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African Journal of Biotechnology

Full Length Research Paper

Polymorphism analysis of kisspeptin (KISS1) gene and its association with litter size in Ethiopian indigenous goat populations

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Polymorphism analysis of the Kisspeptin (KISS1) gene and its association with litter size were conducted in two (Gondar and Woyto Guji) Ethiopian indigenous goat populations. Three new pairs of primers were designed for touchdown PCR condition. A total of 173 and 242 Sanger sequences were employed for further analysis of exon1 (1210 bp) and exon2 (325 bp) regions of the KISS1 gene, respectively. In addition, the identified polymorphic sites were associated with litter size of the animals included in the study. General linear model was carried out for the association analysis. The study revealed that five complete substitutions and 15 polymorphic sites were detected in both exon regions with 2:1 transition and transversion ratio. Most of the polymorphic sites were observed in more than 1% of the sequences qualifying the single nucleotide polymorphisms (SNPs) definition. The overall average codon bias index was 0.301 with 64.09 and 62.47% GC contents in exon1 and exon2, respectively. The overall average H_E was 0.18863±0.21 for exon1 and 0.03155±0.01. Only four loci, which constitute 10 genotypes, from the polymorphic sites showed significant contribution of litter size of the goat populations studied. Heterozygous genotypes (TC and GC at g.950T>C and g.3416G>C, respectively) had performed highest estimates of litter size in three of the loci which contributed for litter size. However, the homozygous genotype observed at locus g.3811C>T showed highest contribution for fecundity trait. 18 to 31% performance differences of litter size were observed due to the influence of the genotypes. In conclusion, the SNP detected in those target regions of the gene confirm the contribution of the KISS1 gene for fecundity trait suggesting importance of the gene for marker assisted selection in goat breeding programs.

Key words: Goat, litter size, KISS1 gene, polymorphism.

INTRODUCTION

Goats, with their diverse genetic resources, play fundamental role for the national economy of Ethiopia. They contribute about 12% of the total annual meat production and together with sheep; they contribute about 90% of the live animal/meat and 92% of the total skin export trade value (FAO, 2004). Goats in Ethiopia are reared by resource poor farmers. Particularly, in rural livelihood, goats are potential livestock species to meet the nutritional needs of the people, and considered as the “Future Animal” not only for rural and but also urban prosperity (Ahlawat et al., 2016).

Recent genomic researched have focused on screening of genes that contribute for expression of economic traits. Reproduction traits are among crucial economic traits in animal husbandry, and are coordinated during normal puberty and the adulthood (Zhang et al., 2011). Among reproduction traits, genetic studies have indicated that litter size and ovulation rate can be genetically determined by the action of genes (Deldar-Tajangookeh et al., 2009). However, it is stated that, not only for fitness traits (for example litter size), the identification of candidate genes that are responsible for variation in continuous traits (for example growth traits) has been a challenge in modern genetics (An et al., 2013). As effect, to date, little has been divulged on the major genes associated with, for instance, litter size in goats. It has been previously shown that KISS1 gene highly contributes for multiple births in goat (An et al., 2013; Othman et al., 2015). KISS1 gene is a gene that encodes Kisspeptin (formerly known as metastin) protein (Gottsch et al., 2009), and is located on the long arm of chromosome 1 (1q32) (Messager et al., 2005). It is expressed in the hypothalamus region of the forebrain (Gottsch et al., 2009) and is a G-protein coupled receptor ligand (GPR54) (Messager et al., 2005). Kisspeptin-GPR54 signaling has an important role in initiating secretion of gonadotropin-releasing hormone (GnRH) (Dungan et al., 2006; Smith et al., 2006). Signaling between kisspeptin and its receptor, GPR54, is now recognized as being essential for normal fertility by regulating the reproductive system (De Roux et al., 2003; Funes et al., 2003; Kirilov et al., 2013). In this line, beside to the *pulse mode*, that is effected by the secretion of the reproductive neuropeptide gonadotropin releasing hormone (GnRH) which is essential for reproductive events in both sexes (like spermatogenesis, follicular development, and sex steroid synthesis), the *surge mode* of GnRH induces ovulation in females (Okamura et al., 2013).

It has also been noted that the central or peripheral administration of kisspeptin stimulates GnRH-dependent luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion in various mammalian species from rodents to humans and it also administers reproductive functions of animals (Gottsch et al., 2004; Navarro et al., 2005; Shahab et al., 2005). Gottsch et al. (2009) also indicated that neurons that express KISS1 play a crucial role in the regulation of pituitary luteinizing hormone secretion and reproduction, and these neurons are the direct targets for the action of estradiol-17β (E_2), which acts via the estrogen receptor α isoform (ERα) to regulate KISS1 expression. Kisspeptin/neurokinin B/dynorphin (KNDy) neurons located in the hypothalamic arcuate nucleus might play a central role in the generation of GnRH pulses in goats, and perhaps in other mammalian species (Okamura et al., 2013). In the arcuate nucleus (Arc) where the dynorphin gene (Dyn) is expressed in KISS1 bearing neurons, E_2 inhibits the expression of KISS1 mRNA; however, E_2 induces the expression of KISS1 in the anteroventral periventricular nucleus (AVPV) (Gottsch et al., 2009). ERα signals through multiple pathways, which can be categorized as either classical involving the estrogen response element (ERE), or non-classical, involving ERE-independent mechanisms. However with these all regulatory functions of the KISS1 gene and its receptor, failure or loss of function or deletion of, for instance, GPR54 causes to hypogonadotropic hypogonadism (De Roux et al., 2003; Chu et al., 2012). It is a deficiency of the pituitary secretion of follicle-stimulating hormone and luteinizing hormone which cause impairment of pubertal maturation and reproductive function.

In general, despite the limited efforts done in small ruminant livestock so far, the expression and regulation of KISS1 gene plays magnificent role in multiple births in goat. This study might serve as an additional input for further evaluation and utilization of the gene in marker assisted breeding program. Therefore, this study was initiated to evaluate the polymorphic nature and role of KISS1 gene on litter size in two Ethiopian indigenous goat populations.

MATERIALS AND METHODS

The study goat populations

Two indigenous goat populations of Ethiopia, Gondar and Woyto Guji, were selected for the study. The two populations are found in

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the extreme north and south corners of the country (Supplementary Figure S1). The two populations are kept under different production systems and agro-ecology. Woyto Guji goat population is found in arid environment. The production system is categorized predominantly by pastoral production system with some crop production (Workneh, 1992; Netsanet, 2014); whereas, Gondar goat population is found in mid to high altitude (1000 to 3000 m.a.s.l.) areas. The area is characterized by mixed crop livestock production system. Gondar goat has 13.58(± 6.44) months of age at first kidding, 8.58±2.02 months kidding interval and 72.18% and 6.38% of frequency of twine and triple births, respectively (Alubel, 2015); whereas Woyto Guji goat has 21.97±0.41months of age at 1st kidding, 6.79 ±0.10 months kidding interval and 1.33 litter size: 1.33 (Netsanet, 2014).

The blood samples were drawn out from the jugular vein with a volume of nine milliliter under aseptic conditions using ethylenediaminetetraacetic acid (EDTA) anticoagulant. The collected samples were brought to the laboratory with ice box and were stored at -20°C until it was subjected to DNA extraction. Salting out protocol was used for the DNA extraction (Shinde et al., 2008). The DNA quality and concentration were tested by nanodrop, and 1.0 to 1.5% agarose gel electrophoresis was used to evaluate the degradation.

Primers employed, target regions and PCR conditions

To amplify the two exon regions of KISS1 gene, three pairs of primers were newly designed from sequences of *Ovis aris (*Acc:HGNC:6341*)* and C*apra hircus (Acc.* GU142847.1) KISS1 genes obtained in the data base, DNA Data Bank of Japan (DDBJ) and were evaluated by Oligo Analyzer 3.1: Integrated DNA technology package (available at: https://eu.idtdna.com/Analyzer/Applications/OligoAnalyzer/default.aspx?err=true) (Supplementary Table S1). The first exon (exon1) has a length of 1,210 bases and exon2 has 325 bases. For both exons, touchdown PCR program was employed with the respective PCR conditions indicated at Supplementary file1 Figure S2. The PCR products, in both directions, were sequenced at Bioscience for eastern and central Africa-International Livestock Research Institute (BecA-ILRI) hub, Nairobi, Kenya. Sample gel image is indicated at Supplementary Figure S3. Sanger sequence products were aligned with complete *cds* of Jining Grey goat population kisspeptin (KiSS-1) gene available in the GenBank (acc. GU142847, ver. GU142847.1).

A total of 173 animals (DNA sequences), 58 of which were from Gondar goat population and 115 from Woyto Guji goat population, were used to analyze exon1; whereas, 250 DNA sequences (Gondar 117 and Woyto Guji 133) were used for exon2, and 242 samples used for the final analysis for the latter exon. The different number of animals which were used for the two exon regions is because some of sequences of exon1 showed poor or low chromatogram peaks and excluded from the analysis. The phenotype data were extracted from the field monitoring activity of the community based breeding program which was started in 2013. The phenotype data (breed type, parity and birth type) were associated with information of the polymorphic regions.

Data management, statistical analysis and packages employed for analysis

CLC main workbench 7.0.4 (CLC Bio-Qiagen) and SeqTrace ver 9.0 (Stucky, 2012) packages were used to generate trace files and visualize the chromatograms. Bioedit and MEGA 6 (Tamura et al., 2013) were employed to correct possible base calling errors and to align the sequences together with the reference sequence of Jining Grey goat sequenced by Cao et al. (2010). Multiple sequence alignments were done in CLC Workbench with ClustalW algorithm (Thompson et al., 1994). Segregating sites (SNPs) were used to analyze the association study using SAS ver.9.1.

General linear models were used to analyze the association of genotype with phenotype performances of the populations studied. (i) Exon1: $Y_{ijklmn} = \mu+P_i+B_j+G1_k+G2_l+G3_m+e_{ijklmn}$; where, P = Parity at i^{th} (i= 1, 2, 3, 4, 5) parity of the doe gave birth; B=Population at j^{th} population (j = 1, 2); G_1= $Site_1$ at k^{th} genotype (k = GG, GC); G_2 = $Site_2$ at l^{th} genotype (l = CC, CT, TT); G_3 = $Site_3$ at m^{th} genotype (m = CC, TC, TT). (ii) Exon2: $Y_{ijkl} = \mu+P_i + B_j +G_k+ e_{ijkl}$. Parities after the fifth parity were merged as the fifth parity because of fewer observations and non-significant influence of parties after the fifth parity. Genotypes other than indicated in the model were excluded from the model since they showed non-significant effects and did not contribute for fitness (coefficient of determination-R^2) of the model.

RESULTS

Detection of single nucleotide and codon usage

In this study, complete substitutions with respect to the reference sequence (Gene bank: acc. GU142847) were observed at five sites (at g.3436 T >C, g.3592C>A, g.3688A>C, g.3878 A >C and g.4023A>C), of which four of them were *transversions*. In addition to the compete substitutions, 12 polymorphic sites in exon1 and three in exon2 were detected (Table 1) in which ten of them (at 945, 950, 3354, 3533, 3649, 3696, 3808, 3811, 3963 and 3989 loci) were *transition* mutations and the remaining five were *transversion* mutations. These polymorphic regions generated 31 haplotypes in which four them (linkage combination at 3649, 3808, 3963 and 3989 loci) showed highest linkage disequilibrium accumulations.

Twelve polymorphic sites were observed in more than 1% of the sequences. Moreover, from the usable nucleotides, in both Gondar and Woyto Guji goat populations, the GC contents were 64.09% for exon1 and 62.47% for exon2. In line with this, the codon bias index was 0.301. Codon observed in five polymorphic sites (g.3354A>G, g.3696C>T, g.3783T>A, g.3927C>G and g.4023A>C) were degenerative codons and codons at five polymorphic sites have small number of observations and excluded from the association analysis though they qualified the SNP definition. Hence, only four loci (g.950T>C; g.3416, g.3811 C>T and g.3963T>C) were remained for the association analysis. From these four screened SNPs 10 genotypes were obtained (Table 2). These are TT and TC genotypes at g.950T>C, CC and GC at g.3416G>C, CC, TC and CC genotypes g.3811C>T and TT, TC and CC genotypes at g.3963T>C.

Amino acid substitutions and their association to litter size

In exon1, from the five sites where complete nucleotide

Table 1. Detected SNPs and IUPAC symbols (N).

Locus (>)	Nucleotide in the reference sequence	IUPAC symbol (N)	Nucleotide substituted (N)	Nucleotide similar with the reference (N)	Total
g.0895G>C	G	-	C(239)	G(3)-	242
g.0945C>T	C	-	T(3)	C(239)	242
g.0950T>C	T	Y(6)	-	C(236)	242
g.3354A>G	A	R(1)	G(172)	-	173
g.3416G>C	G	S(8)	C	G(165)	173
g.3436T>C	T	-	C(173) †	-	173
g.3533A>G	A	R(1)	-	A(172)	173
g.3592C>A	C	M(173)	A†	-	173
g.3649G>A	G	R(51)	A(41)	G(81)	173
g.3688A>C	A	M(173)	C†	-	173
g.3696C>T	C	Y(1)	-	C(172)	173
g.3770G>C	G	S(1)	C	G(172)	173
g.3783T>A	T	W(2)	A	T(171)	173
g.3808G>A	G	R(53)	A(58)	G(62)	173
g.3811C>T	C	Y(22)	T(3)	C(148)	173
g.3878A>C	A	M(173)	C†	-	173
g.3927C>G	C	S(2)	G	C(171)	173
g.3963T>C	T	Y(38)	C(4)	T(131)	173
g.3989G>A	G	R(54)	A(39)	G(60)	173
g.4023A>C	A	M(173)	C†	-	173

†Complete substitution in all sequences of the study populations; IUPAC = International Unit for Pure and Applied Chemistry.

Table 2. Amino acid changes observed in polymorphic sites.

Locus	Amino acids and codons in the reference sequence		Amino acids and codons in sequences studied		Amino acid position	N	Remark
	Amino acid	Codon	Amino acid	Codon			
g.0895G>C	Arginine	CGG	Glycine	GGG	R0298G	3	
g.0945C>T	Tryptophan	UGG	Cysteine	UGU	W0315C	3	
g.0950T>C	Stop codon	UAG	Tryptophan	UGG	*0317W	6	**
g.3354A>G	Cysteine	UGC	Cysteine	UGU	C1118C	1	
g.3416G>C	Alanine	GCC	Glycine	GGC	A1139G	8	**
g.3436T>C	Serine	AGC	Glycine	GGC	S1146G	173	
g.3533A>G	Methionine	AUG	Threonine	ACG	M1179T	1	
g.3592C>A	Alanine	GCC	Serine	UCC	A1198S	173	
g.3649G>A	Cysteine	UGU	Arginine	CGU	C1217R	94	
g.3688A>C	Stop codon	UAG	Glutamic acid	GAG	*1230E	173	
g.3696C>T	Threonine	ACA	Threonine	ACG	T1232T	1	
g.3770G>C	Serine	UCG	Tryptophan	UGG	S1257W	1	
g.3783T>A	Proline	CCA	Proline	CCU	P1261P	2	
g.3808G>A	Histidine	CAC	Tyrosine	UAC	H1270Y	111	
g.3811C>T	Glutamic acid	GAA	Lysine	AAA	E1271K	25	**
g.3878A>C	Valine	GUG	Glycine	GGG	V1293G	173	
g.3927C>G	Threonine	ACG	Threonine	ACC	T1309T	2	
g.3963T>C	Isoleucine	AUA	Methionine	AUG	I1321M	42	**
g.3989G>A	Serine	UCA	Leucine	UUA	S1330L	93	
g.4023A>C	Valine	GUU	Valine	GUG	V1341V	173	

N = number of individuals the mutations observed at the respective locus; **= contributed for litter size.

Table 3. Least square mean standard error (LSM±SE) estimation of fecundity trait.

Exon1			Exon2		
Factors	**N**	**LSM±SE**	**Factors**	**N**	**LSM±SE**
Overall mean	177	1.403±0.12	**Overall mean**	242	1.435±0.09
Parity		***	**Parity**		***
1	40	1.307± 0.14[c]	1	50	1.219±0.10[e]
2	34	1.328± 0.15[c]	2	47	1.300±0.11[d]
3	39	1.614±0.14[a]	3	47	1.696±0.11[a]
4	32	1.448±0.14[b]	4	49	1.537±0.11[b]
5	32	1.319±0.14[c]	5	49	1.425±0.11[c]
Population		***	**Population**		***
Gondar	115	1.681±0.13[a]	Gondar	111	1.688±0.10[a]
Woyto Guji	62	1.126±0.13[b]	Woyto Guji	131	1.182±0.09[b]
Genotype at g.3416G>C		***	**Genotype at g.950T>C**		***
GG	169	1.284±0.09[b]	TT	236	1.349±0.03[b]
GC	8	1.523±0.19[a]	TC	6	1.520±0.18[a]
Genotype at g.3811C>T		***			
CC	151	1.539±0.12[a]			
CT	22	1.355±0.17[b]			
TT	4	1.316±0.25[c]			
Genotype at g.3963T>C		***			
TT	134	1.350±0.14[b]			
TC	38	1.581±0.14[a]			
CC	5	1.279±0.23[c]			

substitution detected, the following amino acid changes were observed on sequences of the populations studied with respect to the reference sequence: Serine to proline at locus g.3436T>C, isoleucine to leucine at locus g.3688A>C, histidine to proline at locus g.3878A>C and glutamine to histidine (Table 2). However, no amino acid changes were observed at loci g.945C>T, g.3354A>G, g.3592C>A, g.3696C>T, g.3783T>A and g.3963T>C because of the degenerative codons (Table 2).

Similarly, the remaining mutations at g.895G>C, g.3533A>G, g.3649G>A, g.3770G>C, g.3808G>A, g.3927C>G and g.3989G>A did not contribute for the fitness of the model suggesting these mutations do not directly influence the litter size though they cause amino acid changes. Therefore, mutations only at g.950T>C, g.3416G>C, g.3811C>T and g.3963T>C were considered for the association study (Table 3). Hence, the analysis of least square mean and standard error (LSM±SE) revealed that there was a 24% increment of litter size in GC genotype individual animals than GG genotypes at locus g.3416G>C in the goat population studied.

Similarly, 18% (among TT and TC genotypes), 22% (among CC and TT genotypes) and 31% (among TC and CC genotypes) litter size differences were observed at loci g.950T>C, g.3811C>T and g.3963T>C, respectively on the animals studied. Genotype TC of locus g.3963T>C is the highest contributor; whereas genotype CC of the same locus is the least contributor.

On the same analysis, highest (LSM±SE =1.614±0.4) and lowest (LSM±SE =1.307±0.14) litter size were obtained in the 3rd and 1st parties in both exons studied, respectively. Similarly, higher estimate of litter size was observed in Gondar goat (LSM±SE=1.681±0.13) than Woyto Guji goat population (LSM±SE =1.126±0.13).

