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Full Length Research Paper

Molecular marker analysis of F_1 progenies and their parents for carotenoids inheritance in African cassava (*Manihot esculenta* Crantz)

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Cassava genotypes were assessed at genomic DNA level to estimate the genetic diversity within and between them using 36 simple sequence repeat markers (SSR). One hundred and forty-seven (147) F_1 progenies derived from crosses amongst the parental genotypes were used to determine the association between three SSR markers and beta-carotene content in cassava. For the diversity study, a total of 131 alleles with an average of 3.7 alleles per locus were found. One yellow fleshed root genotype clustered with a white fleshed root genotype indicating similarity in their genetic background. Three SSR markers were used to screen the parental genotypes and their 147 progenies for a beta-carotene gene. The yellow fleshed root parents and 141 of the F_1 progenies had SSR alleles associated with the presence of beta-carotene gene. The SSR markers identified for beta carotene at CIAT appeared linked to the trait as found in the parents, but evaluation in the progenies indicated that each marker did not account for high phenotypic variance individually. Marker NS 717 (allele 206) accounted for 20% beta carotene content and SSRY 301 (allele 331) accounted for 17%. There are minor QTLs that could probably be involved in beta carotene expression. The markers evaluated therefore do not sufficiently account for beta-carotene expression in the F_1 progenies.

Key words: Cassava, simple sequence repeat (SSR), pro-vitamin A, diversity, validation.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important staple and cash crop in Nigeria; the global leader in the crop's production (Njoku et al., 2011). Cassava production in 2012 was ranked first with 45 million tonnes, followed by yam production at 27 million tonnes, sorghum at 7 million tonnes, millet at 6 million tones and rice at 5 million tonnes (FAOSTAT, 2013). It is also an important staple whose cultivation can provide the nationally required caloric intake for food security which is a minimum of 2400 calories per person per day

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(FAOSTAT, 2013).

Cassava is however, deficient or poor in some important essential food nutrients such as proteins, minerals and vitamins which have affected its utilization as a major food crop. Vitamin A deficiency (VAD) which causes diseases that range from night blindness to *Xerophthalmia* and *Keratomalacia* has been observed as a major health hazard in communities whose nutritional security heavily relies on cassava (Esuma et al., 2012). It is estimated that globally, over 250 million children are at risk of VAD, with 21 of these due to heavy reliance on cassava (WHO, 2009).

The utilization of yellow root cassava in the diets (Sanchez et al., 2006) could offer a possible solution to address this nutritional deficiency. Yellow root cassava is known to have enhanced β-carotene (pro-vitamin A) content (Chavez et al., 2007; Sanchez et al., 2006) and therefore provides sufficient opportunity to sustainably address vitamin A malnutrition through deployment of pro-vitamin A-rich cassava varieties. Global efforts towards breeding cassava for high β-carotene content started in 2002 but progress towards delivery of carotene rich varieties to farmers has been slow probably because of the food quality issues and the apparent negative association between β-carotene and dry matter contents usually, observed in most yellow root cultivars (Akinwale et al., 2010).

Biotechnology tools can enhance efficiency in the breeding process. The selection of target traits can be achieved by indirectly selecting molecular markers that are closely linked to genes for important traits, thus enabling precise identification of genotypes without the confounding effect of the environment (Meuwissen et al., 2001), since selection is based on molecular determination rather than morphological expressions.

QTLs have been identified for mineral concentrations in leaves for phosphorus (Bentsink et al., 2003), caesium (Payne et al., 2004) and potassium (Harada and Leigh, 2006) in *Arabidopsis thaliana* and for zinc in *Thlaspi caerulescens* (Deniau et al., 2006). These studies have demonstrated the presence of allelic variation affecting mineral accumulation, although the identity of the underlying genes remains unknown.

According to Fregene (2006) and Morillo (2009), screening a group of segregating F_1 families from yellow root cassava varieties at CIAT identified two QTLs associated with root colour in cassava in the region between markers SSRY 251 and NS 109 and, SSRY 313 and NS717. The markers were used in screening the parents and progenies for the beta-carotene gene. The objectives of this study were to: 1) examine the relationship among the elite yellow fleshed root and the white fleshed root varieties used in the study using 36 SSR markers commonly used to screen cassava accessions for polymorphism; and 2) to screen parents and F_1 progenies using SSR markers linked to beta-carotene gene expression.

MATERIALS AND METHODS

Plant materials

Six parental varieties and selected progenies were analyzed for beta carotene content. The parents were three varieties with high beta carotene content (pro-vitamin A) and three white storage root (low or no beta carotene) varieties which were crossed to generate segregating F_1 progenies. The white varieties originated from collaborative activities between IITA and NRCRI cassava breeding programs and were released to farmers in 2005 as improved varieties (TMS 98-0002, TMS 98-0505 and TMS 97-0505). The pro-vitamin A cassava varieties TMS 05-0473, TMS 05-1636 and TMS 01-1368 were derived from the IITA breeding collection as elite materials. Pedigree information of the pro-vitamin A parents was not available. There was therefore the need to conduct genetic studies on these varieties to ascertain their relationship. The white varieties were used as female parents while the pro-vitamin A varieties as male parents.

SSR genotyping

Genomic DNA was extracted from young tender leaf tissues from 147 F1 progenies and six parental varieties using the Dellaporta method (Dellaporta et al., 1983). The DNA was checked for quantity and quality using microvolumetric Nanodrop ND-8000 and diluted to 25 ng/100 µl. Three markers were selected as identified in a previous gene tagging study for B-carotene at the International Center for Tropical Agriculture (CIAT), Colombia (Morillo, 2009). Also, subsets of DNA samples of the six parental varieties were assayed with 36 polymorphic SSR markers for diversity study. Amplification was performed in 10 µl reactions containing 50 ng of DNA template, 1 *p*mole of each primer, 1X Taq polymerase buffer, 10 mM $MgCl_2$, 2.5 mM deoxynucleotide phosphates (dNTPs) and 0.075 U Taq polymerase. The PCR profile was run at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, annealing at 55-57°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 30 min.

Based on the fact that amplicons were of different sizes and the forward primers were fluorescently labeled (MWG-Biotech), co-loading of amplicons from the same individual, but at different loci, was therefore possible. Seven co-loading sets were optimized and used for the entire analysis. For each co-loading set, 1 to 2 µl of the different amplicons were mixed and vortexed. Aliquots of 1 µl of the mixture were added to 9 µl of a master mix containing HiDi formamide and GeneScan 500-LIZ size standard (1 ml of HiDi + 12 µl of 500-LIZ). The amplicons were denatured at 95°C for 3 min and subjected to capillary electrophoresis using ABI 3730 DNA sequencer (Applied Biosystems), and allele calls made using GENEMAPPER software version 3.7 (Applied Biosystems).

Screening with markers

One hundred and forty-seven (147) genotypes were derived from crosses involving the six parents and assayed for phenotypic variability and molecular marker genotyping using five amplified SSR markers, but two did not amplified (Table 1).

Quantitative and qualitative analyses

At harvest, three commercial size roots from each plant were selected, washed, peeled, chopped and mixed to obtain a single homogenous sample. The sample was then divided into two sub-samples, one was used for qualitative assessment (colour indicator chart/icheck) and another for quantification of pro-vitamin A

Table 1. The list of three primer sequences used in the SSR marker screening of six parents and 147 selected genotypes.

Name	Left primer	Right primer	Status
SSRY 240	TCGGCTTTTAACATCCTTCG	AGCTAGGAGCAACGCAGTTC	Amplified
SSRY 301	GAACGCTTCAACGGCATAAT	CCAATGCCAACAACACTTCTT	Amplified
NS 717	GCCAAATCGCCAAGGTAATA	GGTGAGTGATAAGGTTACGGC	Amplified
SSRY 215	GTTGATGAGCTGTGGCATTG	CCTAGACGAAGTGGGTCGAA	N/A
NS 313	TGCTGGGGAACTAGTGTGGT	GCAACTTCAAAGGCTGAAGG	N/A

N/A = not amplified.

carotenoids. Carotenoids quantification was by spectrophotometry. The protocol used followed the procedure described in the Harvest-Plus Handbook and in Rodriguez-Amaya and Kimura, (2004). Measurements were made for the six parents and their 147 clones.

Data analysis

PowerMarker (version 3.25) software package was used to estimate genetic diversity parameters using the diallelic data generated from the 36 SSR markers. The diversity parameters estimated included percentage of polymorphic loci, mean number of alleles per polymorphic locus, average observed heterozygosity (Ho) and average gene diversity (He). The principle coordinate analysis was performed using GeneAlex6 software. The simple matching Euclidian distance was used to compute the distance matrix and clustering was done using UPGMA algorithm. This analysis was repeatedly done using the software DARwin5. Phenotypic and genotypic data were analyzed using Microsoft Excel. Data capture was done using the genescan software (Applied biosystems) and the resulting fragments analyzed the alleles scored using the Genemapper software version 4.1 (Applied biosystems) (Ezuma et al., 2012).

RESULTS

Diversity study

Genetic diversity parameters were assessed with 36 SSR markers across six cassava genotypes (three white-storage root and three yellow-storage roots), and the results are presented in Table 2. Out of 36 SSRs, 26 SSR markers were polymorphic for the six genotypes. A total of 131 polymorphic alleles were observed. The number of alleles across loci and groups ranged between 1 and 7 with an average number of allele of 3.81. SSRY 4 had the highest number of alleles followed by SSRY 177 and SSRY 69. The average gene diversity or expected heterozygosity (He) averaged across all the groups and loci ranged from 0.15 in SSRY 34 to 0.81 in SSRY 4 with an average of 0.58, while heterozygosity (observed heterozygosity) ranged from 0.16 to 0.83 with an average of 0.65. Polymorphic information content (PIC) of loci across the two groups was highest in SSRY 4 (0.78) and lowest in SSRY 34 (0.14) with an average of 0.54 (Table 2). Also, both gene diversity and heterozygosity average across the six cultivars were high, 0.58 and 0.65, respectively.

Cluster analysis

In addition, a dendrogram was constructed using the Euclidean distance and unweighted pair group with arithmetic mean (UPGMA) grouping method separated the six cultivars into two major clusters (Figure 1). The marker did not fully discriminate the yellow varieties from the white varieties. Group one (I) contained TMS 05-1636 (yellow pulp) and TMS 97-2205 (white pulp). Group two (II) contained four accessions, TMS 01-1368 (yellow pulp), TMS 05-0473 (yellow pulp), TMS 98-0002 (white pulp) and TMS 98-0505 (white pulp), respectively. Clustering of varieties did not reflect colour. However, the dendogram showed strong affinity/relationship between TMS 05-1636 and TMS 97-2205, and between TMS 98-0002 and TMS 98-0505, probably, they have the same pedigree.

Analysis of molecular variance (AMOVA) showed that most of the variation was distributed within individual populations (95%) and the rest (5%) distributed among populations (Figure 2), supporting the genetic diversity results above.

Phenotypic assessment of Beta-carotene content in the parents and F_1 population

The carotene profile of the six cassava parent varieties selected for crosses is shown in Table 3. Total carotene levels varied among the genotypes, while some genotypes had higher total carotene content than the others.

There was variation in the qualitative and quantitative values of total carotene content among the 147 progenies evaluated. The intensity of the root colour among the segregating progenies ranged from white, light cream, cream, light yellow, yellow, deep yellow, orange to pink (Figure 3). The total carotene content among the progenies also varied from 0.71 to 15.41 ug/g.

Also, Figure 3 illustrates the regression of total carotene content and colour intensity in the roots. A moderate degree of association was found between the total carotene content and color of the root (R2 0.58). This result shows that 58% of the observed variability in the beta-carotene content can relatively be explained by

Table 2. Genetic diversity estimates of six cassava parental materials.

Marker	Allele frequency	Number of alleles	Gene diversity	Heterozygosity	PIC
SSR 4	0.3333	7.0	0.8056	0.6667	0.7818
SSR 240	0.5000	4.0	0.6528	0.8333	0.5994
SSR 181	0.7500	3.0	0.4028	0.5000	0.3633
SSR 179	0.2500	5.0	0.7778	0.8333	0.7409
SSR 177	0.3333	6.0	0.7778	0.6000	0.7456
SSR 171	0.4167	4.0	0.6389	0.8333	0.5689
SSR 169	0.6667	4.0	0.5139	0.5000	0.4760
SSR 161	0.5833	3.0	0.5694	0.8333	0.5045
SSR 155	0.5000	3.0	0.6111	0.6667	0.5355
SSR 151	0.4167	4.0	0.6806	0.8333	0.6218
SSR 148	0.5833	3.0	0.5417	0.5000	0.4598
SSR 147	0.8333	2.0	0.2778	0.3333	0.2392
SSR 135	0.3333	4.0	0.7222	0.6667	0.6713
SSR 110	0.8333	3.0	0.2917	0.3333	0.2723
SSR 108	0.6667	3.0	0.4861	0.5000	0.4235
SSR 100	0.3333	5.0	0.7639	0.8333	0.7260
SSR 64	0.5000	3.0	0.6250	0.5000	0.5547
SSR 63	0.3333	4.0	0.7361	0.8333	0.6874
SSR 59	0.4167	5.0	0.7222	0.5000	0.6800
SSR 52	0.4167	4.0	0.6806	0.6667	0.6218
SSR 51	0.4167	4.0	0.7083	0.6667	0.6589
SSR 34	0.9167	2.0	0.1528	0.1667	0.1411
SSR 21	0.8333	2.0	0.1778	0.3333	0.2392
SSR 20	0.5000	5.0	0.6667	0.5000	0.6221
SSR 12	0.4167	4.0	0.6806	0.6667	0.6218
SSR 9	0.6667	3.0	0.4861	0.3333	0.4235
Mean	0.5289	3.8	0.5827	0.6524	0.5377

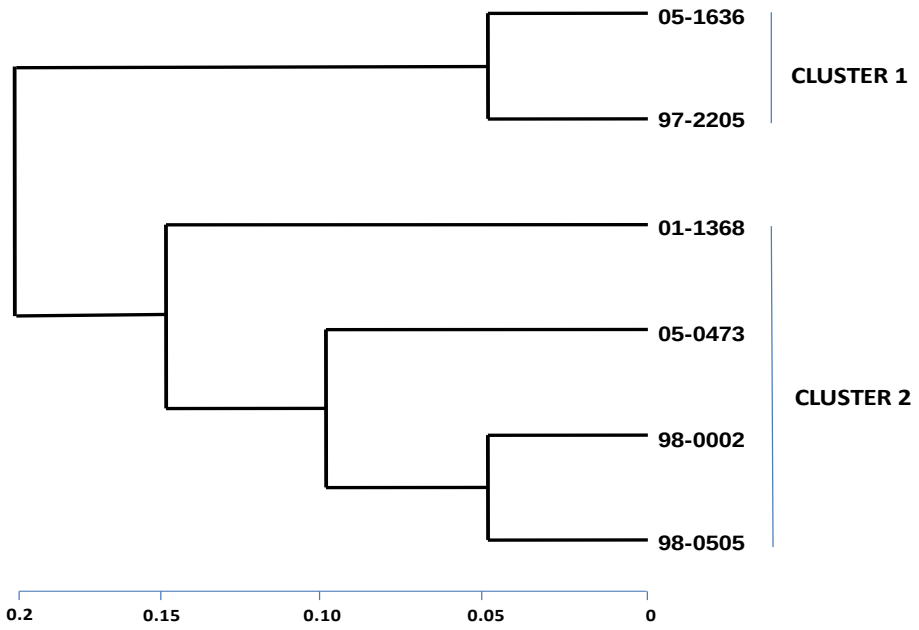

Figure 1. Dendrogram showing cluster analysis of six cassava genotypes based on allelic data at 36 SSR loci using UPGMA.

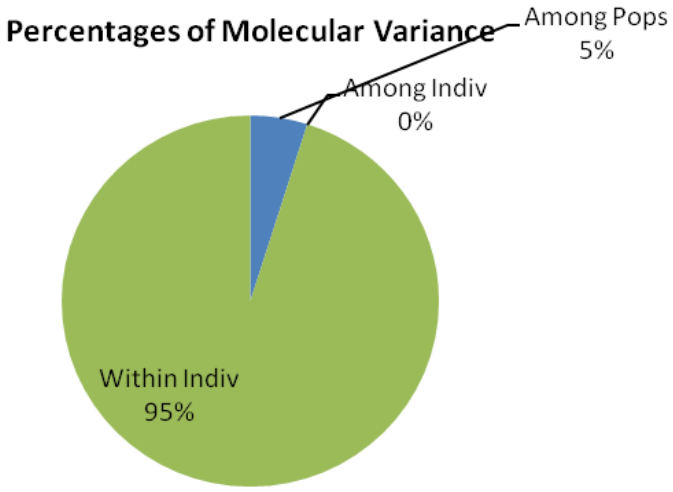

Figure 2. Analysis of molecular variance (AMOVA) of yellow and white fleshed cassava root.

Table 3. Total carotene of parental materials assessed using qualitative and quantitative measurements.

Parent	Pulp colour	Qualitative value	Quantitative value (ug/g)
TMS 01-1368	Deep yellow	6	6.56
TMS 05-1636	Yellow	5	5.51
TMS 05-0473	Light yellow	5	4.19
TMS 97-2205	White	1	1.08
TMS 98-0505	White	1	0.89
TMS 98-0002	White	1	1.12

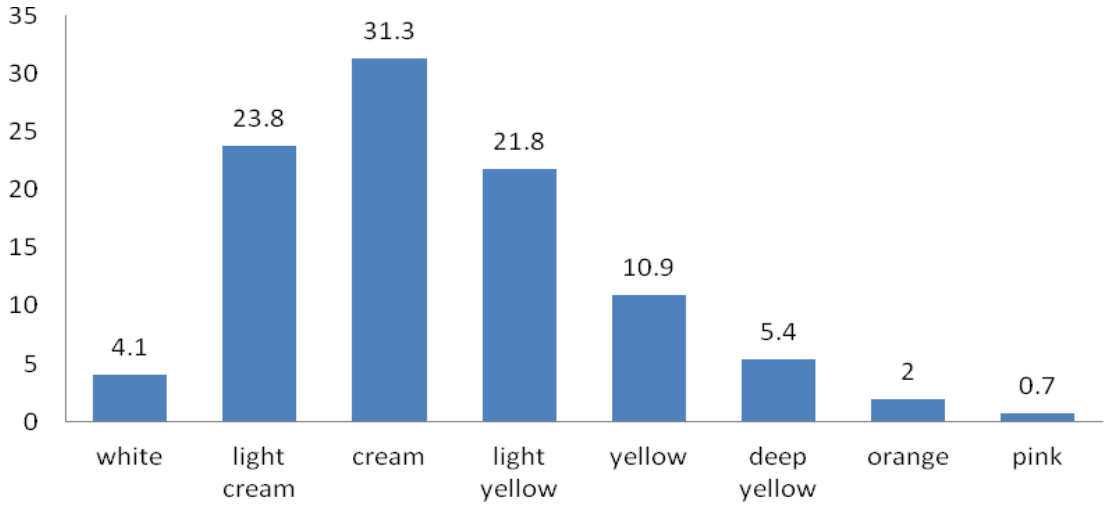

Figure 3. Percentage distribution of frequencies of different root colour classes of F_1 population.

variability in root pulp color, and that any increase in color intensity will result in a proportional increase in the concentration of carotenes (Figure 3).

However, in spite of the apparently good relationship between colour intensity and beta-carotene content as shown in Figure 3, there is still large variation for total

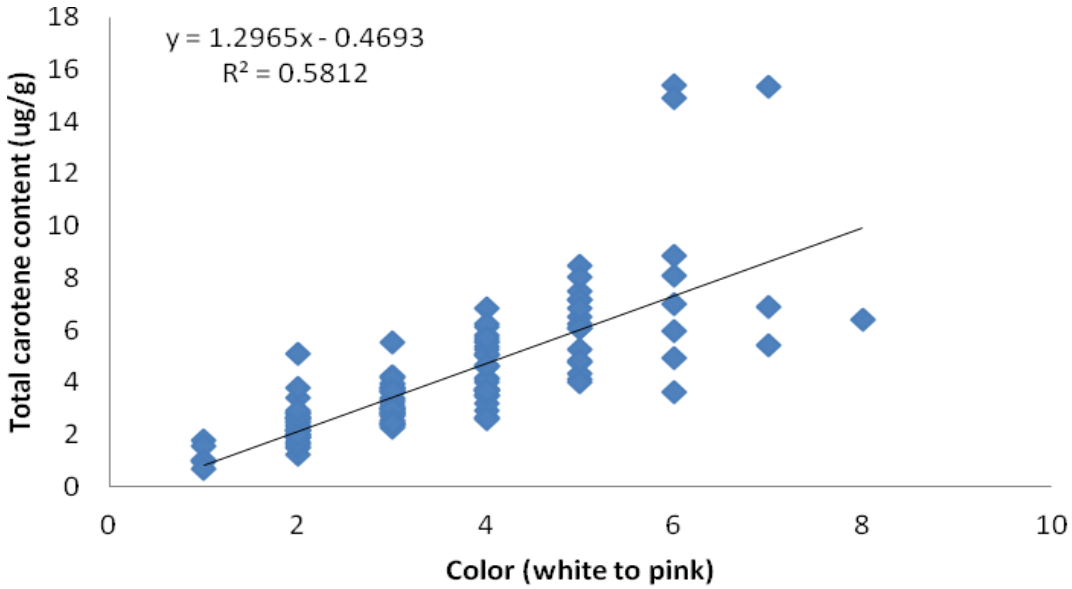

Figure 4. Relationship between colour intensity in the roots (based on visual scale of 1 to 8) and average total carotene content (fresh weight basis) of 147 genotypes.

carotene content as illustrated in Figure 4. For instance, there were several clones on the right side that showed high color intensity but low beta-carotene content. The same thing was observed on the left edge of the figure showing the absence of roots with relatively high content of carotenoids but low or no color intensity in the roots (Figure 3). However, this could be explained if the roots of these clones have other pigments in them different from carotenoids.

