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Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998;

1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001) References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. *Afr. J. Biotechnol.* 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant *Staphylococcus aureus* in community-acquired skin infections. *Emerg. Infect. Dis.* 11: 928-930.

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.

Pelczar JR, Harley JP, Klein DA (1993). *Microbiology: Concepts and Applications.* McGraw-Hill Inc., New York, pp. 591-603.

### Short Communications

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Full Length Research Paper

## Assessment of genetic diversity analysis in contrasting sugarcane varieties using random amplified polymorphic DNA (RAPD) markers

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Sugarcane is an important crop in the country economically, politically and sociologically. It is the second largest agro-industry next to textiles. The selection and combination of parents for crossing rely on an understanding of their genetic structures and molecular diversity. In the present study, 28 sugarcane genotypes were used for genomic diversity analyses based on 30 randomly amplified polymorphic DNA markers (RAPD). These 30 sets of RAPD marker generated a total of 277 discernible and reproducible bands which included 179 polymorphic and 98 monomorphic bands. The unweighted pair group method with arithmetic average (UPGMA) analysis revealed six distinct clusters: I, II, III, IV, V and VI within the 28 genotypes. The polymorphic information content value per locus ranged from 0.21 for the OPA12 locus to 0.53 for OPH05, with an average of 0.40 for all loci. The range of genetic distance or coefficient of similarity among sugarcane genotypes were 0.08-1.00. The analysis of these similarities matrix revealed that greater similarity between Co 05011 and Co 0237, Co 05011 and Co 0241 (1.00), and lowest similarity between CoSe 03234 and CoS8432 (0.08). The knowledge obtained in this study will be useful to future breeding programs for increasing genetic diversity of sugarcane varieties and cultivars to meet the demand of sugarcane cultivation for sugar and bioenergy use.

**Key words:** Sugarcane, random amplified polymorphic DNA (RAPD) marker, genetic diversity, unweighted pair group method with arithmetic average (UPGMA).

### INTRODUCTION

Sugarcane is a commercial crop that belongs to genus *Saccharum officinarum* L. (Poaceae), and contributes nearly 70% sugar production worldwide. The crop is cultivated in more than 90 countries all over the world. Sugarcane is mainly used for white sugar production but

recent emphasis is also being given on production of biofuel like ethanol. It is an alternate source of energy and its production from sugar and or by products like molasses also makes sugarcane a future biofuel plant (Singh et al., 2010). Sugarcane is among the major

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**Table 1.** Characteristics of selected twenty eight sugarcane genotypes.

Variety	Parent	Type
CoS 08272	CoSe 92423 GC cross	Early
CoSe 98239	CoS 7927 x Co 775	Early
CoS 8436	MS 6847 x Co 1148	Early
CoSe 03234	Bo 91 x PCGC cross	Early
Co 98014	Co 8316 x Co 8213	Early
Co 0118	CoS 8347 x Co 86011	Early
CoSe 01424	Bo 91 x Co 453	Early
Co 0238	CoLk 8102 x Co 775	Early
CoC 671	Q 63 x Co	Early
CoJ 64	Co 976 x Co 617	Early
Co 1148	P4383 x Co 312	Unknown
CoS 95255	Co 1158 x Co 62198	Early
UP 9530	CoSe 1084/86 x CoSe 22/85	Midlate
CoSe 1434	Co 880239 x Co 775	Midlate
CoS 08279	CoLk 8102 x Co89803	Midlate
CoS 7250	CoS 8436 x Co 775	Midlate
Co 05011	CoS 8436 x Co 89003	Midlate
CoSe 92423	Bo 91 x Co 435	Midlate
CoS 96275	CoS 8119 x Co 62198	Midlate
CoS 96463	Unknown	Early
UP 49	CoSe 92423 x UP 9742	Midlate
CoS 8432	MS6847xCo1148	Unknown
CoSe 98231	CoS7927xCo775	Unknown
Co 0237	Co 93016 GC	Unknown
Co 0240	Unknown	Midlate
Co 0241	Unknown	Midlate
CoS 6287	Unknown	Midlate
CoS 8276	Unknown	Midlate

economic crops in the world and is the raw material for sugar and ethanol production as also as other by-products having the most efficient energetic balance (input over output) when compared with other energetic crops such as maize, sorghum and wheat (Lam et al., 2009). Brazil is the world leader in sugarcane production next to India with approximately 570 million tonnes of sugarcane, 31 millions of tonnes of sugar and 26 billions of liters of ethanol per year (UNICA, 2011).

Genetic diversity can be estimated based on different methods, morphological traits, pedigree record and molecular markers. The use of molecular markers for evaluation of genetic diversity is receiving much more attention. Molecular marker is potentially a valuable tool for crop improvement. Molecular markers play a role to portray genetic variability in several crops. Nowadays, fingerprinting system based on random amplified polymorphic DNA (RAPD) analysis have been increasingly utilized for detecting polymorphism in those genera which has no prior sequence information. Due to technical simplicity and speed, RAPD methodology has been used for diversity analysis in many plant species (Tonk et al., 2011). RAPD markers have several advantages

over other polymorphism detection techniques including RFLP and other markers. These include quickness, relatively easy assay, and requirement for small amount of template DNA, no requirement of DNA sequence information and use of fluorescence. Because of these advantages, RAPD are commonly used to characterize variability (Vijay et al., 2009). There have been substantial reports of genetic diversity analysis of different crops including sugarcane plants using RAPD molecular markers (Singh et al., 2010a, b; Zhang et al., 2004, 2008; Pandey et al., 2012; Tabasum et al., 2010).

## MATERIALS AND METHODS

### Plant material and DNA isolation

Pot experiment was laid out by planting 28 sugarcane genotypes (Table 1), based upon highly contrasting morphological feature obtained from gene pool of Sugarcane Breeding Institute, Regional Station (SBI-RS) Karnal, Haryana and Sugarcane Research Station, Muzaffarnagar, U.P. India. Fresh leaves collected, were dipped in liquid nitrogen and used to isolate DNA applying CTAB method (Hoisington et al., 1994) with some modifications. 500 mg leaf tissues were ground in liquid N<sub>2</sub> and mixed in 8 ml of

**Table 2.** RAPD primer pairs used in this investigation.

Primer code	Primer sequence	Amplicon size (Kb)	Total bands	Monomorphic bands	Polymorphic bands	% Polymorphism	PIC value
OPH-03	5'-AGACGTCCAC-3'	0.3-2.5	8.0	3.0	5.0	62.5	0.28
OPH-04	5'-GGAAGTCGCC-3'	0.2-1.5	10.0	3.0	7.0	70.0	0.51
OPH-05	5'-AGTCGTCCCC-3'	0.3-2.5	7.0	2.0	5.0	71.4	0.53
OPH-07	5'-CTGCATCGTG-3'	0.2-3.0	11.0	2.0	9.0	81.8	0.44
OPH-09	5'-TGTAGCTGGG-3'	0.4-1.5	8.0	3.0	5.0	62.5	0.33
OPH-12	5'-ACGCGCATGT-3'	0.3-2.0	10.0	5.0	5.0	50.0	0.27
OPH-19	5'-CAAACGTCGG-3'	0.3-1.7	9.0	3.0	6.0	66.7	0.53
OPA-03	5'-AGTCAGCCAG-3'	0.4-1.8	12.0	4.0	8.0	66.7	0.27
OPA-02	5'-GAGGATCCCT-3'	0.2-2.5	8.0	3.0	5.0	62.5	0.33
OPC-19	5'-GTTGCCAGCC-3'	0.2-1.5	11.0	4.0	7.0	63.6	0.29
OPE-02	5'-GGTGC GGAA-3'	0.3-2.8	9.0	3.0	6.0	66.7	0.39
OPA-01	5'-CAGGCCCTTC-3'	0.3-2.5	10.0	4.0	6.0	60.0	0.37
OPA-04	5'-AATCGGGCTG -3'	0.4-1.5	12.0	4.0	8.0	66.7	0.35
OPA-05	5'-AGGGTCTTG-3'	0.2-2.0	10.0	3.0	7.0	70.0	0.37
OPA-07	5'-GAAACGGGTG-3'	0.2-1.5	8.0	4.0	4.0	50.0	0.45
OPA-09	5'-GGTAACGCC-3'	0.2-2.5	5.0	1.0	4.0	80.0	0.43
OPA-10	5'-GTGATTCGAG-3'	0.3-2.5	9.0	4.0	5.0	55.6	0.51
OPA-11	5'-CAATCGCCGT-3'	0.3-3.0	11.0	4.0	7.0	63.6	0.35
OPA-12	5'-TCGGCGATAG-3'	0.2-2.5	8.0	4.0	4.0	50.0	0.21
OPA-13	5'-CAGCACCCAC-3'	0.4-3.0	12.0	5.0	7.0	58.3	0.29
OPA-15	5'-TTCCGAACCC-3'	0.2-2.5	7.0	2.0	5.0	71.4	0.37
OPA-16	5'-AGCCAGCGAA-3'	0.3-2.8	8.0	3.0	5.0	62.5	0.51
OPA-17	5'-GACCGCTTGT-3'	0.2-2.4	10.0	3.0	7.0	70.0	0.55
OPA-18	5'-AGGTGACCGT-3'	0.2-1.8	10.0	4.0	6.0	60.0	0.43
OPA-19	5'-CAAACGTCGG-3'	0.2-2.0	8.0	2.0	6.0	75.0	0.51
OPE-05	5'-TCAGGGAGGT-3'	0.3-2.4	8.0	3.0	5.0	62.5	0.39
OPA-20	5'-GTTGCGATCC-3'	0.3-2.5	10.0	2.0	7.0	70.0	0.51
OPA-20	5'-GTTGCGATCC-3'	0.2-3.0	7.0	3.0	4.0	57.1	0.44
OPAB-8	5'-GTCCACACGG-3'	0.3-3.2	11.0	4.0	8.0	72.7	0.37
OPAB-11	5'-GTAGACCCGT-3'	0.2-2.7	10.0	4.0	6.0	60.0	0.52
Average			9.2	3.3	6.0	64.7	0.40

prewarmed CTAB extraction buffer (2% (w/v) CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), and 0.2% (v/v)  $\beta$ -mercaptoethanol with 2% PVP (w/v) added immediately prior to use and incubated at 65°C for 1 h. The content was mixed with equal volume of chloroform : isoamyl alcohol (24:1) and subjected to centrifugation for 10 min at 12,000 rpm at 4°C. Sample was taken out from ultracentrifuge and aqueous phage was pipetted out in a 25 ml autoclaved tube. Equal volume of chilled iso-propanol was added in aqueous phage to precipitate the DNA. Precipitated threads of DNA were pipetted out in a 2 ml appendoff using wide bore tip followed by centrifugation at 12,000 rpm for 10 min. Milky white pellet was washed with 70% alcohol; air dried, and resuspended in 10 mM Tris, at pH 8.0. To degrade RNA content, RNase treatment was given for 1 h at 30°C. The quality and quantity of the genomic DNA was checked on 0.8% agarose gel and diluted appropriately for working concentration of 25 ng/ $\mu$ l.

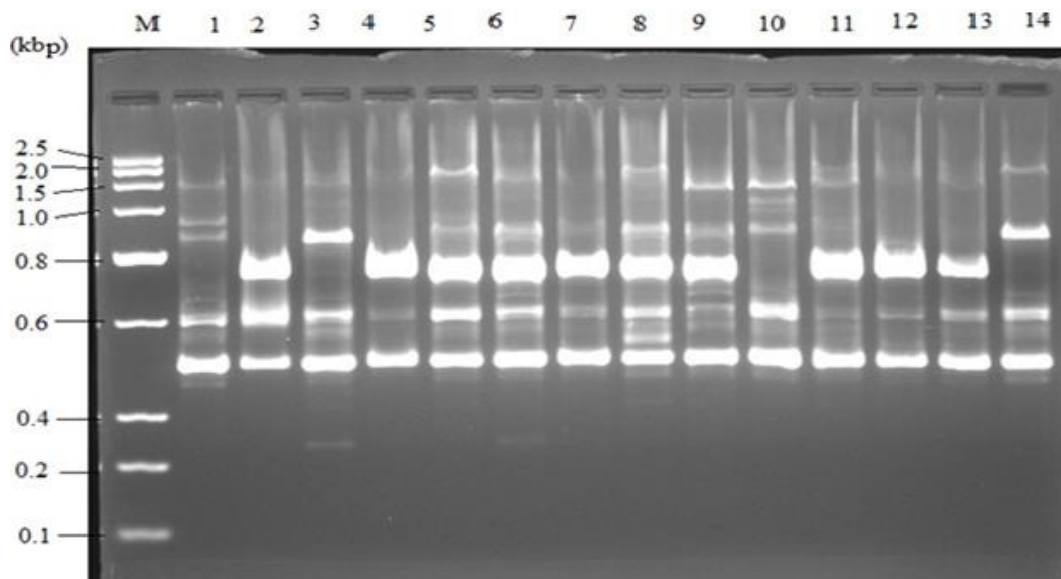
#### RAPD marker genotyping

The polymerase chain reaction was performed in a thermal cycler (MyGene, MG96G<sup>™</sup>) using 30 RAPD primers synthesised by

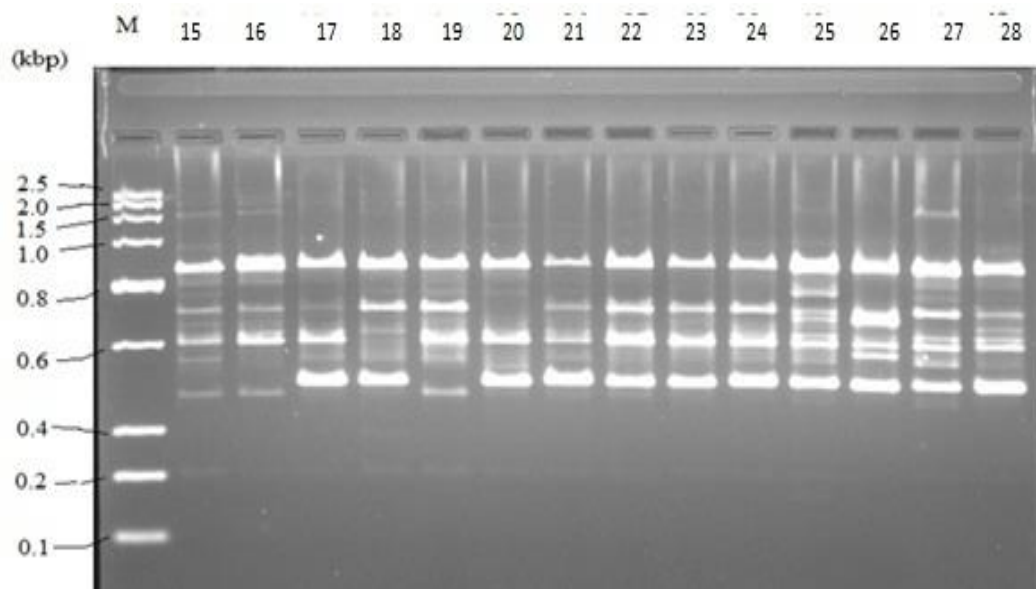
Bangalore Genei, India. The PCR was carried out in 25  $\mu$ l reaction volume containing genomic DNA (50 ng), 1.5 mM MgCl<sub>2</sub>, 1.2 mM dNTPs, 0.7  $\mu$ l (1U/  $\mu$ l) *Taq* DNA polymerase and 30 ng primer (Table 2). The cycling conditions included initial cycle of denaturation at 94°C for 4 min followed by repeated 35 cycles of denaturation at 94°C for 45 s, annealing differ for each primers for 45 s and extension at 72°C for 60 min. After completion of 35 cycles, a final extension at 72°C for 5 min was carried out and finally held at 4°C. The amplified products resulting from different primers were resolved on 1.4% agarose gel and analyzed by standard ethidium bromide staining and finally photographed using gel documentation system (Alfa Innotech, USA). The PCR amplification was performed twice with each primers and band scoring was done accordingly.

#### Amplicon scoring

The bands resulting from different sets of RAPD primers for different populations of *S. officinarum* were scored on agarose gel photograph in gel documentation system for its presence and absence across the populations collected (Figures 1 and 2). The



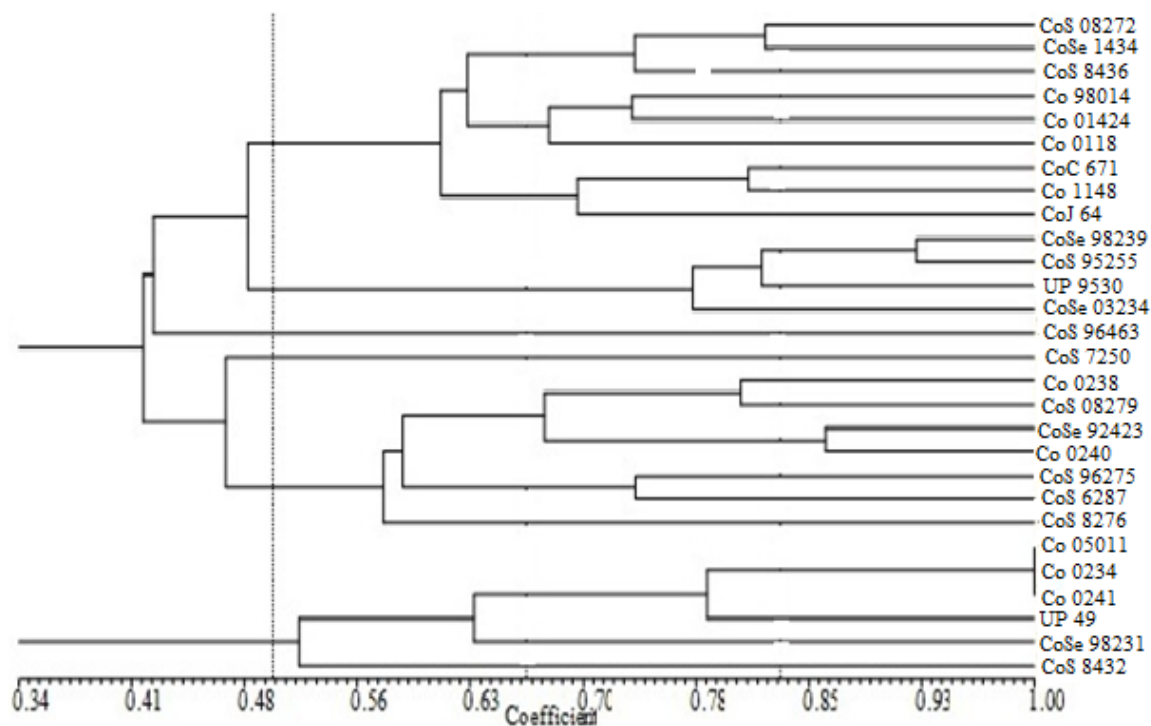
**Figure 1.** RAPD amplification of genomic DNAs of *Saccharum* and related genera used in this study using RAPD primer OPA-19. M, gene ruler 100 bp DNA ladder; Lane 1, CoS 08272; 2, CoSe 98239, 3, CoS 8436; 4, CoSe 03234; 5, Co 98014; 6, Co 0118; 7, CoSe 01424; 8, Co 0238; 9, CoC 671; 10, CoJ 64; 11, Co 1148; 12, CoS 95255; 13, UP 9530; 14, CoSe 1434.



**Figure 2.** RAPD amplification of genomic DNAs of *Saccharum* and related genera used in this study using RAPD primer OPA-19. M, gene ruler 100 bp DNA ladder; Lane 15, CoS 08279; 16, CoS 7250; 17, Co 05011; 18, CoSe 92423; 19, CoS 96275; 20, CoS 96463; 21, UP 49; 22, CoS 8432; 23, CoSe 98231; 24, Co 0237; 25, Co 0240; 26, Co 0241; 27, CoS 6287; 28, CoS 8276.

image profiles of banding patterns were recorded and molecular weight of each bands were determined by DNA ladder. The results were analysed based on the principle that a band is considered to be polymorphic if it is absent in at least one individuals or accessions. Similarity index of bands which were common between

two accessions was estimated by Nei and Li (1979). The final RAPD data generated with 30 RAPD primers were used to calculate pair wise similarity coefficients using Jaccard's coefficient of similarity (Jaccard, 1908). The cluster analysis and dendrogram construction were performed with NTSYS-PC (version 2.02e)



**Figure 3.** Dendrogram for 10 sugarcane genotypes showing the genetic similarities derived from UPGMA cluster analysis using the jaccard similarity coefficient.

software for generating phylogenetic tree using the unweighted pair group method with arithmetic average (UPGMA) method (Nie and Li, 1979).

## RESULTS AND DISCUSSION

For RAPD analysis, a total of 30 primer pairs were screened, and used for polymerase chain reaction (PCR) amplification (Table 2). The PCR product was electrophoresed on 1.4% agarose gels (Figures 1 and 2). Bands were counted and the presence and absence of bands were scored as 1 and 0, respectively. The PCR amplicons sizes ranged from 0.26 to 3.2 Kb. These sets of primers revealed intra-specific variations. A total of 277 bands were scored with 179 polymorphic and 98 monomorphic bands (Table 2). The 28 sugarcane genotypes were clustered based on the matrix of genetic similarities using UPGMA. The cluster analysis and dendrogram construction were performed with NTSYS-PC (version 2.02e). Using 30 RAPD markers, a total of 277 alleles were detected among 28 sugarcane genotypes studied. The average number of allele per locus was 9.2. Dendrogram was constructed based on genetic distance calculated from 277 alleles generated from 28 sugarcane genotypes. The UPGMA cluster tree analysis led to the grouping of the 28 sugarcane genotypes into six major clusters (Figure 3). Cluster I, the largest cluster comprised nine sugarcane genotypes among selected 28 sugarcane genotypes. Cluster II

comprised four sugarcane genotypes. Clusters III and IV were the smallest cluster comprised only one sugarcane genotype, CoS96463 and CoS7250 respectively. Cluster V, the second largest cluster comprised seven sugarcane genotypes among selected 28 sugarcane genotypes while cluster VI the third largest cluster comprised six sugarcane genotypes among selected 28 sugarcane genotypes. The PIC value per locus ranged from 0.21 for the OPA12 locus to 0.53 for OPH05, with an average of 0.40 for all loci (Table 2). The range of genetic distance or coefficient of similarity among sugarcane genotypes were 0.08-1.00. The analysis of these similarities matrix revealed that greater similarity between Co 05011 and 24 Co 0237, Co 05011 and 26 Co 0241 (1.00), and lowest similarity between CoSe 03234 and CoS8432 (0.08) (Figure 3). Those cultivars that display similar coefficient of matrix are genetically close to one another and vice-versa. Similar studies or observation was reported by many researchers worldwide previously (Pandey et al., 2012; Tabasum et al., 2010).

Therefore, the present investigation reported the results of a study on the genetic diversity among 28 accessions of sugarcane revealed by RAPD. Using RAPD as genetic markers, as high as 81.8% polymorphic bands were detected in 28 accessions of sugarcane; similar studies were conducted previously by Burner et al. (1997) and Nair et al. (2002). This study will facilitate in marker-assisted applications in sugarcane breeding. The present investigation is an initial step to evaluate the molecular

diversity of this critically complex genome and polyploidy nature of different sugarcane accessions. Therefore, further work is required to promote molecular markers like genomic simple sequence repeats (gSSRs) and expressed sequence tagged based simple sequence repeats (EST-SSRs) can be utilized. This study will be a boon to conserve sugarcane *in vitro* and *ex-situ* as well identification of parents for breeding improvement programme and also to fulfil the growing demand at national and inter-national market.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

### REFERENCES

- Burner DM, Pan YB, Webster RD (1997). Genetic diversity of North American and Old World *Saccharum* assessed by RAPD analysis. *Genet. Res. Crop Evol.* 44:235-240.
- Hoisington D, Khairallah M, Gonzalez-de-Leon D (1994). Laboratory protocols: CIMMYT applied molecular genetics laboratory, 2nd ed. Mexico: CIMMYT.
- Jaccard P (1908). Nouvelles recherches sur la distribution florale. *Bul. Soc. Vaudoise. Sci. Nat.* 44:223-270.
- Lam E, Shine J, Silva J, Lawton M, Bonos S, Calvino M, Arrer H, Silva-Filho MC, Glynn N, Helsel Z, Ma J, Richard E, Souza GM, Ming R (2009). Improving sugarcane for biofuel: engineering for an even better feedstock. *GCB Bioenergy* 1:251-255.
- Nair NV, Selvi A, Sreenivasan TV, Pushpalatha KN (2002). Molecular diversity in Indian sugarcane cultivars as revealed by randomly amplified DNA polymorphisms. *Euphytica* 127:219-225.
- Nei M, Li WH (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci.* 76:5269-5273.
- Pandey RN, Singh J, Rastogi SP, Sharma ML, Singh RK (2012). Early assessment of genetic fidelity in sugarcane (*Saccharum officinarum*) plantlets regenerated through direct organogenesis with RAPD and SSR markers. *Aust. J. Crop Sci.* 6(4):618-624
- Singh RK, Mishra SK, Singh SP, Mishra N, Sharma ML (2010). Evaluation of microsatellite markers for genetic diversity analysis among sugarcane species and commercial hybrids. *Aust. J. Crop Sci.* 2:115-124.
- Singh S, Gautam RK, Singh RK, Deshmukh R (2010a). Diversity analysis of *Sesbania acculeata* bu using transferable rice SSR markers. *Crop Res.* 39 (1,2 & 3):127-131.
- Singh S, Gautam RK, Singh RK, Deshmukh R, Ojha A (2010b). Molecular diversity in rice genotypes differing in physiological mechanism of salt tolerance through SSR and ISSR markers. *IJABPT* 1 (2):550-560.
- Tabasum S, Khan FA, Nawaz S, Iqbal MZ, Saeed A (2010). DNA profiling of sugarcane genotypes using randomly amplified polymorphic DNA. *Genet. Mol. Res.* 9(1):471-483
- Tonk FA, Giachino RRA, Sonmez C, Yuce S, Bayram E, Telci I, Furan MA (2011). Characterization of various *Hypericum perforatum* clones by hypericin and RAPD analyses. *Int. J. Agric. Biol.* 13(1):31-37.
- UNICA, Unia˜o da indus˜tria de cana-de-ac˜ucar. 2009. Produca˜o do Brasil. Disponı˜vel em: <http://www.unica.com.br/dadosCotacao/estatistica/> Acesso em 16 de Junho de 2011.
- Vijay N, Sairkar P, Silawat N, Garg RK, Mehrotra NN (2009). Genetic variability in *Asparagus racemosus* (Willd.) from Madhya Pradesh, India by random amplified polymorphic DNA. *Afr. J. Biotechnol.* 8(14):3135-3140.
- Zhang HY, Li FS, Liu XZ, He LL, Yang QH, He SC (2008). Analysis of genetic variation in *Erianthus arundinacues* by random amplified polymorphic DNA markers. *Afr. J. Biotechnol.* 7(19):3414-3418.
- Zhang MQ, HongYX, Li QW, Liu SM, Zhang CM, Yang RZ (2004). Molecular polymorphic analyses for the germplasms of *Erianthus arundinacues* collected in China. *J. Plant Resour. Environ.* 13(1): 1-6.

Full Length Research Paper

## Effect of polyethylene glycol (PEG) 6000 on germination and seedling growth of pearl millet [*Pennisetum glaucum* (L.) R. Br.] and LD<sub>50</sub> for *in vitro* screening for drought tolerance

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Millet crop is an important cereal for food security and the fight against poverty and malnutrition in the arid Sahel. It is a staple grain for millions of people in West Africa and India. It has the advantage of tolerating drought-prone environments and low fertility soils. Recent climate change exacerbates the phenomenon of drought; hence, improving pearl millet drought tolerance became a present necessity. The present study was conducted to determine an *in vitro* screening method for drought tolerance, based on the use of polyethylene glycol (PEG) of molecular weight 6000 Da. This experiment was carried out with one millet genotype (HKP), promoted by the agricultural official services. The effect of PEG6000, with varying concentrations from 0 to 40% (w/v), on germination percentage, coleoptiles emergence, shoot and root length, shoot and root weight and root/shoot ratio were analyzed. Data were used to determine the lethal dose for 50% growth inhibition (LD<sub>50</sub>), which was applied for drought tolerance screening. Significant differences were observed for all the characters under different PEG concentrations. LD<sub>50</sub> was 262 g/L for the germination percentage and 242 g/L for coleoptiles emergence.

**Key words:** Pearl millet, water stress, polyethylene glycol, LD<sub>50</sub>.

### INTRODUCTION

Of all domesticated cereals, pearl millet is the most drought tolerant plant (Squire et al., 1987; CGIAR, 2006), with high quality grain. It is the main crop cultivated in the difficult agronomic conditions of the semi-arid tropics of Africa and Asia and contribute to the diet of millions of people (Andrews and Bramel-Cox, 1994; Kumar, 1989).

The productivity in this arid zone is very low, due to the low and erratic rainfall. Given the rapid increase in population in this zone, there is a need for breeding drought tolerant varieties.

Uncertainty of rainfall is critical especially at the beginning of the cycle, after crop emergence. The chal-

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**Table 1.** Pearl millet seed germination percentage as affected by polyethylene glycol (PEG) 6000, three days after incubation.

Treatment	Germination %	Coleoptiles emergence%
PEG 6000 (bars)		
Control (0.0)	96 <sup>a</sup> ±5.2	94 <sup>a</sup> ±5.2
10.0% (-1.0)	95 <sup>a</sup> ± 5.3	92 <sup>a</sup> ± 9.2
20.0% (-3.9)	93 <sup>a</sup> ± 8.2	89 <sup>a</sup> ± 12.0
30.0% (-8.4)	50 <sup>b</sup> ± 18.3	7 <sup>b</sup> ± 0.8
32.5% (-9.8)	7 <sup>c</sup> ± 1.0	0 <sup>c</sup> ± 0
35.0% (-11.3)	8 <sup>c</sup> ± 1.0	0 <sup>c</sup> ± 0
37.5% (-12.9)	2 <sup>c</sup> ± 0.4	0 <sup>c</sup> ± 0
40.0% (-14.7)	0 <sup>d</sup> ± 0.0	0 <sup>c</sup> ± 0

In the same column, values with the same letter are not significantly different (Newman and Keuls Test at 5% level).

lenge is to obtain seed that have a high production capacity, in a short period of time after germination and that develop adequate root biomass to colonize deeper soil layers. Drought constitutes one of the most important environmental stress factors limiting plant growth (Sivakumar, 1992). Large areas of drylands are threatened unproductive due to human activities and recent unfavorable climate change. In order to cultivate plants in such countries, attempts have been made (Saidou, 2011) to develop water stress tolerant lines.

Millet is one of the best stress tolerant plants in the world (ICRISAT, 1987). It is the staple crop of 30 million populations in the dry region of the Sahel and in India. Due to climate change, increasing drought is responsible of significant fraction of yield loss. Breeding and production of improved varieties well adapted to new heavy water stress are urgent requirement. Polyethylene glycol (PEG) of high molecular weight has long been used to simulate water stress in plant growth as described by previous works (Kaufmann and Eckard, 1978; Tazi et al., 2003; Zgallai et al., 2007; Kouakou et al., 2008; Fan et al., 2012). The aim of this work is to measure the effect of PEG on germination and seedling growth of pearl millet, to determine the LD<sub>50</sub> to be used for screening for drought tolerance.

## MATERIALS AND METHODS

The experimental material used was pearl millet, genotype HKP, a cultivar promoted by the National Agronomic Research Institute, for its different interesting traits (Relatively early cycle, good performance, medium tillering, medium cob and large seed). Seeds (mature embryos) were obtained from the Biotechnology and Crop Improvement Laboratory of the National Radio Isotopes Institute of the University of Niamey. The experiment was conducted during April to May 2011, in the Plant Biotechnology laboratory of ULB University of Brussels. Water stress was applied using seven concentrations of PEG6000, corresponding to -1.0; -3.7; -8.0; -9.8; -11.3; -13.0 and -14.7 bars of water potential. These water potential were obtained by adding in 1000 ml of deionized pure water,

respectively: 100; 200; 300; 325; 350; 375 and 400 g of PEG6000, following the method of Lawlor (1970) and Michel (1983).

Seeds were surface sterilized by soaking them first in 70% alcohol for 30 s, and then in 5% calcium hypochlorite for 10 min. Seeds were finally rinsed five times with pure sterile water. Sterilized seeds were germinated in Petri dishes (90 mm diameter) on filter paper soaked with 2 ml of the different concentrations of PEG6000, with 10 seeds per Petri dish. To reduce moist evaporation, the Petri dishes were sealed with sterilized Parafilm. Each Petri dish is a repetition and there are 10 replicates per dose of PEG. The incubation took place for one week, continuously in darkness, at approximately 37°C, to simulate the natural conditions of pearl millet seeds germination, even though it was reported that germination percentage is optimal at 25 to 30°C for *P. glaucum* (Mortmock and Vanderlip, 1989). Polyethylene glycol of high molecular weight is inert, non-ionic. They are small enough to influence the osmotic potential, but large enough to not be absorbed by plant. They could lower the osmotic potential of the nutrient solution without been taken up or been phytotoxic (Lawlor, 1970).

Germination percentage was recorded at the third day of incubation. Root and coleoptiles length as well as their respective weights were evaluated after seven days of culture. A seed is recorded as germinated when the root is visible and at least 1 mm in length.

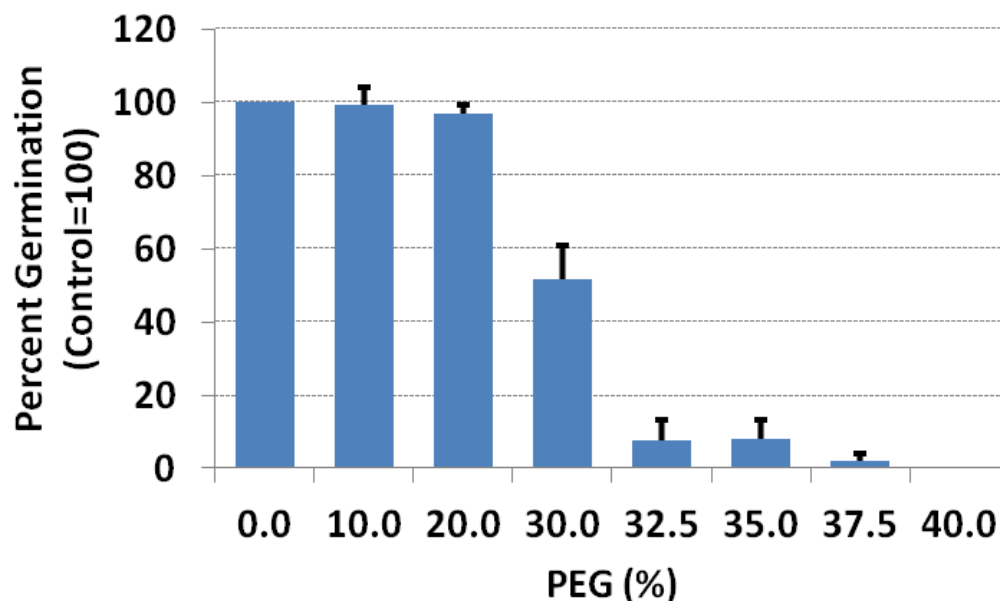
The experiment layout was a randomized complete design with one factor; PEG6000 concentration, with 10 replications. Data were analyzed with one factor analysis of variance (ANOVA), with Excel (2007) and means were separated with Newman and Kheuls test at 5%.

## RESULTS

### PEG and germination percentage

Water stress induced by PEG had significant effects on both the seed germination and the seedling emergence (Table 1, Figures 1 and 2). The germination percentage varied between 0% for the highest dosage (40% PEG = -14.7 bars) to 96% for the control (0 bar). Low PEG dosages (0; 10 and 20%) did not have any significant effect on the germination percentage of the pearl millet seeds, while high dosages of 30 to 40% negatively affected seed germination percentage.





**Figure 1.** Effects of water stress induced by Polyethylene Glycol (PEG) 6000 on germination percentage in pearl millet. The control (free water) is taken as 100% germination.

**Table 2.** Pearl millet shoot and root length and weight and root/shoot ratio as affected by Polyethylene Glycol (PEG) 6000. Seven days after incubation.

Treatment	Shoot length (cm)	Root length (cm)	Shoot Weight (g)	Root Weight (g)	Root/Shoot ratio(w/w)
PEG6000 (bars)					
Control (0.0)	7.66 <sup>a</sup> ± 0.7	14.4 <sup>b</sup> ± 1.1	38.3 <sup>a</sup> ± 5.4	12.9 <sup>a</sup> ± 5.8	0.34 <sup>c</sup> ± 0.15
10% (-1.0)	6.17 <sup>b</sup> ± 0.4	18.6 <sup>a</sup> ± 1.2	23.2 <sup>b</sup> ± 1.0	13.9 <sup>a</sup> ± 4.5	0.60 <sup>b</sup> ± 0.20
20% (-3.9)	3.24 <sup>c</sup> ± 0.3	7.9 <sup>c</sup> ± 1.6	11.1 <sup>c</sup> ± 1.9	13.0 <sup>a</sup> ± 4.5	1.16 <sup>a</sup> ± 0.36
Probability/sign.	0.013	0.029	<0.0001	ns	<0.0001

In the same column, values with the same letter are not significantly different (Newman and Keuls Test at 5% probability level).

### PEG and root length

At 10% PEG the root length was significantly higher than at 20% PEG. The higher concentrations caused a slower development of root (Table 2). Low concentrations of PEG induced a rhizo-stimulation. The mean root length varied from 7.9 cm for the high PEG concentration (20%) to 18.6 cm for the low dosage of PEG.

### PEG and shoot length

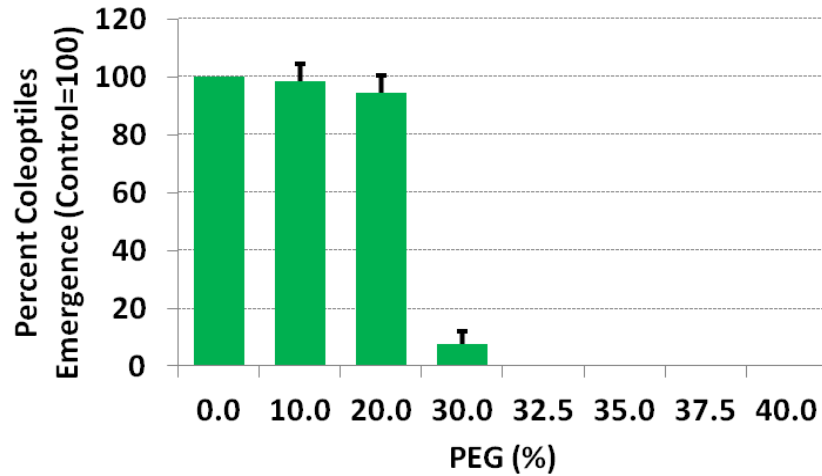
There was no shoot and root emission above 20% PEG and analysis of seedling shoot growth was restricted to the two doses 10 and 20% compared to control (Table 2). Shoot length was significantly lower with an increase in PEG concentration. Shoot length showed reduced values from 7.66 cm for the control to 3.24 cm for the highest concentration (20%).

### PEG and root weight

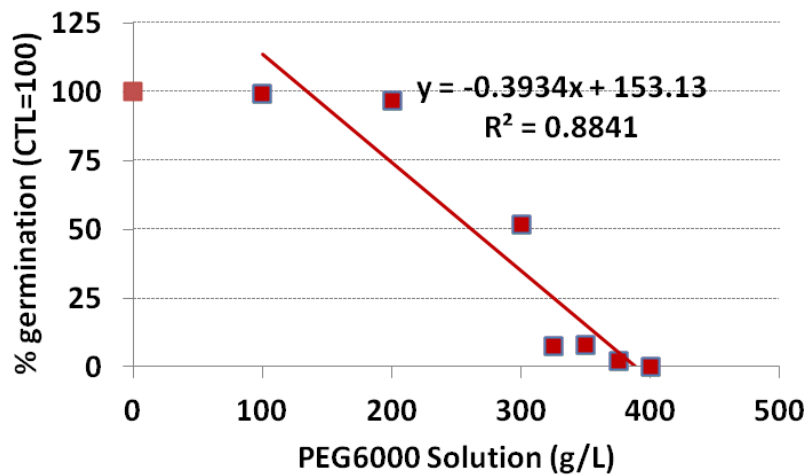
Comparable growth was observed between control, 100 and 200 g/l concentrations of PEG (Table 2). Root weight was not significantly influenced by the treatments between 0 and 200 g/L. The decline in root length with an increase in PEG concentration did not have an effect on the root weight. This means that the roots might have lost volume or became thinner with an increasing in PEG concentration.

### PEG and shoot weight

Mean shoot fresh weight varied significantly from 38.3 g for control (0.0 bar) to 11.1 g for 20% PEG concentration (-3.9 bar). Shoot fresh weight was significantly lower with an increasing in PEG concentration (Table 2). The decrease in water potential by increasing the PEG



**Figure 2.** Effects of water stress induced by polyethylene glycol (PEG) 6000 to emergence percentage in pearl millet. The control (free water) is taken as 100% emergence.



**Figure 3.** Pearl millet germination percentage as affected by Polyethylene Glycol (PEG) of molecular weight 6000, three days after incubation. LD<sub>50</sub> determination.

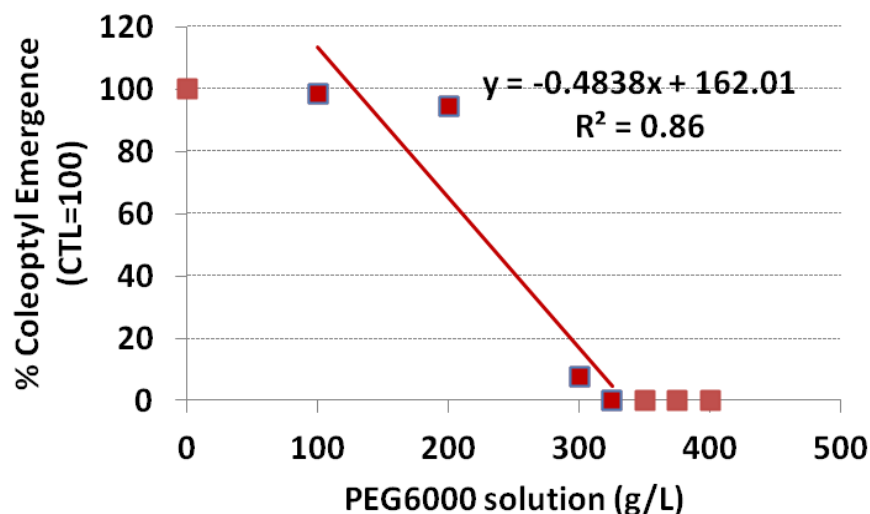
concentration had similar effects on the shoot length and shoot fresh weight. Results showed that the shoots were more sensitive to PEG concentration than the roots; however in field conditions the roots were the first to detect the water stress and to initiate the first signal.

#### PEG and root/shoot ratio

The root/shoot ratio ranged from 0.34 (w/w) for the control to 1.16 (w/w) for the highest PEG concentration (20%) and showed an increase with an increase in PEG concentration. This result shows that the shoot was more sensitive to water deficit induced by PEG.

#### Lethal Dose of 50% growth inhibition determination (LD<sub>50</sub>)

The data concerning germination percentage and coleoptiles emergence were used to calculate the Lethal Dose for 50% growth inhibition. This value was used as screening criteria for drought tolerance in pearl millet crop. The germination percentage as well as the coleoptiles emergence showed negative correlation with increasing concentration of PEG6000 (Figures 3 and 4). Regression analyses revealed that for every one unit increase in dose, germination percentage decreased with 0.393 units while coleoptiles emergence decreased with 0.483 units. The determination coefficients ( $R^2$ ) were 0.88



**Figure 4.** Pearl millet coleoptiles emergence percentage as affected by Polyethylene Glycol (PEG) of molecular weight 6000, seven days after incubation. LD<sub>50</sub> determination.

and 0.86 for germination percentage and coleoptiles emergence, respectively.

$$Y_{\text{ger}\%} = -0.393 X + 151.1$$

$$Y_{\text{col}\%} = -0.483 X + 162.0$$

Where

Y is the relative growth in percent

X is the PEG6000 concentration in % (g PEG/100 g water).

Using the above formula, the LD<sub>50</sub> is the X value calculated for Y equal to 50. The values obtained were: 262 g/L for germination percentage and 242 g/L for coleoptiles emergence. These values indicated the concentrations of PEG6000 that would induce a reduction of 50% in germination and emergence of coleoptiles. The principle of screening is to identify lines or varieties with germination and emergence percentages above 50% with the LD<sub>50</sub>. In this study, the results allowed us to screen for drought tolerant mutants of pearl millet obtained by gamma irradiation mutation induction techniques.

## DISCUSSION

Low PEG dosages (0; 10 and 20%) did not have any significant effect on the germination percentage of pearl millet seeds, while higher dosages (30 to 40%) negatively affected germination. This result was in agreement with several previous reports (Van Der Weerd, et al., 2002;

Radouane, 2007; Farsiani and Ghobadi, 2009; Govindaraj et al., 2010). At 30% PEG, the germination rate was reduced to 50% compared to the control. It was below 10% with PEG concentrations above 30%. At a high PEG dosage (40%), there was no germination. Water hydraulic conductivity is too low and germination process was strongly reduced. The upper limit of the physiological activities (pF 4.2) is perhaps reached. However, working on Tunisian ecotypes of pearl millet, Radhouane (2007) reported more than 50% germination with a high concentration of PEG (equivalent of -2 MPa=-20 bars).

As described by previous authors (Peske et al., 2010), pearl millet seeds emit primary roots at 33% water contents and the PEG effect is delaying the imbibitions process. For the potential of -12 bars and below, the seeds did not absorb more than 28% water, and thus, did not emit primary roots. Our observations are consistent with these results.

In the case of drought conditions, the most powerful root system is one that will increase its volume to allow a better exploitation of deep water reserves (Ousmane, 1996). Thus, root length and weight are important determinants for the choice of cultivar for dryland conditions. In this study, the root length was significantly increased at 10% PEG concentration (-1 bar) compared to the control, but was significantly lower when the concentration increased to 20%. The low concentration of PEG induced a rhizo-stimulation as described by several authors on different growth parameters (Badr et al., 1997; Radouane, 2007; Majeed et al., 2010; Horn et al. 2010; Sumira et al., 2011). This result suggested that a moderate concentration of PEG6000 can be used as root growth regulator in *in vitro* tissue culture.

The effect of low doses of PEG observed for root length was not the same for shoot growth. In this study, the extension of the root system has not a positive effect on the growth of shoot. But in soil conditions, root system could extend to deeper and wetter zones and therefore better support plant growth (Ousmane et al., 1993). This is not evident *in vitro* culture where water and nutrients are homogeneous throughout the Petri dish.

The root/shoot ratio showed comparable root and shoots growth in normal conditions and in water stress situation. This study revealed a significant increase in the root/shoot ratio with increasing PEG concentration. Root growth was maintained against osmotic potential increment. This indicated that the physiological activity of the root system is less sensitive to low relative water content; but sap transfer to leaves require higher water potentials.

## Conclusion

While confirming previous results on the reactions of a plant against water stress, this study showed that PEG of molecular weight 6000 can be used as an *in vitro* simulator of water stress for pearl millet. It was particularly shown that the growth rate of pearl millet root could be maintained at relatively low water potentials (-39 bars), while the sap transfer to plant shoot is strongly inhibited. However, this needs further investigation in order to determine if there is any genotypic effect at this level. In addition, the LD<sub>50</sub> values established for germination percentage and coleoptiles emergence will be used for screening for drought tolerance in pearl millet mutants obtained by induced mutation techniques in a future study.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## REFERENCES

- Andrews DJ, Bramel-Cox P (1994). Breeding cultivars for sustainable crop production in low-input dryland agriculture in the tropics. In: D.A. Buxton (ed.), Chapter 28. p. 211-222., Int. Crop Sci. I. CSSA, Madison, WI.
- Badr HM, Alsadon AA, Al-Harbi AR (1997). Stimulative Effect of Gamma Radiation on Growth and Yield of Two Tomato (*Lycopersicon esculentum*, Mill.) Cultivars. J. King Saud. Univ. Agric. Sci. 9(2):277-286.
- CGIAR (Consultative Group on International Agricultural Research) (2006). Pearl Millet: A hardy Staple for the World's Drylands. CGIAR: Story of the Month-June 2006.
- Fan C, Liu Z, Hu J, Niu B, Huang J, Yao L (2012). Effects of polyethylene glycol 6000 and tripotassium phosphate on protopectinase partition in the aqueous two-phase systems using response surface methodology. Afr. J. Food Sci. 6(4):85-90.
- Farsiani A, Ghobadi ME (2009). Effects of PEG and NaCl Stress on Two Cultivars of Corn (*Zea mays* L.) at Germination and Early Seedling Stages. World Acad. Sci. Eng. Technol. 57:382-385.
- Govindaraj M, Shanmugasundaram P, Sumathi P, Muthiah AR (2010). Simple, Rapid And Cost Effective Screening Method For Drought Resistant Breeding In Pearl Millet. Electronic J. Plant Breed. 1(4):590-599.
- Horn LN, Chikelou MBA, Soleymanne B, Ipinge SNA (2010). Radiosensitivity studies in pearl millet (*Pennisetum glaucum*), Cowpea (*Vigna unguiculata*) and Sorghum (*Sorghum bicolor*) varieties in Namibia. AGRICOLA 2010, pp. 38-40.
- ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) (1987). Proceedings of the International Pearl Millet Workshop, 7-11 April 1986, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.
- Kaufmann MR, Eckard AN (1978). Evaluation of water stress control in Polyethylene Glycol, by analysis of guttation. Plant Physiol. 47:453-8.
- Kouakou TH, Koné M, Koné D, Kouadio YJ et Zouzou M (2008). Réponse physiologique au stade juvénile du génotype R405-2000 de cotonnier (*Gossypium hirsutum* L.) au déficit hydrique induit par le polyéthylène glycol. Sci. Nat. 5(1):81-87.
- Kumar AK (1989). Pearl millet: current status and future potential. Outlook Agric. 18:46-53.
- Lawlor DW (1970). Absorption of PEG by plant and their effects on plant growth. New Phytol. 69: 501-13.
- Majeed A, Khan AUR, Ahmad H, Muhammad Z (2010). Gamma irradiation effects on some growth parameters of *Lepidium sativum* L. ARPJ. Agric. Biol. Sci. 5(1):39-42
- Michel BE (1983). Evaluation of the water potential of Polyethylene Glycol. Plant Physiol. 72:66-70.
- Mortmock MY, Vanderlip RL (1989). Germination and Establishment of Pearl Millet and Sorghum of different seed qualities under controlled high temperature environments. Field Crops Res. 22(3):195-209.
- Ousmane SD (1996). Comportement hydrique de cultivars de mil (*Pennisetum typhoides*) en condition pédoclimatiques contraignantes : réactions de la plante entière à un stress hydrique post-floral. Thèse de Doctorat. Ecole Nationale Supérieure Agronomique de Rennes, septembre 1996. 230p.
- Ousmane SD, Robert E, Marini P (1993). "Rôle du système racinaire dans la résistance à la sécheresse du mil: Analyse de la rhizogénèse post-florale." In: Mils d'Afrique, Diversités génétique et agrophysiologique : potentialités et contraintes pour l'amélioration génétique et l'agriculture. Collection colloques et séminaires de l'ORSTOM, ORSTOM,1993.
- Peske FB, Dionisia A, Coelho L (2010). Condicionamento Fisiologico de Sementes de Milheto. Rev. Bras. Sementes 32(4):132-142.
- Radouane L (2007). Response of Tunisian autochthonous pearl millet (*Pennisetum glaucum* (L.) R. Br.) to drought stress induced by polyethylene glycol (PEG) 6000. Afr. J. Biotechnol. 6(9):1102-1105.
- Saidou AA (2011). Etude moléculaire, évolution et caractérisation de gènes impliqués dans l'adaptation du mil (*Pennisetum glaucum* [L.] R. Br.) aux changements climatiques. PhD dissertation, SupAgro Montpellier, France & Université Abdou Moumouni, Niamey Niger.
- Squire GR, Ong CK, Montieth JL (1987). Crop growth in semi-arid environments. ICRISAT Center, India, Patancheru. pp. 219-231.
- Sumira J, Talat P, Siddiqi T, Mahmooduzzafar (2011). Gamma radiation effects on growth and yield attributes of *Psoralea corylifolia* L. with reference to enhanced production of psoralen. Plant Growth Regul. 64(2):163-171.
- Tazi MR, Berrichi A, Haloui B (2003). Effet du polyéthylène glycol sur la germination et la croissance *in vitro* de l'arganier (*Argania spinosa* L. Skeels) des Beni-Snassen (Maroc oriental). Science et changements planétaires/Sécheresse. Volume 14, Numéro 1, 23-7, Janvier 2003.
- Van Der Weerd L, Claessens MMAE, Efdé C Van As C (2002). Nuclear magnetic resonance imaging of membrane permeability changes in plants during osmotic stress. Plant Cell Environ. 25(11):1539-1549.
- Zgallaï H, Steppe K, Lemeur R (2007) Étude des caractères morphologiques des plantes de tomate soumises à un déficit hydrique en milieu hydroponique. Étude des caractères morphologiques des plantes de tomate soumises à un déficit hydrique en milieu hydroponique. Sécheresse 2007; 18(1): 57-64.

Full Length Research Paper

## Inducing and identifying artificially-induced polyploidy in bananas

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In the present work, polyploidy was induced in the diploid banana varieties 'Malbut', 'Gold', 'Lidi', and 'Thong Dok Mak' through the use of colchicine and oryzalin, and that condition was identified through stomatal analysis, flow cytometry, and chromosome counts. Shoots produced *in vitro* were treated with colchicine at concentrations of 0, 2.5, 7.5 and 12.5 mM for 24 and 48 h, and with oryzalin at 0, 10, 30 and 50 mM for 4 and 7 days. Young leaves were scanned by electron microscopy to determine their stomatal areas (polar diameter × equatorial diameter) and numbers for polyploid identification by stomatal analysis. Polyploid identification by way of flow cytometry analysis used samples of young leaves that were crushed to release their nuclei, with subsequent staining with propidium iodide; ten thousand nuclei were analyzed for each sample. For cytogenetic analyses, root tips were pretreated with 0.002 M 8-HQ for 3 h, fixed in Carnoy solution for 24 h, subjected to conventional squashing techniques, and stained with 10% Giemsa. We identified four tetraploid plants and six mixoploids using these three identification techniques.

**Key words:** Chromosomes duplication, *Musa acuminata*, tissue culture.

### INTRODUCTION

Bananas (*Musa* sp.) are one of the principal fruits eaten in Brazil, providing a nutritious source of potassium and contributing to the regional economy by generating employment and income for farmers as well as for industries that process those fruits to make sweets, jams, yogurts (Silva et al., 2008). Banana breeding programs using chromosome duplication (through treatments with antimetabolic agents such as colchicine and oryzalin) have

been proposed to generate material from diploid hybrids that are resistance to pests and diseases in light of the reduced time and costs involved in using these treatments. Additionally, the high productivities of *Musa* banana strains are directly associated with their ploidy levels, as triploid and tetraploid varieties are more vigorous than diploids (Stover and Simmonds, 1987). Conventional methods of generating polyploid varieties

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that involve backcrossing three diploid hybrids obtained by crossing two diploid species are associated with lengthy development periods with high production costs (Simmonds and Dodds, 1949).

Chromosome doubling using antimetabolic agents requires an efficient system of polyploid induction as well as an effective method for ploidy verification. Polyploidy induction has been undertaken with bananas (Ganga and Chezhiyan, 2002; Rodrigues et al., 2011) using the antimetabolites oryzalin, amiprofos-methyl (AMP), and colchicine at different concentrations and for varying exposure times. Ganga and Chezhiyan (2002) produced 13% tetraploid plants in programs using colchicine, and 17% using oryzalin, and Rodrigues et al. (2011) reported that amiprofos-methyl was most efficient at inducing ploidy in some banana varieties at a concentration of 40  $\mu\text{M}$ . The technique of exposing *in vitro* explants to colchicine has been investigated by a number of researchers (Sakhanokho et al., 2009; Wannakrairoj and Wondyifraw, 2013), but it is then necessary to check the suitability of each species or new variety generated, as their responses reflect their genetic constitutions and their reactions to the mitotic agents employed.

Chromosome counts and stomata morphology have been routinely used to identify polyploids (Campos et al., 2009), although the quickest and most efficient technique for ploidy analysis is flow cytometry of nuclear DNA content (Younis et al., 2013). This technique involves analyzing the optical properties (light scattering and fluorescence) of particles flowing in liquid suspension (Ochatt, 2008). As these particles pass an intersecting laser beam, they generate quantifiable light scattering, fluorescence, or emission signals. This principle can be used, for example, to measure the amount of DNA in a cell (Dolezel and Bartos, 2005).

The present study was part of the banana breeding program "*Embrapa Mandioca e Fruticultura Tropical*" at Cruz das Almas, Bahia State, Brazil, seeking to produce secondary triploid AAA varieties that produce palatable fruits by inducing chromosome doubling in diploid banana plants and subsequently crossing the autotetraploids obtained with diploid elite varieties to obtain new AAA varieties. We induced chromosome doubling using colchicine and oryzalin in four diploid banana varieties 'Malbut', 'Gold', 'Lidi', and 'Thong Dok Mak' and identified polyploids and verified their ploidy through stomatal measurements, flow cytometry analysis, and chromosome counts.

## MATERIALS AND METHODS

### Micropropagation of plant materials and their treatment with antimetabolic agents

Diploid apical meristems from the banana varieties 'Malbut', 'Gold', 'Lidi', and 'Thong Dok Mak' were established *in vitro* and multiplied

for two generations in MS medium (Murashige and Skoog, 1962) supplemented with 30  $\text{g}\cdot\text{L}^{-1}$  sucrose and 4  $\text{mg}\cdot\text{L}^{-1}$  benzylaminopurine (BAP) for shoot proliferation, and subsequently treated with the antimetabolic agents colchicine and oryzalin. Colchicine was used at concentrations of 0, 2.5, 7.5 and 12.5 mM for 24 to 48 h and oryzalin at concentrations of 0, 10, 30 and 50  $\mu\text{M}$  for 4 and 7 days in liquid medium with agitation (60 rpm).

After treatment, the shoots were washed three times with distilled water and transferred to a proliferation medium for further culturing to reduce the frequency of mixoploids (plant material containing cells with chromosome number variations). The plants were then transferred to a rooting medium (MS supplemented with sucrose 30  $\text{g}\cdot\text{L}^{-1}$  and solidified with 7  $\text{g}\cdot\text{L}^{-1}$  agar). The explants were kept in a growth chamber with a light intensity of 36  $\text{micromol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , photoperiod of 16 h, and a temperature of  $25 \pm 2^\circ\text{C}$  during their growth phase. The generation time was thirty days. For rooting, the plantlets were kept under the same conditions for 45 days.