DISCUSSION

The *transition* to *tranversion* ratio, in both exon1 and exon2 of the present study, was 2:1. In former report, G to C *transversion* at site 296, G to T *transversion* at site 454, T to A *transversion* at site 505, G to A *transition* at site 3433 and C to A *transversion* at site 3688 in goats were reported (Cao et al., 2010). Similarly, 2.44:1 SNPs *transition* to *transversion* ratio was detected in the genome wide sequences analysis of three Moroccan goat populations (Benjelloun et al., 2015). *Transition* type substitutions may occur more frequently than *transversion* type substitutions (Kimura, 1980). Contrary to the current study, Feng et al. (2009) and Cao et al. (2010) could not find mutations/base variations in exon2.

Most of the identified SNPs in the current study

qualified the SNP definition. According to Brookes (1999), the bi-allelic form of variation at a specific location in the genome could be a SNP if it is found in more than 1% of the population. Brookes (*ibid*) also stated that SNPs are abundant forms of genome variation, distinguished from rare variation by a requirement for the least abundant allele to have a frequency of 1% or more population. However, in rare cases, there are tri- or tetra-allelic forms for SNPs at a specific location (Kim and Misra, 2007) though this case was not observed in both target regions of the KISS1 gene in the current study.

In the current study, only three polymorphic sites from the total twenty mutations were observed at exon2. However, Feng et al. (2009) and Cao et al. (2010) did not detect any mutation at exon2 in goat. Cao et al. (2010) reported six polymorphisms in the KISS1 gene (G296C, G454T and T505A in intron1; G3433A and C3688A in exon 3 and a 18 bp deletion/insertion in 1960 to 1977 site in intron2) in five Chinese goat breeds. In another study, ten polymorphisms were reported in KISS1 gene of three Chinese goat populations (g.1147T>C, g.1417G>A, g.1428_1429delG, g.2124C>T, g.2270C>T, g.2489T>C, g.2510G>A, g.2540C>T, g.3864_3865delCA and g.3885_3886insACCCC) (An et al., 2013).

In the current study, highest percentages of GC contents in both exon regions (64.09% for exon1 and 62.47% for exon2) were obtained indicating important influence of the codons in the target gene expression (Bernardi et al., 1985; Ikemura, 1985). The CBI (CBI=0.301) estimate shows presence of considerable natural selection pressure in the populations studied (Sharp and Li, 1987). Natural selection favors higher expression and enhanced codon usage optimization in short genes (Fox and Erill, 2010), like KISS1 gene.

In the current study, fours SNPs amongst the detected polymorphic loci in both exons of the KISS1 gene affected litter size of the goats studied (Table 3). However, in the analysis of molecular variance, the relative lower estimate of the fitness model (R^2 ~35%) (Supplementary Table S2) might be because of the fact that multiple birth can be influenced by other genes, like GDF9, BMPR1B, BMP15 genes and also controlled by growth hormones. Growth hormone (GH) of mammals plays an important role in involving cell division, ovarian folliculogenesis, oogenesis and secretory activity (Hull and Harvey, 2002; Ola et al., 2008). By acting through specific receptors within the ovary, GH is expedient in controlling proliferation and apoptosis, oocyte maturation, and the expression and synthesis of receptors to hormones and related substances (Hull and Harvey, 2000; Sirotkin et al., 2003). Silva et al. (2009) also stated that the effect of GH on ovarian function is mainly through inducing the development of small antral follicles in the gonadotrophin-dependent stages and stimulating oocyte maturation. On the other hand, in the absence of the genotypes indicated in the model, it (R^2) was fitted only to 20%. A 15% increment of the fitness model, only by the genotypes observed in the KISS1 gene, implies the significant contribution of the candidate gene on multiple births compared to other genes, growth hormones and other phenotypic fixed effects. This is due to the fact that kisspeptin together with its receptor (GPR54) stimulates the release of LH and FSH in female goats, it is a key regulator and catalyst for the puberty onset, and it is a fundamental gatekeeper of sexual maturation in mammals (Hashizume et al., 2010; Cao et al., 2010 and 2011; Chu et al., 2012).

As a result, the genotypes identified on the coding regions of the KISS1 gene have shown remarkably significant ($P<0.001$) contribution (18 to 31% increment of litter size) on fecundity trait. However, this result is far lower than the finding reported for the CC genotype of Jining Grey goat does (litter size difference estimated to be 0.80 at locus 296) (Cao et al., 2010). In addition, the authors reported that G3433A caused one amino acid change (Ala, A, GCC) to (Thr, T, ACC) at residue 86 (A86T) though non-significant influence of the genotypes (CC, CA, AA) was observed at locus 3688. Complete substitution of A>C (monomorphism) was detected at the latter locus in the current study. In other study, significant ($P < 0.05$) effect of litter size was reported at T2643C locus in goats (Hou et al., 2011). In sheep, KISS1 mRNA expressing cells are found in the arcuate nucleus (ARC) and dorsallateral preoptic area and both appear to mediate the positive feedback effect of estradiol to generate the preovulatory GnRH/LH surge (Smith et al., 2011). The luteinizing hormone (LH) surge has been associated with an increase in the LH response to kisspeptin in humans and sheep (Dhillo et al., 2007; Smith et al., 2009), indicating the surge may be generated by increased kisspeptin output and sensitivity. These all findings indicate that KISS1 gene is an excellent candidate gene for reproductive traits in human and livestock (An et al., 2013).

It is also possible to deduce that, in the ANOVA table (Supplementary Table S2), population as source of variation took the highest share of variation among the fitness model suggesting the genotype component plays a vital role in regulating the reproductive cycles of female animals. Of course, joint consideration of multiple traits can provide additional information compared to information contained in individual traits as suggested by Pei et al. (2009).

Conclusion

In this study, there were 20 mutations, 15 of them were polymorphic, which detected at the two exon regions of the KISS1 gene. Among the polymorphic sites which caused amino acid changes, four of them have significant contribution to litter size of the goat populations studied

confirming relevance of the KISS1 gene for fecundity trait.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Supplementary Table S1. Primers designed for analysis of KISS1 gene.

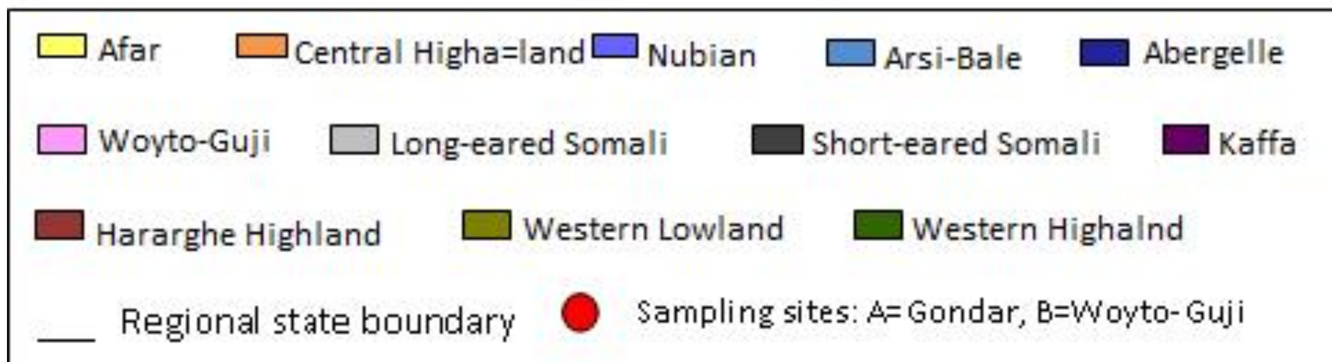
Region	Name given	Sequence	L	GC%	T_m
Exon1-Region1	CH_KISS1_Exon1_F2	5’-TTATGTCACTGCAGCTGG-3’	18	50.0	52.3
	CH_KISS1_Exon1_R1	5'- CTTGCTACTCACTGGCTG -3'	18	55.6	52.9
Exon1-Region2	CH_KISS1_Exon1_F1	5'- AGCGCTGAGCTTCCTAG -3’	17	58.8	54.3
	CH_KISS1_Exon1_R3	5’-GGCAATGGTCAGCATCATC-3’	19	52.6	54.1
Exon2	Chi_KISS1_Exon2_F	5'-CACTGTCCCACTGCATCTC-3'	19	57.9	55.5
	Chi_KISS1_Exon2_R	5’-GTAACGGCAGAAGAGCCTC-3’	19	57.9	55.5

L = length (bp).

Supplementary Table S2. Analysis of variance of exon1 and exon2 regions of KISS1 gene.

Exon1					Exon2				
Source	Df	Type III SS	M. Square	F Val.	Source	Df	Type III SS	M.Square	F Val.
Parity	4	2.46961285	0.61740321	2.93**	Parity	4	6.83085662	1.70771415	8.85***
Population	1	10.60811916	10.60811916	50.34***	Population	1	14.80968402	14.80968402	76.79***
Genotype at G3416C	1	0.41633801	0.41633801	1.98**	Genotype at T950C	1	0.16954025	0.16954025	0.88**
Genotype at C3811T	2	0.38815068	0.19407534	0.92 *					
Genotype at T3963C	2	0.78901259	0.39450630	1.87**					
Error	166	34.98043349	0.21072550			235	45.32326482	0.19286496	
Total	176	52.28248588				241	69.55371901		

R2 = 0.331; CV= 34.87%; ** = P<0.01; ***=P<0.001; R2 =35%; CV = 33.01%.



Supplementary Figure S1. Geographical distribution of goat breeds of Ethiopia. Source: Based on FARM-Africa (1996).

a. Exon2

PCR program:

Step	Time	Cycles
• $95^o{}_C$	5 minutes	
• $94^o{}_C$	15 seconds	5 cycle
• $64^o{}_C$ - $60^o{}_C$	30 seconds	
• $72\,^o{}_C$	45 seconds	
• $94^o{}_C$	15 seconds	35 cycle
• $58^o{}_C$	45 seconds	
• $72^o{}_C$	1 minute	
• $72^o{}_C$	10 minutes	
• $4^o{}_C$	∞	

PCR reaction:

Component	Volume
Primer (10pM/ µl) F	0.4µl
Primer (10pM/ µl) R	0.4µl
BSA	1.0µl
Hi-Di	0.5µl
H_2O (Nuclease free)	17.3µl
Template	0.4µl

b. Exon1_R1(exon1 region1)

PCR program:

Step	Time	Cycles
• $95^O{}_C$	5 minutes	
• $94^O{}_C$	15 minutes	5 cycles
• $62\text{-}58^O{}_C$	30 seconds	
• $72\,^O{}_C$	45 seconds	
• $94^O{}_C$	15 seconds	5 cycles
• $56^O{}_C$	45 seconds	
• $72^O{}_C$	1 minute	
• $72^O{}_C$	10 minutes	
• $4^O{}_C$	∞	

PCR reaction:

Component	Volume
•Primer (10pM/µl)F	0.4µl
•Primer (10pM/µl)R	0.4µl
•BSA (100X:10mg/ml)	2.0µl
•Hi-Di	0.5µl
•H_2O (Nuclease free)	16.3µl
•Template DNA	0.4µl

c. Exon1_R2 (exon1 region2)

PCR program:

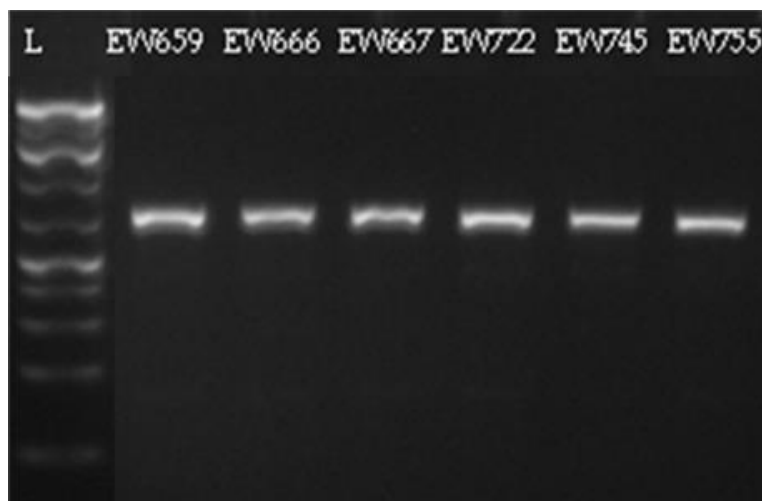
Step	Time	Cycles
• $95^O{}_C$	5 minutes	
• $94^O{}_C$	15 minutes	5 cycles
• $62\text{-}58^O{}_C$	30 seconds	
• $72\,^O{}_C$	1.0 minute	
• $94^O{}_C$	15 seconds	5 cycles
• $56^O{}_C$	1.0 minute	
• $72^O{}_C$	1.0 minute	
• $72^O{}_C$	10 minutes	
• $4^O{}_C$	∞	

Exon1_R2

PCR reaction:

Component	Volume
•Primer (10pM/µl)F	0.4µl
•Primer (10pM/µl)R	0.4µl
•BSA (100X:10mg/ml)	2.0µl
•Hi-Di	0.5µl
•H_2O (Nuclease free)	16.1µl
•Template DNA	0.6µl

Supplementary Figure S2. PCR conditions for exon1 and 2 regions of KISS1 gene amplification.



Supplementary Figure S3. Non purified PCR product gel image of exon2 of KISS1 gene.

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Full Length Research Paper

Establishing sterilization protocol and shoot induction medium for *in vitro* regeneration of sweetbriar (*Rosa rubiginosa* L.)

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***Rosa rubiginosa* L. is popular in Lesotho where rural communities harvest the rosehips for sale to the local companies which process them into various products. There is, however, a challenge in establishing plantations of the species due to deep seed dormancy and so difficulty in germination. In this study, nodal explants of *R. rubiginosa* L. were sterilized with 20 g·L^{-1} Ridomil Gold MZ (RGM) and cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzylaminopurine (BAP). The explants treated with 20 g·L^{-1} RGM showed resistant against fungal contamination giving 73% survival explants. Moreover, addition of BAP in shoot induction medium increased shoot formation as compared to 29% response recorded on MS without BAP. Response of 100% shoot formation was obtained on MS medium with 2 mg·L^{-1} BAP.**

Key words: 6-Benzylaminopurine, nodal explants, ridomil gold mz.

INTRODUCTION

Rosa is a genus belonging to Rosaceae family consisting of over 100 species including *Rosa rubiginosa* L. (Kazaz et al., 2009). *Rosa* is known to have originated from the Northern Hemisphere, Mediterranean, middle Scandinavia and Middle East (Werlemark, 2009), however, it is now found in almost every continent. In Lesotho, it is speculated that *R. rubiginosa* L. was introduced by English missionaries who were feeding rosehips to their horses. There are various commercial applications for *Rosa* species (Ozkan et al., 2004). In Lesotho, the rural communities harvest these rosehips from the wild to sell to the local companies that process them into various products including joint assist, rosehip tea, rosehip powder, rosehip tea blend and cosmetic grade oil extracted from the seeds.

The propagation techniques for *Rosa* species include cuttings, grafting, seeds, suckers and budding (Pavek, 2012). Majority of crops are planted through seeds probably due to simplicity and no technical expertise required. In contrast, seeds of *Rosa* species enter deep

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seed dormancy at maturity due to hard, thick pericarp and high amount of abscisic acid in the seed coat (Gudin et al., 1990) hence require pre-treatment before sowing. Removal of this seed dormancy is a complex process that requires changes in the pericarp, testa and embryo of the seed (Nadeem et al., 2013). Therefore, many researchers have tried different techniques in an attempt to break seed dormancy in *Rosa* species with low success rates. These techniques include macerating enzymes (Yambe and Takeno, 1992), microbial inoculation (Morpeth and Hall, 2000; Kazaz et al., 2010), acid scarification (Bhanuprakash et al., 2004), priming with gibberellic acid (Hosafci et al., 2005), warm and cold stratification (Zlesak, 2007) and hot water treatment (Younis et al., 2007; Haouala et al., 2013).

Consequently, tissue culture can be an alternative method of mass propagation for high-quality *Rosa* plants because it can be used throughout the year, at any location and at reduced costs (Kulus, 2015). It can also be used to overcome the challenge of seed dormancy in *R. rubiginosa* L. However, the success of tissue culture in *Rosa* species is limited by various factors including endophytic and microbial contamination and medium poisoning due to leakage of phenolic exudates that subsequently kill the explants (Shokri et al., 2015).

However, even though many protocols for regeneration of *Rosa* species (Tian et al., 2008; Pawłowska and Bach, 2010; Moallem et al., 2013; Davoudi et al., 2015; Ambros et al., 2016) have been developed little work has been dedicated to *R. rubiginosa* L. (Pawłowska, 2011; Pavek, 2012). Therefore the main objective of this study was to establish sterilization protocol and shoot induction medium for *R. rubiginosa* L. from nodal explants using BAP as the plant growth regulator.

MATERIALS AND METHODS

Sample collection and preparation

The cuttings of *R. rubiginosa* L. were collected in October from the wild in Maseru, Lesotho and shipped to Kenya enclosed in polythene paper bag. The cuttings were received on the third day and used for tissue culture on the fourth day. The explants were prepared by first removing the thorns from the stems. Then approximately 2 cm long cuttings containing a single axillary node were prepared. The explants were disinfected by washing for 30 min in running tap water followed by soaking for 30 min in 10% savlon antiseptic. Following the antiseptic treatment, the explants were rinsed 3 times with sterile distilled water (SDW) and put in 20 $g.L^{-1}$ RGM for 60 min. After this step, the explants were rinsed four times with SDW after which they were surface sterilized in 50% sodium hypochlorite solution for 20 min. The explants were then rinsed four times with SDW before transferring them to 70% ethanol for 10 min. After ethanol treatment, the explants were rinsed four times with SDW and used in the experiment.

Media preparation and culturing

The MS media (Murashige and Skoog, 1962) consisted of BAP (0.0, 1.0, 2.0, 3.0 and 4.0 $mg{\cdot}L^{-1}$) plus 3 $g{\cdot}L^{-1}$ gelrite and 30 $g{\cdot}L^{-1}$ sucrose. The pH of the medium was adjusted to 5.7 before autoclaving at 121°C with pressure of 15 lbs for 20 min. The nodal explants were cultured in a vertical orientation and each treatment was performed in triplicates with each bottle containing one explant. The total number of explants for each treatment was 15. The explants were cultured in the growth room at a temperature of 25°C under 16/8 h photoperiod provided by fluorescent light with intensity of 50 mEm^{-2}/s.

Data collection and analysis

The share of nodal explants forming shoots was recorded after one week of culture. The total number of inoculated explants per treatment was considered 100%. The number of explants contaminated was recorded for a period of 15 days following culture and each type of contamination was classified as fungal or bacterial. The number of shoots per explant and the length of shoots per explant were recorded after 4 weeks of culture. The significant difference among the treatments was determined with Minitab 17 using Turkey's test.

