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SPECIES DIVERSITY AND MOLECULAR CHARACTERIZATION OF ENTOMOPATHOGENIC NEMATODES (HETERORHABDITIDAE, STEINERNEMATIDAE) ISOLATED FROM SOILS OF DIFFERENT ECOSYSTEMS IN NORTHERN POLAND

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K e y w o r d s: entomopathogenic nematodes, LSU, PCR-RFLP, Steinernematidae, Heterorhabditidae, natural occurrence, habitat preference, survey.

A b s t r a c t

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae were isolated from 57 of 91 localities in northern Poland. Of 489 soil samples collected in the field, EPNs were recorded in 27 per cent of them. Steinernema species were more frequent than Heterorhabditis: five of the family Steinernematidae and two of the family Heterorhabditidae were identified. Nematodes *S. feltiae* was the most frequently recorded species in different ecosystems. There were two rare of entomopathogenic nematodes species to the Polish fauna: *S. silvaticum* (only in natural ecosystems) and *S. bicornutum* (only in agrocoenoses). It seems that the presence of a suitable host in the environment is the most important factor for EPNs. Simple PCR-RFLP system was used to differentiate eight EPN species.

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Introduction

Several qualities predispose nematodes of the families Steinernematidae and Heterorhabditidae as biological control agents of pests (EHLERS 2001, SHAPIRO-ILAN et al. 2006). Practical insect management with nematodes is widely used in North America, Europe and Japan (EHLERS 1996, 2001, KAYA et al. 2006, ANSARI et al. 2009). As safe to the environment and warm-blooded animals as well as relatively cost-effective and uncomplicated in mass production, entomopathogenic nematodes (EPNs) are competitive with other insecticides on the market (KAYA et al. 2006). Nematodes can be active in the soil over long periods of time and are highly resistant to adverse conditions in the surrounding environment (LEWIS et al. 2006). A wide range of potential hosts and the occurrence of several insects in the soil can increase the efficiency of pest control with EPNs in a variety of agrocoenoses (EHLERS 2001).

The infection potential of nematodes is high. Laboratory experiments show that nematodes of the family Steinernematidae can infect over 350 insect species of 13 orders (PETERS 1996) but each exhibits specific food preferences for insects occurring in the substrate. *Steinernema carpocapsae* is very attached to pests feeding in agrocoenoses, i.e. beetles of the families Elateridae and Scarabaeidae (ANSARI et al. 2003, 2009) and butterflies of the families Tortricidae and Noctuidae (BRUCK and WALTON 2007, CURTO et al. 2008) while *Steinernema feltiae* is recommended for use in covered crops to control Bibionid flies (Diptera: Bibionidae) (EHLERS 1996, KOWALSKA and KOMOSA 2006). Increased sensitivity to grub of beetles of the family Scarabaeidae (ANSARI et al. 2003) and larvae of Otiorhynchus beetles of the family Curculionidae (EHLERS 1996) is recorded in nematodes of the family Heterorhabditidae. Ecological preferences of local populations of these nematodes occurring naturally in different agro- and bioceonoses should be examined to use EPNs effectively in integrated crop protection programmes. Literature data show that the genetic pool of wild strains of appropriately selected nematodes can give rise to improved biological control (BURNELL and DOWDS 1996).

Many authors worldwide confirm that EPNs of the families Steinernematidae and Heterorhabditidae occur commonly and have been spreading (HOMINICK et al. 1996, HOMINICK 2002). As current European research into the biology and ecology of EPNs shows, these nematodes are rich in species and their environmental preferences vary. New species such as *Steinernema weiseri* (MRÁČEK et al. 2003) or *Steinernema silvaticum* (STURHAN et al. 2005) were discovered during extensive field investigations in the Czech Republic (MRÁČEK et al. 1999, MRÁČEK and BEČVÁŘ

2000, MRÁČEK et al. 2005, PŮŽA and MRÁČEK 2005), Slovakia (STURHAN and LIŠKOVÁ 1999) or Germany (STURHAN 1999) and numerous undetermined nematode isolates from eastern and northern Europe classified as the *glaseri*-group were recognized as one species, *S. arenarium* (STURHAN and MRÁČEK 2001).

Identifying EPNs using the classical method requires considerable experience and is time-consuming. Molecular methods can be an efficient tool confirming isolates determined with the morphometric assessment. PCR-RFLP is an alternative to costly DNA sequencing for molecular identification of nematodes. It is less sensitive and more difficult to gather into databases than the DNA sequencing approach but it is uncomplicated, time-saving and easy to perform in a typical molecular biology laboratory. The identification procedure consists in amplification by PCR using appropriate primers, digesting products with several restriction endonucleases and comparing band patterns. The PCR-RFLP strategy has been used both in taxonomical and phylogenetic research into nematodes (REID et al. 1997, HOMINICK et al. 1997). A homogenous identification system of all known EPN species with the PCR-RFLP method has not been described to date due to the nature of the method, a large number of species and differences in their geographical distribution. At present a total of 75 species of the family Steinernematidae and 19 of the family Heterorhabditidae are listed in the NCBI database. Although numerous nematode sequences, including EPNs, are stored in GenBank, the genome structure of newly discovered species is not available. For instance, only one sequence of *S. silvaticum* is deposited in GenBank.

Nematode species were molecularly characterized most often by analyzing ribosomal DNA (rDNA), which has become a useful DNA region for classifying different eucaryotes at various taxonomical levels (HILLIS and DIXON 1991, after NASMITH et al. 1996). The rDNA is a multi-copy, tandemly repeated array occurring in the nucleolar organizer region at one or several chromosomal sites (LONG and DAWID 1980, after NASMITH et al. 1996). Within the rDNA cistron are coding and non-coding sequences that can be used to study various taxonomical levels, from within species populations to taxa at or above genera. The rDNA coding genes vary in evolutionary conservation from most-conserved 18S (SSU, small subunit), 5.8S to least-conserved 28S (LSU, large subunit).The spacer regions including ETS (external transcribed spacer), ITS (internal transcribed spacer) and IGS (intergenic spacer) are more variable than the gene regions and are generally used for analysis at or below the species level (BECKINGHAM 1982, after NASMITH et al. 1996)

The D2 and D3 expansion segments of the 28S rRNA are often sequenced in studies of nematode phylogenetics due to the availability of conserved primers amplifying DNA from many taxa, and the presence of phylogenetically informative sites (SUBBOTIN et al. 2007). The D2 and D3 segments are useful for analyzing relationships including higher taxonomical levels; examples include studies among orders of the phylum Nematoda (LITVAITIS et al. 2000), within the order Cephalobina (NADLER et al. 2006) as well as within genera of several orders, e.g. *Steinernema* (STOCK et al. 2001).

The aim of this study was to develop a simple and cost-effective system to identify EPNs naturally occurring in the fauna of Poland and Central Europe and to present their biodiversity (MRÁČEK et al. 2005), to establish sequences of the fragment within the 5'-end of the nuclear LSU rDNA that included the D2 and D3 domains for *S. silvaticum* and to compare it to the known sequences of other nematode species.

Materials and Methods

Field collection

Field studies were conducted in 2008–2013 in north-western Poland (Zachodniopomorskie Voivodeship), in a variety of ecosystems encompassing forest complexes, agrocoenoses (crop fields, green sites, orchards), coastal dunes and urbanized areas.

Nematode isolates were obtained from soil samples from 91 sites. Samples were collected using Egner's staff from 100 m^2 of the research area, three times per year, from spring to autumn. From a depth of up to 20 cm were collected 100 unit samples constituting the aggregate sample, with a total volume ca. 600 cm^3. The soil was transported to the laboratory in plastic perforated bags. Each soil sample was thoroughly mixed in the laboratory and placed in six 100 cm^3 plastic containers. The soil was successively wetted with water (5–15 ml H_2O) to obtain suitable moisture (70–80%).

Laboratory examinations
Isolation and multiplication of nematodes

Nematodes were isolated from the soil using the standard *Galleria mellonella* baiting insect method (BEDDING and AKHURST 1975). Morphological and morphometric features of infective-stage larvae (J_3) of these nematodes obtained with the WHITE method (1927) and adult individuals

of the second generation isolated from dead *Galleria* caterpillars by sectioning (NGUYEN and SMART 1997, HOMINICK et al. 1997, NGUYEN 2007a) were used to determine taxonomically individual nematode species.

At least two nematode isolates from different localities determined using morphological features were examined to establish PCR-RFLP profiles characteristic of individual species.

PCR-RFLP

Nucleic acid preparations were extracted from second generation females. For each species 50–100 adults were pooled and used. DNA was extracted with a ready to use set of *Kucharczyk* according to the supplier's procedure.

The rDNA regions were amplified by the PCR in 20 µl reaction. The following were added to each tube: 2 µl of 10 × PCR buffer, 2 µl of $MgCl_2$ (25 mM), 1.6 µl of dNTP mixture (2 mM each), 0.8 µl of forward primer (5 pM/1 µl), 0.8 µl of reverse primer (5 pM/1 µl), 0.2 µl Taq recombinant polymerase (5 U/1 µl), 0.8 µl of DNA (about 15 ng μl^{-1}) and 11.8 µl of distilled water. MBI Fermentas reagents were mostly used.

Three primer sets were used at the initial stage: ITS1-forward ACGAGCCGAGTGATCCACCG (CHERRY et al. 1997, after ADAMS et al. 1998) and ITS1-reverse TTGATTACGTCCCTGCCCTTT (VRAIN et al. 1992, after ADAMS et al. 1998), LSU-forward AGCGGAGGAAAAGAAACTAA (NADLER and HUDSPETH 1998, after STOCK et al. 2001) and LSU-reverse TCGGAAGGAACCAGCTACTA (THOMAS et al. 1997, after STOCK et al. 2001) as well as 18S-forward GCAAGTCTGGTGCCAGCAGC (FOUCHER and WILSON 2002) and 18S-reverse CCGTGTTGAGTCAAATTAAG (FOUCHER and WILSON 2002).

PCR reactions were carried out in a Gene Amp® PCR System 9700 (Applied Biosystems). PCR cycling parameters included denaturation at 95°C for 3 min, followed by 8 cycles of 95°C for 30 sec, 64°C→50°C (touchdown 2°C/1 cycle) for 60 sec, 72°C for 60 sec, followed by next 26 cycles of 95°C for 30 sec, 50°C for 60 sec, 72°C for 60 sec, followed by a postamplification extension at 72°C for 5 min.

After the PCR 5µl of the mixture, mixed with 9µl of distilled water with 1 µl of buffer and 0.5 µl enzyme (10 U μl^{-1}), was used to digest amplification products. Six endonucleases were initially used (AluI, HinfI, HhaI, HpyF3I, PvuII, RsaI). Incubation was conducted overnight in a laboratory incubator at 37ºC.

PCR and PCR-RFLP products were separated by electrophoresis in 1.5% agarose gel. 1· TBE buffer was used for gel preparation and electro-

phoresis. Electrophoresis was run at a constant voltage of 90 V for ca. 90 minutes. Agarose gel was stained with 0.5 μgml ethidium bromide for 5–10 minutes. Electrophoresis products were visualised using UVG:BOX Syngene (Biotech) and documented with GeneSnap7.02 (Synoptics Ltd). Amplification and digestion products were sized with a gene ruler 100 bp DNA Ladder Plus (MBI Fermentas) consisting of 14 fragments in the range 100–3000 bp.

Cloning and sequencing

PCR products were separated in 1% agarose gel. 1×TAE buffer was used. The gel and buffer contained 1 mM l^{-1} guanosine. PCR products were isolated from agarose gel with TOPO TA Cloning® (Invitrogen). Vector pCR®II-TOPO® was used. *E. coli* competence was induced chemically according to the procedure of Invitrogen. LB Agar medium (A&A Biotechnology) and LB-Medium (Carl Roth) were used. GenomeLab DTCS – Quick Start Kit (Beckman Coulter) and M13 primer were used for PCR sequencing. PCR products were treated with Agencourt CleanSEQ® Magnetic Beads (Beckman Coulter). Sequencing was performed in a Beckman Coulter CEQ 8000 Genetic Analysis System. DNA was sequenced in both directions and consensus sequence was received using BioEdit (HALL 1999).

Results

Entomopathogenic nematode fauna

Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae were isolated from 57 of 91 localities, that is 63% of the total number of sites. Of 489 soil samples collected in the field, EPNs were recorded in 131 (27% of the total number of samples) – Table 1. Altogether seven species of EPN were identified: five of the family Steinernematidae and two of the family Heterorhabditidae (Table 1).

Nematodes of the family Steinernematidae recorded in 100 samples (over 76% of the total number of samples containing nematodes) were represented by five species (Table 1). *Steinernema feltiae* (Filipiev): the most frequently recorded species in northern Poland in different ecosystems, recorded in over 47% nematode-containing samples, *Steinernema affine* (Bovien) present in over 14% of nematode-containing samples, often co-occurring with *S. feltiae* in soil, especially in city parks. Its recorded both in open and forested sites. *Steinernema silvaticum* (Sturhan, Spiridonov & Mrácek) relatively rarely occurring in the study area (above 5% of nem-

atode-containing samples), especially in urban forestations and in forest complexes. *Steinernema bicornutum* (Tallosi, Peters & Ehlers) was one of rare species recorded in Poland, too; isolated from orchards and urban green areas (2% of species identified). *Steinernema carpocapsae* (Weiser): the least frequently recorded species in the study area (0.8% of nematode-containing samples).

Nematodes of the family Heterorhabditidae isolated from 35 samples (27% of the total number of nematode-containing samples) were represented by two species (Table 1):

Table 1

The occurrence of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae in northern Poland

Ecosystem	Total number of samples	Number of samples with nematode [%]	Number of samples with more than one nematode species	Number of samples with:							
				Steinernema feltiae	*Steinernema silvaticum*	*Steinernema affine*	*Steinernema carpocapsae*	*Steinernema bicornutum*	*Heterorhabditis bacteriophora*	*Heterorhabditis megidis*	*Steinernema* spp.
Urbincoenoses:											
urban and industrial areas	121	34 (28)	4	15	2	5	0	1	0	15	0
Agrocoenoses:											
orchards	19	12 (63)	0	3	0	1	0	2	0	6	0
crop fields	48	17 (35)	0	12	0	0	1	0	0	5	0
green sites (pastures, meadows)	24	6 (25)	0	0	0	2	0	0	4	0	0
Biocoenoses:											
forests	165	50 (30)	0	27	5	7	0	0	0	0	11
coastal dunes	112	11 (10)	0	5	0	0	0	0	1	4	1
Total	**489**	**131**	**4**	**62**	**7**	**15**	**1**	**3**	**5**	**30**	**12**

Heterorhabditis megidis (Poinar, Jackson & Klein): the most frequently recorded species (23% of nematode-containing samples) beside *S. feltiae*, recorded both in open areas and partly open areas (dunes, lawns, rose bushes) as well as in forestations (orchards, city parks),

Heterorhabditis bacteriophora (Poinar): the least frequently recorded species of the family Heterorhabditidae (3.8% nematode-containing samples) – Table 1; known only from open areas (meadows, dunes).

Molecular examinations

The combination of one primer set (LSU-forward and LSU-reverse) and two endonucleases (HinfI, RsaI), best differentiating the species, was selected based on initial samples with three different primer sets and six restriction enzymes. All eight species of the genera Heterorhabditidae and Steinernematidae were distinguished using this set. A band ca. 950 bp in length was the amplification product in the presence of the primer sets.

The sum of the PCR-RFLP fragments for different species varied (620–1440 bp) and did not always equal the length of the initial product (Figure 1, Table 2). It was smaller in some cases and some digestion products may have been so similar in length that they could not be differentiated on the electrophoretic image display. These fragments possibly include products of digestion with HinfI of the 320 bp LSU amplicon of *S. carpocapsae*. Similarly long fragments obtained with RsaI were recorded for *S. affine* (2 × 200 bp), *S. bicornutum* (2 × 120 bp), *S. feltiae* and *S. kraussei* (2 × 200 bp). If all fragments for the species are of this length, a total length of 920–940 bp would be obtained, which is similar to the initial amplicon.