Analysis of SSR markers for beta-carotene in parent genotypes

Three markers previously identified (Morillo, 2009) for beta carotene content was analyzed in the parents: (a) Marker NS 717: Three marker alleles were identified (based on the base pairs) at this locus. The marker alleles identified were 196, 206 and 211. Allele 211 was only found in the white pulp parents. The other two marker alleles were found in the yellow pulp parents. In the white pulp parents, allele 211 was found in the homozygous state. For two of the yellow varieties (TMS 01-1368 and TMS 05/1636), allele 206 was also found in the homozygous state. In the third yellow parent (TMS 05/0473), marker NS 717 was heterozygous for alleles 196 and 206; (b) SSRY 301: Three marker alleles were identified as alleles 324, 326 and 331. Allele 324 was only found in the white pulp parents in the heterozygous state. Allele 326 was found in the white and yellow pulp parents while allele 331 was found only in the yellow parents. For two of the yellow varieties (TMS 05-1636 and TMS 01-1368), allele 326 and 331 were also found in the heterozygous state, while in the third yellow parent (TMS 05-0473), allele 326 was found in a homozygous state; (c) SSRY 240: In SSRY 240, two marker alleles were identified. The marker alleles identified were 171 and 181. Allele 181 was only found in the yellow parents in the homozygous state. Allele 171 was found only in the white parent TMS 98-0002 (Table 4).

Molecular screening of F_1 populations for beta-carotene gene

One hundred and forty-seven (147) genotypes were screened with three SSR markers. Results show that 96% of the genotypes had at least one marker allele for a beta-carotene gene. Further analysis of the results indicated 17% of the genotypes had one marker allele associated with beta-carotene gene, 27.9% had two informative marker alleles, 27.9% had three informative marker alleles, and 22.5% had four informative marker alleles linked to beta-carotene. Six genotypes had no marker alleles associated with beta-carotene genes (Figure 3).

Marker - trait correlation

Association with colour of root pulp was determined by simple linear correlation of phenotypic data on marker allele means. Among the markers, alleles 206, 181, 326 and 331 (quantitative) were positively correlated with beta-carotene phenotypic values, while allele 196 was negatively correlated with beta-carotene contents. Marker

Table 4. Summary of SSR alleles identified in six cassava clones varying in beta-carotene.

Clone	Pulp color	SSRY 240		SSRY 301			NS 717		
		Allele		Allele			Allele		
		171	181	324	326	331	196	206	211
TMS 98-0002	White	+	-	+	+	-	-	-	+
TMS 97-2205	White	-	-	+	+	-	-	-	+
TMS 98-0505	White	-	-	+	+	-	-	-	+
TMS 05-0473	Yellow	-	+	-	+	-	+	+	-
TMS 05-1636	Yellow	-	+	-	+	+	-	+	-
TMS 01-1368	Yellow	-	+	-	+	+	-	+	-
Total		1	3	3	6	2	1	3	3
Freq		16.7	50.0	50.0	83.3	33.3	16.7	50.0	50.0

Presence (+) or absence (-) of favourable marker allele for beta-carotene gene.

Table 5. Correlation of quantitative and qualitative values with 3 putative markers in 147 F1 genotypes.

Marker	Allele	R-value	
		Quantitative	Qualitative
NS 717	206	0.19969	0.19464; P<0.001
SSRY 240	181	0.08889	0.03804; P< 0.001
SSRY 301	331	0.13587	0.17219; P<0.001

NS 717 with allele 206 has the highest correlation both quantitatively and qualitatively (19%, P<0.001 and 20%, P< 0.001). This was followed by marker 301 with allele 331 giving a correlation values of 13% (quantitative) and 17% (qualitative) beta-carotene values using qualitative standard colour chart assessment (Table 5).

The proportion of phenotypic variation explained by these individual markers ranged from 0.09 to 0.20 (quantitative) and 0.04 to 0.19% (qualitative). For each trait, markers were identified in which the allelic contribution from each of the parents either had an increased effect or lowered the phenotypic value of the trait.

DISCUSSION

Genetic relationship among three yellow root and three white root cassava accessions was evaluated with 36 SSR markers. The set of 36 unlinked SSR loci used in this study have been identified to be adequate for molecular diversity characterization and good estimation of genetic diversity parameters for cassava accessions. The set of markers used in this study were found to be very highly informative and have good distribution coverage of the cassava genome and thus were highly suitable for genetic diversity evaluation (Fregene et al., 2003). The SSR markers showed high mean PIC (52.3%) which demonstrates their ability to discriminate between individual cultivars. The higher the PIC of a marker, the more informative is the marker (Fregene et al., 2003). The most informative marker from the study was SSRY 4 with mean PIC of 0.78, while SSRY 34 was the least informative marker. These results are in agreement with earlier studies in cassava with SSR markers. Kawuki et al. (2009) reported mean PIC of 55, 57 and 61%, respectively, with accessions collected from Africa landraces, Asia and Latin America.

The mean number of alleles (3.743) detected per locus was within that obtained in previous cassava diversity studies (Esuma et al., 2012). The high number of alleles per locus conforms to the nature of cassava as an outcrossing crop. Previous genetic diversity studies in cassava using different types of DNA molecular markers have shown low to medium genetic diversity.

Both expected heterozygosity and observed heterozygosity averaged across the six cultivars and marker loci were high, 57 and 65% respectively. Gene diversity or expected heterozygosity (He = 57%) detected in this study was higher than the average reported for outcrossing species (He = 20.5%) using isozyme markers. It has been suggested that high levels of polymorphism in microsatellite markers are related to the mechanism of mutations and the high rate at which they occur. Heterozygosity is an indication of the probability that two randomly selected alleles from an accession/cultivar of a germplasm are different is 65%.

Expected heterozygosity which explains the probability that two alleles arbitrarily selected are different was found to be high in this study. The results show that the parent materials have high degree of heterozygosity as expected for cassava. Similar observed and expected heterozygosity in cassava has been reported. Raji et al. (2009), using cassava germplasm from various countries in Africa, reported average values of gene diversity and heterozygosity of 0.630 and 0.730, respectively.

The genetic differences found among the six cultivars resulted in their clustering into distinct groups suggesting that those in the same cluster share similar breeding history or pedigree and hence, higher genetic relatedness. Based on the UPGMA analysis of the parents, two groupings were identified. The cultivar TMS 05/1636 and TMS 97/2205 clustered in the same group 1, and the other four cultivars clustered together in group 2, which reflects their similarity in genetic base.

The AMOVA results found no genetic difference between the yellow and white accessions. As expected, between individual variations were most significant and accounted for the majority of the molecular variance. Similar findings have been reported by several previous studies on genetic diversity of sweet potato germplasm (Gruneberg, et al., 2005). Moreover, it is a clear indication that breeders can form in breeding programs different populations with significant levels of genetic difference, which is a prerequisite to exploit heterosis and improvement of populations.

Two deductions from this study are of direct application to cassava breeding. First, the close relationship of TMS 98/0505 and TMS 98/0002, and TMS 05/1636 and TMS 97/2205 cultivars implies that each pair may have been derived from a common source. Second, there is a high genetic diversity among cassava accessions at Umudike which can be exploited for crop improvement.

In addition, the six parents and 147 progenies were phenotyped and genotyped with three SSR markers linked to beta-carotene. The result differentiated eight different distinct phenotypic classes in some of the populations based on eight colour categories. Several phenotypic classes indicate a likely possibility of combination of additive, recessive and/or epistatic effects in the control of beta carotene for the genotypes assessed or more likely variation in root color due to variations in starch content of lines with the same carotene content. Going by these different phenotypic classes, it is obvious that the trait is controlled at least by a few loci, may be about ~2 major genes as was reported in the literature (Grüneberg et al., 2005). None of the loci screened suggest a major dominant gene effects. Marker analyses of the parents indicate that marker allele 181 from SSRY 240 and allele 206 (NS 717) and allele 331 were associated with beta-carotene content. Allele 326 was found in both white and yellow parents. It was found in heterozygous condition in yellow varieties in combination with marker allele 311, indicating that marker allele 311 was dominant to allele 326 and is linked to a beta-carotene gene. The other marker alleles associated with yellow roots were found in heterozygous states also suggesting that they were dominant for beta carotene expression.

The study indicates that 96% of the genotypes showed at least one of the marker alleles associated with a putative beta-carotene gene. In fact, 17% of the genotypes showed one marker allele associated with beta-carotene; 27.9% had two, 27.9% had three informative marker alleles, and 22.5% had four informative marker alleles linked to beta-carotene. Six genotypes had no marker alleles associated with beta-carotene genes, and they were white root genotypes.

There was a good correlation between total carotene content and color intensity in the roots. When qualitative values (color) were correlated with quantitative values (spectrophotometer reading), a relatively high degree of association between beta-carotene content and color values of $R^2 = 0.58$ was obtained. This means that 58% of the observed variability in the beta-carotene content can be explained by variability in the storage root colour. These results agree with reports by other researchers, using different cassava populations (Chavez et al., 2007; Morillo, 2009).

However, 58% correlation shows that the margin of error in using qualitative measurements to assess beta carotene level is still high. Evaluation and analysis based on color assessments are likely to be misleading. Beyond rapid screening at seedling nursery level when population sizes are big, this may not be very good procedure for accurate and precise determination of beta carotene content in germplasm.

The SSR markers identified for beta carotene at CIAT appeared linked to the trait as found in the parents used here, but evaluation of the progenies indicated that each marker did not account for high phenotypic variance individually. The markers evaluated therefore do not sufficiently account for beta-carotene expression in the F_1 progenies. Allele 206 (Marker NS 717) accounted for 20% beta carotene content and allele 331 (Marker SSRY 301) accounted for 17%. It was not possible to determine whether the alleles found in African germplasm were the same ones discovered in Latin American germplasm. However, major genes have been reported for beta carotene expression from classical genetic studies (Iglesias et al., 1997). It could possibly be that the markers evaluated did not capture the significant region of the genome affecting beta carotene expression in the African germplasm as only few markers were used because the objective was to validate the selected markers for MAS in beta carotene expression.

Conclusion

The study shows that SSR marker analysis is a useful tool in studying genetic diversity for cassava improve-

ment. Analysis of the parental cassava genotypes produced two major divergence groups. However, no single group was observed to be unique with yellow colour. Also, screening of the parental genotypes and 147 progenies with three polymorphic markers (SSRY 301, SSRY 240 and NS 717) revealed four marker alleles linked to beta-carotene gene. Yellow root parental genotypes and 141 progenies showed the presence of these marker alleles while the white root parents and six progenies showed absence of these marker alleles.

Though the correlation between the qualitative (colour indicator value) and quantitative values (spectro-photometer value) was high, the correlation values between genotypic and phenotypic data classes for candidate markers were generally low. The results show that these markers do not sufficiently explain all the phenotypic variance observed; at least, they do not explain a high part of it. Therefore some additional mapping (BSA or QTL approach) to find more markers or genomic regions influencing beta carotene inheritance in Africa cassava germplasm is warranted.

Conflict of Interests

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

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Full Length Research Paper

Effects of fermentation and extrusion on the proximate composition and organoleptic properties of sorghum-soya blend

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The effect of extrusion and fermentation on the proximate composition and organoleptic properties of six combinations (100:0, 90:10, 80:20, 70:30, 60:40 and 50:50) of sorghum - soya blend were investigated. A total number of 19 microorganisms were isolated during the fermentation of sorghum-soya extrudates; these comprise of twelve (12) bacteria, four (4) yeast and three (3) moulds. They include *Flavobacterium rigense, Micrococcus icristinae, Enterobacter cloacae, Enterobacter* spp., *Corynebacterium cystitidis, C. pilosun, Staphylococcus albus, Brevibacterium* spp., *Bacillus subtilis, B. cereus, B. brevis, B. megaterium, Candida famata, Saccharomyces cerevisiae, Geotrichum candidium, C. utilis, Aspergillus niger, A. fumigatus and Rhizopus stolonifer.* The pH and the total titratable acidity (TTA) significantly varied during fermentation. The proximate composition of the raw flour blend, unfermented and fermented extrudates was assessed. The crude protein and moisture contents increased in the extruded and fermented samples whereas; extrusion and fermentation reduced the ash and carbohydrate contents. Crude fibre and fat contents varied among all the samples. The sensory evaluation of the unfermented extrudates indicated a good preference for the product in term of colour, texture, aroma and overall acceptability.

Key words: Fermentation, extrusion, sorghum-soya blend, proximate, orgnoleptic.

INTRODUCTION

In Nigeria and many other developing countries of the tropics, protein deficiency in diets is common and is usually found in association with deficiency in calories. Since the diet of an average Nigerian consists of foods that are mostly carbohydrate based, there is a need for strategic use of inexpensive high protein resources that complement the amino acid profile of staple diet in order to enhance their nutritive value. According to Edema et al. (2005) newer protein sources are being explored as protein complements of which oil seeds occupy a prominent place. New food processing technologies can provide a means of improving the nutritional quality of

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Table 1. Level combinations of sorghum and soya bean blends.

Sorghum (g)	Soya flour(g)
100	0
90	10
80	20
70	30
60	40
50	50

food plants, which are the most important dietary sources for meeting the nutritional needs of majority of the population in Nigeria because they are readily available, low in cost and acceptable. Traditionally, cereals and legumes have been processed in homes as part of preparing food for families and one of the oldest ways of processing food at home is by fermentation. The practice of blending locally grown crops in Nigeria partly for aesthetic purposes and nutrient supplementation has been an age long tradition. Fermentation as described by Ojokoh et al. (2003) is a complex chemical transformation of organic substances brought about by the catalytic action of enzymes (either native or elaborated by the microorganisms) fermenting the raw materials.

Extrusion cooking is a relatively recent form of food processing forcing food material through dies with holes of various shapes. Material is continuously metered into an inlet hopper and then transported forward by a rotating screw. As the material approaches the die, there is an increase in pressure and temperature. It involves heat, pressure and shear (Mills et al., 1993). Blended foods prepared by mixing cereal flour (usually maize flour) with soy flour to get a high protein food with good balance of amino acids are widely used in food aid programmes. Blended foods are usually pre-cooked by extrusion so that less cooking time is required and to improve shelf–life. Extrusion cooking technique is used in processing a wide range of products for both human and animal consumption (Guy, 2001). Extrusion cooking was adopted for use in nutrition intervention projects mostly for malnourished individuals in many less developed continents like Asia, Latin America and Africa (Osundahunsi, 2006). Functional ingredients such as soy and botanicals that are relatively unpalatable alone can be incorporated into new food items by extrusion. Traditional foods can be further enhanced by addition of dietary fibre or other ingredients during extrusion. According to Iwe (1998), developing countries have been in search of technologies to produce low cost nutrition food and alternative procedures to process soy based food in less developed countries (LDCs). Extrusion has therefore been found to be versatile, cost effective, and acceptable in the LDCs. Extrusion cooking of corn and soya bean and their various blends has been reported in most available literature; information is lacking on the extrusion of sorghum blended with soya bean flour as combined with fermentation. The objective of the present research was to investigate the combined influence of extrusion and fermentation on the proximate composition and organoleptic properties of sorghum – soya blend

MATERIALS AND METHODS

The sorghum (*Sorghum bicolour*) grains used for the research study was purchased from Oba's market Akure, while soya beans seeds (*Glycogen max*) Variety TGX 1448-2E was obtained from International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria.

Production and fermentation of sorghum-soya blends and extrudate

The dried sorghum grains were sorted by hand to remove leaves, stones and other foreign materials. The cleaned grains were then fed into an attrition mill (ASIRO All Double Grinding Machine) and ground coarsely. The coarsely grinded sorghum was winnowed to remove the bran, milled to fine flour in the attrition mill and then sieved to fineness.

The cleaned soya bean seeds were first coarsely milled, winnowed to separate the coat and finished up in attrition mill. The fine flour was then sieved through a mesh. The fine flour was defatted with n-hexane in cold extraction from 20.57% fat content to 15.17%. The recovered flour was air dried and the clumps broken to fines. The flour samples from sorghum and soya bean were mixed at six level combinations as shown in Table 1. The flour samples were stored in clean sterile transparent polythene bags, tightened at the tips and kept in another bag until used.

The flour blends were hydrated and preconditioned by adding appropriate amount of water and manually mixed in a wide bowl to ensure even moisture distribution and processed in a laboratory single screw extruder (Hobart Corporation Germany) at screw speed of 80 rpm and through a die nozzle diameter of 5 mm at 110°C. The extrudates obtained were allowed to cool and dry before fermentation.

A 100 g portion of each extrudate was weighed and 50 ml of water was added. The samples were allowed to ferment at 30 ± 2°C for 96 h. The fermented samples were oven dried at 500°C for 24 h.

Microbiological analyses of the extrudates

Microbial population of the total bacterial and fungi were determined using nutrient agar (NA) and Potato dextrose agar (PDA) respectively; organisms were enumerated by using appropriate serial dilution and pour plate techniques. The bacterial cultures were incubated at in inverted position at 37±2°C for 24 h while the fungi plates were incubated in an inverted position at 25±2°C for 72 h (3days). The organisms were characterized based on biochemical and morphological observations and tests (Cowan and Steel, 1990).

Physiochemical changes

Determination of pH

The pH of all the fermenting samples (extrudates) was determined

Table 2. Seven-point hedonic scale.

Point score	Point linguistic value
7	Like very much
6	Moderately
5	Like Slightly
4	Neither like nor dislike
3	Dislike slightly
2	Dislike moderately
1	Dislike very much

at twenty-four (24) hour intervals using pocket size digital pH meter (PHC.P (R) Hanna instrument).

Determination of total titratable acidity (TTA)

The total titratable acidity of the fermenting extrudates was determined every twenty–four hour as described by AOAC (1990). A 2 g of macerated sample was weighed, 50 ml of distilled water was added and then filtered. A 10ml of the filtrate was measured and few drops of Phenolphthalein indicator were added. This was titrated with 0.1m sodium hydroxide (NaOH) solution and the titre values in millilitre were read from the burette. The acidity was calculated as follows:

Total titratable acidity was expressed as percent (%) lactic acid, where 1 ml of 0.1 m NaOH is equivalent to 0.09008 lactic acid (Kirk and Sawyer, 1991).

TTA = Titre Value × Volume of Sample × 9 mg/100 g (1)

Proximate analysis

Moisture content was determined by direct oven drying method; the loss in weight after oven-drying was expressed as % moisture content (AOAC, 1990). Crude protein was estimated from the total nitrogen (TN) determined by the micro-Kjeldahl method by multiplying the TN by a factor of 6.25. Crude fat was determined by using the soxhlet extraction method using petroleum ether as the solvent (AOAC, 1990). Ash was measured gravimetrically after ashing at 550°C to constant weight. Carbohydrate was determined by difference.

Sensory evaluation

The design and analysis for evaluation of sensory acceptability as detailed by Ihekoronye and Ngoddy (1985) was employed for the consumer acceptability test. A panel of the judges (untrained but familiar with extruded product such as pasta, noodles, breakfast cereal quality characteristic) was set up. The panellists were asked to express their feeling about the samples based on colour, aroma, texture and general acceptability by scoring them on a seven- point hedonic scale (Table 2).

Statistical analysis

The data obtained were subjected to one way analysis of variance (ANOVA) followed by Turkey's test multiple comparisons using SPSS 17.0 version computer software package. The values were considered significant at $p \leq 0.05$.

RESULTS

Microbial growth during fermentation of sorghum-soya extrudates

Microorganisms isolated during the fermentation of sorghum-soya extrudates

A total number of 19 microorganisms were isolated during the fermentation of sorghum-soya extrudates (Figure 1). These comprise of twelve (12) bacteria, four (4) yeast and three (3) moulds. They include *Flavobacterium rigense, Micrococcus icristinae, Enterobacter cloacae, Enterobacter* spp., *Corynebacterium cystitidis, C. pilosun, Staphylococcus albus, Brevibacterium* spp., *Bacillus subtilis, B. cereus, B. brevis, B. megaterium, Candida famata, Saccharomyces cerevisiae, Geotrichum candidium, C. utilis, Aspergillus niger, A. fumigatus* and *Rhizopus stolonifer.*

Changes in pH during the fermentation of sorghum-soya extrudates

The pH variations during the fermentation of sorghum-soya extrudates are shown in Figure 2. Extrudate A gradually decreased from 6.30±0.1 to 3.34±0.11, extrudate B decreased from 6.43±0.15 to 3.33±0.06. In extrudate C, the initial pH was 6.3±0.00. This decreased to 5.83±0.01 at 72 h and later increased to 6.00±0.00. Extrudate D decreased from 6.33±0.06 to 4.10±0.10 at 72 h and increased to 5.17±0.12 at 96 h. Extrudate E decreased from 6.47±0.12 to 4.23±0.06 at 72 h and increased to 6.17±0.06 at 96 h. Extrudate F decreased from 6.40±0.10 to 5.03±0.06 at 24 h. It increased to 5.93±0.56 at 48 h through 6.83±0.23 at 96 h of fermentation.

Changes in total titratable acidity (TTA) during the fermentation of sorghum-soya extrudates

Variations in titratable acidity (TTA) during fermentation of sorghum-soya extrudates are represented in Figure 3. Extrudate A had TTA of 0.0843 ± 0.0006; this increased to 0.3213 ± 0.002 at 72 h and decreased slightly to 0.3213±0.002. Extrudate B increased from 0.2077 ± 0.002 to 0.2230 ± 0.001 at 24 h, it decreased slightly and increased to 0.3320 ± 0.002 and finally reduced to 0.3120 ± 0.002 at 96 h. Extrudate C increased from 0.0523±0.002 to 0.1560±0.003 at 72 h and decreased to 0.1483 ± 0.001 at 96 h. Extrudate D increased from 0.1123 ± 0.002 to 0.3733±0.12 at 72 h and decreased to0.3607 ± 0.001

Figure 1. Sorghum-soya extrudate.