RESULTS

Contamination of explants was monitored for 15 days and first contamination was recorded on the fourth day of culture. The sterilization protocol resulted in 73% survival explants. The prevalent microbial contaminants were fungal with few incidence of bacterial contamination (Figure 1A and B). For shoot induction, the bud break was observed after one week of culture with more vigorous formation of shoots recorded from the second week of culture. The nodal explants cultured on MS medium without supplementation of BAP had lower shoot response in comparison to the treatments that contained BAP. The highest percentage of shoot formation (100%) was recorded on MS medium supplemented with 2.0 $mg{\cdot}L^{-1}$ BAP (Figure 2). Moreover, after four weeks of culture, the highest number of shoots per explant (1.4 ± 0.3) was recorded on medium supplemented with 2 $mg{\cdot}L^{-1}$ BAP. However, addition of BAP had no significant difference in terms of influencing the shoot length since all the BAP treatments were not significantly different from the control (MS without BAP) (Table 1).

DISCUSSION

In this study, the sterilization protocol for stem segments with single nodes from naturally grown *R. rubiginosa* L. have been successfully developed using antiseptic (savlon), disinfectants (NaOCl and ethanol), and fungicide (RGM). Fungal contamination is among the major enemies in tissue culture of *Rosa* species that may be introduced by the contaminated explants, airborne contaminated culture environment or improper handling of explants during the experiment (Sen et al., 2013). Antibiotics, fungicides, heat and light are normally used in tissue culture to overcome both fungal and bacterial contamination (Leifert et al., 1992).

Alvarenga et al. (2002) used RGM as surface sterilant for microcuttings of cacao and it was observed that a treatment of 1 $g{\cdot}L^{-1}$ RGM increased browning of the explants yet not effective since 100% fungal contamination was obtained. In addition, Sen et al. (2013) used RGM at various concentrations (0.5 to 5%) in

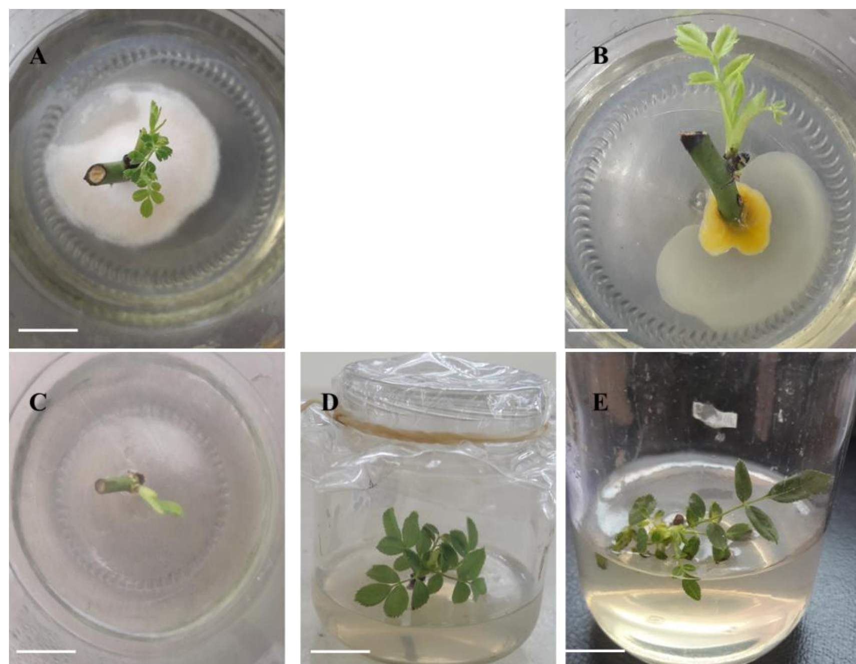

Figure 1. Nodal explants on MS medium supplemented with various concentrations of BAP. Explant contaminated with fungus (**A**) and bacteria (**B**) after 10 days of culture. Shoot response of nodal explants on MS medium supplemented with 2.0 mg·L^{-1} after 6 days (**C**) and four weeks of culture (**D**). Shoots induced on MS medium without BAP (**E**) after four weeks of culture. Bars represent 10 mm.

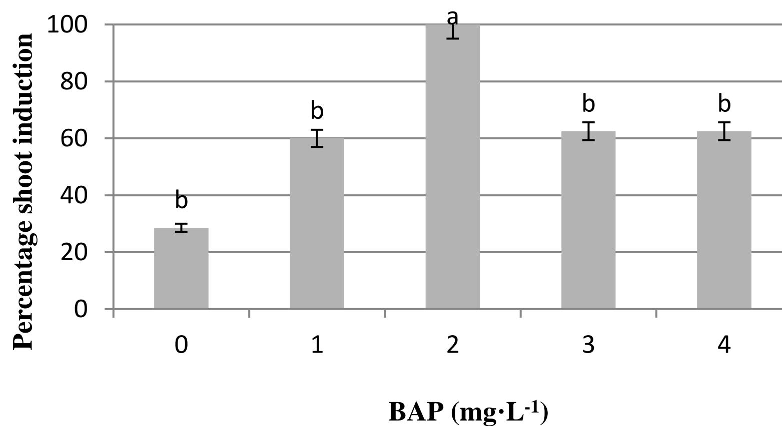

Figure 2. Percentage of shoot response of nodal explants cultured on different concentrations of BAP on MS medium. Values with different letters are significantly different at 95% confidence interval using Turkey's test.

Table 1. Average number of shoots and lengths per explant.

BAP (mg·L^{-1})	Average shoots per explant	Average length (cm)
0.0	0.2 ± 0.1^{b}	1.5 ± 0.5^{a}
1.0	1.0 ± 0.3^{ab}	0.82 ± 0.31^{a}
2.0	1.4 ± 0.3^{a}	0.94 ± 0.62^{a}
3.0	0.8 ± 0.3^{ab}	1.4 ± 0.53^{a}
4.0	0.8 ± 0.3^{ab}	1.0 ± 0.20^{a}

Values with different letters are significantly different at 95% confidence interval using Turkey's test.

combination with mercuric chloride at 0.1% for surface sterilization of nodal explants of *Achyranthes aspera* L. and RGM were not effective when used at concentrations lower than 3 to 5% although it was toxic to the explants at high concentrations.

In this study, the explants were sourced directly from their natural habitat in the wild and many researchers have proven the difficulty in obtaining 100% clean explants from wild grown plants when used directly as source of explants (Odutayo et al., 2007; Rout et al., 2009). In our study a 100% fungal contamination was observed when explants were treated with 3 $g \cdot L^{-1}$ RGM for 30 min. According to the manufacturer, RGM contains Metalaxyl-M as a systemic active compound and Mancozeb which forms a protective layer on the surfaces of the treated plants where it inhibits fungal spores' formation. When duration of explants treatment was extended to 60 min with RGM concentration increased to 20 $g \cdot L^{-1}$ fungal contaminants were significantly reduced by 73% and no browning or shoot inhibition was observed on the explants.

It has been reported that different genotypes of *Rosa* species respond differently to different media and culture conditions (Bressan et al., 1982). In the present study, BAP was effective to induce shoot formation without combination with any other plant growth hormones. In contrary, Moallem et al. (2012) reported that BAP alone was not effective to induce significant shooting in *R. canina* L. rather significant shooting of 100% was only observed in the presence of gibberellic acid (GA_3).

Rosa species are known for high heterogeneity, a trait that makes it difficult to apply a single protocol for regeneration of all *Rosa* species (Khosh-Khui, 2014). Consequently, the results on regeneration of *Rosa* species show diverse responses even under the same culture conditions. It was reported by Zapata et al. (1999), that growth regulators can be omitted on shoot induction medium for *Rosa* species since their meristems have the ability to initiate shoots on their own.

In this study, the stem segments with single axillary buds were used as the starting material for the regeneration of *R. rubiginosa* L. Shoot formation of 29% on nodal explants cultured on MS medium without addition of BAP was observed. This is not surprising based on the report by Zapata et al. (1999), however, it was observed that addition of growth regulator such as BAP increased the rate of shoots formation per treatment with optimum response obtained at 2 $mg \cdot L^{-1}$ BAP. This signifies the importance of adding the plant growth regulator during shoot induction in *Rosa* species.

Conclusions

Treatment of nodal explants of *R. rubiginosa* L. for 60 min with 20 $g \cdot L^{-1}$ RGM was effective in reducing fungal contamination. Based on the results, it is also concluded that 2.0 $mg \cdot L^{-1}$ BAP is optimum concentration for inducing shoot formation from nodal explants of *R. rubiginosa* L. However, further studies are required to determine the optimum rooting medium for shoots of *R. rubiginosa* L.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Biodiesel production from marine microalgae *Nannochloropsis gaditana* by *in situ* transesterification process

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Microalgae is one of the best sources of renewable energy production, such as biofuels. The production of biodiesel from microalgae has several advantages, including the high productivity of lipid and the possibility of cultivating them on marginal land. One of the challanges in using microalgae for biodiesel production is the complexities process of lipids extraction by organic solvents followed by transesterification. The aim of this work is to optimize this process by a single extraction and conversion step. The reaction was carried out for different parameters such as; various oil to methanol ratios, concentration of catalyst, temperature and time reaction. The lipid content of *Nannochloropsis gaditana* microalgae was 0.19 g/g biomass. The best yield of fatty acid methyl ester (65.6%) was obtained at 150 min duration for algae drying, 60% (wt./wt. oil) H_2SO_4 as catalyst concentration, and 1:8 algae biomass to methanol ratio (w/v). The algal biodiesel samples were analyzed with gas chromatography mass spectrometry (GC-MS) and Fourier transform infrared spectroscopy (FT-IR). *N. gaditana* microalgae investigated in this study, proved to be suitable as raw material for biodiesel production, due to their high cetane number (69.68). From the FT-IR result and fatty acid profile, it was implied that marine microalgae, *N. gaditana* in this study can be considered as potential feedstock for biodiesel production to fight the future energy crisis.

Key words: Biodiesel, fatty acid methyl ester, microalgae, *Nannochloropsis gaditana*, transesterification.

INTRODUCTION

The vegetable-oil derivative "biodiesel" offers several advantages as an alternative fuel for diesel engines (Maher et al., 2016). These include improved fuel performance and lubricity, a higher cetane rating than

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petrol-diesel, a higher flashpoint that makes it safe to handle, lower toxicity to plants and animals, reduced exhaust emissions, and the fact that it is simple to phase in and out of use (Sivaprakasam and Saravanan, 2007). It is a local renewable source of energy and highly biodegradable (Meng et al., 2008).

Microalgae are regarded as a promising source of biofuels due to their high lipid contents, high growth rates and requirement of smaller cultivation area. However, the production of bioenergy by the microalgae is still uneconomical. In fact, one of the production steps of transesterification which consumes the most energy is the step of lipid extraction from microalgae cells (Chattip et al., 2012; Meo et al., 2017).

In recent years, many studies tried extensively to improve the transesterification process by the variation of the reaction conditions, for exemple, the choice of catalyst, oil/alcohol ratio, temperature and reaction time (Bradley et al., 2011; Vlada et al., 2014; Verma and Barrow, 2015; Veillette et al., 2017). Although, this research aims to improve the lipid extraction method, others propose a rather interesting alternative approach to *in situ* transesterification without going through a tedious extraction step. Therefore, it would be more interesting to extract and convert the triglycerides from the microalgae into biodiesel in a single step, avoiding the use of large amounts of organic solvents (Chattip et al., 2012).

The *in situ* transesterification methods have been evaluated for biodiesel production from various raw materials, such as vegetable oil (Haas et al., 2004), wastewater sludges (Mondala et al., 2009), rice bran oil (Shiu et al., 2010), cotton seed oil (Qian et al., 2008) and microalgae (Li et al., 2011). It was reported that the direct method may result in higher yields of fatty acid methyl ester (FAME) than those obtained with the two-stage extraction (Bradley et al., 2011).

The *in situ* transesterification process, like conventional reaction, uses acid, basic or anzymatic catalysts. An acid catalyst (sulfuric acid) was chosen for this study because the acid catalyzed reactions were found to be affective in converting the free fatty acids and the triglycerides into FAME. In fact, the use of acid catalyst is the most appropriate method for organic substrates. It give very high yields in FAME (up to 99% of conversion) (Bharathiraja et al., 2014).

The aim of this work is to study the process of direct transesterification by optimizing the extraction and conversions step of marine microalgae, *Nannochloropsis gaditana*. It is important to study the production of microalgae biodiesel in Algeria, where pollution is becoming more and more widespread, due to the waste generation by the petroleum industries. It is also interesting to use the microalgae as an alternative source of biofuel. For the transesterification optimization, variations of the reaction conditions were carried out such as: 1) catalyst concentration; 2) reacting alcohol volume; 3) temperature and 4) reaction time. The study objectives also include the characterization of biodiesel produced by gas chromatography mass spectrometry (GC-MS) and Fourier transform infrared spectroscopy (FT-IR).

MATERIALS AND METHODS

Biological materials

The dried and powdered biomass of *N. gaditana* was used as a biological material in this study. It was provided by the company, Partisano Biotech Algeria in Oran.

Estimation of total lipid

The lipid content of *N. gaditana* was estimated by Soxhlet method of Schafer (1998). 6 g of dried sample was placed in a porous cellulose thimble (25 × 80 mm). The extraction was carried out in Soxhlet apparatus for 4 h. The system was equipped by water-cooled condenser suspended above a 500 ml flask containing 250 ml hexane: 2-propanol (2:1). The solvent was evaporated (55°C) and the lipids content was calculated as a percentage of the dry weight of the algae.

In situ transesterification of microalgal lipid

The transesterification process was conducted simultaneously with oil extraction. This method was based on Nautiyal et al. (2014) protocol with some modifications. The dried algae was subjected to pulverization in mortar for cell distruction. 5 g of resultant powdered was placed in the 100 ml flask with hexane and methanol at various algal biomass to methanol w/v ratios (1:6, 1:8 and 1:10) and different concentration of sulfuric acid H_2SO_4 (40, 60 and 80% by weight of algae). The magnetic stirrer was used to stir the contents in the reactor, the mixture was hearted to 90°C, and the reaction was tested for 30, 90 or 150 min.

The system was equipped with a condenser to maintain the atmospheric pressure inside the reaction and to avoid loss of solvents by evaporation. At the end of the reaction in each experiment, the products were centrifuged at 5000 rpm for 10 min and pouder into a separating funnel. The biodiesel layer (Top layer) was washed with distilled water (30% v/v) and the solvent was evaporated. Biodiesel was heated at 100°C for 15 min to remove water and excess solvents. The gravimetric method was used to determine the biodiesel content and the yield was calculated using Equation 1:

$$\text{Biodiesel yield (\%)} = [\text{Biodiesel produced (grams)} / \text{Oil produced (grams)}] \times 100 \quad (1)$$

Analysis of biodiesel

Gas chromatography mass spectrometry (GC-MS) analysis

FAMEs were analyzed by GC-MS carried out on a PerkinElmer Clarus 500 Gas Chromatograph couplet to Clarus 500 Mass Spectrometer with liquid autosampler (capillary column: 30 m, 0.25 μm diameter). Sample injected (2 μl) took place at 50°C temperature and was held for 3 min. Then, the temperature increased up to 280°C at 10°C min^{-1} and for 3 min. The vector gas used was helium. The average molecular weight of the oil (MM_{oil})

Table 1. Molecular weight and mass contribution of fatty acids of the obtained biodiesel from the promising green microalga, *N. gaditana* using GC-MS.

Fatty acid methyl ester (FAME)	Molecular mass (g/mol)[a] (MM)	Distribution in sample (%)[b]	Molecular mass contribution (g/mol) (MMc) (aXb)/100
(C8:0) Methyl octanoate	158.2	0.23	0.36
(C9:0) Methyl nonanoate	172.2	0.02	0.03
(C10:0) Methyl decanoate	186.2	0.19	0.35
(C11:0) Methyl undecanoate	200.3	ND	ND
(C12:0) Methyl laurate (X_1)	214.3	0.01	0.02
(C13:0) Methyl tridecanoate	228.3	0.97	2,21
(C14:0) Methyl myristate (X_2)	242.4	7	16,96
(C16:0) Methyl palmitate (X_3)	270.4	76.98	208.15
(C16:1) Methyl palmitoleate (X_4)	268.4	ND	ND
(C18:0) Methyl stearate (X_5)	298.5	2.60	7.76
(C18:1) Methyl oleate (X_6)	296.4	0.90	2.66
(C18:2) Methyl linoleate (X_7)	294.4	9.44	27.79
(C18:3) Methyl linolenate (X_8)	292.4	1.62	4,73
Total saturated fatty acids		88.04	
Total unsaturated fatty acids		11.96	
Average molecular mass of constituent fatty acids (MM_{FA})			271.02

was determined using Equation 2:

$$MM_{oil}=[3MM_{FA} + MM_{Glycerol}] - 3MM_{OH,H} \quad (2)$$

Where, $MM_{Glycerol}$ represents the molecular weight of glycerol and $MM_{OH,H}$ represents the molecular weight of OH group and hydrogen (El-Shimi et al., 2013).

The molecular weight of biofuel was determined using Equation 3 (EL-Shimi et al., 2013):

$$MM_{FAME} = MM_{FA} + 15 \quad (3)$$

The cetane number (CN) of biofuel was calculated using Equation 4 (Bamgboye and Hansen, 2008):

$$CN = 61.1 + 0.0088X_2 + 0.133X_3 + 0.152X_4 - 0.101X_5 - 0.039X_6 - 0.243X_7 - 0.395X_8 \quad (4)$$

Where, CN: cetane number, X_1, X_2.....X_8, are % compositions of FAME shown in Table 1.

Fourier transform infrared spectroscopy (FT-IR) analysis

The biodiesel samples were measured on FT-IR alpha Bruker. The resolutions of 26 scans were taken and the sample was recorded in the range of 4000 to 500 cm^{-1}.

Statistical analysis

The experiments were carried out in triplicate. The average of the three values obtened was used to calculate the standard deviation (SD). The final values were represented by mean ± SD. The statistical analysis consists of a parametric test of ANOVA 1 (Tukey HSD) or a non-parametric test of Kruskal-Wallis (Mann-Witney). P values ≤ 0.05 were regarded as statistically significant.

RESULTS AND DISCUSSION

Total lipid content

The total lipid content of *N. gaditana* biomass was found to be 19.18 ± 0.4% which was determined by the method of Shafer (1998). Abubakar et al. (2012) reported that the lipid content in *Chlorella* species presents hight oil yields (10.5%) followed by *Euglena acus* (5.78%), *Nitzschia* (3.63%), *Ankistrodesmus falcatus* (1.58%) and *Scenedesmus acuminatus* (1.58%). So, *N. gaditana* microalgae investigated in this study, representes a good raw material for the production of biofuels, due to their high lipid content, which may enhance the environmental cultivation possibilities without any competition with food crops.