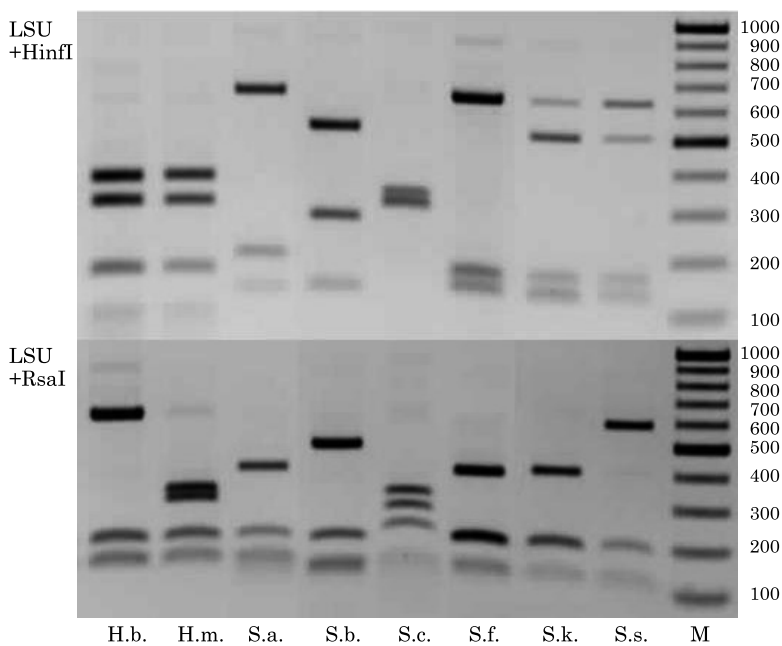

Fig. 1. Images of electrophoretic separation of fragments obtained by digesting LSU-region amplification products using HinfI and RsaI enzymes, characteristic of *Heterorhabditis bacteriofora* (H.b.), *H. megidis* (H.m.), *Steinernema affine* (S.a.), *S. bicornutum* (S.b.), *S. carpocapsae* (S.c.), *S. feltiae* (S.f.), *S. kraussei* (S.k.), *S. silvaticum* (S.s.). M – DNA length marker

Table 2

Fragment lengths (bp) obtained by digesting LSU-region amplification products using HinfI and RsaI enzymes, characteristic of *Heterorhabditis bacteriofora* (H.b.), *H. megidis* (H.m.), *Steinernema affine* (S.a.), *S. bicornutum* (S.b.), *S. carpocapsae* (S.c.), *S. feltiae* (S.f.), *S. kraussei* (S.k.). Fragments probably commigrating in gel are marked in bold

Specification	H.b.	H.m.	S.a.	S.b.	S.c.	S.f.	S.k.	S.s.
HinfI	380	380	630	520	**320**	630	630	630
	300	300	200	280	300	180	500	500
	180	180	140	140	–	140	180	180
	80	80	–	–	–	–	**130**	**130**
Total	940	940	970	940	620	950	1440	1440
RsaI	620	320	400	500	320	400	400	620
	200	300	**200**	200	280	**200**	**200**	200
	140	200	140	**120**	220	120	120	140
	–	140	–	–	140	–	–	–
Total	960	960	740	820	960	720	720	960

An atypical electrophoretic image was also displayed for *S. kraussei* and *S. silvaticum*. The total length of LSU fragments formed after cleaving with HinfI was greater than the length of the undigested amplification fragment. Two products of the same or near the same length are probably produced by the PCR but they differ by the sequence in one of the sites recognized by RsaI. Cleaving with one of them produces fragments 630, 180, 130 bp in length while the second one consists of fragments 500, 180, 130, 130 bp in length.

Discussion

The knowledge on the occurrence of entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae in Poland is fragmentary and rarely describes the nematode/environment correlation. Sites discussed in the literature include selected agrocoenoses (meadows and arable fields) of central and southern Poland (Bednarek 1990, Jaworska and Dudek 1992, Jaworska et al. 1997, Matuska and Kamionek 2008, Ropek and Nicia 2008, Tumialis et. al. 2016), forest complexes in southern and north-eastern Poland (Kamionek et al. 1995) and urban and industrialized areas in central Poland (Pezowicz 2002, Tomalak 2005). Eight species of EPNs have been reported from Poland in the literature: six species of the family Steinernematidae and two species of the family Heterorhabditidae. *Steinernema feltiae* (Bednarek 1990, Jaworska and

Dudek 1992, Dzięgielewska and Kiepas-Kokot 2004, Dzięgielewska 2012, Tomalak 2005, Ropek and Nicia 2008, Tumialis et. al. 2016) and *S. carpocapsae* (Bednarek 1990, Kamionek et al. 1995, Jaworska et al. 1997, Pezowicz et al. 2008, Tumialis et. al. 2016) are the most frequently recorded species of the family Steinernematidae. Nemtodes *S. affine*, *S. bicornutum* and *S. kraussei* are noted less frequently in the Polish fauna (Dzięgielewska 2012, Tomalak 2005, Tumialis et. al. 2014). The occurrence of *S. glaseri* should be confirmed and verified (Kamionek et al. 1995). A new species of the *glaseri*-group, *S. arenarium* (Tomalak 2003, Skrzypek et al. 2011), and *S. kraussei* (Tumialis et al. 2014) have been reported recently. *S. arenarium* is thought to be a European equivalent to *S. glaseri*, a species widespread in North America, especially the United States (Mráček et al. 2005). *Heterorhabditis megidis* and *H. bacteriophora* are the two species of the family Heterorhabditidae identified in Poland (Jaworska et al. 1997, Dzięgielewska 2012, Tomalak 2005, Matuska and Kamionek 2008, Tumialis et. al. 2016).

Six of the nematode species above mentioned have now been confirmed from north-western Poland. Only *S. arenarium*, *S. glaseri* and *S. kraussei* have not been isolated from the area to date. Altogether seven species of the families Steinernematidae and Heterorhabditidae have been recorded in the study area, including *S. silvaticum*. The total number of EPNs identified in Poland after 2000, including six species of the genus *Steinernema* and two of the genus *Heterorhabditis*, is slightly lower than that in other countries of central Europe. Eleven nematode species have been recorded in the Czech Republic, including eight of the genus *Steinernema* and two of the genus *Heterorhabditis* (Mráček et al. 2005). A total of 13 species have been noted in Germany, including ten species of the genus *Steinernema* (Sturhan 1999). Nine species have been identified in Slovakia: eight of the genus *Steinernema* and one of the genus *Heterorhabditis* (Sturhan and Lišková 1999). Ten nematode species have been isolated in northern Europe (England): eight of the genus *Steinernema* and two of the genus *Heterorhabditis* (Hominik et al. 1995).

S. silvaticum was recorded in soil samples collected in urban forestations with *Quercus robur* L. and *Quercus petraea* Mattuschka Liebl. and from large compact forest complexes (Peucedano-Pinetum and Querco roboris-Pinetum) where *Pinus silvestris* L. dominates in the tree stand. It was recorded in sites of mass occurrence of herbivorous Hymenoptera of the families *Diprionidae* and Cynipidae and the butterfly *Bupalus piniastris* L. of the family Geometridae. Although natural hosts of *S. silvaticum* have not been identified to date (Sturhan et al. 2005), it seems highly probable that these insect species can potentially be their hosts, especially Diprion

species (Hymenoptera) or geometers (Lepidoptera) which burrow in the soil to winter during the developmental cycle.

The natural occurrence of *S. silvaticum* nematodes has been relatively recently recorded in Poland (DZIĘGIELEWSKA et al. 2015, LIS et al. 2018). Earlier its has been confirmed in some European countries, i.e. Germany (STURHAN 1999), the Czech Republic (MRÁČEK and BEČVÁŘ 2000, STURHAN and MRÁČEK 2000, MRÁČEK et al. 2005), England and the Netherlands (HOMINIK et al. 1995), Belgium and Sweden STURHAN et al. (2005). Nematodes *S. silvaticum* seems to be attached to forestations and forest communities rather than to open areas. This is also suggested in studies of STURHAN (1999, 2005) and MRÁČEK et al. (2005). Studies using rDNA sequences (ITS1 + 5.SS + ITS2) define *S. silvaticum* and *S. kraussei* nematodes as sister taxa (NGUYEN 2007b). Similarly, the sequencing results for a fragment of the LSU rDNA gene and ITS1 region of the *S. silvaticum* isolate and *S. kraussei* isolate proved very high identity between both of species (DZIĘGIELEWSKA et al. 2015).

The earlier phylogenetic maximum parsimony analysis of alignments of *S. silvaticum* and other Steinernematids with known rDNA sequences (ITS1 + 5.SS + ITS2) consistently placed this species together with *S. feltiae, S. jollieti*, *S. kraussei*, *S. oregonense* and *S. weiseri* (NGUYEN 2007b). Also, the phylogeny of both nuclear and mitochondrial genes indicated close relationships of the Polish *S. silvaticum* isolates with *S. kraussei*, *S. oregonense* and *S. cholashanense* (LIS et al. 2018).

It is expected that further entomopathogenic nematode species new to the fauna of Poland will be recorded. This is supported by the zoogeographical range of the occurrence of nematodes of the family Steinernematidae in Europe. Species likely to occur in Poland, especially of the genus *Steinernema*, have been isolated from the soil in Poland's neighbours such as the Czech Republic, Germany or Slovakia (MRÁČEK et al. 2005, STURHAN 1999, STURHAN and LIŠKOVÁ 1999, STURHAN et al. 2005). These include *Steinernema intermedium* or *S. weiseri* (MRÁČEK et al. 2003, BAZMAN et al. 2008).

It seems, however, that genetic testing will not replace morphological examinations even if more data becomes available and advances in genetic research are made, and will rather serve to support and supplement them. Although DNA sequencing technologies are becoming widespread, PCR-RFLP is still a simple and efficient diagnostic method in research into EPNs.

Our survey of entomopathogenic nematodes in north-western Poland contributes and extends information concerning these nematodes in different habitants. The occurrence of entomopathogenic nematodes will corre-

spond to the occurrence of their insect hosts in the environment. Will also be addicted from abiotic factors specific to the type of habitat (e.g. soil texture, soil pH, soil temperature, soil moisture). Understanding the biotic resources in different ecosystems, including the species diversity of local entomopathogenic nematode populations, will allow more effective use of these beneficial organisms in biological protection against various important plant pests.

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EVALUATION OF GENETIC DIVERSITY IN GERBERA GENOTYPES REVEALED USING SCOT AND CDDP MARKERS

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Key words: genetic diversity, SCoT, CDDP, Gerbera, marker index.

Abstract

Genetic diversity in the germplasm of flowers is a key to genetic improvement of ornamental species. Gerbera cultivars are grown commercially worldwide and their flowers are durable and appealing, having a wide variety of colors. Genetic diversity based on molecular analysis can provide useful information for germplasm management and varietal characterization. In this study, we used start codon targeted (SCoT) and conserved DNA-derived polymorphism (CDDP) to assess the genetic diversity among 22 gerbera cultivars. Our findings showed that average polymorphism information content (PIC) was 0.39 and 0.40 for SCoT and CDDP markers, respectively, indicating that the studied markers were equal in terms of assessing genetic diversity. The results of clustering for both marker systems grouped the genotypes into three clusters. We found a positive significant correlation ($r = 0.73$, $P < 0.01$) between similarity matrix gained by both SCoT and CDDP markers. Cluster analysis for the CDDP and SCoT markers grouped the cultivars in three clusters. Average Marker index (MI) for SCoT and CDDP was calculated 3.40 and 2.45, respectively. This is the first time that the efficiency of SCoT and CDDP markers, as a novel method, have been compared with each other to evaluate genetic diversity in a set of gerbera genotypes. Our results showed that the comparison of different genetic diversity estimation methods could be useful for the improvement and crossing of gerbera genetic resources. This information can be used for the selection of superior genotypes for gerbera breeding programs.

Abbreviations: SCoT – start codon targeted; CDDP – conserved DNA – derived polymorphism; MI – marker index.

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Introduction

Gerbera jamesonii, belonging to *Asteraceae* family, is the fifth most used cut flower in the world originating from South America, Africa, and Asia (NESOM 2004). This plant is perennial and reproduces asexually. Gerbera was domesticated as a result of a cross between *Gerbera jamesonii* and other African *Gerbera viridifolia* species (HANSEN 1985). Gerbera is one of the most popular and beautiful flowers used as a decorative garden plant or as cut flowers and varies in shape, size, and color (white, yellow, orange, red, and pink). Determination of available genetic diversity for the characters of economic importance is very useful in crop breeding. Traditionally, breeding of gerbera is based on hybridization among cultivars and phenotypic selection of the best progenies, followed by clonal dissemination of released cultivars (PRAJAPATI et al. 2014, DE PINHO BENEMANN et al. 2013). However, selection is effective only when the observed traits in population is heritable in nature. Thus, achievement of proper genotype selection depends on important parameters such as variation, estimates of heritability, and genetic progress in gerbera germplasm. Genetic diversity based on molecular analysis can provide useful information for germplasm management and varietal characterization. Researchers have used different tools to evaluate genetic variations such as morphological, isozyme marker, and molecular markers. Morphological analysis is the first step in the classification and description of any plant germplasm (UPADHYAYA et al. 2001, GHAFOOR et al. 2001) which is one of the easiest methods for classical plant breeders in selecting desirable traits. Morphological evaluations are inexpensive and easy but are extremely influenced by environmental conditions. Molecular markers are useful tools to assess genetic diversity and provide an efficient mean to link phenotype and genotype variations (SOUFRAMANIEN and GOPALAKRISHNA 2004, VARSHENY et al. 2005). On the other hand, molecular markers are appropriated tool for measuring diversity of plant species which makes it possible to precisely characterize genotypes and provide measurement of genetic relationships (GHAFOOR et al. 2001). Genetic diversity in gerbera has been analyzed using a wide range of molecular marker systems such as EST-SSR (GONG and DENG 2010), RAPD (PRAJAPATI et al. 2014), and ISSR (LI et al. 2004) and AFLP. However, many of these markers used for genetic diversity and population structure are considered to be not so much efficient. For example, disadvantages of RAPD include being dominant, non-reproducible, and lack of detection of allelic system (MIAH et al. 2013). Although, SSR markers have been utilized to develop diversity fingerprinting in gerbera however, development of SSR markers require sequence information

and may not be suitable across species (CHEN et al. 2016). Thus, new markers such as CDDP and SCoT have been developed which can be considered as proper alternatives for previous markers (GUPTA and RUSTGI 2004).

SCoT markers are one of the reliable techniques and have several advantages over other markers such as higher efficiency, more informative, and even inexpensive. Primers used in this method are designed according to short conserved region surrounding the ATG translation start (or initiation) codon, showing the correlation between functional genes and their corresponding traits. Hence, this method has been successfully applied in different plant species to explore their genetic variability (AGRAWAL et al. 2019, ZHANG et al. 2015). SCoT and CDDP markers are functional markers (FM) which are usually dominant and reproducible (POCZAI et al. 2013). Although SCoT and CDDP are dominant markers, however, a number of co-dominant markers are also generated during amplification which can be used for genetic diversity analysis. SCoTs can be used for assessing genetic diversity and to obtain reliable information about population processes and structure across different plant families (COLLARD and MACKILL 2009a). CDDP and SCoT markers can yield many detectable polymorphic bands. The techniques are based on single primer with a high annealing temperature which leads to improved reproducibility.

CDDP (COLLARD and MACKILL 2009a) and SCoT (COLLARD and MACKILL 2009b) markers were developed based on the conserved regions of genes which have typically functional domains corresponding to conserved DNA sequences within gene regions (POCZAI et al. 2013). SCoT and CDDP have longer primers with higher annealing temperature requirement which will make them more reliable and reproducible than the arbitrary markers such as RAPD. CDDP and SCoT markers have been used to investigate genetic diversity in wide range of plant species (HAMIDI et al. 2014, HAJIBARAT et al. 2015, SAIDI et al. 2017). The use of CDDP and SCoT markers for studying genetic diversity are reported here for the first time for gerbera genotypes. The aims of the present study were to determine the efficiency of CDDP and SCoT markers and the comparison of these markers for estimating genetic diversity and relationships of gerbera genotypes.

Material and Methods

Plant material and genomic DNA extraction

A total of 22 Dutch gerbera cultivars obtained from the National Institute of Ornamental Plants (NIOP), Mahallat, Iran were surveyed in this study (Table 1). Genomic DNA was extracted from 1 g of leaves of each

Table 1

Names of the studied cultivars in this research

No	Genotype	Flower	No	Genotype	Flower
1	Rosalin		11	Duble Dutch	
2	Sorbet		12	Cacharlle	
3	Souvenir		13	Hooper	
4	Dune		14	Nuance	
5	Intense		15	Quote	
6	Aqua melone		16	Esmara	
7	Edelweiss		17	Sazo	–
8	Carambole		18	Pink elegance	
9	Balance		19	Essendre	
10	Stanza		20	Cabana	
21	Klimanjaro		22	Red-417	

cultivar using the DNA isolation method for gerbera based on the modified CTAB method (Lassner et al. 1989). Leaf samples were crushed using 5 ml of extraction buffer (EDTA, 1 M TrisHCl pH 8.0, 3 M NaCl) and, 1% CTAB, 0.7 M NaCl and 5 ml H_2O) and incubated for 1 h at 65°C. The extracted solution was treated with equal volume of Chloroform: Isoproponol mixture (1:1; v/v). DNA pellet was then treated with double volume of ice cold Isopropanol and washed twice with 76% ethanol. The isolated DNA was air dried and stored at −20°C in ddH_2O.

SCoT marker analysis

SCoT markers were amplified through PCR by nine SCoT primers as listed in Table 2. Thermal cycling included 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min annealing at 48°C, 2 min at 72°C and ending by an extension for 10 min at 72°C. The PCR products were separated on 1.5% agarose gel and stained with ethidium bromide. The polymorphic primers were then used for further analysis of 22 gerbera cultivars (Table 2).

Table 2

Primers used in SCoT and CDDP marker systems for study of genetic variation among 22 gerbera cultivars

Type	Primer	sequence 3′ → 5′	% GC	Tm
SCoT	SCoT1	CAACAATGGCTACCACCA	50	50
	SCoT2	CAACAATGGCTACCACCC	55	50
	SCoT13	ACGACATGGCGACCATCG	61	50
	SCoT22	AACCATGGCTACCACCAC	55	50
	SCoT28	CCATGGCTACCACCGCCA	66	50
	SCoT35	CATGGCTACCACCGGCCC	72	50
	SCoT36	GCAACAATGGCTACCACC	55	50
	SCoT 13	ACGGACATGGCGACCATCG	61	50
	SCoT 20	ACCATGGCTACCACCGCG	66	50
CDDP	KNOX-02	CACTGGTGGGAGCTSCAC	67	59
	KNOX-03	AAGCGSCACTGGAAGCC	68	58
	MYB-02	GGCAAGGGCTGCCGG	80	54
	WRKY-R1	GTGGTTGTGCTTGCC	60	51
	WRKY-R2	GCCCTCGTASGTSGT	64	52
	WRKY-R3	GCASGTGTGCTCGCC	65	53
	ERF1	CACTACCCCGGSCTSCG	77	56
	ERF2	GCSGAGATCCGSGACC	77	57
	HEP-VQ	CACGAGGACCTSCAGG	69	51

PCR amplification was performed in 20-µL reactions containing 30 ng of template DNA, 1 × PCR buffer, 0.2 mmol L^{-1} dNTPS, 0.4 µmol L^{-1} of primer, and 500 U of Taq polymerase (Cinaclon, Iran). The PCR reaction was performed in a PCR thermocycler (Master Cycler Gradient, Eppendorf) as follows: 95°C for 4 min, followed by 38 cycles of denaturation at 94°C for 45 s, annealing at 49°C for 45 s, and extension at 72°C for 2 min. A final extension cycle at 72°C for 10 min followed. The PCR products were separated in 1.3% agarose gels and stained with ethidium bromide.

