1.95	6.88	11.00	5.95	3.95	4.51	4.75
600 Gy + 70 mM	26.03	39.00	1.51	7.50	9.72	5.77	4.92	3.77	4.82

mutagenic treatments. The mean values of the treatments are presented in Table 1. The study reveals that, the mean values for plant height, number of primary branches per plant, number of clusters per plant, number of pods per plant, number of seeds per pod and single plant yield decreased below the control in most of the treatments. Similar results were reported in blackgram (Vanniarajan, 1989), cowpea (Palanisamy, 1975), greengram (Krishnaswami, 1977), redgram (Natarajan et al., 1982) and lentil (Sinha and Lal, 2007).

According to Prema et al. (1998), the mean values for plant height, number of pods per plant and single plant yield decreased than the control in all the mutagenic treatments in blackgram. In the present study, the mean single plant yield was more than for the control at 400 Gy + 60 mM and 600 Gy + 50 mM doses. This was in confirmation with the results of Singh et al. (2000) in blackgram. Samiullah et al. (2000) in greengram reported increased mean values for number of branches per plant, Khan et al. (1994) for number of pods per plant and plant height and Tickoo and Chandra (1999) for number of seeds per pod and yield per plant in greengram. Murugan and Subramanian (1993) reported that mean of most of the polygenic traits was shifted in both the direction in cowpea. The changes in the mean values after mutagenic treatments has been reported earlier in many pulse crops including mungbean (Wani et al., 2005, Arulbalachandran and Mullainathan, 2009), lentil (Singh et al., 2006) and urdbean (Deepalakshmi and Ananda Kumar, 2003).

Variability

Knowledge on genetic variability of the available population is very essential for any crop improvement programme, as it positively enhance the efficiency of selection. Gaul (1964) stated that radiation induced variability could be determined in M2 generation whereas Borojevic and Borojevic (1969) opined that variability increased in wheat up to four times in M2 and M3 which decreased in M4 and stabilized around M5 generation. Increase in variability following mutagenic treatments was reported by Kharkwal (2000), Khan et al. (2004) and Sengupta and Datta (2004). In order to know the breeding utility of this variability and selection value of various quantitative traits, it is essential to determine various components and heritable proportion of variability.

In general, PCV values were more than GCV values (Figure 1). This indicates that the apparent variation was not only due to genotypes, but also due to the influence of environment on the expression of the characters. The characters plant height at maturity, number of primary branches, number of clusters per plant and number of pods per plant recorded moderate and high PCV and GCV. The low, moderate and high PCV and GCV were observed for number of seeds per pod, pod length, 100 seed weight and seed yield per plant in the mutated population. Low PCV and GCV were recorded in days to 50% flowering (Table 2). Similar results were reported in blackgram

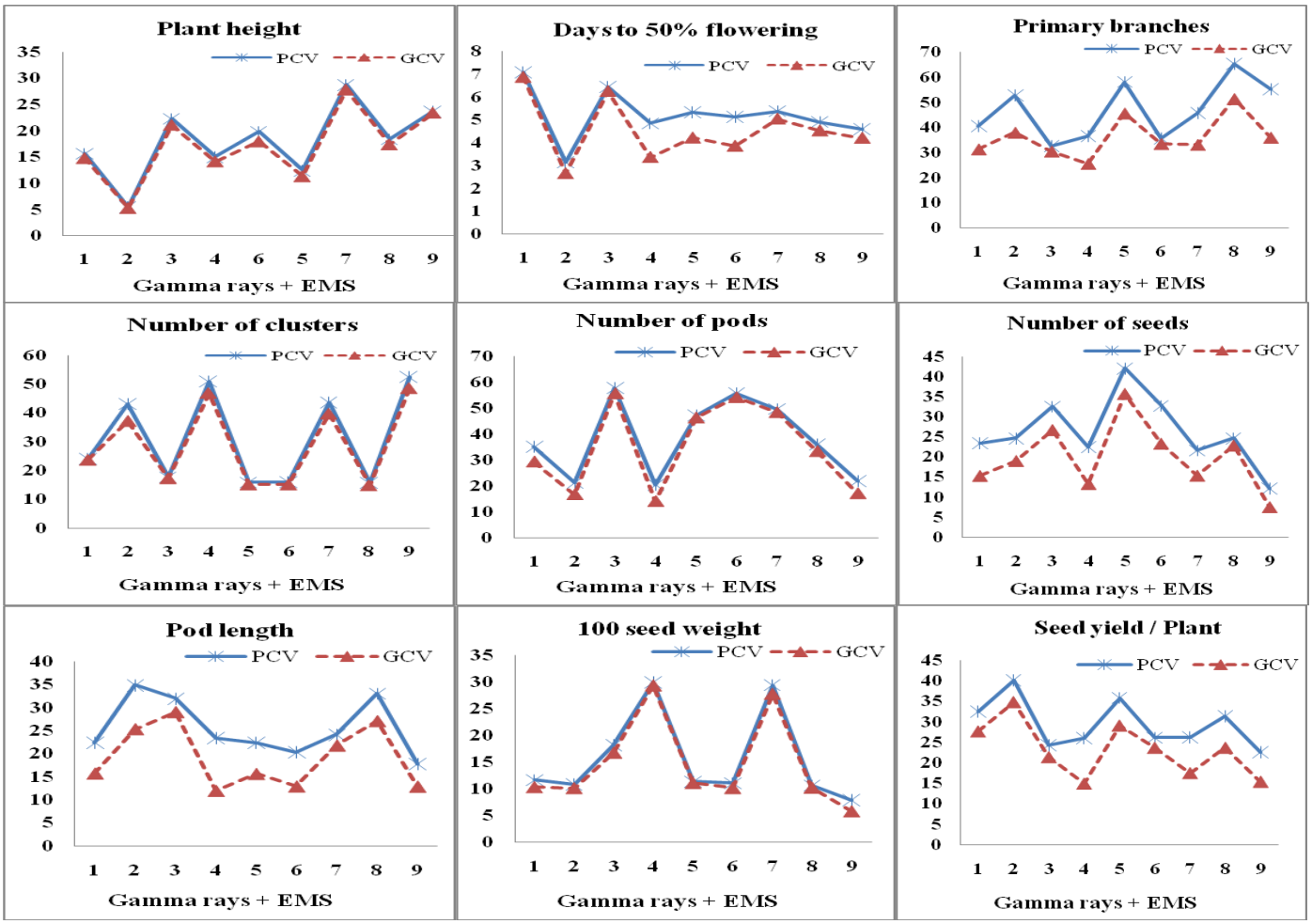

Figure 1. Comparative estimation of genetic parameters for yields and its components in M_2 generation of blackgram. Gamma rays + EMS (x axis) : 1) 400 Gy + 50 mM; 2) 400 Gy + 60 mM; 3) 400 Gy + 70 mM; 4) 500 Gy + 50 mM; 5) 500 Gy + 60 mM; 6) 500 Gy + 70 mM; 7) 600 Gy + 50 mM; 8) 600 Gy + 60 mM and 9) 600Gy + 70 mM.

Table 2. Phenotypic (PCV) and genotypic coefficient of variation (GCV) induced by 400 Gy + EMS in M_2 generation.

Gamma rays + EMS	400 Gy + 50 mM		400 Gy + 60 mM		400 Gy + 70 mM	
	PCV	GCV	PCV	GCV	PCV	GCV
Plant height (cm)	15.67	14.78	5.39	5.25	22.33	21.14
Days to 50% flowering	7.09	6.88	3.14	2.66	6.45	6.26
Number of primary branches	40.66	31.42	52.80	38.07	32.58	30.56
Number of clusters per plant	24.09	23.74	43.10	37.24	17.70	17.39
Number of pods per plant	35.05	29.33	21.40	16.70	57.70	55.64
Number of seeds per pod	23.46	15.33	24.65	19.04	32.62	26.72
Pod length (cm)	22.38	15.74	34.91	25.34	31.95	29.03
100 seed weight (g)	11.63	10.28	10.82	10.10	18.10	16.75
Yield / plant (g)	32.50	27.63	40.13	34.79	24.28	21.36
PCV and GCV induced by 500 Gy + EMS in M_2 generation						
Gamma rays + EMS	**500 Gy + 50 mM**		**500 Gy + 60 mM**		**500 Gy + 70 mM**	
	PCV	**GCV**	**PCV**	**GCV**	**PCV**	**GCV**
Plant height (cm)	15.01	14.16	19.86	17.98	12.41	11.31
Days to 50% flowering	4.85	3.37	5.34	4.22	5.14	3.86

Table 2. Contd.

Number of primary branches	36.70	25.65	58.20	45.70	35.63	33.59
Number of clusters per plant	50.94	46.89	15.83	15.23	15.95	15.23
Number of pods per plant	20.38	14.05	47.12	46.27	55.68	54.13
Number of seeds per pod	22.46	13.33	42.14	35.70	32.78	23.33
Pod length (cm)	23.39	11.97	22.38	15.65	20.33	12.93
100 seed weight (g)	29.90	29.39	11.33	11.06	11.02	10.11
Yield / plant (g)	25.98	14.92	35.77	29.06	26.09	23.65

PCV and GCV induced by 600 Gy + EMS in M_2 generation

Gamma rays + EMS	600 Gy + 50 mM		600 Gy + 60 mM		600 Gy + 70 mM	
	PCV	GCV	PCV	PCV	GCV	PCV
Plant height (cm)	28.80	27.89	18.39	17.41	23.72	23.46
Days to 50% flowering	5.38	5.05	4.91	4.53	4.60	4.21
Number of primary branches	45.85	33.22	65.45	51.45	55.28	36.08
Number of clusters per plant	43.59	39.75	15.66	14.92	52.54	48.61
Number of pods per plant	49.55	48.35	36.00	33.34	21.87	17.09
Number of seeds per pod	21.62	15.38	24.64	22.88	12.10	7.56
Pod length (cm)	24.12	21.78	33.09	27.07	17.74	12.90
100 seed weight (g)	29.37	27.81	10.59	10.18	7.84	5.74
Yield / plant (g)	26.16	17.49	31.30	23.66	22.49	15.39

(Arulbalachandran and Mullainathan, 2009). Similarly, Tabasum et al. (2010) recorded high PCV and GCV for number of cluster per plant, number of pods per plant, seeds per pod, 100 seed weight and seed protein content in blackgram and soybean by Patil and Wakode (2011). Highest magnitude of variability for number of pods per plant in blackgram due to induced mutagenesis was reported by Singh et al. (2000), Anbu Selvam et al. (2010) and Arulbalachandran et al. (2010) in blackgram, Samiullah et al. (2000) in greengram, Waghmare and Mehra (2000), Talukdar and Biswas (2008) in grass pea, Rai et al. (2008) and Lekharam (2010) in French bean, Renuka and Singh (2006) in rice bean and Ali et al. (2011) in chickpea.

Heritability and GA as per cent of mean

Johnson et al. (1955) suggested heritability in combination with genetic advance was more effective and reliable in prediction of resultant effect of selection than heritability alone. This is because, the heritability estimates are subjected to certain estimation errors, that is, overestimation of the parameters would affect the selection efficiency more than the underestimation (Lin et al., 1979) and genotype-environment interaction (Kaul and Garg, 1979). The estimate of heritability acts as a predictive mechanism in expressing the reliability of phenotypic values. Hence, it helps plant breeders to make a selection for a particular character when heritability is high (Arulbalachandran et al., 2010). Base on the fact that heritability is also influenced by the environment, the information on heritability alone may not suffice in pinpointing characters enforcing selection. Therefore, an estimation of heritability, coupled with genetic advance, is also needed to assess the heritable portion of total variation and the genetic gain expected for effective selection. Heritability estimates provide information on the extent to which a particular genetic character can be transmitted to successive generations, whereas genetic advance helps in formulating suitable selection indices. Such estimates facilitate the evaluation of genetic and environmental effects, thereby aiding in selection. Estimates of heritability can be used to predict genetic advance under selection, so that breeders can anticipate improvement from different types and intensities of selection. High value of heritability together with high genetic advance for any character indicates additive gene action and selection will be rewarding for improvement of such traits, whereas high heritability associated with low genetic advance might attribute to the presence of non-additive gene action which indicates epistasis, dominance and genotypic and environmental interaction (Tikka et al., 1977), hence their response to selection would be poor.

Among the characters investigated in the present study, high heritability accompanied with high genetic advance as per cent of mean was observed for the trait like plant height, number of clusters per plant, number of primary branches per plant, number of pods per plant, pod length, number of seeds, 100 seed weight and single plant yield (Table 3). This indicates that these traits are under the control of additive gene action and directional selection for these traits could be effective for

Table 3. Heritability (h^2) and genetic mean (%) induced by 400Gy + EMS in M_2 generation.

Gamma rays + EMS	40 0Gy + 50 mM		400 Gy + 60 mM		400 Gy + 70 mM	
	h^2	GA%	h^2	GA%	h^2	GA%
Plant height (cm)	88.97	8.09	94.89	10.54	89.61	9.59
Days to 50% flowering	94.17	4.07	71.84	3.02	93.94	3.76
Number of primary branches	59.72	42.13	51.84	44.12	87.98	51.48
Number of clusters per plant	97.10	14.34	74.65	27.47	96.51	11.50
Number of pods per plant	70.04	18.13	60.89	14.57	92.98	19.12
Number of seeds per pod	42.72	15.67	59.64	19.29	67.11	23.64
Pod length (cm)	49.49	18.70	52.68	23.82	82.59	29.32
100 seed weight (g)	78.12	18.71	87.19	29.43	85.70	31.95
Yield / plant (g)	72.31	30.26	75.18	31.35	77.40	26.48

Heritability (h^2) and genetic mean (%) induced by 500 Gy + EMS in M_2 generation

Gamma rays + EMS	500 Gy + 50 mM		500 Gy + 60 mM		500 Gy + 70 mM	
	h^2	GA%	h^2	GA%	h^2	GA%
Plant height (cm)	88.94	7.86	81.91	10.27	83.22	7.64
Days to 50% flowering	48.48	2.99	62.59	3.62	56.61	3.40
Number of primary branches	48.65	33.21	61.52	57.59	88.87	54.73
Number of clusters per plant	84.71	28.16	92.58	14.38	91.17	15.57
Number of pods per plant	47.51	12.54	96.39	13.33	94.51	19.77
Number of seeds per pod	35.21	13.15	71.79	30.25	50.66	21.96
Pod length (cm)	26.18	12.19	48.93	18.13	40.45	14.71
100 seed weight (g)	96.57	59.49	95.29	22.24	84.17	19.11
Yield / plant (g)	32.98	17.02	66.02	28.71	82.19	32.20

Heritability (h^2) and genetic mean (%) induced by 600Gy + EMS in M_2 generation

Gamma rays + EMS	600 Gy + 50 mM		600 Gy + 60 mM		600 Gy + 70 mM	
	h^2	GA%	h^2	GA%	h^2	GA%
Plant height (cm)	93.77	10.02	89.71	9.30	97.79	7.42
Days to 50% flowering	88.19	3.78	85.08	3.87	83.57	3.74
Number of primary branches	52.50	42.97	61.70	57.98	42.59	46.21
Number of clusters per plant	83.16	25.23	90.79	15.54	85.62	28.75
Number of pods per plant	95.20	14.96	85.76	19.63	61.08	14.90
Number of seeds per pod	50.59	18.94	86.20	22.02	39.03	10.29
Pod length (cm)	81.52	25.28	66.94	30.26	52.87	17.14
100 seed weight (g)	89.60	54.22	92.37	20.16	53.70	8.67
Yield / plant (g)	44.70	18.13	57.13	24.42	46.81	17.79

desired genetic improvement. This is in conformity with Sinha and Bharati (1990), Deepalakshmi and Ananda Kumar (2004) in blackgram, Khan (1988) in greengram, Gunasekaran et al. (1998) in cowpea and Ali et al. (2011)

in chickpea.

Conclusions

From the results of present study, it may be inferred that the mean values for plant height, number of primary branches per plant, number of clusters per plant, number of pods per plant, number of seeds per pod and single plant yield decreased below the control in most of the treatments. Induced mutations delivered fairly good amount of genotypic coefficient of variation with respect to the number of branches per plant, the number of clusters per plant, pod and seed yield. A high amount of heritability and GA as per cent of mean was noted for plant height, number of clusters per plant, number of primary branches per plant, number of pods per plant, pod length, number of seeds, 100 seed weight and single plant yield. This denoted that these characters are governed largely by additive gene effect, which may be favourably exploited for improving these characters by selection.

Conflict of interests

The authors did not declare any conflict of interest.

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Growth of *Chlorella vulgaris* and *Nannochloris oculata* in effluents of Tilapia farming for the production of fatty acids with potential in biofuels

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The use of microalgae in wastewater treatment and its biotechnological exploitation for the production of biofuels is a potential environmental application. Some species of microalgae are notable due to their lipid composition and fatty acid profile suitable for biofuel production. During the present study, a factorial 2^3 experimental design was conducted, which assessed three factors: i) two species of microalgae (*Chlorella vulgaris* and *Nannochloris oculata*), ii) two types of culture media [wastewater of tilapia farming (WTF) and bold's basal medium (BB)], and iii) two types of lighting (multi-LED lamps and white light). Microalgae were inoculated in photobioreactors in 6 L of medium (WTF or BBM) at an initial concentration of 1.0×10^6 cells ml^{-1} at 20 ± 2°C. The highest average cell density as well as the highest productivity of biomass observed in the treatments was *C. vulgaris* treatment in BBM and multi-LED lighting (8.83×10^7 cells ml^{-1} and 0.0854 g l^{-1} d^{-1}, respectively). Although the majority of lipid productivity was obtained in the exponential phase of *N. oculata* cultivated in multi-LEDs in both treatments (BBM with 58% and WTF with 52%), cultivation of both species was generally maintained in WTF and were those that presented the major lipid productivity (2-18 mg l^{-1} d^{-1}) in comparison with those cultivated in BBM. Palmitic, stearic, oleic, linoleic, linolenic and eicosanoic (C16–C20) fatty acids were present in both species of microalgae in concentrations between 26 and 74%. Based on the results of the present study, we conclude that cultivation of *N. oculata* and/or *C. vulgaris* in WTF illuminated with multi-LEDs is an economic and sustainable alternative for biodiesel production because it can represent up to 58% of lipids with a fatty acid profile optimal up to 74% of the total fatty acids.

Key words: *Chlorella vulgaris, Nannochloris oculata*, production of fatty acids, wastewater of tilapia farming, production of biofuels.

INTRODUCTION

During the last few decades, an energy crisis associated with depletion of irreversible traditional fossil fuel sources is being recognized worldwide. We are aware that its use as a primary source of energy is unsustainable and

contributes to the accumulation of greenhouse gases, which causes global warming and a permanent source of atmospheric environmental pollution (Ahmad et al., 2011; Amaro et al., 2011). Likewise and with current trends of fossil energy consumption, worldwide oil reserves could be depleted by the year 2050 (Demirbas, 2011; Chen et al., 2013). Because of this, international trends of power generation and environmental protection are derived from the research and development of renewable, economically competitive and environmentally friendly alternative sources of energy (Ahmad et al., 2011; Chen et al., 2013; DeJong et al., 2013). Liquid fuels derived from plant matter (also called biofuels) are an alternative to the generation of sustainable energy. In comparison with other renewable forms (for example, wind, tidal and solar), these allow storage for long periods of time due to their chemical structure and can be used in addition to the traditional forms in engines and existing transport infrastructure after mixing to varying degrees with diesel oil (Singh and Gu, 2010; Amaro et al., 2011). The choice of biomass as feedstock for the production of energy depends on social, environmental, economic and industrial factors in addition to its availability and cost. However, generation of biodiesel from grain legume entails the use of large tracts of arable land and fresh water for cultivation. Also, there is the possible competition with food production of direct use to man or animal in addition to seasonal and geographical variations that also affect productivity as well as the use of herbicides and the consequent environmental pollution (Chen et al., 2013).

Therefore, production of biodiesel from microalgae is an important option that should be evaluated as an alternative for the generation of biofuels. This also seems to be a renewable source of fuel that can satisfy the global demand for transport fuels (Chisti, 2007; Demirbas, 2011) but also has the potential to generate large volumes of feedstock without affecting the food supply (Rosch et al., 2012). Microalgae as biofuel producers have different advantages such as high productivity, accumulation of lipids, and ability to grow in wastewater. In addition, microalgae have a higher productivity per area and have the ability to grow in non-arable lands with water unsuitable for agriculture, using CO_2 and other industrial waste (Delrue et al., 2012; Lohrey et al., 2012; Chen et al., 2013; Sánchez et al., 2015). A scarcely exploited alternative for the generation of microalgal biomass is waste water of production aquaculture, which may have as an advantage the biological water treatment and reinstatement into the aquaculture system. This allows the nutrition of microalgae using organic compounds (nitrogen and phosphorus) available in these effluents (Mata et al., 2010; Chávez-Crooker and Obreque-Contreras, 2010; Marinho-Soriano et al., 2011). It is reported that algae produce more lipids in a stress environment or under unfavorable conditions compared to optimal growth conditions. During optimal growth conditions, algae synthesize fatty acids mainly for esterification to glycerol in membrane lipids, which constitute ~5-20% of their dry weight. However, under conditions of stress, by limiting nitrogen or another component, microalgae have a very high production of lipids that can reach up to 77% of its dry weight (Mata et al., 2010; Kirrolia et al., 2013; Josephine et al., 2015).

The present study assessed the production of fatty acids in two freshwater microalgae *Chlorella vulgaris* and *Nannochloris oculata* cultured in Wastewater of Tilapia Farming (WTF) and Bold's Basal Medium (MBB) using two lighting systems in order to determine their potential for the generation of biofuels.

MATERIALS AND METHODS

Strains and growth medium

C. vulgaris (code: CLV2) and *N. oculata* (code: LB2194), were obtained from the collection of the Department of Aquaculture of the Center of Scientific Research and from the Center for Higher Education and Teaching (CICESE) and from the Collection of the University of Texas (UTEX), respectively. Strains were maintained in sterile BBM in flasks of 125 ml under controlled laboratory conditions (Bischoff and Bold, 1963; Nichols, 1973; Andersen, 2005) during a 24 h photoperiod of light without aeration and temperature of 18°C in a light chamber at 18.5 µmol m^{-2} s^{-1}. The large-scale culture of *C. vulgaris* and *N. oculata* was carried out in four 1000 ml Erlenmeyer flasks with 750 ml of sterile BBM and 100 ml of inoculum suspension (1.0×10^6 cells ml^{-1}) for reseeding. Lighting conditions were 24 h photoperiods with constant aeration at a temperature of 18°C in a light intensity of 79.88 µmol m^{-2} s^{-1}.

Photobioreactors, treatment systems

For the cultivation of microalgae, *C. vulgaris* and *N. oculata*, 16 photobioreactors were used, which consisted of 15 x 45 cm acrylic hexagonal units with a capacity of 8 L and closed completely by having the top drilled. Two 5 mm glass tubes were placed, the first to provide oxygen and homogenize cultivation using a pump. The second glass tube was used to take samples to monitor the cultivation. During the study, photobioreactors were placed in two structures in order to have two modules, which are illuminated individually through the following schemes: module 1 lighting the

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Abbreviations: BBM, Bold's basal medium; **WTF,** wastewater tilapia farming; **Nn,** *Nannochloris oculata*; **Ch,** *Chlorella vulgaris;* **LED,** multi-LED lighting; **WL,** white lights.

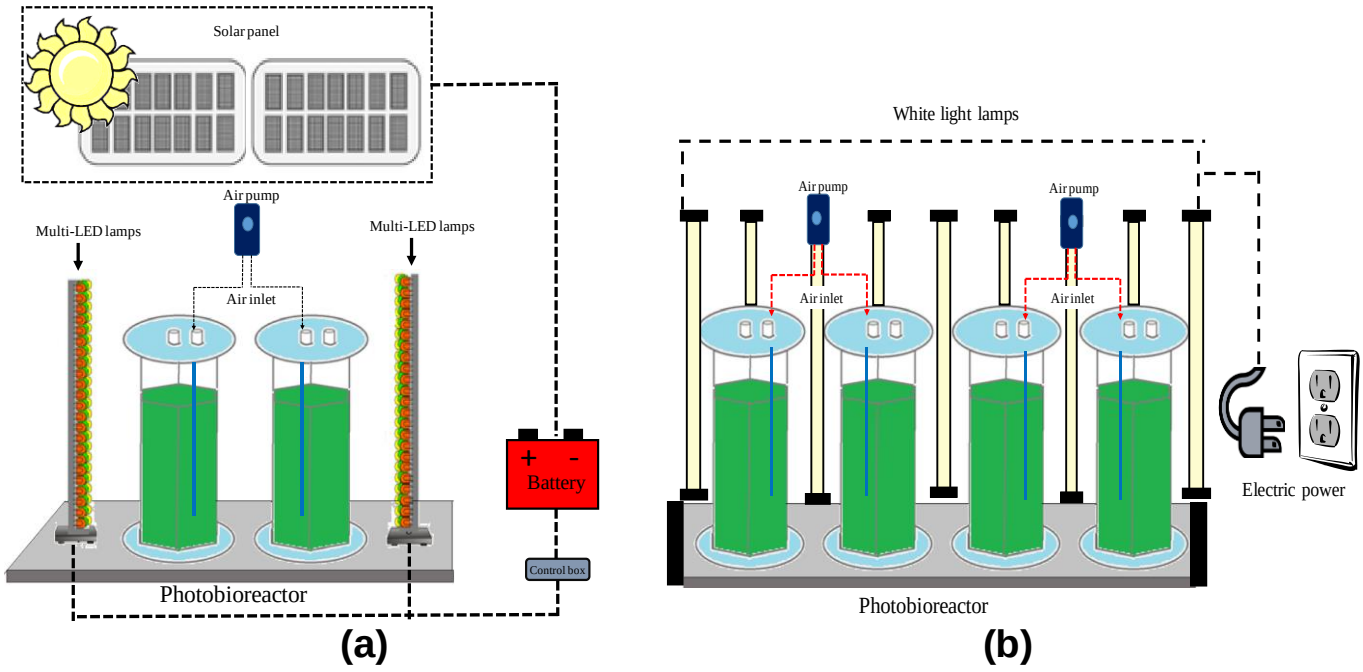

Figure 1. Types of lighting in farming systems. **(a)** Multi-LED lamps. **(b)** White light lamps.

photobioreactors generated from four multi-LED reflectors (RF-240HFS 30W) energized by two batteries (Surrette S-600 deep cycle 6V, 450 Ah) connected to five solar panels (polycrystalline 145 W modules placed in the upper part of the roof of the LLF). The second module consisted of four 40 W white lights energized with direct current (220 V). Both systems (Figure 1) maintained a light intensity of 79.88 µmol m^{-2} s^{-1}.