On the other hand, the lipid level is less than what was found in the other works. Previous study done on *Nannochloropsis* sp. culture under various cultivation time and different photoperiod cycles (24/0, 18/06 and 12/12 h light/dark) showed a 31.3% lipid content (Wahidin et al., 2013). Similar results have been observed with *Nannochloropsis* sp. grown under nitrogen limitation, showing a 68.5% lipid content (Bondioli et al., 2012). This result can be explained by the culture method of microalgae, since the company that supplied the microalgae (*Nannochloropsis gaditana*) aimed at optimizing the production of biomass and not production of lipid. Massart et al. (2010) showed that increasing the growth leads to an oil level reduction.

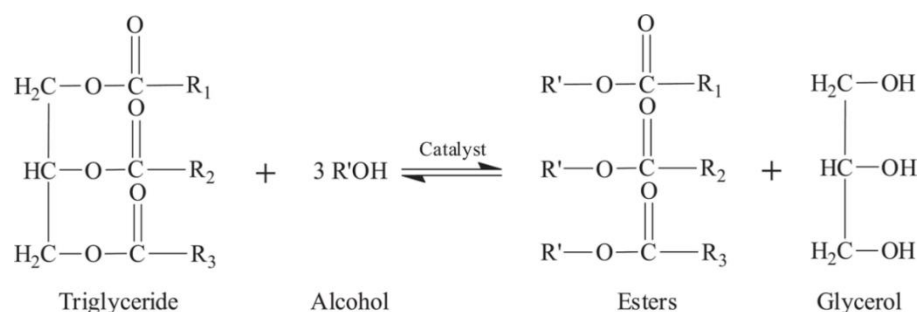

Figure 1. Transesterification reaction.

Figure 2. Influence of the biomass : alcohol ratio on biodiesel yield (at 90°C for 90 min using 60% wt. H_2SO_4). Averages with different indices are significantly different ($p < 0.05$).

Effect of alcohol volume

According to the majority of studies, this ratio appears to be the most important factor governing the speed of the transesterification reaction. The stoichiometry of the reaction involves the use of three moles of alcohol per one mole of triglyceride in order to obtain one mole of glycerol and 3 moles of fatty acid esters (Suganya and Renganathan, 2013).

However, the transesterification reaction is an equilibrium reaction in which a large excess of alcohol is required to promote the reaction in the direction of ester formation (Figure 1) (Refaat, 2009; El-Shimi et al., 2013; Meher et al., 2016).

The present study confirms the importance of using an excess methanol in transesterification process. The results which are presented in Figure 2, illustrate that the increase in the algae biomass: methanol ratio from 1:6 to 1:8 induces the increase in biodiesel yield from 55.4 ± 3.1% to 58.2 ± 2.4 %. Excess alcohol has a positive effect on biodiesel yield, but when the biomass: alcohol ratio is greater than 1:8, it has a negative impact (as observed) (Patil et al., 2012). In fact, the excess alcohol interferes with glycerin by increasing its solubility. When glycerin remains in solution, it helps drive the equilibrium to back to the left, reducing the ester yield (Figure 1) (Choo, 2004; Nautyal et al., 2014; Meher et al., 2016).

Similar results have been observed by several studies, Schwab et al. (1987) showed that to maximize FAME yields, a molar ratio of 1:6 should be used. They also mentioned that a molar ratio greater than 1:6, makes it more difficult to decant the glycerol and to separate the ester from it. Enciner et al. (2002) studied the trasesterification of *Cynara* oil by ethanol for a ratio of 1:3 to 1:15. The best results were obtained for molar ratios between 1:9 and 1:12. At a molar ratio of 1:15, the separation of glycerin becomes difficult. Schwab et al. (1987) reported that when glycerin remains in the reaction medium, it contributes to the shift of equilibrium towards the formation of triglycerides by lowering the yield of the ester.

Effect of catalyst concentration

In addition to the biomass: alcohol ratio, the concentration

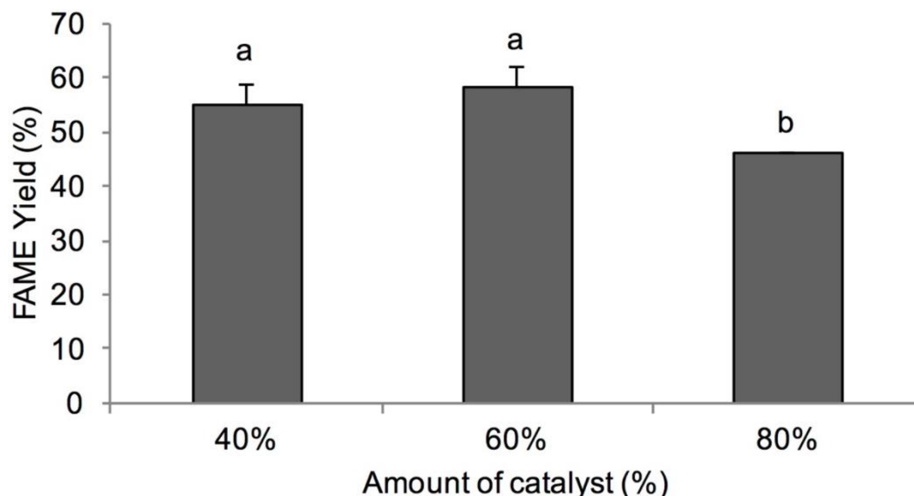

Figure 3. Influence of the catalyst concentration on biodiesel yield (at 90°C for 90 min using biomass to methanol ratio of 1:8). Averages with different indices are significantly different ($p < 0.05$).

of catalyst represents a very important variable in the conversion of the oil into FAME. The *in situ* transesterification process uses acid, basic or enzymatic catalysts. An acid catalyst (sulfuric acid) was chosen for this study because the acid-catalyzed reactions were found to be affective in converting the free fatty acids and the triglycerides into FAME (Ejikeme et al., 2010; Bradley et al., 2011).

In order to study the imparct of the concentration of H_2SO_4 on the FAME yield of *N. gaditana*, the concentration of the catalyst was varied from 40 to 80% (of dry algae biomass). From Figure 3, it can be observed that an increase in H_2SO_4 concentartion from 40 to 60% give an increase in FAME yield up to 58.2 ± 3.8%. Similar results was reported by Nautyal et al. (2014), they investigated the transesterification of *Spirulina platensis* using H_2SO_4 as the acid catalyst. As a result, the maximum production of FAME of 65.6% was obtained with 60% of catalyst concetration.

However, increasing the H_2SO_4 concentration from 40 to 60% induces a decrease in oil conversion into biodiesel (46.0 ± 0.1%). This result can be explained by the negative effect of high concentration in acid catalyst, which may lead to ether formation by alcohol dehydration and, the consequent high use of calcium oxide in the acid neutralization after production with its attendant high production cost and waste generation (Ejikeme et al., 2010).

Effect of reaction time

To study the effect of the reaction time on the conversion rate, three tests (30, 90 and 150 min) were carried out. In this experience, it was observed that when increasing the time reaction up to 90 min, the FAME yield increases from 55.2 ± 4 to 65.6 ± 2.1% (Figure 4). From this, it can be concluded that the biodiesel yield increase with the increase of the time reaction. This results are similar to those obtained by Freedman et al. (1984).

In this work, the method of direct or *in situ* transesterification for the production of FAME from *N. gaditana* oil was studied. A maximum biodiesel yield of 65.6% was arrived at as reported earlier by Shenbaga et al. (2012), who found FAME rates of 66.6 and 68.5% for *Dunaliella salina* and *Nannochloropsis* sp., respectively.

The direct transesterification reaction allows a more interesting production of biodiesel than the two-step transesterification which involves the extraction and conversion of the oil into FAME. The *in situ* transesterification makes it possible to gain more reaction time and also helps to avoid the potential loss of lipids during the extraction step (Johnson and Wen, 2009; Rekha et al., 2012).

Fatty acid profile and properties of microalgae oil and biodiesel

The FAME profile of *N. gaditana* is shown in Table 1. The most abundant fatty acid methyl ester was methyl palmitate, followed by methyl linoleate and methyl myristate, a similar results was reported by Afify et al. (2010). These FAMEs were reported to be common components in biodiesel. Advantageously, among these FAMEs, methyl palmitate whose quantity ranked first in this study was established as one of the biodiesel components that provide highest cetane response (Chattip et al., 2012).

The result shows that the biodiesel extracted of *N.*

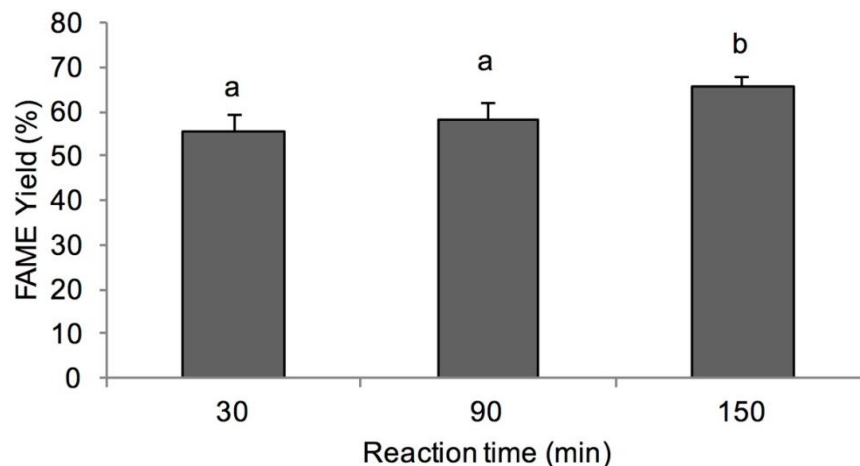

Figure 4. Influence of reaction time on biodiesel yield (at 90°C using biomass to methanol ratio of 1:8, 60% wt. H_2SO_4). Averages with different indices are significantly different ($p < 0.05$).

gaditana is composed of about 88.04% saturated fatty acid and about 11.96% unsaturated fatty acid, a similar trend was reported by Nautyal et al. (2014), who reported that the presence of highly saturated acids leads to increase in the stability of biodiesel.

Sarin et al. (2007) reported that the composition of the palm biodiesel was about 56.6% of unsaturated fatty acids and 43.4% of saturated fatty acids. Similarly, tallow biodiesel was reported to be composed of about 56.7% of unsaturated fatty acids and 42.8% of saturated fatty acids (Alcantara et al., 2000). Therefore, the higher percentage of saturated fatty acid in algae biodiesel makes it more stable as compared to tallow and palm biodiesel.

The average molecular weight of the oil and biodiesel extracted from *N. gaditana* revealed values of 851.10 and 286.02 g/mol, respectively. The same results were reported by El-Shimi et al. (2013), who found that the average molecular weight of the oil and biodiesel of *S. platensis* was 845.19 284 g/mol, respectively.

Cetane number of *N. gaditana* biodiesel was calculated to be 69.38, which is higher as compared to 60, for *S. platensis* (El-Shimi et al*.,* 2013), 45.8 for rapeseed biodiesel (Encinar et al., 2010) and also better than 38 for jatropha biodiesel (Sivaramakrishnan and Ravikumar, 2012). CN of biodiesel is generally higher than conventional diesel because it has longer fatty acids carbons and saturated molecules. The study of biodiesel CN has a high importance; since inadequate CN result in poor ignition quality, delay and excessive engine knock (Bamgboye and Hansen, 2008).

FT-IR analysis

More recently, FT-IR spectroscopy was used for the characterization of biodiesel (Meher et al., 2016). Generally, in the biodiesel samples, FT-IR spectra showed five important absorption bands. It can be observed that the C-H stretching absorbtion occurs at wavelength 2919.90 cm^{-1}, this peak appears strong in microalgae biodiesel samples as shown in Figure 5. Two alkanes peaks which is attributed to the bending absorbtion of methyl ($-CH_3$) and methylene ($-CH_2$) group appear at 1455.90 and 1361.25 cm^{-1}, respectively. Since biodiesel is mainly mono-alkyl ester, the intense C=O stretching band of methyl ester appears at 1739.26 cm^{-1}. One peak observed at 1167.70 cm^{-1} is due to stretching absorption of ester C-O. These results are in agreements with literature (Guil-Guerrero et al., 2004; Ching et al., 2011; Patil et al., 2012).

Ching et al. (2011) reported the same five peaks as *N. gaditana* after analysis of five microalgae biodiesel (*Nannochlororpsis oculata, Dunaliella tertiolecta, Chlorella vulgaris, Selenastrum capricornutum* and *Chlamydomonas reinhardtii)* by FT-IR.

Yadav et al. (2014) reported nine different absorption peaks after analysis of *Hydrodictyon reticulatum* (L) *Lagerheim* green algae biodiesel. Among them, the band at 1740 cm^{-1} which is associated with vibration of C=O shows ester groups, primarily from lipids and fatty acids (Coates, 2000).

Conclusion

The results of this study show that the optimal conditions for maximum FAME yield (65.6%) were determined as: biomass to methanol (w/v) ratio of 1:8, sulfuric acid concentration of about 60% (wt. wt^{-1}. oil), reaction time of 150 min and reaction temperature of 90°C.

The average mass molecular of microalgae oil was calculated to be 851.10 g/mol, reduced to 286.02 g/mol

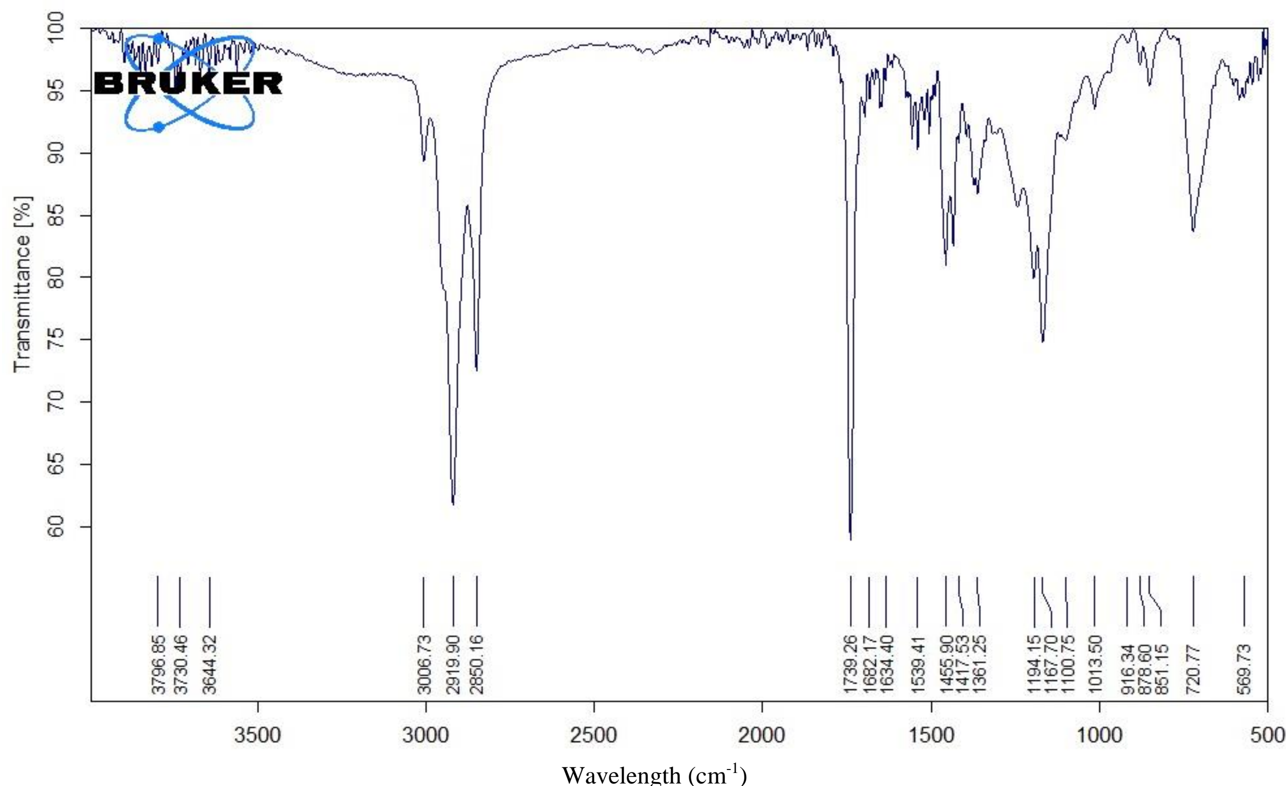

Figure 5. FT-IR analysis of FAME converted *N. gaditana* algal biomass.

for the production of FAME and the cetane number was 69.68, so *N. gaditana* microalgae investigated in this study, is proven to be suitable as raw materials for biodiesel production. Due to the FT-IR result and fatty acid profile, it is indicated that microalgae could produce high quality biodiesel and can be considered as potential feed stock for biodiesel production to fight the future energy crisis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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Full Length Research Paper

Truffle mediated (*Terfezia claveryi)* synthesis of silver nanoparticles and its potential cytotoxicity in human breast cancer cells (MCF-7)

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***Terfezia claveryi* is a species that belongs to the genera of Terfeziaceae or desert truffles, which is a family of truffles. In the present study, silver nanoparticles were synthesized from aqueous extract of *T. claveryi* which are in the range of 25 to 60 nm. The synthesized nanoparticles were characterized by ultraviolet-visible (UV-Vis) spectroscopy, fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). The effect of the silver nanoparticles on human breast cancer cell line has been tested. Peak absorption was recorded at 440 nm in UV-Vis spectra of silver nanoparticles. The XRD data reports that the silver nanoparticles are crystalline in nature and have face centered cubic geometry. FESEM showed the size range of synthesized silver nanoparticles as 25 to 50 nm. The TEM image represents that the majority of silver nanoparticles are in spherical shape with sizes ranging between 40 and 60 nm. The aim of the present study was to report for the first time fruit mediated synthesis of silver nanoparticles using the extract of *T. claveryi* and showed remarkable cytotoxicity activity against human breast MCF-7 cancer cell line.**

Key words: Silver nanoparticles, *Terfezia claveryi*, fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), scanning electron microscopy (FESEM), transmission electron microscopy (TEM), MCF-7 cancer cell line.