CDDP marker analysis

CDDP marker was amplified through PCR using nine CDDP primers as listed in Table 2. PCR amplification was performed in 20-µL reactions containing 30 ng of template DNA, 1 × PCR buffer, 0.25 mmol L^{-1} dNTPS, 0.35 µmol L^{-1} of primer, and 500 U of Taq polymerase (Cinaclon, Iran). The PCR reaction was performed in a PCR (Master Cycler Gradient, Eppendorf) as follows: 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 49°C for 45 s and extension at 72°C for 2 min. A final extension cycle at 72°C for 10 min followed. The PCR products were separated in 1.5% agarose gels and stained with ethidium bromide. These primers (up to 18-mer) and their GC content ranged between 61 and 74%. CDDP primers were selected for final amplification based on GC content of 50–60% and an annealing temperature of 48°C (Table 2). All amplified products were resolved on 1.3% agarose gel made in 1x TBE buffer. The electrophoresis was performed for 45 min at 95 V and visualized with ethidium bromide. The image of banding patterns was captured under UV light using gel documentation system.

Data analysis

The amplified SCoT and CDDP markers were scored for presence (1) or absence (0) of bands. Only clear and reproducible bands were scored. Marker index (MI) was calculated as given by VARSHNEY et al. 2005:

$$MI = PIC \cdot Poymorphic\ band$$

PIC value for each polymorphic locus was estimated according to Roldan-Ruzi (ROLDÁN-RUIZ et al. 2000). PIC = 1 – S(Pij)2; where Pij is the frequency of the ith pattern showed by the jth primer aggregated across all patterns revealed by the primers (BOTSTEIN et al. 1980). NTSYS was applied for analyzing pairwise genetic distances and for making the distance matrix (ROHLF 1998). Genetic similarity among samples was eva-

luated by calculating the Jacard similarity coefficient and dendrogram analysis using the un-weighted neighbor-joining method (UNJ). Mantel's test (MANTEL 1967) for Jaccard coefficients was performed to compare each pair of similarity matrices created by NTSYSpc version 2.0.

Results

SCoT analysis

The nine SCoT primers produced a total of 53 reliable fragments of which 48 bands were polymorphic. Each primer produced an average of eight bands. SCoT1 yielded the maximum number of polymorphic bands and SCoT20 produced the minimum number of fragments (Table 3).

Table 3

Polymorphism detected with 18 CDDP and SCoT primers in fifteen collections of gerbera cultivars

NO	Primer	PB	MB	TB	PIC	MI	PPB%
CDDP	KNOX-02	8	1	9	0.43	3.48	88
	KNOX-03	5	3	8	0.43	2.16	62
	MYB-02	10	0	10	0.44	4.43	100
	WRKY-R1	10	0	10	0.43	4.33	100
	WRKY-R2	4	3	7	0.41	1.64	57
	WRKY-R3	3	0	3	0.28	0.85	100
	ERF1	10	0	10	0.43	4.39	100
	ERF2	6	0	6	0.39	2.35	100
	HEP-VQ	7	0	7	0.41	2.91	100
SCoT	SCoT 1	14	0	14	0.45	6.4	100
	SCoT 2	12	0	12	0.45	5.42	100
	SCoT 11	8	0	8	0.42	3.36	100
	SCoT 13	5	3	8	0.43	2.15	62
	SCoT 20	2	0	2	0.20	0.48	100
	SCoT 22	7	2	9	0.41	2.93	77
	SCoT 28	10	0	10	0.42	4.21	100
	SCoT 35	5	0	5	0.35	1.79	100
	SCoT 36	9	1	10	0.43	3.92	90

TB – total number of amplified bands; PB – polymorphism bands; PPB – percentage of polymorphism bands

Polymorphism percentage ranged from 62 (SCoT13) to 100 (SCoT1, SCoT2, SCoT11, SCoT20, SCoT28, SCoT35) with an average of 92 for the nine primes used. Amplification profile obtained with SCoT13 is presented in Figure 1. SCoT20 primer with 0.2 and SCoT1 and SCoT2 primers with

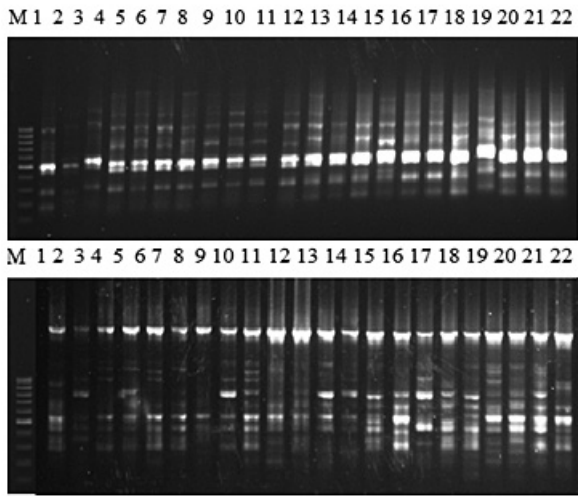

Fig. 1. Amplification profile obtained with KNOX-03 and SCoT13 primers detected in gerbera genotypes

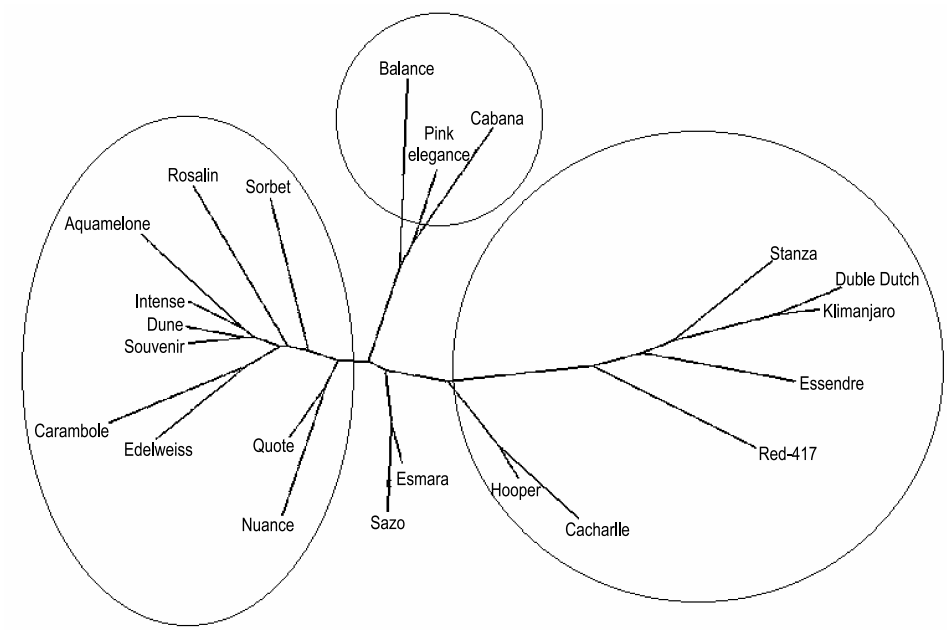

Fig. 2. Dendrogram of the 22 gerbera cultivars based on the dissimilarity matrix developed using SCoT markers

0.45 showed the lowest and highest PIC values among all primers, respectively. Average of PIC was 0.39 per locus. Marker Index (MI) ranged from 0.48 (SCoT20) to 6.4 (SCoT1) – Table 3. The similarity value of the 22 genotypes based on Jaccard's coefficient varied between 0.14 and 0.85. The highest genetic diversity was between Dune and Duble Dutch genotypes. The NJ clustering algorithm from SCoT analysis grouped the 22 genotypes into three clusters. Cluster I contained seven genotypes, cluster II included 11 genotypes, and cluster III included three genotypes (Figure 2). The cluster patterns obtained by SCoT primer is shown in Figure 2. The maximum and minimum number of fragments belonged to SCoT1 (14 bands) and SCoT20 (2 bands), respectively. The amplified bands ranged between 100 and 2500.

CCDP analysis

CDDP primers produced a total of 70 fragments of which 63 fragments were polymorphic bands. The average number of polymorphic bands was 7 per primer ranging from 3 (WRKY-R3) to 10 (MYB-02, WRKY-R1, and ERF1). The polymorphism percentage ranged from 57 to 100 % with a mean of 90%, showing a high polymorphism level. The mean value of PIC was 0.4 per locus which ranged from 0.28 to 0.44 (Table 3). Marker Index (MI) ranged from 0.85 (WRKY-R3) to 4.39 (ERF1) with a mean of 2.94 (Table 3). The similarity value of the 22 genotypes based on Jaccard's coefficient varied between 0.14 and 0.91. The highest genetic diversity was between Balance and Quote genotypes. Neighbor-Net cluster analysis based on CDDP grouped gerbera cultivars into three clusters (Figure 3). Clusters I, II, and III contained four, eight, and seven members, respectively. CDDP clusters had a relatively similar grouping pattern with those obtained by SCoT markers. Amplification profile obtained with KNOX-03 marker is presented in Figure 1. The amplified bands ranged between 150 and 2500.

Correlation between the similarity values using two marker systems

Cophenetic correlation estimated for the two marker systems were 0.86 and 0.86 for SCoT and CDDP, respectively, indicating a good fit for clustering. Positive correlation was observed between the two marker types using mantel test. The correlation coefficient (r) between SCoT and CDDP (significant $P > 0.01$) was 0.73, indicating a high similarity in DNA sequence variation at primer binding.

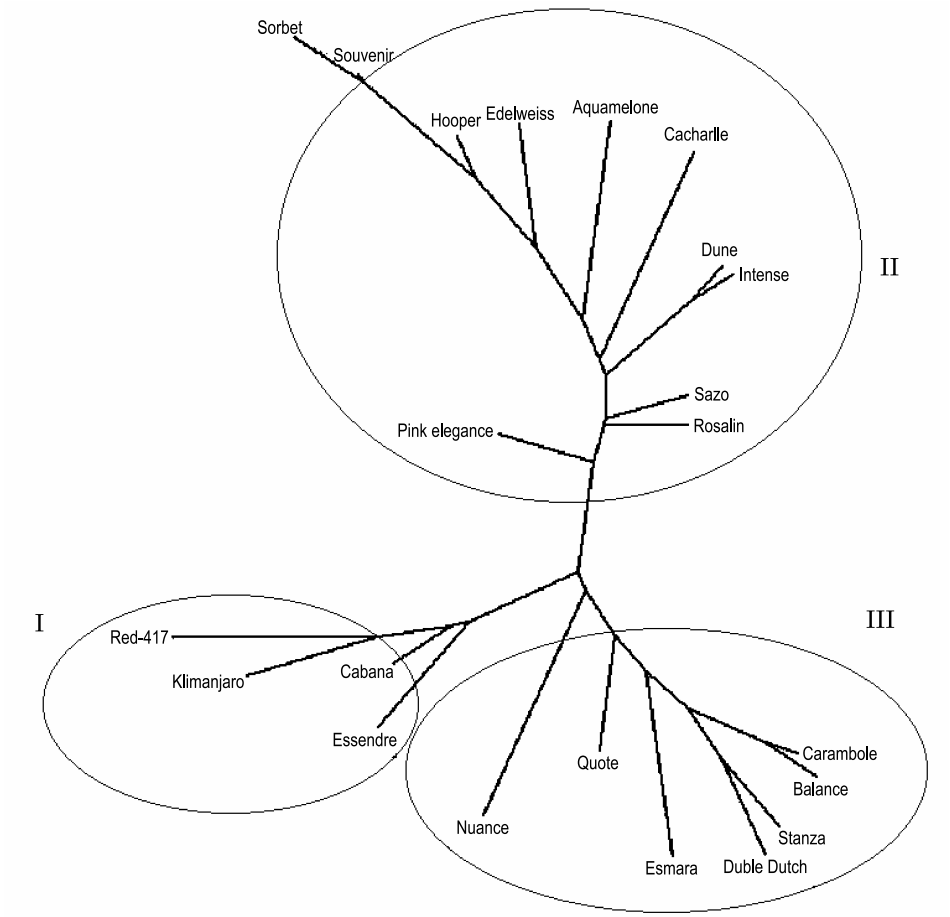

Fig. 3. Dendrogram of the 22 gerbera cultivars based on the dissimilarity matrix developed using SCoT CDDP markers

Discussion

Identification of genetic diversity and classification of genetic resources (germplasm) are important and essential for breeding and management of plant genetic resources. Gerbera is one of the most important cut flowers worldwide therefore breeding of this flower is necessary. Genetic diversity of gerbera is the first step toward flower improvement and researchers utilize molecular markers to assess genetic diversity among horticultural crops. The SCoT and CDDP marker techniques were employed in the present study for many reasons. Both genetic diversity and fingerprinting studies are of useful tools which enable plant breeders to make better decisions regarding selection of germplasm to be used in hybridization.

Our results demonstrated that CDDP and SCoT markers can be used as reliable techniques for detecting levels of DNA polymorphism and genetic relationships in gerbera. Conserved DNA regions sharing the same priming site, but differing in their genomic distribution may yield a large number of easily detectable length polymorphisms.

SCoT markers have many advantages including easy to work with in laboratory conditions, dominant markers, informative, inexpensive, and higher reproducibility compared to arbitrary markers such as RAPD (POCZAI et al. 2013). SCoTs can be utilized either in isolation or in combination with other techniques to evaluate genetic diversity and to obtain reliable information about population processes and structure across different plant families (ABDIN et al. 2017).

In the current study, for the first time two different markers, SCoT and CDDP, were used for analyzing the genetic diversity within a set of 22 diverse gerbera genotypes to know if these marker systems can be effectively used in breeding programs. Our study suggested that SCoT and CDDP markers can produce a highly considerable polymorphism and diversity in the studied gerbera cultivars which is in agreement with those reported in rose and anthurium (SAIDI et al. 2017, SAIDI et al. 2018). Using the combined markers, our findings showed the presence of a significant polymorphism and revealed a high level of variability in surveyed gerbera cultivars which is in agreement with those reported by Poczaie (2013) and Saidi et al. (2018).

CDDP can be used as functional markers (FM) and amplifies conserved DNA regions sharing the same priming site. This marker generated detectable length polymorphisms by high annealing temperature with high reproducibility and efficiency (POCZAI et al. 2013). It has been reported that polymorphism index content (PIC) in anthurium was calculated 0.42 and 0.37 for SCoT and CDDP, respectively. According to our findings, PIC for SCoT and CDDP markers was estimated 0.39 and 0.4, respectively. Average Marker index (MI) for SCoT and CDDP was calculated 3.40 and 2.45, respectively (SAIDI et al. 2018). There has been a report on the analysis of the genetic diversity among landraces and improved safflower genotypes collected from different geographical locations using gene-targeted (SCoT, CDDP, and CBDP) molecular markers. High level of polymorphism using these markers showed that they can be effective in determining the genetic diversity among safflower genotypes (TALEBI et al. 2018).

There have also been attempts to combine other primers in CDDP reactions to amplify polymorphic regions representing DNA stretches between two identical or very similar conserved primer binding sites (HAJIBARAT et al. 2015). Since both markers exhibited a relatively similar

and high PIC and MI values it would be safe to reason that both can be equally valuable in assessing genetic diversity or diagnostic fingerprinting in gerbera. Thus, it can be suggested that the use of SCoT and CDDP markers are more useful for estimating the genetic diversity in gerbera breeding programs.

Although the level of diversity for the two marker systems was nearly equal, however, we predict that the source of recognized diversity might be different. Therefore, each technique amplifies various regions of the genome. Dendograms obtained by SCoT and CDDP could be explained by the similar nature of each maker, extent of polymorphism, number of loci, and specific regions of the genome (Gorji et al. 2011, Saidi et al. 2018). These findings are contrary to the findings reported in potato (Gorji et al. 2011), wheat (Hamidi et al. 2014), and chickpea (Paksereshт et al. 2013).

Mantel correlation coefficient test showed a highly positive correlation between SCoT and CDDP matrices, indicating a stable relationship between genetic distances for both marker systems. This correlation may have been related to similarity in DNA sequence variations at primer binding sites between the SCoT and CDDP markers. The low narrow genetic base and genetic diversity in gerbera leads to slow genetic improvement of this plant. The selection of genotypes for genetic diversity was primarily based on different polymorphisms as obtained by molecular markers. Our findings showed that CBDP and SCoT techniques were highly reproducible and efficient and can be used as powerful tools for assessing genetic diversity among gerbera genotypes. This study showed that SCoT and CDDP techniques are eligible tools to detect the genetic diversity and genetic association of gerbera germplasm. Thus, it is suggested that the use of these markers are appropriate for crop improvement programs particularly in assessing genetic diversity, genotype identification, bulk population, and QTL mapping. SCoT and CDDP markers are based on functional regions of genome and their utilization are easy and not time-consuming. Finally, it is suggested that we need to increase the genetic base of gerbera germplasm through introduction, distance hybridization, and even mutagenesis for effective implication of markers.