The rooted plants were transferred to a greenhouse and placed in cultivation tubes (19 cm  $\times$  5 cm) with a substrate composed of vegetable substrate plantmax<sup>™</sup> and coconut fiber (1:1), with 150 g PG MIX<sup>™</sup> (14-16-18) and 150 g of Osmocote<sup>™</sup> (6-19-10), and irrigated with an intermittent mist system under 50% shading. After 60 days, the plants were transplanted to 20 L plastic pots with the same substrate. Each treatment was replicated five times.

### Calculations of stomatal areas and densities

Stomatal analyzes were performed on all plants one year after their acclimatization. We used fragments of the middle region of the abaxial side of leaf number 1 (youngest fully expanded leaf), without ribs. Fragments (0.5  $\text{mm}^2$ ) were fixed to metallic stubs with carbon tape, desiccated for 48 h, and subsequently coated with gold (20 nm) layer in a BAL-TEC, SCD- 050 sputter coater. The leaf material was examined in a LEO EVO-40 PVX scanning electron microscope (600x) and electromicrographed. To determine stomatal areas (polar equatorial diameter  $\times$  diameter) and densities, five observation fields (five replicates) with the same area (1 $\text{mm}^2$ ) were randomly chosen on the epidermis of the abaxial surface of each sample and five stomata of each were measured. Twenty-six Malbut varieties, 15 Gold, 52 Lidi, and 35 Thong Dok Mak varieties were treated with antimetabolic agents and their survival rates calculated.

The criteria used to identify the tetraploid plants was that all plants that differed statistically in size and density from the stomata of diploid plants were considered possible tetraploids, being subsequently confirmed by flow cytometry analysis and chromosome counts. Analysis of variance (ANOVA) ( $p < 0.05$ ) was performed to evaluate the data; differences between the means were evaluated using the Scott-Knott test ( $< 0.05$ ).

### Flow cytometric analysis

Nuclear DNA contents were determined by grinding approximately 20-30 mg portions of young banana plant and *Pisum sativum* leaves (the latter being the internal reference standard) in 1 ml of cold LB01 buffer to release the nuclei (Dolezel et al., 1998). The nuclei suspension was then aspirated through two layers of gauze using a plastic pipette, and filtered through a 50 micron mesh. The nuclei were stained by adding 25  $\mu\text{L}$  of a 1  $\text{mg}/1\text{ ml}$  propidium iodide solution with 5 $\mu\text{L}$  RNase to each sample. The samples were analyzed after storage in a refrigerator for 1-2 hours. At least 10 billion nuclei were analyzed for each sample. The analyses were performed using a FACSCalibur flow cytometer USA, 2010 (Becton Dickinson) using a logarithmic scale; the histograms were generated

**Table 1.** Anatomical characteristics of the epidermis of diploid Malbut variety banana plants subjected to treatment with colchicine.

Treatment	Anatomical characteristics			Flow cytometry	
	Area ( $\mu\text{m}^2$ )	Stomatal densities ( $\text{n}^\circ/\text{mm}^2$ )	Ploidy	DNA index (pg)	Ploidy
0	204.1 <sup>a</sup>	30.8 <sup>c</sup>	2x	-	-
0	242.6 <sup>b</sup>	27.2 <sup>b</sup>	2x	-	-
2.5 Mm/24 h	260.3 <sup>c</sup>	20.0 <sup>a</sup>	4x	-	-
2.5 Mm/24 h	286.1 <sup>d</sup>	25.8 <sup>b</sup>	4x	-	-
2.5 Mm/24 h	250.8 <sup>c</sup>	23.8 <sup>b</sup>	2x	1.05	2x
2.5 Mm/24 h	237.4 <sup>b</sup>	21.8 <sup>a</sup>	2x	-	-
7.5 Mm/24 h	255.5 <sup>c</sup>	30.4 <sup>c</sup>	2x	-	-
7.5 Mm/24 h	264.1 <sup>c</sup>	25.4 <sup>b</sup>	2x	1.34	2x
12.5 Mm/24 h	270.6 <sup>d</sup>	37.8 <sup>d</sup>	4x	1.34	2x

\*Means followed by the same letter in the same column did not differ statistically by the Scott Knott (<0.05) probability level.

using Cell Quest software and statistically analyzed using WinMDI 2.8 software.

The nuclear DNA contents (pg) of the plants were estimated using the ratios of the fluorescence intensities of the G1 nuclei (nuclei in the G1 interphase stage) to the reference standard (*P. sativum*) and multiplying this ratio by the amount of DNA in the reference standard (9.09 pg).

#### Cytogenetic analyses

For ploidy analysis by chromosome counts, the roots of plants previously selected for stomatal analysis were pretreated with a solution of 0.002 M 8-hydroxyquinoline for 3 h and subsequently fixed in Carnoy solution (3:1) for 24 h. The roots were then washed three times for 10 min in de-ionized water and subsequently hydrolyzed in 1 N HCl at 60°C for 15 min.

The root meristems were excised with the aid of a stereoscopic microscope and then crushed in 45% acetic acid under a cover slip. The slides were air dried and the cover slips removed after immersion in liquid nitrogen (Barbosa et al., 2007), and the slides were subsequently immersed for 30 s in 45% acetic acid and stained with 10% Giemsa for 5 min. The observations and analyses of the slides were performed using a microscope (100 × oil immersion magnification) with digital camera image-capturing system.

## RESULTS

One year after the end of the acclimatization period, banana seedlings survival was found to be influenced by the dosage levels and exposure times to colchicine and oryzalin. Higher doses and longer exposures to antimetabolic agents had negative effects on seedling survival. The survival rate of the 'Lidi', 'Gold', 'Malbut', and 'Thong Dok Mak' varieties were 23.75, 33.75, 46.85 and 30.62% when treated with colchicine and 23.12, 15.625, 36.25 and 22.5% when treated with oryzalin respectively.

Cytogenetic analyses were only performed on mixoploid or tetraploid plants identified by flow cytometry

analyses. Three of the diploid Malbut plants treated with colchicine showed larger and less frequent stomata as compared to control plants (Table 1), and were considered possible tetraploids, although cytometric analyses gave results typical of diploids. Two plants subjected to treatments with oryzalin showed larger stomatal areas and lower stomatal densities, but were likewise considered diploids by flow cytometry analysis (Table 2).

A Gold variety plant treated with colchicine was classified as tetraploid based on flow cytometry and stomatal analyses and had a DNA content of 2.18 pg, although chromosome counts classified it as a mixoploid composed of cells with different ploidy levels (Table 3). No polyploid plants were found after treatments with oryzalin (Table 4). Colchicine treatments were observed to efficiently promote polyploidization in five plants of the Lidi varieties (Table 5), as was confirmed by all three analyses.

The DNA index was approximately 2.18 pg for tetraploids, while mixoploids showed two peaks, one diploid and one tetraploid. A plant treated with oryzalin was classified as a tetraploid (based on chromosome counts and flow cytometry) as it had large stomata at high densities (Table 6).

Colchicine-treated plants of the Thong Dok Mak (TDM) variety (Table 7) were classified as tetraploids by both stomata analyses and chromosomal counts, but showed DNA contents of 1.78 pg by flow cytometry, and therefore must be considered mixoploids. These plants also showed high-density but small stomata. One oryzalin-treated plant was considered tetraploid in all three analyses, and another was classified as a tetraploid only by chromosome counts, as shown in Table 8.

The graphs of the positions of the peaks of diploid, tetraploid, and mixoploid plants are presented in Figure 1. Knowing the relative positions of the peaks of diploid

**Table 2.** Anatomical characteristics of the epidermis in diploid Malbut variety banana plants subjected to treatment with oryzalin.

Treatment	Anatomical characteristics			Flow cytometry	
	Areas ( $\mu\text{m}^2$ )	Stomatal densities ( $\text{n}^\circ/\text{mm}^2$ )	Ploidy	DNA index (pg)	Ploidy
0	246.4 <sup>a</sup>	20.2 <sup>b</sup>	2x	1.27	2x
0	210.6 <sup>a</sup>	23.0 <sup>b</sup>	2x	-	-
10 $\mu\text{M}/4$ dias	285.9 <sup>a</sup>	35.4 <sup>d</sup>	2x	1.23	2x
30 $\mu\text{M}/4$ dias	235.7 <sup>a</sup>	37.8 <sup>e</sup>	2x	1.23	2x
30 $\mu\text{M}/4$ dias	292.2 <sup>a</sup>	27.8 <sup>c</sup>	2x	1.23	2x
30 $\mu\text{M}/4$ dias	239.5 <sup>a</sup>	21.4 <sup>b</sup>	2x	-	-
30 $\mu\text{M}/4$ dias	261.0 <sup>a</sup>	39.4 <sup>e</sup>	2x	1.19	2x
30 $\mu\text{M}/4$ dias	266.4 <sup>a</sup>	25.4 <sup>c</sup>	2x	-	-
30 $\mu\text{M}/4$ dias	229.9 <sup>a</sup>	43.2 <sup>e</sup>	2x	1.27	2x
30 $\mu\text{M}/4$ dias	257.3 <sup>a</sup>	28.2 <sup>c</sup>	2x	-	-
10 $\mu\text{M}/7$ dias	260.4 <sup>a</sup>	23.0 <sup>b</sup>	2x	1.27	2x
10 $\mu\text{M}/7$ dias	239.5 <sup>a</sup>	22.0 <sup>b</sup>	2x	1.19	2x
10 $\mu\text{M}/7$ dias	257.3 <sup>a</sup>	21.2 <sup>b</sup>	2x	1.16	2x
10 $\mu\text{M}/7$ dias	265.4 <sup>a</sup>	27.2 <sup>c</sup>	2x	1.30	2x
10 $\mu\text{M}/7$ dias	292.2 <sup>a</sup>	32.0 <sup>d</sup>	2x	1.27	2x
10 $\mu\text{M}/7$ dias	446.7 <sup>b</sup>	12.8 <sup>a</sup>	4x	1.34	2x
10 $\mu\text{M}/7$ dias	404.3 <sup>b</sup>	17.8 <sup>a</sup>	4x	1.34	2x

\*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

**Table 3.** Anatomical features of the epidermis and cytogenetic analyses of diploid Gold variety banana plants subjected to treatment with colchicine.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas ( $\mu\text{m}^2$ )	Stomatal densities ( $\text{n}^\circ/\text{mm}^2$ )	Ploidy	DNA Index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	260.8 <sup>a</sup>	7.5 <sup>a</sup>	2x	-	-	22	2x
0	242.1 <sup>a</sup>	18.0 <sup>b</sup>	2x	-	-	-	-
2.5 mM/24 h	260.9 <sup>a</sup>	2.6 <sup>a</sup>	2x	1.16	2x	-	-
2.5 mM/24 h	313.7 <sup>c</sup>	15.0 <sup>b</sup>	4x	2.18	4x	31	mixoploid
2.5 mM/24 h	285 <sup>b</sup>	18.4 <sup>b</sup>	2x	-	-	-	-
12.5 mM/24 h	289 <sup>b</sup>	15.4 <sup>b</sup>	2x	1.38	2x	-	-
2.5 mM/48 h	285.3 <sup>b</sup>	16.0 <sup>b</sup>	2x	1.16	2x	-	-
2.5 mM/48 h	353.3 <sup>c</sup>	7.2 <sup>a</sup>	4x	1.54	2x	-	-
2.5 mM/48 h	319.9 <sup>c</sup>	13.8 <sup>b</sup>	4x	1.27	2x	-	-
7.5 mM/48 h	289 <sup>b</sup>	13.5 <sup>b</sup>	2x	-	-	-	-

\*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

material in relation to a reference standard can be used to detect polyploid material. Most of the plants classified as tetraploid by stomatal testing were confirmed as such by flow cytometry analysis, and other characteristics of tetraploid mixoploids were likewise confirmed. In all, six plants were classified by flow cytometry as tetraploid and three as mixoploids. No plant identified as diploid by

stomatal analyses was found to be tetraploid by flow cytometry, demonstrating and confirming the ability of flow cytometry to identify and separate plants into two groups: diploids and mixoploids versus tetraploids.

The chromosome counts described here were performed on 15 plants (5 diploids and 10 polyploids) (Figure 1). A summary of the results of all analyzes are



**Table 4.** Anatomical characteristics of the epidermis of diploid Gold variety banana plants subjected to treatment with oryzalin.

Treatment	Anatomical characteristics			Flow cytometry	
	Areas ( $\mu\text{m}^2$ )	Stomatal densities ( $\text{n}/\text{mm}^2$ )	Ploidy	DNA Index (pg)	Ploidy
0	277.7 <sup>b</sup>	12.8 <sup>a</sup>	2x	-	-
10 $\mu\text{M}/7$ dias	280.0 <sup>b</sup>	14.8 <sup>a</sup>	2x	1.30	2x
10 $\mu\text{M}/7$ dias	337 <sup>c</sup>	16.6 <sup>a</sup>	2x	1.30	2x
30 $\mu\text{M}/7$ dias	324.3 <sup>c</sup>	15.6 <sup>a</sup>	2x	1.30	2x
30 $\mu\text{M}/7$ dias	219.3 <sup>a</sup>	21 <sup>b</sup>	4x	-	-

\*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

**Table 5.** Anatomical features of the epidermis and cytogenetic analyses of diploid Lidi variety banana plants subjected to treatment with colchicines.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas ( $\mu\text{m}^2$ )	Stomatal densities ( $\text{n}/\text{mm}^2$ )	Ploidy	DNA Index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	161.1 <sup>b</sup>	60.6 <sup>e</sup>	2x	-	-	22	2x
0	160.3 <sup>b</sup>	49.2 <sup>d</sup>	2x	-	-	-	-
0	177.0 <sup>b</sup>	45.8 <sup>d</sup>	2x	-	-	-	-
0	161.5 <sup>b</sup>	38.2 <sup>c</sup>	2x	-	-	-	-
2.5 mM/24 h	184.1 <sup>c</sup>	52.8 <sup>e</sup>	2x	1.02	2x	-	-
2.5 mM/24 h	220.6 <sup>d</sup>	43.8 <sup>d</sup>	2x	0.98	2x	-	-
2.5 mM/24 h	193.3 <sup>c</sup>	32.8 <sup>b</sup>	4x	1.13	2x	-	-
2.5 mM/24 h	162.8 <sup>b</sup>	49.8 <sup>d</sup>	2x	1.09	2x	-	-
2.5 mM/24 h	167.7 <sup>b</sup>	47.4 <sup>d</sup>	2x	1.20	2x	-	-
7.5 mM/24 h	229.3 <sup>e</sup>	40.2 <sup>c</sup>	4x	1.05	2x	-	-
7.5 mM/24 h	175.8 <sup>b</sup>	45.8 <sup>d</sup>	2x	1.09	2x	-	-
7.5 mM/24 h	162.1 <sup>b</sup>	39.8 <sup>c</sup>	2x	1.05	2x	-	-
7.5 mM/24 h	175.1 <sup>b</sup>	54.0 <sup>e</sup>	2x	1.20	2x	-	-
12.5 mM/24 h	256.6 <sup>f</sup>	19.8 <sup>a</sup>	4x	2.14	4x	44	4x
12.5 mM/24h	190.2 <sup>c</sup>	32.6 <sup>b</sup>	4x	2.18	4x	31	mixoploid
12.5 mM/24h	210.8 <sup>d</sup>	33.4 <sup>b</sup>	4x	1.16 e 2.18	mixoploid	33	mixoploid
2.5 mM/48 h	194.3 <sup>c</sup>	43.6 <sup>c</sup>	2x	0.98	2x	-	-
2.5 mM/48 h	171.5 <sup>b</sup>	43.4 <sup>c</sup>	2x	-	-	-	-
2.5 mM/48 h	180.5 <sup>c</sup>	37.2 <sup>c</sup>	2x	1.13	2x	-	-
2.5 mM/48 h	170.6 <sup>b</sup>	50.8 <sup>d</sup>	2x	1.16	2x	-	-
2.5 mM/48 h	183.0 <sup>c</sup>	43.2 <sup>c</sup>	2x	-	-	-	-
7.5 mM/48 h	230.5 <sup>e</sup>	39.2 <sup>c</sup>	4x	2.18	4x	44	4x
7.5 mM/48 h	232.9 <sup>e</sup>	40.8 <sup>c</sup>	4x	1.09 e 2.18	mixoploid	44	4x
7.5 mM/48 h	181.2 <sup>c</sup>	55.0 <sup>e</sup>	2x	1.20	2x	-	-
7.5 mM/48 h	195.4 <sup>c</sup>	41.4 <sup>c</sup>	2x	-	-	-	-
7.5 mM/48 h	177.0 <sup>b</sup>	53.4 <sup>e</sup>	2x	1.13	2x	-	-
7.5 mM/48 h	202.3 <sup>d</sup>	38.6 <sup>c</sup>	2x	-	-	-	-
12.5 mM/48 h	104.1 <sup>a</sup>	39.6 <sup>c</sup>	2x	1.09	2x	-	-

\*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

presented in Table 9, and this data shows that there were variations in ploidy determinations among the three

different methods used. Only plants that showed positive results in all three analyses were considered tetraploids,

**Table 6.** Anatomical features of the epidermis and cytogenetic analyses of diploid Lidi variety banana plants subjected to treatment with oryzalin.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas ( $\mu\text{m}^2$ )	Stomatal densities ( $\text{n}^\circ/\text{mm}^2$ )	Ploidy	DNA Index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	128.3a	28.8b	2x	1.09	2x	22	2x
0	182.7c	28.8b	2x	1.20	2x	-	-
0	197.1c	23 a	2x	-	-	-	-
0	213.8c	24.2a	2x	-	-	-	-
10 $\mu\text{M}/4\text{dias}$	163.3b	26.2b	2x	1.09	2x	-	-
10 $\mu\text{M}/4\text{dias}$	284.5e	19.8a	2x	1.05	2x	-	-
10 $\mu\text{M}/4\text{dias}$	234.5d	43.4d	4x	1.05	2x	-	-
10 $\mu\text{M}/4\text{dias}$	201.9c	53.4e	2x	1.13	2x	-	-
50 $\mu\text{M}/4\text{dias}$	156.6b	31b	2x	-	-	-	-
50 $\mu\text{M}/4\text{dias}$	281.2e	27.2b	2x	-	-	-	-
10 $\mu\text{M}/7\text{dias}$	202.2c	22.4a	2x	1.05	2x	-	-
10 $\mu\text{M}/7\text{dias}$	182.4c	38c	2x	1.13	2x	-	-
10 $\mu\text{M}/7\text{dias}$	161.4b	43d	4x	1.05	2x	-	-
10 $\mu\text{M}/7\text{dias}$	194.8c	37c	2x	1.09	2x	-	-
10 $\mu\text{M}/7\text{dias}$	187.8c	44d	2x	1.20	2x	-	-
10 $\mu\text{M}/7\text{dias}$	185.3c	43.2d	2x	1.13	2x	-	-
10 $\mu\text{M}/7\text{dias}$	214.6c	22.8a	2x	1.20	2x	-	-
10 $\mu\text{M}/7\text{dias}$	193.8c	37.8c	4x	1.16	2x	-	-
10 $\mu\text{M}/7\text{dias}$	217.3c	42.2d	4x	1.23	2x	-	-
10 $\mu\text{M}/7\text{dias}$	195.8c	37.6c	2x	1.16	2x	-	-
10 $\mu\text{M}/7\text{dias}$	233.5d	30.8b	2x	1.23	2x	-	-
10 $\mu\text{M}/7\text{dias}$	199.4c	36c	4x	1.23	2x	-	-
10 $\mu\text{M}/7\text{dias}$	237.7d	45d	4x	2.22	4x	44	4x
50 $\mu\text{M}/7\text{dias}$	188.7c	22.4a	2x	0.98	2x	-	-

\*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05)probability level.

**Table 7.** Anatomical features of the epidermis and cytogenetic analyses of diploid Thong Dok Mak variety banana plants subjected to treatment with colchicines.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas ( $\mu\text{m}^2$ )	Stomatal densities ( $\text{n}^\circ/\text{mm}^2$ )	Ploidy	DNA Index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	282.5 <sup>c</sup>	12.4 <sup>a</sup>	2x	-	-	22	2x
0	258.9 <sup>b</sup>	17.8 <sup>b</sup>	2x	-	-	-	-
2.5 mM/24 h	214.4 <sup>a</sup>	29.6 <sup>d</sup>	4x	1.05	2x	-	-
2.5 mM/24 h	267.7 <sup>b</sup>	25.2 <sup>c</sup>	2x	1.31	2x	-	-
2.5 mM/24 h	252.5 <sup>b</sup>	26.6 <sup>c</sup>	2x	1.13	2x	-	-
2.5 mM/24 h	264.6 <sup>b</sup>	25.0 <sup>c</sup>	2x	1.27	2x	-	-
2.5 mM/24 h	236.8 <sup>a</sup>	29.8 <sup>d</sup>	4x	1.31	2x	-	-
2.5 mM/24 h	230.4 <sup>a</sup>	29.8 <sup>d</sup>	4x	1.49	2x	-	-
2.5 mM/24 h	226.0 <sup>a</sup>	29.0 <sup>d</sup>	4x	1.38	2x	-	-
7.5 mM/24 h	346.6 <sup>d</sup>	11.6 <sup>a</sup>	2x	-	-	-	-
7.5 mM/24 h	220.4 <sup>a</sup>	23.4 <sup>c</sup>	2x	-	-	-	-
2.5 mM/48 h	263.4 <sup>b</sup>	30.4 <sup>d</sup>	2x	-	-	-	-
2.5 mM/48 h	270.2 <sup>b</sup>	17.0 <sup>b</sup>	2x	-	-	-	-

Table 7. Contd.

7.5 mM/48 h	326.1 <sup>d</sup>	14.6 <sup>a</sup>	2x	1.31	2x	-	-
7.5 mM/48 h	237.3 <sup>a</sup>	37.8 <sup>e</sup>	4x	1.78	mixoploid	44	4x
7.5 mM/48 h	198.4 <sup>a</sup>	27.8 <sup>d</sup>	4x	-	-	-	-
7.5 mM/48 h	241.0 <sup>a</sup>	26.0 <sup>c</sup>	4x	-	-	-	-
12.5 mM/48 h	283.6 <sup>c</sup>	19.8 <sup>b</sup>	2x	1.27	2x	-	-

\*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

**Table 8.** Anatomical features of the epidermis and cytogenetic analyses of diploid Thong Dok Mak variety banana plants subjected to treatment with oryzalin.

Treatment	Anatomical characteristics			Flow cytometry		Cytogenetic analyses	
	Areas ( $\mu\text{m}^2$ )	Stomatal densities ( $\text{n}^0/\text{mm}^2$ )	Ploidy	DNA index (pg)	Ploidy	Chromosome Numbers	Ploidy
0	273.2 <sup>e</sup>	32.4 <sup>b</sup>	2x	-	-	22	2x
0	202.3 <sup>c</sup>	27.6 <sup>b</sup>	2x	-	-	-	-
0	209.1 <sup>c</sup>	35.2 <sup>c</sup>	2x	-	-	-	-
0	372 <sup>g</sup>	15.2 <sup>a</sup>	2x	-	-	-	-
10 $\mu\text{M}/4\text{dias}$	235.9 <sup>d</sup>	27.6 <sup>b</sup>	2x	1.23	2x	-	-
10 $\mu\text{M}/4\text{dias}$	302.8 <sup>f</sup>	27.2 <sup>b</sup>	2x	1.31	2x	-	-
10 $\mu\text{M}/4\text{dias}$	215.1 <sup>c</sup>	30.4 <sup>b</sup>	2x	1.09	2x	-	-
10 $\mu\text{M}/4\text{dias}$	258.0 <sup>d</sup>	31.8 <sup>b</sup>	2x	-	-	-	-
10 $\mu\text{M}/4\text{dias}$	253.8 <sup>d</sup>	31.4 <sup>b</sup>	2x	1.13	2x	44	4x
10 $\mu\text{M}/4\text{dias}$	206.8 <sup>c</sup>	30.0 <sup>b</sup>	2x	-	-	-	-
10 $\mu\text{M}/4\text{dias}$	241.3 <sup>d</sup>	30.0 <sup>b</sup>	2x	1.16	2x	-	-
10 $\mu\text{M}/4\text{dias}$	172.4 <sup>b</sup>	38.2 <sup>c</sup>	4x	-	-	-	-
10 $\mu\text{M}/4\text{dias}$	127.9 <sup>a</sup>	58.0 <sup>d</sup>	4x	2.18	4x	44	4x
30 $\mu\text{M}/7\text{dias}$	244.0 <sup>d</sup>	29.0 <sup>b</sup>	2x	1.09	-	-	-
30 $\mu\text{M}/7\text{dias}$	242.6 <sup>d</sup>	19.2 <sup>a</sup>	2x	-	-	-	-
30 $\mu\text{M}/7\text{dias}$	228.4 <sup>c</sup>	36.4 <sup>c</sup>	2x	1.23	2x	-	-
50 $\mu\text{M}/7\text{dias}$	226.3 <sup>c</sup>	18.2 <sup>a</sup>	2x	1.34	2x	-	-

\*Means followed by the same letter in the same column did not differ statistically by the Scott Knott test (<0.05) probability level.

the others were considered tetraploid mixoploids.

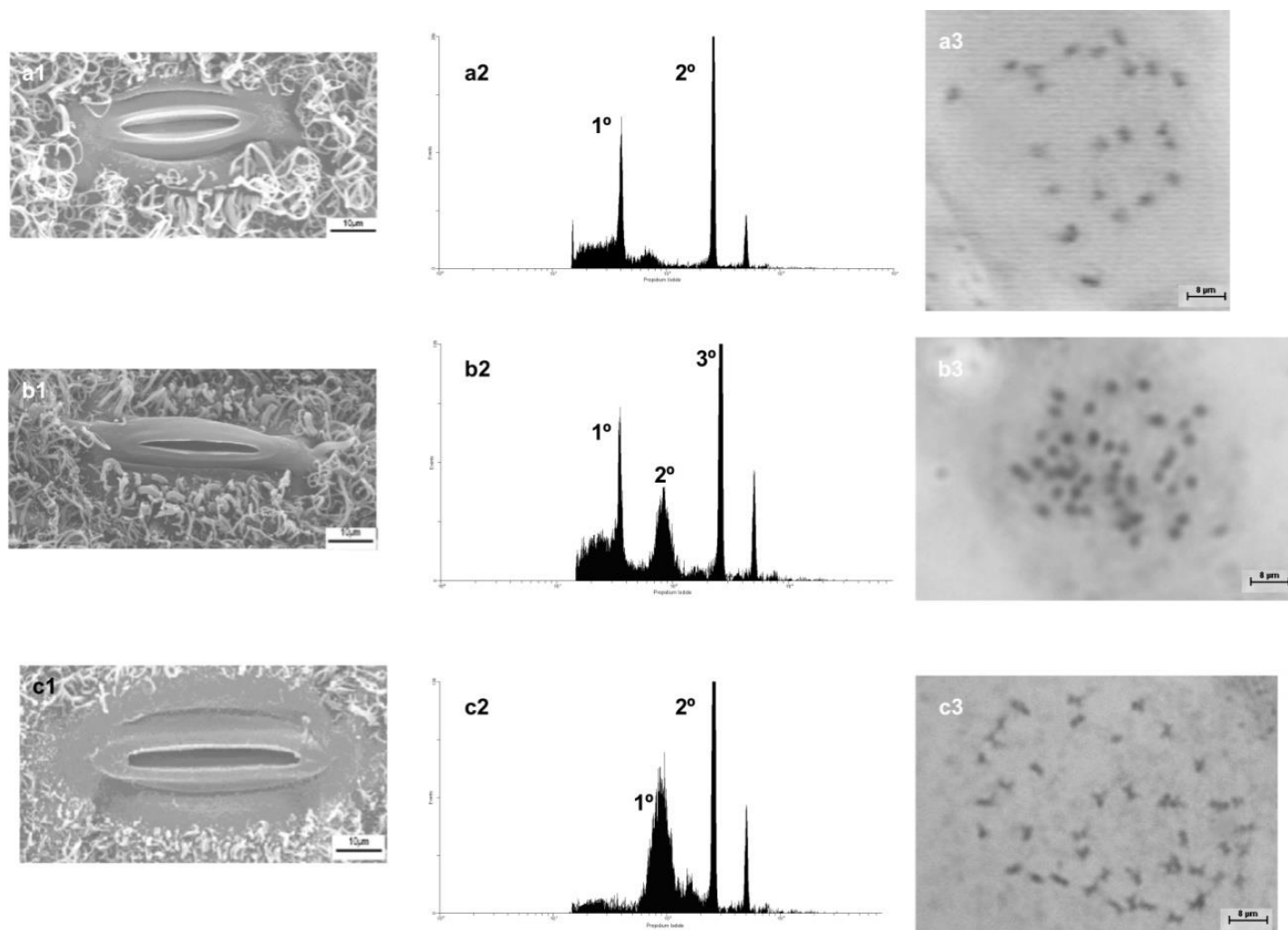
## DISCUSSION

The action of antimetabolic agents is related to their specific effects on chromosome duplication. High concentrations or long periods of exposure to these substances are usually observed to cause phytotoxic effects - generating high mortality among treated seedlings- and tolerance to colchicine and oryzalin varies among species and varieties, depending upon their genetic make up (Vakili, 1962; Ganga and Chezhyan, 2002).

Colchicine toxicity at high dosages was reported by Lone et al. (2010), who found lower survival rates of the protocorms of *Cattleya tigrina* (32%) that had been

treated with 1 g.L<sup>-1</sup> colchicine for 72 h. Regeneration rates of 27.26 % after colchicine exposure were observed in *Tagetes erecta* (Sajjad et al., 2013). Regeneration rates of 37 and 67% were observed in explants of *Aframomum corrorima* treated for 7 days with colchicine and oryzalin respectively (Wannakrairoj and Wondyifraw, 2013).

Both oryzalin and colchicine caused tissue oxidation that resulted in explant death, especially those treated with colchicine for 48 h or oryzalin for seven days. Similar results were observed with *Musa* varieties treated with colchicine for 48 h (Rodrigues et al., 2011) and with *Catharanthus roseus* seeds treated with colchicine for 48 h (Xing et al., 2011). Regenerated plants subjected to these treatments showed slower growth and thicker and smaller leaves when compared with controls - indicating



**Figure 1.** **A**, Diploid material (a1, stomata; b1, Histogram of flow cytometry results with the first and second peaks of the sample and the reference standard; a3, metaphase). **B**, Mixoploid material (b1, stomata; b2, histogram of flow cytometry results with the first and second peaks of the sample and 3rd peak of the reference standard and b3: metaphase). **C**, Tetraploid material (c1, stoma; c2, flow cytometric histogram with the first and second peaks of the sample and the reference standard; and c3, metaphase).

the presence of tetraploid plants. Sajjad et al. (2013) found that colchicine application resulted in the formation of tetraploid individuals of *Tagetes erecta* that showed slower initial growth rates and smaller leaves. Ganga and Chezhiyan (2002) tested different concentrations of colchicine and oryzalin and different exposure periods in attempts to obtain tetraploid bananas, and found that chromosomal replication was similar after exposure to either substance, although the required colchicine concentrations were 125 to 200 times greater than with oryzalin.

Slower initial growth rates of seedlings treated with colchicine have been noted when using this anti-mitotic agent to increase ploidy levels, and its effects on growth are apparently due to reductions in cell division rates (Sajjad et al., 2013). Colchicine inhibits the formation of

linear structures of microtubules in plant cells (polymers of tubulin involved in various cellular functions), thus interfering with the cell cycle (Ade and Rai, 2010). Most of the plants classified as tetraploid in our study had been treated with colchicine. Although a number of authors have reported oryzalin as being efficient at inducing polyploidy in some plants, it is necessary to check these antimitotic ages with each plant type, as their responses can vary as a function of their genetic makeup and the developmental stages of their tissues.

Flow cytometry analysis can process large numbers of plants (several hundred) per day with reliable results, as DNA content is not influenced by external factors such as light intensity, leaf blade development, or plant tissue content water; the analyses are also rapid, and numerous plants can be evaluated in a very short time (Xing et al.,

**Table 9.** Cytogenetic analysis and stomatal analyses of diploid Thong Dok Mak, Gold, and Lidi banana varieties subjected to treatment with colchicine and oryzalin.

Treatment	Stomatal analyses	Flow cytometry analysis	Cytogenetic analysis
TDM - colchicine - control	Diploid	Diploid	Diploid
TDM - oryzalin - control	Diploid	Diploid	Diploid
Lidi - colchicine - control	Diploid	Diploid	Diploid
Lidi - oryzalin - control	Diploid	Diploid	Diploid
Gold - colchicine - control	Diploid	Diploid	Diploid
TDM - colchicine - 7.5 mM/48 h	Tetraploid	Mixoploid	Mixoploid
Lidi - colchicine - 12.5 mM/24 h	Tetraploid	Mixoploid	Mixoploid
Gold - colchicine - 2.5 mM/24 h	Tetraploid	Tetraploid	Mixoploid
Lidi - colchicine - 12.5 mM/24 h	Tetraploid	Tetraploid	Mixoploid
Lidi - colchicine - 7.5 mM/48 h	Tetraploid	Mixoploid	Tetraploid
TDM - oryzalin - 10 $\mu$ M/4 dias	Diploid	Diploid	Tetraploid
Lidi - colchicine - 12.5 mM/24 h	Tetraploid	Tetraploid	Tetraploid
Lidi - colchicine - 7.5 mM/48 h	Tetraploid	Tetraploid	Tetraploid
Lidi - oryzalin - 10 $\mu$ M/4 dias	Tetraploid	Tetraploid	Tetraploid
TDM - oryzalin - 10 $\mu$ M/4 dias	Tetraploid	Tetraploid	Tetraploid

2011). Nuclear DNA content as determined by flow cytometry has been studied in various plants of economic importance, such as *Brachiaria* (Pinheiro et al., 2000), *Triticum* (Kubalaková et al., 2002), *Malus* (Höfer and Meister, 2010), and *Jatropha* (Kaewpoo and Te-chato, 2010). Van Duren et al. (1996) used flow cytometry to identify autotetraploid banana plants induced through chromosomal doubling of diploid material. The phenomenon of mixoploidy was also commonly encountered in this work, and flow cytometry was efficient at detecting and confirming these cases. The use of flow cytometry to identify chromosome duplication has been described in several studies (Costich et al., 2010; Xing et al., 2011; Nemorin et al., 2013).

Pre-checking based on stomatal characteristics was found to be very helpful in identifying populations of tetraploid mixoploids, thus reducing the numbers of plants that had to be examined after *in vitro* cultivation, highlighting the need for care when selecting the morphological characteristics used to confirm plant ploidy levels. The safest method is to compare the material in at least three steps, as performed in the present work.

Motonobu et al. (1997) and Kim and Kim (2003) reported that the numbers of stomata on *Chrysanthemum* and *Cymbidium* were directly proportional to their ploidy levels, but stomata size did not vary among plants with different ploidy levels. Vichiato (2004) found that tetraploid plants of *Dendrobium nobile* showed higher stomatal frequencies because their epidermal cells were smaller than those of diploids.

Thus, while morphological descriptors can be very useful, they should not be used alone for checking ploidy levels as they are subject to genetic effects, so that the

isolated analysis of only certain variables may lead to classification errors for certain genotypes (Souza and Queiroz, 2004; Madon et al., 2005). Lacerda et al. (2008) examined the morphological features of the stomata of Silver Dwarf cultivars obtained from three *in vitro* plant variants, and found that only one showed low stomata densities; they did not determine if this variant was mixoploide or not, however. Studies of chromosome doubling in *Hedychium muluense* by treatments with colchicine and oryzalin demonstrated that ploidy analysis by flow cytometry, coupled with chromosome counting, was more reliable than comparing stomata sizes (Sakhanokho et al., 2009).

Flow cytometry had an important role in identifying mixoploids in the present work as they have been erroneously classified as triploids and tetraploids in other studies. One hypothesis that could explain the occurrence of mixoploidy in the present work is chromosome elimination. Abreu et al. (2006) and Barbosa (2004), working with chromosomal duplication induction in triploid hybrid elephant grass and millet, observed mixoploidy in most of the plants analyzed. According to these authors, there were great variations in chromosome numbers in the metaphases analyzed, and they concluded that these variations were due to chromosome eliminations. Certain mitotic alterations are characteristic of chromosome elimination - such as the lack of chromosome orientation during metaphase, late anaphase segregation, chromatin degradation, chromosome fragmentation, and micronuclei formation (Singh, 2002).

Mixoploids may arise because antimitotic agents may not always reach all of the meristems on a plant (or those

that are actively dividing) (Carvalho et al., 2005). Several authors (Poutaraud and Girardin, 2005; Campos et al., 2009; Wannakraij and Wondyifraw, 2013) have reported different effects of antimetabolic agents when different tissues are targeted or different treatment periods are used. As cytogenetic analyses were performed using root meristems, while stomata and flow cytometric analyses were performed using young leaves, it would not be unexpected if these distinctly different tissues in distinct localities demonstrated different ploidy levels after treatment with antimetabolic agents.

Thus, the possibility of inducing autotetraploidy in banana plants should have a significant impact on breeding programs as this will reduce the time needed, manpower expenditures, and costs involved in obtaining tetraploids as compared to conventional methods. Some types of tetraploids may be impossible to obtain using conventional methods, making antimetabolic agents of great importance to breeding programs. The autotetraploids obtained in this study cannot necessarily be recommended as cultivars, as tetraploids generally have arched leaves and their fruits easily separate and fall, so it will be necessary to obtain triploid plants from them. To that end, future studies will be conducted with the tetraploid plants generated here to cross them with diploid elite varieties in the germplasm bank at Embrapa Cassava. It is expected that crossing tetraploids with diploids will generate triploids that, after agronomic evaluation, can be distributed by Embrapa as new and improved varieties.

Bananas are of significant importance in the Brazilian diet, mainly among low-income families who lack economic resources to purchase non-tropical fruits. The climate in Brazil also favors banana cultivation throughout the year, which ensures an abundant supply of these fruits at low cost. Therefore, research efforts to generate more resistant, productive, nutritious, and good-tasting bananas must be continued.

Additionally, there is a need to promote more balanced and sustainable agriculture techniques that use less agro-defensive products that - and tetraploid induction using antimetabolic agents presents the possibility of inducing disease resistance through the generation of duplicated genetic material, as was observed using disease-resistant Cavendish triploids that had previously been extremely difficult to obtain through conventional breeding. The next step will be to evaluate the agronomic characteristics of plants generated through chromosome duplication to certify their taste, vigor, and resistance to the fungal diseases and pests that are quite common and destructive in Brazilian plantations.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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### REFERENCES

- Abreu JC, Davide LC, Pereira AV, Barbosa S (2006). Mixoploidia em híbridos de capim-elefante x milho tratados com agentes antimetabólicos. *Pes. Agrop. Bras.* 41(11):1629-1635.
- Ade R, Rai MH (2010). Review: Colchicine, current advances and future prospects. *Nus. Biosci.* 2(2):90-96.
- Barbosa S (2004) Micropropagação e duplicação cromossômica de híbridos triploides de capim-elefante e milho. Thesis, Universidade Federal de Lavras, Lavras.
- Barbosa S, Davide LC, Pereira AV, Abreu JC (2007). Duplicação de cromossômica de híbridos triploides de capim-elefante e milho. *Bragantia* 66(3):365-372.
- Campos JMS, Davide LC, Salgado CC, Santos FC, Costa PN, Silva OS, Alves CCS, Viccini LF, Pereira AV (2009). *In vitro* induction of hexaploid plants from triploid hybrids of *Pennisetum purpureum* and *Pennisetum glaucum*. *Plant Breed.* 128:101-104
- Carvalho JFR, Carvalho CR, Otoni WC (2005). *In vitro* induction of polyploidy in annatto (*Bixa orellana*). *Plant Cell Tissue Org.* 80(1):69-75.
- Costich DE, Friebe B, Sheehan MJ, Casler MD, Buckle ES (2010). Genome-size variation in switchgrass (*Panicum virgatum*): flow cytometry and cytology reveal rampant aneuploidy. *Plant Gen.* 3(3):130-141.
- Dolezel J, Bartos J (2005). Plant DNA flow cytometry and estimation of nuclear genome size. *Ann. Bot.* 95(1):99-110.
- Dolezel J, Binarova P, Lucretti S (1998). Analysis of nuclear DNA content in plant cells by flow cytometry. *Biol. Plant.* 31(2):113-120
- Ganga M, Chezhian N (2002). Influence of the antimetabolic agents colchicine and oryzalin on *in vitro* regeneration and chromosome doubling of diploid bananas (*Musa* spp.). *J. Hortic. Sci. Biotechnol.* 77(5):572-575.
- Höfer M, Meister A (2010). Genome size variation in *Malus* species. *J. Bot.* 2010:1-8.
- Kaewpoo M, Te-chato S (2010). Study on ploidy level of micropropagated *Jatropha curcas* L. via flow cytometry. *J. Agric. Technol.* 6(2):391-400.
- Kim MS, Kim JY (2003). Chromosome doubling of a *Cymbidium* hybrid whit colchicine treatment in meristem culture. *Proc. Nioc.* 1:37-40.
- Kubalaková M, Vrana J, Ciháliková J, Simková H, Dolezel J (2002). Flow karyotyping and chromosome sorting in bread wheat (*Triticum aestivum*). *Theor. Appl. Genet.* 104(8):1362-1372.
- Lacerda GA, Silva JOC, Abreu JC, Alves E, Paiva LV (2008). Características morfoanatômicas da epiderme foliar de plantas variantes e não variantes somaclonais de bananeiras (*Musa* sp. Colla cv. Prata-anã) cultivadas *in vitro*. *Acta. Bot. Bras.* 22(1): 85-90.
- Lone AB, Unemoto Ik, Takahashi LSA, Faria RTA (2010). Sobrevivência e desenvolvimento *in vitro* de *Cattleya* (Orchidaceae) submetida a tratamentos com colchicina. *Semina: Ciên. Agric.* 31(4):1337-1342.
- Madon M, Clyde MM, Hasmim H, Mohd YY, Mat H, Saratha S (2005). Poliploidy induction of oil palm through colchicine and oryzalin treatments. *J. Oil Palm Res.* 17:110-123.
- Motonobu E, Kim JS, Inada I (1997). Production and characteristics of chromosome doubling plants of small flowered garden Chrysanthemum, *Dendranthema x gradiflorum* Ramat. Kitam. cv. YS by colchicine treatment of cultured shoot tips. *J. Jpn Soc. Hortic. Sci.* 65:528-833.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15:473-497.
- Nemorin AJ, David E, Maledon E, Nudo J, Dalon G (2013). Microsatellite

- and flow cytometry analysis to help understand the origin of *Dioscorea alata* polyploids. *Ann. Bot.* 112(5):811-819.
- Ochatt SJ (2008). Flow Cytometry in Plant Breeding. *Cytometry Part A* 73:581-598.
- Pinheiro AA, Pozzobon MT, Valle CB, Penteadó MIO, Caneiro VTC (2000). Duplication of the chromosome number of diploid *Brachiaria brizantha* plants using colchicine. *Plant Cell Rep.* 19(3):274-278.
- Poutaraud A, Girardin P (2005). Influence of chemical characteristics of soil on mineral and alkaloid seed contents of *Colchicum autumnale*. *Environ. Exp. Bot.* 54(2):101-108.
- Rodrigues FA, Soares JDR, Santos RR, Pasqual M, Silva SO (2011). Colchicine and amiprofos-methyl (APM) in polyploidy induction in banana plant. *Afr. J. Biotechnol.* 10:13476-13481.
- Sajjad Y, Jaskani MJ, Mehmood A, Ahmad I, Abbas H (2013). Effect of colchicine on *in vitro* polyploidy induction in african marigold (*Tagetes erecta*). *Pak. J. Bot.* 45(3):1255-1258.
- Sakhanokho HF., Rowena KRY, Islam-Faridi KN (2009). Induced polyploidy in diploid ornamental ginger (*Hedychium muluense* R. M. Smith) using colchicine and oryzalin. *Hortscience* 44(7):1809-1814.
- Silva LA, Silva Junior JF, Ledo CAS, Silva SO (2008). Avaliação de genótipos de bananeira na região do baixo São Francisco. *Rev. Bras. Frutic.* 30(3):291-296.
- Simmonds NW, Dodds KS. (1949). Meiose in seeded diploids of *Musa*. *J. Gen.* 49:221-225.
- Singh RJ (2002). *Plant Cytogenetics*. Boca Raton: CRC Press, 2002. 463 p.
- Souza FF, Queiróz MA (2004). Avaliação de caracteres morfológicos úteis na identificação de plantas poliploides de melancia. *Hort. Bras.* 22(3):516-520.
- Stover RH, Simmonds NW(1987). *Bananas*. Third Edition. Longman, London.
- Vakili NG (1962). Colchicine-induced polyploidy in *Musa*. *Nature* 194: 453-454.
- Van Duren M, Morpurgo R, Dolezel J Afza R (1996). Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. *Euphytica* 88:25-34.
- Vichiato MRM (2004). Indução de tetraploides e alongamento de plantas de *Dendrobium nobili* LINDL. (Orchidaceae). Thesis, Universidade Federal de Lavras, Lavras.
- Wannakraij S, Wondyifraw T (2013). *In vitro* chromosome doubling ink [*Aframomum corrorima* (Braun) P.C.M. Jansen] using colchicine and oryzalin. *Kasetsart J. Nat. Sci.* 47:684-694.
- Xing SH, Xin-Bo G, Quan W, Qi-Fang P, Yue-Sheng T, Pin L, Jing-Ya Z, Guo-Feng W, Xiao-Fen S, Ke-Xuan T (2011). Induction and flow cytometry identification of tetraploids from seed-derived explants through colchicine treatments in *Catharanthus roseus* (L.) G. Don. *J. Biomed. Biotechnol.* 1:1-10
- Younis A, Ryu KB, Co VT, Hwang YJ, Jee SO, Kim MS, Kim CK, Lim KB (2013). Analysis of chromosomes and nuclear DNA content in nine genotypes of *Cymbidium*. *Flower Res. J.* 12:158-161.

Full Length Research Paper

## Removal of trace element by isolates of *Aspergillus brasiliensis* EPAMIG 0084 and *Penicillium citrinum* EPAMIG 0086 in biofilters

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Coffee beans processing generates a large volume of wastewater composed of trace elements which can be detrimental to human health. The present study aimed at evaluating the capacity of strains of *Aspergillus brasiliensis* and *Penicillium citrinum* in tolerating and removing trace elements namely: Cu, Mn and Zn from coffee wastewater. The use of fungi in the treatment of polluted wastewater has emerged as a viable alternative to conventional treatment. The fungi were isolated from polluted and unpolluted areas, which were tested on a laboratorial scale and on large scale (aerobic bioreactor) with immobilized biomass. As expected, the strains isolated from polluted areas (*P. citrinum* EPAMIG 0086 and *A. brasiliensis* EPAMIG 0084) were more tolerant to the elements studied than the strains isolated from unpolluted areas (*A. brasiliensis* IBT 26433 and *P. citrinum* INCQS 40011). As for the removal tests conducted on a laboratorial scale, it is worth mentioning that the fungal strains under study responded differently to the tested elements (regardless of their origin). In the tests conducted in bioreactors, the fungus *P. citrinum* EPAMIG 0086 presented a greater removal capacity of the elements in aerobic biofilters (44, 62 and 48% for Cu, Mn and Zn, respectively) than the *A. brasiliensis* EPAMIG 0084 (21.7, 51 and 41.6%, respectively), which indicates that this fungus is an efficient alternative to conventional treatments.

**Key words:** Coffee wastewater, bioreactors, fungi, immobilization, removal, trace elements.

### INTRODUCTION

Increasing population and consumption are placing unprecedented demands on agriculture and natural resources. The productivity increase has led in the production of large quantities of agricultural waste and

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therefore in its disposal in soil and water bodies (Foley et al., 2011). Coffee production is no exception to this rule. According to Melo (2009), wet processing requires large volumes of water in the stages of husk removal, fermentation and washing. For every bag of coffee produced, 3,500 L of coffee wastewater is generated. These residue is rich in organic and inorganic matter, and among the inorganic compounds, there is a large number of trace elements from the use of fertilizers, agricultural lime and pesticides that accumulate in the fruit during development (Kiekens and Cottenie, 1985).

According to Wuana and Okieimen (2011), the predominant trace elements in agricultural areas contaminated with fertilizer and pesticide waste are lead, chromium, arsenic, zinc, cadmium, copper, mercury and nickel. The trace elements and their toxicity are of particular interest to public health, because these substances cannot be processed or chemically destroyed (Davis et al., 2001); are not biodegradable and tend to accumulate in living organisms and many heavy metal ions are known to be toxic or carcinogenic (Fu and Wang, 2011).

According to Ribeiro et al., (2009), however, in addition to the trace elements hazardous to the environment, coffee wastewater also contains N, P, K, Ca, Mg and micronutrients. Thus, if properly treated, it can be reused to supply part of the water and the nutrients demanded by crops. Conventional methods for removal of dissolved trace elements are chemical treatments (like hydroxide precipitation, sulfide precipitation, trace elements chelating precipitation and ion exchange), physical treatments like adsorption (activated carbon adsorbents and carbon nanotubes adsorbents), membrane filtration (ultrafiltration and nanofiltration), coagulation, flocculation, flotation and electrochemical treatment (Fu and Wang, 2011).

Even though conventional technologies adopted for removal of heavy metals from polluted environment tend to be efficient, they are generally expensive and produce huge quantity of toxic chemical products (Zaidi et al., 2011). Moreover, methods such as chemical precipitation and reverse osmosis for removal of trace elements present in low concentrations (below 100 mgL<sup>-1</sup>, such as coffee wastewater) result in incomplete removal of trace elements and excessive use of reagents and energy (Brierley et al., 1985; Kapoor and Viraraghavan, 1995).

The use of biological materials including fungal biomass offers an economical, effective and safe option for removing heavy metals and, therefore, has emerged as a potential alternative method to conventional treatment techniques. Among the various remediation strategies, biosorption of heavy metals by metabolically active or inactive nonliving (dead) biomass of fungal origin is an innovative and alternative technology for removal of metals from contaminated sites (Zaidi et al., 2011). Adsorption is an effective and economic method for heavy metal wastewater treatment. The adsorption process

offers flexibility in design and operation and in many cases will produce high-quality treated effluent. In addition, because adsorption is sometimes reversible, adsorbents can be regenerated by suitable desorption process (Fu and Wang, 2011).

The use of fungal isolates from environments contaminated by trace elements is a very promising alternative in wastewater treatment. As these microorganisms can proliferate in a contaminated environment, they probably have strategies for tolerating and growing in such situation (Lemos et al., 2008; Sprocati et al., 2006). Treatment of trace element contaminated wastewater through microorganisms can be optimized by means of their immobilization on supports inside biofilters. That is because the use of immobilized biomass facilitates its separation from the aqueous medium for subsequent retrieval of elements adsorbed and reuse of the biomass (Tsezos and Deutschmann, 1990).

The present study was conducted in order to assess the removal of the elements copper (Cu), manganese (Mn) and zinc (Zn) present in coffee wastewater by using *Aspergillus brasiliensis* EPAMIG 0084 and *Penicillium citrinum* EPAMIG 0086 isolated from contaminated environments and *A. brasiliensis* IBT 26433 and *P. citrinum* INCQS 40011 from uncontaminated environments. The aim was to compare removal efficiency based on prior exposure of the fungi to the pollutant, through the use of a laboratory-scale and larger scale experiments in aerobic biofilters.

## MATERIALS AND METHODS

All tests were performed in triplicate, and all media were prepared with Milli-Q water (deionized water purified by a Milli-Q system supplied by Millipore Corporation).

### Fungal isolates

The following fungal species were used: *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086, both isolated from Zn, Cu, Pb and cadmium (Cd) contaminated soil from the municipality of Três Marias, MG, Brazil, as well as the same fungal species isolated from uncontaminated regions; *A. brasiliensis* IBT 26433 and *P. citrinum* INCQS 40011.

### Collection of coffee wastewater samples: Qualification and quantification of trace elements

The water derived from the washing of coffee beans, coffee wastewater (CW), was collected from the Coffee Processing Center of Lavras Federal University (CEP - UFLA) for the pH and trace element evaluation. A 0.45 µm membrane was used to filter the water and sent for atomic absorption spectrophotometer (AAS) analyses to check for the presence of the following trace elements described in the literature by Gonçalves (2006) as normally present in the CW: copper (Cu), manganese (Mn) and zinc (Zn). The samples were digested in a digestion tube using nitric acid (HNO<sub>3</sub>) according to the US Environmental Protection Agency (EPA) 3051A

method. Metal concentrations were determined through the use of an atomic absorption spectrophotometer (Perkin Elmer Analyst 300).

#### Trace element tolerance test

Tolerance of the fungi study to the trace elements Cu, Mn and Zn was tested at the following concentrations found in the CW by AAS: 0.5, 1 and 5 mgL<sup>-1</sup> of each element. The isolates were transferred to Petri dishes containing malt extract agar medium (MA) with the addition of copper sulfate, manganese sulfate or zinc sulfate, each one separately. The isolates were also inoculated on dishes containing MA without the addition of any trace element as control. The culture media were adjusted to pH 5 because, according to Zafar et al. (2007), this pH is the best for obtaining the highest rate of metal ion removal by microbial biomass. Moreover, according to Gonçalves (2006), the pH found in CW varies from 4 to 5. The fungi were incubated at 25°C for seven days and the diameter of the colonies was measured. Isolates that exhibited growth at or above the growth of the control were considered tolerant (da Silva et al., 2003).

#### Trace element removal tests on a laboratory scale

The present methodology was adapted from da Silva et al. (2003). The isolates were inoculated in Petri dishes containing MA and incubated at 25°C for seven days. After fungal growth, five discs (0.5 cm diameter) were removed and transferred to 250 mL Erlenmeyer flasks containing 130 mL of 2% malt extract broth (MB) and a solution with trace element salts: copper sulfate, manganese sulfate and zinc sulfate (0.5, 1 and 5 mgL<sup>-1</sup>). Controls were used by means of Erlenmeyer flasks containing the culture medium with the salt solution and without the inoculum, and the culture medium with the inoculum but without the salt solution. These flasks were incubated (100 rpm) at 25°C for 10 days.

After 5 and 10 days, the samples were filtered in 0.45 µm Millipore membranes, the trace element content quantified by AAS and the pH of the solution was evaluated. After 10 days of incubation, the biomass produced was filtered and the dry weight was evaluated. After this sampling period, an aliquot of the biomass was removed with the aid of a platinum loop for analysis in a scanning electron microscope (SEM) with x-ray microanalysis.

#### Trace element removal tests in aerobic biofilters

Three previously disinfected biofilters were used (glass batch reactors) with a 14.6 L capacity, built by Silva (2008). Along the reactors, four taps were installed to allow sampling of the effluent for trace element quantification analyses. The biofilter was continuously oxygenated with a Boyu model U-3600 air pump, with a 4.0 L min<sup>-1</sup> flow rate. The fungal isolates from the region contaminated by trace elements *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086 were transferred to plates containing MA and incubated for 20 days at 25°C.

After this period, 20 mL of sterile distilled water was added to the plate surface and the fungal biomass was scrapped to disaggregate the spores. The spores present in the suspension were counted in a Neubauer chamber and the concentration adjusted to approximately 5x10<sup>6</sup> spores mL<sup>-1</sup>. This suspension (20 ml) was inoculated in bottles containing 250 mL of MA medium (40°C), homogenized and transferred to trays containing support for fungal growth - corrugated pipes (conduits) - previously sterilized in an autoclave. The trays were incubated (25°C) for 20 days for the fungi to grow and colonize the surface and inside of the conduit. As mentioned at the introduction, this test was performed in a period of 20 days rather

than seven days, to evaluate the behavior of the trace element against fungal biomass in a longer period of time on a higher retention time on enlarged scale.

After this period, the support material containing the attached fungal biomass was transferred to the biofilter. Subsequently, 10 L of a synthetic solution of distilled water with the addition of copper sulfate, manganese sulfate and zinc sulfate at a concentration of 5 mgL<sup>-1</sup> Cu, Mn and Zn was added. The pH of the solution was adjusted to pH 6 in order to assess whether at this pH there was greater adsorption of trace elements than at pH 5, previously tested in Erlenmeyer flasks. This pH is also described by Saya et al. (2001) as ideal for the removal of some trace elements tested.

The biofilter was activated and 20 mL was removed from each tap, initially every 12 h, and after the first day, once a day for five days (120 h). The trace element concentration present in the samples was determined by AAS.

#### Scanning electron microscopy with X-ray microanalysis

The use of scanning electron microscope (SEM) helps elucidate the mechanisms involved in biosorption. The SEM can be attached to equipment energy dispersive system (EDS), which allows the determination of the qualitative composition of the trace elements present in the samples from the emission of characteristic X-rays (Calfa and Torem, 2007). In order to perform this test, aliquots of biomass formed in the flask tests with 0 (control) and 5 mgL<sup>-1</sup> trace element concentration were removed. These were washed with distilled water and placed on aluminum supports (stubs) previously covered with carbon tape and observed in a scanning electron microscope (QUANTA 250 - FEI) coupled to a system of X-ray microanalysis (EDS) that uses the emission of X-rays to describe the elements found in the samples. The Genesis program was used for performing the microanalysis under the following conditions: 30,000 kV, spot ranging from 5 to 7 and high vacuum.

These analyses were performed in the Department of Biophysics at Rio de Janeiro Federal University - UFRJ). In order to draw the graphs of the X-ray microanalysis spectrum, a determination was made of the composition of samples per sample area under the following conditions: working distance (WD) of 10 mm, an increase of 1,000 X and voltage of 30 kV.

#### Statistical analyses

Statistical analyses were performed using SISVAR software. Analyses of variance (ANOVA) were conducted using a completely randomized factorial experimental design (DIC).

## RESULTS AND DISCUSSION

### Evaluation of trace elements present in the CW

Table 1 presents the results of the trace element concentrations (Cu, Mn and Zn) detected in CW obtained from the CEP (UFLA). The pH obtained in CW was 4.5. The pH obtained from CW in the present study corroborates the results presented by Gonçalves (2006). In studies conducted by Vasco (1999) and Gonçalves (2006) regarding the presence of trace elements in CW, concentrations of these elements were higher than those found in this study (Table 1); 5 mgL<sup>-1</sup> Cu; 4 mgL<sup>-1</sup> Zn (Vasco, 1999); 1.27 mgL<sup>-1</sup> Cu; 1.33 mgL<sup>-1</sup> Mn and 0.66 mgL<sup>-1</sup> Zn (Gonçalves, 2006).