INTRODUCTION

Truffles which are also called black diamond are a group of desert fungi, which grow in the northern part of Saudi Arabia bordering Kuwait, Iraq and Jordan (Hussain and Al-Ruqaie, 1999). These are rich in antioxidants such as vitamin A, vitamin C and β carotene (Murcia et al., 2002) and are used as convalescent (Janakat and Nassar, 2010). Especially, *Terfezia claveryi* species are rich in carbohydrates and proteins (Bokhary and Parvez, 1993)

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and are commonly used as culinary agent. They look spherical in shape and pale brown in color. Antibacterial characters of *T. claveryi* were already studied as its aqueous extract inhibited the growth of *P. aeruginosa* by Janakat et al. (2004) and Aldebasi et al. (2013). Comparative pathological studies on the healing effect of natural (*Terfezia claveryi*) and synthetic (Vigamox) antimicrobials (Aldebasi et al., 2012) and *in-vivo* effect of *T. claveryi* extract on corneal ulcer of rabbit's eye (Aldebasi et al., 2015). The aqueous extract of *T. claveryi* is hepatoprotective (Janakat and Nassar, 2010). The truffles are rich in flavonoids (Akyuz, 2013) which can be exploited in reducing $AgNO_3$ to Ag^0. The compounds of truffle aqueous extract have important therapeutic roles: anti-inflammatory, anti-carcinogenic, anti-mutagenic, immunesuppressor and anti-microbial properties (Hannan et al., 1989). As the *T. claveryi* is rich in proteins, the reducing activity of the amino acids can be applied for the green synthesis of silver nanoparticles by reducing $AgNO_3$ to Ag^0 (Aldebasi et al., 2014). There are different approaches for the synthesis of silver nanoparticles which include physical, chemical and biological approaches. Among all, biological approach is well preferred because of its eco-friendly, cheap and time saving factors. Nanoparticles are used in paints, waste water treatment (Tiwari et al., 2008), and drug delivery. Particularly in life sciences, nanoparticles have a great importance in drug delivery, gene delivery, photo-dynamics, imaging (MRI) and *in vitro* diagnostics (De Jong and Borm, 2008). The credit of nanoparticles is its non-toxicity which allows them to be used in drug/gene delivery when compared to traditionally method using chemical agents. Presently, silver nanoparticles are applied in diagnostic process in biosensors for quantitative detection (Majdalawieh et al., 2014; Li and Xu, 2014), antibacterial applications in wound dressings and cosmetics, conductive applications in conductive inks, optical applications in metal enhanced fluorescence (MEF) and surface enhanced Raman scattering (SERS). Also, silver nanoparticles can be applicable in the water filters to filter out pathogen free water, in the enhancement of latent finger prints (Sametband et al., 2007) and catalytic degradation of organic dyes (Vidhu and Philip, 2014).

Cancer is a major health problem and it arises from one single cell. According to WHO, if it continue rising without any immediate action, 13.1 million people may die in 2030. Tobacco use, alcohol use, lack of physical activity, low intake of fruit and vegetable are some of the important risk factors; the reason for 30% of worldwide cancer deaths. Diagnosis of tumors in the human body was very difficult (Gurunathan et al., 2013) at their earlier stage and there was a search of new treatment for treating this deadly disease. Radiotherapy, chemotherapy and surgery are some of the cancer treatments which are used to improve the patient's life. Recently, nanoparticles are also used to overcome this problem. The nanoscale devices can easily enter the cells and they made an interaction with DNA, proteins, enzymes and cell receptors. The nanoparticles can detect the cancer disease in a very small volume of cells or tissue (Berrington and Lall, 2012). This study is focused on the cytotoxicity of silver nanoparticles on cultured MCF-7 cell line using different concentrations.

The present study was aimed at reporting for the first time fruit mediated synthesis of silver nanoparticles using the extract of *T. claveryi*. The optical absorption spectrum of synthesized silver nanoparticles is recorded by using UV-visible spectrophotometer. Morphological characterizations are performed using XRD, SEM and TEM. The spherical shaped silver nanoparticles showed excellent cytoxicity against MCF-7 human breast cancer cell lines.

MATERIALS AND METHODS

Preparation of extract

Desert truffles (*T. claveryi)* were collected from Buraidah market, Al-Qassim region Kingdom of Saudi Arabia. The collected fruits were air dried and stored at cool temperature and used when needed. 10 g of fruit was mixed with 150 ml of distilled water and boiled for 8 to 10 min. After cooling, mixture was centrifuged at 5000 rpm for 10 min and the supernatant was collected for Ag nanoparticle synthesis.

Biosynthesis of silver nanoparticles

Fruit (10 g) was mixed with 150 ml of distilled water and boiled for 8 to 10 min and filtered through Whatman No.1 filter paper (pore size 25 µm). The filtrate was further filtered through 0.6 µm sized filters. The solution was decanted and stored at 4˚C; it was used within a week of its preparation. 1 mM aqueous solution of silver nitrate (AgNO3) was prepared and used for the synthesis of silver nanoparticles. 10 ml of extract was added into 90 ml of aqueous solution of 1 mM silver nitrate for bioreduction of $Ag+$ ions in the solution and kept at room temperature for 24 h. The bio-reduction was analyzed by drawing the absorption maxima at 360 to 700 nm using a uv-vis spectrophotometer.

Characterization of Ag nanoparticles

Color change to brown color confirmed the synthesis of silver nanoparticles and were characterized by uv-visible spectroscopy (Thermo Scientific Evolution 201), FESEM ((SUPRA 55)-CARL ZEISS, Germany), XRD (XRD-SMART LAB (9kW)-RIKAGU, JAPAN), TEM (Hitachi H-7500 TEM, Japan) and fourier transforms infrared spectroscopy (FTIR-PERKIN ELMER Spectrum Two model, UK).

Determination of *in vitro* anticancer activity of synthesized AgNPs

Cell culture

Breast cancer cell line (MCF-7) was obtained from National Centre for Cell Science (NCCS), Pune, India. The MCF-7 cells were grown

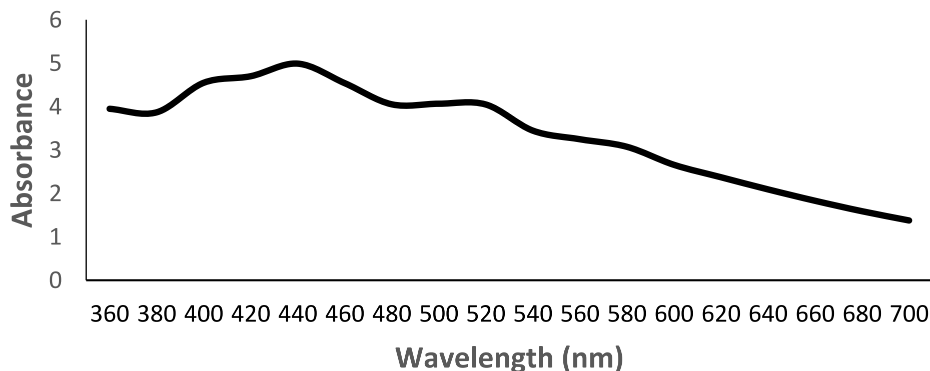

Figure 1. UV-Vis spectrum of *T. claveryi* aqueous extract derived Ag NPs.

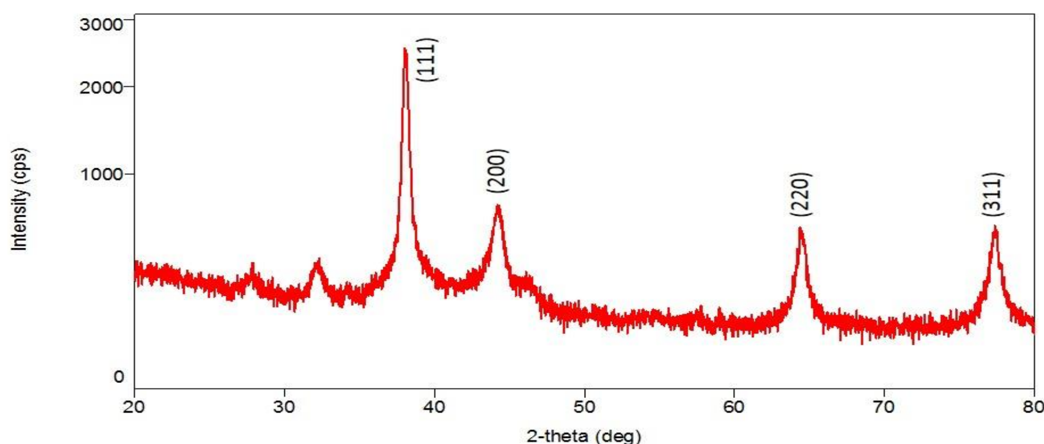

Figure 2. XRD pattern of *T. claveryi* aqueous extract derived Ag NPs.

as monolayer in MEM, supplemented with 10% FBS, 1% glutamine, and 100 U/ml penicillin-streptomycin and incubated at 37°C in 5% CO_2 atmosphere. Stocks were maintained in 75 cm^2 tissue culture flask.

Measurement of cytomorphological changes in MCF-7

MCF-7 cells were pre-treated with different concentration of synthesized AgNPs and incubated for 24 h at 37°C in 5% CO_2 atmosphere. After the incubation of cells, the gross morphological changes in the cells were observed under bright field microscope.

RESULTS AND DISCUSSION

UV-Vis spectrum

The synthesized silver nanoparticles maximum absorption range was measured using UV-visible spectrophotometry. The strong resonance for *T. claveryi* derived silver nanoparticles was clearly observed at 440 nm (Figure 1). The resultant is due to reduction of silver nitrate into silver and which suggests the presence of silver nanoparticles.

Fourier transform infra-red studies

FTIR spectrum of the synthesized AgNPs is shown in Figure 3 which reveals the possible biomolecules present in the fruit extract which is accountable for the reduction of silver ions and its interaction with the AgNPs.

XRD analysis

XRD studies were carried out to study the crystalline characteristics of the *T. claveryi* derived silver nanoparticles. The comparison between the standard and *T. claveryi* derived Ag nanoparticles confirms the crystalline nature of silver nanoparticles as evidenced by the peaks observed at 2θ values of 38.035, 44.21, 46.24, 64.39 and 77.21° corresponding to 111, 200, 220 and 311 Bragg reflections, respectively. The XRD pattern of Ag NPs is shown in Figure 2. The average size of Ag nanoparticles was calculated using XRD data and Scherrer equation and approximately found to be 20 nm. The Scherrer equation followed is, $D = K\lambda / \beta \cos\theta$ where, D is crystallite size of Ag nanoparticles, λ is the

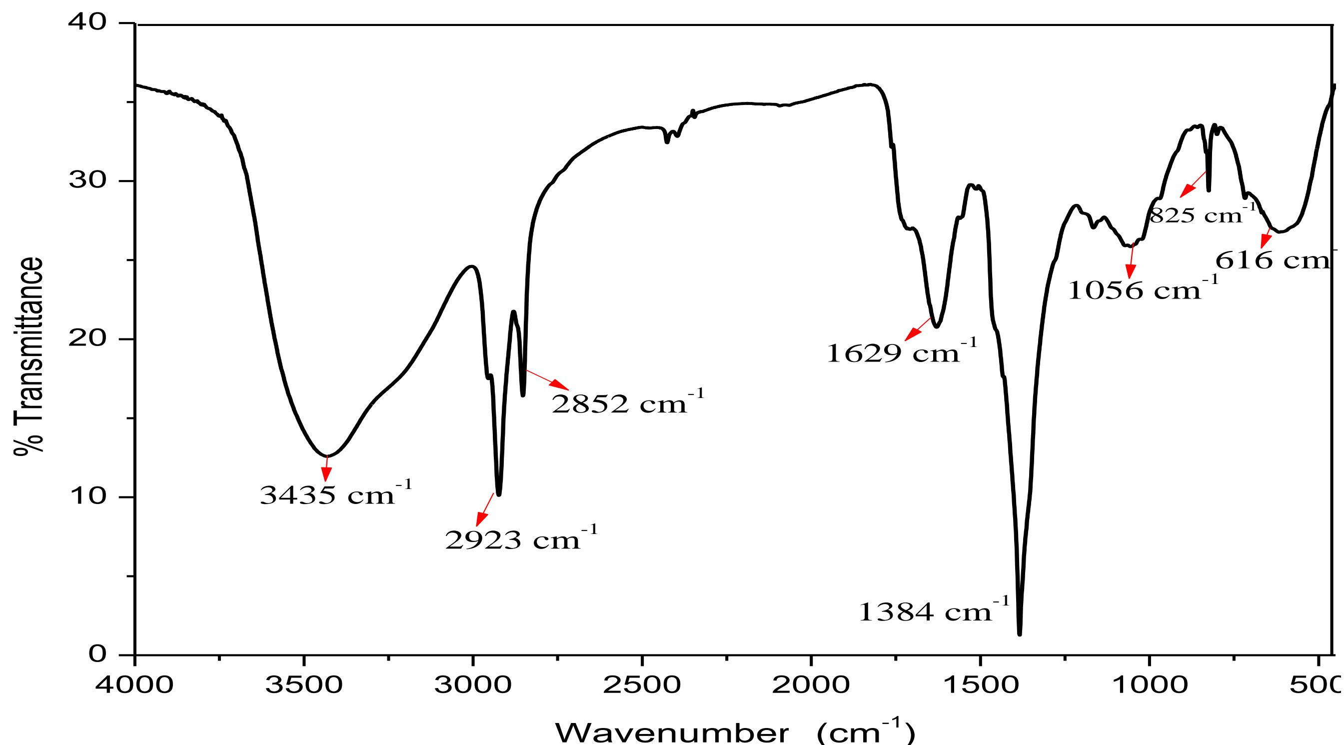

Figure 3. Fourier transforms infrared spectroscopy (FTIR) image of AgNPs synthesized from *T. claveryi.*

wavelength of the X-ray source (1.54056 nm) used in XRD, β is the width at the half maximum of the diffraction peak, θ is the Bragg angle and K is the Scherrer constant (0.94).

Scanning electron microscope (SEM)-EDX

Figure 4a depicts the FESEM image of silver nanoparticles. The size range of synthesized silver nanoparticles was found to be 25 to 50 nm. The silver nanoparticles showed spherical morphology under FESEM observation. The EDX characterization (Figure 4b) has shown absorption of strong silver signal along with other elements, which may originate from the biomolecules that are bound to the surface of silver nanoparticles.

Transmission electron microscope (TEM)

Thin film of sample were prepared on a carbon coated copper grid by just dropping a small amount of sample on the grid and drying it under mercury lamp for 5 min. The TEM image of *T. claveryi* derived Ag nanoparticles shows that the majority of NPs are spherical shaped as presented in Figure 5. Also, TEM image at resolution of 83 nm represents that the Ag NPs size ranges between 40 and 60 nm.

Effect of silver nanoparticles against MCF-7 breast cancer cells

In vitro cytotoxicity of the silver nanoparticles was evaluated against MCF-7 breast cancer cells at different concentrations (10 to 50 g/ml). Our results unveils, that there is direct dose-response relationship with the tested cells at higher concentrations. In relation to cell death, a minimum of 10 g/ml of silver nanoparticles is well enough to induce 50% of cell mortality; shows the cytotoxicity of silver nanoparticles at various time intervals. The cell viability of the silver nanoparticles at different incubation time is depicted by Figure 6. The calculated IC_{50} value of this experiment is 10 mg/ml concentrations. Previously, synthesized AgNPs inducing cytotoxicity were discussed by (Safaepour et al., 2009; Sriram et al., 2010). However, there is no previous investigation data available on *T. claveryi* carrying nanoparticles.

Conclusion

Silver nanoparticles were synthesized from *T. claveryi* aqueous extract whose size falls in the range of 25 to 60 nm. It is convincing here that the aspartic acid, glutamic acid and other amino acids acted as reducing agents to convert $AgNO_3$ to Ag° nanoparticles. In fact, *T. claveryi* is rich in amino acids and also truffles are rich in flavonoids. Initially, the plasmon peak in UV-Vis spectra of 440 nm

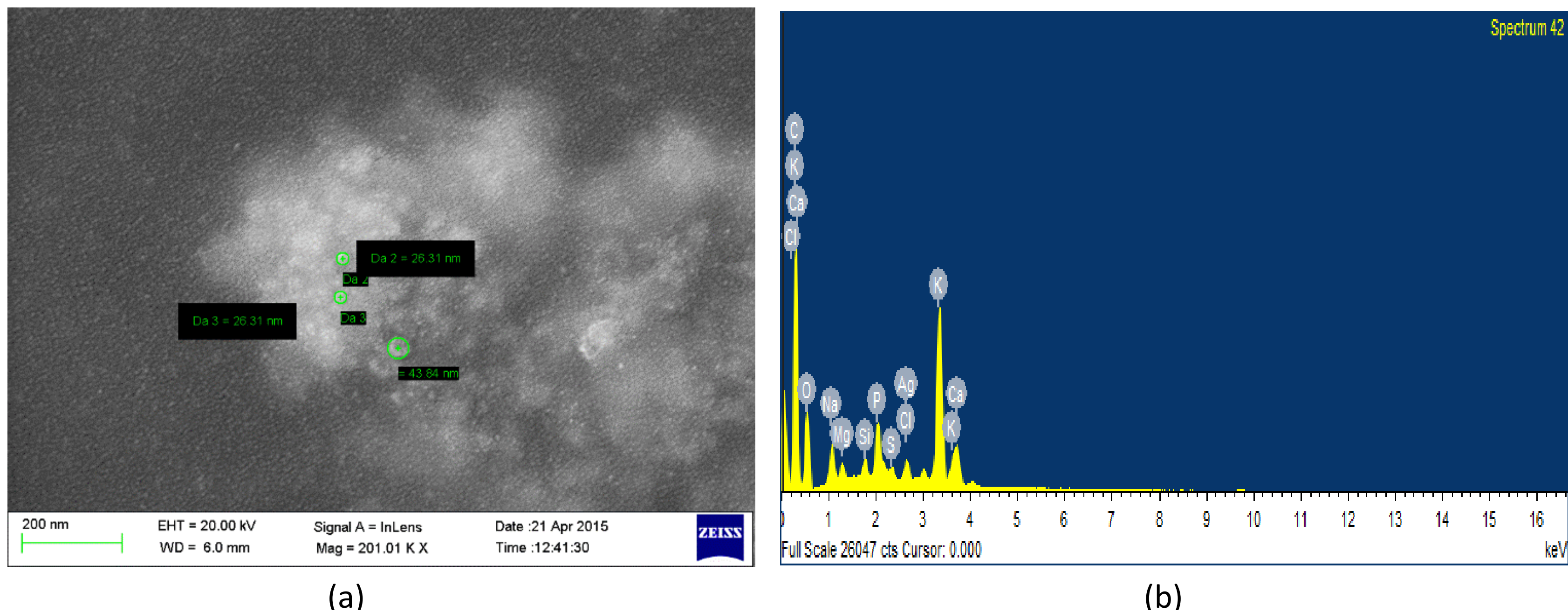

Figure 4. a. FESEM image. **b.** EDX pattern of *T. claveryi* aqueous extract derived Ag NPs.