This paper is the first report on the use of SCoT and CDDP markers on gerbera. Our results demonstrated that CDDP and SCoT markers can be used as reliable techniques for detecting levels of DNA polymorphism and genetic relationships in gerbera. In this study, the two different gene-targeted molecular markers, i.e. SCoT, and CBDP, adopted to study the genetic diversity among gerbera genotypes from different sources demonstrated that they have advantages over the use of dominant random markers (such as ISSR, RAPD, DAFs, and AFLP), as these markers reveal

genetic diversity from the genic region in the genome and this functional diversity can be used in any species (PALIWAL et al. 2013, HEIKRUJAM et al. 2015, ANDERSEN and LUBBERSTEDT 2003). These results demonstrate that SCoT markers are useful for cultivar identification and genetic diversity analyses of gerbera cultivars. This genetic information will support germplasm management and cultivar improvement in gerbera.

Conflict of interest

The authors declare that they have no conflict of interest.

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EFFECT OF *CARICA PAPAYA* MATERIALS ON MICROBIAL AND PHYSICOCHEMICAL QUALITIES OF RIVER, STREAM AND POND WATER*

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Key words: phytochemicals, *Carica papaya*, antimicrobial, physicochemical parameters, water purification.

Abstract

Bacteriological and physicochemical profiles of surface water collected from Chanchaga Local Government area of Niger State were treated with *Carica papaya* leaf, stem, seed and bark powder to ascertain their effectiveness in water treatment. Phytochemical bacteriological and physicochemical analyses were carried out using standard procedures. Phenol, flavonoids, saponins, tannins, alkaloids and steroids were found in all part of *C. papaya*. The stem, seed and bark of *C. papaya* at all concentrations tested completely eliminated the total viable counts (TVC), total coliforms count (TCC), faecal coliforms counts (FCC), and *Salmonella-Shigella* counts (SSC) after 24 hrs. The leaf of *C. papaya* only reduced the TVC and TCC count of stream and pond water after 24 hrs. However, the leaf of *C. papaya* at concentrations of 0.3, 0.4 and 0.5 g l^{-1} completely eliminated the TVC, TCC, FCC and SSC count of the river water after 12 hours of treatment. The minimum inhibitory concentrations (MIC) range between 8 and 64 mg ml^{-1} while the minimum bactericidal concentrations (MBC) of the plant materials range between 16–128 mg ml^{-1} against all organism tested. The plant causes no significant ($p > 0.05$) changes to the total dissolve solid (TDS), hardness, chloride and nitrate content of river water. All part of *C. papaya* significantly reduced chemical oxygen demand (COD), turbidity and nitrate contents of the pond water**.** There were also decreases in dissolved oxygen (DO), TDS, nitrate and turbidity but increase in calcium contents of the stream water. Alum significantly reduced the DO, TDS, hardness, turbidity and pH while calcium hypochlorite reduced the DO and TDS of the water samples. *Carica papaya* plant materials have a double advantage of having phytochemicals of antimicrobial properties and also better potentials for water purification than the synthetic coagulant.

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Introduction

The increasing population and urbanization in urban areas has serious threat to the limited natural resources (SADUZAMAN and JEYANTHI 2013) Nigerians derived their water from surface water (springs, streams, rivers, lakes), hand dug wells, rainwater, pipe borne water and boreholes (GBERIKON et al. 2018). Due to lack of safe public water supply in Minna, Chanchaga Local Government Area, rivers, ponds and the available streams have become a major source of water supply, hence there is need for adequate purification, especially to remove all pathogenic microorganisms before use.

Several chemical coagulants have been used in conventional water treatment processes for portable water production that includes inorganic, synthetic organic polymer and naturally occurring coagulants (OKUDA et al. 2000). However, there is a fear that synthetic or inorganic coagulant like alum, hypochlorite may induce induced undesirable health implications (MARTYN et al. 1989, MILLER et al. 1984). On the other hand, the use of natural materials that are of plant origin to clarify turbid surface waters is not a new idea according to National Research Council (*Moringa*… 2006). There is evidence that the use of extracts from plant species possessing both coagulating and antimicrobial properties are safe for human health (ALI et al. 2004, TSADO et al. 2016, LAWAL et al. 2016, IBRAHIM et al. 2017), these plants have also been reported for medicinal properties and ability to prevent the occurrence of many diseases when consumed in any form (BASHIR et al. 2015, LAWAL et al. 2017). Of the large number of plant materials available on earth, the seeds of *Moringa oleifera* have been shown to be the most studied and one of the most effective primary coagulants for water treatment especially in rural communities (DALEN et al. 2009). Research are very limited on the efficacy of *C. papaya* in surface water purification. The present research therefore investigates the phytochemical profiles of *Carica papaya* seeds, leaf, stem and bark and their purifying properties for domestic water treatment.

Materials and Methods

Samples collection

Fresh leaves, stem, bark and seed samples of *Carica papaya* were obtained from Minna Niger State Nigeria. The plants were identified and authenticated at National Institute for Pharmaceutical Research and

Development, NIPRID Abuja where the vouchers numbers; NIPRD/H/7027 was deposited. Commercial alum was purchased from Bosso market in Minna, Nigeria.

Sample preparation and phytochemical analysis

The plant materials were air dried under ambient temperature for for 2 weeks. The dried plant materials were pulverized into fine powder, filtered using sieve of mesh size 0.8 mm. The powder samples were stored in an air tight containers at ambient temperature until required for use. Preliminary qualitative phytochemical screening which involved performing simple chemical tests to detect the presence of secondary metabolites such as tannins, flavonoids, phenols, alkaloids, saponins, and glycosides, was carried out in accordance with the method described previously (TREASE and EVANS 1983, HARBORNE 1988).

Collection of water samples

Surface water samples were collected from Chanchaga Local Government Area of Minna, Niger State. The samples were collected where people commonly collect water for their domestic activities. Standard sampling methods of APHA (1999) was adopted in the collection of the water samples. Water samples for physicochemical analyses were collected using transparent sterile containers of 2.0 litres capacity. The plastic containers were thoroughly washed with 5% nitric acid (HNO_3) and rinsed with tap water *Guidelines for drinking-water*... 2004). They were later rinsed thoroughly with deionized water and allowed to dry before use.

Screening of plant materials for potential to purify water

In each treatment case, a solution was prepared by dissolving 0.1 g, 0.2 g, 0.3 g, 0.4 g and 0.5 g of each powder sample in 100 ml of sterile distilled water. The solution was shaken for five minutes and poured into 900 ml of water sample, made up to 1litre and allowed to stand for 30 minutes to allow the coagulated particles to settle to the bottom. The supernatant was poured through a filter paper to ensure that any suspended coagulant is (MCCONNACHIE et al. 1999). The supernatant was then subjected to microbial and physicochemical analysis. Based on the volume of water, appropriate different concentration of alum and calcium hypochlorite were introduced into water sample and allowed to stand for some hour, after which the water was subjected to both microbiological and physiological analysis to compare its efficiency of water treatment with the plant materials.

Bacteriological analysis of water

Water samples collected from stream, pond and river were analysed using membrane filtration method. Analyses were carried out to determine total viable counts (TVC), Total Coliform counts (TCC) and Faecal Colifrom counts (FCC). TVC, TCC and FCC were determined by using Lauryl Sulphate broth (MLSB) medium, *Salmonella-Shigella* counts using *Salmonella-Shigella* agar (SSA) (APHA 2009).

Characterization and identification of isolates

Isolates from the plates were identified further by biochemical tests, using the methods of VANDEPITTE et al. (2003) and CHEESBROUGH (2008). Morphological and biochemical tests carried out included Gram staining, catalase, oxidase, indole tests, urease production, methyl red, Voges Proskauer, H_2S production, coagulase, starch hydrolysis, lactose and citrate utilization, mannitol, sucrose and glucose tests.

Physicochemical analyses of the water samples

The physicochemical parameters of the water were analyzed using the standard procedures outlined in the Standard Method for the Examination of Water and Wastewater (Standard Method 1999) to check the pH, conductivity, hardness, turbidity, total dissolved solid (TDS), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Nitrate, Chloride, calcium and magnesium.

Statistical analysis

Data generated were analyzed using statistical package for social sciences (SPSS) and were presented as Mean ± SEM. Differences between groups were compared using analysis of variance, ANOVA ($P < 0.05$) followed by Duncan's Multiple Range Test.

Results

Phytochemical composition

The qualitative phytochemical composition of the seed, leaf, bark and stem of *Carica papaya* are presented in Table 1. Phenol, flavonoids, saponins, tannins, alkaloids and steroids were found in all part of *C. papaya*. Cardiac glycosides, anthraquinones and terpenes were absent in leaf and seed of *Carica papaya*. All other phytochemical tested were present in leaf, stem, seed and bark (Table 1).

Table 1

Phytochemical composition of *Carica papaya* used for water purification

Phytochemical	Seed	Leaf	Bark	Stem
Phenol	+	+	+	+
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+
Alkaloids	+	+	+	+
Cardiac glycosides	–	–	+	–
Anthraquinones	–	–	+	+
Terpenes	–	–	+	+
Steroids	+	+	+	+

Key: + (present), – (absent)

Frequency of occurrence of the bacterial isolates

A total of 13 different bacteria isolates were identified from in river, stream and ponds water sample. *Escherichia coli* recorded the highest percentage frequency of occurrence of 45.00%, 42.50% and 50.0% in river, stream and ponds respectively. *Stapyloccocuss aureus* is the next abundance organism with frequency of occurrence of 15%, 25% and 25% respectively. *Proteus myxofaciens* and *Klebsiella oxytoca* were absent in the ponds sample, while *Klebsiella oxytoca* was absent in river water (Figure 1).

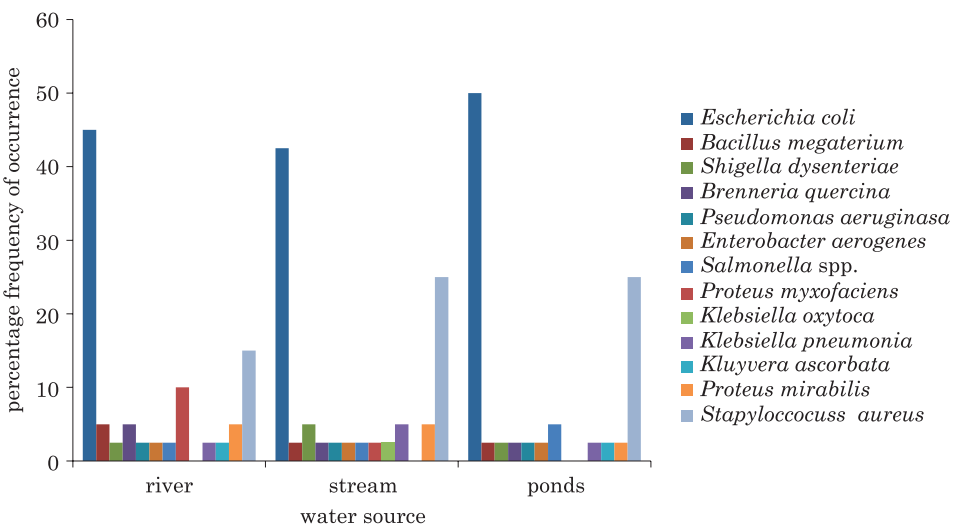

Fig. 1. Percentage frequency of bacteria occurrence in river, stream and ponds water

Bacterial quality of water samples treated with *C. papaya*

Stream water. The stem, seed and bark of *C. papaya* at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 g l^{-1} significantly ($P < 0.05$) reduced the TVC, TCC, FCC and SSC of the stream water after 12 h of treatment, a complete elimination of various groups of bacteria was observed after 24 h of treatment (Table 2). However, the leaf material of *C. papaya* was not able to eliminate viable bacteria of the stream water but significantly ($P < 0.05$) reduced the counts to $1.36\pm0.03 \cdot 10^5$ cfu ml^{-1} and $1.06\pm0.04 \cdot 10^5$ cfu ml^{-1} at concentrations of 0.1 and 0.2 g l^{-1} after 24 h of treatment. Similarly, the leaf material of *C. papaya* was not able to eliminate the coliforms in the stream water but significantly ($P < 0.05$) reduced the counts to $0.49\pm0.02 \cdot 10^3$ cfu ml^{-1} and $0.21\pm0.02 \cdot 10^3$ cfu ml^{-1} at concentrations of 0.1 and 0.2 g l^{-1} respectively after 24 h of treatment (Table 2).

Pond water. The stem, seed and bark materials of *C. papaya* at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 g l^{-1} significantly ($P < 0.05$) reduced the TVC, TCC, FCC and SSC of the pond water after 12 h of treatment. A complete elimination of the bacteria with the exception of *Salmonella* and *Shigella* was observed after 24 h of treatment while and *Salmonella* and *Shigella* were completely eliminated after 12 h of treatment. However, the leaf material of *C. papaya* at a concentration of 0.1 g l^{-1} was able to eliminate the bacteria from the pond water, even after 24 h but significantly ($P < 0.05$) reduced the counts to $0.82\pm0.02 \cdot 10^5$ cfu ml^{-1} after 24 h of treatment (Table 3). Treatment of pond water with alum and calcium hypochlorite completely eliminated the bacteria from the water sample after 24 h.

River water. Treatment of river water with alum and calcium hypochlorite completely eliminated the bacteria after 24 h. The leaf material of *C. papaya* at concentrations of 0.3, 0.4 and 0.5 g l^{-1} completely eliminated the various bacteria from the river water after 12 h of treatment, while at concentrations of 0.1 and 0.2 g l^{-1} a complete elimination of the bacteria was recorded after 24 h of treatment. Similarly, for the seed material a complete elimination of the viable bacteria, coliforms, *Salmonella* and *Shigella* was recorded at concentrations of 0.4 and 0.5 g l^{-1}. The bark material on the other hand, caused a complete elimination of the organisms in the river water at concentrations of 0.2, 0.3, 0.4 and 0.5 g l^{-1} (Table 4).

Table 2

Bacterial counts obtained from treated stream water with *Carica papaya*

Sample [g l^{-1}]	TVC · 10^5 cfu ml^{-1}			TCC ·10^3 cfu ml^{-1}			FCC mpn/100 ml			SSC · 10^3 cfu ml^{-1}		
	0	12	24	0	12	24	0	12	24	0	12	24
Stream water	6.60±0.03	6.60±0.03	6.60±0.03	2.96±0.04	2.96±0.04	2.96±0.04	300.34±7.45	300.46±4.35	300.67±4.49	1.56±0.02	1.56±0.02	1.56±0.02
Calcium hypochlorite	5.78±0.03	2.63±0.03	Nil	2.02±0.03	1.53±0.02	Nil	295.45±4.57	20.13±1.24	Nil	1.63±0.04	1.42±0.02	Nil
Alum	5.52±0.03	3.23±0.03	Nil	2.01±0.02	1.24±0.01	Nil	284.24±2.90	23.67±2.34	Nil	1.62±0.04	1.20±0.02	Nil
Leaf of *C. papaya*												
0.1	6.12±0.02	3.36±0.02	1.36±0.03	2.93±0.04	1.96±0.04	0.49±0.02	292.13±5.43	12.43±1.56	2.34±1.56	1.43±0.02	1.30±0.02	0.50±0.02
0.2	6.21±0.04	3.06±0.02	1.06±0.04	2.23±0.04	1.00±0.04	0.21±0.02	291.09±6.43	10.25±2.35	Nil	1.42±0.02	1.12 ±0.02	Nil
0.3	6.32±0.02	2.75±0.02	Nil	2.54±0.04	1.12±0.03	Nil	299.56±4.89	10.56±0.98	Nil	1.56±0.02	0.96 ±0.02	Nil
0.4	6.22±0.02	1.43±0.02	Nil	2.47±0.04	1.02±0.01	Nil	290.52±3.49	09.12±2.44	Nil	1.56±0.02	0.56 ±0.02	Nil
0.5	6.21±0.02	Nil	Nil	2.72±0.04	1.45±0.04	Nil	289.15±6.78	Nil	Nil	1.50±0.02	Nil	Nil
Stem of *C. papaya*												
0.1	6.10±0.03	2.32±0.02	Nil	2.77±0.04	1.32±0.06	Nil	297.05±5.67	21.78±2.31	Nil	1.51±0.02	1.20±0.02	Nil
0.2	6.29±0.02	2.65±0.03	Nil	2.71±0.04	1.87±0.04	Nil	296.78±7.89	15.34±1.90	Nil	1.51±0.02	1.00±0.02	Nil
0.3	6.10±0.02	2.56±0.02	Nil	2.70±0.04	0.96±0.04	Nil	295.35±6.34	09.90±2.35	Nil	1.49±0.02	0.43 ±0.02	Nil
0.4	6.31±0.02	2.50±0.02	Nil	2.73±0.04	0.86±0.01	Nil	294.23±5.89	08.54±0.78	Nil	1.38±0.02	Nil	Nil
0.5	6.27±0.02	1.56±0.04	Nil	2.65±0.04	1.10±0.04	Nil	294.98±2.67	02.35±0.02	Nil	1.39±0.02	Nil	Nil
Seed of *C. papaya*												
0.1	6.24±0.02	1.50±0.02	Nil	2.89±0.04	0.37±0.04	Nil	270.90±5.47	12.56±1.32	Nil	1.42±0.02	0.65 ±0.02	Nil
0.2	6.24±0.01	1.42±0.02	Nil	2.23±0.04	0.44±0.10	Nil	265.28±3.78	07.64±1.90	Nil	1.51±0.02	0.43 ±0.02	Nil
0.3	6.32±0.01	1.03±0.02	Nil	2.21±0.04	0.16±0.01	Nil	266.93±5.46	05.12±0.32	Nil	1.50±0.02	Nil	Nil
0.4	6.43±0.01	0.56±0.04	Nil	2.16±0.04	Nil	Nil	265.96±3.67	01.97±0.04	Nil	1.51±0.02	Nil	Nil
0.5	6.21±0.02	0.56±0.02	Nil	2.09±0.04	Nil	Nil	265.03±3.09	Nil	Nil	1.53±0.02	Nil	Nil
Bark of *C. papaya*												
0.1	6.28±0.02	2.82±0.05	Nil	2.10±0.04	1.65±0.04	Nil	291.35±5.67	22.34±0.41	Nil	1.49±0.02	0.92±0.02	Nil
0.2	6.28±0.02	1.35±0.02	Nil	2.19±0.04	1.54±0.04	Nil	294.78±3.78	12.12±0.13	Nil	1.47±0.02	0.54±0.02	Nil
0.3	6.27±0.01	1.73±0.03	Nil	2.21±0.04	1.43±0.04	Nil	297.54±6.89	11.89±2.13	Nil	1.30±0.02	0.51 ±0.02	Nil
0.4	6.27±0.03	0.96±0.02	Nil	2.14±0.04	0.96±0.04	Nil	256.47±2.78	05.01±0.56	Nil	1.26±0.02	0.21 ±0.02	Nil
0.5	6.25±0.01	0.26±0.01	Nil	2.04±0.04	0.34±0.04	Nil	296.52±3.49	Nil	Nil	1.26±0.02	Nil	Nil