**Table 1.** Results of the Cu, Mn and Zn concentrations present in coffee wastewater obtained from Lavras Federal University (UFLA-MG).

Trace element present in the CW	Concentration obtained by atomic absorption spectrophotometry (mgL <sup>-1</sup> )
Cu	< 1.0
Mg	< 0.5
Zn	< 0.4

Numbers followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

**Table 2.** Trace element tolerance test: result of fungus growth (cm) in different Cu concentration. Test of means for the fungi factor within the Cu in each concentration level by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Cu concentration (mgL <sup>-1</sup> )		
	0.5	1	5
<i>A. brasiliensis</i> EPAMIG 0084	2.60 <sup>b</sup>	1.63 <sup>a</sup>	1.57 <sup>a</sup>
<i>A. brasiliensis</i> IBT 26433	6.03 <sup>d</sup>	6.25 <sup>d</sup>	6.10 <sup>d</sup>
<i>P. citrinum</i> EPAMIG 0086	2.03 <sup>a</sup>	2.05 <sup>b</sup>	2.00 <sup>b</sup>
<i>P. citrinum</i> INCQS 40011	4.97 <sup>c</sup>	5.05 <sup>c</sup>	4.88 <sup>c</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

**Table 3.** Trace element tolerance test: Result of fungus growth (cm) in different Mn concentration. Test of means for the fungi factor within the Mn in each concentration level by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Mn Concentration (mgL <sup>-1</sup> )		
	0.5	1	5
<i>A. brasiliensis</i> EPAMIG 0084	1.86 <sup>a</sup>	1.67 <sup>a</sup>	1.73 <sup>a</sup>
<i>A. brasiliensis</i> IBT 26433	6.5 <sup>c</sup>	6.10 <sup>d</sup>	6.43 <sup>d</sup>
<i>P. citrinum</i> INCQS 40011	5.28 <sup>b</sup>	5.02 <sup>c</sup>	5.00 <sup>c</sup>
<i>P. citrinum</i> EPAMIG 0086	2.03 <sup>a</sup>	1.97 <sup>b</sup>	2.10 <sup>b</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

**Table 4.** Trace element tolerance test: result of fungus growth (cm) in presence of Zn. Test of means for the fungus factor Zn element by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011. For Zn, the trace element concentration was not significant.

Fungal strain	Fungus growth (cm)
<i>A. brasiliensis</i> EPAMIG 0084	1.67 <sup>a</sup>
<i>A. brasiliensis</i> IBT 26433	6.22 <sup>b</sup>
<i>P. citrinum</i> INCQS 40011	5.03 <sup>ab</sup>
<i>P. citrinum</i> EPAMIG 0086	4.07 <sup>ab</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

### Trace element tolerance test

After variance analysis for the tolerance to trace element, it was concluded that the F test at 5% of significance level indicated that two-way interaction (fungi X concentration) was significant to Cu and Mn. Therefore, the level of fungi in each level of concentration was analyzed (Tables 2 and 3). For the Zn element, there was no significance level in the two-way interaction (fungi X concentration). Then for this element, an individual analysis for the factor fungus was done (Table 4). From Tables 2 to 4, for the most elements tested, it can be observed that fungal strains isolated from contaminated

**Table 5.** Trace element removal test on a laboratory scale: Cu final concentration ( $\text{mgL}^{-1}$ ) in different Cu concentrations tested (0.5, 1 and  $5 \text{ mgL}^{-1}$ ) after 5 days of incubation. Test of means, for the fungi factor within the concentration levels of Cu by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Cu concentration ( $\text{mgL}^{-1}$ )			
	0	0.5	1	5
Control	0.03 <sup>a</sup>	0.49 <sup>bc</sup>	0.95 <sup>cd</sup>	4.54 <sup>d</sup>
<i>A. brasiliensis</i> EPAMIG 0084	0.09 <sup>a</sup>	0.66 <sup>c</sup>	1.22 <sup>d</sup>	4.62 <sup>e</sup>
<i>A. brasiliensis</i> IBT 26433	0.10 <sup>a</sup>	0.38 <sup>abc</sup>	0.52 <sup>ab</sup>	1.34 <sup>a</sup>
<i>P. citrinum</i> EPAMIG 0086	0.01 <sup>a</sup>	0.08 <sup>a</sup>	0.33 <sup>a</sup>	2.40 <sup>b</sup>
<i>P. citrinum</i> INCQS 40011	0.00 <sup>a</sup>	0.08 <sup>a</sup>	0.40 <sup>a</sup>	3.06 <sup>c</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

environments *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086 showed greater tolerance to all the trace elements in the concentrations evaluated, with one exception: the element zinc (Table 4). For this element, the two strains of *P. citrinum* INCQS 40011 and EPAMIG 0086 did not show significant differences regarding tolerance. It was also observed that *A. brasiliensis* IBT 26433 and EPAMIG 0084 had a better growth than *P. citrinum* INCQS 40011 and EPAMIG 0086. However, this fact does not indicate greater tolerance to *A. brasiliensis* strains, since it has a faster natural growth rate than *P. citrinum* strains.

According to Pan et al. (2009), tolerance to trace elements among strains of the same fungal species is due to different mechanisms of detoxification developed by them in response to environmental pressures. These strains promote the reduction of toxicity of trace elements through the complexation of these elements, precipitation or other mechanisms and therefore they can tolerate and grow under such conditions (Saxena et al., 2006).

The results of this study support the assumption that species isolated from environments contaminated with trace elements have a higher tolerance than species isolated from uncontaminated environments (Rouch et al., 1995). Other authors have also studied trace element tolerance of fungi isolated from areas contaminated with these elements. Ezzouhri et al. (2009) evaluated the tolerance of microorganisms isolated from areas contaminated by trace elements in Tangier (Morocco) against the elements Pb, Cr, Cu and Zn. Isolates of *Aspergillus* spp. and *Penicillium* spp. were the most tolerant to all trace elements tested and exhibited the greatest growth in the presence of these elements in relation to other fungi and their controls (medium without addition of trace elements).

Hemambika et al. (2011) also concluded that trace elements resistant isolates show no inhibition of growth for higher concentration of heavy metals, whereas trace elements sensitive isolates show inhibition of growth for higher concentration of heavy metals. These results were similar to that obtained in the present study, in which both

species *P. citrinum* EPAMIG 0086 and *A. brasiliensis* EPAMIG 0084 from contaminated area showed great tolerance to the trace element evaluated.

Probably, this occurs due to the fact that the fungi from uncontaminated areas have no protection mechanisms against these elements as a result of environmental pressure that selects such characteristics. Thus, according to Gadd (1992) once inside the cell, the trace element may be located into organelles, or may be attached to proteins, shifting the ions suitable for cell function from their original positions, promoting cell damage or interfering with the metabolic functions.

#### Trace element removal test on a laboratory scale

Following the variance analysis for the removal of all trace elements tested by the fungi under study, after 5 and 10 days of incubation, it may be concluded that the three way interaction: time X fungus X concentration, was significant by the F test at the 5% significance level for the trace elements Cu and Mn. Therefore, the levels of concentration and time for the trace element Cu (Tables 5 and 6) and Mn (Tables 7 and 8) were analyzed.

When comparing the removal of the copper by the studied strains with their respective controls (Tables 5 and 6), it may be observed that the greatest removal rate was obtained after five days of incubation. Furthermore, it can be inferred that for both species, the greatest removal rate was achieved by strains isolated from uncontaminated areas. *A. brasiliensis* IBT 26433 showed 70.5% removal in a concentration of  $5 \text{ mgL}^{-1}$  for Cu and *P. citrinum* INCQS 40011 and 81% of removal in a concentration of  $0.5 \text{ mgL}^{-1}$  for Cu.

These results seem to contradict the results obtained in the initial tolerance tests when the fungi isolated from contaminated areas showed higher growth and tolerance compared to strains isolated from uncontaminated areas. Therefore, the tolerances to these trace elements are not directly related to the capacity of trace elements removal by these fungi. Shen et al. (2013) evaluated the hypothesis

**Table 6.** Trace element removal test on a laboratory scale: Cu final concentration ( $\text{mgL}^{-1}$ ) in different Cu concentrations tested (0.5, 1 and 5  $\text{mgL}^{-1}$ ) after 5 days of incubation. Test of means, for the fungi factor within the concentration levels of Cu by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Cu concentration ( $\text{mgL}^{-1}$ )			
	0	0.5 $\text{mgL}^{-1}$	1 $\text{mgL}^{-1}$	5 $\text{mgL}^{-1}$
Control	0.001 <sup>a</sup>	0.47 <sup>ab</sup>	0.99 <sup>d</sup>	4.39 <sup>d</sup>
<i>A. brasiliensis</i> EPAMIG 0084	0.05 <sup>a</sup>	0.40 <sup>ab</sup>	1.00 <sup>cd</sup>	4.24 <sup>de</sup>
<i>A. brasiliensis</i> IBT 26433	0.07 <sup>a</sup>	0.41 <sup>ab</sup>	0.86 <sup>bcd</sup>	1.41 <sup>a</sup>
<i>P. citrinum</i> EPAMIG 0086	0.01 <sup>a</sup>	0.15 <sup>a</sup>	0.70 <sup>abc</sup>	2.12 <sup>b</sup>
<i>P. citrinum</i> INCQS 40011	0.01 <sup>a</sup>	0.32 <sup>ab</sup>	0.48 <sup>a</sup>	2.49 <sup>c</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

**Table 7.** Trace element removal test on a laboratory scale: Mn final concentration ( $\text{mgL}^{-1}$ ) in different Mn concentrations tested (0.5, 1 and 5  $\text{mgL}^{-1}$ ) after 5 days of incubation. Test of means, for the fungi factor within the concentration levels of Mn by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Mn concentration ( $\text{mgL}^{-1}$ )			
	0	0.5	1	5
Control	0.05 <sup>a</sup>	0.52 <sup>b</sup>	1.67 <sup>d</sup>	4.55 <sup>e</sup>
<i>A. brasiliensis</i> EPAMIG 0084	0.00 <sup>a</sup>	0.35 <sup>a</sup>	0.73 <sup>b</sup>	3.88 <sup>b</sup>
<i>A. brasiliensis</i> IBT 26433	0.00 <sup>a</sup>	0.34 <sup>a</sup>	0.33 <sup>bc</sup>	4.40 <sup>d</sup>
<i>P. citrinum</i> EPAMIG 0086	0.03 <sup>a</sup>	0.28 <sup>a</sup>	0.66 <sup>b</sup>	3.51 <sup>a</sup>
<i>P. citrinum</i> INCQS 40011	0.07 <sup>a</sup>	0.42 <sup>a</sup>	0.74 <sup>b</sup>	4.03 <sup>c</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

**Table 8.** Trace element removal test on a laboratory scale: Mn final concentration ( $\text{mgL}^{-1}$ ) in different Mn concentrations tested (0.5, 1 and 5  $\text{mgL}^{-1}$ ) after 10 days of incubation. Test of means, for the fungi factor within the concentration levels of Mn by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Mn concentration ( $\text{mgL}^{-1}$ )			
	0	0.5	1	5
Control	0.03 <sup>a</sup>	0.55 <sup>bc</sup>	1.04 <sup>c</sup>	4.45 <sup>d</sup>
<i>A. brasiliensis</i> EPAMIG 0084	0.01 <sup>a</sup>	0.35 <sup>a</sup>	0.73 <sup>ab</sup>	4.14 <sup>b</sup>
<i>A. brasiliensis</i> IBT 26433	0.00 <sup>a</sup>	0.36 <sup>a</sup>	0.75 <sup>ab</sup>	4.44 <sup>bc</sup>
<i>P. citrinum</i> EPAMIG 0086	0.03 <sup>a</sup>	0.29 <sup>ab</sup>	0.78 <sup>ab</sup>	2.96 <sup>a</sup>
<i>P. citrinum</i> INCQS 40011	0.11 <sup>a</sup>	0.40 <sup>a</sup>	0.88 <sup>abc</sup>	4.21 <sup>bc</sup>

that endophytic isolated from trace elements contaminated sites would enhance their host capacity to trace elements removal from environment; but, they observed that there was no significant difference in this capacity by endophytic isolated from contaminated and uncontaminated sites. Then, like the results obtained in the present research, they concluded that growth and heavy metal sorption/absorption and accumulation were not correlated with origin of the endophytic.

*P. citrinum* INCQS 40011 showed the best results for

Cu removal. This phenomenon can be explained by the final pH media. Akar et al. (2009) evaluated Cu adsorption by *Tramaetes versicolor* ATCC 200801 at different pHs, and observed that the maximum removal rate of this element from the solution occurred at pH 5 and, below this pH, adsorption decreased, reaching zero at pH 1. The same phenomenon occurred in the present study, in which *A. brasiliensis* IBT 26433 and EPAMIG 0084 changed the pH of the solution to three, removing the element Cu less efficiently than *P. citrinum* INCQS

**Table 9.** Trace element removal test on a laboratory scale: Zn final concentration ( $\text{mgL}^{-1}$ ) in different Zn concentrations tested (0, 5, 1 and  $5 \text{ mgL}^{-1}$ ) after 5 days of incubation. Test of means, for the fungi factor within the concentration levels of Zn by *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011.

Fungal strain	Zn concentration ( $\text{mgL}^{-1}$ )			
	0	0.5	1	5
Control	0.00 <sup>a</sup>	0.60 <sup>c</sup>	1.02 <sup>bc</sup>	4.57 <sup>d</sup>
<i>A. brasiliensis</i> EPAMIG 0084	0.00 <sup>a</sup>	0.19 <sup>a</sup>	0.73 <sup>a</sup>	4.28 <sup>c</sup>
<i>A. brasiliensis</i> IBT 26433	0.02 <sup>a</sup>	0.25 <sup>ab</sup>	0.71 <sup>a</sup>	4.06 <sup>b</sup>
<i>P. citrinum</i> EPAMIG 0086	0.10 <sup>a</sup>	0.47 <sup>bc</sup>	1.01 <sup>bc</sup>	3.34 <sup>a</sup>
<i>P. citrinum</i> INCQS 40011	0.03 <sup>a</sup>	0.42 <sup>abc</sup>	0.81 <sup>ab</sup>	4.47 <sup>cd</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

40011 and EPAMIG 0086, which maintained the solution pH close to 5. The increased removal rate observed at pH 5 is due to the release of negatively charged binding sites on the fungal cell wall, as a result of proteins denaturation that facilitates binding (Verma et al., 2013).

A higher removal rate observed within five days of incubation compared to 10 days (Tables 5 and 6) is probably due to fungi protection mechanisms that send back trace elements to the solution after long incubation periods. According to Melo and Azevedo (2008), the removal by adsorption process occurs until biomass saturation, in other words, when the active sites are not available anymore and the desorption process of trace elements to solution may occur.

Concerning the Mn, the results from Tables 7 and 8 demonstrate that the best performances were obtained from *A. brasiliensis* IBT 26433 (71% of removal in  $1 \text{ mgL}^{-1}$ , by five days of incubation), followed by *P. citrinum* EPAMIG 0086 (34.9% in  $5 \text{ mgL}^{-1}$ , 10 days of incubation). Therefore, for the Mn trace element, the same pattern was not observed regarding strains isolated of contaminated and uncontaminated areas as occurred previously. Osaizua et al. (2014) studied the trace elements (Al, Fe and Mn) accumulation capacity by fungi isolated of trace elements contaminated areas. They also concluded that the genus *Aspergillus* exhibited the highest bioaccumulation potential. In their study they observed that *A. oryzae* showed the best Mn (II) sorption rate (over 30%).

On the other hand, for the Zn, the F test at 5% of significance level was not significant for the three way interaction: time x fungus x concentration for the Zn removal test. Thus, an analysis of each of the three 2-way interactions was conducted (Table 9). Only the two way interaction: fungus x time was significant by the F test at 5% of significance level for this element. For Zn (Table 9), a greater removal rate after five days of incubation was observed for both species in the study. However, *A. brasiliensis* strains EPAMIG 0084 and IBT 26433 showed a significant percentage of this element ablation compared to *P. citrinum* strains. *A. brasiliensis*

EPAMIG 0084 presented 68% of removal in Zn concentration of  $0.5 \text{ mgL}^{-1}$  while *A. brasiliensis* IBT 26433 showed 60% in the same concentration. For the fungus *P. citrinum* EPAMIG 0086, no significant Zn removal was observed and *P. citrinum* INCQS 40011 showed 26.8% of removal in the concentration of  $1 \text{ mgL}^{-1}$  of Zn; for Zn, the strain origin did not interfere in this element uptake but the species evaluated.

Akhtar et al. (2013) evaluated the zinc removal from industrial effluent by *Aspergillus* sp. They obtained approximate 50% of Zn removal by *Aspergillus flavus* NA9 and observed that the contribution of the functional groups and lipids to zinc biosorption as identified by chemical pretreatment was in the order: carboxylic acids > hydroxyl > amines > lipids. Probably these functional groups and lipids were also responsible by zinc uptake by the *A. brasiliensis* strains EPAMIG 0084 and IBT 26433 used in this study.

Faryal et al. (2006) observed that the optimum pH for Zn removal by *Aspergillus fumigates* RH05 is 5. In the present study, at the beginning of the experiments, the pH reached was 6, a fact that probably influenced significantly the removal of this element by most of the fungi evaluated.

The removal of these trace elements by the fungi under study may be due to biosorption and absorption mechanisms. The phenomenon of biosorption occurs as a result of physical phenomena (regardless of cellular metabolism) and the sorption of these elements to functional groups present on the fungal cell wall such as amino groups, carboxyl, thiol, sulfhydryl and phosphate by electrostatic attraction or bond formation (Pan et al., 2009; Purchase et al., 2009). The adsorption is carried out due to binding of trace element to functional groups present on the cell wall such as the carboxyl, amine, sulfate and phosphate. The absorption occurs when the trace element are transported into the cell and are volatilized or accumulated by binding to organelles or proteins (Aparicio, 2000).

Thus, it may be inferred that different fungi species are capable of adsorbing trace element of solutions by

**Table 10.** Trace element removal test on a laboratory scale: Dry weight obtained from *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011 after 10 days of growth in malt extract plus a solution with trace elements Cu, Mn and Zn in different concentrations.

Fungal strain	Dry weight (g)											
	Cu (mgL <sup>-1</sup> )				Mn (mgL <sup>-1</sup> )				Zn (mgL <sup>-1</sup> )			
	0	0.5	1	5	0	0.5	1	5	0	0.5	1	5
<i>A. brasiliensis</i> EPAMIG 0084	0.66	0.63	0.69	0.68	0.66	0.63	0.69	0.68	0.66	0.63	0.69	0.68
<i>A. brasiliensis</i> IBT 26433	0.60	0.52	0.70	0.58	0.60	0.52	0.70	0.58	0.60	0.52	0.70	0.58
<i>P. citrinum</i> EPAMIG 0086	0.57	0.51	0.51	0.54	0.53	0.51	0.51	0.54	0.53	0.51	0.54	0.54
<i>P. citrinum</i> INCQS 40011	0.49	0.50	0.62	0.49	0.46	0.58	0.62	0.49	0.44	0.52	0.53	0.57

different removal mechanisms and therefore remove them in different quantities; a fact that was observed in this study, even by strains belonging to the same fungal species. These results corroborate those obtained by Adriano (2001), who concluded that species of microorganisms within the same genus, or even strains within the same species, may differ in their sensitivity to trace element.

According to Pan et al. (2009), the greater the variety of trace element present in the solution, the smaller the concentration of trace element adsorbed by the fungi biomass, due to the competition of these elements for active sites. Vivas et al. (2005) in a study of Cd and Zn adsorption by fungal biomass *Glomus mosseae* concluded that these elements have antagonistic effects when together in solution. However, this phenomenon was not observed in this study. Furthermore, the study of removal of the trace element separately would have no interest in the present study because it is not what actually occurs in CW, where there is a wide variety of these elements.

Ren et al. (2009) studied the removal of Cu, Cd, Pb and Zn by *Aspergillus niger* isolated from environments contaminated with such elements. After two days of incubation, there was 84.3% of Cu, 84.4% of Cd, 25% of Pb and 14.4% of Zn removal. In the present study, *A. brasiliensis* IBT 26433 also showed greater efficiency in removal of the element Cu (70.5% - Tables 5 and 6) than Zn (60% - Table 9) and *P. citrinum* INCQS 40011 also removed the element Cu (81% Tables 5 and 6) more efficiently than the element Zn (26.8% - Table 9), indicating that probably the components present on the cell wall of these strains have greater affinity with the trace element Cu.

With regard to dry weight, it was observed that for both fungal species studied, it remained approximately constant in relation to the control for all elements and concentrations tested (Table 10). These results demonstrate that trace elements concentrations are not toxic to the microorganisms assessed because they did not inhibit their growth.

The results allow us to infer that the strains of *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086,

isolated from contaminated region, and *A. brasiliensis* IBT 26433 and *P. citrinum* INCQS 40011, from and uncontaminated region, reacted differently regarding their removal capacity of the trace element Cu, Mn and Zn (Tables 5 to 9). These results indicate that the cell wall of the fungi studied may show a higher affinity to one or another specific trace biosorbent which showed higher affinity towards certain metal in mixed metals solution due to the selectivity of the biosorbent and/or the ambient conditions which may prefer the adsorption of such metal over the others (Sheng et al., 2008).

#### Trace element removal test in aerobic biofilters

An analysis of variance was performed on results of the response variable of trace element removal in aerobic biofilters. The removal of copper by *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086 isolated from contaminated region was used as a response variable. The F test at a 5% significance level was significant for the interaction fungus X time. Thus, unfolding of the fungus factor within time levels was performed (Table 12). Table 12 reveals that the fungus *P. citrinum* EPAMIG 0086 showed the best results in Cu removal for all the times evaluated.

Concerning the removal of manganese and zinc, an analysis of variance was performed for both species studied. The F test at the 5% significance level was not significant for the two way interaction, fungus X time. Therefore, an individual analysis of the time factor and the fungus factor was carried out. Both were significant by the F test at 5% significance level (Table 13). Table 13 shows that the *P. citrinum* EPAMIG 0086 exhibited better results removing manganese and zinc compared with *A. brasiliensis* EPAMIG 0084.

The results of the pH into biofilters are presents in Table 11. It may be observed that in the biofilter inoculated with *A. brasiliensis* EPAMIG 0084, there was a decline of three points in relation to the initial value (pH 6 to 3) after 6 h of activation and maintenance of this pH value after 120 h (Table 11). This pH variation may have influenced Cu removal (Table 12) by this fungus, once Cu

**Table 11.** Trace element removal test in aerobic biofilters: changes in pH into biofilters inoculated with fungi isolated from contaminated area after 120 h of activation.

Fungal strain	pH					
	12 h	24 h	48 h	72 h	96 h	120 h
<i>A. brasiliensis</i> EPAMIG 0084	6	3	3	3	3	3
<i>P. citrinum</i> EPAMIG 0086	6	5	6.5	7	7	7

**Table 12.** Trace element removal test in aerobic biofilters: Cu Concentration after fungi treatment in biofilters ( $\text{mgL}^{-1}$ ). Test of means for the fungus factor within time levels (12 to 120 h) for Cu removal by *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086.

Fungal strain	Time					
	12 h	24 h	48 h	72 h	96 h	120 h
<i>A. brasiliensis</i> EPAMIG 0084	6.21 <sup>b</sup>	6.22 <sup>b</sup>	6.20 <sup>b</sup>	6.16 <sup>b</sup>	6.12 <sup>b</sup>	6.06 <sup>b</sup>
<i>P. citrinum</i> EPAMIG 0086	5.58 <sup>a</sup>	5.19 <sup>a</sup>	4.69 <sup>a</sup>	4.42 <sup>a</sup>	3.47 <sup>a</sup>	3.11 <sup>a</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

**Table 13.** Trace element removal test in aerobic biofilters: Mn and Zn concentration after fungi treatment in biofilters ( $\text{mgL}^{-1}$ ). Test of means for the fungus factor for the elements Mn and Zn by *A. brasiliensis* EPAMIG 0084 and *P. citrinum* EPAMIG 0086.

Fungal strain	Mn concentration	Zn concentration
<i>A. brasiliensis</i> EPAMIG 0084	2.45 <sup>b</sup>	3.53 <sup>b</sup>
<i>P. citrinum</i> EPAMIG 0086	1.87 <sup>a</sup>	2.59 <sup>a</sup>

Values followed by the same letter in columns do not differ from each other by the Tukey test at 5% significance.

was not removed during the incubation period (five days, 120 h). A similar result was achieved in laboratory test, after five days incubation, in which only 21.7% of Cu removal was observed by this strain (Table 5). For Mn and Zn (Table 13), it was observed that time was not significant for the trace element variable removal. The aforementioned elements obtained respectively, 51 and 41.6% removal by *A. brasiliensis* EPAMIG 0084. A much lower rate was obtained for Mn and Zn in laboratory scale after five days incubation (11% of Mn and 6.3% of Zn). This occurred even with the final pH 3 for both tests using this strain. Presumably, the initial pH utilized in the biofilter test (pH 6) and the biomass immobilization process helped Mn and Zn removal in the solution. The pH has been identified as one of the most important parameter in metal biosorption study. It is directly related with competition ability of hydrogen ions to active sites on the biosorbent surface (Lodeiro et al., 2006).

According to Arica et al. (2003), the fungal cell immobilization in supports could also enhance fungal cell performance and adsorptive capacity of the biosorbent system for trace element because they were found to be far more stable during continuous operation in a bio-

reactor than the fungal cells in free forms. Furthermore, Sayer and Gadd (2001) believed that the production of gluconic, citric and oxalic acids by *A. niger* ATCC 210373 helped the removal of trace elements, such as zinc, from solutions due to its complexation with these elements, followed by a connection to the fungal cell wall. This phenomenon may occur with the *A. brasiliensis* species, since it belongs to the same genus of the aforementioned fungus (Varga et al., 2007).

Kapoor et al. (1999) studied the removal of Pb, Ca and Cu by *A. niger* ATCC 11414 at different pHs and compared it with the removal obtained by traditional methods of ion exchange resin and activated carbon. They observed that at pH 5.5 the precipitation of the trace element did not occur, which seemed to indicate that the removal of the trace element obtained in this study was the result of the mechanisms of biosorption and bioaccumulation, and not a process of elements precipitation due to physical phenomena. The same authors concluded that trace element removal by fungal biomass is greater than that obtained with activated carbon, and the removal by ion exchange resins is not effective when the  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{K}^{+}$  ions are present, as occurs in CW. Thus,



treatment of these effluents with fungal biomass would be a very viable alternative. Likewise, the low removal rate of all elements by *A. brasiliensis* EPAMIG 0084 and *A. brasiliensis* IBT 26433 is due to a pH decrease, which makes it possible to obtain a positively charged biomass. This is due to a high proton concentration, which inhibits the trace element binding because of charges repulsion (Kapoor and Viraraghavan, 1995).

Tesekova et al. (2010) studied the  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Pb}^{2+}$  and  $\text{Cd}^{2+}$  removal from wastewater by *A. niger* B 77, immobilized in two different polymers: polyvinyl - alcohol hydrogel (PVA) and Ca - alginate. They also obtained a high removal percentage 72.8% for  $\text{Cu}^{2+}$ ; 55.4% for  $\text{Zn}^{2+}$  and 52.3% for  $\text{Mn}^{2+}$  by Ca - alginate immobilized biomass and by immobilized cubes PVA - biomass: 67.1; 58.5 and 44.6%, respectively. These results are similar than the results obtained in this research by *A. brasiliensis* EPAMIG 0084 (Tables 12 and 13).

Concerning the other fungus in the study, *P. citrinum* EPAMIG 0086 showed one point (pH 6 to 5) in pH decrease in the first 15 h, followed by an increase to 7, which was kept until the end of the experiment (120 h) (Table 11). Probably, the pH variation helped the Cu removal by this fungus (Table 12) (44% Cu removal after 120 h), since the removal of this element was significant with time variable (directly related to the pH variation). For the other elements, this relationship was not observed.

For Mn and Zn, there was no significance between these two variables. However, a significant removal of Mn (62%) and Zn (48%) was also obtained by this fungus (Table 13). These results did not corroborate those obtained in removal tests of trace elements on a laboratory scale after 5 days of incubation, with initial concentration of  $5 \text{ mgL}^{-1}$  (same condition of biofilters removal test). Thus, the following results were found: 21.7% removal of Cu, 6.5% Mn, and 1.6% of Zn (Tables 5, 7 and 9). Probably, this difference is due to the influence of the initial pH used in both tests, pH 5 on a laboratory scale and 6 in the aerobic biofilters. According to Tay et al. (2010), as initial pH increases, the active sites are being deprotonated and strengthened the charge attraction, thus leading to significant increase in Cu (II) biosorption uptake.

The above mentioned results were also obtained by Verma et al. (2013). They observed that the biosorption potential of immobilized biomass was higher than the biosorption potential of free biomass. When compared Cu Biosorption potential of immobilized and free biomass of fungus *P. citrinum* immobilized they obtained 76.2% of Cu removal by immobilized biomass and 74% by free biomass. They also showed that the biosorption increased with rise in pH but can be concluded that no significant change in the amount of Cu (II) removal was observed after pH 5.0 (Verma et al., 2013).

Hemambika et al. (2011) studied the trace elements

biosorption by *Aspergillus* sp. and *Penicillium* sp. isolated from effluent collected from an electroplating industry at India that uses copper, cadmium and lead for plating. They compared the trace elements removal by immobilized cells in sodium alginate and free cells. They achieved 60.94 and 46.91% of Cu removal by *Aspergillus* sp. immobilized and free cells respectively. For the *Penicillium* sp., it showed 97.21% of Cd removal by immobilized cells and 95.27% by free cells. Relative to the best pH to trace elements removal, the authors observed that to *Penicillium* sp. growth and trace elements removal which is pH 6; the same pH value reached in this research (Table 11). Then, it is evidence that the pH medium obtained with the *P. citrinum* EPAMIG 0086 cultivation facilitated the trace elements removal.

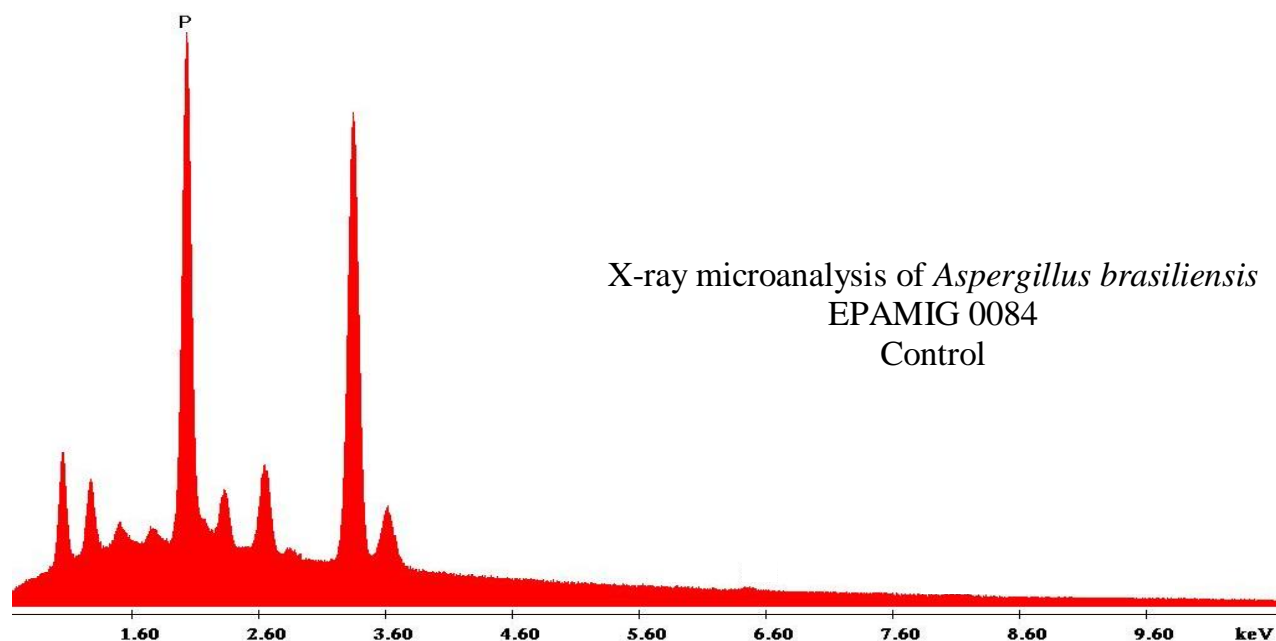
Fourest and Roux (1992) also related a greater trace element removal percentage (mainly Zn) by the filamentous fungus *Rhizopus arrhizus* in pH near neutrality, as well as a decrease of the removal proportional to pH decrease. These authors explain that this phenomenon happens due to the competition by protons and trace elements to the binding sites present on the fungal cell wall. The same was previously observed by Galun et al. (1987) regarding the  $\text{Zn}^{2+}$  uptake by *Penicillium digitatum*. In this study, removal of Zn was highly sensitive to variations in pH, being inhibited at a pH below 3. Probably, this phenomenon occurs also in the present work with fungus *A. niger* EPAMIG 0084, since this fungus changed the media pH from pH 6 to 3 (Table 11) and showed only 29.4% of Zn removal (Table 13) in aerobic biofilters removal test.

Another study evaluated  $\text{Cu}^{2+}$  biosorption by *Penicillium cyclopium*. Removal was considered strongly dependent on pH, on the amount of biomass and on the concentration of Cu ions in the solutions. The biosorption process was fast and, in the first 5 min, up to 75% of the total Cu ions were deposited on the surface of the fungus under study (Tsekova et al. 2006).

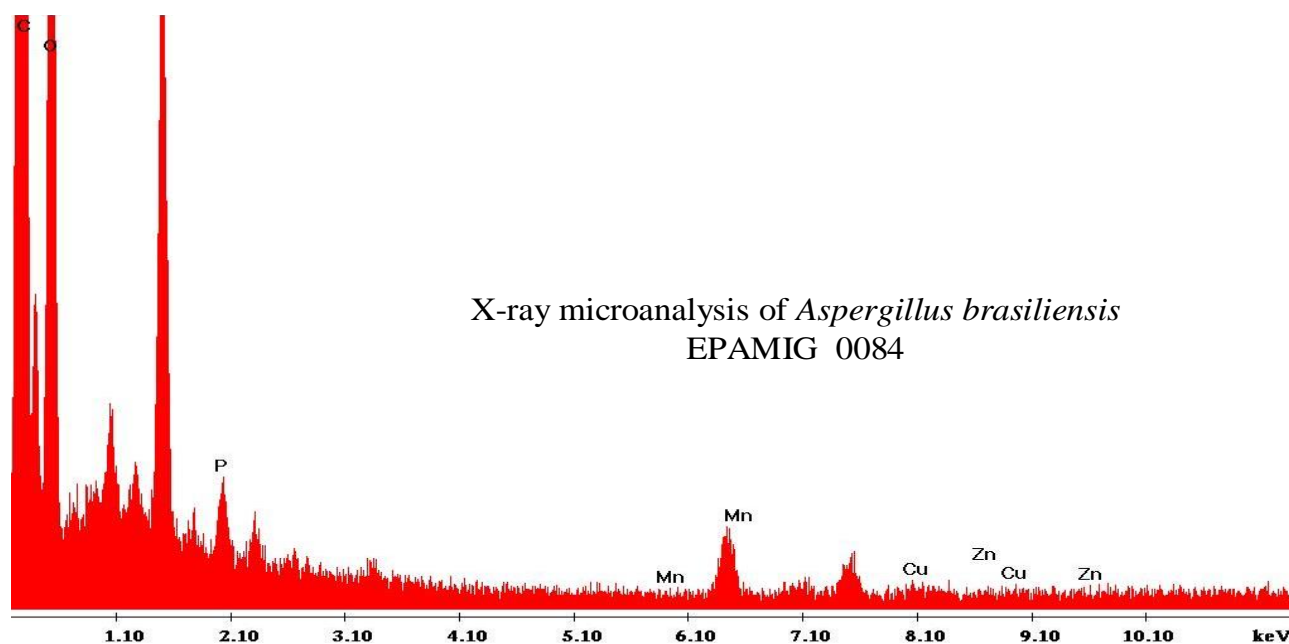
After evaluation of the present results (Tables 12 and 13), it may be concluded that throughout the whole experiment, the fungus *P. citrinum* EPAMIG 0086 more efficiently removed most of the trace elements studied as compared to *A. brasiliensis* EPAMIG 0084. Probably, the trace elements studied are sensitive to pH variation, which occurred in the biofilter inoculated with the fungus *A. brasiliensis* EPAMIG 0084 (Table 11).

### Scanning electron microscopy with X-ray microanalysis

This method of assessing the presence of trace elements detects only those elements that are attached to the fungal cell wall, thus, exhibiting the trace elements that were removed from solution by the biosorption process. However, this analysis, in spite of providing quantitative



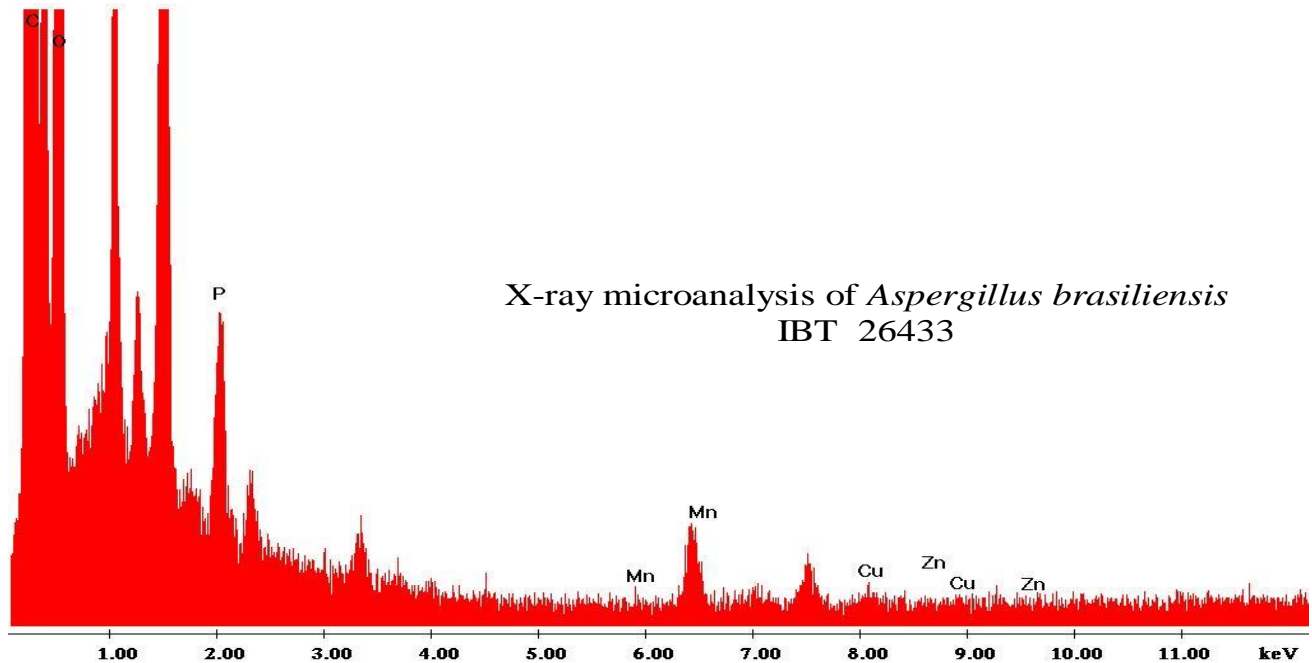
**Figure 1.** Scanning electron microscopy with X-ray microanalysis of the fungus *A. brasiliensis* EPAMIG 0084: control sample - Fungus cultivated without trace elements.



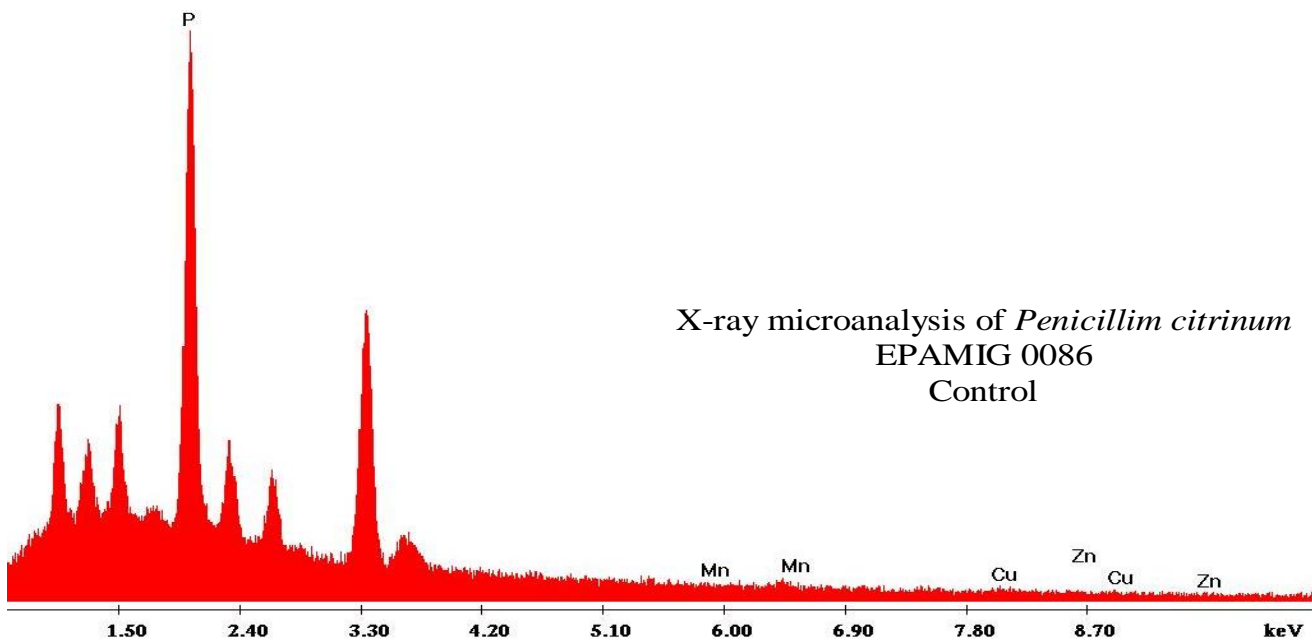
**Figure 2.** Scanning electron microscopy with X-ray microanalysis of the fungus *A. brasiliensis* EPAMIG 0084 isolated from the contaminated region cultivated in the presence of trace elements (Cu, Mn and Zn;  $5 \text{ mgL}^{-1}$ ).

data near the real values, cannot be regarded as a quantitative analysis but only a qualitative one. Figures 1 to 6 present the results of scanning electron microscopy with X-ray microanalysis of *A. brasiliensis* EPAMIG 0084; IBT 26433 and *P. citrinum* EPAMIG 0086; INCQS 40011

after being cultivated in the presence of a trace element solution ( $5 \text{ mgL}^{-1}$ ) for 10 days in 100 rpm at  $25^\circ\text{C}$ . An evaluation of Figures 1 to 6 shows that there was the formation of peaks of the elements Cu, Mn and Zn for the four strains studied, indicating that probably the high



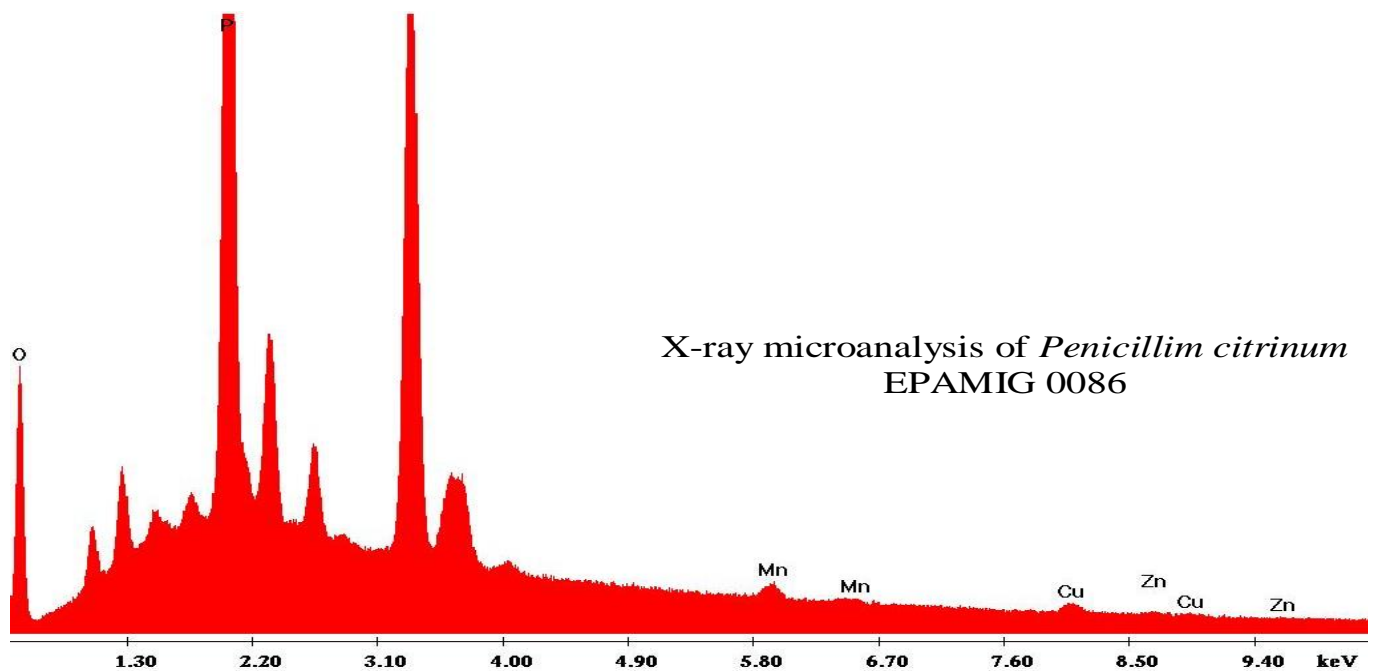
**Figure 3.** Scanning electron microscopy with x-ray microanalysis of the fungus *A. brasiliensis* IBT 26433 isolated from uncontaminated region cultivated in the presence of trace elements (Cu, Mn and Zn; 5 mgL<sup>-1</sup>).



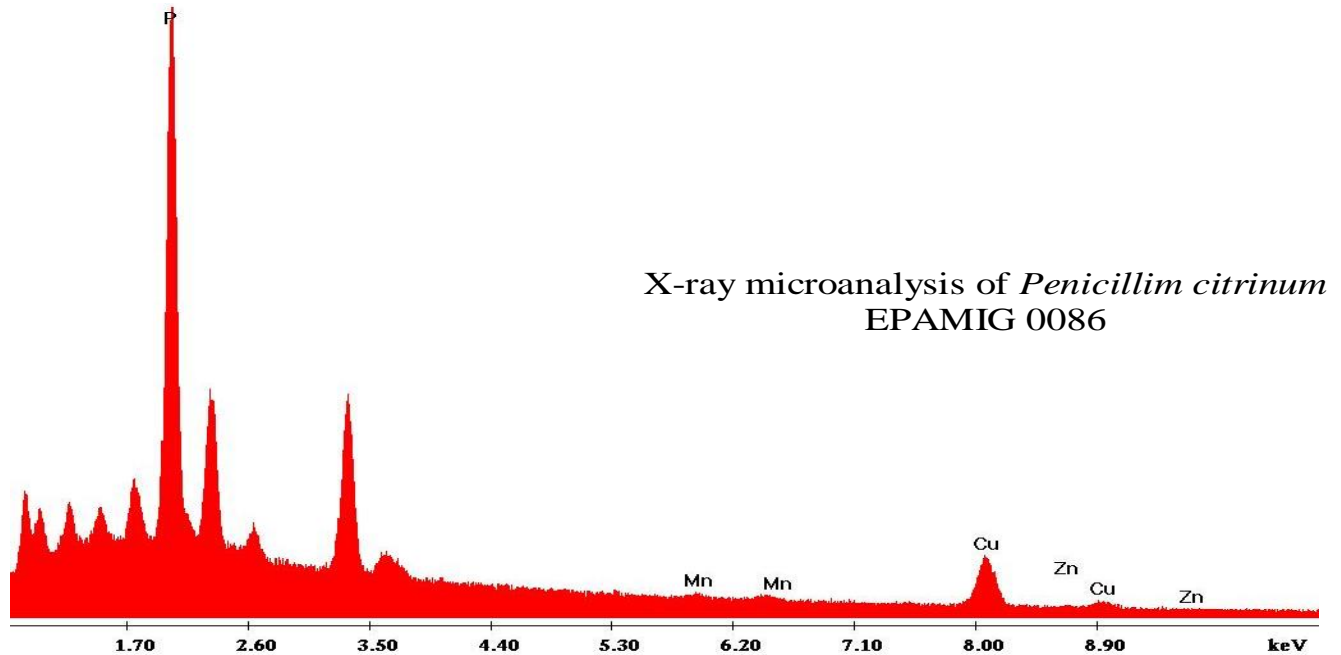
**Figure 4.** Scanning electron microscopy with X-ray microanalysis of the fungus *P. citrinum* EPAMIG 0086: control sample - without trace elements.

percentage trace element removal observed in previous tests could be due to adsorption process of these elements to the fungal cell wall (2008).

Purchase et al. (2009) and Velmurugan et al. (2010) also used scanning electron microscopy with X-ray microanalysis to show Pb adsorption by the fungus



**Figure 5.** Scanning electron microscopy with X-ray microanalysis of the fungus *P. citrinum* EPAMIG 0086 isolated from a contaminated region cultivated in the presence of trace elements (Cu, Mn and Zn; 5 mgL<sup>-1</sup>).



**Figure 6.** Scanning electron microscopy with X-ray microanalysis of the fungus *P. citrinum* EPAMIG 0086, isolated from an uncontaminated region cultivated in the presence of trace elements (Cu, Mn and Zn; 5 mgL<sup>-1</sup>).

*Beauveria bassiana* and Zn by the biomass of *Fusarium* spp. They concluded, through this technique, that the adsorption process is one of the mechanisms responsible

for removal of environment Pb and Zn by these fungi.

Although the results from X-ray microanalyses do not provide qualitative, but quantitative findings, it may be

observed that the peak formed for the Zn element, for all strains tested, was very small in relation to the other trace elements evaluated. This result is in apparent contrast with the previous tests (atomic spectrophotometry) in that Zn was one of the elements removed with greatest efficiency by the fungus being studied (Tables 9 and 12). However, this element must have been absorbed by fungal cells, since it is very important in various enzymatic functions, thus showing that both adsorption and absorption processes play important roles in removal of trace elements from the environment. The same process may have occurred with the other trace elements. However, as can be seen in the Figures 2 to 6, many of these were attached to the fungal cell wall.

## Conclusion

It can be concluded from the present results, that removal is probably related to medium pH. In addition, based on the excellent aerobic biofilters test results, *P. citrinum* EPAMIG 0086 and *P. citrinum* INCQS 40011, regardless of their origin, demonstrated to be very efficient and viable alternatives for solution trace element removal. Therefore, they may be used for coffee wastewater treatment. Since the trace elements are toxic, bioaccumulative and carcinogenic, this study can benefit both the environment close to agricultural regions and public health. Moreover, as a pilot scale project, its applicability in real scale becomes a very close reality, thereby, ensuring environmental and human health quality.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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## REFERENCES

- Adriano DC (2001). Elements in the terrestrial environments: biochemistry, bioavailability and risk of metals. New York, Springer, pp. 866.
- Akar ST, Akar T, Kaynak Z, Anilan B et al. (2009). Removal of copper (II) ions from synthetic solution and real wastewater by the combined action of dried *Trametes versicolor* cells and montmorillonite. Hydrometallurgy 97:98-104.
- Aparicio S (2000). Chromium biosorption by the algal biomass *Sargassum* sp. Tesis - Lulea University of Technology. pp. 45
- Arica MY, Arpa C, Kaya B, Bektas S, Denizli A, Genç O (2003). Comparative biosorption of mercuric ions from aquatic systems by immobilized live and heat-inactivated *Trametes versicolor* and *Pleurotus sajor caju*. Bioresour. Technol. 89:145-154.
- Brierley CL, Kelly DP, Seal KJ, Best DJ (1985). Materials and biotechnology. In: HIGGINS IJ, BEST BJ, JONES J (Ed.). Biotechnology: principles and applications. Oxford: Blackwell Scientific. pp. 163-213.
- Calfa BA, Torem ML (2007). Bioreagents - their use in the removal of heavy metals from liquid streams by biosorption/ bioflotation. Rev. Escol. de Minas 60 (3):537-542.
- Da Silva M, Umbuzeiro GA, Pfenning LH, Canhos VP, Esposito E (2003). Filamentous Fungi Isolated from Estuarine Sediments Contaminated with Industrial Discharges. Soil Sediment. Contam. 12:345-356.
- Davis AP, Shokouhian M, Ni S (2001). Loading estimates of lead, copper, cadmium and zinc in urban runoff from specific sources. Atmosphere 44:997-1009.
- Ezzouhri L, Castro E, Moya M, Espinola F, Lairin K (2009). Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco. Afr. J. Microbiol. Res. 3:35-48.
- Faryal R, Lodhi A, Hameed A (2006). Isolation, characterization and biosorption of zinc by indigenous fungal strains *Aspergillus fumigatus* RH05 and *Aspergillus flavus* RH07. Pak. J. Bot. 38: 817-832.
- Foley JA, Navin R, Kate AB, Emily SC, James SG et al. (2011). Solutions for a cultivated planet. Nature 478:337-342.
- Fourest E, Roux JC (1992). Heavy metal biosorption by fungal mycelial by-products: mechanism and influence of pH. Appl. Microbiol. Biot. 37:399-403.
- Fu F, Wang Q (2011). Removal of heavy metal ions from wastewaters: A review. J. Environ. Manag. 92:407-418.
- Gadd GM (1992). Biosorption. J. Chem. Technol. Biotechnol. 55:302-304.
- Galun M, Galun E, Siegel Bz, Keller P, Lehr H, Siegel Sm (1987). Removal of metal ions from aqueous solutions by *Penicillium* biomass: Kinetic and uptake parameters. Water Air Soil Pollut. 33:359-371.
- Gonçalves M (2006). Characterization and oxidation of organic compounds in wastewater from pulping wet coffee fruits. Dissertation, Lavras Federal University.
- Hemambika B, Rani MJ, Kannan VR (2011). Biosorption of heavy metals by immobilized and dead fungal cells: A comparative assessment. J. Ecol. Nat. Environ. 3:168-175.
- Kapoor A, Viraraghavan T (1995). Fungal-biosorption: an alternative treatment option for heavy metal bearing wastewater: a review. Bioresour. Technol. 53:195-206.
- Kapoor A, Viraraghavan T, Cullimore DR (1999). Removal of heavy metals using the fungus *Aspergillus niger*. Bioresour. Technol. 70: 95-104.
- Kiekens L, Cottenie A (1985). Principles of investigations on the mobility and plant uptake of heavy metals. In: Leschber R et al. (Ed.). Chemical methods for assessing bioavailable metals in sludges and soils. New York: Elsevier. pp. 21-32.
- Lemos JLS, Marion Cony Carlos, Farias YPTMM, Dos Santos RLC

- (2008). Review on the use of microorganisms in bioremediation of industrial wastes containing heavy metals. Rio de Janeiro: CETEM/MCT.
- Lodeiro P, Barriada JL, Herrero R, Sastrede VME (2006). Themarine macroalga *Cystoseira baccata* as biosorbent for cadmium(II) and lead(II) removal: kinetic and equilibrium studies. *Environ. Pollut.* 142:264-273.
- Melo AC de P (2009). Use of wastewater from post-harvest process in coffee production coffee seedlings. Dissertation, Universidade Estadual Paulista "Júlio de Mesquita Filho".
- Melo IS, Azevedo JL (2008). *Environmental Microbiology*. 2. ed. rev. ampl. Jaguariúna: Embrapa Meio Ambiente.
- Osaizua OS, Olateju KS, Oni-Orisan A, Olanike AY (2014). Tolerance, bioaccumulation and biosorption potential of fungi isolated from metal. *Journal of Applied Sciences in Environmental Sanitation*. 9:35-44.
- Pan R, Cao L, Zhang R (2009). Combined effects of Cu, Cd, Pb and Zn on the growth and uptake of consortium of Cu-resistant *Penicillium* sp. A1 and Cd-resistant *Fusarium* sp. A19. *J. Hazard. Mater.* 171:761-766.
- Purchase D, Scholes LN, Revitt DM, Shutes RB (2009). Effects of temperature on metal tolerance and the accumulation of Zn and Pb by metal-tolerant fungi isolated from urban runoff treatment wetlands. *J. Appl. Microbiol.* 106:1163-1174.
- Ren WX, Li PJ, Geng Y, Li XJ (2009). Biological leaching of heavy metals from a contaminated soil by *Aspergillus niger*. *J. Hazard. Mater.* 167:164-169.
- Ribeiro MS, Lima LA, Faria FHS, Rezende FC, Faria LA. Effects of processing coffee wastewater on first year coffee plants growth (2009). *Eng. Agríc.* 29:569-577.
- Rouch DA, Lee BT, Morby AP (1995). Understanding cellular responses to toxic agents: a model for mechanism choice in bacterial metal resistance. *J. Ind. Microbiol. Biotechnol.* 14:132-141.
- Saxena P, Bhattacharyya AK, Mathur N (2006). Nickel tolerance and accumulation by filamentous fungi from sludge of metal finishing industry. *Geomicrobiol. J.* 23:333-340.
- Saya R, Denizli A, Yakup AM (2001). Biosorption of cadmium (II), lead (II) and copper (II) with the filamentous fungus *Phanerochaete chrysosporium*. *Bioresour. Technol.* 76:67-70.
- Sayer JA, Gadd GM (2001). Binding of cobalt and zinc by organic acids and culture filtrates of *Aspergillus niger* grown in the absence or presence of insoluble cobalt or zinc phosphate. *Mycol. Res.* 105:1261-1267.
- Sheng PX, Wee KH, Ting YP, Chen JP (2008). Biosorption of copper by immobilized marine algal biomass. *Chem. Eng. J.* 136:156-163.
- Silva JF (2008). Desenvolvimento de biofiltro para avaliar a capacidade de biossorção de cobre pelo fungo *Cladosporium cladosporioides*. Thesis, Lavras Federal University.
- Sprocati AR, Alisi C, Segre L, Tasso F, Galletti M, Cremisini C (2006). Investigating heavy metals resistance, bioaccumulation and metabolic profile of a metallophilic microbial consortium native to an abandoned mine. *Sci. Total Environ.* 366:649-658.
- Tay CC, Redzwan G, Liew HH, Yong SK, Surif S, Talib SA (2010). Cu (II) biosorption characteristic of *Pleurotus spent* mushroom compost. In: Intern. Conference on Science and Social Research, December 5-7, Kaula Lumpur, Malaysia.
- Tsekova K, Christova D, Ianis M (2006). Heavy Metal Biosorption sites in *Penicillium cyclopium*. *J. App. Sci. Environ. Manag.* 10: 117-121.
- Tsezos M, Deutschmann AA (1990). An investigation of engineering parameters for the use of immobilized biomass particles in biosorption. *J. Chem. Technol. Biotechnol.* 48: 29-39.
- Varga J, Kocsu S, Tóth B, Frisvad JC, Perrone G, Susca A, Meijer M, Samson RA (2007). *Aspergillus brasiliensis* sp. nov., a biseriolate black *Aspergillus* species with world-wide distribution. *Int. J. of Syst. Evol. Micr.* 57: 1925-1932.
- Vasco EZ (1999). Procesamiento de frutos do café por via húmeda y generación de subproductos. In: Seminário Internacional Sobre Biotecnologia Na Indústria Cafeeira 3, Londrina. Anais... Londrina: UFRP/IAPAR/IRD pp. 345-355.
- Velmurugan P, Shim J, You Y, Choi S, Kamala-Kannan S, Lee KJ, Kim HJ, Oh BT (2010). Removal of zinc by live, dead, and dried biomass of *Fusarium* spp. isolated from the abandoned-metal mine in South Korea and its perspective of producing nanocrystals. *J. Hazard. Mater.* 182: 317-324.
- Verma A, Narsi SA, Bishnoi N R, Gupta A (2013). Biosorption of Cu (II) using free and immobilized biomass of *Penicillium citrinum*. *Ecol. Eng.* 61: 486-490.
- Vivas A, Barea JM, Azcon R (2005). *Brevibacillus brevis* isolated from cadmium- or zinc-contaminated soils improves in vitro spore germination and growth of *Glomus mosseae* under high Cd or Zn concentrations. *Microb. Ecol.* 49: 416-424.
- Wuana RA, Okieimen FE (2011). Heavy Metals in Contaminated Soils: A Review of Sources, Chemistry, Risks and Best Available Strategies for Remediation. *ISRN Ecology*. 2011, 20p.
- Zafar S, Aqil F, Ahmad I (2007). Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil. *Bioresour. Technol.* 98: 2557-2561.
- Zaidi A, Oves M, Ahmad E, Khan M S (2011). Importance of Free-Living Fungi in Heavy Metal Remediation, in: Khan M S, Zaidi A, Goel R, Musarrat J. *Biomanag. Metal Contam. Soils* 20:479-494.