Experimental design, sampling and analyses

The experiment consisted of a 2^3 factorial design, where three factors were evaluated: A) type of microalgae, B) type of culture medium, and C) type of lighting at two levels each (8 treatments in total, with a replica by treatment). The experimental units (16 photobioreactors) were then added to 6 L of the microalgae culture medium corresponding to each treatment: eight photobioreactors with sterile BBM (control) prepared previously according to the specifications by Nichols (1973) and the other eight photobio-reactors with sterile WTF collected from a pond cultivation of opencast tilapia located at the Instituto Tecnológico de Boca del Rio. These were previously filtered through a 100 µm packed mesh column for phytoplankton with cotton and polyester fiber. The water was incorporated into the respective photobioreactors and disinfected using 0.8 ml l^{-1} of NaClO and neutralized with 0.75 g l^{-1} of $Na_2S_2O_3.5H_2O$ for 2 h under continuous aeration. The initial concentration of NO_2-N, NO_3-N, NH_3-N and PO_4^{-3} in WTF was determined (1, 8.4, 0.31 and 1.5 mg/l, respectively) using an HANNA Multiparameter (Model HI83099). Each culture medium was inoculated separately with 1.0 × 10^6 cells ml^{-1} of *C. vulgaris* and *N. oculata.* The volume of the inoculum to be used was determined by cell count in a Neubauer chamber (Pica-Granados et al., 2004) using the following equation:

$$V_2 = \frac{(C_1.V_1)}{C_2} \quad (1)$$

Where V_1= volume of reactor operation (6000 ml), C_1= initial cell density in the reactor (1.0 × 10^6 cells ml^{-1}), V_2= volume of inoculum required for the reactor (ml), and C_2= cell density of the inoculum at the time of inoculation of the reactor (cells ml^{-1}).

Duration of the culture was 10 days at 20 ± 2°C. Light intensity of 79.88 µmol m^{-2} s^{-1} with multi-LED lamps or white light was used. Cell density (cells ml^{-1}), lipid content (%), biomass productivity (g l^{-1} d^{-1}), lipid productivity (mg l^{-1} d^{-1}) and fatty acid quantification (%) were evaluated. All results were expressed as mean ± standard deviation. Statistical analysis was done using analysis of variance (ANOVA); $P<0.05$ was accepted as statistically significant. Stat Soft, Inc. (2004) STATISTICA V.7 was used for analysis. A 400 ml sample was used from treatment in the exponential (sixth day) and stationary (eighth day) phases based on growth kinetics. Samples were then filtered to gravimetrically determine dry and wet weight of the biomass. Once dried, samples were taken. We then proceeded to lipid extraction using the Soxhlet method with a mixture of chloroform/ methanol (1:2 v/v) (Halim et al., 2012). The product of the extraction was considered as the lipid content per species and treatment which, after being weighed, was stored in amber vials for later quantification of fatty acid profiles. For quantification of fatty acids, esterification or derivatization of the lipid fraction of the samples was carried out with the addition of 2.63 g of KOH, 30 ml of methanol and 10 ml of water. Evaporation was carried out and cooled to room temperature. Thirty ml of HCl was added to 3% in methanol and again evaporated to salt formation. The sample was rinsed with 20 ml of distilled water and placed in a separation funnel to which 30 ml of hexane was added. The sample was stirred for 1 min and then left to decant to obtain two phases and to scrape the precipitate. The supernatant was heated (40°C) to evaporate the solvent residues and subsequently diluted to 200 µl. Samples were finally stored in amber vials. The supernatant was heated (40°C) to evaporate the residue of the solvent and subsequently gauged to 200 µl to finally keep the samples in amber vials (Lepage and Roy, 1984). Fatty acids were identified and quantified with a gas chromatograph (Perkin Elmer, Model Autosystem, Flame ionization)

Table 1. Cell density average for *Nannochloris oculata* and *Chlorella vulgaris* according to the different treatments.

Microalgae strains	Light conditions	Basal bold's medium	Wastewater tilapia farming
		Cell density (cells ml^{-1})	
Nannochloris oculata	Multi-LED	$2.52 \times 10^7 \pm 1.12 \times 10^7$	$4.75 \times 10^7 \pm 9.63 \times 10^6$
	White light	$6.27 \times 10^7 \pm 1.02 \times 10^7$	$4.52 \times 10^7 \pm 1.17 \times 10^7$
Chlorella vulgaris	Multi-LED	$8.83 \times 10^7 \pm 1.25 \times 10^7$	$5.63 \times 10^7 \pm 1.06 \times 10^7$
	White light	$6.54 \times 10^7 \pm 1.04 \times 10^7$	$3.43 \times 10^7 \pm 9.91 \times 10^6$

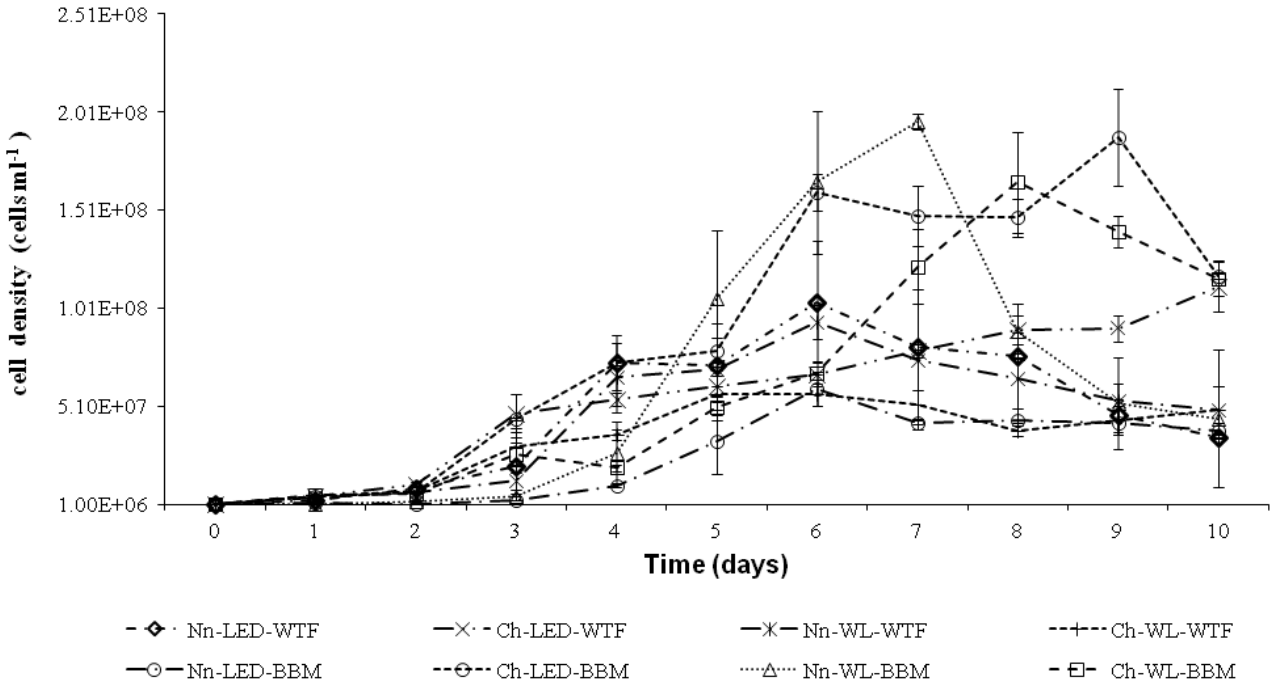

Figure 2. Comparison of cell density of *Nannochloris oculata* (Nn) and *Chlorella vulgaris* (Ch) in Wastewater Tilapia Farming (WTF) and Bold's Basal Medium (BBM) in multi-LED lighting (LED) and white lights (WL).

with an INNOWax capillary column (30 m in length × 0.320 mm in diameter). Nitrogen (N_2) was used as carrier gas. The injector temperature was 250°C and the detector was 300°C. Oven temperature was 150°C (4 min) with a ramp of 5°C min^{-1} to 190°C with a ramp of 2°C min^{-1} to 250°C (11 min). The injection volume was 2 µl per sample.

RESULTS

The results of this study indicate that, in general, the cultivations had a continuous and efficient growth in all tested treatments. It was observed that type of micro-algae and type of culture medium as well as all the interactions among the effects were significant ($P<0.05$). However, the type of lighting had no effect on cell growth. Except for treatment where the microalga *N. oculata* was under BBM white light illumination, most of the microalgae in the different treatments reached the exponential phase between the fifth and sixth day, whereas the first required 7 days and attained a cell density significantly ($P = 0.00004$) higher than that achieved by other algae (1.96×10^8 cells ml^{-1}) under study (Figure 2). The most efficient average cell density was presented during cultivation of *C. vulgaris* in BBM with multi-LED lighting (8.83×10^7 cells ml^{-1}), whereas the less efficient average cell density is presented in the same species in the WTF with white light. The highest average density of *N. oculata* was obtained in BBM with white light illumination (6.27×10^7 cells ml^{-1}), significantly higher than that achieved with the same microalgae but with WTF under the same lighting conditions (Table 1). The total biomass productivity of *C. vulgaris* that occurred during the study regardless of the treatment was in a range of 0.031-0.085 g l^{-1} d^{-1}, whereas for *N. oculata* it was 0.014-0.057 g l^{-1} d^{-1}, presenting significant differences between types of microalgae (Table 2). This

Table 2. Percent lipids, productivity of biomass and lipids of *Nannochloris oculata* and *Chlorella vulgaris* in WTF and BBM under two lighting conditions.

Microalgae strains	Culture media	Light conditions	Growth phase	Lipids (%)	Biomass productivity (g l^{-1} d^{-1})	Lipids productivity (mg l^{-1} d^{-1})
Nannochloris oculata	Bold's basal medium	Multi-LED	Exponential	57.80 ± 10.14	0.0141 ± 0.0002	8.1786 ± 5.7579
			Stationary	28.90 ± 5.13	0.0313 ± 0.0004	9.0313 ± 1.5026
		White light	Exponential	6.21 ± 0.19	0.0357 ± 0.0115	2.2083 ± 0.6482
			Stationary	10.21 ± 0.23	0.0571 ± 0.0074	5.8438 ± 0.8839
	Wastewater of tilapia farming	Multi-LED	Exponential	51.57 ± 7.22	0.0205 ± 0.0025	10.6071 ± 0.2020
			Stationary	33.34 ± 0.34	0.0279 ± 0.0088	7.1875 ± 3.0273
		White light	Exponential	42.49 ± 11.73	0.0219 ± 0.0009	9.2500 ± 2.1802
			Stationary	25.43 ± 3.36	0.0320 ± 0.0047	7.9688 ± 0.1105
Chlorella vulgaris	Bold's basal medium	Multi-LED	Exponential	27.66 ± 5.72	0.0459 ± 0.0028	12.6250 ± 1.8435
			Stationary	21.04 ± 4.99	0.0854 ± 0.0055	17.8281 ± 3.1157
		White light	Exponential	34.94 ± 2.36	0.0384 ± 0.0015	13.3958 ± 0.3830
			Stationary	22.41 ± 2.18	0.0543 ± 0.0132	11.3750 ± 3.6681
	Wastewater of tilapia farming	Multi-LED	Exponential	34.75 ± 2.31	0.0316 ± 0.0033	10.6429 ± 0.4293
			Stationary	27.03 ± 4.99	0.0456 ± 0.0061	12.5000 ± 2.1802
		White light	Exponential	39.38 ± 6.63	0.0446 ± 0.0080	17.2917 ± 0.1768
			Stationary	34.96 ± 4.94	0.0401 ± 0.0021	13.3958 ± 0.3830

may be due to the fact that microalgal biomass productivity is directly dependent on the species studied and on culture conditions (Chojnacka and Marquez-Rocha, 2004; Simionato et al., 2013). On the other hand, the results show that the highest lipid content was reached with *N. oculata* under multi-LED lighting in BBM and WTF (58 and 52%, respectively) in the exponential growth phase. Moreover, lipid productivity for both species was variable, according to medium as well as to type of lighting, reaching 2-18 mg l^{-1} d^{-1} productivities. *C. vulgaris* presented the highest productivity for both culture media (BBM and WTF). However, for *C. vulgaris* in BBM with multi-LED lighting, the highest productivity was in the stationary phase (17.83 mg l^{-1} d^{-1}) unlike in WTF with white light as lighting was in the exponential phase (17.30 mg l^{-1} d^{-1}). Lipid productivity depends mainly on the type of microalgae, type of culture medium, lighting conditions and growth phase. Lipid content was similar to that achieved by *C. vulgaris* in WTF with white lighting of 37% with an average biomass productivity of 0.04 g l^{-1} d^{-1}. For cultivations in WTF as a stress condition for its limitation of nitrogen compared with BBM, results show that for both microalgae, the percentage and lipid productivity for WTF were higher (25-42 %) than in MBB (6-35%), mainly in those treatments using white light illumination (Table 2). In addition, *N. oculata* and *C. vulgaris* showed the highest concentration of mono and polyunsaturated oleic, linoleic, palmitic, stearic, eicosanoic, arachidonic and eicosapentaenoic fatty acids in their lipid composition with fractions of 2-43% and up to 75% total (Table 3).

Table 4 shows the results of the removal efficiencies average of ammonium, nitrites, nitrates and phosphates in the WTF by type of microalgae and lighting. The microalgae *C. vulgaris* in white light presented the greater removal efficiency of nitrogen compounds. The removal efficiency of nitrite with 83% reported highest efficiency, followed by nitrates and ammonium (52 and 23%, respectively). However, higher removal efficiency of phosphates with 66% was in Multi - LEDs. This result is consistent to what was reported for low densities of inoculum (1×10^6 cells ml^{-1}) where the ranks ranged from 63 to 73% (Lau et al., 1995; Jiménez del Río, 1996; Neori et al., 2004; Hanumantha-Rao et al., 2010).

On the other hand, in terms of energy consumption, the treatment with multi-LEDs had an approximate consumption of 79.88 µmol m^{-2} s^{-1} (light intensity), which would amount to 18.94 W m^{-2}, taking as summarized data of equivalence to 1800 µmol m^{-2} s^{-1} ≡ 427 W m^{-2} (Gal et al., 1999).

Comparing energy consumption by type of lighting, white lights was higher than multi-LEDs (40 and 18.94 W, respectively). The photobioreactors operated for 10 days, reached an approximate consumption of 9.6 and 4.32 kWh for white light and multi-LEDs, respectively. The biomass productivity (Table 2) for *C. vulgaris* in both BBM and WTF, stationary phase, multi-LED was of 0.0854 and 0.0456 g l^{-1} d^{-1}, respectively. Therefore, the biomass productivity in 10 day of operation of the photobioreactors (6 l by reactor) was of 5.12 and 2.74 g for BBM and

Table 3. Percent composition (%) of fatty acids in the lipid fraction extracted from *Nannochloris oculata* and *Chlorella vulgaris* grown in photobioreactors in WTF and BBM under two lighting conditions (multi-LED and white light).

Fatty acid	Fatty acid	Treatments							
		Nn[e]-LED[a]-WTF[C]	Nn-LED-BBM[d]	Nn-WL[b]-WTF	Nn-WL-BBM	Ch[f]-LED-WTF	Ch-LED-BBM	Ch- WL - WTF	Ch- WL-BBM
		(%)							
Lauric acid	C12:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Myristic acid	C14:0	0.67	0.00	0.00	0.00	0.00	0.06	0.00	0.00
Palmitic acid	C16:0	11.66	1.58	12.00	9.41	8.32	6.10	1.91	7.29
Stearic acid	C18:0	6.06	1.54	16.97	8.55	19.65	2.08	10.90	3.32
Oleic acid	C18:1	13.68	6.40	18.37	9.25	14.81	13.05	7.06	7.23
Linoleic acid	C18:2	12.15	7.98	12.83	10.26	4.55	1.92	1.44	3.78
Linolenic acid	C18:3	6.97	2.81	0.00	2.59	6.46	3.21	3.64	2.55
Eicosanoic acid	C20:1	12.09	6.11	5.65	30.77	16.01	8.35	8.44	10.24
Arachidonic acid	C20:4	1.26	0.00	3.18	0.00	3.83	7.59	15.09	12.24
	Total	64.54	26.42	69.00	70.83	73.63	42.36	48.48	46.65

[a]Multi-LED lighting; [b]White light; [c]wastewater of tilapia farming; [d]bold's basal medium; [e]*Nannochloris oculata*; [f]*Chlorella vulgaris*.

Table 4. Removal efficiencies average of nitrogen compounds and phosphate in *Chlorella vulgaris* and *Nannochloris oculata* cultured in WTF for both types of lighting.

Treatment			Removal efficiency (%)			
			Ammonium	Nitrite	Nitrate	Phosphate
			(NH_3-N)	(NO_2-N)	(NO_3-N)	(PO_4^{-3})
WTF	Multi-LEDs	*Chlorella vulgaris*	1.32 ± 0.26	30 ± 4.56	41.73 ± 1.56	66.33 ± 6.28
	White light	*Chlorella vulgaris*	22.60 ± 4.12	83 ± 3.49	51.95 ± 2.47	45.00 ± 3.19
	Multi-LEDs	*Nannochloris oculata*	12.50 ± 2.31	0.00 ± 0.00	0.00 ± 0.00	24.96 ± 5.16
	White light	*Nannochloris oculata*	12.50 ± 1.89	0.00 ± 0.00	3.65 ± 0.15	47.48 ± 2.91

WTF, respectively; with energy consumption per gram of 0.84 and 1.6 kWh g^{-1} in BBM and WTF, respectively. The greatest energy consumption per gram of biomass for *N. oculata*, was in white light in BBM (2.80 kW g^{-1}). Comparing the best results among type of lighting in BBM, the requirement of energy per gram of biomass was greater with white light in *N. oculata* (2.80 kWh g^{-1}). However, comparing by type of lighting in WTF, *N. oculata* presented greater energy consumption per gram in white light than *C. vulgaris* in multi-LEDS (2.80 and 1.6 kW g^{-1}, respectively).

DISCUSSION

In this study, microalgae with the highest average density was *C. vulgaris* in BBM with 88.3 × 10^6 cells ml^{-1} during 10 days of cultivation, much higher than the densities obtained in the above-mentioned studies. It is important to note that there were higher densities in all of the treatments reported under different lighting conditions (Figure 2).

De-Bashan et al. (2002) conducted studies on cultures of *C. vulgaris*, *Azopirillum brasilense* and *C. vulgaris/A. brasilense*, showed that the highest density was in discontinuous cultivations with 4 × 10^6 cells ml^{-1}. Cleber et al. (2008) studied chlorophyll content and profile of minerals in the microalga *C. vulgaris* grown in hydroponic wastewater solution. Cultivations were carried out in BBM as control, concentrations of 100% hydroponic residual solution (HRS), 50% residual hydroponic solution and 50% deionized water (HRS50), 25% of residual hydroponic solution and 75% of deionized water (HRS25). The cultivation period was 7 days, obtaining average densities at the end of the period of 10.6 × 10^6, 5.7 × 10^6, 4.2 × 10^6 and 10.1 × 10^6 cells ml^{-1} in BBM, HRS, HRS50 and HRS25, respectively. Furthermore, recent studies to determine the effect that produces leachates of the biosolids on freshwater biota demonstrated an effect on *N. oculata* affecting their growth at concentrations >500 ppm of leachate after being cultivated with 12 h light at 31.5 µmol m^{-2} s^{-1} and 12 h dark for 14 days at concentrations of 50, 200, 500 and 1000 ppm (Flores et al., 2010). Results indicate that the highest and lowest cell density reached 50 and 500 ppm with 1.77 × 10^6 and 0.65 × 10^6 cells ml^{-1}, respectively, con-

cluding that the leachate can function as fertilizer for the growth of *N. oculata* to concentrations not higher than 50 ppm.

As shown in previous studies, various types of waste are used for the cultivation of *C. vulgaris* and *N. oculata* as culture media. Average cell density at the end of the culture for periods between 7 and 12 days with an initial inoculum of 1.0×10^6 cells ml^{-1} was 2.5×10^6 to 10.6×10^6 cells ml^{-1} and 0.65×10^6 to 1.77×10^6 cells ml^{-1} for *C. vulgaris* and *N. oculata*, respectively.

Mata et al. (2010) remark that, under nitrogen limitation, *C. vulgaris* and *N. oculata* maintained a production of 0.02-0.20 and 0.37-0.48 g l^{-1} d^{-1}, respectively. In addition, the stress situation during cultivation caused the microalgae to generate a higher content of lipids (from 75%) in comparison to what is expected (20-50%), with lower biomass productivity (0.02 g l^{-1} d^{-1}) in relation to what is expected (0.40 g l^{-1} d^{-1}). In another study, Chiu et al. (2008) reported that in semicontinuous cultures of *Chlorella* sp. with low and high cell density for CO_2 reduction, biomass productivity of 0.037-0.053 g l^{-1} d^{-1} was obtained; values similar to that obtained in this study for both strains under all culture conditions.

Furthermore, Liang et al. (2009) reported biomass productivity of 0.010-0.254 g l^{-1} d^{-1} in culture of *C. vulgaris* under growth conditions of heterotrophs, autotrophs and mixotrophs with a maximal lipid content of 38% under autotrophic conditions. These results, indicate that under conditions of stress due to a low concentration of nitrogen (primarily nitrates), there is an increase in lipid content in biomass at a range of 15 to 58% (dry weight) depending on the species of microalgae (Mata et al., 2010; Probir et al., 2011; Kirrolia et al., 2013). This behavior was previously observed in other studies (Mata et al., 2010; Kirrolia et al., 2013). Therefore, biomass and lipid productivity can be increased up to 60% under culture conditions with nitrogen deficiency and high concentrations of CO_2 (Chiu et al., 2009; Gouveia and Oliveira, 2009; Liang et al., 2009; Rodolfi et al., 2009; Mata et al., 2010; Borges et al., 2011; Kirrolia et al., 2013).

On the other hand, Guerrero-Cabrera et al. (2014) cultivated three species of microalgae (*Monoraphidium* SP., *Chlorella* SP. and *Scenedesmus* SP.) in three volumes of WTF (1.5, 4 and 9 l, respectively) as culture medium and BBM, and compared the specific rate of growth, volumetric productivity (g l^{-1} d^{-1}), biomass productivity (g l^{-1}), as well as protein and lipid content. They reported that *Scenedesmus sp*. in BBM; it produced a higher specific speed of growth and volumetric productivity (0.332 g l^{-1} d^{-1}) than WTF in 1.5 L of volume. Also reported was *Chlorella sp*, which also showed a higher lipids volumetric productivity (0.011 g L^{-1} d^{-1}) for BBM to WTF to 1.5 L of volume. The maximum lipids concentration in percentage was for *Monoraphidium* SP. in WTF, 1.5 L with 17.8%.

Due to its rapid speed of growth and mainly due to its high content of lipids with a rich fraction of saturated and monosaturated fatty acids, preferably C16–C20 polyunsaturated chain, numerous strains of microalgae have been studied as a potential source of triacylglycerides (TAG), the main raw material for biodiesel production (Delrue et al., 2012; Hoekman et al., 2012; Lohrey et al., 2012; Chen et al., 2013; Wei et al., 2013; Singh et al., 2014; Taher et al., 2014).

Some studies have shown that the highest content of lipids present in *N. oculata* and *C. vulgaris* was achieved in nitrogen deficient cultures with a higher content of mono and polyunsaturated fatty acids (Rodolfi et al., 2009; Mata et al., 2010; Kirrolia et al., 2013; Singh et al., 2014; Taher et al., 2014). Therefore, cultures of *N. oculata* illuminated with multi-LEDs and in WTF may be an attractive and economic alternative for the generation of biodiesel due to the high percentage of lipids and mono and polyunsaturated fatty acids.

Conclusion

The highest biomass productivity and highest cell density was in *C. vulgaris* in BBM and multi-LEDs. *N. oculata* in multi-LEDs reached the highest percentages of lipids in both media. The lipid productivity for both species was variable both for culture medium and lighting condition. Fatty acid composition in the different treatments was mainly saturated, where the highest percentages were in WTF. Based on the results, it is concluded that *N. oculata* and/or *C. vulgaris* in WTF in multi-LEDs are an economic and sustainable alternative in a scheme of cultivation of microalgae with the greatest potential as a generator of biodiesel.

Conflict of interests

The authors did not declare any conflict of interests.

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Full Length Research Paper

Microbiological evaluation and shelf life of seed flour mixes used for infant feeding in rural northern Nigeria

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This study investigates microbial load and shelf life of locally processed cereal-legume-oil seed flour mixes used for infant feeding in rural northern Nigeria. Free fatty acid (FFA), water activity (a_w), pH, total viable count (TVC) and presence of yeast, coliform bacteria and mold were the parameters determined. Hungry rice or *Digitaria exilis*, benne seed or *Sesamum indicum* and soybean or *Glycine max* seeds flours were produced using traditional processing methods, including washing, boiling, fermenting and roasting. The test samples were milled into flour that could pass through a 70 mm mesh sieve and blended on protein basis at 70:30 cereal-legume/oilseed ratios to produce $D_{70}S_{30}$, $D_{70}G_{30}$, $D_{70}S_{15}G_{15}$, $D_{70}S_{20}G_{10}$ and $D_{70}S_{10}G_{20}$ and stored at 30 to 32°C for 60 days. The parameters were determined at day 0 and intervals of 14, 28, 42 and 60 days. At the end of the storage period, all the parameters increased. FFA increased from 0.15 - 0.16% to 0.47 - 0.58%; a_w from 0.46 - 0.48 to 0.72 - 0.80; pH from 3.9 to 4.7 - 4.9 and TVC from <$1.0x10^2$ to $2.2x10^3$ to 3.6×10^4cfu/g for 0 and 60 days, respectively. The differences between the values of the parameters of the formulations were significant ($p<0.05$) especially from day 14. Yeast and coliform were not detected in all the samples but mold was detected from days 14 to 60. The formulation $D_{70}S_{30}$ had best shelf life; however, the samples should be consumed within 14 days of production.