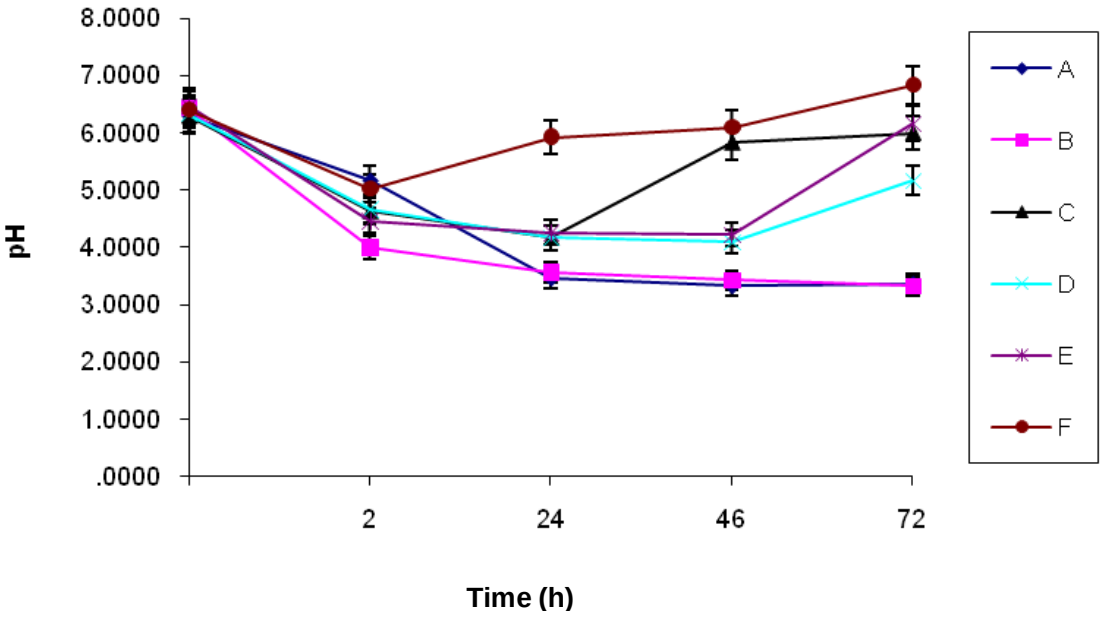

Figure 2. pH variations during fermentation of sorghum-soya extrudates. A = 100 g sorghum flour sample; B = 90 and 10 g sorghum-soya blend sample; C = 80 and 20 g sorghum-soya blend sample; D = 70 and 30 g sorghum-soya blend sample; E = 60 and 40 g sorghum-soya blend sample; F = 50 and 50 g sorghum-soya blend sample.

at 96 h. Extrudate E increased from 0.0637 ± 0.001 to 0.2107 ± 0.11 at 72 h and decreased to 0.1757 ± 0.001 at 96 h. Extrudate F increased from 0.0767 ± 0.002 to 0.3477 ± 0.002 at 72 h and decreased to 0.3403 ± 0.001

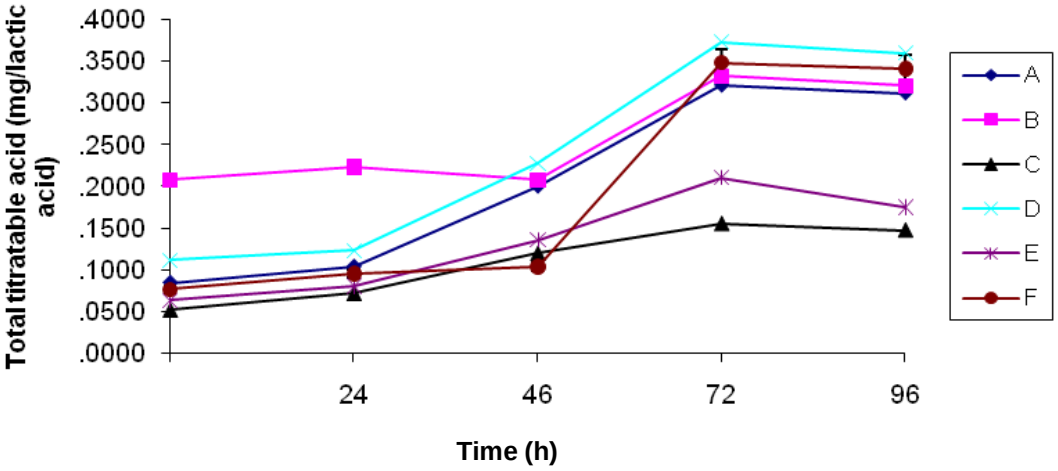

Figure 3. Total titratable acidity (tta) variation during fermentation of sorghum-soya extrudates. A = 100 g sorghum flour sample; B = 90 and 10 g sorghum-soya blend sample; C = 80 and 20 g sorghum-soya blend sample; D = 70 and 30 g sorghum-soya blend sample; E = 60 and 40 g sorghum-soya blend sample; F = 50 and 50 g sorghum-soya blend sample.

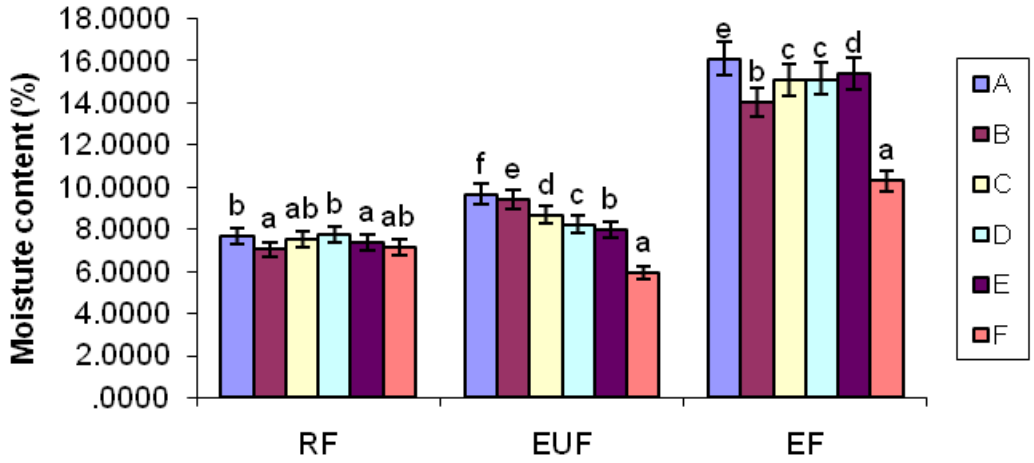

Figure 4. Moisture content of sorghum-soya blends and extrudates. RF = raw flour; EUF = extruded unfermented; EF = extruded fermented; A = 100 g sorghum flour sample; B = 90 and 10 g sorghum-soya blend sample; C = 80 and 20 g sorghum-soya blend sample; D = 70 and 30 g sorghum-soya blend sample; E = 60 and 40 g sorghum-soya blend sample; F = 50 and 50 g sorghum-soya blend sample.

at 96 h of fermentation.

Changes in proximate composition of sorghum-soya blends and extrudates

The moisture composition of sorghum-soya blends and extrudates

The moisture content of sorghum-soya blends and extrudates are represented in Figure 4. Raw flour blends had the lowest moisture content with raw flour (RF) A-F ranging from 7.69 to 7.14 ± 0.27. There were no significant difference (P≤0.05) in the raw flour A and D, between B and E and between C and F. The moisture content of extruded unfermented (EUF) samples ranged between 5.89 ± 0.01 and 9.65 ± 0.01. There was significant difference (P≤0.05) among all the extruded unfermented samples (EUF). Extruded fermented (EF) samples exhibited moisture content of 10.29 ± 0.01 for

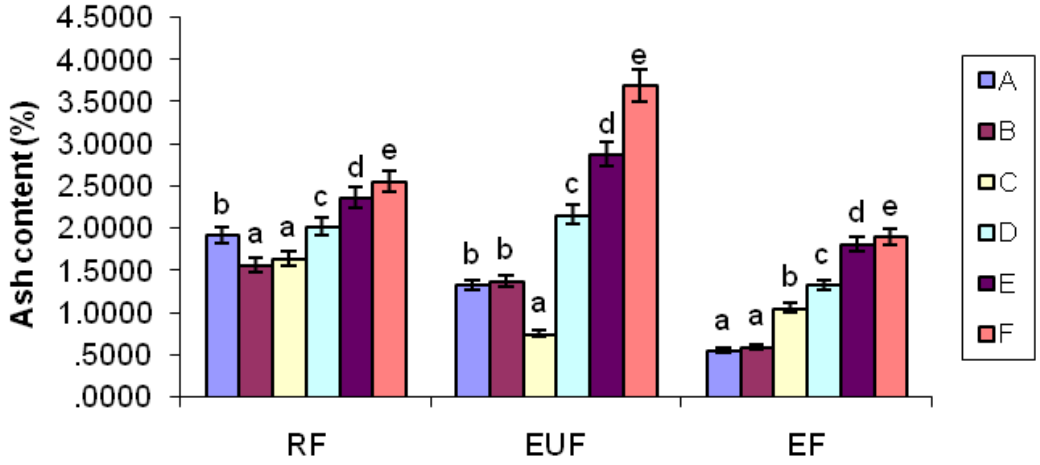

Figure 5. ASH content of sorghum-soya blends and extrudates. RF = Raw flour; EUF = extruded unfermented; EF = extruded fermented; A= A = 100 g sorghum flour sample; B = 90 and 10 g sorghum-soya blend sample; C = 80 and 20 g sorghum-soya blend sample; D = 70 and 30 g sorghum-soya blend sample; E = 60 and 40 g sorghum-soya blend sample; F = 50 and 50 g sorghum-soya blend sample.

extrudate F and 16.11 ± 0.02 for extrudate A. There was no significant difference (P≤0.05) between extrudates C and D, while the moisture content was significantly (P≥0.05) recorded among extrudates A, B, E and F.

The ash composition of sorghum-soya blends and extrudates

The changes in the ash content of the sorghum-soya blends and extrudates are represented in Figure 5. Extruded fermented (EF) samples recorded the lowest ash content between 0.5467 ± 0.04 for extrudate A and 1.90 ± 0.03 for extrudate F. There was no significant difference (P≥0.05) between extrudates A and B, but all other extruded fermented samples were significantly different (P≤0.05) among samples C, D, E and F. There was no significant difference (P≥0.05) between A and B for unfermented extruded (EUF) samples, while C, D, E and F recorded significant (P≤0.05) difference. For the raw flour blends (RF), F had the highest ash composition of 2.02 ± 0.37, B and C had no significant (P≥0.05) difference. Samples A, D, E and F were significantly different(P≤0.05).

The crude fat composition of sorghum-soya blends and extrudates

The crude fat content of the sorghum-soya blends and extrudates are shown in Figure 6. There were significant (p≤0.05) changes in the fat content of the raw flour blends A to F with raw flour blend A having 2.89 ± 0.04 and F, 17.35 ± 0.01. The extruded unfermented (EUF) had no significant (P≥0.05) values among extrudates A, C and E, with 11.80 ± 0.01 for extrudate A and 15.33 ± 0.85 for extrudate E. Extruded fermented (EF) samples were significant (p≤0.05) for extrudates B, C, E and F with lowest significance 8.30 ± 0.01) for extrudate B and highest (12.78 ± 0.02) for extrudate F, while there was no significant difference (p≥0.05) between A and D.

The crude fibre composition of sorghum-soya blends and extrudates

The crude fibre content of the sorghum-soya blends and extrudates are shown in Figure 7. There was no significance (P≥0.05) for sample A (1.70 ± 0.04) and B (1.78 ± 0.02) for raw flour blends (RF). There exists significance among C, D, E and F with lowest signi-ficance for C (1.34 ± 0.03) and highest (2.87 ± 0.04) for sample F. There was significant difference (P≤0.05) for extruded unfermented (EUF) with lowest (1.35 ± 0.01) for B and highest (4.00 ± 0.02) for sample F. Extruded fermented (EF) recorded no significant difference (P≥0.05) between sample C and D and significant difference (P≤0.05) among A, B, E and F with lowest significant for A (0.79 ± 0.02) and highest for E (3.71 ± 0.01).

The crude protein composition of sorghum-soya blends and extrudates

Significant (P≤0.05) values were obtained among samples A to F for raw flour blends (RF) with lowest (9.88

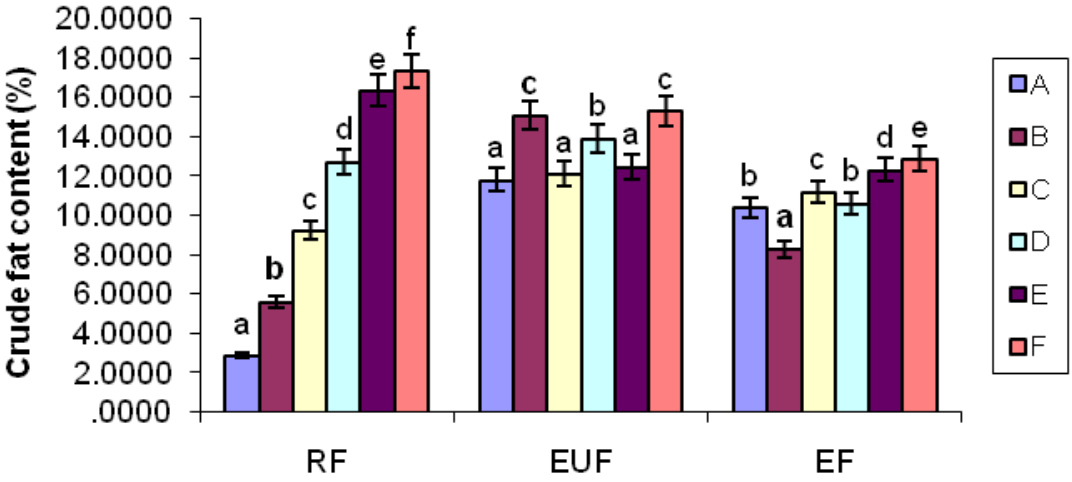

Figure 6: crude fat content of sorghum-soya blends and extrudates. RF = Raw flour; EUF = extruded unfermented; EF = extruded fermented. A = 100 g sorghum flour sample; B = 90 and 10 g sorghum-soya blend sample; C = 80 and 20 g sorghum-soya blend sample; D = 70 and 30 g sorghum-soya blend sample; E = 60 and 40 g sorghum-soya blend sample; F = 50 and 50 g sorghum-soya blend sample.

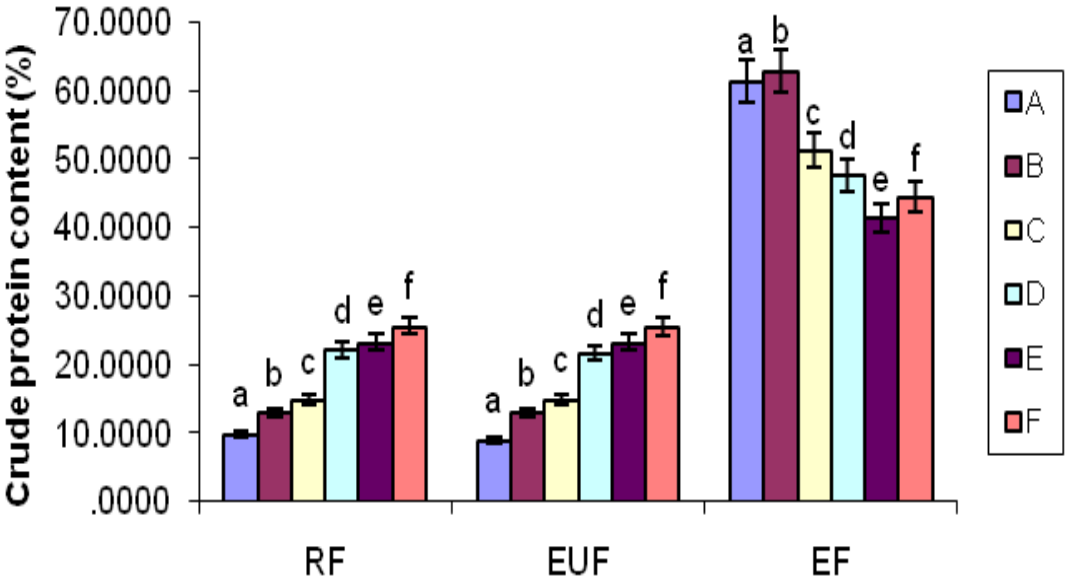

Figure 8. Crude protein content of sorghum-soya blends and extrudates. RF = Raw flour; EUF = extruded unfermented; EF= extruded fermented; A = 100 g sorghum flour sample; B = 90 and 10 g sorghum-soya blend sample; C = 80 and 20 g sorghum-soya blend sample; D = 70 and 30 g sorghum-soya blend sample; E = 60 and 40 g sorghum-soya blend sample; F = 50 and 50 g sorghum-soya blend sample.

± 0.01) for A and highest (25.63 ± 0.02) in F. Extruded unfermented (EUF) samples recorded significant (P≤0.05) changes. Changes exist among A-F (9.01 ± 0.02 for A and 25.10 ± 0.01 for F). Extruded fermented (EF) exhibited significant (P≤0.05) values for samples A-F (Figure 8).

The carbohydrate composition of sorghum-soya blends and extrudates

The raw flour blends (RF) exhibited significant (P≤0.05) values among samples A-F with lowest (44.45 ± 0.26) for

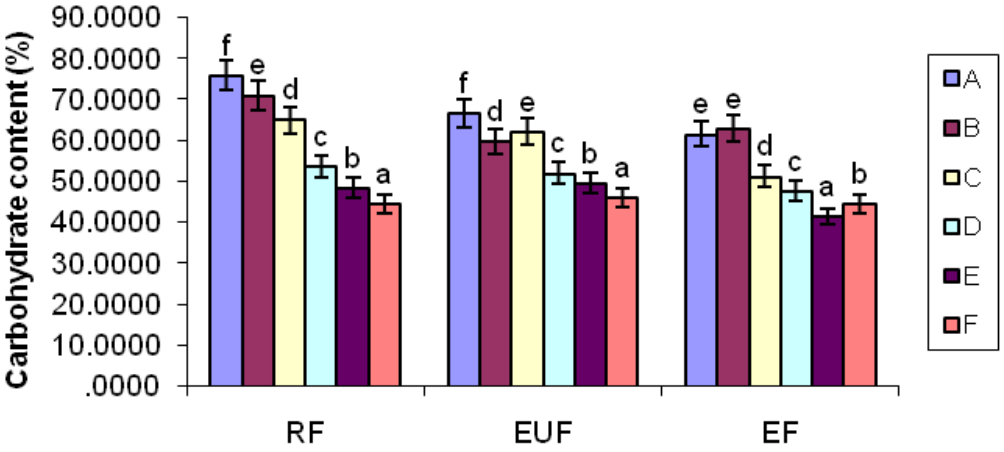

Figure 9. Carbohydrate content of sorghum-soya blends and extrudates. RF = Raw flour; EUF = extruded unfermented; EF= extruded fermented; A = 100 g sorghum flour sample; B = 90 and 10 g sorghum-soya blend sample; C = 80 and 20 g sorghum-soya blend sample; D = 70 and 30 g sorghum-soya blend sample; E = 60 and 40 g sorghum-soya blend sample; F = 50 and 50 g sorghum-soya blend sample.

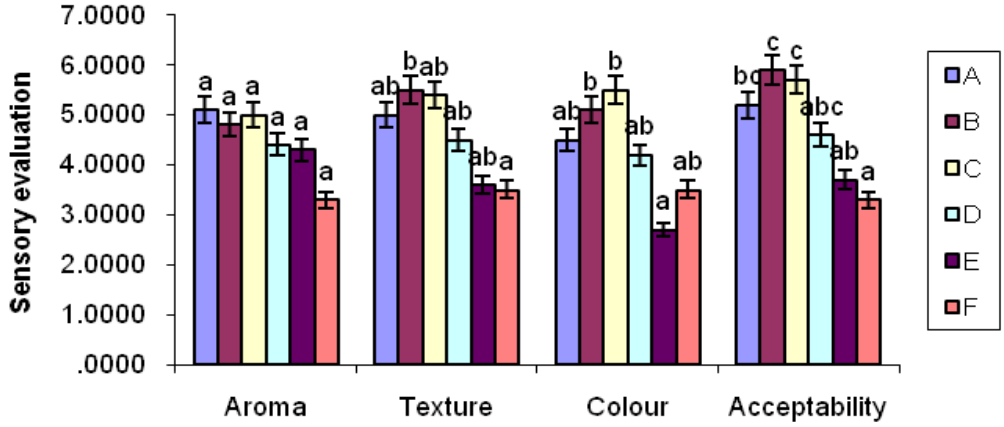

Figure 10. Sensory evaluation of sorghum-soya extrudates. A = 100 g sorghum flour sample; B = 90 and 10 g sorghum-soya blend sample; C = 80 and 20 g sorghum-soya blend sample; D = 70 and 30 g sorghum-soya blend sample; E = 60 and 40 g sorghum-soya blend sample; F = 50 and 50 g sorghum-soya blend sample.

F and highest (75.92 ± 0.31) for A. The values are significantly different (P≤0.05) for extruded unfermented (EUF) among A-F, lowest (46.03 ± 0.84) value for sample F and highest (66.58 ± 0.12) for A. The extruded fermented (EF) samples recorded no significant (p≥0.05) difference between sample A and B, while there were significant values (P≤0.05) among C,D,E and F with lowest (44.46±0.04) (Figure 9).

Sensory evaluation of sorghum-soya extrudates

The result obtained in the sensory evaluation indicated that there was no significant (p≥0.05) difference in aroma for sorghum-soya extrudate unfermented (EUF). Texture was not significantly different among samples A, C, D and E. There was significant lower (3.5 ± 2.17) difference between F and higher (5.00 ± 1.08) for sample B. For colour there was no significant difference among A, D, and F, no significant value between B and C and significant difference was recorded in sample E. Acceptability was not significant between samples B and C. Among A, D and E, difference was noted, but not significant. Also difference was recorded among D, E, and F but not significant. Among A, B, C and E values were significantly different (lowest 3.30 ± 1.70 in F and highest 5.90 ± inB). Sample B had highest acceptability of the six (6) samples. This result is represented in Figure 10.

DISCUSSION

The initial low bacteria count of the extrudates is probably due to the heat treatment given to the samples before fermentation. The increase in bacteria population with time between 0 to 48 h could be attributed to various microorganisms adapting to the fermentation environment and the decrease at later hour (72 to 96) may be due to reduction in pH which would had inhibited some microbial growth in the fermenting media. As fermentation progressed, some extrudates exhibited undulating pH values (though this has not been reported) which could be related to dispersion of different molecules during extrusion cooking prior to fermentation. The lowering of pH could probably be due to the more carbohydrate composition in sorghum-soya blend extrudates which could have degraded to organic acids in extrudates A and B, while the more availability of protein constituents in extrudates E and F might have contributed to the increase in corresponding increase in pH. However, the result suggest that the fermentation research is lactic type since Ezeama and Ihezie (2006) had once reported that cereal fermentation is lactic type where pH of fermenting mass decrease with increase in total titratable acidity (TTA) and vice versa. Omafuvbe et al. (2007) had earlier reported that increase in pH is a common feature in the fermentation of vegetable proteins. The behavioural changes in total titratable acidity (TTA) correspond with changes in pH but the undulating pattern of the total titratable acidity (TTA) in the fermenting mass may be as a result of variations in the composition of soya bean supplementation levels.