Full Length Research Paper

## Phenotypic and molecular characterization of *Salmonella* serotypes in cow raw milk and milk products in Nigeria

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The presence of *Salmonella* and human pathogens in unpasteurized milk remains a public health hazard. The study reported the phenotypic and molecular characterization of *Salmonella* serotypes in cow raw milk, cheese and traditional yoghurt marketed for man's consumption in Nigeria. Isolation of *Salmonella* was done on *Salmonella* and *Shigella* agar by pour plate method at 37°C. Susceptibility to antibiotics was determined by the disc diffusion method. Molecular characterization of the *Salmonella* serotypes and detection of some target genes was done by the polymerase chain reaction. All the isolates were sensitive to ofloxacin and nalidixic acid. Resistance to antibiotics was in varying proportions with amoxicillin (95.2%), tetracycline (90.4%) and augmentin (28.5%). Ten (10) different multiple antibiotic resistance patterns were observed among the serotypes. The serotypes identified include *Salmonella typhi*, *Salmonella typhimurium* and *Salmonella enteritidis*, and the serogroups include D (28.6%), B (52.4%), D<sub>1</sub> (28.6%), respectively. The *invA* genes were amplified in all the *Salmonella* serotypes while *fliC* and *fliB* genes were absent. The *rfbJ* gene was detected only in *S. Typhimurium*. The recovery of multiple antibiotic resistant pathogenic *Salmonella* serotypes in cheese and yoghurt samples in the study is of great health concern.

**Key words:** *Salmonella* serotype, molecular characterization, virulence genes, antibiotics.

### INTRODUCTION

The presence of *Salmonella* and other microorganisms in milk underscore its importance as a vehicle of human

infection. For this reason, the presence of *Salmonella* and human pathogens in unpasteurized milk remains a

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**Abbreviations:** NAL, Nalidixic acid; COT, cotrimoxazole; AMX, amoxicillin; NIT, nitrofurantoin; GEN, gentamicin; AUG, augmentin; TET, tetracycline; MAR, multiple antibiotic resistance; *invA*, invasion gene; *RfbJ*, *FliC*, *FljB*, adherence genes.

public health hazard. Milk-borne illnesses have been recognized since the beginning of the dairy industry.

Many diseases are transmissible via milk products; raw or unpasteurized milk has been a major vehicle for transmission of pathogens (Vasavada, 1988). The use of unclean teats, milking and transporting equipment contributes to the poor hygienic quality of traditional milk products (Altug and Bayrak, 2003). Milk products such as yoghurt, ice cream, and cheese are widely consumed and market for them has existed in many parts of the world for many generations. There are 16 million annual cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths worldwide due to *Salmonella* (Bhunia, 2008). *Salmonella* is the most frequent pathogen associated with outbreaks of foodborne illnesses (80.5% of the outbreaks), followed by *Escherichia coli* (10.1%) (De Buyser et al., 2001). Cooked food products and raw milk were most commonly contaminated with food borne pathogens and many of them were resistant to different antibiotics (Walsh et al., 2005; Normanno et al., 2007; Okpalugo et al., 2008 and Novakova et al., 2010). Contamination of dairy foods with virulent pathogens renders them to be a source of public health hazard. The possible contamination sources are either mastitis in dairy cow or the milk itself (Carter, 1995). Ubiquitous serotypes such as *Salmonella Enteritidis* or *Salmonella Typhimurium* which affect both man and animals generally cause gastrointestinal infections usually less severe than enteric fever. However, they also have the capacity to produce typhoid-like infections in mice and in humans or asymptomatic intestinal colonization in chickens (Velge et al., 2005). Growing concerns over food safety among the consumers call for the manufacturing and processing of foods under extremely hygienic conditions to avoid possible health challenges (Farzana et al., 2009). According to Pui et al. (2011), epidemiologic classification of *Salmonella* is based on the host preferences. The first group includes host-restricted serotypes that infect only humans such as *S. Typhi*. The second group includes host-adapted serotypes which are associated with one host species but can cause disease in other hosts serotypes such as *S. Pullorum* in avian. The third group includes the remaining serotypes. Typically, *S. enteritidis*, *S. typhimurium* and *Salmonella* Heidelberg are the three most frequent serotypes recovered from humans each year (Gray and Fedorka-Cray, 2002; Boyen et al., 2008). Kauffmann-White scheme classifies *Salmonella* according to three major antigenic determinants composed of flagellar H antigens, somatic O antigens and virulence (Vi) capsular K antigens. More than 99% of *Salmonella* strains causing human infections belong to *Salmonella enterica* subspecies *enterica*. The infectious dose of *Salmonella* depends upon the serovar, bacteria strain, growth condition and host susceptibility. However, single-food-source outbreaks indicate that as little as 1 to 10

cells can cause salmonellosis with more susceptibility to infection (Yousef and Carlstrom, 2003; Bhunia, 2008). Pui et al. (2011) in their review article implicated poultry, eggs and dairy products as the most common vehicles of salmonellosis; including contaminated environment with moving animals (swines, cows and chickens) as vectors hence, constituting important risk factor for infection. Salmonellosis continues to be a major public health problem worldwide and contributes to negative economic impacts thus, making research on *Salmonella* gained great interest and concern from scientists.

The present study reported the molecular characterization of *Salmonella* serotypes in cow raw milk, cheese and traditional yoghurt samples in Nigeria and their consequent health hazards in man.

## MATERIALS AND METHODS

### Collection of samples

Thirty samples comprising each of cow raw milk, yoghurt and cheese were collected. Raw milk samples were collected from individual cows marketed at different markets in Ile-Ife, Modakeke, Edun-abon, and Akinlalu, Osun State, Nigeria. The raw milk was collected in a sterile container by placing the container underneath the cow for milking. Yoghurt samples were bought from the hawkers at various markets within the same localities while local cheese (wara) was equally purchased from the same market as above in transparent polythene bags. All cheese and yoghurt samples were transported to the laboratory under refrigeration (4 to 6°C) in thermal boxes containing ice packs and were analyzed bacteriologically within one hour of collection. Raw milk samples were transported to the laboratory at ca.4°C. All samples were collected between June and August, 2011.

### Isolation and characterization of *Salmonella* isolates

Isolation of *Salmonella* species was done on MacConkey agar and *Salmonella-Shigella* agar (SSA) (Oxoid Ltd., Hampshire, England) plates. One milliliter of a five- fold dilution of raw milk and yoghurt was cultured on the sterile agar plates by pour plate technique. For cheese sample, 10 g was aseptically transferred into a sterile mortar and pestle, and homogenized in 90 ml sterile distilled water using stomacher. One milliliter of the 10 fold dilutions of the homogenized cheese was cultured appropriately as above. All plates were incubated at 37°C for 48 h. The isolates were differentiated first on the basis of colonial morphology and microscopic examination. The identity of isolates was confirmed by various biochemical tests with reference to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1994).

### Antibiotic susceptibility

Antibiotic susceptibility of isolates was performed by antibiotic disc diffusion technique on Mueller Hinton agar (Hi Media, Vadhani, India) (Clinical and Laboratory Standards Institute (CLSI) 2008). The antibiotics (oxoid, UK), augmentin (30 µg), nitrofurantoin (200 µg), ofloxacin (5 µg), tetracycline (30 µg), gentamicin (10 µg), amoxicillin (20 µg), cotrimoxazole (25 µg), and nalidixic acid (30 µg) were firmly placed on the agar plates previously seeded with the



**Table 1.** Percentage distribution of *Salmonella* sp. in raw milk, yoghurt and cheese.

Sample	Occurrence (%)
Yoghurt (n= 10)	9 (90)
Cheese (n= 10)	10 (100)
Raw milk (n=10)	1 (10)

**Table 2.** Antibiotic resistance profile of *Salmonella* sp in raw milk, cheese and yoghurt samples in Nigeria.

Antibiotic ( $\mu\text{g}$ )	Resistant	
	Isolates (n)	(%)
Nalidixic acid (30 $\mu\text{g}$ )	0	0
Cotrimoxazole (25 $\mu\text{g}$ )	13	61.9
Amoxicillin (20 $\mu\text{g}$ )	20	95.2
Nitrofurantoin (300 $\mu\text{g}$ )	7	33.3
Gentamicin (10 $\mu\text{g}$ )	11	52.3
Ofloxacin (5 $\mu\text{g}$ )	0	0
Augmentin (30 $\mu\text{g}$ )	6	28.5
Tetracycline (30 $\mu\text{g}$ )	19	90.4

test organisms and incubated at 37°C for 24 h. Sensitivity of the isolates to different antibiotics was indicated by the clear zones of inhibition which were measured in milliliter using a calibrated ruler. The diameters of zone of inhibition were compared with the interpretative chart of zone sizes of susceptibility to antibiotics (CLSI, 2008). Resistance to three or more antibiotics by isolate was defined as multiple antibiotic resistance (MAR).

#### Polymerase chain reaction and amplification of genes

The target genes selected for *Salmonella* sp. were invasive- *invA* (*Salmonella*-specific), adherence- *rfbJ*(B) (serogroup B), *fliC* (I, v) and *fliB* (e, n and z15) using appropriate primers. Oligonucleotide primers were used for the amplification of *invA*, *rfbJ*(B) and *fliB* genes.

A 50  $\mu\text{l}$  reaction mixture contained 1  $\mu\text{l}$  of template DNA (approximately 50 ng), 25 Pmol of each primer, 200  $\mu\text{M}$  deoxyribonucleotide triphosphate mixture, 8  $\mu\text{l}$  of 10X PCR buffer, 1.5 mM  $\text{MgCl}_2$ , 2.5 U of Taq polymerase was used. The standard cycling condition after the initial denaturation step of 5 min at 94°C, were 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C for 35 cycles and final extension step of 5 min at 72°C. For some strains, the annealing temperature was reduced to 54 to 55°C to increase the amount of product that was amplified. PCR products (10  $\mu\text{l}$ ) were separated by electrophoresis in 0.8% agarose gel in Trisborate buffer (0.089 M Tris-base, 0.089 M boric acid, 2.5 mM EDTA- $\text{Na}_2$ , pH 8), with the 1 kbp DNA ladder as a molecular size marker.

#### Statistical analysis

Comparisons of the final values were compared statistically using Chi-square Tests of the statistical analysis system software (SPSS 16.0 version). P value of < 0.05 was considered statistically significant for all the comparisons.

## RESULTS

*Salmonella* sp. were recovered in all the cheese and yoghurt samples but found in only one of the raw milk samples analysed. The percentage occurrence of *Salmonella* sp. in the samples includes raw milk (10 %), yoghurt (90%) and cheese (100%) (Table 1). The antibiotic susceptibility profile of the isolates from cow raw milk, yoghurt and cheese samples is shown in Table 2.

Generally, all the *Salmonella* serotypes were sensitive to ofloxacin and nalidixic acid. Resistance to antibiotics was in varying proportions with 95.2% developing resistance to amoxicillin, 90.4% to tetracycline and 28.5% to augumentin. Resistance was generally higher to amoxicillin than other antibiotics ( $p>0.05$ ).

Ten (10) different multiple antibiotic resistance (MAR) patterns were displayed by the isolates with 41.17% showing resistance to three different classes of antibiotics (Table 3). All the *Salmonella* serotypes were multiple antibiotic resistant.

Table 4 shows the result of the polymerase chain reaction for the detection of *Salmonella* serotypes. The serotypes identified include *S. typhi*, *S. typhimurium*, *S. Enteritidis*, and the serogroups were D, B, D<sub>1</sub> respectively. Out of the 21 strains of *Salmonella* species, 52.4% belong to B serogroup while 20.0 and 28.6% belong to D and D<sub>1</sub> serogroups respectively. The *invA* gene was amplified in all the *Salmonella* serotypes while *rfbJ* was detected only in *S. Typhimurium*. The genes *fliC* and *fliB* were absent in all the serotypes. Figure 1 presents the PCR amplification of *invA* and *rfbJ* genes in *Salmonella* serotype obtained from cow raw milk and milk products.

## DISCUSSION

The detection of *S. typhi*, *S. typhimurium* and *S. enteritidis* in cheese and yoghurt samples analysed may probably be due to contaminated water in processing traditional dairy products, infected people who produce dairy products, contaminated equipment, and lack of public and individual hygiene. In this study, only 4.8% of the raw milk samples yielded *Salmonella* isolate. This may likely be due to observance of simple hygiene during collection. The findings of this present studies agree with the reports of earlier researchers who reported the presence of pathogenic bacteria in cow milk in Czech Republic, cow foremilk in Botswana, pasteurized milk and soya milk in Nigeria (Adeleke et al., 2000; Schlegelova et al., 2002; Guta et al., 2002; Okpalugo et al., 2008).

Previous studies by Fontaine et al. (1980) reported the isolation of *S. typhi* as the causative agents of mastitis in dairy animals which perhaps might have led to the contamination of milk from the udder of infected animals.

*Salmonella* sp. also reside in the intestinal tract where

**Table 3.** Multiple antibiotic resistance patterns among the *Salmonella* isolates in raw milk, cheese and yoghurt samples in Nigeria.

No. of antibiotic	MAR patterns	Isolates	Total (%)
3	COT, AMX, TET	1	7 (41.17)
	AMX, AUG, TET	2	
	AMX, NIT, TET	2	
	AMX, GEN, TET	2	
4	COT, AMX, AUG, TET	1	7 (41.17)
	COT, GEN, AUG, TET	1	
	COT, AMX, GEN, TET	4	
	COT, AMX, AUG, TET	1	
5	COT, AMX, NIT, GEN, TET	1	1 (5.88)
6	COT, AMX, NIT, GEN, AUG, TET	2	2 (11.76)

NAL, Nalidixic acid; COT, Cotrimoxazole; AMX, Amoxicillin; NIT, Nitrofurantoin; GEN, Gentamicin; AUG, Augmentin; TET, Tetracycline; MAR, multiple antibiotic resistance.

**Table 4.** Detection of *Salmonella* serotypes by Polymerase Chain Reaction.

<i>Salmonella</i> serotypes	Serogroup	Antigenic formular	Percentage number (%)	Number of strains positive for target gene			
				<i>InvA</i>	<i>rfbJ</i>	<i>fliC</i>	<i>fliB</i>
<i>Typhi</i>	D	9,12 (Vi):d	4 (20.0%)	4	-	-	-
<i>Typhimurium</i>	B	4,(5),12:i:1,2	11 (52.4%)	11	11	-	-
<i>Enteritidis</i>	D <sub>1</sub>	9,12:g,m	6 (28.6%)	6	-	-	-

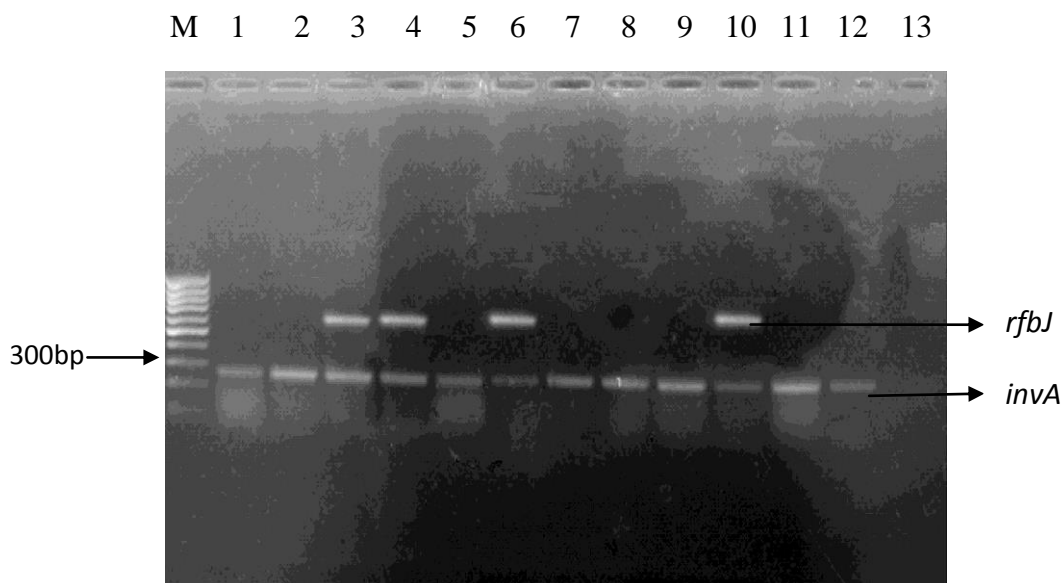
-, Not found.

they cause gastro-enteritis in animals and may have occurred in milk as a result of faecal contamination.

All the *Salmonella* serotypes (*S. typhi*, *S. typhimurium* and *S. enteritidis*) were resistant to more than three classes of antibiotics used. Resistance by isolates to antibiotics may however, have been acquired from over exposure to antibiotics particularly in the veterinary sector or to a lesser extent from natural sources especially when some of the antibiotics tested in this study are commonly used in both the health and veterinary sectors. Studies had reported that of the more than 1 million tons of antibiotics released into the biosphere during the past years, approximately 50% are estimated to flow into the veterinary and agricultural channels (Mazel and Davies, 1999). The application of these antibiotics at sub-therapeutic levels for increased growth and feed efficiencies in farm animals is an integrated part of modern agriculture worldwide, and this leads to the emergence of antibiotic-resistant microbes (Prescott, 2000). When the same antibiotics are used in humans as in animals, resistant microbes which passed on to humans from animal sources could then lead to treatment failure in

humans with serious public health implications. The present study recorded high resistance to amoxicillin, cotrimoxazole and tetracycline, which could be due to misuse of these antibiotics in disease conditions. This study also reveals that all the multi-resistant strains showed a characteristic tetracycline resistance trait. This may account for the indiscriminate use of the antibiotic invariably resulting to selection by the organism. Reduced susceptibility to tetracycline has been reported common in *S. typhimurium* isolates from healthy breeder and broiler flocks in Portugal (Clemente et al., 2014). The multiple resistance recorded in our study was against those antibiotics frequently employed in public health and veterinary sectors. The sensitivity of all the *Salmonella* serotypes to nalidixic and ofloxacin may account for the non-abuse of these antibiotics.

Pui et al. (2011) in their review reported that *Salmonella* strains resistance to one or more antibiotics have increased in the Saudi Arabia, United States, United Kingdom and other countries of the world. This is due to the increased and uncontrolled use as well as easy accessibility to antibiotics in many countries of the world



**Figure 1.** The PCR amplification of *invA* and *rfbJ* genes in *Salmonella* serotype obtained from cow raw milk and milk products. M, 100-bp DNA ladder; lanes 3, 4, 6, 10, *S. typhimurium* isolates; lanes 1, 2, 5, 7, 8, 11; *S. typhi* isolates; lane 9, *S. Enteritidis*; lane 12, positive control; lane 13, negative control.

(Grob et al., 1998; Yoke-Kqueen et al., 2007). In this study, *S. typhi* and *S. typhimurium* were multiresistant to the antibiotics tested, thus confirming the earlier reports in Africa. Emerging resistance in *S. typhi* has been described especially in Africa and Asia and the appearance of *S. typhimurium* DT104 in the late 1980s raised main public health concern, thereby threatening the lives of infected individuals (Grob et al., 1998). Evidence exists to suggest that not only are such antibiotic resistant strains more difficult to control in terms of human infection, they may also be more resistant to heat processes (Davidson and Henson, 1995). This is of great concern because majority of infections with MAR *Salmonella* are acquired through the consumption of contaminated foods of animal origin such as swines and chicken eggs.

Asai et al. (2010) mentioned that cephalosporin and fluoroquinolone-resistant strains of *S. Choleraesuis* have been identified in swines in Taiwan and Thailand. Previous study by Prapas et al., (2008) reported that *S. typhimurium* and *Salmonella* Heidelberg ranked first and second, respectively in multidrug resistance, and is among the most commonly isolated serovars from dairy products.

Due to the use of antibiotics for the promotion of growth and prevention of disease in food animals, there is an increase of human salmonellosis cases caused by food-borne MAR *Salmonella* nowadays (Yang et al., 2010). This indiscriminate and injudicious use of antibiotics in any setting especially in food animals worldwide should be monitored to reduce the transfer risk of MAR

*Salmonella* to humans. Finally, there is a need for continuous surveillance and sharing of antimicrobial susceptibility data for *Salmonella* among countries worldwide to ensure the effectiveness of control programmes.

The amplification of *invA* and *rfbJ* genes by polymerase chain reactions in the *Salmonella* serotypes revealed that the isolates are pathogenic. *S. typhimurium* was the most predominant occurring serotype (52.4 %). The presence of *invA* and *rfbJ* genes may cause *Salmonella* infections to become invasive and result in bacteremia and serious extra intestinal disease since it is the genes code for invasion and attachment. *Salmonella* serotypes have been implicated in several diseases, including enteric or typhoid fever (primarily *S. typhi* and *S. paratyphi*), bacteremia, endovascular infections, focal infections (osteomyelitis), and enterocolitis (typically *S. typhimurium*, *S. enteritidis*, and *S. heidelberg*) (Brenner et al., 2000).

## Conclusion

The recovery of MAR pathogenic *Salmonella* serotypes in cheese and yoghurt samples in the study areas calls for great health concern as these organisms have been associated with salmonellosis and other grave diseases. Good quality raw materials for product processing, adoption of Good Manufactured Practices (GMP) and strict personal hygiene will ensure safety and high quality dairy products.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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## REFERENCES

- Adeleke OE, Adeniyi BA, Akinrinmisi AA (2000). Microbiological quality of local soymilk: a public health appraisal. *Afr. J. Biomed. Res.* 3:89-92.
- Altug G, Bayrak Y (2003). Microbial analysis of caviar from Russia and Iran. *Food Microbiol.* 20:83-86.
- Asai T, Namimatsu T, Osumi T, Kojima A, Harada K, Aoki H, Toshiya S, Takahashi T (2010). Molecular typing and antimicrobial resistance of *Salmonella enterica* subspecies *enterica* serovar *Choleraesuis* isolates from diseased pigs in Japan. *Comp. Immunol. Microbiol. Infect. Dis.* 33(2):109-119.
- Bhunia AK (2008). Food borne microbial pathogens: Mechanisms and pathogenesis. United States of America: Springer Science + Business Media, LLC.
- Boyen F, Haesebrouck F, Maes D, Van Immerseel F, Ducatelle R, Pasmans F (2008). Non-typhoidal *Salmonella* infections in pigs: A closer look at epidemiology, pathogenesis and control. *Vet. Microbiol.* 130 (1-2):1-19.
- Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B (2000). *Salmonella* nomenclature. *J. Clin. Microbiol.* 38: 2465-2467.
- Buchanan RE, Gibbons NE (1994). *Bergey's Manual of Determinative Bacteriology*. 8<sup>th</sup> edition. The Williams and Wilkins company. Baltimore, USA.
- Carter GR (1995). *Veterinary Microbiology*. Academic Press, Philadelphia.
- Clemente L, Correeia I, Themdo P, Neto I, Canica M, Bernardo F (2014). Antimicrobial susceptibility of *Salmonella* enteric isolates from healthy breeder and broiler flocks in Portugal. *Vet. J.* 200(2):276-281.
- CLSI (2008). Performance standards for antimicrobial susceptibility testing. Eighteenth informational supplement. Clinical laboratory standard institute, CLSI document M100-S18, Wayne, PA.
- Davidson PM, Henson TM (1995). Comparison of D55C - values of antibiotic resistant and antibiotic sensitive strains of *Salmonella*. *J. Food Prot.* 58: 40-43.
- De Buyser ML, Dufour B, Maire B, Lafarge V (2001). Implication of milk and milk products in foodborne disease in France and in different industrialized countries. *Int. J. Food Microbiol.* 67:1-17.
- Farzana K, Akhtar S, Jabeen F (2009). Prevalence and antibiotic resistance of bacteria in two ethnic milk based products. *Pak. J. Bot.* 41:935-943.
- Fontaine RE, Cohen ML, Martin WT, Vernon TM (1980). Epidemic salmonellosis from cheddar cheese-surveillance and prevention. *Am. J. Epidemiol.* 111:247-254.
- Gray JT, Fedorka-Cray PJ (2002). *Salmonella*. In Cliver, D. O. and Riemann, H. P. (Eds.). *Food-borne diseases*, San Diego: Academic Press. 2002:55-68.
- Grob U, Tschape H, Bednarek I, Frosch M (1998). Antibiotic resistance in *Salmonella enteric* serotype *Typhimurium*. *Eur. J. Clin. Microbiol. Infect. Dis.* 17: 385-387.
- Guta C, Sebunya TK, Gashe BA (2002). Antimicrobial Susceptibility of *Staphylococcus* sp from Cow Foremilk originating from dairy farms around Gaborone, Botswana. *East Afr. Med. J.* 79(1):1-4.
- Mazel D, Davies J (1999). Antibiotic resistance in microbes. *Cell Mol. Life Sci.* 56:742-754.
- Normanno G, La Salandra G, Dambrosi A, Quaglia NC, Corrente M, Parisi A, Santagada G, Firinu A, Crisetti E, Celano GV (2007). Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *Int. J. Food Microbiol.* 115:290-296.
- Novakova I, Kacaniova, M, Arpasova H, Hascik P, Kunova S, Cubon A (2010). Antibiotic Resistance of Enterococci and Coliform Bacteria in Dairy Products from Commercial Farms. *Scientific Papers. Anim. Sci. Biotechnol.* 43(1):1-3.
- Okpalugo J, Ibrahim K, Izebe KS, Inyang US (2008). Aspects of microbial quality of some milk products in Abuja, Nigeria. *Trop. J. Pharm. Res.* 7(4):1169-1177.
- Prapas P, Zewde B, Tadesse D, Hoet A, Gebreyes W (2008). Characterization of Multidrug-Resistant *Salmonella enterica* Serovar *heidelberg* isolated from humans and animals dairies. *Foodborne Pathog. Dis.* 5:839- 851.
- Prescott JF (2000). Antimicrobial drug resistance and its epidemiology. In: *Antimicrobial Therapy in Veterinary Medicine*, edn 3. Edited by Prescott J. F., Baggot J. D., Walker RD. Ames: Iowa State University Press. pp. 27-49.
- Pui CF, Wong WC, Chai LC, Tunung R, Jeyaletchumi P, Hiayah MS, Ubong A, Farinazlaen MG, Cheah Y, Son R (2011). *Salmonella*: A foodborne pathogen. *Int. Food Res. J.* 18:465-473
- Schlegelova J, Babak V, Klimova E, Lukasova J, Navratilova P, Sustackova A, Sediva K, Rysanek D (2010). Prevalence of and resistance to anti-microbial drugs in selected microbial species isolated from bulk milk samples. *J. Vet. Med.* 49:216-225.
- Vasavada PC (1988). Pathogenic bacteria in milk: A review. *J. Dairy Sci.* 71:2809-2816.
- Velge P, Cloeckert A, Barrow P (2005). Emergence of *Salmonella* epidemics: The problem related to *Salmonella enterica* serotype *Enteritidis* and multiple antibiotic resistance in other major serotypes. *Vet. Res.* 36:267-288.
- Walsh C, Duffy G, Sheridan JJ, Fanning S, Blair IS, Mcdowell DA (2005). Thermal resistance of antibiotic-resistant and antibiotic-sensitive *Salmonella* spp. on chicken meat. *J. Food Saf.* 25: 288-302.
- Yang B, Qu D, Zhang X, Shen J, Cui S, Shi Y, Xi M, Sheng M, Zhi S, Meng J (2010). Prevalence and characterization of *Salmonella* serovars in retail meats of market place in Shaanxi, China. *Int. J. Food Microbiol.* 141 (Suppl 1-2):63-72.
- Yoke-Kqueen C, Learn-Han L, Noorzaleha AS, Son R, Sabrina S, Jiun-Hong S, Chai-Hoon K (2007). Characterization of multiple-antimicrobial resistant *Salmonella enterica* subsp. *enterica* isolated from indigenous vegetables and poultry in Malaysia. *Lett. Appl. Microbiol.* 46:318-324.
- Yousef AE, Carlstrom C (2003). *Salmonella*. In Yousef AE. and Carstrom, C. (Eds.). *Food Microbiology, A laboratory manual*, 2003. New Jersey: John Wiley & Sons, Inc. pp. 167-205.

## Full Length Research Paper

## Reduction of oligosaccharide content of soybeans by the action of *Lactobacillus plantarum* isolated from fermented cereals

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Most African foods used in weaning are usually fermented cereals which supplies mainly carbohydrate. Unless these are supplemented with other nutrient sources, they may lead to excessive intake of carbohydrate which might cause malnutrition in growing children. Minimum dietary requirements of a child for protein could be met through fortification with legumes like cowpea, peanuts or soybeans but they contain raffinose family of oligosaccharide (RFOs), which are responsible for gas formation, bloating and flatulence in children. This work aims at using *Lactobacillus plantarum* and the enzyme  $\alpha$ - and  $\beta$ -galactosidases it produces, to hydrolyse raffinose to simple sugars and hence improve the raffinose-containing weaning foods. Three strains of *L. plantarum* obtained from fermented cereal gruels and identified using both classical and molecular methods were selected after screening for their ability to produce these enzymes in abundance. They were used to ferment different combinations of cereals and legumes. The oligosaccharide profile before and after the various pre-treatment methods and fermentation were determined using gas chromatography with flame ionization detector (GC-FID). Data obtained were analysed using ANOVA at  $p < 0.05$ . Fermentation for 120 h with *L. plantarum* reduced the total RFO content of soybeans to different levels in the samples. Losses of 30, 28 and 37% in stachyose, raffinose and verbascose, respectively were observed in the fermented raw samples; 72, 58 and 41% reduction in the fermented cooked samples and 76, 68 and 71% reduction in the roasted samples relative to the untreated samples. The use of galactosidase enzymes by *L. plantarum* from fermented cereals reduced the raffinose-oligosaccharide profile with simultaneous increase in reducing sugar levels. Adequate weaning foods can be prepared with such and the problem of bloating, gas production and flatulence can be solved by the action of *L. plantarum* in legume-cereal blends.

**Key words:** Raffinose-oligosaccharides, soybeans, fermented cereals, *Lactobacillus plantarum*, alpha-galactosidase, weaning foods.

### INTRODUCTION

Raffinose is a trisaccharide composed of galactose, fructose and glucose. It can be found in beans, cowpea, pigeon pea, Bambara groundnut, other vegetables, and whole grains. It can also be found in honey and brown sugar. Raffinose can be hydrolyzed to D-galactose and sucrose by the enzyme alpha-galactosidase and beta-galactosidase ( $\alpha$  and  $\beta$ -GAL), enzymes not found in the human digestive tract. Alpha-GAL also hydrolyzes other  $\alpha$ -galactosides such as stachyose, verbascose, and galactinol, if present. The enzyme does not cleave  $\beta$ -linked galactose, as in lactose (Storey et al., 1998; Townsend and Pitchford, 2012).

The raffinose family of oligosaccharide (RFOs) is alpha-galactosyl derivatives of sucrose, and the most common are the trisaccharide raffinose, the tetrasaccharide stachyose, and the pentasaccharide verbascose. RFOs are almost ubiquitous in the plant kingdom, being found in a large variety of seeds from many different families, and they rank second only to

sucrose in abundance as soluble carbohydrates (Storey et al., 1998; Townsend and Pitchford, 2012). It is a dextrorotatory trisaccharide, occurring in cotton seed and in the molasses of beetroot, composed of d-galactose, d-glucose, and d-fructose and formed by transfer of d-galactose from UDP-d-galactose (http://www.righthealth.com/corp/doingright).

Production of the enzyme  $\alpha$ -galactosidase is a desirable quality of *L. plantarum* that could be harnessed for the breakdown of Raffinose-oligosaccharide into simple sugars  $\alpha$ -galactosidase enzyme is known to break down raffinose at the  $\alpha$ -1, 4 glycosidic bond while  $\beta$ -galactosidase is able to breakdown the chain at the  $\beta$ -1, 6 positions. The production of  $\alpha$  and  $\beta$  galactosidase by *L. plantarum* can be employed to break down the raffinose oligosaccharide that is present in soybeans through fermentation. Also, Rodriguez et al. (2009) opined that *L. plantarum* is the commercial starter most frequently used in the fermentation of food products of plant origin

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because of its ability to produce the enzyme  $\alpha$ -galactosidase.

*L. plantarum* belong to a group of bacteria called lactic acid bacteria (LAB). They are a group of bacteria characterized by their ability to synthesize lactic acid. Typical LAB are gram-positive, non sporing, catalase - negative, devoid of cytochromes, anaerobic but aerotolerant cocci or rods that are acid-tolerant and produce lactic as the major end product during fermentation (Olaoye and Onilude, 2009). Their use in fermented foods dates back to ancient times because of their contribution to taste, aroma, flavour and increased shelf life of foods that contain them. They are however regarded as safe in foods because of their use as 'probiotics' in food. Lactic acid bacteria have also been isolated from several indigenous and African fermented foods such as *Ogi*, *Foofoo*, *Gari*, *kefir*, *kumiss*, *Tofu*, *Mawe* and drinks such as *Nunu*, Palm wine, *Agadagidi*, and *Burukutu*, etc (Oyewole and Odunfa, 1998).

*L. plantarum* has however been named as one of those good, useful and safe bacteria probiotics, as defined in a FAO/WHO (2002) report, as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Probiotics are beneficial bacteria in that they favourably alter the intestinal microflora balance such as reconstruction of normal intestinal microflora after disorders caused by diarrhoea, antibiotic therapy and radiotherapy. *L. plantarum* inhibits the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Ammor et al., 2006). It also exercises some therapeutic effect in the gut by its ability to breakdown complex oligosaccharide which causes bloating and gas production (Cummings and MacFarlane, 2007).

*L. plantarum* has been implicated in the breakdown of oligosaccharide in the colon, breakdown of unused energy substances and stimulation of cell growth by authors like Cummings and MacFarlane, (2007). It has also been noted that the breakdown of ROF is slower in adults and infants but this can be achieved by the consumption of fermented foods that contains *L. plantarum*. Also, humans cannot digest and absorb carbohydrates like starches, fibre and oligosaccharide without the help of bacteria such as *L. plantarum*. *L. plantarum* also helps people with lactose intolerance to overcome the discomfort they experience by the consumption of such sugars. *L. plantarum* ferments such complex sugars and make it available to host cell in assimilable form as sources of useful energy and nutrient (Gibson and Glenn, 2004).

## MATERIALS AND METHODS

### Oligosaccharide determination in soybean

#### Preparation of inocula

The LAB isolates namely *L. plantarum* isolated from fermented cereal gruels and identified using classical and molecular methods and labelled LV1, LV2 and LV3 that were used for the various tests were prepared by inoculating a colony from a 24 h- old culture of each *L. plantarum* streaked on a plate into a sterile 9 ml MRS broth. This was incubated for 24 h at 30°C.

#### Standardization of inocula

The LAB isolates that were used were standardized according to MacFarland standard using BaCl<sub>2</sub> and HCl at the right proportion. The culture supernatant was also brought to the same optical density (OD) of 0.500 and a colony count of  $3.1 \times 10^3$  cfu/ml, using sterile MRS broth (Olutiola et al., 1993).

### Fermentation of soybeans with the isolates of *L. plantarum*

Two batches each of 1 g, 2 g and 3 g each of the raw, cooked and roasted soybeans were weighed in triplicate into screw capped bottles. The first batch was used for the uncured isolates while the second batch was used for the cured isolates. Sterile distilled water (10 ml) was added to it for the sample to become a paste. 1 ml

each of the standardised inocula of cured and uncured *L. plantarum* were added separately to the two batches and allowed to ferment for five days. Samples were taken for reducing sugar and oligosaccharide content determination every 24 h. A control was set up for the samples with additional 1ml of sterile distilled water in the samples without the organisms.

### Production of enzymes alpha and beta-galactosidases

The method of Mital et al. (1973) as modified by Hassan and Durr (1974) were used for the determination and assay of both enzymes.

### Determination of raffinose, stachyose and verbascose

The method of Beutler (1998) as modified by Black and Bagley (2007) and Townsend and Pitchford (2012) were used. Milled soybean seed, 0.50 g (to pass a 0.5 mm sieve) and fermented samples were weighed accurately into glass test-tubes (18 x 150 mm). Ethanol, 5 ml (95 % v/v) was added to each tube and incubated at 84 to 88°C for 5 min to inactivate endogenous enzymes. The tube contents were quantitatively transferred to a 50 ml volumetric flask and the volume adjusted to the mark with 50 mM sodium acetate buffer (pH 4.5). The samples were allowed to extract over 15 min with occasional swirling. An aliquot (approx. 5 ml) of this slurry was transferred to a glass test tube (16 x 120 mm). Chloroform, 2 ml was added and mixed vigorously on a vortex mixer for 15 s. It was centrifuged at 1 500 g for 10 min to remove most of the lipids from the aqueous phase into the chloroform (lower phase); and the insoluble plant material that concentrated between the phases. The upper (aqueous) phase was analyzed and diluted accordingly.

### Methods

The Oligosaccharide profile of the samples was determined by multiplying the absorbance difference of the blank and samples with the final volume of the mixture and molecular weight of the sample assayed.

The concentration of the oligosaccharides was determined by:

$$c = V \times MW \times \Delta A \text{ [g/L]}$$

Where, V = final volume [mL], MW = molecular weight of the substance assayed [g/mol]  
=  $6300 \text{ [l} \times \text{mol}^{-1} \times \text{cm}^{-1}]$ ; v = sample volume [mL].

Raffinose:

$$\begin{aligned} c &= 2.62 \times 504.5 \times \Delta A \text{ raffinose [g/L]} \\ &= 6300 \times 1 \times 0.2 \\ &= 1.049 \times \Delta A \text{ raffinose [g/L]} \end{aligned}$$

Verbascope:

$$\begin{aligned} c &= 2.62 \times 624.59 \times \Delta A \text{ verbascope [g/L]} \\ &= 6300 \times 1 \times 0.2 \\ &= 1.299 \times \Delta A \text{ verbascope [g/L]} \end{aligned}$$

Starchyose:

$$\begin{aligned} c &= 2.62 \times 666.574 \times \Delta A \text{ stachyose [g/100g]} \\ &= 6300 \times 1 \times 0.2 \\ &= 1.386 \times \Delta A \text{ stachyose [g/L]} \end{aligned}$$

The method of Beutler (1998) as modified by Black and Bagley (2007) was used. The GC-FID is an instrument that measures the concentration of organic substances. It is frequently used as a detector in gas-chromatography. The operating principle is based on the detection of organic compounds formed during the breakdown of complex substances. It can measure organic substance concentration at very low and high levels Model is (Perkin-Elmer).

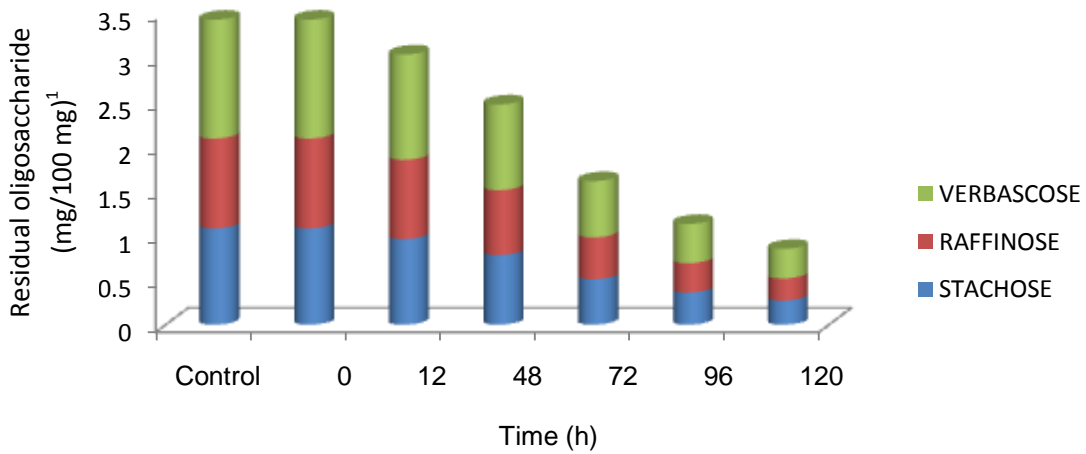
### Production of reducing sugars during fermentation

The total reducing sugars produced was determined using the method of Bernfeld (1955). The readings obtained through a spectrophotometer were subjected to a standard curve of glucose. The other sugars were calculated from the curve also.

### Statistical analysis

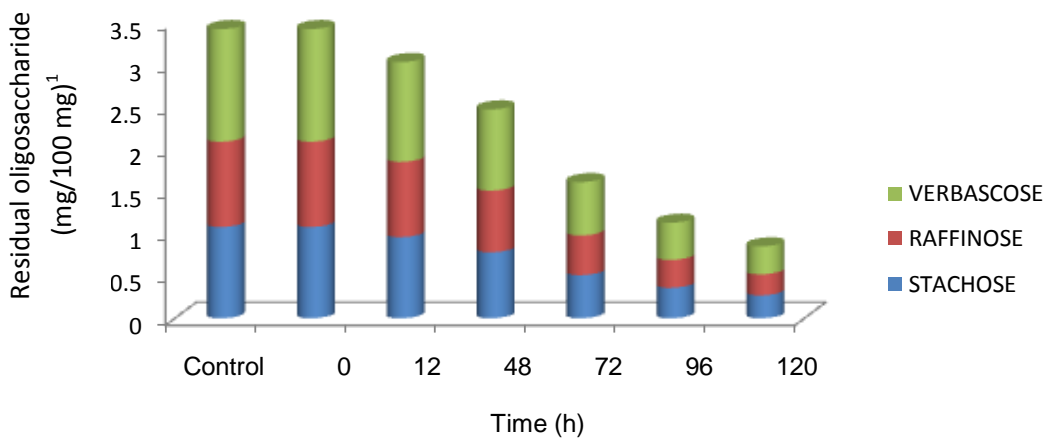
The statistical analyses carried out include analysis of variance,

### FERMENTED RAW



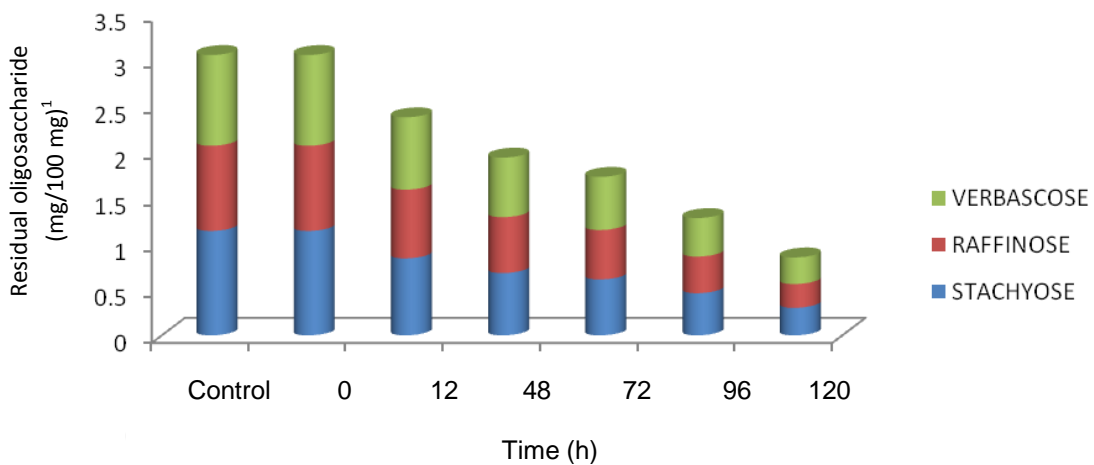
**Figure 1.** Residual oligosaccharide content of raw soybeans during fermentation with *L. plantarum*. Values are means of replicate determinations shows significance in the samples ( $P < 0.05$ ).

### FERMENTED RAW



**Figure 2.** Residual oligosaccharide content of cooked soybeans during fermentation with *L. plantarum*. Values are means of replicate determinations shows significance in the samples ( $P < 0.05$ ).

### FERMENTED ROASTED



**Figure 3.** Residual oligosaccharide content of roasted soybeans during fermentation with *L. plantarum*. Values are means of replicate determinations shows significance in the samples ( $P < 0.05$ ).

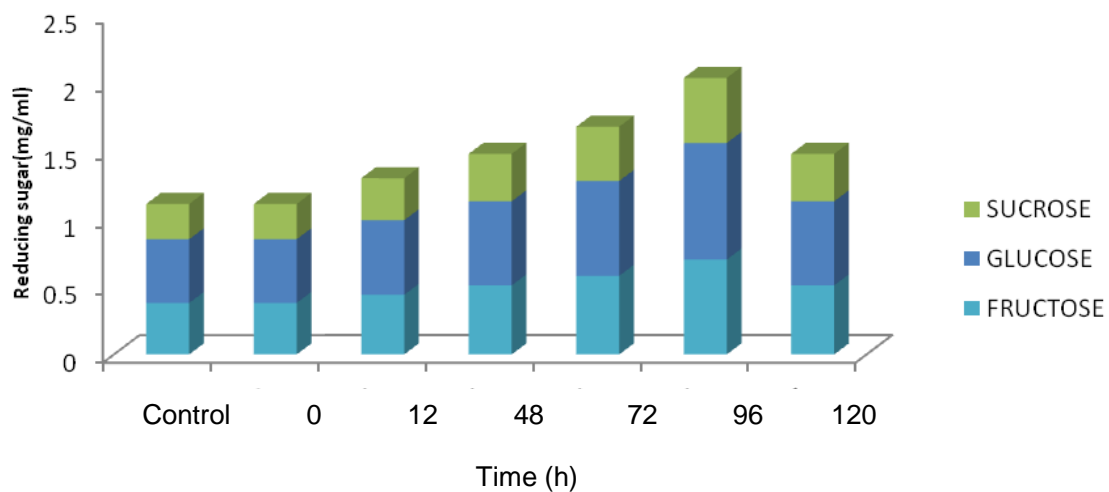
Duncan multiple range of variables, mean, standard deviation and Standard error using SAS Analytical package.

### RESULTS

Figures 1, 2 and 3 show the oligosaccharide content ( $\text{mg}/100 \text{ mg}$ )<sup>1</sup> of the samples as the fermentation

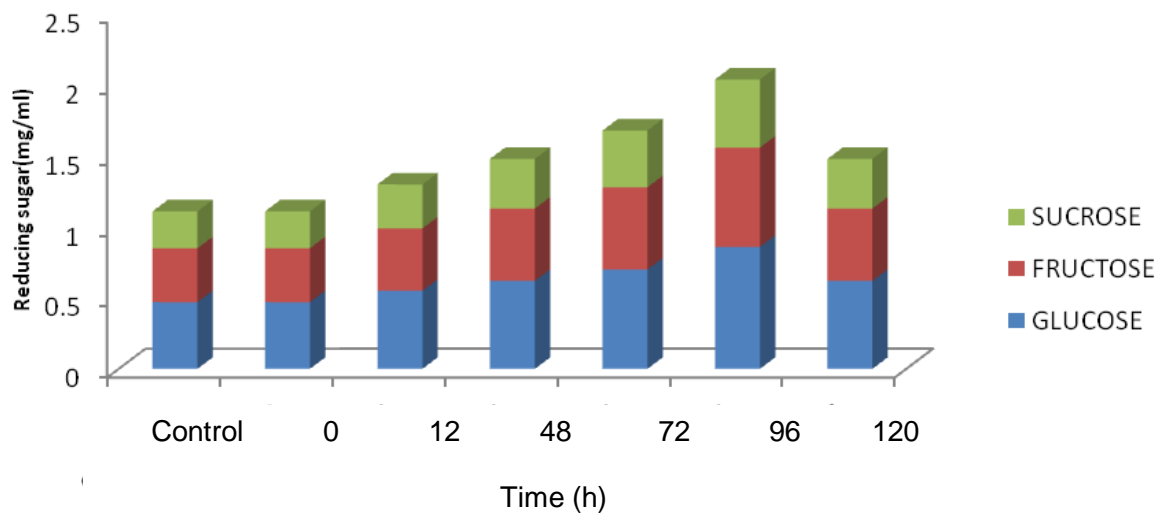
progresses. *L. plantarum* was able to reduce the oligosaccharide content of the samples, even though the different pre-treatment methods have reduced the oligosaccharide content to an extent, fermentation with *L. plantarum* was able to break it down further, and there was reduction in the oligosaccharide content from 0 to 120 h as observed in the samples.

## FERMENTED COOKED REDUCING SUGAR



**Figure 4.** Timed profile of reducing sugar by *L. plantarum* isolate LV1, LV2 and LV3 during fermentation with raw soybeans. Values are means of replicate determinations shows significance in the samples ( $P < 0.05$ ).

## FERMENTED COOKED REDUCING SUGAR



**Figure 5.** Timed profile of reducing sugar by *L. plantarum* isolate LV1, LV2 and LV3 during fermentation with cooked soybeans. Values are means of replicate determinations shows significance in the samples ( $P < 0.05$ ).

Figure 1 shows the comparative oligosaccharide content ( $\text{mg}/100 \text{ mg}$ )<sup>1</sup> from cooked soybeans fermentation by *L. plantarum* isolate. Cooking has reduced the oligosaccharide content of the soybeans before fermentation compared to what was found in raw (3.6) to (1.7). With cooked soybeans, there was a reduction in the oligosaccharide content from 0 hr to 120 h. No significant reduction ( $p < 0.05$ ) was observed between 0 to 24 h, but at 48 h of fermentation, there was a reduction from 1.2 to 1.0 at 48 h, 0.9, 0.8 and 0.6 at 72, 96 and 120 h respectively.

Figure 2 shows the comparative oligosaccharide content ( $\text{mg}/100 \text{ mg}$ )<sup>1</sup> of raw soybeans subjected to fermentation by *L. plantarum*. The oligosaccharide content of the raw sample at 0 h was 3.6, using the isolate; thier reduction was rather slow. No observable change was noticed until the 48th hour (2.6). The oligosaccharide content reduced from 2.0 to 1.7 at 96 h and at 120 h to 1.2.

Figure 3 shows the comparative oligosaccharide content ( $\text{mg}/100 \text{ mg}$ )<sup>1</sup> of the roasted soybeans subjected to fermentation by *L. plantarum*. Roasting reduced the oligosaccharide content a lot before fermentation from 3.6 in raw to 1.4. The highest oligosaccharide content observed was 1.4, it reduced from 1.4 to 1.1 at 24 h, then to 0.9, 0.8, 0.7 and 0.6 at 48, 96 and 120 h, respectively.

There was a significance reduction ( $p = 0.05$ ) in the oligosaccharide content from 0 -120 h when the organism was used. The pre-treatment method had a lot of significant difference ( $p < 0.05$ ) on the oligosaccharide content as it was possible for fermentation to start immediately at 24 h.

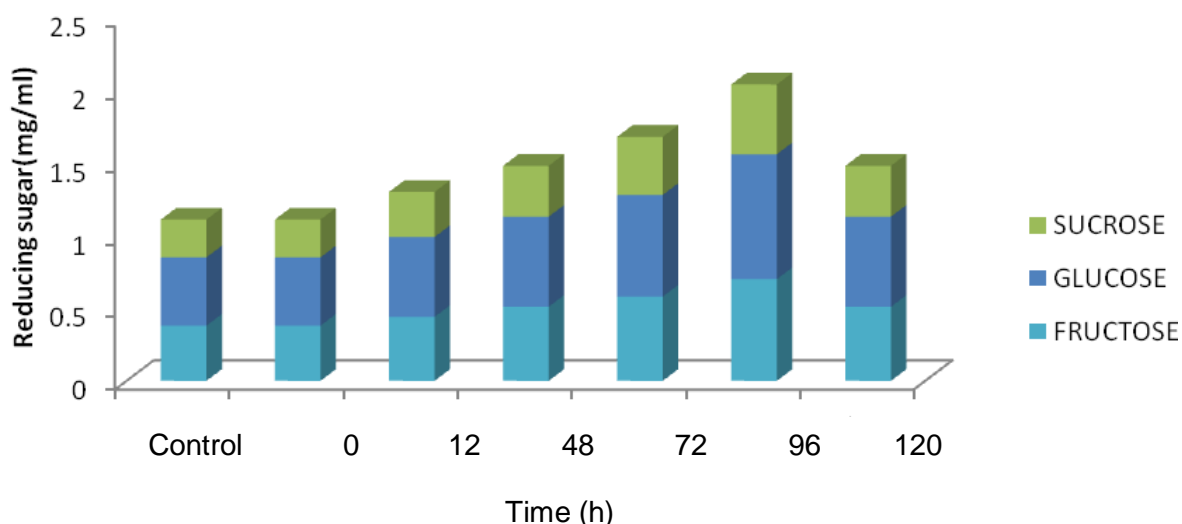
The analysis of variance result shows a significant difference ( $p < 0.05$ ) between the Raw, cooked and roasted soybeans in the reduction of oligosaccharide content, while it was the highest in raw sample (3.6), it was followed by cooked (1.7) and the least was observed in roasted (1.4).

There was a significant difference ( $p < 0.05$ ) in the time interval from 0 - 120 h, also there was a significant difference ( $p < 0.05$ ) in the oligosaccharide content between the *L. plantarum* isolates that was used for fermentation.

Figures 4, 5 and 6 show the effect of fermentation on the reducing sugar ( $\text{mg}/\text{ml}$ ) content of the pre-treated soybeans. It could be observed that there was a reduction in the oligosaccharide content from 0 to 96 h when the *L. plantarum* was used for fermentation. The reducing sugar content of the food blend increased from 0 to 96 h when *L. plantarum* was used for fermentation. The oligosaccharides in the samples were broken down by the organism into reducing sugar. At 120 h, the



## FERMENTED COOKED REDUCING SUGAR



**Figure 6.** Timed profile of reducing sugar by *L. plantarum* isolate LV1, LV2 and LV3 during fermentation with roasted soybeans. Values are means of replicate determinations shows significance in the samples ( $P < 0.05$ ).

**Table 1.** Production of  $\alpha$ - and  $\beta$ -galactosidases by the selected *L. plantarum*.

Isolate code	Alpha-galactosidase	Beta-galactosidase
<i>L. plantarum</i> Lv1	1.818 $\pm$ 0.002	0.958 $\pm$ 0.005
<i>L. plantarum</i> Lv2	1.820 $\pm$ 0.025	0.947 $\pm$ 0.005
<i>L. plantarum</i> Lv3	1.805 $\pm$ 0.010	0.940 $\pm$ 0.003

reducing sugars reduced again.

Table 1 shows the production of alpha and beta galactosidases by *L. plantarum*. The abundant production of these enzymes was a criterion that was used for selecting the organisms for the fermentation of the samples.

Figure 5 shows a trend in the fermentation pattern when the *L. plantarum* was used to ferment the cooked soybeans sample. There was a significant increase ( $p=0.05$ ) in the reducing sugar production (mg/ml) with the *L. plantarum* sample from 0 to 96 h; there was a drop at 120 h. It increased from 0.58 mg/ml at 0 h to 0.98 mg/ml at 72 h and the peaked at 96 h (1.1 mg/ml) but it reduced to 0.84 mg/ml at 120 h.

Figure 4 shows the reducing sugar (mg/ml) production by *L. plantarum* isolate in the same sample when the raw soybeans was used, there was a gradual increase in the reducing sugar produced from 0 to 96 h being the peak (0.52 mg/ml) and 0 h (0.62 mg/ml) 48 h and (0.96 mg/ml) at 96 h. There was a significant reduction ( $p=0.05$ ) at 120 h to 0.85 mg/ml.

Figure 5 shows the reducing sugar production (mg/ml) by the *L. plantarum* isolate in the roasted sample. There was an increase in the reducing sugar from 0 to 72 h respectively, from 0.62 to 0.72 and 1.0 mg/ml. The peak was observed at 96 h (1.8 mg/ml) and a reduction to 0.98 at 120 h. There was also a significant difference ( $p=0.05$ ) in the various time intervals as significant changes took place in the cooked and roasted samples but this was not observed in the raw samples.

## DISCUSSION

The breakdown of raffinose family of oligosaccharide was achieved by the use of *L. plantarum* to ferment the different soybeans sample. Accompanying the breakdown of oligosaccharide is the production of reducing sugars which the complex sugar is broken down into.