Key words: Seed flour, multi-mixes, children, microbial load, shelf life.

INTRODUCTION

Poor child-feeding practices and high rate of infection result in malnutrition and poor health of children (WHO, 2010; UNICEF, 2011). The period from birth to two years is a 'critical window' for the promotion of exclusive breastfeeding (0 to 6 months) and quality/adequate complementary feeding (6 to 24 months) to optimize growth, health, and development in children. In rural northern Nigeria, cereal, legume/oilseed based complementary porridges are local diets fed to children 6 to 24 months of age with continued breast feeding. Rural mothers combine different plant foods because they understand that dietary diversity (multi-mixes) is an

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Abbreviations: FFA, Free fatty acid; **a_w,** water activity; **TVC,** total viable count.

effective approach to improve the quality of young children's plant-base diets especially without access to fortified food products (WHO/UNICEF, 2008). Combining cereals and legumes at the ratio of 70:30 have been shown to have nutrient potentials that complement one another in general (Fernandez et al., 2002) and have amino acid pattern that can compare with that of animal protein. Cereal and legume commonly blended for infant feeding are maize or millet and soybean. Other cereals and legumes/oilseed which are also cheap sources of plant protein but are under exploited and not commonly used in infant feeding include hungry rice (cereal) and benne seed (oilseed). They are under exploited in infant feeding general in Nigeria and in the south in particular; they are however used in rural north.

Hungry rice is one of the nutritious grains in the world (Thiam, 2008). It is particularly rich in many essential amino acids (National Academy of Science, 2006). Benne seed is an oil legume that is rich in micronutrients and phytochemicals (Bedigian, 2003). In the north of Nigeria where hungry rice and benne seed are combined in infant porridge, mothers frequently process these foods to produce complementary porridges. This is a gruesome task and in most cases facilities such as refrigerators to store the processed flour or paste are not available, they therefore store processed flour and pastes for less than seven days. The potentials of storing processed flour in standard household pantry beyond seven days could be exploited to reduce the work load and time for mothers and caregivers.

Shelf life is the length of time that a commodity such as food may be stored without becoming unfit for use or consumption. It is the recommended maximum time for which products can be stored during which the defined quality of a specified proportion of the goods remain acceptable under expected conditions of distribution, storage and display (Gyesley, 1991). A food product within its shelf life should be safe to eat, keep its appearance, colour, texture and flavour and meet any nutritional claims provided on the label (New Zealand Food Safety Authority, 2005). All foods spoil with time but the rate differs from food to food. Factors that affect food quality include microbial (bacteria, yeast and mold) growth, non-microbial spoilage (rancidity, browning and loss of nutrients) and product related spoilage such as water activity, pH and oxygen availability (Fontana, 2008; Sivasankar, 2010).

Free fatty acid in stored foods is used to measure rancidity. Enzymes inherent in foods hydrolyze fat in the food into free fatty acid and glycerol during storage (Morrison, 2006). Light, atmospheric oxygen and moisture also precipitate rancidity which changes the flavour and taste of food (Modi et al., 2004). Water activity of a food is the ratio between the vapour pressure of the food itself, when in a completely undisturbed balance with the surrounding air media and the vapour pressure of distilled water under identical condition (U.S. Food and Drug Administration, 1984). A a_w of 0.80 means that the vapour pressure is 80% of that of pure water. It is the amount of water in a food that is available for microbial use and it helps to predicts microbial growth (Fontana, 2001). Water activity predicts food stability with respect to microbial growth rate of deteriorative reaction and physical properties of foods (texture and shelf life) (Ukegbu and Anyika, 2012). High values support microbial growth, therefore, moist food spoils faster than dry food. A food or solution's pH is a measure of acidity or alkalinity using a numerical scale between 1 and 14. Low pH value shows acidity and it limits the growth of many microorganisms and vice versa (International Commission on Microbiological Standards for Foods, 1996). The pH limit for growth of any microorganism is 0.60 (Fontana, 2008)

Microbial load of a food is determined by the levels of microorganisms (measured in colony forming units per gram) in the food during production, packaging, storage and other handing as well as the type of food in question. The end of shelf life can be based on the quantity of microorganism present (NZFSA, 2005). The objective of this study was to evaluate at intervals (0, 14, 28, 42 and 60 days) microbial load and shelf life of cereal-legume/oilseed multi-mixes used for infant feeding in rural northern Nigeria.

MATERIALS AND METHODS

Digitaria exilis, *Sesamum indicum* and *Glycine max* used for the study were purchased from a local market in Kaduna state in northern Nigeria and processed using traditional methods. *D. exilis* was washed, soaked in water (1:3 ratio W/V), fermented for 48 h (with the water changed every 24 h) and sundried. *S. indicum* seeds were washed, sundried and roasted at 80°C for 10 min. *Glycine max* seeds were also washed and boiled at 100°C for 60 min dehulled manually, soaked in water (1:3 ratio W/V), fermented for 24 h (with the water changed every 24 h) and sundried. The dried *Glycine max* seeds were roasted at 80°C for 10 min. The residual moisture after the processing of the food items was approximately 3%. All the processed seeds were milled (to pass through a 70 mm mesh sieve) separately into flour and analyzed for crude nitrogen (N) before being blended on the basis of their crude nitrogen (N) concentration. Each blend derived 70% of its dietary nitrogen from cereal (hungry rice) and 30% from legume (soybean) and or oilseed (benne seed) and furnished 20 g of protein per day. Five multi-mix formulations were *D. exilis*$_{70}$ and *S. indicum*$_{30}$ ($D_{70}S_{30}$); *D. exilis*$_{70}$ and *G. max*$_{30}$ ($D_{70}G_{30}$)); *D. exilis*$_{70}$ *S. indicum*$_{15}$ *G. max*$_{15}$ ($D_{70}S_{15}G_{15}$)); *D. exilis*$_{70}$, *S. indicum*$_{20}$ and *G. max*$_{10}$ ($D_{70}S_{20}G_{10}$) and *D. exilis*$_{70}$, *G. max*$_{20}$ and *S. indicum*$_{10}$ ($D_{70}G_{20}S_{10}$). 10 g of each mix was packaged in self-sealed polyethylene food bags and stored in a standard household pantry at 30 to 32°C for 60 days.

Free fatty acid (FFA), water activity (a_w), pH and microbiological load of each mix were analyzed in triplicate using standard procedures at days 0, 14, 28, 42 and 60. Free fatty acid was determined by the method of Association of Official Analytical Chemists (AOAC) (2005). The pH was determined using standard electrode pH meter as described by AOAC (1990). Water activity value analyzer model 5803, Germany was used to assess the water activity of the samples. The microbiological load was determined by serial dilution of each sample in ten-fold with sterile 0.1% media by

Table 1. Percentage free fatty acids of seed flour mixes made from *Digitaria exilis Sesamum indicum* and *Glycine max* seed flours.

Sample code	0 day	14 days	28 days	42 days	60 days
$D_{70}S_{30}$	0.15±0.01^{b}	0.26±0.01^{b}	0.27±0.01^{c}	0.37±0.01^{b}	0.47±0.02^{c}
$D_{70}G_{30}$	0.15±0.01^{b}	0.36±0.01^{a}	0.46±0.01^{a}	0.47±0.02^{a}	0.58±0.04^{a}
$D_{70}S_{15}G_{15}$	0.15±0.02^{b}	0.36±0.02^{a}	0.46±0.02^{a}	0.47±0.01^{a}	0.58±0.05^{a}
$D_{70}S_{20}G_{10}$	0.15±0.01^{b}	0.25±0.02^{c}	0.36±0.01^{b}	0.47±0.03^{a}	0.57±0.01^{b}
$D_{70}S_{10}G_{20}$	0.16±0.01^{a}	0.36±0.01^{a}	0.46±0.02^{a}	0.47±0.05^{a}	0.58±0.01^{a}

Means ± SD of 3 determinations; means with different superscript on a column are significantly different. $D_{70}S_{30}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{30}$; $D_{70}G_{30}$ = *Digitaria exilis*$_{70}$/*Glycine max*$_{30}$; $D_{70}S_{15}G_{15}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{15}$/*Glycine max*$_{15}$; $D_{70}S_{20}G_{10}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{20}$/*Glycine max*$_{10}$; D_{70} $S_{10}G_{20}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{10}$ /*Glycine max*$_{20}$.

Table 2. Water activity (a_w) of seed flour mixes made from *Digitaria exilis*, *Sesamum indicum* and *Glycine max* seed flours.

Sample code	0 day	14 days	28 days	42 days	60 days
$D_{70}S_{30}$	0.48±0.01^{a}	0.53±0.01^{e}	0.55±0.01^{d}	0.61±0.01^{c}	0.72±0.01^{e}
$D_{70}G_{30}$	0.47±0.00^{a}	0.55±0.01^{c}	0.60±0.01^{a}	0.61±0.01^{c}	0.80±0.01^{a}
$D_{70}S_{15}G_{15}$	0.47±0.01^{a}	0.58±0.01^{a}	0.58±0.01^{b}	0.60±0.03^{d}	0.78±0.02^{b}
$D_{70}S_{20}G_{10}$	0.48±0.01^{a}	0.54±0.02^{d}	0.57±0.02^{c}	0.65±0.01^{a}	0.75±0.01^{d}
$D_{70}S_{10}G_{20}$	0.46±0.01^{b}	0.57±0.01^{b}	0.58±0.01^{b}	0.62±0.01^{b}	0.77±0.01^{c}

Means ± SD of 3 determinations; means with different superscript on a column are significantly different. $D_{70}S_{30}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{30}$; $D_{70}G_{30}$ = *Digitaria exilis*$_{70}$/*Glycine max*$_{30}$; $D_{70}S_{15}G_{15}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{15}$/*Glycine max*$_{15}$; $D_{70}S_{20}G_{10}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{20}$/*Glycine max*$_{10}$; D_{70} $S_{10}G_{20}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{10}$ /*Glycine max*$_{20}$.

pour plate technique as described by AOAC (1990). Coliform count was determined using nutrient agar with 24 h incubation at 37°C; sabour and dextrose agar was used for yeast/mold with 48 h incubation at room temperature while for TVC it was 24 h incubation at 37°C. One way analysis of variance (ANOVA) and Duncan's multiple range tests were used to separate/compare means obtained between groups. Differences were considered significant at $p<0.05$.

RESULTS

Table 1 presents the percentage of FFA in the seed flour mixes. The FFA values of all the blends increased with storage ($p<0.05$). At day 0, $D_{70}S_{10}G_{20}$ had the highest FFA (0.16%) while the $D_{70}G_{30,}$ $D_{70}S_{30,}$ $D_{70}S_{15}G_{15}$, and $D_{70}S_{10}G_{20}$ had 0.15%. At day 60, the $D_{70}S_{30}$ had the least FFA value (0.47%) while $D_{70}G_{30}$, $D_{70}S_{15}G_{15}$ and $D_{70}S_{10}$ had the highest value (0.58%). There were differences ($p<0.05$) among the FFA values of the samples especially on days 14 and 28. Table 2 shows water activity (a_w) values of the seed flour mixes at different intervals. The values ranged from 0.46 in $D_{70}S_{10}G_{20}$ to 0.48 in $D_{70}S_{30}$ and $D_{70}S_{20}G_{10}$ at day 0. At day 14, $D_{70}S_{15}G_{15}$ had the highest water activity of 0.58, followed by $D_{70}S_{10}G_{20}$ (0.57 a_w), $D_{70}G_{30}$ (0.55 a_w) and $D_{70}S_{20}G_{10}$ (0.54 a_w). $D_{70}S_{30}$ had the least value (0.53 a_w). At day 28 of storage, $D_{70}G_{30}$ had the highest value (0.60 a_w) and the $D_{70}S_{30}$ had the least value (0.55 a_w). The water activity of all the samples were similar at days 28 and 42, but showed significant differences ($p<0.05$) between days 0 and 60. There was a progressive increase in the pH values of the samples with storage (Table 3). At day 0, all the samples had the same pH value (3.9). The pH values at day 14 ranged from 4.0 in $D_{70}S_{10}G_{20}$ to 4.2 in $D_{70}S_{15}G_{15}$ while at day 42, it ranged from 4.5 in $D_{70}S_{30}$ and $D_{70}S_{15}G_{15}$ to 4.8 in $D_{70}G_{30}$ and $D_{70}S_{10}G_{20}$ and 4.9 in $D_{70}G_{30}$. The decrease in acidity was significant ($p<0.05$) from day 0 to day 60. However, there were no significant differences ($P>0.05$) in the pH values of the samples at a particular interval except in days 28, 42 and 60. At day 0, all the samples had the same TVC value of $<1.0\times10^{2}$ cfu/g except for $D_{70}G_{30}$ (Table 4). At day 14, $D_{70}S_{30}$ and $D_{70}S_{20}G_{10}$ had the least (1.0×10^{2}) TVC value and $D_{70}G_{30}$ had the highest (1.4×10^{2} cfu/g). At the end of 60 days of storage, $D_{70}G_{30}$ had the highest (3.6×10^{4}cfu/g) and $D_{70}S_{30}$ the least (2.2×10^{3} cfu/g) TVC value. Only mold (< 100 cfu/g) was detected from the 14 days. At day 60, the $D_{70}G_{30}$ had the highest mold value (2.4×10^{4} cfu/g), followed by $D_{70}S_{15}G_{15}$ (2.6×10^{3} cfu/g), $D_{70}S_{10}G_{20}$ (1.7×10^{3} cfu/g), $D_{70}S_{20}G_{10}$ (2.7×10^{2}

Table 3. pH of seed flour mixes made from *Digitaria exilis*, *Sesamum indicum* and *Glycine max* seed flours measured at intervals.

Sample code	0 day	14 days	28 days	42 days	60 days
$D_{70}S_{30}$	3.9±0.01^{a}	4.1±0.00^{a}	4.4±0.03^{b}	4.5±0.00^{c}	4.7±0.03^{c}
$D_{70}G_{30}$	3.9±0.02^{a}	4.1±0.01^{a}	4.5±0.05^{a}	4.8±0.01^{a}	4.9±0.04^{a}
$D_{70}S_{15}G_{15}$	3.9±0.01^{a}	4.2±0.00^{a}	4.4±0.01^{b}	4.5±0.00^{c}	4.7±0.02^{c}
$D_{70}S_{20}G_{10}$	3.9±0.02^{a}	4.1±0.04^{a}	4.4±0.02^{b}	4.7±0.02^{b}	4.8±0.01^{b}
$D_{70}S_{10}G_{20}$	3.9±0.03^{a}	4.0±0.00^{a}	4.3±0.02^{c}	4.8±0.03^{a}	4.9±0.03^{a}

Means ± SD of 3 determinations; means with different superscript on a column are significantly different. $D_{70}S_{30}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{30}$; $D_{70}G_{30}$ = *Digitaria exilis*$_{70}$/*Glycine max*$_{30}$; $D_{70}S_{15}G_{15}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{15}$/*Glycine max*$_{15}$; $D_{70}S_{20}G_{10}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{20}$/*Glycine max*$_{10}$; D_{70} $S_{10}G_{20}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{10}$ /*Glycine max*$_{20}$.

Table 4. Total viable count (TVC), mold, yeast and coliform count (cfu/g) of seed flour mixes made from *Digitaria exilis*, *Sesamum indicum* and *Glycine max* seed flours.

Parameter	Intervals (days)	$D_{70}S_{30}$	$D_{70}G_{30}$	D_{70} $S_{15}G_{15}$	$D_{70}S_{20}G_{10}$	D_{70} $S_{10}G_{20}$
TVC	0	<1.0x10^{2a}	1.0x10^{2a}	<1.0x10^{2a}	<1.0 x10^{2a}	<1.0 x10^{2a}
	14	1.0×10^{2d}	1.4×10^{a}	1.2×10^{2c}	1.0×10^{2d}	1.3×10^{2b}
	28	2.2×10^{2c}	2.0×10^{2c}	1.5×10^{2c}	2.0×10^{2c}	1.8×10^{2c}
	42	2.0×10^{3c}	2.2×10^{3b}	1.8×10^{3d}	2.3×10^{3a}	2.0×10^{3c}
	60	2.2×10^{3d}	3.6×10^{4a}	2.4×10^{3c}	2.6×10^{3b}	2.1×10^{4e}
Mold	0	N.D	N.D	N.D	N.D	N.D
	14	6.0×10^{1c}	5.0×10a^{d}	8.0×10a	6.0×10^{1c}	7.0×10^{1b}
	28	1.0×10^{2c}	1.1×10^{2b}	1.3×10^{2a}	1.0×10^{2c}	1.1×10^{2b}
	42	2.0×10^{2c}	2.2×10^{2c}	2.3×10^{2b}	2.0×10^{2c}	2.5×10^{2a}
	60	2.5×10^{2e}	2.4×10^{4a}	2.6×10^{3b}	2.7×10^{2d}	1.7×10^{3c}
Yeast	0 - 60	N.D	N.D	N.D	N.D	N.D
Coliform	0 - 60	N.D	N.D	N.D	N.D	N.D

Means ± SD of 3 determinations; means with different superscript on a column are significantly different. $D_{70}S_{30}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{30}$; $D_{70}G_{30}$ = *Digitaria exilis*$_{70}$/*Glycine max*$_{30}$; $D_{70}S_{15}G_{15}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{15}$/*Glycine max*$_{15}$; $D_{70}S_{20}G_{10}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{20}$/*Glycine max*$_{10}$; D_{70} $S_{10}G_{20}$ = *Digitaria exilis*$_{70}$/*Sesamum indicum*$_{10}$ /*Glycine max*$_{20}$. ND = Not detected.

cfu/g). $D_{70}S_{30}$ had the least value (2.5x10^{2} cfu/g). The differences were significant ($p<0.05$) across the different intervals. The same trend was observed in mold. Yeast and coliform were not detected in all the samples throughout the storage period.

DISCUSSION

At day 0, all the samples had FFA value of 0.15% except the $D_{70}S_{10}G_{20}$ which had 0.16%. These values gradually increased during the 60 days of storage probably due to hydrolysis of fat. This is in agreement with the report of Sewald and DeVries (2003) that hydrolysis of glycerides could account for increased values of free fatty acid in stored flours. The low FFA values observed in the $D_{70}S_{30}$ after 60 days of storage relative to the other mixes could be attributed to absence of benne seed in the blend. This implied that $D_{70}S_{30}$ would have a longer keeping quality because long shelf life is associated with low FFA of a food/product (Food Chain, 2001). However, the values of all the samples were below the range (0.5 to 1.5%) that Onwuka (2005) reported when rancidity began to be noticed in most oils and oil rich foods. Also, antioxidant (sesamol and sesamolina) content of benne seed could have contributed to the long shelf life of the mixes (Bedigian, 2003; Ologunde, 2011). The water activity (a_w) of each blend increased with storage. This could be due to concomitant increase in moisture content and as a function of time and temperature (Rong, 2007). The low water activity of the mixes on day 0 relative to the subsequent days could be associated with fermentation

of the staples. This is in agreement with an earlier report by Mba-Anyadioha (2008) that fermentation increased acidity which reduced the water activity and moisture level in which the fermenting organisms thrive successfully. In addition, sun drying and roasting might have contributed to the reduced values obtained. The water activity values of all the mixes were below the value (0.91) in which most food spoilage bacteria do not grow (Sivasankar, 2010). Spoilage bacteria and mold require $0.91a_w$ and $0.70a_w$, respectively for growth (Sivasankar, 2010). The lower limit for growth of mycotoxigenic mold is about 0.78 a_w. Also, yeast was not detected in all the blends throughout the storage period because a_w values were below the value (0.88) that supports yeast growth (Fontana, 2008).

The pH of all the samples was 3.9 on production. This indicated acidity, which might be due to production of weak acid during fermentation of the grains (Mba-Anyadioha 2008). The pH of a food encourages or discourages growth and survival of microflora in that food (Fontana, 2008). Low pH value (acidity) did not favour the growth of pathogens such as *Enterobacteriaceae* and *Aspergillus* which are implicated in infantile diarrhoeal diseases (Rong, 2007). The optimum pH for growth of *Enterobacteriaceae* is in the range of 6.0 to 8.0 (ICMSF, 1996). As the storage progressed, the pH of the samples gradually increased to 4.9 in $D_{70}S_{15}G_{15}$ and $D_{70}S_{15}G_{15}$. Such increase according to Rong (2007) could be attributed to chemical changes. The final pH value (4.9) of the samples was below the value that encourages microbial growth. The low pH of samples at the end of storage period meant that microbial activities in food products were reduced and that helped to extend the shelf-life of the samples.

Yeasts and coliforms were not detected in the mixes throughout the storage period of 60 days probably because of the acidification of the medium by lactic acid fermenters (Mba-Anyadioha, 2008) and storage temperature. The low microbial load of the complementary mixes was due to low water activity and low pH caused by fermentation of the grains (Mensah et al., 1990). Fermentation induced an anti-microbial environment that reduced the risk of microbial contamination as observed by Ayoya et al. (2010) in home-made complementary foods. The low microbial load could also be due to roasting of the food samples. Roasting reduces micro-organisms and enzyme activity and destroys insects to improve keeping qualities (FAO/WHO, 2012). However, the total viable counts of formulated complementary mixes were within the acceptable limits of 10^7 cfu/g (ICMSF, 1996) for flours. The detection of mold at day 14 could be attributed to post production contamination from the environment (including the storage material), temperature, increased water activity and pH. The temperature and pH ranges that favour mold growth are 10 to 35°C and 2 to 9, respectively. However, the mold counts of all the samples were within ICMSF (1996) specifications (10^5 cfu/g) for flours. Microbial proliferations in foods need certain conditions - namely available water (water activity), proper pH, right temperature and nutrients and time. By controlling these conditions one can prevent microbial growth and extend the shelf life of a food. At the end of the storage period, the samples had FFA values below the range that rancidity was observed in a previous study. Also, a_w values were below that which allows for proliferation of microorganisms. The pH was slightly above the value (4.6) at which a product is said to be acidic however, it was below the level which encourages microbial proliferation. Yeasts and coliforms were not detected in the mixes throughout the storage period of 60 days. The presence of mold from day 14 meant that the mixes should be consumed within 14 days of production. Sanitation is a factor in the control of microbial growth. The flours should be prepared under hygienic conditions and stored in air-tight container and in clean and dry pantry that is not dark to discourage growth of mold. Also, porridge made from the flour should be well cooked to further ensure safety.

Conflict of interests

The authors did not declare any conflict of interest.

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Design of experiment approach for the process optimization of ultrasonic-assisted extraction of polysaccharide from mulberry leaves by response surface methodology

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Mulberry is considered as food-medicine herb, with specific nutritional and medicinal values. In this study, response surface methodology (RSM) was employed to optimize the ultrasonic-assisted extraction of total polysaccharide from mulberry using Box-Behnken design (BBD). Based on single factor experiments, a three level, three variable central composite designs were carried out to establish a quadratic regression model for the extraction efficiency of total polysaccharides as a function of extraction time, extraction temperature and material-water ratio. The optimum extraction conditions were obtained as follows: extraction temperature of 70°C, material-water ratio of 1:30 (g/ml), and extraction time of 40 min. Under these conditions, the predicted total polysaccharides extraction efficiency was 3.6%, while the experimental value was 3.56%. Analysis of variance (ANOVA) was used to examine the statistical significance of the developed model. The result indicates that the established model well predicted the extraction efficiency of total polysaccharides from mulberry leaves.

Key words: Mulberry leaves, total polysaccharides, extraction conditions, design of experiment.

INTRODUCTION

Natural products play a significant role in diet based therapies to cure various maladies (Butt et al., 2009). Genus *Morus* (mulberry) is one of such examples that consists of over 150 species, among these *Morus Alba* L. is the dominant one (Srivastavas et al., 2006). Mulberry leaves are composed of mineral, vitamins, food fibers, amino acid, plant sterols, flavones, alkaloids, polysaccharides, and so on. It has demonstrated significant effects as anti-oxidation (Doi et al., 2000; Doi et al., 2001; Butt and Suh, 2007) and anti-tumor (Kim et al., 2000; Masuda et al., 2002); other role of this product includes the capability to reduce low-density lipoprotein

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(LDL) and blood sugar level (Andallu et al., 2001;Andallu et al., 2002; Andallu et al., 2003; Jeszka-Skowron et al., 2014); and the cosmetic application to delaying ageing and beautifying skin (Halliwell, 1992; Tomoyuki et al., 2006; Wang et al., 2011). Numerous studies show that many polysaccharides works as anti-bacteria and anti-viruses, such as HIV, herpes simplex virus, influenza virus, and vesicular viral gastritis (Jia et al., 2012). Mulberry polysaccharides as well as flavonoids, alkaloids and other comonents in the hypoglycemic activity have a certain synergy (Yang et al., 2007). Polysaccharide also has some anti-radiation effect; the mechanism is generally considered for polysaccharide and activation of the hematopoietic system by strengthening the role of phagocytic cells to enhance the body's tolerance to radiation (Zhang et al., 2003).