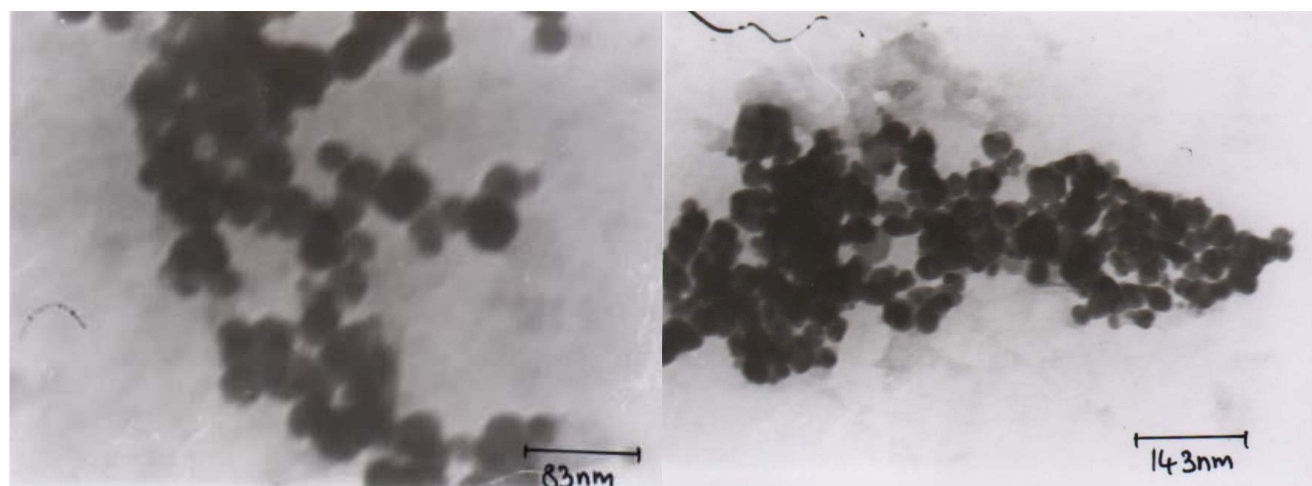

Figure 5. TEM image of *T. claveryi* aqueous extract derived Ag NPs at the resolution of 83 and 143 nm.

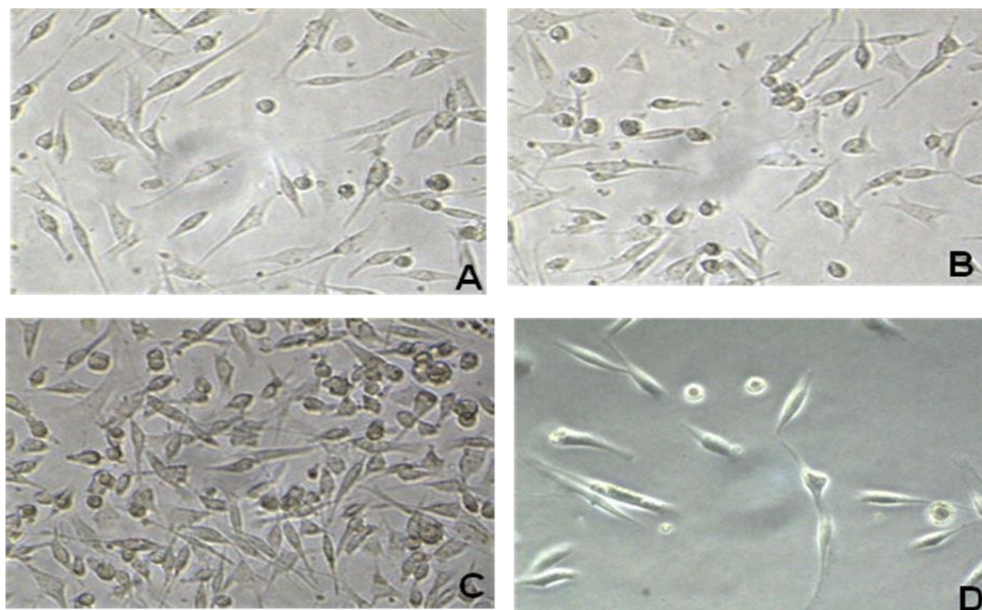

Figure 6. Cytotoxic effect of silver nanoparticles IC_{50} concentration of AgNPs treated on MCF-7 cells. 40x Magnification cytomorphological changes and growth inhibition at different time intervals on the MCF-7 cells (A) 24 h (B) 48 h (C) maximum (D) control.

confirmed the presence of silver nanoparticles, and the XRD derived crystal nature of Ag nanoparticles as face centered cubic. FESEM and TEM images suggest that the silver nanoparticles are in spherical shape. The present study shows significant cytotoxic effects by synthesized silver nanoparticles against MCF-7 breast cancer cells. The method could be exploited for developing economical biosynthesis of Ag nanoparticles in large scales and it would be useful in future nanomedicine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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African Journal of Biotechnology

Full Length Research Paper

Genetic and morphological diversity among sweet potato (*Ipomoea batatas* (L) Lam.) accessions from different geographical areas in Malawi

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An understanding of morphological and genetic diversity of sweet potato landraces is fundamental to any breeding program in a country. Fifty-nine sweet potato accessions from three eco-geographical populations of northern, south eastern and southern Malawi were examined using ten Simple Sequence Repeats (SSR) loci and seven International Board for Plant Genetic Resources (IBPGR) descriptors of sweet potato. The study generated a total of 30 alleles with a mean of 3 alleles per locus and a range of 2 to 5 alleles per locus. The primers were highly polymorphic and discriminatory with Polymorphism Information Content (PIC) mean of 0.55 and a range of 0.29 to 0.75, implying that allelic diversity and molecular relationships revealed by the study are strongly supported. Mean Nei' gene diversity (h=0.30) and Shannon information index (I=0.43) showed moderate genetic diversity of the populations with landraces (h=0.32; I=0.47) exhibiting more genetic diversity than introductions (h=0.25; I=0.38). SSR and morphological markers differently distinguished the accessions as evidenced by poor correspondence of genetic and morphological distance matrices (Mantel' Test, r=0.1095). However, cluster analysis indicated high variability among accessions at morphological (50% dissimilarity) and genetic (64% dissimilarity) level. Therefore, Malawian sweet potato landraces harbour considerably high morphological and genetic diversity warranting use in breeding programs.

Key words: Simple sequence repeats (SSR) loci, morphological diversity, sweet potato accessions, genetic diversity, descriptors, polymorphism.

INTRODUCTION

Sweet potato (*Ipomoea batatas* (L.) Lam) is the seventh most valuable staple crop in the world by fresh weight

and fifth in developing countries after rice, wheat, maize, and cassava (FAO, 2004). In sub-Saharan Africa (SSA) the crop is cultivated on more than 3 million hectares, yielding an estimated ca 13 million tonnes annually (Low and van Jaarswels, 2008).

In Malawi, sweet potato is the second important root crop after cassava and most widely grown in the country. Its production increased by 370% from 1995 to 2006 (FEWS/MoAFS, 1995, 2006) indicating the potential of the crop to alleviate poverty among estimated two million low income small holders farmers who cultivate 0.23 hectare of land on average (Malawi Government, 1999). Sweet potatoes are known to be rich in vitamins (A, C, D and E), highly productive with low demand on labour and inputs as well as tolerant to recalcitrant growing conditions, hence, suitable for marginal lands. These attributes render the crop appealing to low income farmers (Sreekanth et al., 2010) resulting in increasing importance of the crop over other crops in recent years in SSA (Walker et al., 2011).

In general, systematic plant breeding and efficient utilization of agricultural inputs has increased crop productivity in the past century (Warburton et al., 2002). However, increased productivity has often resulted in decreased genetic diversity within gene pools (Fernie et al., 2006) due to many compounding factors including inbreeding. This trend is particularly worrisome among vegetatively propagated crops like sweet potatoes and in particular landraces which have a diverse genetic base but are rarely integrated into the plant breeding programs due to their low production performance. This observation necessitates characterization of sweet potato landraces in Malawi in order to inform rational use and conservation of the present sweet potato genetic resources (Fraleigh, 2006).

Identification and release of sweet potato cultivars in Malawi is mainly based on morphological and agronomical characteristics (Chipungu et al., 1999) making morpho-agronomic characterization the main driver of collection and utilization of sweet potato germplasm in any breeding program in Malawi. While sweet potato morphological descriptors have been variously used (Vimala et al., 2012; Norman et al., 2014; Rahman et al., 2015; Amoatey et al., 2016; Mbithe et al., 2016; Su et al., 2016) and proven useful for preliminary evaluation of accessions due to their considerable discriminatory power, the present trend is to use molecular marker based characterization as a complementary tool to validate morphological characterization findings (Changadeya et al., 2012a; Malviya et al., 2012). Molecular markers have increasingly been employed to investigate sweet potato genetic diversity for germplasm conservation and genetic enhancement (Veasey et al., 2008; Karuri et al., 2010; Moulin et al., 2012; Cruz da Silva et al., 2013; Maquia et al., 2013; Camargo et al., 2013; Ochieng et al., 2015; Naidoo et al., 2016).

Therefore, this study was conducted to assess the level of genetic diversity in Malawian accessions using simple sequence repeats (SSRs) molecular markers and validate the degree of relatedness of the morphologically divergent sweet potato accessions from different geographical sources.

MATERIALS AND METHODS

Accessions collection

A total 268 sweet potato germplasm accessions were collected for morphological characterization from the Northern, South Eastern and Southern (Lower Shire) Regions of Malawi (Figure 1 and Table 1). Prior information on areas of high production and varietal diversity obtained from Karonga, Mzuzu, Blantyre and Shire Valley Agricultural Development Divisions (ADDS) offices facilitated the accession collection (Figure 1).

A total of 59 accessions that showed wide morphological distances within and among geographical populations, namely, the North, South East, the Lower Shire Valley and introductions were sampled for further analysis using SSR markers. Sample leaves for DNA analysis were obtained from the Bvumbwe Agricultural Research Station field where the 268 accessions were planted for morphological characterization. DNA analysis was conducted at University of Malawi, Chancellor College, Department of Biological Sciences, Molecular Biology and Ecology Research Unit (MBERU) DNA Laboratory.

Morphological characterization

Detailed comparisons using morphological descriptors (Table 2) aimed at isolation of potential duplicates among the accessions (Huaman et al., 1999) were carried out at Bvumbwe Agricultural Research Station. Sweet potato vines (25 to 30 cm long) were planted and grown following standard procedures. Characterization of above ground morphology of plants started at 80 to 100 days after planting (Mok and Schmiediche, 1998). Seven IBPGR descriptors for sweet potato (Huaman, 1991; CIP et al., 1991) were used for the discriminatory assessment. The descriptors used had a total of 47 different character states (classes) (Table 2). Morphological indicators on roots were done at harvest (5 months after planting). Data was collected from four randomly sampled plants per accession. These descriptors were qualitatively and quantitatively scored (Huaman, 1991).

Genetic characterization

DNA extraction

Total genomic DNA from freshly harvested leaves was extracted using a modified cetyltrimethylammonium bromide (CTAB) procedure (Doyle and Doyle, 1990; Edwards et al., 1991). Four leaf

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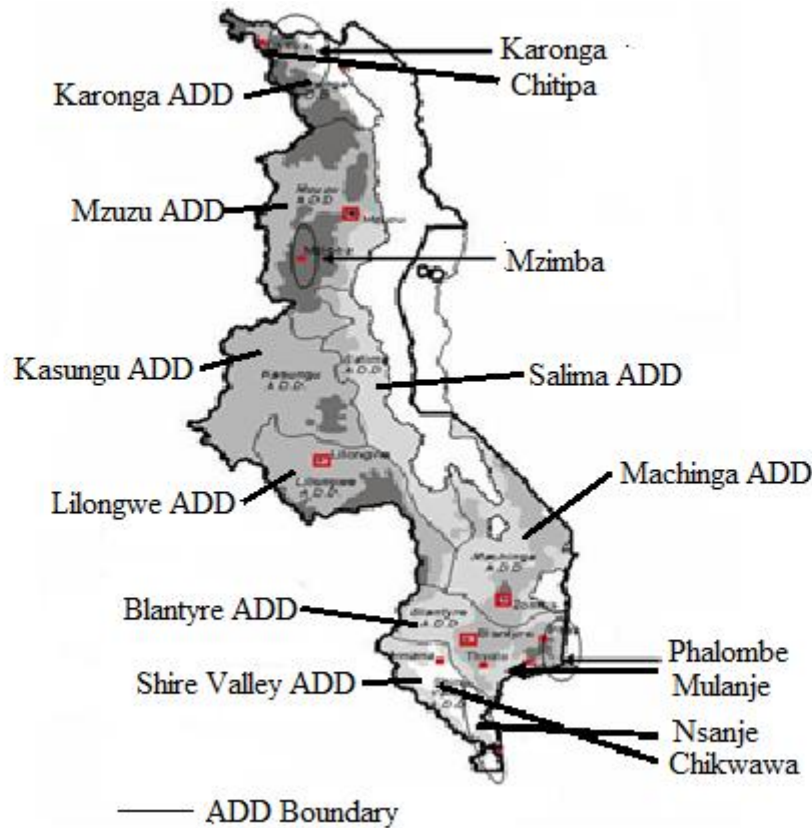

Figure 1. Map of Malawi showing sweet potato collection sites and Agricultural Development Divisions (ADD).

Table 1. Number of sweet potato accessions collected per district and farmer in Malawi.

District	Accessions per district		Number of Farmers per district			Cultivars per farmer	
	Number	%	Interviewed	Donors	%	Mean	Maximum
North							
Chitipa	58	22	20	12	60	4.83	12
Karonga	29	11	20	8	40	3.62	6
Mzimba	46	17	20	13	65	3.54	12
South							
Chikwawa	42	16	20	11	55	3.81	8
Nsanje	77	29	20	12	60	6.41	16
South east							
Phalombe	12	4	20	4	20	3.00	3
Mulanje	4	1	20	2	10	2.00	2
Total	268	100	140	62	44		
Mean	38.26	14.29	20	8.86	44	3.85	8.43
±SD	25.54	-	-	4.34	-	1.40	5.16

SD: Standard deviation.

discs were ground with the aid of carborundum powder in 2 ml microcentrifuge tubes. A total of 500 µl of preheated (60°C) extraction buffer (1.5% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 mM NaCl, 0.2µl β-mercaptoethanol) was added and the mixture

Table 2. Phenotypic classes of morphological traits used for diversity analysis in sweet potato in seven districts in Malawi.

Character	Abbreviation	Character states	IBPGR/CIP code	No. of classes
Vine inter node length	VL	Very short (<3 cm); short (3-5 cm); intermediate (6-9 cm); Long (10-12 cm); very long (>12 cm)	1, 3, 5, 7, 9	5
Vine inter node diameter	VD	Very thin (< 4 mm); thin (4-6 mm); intermediate (7-9 mm); thick (10-12 mm); very thick (>12)	1, 3, 5, 7, 9	5
Vine tip hairiness	TP	None, sparse, moderate, heavy, very heavy	0, 3, 5, 7, 9	5
Leaf lobe number	LN	1, 3, 5, 7, 9	1, 3, 5, 7, 9	5
Petiole pigmentation	PP	Green; Green with purple near stem; Green with purple near leaf; Green with purple at both ends; Green with purple stripes; Purple with green near leaf; Some petioles purple, others green, totally and mostly purple	1, 2, 3, 4, 5, 6, 7, 8, 9	9
Storage root shape	RS	Round; round elliptic; elliptic; obovate; ovate; oblong; long oblong; long elliptic; long irregular or curved.	1, 2, 3, 4, 5, 6, 7, 8, 9	9
Predominant flesh colour	FC	White; Cream; Dark cream; Pale yellow; Dark yellow; Pale orange; Intermediate orange; Dark orange; Strongly pigmented with anthocyanins	1, 2, 3, 4, 5, 6, 7, 8, 9	9
Total classes	-	-	-	47

incubated at 60°C (water bath) for 60 min. An equal volume (500 µl) of chloroform: isoamyl-alcohol (24:1, v/v) was added and the homogenate mixed on shaker for 20 min. The mixture was centrifuged at 15000 rpm for 15 min in a Tomy high speed microcentrifuge. Thereafter, 450 µl of supernatant was transferred into 2.0 ml microfuge tubes, 100 µl of 20% SDS added, mixed and incubated at 65°C for 10 min in a water bath. Potassium acetate (500 µl; 5 M) was added and mixture incubated at 4°C for 20 min and centrifuged at 15 000 rpm for 10 min. The DNA in the supernatant was precipitated in 700 µl cold isopropanol at -20°C for 1 to 2 h. After centrifugation at 15000 g for 15 min, the alcohol was decanted, and the DNA pellets were rinsed with 70% cold ethanol and centrifuged again for 5 min. The DNA pellets were air dried for 15 min before suspension in 50 µl Tris-EDTA buffer (pH 8.0). The DNA extracts were further purified by repeated phenol-chloroform and chloroform: isoamylalcohol procedures in order to remove PCR inhibitors before resuspension in 50 µl TE after air drying.

DNA amplification and visualisation

The Polymerase Chain Reaction (PCR) using ten SSR primers (Table 3) was carried out in a mini-cycler model PTC-150 (MJ Research Inc, Watertown, USA). PCR final volume for each tube was 13.11 µl, comprising 2 µl of 25 ng/µl genomic DNA, 5.7 µl double distilled water, 1 µl of 10 mM dNTP mix, 1.25 µl of 10X PCR buffer, 1.6 µl of 25 mM Magnesium Chloride ($MgCl_2$), 0.75 µl of 15 pmol of both forward and reverse primers and 0.06 µl of 5 u/µl *Taq* DNA polymerase stored in buffer A (Promega, 2000), was used.

PCR steps included the following: initial denaturing at 94°C for 2 min, then 30 amplification cycles of denaturing at 94°C for 30 s, annealing at an optimal temperature for a specific primer pair for 15 s and extension at 72°C for 30 s. The final extension was at 72°C for 20 min followed by a soaking temperature of 4°C. The amplified products of PCR were resolved using 6% polyacrylamide gel electrophoresis in BIORAD Sequi-Gen® GT Nucleic Acid Electrophoresis Cell where pGem DNA marker (Promega, 2000) and ϕ X174 DNA/*Hinf* 1 (Promega, 2000) were used as band size standard markers.

Data analysis

Statistical analysis for loci variability

In order to investigate the genetic variation among sweet potato accessions in the study, the 59 accessions were assigned to five population groups, namely, North (1), South East (2), Shire Valley(3), Landraces (a combination of North, South East and Shire valley) (4) and introductions (5). Allelic variation was estimated by the total number of alleles amplified per loci and population. Polymorphism Information Content (PIC), a measure of variability of each locus was calculated as described by Saal and Wracke (1999):

$$\mathrm{PIC} = 1 - \sum_{i=1}^{n} p_i^2$$

where p_i is the frequency of the ith allele out of the total number of alleles at a SSR locus and n is the total number of different alleles for that locus.

Analysis of genetic variation

Owing to difficulty in estimation of exact number of copies of individual alleles among polyploids like sweet potatoes, allelic data is usually analyzed at binary data matrix and SSRs are considered as dominant markers (Lian et al., 2003). Therefore, each allelic band was considered as a binary character and was scored as 1 (present) or 0 (absent) for each sample, hence, generating a data matrix usable in POPGENE freeware version 1.31 (Yeh et al., 1999). Two measures of genetic diversity; Nei's genetic

Table 3. Microsatellite (SSR) primers used in the study.