Data are MEAN ± SEM of triplicate determinations. TVC – total viable counts; TCC – total coliforms counts; g l^{-1} – gram per litre; FCC – faecal coliforms counts; SCC – *Salmonella-Shigella* counts; g l^{-1} – gram per litre; Nil – not detected

Table 3

Bacterial counts obtained from treated pond water with *Carica papaya*

Sample [g l^{-1}]	TVC · 10^5 cfu ml^{-1}			TCC · 10^3 cfu ml^{-1}			FCC mpn/100 ml			SSC · 10^3 cfu ml^{-1}		
	0	12	24	0	12	24	0	12	24	0	12	24
Pond water	6.10±0.04	3.30±0.56	3.30±0.03	2.96±0.24	2.96±0.03	2.96±0.03	250.34±0.89	250.24±3.45	250.56±2.87	1.68±0.03	1.68±0.03	1.68±0.03
Calcium hypochlorite	5.78±0.03	2.63±0.03	Nil	2.02±0.03	1.53±0.03	Nil	224.00±0.00	20.03±0.52	Nil	1.63±0.03	1.20±0.02	Nil
Alum	5.52±0.03	3.23±0.03	Nil	2.01±0.02	1.24±0.03	Nil	240.00±0.00	23.50±0.05	Nil	1.62±0.03	Nil	Nil
Leaf of *C. papaya*												
0.1	6.00±0.02	3.30±0.36	0.82±0.02	2.93±0.03	1.96±0.24	0.49±0.02	243.23±0.56	22.32±3.05	2.67±0.05	0.90±0.12	0.90±0.03	0.60±0.03
0.2	5.98±0.41	3.28±0.03	Nil	2.23±0.21	1.00±0.03	Nil	242.45±0.89	20.54±3.02	Nil	0.60±0.03	0.52±0.03	Nil
0.3	5.97±0.56	3.26±0.03	Nil	2.54±0.90	1.12±0.24	Nil	232.24±0.75	15.23±3.07	Nil	0.20±0.03	0.12±0.03	Nil
0.4	5.97±0.03	3.25±0.04	Nil	2.47±0.23	1.02±0.03	Nil	230.33±0.95	14.56±3.01	Nil	Nil	Nil	Nil
0.5	5.95±0.03	3.22±0.02	Nil	2.72±0.03	1.45±0.03	Nil	224.43±0.99	12.65±3.01	Nil	Nil	Nil	Nil
Stem of *C. papaya*												
0.1	6.10±0.03	3.26±0.03	Nil	2.77±0.03	1.32±0.25	Nil	234.43±0.98	45.56±3.98	Nil	0.73±0.02	0.53±0.02	Nil
0.2	6.10±0.67	3.22±0.43	Nil	2.71±0.03	1.87±0.03	Nil	234.67±0.92	41.32±3.09	Nil	0.30±0.13	0.12±0.03	Nil
0.3	5.96±0.12	3.22±0.03	Nil	2.70±0.45	0.96±0.25	Nil	231.23±0.96	35.89±3.43	Nil	Nil	Nil	Nil
0.4	5.95±0.03	3.20±0.21	Nil	2.73±0.03	0.86±0.53	Nil	229.67±0.47	30.53±1.89	Nil	Nil	Nil	Nil
0.5	5.95±0.03	3.11±0.08	Nil	2.65±0.45	0.10±0.03	Nil	225.58±0.98	15.57±3.12	Nil	Nil	Nil	Nil
Seed of *C. papaya*												
0.1	5.97±0.03	3.00±0.03	Nil	2.89±0.03	0.37±0.03	Nil	230.32±0.82	12.23±3.43	Nil	Nil	0.20±0.03	Nil
0.2	5.93±0.32	2.96±0.03	Nil	2.23±0.43	0.44±0.56	Nil	228.89±0.98	08.09±3.12	Nil	Nil	Nil	Nil
0.3	5.92±0.03	2.93±0.08	Nil	2.21±0.03	0.16±0.03	Nil	223.65±0.89	04.05±3.15	Nil	Nil	Nil	Nil
0.4	5.85±0.03	2.90±0.03	Nil	2.16±0.74	Nil	Nil	219.78±0.91	01.99±3.09	Nil	Nil	Nil	Nil
0.5	5.82±0.56	2.89±0.32	Nil	2.09±0.45	Nil	Nil	210.43±0.96	Nil	Nil	Nil	Nil	Nil
Bark of *C. papaya*												
0.1	5.98±0.32	3.19±0.03	Nil	2.10±0.34	1.65±0.03	Nil	245.45±0.02	40.27±4.89	Nil	0.30±0.2	0.62±0.32	Nil
0.2	5.97±0.03	3.16±0.03	Nil	2.13±0.32	1.54±0.21	Nil	243.56±0.02	36.48±3.89	Nil	0.10±0.11	0.22±0.34	Nil
0.3	5.92±0.16	3.13±0.03	Nil	2.21±0.03	1.43±0.33	Nil	241.46±0.02	30.35±2.99	Nil	Nil	Nil	Nil
0.4	5.90±0.03	3.10±0.03	Nil	2.04±0.21	0.96±0.03	Nil	222.25±0.02	15.89±2.34	Nil	Nil	Nil	Nil
0.5	5.87±0.34	3.10±0.03	Nil	2.04±0.43	0.34±0.78	Nil	220.62±0.02	2.45±3.02	Nil	Nil	Nil	Nil

Data are MEAN ± SEM of triplicate determinations. TVC – total viable counts; TCC – total coliforms counts; g l^{-1} – gram per litre; FCC – faecal coliforms counts; SCC – *Salmonella-Shigella* counts; g l^{-1} – gram per litre; Nil – not detected

Table 4

Bacterial counts obtained from treated river water with *Carica papaya*

Sample [g l^{-1}]	TVC · 10^5 cfu ml^{-1}			TCC · 10^3 cfu ml^{-1}			FCC mpn/100 ml			SSC · 10^3 cfu ml^{-1}		
	0	12	24	0	12	24	0	12	24	0	12	24
River water	8.62±0.03	.8.62±0.54	8.62±0.03	4.32±0.03	4.32±0.03	4.32±0.03	360.45±0.03	360.89±0.45	360.89±0.45	1.63±0.03	1.63±0.03	1.63±0.03
Calcium hypochlorite	8.56±0.03	0.26±0.34	Nil	4.02±0.03	0.15±0.03	Nil	350.43±0.03	20.32±0.52	Nil	1.63±0.03	1.42±0.02	Nil
Alum	8.52±0.03	0.32±0.04	Nil	4.11±0.03	0.12±0.03	Nil	340.43±0.03	23.23±0.67	Nil	1.62±0.03	1.20±0.02	Nil
Leaf of *C. papaya*												
0.1	8.62±0.03	0.84±0.03	Nil	4.32±0.02	00.36 ±0.03	Nil	358.43±0.03	Nil	Nil	1.63±0.03	3.03±0.02	Nil
0.2	8.62±0.03	0.62±0.03	Nil	4.32±0.02	00.02±0.03	Nil	356.67±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.3	8.62±0.03	Nil	Nil	4.32±0.02	Nil	Nil	355.54±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.4	8.62±0.03	Nil	Nil	4.32±0.02	Nil	Nil	352.23±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.5	8.62±0.03	Nil	Nil	4.32±0.02	Nil	Nil	350.45±0.03	Nil	Nil	1.63±0.03	Nil	Nil
Stem of *C. papaya*												
0.1	8.62±0.03	1.83±0.04	Nil	4.32±0.02	0.89 ±0.02	Nil	357.45±0.03	17.78±0.01	Nil	1.63±0.03	0.68 ±0.02	Nil
0.2	8.62±0.03	1.69 ±0.02	Nil	4.32±0.02	0.62 ±0.02	Nil	354.34±0.03	6.34±0.01	Nil	1.63±0.03	0.39 ±0.02	Nil
0.3	8.62±0.03	1.10 ±0.05	Nil	4.32±0.03	0.30 ±0.02	Nil	351.45±0.03	Nil	Nil	1.63±0.03	0.39 ±0.02	Nil
0.4	8.62±0.03	0.69 ±0.03	Nil	4.32±0.02	Nil	Nil	350.24±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.5	8.62±0.03	Nil	Nil	4.32±0.03	Nil	Nil	348.56±0.03	Nil	Nil	1.63±0.03	Nil	Nil
Seed of *C. papaya*												
0.1	8.62±0.03	1.69 ±0.09	0.69 ±0.02	4.32±0.02	0.92 ±0.02	0.52 ±0.02	350.87±0.03	7.79±0.12	4.00±0.00	1.63±0.03	0.70 ±0.02	Nil
0.2	8.62±0.03	1.31 ±0.05	0.31 ±0.02	4.32±0.02	0.74 ±0.02	0.44 ±0.02	342.32±0.03	3.34±0.23	Nil	1.63±0.03	0.31 ±0.02	Nil
0.3	8.62±0.03	1.10 ±0.03	0.10 ±0.02	4.32±0.03	0.36 ±0.02	0.21 ±0.02	337.09±0.03	1.57±0.42	Nil	1.63±0.03	0.26 ±0.02	Nil
0.4	8.62±0.03	0.39 ±0.01	Nil	4.32±0.01	0.10 ±0.02	Nil	332.67±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.5	8.62±0.03	0.16 ±0.02	Nil	4.32±0.01	0.06 ±0.02	Nil	328.56±0.03	Nil	Nil	1.63±0.03	Nil	Nil
Bark of *C. papaya*												
0.1	8.62±0.03	0.52±0.03	Nil	4.32±0.03	0.35 · 10^2	Nil	338.45±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.2	8.62±0.03	Nil	Nil	4.32±0.03	Nil	Nil	345.89±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.3	8.62±0.03	Nil	Nil	4.32±0.03	Nil	Nil	343.87±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.4	8.62±0.03	Nil	Nil	4.32±0.03	Nil	Nil	331.67±0.03	Nil	Nil	1.63±0.03	Nil	Nil
0.5	8.62±0.03	Nil	Nil	4.32±0.03	Nil	Nil	327.78±0.03	Nil	Nil	1.63±0.03	Nil	Nil

Data are MEAN ± SEM of triplicate determinations. TVC – total viable counts; TCC – total coliforms counts; g l^{-1} – gram per litre; FCC – faecal coliforms counts; SCC – *Salmonella-Shigella* counts; g l^{-1} – gram per litre; Nil – not detected

Effect of *Carica papaya* treatment on physicochemical parameters of water samples

Pond water. *Carica papaya* stem, seed, leaf and bark material significantly ($p < 0.05$) reduced turbidity from 9.61±0.45 NTU to 9.43±0.78 NTU, 8.54±0.84 NTU, 8.52±0.76 NTU and 8.22±0.49 NTU respectively. However, only the seed and leaf material of *Carica papaya* caused a significant ($p < 0.05$) reduction in pH to 7.45±0.21 and 8.45±0.69 respectively. Similarly, all parts of *C. papaya* tested significantly ($p < 0.05$) reduced COD and nitrate contents of the pond water, while seed and leaf materials decreased the chloride content. The stem, leaf and bark materials decreased the conductivity of the pond water (Table 4). Alum significantly reduced the DO, TDS, water hardness, turbidity and pH. However, calcium hypochlorite did not significantly reduce the turbidity, hardness and pH but reduced the DO and TDS of the pond water (Table 5).

River water. Treatment of river with *Carica papaya* caused no significant ($p > 0.05$) changes to the Temperature, TDS, hardness, chloride, nitrate, when compared with the untreated river water. However, the plant seed material significantly ($p < 0.05$) reduced the pH, the seed and stem material increased the conductivity, the leaf material increased the calcium content of the river water (Table 4). Treatment with alum significantly ($p < 0.05$) decreased the pH but increased the Conductivity and chloride content of the water, while calcium hypochlorite significantly ($p < 0.05$) increased the TDS, calcium, chloride when compared with the untreated river water (Table 6).

Stream water. Treatment of stream water with alum significantly ($p < 0.05$) decreased pH (6.25±3.50), DO (6.25±3.50 mg l^{-1}), nitrate (12.53±2.56 mg l^{-1}), turbidity (5.38±2.58 NTU) but increased the conductivity (365.50±3.80 μS cm^{-1}), calcium (97.50±3.80 mg l^{-1}) and magnesium (78.25±1.75 mg l^{-1}) contents when compared with the untreated stream water. Calcium hypochlorite on the other hand decreased the DO (6.73±0.56 mg l^{-1}), nitrate and increased conductivity (365.50±3.80 μS cm^{-1}) and calcium (85.41±3.41 mg l^{-1}) of stream water when compared with untreated water. Treatment of the stream water with stem, seed, leaf and bark material of *Carica papaya* decreased DO, TDS, nitrate and turbidity but increased calcium contents of the stream water (Table 7).

Table 5

Physicochemical properties of pond water samples treated with *Carica papaya*

Parameters	Untreated	Alum	Calcium hypochlorite	Stem	Seed	Leaf	Bark
pH	9.30 ± 0.02^{b}	8.20 ± 1.30^{a}	9.10 ± 0.46^{b}	9.43 ± 0.78^{b}	7.45 ± 0.21^{a}	8.45 ± 0.69^{ab}	9.42 ± 0.23^{b}
DO [mg l^{-1}]	8.67 ± 0.45^{b}	6.73 ± 2.50^{a}	6.73 ± 0.56^{a}	9.52 ± 2.65^{b}	9.23 ± 4.87^{b}	8.53 ± 2.90^{ab}	7.94 ± 2.57^{a}
TDS [mg l^{-1}]	134.78 ± 2.34^{b}	113.20 ± 2.50^{a}	113.24 ± 4.97^{a}	145.04 ± 4.90^{c}	110.23 ± 2.87^{b}	90.43 ± 2.54^{a}	120.21 ± 2.65^{b}
Conductivity [µS cm^{-1}]	184.90 ± 2.34^{b}	187.90 ± 2.50^{b}	187.21 ± 4.32^{b}	138.52 ± 2.25^{a}	275.07 ± 2.76^{c}	140.32 ± 1.54^{a}	160.09 ± 4.90^{a}
Temp. [^{O}C]	33.96 ± 2.45^{a}	32.25 ± 2.70^{a}	32.62 ± 2.32^{a}	34.22 ± 1.57^{a}	32.52 ± 0.55^{a}	32.72 ± 0.76^{a}	32.52 ± 0.98^{a}
Hardness [mg l^{-1}]	694.97 ± 8.34^{b}	342.50 ± 2.60^{a}	654.32 ± 5.43^{b}	431.09 ± 2.67^{b}	324.34 ± 2.89^{a}	402.21 ± 4.43^{b}	302.07 ± 1.76^{a}
Calcium [mg l^{-1}]	89.24 ± 2.45^{a}	184.30 ± 1.65^{b}	85.41 ± 3.43^{b}	88.45 ± 2.87^{b}	68.24 ± 4.52^{a}	68.32 ± 2.60^{a}	88.32 ± 2.26^{b}
Chloride [mg l^{-1}]	20.01 ± 2.34^{a}	19.55 ± 2.50^{a}	20.54 ± 2.35^{a}	20.52 ± 0.76^{b}	16.52 ± 1.76^{a}	16.52 ± 2.59^{a}	21.52 ± 1.76^{b}
Nitrate [mg l^{-1}]	13.53 ± 1.45^{b}	12.50 ± 2.40^{b}	12.53 ± 3.45^{b}	10.32 ± 0.70^{a}	10.23 ± 0.68^{a}	10.52 ± 0.45^{a}	13.12 ± 0.86^{b}
Magnesium [mg l^{-1}]	65.78 ± 1.78^{ab}	73.43 ± 1.60^{b}	64.21 ± 1.56^{a}	73.32 ± 4.56^{b}	66.32 ± 2.75^{ab}	66.23 ± 1.54^{ab}	58.43 ± 1.54^{a}
Turbidity (NTU)	9.61 ± 0.45^{b}	7.78 ± 1.58^{a}	9.61 ± 0.32^{b}	8.53 ± 0.52^{a}	8.54 ± 0.84^{a}	8.52 ± 0.76^{a}	8.22 ± 0.49^{a}
COD [mg l^{-1}]	88.90 ± 1.56^{b}	86.09 ± 0.80^{b}	84.02 ± 2.45^{b}	69.22 ± 4.53^{a}	65.23 ± 2.90^{a}	65.22 ± 4.53^{a}	69.22 ± 2.52^{a}