In order to speed up investigations of polysaccharides and subsequent application, it is necessary to optimize the polysaccharides extraction process. Microwave-assisted extraction of polysaccharides has been used widely (Soria et al., 2014). To the best of our knowledge, the optimization of the polysaccharide extraction from mulberry leaves have only a few been reported (Ying et al., 2011; Liu et al., 2015; Thirugnanasambandham et al., 2015). In this study, Box-Behnken design (BBD) was selected to optimize the extraction parameters by response surface methodology (RSM) (Bezerra et al., 2008). Firstly, single-factor experimental designs (that is, extraction time, extraction temperature, ratio of water volume to raw material weight) were carried out before BBD experiments. Secondly, the more significant factors (that is, extraction time, extraction temperature, and ratio of water volume to raw material weight), with three levels, were chosen for further extraction optimization of polysaccharides by BBD experiment and RSM analysis. This method has been extensively used to optimize chemical and biochemical processes (Ge et al., 2014; Wang et al., 2014). This paper defined three factors namely extraction time, extraction temperature and material mass concentration by response surface methodology (RSM) to gain the optimum extraction condition.

MATERIALS AND METHODS

Pretreatment of mulberry leaves

Fresh mulberry variety Yu711 (*Morus multicaulis*) leave was harvested by hand from the National Mulberry Gene Bank of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu Province, China, and then were wind-dried. Dried mulberry leaves were crushed and passed through a 2.36 mm sieve, and used as the experimental sample.

Extract liquid preparation and glucose determination

The 3.0 g mulberry leaves powder was added to 120 ml 80% ethanol, and extracted for 40 min at 50°C by ultrasonic extraction method. After extraction, the residue was dried in an oven at 50°C, and each pretreated sample (1 g) was submitted to polysaccharide extraction according to the design of the experiment taking in consideration extraction times, temperatures and the ratio of water to raw material. After each reaction, samples were centrifuged for 5 min at 8000 rpm at room temperature. An amount of 1 ml of each supernatant was mixed with 5 ml 10% sulfuric acid anthrone solution. The mixture was strongly vortexed, and was heated until water boiled, held for 10 min, then water-cooled and stabilized 10 min at room temperature. The contents of D-(+)-Glucose were determined by sulfuric acid anthrone colorimetric method. D-(+)-Glucose was used as a standard for measurement of the total sugar content (Dubois et al., 1956). The absorbance of samples was measured by 722 UV-VIS spectrophotometry at 625 nm. The polysaccharide yield (%) was calculated using the following equation:

$$\text{Polysaccharides yield (\%)} = \frac{C \times N \times V}{W \times 1000} \times 100\%$$

Where, C is the concentration of polysaccharide calculated from the calibrated regression equation (mg/mL); N is the dilution factor; V is the total volume of extraction solution (mL); and W is the mass of raw material (g).

Single factor experiments

Effect of the ratio of water to raw material on polysaccharides

Adjustment of extraction time to 40 min, extraction temperature to 50°C and extract polysaccharides from mulberry leaves with different raw material to water ratio of 1:5, 1:15, 1:25, 1:35 and 1:45 g/ml, respectively were done. The extraction rate was determined with the above method described.

Effect of extraction time on polysaccharides

Fixed extraction temperature of 50°C, water-material ratio of 1:15 g/ml and different extract time of 10, 20, 30, 40 and 50 min, respectively were used. The extraction rate was determined with the above method described.

Effect of extraction temperature on polysaccharides

Fixed water-material ratio of 1:15, extraction time 20 min and extract polysaccharides from mulberry leaves with different temperatures were 40, 50, 60, 70 and 80°C，respectively were used. The extraction rate was determined with the above method described.

Experimental Box-Behnken design

After determining the preliminary range of the parameters by a single-factor experiment for the polysaccharides production, the extraction parameters were optimized using RSM. Based on the results of preliminary experiments, two levels, three variables Box-Behnken central composite design was carried out to establish a quadratic regression model for the extraction efficiency (y) of total polysaccharides as a function of extraction temperature (x_1), extraction time (x_2) and material-water ratio (x_3) as presented in Table 1. Based on the results of Box-Behnken design, optimization Box-Behnken design plan of extraction technology or predicted

Table 1. The factors and levels of Box-Behnken central composite design of extraction technology of polysaccharides from mulberry leaves by utrasonic-assisted

Level	Factor		
	X_1 temperature (°C)	X_2 time (min)	X_3 Material-water ratio (g /ml)
-1	60	10	1：15
0	70	20	1：25
+1	80	30	1：35

Table 2. Comparison between predicted and actual value on the yield of polysaccharides from mulberry leaves by Box-Behnken design.

Order	(X_1)	(X_2)	(X_3)	Actual value (y,%)	Predicted value (y,%)
1	-1	-1	0	1.94	1.92
2	0	0	0	3.52	3.35
3	-1	1	0	2.17	1.95
4	1	0	-1	0.95	0.84
5	0	1	1	1.99	2.11
6	0	0	0	3.53	3.35
7	0	0	0	3.43	3.35
8	0	-1	1	3.02	2.94
9	-1	0	-1	1.79	1.93
10	0	0	0	3.16	3.35
11	1	0	1	3.11	2.97
12	0	0	-1	1.33	1.41
13	1	-1	0	1.42	1.63
14	0	0	0	3.11	3.35
15	1	1	0	1.41	1.44
16	0	-1	-1	0.85	0.74
17	-1	0	1	2.58	2.69

value and actual value on the yield of polysaccharides from mulberry leaves was analyzed (Table 2).

Data analysis

Polysaccharide concentration was determined according to the colorimetric method describe aboved and the data were evaluated by using the Expert Design 8.0.6 for Windows software (SPSS Inc., USA). The values were calculated as the mean of individual experiments in triplicate. The statistical significance was analyzed by Student's t test and regression analysis and the mathematical model was performed and expressed as response of surface.

RESULTS

Effect of extraction temperature on polysaccharides

Extraction temperature range was 40 to 80°C, higher temperature leads to low extraction rate. Based on the extraction condition of polysaccharides from mulberry leaves, material-water ratio of 1:15, and time 20 min, temperature was set as 40, 50, 60, 70 and 80°C, respectively. Figure 1A shows that the extraction rate of polysaccharides from mulberry leaves reached the highest when temperature was set as 70°C.

Effect of water-material ratio on polysaccharides

As shown in Figure 1B, material-water ratio was selected as 1:5, 1:15, 1:25, 1:35, and 1:45 while the other factors were set as temperature of 50°C, and time of 20 min. The optimal material-water ratio for extraction rate of polysaccharides was 1:25.

Effect of extraction time on polysaccharides

With respect to time, the other factors were set as material-water ratio of1:15, and temperature 50°C and time was selected as 10, 20, 30, 40 and 50 min .The result in Figure 1C shows that the extraction rate increased rapidly from 10 to 20 min and the remaining little changed at later time. Therefore, the appropriate time was selected at 20 min.

Improvement of the extraction conditions

The response selection is a critical stage in optimization. The responses were directly related to the parameters that define the extraction yield of polysaccharides. Based

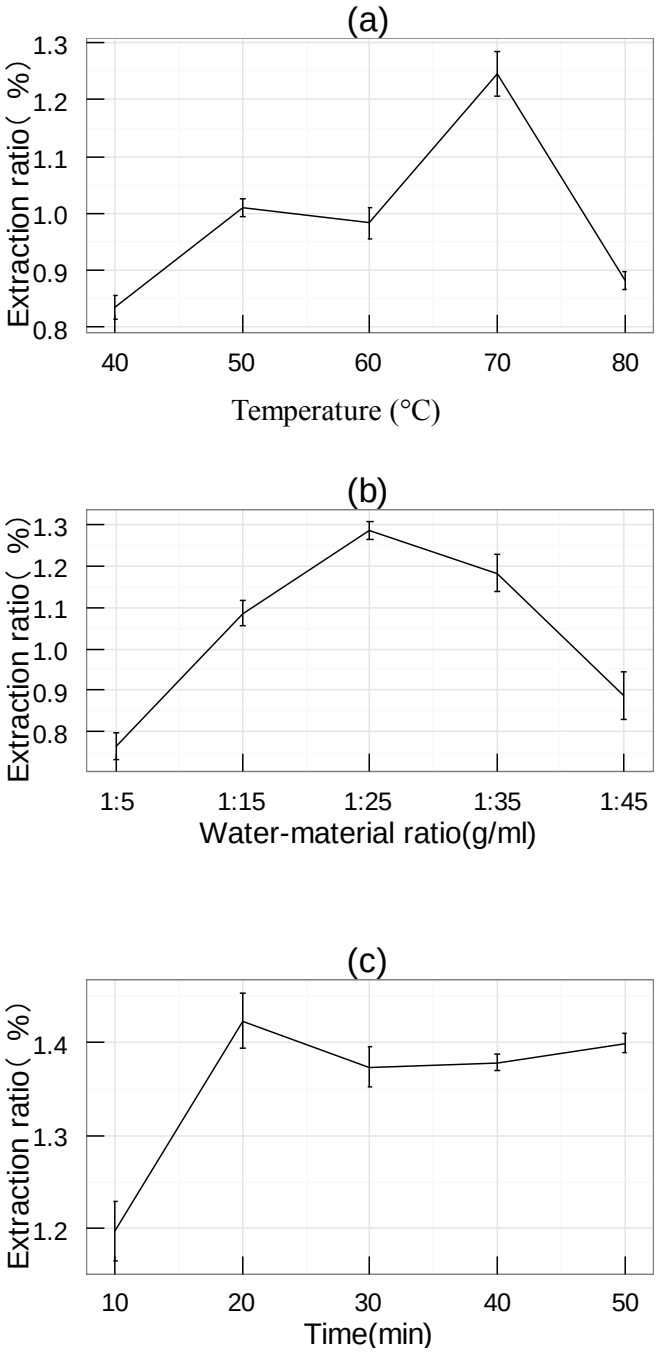

Figure 1. Effect of temperature, water-material ratio and extraction time on the yield of polysaccharides from mulberry leaves respectively. **(A)** extraction temperature. **(B)** Extraction water-material ratio. **(C)** Extraction time.

on the single factor experiments and the principle of Box-Beahnken design, temperature (x_1), time (x_2), and material-water ratio (x_3) were chosen to be as independent variables. Meanwhile, polysaccharide yield was regarded as response value. The response surface analysis was adapted to improve the process condition of extraction polysaccharides from mulberry leaves (Figure 2). According to statistical analysis, the result of regression analysis was shown in Table 3. By applying multiple analyses, the results were fitted to a second-order polynomial equation and the model was obtained as follows:

$$Y=3.35-0.20X_1-0.040X_2+0.72X_3-0.058X_1X_2+0.34X_1X_3-0.38X_2X_3-0.65X_1^2-0.96X_2^2-0.59X_3^2$$

Table 3 shows the significance of the linear relationship between dependent variable and independent one. The model could account for 97.46% of the response value, showing a good fit of the model. The non-significance of lack of fit showed that the quadratic regression equation could predict the response value. The significant terms of x_1, x_3, x_1x_3, x_2x_3, x_1^2, x_2^2,and x_3^2 shows that they could have a considerable influence on the response value. The actual response value was close to the predicted one, suggesting that the regression model for the design was available. The model “Prob > F” value was less than 0.001, showing that the model test is remarkable. Lack of fit was not significant. Model calibration coefficient *Adj* R^2 (0.9419) means that the model can explain the change in 94.19% response value, and only about 5.81% of the total variance was not explain with this model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 15.174 indicates an adequate signal (Li et al., 2012). This model can be used to navigate the design space. x_1^2, x_2^2 and x_3^2 are significant model terms in Table 3.The results suggest the range of extraction time (x_2), extraction temperature (x_1), and material-water ratio (x_3).

In order to estimate the polysaccharides extraction factors, plotted graphs were made among the influencing parameters. The response surface graph depicted by the two out of three factors formed a series of approximate circles with one center regardless of diverse gradients; simultaneously vaults hanging down took shape at the three-dimensional chart and reached the maximum. The response values from exertion would be calculated by first order local deviation with equivalence to zero, when x^1, x^2 and x^3 were set to 70°C, 20 min and 1:30 (g/ml), respectively.

DISCUSSION

Morus is a genus of deciduous trees native to warm, temperate, and subtropical regions of Asia, Africa, North America, and Southern Europe. Mulberry leaves have historically been used for its foliage, to feed silkworm (*Bombyx mori* L.). In traditional Chinese medicine, Mulberry leaves has been used as a tonic and sedative, as dried mulberry fruits and root bark of the mulberry. Polysaccharides existed in mulberry leaves and exhibit anti-tumor, inhibition of enzymatic activity, anti-virus and

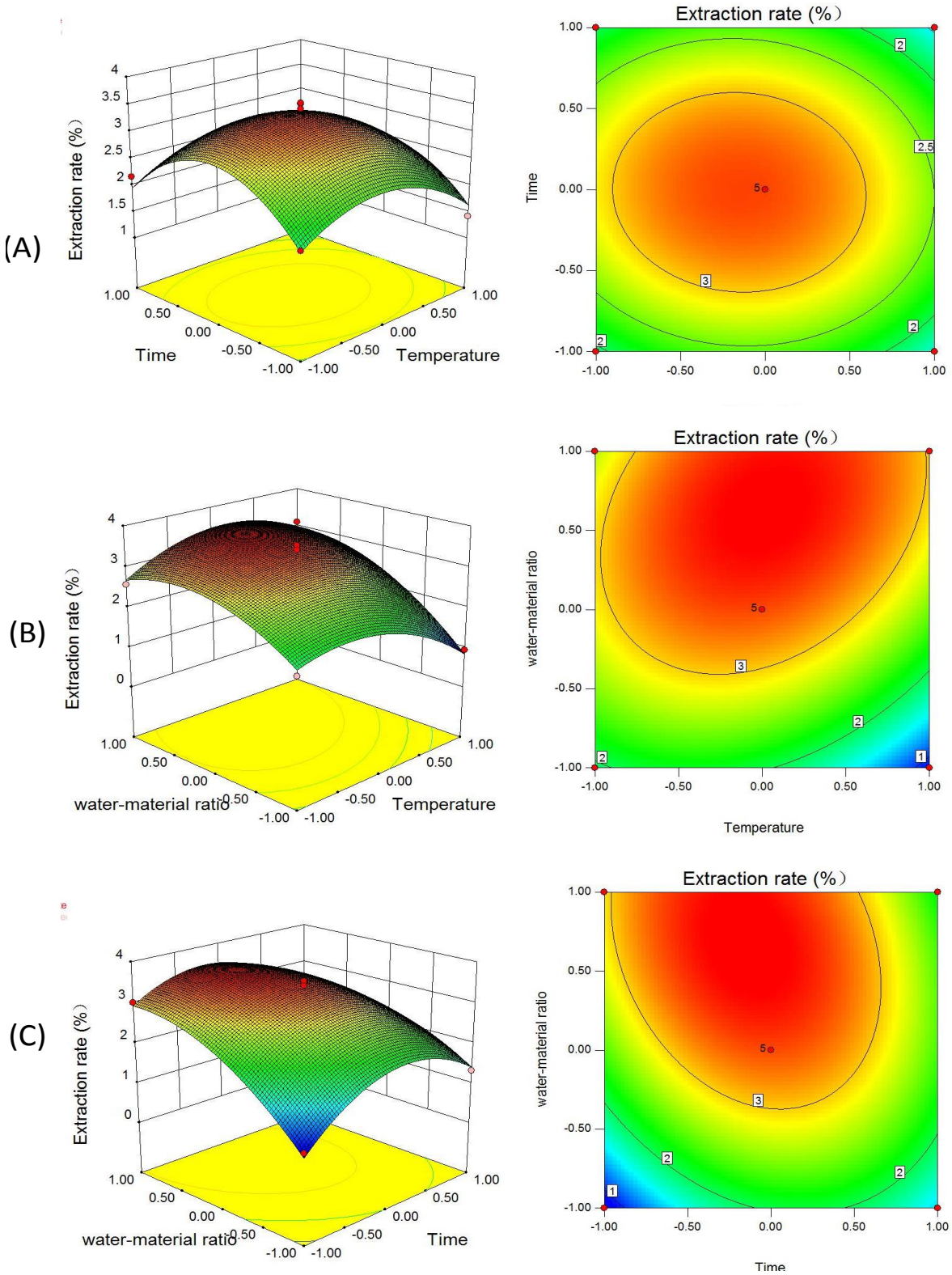

Figure 2. Response surface and contour plot showing the interactive extraction effects. **(A)** Temperature and time. **(B)** Water-material ratio and temperature. **(C)** Water-material ratio and time.

other biological activities, suggesting that the mulberry leaves have compounds with physiological functions that

Table 3. ANOVA for polysaccharide yield

Source	Sum of squares	df	Mean square	F value	*p*-value Prob > F	Significant
Model	13.48	9	1.50	29.83	< 0.0001	**
X_1	0.32	1	0.32	6.41	0.0391	*
X_2	0.013	1	0.013	0.26	0.6278	
X_3	4.18	1	4.18	83.15	< 0.0001	**
X_1X_2	0.013	1	0.013	0.27	0.6215	
X_1X_3	0.47	1	0.47	9.35	0.0184	*
X_2X_3	0.56	1	0.56	11.23	0.0122	*
X_1^2	1.79	1	1.79	35.61	0.0006	**
X_2^2	3.89	1	3.89	77.44	< 0.0001	**
X_3^2	1.46	1	1.46	29.10	0.0010	**
Residual	0.35	7	0.050		0.3208	Not significant
Lack of fit	0.19	3	0.064	1.61		
Pure error	0.16	4	0.040			
Cor total	13.83	16				
R-squared	0.9746					
Adj-squared	0.9419					
Adeq precision	15.174					

**Extremely significant difference at $p<0.01$, *significant difference at $p<0.05$.

offer protection to humans (Jeszka-Skowron et al., 2014).

There are a few reports about mulberry polysaccharide extraction. A water-soluble polysaccharide was extracted from *Morus abla* L. leaves by method of boiling water and ethanol deposition by Liu (2005). *M. abla* L. leaves polysaccharides were shown to be a homogenous component by the column chromatography and electrophoresis (Liu, 2005). Experiments showed that mulberry leaves polysaccharides are composed of a single polysaccharide，but the extraction temperature and extraction time was too long. Yin et al. (2011) studied the ultrasound-assisted extraction of polysaccharides from mulberry leaves and the results showed that optimum conditions were extraction temperature of 60°C, extraction time of 20 min and ratio of water to raw material of 15:1 (ml/g). Thirugnanasambandham et al. (2015) investigated the effects of three microwave-assisted extraction factors on the yield of polysaccharides using RSM, showing that optimum extraction conditions were determined as follows: weight of the sample of 20 g, microwave power of 170 W and extraction time of 10 min. In this paper, the optimum extraction conditions were obtained as follows: extraction temperature of 70°C, material-water ratio of 1:30 (g/mL), and extraction time of 40 min. Under these conditions, polysaccharide yield was found to be 3.56%; no significant difference was observed between the predicted yield and experimental one when the Student *t*-test was conducted, indicating that the model was satisfactory and adequate for reflecting the expected optimization.

In conclusion, based on single factor experiments, a 2 level, three variable central composite designs was carried out to establish a quadratic regression model for the extraction efficiency of total polysaccharides as a function of extraction time, extraction temperature and water-material ratio. The optimum extraction conditions were obtained as follows: extraction temperature of 70°C, material-water ratio of 1:30 g/mL and extraction time of 40 min. Under these conditions, the predicted total polysaccharides extraction efficiency was 3.6% and the experimental value was 3.56%. The result indicates that established model predicted the extraction efficiency of total polysaccharide from mulberry leaves and the optimized extraction conditions are a good reference for practice.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Separation and purification of chlorogenic acid from tobacco by-products by polyamide and silicagel column chromatography

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Methods for separation of chlorogenic acid (CA) from tobacco by-products were established. The liquid chromatography tandem mass spectrometry (LC-MS) method for identification and analysis of chlorogenic acid from tobacco materials has been developed. CA was isolated by polyamide and further purified by silicagel column chromatography. Results reveal that polyamide is suitable for separation of CA from tobacco extract. After one run treatment with polyamide, the content of chlorogenic acids was 40.3%. The product was further purified using silicagel column chromatography; the content of total chlorogenic acid was increased 2.29-fold from 40.3 to 92.2%. The LC-MS results showed that total chlorogenic acids were made up of four components: 5-CQA, 3-CQA, 4-CQA and cis-5-CQA. The content of 5-CQA was the most (78.2%) and other three chlorogenic acid derivatives were 1.9, 10.1 and 2.0%, respectively.

Key words: Tobacco by-products, chlorogenic acid, polyamide, silica gel column, LC-MS.

INTRODUCTION

Tobacco, an herbaceous plant, is one of the important economic crops in the world. The leaves of tobacco are the most essential material for cigarette production (Chen et al., 2013); but, more than 20% of the tobacco are discarded during production, and not used for other purposes (Zhang et al., 2012). Therefore, it is urgent to dispose the tobacco by-products. Chlorogenic acid, which is formed by esterification of caffeic acid and quinic acid, is the major phenolic compound in tobacco by-products. It is possessed of physiological effects such as anti-bacterial, antivirus and antioxidant (Sato et al., 2011; Zhao et al., 2010; Yun et al., 2012; Yuan et al., 2012;

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Abbreviations: CA, Chlorogenic acid; **LC-MS,** liquid chromatography tandem mass spectrometry; electrospray ionization (ESI).

Wang et al., 2009; Danino et al., 2009). It is feasible to utilize tobacco by-products as the material for separating chlorogenic acids. Generally, an ultrasonic method is used for the extraction of chlorogenic acid (Li et al., 2005; Mazvimba et al., 2012). Many materials, for example polyamide, macroporous resins (Wan et al., 2014; Zhang et al., 2008), silica gel column are used in separating and purifying chlorogenic acid. In recent years, polyamide has been widely used in separating plant effective components such as phenolic acids, flavonoids, quinones (Guo et al., 2011; Sun et al., 2013) and good results are obtained. Silica gel column chromatography is another material for separation according to different absorption capacity.

In the present study, we investigate the adsorption and desorption properties of chlorogenic acids on polyamide and developed an efficient method for the preparative separation of chlorogenic acids from tobacco by-products. In addition, chlorogenic acids in the final products were identified by liquid chromatography tandem mass spectrometry.

MATERIALS AND METHODS

Materials and reagents

Tobacco by-products provided by a company in Anhui province of China and were smash into 40 mesh by a pulverizer (selected by sieve), and then were kept at room temperature. The tobacco by-products extracts were prepared by ultrasonic-assisted procedure with 10-fold 95% ethanol (pH = 4.0) at 50°C for 1 h twice. The extracting solution was filtered and then condensed, stored at 4°C in the dark for the subsequent experiments. Ethanol, phosphoric acid (analytical grade) was purchased from Tingbest Co. (Nangjing, China), methanol (HPLC grade) was purchased from TEDIA Co. (USA), and all aqueous solutions were prepared with pure water produced by Milli-Q system (Bedford, MA, USA).

HPLC analysis

Quantitative analysis of the concentration of chlorogenic acid was carried out by HPLC on Agilent 1100 HPLC system composed of two quaternary pumps with a degasser, a thermostatted column compartment, a variable wavelength detector, autosampler and 1100 ChemStation software. Sample analysis was carried out on Ultimate C18 column (250 mm × 4.6 mm I.D., 5 µm) at a column temperature of 30°C. Mobile phase used was a mixture of 10 mmol/L phosphate buffer (pH4.0): methanol (25:75,v/v) at a rate of 1.0 mL/min. Injection volume was 10 µL and UV detection was at 326 nm. Each analysis was repeated for three times. All samples were filtered through 0.45 µm membrane before HPLC analysis. Standard curve method was used for quantification.

LC-MS analysis

HPLC–MS was conducted on an Agilent 6460 HPLC (Agilent, California, USA), coupled to negative electrospray ionization (ESI) tandem mass spectrometry (MS/MS) method. Chlorogenic acid separations were achieved on a C18 reverse phase column (100 × 2.1 mm; ZORBAX Eclipse Plus; Agilent, USA). The mobile phase, a solution of methanol and 0.1% aqueous formic acid (20:80,v/v), was set at a flow-rate of 200 µl/min. Mass spectra in the negative ion mode were operated under the following conditions: fragmenter voltage = 5 eV; voltage = 3500 V; nebulizer pressure = 45 psi; capillary temperature = 300°C; m/z range = 50 to 800.

The polyamide adsorption capacity for chlorogenic acid

Static adsorption test

1 g polyamide was put into a 150 mL conical flask and 20 mL of solution of tobacco extracts (1.15 mg/mL chlorogenic acid) was added. The flask was shaken on an incubation shaker (120 rpm) at 25°C for 8 h to reach adsorption equilibrium. The solution after adsorption was analyzed by HPLC, and then the ratio adsorption amount was calculated.

$$Qe = V_0 (C_0 - Ce)/W \quad (1)$$

Where, Qe is adsorption amount, which represents the mass of adsorbed on 1 g of polyamide resin at adsorption equilibrium; C_0 and Ce is the initial and equilibrium concentration of chlorogenic acid in the solutions, respectively; V_0 is the initial volume of solution added into the flask, and W is the weight of the polyamide resin.

Dynamic adsorption

Dynamic adsorption experiment was carried out on glass column (10 × 400 mm) wet-packed with polyamide at 25°C and the bed volume (BV) of the resin was 14 mL (equal to 3 g of dry resin). Sample solution containing certain concentration of chlorogenic acid (pH 4.0, adjusted by 2M H_3PO_4 solution) flowed through the glass column at different flow rates, and the chlorogenic acid in the eluent was determined by HPLC.

Dynamic desorption test

While adsorption of chlorogenic acid was confirmed, dynamic desorption test was carried out. A gradient program was adopted: the adsorbate-laden column was eluted with different concentration ethanol solution, and collected at 1 BV intervals. According to the monitoring of the concentration of chlorogenic acid in every interval, effect of ethanol concentration on the efficiency of elution had been investigated.

RESULTS AND DISCUSSION

Static adsorption on polyamide

Based on the HPLC analysis, the initial concentration of chlorogenic acid in the extraction solution was 4.68 mg/mL, and then diluted to a concentration of 1.15 mg/mL for static desorption test. The results of static adsorption were summarized in Table 1. As seen in Table 1, the polyamide adsorption capacity for chlorogenic acid was 9.78 mg/g.

Effect of the flow rate on dynamic adsorption

Sample solution containing same concentration of chlorogenic acid flowed through the glass column at

Table 1. Adsorption capacity of chlorogenic acid of tobaoo by-products on polyamide.