The effect of processing on the sugars and total RFO

content of the legume revealed that cooking in water and roasting resulted in a loss of RFO to an extent when compared with the raw samples. The results agree with those earlier reported by Burbano et al. (1990) and Hymowitz (2012) who established that oligosaccharide content of legumes was influenced and reduced by different pre-treatments methods and environmental factors; but fermentation is able to reduce it to a level that is safe for consumption. Both processes involved the use of heat.

Hence, the reductions may have been due to heat induced hydrolysis of the oligosaccharide to simple disaccharides. These findings are in agreement with those reported for cowpeas and other legumes in the work of Oboh et al. (2000). Cooking also resulted in slight loss of sugar which is in agreement with the result of Somlari and Balogh (1993).

Fermentation for 120 h with *L. plantarum* reduced the total RFO content of soybeans to different levels in the samples. Losses of 30, 28 and 37% in stachyose, raffinose and verbascose respectively were observed in the fermented raw samples. A 72, 58 and 41% reduction in stachyose, raffinose and verbascose respectively was observed in the fermented cooked samples. Fermented roasted samples showed a 76, 68 and 71% reduction in stachyose, raffinose and verbascose respectively when compared to raw seeds. These losses were significant at the different fermentation time. This is also similar to the findings of Mulimani and Ramaligam (1995) who reported complete hydrolysis of stachyose and raffinose to more than 60% hydrolysis due to fermentation.

The reduction of RFO in the raw sample was small when compared to the other samples. This may be as a result of the relative hardness of the seed coat which limits the uptake of water and may prevent thorough fermentation of the samples. Significant reduction in the RFO content has been reported during fermentation and other forms of pre-treatment by different authors (Akinyele and Akinlosotu, 1991). The pre-treatment was however necessary to make the samples amenable to LAB

fermentation. This is because it was not easy to ferment the raw samples with the hard seed coat but the pre-treatment methods have solved this problem.

The changes in total RFO content due to fermentation with *L. plantarum* indicated a significant reduction in all the samples due to the metabolic activity of *L. plantarum*. Similar findings have been reported for related leguminous plants such as cowpea, African yam beans, Jack beans and Pigeon peas by Akinyele and Akinlosotu (1991) and Oboh (2000). The relative reduction in the RFO observed in the study could be attributed to the presence of both alpha and beta-degradation of the sugars at alpha 1, 4 and beta - 1, 6 positions, respectively. This is similar to that obtained by Oboh and co-workers (2000). This is because *L. plantarum* produce the enzyme alpha-galactosidase in the medium of growth which is able to breakdown the oligosaccharides of the RFO.

A reduction rate of about 20% was observed in all the sugars at every 24 up to 96 h. This is similar to the findings of Leblanc et al. (2004) who reported that *L. plantarum* was able to eliminate raffinose, a non-digestible alpha-oligosaccharide (NDO) found in soy products. The combination of the various pre-treatment methods and fermentation of soybeans have caused a reduction in the RFO and ANF. This has led to increase in the nutritional composition of the food in which it is added, it also increased the availability of minerals, for example  $\text{Ca}^{2+}$  and  $\text{Fe}^{2+}$ . This observation are in agreement with earlier studies by Onilude et al. (1999) and Wakil and Onilude (2009).

There was however, a relative increase in the reducing sugars profile, a 61, 53 and 32% increase was observed in the glucose, fructose and sucrose after the raw samples were fermented with *L. plantarum*. The fermented cooked samples experienced a 78, 64 and 44% increase in glucose, fructose and sucrose, respectively while the fermented roasted samples experienced a percentage increase of 87, 71 and 52, respectively during fermentation of all the samples with *L. plantarum* from 0 to 96 h. Similar findings was reported by Muzquiz et al. (1993) who reported increase in the reducing sugar profile as the total RFO reduced due to its breakdown during fermentation by the enzymatic activities of microorganisms. The reducing sugar in the fermenting soybeans increased from 24 to 96 h and decreased thereafter in all the fermentation procedures. The period of rapid increase coincides with the period of increased total reducing content, reduced RFO, and alpha and beta galactosidase activity. There was a reduction at 120 h. The decrease in reducing sugar level of the fermenting powdered samples indicates that they are being used by *L. plantarum* for metabolism. Similar result was reported by Omafuvbe et al. (2007).

The use of intracellular alpha-galactosidase from *L. plantarum* to breakdown raffinose, stachyose and verbascose in raw, cooked and roasted soybeans was achieved. The optimum conditions for the enzymic hydrolysis of raffinose, stachyose and verbascose was pH 5.5 at 50°C for 24 h to 96 h. Alpha-galactosidase showed optimum activity at pH 5.0 and 50°C, with the substrate p-nitrophenyl-alpha-D-galacto-pyranoside (PNGP). The enzyme activity showed detectable loss in reducing sugar production after 96 h. This is also similar to the findings observed in work of Mulimani and Ramalingam (1995) on enzymic hydrolysis of raffinose and stachyose in soymilk by alpha-galactosidase.

Another important quality of a desired food weaning blend is one with a low raffinose-oligosaccharide content in which the complex sugar has been reduced to a level that is digestible or metabolisable by the infants as well as making the reducing sugar readily available for energy production. This was achieved in the food blend formulated in this work. With the use of *L. plantarum*, there was a reduction in the oligosaccharide content of roasted soybeans from 0 to 96 h. Furthermore, there was

an increase in the reducing sugar content of the soybeans during fermentation. This corroborates the work of Esponsa and Ruperez (2006) which reported a reduction of galacto- oligosaccharide content of soybeans during fermentation. The reduction of RFO, ANF and other unwanted or toxic substances during fermentation and improvement of the nutritional composition of the food by *L. plantarum* is one of the attributes of a good starter culture. The results of this study agrees with that of Le Blanc et al. (2004) on selection of the optimum growth conditions of *L. plantarum* with elevated levels of alpha-gal to be used in the reduction of non-reducing-oligosaccharide in soy products when used as starter cultures (Amankwah et al., 2012; Nagai et al., 2012).

The presence of trisaccharide- oligosaccharide raffinose, a member of RFO in soybeans also received a good treatment. *L. plantarum* produces enzyme  $\alpha$ - and  $\beta$ -galactosidase that are able to break down the foods in which the consumption of raffinose is high (Hymowitz, 2012). Apart from the discomfort that is usually experienced, it results in vomiting, diarrhoea, weight loss and malnutrition, because the little child cannot metabolise the complex sugar if consumed on a regular basis, malnutrition sets in. *L. plantarum* was thus able to metabolise the complex sugar and break it down to simple sugars in a manner that is readily available and metabolisable by the growing infants so that they can use such for growth.

*L. plantarum* is able to metabolize the large amount of complex sugars that are present in soybeans and therefore reduce or remove the limited use and the barrier to wide use of the leguminous food plant.

## Conflict of Interest

The author(s) have not declared any conflict of interests.

## REFERENCES

- Akinyele IO, Akinlosotu AA (1991). Effect of soaking, dehulling and fermentation on the oligosaccharides and nutrient content of cowpeas (*Vigna unguiculata*). Food Chem. 41:43-53.
- Amankwah EA, Barimah J, Nuamah AKM, Oldham JH, Nnaji CO (2012). Formulation of Weaning Food from Fermented Maize, Rice, Soybean and Fishmeal. Pak. J. Nutr. 8:1747-1752.
- Ammor S, Tauveron G, Dufour E, Chevallier I (2006). Antimicrobial activity of Lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility-screening and characterisation of the antimicrobial compounds. Food Contr. 17:454-465
- Bernfeld P (1955). Methods in Enzymology. Ed. By Kaplan MO and Colowity SP vol. 1 Academy Press, N.Y. p. 149.
- Beutler HO (1988). Raffinose. In Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), 3rd ed., Vol. VI, VCH Publishers (UK) Ltd., Cambridge, UK. pp. 90-96.
- Black LT, Bagley EB (2007). Determination of oligosaccharides in soybeans by high pressure liquid chromatography using an internal standard. J. Amer. Oil Chem. Soc. 55:178-185.
- Burbano C, Muzquiz M, Ayet G, Cuadrado C, Pedrosa MM (1990). Evaluation of antinutritional factors of selected varieties of *Phaseolus vulgaris*. J. Sci. Food Agric. 79:1168-1472.
- Cummings JH, MacFarlane GT (1997). Role of intestinal bacteria in nutrient metabolism. Clin. Nutr. 16:3-9.
- Esponsa MY, Ruperez P (2006). Soybean oligosaccharide potential as new ingredients in functional food. Nutri. Hosp. 21:43-50.
- Gibson, Glenn R (2004). Fibre and the effects on probiotics (the prebiotics concept). Clin. Nutri. Suppl. 1(2):25-31.
- Gray SL, Lackey BR, Tate PL, Riley MB, Camper ND (2012). Determination of raffinose- oligosaccharides in different sources of food. J. Exper. Biol. 229:560-568.
- Hassan N, Durr IF (1974). Utilization of lactose, Glucose and Galactose by a mixed culture of lactic acid bacteria isolated from milk. Appl. Environ. J. 32:951-958.
- Hymowitz T (2012). "Soy benefits". National Soybean Research Laboratory. [http://www.nsr.luiuc.edu/soy\\_benefits.html](http://www.nsr.luiuc.edu/soy_benefits.html). Retrieved February 16, 2012. J. Nutr. 107:1859-1862.
- LeBlanc JG, Garro MS, Silvestroni A, Connes C, Piard JC, Sesma F, Savoy GG (2004). J. Appl. Microbiol. 97:876-881.
- Mital R, Worthington J, Larry RBC (1973). Alpha-Galactosidase activity of fungi on intestinal Gas-forming peanut oligosaccharide. J. Agric. Food Chem. 22:816-914.
- Mulimani VH, Ramalingam P (1995). Enzymic hydrolysis of raffinose and stachyose in soymilk by alpha-galactosidase from *Gibberella fujikuroi*. Biochem. and Molec. Biol. Inter. 36:897-905.

- Muzqizm RC, Cuadrado C, Fenwick GR (1993). Effect of Germination on the Oligosaccharide content of Lupin Species. *J. Chrom.* 607:349-352.
- Nagai TO, Staatz JM, Bernsten, RH, Sakyi-Dawson EO, Annor GA (2012). Locally Processed Roasted-Maize-based Weaning Foods Fortified with Legumes: Factors Affecting Their Availability and Competitiveness in Accra, Ghana. *Afr. J. Food Agri. Nutr. Deve.* 9:1927-1944.
- Oboh HA, Muzquiz M, Burbano C, Cuadrodo C, Pedrosa MM, Ayet G, Osagie AU (2000). Effect of soaking, cooking and germination on raffinose oligosaccharide content of raw seeds of Lima beans, Pigeon peas, African Yam beans and Jack beans. *Plt foods Human Nutr.* 55:97-110.
- Olutiola PO, Famurewa O, Sonntag HG (1993). An introduction to General Microbiology. Heidelberg verlagsanstalt und Duckeneil GMBH Heidelberg pp. 94-126.
- Omafuvbe BO, Esosukpo EO, Oladejo V, Teye, AA (2007). Effect of Soaking and Roasting Dehulling methods of Soybean on *Bacillus* Fermentation of Soy-Daddawa. *Am. J. Food Technol.* 2:57-264.
- Onilude AA, Sanni AI, Ighalo M (1999). Effect of process improvement on the physicochemical properties of infant weaning food from fermented composite blends of cereal and soybeans. *Plt. Foods Human Nutr.* 54:239-250.
- Oyewole OB and Odunfa SA (1990). Characterization and distribution of Lactic acid bacteria in cassava fermentation during fufu production. *J. Appl. Bacteriol.* 68:49-59.
- Rodriguez H, Curiel JA, Landete JM, de la Rivas B, Lopez FF, Gomez CC, Mancheno JM, Munoz R (2009). Food phenolics and Lactic Acid Bacteria. *Inter. J. Food Microbiol.* 30:79-90.
- Townsend E, Pitchford NJ (2012). Sugar Variation in Soybean Seed Assessed with a Rapid Extraction and Quantification Method. *Int. J. Agr.* 209:571-579
- Wakil SM, Onilude AA (2009). Microbiological and chemical changes during production of malted and fermented cereal - legume weaning foods. *Adv. Food Sci.* 31:139-145.

Full Length Research Paper

## Sensory, physicochemical and microbiological characteristics of Greek style yogurt flavored with pequi (*Caryocar Brasiliense*, Cambess)

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The aim of this study was to develop Greek yogurt flavored with pequi and to assess its physicochemical and microbiological characteristics, as well as its shelf-life, sensory acceptance and purchase intent. Four yogurt formulations were prepared: no addition of Pequi pulp (control - treatment 1), 1% pequi pulp (treatment 2) 2% pequi pulp (treatment 3) and 3% pequi pulp (treatment 4). The levels of fat, moisture and ash showed no difference between formulations. For protein, Greek style yogurt with 2% pequi pulp showed the highest values. Clear yellowish color was observed in yogurts, with a tendency to red and green. All yogurt samples prepared with up to 22 days of storage showed pH and acidity values in accordance with Brazilian legislation. Microbiologically, yogurts were stable and with satisfactory sanitary conditions for consumption. Greek style yogurt with type 2% pequi pulp showed the greatest acceptance by consumers. In conclusion, Greek style yogurt flavored with pequi is a good food alternative, because in addition to market innovation, it has high nutritional value and good acceptance by consumers.

**Key words:** Fruits from the cerrado region, sensory acceptance, concentrated yogurt, fruit pulp, physicochemical characteristics.

### INTRODUCTION

Consumers have shown preferences for healthy and innovative foods due to health concerns. The quality of food products and their influence on human nutrition and health deserve a prominent place in the scientific

community (Lazaro Filho et al., 2007).

In this context, yogurt stands out, which is a milk derivative, obtained by fermentation produced by symbiotic cultures such as *Lactobacillus delbrueckii*

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**Figure 1.** Pequi fruit (1) and Greek style yogurt (2).

subsp. *Bulgarius* and *Streptococcus thermophilus* (Leal et al., 2013).

Machado et al. (2012) reported that the consumption of yogurt has increased and attributed this to benefits such as facilitated absorption of calcium, phosphorus, iron and the action of proteins and digestive enzymes.

Considered as an intermediate product between traditional fermented milks and non-matured cheeses with high moisture content such as quark, boursin and petit suisse cheeses, concentrated yogurt is produced in several countries with different names: labneh (East), skyr (Island), shrikhand (India) and Greek style yogurt (Greece and Brazil) (Ramos et al., 2009).

Greek style yogurt is a thick, creamy and semi-solid product due to the high concentration of total solids obtained by coagulation and decrease of milk pH added of soluble solids and other food substances and submitted to acid fermentation through the action of specific microorganisms for periods of time longer than traditional yogurts.

The sensory characteristics of yogurts may be improved by adding flavorings and fruit pulps, providing a distinctive combination of lactic acid flavor and fruit flavor (Miguel et al., 2010). Fruits from the cerrado region of Brazil have become an option for adding value to yogurts due to their high nutritional value and sensory attractives such as, color, flavor and unique and intense aroma (Almeida et al., 2008).

Although the consumption of fruits from the cerrado region has grown substantially in recent times, they are still not commercially exploited, either by lack of knowledge or lack of incentives for their commercialization and market consolidation. Their use in cooking has aroused interest in many segments of society such

as farmers, industry, research institutions and health and food agencies (Oliveira et al., 2008).

*Caryocar Brasiliense*, Cambess is a fruit of high nutritional value, rich in vitamins A, E, C, fatty acids, phosphorus, potassium and magnesium, which are substances responsible for the proper functioning of the body (Sousa et al., 2012).

The golden yellow pulp of pequi provides an attractive color in Brazilian cuisine, enjoyed by much of the population mainly due to the peculiarities of the exotic flavor, and characteristic strong aroma present in the fruit.

The production of concentrated yogurt using fruits from the cerrado region is still inexistent and can add greater value to the final product, allowing an alternative income to producers, also contributing to higher consumption of fruits from this region.

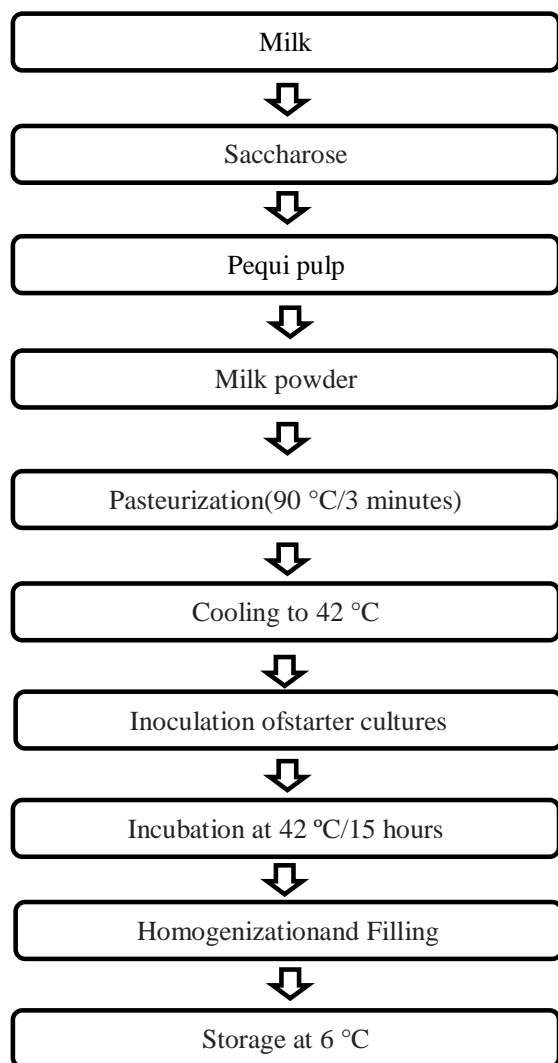
The aim of this study was to develop Greek style yogurt flavored with pequi and to assess its physicochemical and microbiological characteristics, as well as its shelf-life, sensory acceptability and purchase intent.

## MATERIALS AND METHODS

This project was submitted to the Ethics Research Committee of the Federal Institute of Education, Science and Technology of Goiás, Brazil and approved under Protocol n°. 020/2013.

### Ingredients and cultures

Refrigerated bovine milk, 10% of powdered whole milk per 1000 mL, 12% of sucrose per 1000 mL, starter cultures and cooked pequi pulp (Figure 1) were used for the development of Greek style yogurt (Figure 1). Refrigerated milk was obtained from the



**Figure 2.** Production flowchart of Greek style yogurt added of pequi pulp.

Department of Livestock, Goiás Federal Institute - Rio Verde Campus - GO, with 2.91% protein, 3.47% fat, 3.99% lactose, 11.35% total dry extract, 7.88% nonfat dry extract, 599 thousands CS/mL somatic cell count and 781 thousands CFU/mL total bacterial count. Due to the fact that processing involves heat treatment step (pasteurization), which aims to eliminate pathogenic and spoilage microorganisms, it was possible to use milk without any risk to the final product. The fulfillment of this step minimizes the exposure of consumers to high risks of contamination by a variety of pathogenic microorganisms present in the raw material (Fai et al., 2009).

Powdered whole milk and sucrose were purchased at a local market in Rio Verde, GO. The starter culture used was obtained by pricking whole commercial yogurt containing pro-symbiotic cultures of *S. thermophilus* and *L. delbrueckii* subsp. *Bulgaricus*. Pequi pulp was obtained from fruits harvested in farm located at the municipality of Lagoa Santa, MG. The fruits were selected, cleaned in a 200 ppm sodium hypochlorite solution, peeled and manually pulped using previously sanitized stainless knives.

After pulping, pequi pulp was submitted to cooking at a ratio of 1:3 (pequi pulp: mineral water) for 120 min, homogenized in an industrial blender and sieved. The pequi pulp used presented 1.57% protein, 75.15% moisture, 0.32% ash, ether extract 16.76%, total soluble solids 17.00, pH 6.00, acidity 0.08% citric acid, 61.70 luminosity, 13.98 red coloration and 56.81 yellow coloration.

### Yogurt processing

Figure 2 shows the production flowchart of Greek style yogurt added of pequi pulp. Four yogurt formulations were prepared: No addition of pequi pulp (control - treatment 1), 1% pequi pulp (Treatment 2), 2% pequi pulp (Treatment 3) and 3% pequi pulp (Treatment 4).

Greek style yogurt was experimentally developed in the Food Engineering Unit, Federal Institute of Goiás - Rio Verde Campus, GO.

Refrigerated milk was added to sucrose, powdered milk and pequi pulp (treatments 2, 3 and 4), homogenized and submitted to pasteurization at temperature of 90°C for 3 min. Then, it was then cooled to 42°C and added to starter culture, followed by homogenization. After the addition of the culture, the milk was kept at 42°C in BOD (refrigerated incubator) for 15 consecutive hours. After the incubation period, the curd was transferred to polyethylene pots (250 mL) and stored under refrigeration at temperature of 6°C for eight days for analyses.

### Physicochemical analyses

Pequi pulp was submitted to the following analyses: moisture, pH, titratable acidity, lipids, protein, ash and soluble solids content, which were determined in accordance with the Association of Official Agricultural Chemists (AOAC, 2000) and analysis of color in colorimeter (ColorQuest II, Hunter Lab Reston, Canada) and the results were expressed in L\*, a\* and b\*, where, L\* values (brightness) can vary from black (0) to white (100), chroma a\* from green (-60) to red (+60), and chroma b\* from blue (-60) to yellow (+60), as reported by Paucar - Menacho et al. (2008).

The analyses of chemical composition were determined by differential absorption of the infrared waves milk components, using equipment Milkoscan 4000 (Foss Electric A/S. Hillerod, Denmark). Somatic cells count (SCC) whose principle is based on flow cytometry was performed using equipment Fossomatic 5000 Basic (Foss Electric A/S. Hillerod, Denmark) and total bacterial count (TBC) through the device Bactoscan FC (Foss Electric A/S. Hillerod, Denmark), of refrigerated bovine milk were performed at the Laboratory of Milk Quality, Research Center at the School of Veterinary and Animal Science, Federal University of Goiás.

Greek style yogurt flavored with pequi was analyzed for moisture, protein, ash, and fat by AOAC (2000) and color on the eighth day of storage.

The shelf-life of each yogurt formulation was evaluated in the first day and after 8, 15, 22 and 29 days of storage regarding analysis of pH using a digital potentiometer (Quimis) previously calibrated with pH 4.0 and 7.0 buffer. Titratable acidity was determined according to AOAC (2000) and expressed as percentage (%) of lactic acid. All analyses were performed in triplicate.

### Microbiological analyses

Microbiological analyses were performed on pequi pulp and Greek style yogurt flavored with pequi on the fifth day of storage in order to verify the quality of yogurts using plating technique for the count

**Table 1.** Physicochemical analyses of Greek style yogurt flavored with pequi.

Parameter	Treatment				CV (%)	F value
	1	2	3	4		
Protein (%)	5.66 ± 0.21 <sup>ab</sup>	5.46 ± 0.29 <sup>ab</sup>	6.14 ± 0.46 <sup>a</sup>	5.34 ± 1.02 <sup>b</sup>	10.40	3.2*
Fat (%)	5.89 ± 0.60 <sup>a</sup>	6.44 ± 0.88 <sup>a</sup>	6.44 ± 0.88 <sup>a</sup>	6.44 ± 0.53 <sup>a</sup>	11.75	1.27 <sup>ns</sup>
Moisture (%)	72.60 ± 0.14 <sup>a</sup>	68.3 ± 10.48 <sup>a</sup>	77.86 ± 11.6 <sup>a</sup>	73.46 ± 0.76 <sup>a</sup>	10.71	2.24 <sup>ns</sup>
Ash (%)	1.24 ± 0.09 <sup>a</sup>	1.23 ± 0.16 <sup>a</sup>	1.23 ± 0.11 <sup>a</sup>	1.15 ± 0.11 <sup>a</sup>	9.96	1.08 <sup>ns</sup>
L*	83.72 ± 0.72 <sup>a</sup>	82.62 ± 0.4 <sup>5b</sup>	82.02 ± 0.22 <sup>c</sup>	81.49 ± 0.58 <sup>d</sup>	0.64	241.27**
a*	-0.67 ± 0.33 <sup>c</sup>	-0.42 ± 0.14 <sup>b</sup>	0.33 ± 0.17 <sup>a</sup>	0.24 ± 0.21 <sup>a</sup>	-171.18	344.38**
b*	5.36 ± 0.95 <sup>d</sup>	9.14 ± 0.21 <sup>c</sup>	11.39 ± 0.55 <sup>b</sup>	12.93 ± 0.50 <sup>a</sup>	6.33	2063.80**

Averages in the same row followed by the same letter do not differ. \*\*Significant at 1% probability. \*Significant at 5% probability. <sup>ns</sup>not significant

of molds and yeasts, as well as mesophilic, *psychrotrophic* and coliforms at 30°C, according to methodology recommended by Silva and Junqueira (2001).

### Sensory analysis

The sensory acceptance of Greek style yogurt flavored with Pequi was assessed at the Laboratory of Sensory Analysis - Food Engineering Unit, Federal Institute Goiás - Rio Verde Campus, GO.

The samples were evaluated by 78 untrained panelists, consumers or not of yogurt and within the age group of 18-53 years. Acceptance testing was carried out in individual booths.

The four samples were served in 50 mL disposable cups previously coded with random three-digit numbers and presented under ambient light. The panelists evaluated the samples using a 5-point hedonic scale, ranging from 1 (dislike) to 5 (liked), indicating how much they liked or disliked samples of Greek style yogurt flavored with pequi.

To better understand the likely consumers of the yogurt developed, panelists were requested to answer a structured questionnaire on the purchase intention of Greek style yogurt with pequi.

### Statistical analysis

The results of physicochemical analyses and acceptance test of Greek style yoghurt were obtained using a completely randomized design (CRD), in triplicate with three replicates for all analyses and submitted to analysis of variance (ANOVA) and Tukey's test with significance level of 5% using the Sisvar Software (Ferreira, 2003).

For the results of physicochemical analyses of raw materials (milk and cooked pequi pulp), descriptive statistical analysis was used.

The results of the shelf- life and purchase intent study were submitted to analysis of variance and regression through Microsoft Excel 2010.

## RESULTS AND DISCUSSION

The results of physicochemical analyses of Greek style yogurt flavored with pequi are shown in Table 1. As

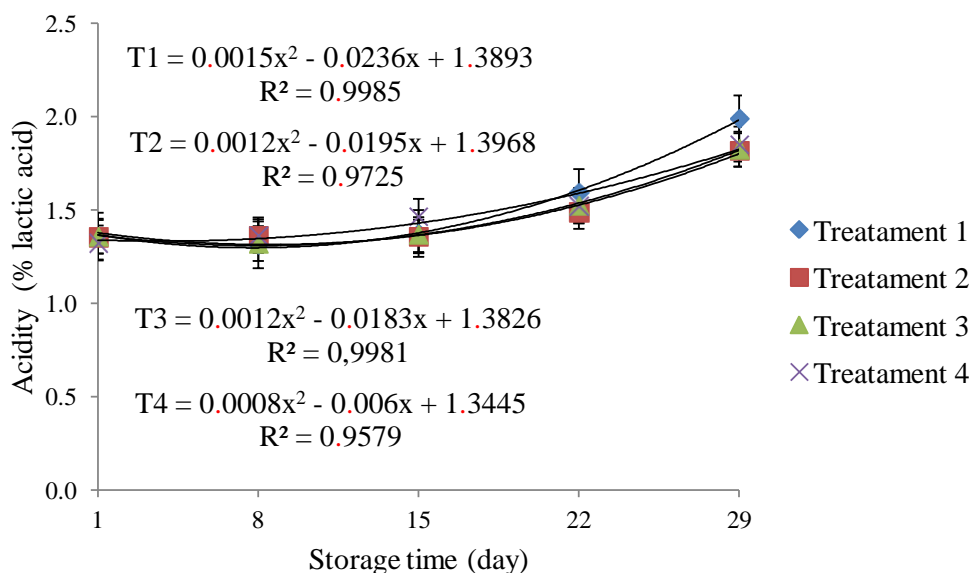
shown in Table 1, the protein values of yogurts ranged from 5.34 to 6.14%. Greek style yogurt with 2% pequi pulp (treatment 3) had higher protein content (6.14%), significantly differing ( $p \leq 0.05$ ) from Treatment 4 (3% pequi pulp). The protein contents in this study were higher than those obtained by Medeiros et al. (2009) in different jackfruit yogurts (4.83 and 4.97%) but in accordance with Brazilian legislation, which establishes minimum protein contents in yogurt of 2.90% (Brasil, 2000).

No significant difference ( $p > 0.05$ ) in parameters fat, moisture and ash was observed; however, Treatment 1 (Greek style yogurt with no pequi pulp) showed lower fat content compared to the other treatments. Although they have been added in increasing levels of pulp of pequi in treatments 2, 3 and 4, no significant differences in fat content were observed, but treatment 1 without addition of pulp showed lower fat content.

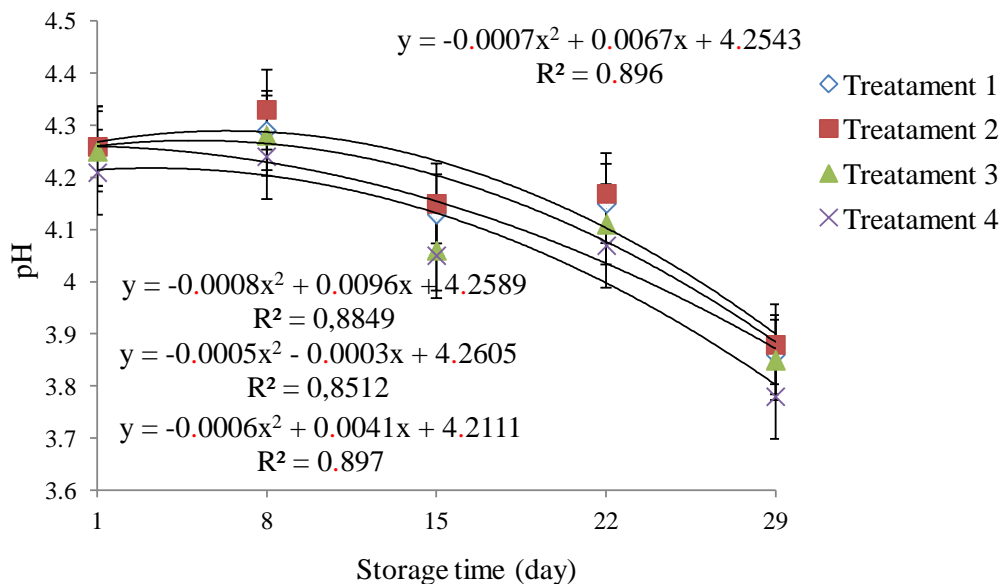
Similar fat and protein results were observed by Özer and Robinson (1999), who studied the behavior of starter cultures in concentrated yoghurt (Labneh) produced using different techniques. In general, Greek style yogurts showed clear yellowish color (high brightness values and positive  $b^*$  component), with a tendency to red (slightly positive  $a^*$  component) in treatments 3 and 4 (2% and 3% pequi pulp) and green (negative  $a^*$  component) in treatments 1 and 2 (0 and 1% pequi pulp). Higher  $L^*$  value was observed in Treatment 1 (0% pequi pulp) and reduced brightness values ( $L^*$ ) with the addition of different pequi pulp concentrations.

Carniato et al. (2013) studied the development of strawberry yogurt with quinoa and amaranth and found results similar to those of this study, observing brightness values of 78.28 and 68.64, chroma  $a^*$  values of 1.88 and 2.63 and chroma  $b^*$  values of 4.0 and 5.41.

The shelf- life results of Greek style yogurt flavored with pequi are shown in Figures 3 and 4. The titratable acidity (Figure 2) of Greek style yogurt flavored with pequi ranged from 1.31 to 1.99% lactic acid. The acidity of



**Figure 3.** Characteristics of acidity kind of Greek yogurt flavored with pequi (*Caryocar Brasiliense*, Cambess) during refrigeration at 6°C for 29 days storage.



**Figure 4.** Characteristics of pH of the Greek type yogurts flavored with pequi (*Caryocar Brasiliense*, Cambess) during refrigeration at 6°C for 29 days storage.

yogurts increased during storage, regardless of the addition of Pequi pulp. The increased acidity is related to the continued fermentation process by lactic acid bacteria during the storage period as the result of post-acidification of products with lactic acid production (Aportela - palacios et al., 2005), in which lactose is also converted into lactic acid (Pereira et al., 2012).

Preci et al. (2011) observed the same behavior when developing light yogurt with yerba mate extract (*Ilex paraguariensis* St. Hil.) with added probiotics and Pimentel et al., (2012) during storage of probiotic yogurt with inulin-type fructan.

The mean pH values (Figure 3) of formulations ranged from 4.29 to 3.78, decreasing during the storage period,



**Table 2.** Results of microbiological analyses of Greek style yogurts flavored with Pequi carried out on the fifth day of storage.

Microorganism	Treatment			
	1	2	3	4
Total coliforms	aus.	aus.	aus.	aus.
Mesophilic (log <sub>10</sub> )	1.57	1.45	1.26	1.20
Yeasts and molds (log <sub>10</sub> )	1.46	1.36	1.70	1.18
Psychrotrophic (log <sub>10</sub> )	1.86	1.34	1.58	1.58

**Table 3.** Acceptance results of Greek style yogurts flavored with pequi.

Parameter	Treatment				CV (%)	F value
	1	2	3	4		
Color	5.86 ± 1.06 <sup>b</sup>	6.08 ± 1.0 <sup>ab</sup>	6.47 ± 1.35 <sup>a</sup>	6.12 ± 1.31 <sup>ab</sup>	19.55	3.53*
Aroma	6.18 ± 1.30 <sup>b</sup>	6.62 ± 1.2 <sup>ab</sup>	7.08 ± 1.28 <sup>a</sup>	6.55 ± 1.33 <sup>b</sup>	19.23	6.56**
Texture	5.83 ± 1.10 <sup>b</sup>	6.38 ± 1.1 <sup>a</sup>	6.67 ± 1.26 <sup>a</sup>	6.23 ± 1.26 <sup>ab</sup>	18.56	6.94**
Flavor	5.99 ± 1.28 <sup>c</sup>	6.55 ± 1.3 <sup>b</sup>	7.37 ± 1.28 <sup>a</sup>	6.79 ± 1.44 <sup>b</sup>	19.78	14.73**

Averages in the same row followed by the same letter do not differ. \*Significant at 5% probability. \*\*Significant at 1% probability.

corroborating with the results of Oliveira and Damin (2002), who observed slight decrease in pH studying the viability of yogurt bacteria and probiotic cultures in fermented milk under refrigeration at 4°C during the storage period.

Yogurt cultures such as *L. bulgaricus* and *S. thermophilus* remain active even at refrigeration temperature and are able to produce small quantities of lactic acid through lactose fermentation, resulting in noticeable decrease in pH (Soares et al., 2011). Beal et al. (1999) in a study on the combined effects of culture conditions and storage time on acidification and viscosity of yogurt reported that yogurt will always show a decrease in pH and increase in acidity during storage under refrigeration.

The results indicate that all treatments showed the same behavior throughout the storage period. All samples of Greek style yogurt with or without pequi pulp with storage period of up to 22 days showed pH and acidity values in accordance with Brazilian law (Brasil, 2007), which recommends minimum values of 0.6 and maximum of 15 g of lactic acid per 100 g of product.

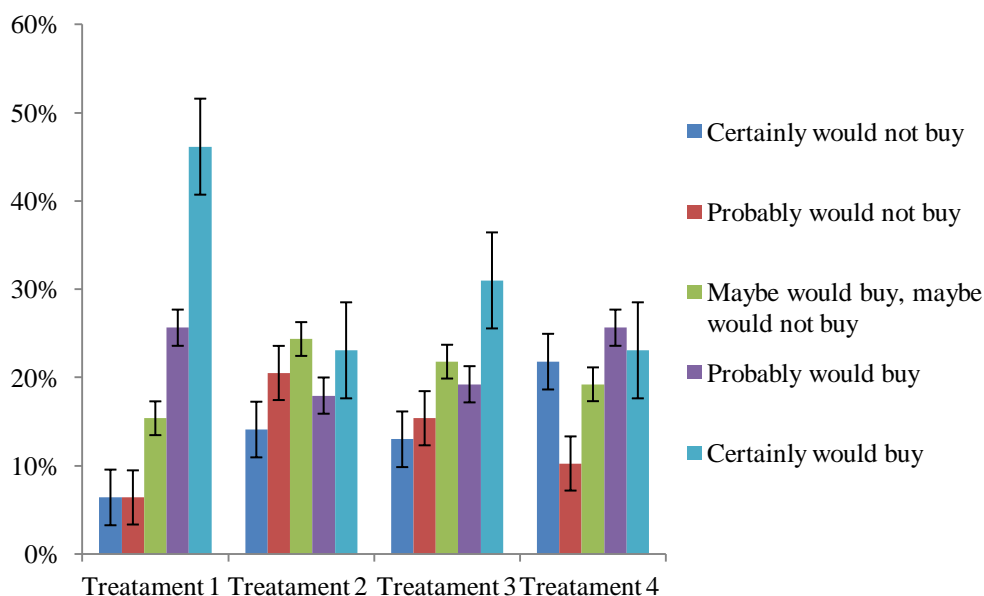
Pequi pulp was microbiologically stable, showing total coliform count (absence), mesophilic (< 10<sup>2</sup>) and yeasts and molds (< 10<sup>2</sup>) below microbiological standards established by Brazilian legislation (10<sup>4</sup> CFU g<sup>-1</sup>) for ready-to-eat products (Brasil, 2001).

Table 2 shows the results of microbiological analyses of Greek style yogurts flavored with pequi prior to the sensory analysis (fifth day of storage). Microbiological analyses of Greek style yogurts flavored with pequi (Table 2) showed that the counts of yeasts and molds,

total coliforms, mesophilic and psychrotrophic bacteria were below the limits established by Brazilian legislation (Brasil, 2007), indicating that the products have satisfactory sanitary conditions for consumption. Oliveira et al. (2008) reported similar microbiological results when studying the development of araticum yogurt.

The sensory acceptance results of Greek style yogurts flavored with pequi are shown in Table 3. The sensory acceptance results for color showed a significant difference (p < 0.05) between samples of Greek style yogurts flavored with pequi. Samples of treatment 3 (2% pequi pulp) had greater acceptance, with significant difference from samples of treatment 1 (0% pequi pulp), which did not differ from samples of treatments 2 and 4 (1 and 3% pequi pulp), respectively. For parameter aroma, treatment 3 showed the highest value (p < 0.01), significantly differing from Treatments 1 and 4 (0 and 3% pequi pulp), respectively.

In relation to texture, treatments 2, 3 and 4 showed higher means than treatment 1 (p < 0.01), indicating good acceptance by consumers. For attribute flavor, treatment 3 showed greater acceptance (p < 0.01) compared to other formulations (0, 1 and 3% pequi pulp). This results indicate greater acceptance by consumers of Greek style yogurt with 2% pequi pulp, showing through the purchase intention test (Figure 5) that over 30% of panelists would buy Greek style yogurt flavored with 2% pequi pulp (treatment 3); however, Rocha et al. (2008) studied the development and evaluation of yogurt flavored with fruits from the cerrado region and reported that yogurt flavored with pequi had low acceptance by judges, but were not rejected.



**Figure 5.** Purchase Intention of Greek type yogurts flavored with pequi.

## Conclusion

The Greek yogurt formulated with 2% pequi pulp showed better sensory acceptance and higher protein content. The addition of the pequi pulp decreased Greek yogurt brightness, but increased the intensity of the yellow color, making it a visually attractive for the consumer.

Based on the results of pH and titratable acidity, Greek yogurt enriched with pequi showed consumption the viability in until 22 days of storage. Pequi a typical fruit of the Brazilian savanna, has color, odor and flavor characteristic; it constitutes an alternative for inclusion in firm yogurts, however, work must be performed with the use of pulp at lower concentrations.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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## REFERENCES

Almeida SP, Costa TSA, Silva JA (2008). Frutas nativas do cerrado: caracterização físico-química e fonte potencial de nutrientes. Embrapa Cerrados, Embrapa Informação Tecnológica (1):351-381.

Aportela-palacios A, Aosa-Morales ME, Vélez-Ruiz JF (2005).

Rheological and physicochemical behavior of fortified yogurt, with fiber and calcium. *J. Texture Stud.* 36(3):333-349.

Association of Official Agricultural Chemists (AOAC) (2000). Official methods of the Association of the Agricultural Chemists. (2): 1175.

Beal C, Skokanova J, Latrille, Martin N, Corrieu G (1999). Combined effects of culture conditions and storage time on acidification and viscosity of tirred yogurt. *J. Dairy Sci.* 82(4):673-681.

Brasil (2001). Agência Nacional de Vigilância Sanitária. Aprova o Regulamento Técnico Sobre os Padrões Microbiológicos para Alimentos. Resolução - RDC nº 12, de 2 de janeiro de 2001.

Brasil (2007). Ministério da agricultura, pecuária e abastecimento. Regulamento Técnico de Identidade e Qualidade de Leites Fermentados. Instrução Normativa nº 46, de 23 de outubro de 2007.

Brasil (2000). Ministério de Agricultura, Pecuária e Abastecimento. Oficializa os padrões de identidade e qualidade (PIQ) de leites fermentados. Resolução - RDC nº 5, de 13 de novembro de 2000.

Carniato KV, Santos JL, Scheik LK, Oliveira VK, Gularte MA (2013). Desenvolvimento de iogurte de morango acompanhado de Quinoa e amaranto e sua aceitação pelos consumidores. XXII congresso de iniciação científica da universidade federal de pelotas.

Fai AEC, Oliveira AM, Soares DS (2009). Avaliação da qualidade de queijos de coalho comercializados no entorno da Cidade Universitária. *Higiene Alimentar.* 23:160-165.

Ferreira DF (2003). Sisvar: versão 4.3 (Build 43). Lavras: Departamento de Ciências Exatas, Universidade Federal de Lavras.

Lazaro Filho GC, Mendes JA, Neto JAR, Souza LBS, Santana WJ (2007). Importância nutricional dos *Lactobacillus*. *Higiene Alimentar.* 154(18):39-43.

Leal NS, Zambrano F, Seraphim LC, Dibbern LS, Almeida LCF, Fernandes S (2013). Aceitabilidade de iogurte de leite de ovelha com adição de alecrim (*rosmarinus officinalis* L.). *Veterinária e Zootecnia.* 20(1):131-139.

Machado MS, Conceição ALS, Santos DB, Belo GO, Cardoso RL (2012). Elaboração, processamento e análise sensorial de iogurte de leite de cabra com pinha (*annonasquamosal*). *Enciclopédia biosfera, Centro Científico Conhecer.* 8(15): 842 2012.

Miguel PR, Marmitt T, Schlabit C, Hauschild FAD, Souza CFV (2010). Desenvolvimento e caracterização de "iogurte" de soja sabor morango produzido com extrato de soja desengordurado enriquecido com cálcio. *Alimentos e Nutrição.* 21(1):57-63.

- Oliveira KAM, Ribeiro LS, Oliveira GV, Pereira JMATK, Mendonça RCS, Assumpção CF (2008). Desenvolvimento de formulação de iogurte de araticum e estudo da aceitação sensorial. *Alimentos e Nutrição* 19(3):277-281.
- Oliveira MO, Damin MR (2002). Efeitos do teor de sólidos e da concentração de sacarose na acidificação e na viabilidade de bactérias do iogurte e das probióticas em leite fermentado. In: Congresso Brasileiro de Ciência e Tecnologia de Alimentos 17:3015-3018.
- Özer BH, Robinson RK (1999). The Behaviour of Starter Cultures in Concentrated Yoghurt (Labneh) Produced by Different Techniques. *Lebensm.-Wiss. u.-Technol.* 32(7):391-395.
- Paucar-Menacho LM, Silva LHD, Barretto PADA, Mazal G, Fakhouri FM, Steel CJ, Collares-Queiroz FP (2008). Desenvolvimento de massa alimentícia fresca funcional com a adição de isolado protéico de soja e polidextrose utilizando páprica como corante. *Ciência e Tecnologia de Alimentos* 28(4):767-778.
- Pereira GG, Rafael LM, Gajo AA, Ramos TM, Pinto SM, Abreu LR, Resende JV (2012). Influência do pH nas características físico-químicas e sensoriais de frozenyogurt de morango. *Semina: Ciências Agrárias* 33(2):675-686.
- Pimentel TC, Garcia S, Prudencio SH (2012). Iogurte probiótico com frutanos tipo inulina de diferentes graus de polimerização: características físico-químicas e microbiológicas e estabilidade ao armazenamento. *Semina: Ciências Agrárias* 33(3):1059-1070.
- Preci D, Cichoski AJ, Valduga AT, Oliveira D, Valduga E, Treichel H, Toniazzo G, Cansian RL (2011). Desenvolvimento de iogurte light com extrato de erva-mate (*Ilex paraguariensis* Hil.) e adição de probióticos. *Alimentos e Nutrição* 22(1):2011.
- Ramos TM, Gajo AA, Pinto SM, Abreu LR, Pinheiro AC (2009). Perfil de textura de *labneh*. *Revista do Instituto de Laticínio "Cândido Tostes"* 64(369):8-12.
- Rocha C, Cobucci RMA, Maitan, VR, Silva OC (2008). Elaboração e avaliação de iogurte sabor frutos do cerrado. *Boletim Ceppa* 26(2):255-266.
- Silva N, Junqueira VCA, Silveira NFA (2001). Manual de Métodos de Análise Microbiológica de Alimentos.
- Soares DS, Fai AEC, Oliveira AM, Pires EMF, Stamford TLM (2011). Aproveitamento de soro de queijo para produção de iogurte probiótico. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* 63(4).
- Sousa FC, Silva LMM, Sousa EP, Lima AKVO, Figueiredo RM (2012). Parâmetros físicos e físico-químicos da polpa de pequi. *Revista Verde* 6(1):12-15.

## Full Length Research Paper

## Effects of jaboticaba (*Myrciaria jaboticaba*) peel on blood glucose and cholesterol levels in healthy rats

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The jaboticaba (*Myrciaria jaboticaba* (Vell.) Berg) peel was lyophilized and the proximate composition, total anthocyanins and polyphenolic content were determined. The effect of the freeze-dried jaboticaba peels (FJP) in the plasmatic levels of glucose, lipid fractions, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in *Wistar* adult male rats was investigated. The animals were distributed in four groups G0 (control), G1, G2, G4, which received 0, 1, 2 and 4% FJP powder added to normal diet, respectively. The chemical analyses showed that FJP is a source of fiber, anthocyanins, gallic and ellagic acids. The FJP supplementation was responsible for a reduction in the plasmatic glucose levels in G2 group. Total triglycerides and cholesterol levels were reduced in the G1 animals as compared to G0, but the animals treated with 2 and 4% FJP showed increased total cholesterol level and LDL-cholesterol fraction. HDL-cholesterol and hepatic probes showed no significant changes in the experimental groups G1, G2 and G4 in comparison to G0.

**Key words:** Polyphenols, glucose and cholesterol levels, Jaboticaba, *Myrciaria jaboticaba* (Vell.) Berg.

### INTRODUCTION

The consumption of exotic fruits and their byproducts has been strongly associated with reduced risk for developing chronic diseases such as obesity, cardiovascular diseases, type 2 diabetes, insulin resistance, neurodegenerative diseases, cancer and others (Dragano et al., 2013; Lenquiste et al., 2012; Leite-Legatti et al., 2012; Papandreou et al., 2009). There are strong evidences that these properties are related to phytochemicals that may be able to combat the formation

of free radicals and the increased oxidative stress. Moreover, these compounds present in seeds, peels and pulps of these fruits have anti-inflammatory, hypoglycemic and hypolipidemic effects (Dragano et al., 2013; dos Santos et al., 2010; Esteves et al., 2011).

The berries are highlighted in this context, because they are rich in polyphenols and have significant amounts of fiber (Kim et al., 2010). These compounds purified or within the whole food, seem to promote beneficial effects

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**Abbreviations:** AIN-93M, American Institute of Nutrition 93- adult maintenance diet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHT, 2,6-di-tert-butyl-4-methylphenol; FJP, freeze-dried jaboticaba peels; HDL, high-density lipoprotein; HPLC-DAD, high performance liquid chromatography - diode array detector; LDL, low-density lipoprotein; ORAC, oxygen radical absorbance capacity; TEAC, trolox equivalents antioxidant capacity.

to the body, both in the treatment and prevention of chronic diseases (Tsuda et al., 2003; Guo et al., 2012).

Studies have shown that diets rich in polyphenols can attenuate the development of type 2 diabetes, since they could protect the pancreas against oxidative stress, preserve  $\beta$  pancreatic cells, increase the excretion of insulin by these and inhibits the glucose absorption from intestine (Lenquist et al., 2012; Jayaprakasam et al., 2006). Besides phenolic compounds, the dietary fibers also exhibit important role in reducing blood glucose (Esteves et al., 2011; Kim et al., 2010).

The bioactive compounds from products of blueberries are related to the reduction of cholesterol and bile acids hepatic synthesis, increasing fecal excretion of lipids and thereby reducing plasma cholesterol levels (Kim et al., 2010). Pigs fed with a diet containing cereals/grains supplemented with fiber source and 1.5% blueberries resulted in reduced total cholesterol (Kalt et al., 2008). Thus, it is possible that consumption of foods containing a combination of phenolic compounds and fibers provide improvements of glycemic and lipids control *in vivo*.

Jaboticaba is a tropical wild berry from Southeastern Brazil. *Myrciaria jaboticaba* (Vell) Berg. and *Myrciaria caulifolia* (DC) Berg are the varieties more suitable for 'in natura' consumption, as well as for food industry applications (Pinto et al., 2011). The berry diameter is about 3-4 cm; it has one to four seeds inside, and the peel is very purple. The pulp is white and sweet. Normally, the peel is not consumed and discarded. However, recent researches have been performed with the purpose of adding value to the use of this byproduct by industry (Pinto et al., 2011).

The polyphenols from *M. jaboticaba* were not extensively explored. However, some bioactive compounds were already described: anthocyanins, mainly cyanidin and delphinidin 3-glucoside; ellagitannins, ellagic acid, vitamin C, limonene, terpenes, dietary fibers and others (Leite-Legatti et al., 2012; Abe et al., 2012; Lima et al., 2011; Alezandro et al., 2013). In our studies, 1 and 2% of FJP in diets increased systemic antioxidant status. Antioxidant assays performed in the plasma of animals increased 70 and 30% of antioxidant activity by ORAC and TEAC (Leite et al., 2011).

We hypothesized that the antioxidant capacity of plasma from rats fed FJP could contribute to the improvement of glycemia and lipid profile control. The aim of this study was to investigate the chemical composition and the effects of the addition of a jaboticaba byproduct to the diet of adult Wistar rats concerning biochemical parameters.

## MATERIALS AND METHODS

### Preparation and chemical composition of the freeze-fried jaboticaba peels

Jaboticaba fruits (*M. jaboticaba* (Vell.) Berg) were bought on a local market in Campinas in September, 2008. The fruits were washed,

manually peeled and the peels were frozen at  $-18^{\circ}\text{C}$ . The peels were then lyophilized in a freeze-dryer (Liobras, Brazil) at  $30^{\circ}\text{C}$ , 300  $\mu\text{m Hg}$  for 95 h. The freeze-dried product (FJP powder) was stored at  $-80^{\circ}\text{C}$ .

### Proximate composition

The contents of protein (Kjeldahl method), moisture, ash, lipids, fiber (acid hydrolysis) and total sugars of freeze-dried jaboticaba peels were determined (AOAC, 1995; Bligh and Dyer, 1959; Lane and Eynon, 1923; IAL, 1985).

### Extraction of bioactive compounds

The FJP powder was weighed into a centrifuge tube (250 mg) and extracted with 15 mL of ethanol/water (60:40, v v<sup>-1</sup>). The sample was shaken in a vortex for 30 s and then allowed in water bath at  $70^{\circ}\text{C}$  for 1 h, under agitation for each 15 min. The extract was filtered and allowed in an amber bottle. A second extraction was performed in the residue with 10 mL ethanol/water using the same procedure, and the supernatants combined and used to the following analysis (Colomeu et al., 2014). Each analysis was performed in triplicate.

### Folin-Ciocalteu reagent reducing substances (FCRRS)

The Folin-Ciocalteu method was used to determine total polyphenols contents and was defined as FCRRS, since it is known to be affected by several interfering substances (Silva et al., 2013; O'Brien, et al., 2013). The total phenolic content was determined by using the adapted Folin-Ciocalteu method (Swain and Hillis, 1959). Water, Folin-Ciocalteu reagent and sodium carbonate were added to the extract or standard solutions, and after 2 h in the dark at room temperature the absorbance of samples and standard curve was read at 725 nm. The measurements were done using 96-well microplate and a microplate reader (Sinergy HT, Biotek, Winooski - USA). The results were expressed as gallic acid equivalents (GAE  $\text{mg g}^{-1}$  FJP).

### Total anthocyanins analysis

The total anthocyanins were also quantified in the ethanol extract according to the pH-differential method described by Wrolstad (Wrolstad, 1993).

### Oxygen radical absorbance capacity (ORAC) assay

The ORAC test (Davalos et al., 2004) was carried out adding 20  $\mu\text{L}$  of samples extract or standard solutions, 120  $\mu\text{L}$  of fluorescein diluted in potassium phosphate buffer (pH 7.4), and 60  $\mu\text{L}$  of AAPH (2,2'-azobis(2-methylpropionamide) dihydrochloride) to black microplates, in the dark. Trolox (( $\pm$ )-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as standard and the microplate reader (Synergy HT, Biotek, Winooski, USA) was set using fluorescent filters: excitation wavelength, 485 nm; emission wavelength, 520 nm. ORAC values were expressed in  $\mu\text{mol}$  trolox equivalent ( $\mu\text{mol TE}$ ) per gram of FJP by using the standard curves ( $2.5 - 80.0 \mu\text{mol TE L}^{-1}$ ) for each assay. The fluorescence readings were used to the appropriate calculations.

### HPLC-DAD analysis

For chromatographic analysis, 1 g of FJP was extracted in duplicate with 25 mL of 80% methanol at  $37^{\circ}\text{C}$  for 3 h in a shaking water bath

bath. The analysis of the phenolic compounds from the methanol extracts was carried out in a high performance liquid chromatography (HPLC) (Agilent 1100), with manual injection (three injections each extract), 20  $\mu\text{L}$  sample loop and ternary pump, coupled to a diode array detector (DAD) (Agilent G1315B), at room temperature. The data was obtained and processed using the software ChemStation (Hewlett Packard, Germany). A reverse phase chromatographic column (C18 Eclips ODS (250 mm  $\times$  4.5 mm, Agilent) was used. The mobile phase was 1% orthophosphoric acid in water (v.v<sup>-1</sup>) (A) and acetonitrile (B). The elution gradient started at 95: 05 (A: B) at 0.7 mL min<sup>-1</sup>. This condition was maintained for 5 min and the concentration of A was decreased so that at 25 min it reached 60:40 (A:B) followed by a linear increase of solvent A to 95% until 35 min. The detection was done at 210, 254, 280, 300 and 340 nm, which allowed the simultaneous quantification and the tentative identification of the phenolic compounds separated by the HPLC. The comparison parameters were elution time, spectra of absorption and sample fortification. The identification was carried out using the chromatograms obtained at each injection and compared with the absorption spectra and retention time of the standards. Co-chromatography was performed to confirm the identity of the compounds. The concentrations of the identified compounds were calculated from the analytical curves obtained using commercial products (ellagic acid, quercetin and gallic acid from Sigma, St Louis, MO, USA) under identical chromatographic conditions (Port's et al., 2013).

### ***In vivo* experiment**

The *in vivo* assay was performed with 32 Wistar adult rats (individual weight of approximately 250 g), from Unicamp Biotherium Center (CEMIB), divided into four groups with eight animals each. The animals were kept in individual cages and received AIN-93M diet (Reeves et al., 1993) added with FJP.

The amount of proteins was reduced to 12% and the amount of sucrose in the diets supplemented with FJP was modified to make them isocaloric. The ingredients used and distributed equally among the diets were: starch (46.57%); dextrin (15.5%); casein (14.2%); cellulose (5.0%); soy oil (4.0%); mineral mix (3.5%); vitamins mix (1.0%); L-cystine (0.18%); choline substrate (0.25%); and BHT (0.0008%). The groups denominated G0, G1, G2 and G3 received diets added by 0, 1, 2, and 4% FJP powder (described above) in diet, respectively and the amount of sucrose was adjusted to 10, 9.31, 8.62 and 7.24% for the same groups.

The *in vivo* experiment was carried during 28 days. The animals were kept under controlled temperature (22  $\pm$  2°C) during the whole experiment, with alternated periods of 12 h in light and dark. All animals were weighed at every two days and the diet consumed was monitored. At the end of the feeding period, the animals were fasted (12 h), killed by decapitation, the blood was collected in heparin-coated tubes and the plasma was obtained by centrifugation. The livers were carefully removed, rinsed with saline solution and weighted. The plasma samples were stored at -80°C.

The experiment was performed according to ethical principles for experiments with animals adopted by the Brazilian College for Animal Experimentation (COBEA) and it was previously approved by the Committee for Ethics in Animal Research - CEUA/ Unicamp, University of Campinas, Brazil (#1627-1).

### **Blood analysis**

Plasma lipids and glycemia were determined by using colorimetric enzymatic methods that employed the commercial kits Laborlab for quantifying blood glucose (CAS #02200), triglycerides (CAS #02700), total cholesterol (CAS #01400), HDL-cholesterol (CAS

#08900), ALT (#00200) and AST (#00300), and Wienerlab (São Paulo, Brazil) for LDL-cholesterol (CAS #1220104) were determined in a spectrophotometer (Beckman®, DU640, Corona, USA).

### **Statistics**

Shapiro Wilk was used as normality test. ANOVA and Tukey test were carried out. A 5% significance level was used and GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA, USA) was used as software.

## **RESULTS**

The FJP powder is constituted mostly of sugars, fibers, polyphenols and anthocyanins with significant antioxidant activity (Table 1). A linear trend line showed a correlation between the total antioxidant activity and the anthocyanins values ( $r^2 = 0.9744$ ;  $r = -0.9871$ ;  $P = 0.1023$ ;  $y = 906.03 - 0.1789x$ ). The chromatographic analysis showed that FJP possessed four identified compounds: ellagic and gallic acid, cyanidin 3-glucoside and quercetin (Table 1, Figure 1).