The microbial flora of the fermenting media were heterogeneous comprising of 12 genera of bacteria (*F. rigense*, *M. icristinae*, *Enterobacter* spp., *E. cloacae*, *C. cystitidis*, *C. pilosun*, *S. albus*, *Brevibacterium* spp., *B. subtilis*, *B. cereus*, *B. brevis and B. megaterium*), four yeast (*C. famata*, *S. cerevisiae*, *G. candidium* and *C. utilis*) and three moulds (*A. niger*, *A. fumigatus* and *R. Stolonifer*). This is similar to the findings of Efiuvwevwere and Akoma (1997) that soya bean supplemented products had greater microbial diversity and higher microbial population. *Enterobacter species* were identified at 0 and 24 h of fermentation in extrudate B and C, however as fermentation progressed, these less desirable bacteria disappeared. The involvement of *Enterobacter* species may be attributed to handling and normal contaminants. At 48 h, *Bacillus species* constituted the major bacteria in extrudate B to F, while *R. stolonifer* was the dominant fungi in extrudates E and F. This could be due to the microbial flora associated with soya bean grain as these have higher percentage of soya bean supplementation. Since the major components of these extrudates are protein, fat and carbohydrate, the organisms responsible for the fermentation must have the ability of utilizing these three components and most of the isolated organisms from the fermenting extrudates have such characteristics which include *Bacillus*, *Micrococcus* and *Staphylococcus* species. As noted earlier, *Bacillus* species were the dominant bacteria in all extrudates at 48 h except in extrudate A with no soya bean supplement. Enujiugha (2009) confirmed that *Bacillus* species are known to have proteolytic ability and are also able to break down oil. Although other bacteria were isolated, they appear not to have great influence in the fermentation process as *Bacillus* species constitute 99% of the total bacteria at 48 h. This could be due to the fact that *Bacillus* cells exhibit very high protease activities as compared with all other bacteria isolated.

Moisture content is one of the most important and commonly measured properties of different food products. It is measured for various reasons including legal and label requirements, economic importance, food quality, better processing operations and storagability. The non-significant difference ($p \geq 0.05$) of extrudate A and D, B and E and significant difference ($p \leq 0.05$) of C and F of raw flour blends (RF) indicate to a great extent the storagability and stable shelf life of the raw flour before the commencement of extrusion and fermentation processes. The increase in moisture content after extrusion and fermentation is due to the hydration of the flour prior to extrusion and addition of water before fermentation. The increased significant difference ($p \leq 0.05$) among extruded unfermented A to F indicate little reconstitution in just a little water prior to consumption thereby causing reduction in cooking time and fuel consumption.

The ash content is an inorganic residue remaining after the removal of the water and organic matter which provides a measure of total amount of minerals in the food component. Figure 5 shows the significantly ($p \leq 0.05$) lower ash content values recorded in the extruded fermented (EF) samples as compared with that of raw flour blends (RF) and extruded unfermented (EUF) which could be as a result of the retting of the extrudates there by encouraging the leaching of water soluble mineral content of the extrudates during the fermentation process and this loss of minerals could have served as the mineral source for the fermenting microorganisms. The low ash content in fortified meals does satisfy the recommended minimum composition in accordance to Agunbiade and Ojezele (2010) report.

Crude fibre is a measure of the quantity of indigestible cellulose, pentosans, and lignins present in food products. Crude fibre has little food value but it gives bulk to the food and equally aids in regulating physiological functions. The statistical results as presented in Figure 7 show crude fibre composition of sorghum-soya blends and extrudates. The significantly ($p \leq 0.05$) lower fibre content of extrudates A to D in extruded unfermented (EUF) samples as compared to that of raw flour blends (RF) could be as a result of mechanical shear effects of the extruding screw employed during the extrusion procedure.

Fermentation caused a significant (p≤0.05) reduction in the fibre content of the extrudates. This could probably be associated with the activities of the fermenting micro-oganisms which would have synthesized cellulose enzyme thereby breaking down the cellulose in the fermented extrudates. Crude fat (including oil) is one of the major components of the food that provides energy and essential lipids nutrients in many food products. Lipid constituent is a major determinant of overall physical characteristics, such as aroma, texture, the mouth-feel and appearance. Statistical analysis showed in Figure 6 indicates signi-ficant difference (p≤0.05) among extrudates A to F in raw flour blends. Fat increased with soya bean level supple-mentation but as extrusion took over, extrudates A, C and E had no significant difference (p≥0.05) but the increase in fat in extrudates A, B, C and D could be as a result of melting of the oils due to heat treatment applied while the drastic reduction in extrudates E and F could be attri-buted to physical loss in oil droplets which is similar to the findings of Nwabueze (2006). Declination in fat content after extrusion may also be as a result of starch/lipid com-plexes formation which resists the extraction of the oil. This is equally familiar with Camire (2001) reports. How-ever decrease in fat in extruded fermented (EF) could be attributed to the hydrolysis of glyceride to free fatty acids and glycerol.

Proteins are polymers of amino acids and their amount in a sample represents their quality index. Statistical results showed a significant difference (p≤0.05), an indication that supplementation level affected the protein content of the raw flour blends (RF) and extruded unfermented (EUF). However, interaction between the extruded fermented (EF) exhibited a discrete behaviour with extrudate A and B having the highest values. The decrease in protein value in some of the extruded unfermented (EUF) could be as a result of interaction of amino acids in maillard reactions. This fact had been reported by Onyango et al. (2005). The reduction in protein in extruded unfermented (EUF) could as well be attributed to excessive heating during extrusion cooking. This finding supports the earlier report by Onyango et al. (2004) that heat denatures the protein of cytoplasm and that of the cell membrane. The increase in proportion of extruded fermented (EF) probably may be as a result of the activities of micro-organisms involved in the fermentation of the mass which might have secreted extracellular enzymes that cones-quently increased the protein contents. This is supported by the findings of Ojokoh et al. *(*2003). More also, the microorganisms made use of carbohydrate as energy source thereby producing carbon dioxide as a by-product leading to nitrogen concentration thereby increasing the protein in the fermenting mass as carbon ratio decrease resulting in the redistribution of the nutrient percentages. This is supported by the reports of Onyango et al. (2004).

Carbohydrate varied among the raw flour blends (RF), extruded fermented (EUF) and extruded fermented (EF). The significant difference (p≤0.05) in carbohydrate decrease with decrease in proportion of sorghum flour incorporation. The slight decrease in the extruded unfer-mented (EUF) could be attributed to the heating tech-niques applied in the cooking extrusion process. Apart from extrudate B, all extruded fermented (EF) samples showed decrease in carbohydrate (Figure 9). The lower carbohydrate content may be due to the activities of microorganisms on the starch components. This could be linked with enzymatic action on the starch component leading to break down of starch into various sugars. Since *Bacillus* species which dominated at 48 h of fermentation are amylolytic in action, this is substantiated by Fadahunsi and Duyilemi (2010) that *Bacillus* species are amylolytic. It had been reported by Achi (2005) that *Bacillus* species have certain enzymes such as amylase, galactanase, galactosidase, glycosidase and fructofuranosidase which are involved in the degradation of carbohydrate. Microbial amylases hydrolyze carbohydrate into sugars which are then readily digestible by humans. However the variations in the proximate composition could be associated with the processing techniques employed.

The tested organoleptic assayed for extruded unfer-mented such as aroma, texture, colour and acceptability indicated that all the samples were all acceptable. The preferred aroma may be likened to the fat component of the sample. Further studies should be carried out for comparison of sensory properties between extruded unfer-mented (EUF) and extruded fermented (EF) including tasting.

Conclusion

Fermentation of the sorghum-soya blend after extrusion improved the protein content compared to the raw samples. Evidence from the results of this research work showed that the sorghum-soya extrudates presents an exciting opportunity in processing of cereal-legume into a convenience ready-to eat inexpensive food products which can store well without refrigeration that require only reconstitution in warm water.

Conflict of Interest

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

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Full Length Research Paper

Sugar cane juice for polyhydroxyalkanoate (PHA) production by batch fermentation

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Clarified sugar cane juice was evaluated as an alternative substrate for the batch production of polyhydroxyalkanoates (PHAs) by *Alcaligenes latus*, and a mineral salt broth was used as the control. The study included the physicochemical characterization of the juice, measurement of the fermentation kinetic parameters and identification of the polymer type by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. Batch-type aerobic fermentations were performed (33°C at 200 rpm and pH 6.5-7 for 60 h) and set at 20 gL^{-1} of fermentable sugar and a carbon/nitrogen ratio of 28.3/1. A 10% v/v ratio of inoculum/substrate was used. The alternative substrate presented a greater concentration of magnesium and micronutrients such as Fe, B, Zn, Mg and Cu compared to the control. The biomass yield ($Y_{P/X}$) of the juice was 1.27 $g.g^{-1}$ (0.414 $g.g^{-1}$ in control medium), which was 69% more than the control medium; the product-substrate yield ($Y_{P/S}$) was 0.10 $g.g^{-1}$, which was similar to the control medium (0.15 $g.g^{-1}$ control medium). The production of PHB was of 1.3 gL^{-1}, less than half of the concentration obtained in commercial substrate. Infrared spectroscopy indicated that the polymer obtained in the evaluated substrates was polyhydroxybutyrate (PHB). The clarified juice of sugar cane, without the addition of nutritional supplements can be used for the production of biomass of *A. latus*, first step in the production of PHB.

Key words: Alternative substrate, *Alcaligenes latus,* polymer, polyhydroxybutyrate (PBH), fermentation.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are lipidic materials that are accumulated by a great variety of microorganisms in the presence of an excess of carbon. The assimilated carbon sources are biochemically transformed into units of hydroxyalkanoates, which are polymerized and stored in the form of insoluble inclusions in the cell cytoplasm (Brito et al., 2011). PHA is a natural polyester that is biocompatible and 100% biodegradable under aerobic

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Abbreviations: PHA, Polyhydroxyalkanoate; **PHB,** polyhydroxybutyrate; $Y_{P/X}$:, biomass yield; $Y_{P/S}$, substrate yield; $Y_{X/S}$, biomass-substrate yield; **ATR-FTIR,** attenuated total reflectance Fourier transform infrared spectroscopy; **%w/v,** weight/volume percentage; **Ppm,** parts per million; **OD,** optical density; **DCW,** dry cell weight; **FS,** final concentration of fermentable sugars.

and anaerobic conditions (Verlinden et al., 2011), and it has characteristics similar to polyethylene and polypropylene, which is why it has been considered as a substitute of conventional plastics.

PHAs are produced by at least 75 different bacterial genera including Gram-positive and Gram-negative bacteria, such as *Cupriavidus necator (Ralstonia eutropha), Alcaligenes latus, Bacillus megaterium, Klebsiella aerogenes, Pseudomonas putida, Pseudomonas oleovorans,* and *Sphaerotilus natans.* When these polymers are intracellularly stored under stress conditions, such as phosphorous and nitrogen stress, limited oxygen, and excess carbon, they can represent up to 80% of the dry weight of the biomass obtained in the fermentation (Gonzalez et al., 2013). Depending on the carbonate substrate and metabolism of the microorganism, different monomers, polymers and co-polymers can be obtained (Gonzalez et al., 2013).

To date, a satisfactory PHA production process has not been achieved. One of the major problems faced by the industrial-scale production of this polymer is the high cost of production, especially the high prices of raw materials (for example, carbon and energy sources for growth of microorganisms), which make them uncompetitive compared to petrochemical (Chen, 2009). In addition, the selection of an adequate carbon source is an important criterion because it determines not only the PHA content but also the polymeric composition, which affects the final properties of the polymer. Fifty percent of the final cost of the materials corresponds to the carbon source (Brigham et al., 2011).

According to Ntaikou et al. (2009), cited by Sharifzadeh et al. (2009), an economy source for the fermentation includes a culture medium that contains sugar cane honey, corn steep liquor and effluents of palm oil and olive oil. Cane molasses contains vitamins and minerals and is a source of calcium, magnesium, potassium and iron, which are considered as impurities in no refined sugar (Akaraonye et al., 2010), therefore, this raw material can be used for the production of PHAs. Albuquerque et al. (2007) obtained 30% of P (3HB-co-3HV) and 3.5 gL^{-1} of biomass concentration, from a mixed bacterial culture using sugar cane molasses. Chaijamrus and Udpuay (2008), obtained 43% w/w of *B. megaterium*, (after 45 h of growth), when 4% of molasses was used. Gouda et al. (2001), cited by Bello et al. (2009) found that the greater accumulation of PHB with respect to cell dry weight, was obtained with substrate supplemented with molasses 0.5% (w/v). Similar results were obtained by Waranya et al. (2011), which evaluated the juice of sugar cane to produce PHB with *A. eutrophus.* Oehmen et al. (2014) evaluated the effect of pH control on the volumetric productivity of PHA, using molasses, and a mixture of PHA-producing microorganisms. They found that controlling the pH of the fermentation to 8, the volumetric productivity is increased.

A. latus (*Azohydromonas australica*) is a microorganism producer of intracellular PHB (Wang et al., 2012) and is known for its ability to use sucrose as a carbon source (Yezza et al., 2007; Zafar et al., 2012); therefore, the goal of this work was to evaluate sugar cane juice as an alternative substrate for the production of PHA by *A. latus* in batch fermentation.

MATERIALS AND METHODS

Microorganism and maintenance

A. latus obtained from the American Type Culture Collection was used as the lyophilized culture. The strain was reconstituted and preserved in vials with a mineral salt broth at -20°C. The work was performed by keeping the culture in a slant with the mineral salt medium at 4°C (Grothe and Chisti, 2000).

Fermentation substrates

Two fermentation substrates were used: sugar cane juice and mineral salt medium. The cane juice was selected as a substrate because it provides a good source of carbon, vitamins and minerals, which are required for the growth of *A. latus*, and can be used as a low-cost substrate for the growth of bacteria and production of PHA.

The sugar cane juice was obtained in a Colombian sugar mill at an industrial scale, and sugar cane variety CC8592 was used. The cane was weighed (Fletcher, USA), and to facilitate the juice extraction and improve its efficiency, the cane was shredded in a shredding machine (Dedini, Brazil) and milled in four-roller mills (Fulton mill, USA).

The juice extracted in the mills was weighed (Fletcher, USA) and then neutralized with lime (pH 6.5 +/- 0.5) to help in the separation of the solids. The juice was heated to 105°C in order to accelerate the separation of the non-sugar solids, and the clarified and sediment-free juice was used.

The cane juice was characterized based on the content of macronutrients (nitrogen, sodium, potassium and magnesium) and micronutrients (iron, boron, zinc, manganese, and copper). The clarified juice samples were subjected to acid digestion (500 ml of HNO_3 and 250 ml of $HClO_4$ solution in a 2:1 proportion) and then stirred with a stir plate (Dari, Cali Colombia) at 360°C for 28 min. The sample was filtered in Teflon filters and diluted to approximately 50 ml with distilled water. The macronutrients were determined by atomic absorption (AOAC 985.35) in an atomic absorption spectrophotometer (Perkin Elmer, model 2380, USA); the phosphorous content was analyzed by visible-light colorimetry (AOAC 995.11). The results were expressed in weight/volume percentages (%w/v) for macronutrients and in parts per million (ppm) for micronutrients.

To formulate the substrate, the cane juice was adjusted to 20 ($g.l^{-1}$) of fermentable sugar, and ammonium sulfate was added as a nitrogen source to maintain a carbon-to-nitrogen ratio of 28.3/1 (C:N 28.3/1), which is the commercial substrate ratio. The substrate was adjusted to pH 6.5 +/- 0.5 with 2N NaOH/2N HCl.

The mineral salt substrate was used as a commercial substrate (control substrate), which is composed of (in $g.l^{-1}$) sucrose 20; $(NH4)_2SO_4$ 1.4; KH_2PO_4 1.5; $Na_2HPO_4.12H_2O$ 3.6; and $MgSO_4.7H_2O$ 0.2. In addition, 1 ml/lof trace element solution (TES) was included, and the TES is composed of (in $g.l^{-1}$) ammonium ferric citrate 60; $CaCl_2.2H_2O$ 10; H_3BO_3 0.3; $CoCl_2.6H_2O$ 0.2; $ZnSO_4.7\ H_2O$ 0.1; $MnCl_2.4\ H_2O$ 0.03; $Na_2MoO_4.2H_2O$ 0.03; $NiSO_4.7H_2O$ 0.02 and $CuSO_4.5H_2O$ 0.01 (Grothe and Chisti, 2000). The pH of the medium was adjusted to 6.5 +/- 0.5 with 2 N NaOH/2 N HCl.

Inoculum preparation

For the inoculum preparation, 800 ml of mineral salt broth was prepared in a 2000 ml Erlenmeyer flask. The broth was inoculated under sterile conditions with four slants of *A. latus* (from the described workbench). The Erlenmeyer flask was incubated under aerobic conditions in a rotary shaker (Innova 44, USA) at a temperature of 33°C and rpm of 200 for 48 h (Grothe and Chisti, 2000). The broth was then used as inoculum for the fermentations of the sugar cane juice and mineral salt medium.

Fermentation

Six batch fermentations were performed in 2000 ml Erlenmeyer flasks, which had an effective working volume of 1000 ml (three fermentations per substrate). The Erlenmeyer flasks remained stirring at 200 rpm, without aeration, in the rotary shaker for 60 h at 33°C. The pH of the substrate was measured and adjusted every two hours, to 6.5-7.0 using 2 N NaOH. The procedure was performed in laminar flow cabin, in order to ensure sterility. The substrate fermentation were inoculated with the *A. latus* inoculum (described above), maintaining a 10% v/v ratio with respect to the substrate volume. Dissolved oxygen was not controlled during fermentation. The flasks were opened every 2 h for pH control, therefore, the headspace oxygen was renewed every 2 h.

For each substrate, at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 h of fermentation, 30 ml of fermented media was aseptically collected in the laminar flow to measure the fermentation kinetic parameters (time 0 corresponded to the initial conditions of each substrate) and by Gram stain purity monitoring fermentation every 6 h.

Determination of kinetic parameters

The following parameters were determined: biomass concentration (X), substrate consumption (carbon source), nitrogen consumption, biomass-substrate yield ($Y_{X/S}$), product-substrate yield ($Y_{P/S}$), product-biomass yield ($Y_{P/X}$) and PHB productivity. The biomass concentration was determined by a previously constructed optical density (OD) vs. dry biomass weight (DCW) calibration curve which replaced the optical density value in the equation of the described curve.

To construct the calibration curve, an *A. latus* culture was created; 400 ml of mineral salt broth was inoculated with two slants of *A. latus* and incubated at 33°C (Binder, USA) for 48 h at 200 rpm in a rotary shaker (Grothe and Chisti, 2000). After finalization of the growth time, 90 ml of the cell suspension was distributed in nine glass test tubes with known initial weight. The tests tubes with 10 ml of cell suspension were placed in an oven (Binder, USA) at 105°C until reaching a constant weight and then cooled in a vacuum desiccator for one hour. Subsequently, they were weighed in an analytical balance (Mettler Toledo xs-204, USA). The values obtained from the nine tubes were averaged, and the dry biomass weight concentration in the initial culture (g/10 ml) was obtained.

With the remaining culture, dilutions in sterile distilled water were prepared (1/5, 1/6, 1/7, 1/9, 1/15, 1/20 and 1/30), and the absorbance of each dilution was measured at 600 nm (Spectronic Genesys 2PC, USA) using distilled water as the blank. The calibration curve was constructed with these results, and the absorbance and grams of dry biomass per liter ($g.l^{-1}$) were correlated (Grothe et al., 1999; Patwardhan and Srivastava, 2004).

The substrate consumption (carbon source) was calculated with Equation 1, using the concentration data of fermentable sugars collected at each of the times mentioned above. The concentration of sugars was measured by high-performance liquid chromatography (HPLC - Shimadzu, USA; LC-10ADvp pump, RID-10A refraction index detector, SCL-10Avp controller, SILC-10AFautosampler, Zstar software,and a Hi-PlexCa column at 300 × 7.7 mm coupled to a YOYO pre-column with WAT015209 insert (Sugar-Pak) USA). The operation conditions were as follows: 75°C column temperature; deionized reverse osmosis water (deionizer, Simplicity, USA) mobile phase; 0.6 ml/min mobile phase flow; and 10 µl injection volume of samples and standards. The samples were filtered (EMD Millipore Durapore PVDF filters, 0.22 µM pore, 47 mm diameter), and the filtrate was injected into a HPLC system. The calibration standards with sucrose concentrations of 0.09, 0.1, and 0.11 $g.l^{-1}$ and glucose and fructose concentrations of 0.009, 0.01 and 0.011 gL^{-1} were prepared in the HPLC-grade water.

The nitrogen consumption as the free amino nitrogen (FAN) (in ppm) was calculated using equation 2. The concentration of ammonia was determined semi-quantitatively by the ammonium (NH_4^+) test by Merck, which is a colorimetric method where the ammonium ions form a yellow-brown compound with the NeBler reactant. The reaction region of the test strip was compared with the regions of a calorimetric scale (10-30-60-100-200-400 mg/l of NH_4^+). The values for $Y_{X/S}$, $Y_{P/S}$ and $Y_{X/P}$ were determined by Equations 3, 4 and 5, respectively.

$$Substrate\ consumption = \frac{\mathrm{FS}}{\mathrm{FS_0}} * 100\% \quad (1)$$

Where FS is the final concentration of fermentable sugars ($g.l^{-1}$) and FS_0 is the initial concentration of fermentable sugars ($g.l^{-1}$) (Serna et al., 2010).

$$FAN\left(\frac{mg}{l}\right) = \frac{ml NaOH\ 0.1\ N\ required\ in\ the\ titration * 1.4 * 1000}{100\ ml\ of\ collected\ sample} \quad (2)$$

The basic ratio used for the calculations is 1 ml of NaOH 0.1 N= 1.4 mg of nitrogen.

$$Y_{X/S} = \frac{X - X_0}{So - S} = g.g^{-1} \quad (3)$$

$$Y_{P/S} = \frac{P}{\mathrm{So - S}} = g.g^{-1} \quad (4)$$

$$Y_{P/X} = \frac{P}{Xo - X} = g.g^{-1} \quad (5)$$

Where, S is the concentration of fermentable sugars ($g.l^{-1}$) at each fermentation time; X is the final concentration of biomass (gL^{-1}); X_0 is the initial concentration of biomass (gL^{-1}); S_0 is the initial concentration of fermentable sugars ($g.l^{-1}$); S is the final concentration of fermentable sugars (gL^{-1}); and P is the final concentration of PHA (gL^{-1}). The volumetric productivity was calculated as the grams of PHB produced per liter per hour of culture time ($gL^{-1}h^{-1}$) (Wang and Lee, 1997).

Extraction and quantification of the PHB

The extraction and quantification of PHB was performed by a modification of the technique proposed by Kim et al. (1994) as follows: a mixture of 12.5 ml of 30% sodium hypochlorite (v/v) and 12.5 ml of chloroform was prepared; this solution was then mixed with 1 g of wet biomass and centrifuged in 50 ml Eppendorf tubes and then placed in a bain-marie at 30°C for 90 min and centrifuged for 15 min at 4000 rpm. Three phases were formed: hypochlorite in the upper phase, cell debris in the middle phase, and PHB with chloroform in the bottom phase. The PHB was extracted with a pipette. For the separation of the chloroform and PHB, the PHB was precipitated using 7:3 methanol and water (v/v) and then filtered (0.45 µ). The mixture was placed in an exhaust hood for 48 h to volatilize the methanol excess. Finally, the PHB pellet was

Table 1. Physicochemical characterization of the sugar cane juice (pure).