Initial mass of CA (mg)	Average equilibrim mass of CA (mg)	Mass of CA adsorbed (mg)	Qe (mg/g resin)
22.92	13.14	9.78	9.78

Table 2. Effect of different flow rate on chlorogenic acid adsorption.

Flow rate (mL/min)	Initial mass of CA (mg)	Content of CA in out flow liquid (mg)	Qe (mg/g resin)
0.15	22.92	8.38	14.54
0.32	22.92	7.52	15.40
0.75	22.92	5.27	17.65
1.5	22.92	5.83	17.09

Table 3. Effect of ethanol concentration on the efficiency of elution.

Fraction	Eluant (% ethanol)	Concentration (mg/mL)	Desorption ratio (%)
1	0	0.00	0.0
2	10	0.33	16.5
3	20	0.55	26.9
4	30	0.56	27.5
5	40	0.20	10.0
6	50	0.05	2.3
7	60	0.00	0.0

different flow rate to test dynamic adsorption. As shown in Table 2, the adsorption capacity increased with the increase of flow rate and reached the maximum adsorption capacity (17.65 mg/g) when the flow rate was 0.75 mL/min. The adsorption capacities decreased slightly at higher flow rate (1.5 mL/min) due to lack of sufficient time to adsorb by polyamide. Therefore, the flow rate was adjusted to 0.75 mL/min for all later experiments.

Effect of ethanol concentration dynamic desorption

Adding at the flow rate of 0.75 mL/min, the extract was absorbed on polyamide column for 30 min. Firstly, the column was eluted with 1 BV distilled water, then gradient eluted with 10, 20, 30, 40, 50 and 60% ethanol, and 7 elution fraction (1 BV for each) were collected for calculating the concentration and desorption ratio. As shown in Table 3, chlorogenic acid does not exist in fraction 1. In water solution, the chemical complexation between phenolic hydroxyl groups and the amide groups of the polyamide through the association of the hydrogen bond was so strong that chlorogenic acid is difficult to be eluted. Meanwhile, lots of impurities were removed with water, which was convenient for further isolation. Chlorogenic acid was mainly centered in five fractions (from 10 to 50% ethanol eluant), and the total desorption ratio was 83.2%. According to desorption ratios of the 5 fractions, 10, 40 and 50% ethanol were found to be not favorable for the eluant due to their low desorption ratios, but the difference between the desorption ratio of 30 and 20% ethanol is 0.6% only. As shown in Figure 1 to 2, the unknown peak, whose retention time was earlier than that of the peak represented chlorogenic acid (the highest peak), almost vanished in the 30% ethanol eluant. To decide the concentration of ethanol for further experiments, it was necessary to select 20 and 30% ethanol as eluant for dynamic desorption, respectively.

The dynamic desorption curve on polyamide was shown in Figure 3. By comparison, it can be seen that the shape of elution peak with 30% ethanol eluant is relatively concentrated and more symmetric than that of elution peak with 20% ethanol eluant. Approximately, 4 BV of desorption solution desorbed chlorogenic acid completely from polyamide resin. The two fractions of desorption solutions were combined and evaporated by rotary vaporization at 45°C under reduced pressure, then freeze-dried. The dried product was weighed and the contents, yields of chlorogenic acid were calculated (Table 4). Based on an overall consideration of various factors (the volume of eluant, yield and purity), 30%

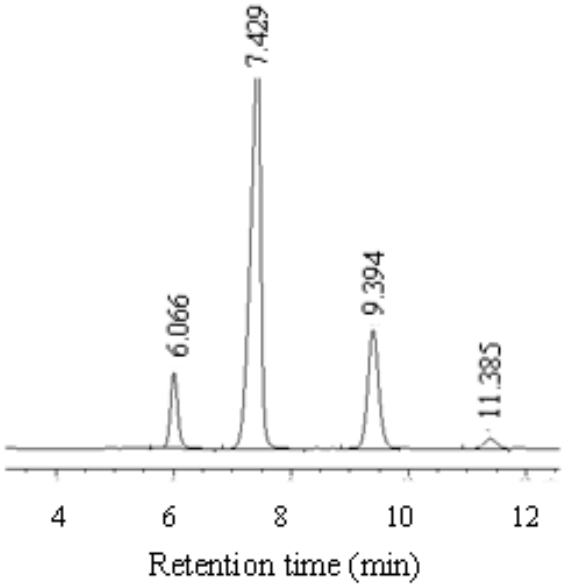

Figure 1. Chromatography of the 20% ethanol eluant (left).

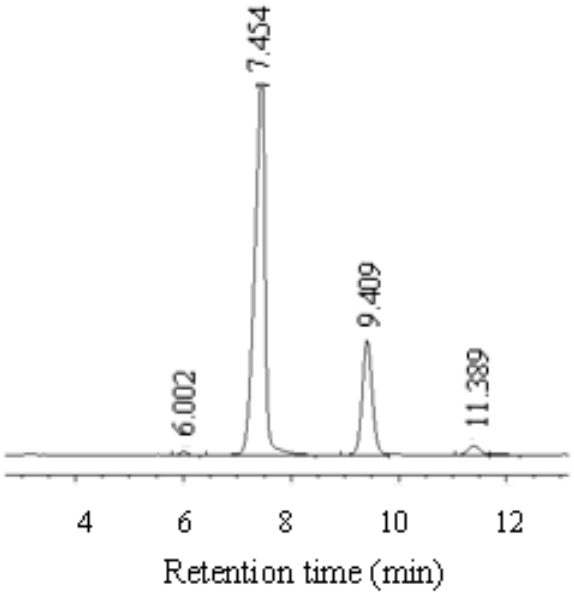

Figure 2. Chromatography of the 30% ethanol eluant (right).

ethanol was used as the eluant for further experiment.

Column chromatography

Polyamide (80 to 100 mesh) was packed in a glass column (10 mm × 40 mm i.d). Adjust the volume of the extract according to the volume of column packing.

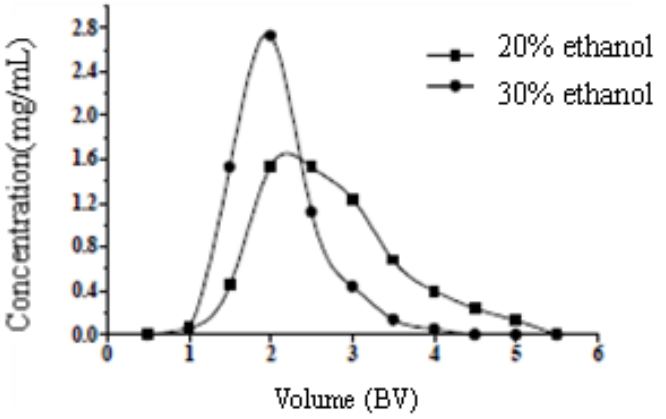

Figure 3. Elution curves of CA with two concentration of ethanol (left).

Chromatographic zone was set on top of the column packing to reach saturated adsorption equilibrium. The resin was first washed by pure water (1 BV) and then desorbed with 30% ethanol (5 BV). As shown in Figure 4, results of chromatography showed 3 bands significantly. A band moved the quickest, the color of the eluate is red, while the color of B band is dark, and C is yellow. The content of chlorogenic acid in the eluent of C band is 75.2%, while the content is 23.1% in B band, in which impurities took the most part. However, 23.1% of chlorogenic acid would be losing if the B band section was abandoned directly. This section was further purified by polyamide column with the same solvent system. This collecting eluate was merged with C band eluate then freeze-dried. The crude product was obtained and the content of chlorogenic acid was calculated. After treatment with polyamide twice, the content of chlorogenic acid reached 40.3% in the product, which is 5.7-fold as much as that in tobacco by-products.

Purification on silicagel column

The product after polyamide isolation was dissolved and then subjected to further chromatographic purification on silicagel column (300 × 25 mm id). The total content of chlorogenic acid after separation was 92.2%.

Quantitative determination

After detected by LC-MS in the negative ion mode, four peaks were separated on the total ion chromatogram (TIC) (Figure 5). Four quasi-molecular ions were 353.1[M-H]- and their peaks were rather strong in the mass spectrum.

Figure 6 shows the ion scan spectrum of this compound, with characteristic fragment ions at m/z191, 179,

Table 4. Effect of ethanol elution concentration on the product purity.

Sample	Eluant (% ethanol)	Yield (%)	Purity (%)
1	20	35.6	26.6
2	30	33.8	27.7

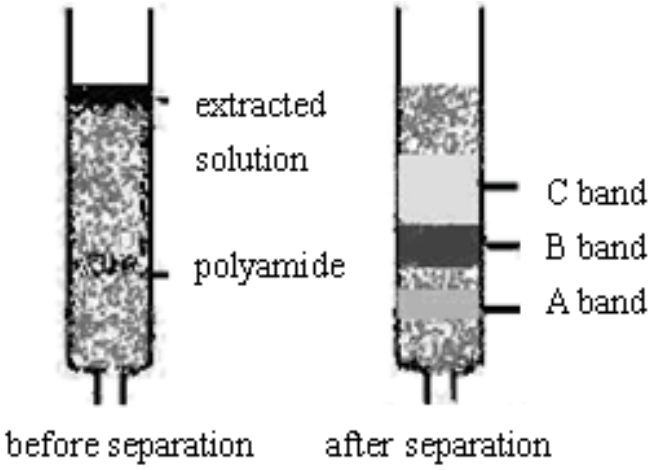

Figure 4. Column chromatography of polyamide (right).

173, 161 and 135. Ions at m/z 191 or 173 indicated fragments of the quinic moiety, and ions at m/z 179 or 161 indicated fragments of the caffeoyl moiety, and they were [quinic-H]-, [quinic-H-H_2O]-, [caffeic- H]-, [caffeic-H_2O-H]-, respectively. Ions at m/z135 indicated another fragment of caffeic acid. Except the difference of type among the fragments, the relative intensities of the fragment ions were quite different, which was related to the peak-heights. Along with relevant references (Zhang et al., 2013; Rodrigues and Bragagnolo, 2013; He et al., 2010), four peaks were identified as four isomers of caffeoylquinic acid [3- CQA (I), 5-CQA (II), 4-CQA (III) and cis-5- CQA (IV)], respectively. In the product, 5-CQA is the dominant compound, of which the content was 78.2% and other three chlorogenic acid isomers were 1.9% (3-CQA), 10.1% (4-CQA) and 2.0% (cis-5-CQA), respectively. IUPAC numbering system was used in this work for the structure of chlorogenic acids.

Conclusions

The separation and purification process of chlorogenic acid with polyamide and silicagel has been successfully developed in this study, and products with different purities (40.3, 92.2%) can be gained after different procedure. The LC-MS/MS system used in this work appeared to an excellent tool for identifying structures of different components in the high purity product, especially the isomers. The identification by LC-MS showed that the discarded tobacco leaves are rich in chlorogenic acid (5-CQA) as well as other isomers (3-CQA, 4-CQA, cis-5-CQA), which possess the similar pharmaceutical bioactivities. Thus, the tobacco by-products can be used as one of alterative materials for extracting chlorogenic acids. It is a great significance to explore the variety of materials and reduce the costs of large-scale separation process of chlorogenic acids.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Acute toxicity and behavioral changes of Caspian kutum (*Rutilus frisii Kutum* Kamensky, 1991) and Caspian roach (*Rutilus rutilus caspicus* Jakowlew, 1870) exposed to the fungicide hinosan

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Pesticides are used in agriculture to control pest and protect human health and animals. Excessive use of pesticides caused risk for human health and threated non-target organisms, polluted water, soil and air. Hinosan is a component of organophosphate pesticide which is used as a fungicide in agricultural fields. In the study, lethal concentration (LC_{50}) of Hinosan was calculated for *Rutilus frisii kutum* and *Rutilus rutilus caspicus* with a mean weight of 3 ± 1 g [mean ± SD]. The experiment were carried out in static condition and based on instructions of O.E.C.D in four days under controlled water physicochemical factors with pH = 7 to 8.5, dissolved oxygen = 200 mg L^{-1} ($CaCO_3$) and temperature = 20 ± 1°C. Fishes were acclimatized in 70x40x30 cm aquarium for 10 days. Five treated aquariums with concentration ranges 1, 2, 4, 8, 16 ppm of hinosan (Technical 95 Edifenphos) with one control group (no toxic concentration), were performed. Data were analysed using the probit analysis. LC_1, LC_{10}, LC_{30}, LC_{50}, LC_{70}, LC_{90} and LC_{99} were calculated in 24, 48, 72 and 96 h. Our results indicate that LC50 96 h hinosan for *R. frisii Kutum* and *R. rutilus caspicus* were obtained 3.61 and 2.88 ppm, respectively. These findings suggest that hinosan is medium toxicity for these two species. Clinical symptoms including irregular protrusion of the eyes and irregular swimming were observed.

Key words: Organophosphore, hinosan, *Rutilus frisii kutum*, *Rutilus rutilus caspicus,* LC_{50}, pollution.

INTRODUCTION

Aquatic ecosystems as the largest environments are constantly faced with the threats such as genetically restrictions and biological diversity. However, these environments are not the target for pesticides; nevertheless some results of studies sighted the presence of pesticides and their metabolites in surface water (Mansingh and Wilson, 1995; Tsuda et al., 1996; Van-Der Geest et al., 1997). Organophosphorus fungicide compounds are widely use in agricultural and thus permeate into fish farms and aquatic ecosystems which causes the contamination (Cossarini-Dunier et al., 1990). Annually dozens tone of organophosphorus fungicides penetrate into the global environment (Melnikov et al., 1977). These compounds, along with

agricultural pesticides, are highly toxic and can cause mortality in fish population (Gelman and Herzberg, 1979). Organophosphate compounds act as inhibitors of AChE activity and in some cases, curb the activity of nervous system (Repetto et al., 1988). Laboratory conditions indicates that organophosphate exposure causes impact on immune system through influence on antigens and antibodies, lymphocyte proliferation and cytokine, production T toxic lymphocytes and hydrogen peroxide production by macrophages, and disrupting activity of the nervous system (El-Gendy et al., 1988).

Hinosan (Edifenphos), which is chemically known as O-ethyl S,S-diphenyl phosphorodithioate ($C_{14}H_{15}O_2PS_2$) was introduced into the world of agriculture in 1966. The members of organophosphate toxins are used as fungicides in rice agricultural fields. Studies on similar compounds (parathion, malathion Sarin, Tabun, and soman) indicates that all these toxins when used at destructive doses significantly stop the antibodies activity (Casale et al., 1983). Organophosphates pesticides generate free radicals under various metabolic processes in living organisms. These radicals often harm the structure biomolecules such as proteins, genetic material (damage DNA) and there (Mohanty et al., 2011). Edifenphos (hinosan) is an organophosphate fungicide cutinase inhibitors that displays a specific antipenetrant action, but in practice its therapeutic activity may also involve direct fungitoxicity (Sisler, 1986). Several studies have been conducted on fish health effects of organophosphate fungicides and the removal process of these pesticides from water resources (Studnicka and Sopinska, 1983; Ahmad, 2011; Rauf and Arain, 2013; Shahbazi et al., 2015). In some species, the destructive actions of the toxin on the hepatic tissues have been reported, but the effects of these toxins on fish tissue are needed for further studies. However, a few studies have been conducted to evaluate adverse effect of edifenphos on fishes. El-Gendy et al. (1996) stated that edifenphos causes elevation of catalase activity in tissues of *Oreochromis niloticus* and had depressive effect on activates of of acetylcholinesterase (AChE), adenosine triphosphatase (ATPase) and glutathion-S transferase (GST).

Rutilus is a genus of fishes in the family Cyprinidae, which are found in Europe and western Asia where there are about 15 species (Coad, 2014). *Rutilus frisii Kutum* Kamensky, 1991, (Caspian Kutum) and *Rutilus rutilus caspicus* Jakowlew, 1870, (Caspian roach) are native of Caspian Sea and rivers leading to. *R. frisii Kutum* main habitat is the southern part of the catchment area, particularly the coast of Iran (Tamarin and Kuliev, 1989; Raeisi et al., 2014). In March and April, *R. frisii kutum* species migrate from Iranian waters (southern part of Caspian Sea) into estuaries and rivers for spawning (Ghadirnejad, 1996). Overfishing, pollutants, overexploitation of bottom sediments in the rivers and dams chang or block natural spawning locations of *R. frisii kutum* (Heyrati et al., 2007). *R. rutilus caspicus* is widely distributed in the Caspian Sea because of over fishing and deterioration of its spawning grounds; this species is considered for listing as a threatened species for the region (Abdoli, 2000; Raeisi et al., 2014). Sensitivity of various fish species is different on toxic substances, so toxicology tests are needed for different fish (Finney, 1971). For this purpose, LC_{50} 96 h is required for any ecotoxicology studies.

The present study was conducted to determine the acute toxicity of the hinosan in *R. frisii Kutum* and *R. rutilus caspicus.* Most migration route and hatchery pools of these two species are in the rivers adjacent to the rice paddies. These paddies use hinosan widely as a fungicide to combat rice blast.

Although, these two species are harvested commercially and several studies have been conducted on these two species but limited data are available on their sensitivity to pesticides. The aim of this study was to investigate the adverse effect of this toxin to *R. frisii Kutum.*

MATERIALS AND METHODS

200 number live specimens of *R. frisii Kutum* and *R. rutilus caspicus* were obtained from Shahid Rajaee Center, Sari, Iran. Samples weighted 3±1 g and acclimatized in 70*40*30 cm aquarium for 10 days. In order to measure biological capability and determine survival, fishes were kept in natural and toxin-free environment to determine natural mortality. Dissolved oxygen was fixed on 7 to 7.5 ppm, pH: 7 to 7.5, temperature: 20±1 and hardness: 200 ppm. Physical and chemical parameters of water are coincided with Stephenson (1982), Aydin et al. (2005) and Yilmaz et al. (2004). Fishes were fed twice daily with Biomar feed at 2% body weight, before the test, feeding was stopped 24 h prior to the test and throughout the test. All Experiments were performed 16 h light and 8 h of darkness. Fish behavior and clinical signs were recorded. Static acute toxicity test was performed following guideline the OECD standard method (OECD, 1989). 5 treated aquariums with concentration ranges 1, 2, 4, 8, 16 ppm of Hinosan (Technical 95 Edifenphos) with one control group (no toxic concentration), were performed. Mortality rates were recorded after 24, 48, 72 and 92 h and dead fishes were quickly removed from the aquarium. The nominal concentration of toxin causing mortality (LC_1, LC_{10}, LC_{30}, LC_{50}, LC_{70}, LC_{90} and LC_{99}) within 24, 48, 72 and 92 h was calculated. LC_{50} values for 24, 48, 72 and 96 h exposures were computed on the basis of probit analysis version 16/0

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Abbreviations: MAC, Maximum allowable concentration; **LOEC,** the lowest observable effect concentration; **NOEC,** no observable effect concentration.

Table 1. Cumulative mortality of *Rutilus rutilus caspicus* (n=30, each concentration) exposed to acute hinosan.

Concentration (ppm)	Mortality (Number)			
	24 h	48 h	72 h	96 h
Control	0	0	0	0
1	0	0	0	0
2	0	0	0	2
4	15	21	28	30
8	30	30	30	30
16	30	30	30	30

Table 2. Lethal concentrations (LC_{1-99}) of hinosan depending on time (24-96 h) for *Rutilus rutilus caspicus* (estimate ± lower and upper bound).

Point	Concentration (ppm)			
	24 h	48 h	72 h	96 h
LC1	2.50 ± 0.9	2.41 ± 0.8	1.73 ± 0.2	1.54 ± 0.3
LC10	3.16 ± 0.9	3.01 ± 0.8	2.39 ± 0.2	2.15 ± 0.3
LC30	3.64 ± 0.9	3.43 ± 0.8	2.87 ± 0.2	2.58 ± 0.3
LC50	3.97 ± 0.9	3.73 ± 0.8	3.21 ± 0.2	2.88 ± 0.3
LC70	4.30 ± 0.9	4.03 ± 0.8	3.54 ± 0.2	3.19 ± 0.3
LC90	4.78 ± 0.9	4.46 ± 0.8	4.02 ± 0.2	3.62 ± 0.3
LC99	5.44 ± 0.9	5.05 ± 0.8	4.68 ± 0.2	4.22 ± 0.3

Table 3. Cumulative mortality of *Rutilus frisii Kutum* (n=30, each concentration) exposed to acute hinosan.

Concentration (ppm)	Mortality (number)			
	24 h	48 h	72 h	96 h
Control	0	0	0	0
1	0	0	0	0
2	0	2	2	10
4	2	4	9	15
8	30	21	30	30
16	30	30	30	30

(Finney, 1971). Eventually, maximum allowable concentration (MAC), the lowest observable effect concentration (LOEC) and no observable effect concentration (NOEC) were also determined.

RESULTS

No mortality was observed during acclimation. Result shows that within 96 h test, LC_{50} value declined with increasing toxin concentration and duration of exposure. It means that an LC_{50} value in the first 24 h of the experiment always was higher than LC_{50} at 96 h. According to the results LC_{50} 96 h hinosan for *R. frisii Kutum* and *R. rutilus caspicus* were obtained as 3.61 and 2.88 ppm, respectively. The nominal concentration of toxin causing mortality (LC_1, LC_{10}, LC_{30}, LC_{50}, LC_{70}, LC_{90} and LC_{99}) within 24, 48, 72 and 92 h for each toxin was calculated (Table 1 to 4). Hundred percent mortality of fishes were occurred only hours after exposure in 8 and 16 ppm concentration. Fish exposed to toxicant showed abnormal behavior such as faster opercular activity, swimming erratically with jerky movements, protrusion of the eyes and bruise in the caudal fin. Exposed fish incurred curvature in vertebra and their gill pigmentation was decreased. Behavioral changes and clinical symptoms at doses of 2 and 4 were observed 5 h after exposure, but at higher doses, about 2 h after the experiment, symptoms were detected. Control and 1 ppm concentration groups showed normal behavior during

Table 4. Lethal Concentrations (LC_{1-99}) of Hinosan depending on time (24-96 h) for *Rutilus frisii Kutum* (estimate ± lower and upper bound).

Point	Concentration (ppm)			
	24 h	48 h	72 h	96 h
LC_1	2.37 ± 0.33	1.67 ± 0.11	1.26 ± 0.12	0.29 ± 0.09
LC_{10}	4.31 ± 0.33	3.20 ± 0.11	2.74 ± 0.12	1.46 ± 0.09
LC_{30}	4.98 ± 0.33	4.30 ± 0.11	3.82 ± 0.12	2.73 ± 0.09
LC_{50}	5.45 ± 0.33	5.07 ± 0.11	4.56 ± 0.12	3.61 ± 0.09
LC_{70}	5.92 ± 0.33	5.83 ± 0.11	5.30 ± 0.12	4.50 ± 0.09
LC_{90}	6.59 ± 0.33	6.94 ± 0.11	6.37 ± 0.12	5.77 ± 0.09
LC_{99}	7.52 ± 0.33	8.46 ± 0.11	7.85 ± 0.12	7.53 ± 0.09

experiment. Fishes at concentrations of 8 and 16 ppm had a more mobility than the control group.

DISCUSSION

Exposure time is one of the effective factors of organophosphorus toxic ratios (Larkin and Tjeerdema, 2000). When fish are exposed to a constant concentration of the toxin, fish tolerance is diminished over time and the toxin has more effect. However, where the toxin accumulates in fish tissue there is increase adverse effects on the body and thereby causes decrease in LC_{50} 96 h. Overall, LC_{50} for Hinosan in *R. frisii Kutum* and *R. rutilus caspicus* showed a decreasing trend over 96 h and in listed physicochemical conditions. Result of LC_{50} 96 h for toxin showed that the rate of LC_{50} decreased with increase in toxin concentration and duration of exposure. The results of the acute toxicity of diazinon and Deltamethrin on *Ciprinus carpio* (common carp) concedes decreasing trend in LC_{50} 96 h (Svoboda et al., 2001; 2003). Contrasting results are limited on toxicity of hinosan in fishes. The influence of hinosan on fish toxicity was evident. El-Gendy et al. (1998) studied the effects of hinosan on the immune response and protein biosynthesis. They declared that hinosan and glyphosate with 1/1000 concentration exhibit changes in the electrophoretic pattern of serum fish proteins. Similar electrophoretic findings are in agreement with those of Shimaila (1989). Gaafar et al. (2010) showed that exposure of organophosphate hinosan pesticide to an *O. niloticus*, had adverse effect on some serum parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), cholinesterase activity, total protein, blood urea nitrogen and creatinine. They reported that LC_{50} 96 h for *O. niloticus* is 1 ppm. Dubey et al. (2014) stated that these changes may be attributed to direct toxic effect of Hinosan on hepatocytes since the hepatopancres is the site of detoxification of all types of toxins and chemicals (Robert, 2001). Shamooshaki et al. (2008) determined LC_{50} values of hinosan for *Acipenser nudiventris* 28 ppb and reported that *A. nudiventris* is severely irritating to hinosan. Also, Alinezhad et al. (2005) determined LC_{50} values of hinosan for *Acipenser persicus* and *Acipenser stellatus* as 307 and 0.206 ppb, respectively. Behaviour is considered as a promising tool in ecotoxicology (Drummond and Russom, 1990; Cohn and MacPhail, 1996). Pesticides are lipophilic and rapidly absorbed in fish gills which cause respiratory limitations (Masud and Singh, 2013). Fishes exposed to hinosan had respiratory disorders which quickly opened and closed their gill cover. Fishes were anxious, had anharmonic breathing and unusual semi-circular swimming. At doses of 16 ppm, some fishes had curvature in vertebral column and collapsed to the bottom of the aquarium. These findings coincided with those of other authors who studied acute toxicity of other organophosphorus pesticides (Rao et al., 2005; Rao, 2006; Pandey et al., 2005).