Primer	Primer sequences (5´-3´)	Primer source	Observed allele size range	Expected allele size range	Ta[1] (°C)
IB-S09	F GCTGCTCAATCCCTCTCCTT R GGAACTCGATACAGCGTGGT	Benavides (2002-2203)	46-52	*	60
IB-S10	F CTACGATCTCTCGGTGACG R CAGCTTCTCCACTCCCTAC	Benavides (2002-2203)	350-396	*	60
IB-R13	F GTACCGAGCCAGACAGGATG R CCTTTGGGATTGGAACACAC	Karuri et al. (2010)	222-226	*	60
IB-R16	F GACTTCCTTGGTGTAGTTGC R AGGGTTAAGCGGGAGACT	Karuri et al. (2010)	218-240	131-237	60
IB-R19	F GGCTAGTGGAGAAGGTCAA R AGAAGTAGAACTCCGTCACC	Karuri et al. (2010)	218-222	190-208	60
IB-CIP-5R	F CCTCAACGAATTTGACCTC R GATGACGGTGTGTCTGAAG	Yanez (2002)	120-128	*	65
IB-242	F GCGGAACGGACGAGAAAA R ATGGCAGAGTGAAAATGGAACA	Buteler et al. (1999)	124-132	95-135	58
IB-286	F AGCCACTCCAACAGCACATA R GGTTTCCCAATCAGCAATTC	Buteler et al. (1999)	100-106	90-122	57
IB-297	F GCAATTTCACACACAAACACG R CCCTTCTTCCACCACTTTCA	Buteler et al. (1999)	126-138	130-200	58
IB-324	F TTTGGCATGGGCCTGTATT R GTTCTTCTGCACTGCCTGATTC	Tseng et al. (2002)	126-134	*	56

Ta[1]=Optimal annealing temperature, *Required information the fields not available.

diversity (h) (Nei, 1973a, b) and Shannon's information index (I) (Lewontin, 1974) were computed in POPGENE.

Pearson's correlation coefficient was calculated to estimate the degree of association among indices. The significance of the coefficients was calculated at P<0.05 using the t- statistics (Sokal and Rholf, 1969).

Cluster analysis comparison using SSR and morphological markers

The data on morphological traits and SSR of the 59 accessions were transformed into binary data matrixes. The presence of a SSR allele at a particular locus and a character state in a particular class for morphological traits was recorded as 1 and 0 for present and absent, respectively. Based on the presence/absence, dissimilarity coefficients were generated using the SIMINT module (NTSYS pc 2.11c software (Rholf, 2001)). The default parameter DIST (average genetic distance) was used to generate the binary data matrix. Dendrograms were generated from the sequential, agglomerative, hierarchical, and nested (SAHN) clustering method using the Unweighted Pair Group Method and Arithmetic Average (UPGMA) (Sneath and Sokal, 1973; Rholf, 2001) using NTSYS pc 2.11. Correlations between similarity matrices from morphological and SSR coefficients were calculated by Pearson's product-moment. The significance of the correlation was tested by Mantel's test (Mantel, 1967) using the NTSYS program (MXCOMP option).

RESULTS AND DISCUSSION

Variation of SSR markers

Number of alleles and size range

The total number and size range of alleles at each locus among the five populations are presented in Table 4. The total number of alleles scored varied among the ten loci and five populations. The highest number scored with reference to all populations was at locus IB-297 (5

Table 4. Number of alleles (A) and size ranges (SR) in base pairs (bp) in 59 sweet potato accessions at ten SSR loci.

Population	Locus IB-R16		Locus IB-324		Locus IB-297		Locus IB-242		Locus IB-286	
	A	SR	A	SR	A	SR	A	SR	A	SR
Shire Valley	2	218-240	3	126-134	5	126-138	4	124-132	4	100-106
South East	2	218	3	126-134	5	126-138	4	124-132	4	100-106
North	2	218-240	3	126-134	5	126-138	4	124-132	4	100-106
*Landraces	2	218-240	3	126-134	5	126-138	4	124-132	4	100-106
Introductions	2	218-240	3	126-134	5	126-138	4	124-132	4	100-106
All populations	2	218-240	3	126-134	5	126-138	4	124-132	4	100-106

Population	Locus IB-R19		Locus IB-R13		Locus IB CIP-5R		Locus IB-SO9		Locus IB-S10	
	A	SR	A	SR	A	SR	A	SR	A	SR
Shire Valley	2	218-222	2	222-226	3	120-128	3	46-52	2	350-396
South East	2	218-222	2	222-226	3	120-128	3	46-52	2	350-396
North	2	218-222	2	222-226	3	120-128	3	46-52	2	350-396
*Landraces	2	218-222	2	222-226	3	120-128	3	46-52	3	350-396
Introductions	2	218-222	2	222-226	3	120-128	3	46-52	2	350-396
All populations	2	218-222	2	222-226	3	120-128	3	46-52	2	350-396

*Landraces = combination of South East, North and Shire valley populations.

alleles) and the least at loci IB-R16 (2 alleles), IB-R19 (2 alleles), IB-R 13(2 alleles) and IB-S10 (2 alleles). A total of 30 allele sizes with a range of two to five alleles and a mean of three alleles per locus were observed in the study. Gichuru et al. (2006) also generated two to five alleles in 57 sweet potato landraces from Kenya, Uganda and Tanzania using four SSR primers. Another study on sweet potato by Kiarie et al. (2016) which used ten SSR markers revealed a total number of alleles of 18 with an average of 3 alleles per locus. Low total numbers of alleles (23) were also recorded among Kenyan sweet potato in a study by Karuri et al. (2010) which employed six SSR markers. The average number of alleles per locus in their study was 3.67. Such findings from Kenya, which is a secondary centre of sweet potato diversity implies the Malawian accession are equally genetically diverse given that Karuri et al. (2010) genotypes revealed high levels of observed heterozygosity ranging from 0.21 to 1.0. High genotypes diversity among Kenyan sweet potato has been previously observed by other researchers (Gichuru et al., 2006; Njuguna, 2005; Karuri et al., 2009). A study by Roullier et al. (2013c) of 369 landraces in Papua New Guinea, another secondary centre of sweet potato diversity, revealed 16 alleles at six SSR loci with a mean of 6.7 alleles per locus. A higher number of total alleles was however reported by Zhang et al. (2000) who reported 70 SSR variants from six loci in 113 accessions from three geographic origins, averaging 11.67 variants per loci. The high number of variants generated in this study could be attributed to the large number of accessions and the wide geographical sampling range (Zhang et al., 2000). Random mutations that occur over time as a result of asexual propagation of sweet potato via vines can explain the allelic diversity observed in the present study (Villordon and LaBonte, 1995; Zohary, 2004; Purugganan and Fuller, 2009; Roullier et al., 2011; Roullier et al., 2013b). Such mutations are also the cause of allelic diversity among banana cultivars which are also vegetatively propagated (Changadeya et al.,

Table 5. Polymorphism information content (PIC), Nei's (h) gene diversity measure and Shannon information index (I) at six SSR loci for all sweet potato accessions.

Population	N	Primer PIC values												h	I
		IBR-16	IB-324	IB-297	IB-242	IB-286	IB-R19	IB-R13	IB-CIPR5	IB-S09	IB-S10	Mean PIC	± SE		
Shire Valley	19	0.42	0.66	0.75	0.71	0.46	0.45	0.46	0.63	0.66	0.46	0.57	0.02	0.37	0.49
South East Region	7	0.00	0.60	0.75	0.67	0.67	0.42	0.44	0.62	0.63	0.46	0.53	0.04	0.25	0.36
Northern Region	24	0.38	0.66	0.77	0.69	0.39	0.48	0.46	0.64	0.66	0.47	0.56	0.03	0.32	0.47
Introductions	9	0.30	0.57	0.73	0.69	0.42	0.46	0.47	0.59	0.63	0.38	0.52	0.01	0.25	0.38
Landraces	50	0.37	0.66	0.77	0.70	0.46	0.46	0.46	0.64	0.66	0.47	0.57	0.04	0.32	0.47
Mean	59	0.29	0.63	0.75	0.69	0.48	0.45	0.46	0.62	0.65	0.45	0.55	-	0.30	0.43
± SE	-	0.01	0.04	0.05	0.03	0.06	0.04	0.03	0.02	0.02	0.03	0.04	-	0.05	0.06

SE: Standard error.

2012b). Ultimately, genetic diversity of the studied materials is the most important factor limiting average number of alleles identified per SSR locus during screening. However, factors such as number of SSR loci and repeat types and methodologies employed for detection of polymorphic markers influence allelic differences (Legesse et al., 2007). This study used Polyacrylamide Gel Electrophoresis (PAGE) which is considered second best to Automated Sequencer Capillary Electrophoresis (ASCE) in terms of efficiency of resolving allelic variations at a finer scale than Metaphor® Agarose Gel Electrophoresis (MAGE) (Sanchez-Perez et al., 2006).

Polymorphism Information Content (PIC) of the six SSR loci

A summary of PIC, Nei's gene diversity (h) and Shannon information index (I) is presented in Table 5.

Mean PIC for the primers ranged from 0.29 (IB-R16) to 0.75 (IB-297) with mean value of 55. On average the primers revealed the highest polymorphism in Shire valley and landraces populations (PIC, 0.57) and the lowest in introduction population (PIC, 0.52). The primers mean PIC of 0.55 implies that the loci used in the study were highly polymorphic and discriminatory since any PIC value > 0.5 indicates highly polymorphic locus (Botstein et al., 1980). Therefore, the allelic diversity and molecular relationships in this study are strongly supported. The mean PIC value reported in this study is higher than 0.46, 0.28, 0.39, 0.47, 0.27, 0.42, and 0.36 reported for sorghum (Geleta et al., 2006), cucumber (Danin-Poleg et al., 2001), potato (Ashkenazi et al., 2001), sweet potato (Karuri et al., 2010), sweet potato (Ochieng et al., 2015), sweet potato (Naidoo et al., 2016), sweet potato (Kiarie et al., 2016), respectively. Hao et al. (2006) recommended that any objective evaluation of genetic diversity among germplasm collections needs to consider, both, the number of alleles per locus and their respective PIC values in combination. The PIC values per locus in the current study showed a significant and positive correlation with the number of alleles per locus ($r = 0.81$, $P < 0.05$). The results are consistent with those of Yu et al. (2003) and Jain et al. (2004) in rice ($r = 0.62$, 0.72, respectively) and by Vaz Patto et al. (2004) in maize ($r = 0.85$). The findings, therefore, suggest that in general the sweet potato accessions harbour high genetic divergence and the highest are exhibited by landraces and Shire valley accessions and the lowest are in introductions. This observation also indicates that local allelic diversity in landraces can be relied upon in breeding programs other than imported diversity in introductions.

Genetic diversity among geographical populations

Genetic diversity among the populations as measured by Nei's gene diversity (mean h=0.30) measure and Shannon information index (mean I=0.43) showed that the populations were moderately diverse (Table 5). The two indices

were positively and significantly correlated (r=0.84) and the differences among populations for h and I indices were significant at p< 0.05. The indices confirmed the findings from individual population PIC indicating that Shire valley (h=0.37; I=0.49) and Landraces (h=0.32; I=0.47) accessions were the most genetically diverse and introductions (h=0.25; I=0.38) and south east region (h=0.25; I=0.36) accessions were the least diverse (Table 5). Ochieng et al. (2015) in their study of 68 sweet potato accessions and 12 SSR loci reported similarly moderate mean gene diversity (h=0.34). Kiarie et al. (2016) using ten SSR loci on 18 sweet potato accessions recorded moderate gene diversity of 0.41. Crus da Silva et al. (2013) detected moderate mean gene diversity (h=0.27) using RAPD molecular markers on Northeastern Brazilian sweet potato. Similar moderate to low gene diversity values have been registered in other crops such in mulberry population (h= 0.20) (Zhao et al., 2006) and *Medicago citrina* populations (h= 0.15) (Juan et al., 2004). However, some sweet potato studies in some parts of the world, have documented very high gene diversity (h); Mesoamerica (h=0.71), Venezuela-Colombia (h=0.70) and Peru-Ecuador (h= 0.52). Such findings are an indication of the richness of the Latin American gene pool as a centre of sweet potato diversity (Zhang et al., 2000).

Comparison between morphological and SSR data

UPGMA-based cluster analyses on binary data of seven morphological traits and 59 sweet potato accessions are shown in Figure 3. The morphological clustering grouped the accessions into three main clusters A, B and C consisting of a singleton accession in clusters B, 27 in cluster A and 31 accessions in cluster C. The clusters A and C comprised of accessions from all sources under study namely North, South East, Shire Valley and introductions while the singleton cluster contains accession Tchubatchuba from the Northern population. The clusters A and C were further sub grouped to establish any possibilities of the accessions to cluster according to sources of origin.

The composition of sub clusters I, II, III and IV of main cluster A contained accessions from all sources of origins while sub cluster V contained accessions from the Northern population and included Yoyera which was also sampled in the Shire Valley. While sub cluster I of main cluster C contained accessions from all sources of origins, sub cluster II contained accessions from the North including Tsambalimodzi which was also sampled from the Shire valley and an introduction A45, which originates from the Republic of South Africa. All the accessions in sub cluster III of C originated from the Shire valley.

In SSR analysis, a dendrogram for landraces (north, south east and Shire valley) (50) excluding nine introductions was generated. SSR clustering grouped the 50 landrace accessions into two main groups A and B composed of 16 and 34 accessions, respectively (Figure 3). Groups A and B generated sub clusters I to II and I to IV, respectively. The accessions in group A and its sub clusters I and II did not show the tendency to cluster according to the three eco-geographical sources. However, sub clusters I and III of main cluster B grouped accessions according to eco-geographical origins. Sub cluster I contained accessions from the North while sub cluster III contained accessions from the Shire valley.

Generally, morphological clustering of sweet potato was different from SSR clustering in the present study as different clusters contained different accessions. This implies that the two methods distinguished the genotypes in the accessions differently. This was further evidenced by the Mantel (1967) matrix correspondence test that demonstrated that there was low correspondence between the distance matrices generated from SSR and morphological traits (r = 0.1095). Low association between SSR and morphological data has been reported in different crops indicating independent nature of morphological and genetic variation since SSR loci are part of non-coding DNA which is not expressed and therefore not subjected to the same forces of selection which shape phenotypic characters (Kjaer et al., 2004; Vieira et al., 2007).

High variability was detected at both morphological (50% dissimilarity) and genetic (64% dissimilarity) level as expressed in the clustering patterns. However, both cluster (Figures 2 and 3), accessions exhibited some degree of clustering according to eco-geographical associations, suggesting a genetic distinction. This observation is contrary to what Gichuru et al. (2006) showed where morphological clustering was irrespective of geographical origin but SSR analysis tended to cluster Tanzanian landraces together from the Kenyan and Ugandan accessions. The tendency of sweet potato to cluster according to geographical source was also reported using other molecular methods such as random amplified polymorphic DNA, RAPD (Gichuki et al., 2003), Amplified Fragment Length Polymorphism (AFLP) (Zhang et al., 1998) and Selective Amplification of Microsatellite Polymorphic loci (SAMPL) (Tseng et al., 2002). The pattern of some accessions in this study to cluster irrespective of eco-geographical origin implies some similarity among them which could be due to gene flow which is facilitated by long term tradition of sharing vines among farmers as well as recent increased efforts by NGOs to distribute massively sweet potato vines especially during years of drought. Other studies have documented human mediated sweet potato gene flow since prehistorical era (Roullier et al., 2013a).

Conclusions

Morphological and SSR markers displayed considerably high genetic diversity of the sweet potato accessions as

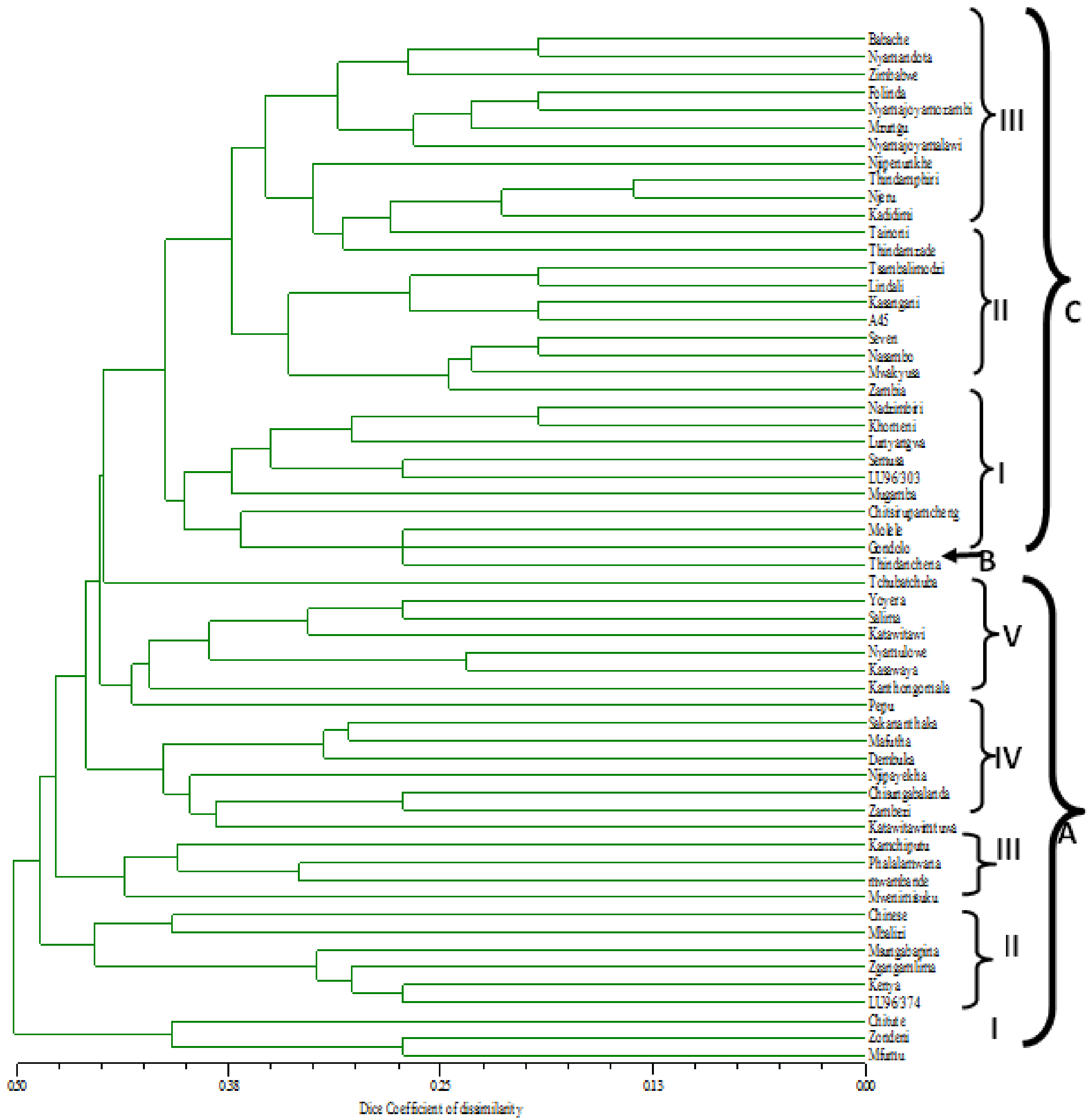

Figure 2. Cluster analysis of 59 sweet potato accessions on seven IBPGR morphological traits.