Data are MEAN±SEM of triplicate determinations. Values followed by different superscript are significantly different ($p < 0.05$).
COD – chemical oxygen demand; TDS – total dissolved solid; DO – dissolved oxygen; NTU – nephelometric *turbidity* units; µS cm^{-1} – micro-Siemens per centimetre; mg l^{-1} – milligram per litre

Table 6

Physicochemical properties of river water samples treated with *Carica papaya*

Parameters	River	Alum	Calcium Hypochlorite	Stem	Seed	Leaf	Bark
pH	9.57 ± 1.75^{b}	7.45 ± 0.58^{a}	9.10 ± 0.46^{b}	8.55 ± 0.87^{b}	7.54 ± 0.65^{a}	8.53 ± 0.76^{b}	8.53 ± 0.54^{b}
DO [mg l^{-1}]	6.38 ± 2.17^{a}	6.75 ± 0.55^{a}	6.73 ± 0.56^{a}	9.57 ± 0.90^{b}	9.54 ± 0.87^{b}	8.54 ± 0.89^{a}	8.33 ± 0.65^{a}
TDS [mg l^{-1}]	115.21 ± 1.02^{a}	113.07 ± 0.77^{a}	113.24 ± 4.97^{b}	131.32 ± 2.65^{a}	127.32 ± 2.99^{a}	150.32 ± 3.65^{a}	149.32 ± 1.88^{a}
Conductivity [$\mu S\ cm^{-1}$]	169.55 ± 2.83^{a}	262.06 ± 0.57^{b}	187.21 ± 4.32^{ab}	168.23 ± 1.76^{a}	178.45 ± 2.54^{b}	178.23 ± 2.87^{b}	168.21 ± 1.65^{a}
Temp. [^{O}C]	33.42 ± 2.45^{a}	32.24 ± 1.57^{a}	32.62 ± 2.32^{a}	32.55 ± 0.89^{a}	32.21 ± 0.76^{a}	32.54 ± 0.99^{a}	33.23 ± 0.54^{a}
Hardness [mg l^{-1}]	652.23 ± 2.56^{b}	312.30 ± 2.58^{a}	654.32 ± 5.43^{b}	416.32 ± 4.90^{a}	326.21 ± 1.67^{a}	416.34 ± 2.76^{a}	411.21 ± 3.56^{a}
Calcium [mg l^{-1}]	85.40 ± 2.89^{a}	85.05 ± 0.78^{a}	120.41 ± 3.43^{b}	82.54 ± 2.65^{bc}	69.24 ± 1.65^{a}	95.34 ± 2.54^{c}	75.34 ± 3.55^{a}
Chloride [mg l^{-1}]	19.52 ± 1.74^{a}	87.08 ± 3.87^{b}	86.54 ± 2.35^{b}	19.54 ± 0.90^{a}	20.34 ± 0.89^{a}	20.52 ± 1.65^{a}	20.22 ± 1.89^{a}
Nitrate [mg l^{-1}]	10.61 ± 1.45^{a}	12.53 ± 0.54^{a}	12.53 ± 3.45^{a}	7.85 ± 0.76^{a}	19.23 ± 2.59^{b}	7.84 ± 0.87^{a}	8.83 ± 0.90^{a}
Magnesium [mg l^{-1}]	63.12 ± 1.20^{a}	64.47 ± 0.59^{a}	64.21 ± 1.56^{a}	61.54 ± 2.65^{a}	82.32 ± 2.90^{c}	62.32 ± 3.65^{a}	74.32 ± 4.65^{b}
Turbidity (NTU)	9.14 ± 2.30^{b}	4.38 ± 0.87^{a}	9.61 ± 0.32^{b}	9.53 ± 0.76^{b}	8.55 ± 0.76^{ab}	7.53 ± 0.89^{a}	7.53 ± 0.90^{a}
COD [mg l^{-1}]	81.23 ± 2.50^{b}	64.08 ± 0.76^{a}	84.02 ± 2.45^{b}	61.54 ± 1.54^{a}	62.32 ± 2.99^{a}	61.33 ± 1.87^{a}	65.42 ± 3.55^{a}

Data are MEAN±SEM of triplicate determinations.Values followed by different superscript are significantly different ($p < 0.05$).
COD – chemical oxygen demand; TDS – total dissolved solid; DO – dissolved oxygen; NTU – nephelometric *turbidity* units; $\mu S\ cm^{-1}$ – micro-Siemens per centimeter, mg l^{-1} – milligram per litre

Table 7

Physicochemical properties of stream water samples treated with *Carica papaya*

Parameters	Stream	Alum	Calcium Hypochlorite	Stem	Seed	Leaf	Bark
pH	9.26±1.98^b	6.25±3.50^a	9.10±0.46^b	7.54±0.55^a	7.33±0.56^a	9.54±0.42^a	8.54±0.45^b
DO [mg l^{-1}]	8.36±1.19^b	6.25±3.50^a	6.73±0.56^a	8.44±0.77^a	9.43 ±0.54^b	8.42±4.56^a	8.44±3.54^a
TDS [mg l^{-1}]	140.35±2.87^b	328.35±2.36^b	113.24±4.97^a	141.21±3.54^a	144.32±2.67^a	140.32±3.54^a	143.32±2.55^a
Conductivity [µS cm^{-1}]	185.73±2.76^a	365.50±3.80^b	187.21±4.32^a	187.45±2.67^a	197.32±3.65^b	194.55±2.45^b	187.23±4.67^a
Temp. [^{O}C]	33.45±1.98^a	32.27±3.59^a	32.62±2.32^a	33.22±3.54^a	32.33±2.56^a	32.55±2.67^a	33.53±2.31^a
Hardness [mg l^{-1}]	351.46±2.12^a	256.55±3.50^a	654.32±5.43^b	334.34±1.52^a	314.32±0.55^a	342.44±0.76^a	354.32±0.89^a
Calcium [mg l^{-1}]	86.31±2.24^a	97.50±3.80^b	85.41±3.43^b	87.54±2.14^b	85.54±2.43^b	86.43±4.55^b	78.65±2.99^a
Chloride [mg l^{-1}]	20.51±1.72^a	19.55±3.77^a	20.54±2.35^a	20.45±2.34^a	20.23±2.76^a	19.23±2.65^a	20.13±2.90^a
Nitrate [mg l^{-1}]	18.58±0.70^b	12.53±2.56^a	12.53±3.45^a	13.33±4.87^a	13.34±2.43^a	13.23±4.35^a	13.33±3.50^a
Magnesium [mg l^{-1}]	66.11±2.01^a	78.25±1.75^b	64.21±1.56^a	67.76±4.65^a	67.87±2.43^a	67.44±2.43^a	65.43±3.76^a
Turbidity (NTU)	9.53±1.09^b	5.38±2.58^a	9.61±0.32^b	9.34±3.77^a	9.14±2.43^a	8.53±2.76^a	13.13±3.66^b
COD [mg l^{-1}]	84.09±29^b	86.07±3.87^b	84.02±2.45^b	69.23±2.90^a	62.23±4.54^a	63.24±4.56^a	65.23±4.55^a

Data are MEAN±SEM of triplicate determinations. Values followed by different superscript are significantly different ($p < 0.05$). COD – chemical oxygen demand; TDS – total dissolved solid; DO – dissolved oxygen; NTU – Nephelometric *Turbidity* Units, µS cm^{-1} – micro-Siemens per centimetre; mg l^{-1} – milligram per litre

Table 8

Minimum inhibitory and bactericidal concentrations of the plant materials against the isolated organism

Plant	Part	E. coli	*B. megaterium*	*S. dysenteriae*	*B. aquercina*	*P. aeruginasa*	*E. aerogenes*	*S. gallinerum*	*P. myxofaciens*	*K. oxytoca*	*K. pneumonia*	*K. ascorbata*	*P. mirabilis*	*S. aureus*
Minimum inhibitory concentrations (MBC)														
Carica papaya	Seed	32	16	32	32	32	16	32	16	16	16	16	16	16
	Leaf	16	32	16	16	16	16	16	32	32	16	16	16	64
	Bark	16	32	64	32	64	16	64	16	16	16	16	16	16
	Stem	16	16	64	64	64	32	32	32	32	32	32	32	16
Minimum bacterial concentrations (MBC)														
Carica papaya	Seed	32	16	32	32	32	16	32	16	32	32	16	32	32
	Leaf	16	32	16	16	16	16	16	32	32	16	16	16	64
	Bark	16	32	64	32	64	16	64	32	16	16	32	16	32
	Stem	32	16	64	64	64	32	32	32	32	32	32	32	16

mg ml^{-1} – milligram per milliliter

Minimum inhibitory concentrations and minimum bactericidal concentrations

The minimum inhibitory concentrations (MIC) of the plant materials against the isolated organism are presented in Table 8. The MIC range between 8 and 64 mg ml^{-1} against all the isolates tested. The minimum bactericidal concentrations (MBC) of the plant materials (seed, leaf, bark and stem) against the isolated organism range between 16–128 mg ml^{-1} against all organism tested (Table 8).

Discussion

The result for phytochemical screening of *Carica papaya* showed that the plants contained some phytochemical compounds which possess good antimicrobial properties on the isolates obtained from the water sample. Although, the stem and bark material of *C. papaya* contains all the phytochemical tested (Table 1). Previous study on *C. papaya* reported that the leaves contain anthraquinones, phenols, glycoside amino-acid, terpenoid, reducing sugar, saponin, tannin, alkaloids and flavonoids. On the other hand, the stem material contains alkaloid, saponin, flavonoids and reducing sugar (MARSHALL et al. 2015). The absence of cardiac glycosides, anthraquinones and terpenes in leaf and seed of *Carica papaya* could be associated with the earlier findings, who reported that not all phytochemicals are present in all part of a plant (LAWAL et al. 2014). The presence of saponins supports the fact that pawpaw has cytotoxic effects such as permeabilization of the intestine as saponins are cytotoxic (OKWU and OKWU 2004). Another important action of saponins is their expectorant action through the stimulation of a reflex of the upper digestive tract (DAVID 1983). The presence of alkaloids in the leaves shows that these plants can be an effective anti-malarial, since alkaloids consist of quinine, which is an anti-malaria (ROBINSON 1985).

Bacterial species identified from the river, stream and ponds water sample were mostly members of *Enterobacteriaceae* family. These bacterial includes *Salmonella*, *E. coli, Klebsiella*, *Proteus*, *Pseudomonas*, *Enterobacter*. These bacterial genera isolated were found to be the same with those that are commonly seen in contaminated water bodies and other aquatic environments and it is in line with the research work of OKONKWO et al. (2008) where similar bacterial species were isolated from surface water sample. Similar study by PRASAI et al. (2007), also identified *Escherichia coli* (26.4%), *Enterobacter* spp. (25.6%), *Citrobacter* spp. (22.6%), *Pseudo-*

monas aeruginosa (6.3%), *Klebsiella* spp. (5.4%), *Shigella* spp. (3.78%), *Salmonella typhi* (3.3%), *Proteus vulgaris* (2.9%), *Serratia* spp. (2.52%) and *Vibrio cholerae* (0.84%) in water samples from Kathmandu Valley, Nepal.

The mean viable counts for the river, stream and pond water samples recorded in this study were generally higher exceeding the set standard stated for water meant for drinking purposes. The presences of these bacterial species in the water samples possibly suggest that the water bodies in the study area have been contaminated with wastes either of human or animal origin. This means that using this water without treatment could be hazardous to human and animal health. This observation is line with similar studies conducted on surface water in Malawi by PRITCHARD et al. (2009), in Bamenda, Cameroun by YONGABI et al. (2011), in Abeokuta, Ogun State by OJEKUNLE et al. (2014) and in Guma Local Government Area, Benue State, Nigeria by ADEOTI et al. (2018).

The total coliform group consists of several genera of bacteria belonging to the family Enterobacteriaceae; they are a group of bacteria found in the intestines of humans and other animals, also occur naturally in the soil, on vegetation and in surface waters. Most members of the coliform group do not cause disease. Effect of treatment on bacteria loads of water samples showed that control water samples from the river, pond and stream had much higher total coliform count (TCC) than those of the treated samples. The mean bacteria count recorded in untreated water were generally high exceeding the limit stated by WHO (2011).WHO (2011) recommends a zero value total coliform counts in drinking water. This is in line with earlier observation that stream, river and pond water without treatment are not safe for drinking. The high total coliform counts for drinking water have been found in several studies (MOHAMMAD 2015, PRITCHARD et al. 2009). The presence of these rates of bacteria may be due to the soil and Agricultural runoff, effluent from septic system or domestic sewage discharge and infiltration of grazing animal faecal matter, in addition to, atmospheric deposition leading to increase of various microbial densities (AMAGLOH and BENANG, 2007). Drinking water contaminated with these organisms can cause stomach and intestinal illness including diarrhea and nausea, and even lead to death. These effects may be more severe and possibly life threatening for babies, children, the elderly or people with immune deficiencies or other illnesses (PRITCHART et al. 2007).

Following treatments, with the *Carica papaya* plant material, there was drastic reduction in the total coliform count recorded for all the water samples. This shows that the plant material used impacted significantly on the bacteria loads of the water samples thus making the water safe for consumption. This activity could be attributed to its phytochemicals com-

position particularly the alkaloid, flavonoid and tannins (Table 1) which have been reported for antimicrobial activity (MARSHALL et al. 2015). The significant activities of *C. papaya* against the microbial population of stream water reported in this study, is In line with the several other studies who reported that *Carica papaya* leaves stem, and seed have antimicrobial potentials (ANIBIJUWON and UDEZE 2009, BASKARAN et al. 2012). The reports of IFESAN et al. (2013) have also shown that *Carica papaya* have significant antibacterial activity in various material from different tree parts.

Hydrogen ion concentration (pH) recorded for the untreated water samples exceeded the maximum permissible limit of the standards. However, the alum treated water moved towards more acidity attributable to the fact that alum produces sulphuric acid in solution which lowers the pH values. This tendency towards increase in acidity could also be due to trivalent cation aluminium which serves as a lewis acid that can accept a lone pair of electrons (MILLER et al. 1984). DOER (2005) reported that the action of plant as a coagulant lies in the presence of water soluble cationic proteins. This suggests that in water, the acidic amino acids present in proteins of plant materials under investigation would donate a proton from water resulting in the release of a H^+ group making the solution acidic. This accounted for the slight tendency towards acidic pH values observed when the *C. papaya* materials were used. The observed decrease pH following treatment with the *C. papaya* materials disagrees with the earlier study by MUYIBI and EVISON (1994) who observed *Moringa* in the complete removal of total hardness in water produce no significant increases in pH values. However, as earlier mentioned, high dosage of alum in water treatment even though a better coagulant may lead to high acidity, raising health concerns about alum related diseases reported by several investigators (MILLER et al. 1984, MARTYNS et al. 1998 and NAJM et al. 1998).

The U.S. Environmental Protection Agency (EPA) has classified hardness into four categories namely, soft (0–50 mg l^{-1}), moderately hard (50–150), hard (150–300), very hard (> 300). Based on this classification, the river, stream and pond water used in this study are very hard. The high values, exceeded the maximum permissible limit of the standards (*Guidelines for drinking-water*… 2004). By indication the use of this water for laundry purpose will not be adequate as it will not lather with soap. The observation with calcium hypochlorite as opposed to alum when used to remediate river, pond and stream water is an indication that purification of water with calcium hypochlorite does not improve the hardness quality of the water. However, the observed significant reductions in the

level of water hardness following treatment with the *C. papaya* materials reflect the water remediation quality of the plant. This reduction in water hardness will improved the usefulness of the water for laundry purposes, it will also reduce the cost of laundry as lesser amount of soap will be required for ladder formation.

The nitrates levels of water samples in this study exceeded the maximum permissible limit of the standards. Thus consumption of this high nitrate containing water could interfere with the ability of red blood cells to transport oxygen. This high nitrate containing water could leads to blue baby disease to infants and difficulty in breathing since their bodies are not receiving enough oxygen. Treatments of these water with alum and calcium hypochlorite does not eliminate the threat of nitrate intoxication when consumed as it does not reduced the concentration of the nitrate, thus the water is still considered unhealthy and unfit for human consumptions. The dissolve oxygen, TDS and conductivity in pond, stream and river water reported in this study, correspond with the maximum permissible limit of the standards as recorded by WHO and FEPA (*Emerging issues*… 2011).

The conventional method of water treatment using expensive chemicals such as alum and calcium hypochlorate (chlorine) is expensive thereby making portable drinking water beyond the reach of rural dwellers that depend mainly on contaminated water sources such as wells, ponds, streams, rivers e.t.c. exposing them to water borne diseases. The satisfactory results obtained from the treatment of water with *C. papaya* materials will reduced the alum requirement in water treatment and by implications the risk of alum related diseases as well as the cost of water treatment (Muyibi and Okuofu 1995).

Conclusion

In conclusion, surface water in the study area are contaminated with bacterial strains which are capable of causing infectious diseases as a result, human health is continuously being threatened. *Carica papaya* plant materials have a double advantage of having phytochemicals of antimicrobial properties and also better potentials for water purification than the synthetic coagulant.