Maximum Allowable Concentration (MAC) in natural environments according to LC_{50} 96 h is 0.1 LC_{50} level. The value of MAC for hinosan in mean temperature of 20±1°C was calculated as 0.361 ppm for *R. frisii Kutum* and 0.288 ppm for *R. rutilus caspicus*. The lowest observable effect concentration (LOEC) was also determined. The LOEC is analogous to the “limit of detection” of the conventional methods of analysis. LOEC represents the initial toxicity threshold of a chemical while NOEC represents the concentration of toxicant that will not cause any effect. Sensitivity of bioassays, Toxicity evaluation and comparative evaluation of the effects of pesticides was evaluated using the LOEC values (Fernández-Alba et al., 2002). The acute toxicity response, LOEC and NOEC is shown in Figures 1 and 2.

Conclusion

According to Table 5 (determination of toxicity in different pesticides), hinosan for *R. frisii Kutum* and *R. rutilus caspicus* are medium toxicity. Due to the vicinity of these two species location to farmland and orchards, further studies should be conducted on acceptable level of this fungicide.

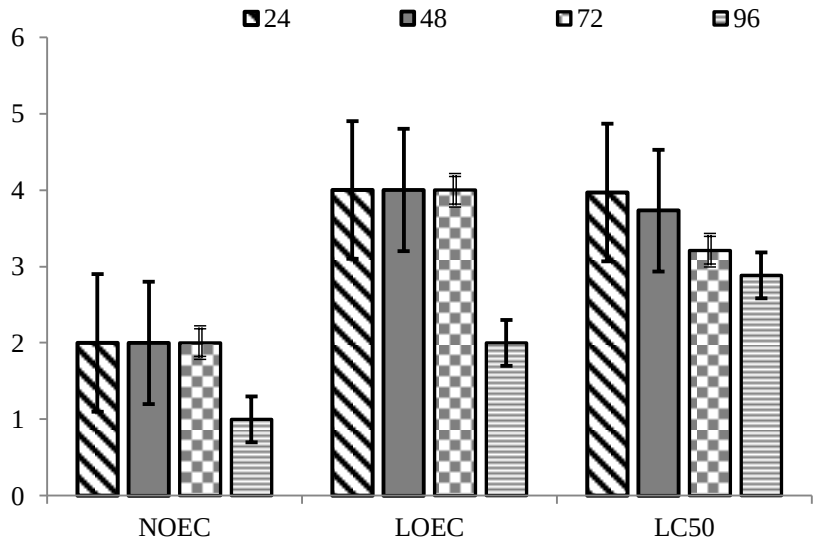

Figure 1. Acute toxicity testing statistical endpoints in *Rutilus rutilus caspicus* exposed to crude hinosan at different times.

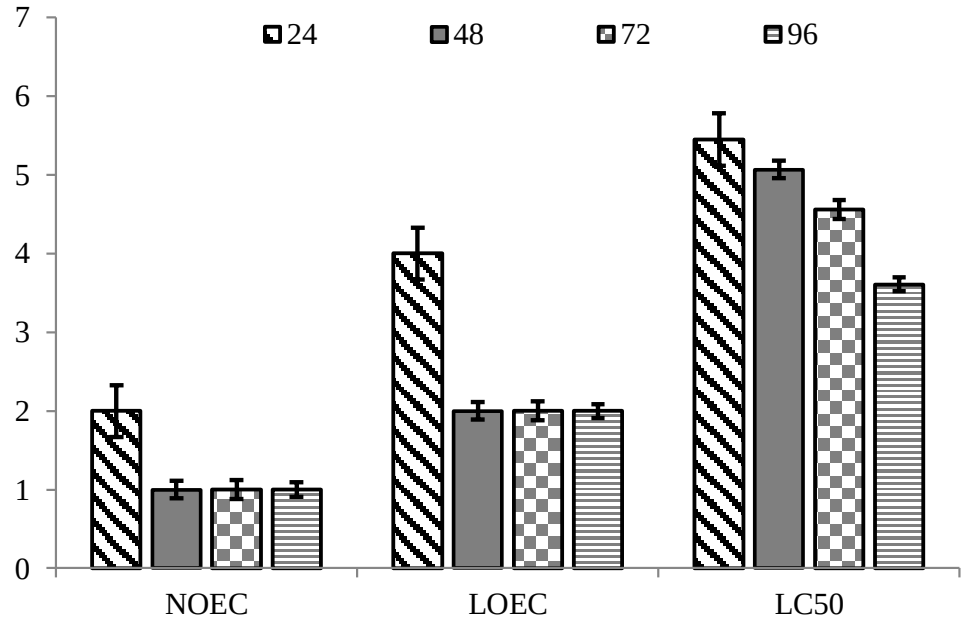

Figure 2. Acute toxicity testing statistical endpoints in *Rutilus frisii Kutum* exposed to crude hinosan at different times.

Table 5. Determination of toxicity in different pesticides (Wasserschadstoff -Katalog, 1975).

LC_{50} (mg/L)	Degree of toxicity
Up to 100	Nearly no poison
10-100	Toxicity low
1-10	Toxicity medium
0.1-1	Toxicity high
Less to 0.1	Toxicity very high

Conflict of Interest

The authors did not declare any conflict of interest.

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Full Length Research Paper

Physical-chemical characteristics and antioxidant potential of seed and pulp of *Ximenia americana* L. from the semiarid region of Brazil

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Ximenia americana **popularly known as wild plum grow wildly in Brazilian semiarid region and its fruit were harvested in two maturity stages and evaluated for quality. The experimental design was completely randomized with three treatments (immature, mature pulp and seeds), and treatment effect was evaluated for pulp fraction (composed of both mesocarp and exocarp or peel) in maturation stages: immature (largest size with green colored peel), mature (largest size with yellow colored peel) and seeds of mature fruits, from eight repetitions of 25 fruits each, totaling 200 fruits per treatment. Fruit were separated and evaluated for physical and physicochemical variables, mineral composition, bioactive compounds content and total antioxidant activity (TAA). The *X. americana* grown in Brazilian semiarid region presents a great potential to further commercial exploitation. The pulp, aside its maturation stage contains high levels of lipids, proteins, sugars, starch, titratable acidity, vitamin C, yellow flavonoids, polyphenols and antioxidant activity. The seed also presents high levels of lipid, protein, starch, total extractable polyphenols and antioxidant activity. Imature pulp stands out for acidity, polyphenol, flavonoid and anthcyanin levels, while mature fruit pulp has higher yield, sugar and vitamin C levels. Seeds have higher starch, protein and lipi levels. The antioxidant activity found for wild plum pulp could be attributed to polyphenol and vitamin C contents; meanwhile, the antioxidant activity of seeds was dependent only on polyphenolic content. Pulp had higher Na, K, Mg, Ca and Fe levels, however, both seed and pulp fractions have substantial contents of P, K, Cu and Mn.**

Key words: Development, bioactive compounds, antioxidant activity, minerals, quality.

INTRODUCTION

A significant part of Brazil's large biodiversity is in the Northeastern semiarid region known as Caatinga, an ecosystem unique to this country. In spite of the large number of plant species present in Caatinga, among those unexploited species is the wild plum, with potential for further commercial exploitation (*Ximenia americana*

Linnaeus).Although, in Brazil, its occurrence is mainly in the semiarid region, it is also found in Africa, India, New Zealand, Central America and in other South American countries (Sacande and Vautier, 2006; Souza, 2008), where reports show it is broadly used in non-traditional medicine (Gronhaug et al., 2008; James et al., 2008; Le et al., 2012).

The consumption of tropical fruits has increased in domestic and foreign markets due to the growing enlightement of their nutritional and therapeutic properties (Rufino et al., 2010). Tropical fruits may present unique sensorial characteristics and high concetrations of nutrients (Souza et al., 2012) especially when it comes to wild or native species (Genovese et al., 2008). These are outstanding sources of antioxidant compounds, which are associated with anti-aging and health promoting properties due to their potential to lower or inhibit oxidative stress (Hassimoto et al., 2005). These antioxidants differ in nature as minerals, dietary fibers, phytochemicals as phenolics and vitamin C and pigments as carotenoids and chlorophyll, which overall are more abundant in immature fruit.

Previous reports show that *X. americana* are rich in antioxidants such as vitamin C and phenolics, thus representing a good source of such compounds for humans (Rezanka; Sigler, 2007; Silva et al., 2008; Lamien-Meda et al., 2008; Mora et al., 2009). Besides the pulp, fruit may also be explored for their seeds yet, another study shows that wild plums seeds are very tasty and have been used as a food spice, despite their purgative effects (Brasileiro et al., 2008).

The characterization of bioactive compounds is important to determine the nutritional quality and commercial value of a fruit and its subproducts. However, the wild plum's potential as source of energy, minerals, carbohydrates, and other bioactive health-promoting compounds have not yet been investigated. Thereby, by characterizing the wild plum and its seed, we hope to enable its commercial exploitation as fresh or processed product, as source of additives and natural ingredients. Thus, this work aimed to characterize the physical, mineral, chemical and antioxidant potential of seed and pulp from *X. americana* grown native to semiarid region of Brazil, as a means to enhance its consumption and production.

MATERIALS AND METHODS

Sample preparation

X. americana plants grow wildly in Mossoró-Assu, RN, Brazilian semiarid region (5° 16'' 52"" S and 37° 11'' 46"" W) where the climate, according to Köppen classification is "BSwh", dry and very hot with two seasons: dry, from June to January and rainy, from February to May (Carmo Filho and Oliveira, 1995). Fruit were harvested on December of 2011, when relative humidity was 62.49%, average temperature was 28°C and rainfall was 28.95 mm, as reported by the weather station of Universidade Federal Rural do Semi-Árido-UFERSA.

Fruit harvested in two maturation stages, immature (largest size with green colored peel) and mature (largest size with yellow colored peel) were selected for uniformity of maturation and no damage marks and then, washed in tap water. Afterwards, fruit in both developmental stages were divided into two fractions, pulp (composed of both mesocarp and exocarp or peel) and seeds. Only mature seeds were evaluated.

For treatment, effect was evaluated for pulp fraction (composed of both mesocarp and exocarp or peel) in maturation stages: immature (largest size with green colored peel) and matures (largest size with yellow colored peel) and seeds of ripe fruits, from 8 repetitions of 25 fruits each, totaling 200 fruits per treatment. Pulp tissue was homogenized in Ultra-Turrax® (IKA, Germany) homogenizer, meanwhile seeds were crushed in a Wiley® stainless-steel (Thomas Sci., USA) mill and both samples were stored in a domestic freezer (-20°C) until the analysis were performed.

Physical characteristics

Evaluations were done with eight replications with 25 fruit each. Fruit were individually measured for their longitudinal and transverse diameters with a digital stainless hardened caliper (Shan, China) to determine shape (Lopes, 1982); fresh mass with an analytical balance (model FA 2104N by Celtac, China), and mass yield of different fractions (seed, peel and pulp) obtained by the difference between total fruit mass and that of the different constituents per si.

Chemical and mineral composition

Humidity was determined as samples were dehydrated at 65°C until reaching a constant weight. Lipid content was determined by Soxhlet extraction method and ashes were determined at 550°C according to Silva and Queiroz (2002). Protein content was determined by Kjeldahl method using a conversion factor of 6.25 (Silva, 2009). pH was determined using a pH meter (Model mPA-210 by Tecnal®, Brazil) with automatic temperature adjustment as described by AOAC (2002). Titratable acidity was determined according to AOAC (2002) using an automatic titrator (Titrette® model Class A precision by BRAND, USA) and results were expressed as mEq H_3O^+/100 g. Soluble solids were determined with a digital refractometer (Palette model PR – 100, by Atago, Japan) (AOAC, 2002). Total sugar content determined by the anthrone (Vetec, Brazil) method according to Yemn and Willis (1954), reducing sugars by DNS (3,5-dinitro salicylic acid, Vetec, Brazil) method according to Miller (1959) and starch content according to the method of AOAC (2002) and Miller (1959). Absorbances were monitored with UV-VIS spectrophotometer (model UV-1600 by Pró-Análise®, Brazil).

Minerals were quantified as following: potassium and sodium

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by flame emission photometer and phosphorus by spectrophotometry (model SP 1105 by BEL Photonics, Brazil). Calcium, magnesium, copper, manganese and iron were determined by atomic absorption spectrophotometry (model AA240FS by Varian, USA) after nitric acid was submitted to microwave (MD3113 series by CEM II®, USA) digestion of organic matter (Silva, 2009). Results were expressed on fresh weight basis. All other reagents were of analytical grade and were also supplied by Vetec, Brazil.

Bioactive compounds content

The total vitamin C was determined by titration with 0.02% 2,6-dichloro-indophenol (DFI) (as proposed by Strohecker and Henning (1967). One gram of pulp was diluted to 100 mL of 0.5% oxalic acid and homogenized. Then, 5 mL of this solution was diluted to 50 mL with distilled water and titrated and results were expressed as mg/100 g FW (fresh weight).

Anthocyanins and yellow flavonoids were extracted and determined as described by Francis (1982). One gram of pulp was extracted with a 95% ethanol/1.5 N HCl (85:15) solution, vortexed for 2 min and then, brought to 50 mL with the extracting solution. Protected from the light, the mixture was refrigerated at 4°C for 12 h, then filtered on Whatman N.1 paper and the filtrate was gathered. The absorbance of the filtrate was measured at 535 nm for the total anthocyanin content using an absorption coefficient of 98.2 mol/cm and at 374 nm for the total yellow flavonoid content using an absorption coefficient of 76.6 mol/cm. Both results were expressed as mg/100 g FW.

The total phenol content of acerola was measured by a colorimetric assay using Folin-Ciocalteu reagent as described by Obanda and Owuor (1997). Before the colorimetric assay, the samples were subjected to extraction in 50% methanol and 70% acetone as described by Larrauri et al. (1997). Extracts were added to 1 mL Folin Ciocalteau reagent (1 N), 2 mL Na_2CO_3 at 20% and 2 mL of distilled water. Results were expressed as gallic acid equivalents (GAE) mg/100 g FW.

Total carotenoids were measured as described by Higby´s (1962). Five grams of pulp were homogenized with 15 mL alcohol and 5 mL hexane and then, let to stand for 90 min. The mixture was filtered into a 25 mL flask, then 2.5 mL acetone was added and volume completed with hexane. Absorbance was monitored at 450 nm and results were expressed as mg/100 g FW.

Total antioxidant activity - $ABTS^{\bullet+}$ assay

The total antioxidant activity (TAA) was determined using 2,2-azinobis-3-ethylbenzthiazoline-6-sulphonic acid radical cation ($ABTS^{\bullet+}$, Sigma) method as described by Miller et al. (1993). Before the colorimetric assay, the samples were subjected to a procedure of extraction in 50% methanol and 70% acetone. Once the radical was formed, the reaction was started by adding 30 µL of extract in 3 mL of radical solution, absorbance was measured (734 nm) after 6 min and the decrease in absorption was used to calculate the total antioxidant activity (TAA). A calibration curve was prepared and different trolox concentrations (standard trolox solutions ranging from 100 to 2000 µM) were also evaluated against the radical. Antioxidant activity was expressed as trolox equivalent antioxidant capacity (TEAC), µmol trolox/g FW.

Total antioxidant activity - $DPPH^{\bullet}$ assay

The free radical-scavenging by DPPH assay is based on the sequestering of DPPH radical (2,2-diphenyl-1-picryl-hydrazyl, Sigma) by antioxidants, decreasing absorbance at 515 nm and was proposed by Brand-Williams et al. (1995) and modified by Sánchez-Moreno et al. (1998). A methanol solution containing 0.06 mM DPPH was prepared and aliquots of 100 mL were added and absorbance monitored at 515 nm, until stabilization (110 min for immature fruit pulp, 85 min for mature and 30 min for seed 30). The antioxidant activity was expressed as the concentration of antioxidant able to reduce the free radicals by 50% (EC_{50}) and expressed in g /g DPPH.

Statistical analysis

The experimental design was completely randomized with 3 treatments (immature, mature pulp and seeds) with eight replicates consisting of 25 fruit, each, totaling 200 fruits per treatment. The data obtained was subjected to analysis of variance (ANOVA) using a Sisvar 5.1 Build 72 program (Ferreira, 2011) and the means were compared by the Tukey test at 5% probability. Pearson's correlation analysis to the 5% level of significance between mean values found for bioactive compounds and total antioxidant activity during maturation was performed using BioEstatística 5.0 program.

RESULTS AND DISCUSSION

Physical analysis

There was significant differences between the physical characteristics evaluated (Table 1). There was a statistical difference for the physical characteristics evaluated for yield ripe fruit highlighted with higher value (Table 1). *X. americana* fruit here evalauted were yellow-orange drupes when mature and green colored when immature with an aromatic bittersweet pulp involving a seed with white nut. The values for transversal diameter were slightly lower than longitudinal diameter indicatives of an spherical shape ($0.9 \leq$ ratio ≤ 1.1), while the seeds were oblong ($1.1 <$ ratio ≤ 1.7) (Table 1).

For local population, the yellow colored fruit is regarded mature when it is rounded in shape and present a characteristic aroma. Immature fruits presented an average weight of 4.5 g, while mature average mass was 4.4 g, the seeds weighted 0.89 g and pulp consisting of mesocarp and exocarp constituted the main portion yielding an average of 78.55% for immature fruits and 79.87% for mature fruits (Table 1). As fruit ripens, mass and yield increase and seeds correspond to approximate 20% of whole fruit mass. Mora et al. (2009) reported that wild plums from Mexico presented the same rounded shape with mass ranging from 4.2 to 6.5 g, mesocarp and exocarp yield ranges between 70.8 and 78.4% while seeds were from 29.2 to 21.6%.

Chemical composition

There was a significant difference for all variables evaluated

Table 1. Physical characterization of *Ximenia americana* from the semiarid region of Brazil.

Variable	Seed	Pulp	
		Immature	Mature
Longitudinal diameter (mm)	13.57 ± 0.60[b]	18.60 ± 0.65[a]	18.51 ± 0.38[a]
Transversal diameter (mm)	10.42 ± 0.27[b]	17.88 ± 0.45[a]	17.61 ± 0.57[a]
Format (Ratio LD/TD)	1.30 ± 0.07[a]	1.04 ± 0.03[b]	1.05 ± 0.03[b]
Fresh mass (g)	0.89 ± 0.08[b]	4.57 ± 0.58[a]	4.43 ± 0.41[a]
Yield (%)	20.13 ± 0.56[c]	78.55 ± 1.33[b]	79.87 ± 0.56[a]

The same letters in the same row indicate no significant difference at a level by the Tukey test at 5% probability. Data expressed as mean values ± standard deviation.

Table 2. Chemical composition of *Ximenia americana* from the semiarid region of Brazil based on fresh matter.

Variable	Seed	Pulp	
		Immature	Mature
Moisture (%)	8.07 ± 3.15[b]	65.99 ± 2.01[a]	64.91 ± 2.65[a]
Total soluble solids (°Brix)	17.95 ± 2.93[b]	26.89 ± 1.62[a]	26.22 ± 1.84[a]
Total soluble sugars (%)	3.66 ± 0.83[c]	9.49 ± 0.66[b]	10.64 ± 1.01[a]
Reducing sugars (%)	2.74 ± 0.60[b]	8.71 ± 0.86[a]	9.60 ± 1.57[a]
Starch (%)	11.00 ± 2.51[a]	5.70 ± 1.59[b]	5.17 ± 0.59[b]
Titratable acidity (mEq H_3O^+/100 g)	12.92 ± 2.15[c]	75.39 ± 4.72[a]	65.33 ± 7.39[b]
pH	4.16 ± 0.31[a]	3.03 ± 0.05[b]	2.96 ± 0.12[b]
Protein (%)	8.99 ± 0.57[a]	5.50 ± 0.34[b]	5.91 ± 1.08[b]
Lipid (%)	45.70 ± 4.40[a]	23.33 ± 2.37[b]	22.85 ± 3.25[b]
Ashes (%)	1.31 ± 0.11[b]	3.51 ± 0.45[a]	3.65 ± 0.83[a]

The same letters in the same row indicate no significant difference at a level by the Tukey test at 5% probability. Data expressed as mean ± standard deviation.

between pulps and seeds (Table 2). However, between maturity stages, mature pulp presented signficantly higher soluble sugar content while immature pulp presented signficantly higher titratable acidity. The pulp of the wild plum showed little variation in humidity values during maturation, 65.99% for immature and 64.91% for mature fruit, while the seed presented 8.07% of humidity (Table 2); these values agree with those reported for pulps from native Brazilian pulps; 37.7 to 90.2% (Gonçalves et al., 2010). The high soluble solids content (26 °Brix) observed for wild plum ranges among the recommended values for fruit processing. This almost surely certifies a more natural taste as greater contents of these constituents implies a reduced sugar addition, less time to evaporate water, lower energy consumption and higher product yield, resulting in a more economical process (Pereira et al., 2012). Mora et al. (2009) observed lower soluble solids contents (10.9 to 17%), for wild plums from Mexico.

The total soluble sugars and reducing sugars content were three times higher in pulp than in seed (3.6 and 2.7%, respectively), whereas for starch content, seeds (11%) had twice the amount found for pulp (Table 2). These parameters varied as maturation of *X. americana* progressed and levels of total sugars, reducing sugars and starch were similar to those reported for banana (Oliveira et al., 2013; Ribeiro et al., 2012), considered a rich source of carbohydrates. However, wild plum seeds presented lower levels of carbohydrates than seeds from other wild species as chicken lard (18.41%, *Swartzia langsdorfii* Radlk.) and 'cagaita' (17.84%, *Eugenia dysenterica* DC.) (Roesler et al., 2007).

Both pulp and seeds of *X. americana* present high titratable acidity, 65 and 12 mEq H_3O^+/100 g, and low pH, 2.9 and 4.1, respectively (Table 2). These results indicate that *X. americana* pulp is acidic. These are desirable characteristics for the processing industry as acidity contributes to an enhanced flavor, which promotes a high dilution factor in the formulation of juices leading to a greater yield while low pH dismisses any acidification during processing. Silva et al. (2008) also reported a pH value of 2.6 for pulp of mature wild plum and similar

results to those here presented (pH 4.1) were found for seeds of ‘cagaita’ (pH 4.3) which were lower than those of seeds from chicken lard (pH 6.5), ‘araticum’ (pH 5.7, *Annona crassiflora* Martius) and ‘lobeira’ (pH 5.7, *Solanum lycocarpum* A. St.-Hil.) (Roesler et al, 2007).

The protein content was high in both seeds (8.9%) and pulp (5%) (Table 2), therefore wild plums represent an important source of protein when compared to other tropical fruit as yellow guava (4.24%, *Psidium cattleyanum* Sabine), ‘guabiroba’ (5.53%, *Campomanesia xanthocarpa* O. Berg) and ‘mandacaru’ (4.05%, *Cereus hildmannianus* K. Schum.) (Pereira et al., 2012; Pereira et al., 2013). Wild plum seeds present higher protein content than seeds of ‘banha’ (2.7%) and ‘cagaita’ (4.42%) (Roesler et al., 2007), ‘pequi’ almond (25.27%, *Caryocar brasiliense* Camb.) and in brown (19.1%) and gold flax seeds (21.6%) (Barroso et al., 2014; Lima et al., 2007).

X. americana seed stands out for the lipid content (45.7%), twice that of pulp (Table 2). It is considered high when compared to seeds from pequi (18%, *Caryocar brasiliense* Camb.), tucumã (19%, *Astrocaryum vulgare*) and avocado (8.4%, *Persea americana* Mill), as well as many other traditional and non-traditional fruit (Table 3). However, Saeed and Bashier (2010) reported lipid content values greater than 51% for wild plum seeds from Western Sudan. Ashes content of mature wild plum pulp was 3.65 and 1.31% for seed (Table 2), these were higher than found in pulp (0.93%), seed (0.93%) and exocarp (1.05%) of noni (*Morinda citrifolia* Linn.) (Costa et al., 2013), although lower than in cashew nut (2.6%, *Anacardium occidentale* L.) and raw nut (2.1%, *Carya illinoinensis* K. Koch.) (Taco, 2011).

X. americana pulp presented outstanding results when compared to other traditionally and non-traditionally marketed fruit (Table 3), suggesting a great exploitation potential esspecially, regarding their soluble solids, acidity, ashes, proteins and lipids contents in comparison to other native exotic fruit from semiarid Brazil as mandacaru/cardeiro (*Cereus jamacaru* DC); batinga (*Eugenia* sp); xique-xique (*Pilosocereus gounellei* Byles and G.D. Rowley); facheiro (*Pilosocereus pachycladus* F. Ritter); pirim (*Psidium schenckianum* Kiaersk); quixaba (*Sideroxylon obtusifolium* T.D. Penn); cumbeba (*Tacinga inamoena* N.P. Taylor and Stuppy.) and juá (*Ziziphus joazeiro* Mart) (Nascimento et al., 2011).

Bioactive compounds and total antioxidant activity

There was a significant difference among pulps and seeds regarding the bioactive compounds contents which were mostly higher in immature fruit pulp (Table 4). However, mature fruit pulp showed higher total vitamin C content of 187.98 mg/100 g, than immature pulp or seed. However, even higher total vitamin C levels (251.21 mg/100 g), were observed in preliminary studies with wild plums developed by our group (Silva et al., 2008). Thereby, wild plum represents a good source of vitamin C as its content is four times greater than recommended for daily intake of children and adults (45 mg). This is even more expressive when compared to other fruit listed on Table 3, as orange (73.3 mg/100 g, *Citrus sinensis*), tangerine (112 mg/100 g, *Citrus reticulata*) and açai (84 mg/100 g) (Rufino et al., 2010; Taco, 2011).

Immature *X. americana* pulp showed higher contents of total carotenoids, yellow flavonoids and anthocyanins than seeds or mature fruit (Table 4). Carotenoids are antioxidants as well as precursors of important vitamin A and *X. Americana* pulp presented higher contents than cashew apple (0.4 mg/100 g), ‘camu camu’ (0.4 mg/100 g), carnaúba (0.6 mg/100 mg, *Copernicia prunifera*), jaboticaba (0.32 mg/100 g, *Myrciaria cauliflora*), jambolan (0.51 mg/100 g, *Myrciaria cauliflora*), mangaba (0.3 mg/100 g, *Hancornia speciosa*) and myrtle (0.5 mg/100 g, *Blepharocalyx salicifolius*) (Rufino et al., 2010). The content found for *X. americana* seeds (0.59 mg/100 g) was higher than found for ‘pequi’ almond (0.295 mg/100 g) (Lima et al., 2007).