substantiated by diversity measures used in the study, therefore the landraces can be used in breeding programs. Each method of characterization distinguished the genotypes in the accessions differently thus can be used effectively in any sweet potato characterization program regardless of low correlation between morphological and SSR markers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

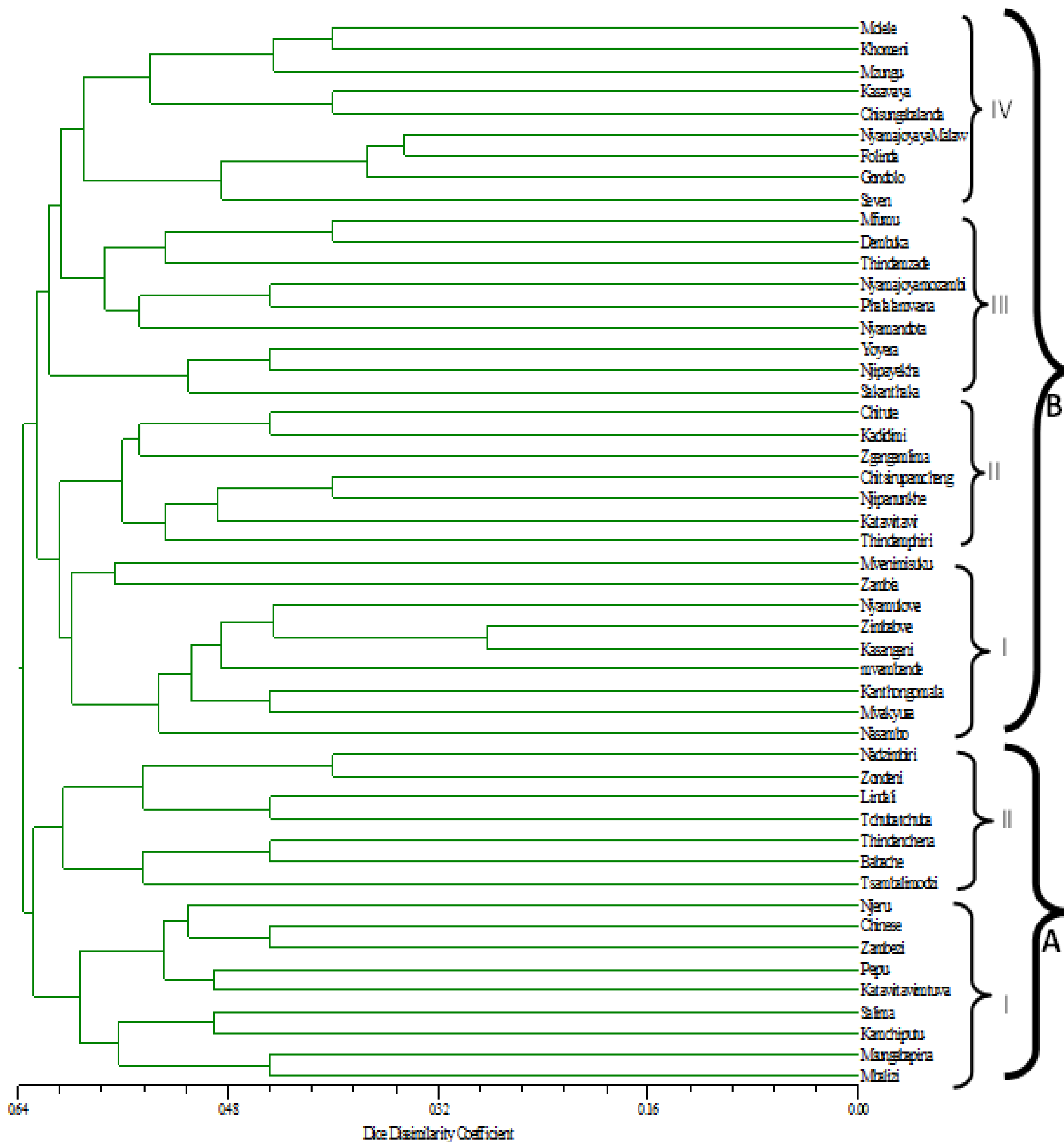

Figure 3. Cluster analysis of 50 landrace sweet potato accessions using ten SSR loci.

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Postpartum serum biochemical profile of Sudanese cystic ovarian crossbred dairy cattle

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Cystic ovarian disease (COD) is an ovarian dysfunction in cows resulting in a serious economic loss in the dairy industry. This study was conducted to examine the hemoglobin (Hb) concentration, serum total protein (TP), phosphorus (P), copper (Cu), zinc (Zn), iron (Fe) and manganese (Mn) levels of Sudanese crossbred (Friesian x Kenana) cows with COD in semi-closed condition. Forty-five dairy cows were divided into two groups. Group A (n= 30) were the cows with COD, and group B (n= 15) were healthy normal cycling cows (NC) that served as healthy control. Diagnosis of COD was based on history of frequent prolonged signs of estrus and per rectal palpation. Per rectal palpation for the uterus and ovaries was done weekly. A cow having a large follicle in the ovary that remained at the same position for three successive palpations or more was considered having COD. Results of the blood analysis showed that the serum levels of P, Cu, Zn and Mn of cows with COD were significantly lower ($P<0.05$) than those of NC cows (5.2 ± 1.3 vs. 6.7 ± 2.5 mg/dl, 0.41 ± 0.3 vs. 0.72 ± 0.3 ppm, 0.5 ± 0.3 vs. 0.7 ± 0.3 ppm and 0.4 ± 0.2 vs. 0.6 ± 0.2 ppm, respectively). No differences ($p > 0.05$) in Hb concentration (7.5 ± 1.2 vs. 7.4 ± 1.1 g/dl), serum TP (6.8 ± 1.2 vs. 6.5 ± 0.7 g/dl) and Fe (3.7 ± 1.3 vs. 3.7 ± 1.9 ppm) were observed between the two groups. This study reported reduced serum minerals (P, Cu, Zn and Mn) levels in Sudanese crossbred dairy cows with COD as compared to NC cows. Future studies are still needed to highlight the contribution of these minerals in inducing COD.

Key words: Cystic ovarian disease, deficiency of minerals, dairy cow.

INTRODUCTION

Over the past few decades, milk yield per cow has relatively increased due to a continuous genetic selection, improvement of nutrition and herd management (Oltenacu and Broom, 2010). Simultaneously, dairy cow fertility has significantly declined (Butler, 2003). Reproductive performance is an essential factor for assessing the dairy

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Table 1. Proximal composition of supplementary feed provided to dairy cows.

DM	CP	EE	NFE	Ash	Moisture
92.40	20.80	6.40	52.20	4.90	7.60

Table 2. Proximal composition of forages of Abu 70 (*Sorghum vulgare*) and Alfalfa/Burseem grown at river Nile state as DM% (AbuDamir et al., 1983).

Feed	DM (%)	CP (%)	CF (%)	EE (%)	NFE (%)	Ash (%)
Abu70	93.50	10.73	40.14	1.37	37.15	10.66
Burseem	93.48	22.93	30.40	1.63	32.70	12.35

Table 3. Mineral composition of forages of Abu 70 and Barseem grown at river Nile state as DM% (AbuDamir et al., 1983).

Feed	Ca	Mg	P	Na	K	Cu	Zn	Mn
Abu70	0.44	0.30	0.24	0.02	2.64	7.84	25.4	53.1
Burseem	2.1	0.28	0.22	0.034	3.18	10.38	22.0	55.6

cow profitability. It is known that the end product of the reproductive process is a result of a close and well-orchestrated interaction between hypothalamus, pituitary, ovary and the uterus (Carruthers et al., 1980). The complexity of fertility suggests that any factor that interferes with the function of one or more organ would be influential to the general reproductive health (Christensen et al., 2012). One of the most common ovarian dysfunctions during early postpartum period (PPP) is ovulation failure, and consequent formation of ovarian cyst (Opsomer et al., 1998). The cystic ovarian disease (COD) is an important cause of subfertility in dairy cows as it extends the calving interval (Vanholder et al., 2006). This extension, in addition to the treatment cost and the increasing involuntary culling rate, would result in considerable loss for the dairy farmers (Bartlett et al., 1986; Vanholder et al., 2006). It has become clear that COD is the consequence of malfunction of the hypothalamic-pituitary-gonadal axis (Peter, 2004). The cows with high milk yield were more susceptible to develop infertility (Lucy, 2001). High milk production associated with negative energy balance (NEB) during the early PPP, was reported as a predisposing factor for the COD (Vanholder et al., 2006). The role of the NEB in cyst formation remains inconclusive (Butler, 2003). However, it was suggested that cows with a longer period of NEB, poor liver functions and low circulating insulin-like growth factor-I (IGF-I) concentrations in the early PPP were likely to develop inactive or cystic ovaries and persistent corpus luteum (Zulu et al., 2002a; Zulu et al., 2002b). The genetic factor may also be involved (Hooijer et al., 2001). Certain animal lines such as the Holstein Friesian were genetically predisposed to develop COD (Vanholder et al., 2006). Moreover, it has been reported that single or combined mineral (Cu, Co, Se, Mn, I, Zn and Fe) deficiency can induce reproductive failure (Hidiroglou, 1979; Ahmed et al., 2017).

In the Sudan, to fill the gap of shortage in milk production, some local cow breeders had imported Holstein-Friesian cattle since 1976 (Rahman and Alemam, 2008). Due to the widespread distribution of crossbred dairy cows, more research is required to investigate the incidence and prevalence of all infertility problems. This study was conducted to estimate some minerals serum levels in Sudanese crossbred dairy cows with COD.

MATERIALS AND METHODS

This study was carried out in the River Nile State, Sudan, during the year 2014. Forty-five crossbred (Friesian x Kenana) dairy cows were included. They were under semi-closed system as they were allowed to graze from 7 to 10 am. Their ages ranges between 5 and 11 years, and their body condition scores (Wildman et al., 1982) were from 3.0 to 3.50. The cows were milked twice a day. They were fed roughages, composed of Abu 70 (*Sorghum vulgare*) and Alfalfla (AbuDamir et al., 1983) (Tables 2 and 3), in addition to a supplementary feed that was prepared to meet their production requirements (Table 1). The cows were divided into two groups. Cows in group A (n=30) were diagnosed having cystic ovarian disease (COD), whereas group B cows (n=15) were healthy and normally cycling (NC) that served as control. The COD was diagnosed based on history of frequent prolonged signs of estrus, and further by per rectal palpation (Hafez and Hafez, 2000; Noakes et al., 2001). Per rectal palpation for the uterus and ovaries was done every week starting from the third postpartum week as a routine practice for each cow. A cow having a large sac-like fluid filled structure in the ovary that remained at the same position for

Table 4. Hemoglobin (Hb) concentration (g/dl), serum total protein (TP) (g/dl) and serum levels of P, Cu, Zn, Fe, Co and Mn in cows with COD (group A) and NC cows (group B).

Parameter	Group A	Group B
Hb (g/dl)	7.4 ±1.1[a]	7.5 ± 1.2[a]
TP (g/dl)	6.5 ± 0.7[a]	6.8 ± 1.2[a]
P (mg/dl)	5.2 ± 1.3[a]	6.7 ± 2.5[b]
Cu (ppm)	0.4 ± 0.3[a]	0.7 ± 0.3[b]
Zn (ppm)	0.5 ± 0.3[a]	0.7 ± 0.3[b]
Fe (ppm)	3.7 ±1.3[a]	3.7 ± 1.9[a]
Mn (ppm)	0.4 ± 0.2[a]	0.6 ± 0.2[b]

Results are shown as mean ± standard deviation (SD). Values with different superscripts in the same row differ significantly (P < 0.05).

three or more successive palpations was considered having COD.

Collection of blood samples

Ten milliliters of blood were collected from the jugular vein of each cow. Two milliliters in heparinized tube was used for estimation of Hb concentration, and 8 ml in sterile tube for estimation of TP and the minerals (P, Cu, Zn, Fe and Mn). The blood in the 8 ml-tube was allowed to clot by leaving it undisturbed at room temperature for about 30 min. The clot was then removed by centrifugation at 2000 *g* for 10 min and sera were stored at -20°C until analysis.

Measurement of Hb, total protein and minerals serum levels

The hemoglobin concentration was estimated within two hours from blood collection using the standard Sahli's method. The serum total protein concentration was estimated with a commercial kit (Biuret Colorimetric kit, Spinreact, Spain). The serum levels of P, Cu, Zn, Fe and Mn were measured using the Phoenix -986 atomic absorption spectrophotometer.

Statistical analysis

The statistical analysis was performed using the SPSS version 20.The Independent Sample T test was used to compare the means between the two groups. Results were expressed as mean ± standard deviation (SD). Significant difference was considered at p<0.05.

RESULTS

The means ± standard deviation (SD) of Hb concentration, serum total protein and serum minerals levels of group A and group B are shown in Table 4. No differences (p > 0.05) were observed in Hb concentration (7.4 ± 1.1 vs. 7.5 ± 1.2 g/dl), serum TP (6.5 ± 0.7 vs. 6.8 ± 1.2 g/dl) and the level of serum Fe (3.7 ± 1.3 vs. 3.7 +1.9 ppm) between the two groups. However, the serum levels of P, Cu, Zn, and Mn were lower (p < 0.05) in COD (group A) than those of NC cows (group B) (5.2 ± 1.3 vs. 6.7 ± 2.5 mg/dl, 0.41 ± 0.26 vs. 0.72 ± 0.29 ppm, 0.5 ± 0.3 vs. 0.7 ± 0.3 ppm and 0.4 ± 0.2 vs. 0.6 ± 0.2 ppm, respectively).

DISCUSSION

Cystic ovarian disease (COD) is one of the most important infertility problems in dairy cows. It occurs most frequently during the PPP one to two months after calving at a time when ovarian function usually restarts (Vanholder et al., 2006). It is characterized by the presence of one or more large anovulatory follicular cysts in the ovary, unilateral or bilateral, as well as abnormal pattern of estrus (Peter, 2004). The existence of such ovulatory follicular cysts would extend the calving-to-conception and calving intervals resulting in economic losses for dairy industry.

In an earlier study (Nadaraja and Hansel, 1976), COD was induced by suppressing bovine luteinizing hormone (LH) using either estradiol or antibodies against LH. Furthermore, exogenous cortisol was used to suppress the LH surge, ovulation and the behavior of estrus (Stoebel and Moberg, 1982). It has been proposed that there is a metabolic signal required for an efficient LH surge, and poor nutrition (stress) and NEB would interrupt this signal (Mwaanga and Janowski, 2000; Johnson, 2004). In the status of NEB, some hormonal and metabolic changes might increase the COD formation at the hypothalamus-pituitary as well as ovary-follicle levels (Diskin et al., 2003). During NEB, there are decreased blood levels of glucose, IGF-I, insulin and leptin (Beam and Butler, 1999; Block et al., 2001), and increased concentrations of metabolites such as non-esterified fatty acids and β-hydroxybutyrate (Vanholder et al., 2006). The IGF-I and insulin stimulate follicular development by enhancing the steriodogenesis, and differentiation of granulosa cell (Davoren et al., 1986; Zulu et al., 2002a). Leptin is a hormone produced by adipose cells, and is required to induce the first postpartum LH surge (Elias and Purohit, 2013). Nutrition and suckling were the two critical factors that delayed the onset of estrous cycles in postpartum cows (Lamb, 2012). It was reported that the postpartum period was found to be extended in Sudanese crossbred dairy cows due to many reasons and COD was one of them (Elzubeir and Elsheikh, 2004).

The result of this study shows that the serum TP of cows with COD is not different from that of the NC cows (Table 4). This result is consistent with a recent study by Yotov et al. (2014). Moreover, the Hb concentration is also not significantly different between the two groups (Table 4), which agrees with an earlier study (Larson et al., 1980). The serum Fe of cystic and control cows were nearly the same (Table 4). This result was expected as the Hb concentrations of the cystic cows were normal (Table 4), and neither anemia nor hemorrhage was

observed in both groups.

The serum phosphorus of the COD cows was significantly lower than that of the NC cows (Table 4). Similar results were reported by some recent studies (Bindari et al., 2014; Phiri et al., 2007; Yotov et al., 2014). Phosphorus is essential in every metabolic pathway, energy utilization and transfer as well as being part of nucleic acids structure (Murray et al., 2003).

This study also revealed that serum Cu of the cows with COD was lower than that of the NC cows (Table 4). This came in line with a previous research (Yasothai, 2014). Cu deficiency is associated with subfertility and delayed estrus or anestrus (Kumar et al., 2011; Yasothai, 2014). It is a co-factor for important enzymes like the amine oxidase, copper-dependent superoxide dismutase, cytochrome oxidase and tyrosinase (Murray et al., 2003). It was reported that Cu and gonadotropin releasing hormone (GnRH) complexes were more efficient in stimulating the secretion of the LH and FSH than the GnRH alone (Michaluk and Kochman, 2007).

The results also show that cows with COD had significantly lower serum Zn level than the NC cows (Table 4). Earlier studies reported that Zn deficiency was associated with reduced fertility, and that Zn supplementation was successfully used to increase the conception rate (Marai et al., 1992; Moellers and Riese 1988). Zinc was also found to be essential for recovery of the endometrium after calving and the accelerated return to estrus and normal reproductive performance (Yasothai, 2014). These different effects may be due to its metabolic effect on estrogen, progesterone and prostaglandins (Favier, 1992). Moreover, the nuclear steroid receptors are all Zn finger proteins (Favier, 1992). In addition, Zn has anti-apoptotic and antioxidant properties (Ebisch et al., 2007).

The results also showed that Mn serum level of cows with COD was significantly lower than that of their respective NC cows. This result was quite consistent with the results of previous studies (Corah, 1996; Yasothai, 2014). Deficiency of Mn was associated with occurrence of COD and poor follicular development with delayed ovulation (Corah, 1996). Mn can influence the reproductive efficiency in several ways. First, Mn is involved in all metabolic processes (Davis et al., 1990; Hansen et al., 2006; Tuormaa, 1996). Second, it acts as a co-factor for the enzymes that catalyze the biosynthesis of cholesterol (Tuormaa, 1996). Cholesterol is a precursor for all steroid hormones including the sex hormones (Murray et al., 2003). Mn was also reported to induce the hypothalamic secretion of the luteinizing hormone releasing hormone (Lee et al., 2007).

Conclusion

The current study examined the Hb concentrations and serum total protein (TP), phosphorus (P), copper (Cu), zinc (Zn), iron (Fe) and manganese (Mn) levels of 30 dairy cows diagnosed having COD and compared with 15 normal cyclic (NC) cows. Results show that there were no differences in the Hb concentrations and the serum levels of TP and Fe between the two groups ($p > 0.05$). However, the serum levels of P, Cu, Zn and Mn of cows with COD were significantly lower than those of the NC cows ($p < 0.05$). This study reported decreased serum minerals (P, Cu, Zn and Mn) levels in Sudanese crossbred dairy cows with COD as compared to NC cows. Future studies with a larger sample size are recommended to highlight the contribution of these minerals in inducing COD in these cows.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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