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Conflict of interest

The authors declared no conflict of interest exist.

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AMINO ACID PROFILES, ANTINUTRIENTS, CONCENTRATIONS OF MINERALS AND ANTINUTRIENT-MINERAL MOLAR RATIOS OF "AKIDIAGWORAGWO" AND "NWAGBARAOTI" TRADITIONAL FOODS

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Key words: amino acid profiles, antinutrients, Mbano people, minerals, molar ratios, traditional foods.

Abstract

Amino acid profiles, anitnutrients, concentrations of minerals and antinutrient-mineral molar ratios of "akidiagworagwo" and "nwagbaraoti" traditional foods were investigated with standard methods. Results obtained for amino acids showed that the food samples contained nine of the ten essential amino acids and eight of the ten non-essential amino acids. Values of total amino acid groups showed that TAAs, TNEAAs, TEAAs with His, TEAAs without His, TNAAs, TNAAs, TBAAs, TBCAAs, and TArAAs were higher in "akidiagworagwo" than "nwagbaraoti". Higher leucine to isoleucine values and TBAA to TAAA ratio of less than one were observed for the foods. The food samples fall short of amino score requirements for isoleucine, sulphur containing amino acids, threonine and valine. Tryptophan was not found in both food samples. Minerals found in the studied foods were calcium, magnesium, zinc and iron with phytate and oxalate as antinutrients. The estimated bioavailability of the antinutrents to

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mineral ratios depicted bioavailability of minerals in the food samples to the body following consumption. The study has investigated amino acid profiles, antinutrients, concentrations of minerals and antinutrient-mineral molar ratio of "akidiagworagwo" and "nwagbaraoti" traditional foods.

Introduction

Food substances are known to furnish the body with nutrients that nourish it and are very important to the body (OLUSANYA 2008). The knowledge of food constituents is dated back to the days of our forefathers, who searched for foods with different constituents to eat (DURU et al. 2013a). The search for foods with different constituents then could be due to the inherent effects of such foods in the body following consumption (DURU et al. 2013b). Foods in their natural form are known to contain both nutritive and non-nutritive constituents (OKAKA and OKAKA 2005). The nutritive constituents found in foods are carbohydrates, proteins, vitamins, fats and oils, minerals, and water (OKAKA and OKAKA 2005, OLUSANYA 2008, DURU et al. 2013a, AGOMUO et al. 2017), while the non-nutritive constituents are phytochemicals and antinutritional factors (OKAKA and OKAKA 2005, OLUSANYA 2008, DURU et al. 2013b, AGOMUO et al. 2017). Nutritionally, carbohydrates, proteins, fats and oils are known as primary food substances whereas welfare food substances constitute vitamins and minerals (OLUSANYA 2008). Functionally, a lot has been reported on both the primary food substances as well as welfare food substances (OKAKA and OKAKA 2005, OLUSANYAN 2008, DURU et al. 2013a). The phytochemicals found in foods have been implicated to possess certain healthy and healing physiological activity to the body though depending on their concentrations while antinutritional factors have been reported to influence the bioavailability of minerals and other nutrients in foods to the body (SHAHIDI 1997, URGA and NARASIMBA 1998). In recent times, there is a renewed interest on traditional foods. Different authors have attempted to define traditional foods (ALBAYRAK and ERODOGAN 2010, DURU et al. 2015, AMADI et al. 2017, AMADI et al. 2018), and evaluate the constituents of those that have not gone into extinction (DURU et al. 2013b).

"Akidiagworagwo" and "nwagbaraoti" traditional foods are among those traditional foods that have not gone into extinction and deserve to be evaluated for their constituents. The foods are common to Mbano people of Imo State, Southeastern Nigeria. Mbano people transversed Isiala and Ehime Mbano Local Government Authorities in Imo State, located within coordinates 5.6677° north and longitude 7.2034° east. The people of Mbano

are mostly farmers though few are into trading. They speak Igbo language as native dialect. "Akidiagworagwo" and "nwagbaraoti" traditional foods are among the most important elements of cultural identity, unification and heritage for the people of Mbano. The foods also have positive effects on rural economies, and serve as effective instrument in preventing unfair competition and brand creation since some village dwellers prepare and sale the foods as sources of income within the locality.

According to ALBAYRAK and ERODOGAN (2010) protection of traditional foods allows the protection of cultural heritage, consumers, and local producers. The same authors also noted that protection of traditional foods allow job creation, and especially an increase of women's contribution to the economy. With the renewed interest on traditional foods, there is need to extend the study on such foods to accommodate "akidiagworagwo" and "nwagbaraoti" as to derive the positive effects of protecting traditional foods as noted by ALBAYRAK and ERODOGAN (2010) and as well to prevent them from going to extinction by informing the owners of the foods and other interested individuals with the outcome of the study and the need for their continued consumption.

The present study therefore evaluated the amino acid profiles, antinutrients, concentrations of minerals and antinutrient-mineral molar ratios of "akidiagworagwo" and "nwagbaraoti" traditional foods.

Materials

Collection of materials used in the preparation of "akidiagworagwo" and "nwagbaraoti" traditional foods

The study on "akidiagworagwo" and "nwagbaraoti" traditional foods was carried out within Isiala and Ehime Mbano Local Government Areas of Imo State, Southeastern, Nigeria where the foods are produced for domestic consumption. The major raw materials and ingredients used in the preparation of "akidiagworagwo" and "nwagbaraoti" traditional foods for this study were purchased from three local markets (Orie Amaraku, Ekezeala and Orie Nsu markets) within Mbano locality.

Preparation of "akidiagworagwo" traditional food

One hundred and fifty (150) grams of "ugbakala" (sliced fermented cooked seed of *Pentaclethra macrophylla*), 700 g of "akidi" (*Vigna unguiculate*), 250 g of fresh yellow maize (*Zea mays*); ten grams of "ose nkirisi"

(*Capsicum* spp.), 150 mL of red palm oil (*Elaeis guineensis* oil), and 5000 mL of water were used for the preparation of "akidiagworagwo". Required quantity of "akidi" was put in cooking pot with 5000 mL of water and cooked by application of heat for one and half hour till the "akidi" was confirmed soft. Fresh yellow maize was added, and the cooking continued for thirty minutes more before the remaining ingredients were added and mixed properly to form "akidiagworoagwo" ready to be served (Figure 1).

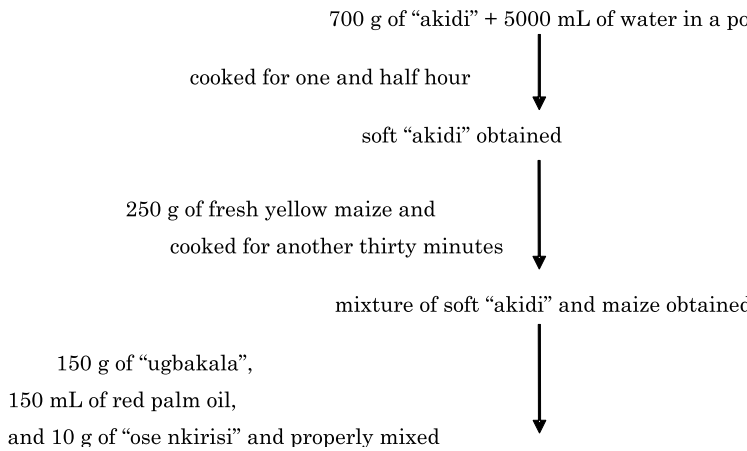

Fig. 1. Flow-chart showing preparation of "akidiagworoagwo"

Preparation of "nwagbaraoti" traditional food

Five hundred (500) grams peeled "egusi" (*Citrullus* spp.); 50 g of "eru-usu"; 30 g of "ose nwabakala" (*Capsicum* spp.); and water were used for the preparation of "nwagbaraoti". The measured peeled "egusi", "ero--usu", and "ose nwabakala" were mixed and pounded together with local mortar and pestle into a smooth oily paste. 200 mL of water were added into the mixture as the pounding continue to produce a smooth and softer oily paste which was mixed properly. The smooth and softer oily paste was then rolled into slightly flattened small balls for faster and even distribution of heat during cooking. The balls were wrapped with "ugu" (*T. occidentalis*) leaves and cooked by steaming in a cooking pot under medium heat. The cooking lasted for one hour thirty minutes, after which delicious and tasty "nwagbaraoti" was ready to be served (Figure 2).

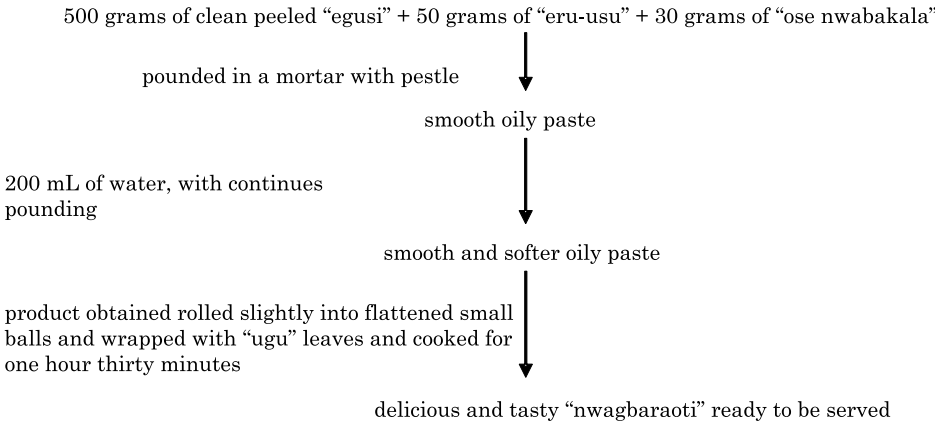

Fig. 2. Flow-chart for the preparation of "nwagbaraoti"

Fig. 3. Prepared "akidiagworagwo" in a glass plate sourrended by the raw the materials used in its preparation

Fig. 4. Prepared balls of "nwagbaraoti" inside ceramic plate sourrounded by raw materials used in its preparation

Methods

Preparation, preservation, and analysis of food samples

The prepared samples of "akidiagworagwo"and "nwagbaraoti" foods were dried in an oven at 55ºC for 48 hours. The dried samples were ground with a hand mill into powdered form and stored in air tight container at 40ºC until required for analysis.

Amino acid determination

The total amino acid (AA) compositions of the food samples were quantified using the ion-exchange chromatography-based Technicon Sequential Muti-sample (TSM) amino acid analyser (Technicon Instruments Corporation, New York) method described by SPACKMAN et al. (1958). Dried ground samples of the foods were defatted, hydrolysed, evapourated in a rotary evapourator and then loaded into the Technicon Sequential Muti-sample (TSM) amino acid analyser. Two grams each of the samples were weighed into the extraction thimbles and the fats were extracted with chloroform/methanol (2:1 mixture) using soxhlet extraction apparatus as described by AOAC (2006). The extraction lasted for 15 hours. The defatted samples weighing 0.7491 g were put into a glass ampoules and 7 mL of 6 M HCl was added. Oxygen was liberated by passing nitrogen into the ampoules (this was to avoid possible oxidation of some amino acids during hydrolysis). The glass ampoules were then sealed with Bunsen burner flame and put in an oven preset at 105±5°C for 72 hours. The ampoules were allowed to cool before breaking them open at the tip and the content filtered to remove the humus. Their filtrates were then evapourated to dryness at 40°C under vacuum in a rotary evapourator. The residues obtained were dissolved with 5mL of acetate buffer (pH 2.0) and stored in plastic specimen bottles and kept in the freezer till needed for analysis. Ten micro litres (10 μL) of each sample was dispensed into the cartridge of the analyser. The TSM analyser is designer to separate and analyse free acidic, neutral and basic amino acids of the hydrolysate. The values for the individual amino acids expressed in g/100 g protein were calculated using chromatogram peaks generated.

Estimation of total amino acid groups, percentages of amino acid groups, and amino acids scores

Total Amino Acid (TAA) group was estimated by summing up all the amino acids observed in the food samples. Total Non-Essential Amino Acid (TNEAA) was estimated by adding all the observed non-essential amino

acids in the food samples. Total Essential Amino Acid with histidine (TEAA with His) was calculated by adding all the observed essential amino acids in the food samples with histidine. Total Essential Amino Acid without histidine (TEAA without His) was estimated by adding all the observed essential amino acid in the studied samples without histidine. Total Neutral Amino Acid (TNAA) was estimated by adding all the neutral amino acids (glycine, alanine, valine, leucine and isoleucine) of the studied samples. Total Acidic Amino Acid (TAAA) was calculated by adding all the acid amino acids (aspartic acid and glutamic acid) observed in the samples. Total Basic Amino Acid (TBAA) was estimated by adding all the basic amino acids (lysine, arginine, and histidine) observed in the studied samples. Total Sulphur-containing Amino Acid (TSAA) was calculated by adding all the sulphur-containg amino acids (methionine and cysteine) observed in the food samples. Total Branched Chain Amino Acid (TBCAA) was calculated by all the branched amino acids (leucine, isoleucine and arginine) observed in the food samples. Total Aromatic Amino Acid (TArAA) was estimated using all the aromatic amino acids (phenylalanine, tyrosine, and tryptophan) of the food samples. Percentages of amino acid groups were estimated following the methods as described by IBEGBULEM et al. (2012). Estimation of amino acid scores of the food samples were calculated as the ratio of the actual mount (mg) of each amino acid involved per g of the protein to the required amount [mg] of the amino acid [mg] per g of a reference protein as described by FAO/WHO (1973) and WARDLAW and KESSEL (2000) using FAO/WHO/UNU provisional scoring pattern as provided by HARPER (2017).

Determination phytate

The method of GRIFFITH and THOMAS (1981) was used for phytate determination. The two grams of defatted sample was extracted for 1 hour in 50 mL of 0.18 M trichloro acetic acid (TCA) at room temperature. The suspension was centrifuged and an aliquot (10 mL) was added to 5 mLs of 0.036 M ferric chloride solution and placed in boiling water. After 45 minutes, the precipitated ferric phytate was collected by centrifugation, washed twice with 30 mL of the trichloroactic acid and once with 50 mL of water. The precipitate obtained was suspended in 3 mL of 1.5 M NaOH, diluted to 30 mL with water, and the resulting ferric hydroxide coagulated by heating. The ferric hydroxide was then centrifuged, washed with water and dissolved in 50 mL of 3.2 M HNO_3 and made up to 100 mL with water. The iron content was then determined by spectrophotometry. This was repeated for each studied sample. Amount of phytate in the sample was calculated.

Determination of oxalate

The method as described by ONWUKA (2005) was adopted. Two grams of the sample were extracted three times by warming (50ºC) and stirring with magnetic stirrer for one hour in 20 mL of 3 M HCL. The combined extract was diluted to 100 mL with water and used for total oxalate estimation. The extract of 5 mL volume was made alkaline with 1 mL of 5 M ammonium hydroxide. This was made acid to phenolphalein (2 or 3 drops of this indicator) by drop wise addition of glacial acetic acid. Then, 1 mL of 5% calcium chloride was added and the mixture was allowed to stand for 3 hours after which it was centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded and the precipitate washed 3 times with hot water with thorough mixing and centrifuging each time. To each tube was added 2 mL of 3 M H_2SO_4 and the precipitate dissolved by warming in a water-bath (70ºC). The content of each tube was titrated with freshly prepared 0.01 M $KMnO_4$. Titration was carried out at room temperature until the pink colour appeared throughout the solution which was allowed to stand until the solution was colourless. The solution was then warmed to 70ºC and titration was continued until a pink colour persisted for at least 30 seconds indicating end point. Oxalate in samples was calculated using molarity relationship.

Determination of calcium, magnesium, iron, and zinc in the food samples; and estimation of antinutrient-mineral molar ratios of the food samples

Two (2) grams of each sample were wet-digested with heat and concentrated HNO_3/H_2SO_4 (7.5 mL and 5 mL respectively) solution. After the materials begin to char; digestion continued with only HNO_3 until a light yellow liquid was obtained. Calcium, magnesium, iron, and zinc concentrations were determined with the aid of atomic absorption spectrophotometer (Analyst 700 series, Parkin Elmer. Germany) according to the manufacturer`s instruction. Antinutrient-mineral ratios were estimated following the relationships as described by IGWE et al. (2013) and GEMEDE et al. (2016).

Statistical analysis

Results for amino acid constituents, total and % total amino acid groups, ratios of some amino acids and amino acid groups, minerals and antinutrients of “akidiagworagwo” and “nwaogbaraoti” traditional foods were presented as mean and standard deviations of six determinations while

amino acid scores and antinutrient-mineral molar ratios were presented as mean of six determinations. Data were analyzed using Students` t-distribution test of significance. Values were considered significant at ($p < 0.05$).