Nutrient	Parameter	Value
General chemical and nutritional properties	°Brix (%)	14.94
	Purity (%)	86.4
	Fermentable sugar (%)	13.54
	Volatile Acidity (ppm)	306
	Lactic Acidity (ppm)	100
	pH	6.78
	Free Amino nitrogen (FAN) (ppm)	45
Macronutrients	N- Total%	0.033
	P_2O_5%	0.023
	CaO%	0.41
	MgO%	0.38
	K_2O%	0.37
	Na_2O%	0.07
	S%	0.15
Micronutrients	B (ppm)	14.72
	Cu (ppm)	5.39
	Fe (ppm)	105.5
	Mn (ppm)	16.86
	Zn (ppm)	12.36

weighed (Mettler Toledo xs-204, USA), and the biomass yield was calculated by Equation 6.

$$\%\ of\ accumulation = \frac{Final\ polymer}{Final\ dry\ biomass} * 100 \quad (6)$$

Analysis of biopolymers using ATR-FTIR spectroscopy

The biopolymer samples obtained after fermentation in the evaluated substrates, clarified juice and mineral salt commercial medium were analyzed using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy.

A small amount of each of the biopolymer samples was collected for the ATR-FTIR analysis and directly placed on the germanium crystal of the sample carrier (*Thermo Scientific iS10*, USA). The ATR-FTIR spectra were collected in a spectrophotometer (*Thermo Scientific iS10*, USA), and the results of the functional group analysis was compared with the functional groups of the polyhydroxybutyrate (PHB) standard reported in the literature.

Experimental design

A completely random unifactorial design was used, and the measurements were repeated 11 times by triplicate. The unifactorial design was composed of the type of substrate with two levels: (1) sugar cane juice and (2) commercial substrate.

The response variables were measured at times of 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 h of fermentation (time 0 corresponded to the initial fermentations value). The response variables were biomass concentration (X), carbon source consumption (FS), and nitrogen source consumption. The determined parameters were PHB productivity, biomass yield($Y_{X/S}$), product yield ($Y_{P/S}$), product-biomass yield, and volumetric productivity, which were calculated after 60 h of fermentation.

The results were analyzed by a variance analysis using the mixed procedure of the SAS (Statistical Analysis System) statistical package, version 9.3. To test the hypothesis of the differences between treatments, the treatment time and treatment type according to the interaction period, structure of the compound symmetry covariance and first-order autoregressive covariance were used based on the characteristics of the repeated measurements in the experiment. In addition, the averages of the treatments, treatment time and treatment type according to the interaction period were compared by a least squares analysis using the LSMEANS statement used in the previous procedure.

RESULTS AND DISCUSSION

Characterization of the sugar cane juice

Table 1 presents the characterization of the sugar cane juice before its use in the formulation of the fermentation substrate. As observed in Table 1, the sugar cane juice presents an adequate content of essential macronutrients and micronutrients, which can influence the growth of bacteria. When comparing these values with those of the commercial substrate, the alternative substrate presents a greater concentration of all micronutrients and of the Mg macronutrient. Mg is necessary for the proper functioning of most metabolism enzymes and is an activator of glycolytic enzymes, stimulator of fatty acid synthesis, and participant with K in slowing phosphate penetration and it is involved in the structure of ribosomes, cellular membranes and nucleic acids (Bouix, 2000). Therefore, because it has a greater amount of magnesium, the clarified juice provides a metabolic advantage to the microorganisms that are cultured in this substrate. In the studies performed by Gahlawat and Srivastava (2012), the substrate nutrients were optimized to increase the production of PHB by *A. latus*. In that research, the Plackett - Burman protocol and surface response methodology were evaluated, and the Mg, P and micronutrients were found to have an important effect on the production of biomass and PHB. This result can be explained by the important role that mineral nutrients (Mg, K and P) play in sustaining the buffer capacity of the culture medium, which is required for bacterial growth and PHB production. In addition, the sugar cane juice is an economical and available carbon source for use in the scaling of the PHA production that decreases the production costs of bioplastics and provides an alternative use of this raw material derived from the production of sugar.

Fermentation kinetic parameters

Figures 1, 2 and 3 show the kinetics of the biomass production, substrate consumption (carbon source) and

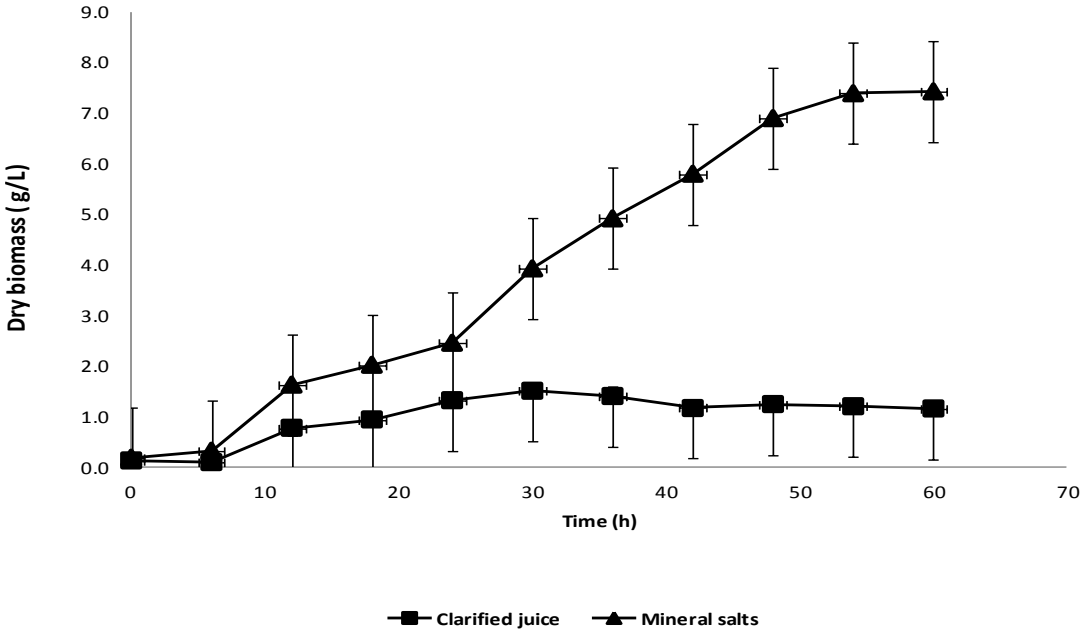

Figure 1. Biomass concentration of *Alcaligenes latus* obtained in mineral salt broth (commercial substrate) and clarified sugar cane juice.

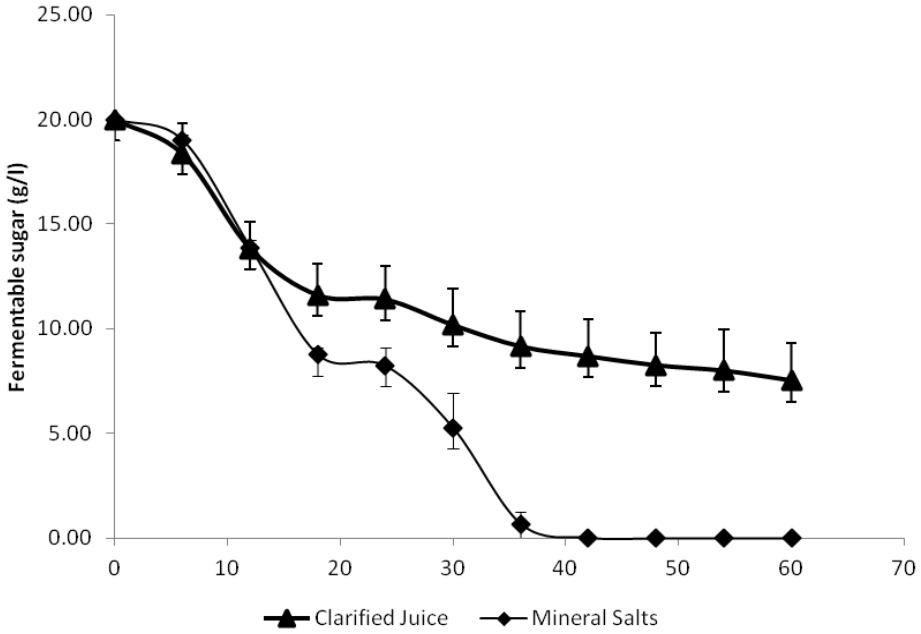

Figure 2. Substrate consumption (carbon source) of *Alcaligenes latus* in mineral salt substrate (commercial substrate) and in sugar cane juice during batch fermentation.

nitrogen source consumption, which were obtained in batch fermentations of the sugar cane juice substrate and mineral salt commercial substrate. The greatest biomass production occurred at 30 h of fermentation for the sugar cane juice (1.50 gL^{-1}) and 60 h of fermentation for the mineral salt substrate (7.42 gL^{-1}) (Figure 1). This result was obtained because the mineral salt medium presented an adequate nutritional balance (essential nutrients) for the reproduction and accumulation of PHA by the microorganism. In the clarified juice substrate was

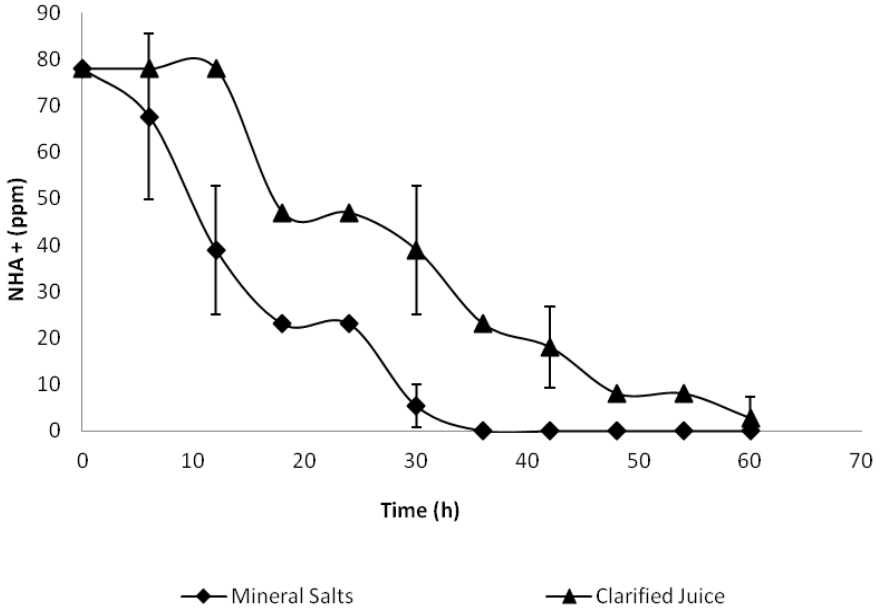

Figure 3. Consumption of the nitrogen source (ammonium sulfate) by *Alcaligenes latus* in the mineral salt substrate (commercial substrate) and sugar cane juice during batch fermentation.

not added any type of nutritional supplementation. In the work performed by Waranya et al. (2011) in which sugar cane juice was evaluated as a substrate for the production of PHA using *A. latus* bacteria, the greatest biomass production occurred at 36 h, which was relatively low. In addition, this result was corroborated by the preference of the bacteria in consuming glucose instead of sucrose as the main carbon source. The statistical analysis demonstrated that there was a significant difference (P< 0.1) in favor of the mineral salt substrate.

Similarly, the greatest substrate consumption (fermentable sugar) occurred in the commercial substrate, which was 100% at 48 h of fermentation. In the sugar cane juice broth, 62.45% of the substrate was consumed at 60 h of fermentation, and this result can be associated with the high temperatures to which the alternate substrate is subjected; these temperatures generate a browning that indicates a thermal decomposition of the sucrose, glucose, and fructose content and reaction of these hydrocarbons with amino-nitrogenated compounds to produce colored polymers, such as melanoidins, and furfurals, such as 5-(hydroxymethyl)-2-furaldehyde,which indicate the growth of bacteria and possible accumulation of PHA (Waranya et al., 2011; Rein, 2012). The greatest nitrogen consumption occurred in the commercial substrate, which was 100% at 36 h of fermentation, and sugar cane juice broth, which showed 96% nitrogen consumption at 60 h. The statistical analysis showed a significant difference from hour 12 to 36 h of fermentation (P< 0.1) in favor of the mineral salt substrate medium. Grothe et al. (1999), found that the standards of sucrose and nitrogen consumption were consistent with the standards of lag-exponential-stationary growth of biomass; in addition, different nitrogen sources, such as urea and ammonium sulfate, were evaluated, and they confirmed that *A. latus* presents a higher affinity towards ammonium sulfate. The rapid depletion of ammonium sulfate in the commercial substrate is primarily a result of the balance of essential macronutrients and micronutrients for bacterial reproduction in the medium.

Table 2 shows the performance kinetic parameters obtained in the evaluated substrates. The polymer accumulation obtained in the commercial substrate was 14% greater than that reported by Grothe and Chisti (2000), who evaluated PHA production in the mineral salt medium with *A. latus* and obtained a PHA accumulation of 63% in 93 h. Yezza et al. (2007) showed that *A. latus* accumulated up to 77% (weight-weight) of PHB in an evaluation of maple sap as an economic carbon source, which contains 10 - 30 $g.l^{-1}$ of sucrose under nitrogen limitation. The kinetic parameters and yields obtained in the sugar cane juice alternative substrate were superior to the results obtained in the work performed by Waranya et al. (2011) except for the $Y_{X/S}$ value and productivity, for which different sugar concentrations were evaluated for the production of PHA in batch fermentation using sugar cane juice as a substrate ($Y_{X/S}$=0.163 $g.g^{-1}$, $Y_{P/S}$=0.05 $g.g^{-1}$, $Y_{P/X}$=0.306 $g.g^{-1}$, and productivity= 0.031 $gL^{-1}.h^{-1}$).The greatest biomass product yield was obtained in the

Table 2. Kinetic parameters obtained in batch fermentations using the following substrates: mineral salt broth (commercial substrate) and substrate formulated with clarified sugar cane juice.

Parameter	Commercial substrate	Clarified juice substrate
$Y_{X/S}$ (g.g^{-1})	0.362	0.082
$Y_{P/X}$ (g.g^{-1})	0.414	1.27
$Y_{P/S}$ (g.g^{-1})	0.15	0.104
Productivity (g.l^{-1}.h^{-1})	0.05	0.022
Polymer production (g.l^{-1})	3.0	1.3
% of polymer accumulation	74	63

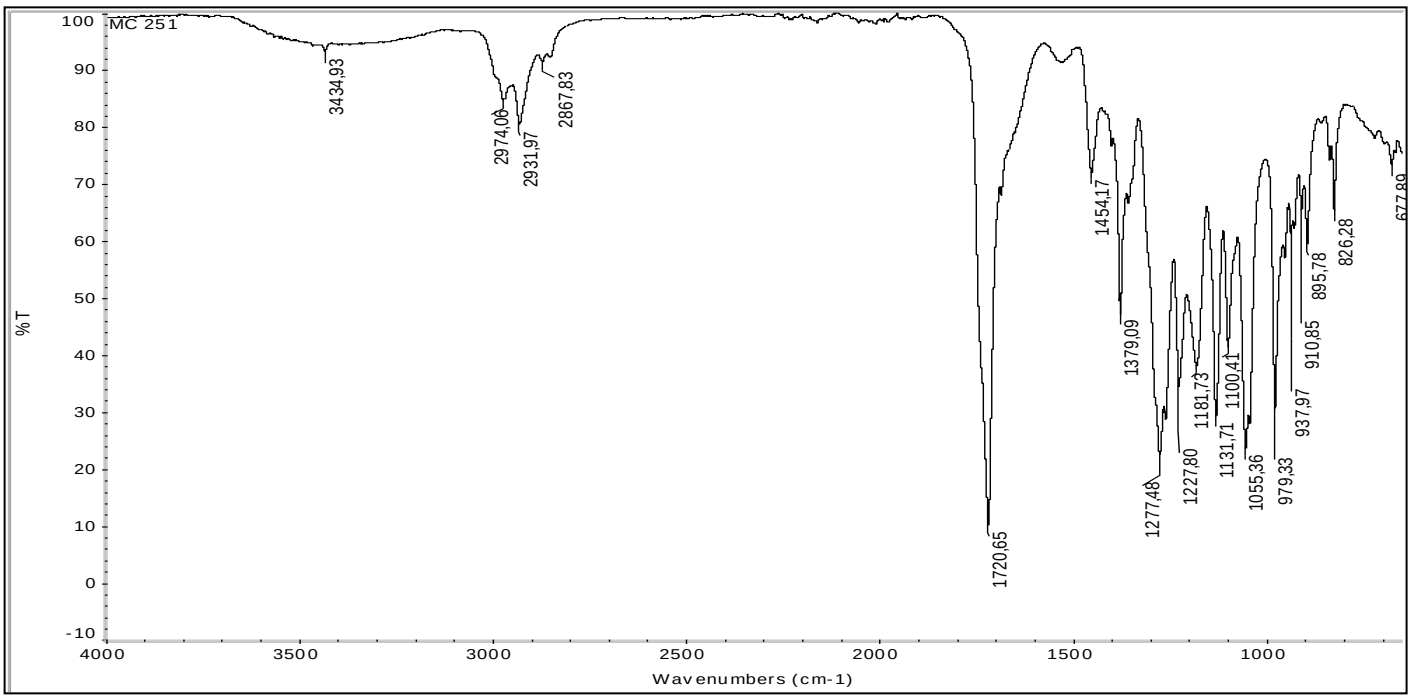

Figure 4. Infrared spectroscopy of the polymer produced by *A. latus* in the substrate formulated with clarified sugar cane juice.

sugar cane juice substrate, which was 69% greater than that obtained for the commercial substrate. The sugar cane juice substrate presented a greater PHB accumulation, greater $Y_{P/S}$, and greater volumetric productivity than in the studies of (Kumalaningsih et al., 2011), who used soybean curd waste as the alternative substrate with *A. latus* and obtained PHB of 0.68 gL^{-1}, $Y_{P/S}$v of 0.39 g.g^{-1} and 0.0125 gL^{-1}h^{-1}. This result may have been primarily because cane juice is rich in amino acids such as aspartic (0.11%), glutamic (0.05%), alanine (0.06%), valine (0.03%), gamma-aminobutyric (0.03%), threonine (0.02%), isoleucine (0.01%), and glycine (0.01%) and group B vitamins (Chen, 1991). These amino acids are normally used as growth factors for microorganisms, and although they are required in small amounts, they may be vital for the optimal growth and performance of the microorganisms (Madigan et al., 2000).

Identification of the polymer produced by *A. latus*

By means of infrared spectroscopy (Figure 4), the polymer produced by *A. latus*in both the commercial substrate and clarified sugar cane juice-formulated substrate was a polyester-type polymer. This polymer was identified in the presence of the carbonyl group band (1720 cm^{-1}), aliphatic chain methyl bands (2995 to 2975 cm^{-1}), aliphatic chain methylene bands (2865 to 2855 cm^{-1}), and ester C-O stretching band (1279 cm^{-1}) (Table 3). The obtained results coincide with those reported by

Table 3. Signals for the PHB sample obtained in clarified juice.

Signal (cm^{-1})	Assignment
2995-2975 range	C-H methyl asymmetric stretch
2933	C-H methylene stretch
2865-2855 range	C-H methyl symmetric stretch
1720	C=O carbonyl stretch
1378	Methyl symmetric bend
1279	C-O ester asymmetric stretch
1228	C-O ester symmetric stretch

Hong et al. (1999) and Bayari and Severcan (2005) and were cited by Liuet al. (2011), who revealed the presence of intense absorption bands at 1724 and 1281 cm^{-1} that correspond to the ester carbonyl (C=O) group and the –CH group, respectively.

The identification performed by FT-IR suggests a 90% agreement with the spectrum reported in the scientific literature for poly(3-hydroxybutyrate). This consistency occurs when the position and intensity of the signals presented in the FT-IR spectra are observed, and these results are consistent with those reported by Kansiz et al. (2000).

Conclusions

The present study shows that *A. latus* can grow and produce polyhydroxyalkanoate in a clarified sugar cane juice substrate at a concentration of 20 gL^{-1} of fermentable sugars and C:N ratio of 28.3:1; in addition, the biomass yield was superior to those obtained with the commercial substrate. Therefore the clarified juice of sugar cane, without the addition of nutritional supplements can be used for the production of biomass of *A. latus*, first step in the production of PHB. The results of the physicochemical analyses showed that the composition in macronutrients and micronutrients of the clarified sugar cane juice is comparable with the composition of the mineral salt broth. The infrared spectroscopy results showed that the polymer obtained from the fermentation of the clarified sugar cane juice was of a PHB type. The sugarcane juice is a commercial alternative for the PHB bacteria reproduction, and for the production of PHB, however, the process must be optimized. Additionally, the use of sugar cane juice is an option for product diversification in the sugar mills.