There was no statistical difference in food intake among G0, G1, G2, and G3 rats (data ranging from 21.2  $\pm$  1.4 to 22.8  $\pm$  1.1 g per day). The intake of jaboticaba peel by the experimental groups showed a dependent-dose response (214, 456 and 884 mg day<sup>-1</sup> or 98.43, 130.29 and 252.57 mg 100g<sup>-1</sup> day<sup>-1</sup> for G1, G2 and G3, respectively). As expected, the results showed no differences in the weight gain among animals from groups G0 and G1, G2 and G3. The final body weight was similar among the groups with means ranging from 356.9  $\pm$  27.74 to 356.9  $\pm$  22.96. The weight of livers from G1 rats (2.81  $\pm$  0.12 g%) were smaller than the G0 group (3.31  $\pm$  0.25 g%).

The reduction of the blood glucose in group G2 was significant when compared to the control (G0). According to these results (Figure 2A), the addition of 2% FJP to the diet of rats decreased 19.8% in plasma glucose in comparison to G0 (117.4 mg dL<sup>-1</sup> in G2 and 94.15 mg dL<sup>-1</sup> in G0).

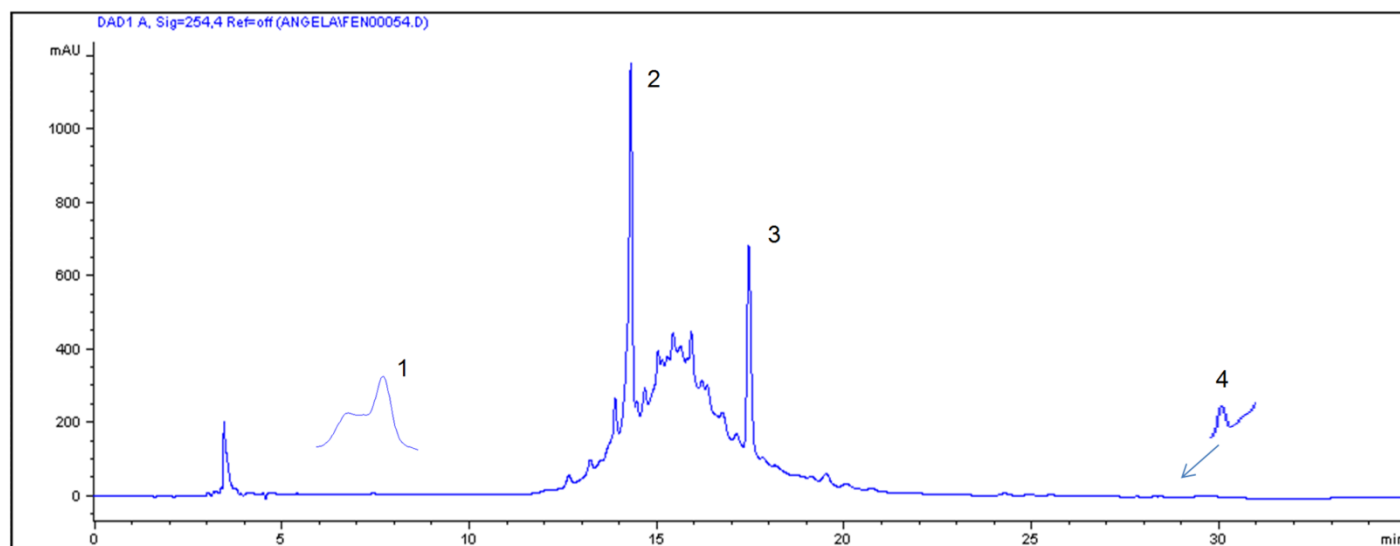
The G1 group showed a reduction in total cholesterol and triglycerides levels (Figure 2B and C). The intake of 1% FJP caused a significant decrease (70.75%) in the total cholesterol levels of animals in comparison to G0. However, the trend was not linear: cholesterol level increased 61.94% in G3 compared to control group, reaching 47.37  $\pm$  6.28 mg dL<sup>-1</sup>, which was an unexpected result, and could indicate that jaboticaba peels, when consumed in excess, may generate undesirable effects. A similar effect was showed in the LDL-cholesterol analysis (Figure 2D). Although, no change was observed in the levels of HDL of the experimental groups (Figure 2E).

There was no statistical difference in the ALT activity among the groups (Figure 2F). The AST enzyme

**Table 1.** Proximate composition and polyphenol compounds of the freeze-dried jaboticaba peel.

Component	Content (%)	SD <sup>a</sup>
Moisture	22.72	0.23
Lipids	1.27	0.07
Ash	3.01	0.07
Crude protein	3.90	0.12
Crude fiber	6.45	0.28
Total sugars <sup>b</sup>	59.04	0.99
Reducing sugars	40.95	0.57
Sucrose	18.09	0.01
Gallic acid (mg/ 100 g)	3.87	0.15
Ellagic acid (mg/ 100 g)	362.43	3.67
Quercetin (mg/ 100 g)	5.17	0.05
Total anthocyanins (mg/ 100 g)	926.78	16.55
FCRRS (mg GAE/g)	86.67	1.05
ORAC (μmol TE/g)	807.00	4.83

<sup>a</sup>Standard deviation. <sup>b</sup>Total sugars were calculated by the sum of reducing sugars and sucrose.



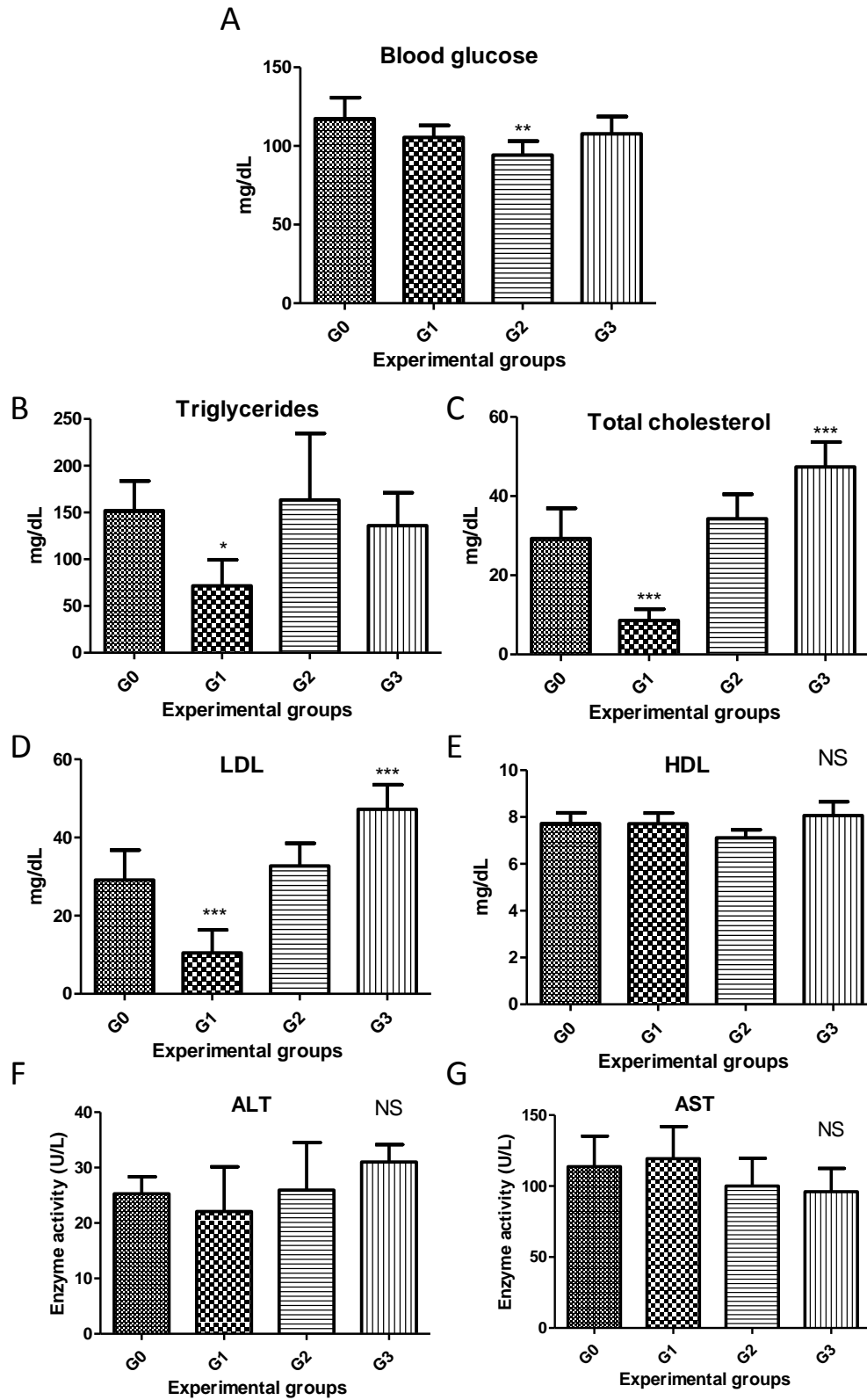
**Figure 1.** Typical chromatogram of 20 mg mL<sup>-1</sup> methanolic extract of freeze-dried jaboticaba peel by HPLC-DAD (254 nm). Compounds: 1) gallic acid; 2) cyanidin 3-glucoside; 3) ellagic acid; 4) quercetin.

presented a slight tendency for activity reduction as FJP level increases, with no significance ( $P > 0.05$ ) (Figure 2G).

## DISCUSSION

Jaboticaba peels have not been largely used by the food industry in commercial products. Although, the interest in this byproduct have increased since several bioactive

compounds were described by some authors (Pinto et al., 2011; Wu et al., 2013). The freeze-dried jaboticaba peel is a rich source of polyphenols, mainly flavonoids, anthocyanins and dietary fibers (Leite et al., 2011). Corroborating this study, ellagic acid, gallic acid, tannins, quercetin derivatives and anthocyanins were also identified by other authors (Abe et al., 2012; Alezandro et al., 2013). The polyphenols of FJP might be responsible for its antioxidant, antimutagenic, anti-inflammatory and antitumoral activities (Dragano et al., 2013; Leite-Legatti



**Figure 2.** Plasmatic glucose levels (A), total triglycerides (B), cholesterol (C), LDL-cholesterol (D), HDL-cholesterol (E) and plasmatic ALT (F) and AST enzymes (G) of freeze-dried jaboricaba peeled *Wistar* rats. The groups G0, G1, G2 and G4 received 0.00, 0.01, 0.02 and 0.04 g jaboricaba peel  $\text{kg}^{-1}$  in diet, respectively. Data were expressed as the mean  $\pm$  SD values ( $n=8$ ). The statistical significance among the groups was evaluated using ANOVA and Tukey test (\* $P<0.05$ ; \*\* $P<0.01$ ; and \*\*\* $P<0.001$  when compared to G0). NS= non-significant.



et al., 2012; Alezandro et al., 2013).

Polyphenol-rich fruits, as jaboticaba, is strongly effective in increasing insulin secretion. This could explain the plasma glucose reduction found in the present study, possibly caused by the increase in insulin secretion in FJP-fed animals (Lenquiste et al., 2012). The fibers from FJP (Table 1) could also be responsible to enhance hypoglycemic effect, acting in synergy with polyphenols from the peel (Kim et al., 2010). In addition, studies have shown that the intake of jaboticaba peel is related to an increased insulin sensitivity in liver and adipose tissue (Dragano et al., 2013; Lenquiste et al., 2012). Studies with polyphenol and fiber-rich meals corroborate this result: reduction in plasma glucose levels by 13.04% (Esteves et al., 2011).

The intake of 1% FJP caused a significant decrease (48.69%) in the total cholesterol and triglycerides levels of G1 animals. Although, the rats that consumed 4% of FJP showed an unexpected increase in total cholesterol, suggesting that there is no dose-response trend. However, other studies using different animal models, but the same doses of FJP did not confirm this data (Lenquiste et al., 2012). Corroborating this study, Alezandro et al. (2013) showed that daily 1 and 2g kg<sup>-1</sup> doses of aqueous extract of jaboticaba peel were responsible for plasma triglycerides and cholesterol lowering levels.

In another way, as in other studies, no significant differences in HDL-cholesterol levels were found in the experimental groups in comparison to the control (Alezandro et al., 2013). Kwon et al. evaluated the effect of black soy anthocyanins addition on an hyperlipidic diet of rats and observed 23 and 20% reduction in triglycerides and cholesterol levels, respectively, and an increase of 37% in the HDL-cholesterol fraction (Kwon, et al., 2007). In contrast to the present, a recent study showed that FJP could enhance HDL-cholesterol in obese rats (Lenquiste et al., 2012).

Like polyphenols, the fibers of jaboticaba may have also played the hypocholesterolemic role. The polyphenol and fiber-rich diets was mainly related to an up-regulation of CYP7A1 expression, suggesting an increase in the conversion of hepatic cholesterol to bile acid, resulting in a decrease in the plasma cholesterol levels (Kim et al., 2010; Villanueva et al., 2011). The intake of these diets also indicate a decreased hepatic cholesterol synthesis by down-regulation of CYP 51, and higher fecal lipid excretion, corroborating the plasma cholesterol-lowering effect (Kim et al., 2010).

Kalt et al. verified that pigs fed with rich fibers diet supplemented with 2% freeze-dried blueberries showed a reduction of 11.7% for total cholesterol and 15.1% for LDL-cholesterol (Kalt et al., 2008). In our study, the addition of 1% FJP was sufficient to reduce total cholesterol to 70%. In view of the increase in total cholesterol and LDL-cholesterol fractions in G2 and G3 groups, we may suppose that 2% FJP should represent

an upper limit for dietary intake. Thus, even that other studies showed good results with the same doses (Dragano et al., 2013; Lenquiste et al., 2012), more investigations are necessary in order to elucidate the mechanisms that may cause undesirable effects.

Aminotransferases (ALT and AST) are enzymes present in high levels in the muscle, liver and brain. Increased levels of ALT are therefore relatively specific to hepatobiliary disease, although the AST levels are likely to be higher increased in the diseases of other organs. Increases over 10 times above the upper limit of normal variation normally are considered hepatic or biliary pathologies (Motta, 2003). The slight increase of ALT in the present study is therefore irrelevant and not related to a possible liver damage, since the AST level remained unchanged.

## Conclusions

Freeze-dried jaboticaba peel showed high amounts of fibers and polyphenols, as ellagic, gallic acid and anthocyanins. Significant reductions in the glucose levels were observed in rats fed diets containing 2% FJP. The rats fed 1% FJP also showed reduced levels of total triglycerides, cholesterol and LDL cholesterol. Thus, in the same conditions of this study, we can conclude that FJP do not represent toxicity to the liver, since the ALT and AST activity was similar among the groups. In addition, the 2% FJP in diet is the upper limit dose in which we have observed functional role without any health undesirable effects. Further investigations about higher and lower doses of phenolic compounds in the human diet are needed. It is, therefore, very important to investigate the metabolism of such compounds *in vivo* and their effective dosages.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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## REFERENCES

- Abe LT, Lajolo FM, Genovese MI: Potential dietary sources of ellagic acid and other antioxidants among fruits consumed in Brazil: Jaboticaba (*Myrciaria jaboticaba* (Vell.) Berg) (2012). *J. Sci. Food Agric.* 92: 1679-87.
- Alezandro MR, Dubé P, Desjardins Y, Lajolo FM, Genovese MI (2013). Comparative study of chemical and phenolic compositions of two species of jaboticaba: *Myrciaria jaboticaba* (Vell.) Berg and *Myrciaria cauliflora* (Mart.) O. Berg. *Food Res Int* 54:468-477.
- Alezandro MR, Granato D, Genovese MI (2013). Jaboticaba (*Myrciaria*

- jaboticaba (Vell.) Berg), a Brazilian grape-like fruit, improves plasma lipid profile in streptozotocin-mediated oxidative stress in diabetic rats. *Food Res. Int.* 54(1):650-659.
- AOAC (1995). Official methods of analysis of Association of Official Analytical Chemists international (1995). 16 edn. Arlington: AOAC.
- Bligh EG, Dyer WJ(1959). A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Phys.* 37: 911-17.
- Colomeu TC, Figueiredo D, Cazarin CBB, Schumacher NSG, Maróstica Jr MR, Meletti LMM, Zollner RL (2014). Antioxidant and anti-diabetic potential of *Passiflora alata* Curtis aqueous leaves extract in type 1 diabetes mellitus (NOD-mice). *Int. Immunopharmacol.* 18: 106-15.
- Davalos A, Gomez-Cordoves C, Bartolome B (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *J. Agric. Food Chem.* 52:48-54.
- dos Santos HM, Oliveira DF, de Carvalho DA, Pinto JMA, Campos VAC, Mourao ARB, Pessoa C, de Moraes MO, Costa-Lotuf LV (2010). Evaluation of native and exotic Brazilian plants for anticancer activity. *J. Nat. Med Tokyo* 64:231-238.
- Dragano NRV, Marques AC, Cintra DEC, Solon C, Morari J, Leite-Legatti AV, Velloso LA, Maróstica Júnior MR (2013). Freeze-dried jaboticaba peel powder improves insulin sensitivity in high-fat-fed mice. *Br. J. Nutr.* 110:447-455.
- Esteves EA, Oliveira LG, Pires ST, Batista AG, Dessimoni-Pinto NAV, Santana RC (2011). Nutritional composition of *Copaifera langsdorffii* Desf. aril flour and its effect on serum lipids and glucose in rats. *Food Res. Int.* 44:2357-2361.
- Guo HH, Xia M, Zou T, Ling W, Liu GL, Zhong RM, Zhang W (2012). Cyanidin 3-glucoside attenuates obesity-associated insulin resistance and hepatic steatosis in high-fat diet-fed and db/db mice via the transcription factor FoxO1. *J. Nutr. Biochem.* 23:349-60.
- IAL (1985). Normas analíticas. Métodos químicos e físicos para análise de alimentos. 2 edn. São Paulo-SP: Instituto Adolfo Lutz.
- Jayaprakasam B, Olson LK, Schutski RE, Tai MH, Nair MG (2006). Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (*Cornus mas*). *J. Agric. Food Chem.* 54:243-248.
- Kalt W, Foote K, Fillmore SAE, Lyon M, Van Lunen TA, McRae KB (2008). Effect of blueberry feeding on plasma lipids in pigs. *Br. J. Nutr.* 100:70-78.
- Kim H, Bartley GE, Rimando AM, Yokoyama W (2010). Hepatic Gene Expression Related to Lower Plasma Cholesterol in Hamsters Fed high-fat diets supplemented with blueberry peels and peel extract. *J. Agric. Food Chem.* 58:3984-3991.
- Kwon SH, Ahn IS, Kim SO, Kong CS, Chung HY, Do MS, Park KY (2007). Anti-obesity and hypolipidemic effects of black soybean anthocyanins. *J. Med. Food* 10:552-556.
- Lane H, Eynon L(1923). Determination of reducing sugar by means of Fehling's solution with methylene blue as internal indicator. *J. Soc. Chem. Ind.* 42:32T-37.
- Leite AV, Malta LG, Riccio MF, Eberlin MN, Pastore GM, Maróstica MR (2011). Antioxidant Potential of Rat Plasma by Administration of Freeze-Dried Jaboticaba Peel (*Myrciaria jaboticaba* Vell Berg). *J. Agric. Food Chem.* 59: 2277-83.
- Leite-Legatti AV, Batista AG, Dragano NRV, Marques AC, Malta LG, Riccio MF, Eberlin MN, Machado ART, Carvalho-Silva LB, Ruiz ALTG, Carvalho JE, Pastore GM, Maróstica Júnior MR (2012). Jaboticaba peel: Antioxidant compounds, antiproliferative and antimutagenic activities. *Food Res. Int.* 49:596-603.
- Lenquiste SA, Batista AG, Marineli RD, Dragano NRV, Maróstica MR (2012). Freeze-dried jaboticaba peel added to high-fat diet increases HDL-cholesterol and improves insulin resistance in obese rats. *Food Res. Int.* 49:153-160.
- Lima ADB, Correa AD, Saczk AA, Martins MP, Castilho RO (2011). Anthocyanins, Pigment Stability and Antioxidant Activity in Jaboticaba [*Myrciaria cauliflora* (Mart.) O. Berg]. *Rev. Bras. Frutic.* 33: 877-887.
- Motta VT (2003). *Bioquímica Clínica para o Laboratório: princípios e interpretações*. 4 edn: Robe.
- Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P, Klimis-Zacas D, Margarity M, Lamari FN (2009). Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. *Behav. Brain Res.* 198:352-358.
- Pinto NAVD, Moreira WA, Cardoso LM, Pantoja L (2011). Jaboticaba peel for jelly preparation: an alternative technology. *Ciência e Tecnologia de Alimentos* 31:864-69.
- Port's PdS, Chisté RC, Godoy HT, Prado MA (2013). The phenolic compounds and the antioxidant potential of infusion of herbs from the Brazilian Amazonian region. *Food Res. Int.* 53(2):875-881.
- Reeves PG, Nielsen FH, Fahey GC (1993). *Ain-93 Purified Diets for Laboratory Rodents - Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the Ain-76a Rodent Diet*. *J. Nutr.* 123:1939-51.
- Silva FGD, O'Callaghan Y, O'Brien NM, Netto FM (2013). Antioxidant Capacity of Flaxseed Products: The Effect of In vitro Digestion. *Plant Food Hum. Nutr.* 68:24-30.
- Swain T, Hillis WE (1959). The phenolic constituents of *Prunus domestica*. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* 10:63-68.
- Tsuda T, Horio F, Uchida K, Aoki H, Osawa T(2003). Dietary cyanidin 3-O-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *J. Nutr.* 133: 2125-30.
- Villanueva MJ, Yokoyama WH, Hong YJ, Bartley GE, Ruperez P (2011) Effect of high-fat diets supplemented with okara soybean by-product on lipid profiles of plasma, liver and faeces in Syrian hamsters. *Food Chem.* 124:72-79.
- Wrolstad RE (1993). *Color and Pigment Analyses in Fruit Products*. Corvallis: Oregon Agricultural Experimental Station.
- Wu SB, Wu J, Yin ZW, Zhang JZ, Long CL, Kennelly EJ, Zheng SP (2013). Bioactive and Marker Compounds from Two Edible Dark-Colored *Myrciaria* Fruits and the Synthesis of Jaboticabin. *J. Agric. Food Chem.* 61: 4035-43.

Full Length Research Paper

## Improving oxidative stability of ghee using natural oxidants from agri-industrial wastes

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**Bioactive compounds found in peanut skin (PS), pomegranate peels (PP) and olive pomace (OP) cake were extracted using ethanol (80%), ethyl acetate and hexane. The ethanol extract showed slightly better antioxidant characteristics than ethyl acetate and hexane extracts. Extracts showed varying degrees of antioxidant potential in different test systems in a dose-dependent manner. In general, it was observed that extracts with higher antioxidant capacity were in parallel to their higher phenolic contents. Total phenolic compounds (as gallic acid equivalent, GAE) ranged between 0.89 to 16.6, 1.83 to 261 and 1.56 to 124 mg GAE/g extract for OP, PS and PP, respectively. Ethanol extracts of different by-products were added to ghee at concentrations of 200, 400 and 600 ppm, respectively. BHA was also added to ghee at a concentration of 200 ppm. All samples were incubated at 63°C/21days. Ethanol extracts of PS, OP and PP gave good antioxidant activity during accelerated oxidative incubation of ghee. It could be concluded that ethanol extracts under study, at a concentration of 200 ppm, can retard fat auto-oxidation.**

**Key words:** Ghee, agri industrial by-products, natural antioxidants, stability indices.

### INTRODUCTION

Antioxidants (natural and synthetic) play a significant role in retarding lipid oxidation reactions in food products. The detrimental effects of excessive lipid oxidation such as formation of off-flavors and undesirable oxidized chemical compounds (aldehydes, ketones and organic acids etc) are well known (Saad et al., 2007). Synthetic antioxidants [for example, tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated

hydroxytoluene (BHT)] are widely used as food additives, but their application has been reassessed because of possible toxic or carcinogenic components formed during their degradation (Jo et al., 2006; Pitchaon et al., 2007). Consequently, the search for endogenous protective ingredients in foods has been intensified wherein their utilization requires only manipulation of food formulations. A number of natural antioxidants have been added during

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food processing and have elongate the shelf life and oxidative stability of stored products (Chenn et al., 2008; Ebrahimabadi et al., 2010; Jang et al., 2012; Xiaowei et al., 2011).

A huge amount of plant biomass wastes is produced yearly as by-products from the agro-food industries. These wastes are attractive sources of natural antioxidants. The high concentration of phenolic compounds present in peels, skins and seeds supports the utilization of these residues as a source of natural antioxidants. Phenolic compounds exhibit a wide range of physiological properties such as anti-allergenic, anti-arthrogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thermotoxic, cardio protective and vasodilatory effects (Balasundram et al., 2006). Phenolics could be extracted by water or solvents and the extraction conditions need to be optimized with respect to solvent polarity and physical conditions (Nepote et al., 2005). In addition, research has indicated that natural phenolic compounds can be extracted from raw materials or waste products of food industry (Peschel et al., 2006).

Studies were conducted to investigate antioxidants properties of peanut, peanut kernels, peanut hulls and peanut-based products (Yu et al., 2005; Wang et al., 2007). Peanut skins were demonstrated to be rich in phenolics and other health promoting compounds (Yu et al., 2005; Wang et al., 2007; Monagas et al., 2009). The olive oil industry generates large quantities of a deleterious by-product known as olive pomace. Olive pomace has broad spectrum toxicity against some microorganisms, plants, insects, animals and human cells (Obied, 2007; Aldini et al., 2006). Nevertheless, olive pomace has been considered as a potential source of natural antioxidants (Niaounakis and Halvadakis, 2004; Aldini et al., 2006). A wide range of phenolic compounds has been identified in virgin oil (Suarez et al., 2010) wherein only ca. 2% of the total phenols found in olive fruits are transferred to the extracted olive oil. The rest of phenolic compounds (98%) are retained in the olive waste cake. Pomegranate has been used extensively in the folk medicine of many cultures and its consumption has grown tremendously especially in the last decades (Li et al., 2006; Cam et al., 2009). The peels of some fruits have higher antioxidant activity than pulps (Guo et al., 2003; Fuhrman et al., 2005). Pomegranate is a good example for this type of fruits wherein their peels constitute approximately 40% of the whole fruit and are rich in ellagic acid derivatives (Cerda et al., 2003; Seeram et al., 2005).

From an environmental and economic perspective, it is very important that plant by-products produced by agro-food industry be used. Therefore, the objectives of this study were; (1) to evaluate different extracts from peanut skin (PS), pomegranate peels (PP) and olive waste cake (OP) as a source of natural antioxidants, (2) to characterize the composition and content of phenolics in different extracts and (3) to evaluate the efficiency of using

agro food wastes ethanolic extracts in improving the quality, overall acceptance and oxidative stability of ghee during storage under thermal oxidative conditions.

## MATERIALS AND METHODS

Plant biomass wastes, as a by-products of food industries, commonly found in Egypt, were used in this investigation. Olive (*Olea europaea* L.) pomace (OP) was obtained from Food Technology Research Institute (Agricultural Research Center, Giza, Egypt). Peanut (*Arachis hypogaea* L.) skins (PS) were obtained from the 10<sup>th</sup> of Ramadan City. Pomegranate (*Punica granatum* L.) fruits (PP), were obtained from local market (Zagazig, Egypt), washed with distilled water and manually peeled. Pomegranate peels (PP) were collected then rinsed with distilled water and considered as a by-product. The starting materials were dried in an air draft drying oven (40°C) until the moisture content became 12% or less. By-products were ground and sieved through 60 mesh sieve and finally cooled or kept at 4°C until the extractions were carried out. Butylated hydroxyl anisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid and quercin were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were of the highest purity available.

### Preparation of extracts

Dried materials were extracted with different solvents, named ethanol (80%), ethyl acetate and hexane at a ratio of 10:1 (v/w, 10 mL solvent: 1 g raw material) in closed vessels by stirring at room temperature (25°C) for 4 h followed by filtration through Whatmann no. 1 filter paper. The residues were re-extracted again under the same conditions.

All vessels were wrapped with aluminum foil to prevent light degradation during extraction (Yu et al., 2005). N-Hexane and ethyl acetate extracts were evaporated in a rotary evaporator (Buchi-water bath-B-480, Switzerland) below 40°C, while ethanol 80% extracts were freeze-dried (Thermo Electron Corporation-Heto Power Dry LL 300 Freeze Dryer, Gzechoslovak). The dried extracts after evaporation of solvents were weighed to determine the yield and stored at -20°C until used.

### Determination of total phenolic compounds (TPC)

The concentration of TPC in different extracts was measured using UV spectrophotometer (Jenway-UV-VIS Spectrophotometer), based on a colorimetric oxidation/reduction reaction, as described by Škerget et al. (2005) using Folin-Ciocalteu reagent. Specifically, 0.5 ml of diluted extract (10 mg in 10 ml solvent) was mixed with 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with distilled water) and 2 ml of Na<sub>2</sub>CO<sub>3</sub> (75 mg/ml). The sample was incubated for 5 min at 50°C then cooled. For a control sample, 0.5 ml of distilled water was used. The absorbance was measured at 760 nm. Total phenolic content expressed as gallic acid equivalent (GAE) was calculated, and the results were expressed as a mg GAE /g extract.

### Identification of phenolic acids using HPLC

Phenolic acids of the dried extracts were identified according to the method described by Mattila et al. (2000). HPLC (Hewlett Packard series 1050, USA) equipped with auto sampling, injector, solvent degasser, UV detector set at 330 nm and quarter HP pump (series 1050) was used. Column (C<sub>18</sub> hypersil BDS) with particle size 5 µm was used. The separation was carried out with methanol and

**Table 1.** Yield of extracts (g/100 g) for different plant wastes-by products of food processing.

Plant waste	Hexane extract	Ethylacetate extract	Ethanol 80% extract
Olive pomace	5.23	1.00	9.43
Pomegranate peels	0.50	0.90	43.84
Peanut skin	9.47	7.67	14.56

**Table 2.** Total phenolic compounds (mg gallic acid /g extract) in different extracts.

By-product	Concentration (mg gallic acid/ g extract)		
	Hexane	Ethyl acetate	Ethanol 80%
Olive pomace (OP)	16.63	0.89	12.23
Pomegranate peels (PP)	1.56	12.49	124.23
Peanut skins (PS)	1.83	5.69	261.69

acetonitrile as a mobile phase at a flow rate of 1mL/min. The column temperature was performed at room temperature (25°C) throughout the experiment. Identification and quantification were carried out based on calibrations of the standards prepared from phenolic acids dissolved in a mobile phase. Retention time and peak area were used for calculation of phenolic acid compounds by the data analysis of Hewlett Packard Software.

#### Radical scavenging activity (RSA) of extracts

The electron donation ability of the obtained extracts was measured by bleaching of the purple colored solution of DPPH according to the method of Hanato et al. (1988). 100 µL of each extracts (10 mg extract/10 ml solvent) was added to 3 ml of 0.1 mM DPPH dissolved in ethyl acetate, ethanol and hexane according to the solvent used for extraction. After an incubation period of 30, 60 and 120 min at room temperature, the absorbance was determined against a control at 517nm (Gulcin et al., 2004). Percentage of antioxidant activity of DPPH was calculated as follows:

$$\text{Antioxidant activity (Inhibition) \%} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

Where,  $A_{\text{control}}$  is the absorbance of the control reaction and  $A_{\text{sample}}$  is the absorbance in the presence of the extract. BHA was used as a positive control. Samples were analyzed in triplicate.

#### Stability of ghee enriched with ethanol 80% extract

The butter, used for preparing ghee in the present study, was made from pasteurized and un-ripened buffaloes' cream. The butter was converted into ghee by boiling according to the method described by Fahmi (1961). Ghee samples were divided into 11 portions as follows: Portion 1 was kept without additives as control throughout the experimental period and was considered to be as a negative control (C); Portion 2 was treated with 200 ppm BHA throughout the experimental period of three weeks and was considered to be as positive control (C<sub>1</sub>); portions 3, 4 and 5 were treated with 200, 400 and 600 ppm of PS ethanolic extract, respectively, (T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>).

Portions 6, 7 and 8 were treated with 200, 400 and 600 ppm of PP ethanolic extract, respectively, (T<sub>4</sub>, T<sub>5</sub> and T<sub>6</sub>). Portions (9, 10 and 11) were treated with 200, 400 and 600 ppm of OP ethanolic extract, respectively, (T<sub>7</sub>, T<sub>8</sub> and T<sub>9</sub>).

All samples were incubated in an oven at 63±1°C to accelerate

the oxidation for 21 days. Samples were analyzed every three days for peroxide value (PV), acid value (AV), and 2-thiobarbituric acid (TBA) value. AV and PV were determined according to AOAC (1984). Determination of lipid oxidation was assessed in triplicates by the TBA method according to Fernandez-Lopez et al. (2005).

#### Oxidation stability test of Ghee

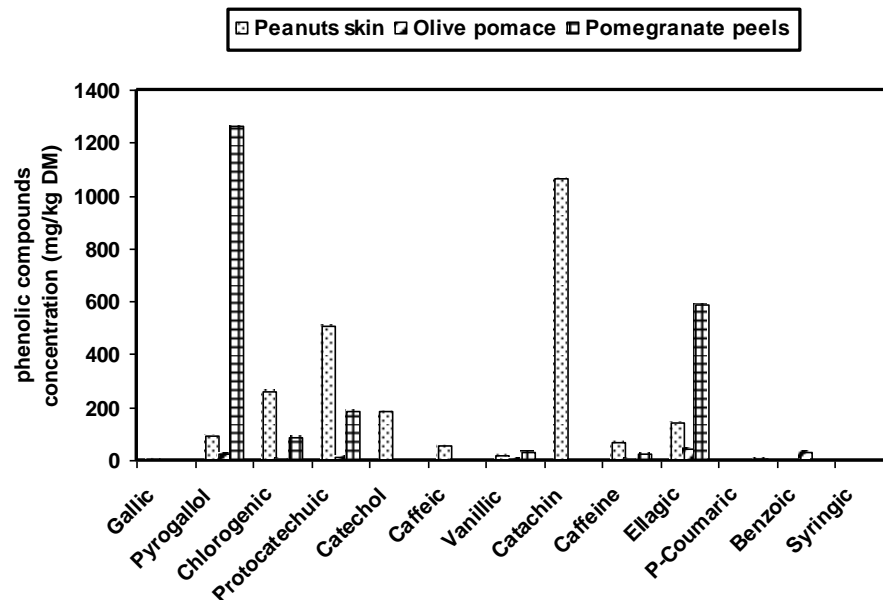
Determination of an oxidative stability of ghee by Rancimat equipment (USA, model 617), was based on volatile acids from oxidation reaction passed through DI water, in which conductivity values were detected. Heating block was held constant at 130°C. A rate of air flow through liquid butter oil (ghee) was 10 L/h. Prior to the testing, frozen samples of ghee were thawed at 40-50°C, and a 3±0.002 g of ghee sample was taken for the analysis according to AOCS (1997).

## RESULTS AND DISCUSSION

### Characterization of phenolic compounds TPC in different extracts

The yield of extracts with different solvents varied from 0.1 to 42.5 g extract/100 g wastes (Table 1). PS, PP and OP had the highest yield when extracted with ethanol 80% followed by hexane and ethyl acetate, respectively. Variation in the extraction yields of different extracts might be attributed to differences in polarity of compounds found in plants such differences have been reported (Jaya et al., 2001).

The amount of TPC varied in the different extracts, ranging from 0.89 to 261 mg GAE/g extract (Table 2). In general, the results stated that ethanol 80% and ethyl acetate were better than hexane in extracting phenolics from PP and PS owing to their higher polarity and good solubility (Siddhuraju and Becker, 2003; Kequan and Liangli, 2004). On the other side, hexane extracted the highest amount of phenolics from OP followed by ethanol 80% as shown in Table 2.



**Figure 1.** Levels of phenolic compounds in plant wastes-by products of processing food extract as determined by HPLC.

Figure 1 shows the percentage of identified phenolic compounds in PS, PP and OP. There was a great variation among the components identified in each waste by-product. Phenolic compounds were identified in PS, namely pyrogallol, protocatechuic, catachin and ellagic acid, with amounts ranging from 0.07 to 10.64 mg/g. The main phenolics identified in OP were pyrogallol, ellagic acid, chlorogenic, protocatechuic with amount ranging from 0.01 to 0.46mg/g. The major phenolic compounds identified in PP were pyrogallol, ellagic acid, chlorogenic, protocatechuic with amount ranging from 0.05 to 12.64 mg/g. Balasundran et al. (2006) stated that the antioxidant activity of phenolic compounds depends on the structure, in particular the number and positions of the hydroxyl groups of the nature of substitution on the aromatic rings. Moure et al. (2001) reported that the antioxidant compounds from residual sources could be used for increasing the stability of foods by preventing lipid peroxidation and also for protecting oxidative damage in living systems by scavenging oxygen radicals. It is well known that total antioxidant activity of waste extracts was line early proportional to the concentration of total phenolics (Meftahizade et al., 2011).

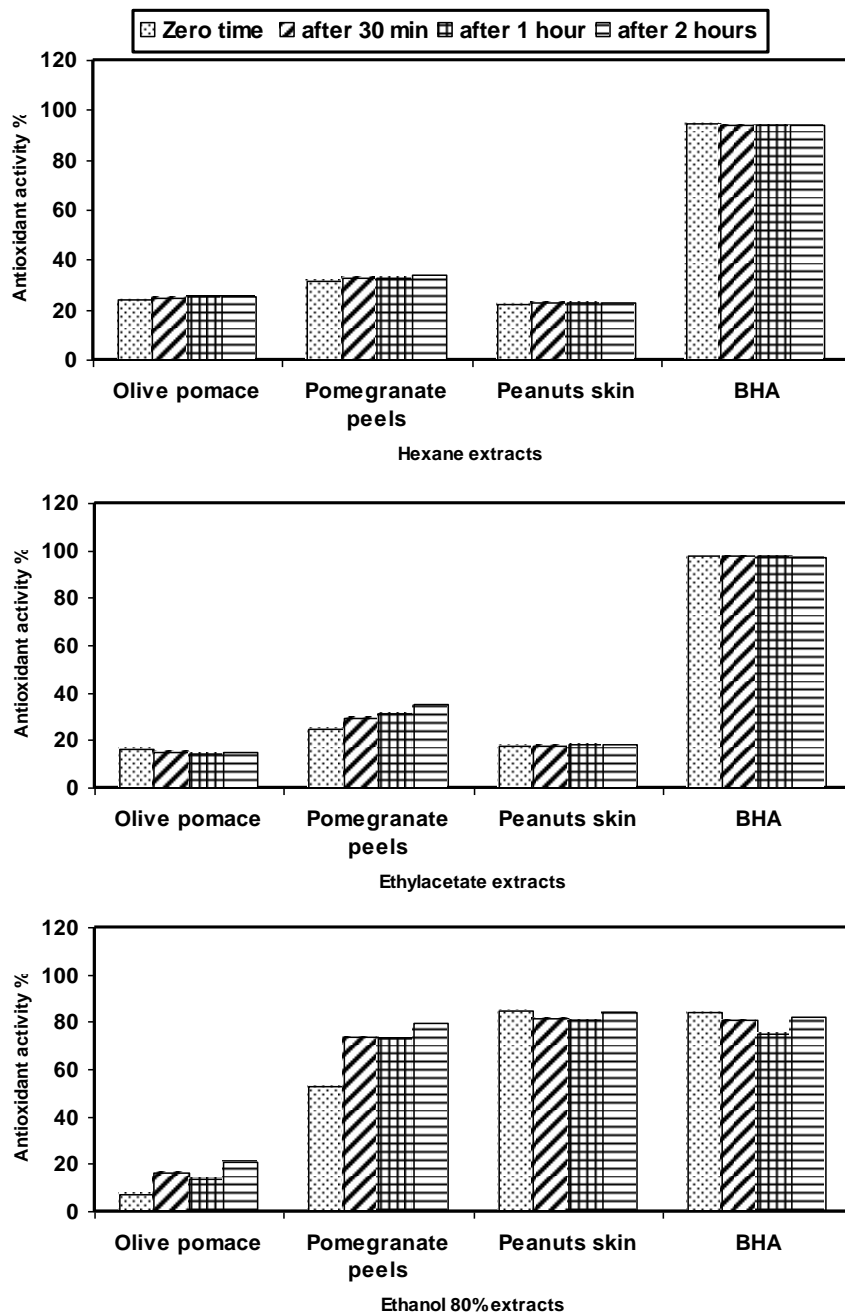
#### RSA against DPPH•

The results of RSA of various extracts are represented in Figure 2. The results clearly indicate that all extracts exhibited antioxidant activity. The extracts that contained a high amount of TPC (Table 2) showed high RSA. In general, ethanol 80% followed by hexane then ethyl

acetate extracts showed RSA as strong as that of BHA (Figure 3A, B and C). It has been proven that the antioxidant activity of plant extracts is mainly ascribable to the concentration of phenolic compounds in the plant (Heim et al., 2002). The extracts RSA with different solvents varied from 91.4 to 5.50% after 120 min of incubation.

The highest RSA was observed with PP ethanol 80%, hexane and ethyl acetate extracts with respective values of 80, 38 and 35%, respectively. Ethanol 80%, hexane and ethyl acetate extracts of PS had values of 85, 22 and 19%, respectively. In addition, hexane, ethanol 80% and ethyl acetate extracts of OP had values of 24, 20 and 18%, respectively.

The results of the DPPH• radical scavenging assay suggest that components involving the extracts are capable of scavenging free radicals *via* electron- or hydrogen-donating mechanisms and thus might be able to prevent the initiation of deleterious free radical mediated chain reactions in susceptible matrices. This further shows the capability of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathological damage. The effect of antioxidants on DPPH radical-scavenging is thought to be due to their hydrogen-donating ability, DPPH• is a stable free radical and accepts an electron or hydrogen radical to become a stable molecule (Gulcin et al., 2004). Free radicals involved in the process of lipid peroxidation are considered to play a major role in numerous chronic pathologies such as cancer and cardiovascular diseases (Dorman et al., 2003).



**Figure 2.** RSA of hexane (a), ethyl acetate (b) and ethanol 80% (c) wastes extracts in DPPH• radical scavenging activity system compared with BHA.

### Stability of enriched-ghhee during storage under accelerated oxidative conditions

#### Peroxide values

Data illustrated in Figure 3 shows that the PV of control ghee samples increased during the accelerated incubation up to 21 days. The others samples enriched

with BHA and natural antioxidants had the lower PV values than the control sample during storage under accelerated incubation at 63°C/21days. The results obtained in this work reflected the impact of these extracts, as natural antioxidants, in the retarding of ghee oxidation. These results are in agreement with those of Puravankara et al. (2000) and Pankaj et al. (2013). The order of efficiency in inhibiting oxidation was in the order PS, PP and finally OP extract.

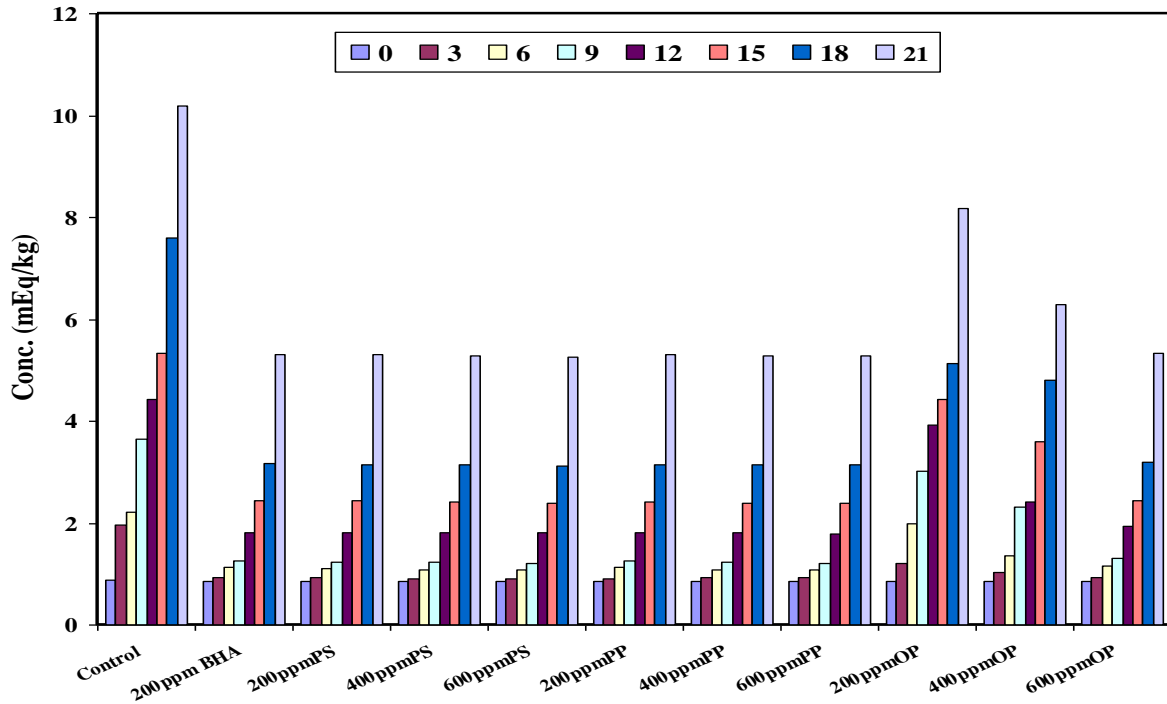


Figure 3. Effect of ethanol extracts at different concentration on the peroxide value of Ghee during storage.

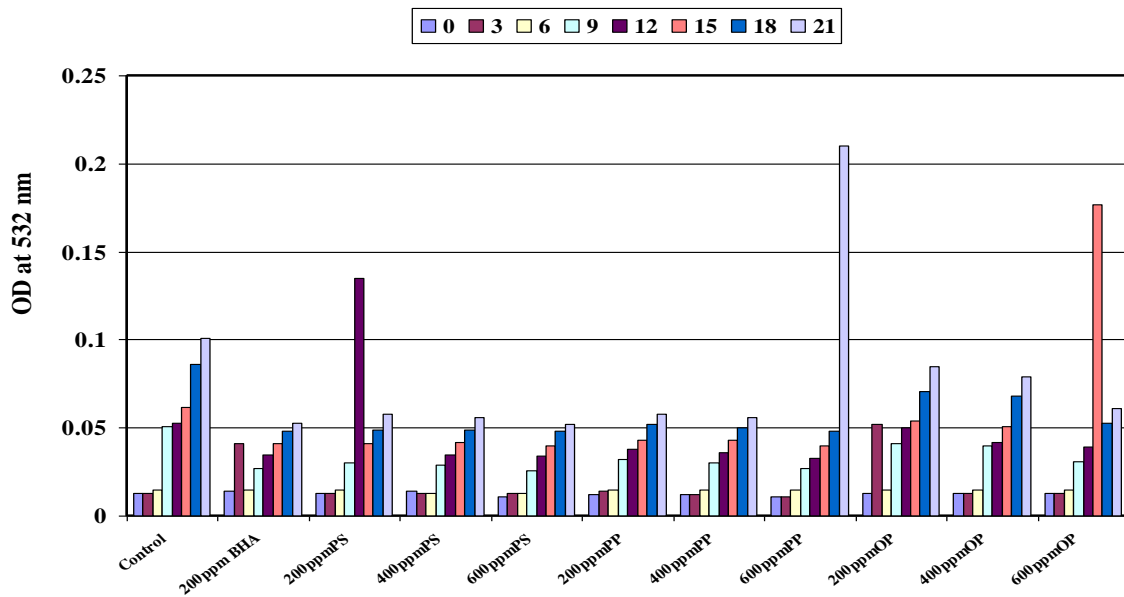


Figure 4. Effect of ethanol extracts at different concentration on the TBA of Ghee during storage.

**TBA**

It is well known that TBA values are taken as an index to evaluate the advance of oxidation changes occurred in oil and fats. The addition of extracts as natural antioxidants to ghee retarded the oxidative changes during accele-

rated storage (Figure 4). This means that the formation of malonaldehyde, which affect the formation of pink colour intensity from the reaction of TBA material with malonaldehyde, took place at a relatively lower rate in treated ghee samples. However, the control ghee samples showed higher TBA values throughout the



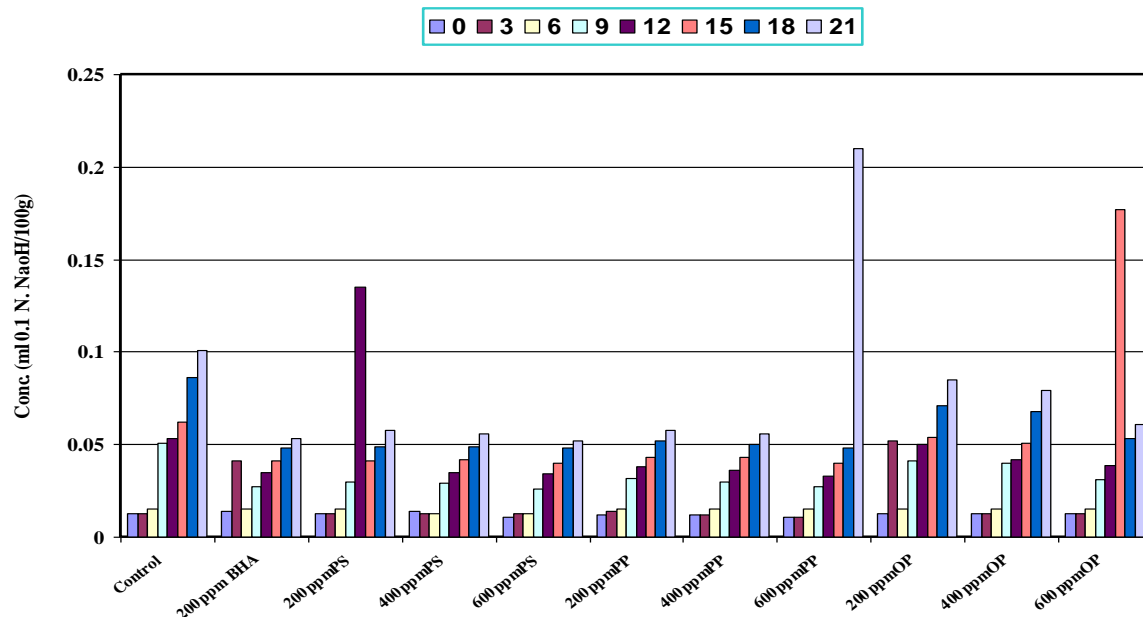


Figure 5. Effect of ethanolic extracts at different concentrations on acid value of Ghee during storage.

accelerated incubation period. These structural requirements were supported by the powerful antioxidant activity of the well-known BHA. Phenolic compounds act as hydrogen or electron donors to the reaction mixture and therefore the formation of hydro peroxides are decreased. The slow formation of conjugated dienes and consequently the secondary products by extracts and their major compounds indicated that these materials may act as hydrogen donors to proxy radicals, thus, retarding the autoxidation of linoleic acid by chain radical termination (Farag et al., 1989; Özkanlı and Kaya, 2007; Mohdali, 2010).

### Acid value

Data illustrated in Figure 5 shows that the AV remained without noticeable changes within the first six days of storage at 63°C for all treatments including the control. Slightly increases in AV were observed until 15 days of storage period, then considerable increase in AV were recorded till the end of storage period (21 days) for all samples including the control. Data presented in Figure 3 show clearly that the AV of stored ghee was noticeably affected by enrichment with by-products ethanolic extracts. PS ethanol 80% extracts (200 ppm) showed the lowest increase in AV compared with other extracts and BHA at same ratio (200 ppm). These results are in agreement with those of Siddhuraju and Becker (2003).

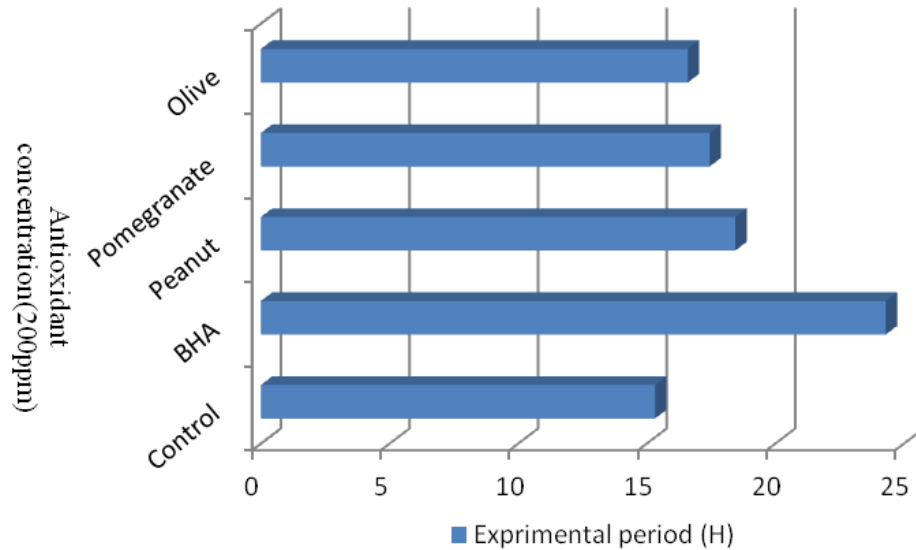
### Rancimat

The results of induction period are illustrated in Figure 6.

Induction time was the highest (24.3 h) for ghee enriched with BHA, followed by ghee enriched with PS (18.4 h) and was the lowest for control (15.3 h). These results agree with those of Marian Kucera et al. (2011) and Suwarat and Tungjaroenchai (2013). As can be seen from Figure 6, the logarithm of TRanc shows linear dependence on exothermic temperature and can be described by the following equation: ( $\text{Log TRanc} = a \cdot t + b$ ), where: a and b are adjustable coefficients and T the temperature in degree celsius (°C). The oil stability index OSI of ghee samples varied from  $14.85 \pm 0.17$  to  $24.80 \pm 0.34$  h; the higher the temperature and longer period of time, the higher were values of OSI in hours (Figure 6). Although some papers reported that high temperature with prolong period of time affected high yield, color, oxidative stability tended to changed continuously. Degradation of oil was normally induced by moisture, high temperature, crust formation and various structural, textural and chemical changes in the product, and degradation of frying medium (Paul et al., 1997). The oxidative stability of oils can be ranked based on the rule "the longer the induction time the more stable is the oil". The relative resistances of the oils on their thermal oxidative degradation depend on temperature.

### Conclusion

Peanut skin, pomegranate peels and olive pomace extracts were prepared using different solvents, and the *in vitro* antioxidant activity of each extract was investigated. In general, it was observed that extracts with higher antioxidant capacity were in parallel to their



**Figure 6.** Rancimat of ghee treated with different plant wastes-by products of food processing extracts at 130°C.

higher phenolic contents. It could be concluded that the obtained extracts using higher-polarity solvents were more effective radical scavengers than those obtained using lower-polarity solvents. Ethanol 80% showed slightly better characteristics than hexane and ethyl acetate as a solvent for phenolic compounds extraction. Thus, for use in the food industry, ethanol 80% would be a more appropriate solvent. Furthermore, it is notable that PS extracts exhibited a strong antioxidant capacity in all assays used, followed by PP and OP extracts. Overall, ethanol 80%, ethyl acetate and hexane extract showed relatively comparable activity to BHA. Therefore, these extracts could be used as preservative ingredients in the food and/or pharmaceutical industries.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

### REFERENCES

- Aldini G, Piccoli A, Beretta G, Morazzoni P, Riva A, Marinello C, Facino R (2006). Antioxidant activity of polyphenols from solid olive residues of Cv. Coratina. *Fitoerapia* 77:121-128.
- AOAC (1984). Official Methods of analysis 14th ed. Association of official analytical chemists - Washington.
- Balasundram N, Sundram K, Samman S (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence and potential uses. *Food Chem.* 99:191-203.
- Cam M, Hisil Y, Durmaz G (2009). Classification of eight pomegranate juices based on antioxidant capacity measured by four methods. *Food Chemistry*. 112:721-726.
- Cerda B, Liorach R, Ceron JJ, Espin JC, Tomas-Barberan FA (2003). Evaluation of the bioavailability and metabolism in the rat of punicalagin, an antioxidant polyphenol from pomegranate juice. *Europ. J. Nutr.* 42: 18-28.
- Chenn G, Xiong YL, Wang L, Gomez-Basauri J, Nicastro F (2008). Effect of preventox on the storage stability of raw and precooked pork patties. *J. Muscle Foods*. 19:1-16.
- Dorman HJD, Kosar M, Kahlos K, Holm Y, Hiltunen R (2003). Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. *J. Agric. Food Chem.* 51:4563-4569.
- Ebrahimabadi AH, Diafari-Bidogoli Z, Mazoochi A, Kashi FJ, Batooli H (2010). Essential oil composition, antioxidant and antimicrobial activity of the leaves and flowers of *Chaerophyllum macropodium* Boiss. *Food Control*. 21:1173-1178.
- Fahmi AH (1961). *Sammah*, Book in Arabic Language Published by commercial modern publisher. Cairo University, Egypt.
- Frag RS, Badel AZM, El-Baraty GSA (1989). Influence of thyme and clove essential oils on cotton seed oil oxidation. *J. Am. Oil Chem. Society*. 66(6):800-804.
- Fernandez-Lopez J, Zhi N, Aleson-Carbonell L, Perez-Alvarez JA, Kuri V (2005). Antioxidant and antibacterial activities of natural extracts: Application in beef meat balls. *Meat Sci.* 69:371-380.
- Fuhrman B, Volkova N, Aviram M (2005). Pomegranate juice inhibits oxidized LDL uptake and cholesterol biosynthesis in macrophages. *J. Nutr. Biochem.* 16:570-576.
- Gulcin I, Kufrevioglu OI, Oktay M, Buyukokuroglu ME (2004). Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.). *J. Ethnopharmacol.* 90:205-215.
- Guo C, Yang J, Wei J, Li Y, Xu J, Yiang J (2003). Antioxidant activities of peel, pulp, and seed fractions of common fruits as determined by FRAP assay. *Nutr. Res.* 23:1719-1726.
- Hanato T, Kagawa H, Yasuhara T, Okuda T (1988). Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chem. Pharm. Bull.* 36:2090-2097.
- Heim KE, Taigliaferro AR, Bobilya DJ (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.* 13:572-584.
- Jang HA, Kim YP, Kim HS (2012). Effect of natural antioxidants on the oxidation of microencapsulated seed oil. *Food Control*. 23:528-534.
- Jaya PGK, Singh RP, Sakariah KK (2001). Antioxidant activity of grape seed (*Vitis vinifera* L.) extracts on peroxidation models *in vitro*. *Food Chem.* 73:285-290.
- Jo SC, Nam KC, Min BR, Ahn DU, Cho SH, Park WP, Lee SC (2006). Antioxidant activity of *Prunus mume* extract in cooked chicken breast meat. *Int. J. Food Sci. Tech.* 41:15-19.