Yellow flavonoid contents of immature *X. americana* pulp were highest, followed by mature pulp (38.29 mg/100 g) and seeds (15.26 mg/100 g). It was also higher when compared to wild plum pulp from Burkina Faso (30 mg/100 g) (Lamien-Meda et al., 2008), acerola and camu-camu (Table 3). Total anthocyanin concentration was statistically higher in immature pulp (4.36 mg/100 g) followed by mature pulp (2.8 mg/100 g) and seeds (1.79 mg/100 g) and also, higher than reported for ‘mangaba’ (0.4 mg/100 g), murici (0.5 mg/100 g, *Byrsonima dealbata*), uvaia (1.13 mg/100 g, *Eugenia pyriformis*) and bacuri (0.3 mg/100 g, *Platonia insignis*) (Rufino et al., 2010). However, *X. americana* seeds contained less anthocyanins than nuts (18.02 mg/100 g), pistachio (6.06 mg/100 g), hazelnut (6.71 mg/100 g) and kernels (2.46 mg/100 g) (Bolling et al., 2011).

The content of total extractable polyphenols was statistically higher in immature pulp (4025 mg/100 g), followed by mature pulp (3002.08 mg/100 g) and seeds (2245.69 mg/100 g) (Table 4). According to Vasco et al. (2008), *X. americana* may be classified as a good source of phenolics as their contents were greater than 500 mg GAE/100 g. The phenolic content of wild plums reported here was higher than reported by Mora et al. (2009) for wild plums from Mexico (2960 mg GAE/100 g) and by Lamien Meda et al. (2008) for wild plums from Africa (2086.67 mg GAE/100 g). It was also higher than reported for blackberry (2167 mg GAE/100 g), bacuri (23.8 mg GAE/100 g) (Rufino et al., 2010; Vasco et al., 2008) and for pulp of other important fruit species (Table 3). These results indicated that wild plums are relevant sources of polyphenols, including its seeds with values

Table 3. Chemical composition and antioxidant activity of traditional and non-traditionally marketed fruit pulp.

Fruit	Moisture (%)	Protein (%)	Lipid (%)	Total Vitamin C (mg/100g)	Total Carotenoid (mg/100 g)	Total Anthocyanin (mg/100 g)	Yellow flavonoids (mg/100 g)	Total Polyphenol (mg/100 g)	DPPH EC50 (g/g)	ABTS (µmol Trolox/g)	Reference
Traditional											
Banana *Musa* spp.	77.7	1.2	0.1	-	-	-	-	-	-	48.3	TACO, 2011 Leong and Shui (2002)
Mango *Mangifera indica*	75 - 84	-	19 - 20	-	-	-	544.9	600.0	-	12.9 3.1	Kuskoski et al. (2006) Vasco et al. (2008)
Papaya *Carica papaya*	88.6	0.5	0.1	82.2	-	0.69	-	53.2	2.24	7.6	Almeida et al. (2011) Taco, (2011)
Acerola *M. emarginata*	91.0	0.9	0.2	1357.0	1.4	18.9	9.6	1063.0	670.0	96.6	Rufino et al. (2010) Taco 2011
Cashew Apple *A.occidentale*	86.9	1.0	0.3	190.0	0.4	9.5	63.8	830.0	906.0	79.4	Rufino et al. (2010) Taco 2011
Non-traditional											
Camu-camu *Myrciaria dubia*	89.8			1882.0	0.4	42.2	20.1	1176.0	478.0	153.0	Rufino et al. (2010)
Araticum-do-mato	78.6	-	-	32.0	-	-	-	531.7	15946.5	3.85	Pereira et al. (2013)
Yellow Guava *P. cattleyanum*	83.3	-	-	30.0	-	-	-	3713.2	389.7	242.30	Pereira et al. (2012)
Gabiroba *C. adamantium*	80.9	1.06	0.55	-	-	-	-	1.222.6	-	107.96	Alves et al. (2013)
Marolo *Annona crassiflora*	80.2	0.92	1.84	59.1	0.57	-	-	739.4	-	131.58	Souza et al. (2012)

higher than sprocket (206 mg/100 g) and nut (1602 mg/100 g) (Bolling et al., 2011).

According to both DPPH and ABTS methods, the total antioxidant activity of *X. americana* is high, although by DPPH method, there was no significant difference among treatments and by ABTS method, seeds had higher the total antioxidant activity (Table 4). Seeds are important sources of natural antioxidants and often have greater antioxidant activity than edible fruit portion itself (Guo et al., 2003; Oliveira et al., 2009; Morais et al., 2013). However, total antioxidant activity of wild plums was high compared to other fruit species (Table 3) and therefore, can be recommended for both fresh consumption and for uses in pharmaceutical, cosmetic and nutritional industries due to benefitial property of free radical neutralization, thereby reducing the inicidence of degenerative diseases. Lamien-Meda et al. (2008) reported that wild plum pulp from Burkina Faso also present high antioxidant capacity.

Table 4. Bioactive compounds and antioxidant activity of *Ximenia americana* from the semiarid region of Brazil based on fresh matter.

Variable	Seeds	Pulp	
		Immature	Mature
Total vitamin C (mg/100 g)	72.23 ± 6.99[c]	170.30 ± 16.01[b]	187.98 ± 10.51[a]
Total carotenoids (mg/100 g)	0.59 ± 0.09[b]	0.99 ± 0.12[a]	0.88 ± 0.09[a]
Yellow flavonoids (mg/100 g)	15.26 ± 2.69[c]	45.69 ± 4.81[a]	38.29 ± 7.33[b]
Total anthocyanins (mg/100 g)	1.79 ± 0.36[b]	4.36 ± 1.44[a]	2.81 ± 0.18[b]
Total polyphenols (mg/100 g)	2245.69 ± 287.58[c]	4025.86 ± 551.64[a]	3002.08 ± 790.04[b]
DPPH (EC_{50}) (g /g DPPH)*	335.00 ± 15.86[a]	293.89 ± 61.55[a]	325.85 ± 32.19[a]
ABTS (µmol Trolox/g)	304.48 ± 48.51[a]	158.57 ± 32.70[b]	187.57 ± 47.05[b]

The same letters in the same row indicate no significant difference at a level by the Tukey test at 5% probability. Data expressed as mean ± standard deviation.*Concentration of antioxidant required to reduce the original amount of free radicals by 50%.

Table 5. Correlation between bioactive compounds and total antioxidant activity from wild plums from the semiarid region of Brazil.

	Flavonoids	Anthocyanins	Polyphenols	Carotenoids	Vitamin C	DPPH
Pulp						
Anthocyanins	0.5927*					
Polyphenols	0.2125	0.3627				
Carotenoids	-0.3980*	0.0675	0.2754			
Vitamin C	-0.3496	0.0533	0.4414*	0.0089		
DPPH	-0.2055	-0.3067	-0.7295*	-0.2641	-0.4777*	-
ABTS	0.1774	0.3123	0.8491*	0.1856	0.4165*	0.7725*
Seeds						
Anthocyanins	0.4371					
Polyphenols	0.2439	-0.2772				
Carotenoids	0.3036	0.6168*	-0.0104			
Vitamin C	0.0951	-0.4204	0.5016	-0.0810		
DPPH	0.1422	0.6461*	-0.8090*	0.4095	-0.5096	
ABTS	-0.2772	-0.5224	0.4870	-0.3591	0.2129	0.5956*

*Significant at $p < 0.01$.

According to the Pearson's correlation analysis between bioactive compounds and total antioxidant activity for wild plums (Table 5), there were significant interaction between total antioxidant activity and polyphenolic and vitamin C contents for mature pulp. For the DPPH method, correlation was negative since this method evaluates the amount of sample necessary to neutralize DPPH radical, the smaller values represent a higher antioxidant activity (inversely proportional to the antioxidant activity). Therefore, polyphenols and vitamin C are mainly responsible for the high antioxidant activity of wild plum pulp. However, for wild plum seeds, antioxidant activity was significantly correlated only to total antioxidant activity determined by DPPH method. Alves et al. (2013) also observed that the total phenolic content was positively correlated with the antioxidant activity of gabirobeira (*Camponesia adamantium*).

Mineral composition

There was a significant difference in sodium, potassium, magnesium, calcium and iron contents were generally higher in *X. americana* pulp (Table 6). Sodium levels were three times higher in pulp than seeds and considerably higher than reported for mature açai pulp (6.8 mg/100 g, *Euterpe oleracea* Mart.) (Gordon et al., 2012). Pulp potassium content (101.5 mg/100 g) was twice that of seed, representing 50% of the average levels reported for banana (263 mg/100 g) (Taco, 2011),

Table 6. Mineral content of wild plums from the semiarid region of Brazil.

Minerals (mg/100 g)*	Seeds	Pulp	
		Immature	Mature
Sodium	11.22 ± 1.56[b]	29.16 ± 5.04[a]	36.22 ± 12.32[a]
Potassium	56.90 ± 2.34[b]	110.12 ± 11.62[a]	101.56 ± 19.78[a]
Phosphorus	2367.77 ± 51.67[a]	2484.24 ± 223.84[a]	2425.22 ± 285.16[a]
Magnesium	0.95 ± 0.07[b]	1.23 ± 0.18[a]	1.20 ± 0.24[a]
Calcium	2.40 ± 0.09[b]	4.41 ± 0.29[a]	4.14 ± 0.51[a]
Copper	0.025 ± 0.00[a]	0.028 ± 0.02[a]	0.030 ± 0.02[a]
Zinc	0.093 ± 0.01[a]	0.110 ± 0.02[a]	0.131 ± 0.06[a]
Iron	0.21 ± 0.06[b]	0.347 ± 0.06[a]	0.367 ± 0.16[a]
Manganese	1.51 ± 0.30[a]	1.334 ± 0.27[a]	1.297 ± 0.26[a]

The same letters in the same row indicate no significant difference at a level by the Tukey test at 5% probability. Data expressed as mean ± standard deviation. *Expressed in mg/100 g dry matter.

which is well known as a good potassium source. Lime orange (130 mg/100 g, *Citrus sinensis* (L.) Osbeck) and mango (138 mg/100 g, *Mangifera indica* L. cv. Tommy Atkins) have potassium levels similar to those found for *X. americana* (Taco, 2011).

Phosphorus content present in the seed (2367 mg/100 g) and pulp (~2400 mg/100 g) of *X. americana* were higher than acerola (*Malpighia glabra* L.), pineapple (*Arabic comosus* (L.) Merril), cocoa (*Theobroma arábi* L.), guava (*Psidium guajava* L.), soursop (*Annona muricata* L.), Brazil nut (*Bertholletia excelsa* HBK), sesame seed (*Sesamum indicum* L.), flax (*Linum usitatissimum* L.) and walnut (*Carya illinoinensis* K. Koch.) (Taco, 2011). Magnesium were lower than reported for marolo pulp (26.28 mg/100 g*, Annona crassiflora* Mart.), genipap (8.17 mg/100 g, *Genipa americana* L.), murici (10.14 mg/100 g*, Byrsonima crassifolia* L. Rich), soursop (10.61 mg/100 g, *Annona muricata* L.) and sweet passion fruit (19.82 mg/100 g, *Passiflora alata* Dryand) (Souza et al., 2012).

Pulp had higher calcium levels (~4.41 mg/100 g) than seeds (2.4 mg/100 g) and could be compared to murici pulp (5.50 mg/100 g, *Byrsonima crassifolia* L. Rich) and sweet passion fruit (4.76 mg/100 g, *Passiflora alata* Dryand) (Souza et al., 2012). Although, it was lower than acerola (13 mg/100 g), pineapple (22 mg/100 g), banana (8 mg/100 g, *Musa acuminata* Colla x *Musa balbisiana* Colla, AAB cv. Prata) and cocoa (12 mg/100 g, *Theobroma arabi* L.) (Taco, 2011).

There was no significant difference for copper, zinc and magnesium levels between pulps and seed (Table 6). The copper content present in the seed and pulp (>0.024 mg/100 g) of wild plum was higher than recommended for daily intake of a human adult (Brasil, 2005). The zinc content of the pulp, ~0.1 mg/100 g, was similar to those reported for different banana cultivars (0.1 - 0.3 mg/ 100 g) (Taco, 2011). Manganese content was similar between wild plum seeds and pulp (~1.3 mg/100 g), and such concentrations meet 75% of the recommended daily intake for adults (2.5 mg/day) (Brasil, 2005). These values were higher than found in acerola (0.07 mg/100 g), banana cv. Prata (0.42 mg/100 g), cocoa (0.04 mg/100 g), cashew (0.12 mg/100 g), guava (0.08 mg/100 g), soursop (0.08 mg/100 g) and papaya (0.01 mg/100 g) (Taco, 2011). Iron levels were higher in pulp and also considered high when compared to acerola (0.2 mg/100 g), cashew apple (0.2 mg/100 g), guava (0.2 mg/100 g), soursop (0.2 mg/100 g) and papaya (0.20 mg/100 g) (Taco, 2011).

Conclusions

The *X. americana* grown in Brazilian semiarid region presents a great potential to further commercial exploitation. The pulp, aside its maturation stage contains high levels of lipids, proteins, sugars, starch, titratable acidity, vitamin C, yellow flavonoids, polyphenols and antioxidant activity. The seed also presents high levels of lipid, protein, starch, total extractable polyphenols and antioxidant activity. Imature pulp stands out for acidity, polyphenol, flavonoid and anthcyanin levels, while mature fruit pulp has higher yield, sugar and vitamin C levels. Seeds have higher starch, protein and lipid levels. The antioxidant activity found for wild plum pulp could be attributed to polyphenol and vitamin C contents; meanwhile, the antioxidant activity of seeds was dependent only on polyphenolic content. Pulp had higher Na, K, Mg, Ca and Fe levels, however, both seed and pulp fractions have substantial contents of P, K, Cu and Mn. This work indicate promising perspectives for the exploitation of this fruit and dissemination of data on its constituents is critical and enables it to be introduced in production systems and consequentely, marketed.

Conflict of interests

The authors did not declare any conflict of interest.

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Short communication

Cellulolytic activity of gut extract of subterranean termite, *Odontotermes obesus* Rambur: A pretreatment tool for conversion of lignocellulosic biomass to fermentable sugar for biorefinery industry

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Lignocellulosic biomass is a chief and cheap raw material for bioethanol production. However, pretreatment is a critical and most expensive step in lignocellulosic biomass to biofuel conversion. Biological pretreatments offer an alternative for lignocellulosic biomass conversion using enzyme hydrolysis. Termites are well known for the ability to digest lignocelluloses, using it as a sole food source. To effectively digest ligonocellulose/wood, termites produce an array of enzymes along with the help of microbial and protist symbionts. Subterranean termite, like *Odontotermes obesus*, devoid of protist symbiont in their hind gut capable of digesting cellulose using endogenous cellulases produced naturally by them. The cellulolytic activity of gut extracts of *O. obesus* was evaluated with commercially available carboxymethylcellulose (CMC) for their efficacy in conversion of cellulose to fermentable sugar. It is found that the gut extracts of *O. besus* potentially convert 49 to 75% of CMC into glucose. Hence, we look for novel hydrolytic enzymes in the gut extracts of *O. obesus* for efficient conversion of lignocellulosic biomass to fermentable sugars.

Key words: Termite gut, *Odontotermes obesus*, enzymes, lignocelluloses, hydrolytic enzymes.

INTRODUCTION

Petroleum products are the main transportation fuel. It has been recognized for some time that current use of fossil fuels will not only deplete the world´s oil reservoir but also have serious impact on the environment, leading to increased health risk and global climate change (Panwar et al*.,* 2010). It has been estimated that fossil fuels will be depleted by the year 2100 which makes the need for alternative fuels solutions significant (Saxena et al*.,* 2007). Global warming can mainly be attributed to an increase in CO_2 emissions which have increased by 30% in the past 200 years. Renewable energy causes little or no pollution (European Renewable Energy Council, 2008). Fuel produced by the activity of microorganisms, such as ethanol, methane and hydrogen, are called

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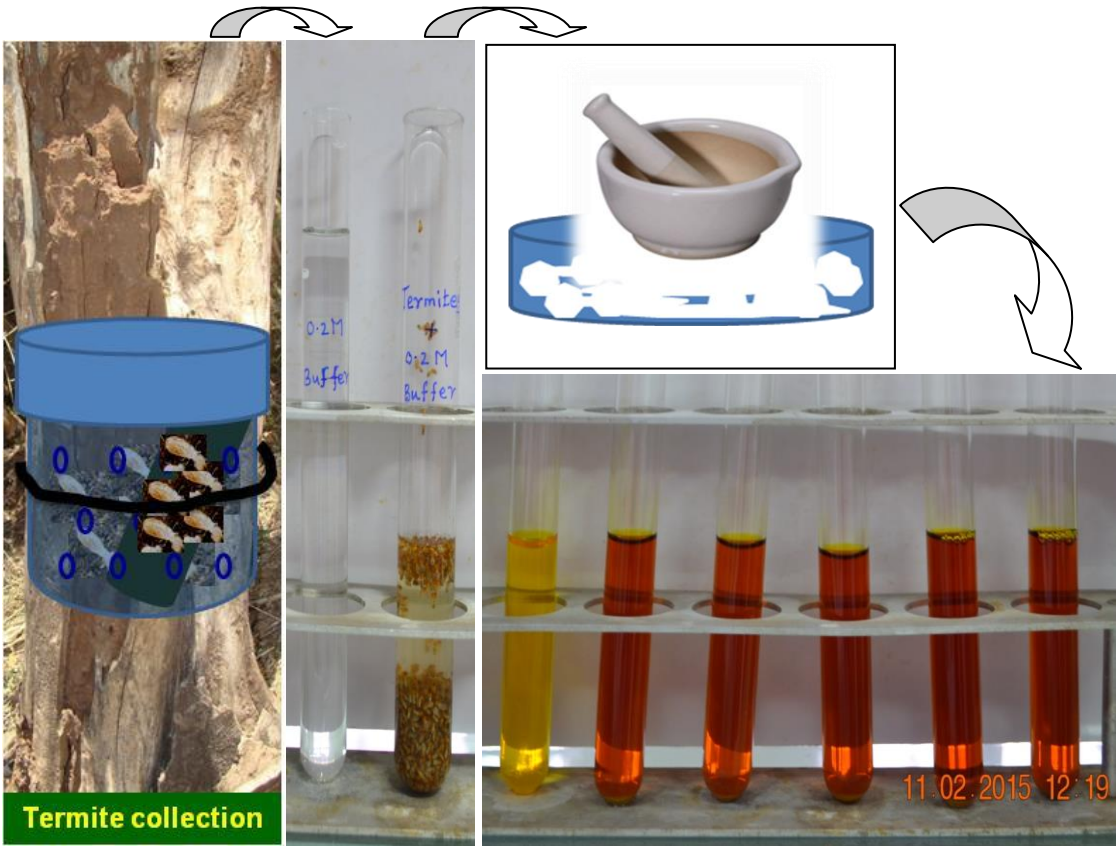

Plate 1. Termite collection and extraction of enzymes and enzyme assay.

biofuels (Drapcho et al., 2008). The use of biomass for biofuel production which can also be used for human consumption (for example, sugar beets, sugar cane and corn) is very controversial and has caused a food-versus-fuel debate worldwide since the amount of such biomass is limited. Therefore, the interest in other types of biomass has emerged in recent years. Lignocellulosic biomass is an example of such biomass. It is available in almost all plants (Hahn-Hägerdal et al., 2007). The production of lignocellulose on earth is about 2 to 5 $\times 10^{12}$ tons every year (Wyman et al., 2005). Pretreatment is a crucial and most expensive step in lignocellulosic biomass to biofuel conversion. Therefore, pretreatment of lignocellulosic biomass by alternative cost effective method is appreciable. Biological conversion of cellulosic biomass to fuels and chemicals offers the high yields to products vital to economic success and the potential for very low costs. Biological pretreatments offer an alternative for lignocellulosic biomass conversion using enzyme hydrolysis (Noah et al*.,* 2013) hence, exploration of such system in nature is needed. Insects include termites have the unique ability to digest lignocellulose with high efficiency, often using it as a sole food source. Termites can digest 74-99% lignocelluloses (Ni and Tokuda, 2013). Earlier studies indicated that novel enzymes with high lignocellulose degradation potential may reside in termite guts (Ni and Tokuda, 2013). A better understanding of lignocellulose digestion by termites may help to overcome challenges in the conversion of lignocellulosic biomass into soluble sugars. Thus, enzyme profiling combined with enzyme activity with reference to cellulose degradation is attempted.

MATERIALS AND METHODS

Termite collection

O. obesus termites were field collected on the *Eucalyptus camadulensis* tree at Forest Campus, Coimbatore, Tamilnadu, India as described in Smith and Koehler (2007) (Plate 1). Its identity was confirmed at National Forestry Insect Collection (NFIC), Forest Research Institute, Dehradun. Briefly, a PVC bucket with holes to allow termite access was tied on the trunk of a tree covered with a PVC lid. A small wood piece of *E. camadulensis* was placed into the bucket as a food source. Termites were migrated into the wood piece placed in the bucket. The bucket containing termite colony was brought into the laboratory. Termites were separated from the wood piece, frozen and kept under refrigeration until dissection. Collections were restricted to a single colony.

Table 1. Cellulase activity of gut extracts of *O. obesus* on CMC.

Parameter	Carboxymethylcellulose (CMC) substrate concentration (mg/ml)				
	1	2	3	4	5
Concentration of crude enzyme (ml)	0.1	0.2	0.3	0.4	0.5
Concentration of product (glucose) mg/ml	0.49 (49%)	1.08 (54%)	1.83 (61%)	2.72 (68%)	3.75 (75%)
Remaining substrate (mg/ml)	0.51	0.46	0.39	0.32	0.25
Specific activity of enzymes (nmol reducing sugar/ termite equivalent/ min)	1.77	2.91	3.06	4.18	5.37

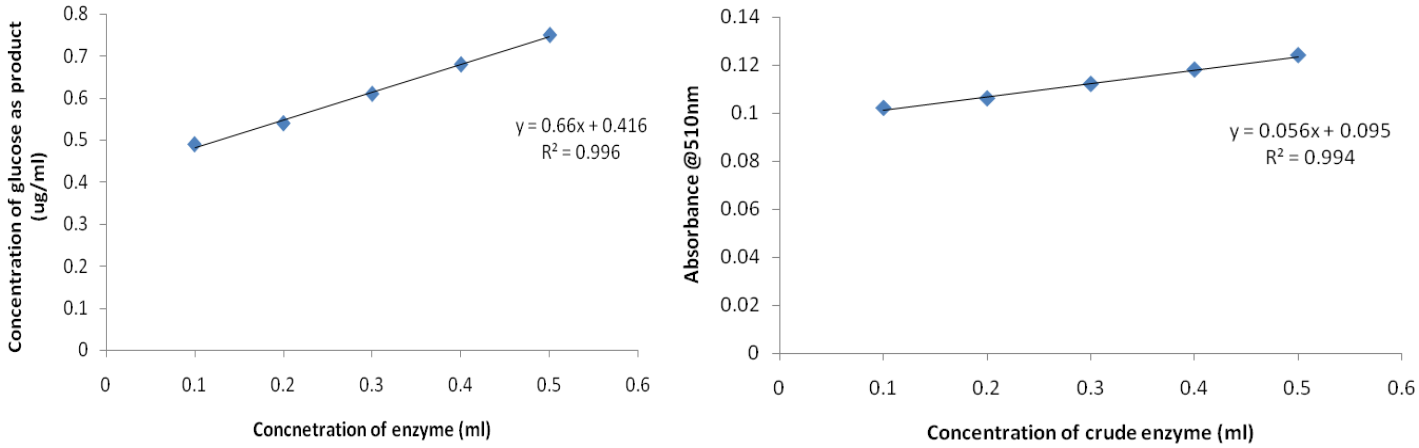

Figure 1. Determination of enzyme activity of gut extracts of *O. obesus*

Termite dissection, enzyme extraction and activities

Enzymes were extracted from collected termites sample as described by Smith et al. (2009). Briefly, each termite's gut was removed in sodium acetate buffer (0.1 M, pH 5.5 for endogluconase assay). An enzyme extract was prepared for whole gut region from 50 termites (100 mg weight) for the endoglucanase assay (Smith et al., 2009). The gut regions were placed into mortar and pestle containing the appropriate buffer after removing the gut contents, and kept on ice. The gut regions were manually homogenized on ice. The homogenates were centrifuged at 20000 *g* at 4°C for 15 min. The supernatants were collected, frozen, and kept at -20°C until enzyme assays. Final concentrations were equivalent to 50 termite gut regions per 10 mL. For the endoglucanase assays, 0.1 to 0.6 ml of tissue extract was combined with 0.9 to 0.4 ml of 2% carboxymethylcellulose solution (CMC, Sigma-Aldrich, in 0.1 M sodium acetate buffer, pH 5.5) in each test tube and allowed to react for 70 min at 23°C. A 1 ml volume of 1% 3,5-dinitrosalicylic acid (DNSA), 0.4M sodium hydroxide and 30% sodium potassium tartrate was added to each tube. The tubes were immediately placed in boiling water for 10 min and then on ice for 15 min. Each cooled test tubes were read at 510 nm using an UV-vis spectrophotometer (VWR Scientific, Hitachi). Control tubes were allowed to react for 10 min to allow for passive mixing of solutions before boiling with DNSA solution (Zhou et al. 2007); standards used were dilutions of glucose. For all replicates, control plates were used to adjust for 510 nm absorbance. The standard graph was prepared using glucose as standard. Based on the standard graph, regression equation was arrived at and concentration of the enzyme was calculated.

Data analysis

Experiments were set up as one-factor designs with a single homogenization for each gut /substrate combination. Thus, the experiment had a single biological replication, a previously accepted method (Nakashima et al., 2002), with technical replicates being used for statistical analysis. The endoglucanase assays had four technical replicates. Enzymatic activities were calculated using the formulae presented in Smith et al. (2009).