Conflict of Interests

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

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Induced spawning of *Liza ramada* using three different protocols of hormones with respect to their effects on egg quality

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Two intramuscular injection strategies were performed for females of *Liza ramada*. The first strategy was applied with two injections; priming dose (CPE, 20 mg per fish). Then, the resolving dose (200 µg/kg of LHRH-a) was given after 24 h later. The second strategy was applied with two injections; the priming dose (3500 IU HCG per fish). Then, the resolving dose (200 µg/kg LHRH-a) was given after 24 h later. The three successful spawning attempts occurred within 3 days when the female received an extra dose of LHRH-a 100 µg/kg (3rd injection) from the second breeding protocol. In both strategies, the males received a single dose of LHRH-a. Ovarian biopsy of hormonal treated and control females were taken at different times after each treatment in order to monitor oocyte development and to determine the time of ovulation, since voluntary spawning was not expected. Measurements of oocyte diameters were carried out at 24 h intervals (0, 24, 48, and 72 h). Diameters of oocytes were measured with a hemacytometer under a light microscope. Mature oocytes with a diameter of 600 ± 50 were more appropriate to injection and spawning. After the injection of the first strategy, the egg diameters ranged from 650 to 680 µm with clear oocyte center and final dose of the resolving dose of the egg diameters were 700 ± 50 µm. After 48 h from the second injection of the first strategy, only one fish spawned. The total number of the spawned eggs ranged from 1 to 1.2 million/fish with no signs of fertilization having a diameter that ranged from 700 to 750 µm. The spawned unfertilized eggs were rounded colourless and transparent. In the second strategy and after the final injection of the resolving dose, the egg diameters were 800±30 µm. After the resolving dose of the first strategy, there was no response of spawning. It showed more successful spawning rather than the first one which showed deformed unfertilized eggs. At the second breeding protocol, fish spawned during the 48 h after the third injection dose. After 48 h, the first three fish were successfully spawned with fertilization rates of 1, 1.8 and 1.6 million eggs/spawn and the percentage of fertilization were 52, 75 and 64%, respectively, but without hatching, and all the fertilized eggs reached the gastrula stage. Control non-injected females were subjected to the same rearing conditions but did not spawn. Two replicate samples of 1 ml of eggs were taken for both control and injected fish. Regarding fatty acids profile, the results reported that the mono-unsaturated fatty acid (MUFA), oleic acid, was highly recorded in the fertilized eggs of the treated females with the second strategy injection while in the first strategy this was not detected except in the unfertilized eggs. The females that was treated with the second strategy injection had more fatty acids particularly the saturated fatty acid, highly unsaturated fatty acid and polyunsaturated fatty acid, (eicosapentaenoic acid (20:5n-3,EPA), docosahexaenoic acid (22:6n-3,DHA) and arachidonic acid (20:4n-6,ARA). The most significant depletions were observed in polyunsaturated fatty acids. Our results suggested that CPE, HCG and LHRH-a promote ovulation and spawning process for both scheduled induction and the frequency of hormone injection influence the fatty acid composition of normal, injected gonad and fertilized eggs of *L. ramada* in relation to egg quality.

Key words: Induced spawning, luetunizing hormone releasing hormone analogue (LHRH-a), gonadotropin hormone (HCG), carp pituitary extract, fatty acids, *Liza ramada*.

INTRODUCTION

Liza ramada is an attractive species for farming in sea, brackish and fresh water (Pillay, 1990). It is considered as an excellent candidate for aquaculture because of the rapid growth rate, ability to efficiently utilize a wide range of natural and artificial foods, tolerance of a wide range of environmental conditions and resistance to disease and stresses. Thin-lipped mullet, *L. ramada,* is an economically important species of fish found in Egypt. However, its supply is almost dependent on the wild. Recently, numbers of wild thin-lipped mullet have been gradually declining. All farming is carried out using the fries in Egypt which increase fears of a further decline in this resource and sharp rise in price. Therefore, the establishment of a method of artificial propagation for thin-lipped mullet is needed to support the supply. Induced spawning using different hormonal injection is the common spawning induction technique in numerous fish species in aquaculture. Failure of the hormonal treatment has been observed in many cultured broodstock (Mylonas and Zohar, 2001), and has been attributed to the low levels of the GnRH-a in circulation due to the rapid metabolism and clearance of this peptide after injection (Gothilf and Zohar, 1991). Other studies reported that the presumed lack of gonadotropin in the circulation of captive fish could result from an insufficient amount of gonadotropin in the pituitary, inadequate secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus, or a combination of these reasons (Hill et al., 2005; Monbrison et al., 2003). To overcome possible failure along the endocrine axis controlling gonadal function, various hormonal treatments have been adopted to induce maturation and spawning. The effectiveness of injections to induce ovulation was evaluated by monitoring the percentage of ovulated fish, the ovarian maturation and ovulation after hormone treatment, fecundity and egg quality (Montchowui et al., 2011). The effect of injection on gonadal maturation was reported by Park (2002) in captivity. Higher or lower doses affected the egg quality, led to spawning failure or low output of hatching. Kagawa et al. (2013) documented variation in egg quality obtained from the female eels injected with exogenous hormones.

Lipids and their constituent fatty acids are utilized as energy sources throughout embryogenesis, and particularly in the later stages of development prior to hatching (Yanes-Roca et al., 2009). Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) and Archidonic acid (ARA) are the major fatty acids in the total lipid of eggs of most fish and these fatty acids markedly influence the reproductive parameters. There was a marked relationship between egg quality and fatty acid content of eggs. Successful fertilization of eggs and subsequent development of the offspring depend greatly on the quality of gametes produced by the parent fish. Fatty acids and particularly polyunsaturated fatty acids (PUFA) are functionally essential for normal growth, development and reproduction in fish (Sargent et al., 2002). Hormonal induction of final oocyte maturation and ovulation, however, can result in reduced egg quality. Ovulated eggs from fish injected with high dose of GnRH-a had lower fertility, survival to eyeing (eye-pigment formation stage) and hatching rates compared to eggs from fish injected with lower GnRH-a doses or from control fish (Mylonas et al., 1992).

The aim of the present study was to induce spawning of *L. ramada* and study the effect of multiple hormonal inductions on oocyte diameter and spawning performance and also, to study the fatty acid composition of captive normal and injected mullet ovaries with special reference to its effect on egg quality at different stages.

MATERIALS AND METHODS

Broodstock and broodstock management

The broodfish of *L. ramada* were captured in natural water of Mallahat Port Fouad during their migration to the spawning grounds in the Mediterranean Sea in December and transported to the marine hatchery. The broodfish were acclimatized indoors and stocked in well circulating aerated fiberglass tanks (3 m^3) with sea water supply of 15 L/min. Fifteen ripe broodfish samples (nine females and six males) were selected carefully. The total length of the females ranged from 34 to 47 cm, and total weight from 750 to 950 g. This study was carried out in the marine hatchery in the National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. Throughout the experimental period, the water temperature was 15 ± 2°C and salinity of sea water was 32 ± 1.5 ppt. Control non-injected females were subjected to the same rearing conditions. Air and fresh sea water were introduced into the bottoms of the tank. Roofing sheets were added to serve as shelter. Fish were not fed during the entire period of the experiment. A ripe female is deemed as ready for hormone injection when the mean diameter of the eggs is at least 600 µm (when they are in prime spawning condition).

Experimental design and hormone injection strategies

Two intramuscular injection strategies were adopted for *L. ramada* females in the present work. The first strategy was applied by two injections; the first one (priming dose) was carp pituitary extract of 20 mg per fish. After 24 h later, the second dose (resolving dose)

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Abbreviations: **EPA,** Eicosapentaenoic acid; **DHA,** docosahexaenoic acid; **ARA,** archidonic acid; **PUFA,** polyunsaturated fatty acids; **HCG,** gonadotropin hormone; **LHRH-a,** luetunizing hormone releasing hormone analogue; **CPE,** carp pituitary extract.

Table 1. The dosage, number of injections and egg diameters in the two different induction strategies.

S/N	Body weight (g)	Initial Egg diameter (0 h)	First strategy				Second stategy						Total No. of spawnd egg Million	% of Fertilized egg
			Dose		Egg diameter after 1st injection (µm) (24 h)	Egg diameter after 2nd injection (µm) (48 h)	Dose			Egg diameter after 1st injection (µm) (24 h)	Egg diameter after 2nd injection (µm) (48 h)	Egg diameter after 3rd injection (µm)		
			CPE	LHRH			HCG	LHRH 2 nd	LHRH 3 rd					
1	950	650	20	200	680	700	-	-	-	-	-	-	deformed	-
2	900	660	20	200	700	750	-	-	-	-	-	-	1.2 million (unfertilized)	-
3	750	650	-	-	-	-	3500	200	-	700	730	-	-	-
4	850	700	-	-	-	-	3500	200	100	750	790	830	1.8 million	75
5	880	700	-	-	-	-	3500	200	100	760	780	820	1.6 million	64
6	860	720	-	-	-	-	3500	200	100	760	780	800	1 million	52

was given as 200 µg/kg of luteinizing hormone releasing hormone (LHRH-a) (Table 1). The second strategy was also applied by two injections; the first one (priming dose) was 3500 IU of human chorionic gonadotropin (HCG) / fish. After 24 h later, the second dose (resolving dose) was given consisted of 200 µg/kg of LHRH-a (Table 1) and due to signs of incomplete spawning process after the resolving dose of the second strategy. The three successful spawning attempts occurred within 3 days when the female received an extra dose of LHRH-a (3rd injection) from the second breeding protocol (Table 1), thus all females were injected with 100 µm/kg LHRH-a as an extra dose of hormone for performing spawning. During the time of the resolving dose in both strategies, the males received only single dose of LHRH-a (100 µg/kg). Before injections, fish were anaesthetized using phenoxy-2 ethanol (40 mg/L) Sigma. After the resolving dose of the two strategies, the females were transferred with spermiating males (sex ratio 1:3 males to females in all spawning trials to optimize fertilization rates) to an indoor spawning tank and divided into two groups according to the two different breeding protocols of the injected females. Afterwards, the females were maintained together with active males and then some females spawned after 48 h. Prior to stocking of the fertilized eggs, production tanks were set up with filtered seawater adjusted to a temperature similar to that in the broodstock tanks. The fertilized eggs were added to production tanks with very light aeration and no water circulation in order to reduce water movement to the minimum.

The ripe females were characterized by the swelling of the soft and red abdomen and the protrusion of the genital papilla. Ovulation was checked by applying gentle pressure to the abdomen of female broodstock at intervals. At this time, the males were characterized by slim body shape and when squeezed on its abdomen the semen appeared and swim in unison with the females or circle around them. After spawning, floating fertilized eggs were gathered by egg collectors which are placed on the outside of each tank and then removed and incubated in circular incubators with extra oxygen which can be provided by aeration and the different embryonic developmental stages were documented by compound microscope with camera. Five samples of 1 ml from the spawning tanks containing fertilized eggs were examined under a light microscope to assess their fertilization rate and egg diameter and embryonic development. The spent breeders were removed from the tank or aquarium as soon as the spawning is over.

Gonadal biopsy

After anesthesia, the ovary of hormonal treated and control females were biopsied at different times after each treatment in order to monitor oocyte development and to determine the time of ovulation, since voluntary spawning was not expected. Measurements of oocyte diameters were carried out at 24 h intervals (0, 24, 48, and 72 h). Diameters of oocytes were measured with a hemacytometer under a light microscope. Fully-grown females were selected by using a polyethylene cannula (small tube) of 0.85 mm diameter that inserted into the oviduct of the anaesthetized female and a sample of the eggs withdrawn from the middle zone of the ovary using gentle aspiration. The sample of the eggs were fixed in a clearing fixative solution of ethanol, formalin, and acetic acid (6:3:1) allowing follicle separation and observation of cellular content and examined under a compound microscope in order to select fully grown oocyte females for hormonal treatment.

Fatty acid analysis

Two replicate samples of 1 ml of gonads were taken for both control and injected fish. Samples of gonads were carried out at first and second strategy during 1st , 2nd and 3rd injection. All samples were immediately freeze dried at -40°C. Before the assay was performed, dried tissues were ground to a powder individually. TL of each sample was extracted with chloroform–methanol (2:1, v/v), according to the method of Folch et al. (1957). Fatty acid methyl esters were prepared by transesterification with 0.4 M KOH–methanol, and then detected by gas chromatograph (GC-6890A, USA) following Huang et al. (2010). Fatty acid

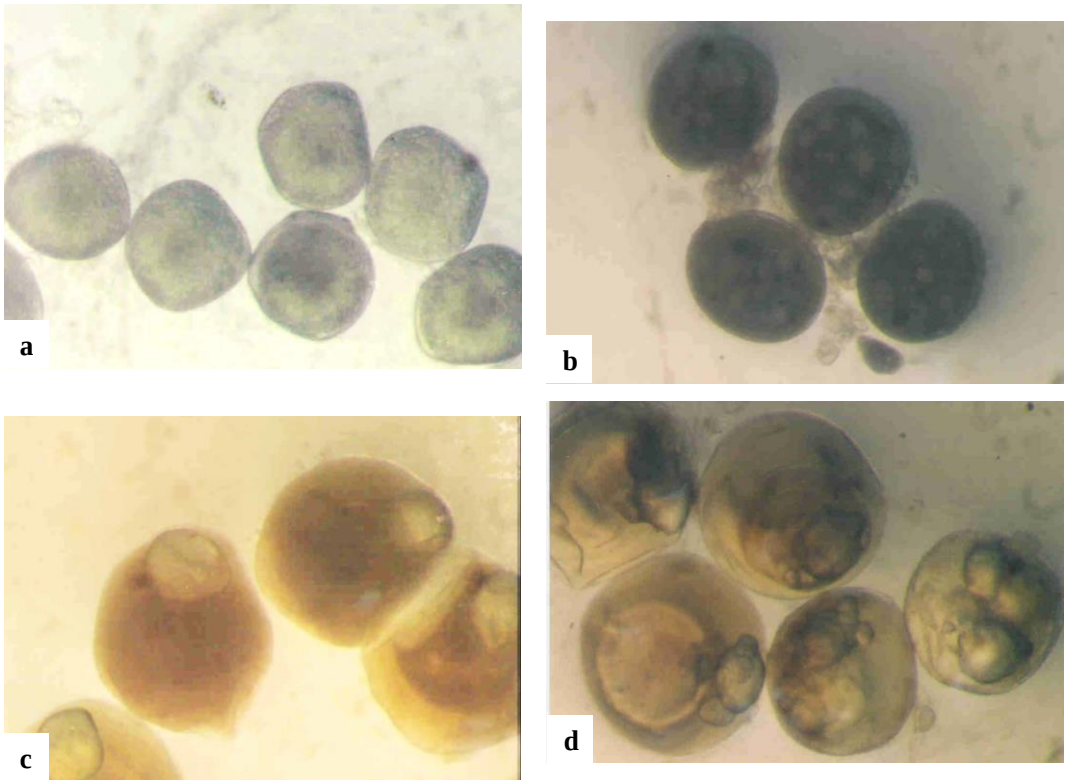

Figure 1. Photograph of cannulated eggs of *Liza ramada* from the ovary showing: a) cannulated eggs with diameters less than 600 µm, b) devoid eggs of control group, c) cannulated eggs of first strategy, and d) cannulated eggs of second strategy.

content was determined using the normalisation method. All measurements were taken in triplicate, and the fatty acids content was expressed as area percentage.

Statistical analysis

Statistical analysis was performed using ANOVA to detect significant differences between means of analytical data on first strategy and second strategy hormone treatment groups for both males and females. All results are presented as mean ± standard deviation.

RESULTS

At the beginning of the experiment, ovarian biopsy in some samples contained grown oocytes (post-vitellogenic stage) with diameters of 475 ± 40 µm having a central and opaque germinal vesicle and a granular cytoplasm (Figure 1a). In other fish samples, the biopsies contained oocytes with diameters of 560 ± 30 µm, having a heterogeneous cytoplasm and a coalescent central yolk vesicle and mature oocytes with a diameter of 600 ± 50 µm having a translucent cytoplasm, a migrating germinal vesicle, and a round yolk vesicle which are more appropriate to injection and spawning (Figure 1b). Accordingly, fish with egg diameters of 600 ± 50 µm were stocked in fiberglass aquaria of 3 m^3 sea water, while those less than 600 µm were excluded. Females were injected according to the schedule shown in Table 1. After first injection of the first strategy, the egg diameters ranged from 650 to 680 µm with clear oocyte center. After 24 h of the resolving dose, all fish contained ovulating oocytes with egg diameter of 700 ± 50 µm. Also, it showed the appearance of large oil droplets (Figure 1c). No response of spawning was noticed in all broodstock, but signs of ovulation were started in the abdomen of the females. After 48 h of the second injection of the first strategy, numbers of fish were spawned. The numbers of spawned eggs varied between 1 to 1.2 million/fish with no singe of fertilization. The spawned eggs varied in diameter between 700 ± 30 to 700 ± 50 µm respectively (Figure 2a, b and c). During the next 2 h, the cytoplasm become dense and more or less yellow in colour, all eggs were demerit in the bottom of the tank.

In the 2nd strategy, the females were having a minimum oocytes diameter of 790 µm. After 24 h from the third injection of an extra dose of hormone, cannulated ova ranged from 800 to 850 µm. The ovulating oocytes were presented with a migrating germinal vesicle, and a heterogeneous cytoplasm showing the fusion of oil droplets to a single oil droplet and more or less a coalescent central yolk vesicle (Figure 1d). After 48 h,

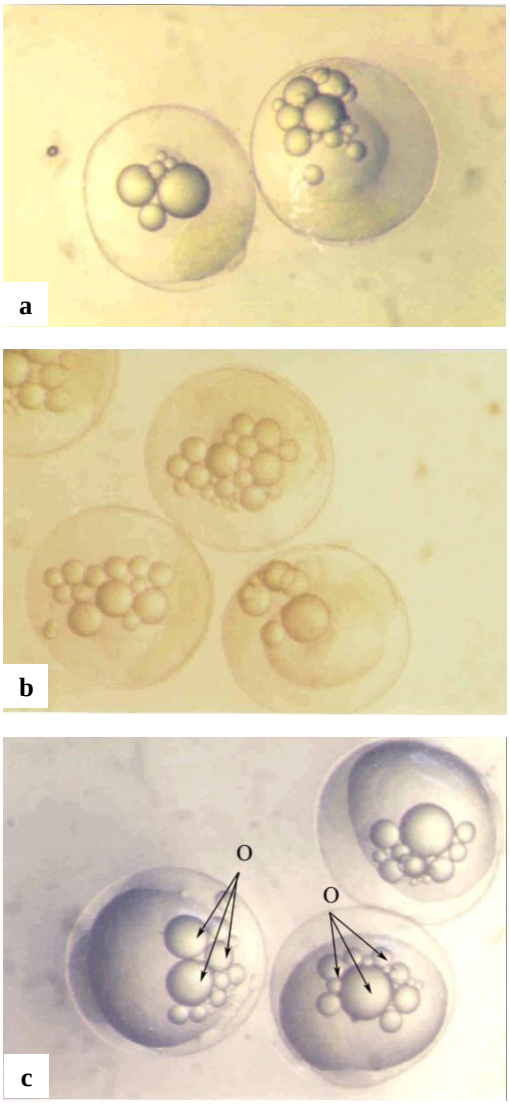

Figure 2. Photograph of spawned unfertilized eggs (a, b and c) having multi numbers of oil droplet (o) with egg diameter of 700 ± 50 μm (a, b) and of 700 ± 30 μm (c), after 48 h of the second injection of the first strategy.

the first three fish were successfully spawned with fertilization rates which were 1, 1.8 and 1.6 million eggs/spawn and the percentage of fertilization were 52, 75 and 64%, respectively, without hatching. All the fertilized egg reached the gastrula stage (Table 1). Control non-injected females subjected to the same rearing conditions did not spawn.

Embryonic development stages

The spawned fertile eggs of *L. ramada* were rounded, colorless and transparent with one oil globule. Thin-lipped mullet has a synchronous ovarian maturation and usually spawns only once a year. The surface of the fertilized egg shell is smooth, however; the yolk was unsegmented. After 20 min from fertilization the egg membrane swells up and separated from the previtelline space. The egg diameter was 800 ± 30 □m and water salinity was 34 ppt. The percent of fertilization varied between 52 and 75% without hatching. The stages take place as follows:

Germinal disc stage

After about 30 min from fertilization, the germinal disc (blastodisc) was in the form of cap-shaped at the animal pole and visible previtelline space.

Cleavage and morula stage

The first cleavage takes place at about 45 min from fertilization which divided the blastodisc into two blastomeres of approximately the same size (Figure 3a). After $1^{1/2}$ h, the second cleavage takes place at right angle to the first one to give four blastomeres. The third cleavage took place at age of about $2^{1/2}$ h after fertilization. This cleavage is parallel to the first one and producing eight blastomeres which were arranged in the 2 rows (Figure 3b). Cleavage continues but with irregular pattern forming a multicellular cap (morula stage (Figure 3c) at age of about 5 h after fertilization). The mean total diameter of egg was 850 ± 20 μ, the height of blastodermal cap was about 265 μm and the oil globule was 350 μ.

Blastula stage

After about 7 h of fertilization, the blastoderm was flattened out over the yolk forming a cellular cap (340 μm) and the total egg diameter was 865 ± 10 μm (Figure 3d).

Gastrula stage

This stage is characterized by cell movement by the epiboly process. The cells of blastoderm thickened forming embryonic shield and moved over the whole surface of the yolk (Figure 3e). The egg diameter was 880 μm, while at the end of gastrula stage, the egg diameter was 900 μm; the egg was recognized with a fold at the animal pole.