- Kequan Z, Liangli Y (2004). Effects of extraction solvent on wheat bran antioxidant activity estimation. *LWT. Food Sci. Technol.* 37:717-721.
- Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S (2006): Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chem.* 96:254-260.
- Marian K, Mirslav R (2011). Determination of the oxidative stability of vegetable oil - based lubricatis - Ph.D. Thesis. *Acta Facultatis Technicae Zavlén - Slovakia.* 121-133.
- Mattila P, Astola J, Kumpulainen J (2000). Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. *J. Agric. Food Chem.* 48:5834-5841.
- Meftahizade H, Moradkhani H, Barjin AF, Naseri B (2011). Application of *Lavandula officinalis* L. antioxidant of essential oils in shelf life of confectionary. *Afr. J. Biochem.* 10(2):196-200.
- Mohdali AA (2010). Evaluation of some food processing by-products as sources for natural antioxidants. Ph.D. Thesis. Faculty III - Process Science, Institute of Food Technology and Food Chemistry, Technical University of Berlin, Germany. 69-77.
- Monagas M, Garrido I, Lebron-Aguilar R, Gomez-Cordoves MC, Rybarczyk A, Amarowicz R, Bartolome B (2009). Comparative flavan-3-ol profile and antioxidant capacity of roasted peanut, hazelnut, and almond skins. *J. Agric. Food Chem.* 57:10590-10599.
- Moure A, Cruz JM, Francob D, Dominguez JM, Sineirob J, Dominguez H, Nunez MJ, Paraj JC (2001). Natural antioxidants from residual sources-Review. *Food Chem.* 72:145-171.
- Nepote V, Grosso N, Guzman C (2005). Optimization of extraction of phenolic antioxidants from peanut skins. *J. Sci. Food Agric.* 85:33-38.
- Niaounakis M, Halvadakis CP (2004). *Olive Mill Waste Management*, First ed. Trophito-George Dardanos, Athens.
- Obied HK, Bedgood DR, Prenzler PDR obards K (2007). Bioscreening of Australian olive mill waste extracts: Biophenol content, antioxidant, antimicrobial and molluscicidal activities. *Food Chem. Toxicol.* 45:1238-1248.
- Özkanlı O, Kaya A (2007). Storage stability of butter oils produced from sheep's non-pasteurized and pasteurized milk. *Food Chem.* 100:1026-1031.
- Pankaj P, Khamrui K, Devaraja HC, Singh RRB (2013). The effects of alcoholic extract of Arjuna (*Terminalia arjuna* Wight & Arn.) bark on stability of clarified butterfat. *J. Medi. Plants Res.* 7(35):2545-2550.
- Paul S, Mittal GS, Chinnan MS(1997). Regulating the use of degraded oil/fat in deep-fat/oil food frying. *Critical Reviews Food Sci. Nutrit.* 37:635-662.
- Peschel W, Rabaneda FS, Diekmann W, Plescher A, Gartzia I, DImenez J, Raventos RL, Buxaderas S, Codina G (2006). An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chem.* 97:137-150.
- Pitchaon M, Maitree S, Rungnaphar P (2007). Assessment of phenolic content and free radical-scavenging capacity of some *Thai* indigenous plants. *Food Chem.* 100:1409-1418.
- Puravankara D, Boghra V, Sharma RS (2000). Effect of antioxidant principles isolated from mango (*Mangifera indica* L) seed kernels on oxidative stability of buffalo ghee (butter-fat). *J. Sci. Food Agric.* 80:522-526.
- Saad B, Yong YS, Mohd AN, NoorHasani H, Abdussalam SMA, Muhammad IS, Saida FS, Khariuddin MT, Kamarudzaman A (2007). Determination of synthetic phenolic antioxidants in food items using reversed-phase HPLC. *Food Chem.* 105:389-394.
- Seeram N, Lee R, Hardy M, Heber D (2005). Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry. *Sep. Purif. Technol.* 41:49-55.
- Siddhuraju P, Becker K (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringaoleifera Lam.*) leaves. *J. Agric. Food Chem.* 51:2144-2155.
- Škerget M, Kotnik P, Hadolin M, Riner-Hra ĆA, Simonič M, Knez Ć. (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chem.* 89:191-198.
- Suarez M, Romero M, Motilva M (2010). Development of phenol enriched olive with phenolic compounds from olive cake. *J. Agric. Food Chem.* 58:1036-1043.
- Suwarat N, Tungjaroenchai W (2013). Characteristic of Ghees Obtained from Different Post-Clarification Temperatures. *Int. J. Biosci. Biochem. Bioinform.* 3(4):332-334.
- Wang J, Xiaoping Y, Zhengyu J, Yuan T, Huanlu S (2007). Free radical and reactive oxygen species scavenging activities of peanut skins extract. *Food Chem.* 104:242-250.
- Xiaowei M, Hongxia W, Liain L, Quansheng Y, Songbiao W, Rulinzhan S, Shanshan X, Yigang Z (2011). Polyphenolic compounds and antioxidant properties in mango fruits. *Scientia Horticulturae* 129:102-107.
- Yu J, Ahmedna M, Goktepe I (2005). Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chem.* 90:199-206.

Full Length Research Paper

## Role of folic acid in chlorpyrifos induced teratogenicity in mice

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Folic acid is known to reduce the incidence of neural tube defects, in animal experiments however, it has not been effective in reducing the congenital anomalies caused by antiepileptics and many other chemicals. Pesticides of organophosphate group such as chlorpyrifos are widely used in agriculture and household, and are shown to be teratogenic in animal studies. This study was designed to explore the effect of folic acid on chlorpyrifos induced teratogenicity. Pregnant mice were divided into groups of 8 animals each, and were exposed to oral dose of chlorpyrifos and/or folic acid on gestation days (GD) 6 and 7 or GD 1 to 15. Fetuses were recovered on 15<sup>th</sup> day of gestation. On morphological and morphometric examination it was found that chlorpyrifos is teratogenic to mice fetuses in an oral dose of 100 ug/gbw when given on GD 6 and 7. It was embryo toxic and caused growth retardation and morphological anomalies of skull, body curvature, skin, limbs, eyes and ears. Folic acid in a dose of 2 ug/gbw, when given at the time of chlorpyrifos exposure (GD 6 and 7), lead to significant improvement in crown-rump (CR) length and weight of fetuses, reduction in the number of resorptions and frequency of anomalies. However the CR length and weight of fetuses given folic acid and chlorpyrifos were significantly less than the control. There was no additional benefit of giving folic acid in the same dose from GD1 to 15. Thus this study was aimed at confirming the teratogenic potential of chlorpyrifos in mice and assessing the role of folic acid in the prevention of teratogenesis induced by chlorpyrifos.

**Key words:** Folic acid, organophosphates, teratogenesis, chlorpyrifos.

### INTRODUCTION

Based on animal studies, interventional trials and epidemiological studies, maternal folic acid is known to be protective in neural tube defects (Blencowe et al., 2010; Hamner et al., 2009). Consumption of folic acid by women of childbearing age is an important public health goal in US (Tinker et al., 2010). Role of folic acid in the

prevention of other congenital anomalies is still controversial. Folic acid has shown to reduce the incidence of teratogenesis induced by ethanol, arsenic, retinoic acid, valproic acid and ochratoxin A, in animal experiments, but has failed to decrease the congenital anomalies caused by antiepileptics and many other chemicals

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Abbreviations: **GD**, Gestational day; **gbw**, gram body weight; **CR**, crown-rump.

**Table 1.** Dose schedule of chlorpyrifos and folic acid.

Group	Chemical given	Dose (ug/gbw)	Time of administration
Group A (Experimental Group)	Chlorpyrifos	100	GD 6 and 7
Group B (Experimental Group)	Chlorpyrifos and folic acid	100 and 2	GD 6 and 7, GD 6 and 7
Group C (Experimental Group)	Chlorpyrifos and folic acid	100 and 2	GD 6 and 7, GD 1 to 15
Group D (Folic acid control)	Folic acid	2	GD 1 to 15
Group E (Plain Control)	Food and Water	<i>ad libitum</i>	--

(Yanaguita et al., 2008; Katagiri et al., 2007). Pesticides of organophosphate group are widely used in agriculture and household. Human population, including the pregnant women is exposed to its harmful effects (Eskenazi et al., 2004). Organophosphates, such as chlorpyrifos have been shown to be highly teratogenic in animal studies (Tian et al., 2005; Chanda et al., 1995; Ahmad and Asmatullah, 2007). The present study was designed to explore, whether folic acid has any protective role in teratogenesis induced by chlorpyrifos.

## MATERIALS AND METHODS

The present research was conducted on Swiss Webster strain of albino mice *Mus musculus*. Animals were reared in the animal house of the Department of Zoology, University of the Punjab, Lahore. They were kept under optimum conditions and fed commercially prepared chick feed No.3 and water *ad libitum*. Stage of estrous cycle was determined. The females found to be in late pro-estrous and estrous were kept overnight with males in separate cage in 2:1 female to male ratio. Presence of vaginal plug in the morning or sperms in vaginal smear confirmed successful mating and that day was taken as day one of pregnancy.

Pregnant female mice were separated from males and divided into various groups of eight animals each. They were labeled and given oral doses of Chlorpyrifos and/or Folic acid with the help of a plastic syringe, according to the schedule in Table 1. Pregnant female mice were sacrificed on the 15th day of gestation, under ether anesthesia. The two horns of gravid uteri were dissected out. Implantation sites were carefully observed and resorptions noted. Number of live fetuses in each horn of uterus was noted. The uteri were cut open and the fetuses were carefully recovered. These fetuses were then put in Bouin's fixative for 48 h, and washed in 70% alcohol to remove the fixative. The separated fetuses were preserved in 80% alcohol.

Morphometric and morphological study of 50 fetuses per group was done. Crown-rump length of fetuses was measured with a millimeter scale and the weight was determined in milligrams on an electric balance. Detailed morphological study was carried out under dissecting microscope using a magnification of 10 x. Head, ear, eye, trunk, tail and skin characteristics were carefully examined and compared in different experimental and control groups. Anteroposterior and transverse diameter of skull and length of tail was taken with a digital vernier caliper. The head circumference was calculated from skull dimensions. Head circumference to CR length ratio and tail length to CR length ratio was calculated and compared. Any abnormality noticed in morphological examination, was recorded. Selected fetuses were photographed with a digital camera.

## RESULTS

### Control groups

#### *Group E (plain control)*

The fetuses of control group E had a semi curved body which was distinctly divisible into head, trunk and tail regions. Skull had acquired a rounded smooth contour and suture lines were faintly visible under the dissecting microscope. Eyes were well formed showing an elliptical aperture and bulging rounded lens in the middle. Upper and lower eyelids had also reached a considerable state of development but still were unable to completely close the eyes.

External ears were also advanced in development. An external auditory meatus could be clearly seen almost completely hidden by a large well developed pinna. The snout had also taken its typical protruding shape, with two nostrils located quite close to each other at its anterior tip.

Upper and lower lips were also clearly defined. Multiple rows of vibrissae were visible at the sides of the two nostrils. Trunk part of the fetus was divisible into a relatively flat thorax and a protuberant abdomen. The outline of the liver could be made out easily. Tail was quite long and reached till the snout area. It showed the typical curve towards the cranium which is quite peculiar to this stage of development (Figure 1).

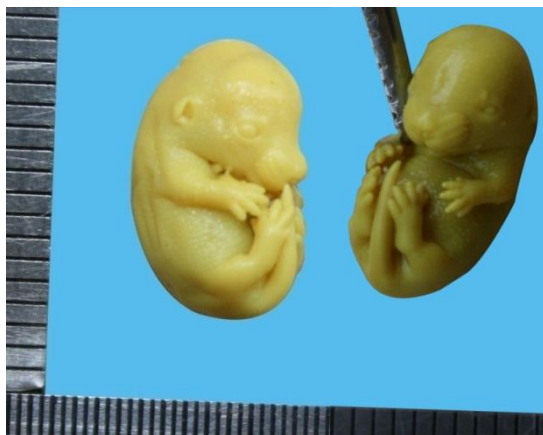
#### *Group D (folic acid control)*

The control fetuses of group D (given only folic acid on GD 6 and 7) were similar to group E control fetuses in morphological appearance. The morphometric results are shown in Tables 2 and 3.

### Experimental groups

#### *Group A (given 100 ug/gbw chlorpyrifos on GD 6 and 7)*

The general morphological appearance of fetuses of this group varied considerably. The small fetuses appeared to



**Figure 1.** Photograph of lateral and anterior view of fetus of control Group showing normal development of skull, eye, pinna, snout, upper limb, lower limb, trunk and tail.

**Table 2.** Quantitative data of morphological features of fetuses of various groups.

Group	Number of fetuses recovered	Number of resorptions	Variation in litter size	Mean CR length( mm)	Mean Wt. in mg	Mean head circum (mm)	Head circ.: CR length (mm)
Group A	30	All fetuses resorbed in two mice	2 to 9	8.84	133.47	13.88	1.57:1
Group B	47	15	3 to 10	11.22	247.51	16.17	1.45:1
Group C	42	17	6 to 8	11.32	249.4	16.01	1.42:1
Group D	66	1	8 to 10	13.63	377.10	19.60	1.44:1
Group E	61	0	8 to 12	14.32	399.68	20.26	1.42:1

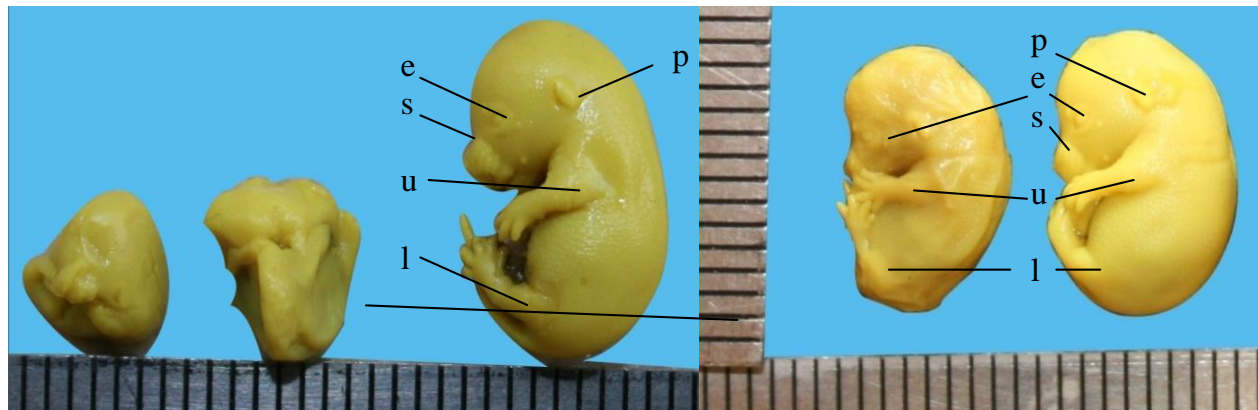
**Table 3.** Morphological features of fetuses of various groups.

Group	Skull	Wide Fontanellae (%)	Hges. (%)	Disturb body axis (%)	Eyes absent (%)	Rounded eyes (%)	Small eyelids (%)	Small pinna (%)	Low set ears (%)
Group A	Small	43.3	16.6	3.3	30	66.6	56.6	80	33.3
Group B	Small in all	23.4	17	8.5	2.1	59.5	23.4	68.1	4.2
Group C	Small in 50%	28.5	2.3	2.3	0	26.2	19	40	7.8
Group D	Round smooth	0	Nil	Nil	Nil	Nil	nil	nil	Nil
Group E	Round smooth	0	Nil	Nil	Nil	Nil	Nil	Nil	Nil

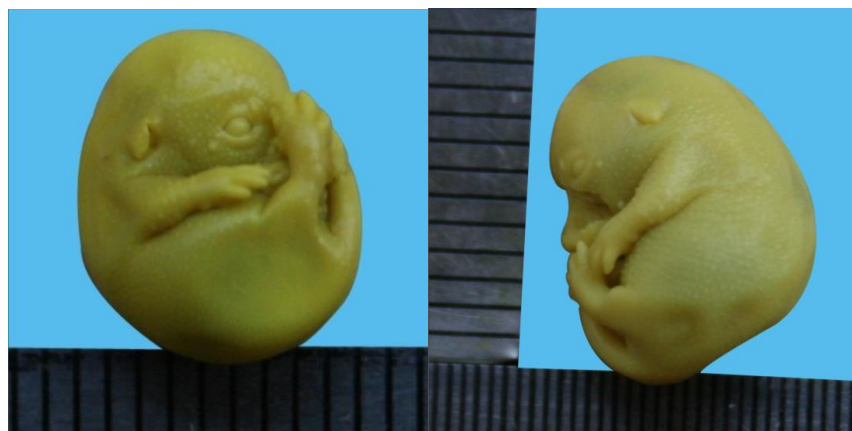
be a curved mass of tissue in which head and tail ends could be made out with difficulty. Lower half of the body was almost as thick as the cranial end and tail was not distinguishable from the rest of the body (Figure 2). There were absolutely no impressions in the head area indicative of ear and eye differentiation. Limb buds had just started forming. These buds were very small and not discernable into parts. In one fetus only one upper limb bud had formed. Various levels of developmental arrest

and malformations were observed in fetuses of this group. Body axis was abnormal in 4 fetuses, showing scoliosis, a twist in the spine or increased curvature (Figure 3).

Tail was very variable in size and appearance. Mean length of the tail was 4.79 mm. It was so long in few fetuses that it was extending beyond the snout. In few fetuses it was twisted (Figure 4a). In small fetuses the tail appeared almost as thick as the rest of the body. 16.6%



**Figure 2.** Photographs of group A fetuses (given 100 ug/gbw chlorpyrifos on GD 6 and 7) with different sizes and developmental status. e, Eye; s, snout; p, pinna; u, upper limb; l, lower limb.



**Figure 3.** Photograph of fetuses with abnormal body axis. Note scoliosis.

fetuses showed hemorrhagic spots in skin at different regions (Figure 4b). Tail: CR length ratio was 0.54:1 in group A fetuses, which was not significantly different from control. Skull of almost all fetuses was found to be small and in 43.3% fetuses, fontanellae were wide. The contour of the skull was not round and smooth as in control. Two fetuses showed high degree of convexity in the cervical region whereas another two showed a raised skull in parietal region. (Figures 4 to 5).

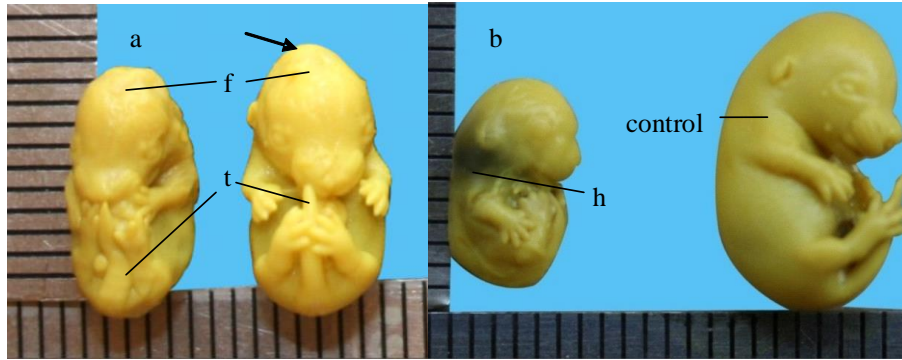
In 20 fetuses, eyes were round indicating immaturity in the stage of development. In 17 fetuses eyes were very small in size in comparison to control, whereas there were faint impressions in the region of eyes showing the initiation of formation of eyes in five fetuses. In another four fetuses, eyes were not formed at all. In 80% fetuses, pinna was formed but was quite small as compared to control fetuses. In three fetuses there was just a faint pit indicating the ear region and no indication of pinna formation. In 33.3% fetuses, pinna was located at a lower level in comparison to control (Figures 4 to 6).

#### **Group B (given chlorpyrifos and folic acid on GD 6 and 7)**

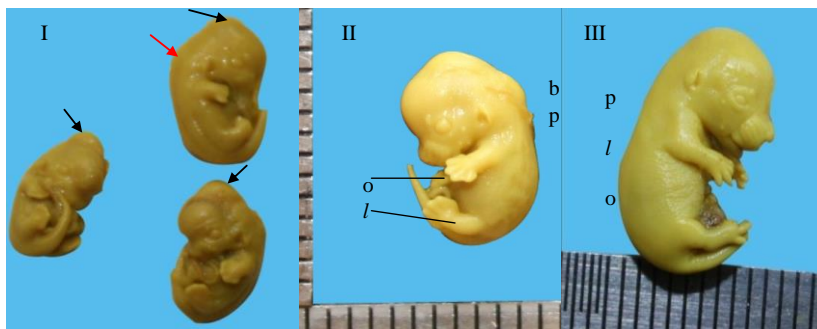
In most of the fetuses, eyes were normal in position. In 23.4% fetuses eyes were small. In one of the fetus, right eye was smaller than the left. In another fetus eyes were not visible at all. In 59.5% fetuses the shape of the eyes was round indicating immaturity in development. Pinna was quite well formed in 31.9% fetuses however in 20 fetuses it was smaller in size in comparison to control. In one fetus the size of pinna was different on two sides, the left one being smaller. The location of the pinna was normal in majority of the fetuses of this group (Figure 7).

#### **Group C (given chlorpyrifos on GD 6 and 7 and folic acid on GD 1 to 15)**

The skull showed protuberance in parietal region in two fetuses, whereas another one showed a bulge in occipital



**Figure 4. (a).** Group A fetuses Left- with f. prominent fontanella and t. twisted tail, Right- with f. prominent fontanella and bulge in the parietal area of the skull (arrow); t, tail extending straight upto snout. **(b).** Lt. Deformed group A fetus with h. hemorrhagic spots. Rt, Control fetus.



**Figure 5.** Photographs of deformed fetuses. **(I)** Parietal bulge (black arrow), cervical bulge (red arrow). **(II)** l, Paddle shaped limb buds; b, occipital and cervical bulges; p, small pinna and o, omphalocele. **(III)** p, small pinna; l, straight limb bud; o, omphalocele.

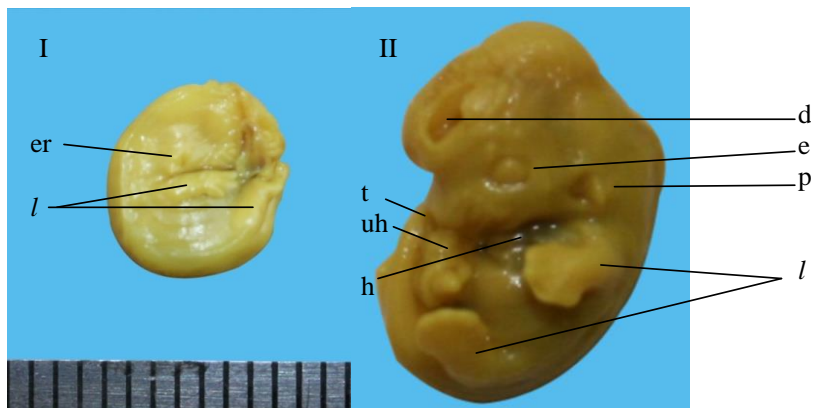


**Figure 6.** Photograph of affected fetuses. **(I)** The small size of pinna, faint impressions of closed eyes, variation in tail, limb buds and body curvature. **(II)** Photograph of affected fetuses of group A. Note the level of pinna, variation in length of tail and paddle shaped limb buds. Boxed fetus shows amelia of the upper limb.

region. In most of the fetuses, eyes were normal in position, but the shape and size revealed variation. The eyes were round in 11 out of 42 fetuses whereas the size

was quite small in comparison to control in 8 out of 42 fetuses. Pinna formation was apparently normal in most of the fetuses. It was quite small in comparison to control

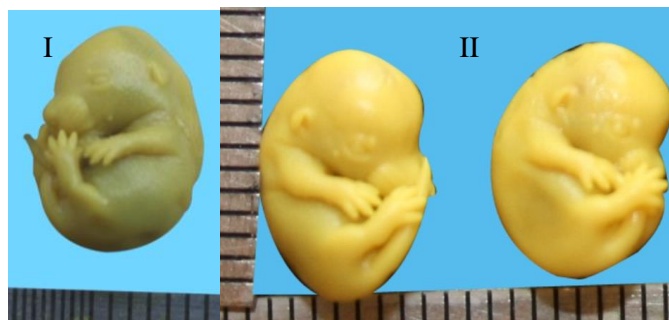




**Figure 7.** Photographs of malformed fetuses of group B (given 100 ug/gbw chlorpyrifos and 2 ug/gbw folic acid on GD 6 and7) showing: (I) er, ear pit; *l*, malrotated limb buds; (II) p, very small pinna; *l*, Paddle shaped limb buds; e, rounded eyes; d, deficient skull bones; t, long tail uh, umbilical hernia; h, thin skin with hemorrhages.



**Figure 8.** Photograph of a litter recovered from group C mouse (given 100 ug/gbw chlorpyrifos on GD 6 and 7 and 2 ug/gbw folic acid on GD 1-15). The fetus on extreme right is from the control group. Note the difference in size and level of development.



**Figure 9.** Photographs of group C fetuses (given 100 ug/gbw chlorpyrifos on GD 6 and 7 and 2 ug/gbw folic acid on GD 1-15). I. Increased curvature and absent elbow bend. II. Increased curvature and long tail extending beyond snout.

in 13 out of 42 fetuses. In three fetuses pinna was located at a lower level. (Figures 8 to 9).

Histograms showing the comparison of mean CR

length, mean weight of fetus and mean head circumference to CR length ratio are shown in Figures 10 to 12.

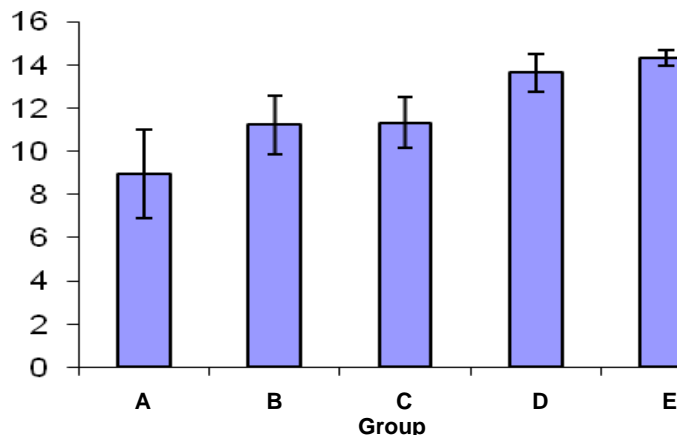


Figure 10. Histogram showing comparison of mean CR length.

Table 4. Statistical analysis of data for variations in skin features and body axis of fetuses.

Group	Skin					Body Axis		
	Normal	Hge	Wrinkles	others	total	Normal	Distorted	Total
A	24	5	1	0	30	26	4	30
B	38	8	0	1	47	43	4	47
C	41	1	0	0	42	41	1	42
D	46	4	0	0	50	50	0	50
E	50	0	0	0	50	50	0	50
Total	199	18	1	1	219	209	9	218

Chi-square likelihood ratio 24.679 and 14.622, P-value 0.006.

## Statistical analysis

### Morphometric parameters

ANOVA test revealed significant difference between experimental and control groups. For comparison between different groups the data was further subjected to Bonferroni analysis. Group A fetuses, (given chlorpyrifos on GD 6 and 7) when compared with other experimental and control groups revealed significant difference (p-value <0.001). The difference between the values from fetuses of group B (given chlorpyrifos and Folic acid on GD 6 and 7) and C (given chlorpyrifos on GD 6 and 7 and Folic acid on GD 1 to 15) was not significantly statistically. (P-value of 1.0). Fetuses of groups B and C however were significantly different from those of control groups (D and E), with p-value <0.001. The fetuses of two control groups (D and E) were quite similar with insignificant statistical difference. Tukey test revealed that fetuses of group A were affected the most.

The difference between control group (E) and experimental groups (A, B and C) was statistically significant in following parameters (Tables 4 to 7): 1. CR length, weight, head circumference, tail length, size and

shape of eyes; 2) The difference in head circumference: CR length ratio was statistically significant only between group A and all other groups including control; 3) As regards skin hemorrhages, body axis and presence of protuberance in different regions of skull, the occurrence of these abnormalities was statistically significant in fetuses of group A and B, and 4) A statistically significant difference of group A and C fetuses from control was noted in location of eyes and ears at lower level and a small size of pinna.

## DISCUSSION

It has become quite clear from this study, that chlorpyrifos is teratogenic to mice fetuses in a single oral dose of 100 ug/gbw when given on 6<sup>th</sup> and 7<sup>th</sup> day of gestation. On giving chlorpyrifos, there was decrease in the litter size and increase in the number of resorptions and malformations. The CR length and weight of chlorpyrifos treated fetuses was significantly reduced in comparison to control.

When 2 ug/gbw dose of folic acid is given at exposure to chlorpyrifos, it leads to reduction in the number of

**Table 5.** Statistical analysis of data for variations in fontanellae and skull bulge in fetuses.

Group	Fontanella				Skull Bulge		
	Approx (normal)	Open (Prominent)	Wide (v.prominent)	No comments	parietal region	cervical region	occipital region
A	17	5	7	1	2	2	0
B	29	11	6	1	10	0	0
C	27	12	3	0	2	0	1
D	50	0	0	0	0	0	0
E	50	0	0	0	0	0	0
Total	172	28	16	2	14	2	1

Chi-square likelihood ratio 72.282 and 35.851, P-value<0.001.

**Table 6.** Statistical analysis of data for variations in position, size and shape of eyes of fetuses.

Group	Position of eyes			Size of eyes				Shape of eyes		
	normal	lower	No comments	Normal	small	Very small	Not formed	elliptical	Round	No comments
A	20	6	4	0	9	17	4	0	20	10
B	46	0	1	34	11	1	1	18	28	1
C	38	4	0	34	6	2	0	31	11	0
D	50	0	0	50	0	0	0	50	0	0
E	50	0	0	50	0	0	0	50	0	0
total	204	10	5	168	26	20	5	149	59	11

Chi-square likelihood ratio 40.585, 156.517 and 176.811, P-value <0.001.

**Table 7.** Statistical analysis of data for variations in size and position of pinna of fetuses.

Group	Size of pinna						Position of pinna			
	Very well formed	Well formed	small	Very small	Not formed	Different on two sides	normal	low	No comments	
A	0	1	2	24	3	0	17	10	3	
B	15	10	20	0	1	1	45	1	1	
C	17	12	11	2	0	0	39	3	0	
D	50	0	0	0	0	0	50	0	0	
E	50	0	0	0	0	0	50	0	0	
total	132	23	33	26	4	1	201	14	4	

Chi-square likelihood Ratio =255.401 and 47.301, P-value < 0.001.

resorptions and frequency of anomalies. There was also significant improvement in CR length and weight of fetuses on giving folic acid along with chlorpyrifos on GD 6 and 7. However the CR length and weight of fetuses given folic acid and chlorpyrifos were significantly less than the control. When folic acid was continued for longer period, from GD 1 to 15 in the same dose, there was no additional benefit.

Tian et al. (2005) evaluated the potential teratogenicity and developmental toxicity of chlorpyrifos in mice. A

single intraperitoneal injection of 80 mg/kg on GD10 resulted in significant reduction in number of live fetuses and increase in resorptions, as compared to control fetuses. They also observed, external and skeletal malformations and cleft palate with this dose of chlorpyrifos, however there was no indication of maternal toxicity. Chanda et al. (1999) and Ahmad and Asmatullah (2007) have also reported chlorpyrifos to be teratogenic to mice embryos. The results of present study are quite similar to these studies. In the present study a high dose

of chlorpyrifos, proved to be embryotoxic and teratogenic in mice when given during the period of organogenesis. The number of resorptions was significantly increased (19 resorptions) as compared to control (1 resorption). External malformations of eyes and ears were noticed.

A dose of 100 ug/gbw on GD 6 and 7 did not produce any overt toxicity in mothers in the present study. Two mothers showed transient hyper salivation and restlessness after the second dose, which was completely reversible. Ahmad and Asmatullah (2007) calculated the LD 50 of chlorpyrifos in the range of 144 ug/gbw. A dose lower than LD50, was given to mothers in the present study. This may be the reason why no significant toxic effects were observed in mothers.

Calvert et al. (2007) reported the birth of three infants with congenital anomalies, in Collier County, Florida. They were born within 8 weeks of one another and their mothers worked for the same tomato grower. All three mothers worked during the period of organogenesis in fields recently treated with several pesticides. Although it was difficult to pin point which particular pesticide was responsible, but this incident definitely highlights the need to reduce the pesticide exposure to the pregnant population. There is also a further need for epidemiological studies to examine the role of pesticide exposure in the etiology of birth defects. At the same time the use of nutrients which may reduce the incidence of congenital anomalies must be encouraged. Folic acid is one of such nutrients.

Role of folic acid in the prevention of neural tube defects is a well established fact and is backed by many experimental and epidemiological studies. Folic acid has been shown to reduce the incidence of neural tube defects caused by valproic acid (Dawson et al., 2006; Padmanabhan and Shafiullah, 2003) retinoic acid (Reynolds et al., 2003; Firat et al., 2005), ochratoxin A (Katagiri et al. 2007), Fumonisin B1 (Sadler et al., 2002), ethanol (Yanaguita et al., 2008), arsenic (Gefrides et al., 2002) and hyperthermia (Shin and Shiota, 1999; Li et al., 2003). Its role in the prevention of other congenital defects has not yet been fully explored. Since chlorpyrifos has been found to be teratogenic to many organs and systems. Thus, the role of folic acid in the prevention of chlorpyrifos induced teratogenicity becomes quite important.

In the present study, oral dose of 2 ug/gbw folic acid was given with an intention to reverse or decrease the teratogenic effects of chlorpyrifos. When given on GD 6 and 7, folic acid was able to decrease the frequency of congenital malformations induced by chlorpyrifos. When once daily dose of folic acid was continued over a period of 15 days, there was no further reduction in the frequency and severity of birth defects. Moreover it is interesting to note that in the present study there are indications that folic acid therapy from GD1 till the exposure to chlorpyrifos on GD 6 and 7, was unable to prevent the teratogenic damage. Once the damage was

done, further doses of folic acid were unable to reverse the damage.

In an attempt to observe the effects of ethanol and folic acid deficiency on outcome, the pregnant mice with different folate status when exposed to different doses of ethanol from 7 to 9th GD. The animals were sacrificed on 18th GD. The results of this experiment revealed that in animals receiving a commercial diet, a high dose of ethanol was deleterious to pregnancy, including congenital anomalies, intrauterine growth restriction, reduction of the placenta and increased late fetal deaths and resorptions, events that did not occur with the low dose of ethanol. However, with a folate free diet, a low ethanol dose was as deleterious as a high dose.

Yanaguita et al. (2008) concluded that supplementation with the recommended dose of folic acid (2 mg/kg), was not effective in preventing the deleterious teratogenic effects induced by ethanol, indicating the need for an increased dose. Firat et al. (2005) in their experiment on rats, administered Retinoic acid in different doses, orally on GD8. Folic acid of 4.0 mg/kg was injected intraperitoneally on 7th to 9th GD. Folic acid administration prevented the decrease in mean fetal weight and height of the embryos treated with 40 mg/kg retinoic acid. In addition there was a marked decrease in the number of degenerated chondrocytes and an improvement in the structure of granular endoplasmic reticulum along with intact nuclei.

Folic acid of 4.0 mg/kg used in this study was reported to be in the therapeutic range since there was no pathological alteration neither in body weight and height nor in histological appearance of Meckel's cartilage of the embryos born to mothers treated with Folic acid alone (Firat et al., 2005). We have seen that even 2 ug/gbw (equal to 2 mg/kg) folic acid has been effective in improving the fetal weight and height of chlorpyrifos treated fetuses. In the present study, a dose of 2 mg/kg folic acid did not produce any toxic effects neither in mothers nor in fetuses. This dose was also not teratogenic to the fetus. The CR length and weight of fetuses given folic acid was more than the mean values of control fetuses but the difference was statistically insignificant. Thus there was no harmful effect of giving folic acid throughout pregnancy, on the fetal development. It should be noted that the handling of mothers for giving the daily doses of folic acid did not adversely affect the fetal development either or folic acid therapy was able to counteract the effect of stress of experimentation.

It has been confirmed at multiple levels that folic acid consumption decreases the incidence of neonatal deaths mainly by decreasing neural tube defects (NTDs) (Blencowe et al., 2010; Hamner et al., 2009). In countries like US, in order to prevent NTDs, folic acid fortified food items are made available to general population and there are various public awareness programs emphasizing the usefulness of folic acid consumption in women of child-

bearing age. In spite of all the efforts, it was found out in a study conducted in Atlanta from 2003 to 2006, that only 24% of non-pregnant U.S women of childbearing age consumed the recommended dose of folic acid (Tinker et al., 2010).

Pakistan is an agricultural community where organophosphate pesticides are widely used. People are generally indifferent to instructions due to illiteracy and pregnant population is exposed to harmful effects of chemicals including pesticides. Lassi and Butta (2012) have recently discussed the possibility of adding folic acid to oral contraceptives in this country. An evaluation of present health strategies needs to be done and measures must be employed to make women aware of importance of avoiding exposure to pesticides and promote the use of folic acid.

## Conclusion

Chlorpyrifos is teratogenic to mice fetuses when given on GD 6 and 7 of gestation. Administration of folic acid on GD 6 and 7 does ameliorate the adverse effects of this pesticide to a considerable extent however, administration of folic acid for a longer duration does not appear to have an additional benefit in alleviating the adverse effects of chlorpyrifos. It is further concluded that folic acid administration to pregnant mice in a dose of 2 $\mu$ g/gbw from gestational day 1 to 15 does not have any toxic or teratogenic effect.

## Conflict of Interest

The author(s) have not declared any conflict of interests.

## REFERENCES

- Ahmad KR, Asmatullah (2007). Embryotoxic and teratogenic effects of chlorpyrifos in mice: maternal exposure at the inception of organogenesis. *Proc. Pakistan Cong. Zool.* 27:23-24.
- Blencowe H, Cousens S, Modell B, Lawn J (2010). Folic acid to reduce neonatal mortality from neural tube disorders. *Int. J. Epidemiol.* 39(1):110-121.
- Calvert GM, Alarcon WA, Chelminski A, Crowley MS, Barrett R, Correa A, Higgins S, Leon HL, Correia J, Becker A, Allen RH, Evans E. (2007). Case report: three farm workers who gave birth to infants with birth defects closely grouped in time and place. Florida and North Carolina, 2004-2005. *Environ. Health. Perspect.* 115:787-791.
- Chanda SM, Harp P, Liu J, Pope CN (1995). Comparative developmental and maternal neurotoxicity following acute gestational exposure to chlorpyrifos in rats. *J. Toxicol. Environ. Health* 44:189-202.
- Dawson JE, Raymond AM, Winn LM (2006). Folic acid and pantothenic acid protection against valproic acid induced neural tube defects in CD-1 mice. *Toxicol. Appl. Pharmacol.* 211:124-32.
- Eskenazi B, Harley K, Bradman A, Weltzien E, Jewell NP, Barr DB, Barr DB, Furlong CE, Holland NT (2004). Association of in utero organophosphate pesticide exposure and fetal growth and length of gestation in agricultural population. *Environ. Health. Perspec.* 112:1116-24.
- Firat D, Kuntsal L, Sirin Y (2005). Protective effects of prenatal administration of folic acid on retinoic acid-induced cellular damages of Meckel's cartilage in rats. *Tohoku J. Exp. Med.* 205:27-36.
- Gefrides LA, Bennett GD, Finnell RH (2002). Effects of folate supplementation on the risk of spontaneous and induced neural tube defects in splotch mice. *Teratology* 65:63-69.
- Hamner HC, Mulinare J, Cogswell ME, Flores AL, Boyle CA, Prue CE, Wang CY, Carriquiry AL, Devine O (2009). Predicted contribution of folic acid fortification of corn masa flour to the usual folic acid intake for the US population: National Health and Nutrition Examination Survey 2001-2004. *Am. J. Clin. Nutr.* 89(1):305-315.
- Katagiri R, Kurome M, Teshima Y, Ueta E, Naruse I (2007). Prevention of ochratoxin A-induced neural tube defects by folic acid in the genetic polydactyly/rhinencephaly mouse, Pdn/Pdn Congenit. Anom. (Kyoto). 47:90-96
- Lassi ZS, Bhutta ZA (2012). Clinical utility of folate-containing oral contraceptives. *Int. J. Women's Health.* 4:185-90.
- Li H, Wang B, Zhao D, Han L (2003). Protective effects of vitamin B12, ginseng saponin and folic acid against murine fetal deformities caused by hyperthermia. *Chin. Med. J.* 116:1776-1778.
- Padmanabhan R, Shafiullah MM (2003). Amelioration of sodium valproate induced neural tube defects in mouse fetuses by maternal folic acid supplementation during gestation. *Congenit. Anom.* 43:29-40.
- Reynolds PR, Schaalje GB, Seegmiller RE (2003). Combined therapy with folic acid and methionine in the prevention of retinoic acid induced cleft palate in mice. *Birth Defects Res. A. Clin. Mol. Teratol.* 67:168-173.
- Sadler TW, Merrill AH, Stevens VL, Sullards MC, Wang E, Wang P (2002). Prevention of Fumonisin B1-induced neural tube defects by folic acid. *Teratology* 66:169-176
- Shin JH, Shiota K (1999). Folic acid supplementation of pregnant mice suppresses heat-induced neural tube defects in the offspring. *J. Nutr.* 129:2070-3.
- Tian Y, Ishikawa H, Yamaguchi T, Yamauchi T, Yokoyama K (2005). Teratogenicity and developmental toxicity of chlorpyrifos: maternal exposure during organogenesis in mice. *Reprod. Toxicol.* 20: 267-70.
- Tinker SC, Cogswell ME, Devine O, Berry RJ (2010). Folic acid intake among U.S women aged 15-44 years, National Health and Nutrition Examination Survey, 2003-2006. *Am. J. Prev. Med.* 38(5):534-42.
- Yanaguita MY, Gutierrez CM, Ribeiro CN, Lima GA, Machado HR, Peres LC (2008). Pregnancy outcome in ethanol-treated mice with folic acid supplementation in saccharose. *Childs Nerv. Syst.* 24:99-104.

Full Length Research Paper

## Intestine histology, nutrient digestibility and body composition of Nile tilapia (*Oreochromis niloticus*) fed on diets with both cotton and sunflower seed cakes

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**Physiological response of Nile tilapia to diets with both sunflower (SFSC) and cotton seed cakes (CSC) at increasing proportions (10%CSC25%SFSC; 15%CSC20%SFSC; 20%CSC15%SFSC; 25%CSC10%SFSC and a control that was a commercial tilapia diet) was investigated in two trials using diets containing 25% crude protein. Trial 1 run for 120 days in 15 happas of 1 m<sup>3</sup> stocked with 35 fish of 5.72 g. Each treatment had three replicates. Fish were fed 4% of their body weight per day. The number and length of intestinal folds was investigated. Trial 2 was run for 60 days in 24 tanks with 60 L of water and 30 fish of 3 g. Each treatment had six replicates fed to apparent satiation. Nutrient digestibility and body composition were determined. The test diets did not negatively affect the number and length of intestinal folds as they were similar to the control. The diets with 10%CSC25%SFSC and 20%CSC15%SFSC had the most efficiently digested protein, and highest protein deposition implying that the dietary protein was effectively used. The formulations with 10%CSC25%SFSC and 20%CSC15%SFSC could be used for making Nile tilapia diets.**

**Key words:** Plant protein, intestine histology, digestibility, nutrient retention, feed.

### INTRODUCTION

Nile tilapia is a species that is popularly farmed in the tropics because it grows fast, feeds on a wide range of foodstuffs and tolerates stress (El-Sayed, 1999; Mahmoud, 2009). Plant protein sources are increasingly being used in diet of Nile tilapia because they are

relatively cheaper than conventional protein sources like fish meal and soya beans (Garcia-Abiado et al., 2004; Agbo et al., 2011; Munguti et al., 2012). Sunflower and cotton seed cakes (SFSC and CSC respectively) are among the plant protein sources that have been

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demonstrated to enhance the performance of Nile tilapia (Jauncey, 1998; Tacon et al., 2009; Mbahinzireki et al., 2001; Olvera-Novoa et al., 2002; Rincharde et al., 2002). Although Nile tilapia is adapted to feeding on plant protein (Jauncey, 1998), the inclusion levels are restricted because CSC and SFSC contain high fiber content of 18-23% which could decrease palatability, food intake and nutrient digestibility thereby reducing growth (El-Sayed, 1999; Olvera-Novoa et al., 2002; Silvia et al., 2010). Moreover, fiber does not have any nutritional value in fish (Maina et al., 2002). Cotton also contains 400 to 800 mg/kg of an anti-nutritional factor called gossypol (Gatlin et al., 2007) which can impair fish growth (Luo et al., 2006; Meric et al., 2011; Renuka et al., 2005). Renuka et al. (2005) noted that cotton seed contains more gossypol (0.08%) compared to the leaf (0.05%), pod (0.04%), stem (0.03%) and root (0.02%). Tilapia can tolerate up to 0.18% free gossypol without adverse effects on growth (Robinson and Li, 1995; Evans et al., 2010). Sunflower contains low quantities of anti-nutritional factors namely: protease inhibitors, saponins, arginase inhibitor (Francis et al., 2001; Silvia et al., 2010). Heat treatment can however reduce the effects of the anti-nutritional factors (Olvera-Novoa et al., 1998; Evans et al., 2005) thereby making it possible to increase the quantities of CSC and SFSC that could be used in tilapia diets (Jauncey, 1998). Teichert-Coddington et al. (1997) noted that incorporation of up to 15% CSC in tilapia diets causes no gossypol toxicity while Jauncey (1998) and Hecht (2007) recommend a maximum inclusion of 30%. On the contrary, Agabo et al. (2011) observed that CSM could substitute upto 50% of fish meal protein in the diet of Nile tilapia without negative effects on growth and feed utilization. For SFSC, Olvera-Novoa et al. (2002) recommends the use of up to 20% sunflower in the diet of *Tilapia rendalli*. Jauncey (1998) recommends an inclusion level of SFSC of up to 25% for Nile tilapia while Maina et al. (2002) reports that it should not exceed 30% of the total crude protein. But the supply and price of cotton and sunflower fluctuates especially in developing countries thus the need to combine SFSC and CSC in the same diet to complement one another. However, there is scanty information on the physiological effects of diets containing both SFSC and CSC incorporated at different quantities. This information is vital in order to produce feed that does not cause adverse effects on Nile tilapia. Most studies conducted have evaluated CSC and SFSC independently. For instance, Garcia-Abiado et al. (2004) determined effect of diets with 0, 25, 50, 75 and 100% CSM as fish meal replacement and noted similar growth between fish fed on 0% CSM and 25-50% CSM and a decline in growth with 75-100% CSM. However, significant pathological effects on blood plasma were observed with diets formulated with 25-100% CSM. Although El-Saidy and Gaber (2003) substituted fish meal with 75 and 100% of a plant protein mixture (PPM) comp-

posed of 25% soya bean, 25% cotton seed meal, 25% sunflower meal, and 25% linseed and noted good growth performance, the physiological effect of CSM and SFSC in the diet was not evaluated. Soltan and El-Bab (2008) also investigated effect of replacing fish meal with a PPM comprising 20% cotton seed, 20% sunflower, 20% canola, 20% seasmee, and 20% linseed meal. At 15-45% substitution of fish meal, the growth was similar to the control that contained only fish meal but all the fish fed on PPM had significant adverse pathological effects on the blood. The pathological defects intensified with increasing dietary levels of PPM whereas Aanyu et al. (2012) investigated the performance of Nile tilapia fed on diets with increasing quantities of SFSC and CSC and observed the highest absolute growth with 15% SFSC and 20% CSC, the physiological effect of the diets were not investigated. Physiological effect of diets such as histological response of the digestive system, nutrient digestibility and retention are some of the vital aspects for guiding feed manufacturers to manipulate feed formulae to improve the quality of feed.

The histology of the gastro-intestinal tract is influenced by the type of feed eaten by the fish (El-Bakary and El-Gammal, 2010; Delashoub et al., 2010). The intestine plays a vital role in absorbing nutrients that are used for growth (Rodrigues et al., 2009). Harmful effects on the intestinal anatomy can decrease its efficiency in nutrient absorption and fish growth (Mahmoud, 2009; Delashoub et al., 2010; Rašković et al., 2011). Hence, histological changes in the intestine can give insights on the performance of the fish when fed on a specific diet (Hu et al., 2007; Rašković et al., 2011). Besides, apparent digestibility coefficient measurements indicate the extent to which the nutrients in a diet are digested and made bio-available for the fish (Koprucu and Ozdemir, 2005; Jimoh et al., 2010). When feed with a good nutritional composition cannot be efficiently digested then fish performance is likely to be poor (Deganp and Yehuda, 1999; Cook et al., 2000). Knowledge of the body composition of the fish as influenced by the type of diet eaten is also vital (Maina et al., 2003; Rust, 2003). Digested nutrients are first used for maintenance of body systems and the balances are deposited in the body (Jobling, 1994). Excess protein is used for growth while excess fat leads to fatty fish. Moreover, fatty fish are not preferred by consumers (Jauncey, 1998). The objective of this study was therefore to determine the effect of diets with both cotton and sunflower on the histology of the fore intestine, nutrient digestion, and retention by Nile tilapia. This information will guide feed producers as they manipulate feed formulae to improve feed quality.

## MATERIALS AND METHODS

An experiment comprising two feeding trials was conducted to investigate the effect of diets with cotton and sunflower seed cakes

**Table 1.** Proximate composition of feed ingredients used for formulating the test diets for trial 1 and 2.

<b>Trial 1 Ingredient</b>	<b>Dry matter</b>	<b>Crude protein</b>	<b>Crude lipid</b>	<b>Crude fibre</b>	<b>Ash</b>
Cotton seed cake	91.88	33.26	6.92	22.56	21.19
Sunflower seed cake	91.61	30.84	13.25	14.46	22.43
Soy beans	90.70	35.84	17.26	1.53	16.59
Blood meal	90.19	72.00	0.52	0.0	11.50
Maize bran	88.59	7.27	5.35	0.0	10.50
Wheat pollard	91.05	14.34	4.55	6.65	15.24
Wheat flour	88.26	10.84	1.97	0.0	9.17
<b>Trial 2 Ingredient</b>					
Cotton seed cake	92.81	34.69	0.84	17.96	6.49
Sunflower seed cake	93.38	24.33	14.76	31.71	4.32
Soy beans	90.76	32.29	15.32	13.91	8.87
Blood meal	88.42	76.84	0.77	0.92	7.02
Maize bran	88.13	10.06	10.55	2.68	2.98
Wheat pollard	90.30	14.65	4.86	8.78	3.53

on: i) the histology of the intestine (trial 1) and 2) nutrient digestibility and body composition of Nile tilapia (trial 2).

### Experimental diets, fish and feeding

The experimental diets were formulated using sunflower and cotton seed cakes, wheat pollard, maize bran, soya meal, blood meal, sunflower oil, wheat flour, vitamin and mineral premix, salt. To inactivate anti-nutritional factors, soya beans and cotton seed cake were heat treated at 40-50°C for 10 min. Ingredients were ground and sieved using a 0.2 mm mesh size sieve. The proximate composition of ingredients used in trial 1 and 2 are provided in Tables 1 and 2.

For each trial, 4 experimental diets containing 25% crude protein were formulated with varying proportions of cotton (CSC) and sunflower seed cakes (SFSC) as the main protein sources using WinPas software. The diets include 10%CSC25%SFSC; 15%CSC20%SFSC; 20%CSC15%SFSC; 25%CSC10%SFSC. The control for trial 1 was a commercial tilapia diet with 25% crude protein from Ugachick Poultry Breeders Ltd in Uganda. Table 2 shows the formulae for trial 1 and 2. An inert marker (chromic oxide) was incorporated into the diets for trial 2 to enable determination of the apparent digestibility of the nutrients. The proximate composition of the formulated diets is provided in Table 3.

Nile tilapia fingerlings were obtained from the Aquaculture Research and Development Centre, conditioned for 2 weeks and size graded to obtain fish with no significant differences in initial weights between treatments. The fish in trial 1 were fed on their respective diets three times a day between 9 - 10 am, 12 noon - 1 pm and 4 - 5 pm at a feeding rate of 4% of the live body weight per day. In trial 2, fish were fed to apparent satiation twice a day from 9:00-10:00 am and 4:00-5:00 pm.

### Experimental facilities and design

Trial 1 was run for a period of 120 days in a pond with a water surface area of 1,450 M<sup>2</sup> (44 x 33 m) each. The water depth at the

inlet side was 1 and 1.5 m at the outlet side. Fifteen (15) happas were placed in one pond. Fifteen happas were used because the five different diets (four formulated diets and a commercial feed) represented a treatment and each treatment was replicated three times. Treatments were assigned using a randomized complete block design. Each happa measured 1x1x1 m (length, width and height) and had a mesh size of 1 mm. The happas were supported using wooden poles and 70 cm depth of the happas was submerged in the pond water. The happas were installed in 3 rows and each row had 5 happas with each treatment represented in each row. The happas within the same row were installed 3 m away from each other while the happas at the end of each row were installed 8 m away from the pond dyke. The distance between the rows was 10 m. At the inlet side, the first row of happas was installed 10 m away from the rear pond dyke while at the outlet side; the last row of happas was installed 11 m away from the hind pond dyke. A 2 inch net was placed on top of each happa to prevent birds from preying on the fish. The happas were each stocked with 35 Nile tilapia fingerlings of 5.7 g. In order to minimise clogging of the happas with organic matter, they were cleaned after every three days using a brush.

Trial 2 was carried out in a flow through culture system for a period of 60 days in 24 plastic tanks each with a water volume of 60 L. Each tank had 30 fish of 3 g, aerated using an air stone and covered with a gill net mesh of 1 inch to control the stocked fish from jumping out. The treatments were randomly distributed.

### Data collection

Samples for histological examination of the fore intestine were collected at the end of the trial 1. The fore intestine was selected because it is where most of the nutrient absorption takes place (Rust, 2003). Three fish were randomly picked from each happa and those from the same treatment pooled together. The abdomen was opened and the digestive system was carefully dissected to cut out the intestine. Two (2) cm sections were cut from the anterior part of the intestine. The anterior intestine was defined as the portion that is three quarters the total length of the intestine from the stomach and posterior intestine as the remaining portion which



**Table 2.** Proportions (%) of the ingredients used for making diets for trial 1 and 2

<b>Trial 1 ingredient</b>	<b>10%CSC 25%SFSC</b>	<b>15%CSC20%SFSC</b>	<b>20%CSC15%SFSC</b>	<b>25%CSC10%SFSC</b>
Sunflower seed cake	25	20	15	10
Cottonseed cake	10	15	20	25
Soybean meal	10	10	10	10
Blood meal	7	7	7	7
Wheat pollard	34.1	34.1	34.1	34.1
Maize bran	7	7	7	7
Sunflower oil	2	2	2	2
Vitamin and mineral premix	2	2	2	2
Wheat flour	2.5	2.5	2.5	2.5
Salt	0.4	0.4	0.4	0.4
<b>Trial 2 Ingredient</b>				
Sunflower seed cake	25	20	15	10
Cottonseed cake	10	15	20	25
Soybean meal	2.5	2	3	3
Blood meal	10	10	10	9
Wheat pollard	42	43.1	40.1	41.1
Maize bran	3.6	3	5	5
Sunflower oil	1.5	1.5	1.5	1.5
Vitamin and mineral premix	2	2	2	2
Wheat flour	2	2	2	2
Salt	0.4	0.4	0.4	0.4
Chromium oxide	0.5	0.5	0.5	0.5

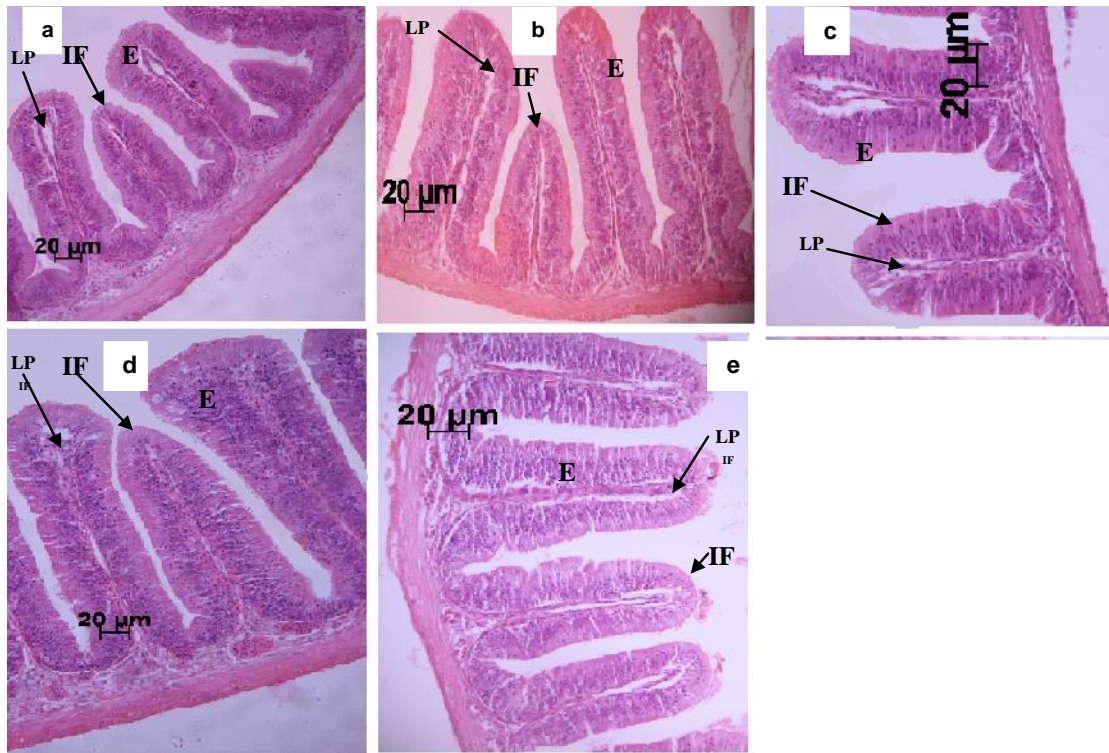
Vitamin and mineral premix contained: Vitamin A, 7,000,000 I.U; Vitamin D3, 2,000,000 I.U; Vitamin E, 10,000 mg; Vitamin K3 STAB, 200 mg; vitamin B1, 300 mg; Vitamin B2, 800 mg; vitamin B6, 400 mg; vitamin B12, 2 mg; niacin, 3,000 mg; pantoic acid, 1,000 mg; folic acid, 100 mg; biotin, 75 mg; choline, 35,000 mg; manganese, 6,000 mg; iron, 4,000 mg; zinc, 5,000 mg; copper, 800 mg; cobalt, 30 mg; iodine, 100 mg; selenium 1%, 20 mg; antioxidant, 20,000 mg; olaquinox 10%, 20,000 mg; salox 12%, 50,000 mg; ronozyme p, 5,000 mg; ronozyme g2, 12,000 mg; carophyl yellow, 2,500 mg; carophyl red, 500 mg

**Table 3.** Analyzed proximate composition (%) of the diets fed to Nile tilapia during trial 1 and 2.

<b>Trial 1</b>	<b>Diet</b>				<b>Control</b>
	<b>10%CSC25%SFSC</b>	<b>15%CSC20%SFSC</b>	<b>20%CSC15%SFSC</b>	<b>25%CSC10%SFSC</b>	
Crude protein	25.23	25.26	24.68	25.22	24.52
Crude lipid	7.52	8.26	8.03	7.51	5.95
Nitrogen free extract	48.42	45.53	50.97	45.13	49.85
Crude fibre	1.88	2.15	1.93	3.64	2.35
Ash	16.96	18.69	14.39	18.50	17.33
<b>Trial 2</b>					
Crude protein	25.81	24.89	25.71	25.89	
Crude lipid	7.48	4.38	3.38	2.37	
Nitrogen free extract	49.23	51.30	51.51	53.65	
Crude fibre	11.41	12.74	12.85	11.60	

extended distally from the anterior intestine to the anus. All tissues were fixed in Bouin's fixative and embedded in paraffin wax.