RESULTS AND DISCUSSION

Cellulase activity of whole gut extracts of *O. obesus* on commercially available cellulose CMC was evaluated and the results are tabulated in Table 1. The activity of termite gut enzymes in terms of product delivery (glucose) was calculated based on the standard graph made using glucose as standard. The crude enzyme activity was also measured in terms of concentration of glucose as end product of CMC (Figure 1). It was found that the crude enzyme from *O. obesus* digested 49 to 75% of the substrate

within 35 min of reaction time. The activity increases with increase in enzyme concentration. 0.5 ml of crude enzyme digested 3.75 mg/5 mg of CMS within 35 min of reaction period. It was also found that the specific activity of crude enzyme extracted from whole gut of *O. obesus* ranges from 1.77 to 5.37 nmol reducing sugar/ termite equivalent/ min. It was supported with the earlier study made by Smith and Koehler (2007) on the enzyme activity of gut extracts from subterranean termite, *Reticulitermes flavipes* (Kollar) and found specific activity of 2.81 to 5.21 nmol reducing sugar/ termite equivalent/ minute. The activity especially endoglucanase activity was high in fore and hind gut than in the mid gut. The endoglucanase activity started at foregut where amorphous region of cellulose was cleaved followed by crystalline structure at hindgut (Smith and Koehler, 2007). Endoglucanase activities were mainly confined to the hindgut (Smith et al., 2009). The patterns of cellulolytic enzymes in the *O. obesus* appeared to indicate degradation of amorphous and crystalline cellulose along the gut. Endoglucanase activity was observed to be higher in the fore gut than in the mid gut and appeared to increase progressively in the hind gut. Similar endoglucanase patterns have been observed in *Coptotermes lacteus* (Hogan et al., 1988). This indicated that the amorphous and crystalline cellulose were degraded throughout the gut ecosystem of *O. obesus*. Subterranean termite, *O. obesus* is not only more aggressive, but their digestive systems are apparently more capable of digesting wood, particularly crystalline cellulose. This would suggest the possibility of natural cellulose source to wood digestion being available in their gut range. Our findings suggest a processive mechanism of amorphous cellulose degradation started at foregut and digestion of crystalline cellulose at hindgut. This corroborates the need of biotechnological interventions to study in detail about digestion in gut of *O. obesus* for *in vitro* production of endogenous cellulolytic enzymes.

Conclusion

One of the major constraints in bioethanol industry is conversion of lignocellulosic biomass to fermentable sugar, since it is very expensive process. Biological pretreatment methods are cost effective and environment friendly. Hence, use of enzymes from natural resources is warranted. Lower animals especially termites produce array of enzymes to digest lignocelluloses to sugars for their energy needs. We extracted enzymes from gut of subterranean termite, *O. obesus* and evaluated the activity of the extracted enzymes for cellulose degradation. It was found from the study that *O. obesus* can be able to digest 49 to 75% of cellulose into glucose. The specific activity of crude enzyme extracted from whole gut of *O. obesus* ranges from 1.77 to 5.37 nmol reducing sugar/termite equivalent/minute. Therefore, use of cellulolytic enzymes in gut extracts of termite, *O. obesus* in biorefinery industry would be through biotechnological interventions.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Physicochemical and microbiological meat quality, growth performance and carcass characteristics of feedlot-finished cull Santa Inês ewes and Moxotó goats[1]

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This study aimed at assessing the physicochemical and microbiological quality of feedlot-finished, culled Santa Inês ewes and Moxotó goats, and compares the intake, weight gain and carcass characteristics. Three Santa Inês ewes and Moxotó goats with similar ages were confined in double and/or individual pens and then euthanized after 40 days of confinement. The average intake, daily weight gain, hot carcass yield, cold carcass yield, weight loss by cooling and rib eye area (94.45%, 0.220 kg/day, 41.37%, 40.14%, 2.99%, 16.1 cm², respectively) of the Santa Inês ewes were higher than that of the Moxotó goats (84.01%; 0.06 kg/day; 41.55%; 39.81%; 4.12%; and 11.35 cm², respectively). The chevon (goat meat) showed higher protein levels (23.09 g/100 g), lower lipid levels (1.43 g/100 g) and higher red-color intensity (11.62), although it had lower tenderness (8.98 kgf shear strength). The superiority of the culled ewes was assessed relative to that of the culled goats, and the results showed that the Santa Inês sheep has better performance than the Moxotó goats. However, mutton and chevon derived from the culled animals are promising alternatives for small farmers because culled meats add value to the standard meat obtained from these animals, broaden sales alternatives and increase profitability.

Key words: Performance parameters, physicochemical parameters, microbiological parameters, cull animals.

INTRODUCTION

The search for healthier foods has influence changes in eating habits of meat consumer in recent years. The interest in mutton (sheep meat) and chevon (goat meat) has grown substantially, partially because of the increased interest in their possible beneficial effects on consumer health, which is related to their characteristics as natural resources with high-quality protein production (Carvalho et al., 2007) and lower levels of cholesterol, saturated fat and calories than other red meats (Madruga, 2004). Goat meat is considered a lean meat and its chemical composition is in agreement with current consumer requirements. On the other hand, sheep meat is smoother and juicier (Costa et al., 2008).

Culling is a common practice in farms with full production cycles, and the sale of culled animals is often complicated by low acceptability of the meat by the

Table 1. Bromatological composition of the concentrate feed and corn silage provided to Santa Inês ewes and Moxotó goats.

Parameter	Diet	
	Concentrate feed	Corn silage
DM (%)	93.1	30.0
MM (%)	6.39	3.33
CP (%)	15.92	5.55
NDF (%)	32.58	63.63
ADF (%)	15.74	27.95
TDN (%)	83.05	61.93

DM, dry matter; MM, mineral matter; CP, crude protein; NDF, neutral-detergent insoluble fiber; ADF, acid-detergent insoluble fiber; TDN, total digestible nutrients.

consumer market (Pelegrini et al., 2008). Culled meat is difficult to sell because fat accumulation increases in the carcass with age (François, 2009). Fat has the greatest variability among animal tissue, both quantitatively and with regard to its distribution (Pelegrini et al., 2008). The composition of fatty acids in lipids directly affects the nutritional and sensory qualities of the meat, and greater saturation reduces quality, especially considering its negative effects to human health (Mahgoub et al., 2002).

Confinement is an alternative for consideration among production systems because it promotes higher weight gain and better quality carcasses (Hashimoto et al., 2007). Neres et al. (2001) indicated that good productivity and medium-sized ruminant carcass quality are difficult to achieve on native pasture systems primarily because of nutrient deficiencies; thus, the use of cultivated pastures and supplementation in grazing and/or confinement are required to fully exploit the genetic potential of animals.

Thus, this study aimed at checking the composition of meat of different species assessing the physicochemical and microbiological quality of sheep and goat meat and compares the intake, weight gain and carcass characteristics of feedlot-finished, culled Santa Inês ewes and Moxotó goats.

MATERIALS AND METHODS

This research project was submitted to the Research Ethics Committee (Comitê de Ética em Pesquisa, CEP) of the Federal Institute of Education, Science and Technology of Goiás (Instituto Federal de Educação, Ciência e Tecnologia Goiano) and is in compliance with the National Health Council (Conselho Nacional de Saúde, CNS) Resolution 196/96, and it received an approval protocol (No. 022/2013) prior to conducting the experiment.

The experiment was conducted from October to December 2013 at the Sheep Farming Center of the Department of Animal Science of the Federal Institute of Goiás (Instituto Federal Goiano), Rio Verde Campus, Goiás (GO), which is located in southwest GO at 17° 47' 53" S, 51° 55' 53" W and 815 m average altitude.

The animals were confined for a 40-day period, and the first 10 days were used for adaptation to the facilities and experimental diet. Three Santa Inês ewes and three Moxotó goats of similar ages (full mouth with visible wear of incisors) were used, and the animals were previously tagged, wormed and randomly distributed in confinement systems according to the animal species (goat and sheep). The animals were housed in 1.20 × 2.10 m (2.52 m^2) roofed pens with concrete floors, and the pens were equipped with feeders and drinkers that were cleaned every two days. The feces were collected daily and disposed in a compost bin.

The diet provided to the confined animals consisted of 50% corn silage and 50% concentrate feed, and it was supplied twice daily at 8 AM and 6 PM. The roughage and concentrate were weighed on an electronic scale accurate to 5 g and mixed when supplying the feed. The amount supplied was adjusted according to the leftovers, which were collected and weighed and should have been a maximum of 10% of the amount supplied. Water was supplied ad libitum.

The concentrate feed used in the animal diet was purchased from a local shop in Rio Verde – GO, and the corn-based roughage feed (silage) was produced at the Federal Institute of Goiás (Instituto Federal Goiano), Rio Verde Campus.

Samples of the feed and silage were ground in a Wiley mill, sieved through 1 mm mesh and placed in a convection oven at 105°C for approximately 12 h to determine the dry matter (DM) content according to the method of Silva and Queiroz (2002), incinerated in a muffle furnace at 600°C to determine the mineral matter (MM), treated with the semi-micro-Kjeldahl method to determine the crude protein (CP), and assessed using the Filter Bag Technique in a fiber measuring device (model TE-149 – Tecnal®) to determine the neutral detergent fiber (NDF) and acid detergent fiber (ADF) content according to the method of Van Soest et al. (1994). The silage and concentrate bromatological compositions are outlined in Table 1.

The average daily intake per animal was assessed by calculating the difference between the amount of feed supplied and rejected (leftovers). The animals were weighed at the start of confinement (initial weight, IW) and prior to slaughter (slaughter weight, SW). Intermediate weighing assessments between IW and SW were performed regularly every seven days to monitor performance. All weighing assessments were performed in the morning after fasting of solids for 14 h. The total weight gain (TWG) was assessed by calculating the difference between the SW and IW, and the daily weight gain (DWG) was assessed by calculating the difference between two weighing and dividing that figure by the number of days between weighing assessments.

The ewes and goats were subjected to 16 h water diets and weighed again prior to slaughter, and the live weight at slaughter (LWS) was calculated at day 40. The slaughter was conducted at

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Abbreviations: CV, coefficient of variation; **ADI,** average daily intake; **TWG,** total weight gain; **DWG,** daily weight gain; **HCY,** hot carcass yield; **CCY,** cold carcass yield; **WLC,** weight loss by cooling; **REA,** rib eye area; **DM,** dry matter; **MM,** mineral matter; **CP,** crude protein; **NDF,** neutral-detergent insoluble fiber; **ADF,** acid-detergent insoluble fiber; **TDN,** total digestible nutrients.

Table 2. Mean values for the intake, weight gain, hot and cold carcass yield, weight loss by cooling and rib eye area of the culled Santa Inês ewes and Moxotó goats.

Parameter	Mean[(1)]	SD	CV (%)	Mean[(1)]	SD	CV (%)
	Ewes			Goats		
ADI (%)	94.45	± 2.08	1.2	84.01	± 1.43	0.82
TWG (kg)	8.73	± 4.10	2.37	2.47	± 1.19	0.48
DWG (kg)	0.22	± 0.10	0.06	0.06	± 0.03	0.02
HCY (%)	41.37	± 0.14	0.08	41.55	± 3.97	0.10
CCY (%)	40.14	± 0.41	0.24	39.81	± 3.34	1.93
WLC (%)	2.99	± 1.12	0.65	4.12	± 1.16	0.67
REA (cm²)	16.1	± 3.20	1.85	11.35	± 2.68	2.68

[(1)]Means from the analyses. SD, standard deviation; CV, coefficient of variation; ADI, average daily intake; TWG, total weight gain; DWG, daily weight gain; HCY, hot carcass yield; CCY, cold carcass yield; WLC, weight loss by cooling; REA, rib eye area.

the municipal slaughterhouse of the city of Rio Verde, GO and supervised by the Municipal Inspection Service (Serviço de Inspeção Municipal), using the humane slaughter method. The following samples were collected from the slaughtered animals: blood, skin, guts, internal organs, feet (severed at the level of the tarsal-metatarsal and carpal-metacarpal joints) and head (severed at the level of the atlantooccipital joint). The remainder of the animal's body was identified and weighed to assess the hot carcass weight (HCW) and hot carcass yield (HCY).

The HCY was assessed using the equation below:

$$\text{HCY (\%)} = \frac{\text{HCW}}{\text{SW}} * 100$$

HCY (%) = hot carcass yield expressed as percentage; HCW = hot carcass weight (kg); SW = final fasting weight or slaughter weight (kg).

The hot carcass was transported to the cold room suspended by the joints on overhead rails and kept 17 cm apart from the other carcass; the carcasses were maintained at a temperature of 2°C for a 24 h period and then weighed again to assess the cold carcass weight (CCW).

The cold carcass yield (CCY) was calculated after assessing the CCW as follows:

$$\text{CCY (\%)} = \frac{\text{CCY}}{\text{SW}} * 100$$

Where, CCY (%) = cold carcass yield expressed as percentage; CCW = cold carcass weight (kg); SW = final fasting weight or slaughter weight (kg).

The weight loss by cooling was assessed using the following equation:

$$\text{WLC (\%)} = \frac{(\text{HCW} - \text{CCW})}{\text{HCW}} * 100$$

Where, WLC (%) = weight loss by cooling expressed as percentage; HCW = hot carcass weight (kg); CCW = cold carcass weight (kg).

A cross-section of the *Longissimus dorsi* muscle was performed between the 12th and 13th ribs to assess the rib eye area (REA) according to the method of Osório et al. (1998), and color, shear strength and microbiological analyses were performed to determine the protein, lipid, moisture, ash and pH levels according to the Association of Official Agricultural Chemists (AOAC, 2000). All of the analyses were performed in triplicate.

The color analysis was performed using a Hunter Lab Colorimeter, model Color Quest II, and the values were expressed as L*, a* and b*, where L* (luminosity or brightness) may range from black (0) to white (100), a* (chroma) may range from green (-60) to red (+60) and b* (chroma) may range from blue (-60) to yellow (+60) as reported by Paucar-Menacho et al. (2008).

The shear strength was assessed using a Stable Micro Systems® texturometer model TAXT, in addition to a texture analyzer with blade set that included a knife (blade and guillotine), which was applied at a temperature of 25°C. Samples of 2.0 cm diameter and 1 cm mean height were used for this analysis. The pre-test speed was 10 mm/s, test speed was 5 mm/s and post-test speed was 10 mm/s.

Microbiological analyses were performed in mutton and chevon using the most probable number (MPN) method to count the total coliforms and fecal coliforms. The MPN is a classic culture method to assess *Salmonella* and analyze *Staphylococcus aureus* by performing direct counts in plates according to the method recommended by Silva and Junqueira (2001).

The data on the performance and carcass characteristics were analyzed using a descriptive statistical analysis (Borba, 2014), and the results from the physicochemical analysis were assessed through a completely randomized design (CRD). Three replicates were performed in triplicate for all analyses using the Software Sisvar (Ferreira, 2003) at the 5% significance level according to Tukey's test.

RESULTS AND DISCUSSION

Performance and carcass characteristics

The culled Santa Inês ewes showed a 94.45% average daily intake, 0.220 kg/day daily weight gain, 41.37% hot carcass yield, 40.14% CCY, 2.99% weight loss by cooling and 16.1 cm² rib eye area (Table 2) after the 40 experimental days. The culled Moxotó goats showed an 84.01% average daily intake, 0.06 kg/day daily weight gain, 41.55% hot carcass yield, 39.81% CCY, 4.12% weight loss by cooling and 11.35 cm² rib eye area (Table 2).

Superiority of the culled ewes over the culled goats was observed in all of the evaluated parameters, indicating. that Santa Inês sheep have better performance than Moxotó goats (Table 2).

Table 3. Physicochemical results for the culled Santa Inês mutton and Moxotó chevon.

Parameter	Mutton	Chevon	CV (%)	F Value
Protein (g/100 g)	20.38 ± 1.9^{B}	23.09 ± 1.6^{A}	8.22	10.40*
Lipids (g/100 g)	2.65 ± 0.99^{A}	1.43 ± 0.35^{B}	12.06	36.33*
Moisture (g/100 g)	70.65 ± 1.8^{B}	74.11 ± 1.2^{A}	2.13	22.74*
Ash (g/100 g)	0.96 ± 0.03^{B}	0.92 ± 0.02^{A}	2.67	11.62*
pH	5.94 ± 0.10^{B}	6.06 ± 0.08^{A}	1.53	7.11*
L	41.31 ± 4.95^{A}	43.53 ± 2.21^{A}	9.04	4.03^{ns}
a*	9.69 ± 0.86^{B}	11.62 ± 1.11^{A}	9.3	45.4*
b*	7.26 ± 0.82^{B}	10.22 ± 1.35^{A}	12.8	84.3*
Shear (kgf)	4.74 ± 3.96^{B}	8.98 ± 3.34^{A}	53.4	7.4*

Different letters in the same row indicate significant differences (P<0.01) according to Tukey's test. *Significant at the 5% probability level. ^{ns}Not significant.

François (2009) obtained lower values than those indicated here and reported that culled ewes presented a 0,140 kg/day weight gain over 75 days, and Brum et al. (2008) also obtained lower results, reporting 0.151 kg/day daily weight gain when evaluating Corriedale ewe lambs maintained on cultivated millet pasture with 10% supply; the differences in the study results are attributed to the different diets provided. The average daily weight gain values recorded in this experiment were close to the values reported by Dantas et al. (2008), who studied the carcass characteristics of pasture-finished Santa Inês sheep submitted to different levels of supplementation and found that the supplemented animals showed a daily weight gain of 192 and 148 g.

Similar weight gain results to those of the Moxotó goats highlighted in the present study were reported by Menezes et al. (2004), who examined the intake of goats when corn was replaced by cassava peel and indicated that the average daily weight gain of the animals was unsatisfactory, even for the treatment without cassava peel (0.098 kg/day). Similar weight gains were also reported by Bueno et al. (2000), who observed 0.058 kg/day weight gain in growing goats fed a diet of dried citrus pulp as a replacement for corn.

The HCY and CCY observed in this experiment were close to those assessed by Pelegrini (2007), who observed 47.25 and 45.20% HCY in Texel and Ideal ewes, respectively, and 43.72% and 45.95% CCY for Texel and Ideal ewes, respectively. The higher HCY and CCY of the ewes was a result of their greater SW.

Bernardo et al. (2008) reported 45.60%, 45.86% and 43.34% HCYs and 44.73%, 44.96% and 42.34% CCYs when studying the carcass characteristics of Anglo-Nubian, Boer and mongrel crossbred goats, respectively, which is consistent with the results of this study.

The results presented here are important because they show that the performance of culled ewes and goats would not preclude their commercial viability because they showed similar DWG values to those reported in the literature.

Meat characteristics

The protein levels ranged from 20.38 to 23.09 g/100 g for mutton and chevon, as shown in Table 3. Chevon showed higher protein values (23.09 g/100 g) that were significantly different (p≤0.05) from those of mutton (20.38 g/100 g).

The mean protein levels of the two meats were similar, although they may change with animal age at slaughter, with a trend towards increased protein with age (Oliveira, 2011). The higher protein levels of chevon are related to genetic factors and lower lipid levels.

A significant difference in lipid levels was observed between the species, with mutton showing higher values (2.65 g/100 g) and chevon showing lower values (1.43 g/100 g). Fat is a key factor for meat quality, and it directly affects the sensory properties and nutritional value. Fat is the most variable basic meat component, and it is directly affected by several factors, including species, breed, age, sex, nutrition and cut (Lawrie, 2005).

The meat moisture level of the different species was higher in chevon (74.11 g/100 g), and the value was significantly different (p<0.05) from that of mutton (70.65 g/100 g). The moisture levels are directly related to the fat levels, with higher fat levels corresponding to lower water levels. Mutton showed higher levels of inorganic residue (ash; 0.96 g/100 g) compared with that of chevon (0.92 g/100 g).

pH is associated with pre-slaughter and slaughter conditions, animal excitability, muscle glycolytic potential and carcass cooling temperature (Lima, 2009). The final muscle pH also varies according to animal species and muscle type. The pH values may range from 5.5 to 5.8 in muscles with predominantly fast-twitch fibers (white fibers), whereas values ranging from 5.8 to 6.4 are observed in slow twitch muscles (red fibers). The pH values were similar between the different species, although there were slight differences (mutton 5.94; and chevon 6.06).

The final muscle pH is a factor that affects several meat

Table 4. Results from the microbiological analyses of culled Santa Inês mutton and Moxotó chevon.

Microorganism	Mutton	Chevon
Total coliforms (log_{10})	4.38	4.38
Thermotolerant coliforms (log_{10})	4.38	4.38
Salmonella	abs.	abs.
S. aureus	abs.	abs.

quality aspects, including the water holding capacity, cooking weight loss, shear strength, tenderness, juiciness and color (Lawrie, 2005). The meat chemical composition of the different species showed similar values to those reported in the literature, and this finding corroborates Beserra et al. (2000), who recorded values ranging from 15.9 g/100 g to 19.08 g/100 g for protein levels; 77.80 g/100 g to 80.25 g/100 g for moisture levels; 1.12 g/100 g to 1.21 g/100 g for fat levels; and 1.29 g/100 g to 2.03 g/100 g for ash levels in a study of the chemical characteristics of cabrito from the Moxotó breed and Alpine brown x Moxotó cross-bred animals.

Similar values to those presented here were reported by Dias et al. (2002), who recorded 76.54 g/100 g, 1.08 g/100 g, 2.06 g/100 g and 23.27 g/100 g for moisture, ash, lipid and protein levels, respectively, when examining the physical and physicochemical characteristics of culled Moxotó chevon, as well as those reported by Duarte (2003), who recorded values of 75.13 g/100 g, 22.69 g/100 g, 2.55 g/100 g and 0.98 g/100 g for moisture, protein, fat and ash levels, respectively, when analyzing shoulder cuts from crossbred Boer goats slaughtered at 228 days.

Pinheiro et al. (2008) found values of 74.05 g/100 g moisture, 5.36 g/100 g fat, 18.85 g/100 g proteins and 1.15 g/100 g ash in a study of the chemical composition and yield of mutton *in natura*, which is consistent with the results shown for the meat physicochemical characteristics. Different values were reported by Santos Júnior et al. (2009), who recorded values of 76 g/100 g moisture, 19.18 g/100 g protein, 5.40 g/100 g lipids, 1.18 g/100 g ash and 5.56 pH in a study of the physicochemical characteristics of culled mutton used to prepare hamburgers supplemented with oat flour.

The meats from different species mostly showed dark (intermediate values of L*), red (positive component a*) and yellowish (positive component b*) coloration. Chevon showed values of 43.53 L*, 11.62 a* and 10.22 b*, with similar values to mutton for L*(41.41) and different values for a* (9.69) and b* (7.26). Chevon is noticeably similar to mutton in color despite showing higher L* values; however, its color is a brighter brick-red color (11.62 a*) compared with that of mutton (9.69).

The meat color is dependent on its total myoglobin content and is affected by the species, sex, age, nutrition and slaughter conditions. Similar culled mutton results to those presented here were reported by Santos Júnior et al. (2009), who assessed values of 39.75; 13.08 and 11.78 for the parameters L*, a* and b*, respectively. Lower L*, red and yellow parameters were reported by Sobrinho et al. (2005) when assessing the mutton quality characteristics of different genotypes and different ages at slaughter; these authors found L* values ranging from 37.26 to 37.91, a* values ranging from 7.71 to 7.91 and b* values ranging from 4.19 to 4.39. The mutton red (a*) levels were lower than those reported by Esteves (2011), who identified the color of mutton at different ages and observed values of 36.37 L*, 16.74 a* and 7.34 b*.

Dias et al. (2002) reported different L* (24.98) and yellow intensity (1.24) in the color analysis of culled chevon from the Moxotó breed, although similar red intensity values (15.29) were observed and confirmed the intense color of the meat.

The shear strength value (Table 3) of mutton was 4.74 kgf and chevon was 8.98 kgf. Thus, chevon showed a higher value from that of mutton. Genetics, breed, slaughter age, sex, nutrition, muscle fiber bundle size, connective tissue, contracted muscle fibers post-mortem and meat lipid levels stand out among the factors affecting meat tenderness (ALVES et al., 2005).

Pinheiro et al. (2009) and Zeola et al. (2005) recorded mean values of shear strength from 2.35 kgf to 4.08 kgf and 4 kgf in a study of Santa Inês sheep at different physiological stages and culled ½ Ile de France x ½ Ideal crossbred ewes, and these values corroborate the shear strength results assessed in this study.

Conversely, Dias et al. (2002) found a lower shear strength value of 4.97 kgf for chevon from culled animals, which were related to the higher fat levels.

The microbiological analysis of the meats showed an absence of *Salmonella* and *S. aureus*. However, the analysis of total and thermotolerant coliforms showed a count of 4.38 log_{10} for both microorganisms (Table 4). The total coliform value indicates contamination during the slaughter process and deficient handling, cleaning and sanitation. Thermotolerant coliforms indicate fecal contamination because that group originates exclusively from the intestinal tracts of warm-blooded animals.

Resolution 12 (RDC12) of the National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária; Brasil, 2001) defines the technical regulations related to the microbiological standards for food, including

beef, pork and other mammal meats that are refrigerated or frozen "*in natura*" (whole or cut carcasses, quarters or cuts). The 25 g samples of ground meats and viscera from cattle, pigs and other mammals did not include *Salmonella*. However, the values shown meet the standards required by the current legislation.

Conclusion

The results indicate that culled Santa Inês ewes have a better intake, weight gain and carcass yield than culled Moxotó goats. Chevon showed higher protein levels, lower lipid levels and higher red color intensity, although it also had lower tenderness. The microbiological quality of the meat indicated contamination during the slaughter process and deficient handling, cleaning and sanitation. Chevon and mutton derived from culled animals is a promising alternative for small farmers because culled meats add value to the standard meat obtained from these animals, broaden sales alternatives and increase profitability.

Conflict of interests

The authors did not declare any conflict of interest.

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