Organogenesis stage

At early stage of organogenesis the embryonic shield was clearly recognized with a thickening along the dorso-

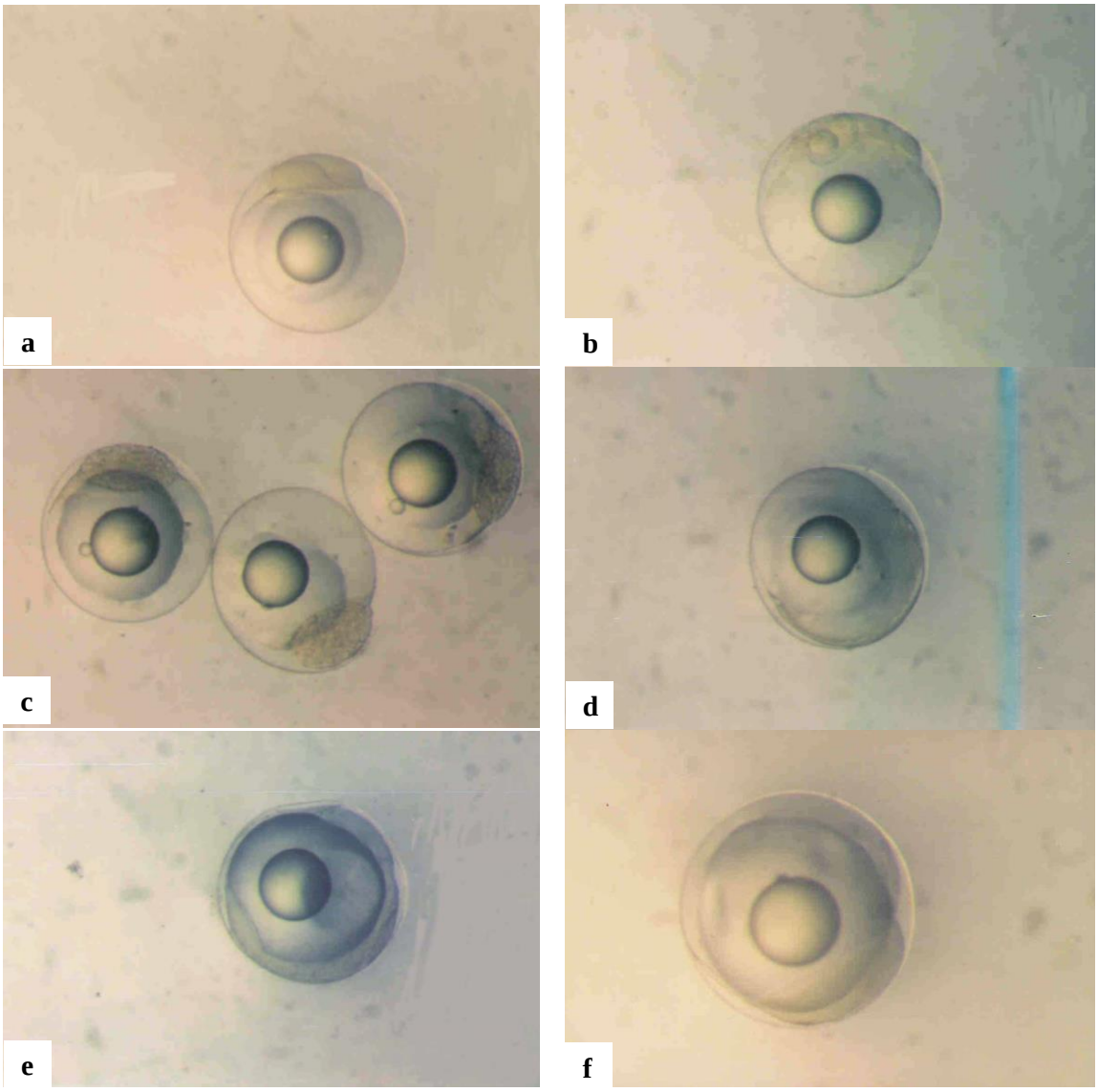

Figure 3. Photomicrograph of early embryonic stages in fertilized eggs with single oil droplet (until gastrula stage). **a,** First cleavage in fertilized eggs divided the blastodisc into two blastomeres of approximately the same size(x10). **b,** Third cleavage took place and producing eight blastomeres which were arranged in the 2 rows(x10). **c,** Cleavage continues with irregular pattern forming a multicellular cap, morula stage and blastomer formation (x10). **d,** Blastula stage: blastoderm was flattened out over the yolk forming a cellular cap(x10). **E,** Gastrula stage: The cells of blastoderm thickened forming embryonic shield and moved over the whole surface of the yolk.(x10). **F,** End of gastrula stage and beginning of early stage of organogenesis: the embryonic shield was clearly recognized. (x10)

lateral margins of the yolk after 8 h of post fertilization and with aggregation of cells but did not form any clear cut division or boundaries (Figure 3f). After this stage, shrinkage of the eggs and sinking at the bottom of the tank were observed.

Fatty acids profile in the gonads and eggs at the first and second strategy protocols in treated fish

Fatty acid compositions of *L. ramada* were determined during different stages of injected gonad (ovary and testes) and spawned eggs (fertilized and unfertilized eggs) at the first and second strategy as shown in Tables 2, 3, 4.

Tables 2 and 3 show the overall average fatty acid composition in ovary and spawned ova of *L. ramada* in the two injections strategies together with the range of individual fatty acids observed. Linolinic acid (ω3) and highest value of Σ ω6 fatty acid were recorded only in fertilized egg. The essential fatty acids (EPA, DHA, ARA, and PUFA) represented in un-respond ovary were

Table 2. Fatty acid composition of ovary and spawned ova of *Liza ramada* in the first injection strategies.

Fatty acid	Control (%)	1st Strategy (%)	Unrespond (%)	Unfertilized (%)
Leuric acid	0.15±0.005	0.25±0.006	ND	0.35±.02
Myristic acid	0.83±0.006	4.53±0.52	4.04±0.05	12.68±0.11
Palmitic acid	8.57±0.2	9.90±1.36	8.38±0.09	16.64±0.62
Margarinic acid	2.21±0.008	ND	0.92±0.02	3.97±0.40
Stearic acid	10.16±0.75	4.120.09	16.47±0.96	0.003±0.001
∑ Saturated acid	21.91	18.80$	29.81*	33.64**
Oleic acid	1.82	24.85	5.16	ND
∑ MonoSaturatedacid	1.82	24.85	5.16	ND
Linoleic acid ω6	ND	1.01	ND	2.58±
Linolinic acid ω3	ND	ND	ND	ND
Arachidonic acid ω6	2.17	ND	4.97±	1.98±0.40
Eicosapentaenoic acid ω3	ND	3.64±0.37	ND	1.06±0.14
Docosahexanoic acid ω3	0.23±0.003	0.24±0.02	0.05±	0.35±0.001
∑Polyunsaturated acid	2.41	4.90$	5.03**	5.97$
∑ω6	ND	1.01	4.97	4.56
∑ω3	0.23	3.89$	0.05*	1.41$
DHA/EPA	ND	0.07	ND	0.33
ARA/EPA	ND	ND	ND	1.87
∑ ω3 / ∑ ω 6	ND	3.85	0.01	0.31

$, Not significant; *significant; ** highly significant (p<0.05); nd, not detected.

Table 3. Fatty acid composition of ovary and spawned ova of *Liza ramada* in the second injection strategies.

Fatty acid	Control (%)	2nd strategy (%)	Spawn (%)	Fertilized (%)
Leuric acid	0.15±0.005	1.41±0.02	1.68±0.02	0.29±0.11
Myristic acid	0.83±0.006	ND	3.23±0.08	0.29±02
Palmitic acid	8.57±0.2	14.01±	26.27±1.08	8.62±0.73
Margarinic acid	2.21±0.008	1.60±0.05	3.13±0.2	2.24±0.66
Stearic acid	10.16±0.75	24.14±1.02	25.67	5.21±0.53
∑ Saturated acid	21.91	41.16**	59.97**	22.87*
Oleic acid	1.82	6.27	2.85	21.22
∑ MonoSaturatedacid	1.82	6.27	2.85	21.22
Linoleic acid ω6	ND	ND	1.37	0.87±0.02
Linolinic acid ω3	ND	ND	ND	0.17±0.0
Arachidonic acid ω6	2.17	3.07±	2.80	0.17±
Eicosapentaenoic acid ω3	ND	ND	1.24±	2.49±
Docosahexanoic acid ω3	0.23±0.003	0.35±0.009	0.23±0.3	0.39±
∑Polyunsaturated acid	2.41	3.42**	5.64$	10.90$
∑ω6	ND	3.07	4.17	7.86
∑ω3	0.23	0.35*	1.47$	3.04$
DHA/EPA	ND	ND	0.18	0.14
ARA/EPA	ND	ND	2.26	2.81
∑ ω3 / ∑ ω 6	ND	0.11	0.35	0.39

$, Not significant; *significant; ** highly significant (p<0.05); nd, not detected.

recorded as 1.06, 0.35, 1.98 and 5.97%, respectively (Table 2). Arachidonic acid recorded the highest value in un-respond ovary. The results show no significant in the un-respond ovary and unfertilized eggs in case of 1st

Table 4. Fatty acid composition of testes of *Liza ramada* in the second injection strategies.

Fatty acid	1st injection testes (%)	2nd injection testes (%)	Spawned (%)
Leuric acid	0.54±0.06	0.97±0.07	1.06 ± 0.47
Myristic acid	0.33±0.18	0.35±0.05	0.31 ± 0.10
Palmitic acid	33.14±2.77	12.22±1.07	1.55 ± 0.41
Margarinic acid	1.36±0.33	-	3.13 ± 0.81
Stearic acid	11.17±0.76	21.23±2.67	57.45 ± 5.62
∑ Saturated acid	46.51	34.77$^{\$}$	65.50$^{\$}$
Oleic acid	46.07 ± 1.02	-	3.48 ± 0.11
∑ Monosaturatedacid	46.07	-	3.82
Linoleic acid ω6	0.48 ± 0.07	0.43±0.07	-
Linolinic acid ω3	-	0.83±0.18	
Arachidonic acid ω6	2.91 ± 0.93	3.23 ± 2.55	-
Eicosapentaenoic acid ω3	2.24 ± 1.51	-	-
Docosahexanoic acid ω3	-	-	1.62 ± 0.62
∑Polyunsaturated acid	5.63	4.49$^{\$}$	1.62$^{\$}$
∑ω6	3.39	3.66$^{\$}$	-
∑ω3	2.24	0.83$^{\$}$	1.62$^{\$}$
DHA/EPA	-	-	-
ARA/EPA	1.29	-	-
∑ ω3 / ∑ ω6	0.66	0.22	-

strategy except in ARA and in MUFA (Table 2). In unfertilized eggs, the MUFA value was not detected while linoleic acid ω6 recorded the highest value (Table 2).

The results reported that essential fatty acids that affected egg quality such as EPA, DHA, ARA, PUFA and MUFA were recorded as 2.49, 0.39, 0.17, 10.90 and 21.22%, respectively, in the fertilized eggs of second strategy injection (Table 3). While fatty acid results revealed that mono-saturated fatty acid, oleic acid, was highly recorded than polyunsaturated fatty acid and highly unsaturated fatty acid. In the present study spawned ovary had a high record of saturated fatty acid (Table 3). Highly significant differences were recorded in first and second strategies between fertilized and unfertilized eggs particularly in saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acids and Eicosapentanoic acid (EPA) ω3 (Table 2, 3 and Figure 4). There are no differences in the level of DHA during different injection periods. The highest value of Σ ω6 fatty acid was recorded in the fertilized ova, while it does not record in control fish (Table 3). The ratio of (n3) / (n6) PUFA in the fertilized eggs and unfertilized eggs were 0.39 and 0.31, respectively (Table 2, 3 and Figure 4). Fatty acid results in the first strategy treated females were lower than that in the second strategy. The lowest PUFA was (22:6n-3, DHA) in both strategies (Tables 2 and 3).

Fatty acid composition of testes in male fish were determined during different stages and spawning testes of the second injection strategy only as shown in (Table 4 and Figure 5). The present results report that the mono-saturated fatty acid, recorded in the 1st injection stage 46.1% and spawned stage only 3.8%, while saturated fatty acid results revealed high level in 1st, 2nd injection and in spawned testes as (46.51, 34.7 and 65.5%, respectively). After 1st injection, the major essential fatty acid that affected the quality of EPA, ARA, and PUFA were recorded as 2.25, 2.91 and 5.63% respectively. There was no DHA value detected in this treatment. Docosahexanoic acid ω3 just detected with a low level in spawned male. In the present study, the level of arachidonic acid in 1st and 2nd injection was 2.91 and 3.23%, respectively. There was no trace of arachidonic acid in the spawned testes (Table 4 and Figure 5).

DISCUSSION

The present study demonstrates the effect of different hormonal protocols injection on spawning of *L. ramada* and fatty acid composition in gonads and eggs during experiment. Hormonal injections have been adopted to induce ovulation and spawning in *L. ramada* fish. Human chorionic gonadotropin hormone (HCG), Luetunizing hormone releasing hormone analogue (LHRH-a) and Carp pituitary extract (CPE) were used in various combinations and tested as spawning agents to increase the final oocyte maturation (FOM) and ovulation in captivity as reported by Duncan et al. (2003). Dosage of HCG and LHRH-a used in the present study were based on results of previous investigations as indicated by Monbrison et al. (2003). Other experiments revealed that

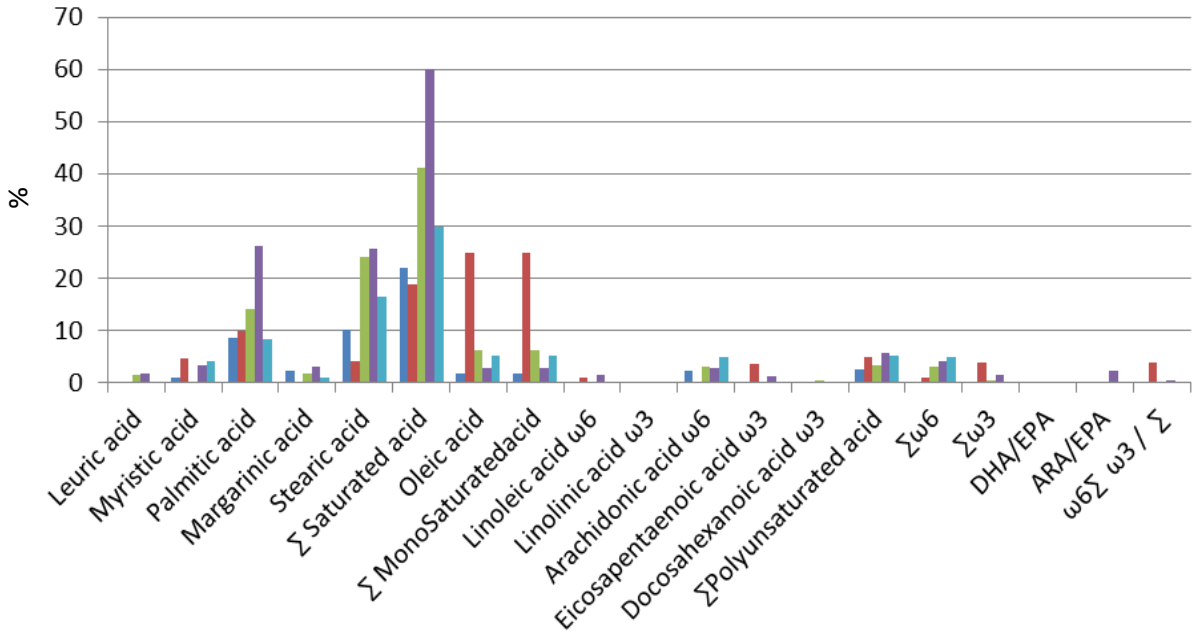

Figure 4. Fatty acid composition of ovaries of *Liza ramada* during different stages and spawned eggs in the two injection strategies.

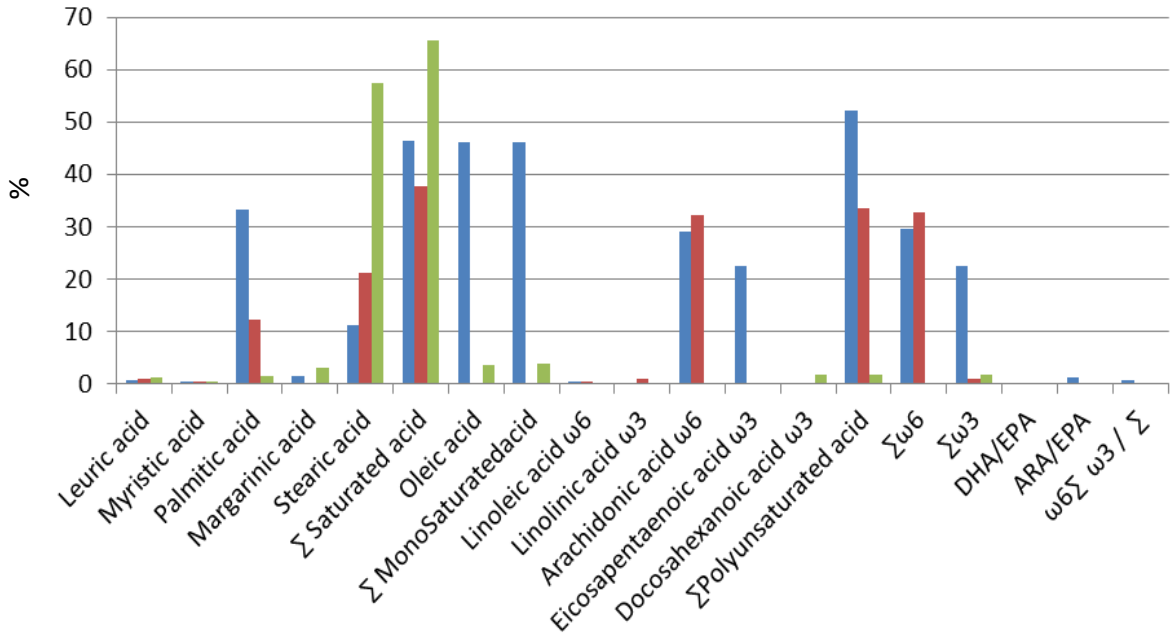

Figure 5. Fatty acid composition of testes of *Liza ramada* during different stages and spawning male in the second injection strategies only.

females grow faster than males because spermatogenesis occurs much faster than ooogensis during gonadotropin treatment (Ohta et al., 1997). Therefore, in our study, the males received only half dose of the females and it was necessary to start hormonal treatment in males later, after females have started.

Monbrison et al. (2003) indicated that the presumed lack of gonadotropin in the circulation of captive fish could result from an insufficient amount of gonadotropin in the pituitary, inadequate secretion of gonadotropin

releasing hormone (GnRH) from the hypothalamus, or a combination of these reasons to overcome possible failure along the endocrine axis controlling gonad function. The minimum egg diameter for successful induc-tion of spawning in grey mullet in our study was 600 µm as reported by El-Gharabawy and Assem (2006). They reported that female mullet were first observed to possess early vitellogenic oocyte in November and weeks later, vitellogenic had progressed to the point where spawning could be induced when mean oocyte diameter reach at least 600 µm. In contrast, Monbrison et al. (2003) indicated that once oocytes of grey mullet, *Mugil cephalus* reached the diameter of 500 µm females were induced to spawn with hormonal treatment.

In this study, the hormonal injection frequency had an influence on increasing the oocyte diameter particularly after 48 h of the last injection in the second strategy. The cannulated ova ranged from 800 to 850 µm and the ovulating oocytes were presented with a migrating germinal vesicle, with a single oil droplet. These results indicate that hormones injection frequency affects efficiency of nutrient transfer from liver to gonads (Naz, 2009). The nutrient reserves of the fish eggs are used by developing larvae both as substrate for energy metabolism and as structural component in membrane biogenesis (Sargent, 1995).

Marino et al. (2003), reported that in the induced breeding of the dusky grouper, the batch of the larger vitellogenic oocytes grew from 350-450 to 800-830 µm as a result of GnRH-a effect, and underwent completion of vitellogenesis, FOM and ovulation within 70 to 80 h, while a batch of smaller vitellogenic oocytes continued their vitellogenic growth and increased in diameter as a results of hormonal injection.

In this study, after 48 h from the second injection dose (resolving dose) in the first breeding protocol, only one fish spawned. The ovulated ova were about 1,000,000 their diameter varied between 700 and 730 µm. The spawned deformed unfertile eggs of *L. ramada* were rounded, colorless and transparent with more than one oil droplet. The survival of eggs which initially contained multiple oil droplets was always low (El-Gharabawy and Assem, 2006). After 48 h from the 3rd injection, the injected females were successfully spawned. The spawned fertile eggs were rounded, colorless and transparent with one oil globule with fertilization rates that varied between 1 and 1.8 million eggs/spawn and with average diameters of spawned eggs that ranged between 800 and 830 µm and the percentage of fertilization varied between 52 and 75 % but without hatching (until gastrula stage). In agreement with the present results, Lee et al. (1987) reported that some spawned egg diameter with more than 800 µm do not have big fertilization rate or no hatching rate. Ohta et al. (1996) showed that over-ripening after ovulation is one of the main factors affecting quality, but the percentage of buoyant eggs and fertilized eggs is usually low even if the eggs are stripped immediately after ovulation. These findings suggest that the cause of the poor quality is not only over-ripening after ovulation. In contrast with the present result, Lee et al. (1987) found that 94% of spawning success rate of fertilized ova was achieved with first strategy in which CPE is a priming dose followed by LHRH-a in a resolving dose. The spawned fertile eggs of *L. ramada* were rounded, colorless and transparent with one oil globule. The percent of fertilization varied between 52 and 75% but without hatching. The egg diameter was 880 µm, while at the end of gastrula stage the egg diameter reached 900 µm and the egg recognized with a fold at the animal pole. After this stage, shrinkage of the eggs and sinking at the bottom of the tank was observed then all eggs were died. This sudden egg mortality was believed to be due to excessive water turbulence in the incubation system (Main et al., 2004).

In the present work, during the time of the resolving dose in both strategies, the males received only single dose of 100 µg/kg (LHRH-a). In contrast with the present results, Shehadeh et al. (1973) concluded that grey mullet males were able to complete spermatogenesis without hormonal treatment. This information is very important for commercial hatcheries for optimum collec-tion of food quality eggs leading to higher larvae production (Sahoo et al., 2005). The present study demonstrates that the fatty acid compositions in gonads and eggs from second strategy group were significantly higher than the first strategy group ($p < 0.05$). The major fatty acid in the injected ovary that was treated with the second strategy injection were saturated fatty acid, monounsaturated fatty acid, polyunsaturated fatty acid, highly unsaturated fatty acid, (eicosapentaenoic acid (20:5n-3,EPA), docosahexaenoic acid (22:6n-3,DHA) and arachidonic acid (20:4n-6,ARA). It was determined that saturated fatty acid ratio is high in comparison with unsaturated fatty acid. Evans et al. (1998) suggested that the decline in the unsaturated to saturated fatty acids ratio, and total lipid indicates a reduction in egg membrane fluidity. Most significant depletions were observed in polyunsaturated fatty acids. Ballestrazzi et al. (2003), reported that the content of unsaturated fatty acids, particularly the ω3 fatty acid series (EPA and DHA) significantly decreased with increasing levels of saturated fatty acids. Among the PUFA, eicosapentaenoic acid (EPA, C20:5 n-3), docosahexaenoic acid (DHA, C22:6 n-3) and arachidonic acid (ARA, C20:4 n-6)), have been shown to play pivotal role in regulation of oocyte maturation and ovulation (Pickova et al., 2007).

In our study, the mono-unsaturated fatty acid was highly recorded in the fertilized eggs of the treated females with the second strategy injection. Results obtained in the study are supported by those of Bulut (2004). The high levels of the major fatty acids (mainly MUFA) found in the common snook shows their importance as energy store for embryonic development (Almansa et al., 2001). Fatty acid that results in the first

strategy treated females were lower than that in the second strategy and MUFA value was not detected in unfertilized eggs. In contrast, in the second strategy, fatty acid results revealed that the fertilized eggs contain more mono-unsaturated fatty acid and n-6 fatty acid than highly unsaturated fatty acid. Furuita et al. (2003), reports that higher levels of ω6 fatty acids have negative effect on egg quality. The ratio of (n3)/(n6) PUFA in the fertilized eggs and unfertilized eggs were 0.39 and 0.31, respectively. This ratio was very low compared to similar ratio in many other marine species (Naz, 2008; 2009). It is well documented that the (n3/n6) PUFA ratios in snook eggs is lower (2.52%) than the typical marine fish (2.9%) (Yanes-Roca et al., 2009). Deficiency or excess of ω3 highly unsaturated fatty acids (HUFA) has been found to depress egg quality of several species (Furuita et al., 2000; 2002). May be the hormonal manipulation impaired ovarian function that cause the result of the diminished of ω3 fatty acid particularly absence of linolenic acid which is the precursor of ω3 fatty acid series and reflecting the diminished ratio of ω3/ ω6 fatty acid. These fatty acids play an important structural role as components of phospholipids in fish bio-membranes and are associated with the membrane fluidity and correct physiological functions for bound membrane enzymes and cell functions in marine fish (Bell et al., 1986). Considering the relative low proportion of ratio of (n3)/(n6) PUFA, the low value should be attributed to the low value of 22:6n-3, DHA in the n3 PUFA in both strategies. Ferna´ndez-Palacios et al. (1995) observed poor hatching rates and survival in sea bream, linked to low fatty acid content. Also, Furuita et al. (2002; 2003) observed the same thing in Japanese flounder. In marine fish, EPA and especially, DHA are regarded as EFAs due to their necessity for good development and growth.