Sections of 5 µm were cut and stained with Harris hematoxylin and eosin (H&E).



**Figure 1.** Light micrograph of the anterior part of the intestine of Nile tilapia. The intestine is arranged into intestinal folds (IF) with an epithelial sheet (E) covering the folds and it bears a lamina propria (LP) of loose connective tissue. **A.** 10%CSC25%SFSC; **b.** 15%CSC20%SFSC; **c.** 20%CSC15%SFSC; **d.** 25%CSC10%SFSC; **e.** Control diet. Magnification  $\times 200$ .

At the beginning of trial 2, a total of 20 fish were removed from the pool of fish and 5 fish were removed from each happa at the end of the trial for determining the initial and final body composition of the fish from different treatments. The fish were frozen at  $-20^{\circ}\text{C}$  until they were analysed.

After two weeks of the trial 2, faecal samples were collected daily from each tank by siphoning the bottom of the tank. Faecal collection started one hour after fish feeding and stopped after 3 h. The samples were dried in an oven at  $40^{\circ}\text{C}$  for 12 h. Samples from each tank were pooled together, ground and homogenized before proximate analysis.

#### Laboratory analysis

Proximate composition (crude protein, crude lipid, crude fibre, ash, moisture, Nitrogen free extract) of the fish and diets was determined based on the method described by the AOAC (1990). Crude protein was measured by determining the nitrogen content of the ingredient using the micro-Kjeldahl method and calculating the crude protein level by multiplying the nitrogen content by 6.25. Crude lipid was determined by ether extraction method using soxhlet apparatus. Ash content was measured by placing a sample of known weight in a furnace of  $470\text{-}550^{\circ}\text{C}$  for 3 h and the remaining weight was considered the ash. Moisture content was measured by placing a sample of known weight in an oven set at  $105\text{-}110^{\circ}\text{C}$  until the sample attained a constant weight. The lost weight from the sample was considered the moisture content and the remaining weight dry matter.

Photomicrographs were taken using a light microscope (Carl Zeiss) with an Axio-Vision 2.05 image analysis system. Samples from the anterior intestine of the fish were compared between treatments. The intestinal sections were evaluated according to criteria described by Dimitroglou et al. (2010) which include among others: intestinal fold length, width, and number.

#### Data analysis

The effect of the experimental treatments on the digestibility of dry matter, crude protein, fat, ash and fiber in the faeces; composition of protein, fat and ash in fish carcass; and the number, length and width of intestinal fold was compared using SPSS one way analysis of Variance (ANOVA). Differences between treatments means were determined using Tukey's multiple comparison test. Significant differences were considered at  $p < 0.05$ .

## RESULTS

### Histology of the anterior intestine

Figure 1 shows histology images of the fore intestine of Nile tilapia fed on the experimental diets. All the diets had similar morphology of the intestinal folds. However, fish fed on 20%CSC15%SFSC had the highest number and

**Table 4.** Number, length and width of intestinal folds of the fore intestine of Nile tilapia fed on different diets with increasing quantities of cotton and sunflower seed cakes.

Parameter	10%CSC	15%CSC	20%CSC	25%CSC	Control	P value
	25%SFSC	20%SFSC	15%SFSC	10%SFSC		
Number of intestinal folds	46.00±4.00	37.70±5.86	48.00±2.00	44.50±0.50	42.00±5.00	NS
Length of intestinal folds (µm)	27.18±4.35	29.86±9.38	31.45±4.79	29.87±5.12	30.44±4.00	NS
Width of intestinal folds (µm)	11.08±1.85 <sup>a</sup>	13.38±3.87 <sup>b</sup>	13.92±2.49 <sup>b</sup>	13.45±3.75 <sup>b</sup>	14.82±2.41 <sup>b</sup>	0.000

Values on the same row with different superscripts (a, b, c, d) are significantly different (P<0.05). NS is no significant difference.

**Table 5.** Initial and final body composition of the Nile tilapia fed on diets containing increasing quantities of cotton and sunflower seed cakes.

Parameter	Diet					P value
<b>Initial body composition (%):</b>						
Ash	15.26					
Crude protein	59.02					
Fat	11.57					
<b>Final body composition (%):</b>						
	10%CSC	15%CSC	20%CSC	25%CSC		
	25%SFSC	20%SFSC	15%SFSC	10%SFSC		
Ash	12.44±0.27 <sup>b</sup>	11.38±0.14 <sup>a</sup>	12.20±0.11 <sup>b</sup>	12.42±0.27 <sup>b</sup>		0.001
Crude protein	59.91±0.75 <sup>b</sup>	57.25±0.14 <sup>a</sup>	59.79±0.40 <sup>b</sup>	56.33±0.60 <sup>a</sup>		0.000
Fat	18.67±0.27 <sup>a</sup>	26.27±1.03 <sup>c</sup>	22.03±0.18 <sup>b</sup>	23.45±0.78 <sup>b</sup>		0.000

**Table 6.** Apparent digestibility coefficients of the nutrients in diets containing increasing quantities of cotton and sunflower seed cake fed to Nile tilapia.

Parameter	10%CSC	15%CSC	20%CSC	25%CSC	P value
	25%SFSC	20%SFSC	15%SFSC	10%SFSC	
Dry matter	97.45±0.01	97.59±0.02	97.61±0.01	97.44±0.05	NS
Crude protein	99.60±0.47 <sup>b</sup>	98.36±0.04 <sup>a</sup>	98.34±0.02 <sup>a</sup>	98.46±0.01 <sup>a</sup>	0.000
Crude fat	99.78±0.25 <sup>c</sup>	98.93±0.01 <sup>b</sup>	99.06±0.02 <sup>b</sup>	98.22±0.03 <sup>a</sup>	0.000
Crude ash	98.46±1.77	97.15±0.31	97.82±0.17	97.59±0.20	NS
Crude fibre	96.93±3.50	94.81±0.09	95.74±0.23	95.77±0.10	NS

length of intestinal folds although not significantly different from the other treatments (Table 4). The width of the intestinal folds was significantly lower with fish fed on 10%CSC25%SFSC.

### Body composition

There was a reduction in the amount of ash in the fish after feeding on the experimental diets (Table 5). Higher protein content was obtained with diets containing 10%CSC25%SFSC and 20%CSC15%SFSC. Fish fed on 15%CSC20%SFSC had a significantly lower amount of ash but higher fat content (P<0.05) compared to the other treatments. The lowest fat content was obtained with fish fed 10%CSC25%SFSC.

### Digestibility of nutrient

There was no significant difference in the digestibility of dry matter, ash and fibre between treatments. Crude protein and fat were more efficiently digested by fish fed on 10%CSC25%SFSC and 20%CSC15%SFSC (Table 6).

### DISCUSSION

Besides assessing the impact of a diet on growth and feed utilization efficiency in fish, it is also vital to know the mechanisms responsible for the observed performance. These include among others nutrient digestibility, absorption in the digestive system and retention in the body.

The efficiency at which the diets ingested are digested largely determines growth performance (Deganp and Yehuda, 1999; Guillaume and Choubert, 1999). This is because the nutritive value of food depends not only on its nutrient content but also on the capacity of the animal to digest and absorb the nutrients (Cook et al., 2000; Rust, 2003).

In this study, the apparent digestibility of crude protein, fat, ash and fibre for all the experimental diets ranged between 94 and 99% despite the high fibre content in SFSC and CSM (Table 1). This high level of nutrient digestibility is attributed to the fact that the feed was cooked before pelleting making it easier for the fish to digest (Jauncey, 1998; Engin and Ozkan, 2008). Deganp and Yehuda (1999) also found high digestibility values for protein in sunflower seed meal (78%), in rapeseed meal (86%) and in cottonseed meal (79%). In this study, 10%CSC25%SFSC had the most efficiently digested crude protein, fat, ash and fibre suggesting that the diet had more bio-available nutrients for the fish (Rust, 2003).

The efficiency at which digested nutrients are absorbed can be assessed using the histology of the intestine because it is the main site for nutrient absorption (Rodrigues et al., 2009). Negative effects on the anatomy of the intestine reduce the efficiency of its performance (Hu et al., 2007; Rašković et al., 2011). Borgeson et al. (2006) noted a decrease in intestinal fold length (villi) with decreasing fish meal and increasing plant protein in the diet of Nile tilapia and this corresponded with reduced growth.

In this study, the diets caused no significant negative changes in the histology of the intestine of Nile tilapia in terms of the number and length of intestinal folds when compared to the control diet that contained fish meal. An increase in the number, length and width of intestinal folds is associated with an increase in the surface area for absorption of nutrients vital for fish growth (Delashoub et al., 2010; Dimitroglou et al., 2010; El-Bakary and El-Gammal, 2010). The diet with 20%CSC15%SFSC had the highest absolute number, length and width of intestinal folds implying that the nutrients in this diet could have been more efficiently absorbed by the fish.

Nutrients absorbed in the digestive system are first used for maintenance of body functions and the surplus is retained in the body. Excess protein is deposited in the body for growth while excess energy is stored as fat (Jobling, 1994; Cook et al., 2000). This study observed a higher crude protein deposition and lower fat content in the carcass of fish fed 10%CSC25%SFSC and 20%CSC15%SFSC.

The lowest crude protein retention and highest fat deposit was obtained with fish fed 15%CSC20%SFSC. The results suggest that the diets with 10%CSC25%SFSC and 20%CSC15%SFSC were the most efficiently assimilated. Deposition of protein in fish is known to result into fish growth. The higher the protein

deposition, the higher the weight gained (Sveier et al., 2000; Lupatsch et al., 2003). This observation is in agreement with findings by Aanyu et al. (2012) where the diet with 20%CSC15%SFSC had the best growth performance although it was not significantly different from the diet with 10%CSC25%SFSC while the diet with 15%CSC20%SFSC had a significantly lower growth.

In conclusion, the test diets did not negatively affect the number and length of intestinal folds as they were similar to the control. The diets with 10%CSC25%SFSC and 20%CSC15%SFSC had the most efficiently digested protein, and highest protein deposition implying that dietary protein was effectively used. Feed formulations with 10%CSC25%SFSC and 20%CSC15%SFSC could be used for making Nile tilapia diets. Future research should analyze Nile tilapia diets formulated based on the amino and fatty acid profiles of cotton and sunflower seed cakes. In this study, the feed was formulated based on the proximate composition of the ingredients. More factors relating to the intestine morphology also need to be assessed since the intestine is the key site for nutrient absorption.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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### REFERENCES

- Aanyu M, Carpaj C, Widmer M (2012). Effect of diets with graded levels of inclusion of cotton and sunflower seed cakes on the growth performance and feed utilization efficiency of Nile tilapia, *Oreochromis niloticus*. *Livest. Res. Rural Dev.* 24:5.
- Agbo NW, Madalla N, Jauncey K (2011). Effects of dietary cottonseed meal protein levels on growth and feed utilization of Nile tilapia, *Oreochromis niloticus* L. *J. Appl. Sci. Environ. Manage.* 15: 235 -239.
- AOAC (1990). Official methods of analysis. 15th Edition. Association of official Analytical Chemists. Washington D.C. USA.
- Borgeson TL, Racz VJ, Wilkie DC, White LJ, Drew MD (2006). Effect of replacing fish meal and oil with simple or complex mixtures of vegetable ingredients in diets fed to Nile tilapia, *Oreochromis niloticus*. *Aquacult. Nutr.* 12: 141-149.

- Cook JT, McNiven MA, Richardson GF, Sutterlin AM (2000). Growth rate, body composition and feed digestibility/conversion of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). *Aquaculture* 188:15-32.
- Deganp G, Yehuda Y (1999). Digestibility of protein sources in feed for *Oreochromis aureus* x *O. nilotica*. *Indian J. Fish.* 46: 33-39.
- Delashoub M, Poust I, Khojasteh B (2010). Histology of Big head Carp (*Hypophthalmichthys nobilis*) intestines. *GV* 5:302-306.
- Dimitroglou A, Merrifield DL, Spring P, Sweetman J, Moate R, Davies SJ (2010). Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilization, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*); *Aquaculture* 300:182-188.
- EI-Bakary NER, El-Gammal HL (2010). Comparative Histological, Histochemical and Ultrastructural Studies on the proximal Intestine of Flathead Grey Mullet (*Mugil cephalus*) and Sea Bream (*Sparus aurata*). *J. World Appl. Sci.* 8:477-485.
- EI-Saidy DSD, Gaber AMM (2003). Replacement of fish meal with a mixture of different plant protein sources in juvenile Nile tilapia, *Oreochromis niloticus* (L.) diets. *Aquac. Res.* 34:1119-1127.
- EI-Sayed AM (1999). Alternative dietary protein sources for farmed tilapia, *Oreochromis* spp. *Aquaculture* 179:149-168.
- Engin K, Ozkan F (2008). Apparent nutrient digestibility of balanced diets with soy bean or meat and bone meal in the Nile tilapia (*Oreochromis niloticus* L.) infected with *Vibrio anguillarum*. *Turk. J. Vet. Anim. Sci.* 32:173-181.
- Evans JJ, Pasnik DJ, Peres H, Lim C, Klesius PH (2005). No apparent differences in intestinal histology of channel catfish, *Ictalurus punctatus* fed heat-treated and non heat-treated raw soybean meal. *Aquacult. Nutri.* 11: 123-129.
- Evans JJ, Pasnik DJ, Yildirim-Aksoy M, Lim C, Klesius PH (2010). Histologic changes in channel catfish, *Ictalurus punctatus* Rafinesque, fed diets containing graded levels of gossypol acetic acid. *Aquacult. Nutr.* 16: 385-391.
- Francis G, Makkar HPS, Becker K (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199: 197-227.
- Garcia-Abiado MA, Mbahinzireki G, Rinchar J, Lee KJ, Dabrowski K (2004) Effect of diets containing gossypol on blood parameters and spleen structure in tilapia, *Oreochromis* sp, reared in a recirculating system. *J. Fish Dis.* 27:359-368.
- Gatlin DM, Barrows FT, Brown P, Dabrowski K, Gaylord TG, Hardy RW, Herman E, Hu G, Krogdahl A, Nelson R, Overturf K, Rust M, Sealey W, Skonberg D, Souza EJ, Stone D, Wilson R, Wurtele E (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquac. Res.* 38:551-579.
- Guillaume J, Choubert G (1999). Digestive physiology and nutrient digestibility in fishes. In: *Nutrition and feeding of fish and crustaceans*. Guillaume J, Kaushik S, Bergot P, Metaille R (editors) Springer-Praxis. pp. 27-56.
- Hecht T (2007). Review of feeds and fertilizers for sustainable aquaculture development in sub-Saharan Africa. In *Study and analysis of feeds and fertilizers for sustainable aquaculture development*. Hasan MR, Hecht T, De Silva SS, Tacon AGJ (editors). FAO Fisheries Technical Paper. No. 497. Rome, FAO. pp. 77-109.
- Hu CH, Xu Y, Xia MS, Xiong L, Xu ZR (2007). Performance, microbial ecology and intestine morphology of Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 270:200-206.
- Jauncey K (1998). Tilapia feeds and feedings. *Pisces Press LTD*, Stirling, Scotland.
- Jimoh WA, Fagbenro OA, Adeparusi EO (2010). Digestibility coefficients of processed jackbean meal *Cannavalia ensiformis* (L.) DC for Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) diets. *Int. J. Fish Aquac.* 2:102-107.
- Jobling M (1994). *Fish bioenergetics*. London Chapman and Hall. pp. 93-168.
- Kopruca K, Ozdemir Y (2005). Apparent digestibility of selected feed ingredients for Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 250:308-316.
- Luo LM, Xue M, Wu X, Cai X, CAO H, LIANG Y (2006). Partial or total replacement of fishmeal by solvent-extracted cottonseed meal in diets for juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Nutr.* 12:418-424.
- Lupatsch I, Kissil GW, Sklan D (2003). Comparison of energy and protein efficiency among three fish species gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) and white grouper (*Epinephelus aeneus*): energy expenditure for protein and lipid deposition. *Aquaculture* 225:175-189.
- Mahmoud SA (2009). Effect of different artificial diets on growth rate, condition and histological structure of Nile tilapia (*Oreochromis niloticus*). *J. Fish Hydrobiol.* 4:29-34.
- Maina JG, Beames RM, Higgs D, Mbugua PN, Iwama G, Kisia SM (2002). Digestibility and feeding value of some feed ingredients fed to tilapia *Oreochromis niloticus* (L.). *Aquac. Res.* 33:853-862.
- Maina JG, Beames RM, Higgs D, Mbugua PN, Iwama G, Kisia SM (2003). Partial replacement of fish meal with sunflower cake and corn oil diets in tilapia *Oreochromis niloticus* (Linn): effect on whole body fatty acids. *Aquac. Res.* 34:601-608.
- Mbahinzireki GB, Dabrowski K, Lee KJ, El-Saidy D, Wisner ER (2001). Growth, feed utilization and body composition of tilapia (*Oreochromis* sp.) fed with cottonseed meal-based diets in a recirculating system. *Aquacult. Nutr.* 7:189-200.
- Meric L, Wuertz S, Kloas W, Wibbelt G, Schulz C (2011). Cottonseed Oilcake as a Protein Source in Feeds for Juvenile Tilapia (*Oreochromis niloticus*): Anti-nutritional Effects and Potential Detoxification by Iron Supplementation. *Isr. J. Aquac. Bamidgheh IIC:63.2011.588*, 8 pages.
- Munguti J, Charo-Karisa H, Opiyo MA, Ogello EO, Marijani E, Nzayisenga L and Liti D (2012). Nutritive value and availability of commonly used feed ingredients for farmed Nile tilapia (*Oreochromis niloticus* L.) and African catfish (*Clarias gariepinus*) in Kenya, Rwanda and Tanzania. *Afr. J. Food Agric. Nutr. Dev.* 12: 6135-6155.
- Olvera-Novoa MA, Martinez PCA, Galván CR, Chávez SC (1998). The use of seed of the leguminous plant *Sesbania grandiflora* as a partial replacement for fish meal in diets for tilapia (*Oreochromis niloticus*). *Aquaculture* 71:51-60.
- Olvera-Novoa MA, Olivera-Castillo L, Martinez-Palacios CA (2002). Sunflower seed meal as a protein source in diets for *Tilapia rendalli* (Boulanger, 1896) fingerlings. *Aquacult. Res.* 33:223-229.
- Rašković BS, Stanković MB, Marković ZZ, Poleksić VD (2011). Histological Methods in the assessment of different feed effects on liver and intestine of fish. *J. Agric. Sci.* 56:87-100.
- Renuka CK, Kumarmath P, Skadokol JC, Hosamani SV (2005). Chemical Composition and Antinutritional Factors in Different Parts and Whole Cotton (*Gossypium hirsutum*) Plant. *Karnataka J. Agric. Sci* 18: 114-117.
- Rinchar J, Mbahinzireki G, Dabrowski K, Lee KJ, Garcia-Abiado MA, Ottobre J (2002). Effect of dietary cotton seed meal protein level on growth, gonad development and plasma sex steroid hormones of tropical fish tilapia *Oreochromis* sp. *Aquac. Int.* 20: 11-28.
- Robinson EH, Li MH (1995). Use of cottonseed meal in aquaculture feeds In: *Nutrition and utilization technology in aquaculture* Editors: Lim CE and Sessa DJ. pp. 157-161.
- Rodrigues APO, Pauletti P, Kindlein L, Cyrino JEP, Delgado EF, Machado-Neto R (2009). Intestinal morphology and histology of the striped catfish *Pseudoplatystoma Fasciatum* (Linnaeus, 1766) fed dry diets. *Aquac. Nutr.* 15:559-563.
- Rust MB (2003). Nutritional physiology. In: Halver JE, Hardy RW (eds). *Fish nutrition*, 3rd Edition. New York, Academic Press Inc. pp. 367-452
- Silvia NM, Ana T, Silvia M, Miguel JC (2010). Sunflower meal as a partial substitute in juvenile sharpnose sea bream (*Diplodus puntazzo*) diets: Amino acid retention, gut and liver histology. *Aquaculture* 298: 275 - 281.
- Soltan MA, El-Bab AFF (2008). Effect of dietary replacement of fish meal by mixture of different plant protein sources on growth performance and some blood parameters of Nile tilapia, *Oreochromis niloticus*. *Egypt. J. Aquat. Biol. Fish.* 12: 1 -16 ISSN 1110-1131.
- Sveier H, Raae AJ, Lied E (2000). Growth and protein turnover in Atlantic salmon (*Salmo salar* L.); the effect of dietary protein level and

- protein particle size. *Aquaculture* 185: 101-120.
- Tacon AGJ, Metian M, Hasan MR (2009). Feed ingredients and fertilizers for farmed aquatic animals: sources and composition. *FAO Fisheries and Aquaculture Technical Paper*. No. 540. Rome, FAO. 209pp.
- Teichert-Coddington DR, Popma TJ, Lovshin LL (1997). Attributes of tropical pond-cultured fish In: Enga HS, Boyd CE (eds.) *Dynamics of Pond Aquaculture*. CRC Press, Boca Raton, Florida, USA. pp. 183-198.

Full Length Research Paper

## Chemical composition of medicinal plants used as auxiliary treatments for obesity

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The objective of this study was to find substances of pharmacological interest in a variety of medicinal plants, such as *Aloe vera* (L.) Burm. (aloe), *Simaba ferruginea* St. Hil. (calunga), *Baccharis trimera* (Less.) DC (carqueja), *Garcinia cambogia* Desr. and *Tournefortia paniculata* Cham. (Marmelinho), to aid in the treatment of obesity and other diseases. To reach this goal, phytochemical screenings were performed, percentage and mineral compositions were determined, and the content of a number of bioactive compounds in the medicinal plants were studied. Important substances with therapeutic potential, especially phenolic compounds, saponins and dietary fiber, were found in all plants, and significant levels of calcium were found in *G. cambogia* and *S. ferruginea*. The studied plants showed great diversity with regard to phytochemicals and have the potential to be used in pharmaceutical formulations that have possible health benefits. However, more studies must be conducted on these plants, because recommendations regarding the possible risks and benefits for human health would be premature at present; additional studies on toxicity, efficiency and safety are necessary, particularly in relation to the saponins found in all plants and to the high levels of phenolic compounds in *T. paniculata* (36.19 g 100 g<sup>-1</sup> dry matter).

**Key words:** Phytochemical screening, percent composition, minerals, bioactive compounds, medicinal plants, obesity.

### INTRODUCTION

The prevalence of obesity has increased steadily and is considered an important public health problem in developed countries and a global epidemic by the World Health Organization (WHO, 2010). A treatment for

obesity is essential, because the condition is associated with various diseases, such as diabetes, some cancers and cardiovascular diseases, among others (Guh et al., 2009).

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Despite being one of the oldest diseases known to man, pharmacological options for the treatment of obesity are still limited and have many side effects (Mahan and Scott-Stump, 2008). The use of medicinal plants is being widely explored by consumers because of easy access, low cost, no need for prescriptions, and a belief in the absence of toxic effects. In addition, the pharmaceutical industry is interested in these plants as a viable alternative for the future development of drugs that effectively and safely induce weight reduction (Mayer et al., 2009). Studies show that several natural products, including extracts and compounds isolated from plants, can be used for reducing body weight and preventing obesity (Souza et al., 2011; Simão et al., 2012).

Various medicinal plants such as *Aloe vera* (L.) Burm. (aloe), *Simaba ferruginea* St. Hil. (calunga), *Baccharis trimera* (Less.) DC (carqueja), *Garcinia cambogia* Desr. and *Tournefortia paniculata* Cham. (marmelinho) are used in the treatment of obesity (Souza et al., 2011; Simão et al., 2012, 2013). However, there is no scientific evidence for most of these plants regarding their efficacy and safety in the treatment of this disease; their use is solely based on popular beliefs, which emphasizes the need for scientific studies to elucidate the chemical constituents of these plants. The pharmacological/toxic understanding regarding their use in the treatment of obesity and other diseases is highly important.

The aims of this study were to perform phytochemical screening, determine chemical and percent composition as well as the bioactive compounds from the following medicinal plants: *A. vera*, *B. trimera*, *S. ferruginea*, *G. cambogia* and *T. paniculata*, and examine the potential for these substances as auxiliaries in the treatment of obesity.

## MATERIALS AND METHODS

### Sample collection and preparation

*B. trimera* (Less.) DC (carqueja) and *T. paniculata* Cham. (marmelinho) leaves as well as the stem bark of *S. ferruginea* St. Hil. (calunga) were acquired in the municipal market of Belo Horizonte, Minas Gerais in January 2011. The *B. trimera* and *T. paniculata* leaves were washed with tap and distilled water and then placed with the *S. ferruginea* stem bark in forced air circulating ovens to dry for 48 h at  $\pm 35^{\circ}\text{C}$ . After drying, the leaves and the bark were individually ground in a Wiley-type mill, and the powders were stored in hermetically sealed flasks until analyzed. Commercially available samples of *Aloe vera* (L.) Burm. (aloe) and of *Garcinia cambogia* Desr., were acquired from FLORIEN, a distributor of pharmaceutical raw materials. The *A. vera* and *G. cambogia* samples were further processed by lyophilization of the plant mucilage (*A. vera*) and by spray drying (*G. cambogia*).

### Analyses

#### Phytochemical screening

The powders obtained from the medicinal plants (FMP) were phyto-

chemically screened. Specific reagents were used for each chemical group to induce chemical reactions that developed distinct colours and/or precipitates, which were characteristic for each class of substances (Matos, 1997). The specific groups of chemicals analyzed were the following: organic acids, reducing sugars, alkaloids, anthraquinones, azulenes, carotenoids, catechins, depsides and depsidones, coumarin derivatives, steroids and triterpenoids, flavonoids, cardiotoxic glycosides, sesquiterpene lactones and other lactones, polysaccharides, proteins and amino acids, saponins and tannins.

#### Percent composition

The samples were placed in an oven at  $105^{\circ}\text{C}$  until a constant weight was reached to determine moisture content. The ether extract was determined using a Soxhlet continuous extractor. The crude protein was measured by the Kjeldahl method using the conversion factor 6.25 ( $\text{N} \times 6.25$ ). The percentage ash and fixed mineral residue were obtained from a defined quantity of samples by incineration ( $550^{\circ}\text{C}$ ) in a muffle furnace. The total soluble and insoluble dietary fiber was determined using an enzymatic method. The non-nitrogen extract was determined by the difference between 100 and the sum (in dry matter) of the ether extract, protein, ash and total dietary fiber. All methods used to determine percent composition in this study were performed using the methodology described by the Association of Official Analytical Chemists - AOAC (2005).

#### Mineral composition

The samples were subjected to a nitroperchloric digestion in digester blocks with temperature control to quantify the minerals (Fe, Zn, Mn, Cu, Ca, Mg, P, K and S). Colorimetry was used to quantify P and S, flame photometry was used for K and atomic absorption spectrophotometry was used to determine amounts of Ca, Mg, Cu, Mn, Zn and Fe. The procedures used to analyze metal composition are described by Malavolta et al. (1997).

#### Phenolic compounds

The phenolic compounds were extracted with 50% methanol under reflux with three consecutive washes at  $80^{\circ}\text{C}$ . The extracts were collected and evaporated to 25 mL. The phenolic compounds were measured using the Folin-Denis reagent and tannic acid as a standard (AOAC, 2005).

#### Oxalic acid

The method developed by Loures and Jokl (1990) was employed to measure the oxalic acid. A hot extraction was performed with hydrochloric acid, and the oxalic acid was precipitated and quantified by titration of calcium oxalate with potassium permanganate.

#### Nitrate

Nitrate was extracted from the samples with distilled water at  $45^{\circ}\text{C}$ . Typically, a complex is formed by the nitration of salicylic acid under highly acidic conditions, and a distinct peak can be measured using a spectrophotometer at 410 nm in basic solutions (pH greater than 12). The absorbance of the material was directly proportional to the amount of nitrate present as long as no ammonium, nitrite, or



**Table 1.** Qualitative phytochemical screening of medicinal plants.

Constituent	<i>Aloe vera</i>	<i>Baccharis trimer</i>	<i>Simaba ferruginea</i>	<i>Garcinia cambogia</i>	<i>Tournefortia paniculata</i>
Organic acids	-	-	-	-	+
Reducing sugars	+	+	+	+	+
Alkaloids	-	-	-	-	-
Anthraquinones	-	-	-	-	+
Azulenes	+	+	+	-	-
Carotenoids	-	+	-	-	+
Catechins	-	-	-	+	+
Depsides and depsidones	-	+	+	-	+
Coumarin derivatives	-	-	-	-	-
Steroids and triterpenoids	-	+	+	-	+
Flavonoids	-	+	+	-	+
Cardiac glycosides	-	-	-	-	-
Sesquiterpene lactones and other lactones	-	-	-	-	+
Polysaccharides	+	+	-	-	-
Proteins and amino acids	+	+	+	+	+
Saponins	+	+	+	+	+
Tannins	-	-	-	+	+

The signs indicate the presence (+) or the absence (-) of the metabolite.

chlorine ions were present. Potassium nitrate was used as a standard (Cataldo et al., 1975).

#### Trypsin inhibitor

Trypsin inhibitors were extracted with 0.1 mol L<sup>-1</sup> NaOH under magnetic stirring. After centrifugation at 10,000 × g for 60 min, an aliquot of the supernatant was used in the enzyme assay (Kakade et al., 1974). The trypsin activity was measured according to the methodology proposed by Erlanger et al. (1961). In total, 200 µL plant extracts and 200 µL enzyme were incubated in a water bath at 37°C for four time periods after the addition of 800 µL benzoyl-DL-arginine-p-nitroanilide (BAPNA). The BAPNA substrate solution (pH 8.2) was prepared in TRIS buffer (tris (hydroxymethyl) aminomethane) at 0.05 mol L<sup>-1</sup> with 20 mmol L<sup>-1</sup> CaCl<sub>2</sub>. The reaction was stopped using 200 µL 30% acetic acid, and the product was measured using a spectrophotometer at 410 nm.

#### Saponins

The saponins were extracted with ethanol under stirring at room temperature for 60 min. The total saponin content was determined by the reaction of saponins with anisaldehyde, and digitonin was used as a standard (Baccou et al., 1977).

#### Statistical analysis

All data were collected in triplicate and presented as the mean ± standard deviation. The data were statistically evaluated by analysis of variance, and the means were compared using the Scott Knott test (P < 0.05) with the aid of the R software (R Development Core Team, 2011).

## RESULTS AND DISCUSSION

Phytochemical screening is characterized by the identification of chemical compounds present in plant materials. The phytochemical screening results of substances FMPs are shown in Table 1.

The results indicated the presence of different metabolic groups of pharmacological interest in the plants examined, such as tannins (*G. cambogia* and *T. paniculata*), depsides and depsidones (*B. trimer*, *S. ferruginea* and *T. paniculata*), carotenoids (*B. trimer* and *T. paniculata*), and triterpenoids (*B. trimer*, *S. ferruginea* and *T. paniculata*), among other groups of metabolites.

Alkaloids and cardiac glycosides were not detected in any of the analyzed plants. The preliminary phytochemical screening provided a qualitative view of the chemical groups found in the plants, but further studies are needed to determine the concentration and characterization of these substances.

The results of the phytochemical screening are in agreement with other studies conducted on these plants, such as the study by Vasquez et al. (1996) on *A. vera* gel and its extracts, which showed the presence of saponins and the absence of tannins, flavonoids and alkaloids. Studies conducted by Rodrigues et al. (2009) on *B. trimer* showed the presence of flavonoids and saponins and the absence of alkaloids, anthraquinones, coumarins and cardiac glycosides. Moraes and Souza (2007) examined *T. paniculata* leaves and reported the presence of flavonoids and tannins as well as the absence of

**Table 2.** Percent composition of medicinal plants in g 100 g<sup>-1</sup> dry matter.

Constituent	<i>Aloe vera</i>	<i>Simaba ferruginea</i>	<i>Baccharis trimera</i>	<i>Garcinia cambogia</i>	<i>Tournefortia paniculata</i>
Ether Extract	ND <sup>2</sup>	1.97±0.11 <sup>c</sup>	2.49±0.20 <sup>b</sup>	ND	3.88±0.21 <sup>a</sup>
Crude Protein	1.54±0.11 <sup>d</sup>	8.96±0.11 <sup>b</sup>	7.15±0.22 <sup>c</sup>	1.78±0.25 <sup>d</sup>	10.78±0.35 <sup>a</sup>
Ash	3.30±0.08 <sup>e</sup>	8.53±0.04 <sup>b</sup>	6.18±0.05 <sup>c</sup>	34.18±0.24 <sup>a</sup>	3.61±0.01 <sup>d</sup>
Insoluble Fiber	5.20±0.18 <sup>d</sup>	45.20±0.43 <sup>c</sup>	64.67±0.50 <sup>a</sup>	3.09±0.18 <sup>d</sup>	46.71±0.80 <sup>b</sup>
Soluble Fiber	5.77±0.33 <sup>b</sup>	3.74±0.22 <sup>c</sup>	1.60±0.07 <sup>d</sup>	30.11±1.19 <sup>a</sup>	1.16±0.05 <sup>d</sup>
Total Fiber	10.97±0.42 <sup>d</sup>	48.94±0.62 <sup>b</sup>	66.26±0.53 <sup>a</sup>	33.08±1.02 <sup>c</sup>	47.87±0.86 <sup>b</sup>
NNE <sup>1</sup>	84.18±0.51 <sup>a</sup>	31.60±0.64 <sup>c</sup>	17.93±0.60 <sup>d</sup>	30.96±1.17 <sup>c</sup>	33.86±0.11 <sup>b</sup>

All data were collected in triplicate and represent the mean ± standard deviation. The same letter in rows indicates that the values do not differ by the Scott-Knott test ( $P < 0.05$ ). <sup>1</sup>NNE: Non-nitrogen extract. <sup>2</sup>ND: Not detected. The moisture content in the powders of the medicinal plants in g 100 g<sup>-1</sup>: *Aloe* = 8.53; *Simaba* = 8.42; *Baccharis* = 8.56; *G. cambogia* = 3.94; *Tournefortia*: 9.90.

alkaloids. Studies performed by Subhashini et al. (2011) on *G. cambogia* revealed the presence of saponins, tannins, sugars, and proteins and the absence of flavonoids as well as a positive result for the alkaloids test. There were no studies found on the phytochemical screening of *S. ferruginea*.

The results of the chemical composition FMPs are shown in Table 2. In general, the contents of the ether extract were low, and *T. paniculata* had the highest content of ether extract (3.88 g 100 g<sup>-1</sup> dry matter - DM). Ether extract was not detected in *A. vera* and *G. cambogia*. In addition, the contents of crude protein were relatively low, and the ash content was very high in *G. cambogia* (34.18 g 100 g<sup>-1</sup> DM).

The highest content of dietary fiber (DF) was found in *B. trimera* (66.26 g 100 g<sup>-1</sup> DM), and the lowest amount in *A. vera* (10.97 g 100 g<sup>-1</sup> DM). In addition, a high content of soluble DF was found in *G. cambogia* (30.11 g 100 g<sup>-1</sup> DM), and insoluble DF was found in *B. trimera* and *T. paniculata* at 64.67 and 46.71 g 100 g<sup>-1</sup> DM, respectively.

Epidemiological studies suggest that dietary fiber is capable of preventing obesity and weight gain and reduces the risk for developing diabetes and cardiovascular diseases, among others (Liu et al., 2003).

In general, soluble DF helps in the treatment of obesity because it slows gastric emptying, glucose absorption, and reduces cholesterol in blood serum (Rique et al., 2002; Mello and Laaksonen, 2009). Conversely, insoluble DF accelerates intestinal transit and increases feces weight (Rique et al., 2002). Thus, the presence of DF in the composition of the analyzed plants can aid in the treatment of obesity.

The non-nitrogen extract or glicidic fraction consisted primarily of sugars. The highest content of sugars was found in *A. vera* (84.18 g 100 g<sup>-1</sup> DM) followed by *T. paniculata* (33.86 g 100 g<sup>-1</sup> DM), *S. ferruginea* (31.60 g 100 g<sup>-1</sup> DM) and *G. cambogia* (30.96 g 100 g<sup>-1</sup> DM).

In addition to the influence of macronutrients on the

development of obesity, micronutrients, especially minerals, have received much attention because of their influence on body weight control. Table 3 shows the mineral contents FMPs examined in this study, which can be used as auxiliaries in the treatment of obesity. High levels of certain minerals were found in *A. vera*, such as potassium (1,009.44 mg 100 g<sup>-1</sup> DM) and manganese (47.07 mg 100 g<sup>-1</sup> DM). High levels of potassium (1,383.13 mg 100 g<sup>-1</sup> DM), calcium (2,660.70 mg 100 g<sup>-1</sup> DM) and iron (55.67 mg 100 g<sup>-1</sup> DM) were found in *S. ferruginea*, and potassium (2,336.69 mg 100 g<sup>-1</sup> DM) and iron (39.98 mg 100 g<sup>-1</sup> DM) were found in *B. trimera*. In addition, high levels of calcium (7,273.23 mg 100 g<sup>-1</sup> DM), sulfur (999.38 mg 100 g<sup>-1</sup> DM) and iron (73.69 mg 100 g<sup>-1</sup> DM) were found in *G. cambogia*.

Minerals play important roles in the human body, and one of them is regulation of metabolism. The absence of some minerals can cause metabolic problems, such as a slowing of the metabolism, which may lead to weight gain. In addition, some minerals can participate in the digestion of carbohydrates, fats and proteins and can act as aids in weight reduction.

The high levels of calcium shown in *G. cambogia* and *S. ferruginea* can be extremely effective for the treatment of obesity because calcium intake is involved in the regulation of body weight (St-Onge, 2005). Variations in the concentration of circulating calcium can affect food intake. High calcium content is known to decrease food intake because of the greater availability of calcium to ion channels. Several studies have shown that obese patients submitted to diets with high contents of calcium show a reduction in body fat (Heaney, 2003; Moore et al., 2004; Zemel et al., 2004).

Bioactive compounds present in plants can aid in health maintenance and in reducing the risk of disease; however, these compounds can cause damage to health if their concentration levels are too high. Thus, it is important to perform characterization studies on these

**Table 3.** Mineral composition of medicinal plants in g 100 g<sup>-1</sup> dry matter.

Mineral	<i>Aloe vera</i>	<i>Simaba ferruginea</i>	<i>Baccharis trimera</i>	<i>Garcinia cambogia</i>	<i>Tournefortia paniculata</i>
P	44.40±0.00 <sup>c</sup>	83.84±6.31 <sup>a</sup>	83.84±6.31 <sup>a</sup>	76.44±0.00 <sup>b</sup>	44.40±0.00 <sup>c</sup>
K	1,009.44±6.31 <sup>c</sup>	1,383.13±27.48 <sup>b</sup>	2,336.69±49.31 <sup>a</sup>	742.59±69.31 <sup>d</sup>	495.75±12.82 <sup>e</sup>
Ca	845.45±6.31 <sup>c</sup>	2,660.70±16.68 <sup>b</sup>	612.42±0.00 <sup>d</sup>	7,273.23±33.46 <sup>a</sup>	381.06±23.10 <sup>e</sup>
Mg	258.74±6.31 <sup>b</sup>	163.79±0.00 <sup>c</sup>	142.17±0.00 <sup>d</sup>	419.88±6.01 <sup>a</sup>	147.98±6.41 <sup>d</sup>
S	258.74±27.51 <sup>e</sup>	731.60±10.92 <sup>b</sup>	630.65±12.63 <sup>c</sup>	999.38±63.32 <sup>a</sup>	527.19±23.54 <sup>d</sup>
Cu	13.12±0.21 <sup>a</sup>	3.10±0.32 <sup>c</sup>	7.81±0.76 <sup>b</sup>	3.38±0.38 <sup>c</sup>	2.04±0.27 <sup>d</sup>
Mn	47.07±0.49 <sup>a</sup>	5.24±0.11 <sup>e</sup>	13.47±0.27 <sup>c</sup>	18.75±0.11 <sup>b</sup>	7.00±0.17 <sup>d</sup>
Zn	1.57±0.23 <sup>d</sup>	2.20±0.16 <sup>c</sup>	3.84±0.00 <sup>b</sup>	6.51±0.15 <sup>a</sup>	1.40±0.05 <sup>d</sup>
Fe	2.58±0.38 <sup>e</sup>	55.67±0.16 <sup>b</sup>	39.38±1.44 <sup>c</sup>	73.69±0.65 <sup>a</sup>	11.51±0.43 <sup>d</sup>

All data were collected in triplicate and represent the mean ± standard deviation. The same letters in a row indicate that the values do not differ by the Scott-Knott test ( $P < 0.05$ ). The moisture content in the powders of the medicinal plants, in g 100 g<sup>-1</sup>: *Aloe* = 8.53; *Simaba* = 8.42; *Baccharis* = 8.56; *G. cambogia* = 3.94; *Tournefortia*: 9.90.

**Table 4.** Contents of bioactive compounds of medicinal plants in dry matter.

Medicinal plant	Phenolic compounds (g 100 g <sup>-1</sup> )	Oxalic acid (g 100 g <sup>-1</sup> )	Nitrate (g kg <sup>-1</sup> )	Trypsin inhibitor (TIA mg <sup>-1</sup> ) <sup>1</sup>	Saponins (g 100 g <sup>-1</sup> )
<i>Aloe vera</i>	0.15±0.02 <sup>d</sup>	ND <sup>2</sup>	0.77±0.01 <sup>d</sup>	ND	0.07±0.00 <sup>d</sup>
<i>Simaba ferruginea</i>	1.62±0.03 <sup>c</sup>	0.97±0.09 <sup>a</sup>	0.81±0.02 <sup>c</sup>	0.17±0.02 <sup>c</sup>	0.13±0.00 <sup>c</sup>
<i>Baccharis trimera</i>	4.03±0.21 <sup>b</sup>	0.91±0.03 <sup>a</sup>	2.68±0.12 <sup>b</sup>	6.26±0.32 <sup>b</sup>	0.54±0.03 <sup>b</sup>
<i>Garcinia cambogia</i>	0.09±0.01 <sup>d</sup>	ND	0.16±0.01 <sup>e</sup>	ND	0.07±0.00 <sup>d</sup>
<i>Tournefortia paniculata</i>	36.19±0.91 <sup>a</sup>	0.93±0.02 <sup>a</sup>	6.96±0.13 <sup>a</sup>	22.01±2.40 <sup>a</sup>	1.00±0.09 <sup>a</sup>

Data are the mean of three replicates ± standard deviation. The same letter in the columns indicates that the values do not differ by the Scott-Knott test ( $P < 0.05$ ). <sup>1</sup>TIA: trypsin inhibitor activity, in nmol min<sup>-1</sup> mg<sup>-1</sup>. <sup>2</sup>ND: Not detected. Moisture contents in the flours from medicinal plants, in g 100 g<sup>-1</sup>: *Aloe* = 8.53; *Simaba* = 8.42; *Baccharis* = 8.56; *G. cambogia* = 3.94; *Tournefortia*: 9.90.

compounds in plant extracts. The bioactive compounds in samples FMPs are shown in Table 4.

Phenolic compounds were found in all plants; *T. paniculata* showed the highest content of phenolic compounds (36.19 g 100 g<sup>-1</sup> DM), followed by *G. cambogia* (0.09 g 100 g<sup>-1</sup> DM) and *A. vera* (0.15 g 100 g<sup>-1</sup> DM) showed the lowest content. The maximum dose of phenolic compounds suggested for humans is approximately 1 g day<sup>-1</sup> (Scalbert et al., 2005); thus, the daily limit is reached with only 3 g of *T. paniculata*. These plants are not typically used in food but have been used for the treatment of obesity. *T. paniculata* is notable among the plants examined in this study because of the health risks associated with its consumption, due to its high content of phenolic compounds concentrated in small quantities of the plant.

The concentrations of phenolic compounds found in the leaves of *B. trimera* (4.03 g 100 g<sup>-1</sup> DM) were higher than those observed in other studies with this plant, whose levels ranged from 0.045 to 2.67 g 100 g<sup>-1</sup> DM (Freitas et al., 2004; Souza et al., 2011; Oliveira et al., 2012). These

differences may result from the methods of preparation (maceration and infusion) and by the use of other extraction solvents, such as ethanol, ethyl acetate, and butanol, among others.

The amount of phenolic exceeded the amount recorded by Moniruzzaman et al. (2012) for *A. vera*, which was 0.0008 g 100 g<sup>-1</sup> DM. The authors also found that the leaves of *A. vera* have higher phenolic content than the gel, which indicates the leaves can be used as antioxidants. Conversely, the amount of phenolic for *G. cambogia* were lower than the amount reported by Subhashini et al. (2011), which was 7.5 g pyrocatechol 100 g<sup>-1</sup> DM and also those recorded by Jantan et al. (2011) in 22 methanol extracts of different parts (leaves, trunks, bark and fruits) of nine *Garcinia* species showed amounts ranging from 0.44 to 6.28 g gallic acid 100 g<sup>-1</sup> DM.

The different results could have been caused by the pattern used in the dosage, different extragents, different species; the parts of the plant used and the origin of the samples. No records were found in the literature on the

phenolic content of *S. ferruginea* and *T. paniculata*.

Some phenolic compounds, such as tannins, can inhibit certain digestive enzymes, such as amylase and trypsin, which can result in weight loss and help in the treatment of obesity (Monteiro et al., 2005). They also have multiple biological effects, such as antioxidants, anti-allergic, anti-inflammatory, anti-bacterial, anti-thrombotic, vasodilating and cardioprotective (Balasundram et al., 2006); these multiple biological effects have a broad field of application for the phenolics of these plants.

There was no significant difference between the plants *B. trimera*, *S. ferruginea* and *T. paniculata* regarding the content of oxalic acid, and oxalic acid was not detected in *A. vera* and *G. cambogia*. Oxalic acid content higher than 10 g are considered toxic to human health (Nappi et al., 2006); therefore, the amount of oxalic acid found in the plants examined in this study posed no health risk. The toxic effect of oxalic acid in the body has been associated with the reduction of bioavailability of some essential minerals, such as calcium, and the primary consequences are hypocalcemia and rickets, although absorption of iron, magnesium and zinc are also an issue (Siener et al., 2005).

Nitrate was also found in all plants with amounts ranging from 0.16 to 6.96 g kg<sup>-1</sup> DM. The acceptable daily intake of nitrate is 5 mg kg<sup>-1</sup> body weight (WHO, 2003). The excessive consumption of this compound can lead to cyanosis through the formation of metmyoglobin and neoplasms from the formation of N-nitroso compounds (Faquin and Andrade, 2004). Great quantities of the analyzed plants would have to be consumed by a 60kg person to reach 300 mg nitrate. Therefore, the contents of nitrate found in these medicinal plants should not be a health risk.

*T. paniculata* showed the highest potential for trypsin inhibition (22.01 trypsin inhibitor activity in nmol min<sup>-1</sup> (TIA) mg<sup>-1</sup> DM), followed by *B. trimera* (6.26 TIA mg<sup>-1</sup> DM), and *S. ferruginea* (TIA 0.17 mg<sup>-1</sup> DM). The presence of trypsin inhibitors was not detected in the plants *A. vera* and *G. cambogia*. Souza et al. (2011) observed the presence of trypsin inhibitors in aqueous and methanolic extracts of *B. trimera* leaves, which confirmed the results in this study that trypsin inhibition was found in *B. trimera*. However, these authors expressed their results in percentage of trypsin inhibition instead of in TIA mg<sup>-1</sup> DM, which made it impossible to make comparisons with the activity observed in this study.

The presence of trypsin inhibitor, particularly in *T. paniculata*, resulted in specific inhibition of proteolytic enzymes, which can lead to decreased protein digestion and a decrease in the weight of animals. Saponins were found in all the studied species, and *T. paniculata* showed the highest amount (1.00 g 100 g<sup>-1</sup> DM). The content of saponins recorded in this study for *B. trimera* (0.54 g 100 g<sup>-1</sup> DM - ethanol extract) were within the

range reported by Souza et al. (2011) for this plant, which ranged from 0.23 (aqueous extract) to 0.75 (methanol extract) g 100 g<sup>-1</sup> DM. In the same study, these authors also found no hemolytic effect in tests conducted with extracts of this plant. The results in that study indicated a low toxicity of the saponins present in these leaves.

Saponins can cause many side effects, such as changes in reproduction and growth and a reduction in nutrient absorption because of changes in the permeability of cell membranes (Francis et al., 2002); this information highlights the need for studies to verify the toxicological potential of this phytochemical present in plant extracts. However, saponins may aid in the treatment of obesity because they can inhibit digestive enzymes and act on lowering cholesterol in human plasma by forming micelles in the small intestine with bile acids, thus preventing their reabsorption (Pereira and Cardoso, 2012).

Few articles related to the bioactive compounds found in the plants analyzed in this study were found in the literature (Souza et al., 2011; Moniruzzaman et al., 2012; Oliveira et al., 2012). Thus, there is a great need for more studies on these plants, and in particular, to examine any harmful effects resulting from their consumption by humans.

## Conclusion

The plants examined showed high levels of substances, such as dietary fibers, some minerals, phenolic compounds, saponins and trypsin inhibitors, that have potential applications for weight loss. However, it is premature to recommend the use of these plants because of the possible risks to human health.

Additional studies on toxicity, efficacy and safety are necessary, particularly because of the saponins found in all plants examined and the high levels of phenolic compounds in *T. paniculata*.

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## Conflict of Interests

The author(s) have not declared any conflict of interests.

## REFERENCES

Association of Official Analytical Chemists (2005). Official methods of analysis of the association of the analytical chemists. 17. ed. Washington.

- Baccou JC, Lambert F, Sauvaire Y (1977). Spectrophotometric method for the determination of total steroidal sapogenin. *Analyst* 102(1215):458-465.
- Balasundram N, Sundram K, Sammar S (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* 68(1):191-203.
- Cataldo DA, Maroon M, Schrader LE, Youngs VL (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* 6(1):71-80.
- Erlanger BF, Kukowsky N, Cohen W (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* 95:271-278.
- Faquin V, Andrade AT (2004). Acúmulo de nitrato em hortaliças e saúde humana. Lavras: UFLA/FAEPE, 2004. pp 88.
- Francis G, Kerem Z, Makkar HP, Becker K (2002). The biological action of saponins in animal systems: a review. *Br. J. Nutr.* 88(6):587-605.
- Freitas MSM, Martins MA, Carvalho AJC, Carneiro RFV (2004). Growth and yield of total phenols in coot [*Baccharis trimera* (Less.) DC.] In response to inoculation of mycorrhizal fungi in the presence and absence of mineral fertilizer. *Rev. Bras. Plantas Med.* 6(3):30-34.
- Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH (2009). The incidence of co-morbidities related to obesity and overweight: A systematic review and meta-analysis. *BMC Public Health* 9(88):1-20.
- Heaney RP (2003). Normalizing calcium intake: projected population effects for body weight. *J. Nutr.* 133(1):268-270.
- Jantan I, Farra AJ, Fadlina CS, Khalid R (2011). Inhibitory effects of the extracts of *Garcinia* species on human low-density lipoprotein peroxidation and platelet aggregation in relation to their total phenolic contents. *J. Med. Plant. Res.* 5(13):2699-2709.
- Kakade ML, Simons N, Liener IE (1974). Determination of trypsin inhibitor activity of soy product: A collaborative analysis of an improved procedure. *Cereal Chem.* 51:376-382.
- Liu S, Willett WC, Manson JE, Hu FB, Rosner B, Colditz G (2003). Relation between changes in intakes of dietary fiber and grain products and changes in weight and development of obesity among middle-aged women. *Am. J. Clin. Nutr.* 78(5):920-927.
- Loures A, Jokl L (1990). Microtécnica para determinação de ácido oxálico em folhas e derivados. In: Encontro Nacional de Analistas de Alimentos, 6, 1990, Curitiba. Resumos... Curitiba: Instituto de Tecnologia do Paraná. pp 59.
- Mahan LK, Escott-Stump S (2008). Krause's food, nutrition, and diet therapy. 12th ed. Philadelphia: WB Saunders, pp 1359.
- Malavolta E, Vitti GC, Oliveira AS (1997). Avaliação do estado nutricional das plantas. Piracicaba: Potafos. pp 319.
- Matos FJA (1997). Introdução à fitoquímica experimental. 2.ed. Fortaleza: UFC pp 141.
- Mayer MA, Hocht C, Puyo A, Taiara CA (2009). Recent advances in obesity pharmacotherapy. *Curr. Clin. Pharmacol.* 4(1): 53-61.
- Mello VD, Laaksonen DE (2009). Dietary fibers: current trends and health benefits in the metabolic syndrome and type 2 diabetes. *Arq. Bras. Endocrinol. Metabol.* 53(5):509-518.
- Moniruzzaman M, Begum R, Sohel A, Amrita B, Ibrahim K, Siew H (2012). *In vitro* antioxidant effects of *Aloe barbadensis* Miller extracts and the potential role of these extracts as antidiabetic and antilipidemic agents on streptozotocin-induced type 2 diabetic model rats. *Molecules* 17(11):12851-12867.
- Monteiro JM, Albuquerque UP, Araújo EL (2005). Tannins: an approach to chemical ecology. *Quím. Nova.* 28(5):892-896.
- Moore LL, Singer MR, Bradlee ML, Ellison RC (2004). Dietary predictors of excess body fat acquisition during childhood. *Circulation* 109(7): 5.
- Moraes LD, Souza OV (2007). Reviews of Qualitative and Quantitative Variation of Secondary Metabolites in *Tournefortia paniculata* Cham (Boraginaceae). *Rev. Bras. Biociên.* 5(2): 1032-1034.
- Nappi GU, Ribeiro-Cunha MR, Coelho JV, Jokl L (2006). Validation methods to determine phytic and oxalic acids in "multimisturas". *Ciênc. Tecnol. Aliment.* 26(4):811-820.
- Oliveira CB, Comunello LN, Lunardelli A, Amaral RH, Pires MGS, Silva GL, Manfredini V, Vargas CR, Gnoatto SCB, Oliveira JR, Gosmann G (2012). Phenolic enriched extract of *Baccharis trimera* presents anti-inflammatory and antioxidant activities. *Molecules* 17(1): 1113-1123.
- Pereira RJ, Cardoso MG (2012). Vegetable secondary metabolites and antioxidants benefits. *J. Biotechnol. Biodivers.* 3(4): 146-152.
- R Core Team. R: A language and environment for statistical computing (2011). Viena: R Foundation for Statistical Computing; 2012. ISBN 3-900051-07-0. Available: <http://www.R-project.org/>
- Rique ABR, Soares EA, Meirelles CM (2002). Nutrição e exercício na prevenção e controle das doenças cardiovasculares. *Rev. Bras. Med. Esporte.* 8(6):1-11.
- Rodrigues CRF, Dias JH, De Melo RN, Ritcher RF, Picada JN, Ferraz A (2009). B. Genotoxic and antigenotoxic properties of *Baccharis trimera* in mice. *J. Ethnopharmacol.* 125(1): 97-101.
- Scalbert A, Johnson IT, Saltmarsh M (2005). Polyphenols: antioxidants and beyond. *Am. J. Clin. Nutr.* 81(1):215-217.
- Siener R, Hunow R, Seidler A, Voss S, Hesse A (2005). Oxalate contents of species of the Polygonaceae, Amaranthaceae and Chenopodiaceae families. *Food Chem.* 98(2):220-224.
- Simão AA, Corrêa AD, Chagas PMB (2012). Inhibition of digestive enzymes by medicinal plant aqueous extracts used to aid the treatment of obesity. *J. Med. Plant. Res.* 6(47): 5826-5830.
- Simão AA, Lage FF, Chagas PMB, Fraguas RM, Freire JM, Marques TR, Corrêa AD (2013). Antioxidants from Medicinal Plants Used in the Treatment of Obesity. *Eur. J. Med. Plant.* 3(3):429-443.
- Souza SP, Pereira LLS, Souza AA, Santos CD (2011). Inhibition of pancreatic lipase by extracts of *Baccharis trimera* (Less.) DC. Asteraceae: evaluation of antinutrients and effect on glycosidases. *Rev. Bras. Farmacogn.* 21(3):450-455.
- St-Onge MP (2005). Dietary fats, teas, dairy, and nuts: potential functional foods for weight control? *Am. J. Clin. Nutr.* 81(1): 7-15.
- Subhashini N, Nagarajan G, Kavimani S (2011). *In vitro* antioxidant and anticholinesterase activities of *Garcinia combogia*. *Int. J. Pharm. Pharm. Sci.* 13(3): 129-132.
- Vasquez B, Avila B, Segura D, Escalante B (1996). Antiinflammatory activity of extracts from *Aloe vera* gel. *J. Ethnopharmacol.* 55(1):69-75.
- World Health Organisation (2010). WHO Global database on Body Mass Index. WHO: Geneva.
- World Health Organization (2003). WHO Food additives series No 50. Safety evaluation of certain food additives. Fifty-ninth report of the joint FAO/WHO Committee on Food Additives. Geneva.
- Zemel MB, Thompson W, Milstead A, Morris K, Campbell P (2004). Calcium and dairy acceleration of weight and fat loss during energy in obese adults. *Obes. Res.* 12(4):582-590.



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