The results of the previous and the present experiment suggest that high levels of ω6 fatty acids, low levels of ω3 and low ratio of ω3/ ω6 may be the reasons of the negative effect on egg quality of *L. ramada* fish. In our results in males, mono-unsaturated fatty acid was recorded in the 1st injection and spawned stages. It was determined that monounsaturated fatty acid ratio is high in comparison with the spawned stage. In the same stage, the most significant depletions were observed in polyunsaturated fatty acids and DHA. Eicosapentanoic acid (EPA) (ω3) and DHA were recorded only in one different stage. In the present study, the level of arachidonic acid in 1st and 2nd injection were 2.91 and 3.23%, respectively and there was no trace of arachidonic acid in the spawned testes.

Izquierdo et al. (2001) reported that EPA and ARA modulate steiodogenesis in the testes and the timing of spermiation may be delayed and the fertilization rate reduced in the conditions of deficiency of these fatty acids. ARA stimulates testicular testosterone production in goldfish testes and ovaries by conversion to prostaglandin (Mercure and Van Der Kraak, 1996). In our study, the lower EPA, and DHA in both females and males of the 1st and 2nd injection strategy groups are indication of the negative effect of the embryonic development process. In general, a certain level and correct balance between the three essential fatty acids EPA, DHA and ARA seem to be important for successful reproduction and embryonic development. This finding can be used as an important and quick, diagnostic marker to predict the egg viability of wild *L. ramada* in order to save time and money (Bell et al., 1997).

Conclusion

In conclusion, in both strategies, human chorionic gonadotropin carp pituitary extract and LHRH can influence the developmental process and induce spawning of *L. ramada* and they have an effect on the biochemical composition during the development stages at different hormone injection protocols, spawning and fertilized eggs. The second intramuscular injection strategy (the priming dose (3500 IU HCG per fish and the resolving dose (200 then100 µg/kg LHRH-a) showed the best results for successful induce spawning with fertilized eggs and embryonic development but without hatching. Poor survival may be linked to low fatty acid content. In the second strategy, fatty acid results revealed that the fertilized eggs contain more mono-unsaturated fatty acid and n-6 fatty acid than highly unsaturated fatty acid that may have negative effect on egg quality. Deficiency or excess of highly unsaturated fatty acids ω3 (HUFA) has been found to depress egg quality of several species. The hormonal manipulation may be impaired ovarian function that cause the result of the diminished ω3 fatty acid particularly the absence of linolenic acid which is the precursor of ω3 fatty acid series and reflects the diminished ratio of ω3/ ω6 fatty acid. This finding can be used as an important and quick, diagnostic marker to predict the egg viability of wild *L. ramada.* Therefore, egg composition can be used as a possible index to evaluate broodstock physiological condition and subsequent egg quality. Further work may be needed to determine the whole fatty acid requirements of wild L. *ramada* fish at further production stages.

Conflict of Interests

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

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Full Length Research Paper

Pharmacognostic evaluation and antisickling activity of the leaves of *Securinega virosa* Roxb. ex Willd. (Euphorbiaceae)

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Securinega virosa **(Euphorbiaceae) together with condiments from natural sources serve as antisickling remedies in Nigeria. This study was aimed at establishing the pharmacognostic profile as well as the antisickling activity of the leaves of *S. virosa* Roxb. ex Willd (Euphorbiaceae). Evaluation of the fresh, powdered and anatomical sections of the leaves were carried out to determine the macromorphological, micromorphological and chemomicroscopic characters. Chemical tests were employed in phytochemical investigations. Evaluation of the antisickling activity involved the inhibition of sodium metabisulphite-induced sickling of the HbSS red blood cells obtained from confirmed sickle cell patients who were not in crises. Concentrations of the crude extract and its fractions were tested with normal saline and p-hydroxybenzoic acid serving as controls. Microscopical studies showed anomocytic stomata arrangement and glandular trichomes. Phytochemical evaluation revealed the presence of tannins, flavonoids, alkaloids, saponins and cardiac glycosides. Percentage sickling inhibitions of the aqueous methanol extract of *S. virosa* as well as all the fractions, except the petroleum ether fractions were significant all through the period of assay p < 0. 05 compared to normal saline. These results are suggestive of a potential role for *S. virosa* in the management of sickle cell disorders and a candidate for further investigations.**

Key words: *Securinega virosa*, euphorbiaceae, pharmacognostic standardization, sickle cell disorders.

INTRODUCTION

Despite all the progress in synthetic chemistry and bio-technology, plants are still an indispensable source of medicinal preparations, both for prevention and cure. Lifestyle and eating habit alterations among the people make it vital to refer to herbal medicines as an alternative or complementary therapeutic measure. Nearly 70% of the world's population (mainly in the developing countries) relies entirely on such traditional medical therapies as their primary form of health care. Various herbs are also part of the socio-cultural and socio-economic heritage. Even in the present times, rural populations turn to herbal medicine as the most preferred therapeutic source (Meena et al., 2012).

Sickle cell disease (SCD) is a potentially devastating

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condition that is caused by an autosomal recessive inherited hemoglobinopathy, which results in the hallmark clinical sequale of vasoocclusion. It is seen worldwide, but occurs most frequently in Africa and less commonly in those of Mediterranean, Latino, East Indian and Arab descent. It is estimated that 16% of the population in Africa has a sickle hemoglobinopathy which is the highest proportion worldwide. The Americas and the East Mediterranean region represent the next highest proportion of sickle cell hemoglobinopathy as delineated by the World Health Organisation (Angastiniotis and Modelle, 1998).

Despite a variety of antisickling agents acting at different levels of the sickling mechanism, there is still a paucity of antisickling medicines. This is because of the potential toxicities associated with most of these agents. Apart from the general mutagenic and carcinogenic tendencies of gene modifiers, hydroxyurea, a classical example that possesses antisickling activity, causes bone marrow suppression which greatly limits its use (Strouse et al., 2008). On the other hand, this is gradually paving way for the consideration of condiments from natural sources as antisickling remedies. The increasing interest in these condiments is not unconnected with the general innocuous nature of their sources, which most often are herbs and even at times food crops.

The genus *Securinega* (Family Euphorbiaceae) comprises more than 20 species, including *Securinega virosa* Roxb. ex Willd, which is found growing in moderately fertile, well drained soil in temperate and subtropical regions of the world. Common names include bushweed (English), Iranje (Yoruba), Njisinta (Ibo), Gussu (Hausa) and Kartfi-Kartfi (Shuwaarabs).

S. virosa has been investigated for its efficacy in other studies. Studies of the methanol extract of *S. virosa* leaves on streptozotocin-induced diabetic rats showed significant reductions in blood glucose levels (Tanko et al., 2008). The anti-diarrheal activity of the methanol extracts of the leaves, stem and root barks on castor oil-induced diarrheal model showed the leaves and root bark extracts possess pharmacological activity against diarrhea (Magaji et al., 2007). The n-butanol fraction of *S. virosa* root bark contains bioactive principles that possess anticonvulsant activities which may be beneficial against absence seizure, further lending credence to its ethnomedicinal use in the management of epilepsy (Magaji et al., 2013). The leaf extracts of *S. virosa* have high antioxidant activities, with the ethanol extract having the greatest antioxidant activity compared to the hexane and ethyl acetate extracts (Uzama et al., 2013). Two cytotoxic alkaloids, virosecurinine and viroallosecurinine have been isolated from the leaves of *S. virosa* (Kuo-Hsiung et al., 1991).

This study is the pharmacognostic and biological evaluation of *S. virosa* Roxb. ex Willd (Euphorbiaceae), one of the recipes which has been used with acclaimed success by traditional healers in Nigeria in managing Sickle Cell Anaemia.

MATERIALS AND METHODS

Preparation of plant extract

The leaves of *S. virosa* Roxb. ex Willd (Euphorbiaceae) were collected in Benin City, Edo State, Nigeria. The plants were authenticated by the curator at the Forest Research Institute of Nigeria (FRIN), Ibadan where voucher specimens were deposited with the Herbarium specimen number FHI109685. The fresh leaves were air-dried for 72 h and powdered using an electric mill.

Macroscopic and microscopic examination

The following macroscopic characters for the fresh leaves were noted: size and shape, colour, surfaces, venation, presence or absence of petiole, the apex, margin, base, lamina, texture, odour and taste (Wallis, 1985; Evans, 2006).

The outer epidermal membranous layer (in fragments) were cleared in chloral hydrate, mounted with glycerin and observed under a compound microscope. The presence/absence of the following was observed: epidermal cells, stomata (type and distribution) and epidermal hairs (types of trichomes and distribution). The transverse sections of the fresh leaves through the lamina and the midrib as well as a small quantity of the powdered leaves were also cleared, mounted and observed (African Pharmacopoeia, 1986).

Examination of the powder for starch grains, lignin, mucilage, calcium oxalate crystals, cutin and suberin were carried out using standard techniques (Evans, 2006).

Phytochemical investigation

Chemical tests were employed in the preliminary phytochemical screening for various secondary metabolites such as tannins (phenazone; iron complex; formaldehyde and modified iron complex tests were carried out on the aqueous extract to detect the presence of hydrolysable, condensed and pseudo tannins), cardiac glycosides (Keller - Killiani and Kedde tests were carried out on the methanolic extract to detect the presence of a deoxy sugar, whose natural occurrence is to date, known only in association with cardiac glycosides and to indicate the presence of a lactone ring on the cardenolides respectively), alkaloids (Mayer's, Dragendorff's, Wagner's and 1% picric acid reagents to detect the presence of alkaloidal salts and bases), saponins glycosides (frothing of the aqueous extract when shaken and haemolysis test on blood agar plates were carried out to indicate and confirm the presence of saponins), anthracene derivatives (Borntrager's test for combined and free anthraquinones, where aglycones were extracted using chloroform and shaken with dilute ammonia) and cyanogenetic glycosides (sodium picrate paper test were used to test for the presence of hydrocyanic acid in the sample. Conversion to sodium isopurpurate indicates the presence of cyanogenetic glycosides) (Evans, 2006; Brain and Turner, 1975; Ciulei, 1981; Harborne, 1992).

Extraction and fractionation

The powdered leaves of *S. virosa* (3.60 kg) were extracted with MeOH-H_2O (50:50). Evaporating the solvent yielded an extract (0.52 kg) which was subsequently re-suspended in water and successively partitioned into petroleum ether (3 X 2L), Chloroform (3 X 2L) and n-BuOH (3 X 2L).

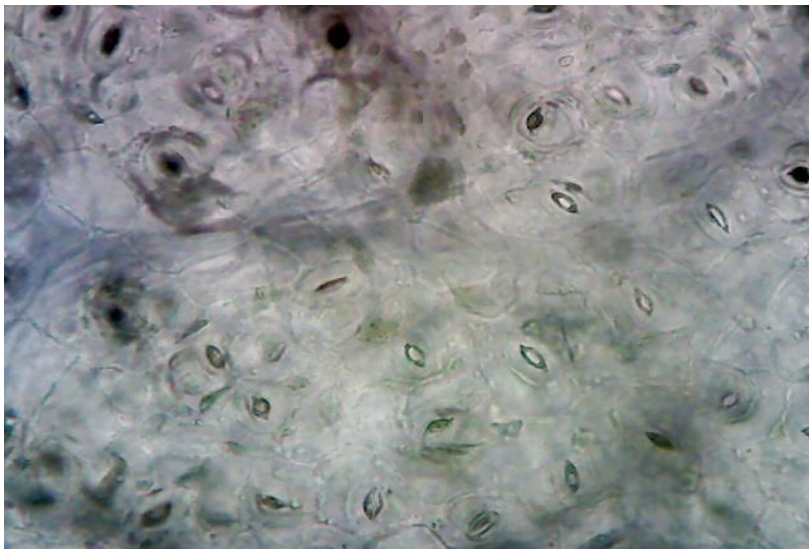

Figure 1. Diagnostic epidermal characters of *S. virosa* showing epidermal cells and stomata arrangement.

HbSS blood samples

HbSS Blood samples were collected by venipuncture from confirmed sickle cell patients not in crises on their clinic days at the Consultant Outpatient Department (COPD) of the University of Benin Teaching Hospital, Benin City, Nigeria. None of the patients used was recently transfused with HbAA blood.

Antisickling activity evaluation

The evaluation of the leaf extract and fractions of *S. virosa* for antisickling activities was carried out using a modified method of Moody and co-workers (Moody et al., 2003). Venipuncture blood samples from sickle cell anaemia patients not in crises were collected into EDTA bottles. Collected samples were centrifuged to remove the serum. The resulting packed erythrocytes were washed three times with sterile normal saline and centrifuged each time to remove the supernatant. A 0.5 ml sample of the washed erythrocytes were mixed each with 0.5 ml of the different concentrations of the aqueous methanol extract of *S. virosa* (100, 300 and 500 mg/ml) or fractions (500 mg/ml) in uncovered test tubes. A 5 mg/ml solution of ρ-hydroxybenzoic acid (PHBA) in normal saline was used as the positive control while normal saline served as negative control. Samples were taken from the different mixtures and the remaining portions of the mixtures incubated for 3 h, shaken twice during the period.

A 0.5 ml sample of 2% sodium metabisulphite was added to each mixture to deoxygenate the system, mixed thoroughly and sealed with liquid paraffin. Samples were taken in quadruplicates from the different mixtures at 0 min and at subsequent 30 min interval until seven readings were obtained.

Each sample was smeared on a microscopic slide, fixed with 95% methanol, dried and stained with giemsa stain. Each slide was examined under the oil immersion light microscope and counting of 100 red cells in each sample. The numbers of both sickled and unsickled red blood cells were counted and the percentage of unsickled cells determined.

Statistical analysis

Data are expressed as mean ± SEM. The differences between the means were analyzed using one way analysis of variance (ANOVA). Values of $P < 0.05$ were taken to imply statistical significance between compared data.

RESULTS

Macroscopic description

S. virosa leaves are simple, petiolated with variable shapes, obovate or orbicular. The margin was entire, apex acuminate, base cuneate and venation was reticulate. Average leaf size was 6. 3 cm ± 0. 8 (length) and 3. 5 cm ± 0. 3 (breadth). The taste and odour of the fresh leaf was characteristic.

Microscopic description

Micromorphological features revealed that anticlinal walls were thick and straight that is have straight epidermal cells. Stomata were present in both lower and upper epidermi. The stoma had two subsidiary cells with their long axis parallel to the pore and sometimes a 3rd subsidiary cell, indicating paracytic stomata arrangement (Figure 1). Glandular trichomes (Figure 2) were present on both surfaces.

A transverse section of the leaf across the mid-rib showed an upper and lower epidermi consisting of cells of similar sizes. It had an isobilateral structure that is both surfaces are identical. The mesophyll, consisting of upper and lower palisade layers and a median spongy mesophyll embedded a crystal sheath. The mid-rib bundle was surrounded by a zone of collenchyma cells on both surfaces. The phloem vessels embedded the

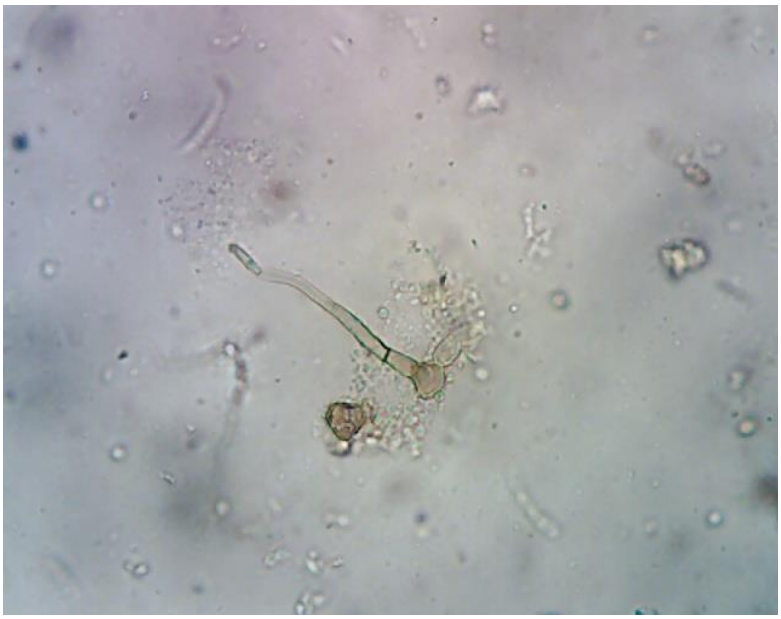

Figure 2. Glandular trichomes of *S. virosa*.

Table 1. Phytochemical constituents of *S. virosa* leaves.

Classes of secondary metabolites	Inferences
Alkaloids	+
Tannins	+
Flavonoids	+
Anthracene derivatives	-
Saponin glycosides	+
Cardiac glycosides	+
Cyanogenetic glycosides	-

- = Absent; + = present.

xylem vessels. Chemomicroscopic examination of the leaves revealed the presence of starch, calcium oxalate crystals, mucilage, tannins and cellulose.

Phytochemical screening

Phytochemical screening of the leaves of *S. virosa* for secondary plant metabolites revealed the presence of alkaloids, tannins, flavonoids, saponins and cardiac glycosides (Table 1).

Sickling inhibitory activities of crude extracts and fractions of *S. virosa*

Percentage sickling inhibition of the various doses of *S. virosa* extracts and fractions were significant all through the period of assay $p < 0.05$ compared to normal saline, except for petroleum ether fraction (Table 2); $n = 4$.

DISCUSSION

The types and distributions of Pharmacognostic characters in plants aid in their classification and identi-fication. Before any crude drug can be included in a Herbal Pharmacopoeia, pharmacognostic parameters and standards must be established (Abere et al., 2007). The results of these pharmacognostic investigations could, therefore serve as a basis for proper identification, collection and investigation of the plant. The macro- and micromorphological features of *S. virosa* described, distinguishes it from other members of the genera. These features described in this study are in tandom with those found in the Euphorbiaceae family (Inamda and Ganggadhara, 1978).

The Pharmacological activities of a given plant are associated with the type and nature of secondary plant metabolites present. The need for phytochemical screening has become imperative, since many plants accumulate biologically active chemicals in their tissues. Phytochemical evaluation of *S. virosa* revealed the presence of tannins, flavonoids, alkaloids, saponins and cardiac glycosides. These compounds detected in the plant are known to possess medicinal properties and health promoting effects (Danlami et al., 2013).

The *in-vitro* technique adopted in the antisickling efficacy bioassay was based on the simulation of the major *in-vivo* sickling-precipitating factor (that is, reduction of oxygen tension), using sodium metabisulphite as a physiologically acceptable reducing agent. The use of erythrocyte suspension instead of whole blood was particularly essential in ruling out the possibility of interactions of plasma component and products of their

Table 2. The sickling inhibitory activities of *Securinega virosa* crude extracts and fractions.

Time of incubation (min)	Percentage inhibition (%)							
	A	B	C	D	E	F	G	H
0	32.0±0.12	55.6±1.04	53.5±0.24	65.5±1.59	75.0±0.32	60.9±1.02	86.4±0.49	87.6±1.12
30	31.0±0.26	60.2±0.23	52.5±1.77	63.5±1.38	71.5±0.78	67.1±1.73	88.0±1.36	75.5±1.45
60	30.0±0.37	54.0±1.15	54.5±0.40	69.3±0.73	70.5±0.53	80.0±0.21	87.5±1.05	81.6±0.67
90	29.0±1.01	50.0±0.09	62.2±1.13	70.1±0.18	76.0±1.80	76.0±0.44	89.6±1.30	85.0±1.80
120	27.0±0.20	46.0±0.74	55.5±0.39	67.5±1.41	65.0±0.47	74.0±0.83	86.7±0.91	70.3±0.22
150	25.0±0.71	38.2±1.16	54.5±0.14	69.5±1.12	67.0±0.90	79.0±1.05	76.0±1.15	69.3±0.18
180	25.0±1.22	32.4±1.70	51.0±1.52	56.0±0.35	74.4±1.04	73.0±1.30	79.0±0.33	73.5±1.05

A= Blood + normalsaline + sodium metabisulphite; B = blood + PHBA + sodium metabisulphite; C = blood + crude extract of *S. virosa* leaf at 100 mg/ml + sodium metabisulphite; D = blood + crude extract of *S. virosa* leaf at 300 mg/ml + sodium metabisulphite; E = blood + crude extract of *S. virosa* leaf at 500 mg/ml + sodium metabisulphite; F = blood + chloroform fraction + sodium metabisulphite; G = blood + N-butanol fraction + sodium metabisulphite; H = blood + aqueous fraction + sodium metabisulphite. The results of the petroleum ether fraction were indeterminable.

several immunological reactions and certain metabolic co-factors in general with the red blood cells (Coker et al., 2007). Such interactions could significantly affect the shape and size of red blood cells and in the process inadvertently produce false negative or false positive results. The aqueous methanol extracts of *S. virosa* showed significantly inhibitory effect at the concentrations (100, 300 and 500 mg/ml) on sodium metabisulphite-induced sickling.

The fractions of the crude extract of *S. virosa* inhibited sodium metabisulphite induced sickling of HbSS red blood cells to varying degrees. The inhibitory activity of *S. virosa* could be due to the presence of bioactive compounds. Phenolic compounds which are present in *S. virosa* have been reported to possess antioxidant activity. Antioxidants have been reported to be major components of medicinal plants with known antisickling activity (Tatum and Chow, 1996). The antisickling activity could be linked to their ability either to inhibit *in-vitro* polymerization of haemoglobin or to some structural modification linked to the environment of haemoglobin by the extracts (Bianchi et al., 2007).

Conclusion

The pharmacognostic parameters of *S. virosa* which have been reported could be useful in its standardization. On the basis of the biological results, aqueous methanol extracts as well as the chloroform, n-butanol and aqueous fraction of *S. virosa* have been found to possess an antisickling activity, indicating that it has a role in the treatment of sickle cell disorders and a good candidate for further investigations.

Conflict of Interests

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

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