Results

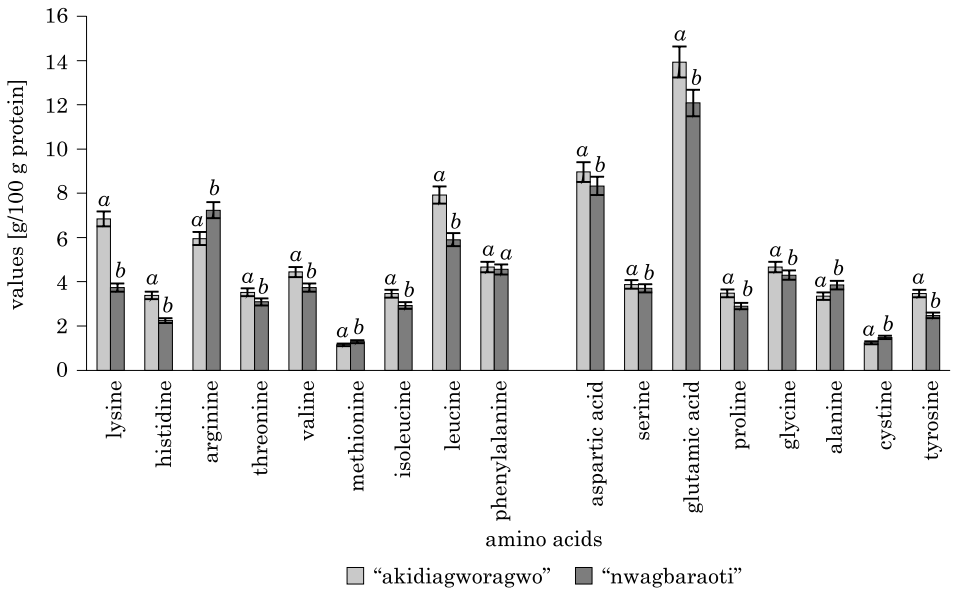

Fig. 5. Amino acid constituents of "akidiagworagwo" and "nwagbaraoti" traditional foods

Amino acid constituents of "akidiagworagwo" and "nwagbaraoti" traditional foods as presented in Figure 5 revealed the presence lysine (6.84–3.74 g/100 g protein), histidine (2.25–3.39 g/100 g protein), arginine (5.95–7.23 g/100 g protein), threonine (3.09–3.52 g/100 g protein), valine (3.74–4.44 g/100 g protein), methionine (1.15–1.29 g/100 g protein), isoleucine (2.93–3.46 g/100 g protein), leucine (5.90–7.92 g/100 g protein), phenylalanine (4.65–4.66 g/100 g protein), aspartic acid (8.33–8.96 g/100 g protein), serine (3.70–3.88 g/100 g protein), glutamic acid (12.08–13.93 g/100 g protein), proline (2.90–3.48 g/100 g protein), glycine (4.30–4.66 g/100 g protein), alanine (3.35–3.85 g/100 g protein), cystine (1.24–1.48 g/100 g protein), and tryrosine (2.48–3.47 g/100 g protein).

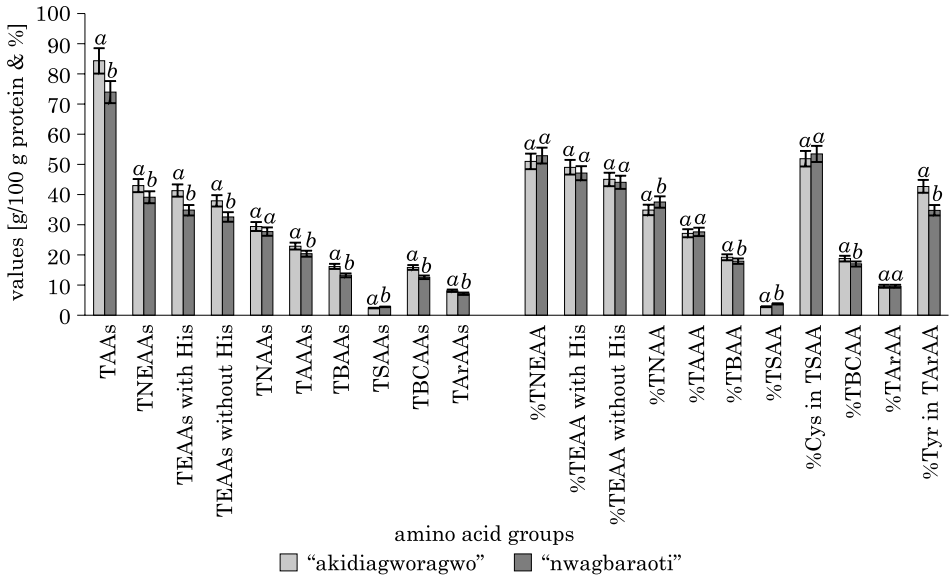

Fig. 6. Total and %total amino acid groups of "akidiagworagwo" and "nwaogbaraoti" traditional foods

TAAs – total amino acids; TNEAAs – total non-essential amino acids; TEAAs with His – total essential amino acids with histidine; TEAAs without His – total essential amino acids without histidine; TNAAs – total neutral amino acids; TAAAs – total acidic amino acids; TBAAs – total basic amino acids; TSAAs – total sulphur containing amino acids; TBCAAs – total branched chain amino acids; and TArAAs – total aromatic amino acids; % TNEAA – percentage total non-essential amino acid; % TEAA with His – percentage total essential amino acid with histidine; % TEAA without Histidine – percentage essential amino acid without histidine; % TNAA – percentage total neutral amino acid; % TAAA – percentage acid amino acid; % TBAA – percentage basic amino acid; % TSAA – percentage sulphur-containing amino acid; % Cys in TSAA – percentage cystine in total sulphur amino acid; % TBCAA – percentage branched-chain amino acid; % TArAA – percentage total aromatic amino acid; % Tyr in TArAA – percentage tyrosine **i**n total aromatic amino acid.

Figure 6 is based on Figure 5; bars of an amino group with different letters of alphabet are statistically significant at ($p < 0.05$).

Results of total and % total amino acid groups of "akidiagworagwo" and "nwaogbaraoti" traditional foods as presented in Figure 6 revealed TAAs (73.94–84.30 g/100 g protein), TNEAAs (39.12–42.97 g/100 g protein), TEAAs with His (34.82–41.33 g/100 g protein), TEAAs without His 32.57–37.94 g/100 g protein), TNAAs (27.74–29.41 g/100 g protein), TAAAs (20.41–22.89 g/100 g protein), TBAAs (13.22–16.18 g/100 g protein), TSAAs (2.39–2.77 g/100 g protein), TBCAAs (12.57–15.82 g/100 g protein), TArAAs (7.13–8.13 g/100 g protein), % TNEAA (50.97–52.91%), % TEAA with His (47.09–49.03%), % TEAA without His (44.05–45.01%), % TNAA (34.89–37.52%), % TAAA (27.15–27.67%), % TBAA (17.88–19.19%), % TSAA (2.84–3.75%), % Cys in TSAA (51.88–53.43%), % TBCAA (17.00––18.77%), % TArAA (9.64–9.64%), and % Try in TArAA (34.78–42.68%).

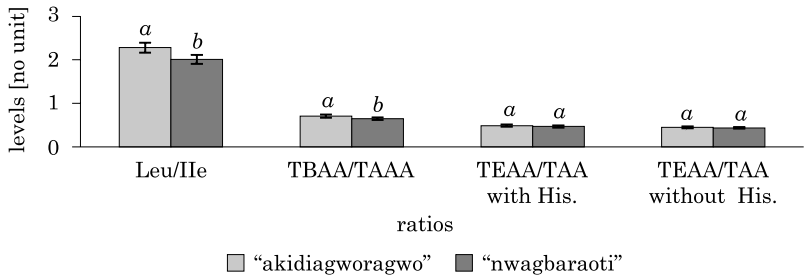

Fig. 7. Ratios of some amino acids and amino acid groups of "akidiagworgawo" and "nwagbaraoti" traditional foods

Leu/IIe – leucine: isoleucine; TBAA/TAAA – total basic amino acids: total acid amino acids; TEAA/TAA with His – total essential amino acid: total amino acids with histidine; TEAA/TAA without His – total essential amino acids: total amino acid without histidine.

Figure 7 is based on Figure 5 and Figure 6; bars of an amino ratio with different letters of alphabets are statistically significant at ($p < 0.05$).

Ratios of some amino acids and amino acid groups of "akidiagworgawo" and "nwagbaraoti" traditional foods presented in Figure 7 reveals Leu/IIe (2.01–2.28), TBAA/TAAA (0.65–0.71), TEAA/TAA with His (0.47–0.49), and TEAA/TAA without His (0.44–0.45).

Table 1

Amino acid scores of "akidiagworagwo" and "nwagbaraoti" traditional foods based on FAO/WHO/UNU (1981) provisional amino acid scoring pattern [mg g^{-1}]

Amino acid	"Akidiagworagwo"	"Nwagbaraoti"	FAO/WHO/UNU (1981)
Isoleucine	0.88	0.73	40
Leucine	1.13	0.84	70
Lysine	1.24	0.68	55
Methionine + cystine	0.68	0.79	35
Phenylalanine + tyrosine	1.36	1.19	60
Threonine	0.88	0.77	40
Tryptophan	NA	NA	10
Histidine	NC	NC	NA
Valine	0.89	0.75	50
Total	7.06	5.75	360

Results are mean of six determinations. Legend: NA – not available; NC – not considered

Table of amino acid scores of "akidiagworgawo" and "nwagbaraoti" traditional foods (Table 1) revealed isoleucine (0.73–0.88 mg g^{-1}), leucine (0.84–1.13 mg/100 g), lysine (0.68–1.24 mg/100 g), methionine + cystine (0.68–0.79 mg g^{-1}), phenylalanine + tyrosine (1.19–1.36 mg g^{-1}), threonine (0.77–0.88 mg/100 g), and valine (0.75–0.89 mg/100 g).

Table of minerals, antinutrients, and antinutrients-mineral molar ratios of "akidiagworagwo" and "nwagbaraoti" traditional foods (Table 2) showed the presence of Ca (30.00–96.00 mg/100 g), Mg (9.14–18.63 mg/100 g), Zn (0.71–1.40 mg/100 g), phytate (0.80–1.78 mg/100 g), oxalate (0.12–0.32 mg/100 g), [Oxa.]/[Ca] ($1.52 \cdot 10^{-3}$–$1.82 \cdot 10^{-3}$), [Oxa.]/[Ca+Mg] ($1.31 \cdot 10^{-3}$–$9.00 \cdot 10^{-4}$), [Phyt.]/[Fe] ($3.76 \cdot 10^{-2}$–$7.17 \cdot 10^{-2}$), [Phyt.]/[Ca] ($5.06 \cdot 10^{-4}$–$3.60 \cdot 10^{-3}$), [Phyt.]/[Zn] ($1.259 \cdot 10^{-1}$–$1.89 \cdot 10^{-1}$) and [Ca×Phyt.]/Zn ($9.4 \cdot 10^{-2}$–$4.5 \cdot 10^{-1}$ mol kg^{-1}).

Discussion

The studied food samples possessed nine of the ten essential amino acids (Figure 5). They also possessed eight of the ten non-essential amino acids. These amino acids become very important when their functions are considered in the body, following consumption. Different authors have reported the functions of these amino acids in the body (Uwakwe and Ayalogu 1998, Wardlaw and Kessel 2002, Olusanya 2008, Wu 2009, Connolly 2011, Ibegulem et al. 2012, Westerterp-Plantenga et al. 2012, Wu et al. 2013). Essential amino acids such lysine, histidne, valine, isoleucine, and leucine as well as non essential amino acids such as aspartic acid, serine, glutamic acid, proline, glycine, and tryrosine were significantly ($p < 0.05$) higher in "akidiagworagwo" when compared to those of "nwagbaraoti". It could be that "akidiagworagwo" may offer these amino acids to the body more than "nwagbaraoti" when consumed. The studied food samples have more TNEAAs than TEAAs with or without His. This could imply that the foods may not be good sources of essential amino acids to the body when compared to other traditional foods, though they surpassed the 1.7 g essential amino acids of egg white as reported by Connolly (2011). TNEAAs of the food samples also surpassed the 2.1 g of egg white (Connolly 2011). The benefits of amino acids under the acronym TEAAs, TNAAs, BCAAs have been reported by different authors (Fernstrom 1994, Fernstrom 2013, Layne 2017). The obtained values of total amino acid groups (Figure 6) showed that TAAs, TNEAAs, TEAAs with His or without His, TNAAs, TAAAs, TBAAs, TBCAAs, and TArAAs were significantly higher ($p < 0.05$) in "akidiagworagwo" than "nwagbaraoti". Percentages of amino acid groups placed the food samples as having more % TNEAA than % TEAA with or without His. It has been noted that 40 to 45% of total amino acid content in most high quality proteins represent essential amino acids. With or without His, the % total essential amino acids (% TEAA) of the two traditional foods were higher than 40%.

Table 2

Minerals, antinutrients and antinutrient-mineral molar ratios of "akidiagworagwo" and "nwagbaraoti" traditional foods

Specification	Minerals [mg/100 g]				Antinutrients [mg/100 g]		Antinutrient-mineral molar ratios					
Food	Ca	Mg	Fe	Zn	Phyt.	Oxa.	[Oxa.]/[Ca]	[Oxa.]/[Ca + Mg]	[Phyt.]/[Fe]	[Phyt.]/[Ca]	[Phyt.]/[Zn]	[Ca · Phyt.]/Zn (mol./kg)
"Akidiagwo-ragwo"	96.00 ± 3.03^a	9.14 ± 0.47^a	1.80 ± 0.10^a	0.71 ± 0.04^a	0.80 ± 0.03^a	0.32 ± 0.02^a	$1.52 \cdot 10^{-3}$	$1.31 \cdot 10^{-3}$	$3.76 \cdot 10^{-2}$	$5.06 \cdot 10^{-4}$	$1.89 \cdot 10^{-1}$	$4.5 \cdot 10^{-1}$
"Nwagba-raoti"	30.00 ± 1.61^b	18.63 ± 0.96^b	2.10 ± 0.11^b	1.40 ± 0.59^b	1.78 ± 0.10^b	0.12 ± 0.01^b	$1.82 \cdot 10^{-3}$	$9.00 \cdot 10^{-4}$	$7.17 \cdot 10^{-2}$	$3.60 \cdot 10^{-3}$	$1.259 \cdot 10^{-1}$	$9.4 \cdot 10^{-2}$

Results are mean and standard deviation of six determinations. Values with different letters of alphabets along the same column are statically significant at ($p < 0.05$). Phyt. – Phytate; Oxa. – Oxalate

This could be indication that the foods may contain a complete protein. The % total neutral amino acids (% TNAA) of the food samples (Figure 2) were below 50% and could be indication that their proteins maybe charged. The observed values were lower than 59.36, 59.33 and 59.98% reported for raw, boiled, and fermented *P. african* respectively by IGWE et al. (2013). Values of % TNEAA, % TEAA with His or without His, % Cys in TSAA, % TAAA and % TArAA in "akidiagworagwo" were insignificantly ($p > 0.05$) affected when compared to those of "nwagbaraoti". The values of % cyteine for the studied food samples are in line with the statement of ADEYEYE et al. (2007), who noted that protein from plant source contribute substantially more cysteine than methionine in TSAA. The % TBCAA of 18.77% for "akidiagworagwo" and 17% of "nwagbaraoti" could be indication that upto 20% of their amino acid contents could be available for energy production. This is more than required 10% from proteins as noted by WARDLAW and KESSEL (2002). The % TBCAA value of "akidiagworagwo" in the present study is comparable to that of *R. hookeri* but lower than that *E. guineensis* (IBEGBULEM et al. 2012) whereas that of "nwagbaraoti" is lower than those of *R. hookeri* and *E. guineensis* (IBEGBULEM et al. 2012). The values of both % TArAA and % Tyr in TArAA could be indication that over 40% of phenylalanine contents of the studied food samples undergo sparing action by tyrosine. % tyrosine is higher in "akidiagworagwo" than "nwagbaraoti" traditional food. The observed values for % tyrosine in the present study were higher than those of *R. hookeri and E. guineensis* wines (IBEGBULEM et al. 2012).

The ratios of leucine to isoleucine revealed higher leucine contents for the food samples (Figure 7). According to IGWE et al. (2013), metabolic antagonism especially on trypsin and niacin may set in. TBAA to TAAA ratio of less than one for the two food samples could be indication that the foods may contain acid proteins (Figure 7) and their amino acids could also serve as acids at physiological pH (SCHULTZ et al. 2006). The less than one observation of TBAA/TAAA in the present study is in line with the earlier report by IBEGBULEM et al. (2012) on wines of *R. hookeri and E. guineensis*. IBEGBULAM et al. (2012) noted that edible material with TEAA/TAA ratio of more than 50% could support protein synthesis. The observed TEAA/TAA ratios (with or without His) for "akidiagworagwo" and "nwagbaraoti" were lower than 50%, and may not support protein synthesis as noted by IBEGBULAM et al. (2012).

The amino acid score determines the effectiveness with which absorbed dietary nitrogen can meet the indispensable amino acid requirement at safe level of protein intake (FAO/WHO/UNU 2002). In recent times, prediction of protein quality using Protein Digestibility Corrected Amino Acid

Score (PDCAAS) has been established. PDCAAS is a method of evaluating the protein quality based on both the amino acid requirements of humans and their ability to digest it. PDCAAS has a relationship with digestibility and amino acid score (FAO/WHO/UNU 2002). The amino acid scores as presented in Table 1 revealed that leucine, lysine and aromatic amino acids in "akidiagworagwo" surpassed their requirement by 13, 24 and 36% respectively, while aromatic acids in "nwagbaraoti" surpassed its requirement by 19%. Isoleucine, sulphur containing amino acids, threonine, and valine fall short of their requirements in "akidiagworagwo" by 12, 32, and 11% respectively whereas isoleucine, leucine, lysine, aromatic amino acids, threonine, and valine fall short of their requirements in "nwagbaraoti" traditional food by 27, 16, 32, 21, 23, and 25% respectively. Aromatic amino acids for "akidiagworgawo" and lysine for "nwagbaraoti" are the limiting amino acids for having the least provisional scores (AMADI and DURU 2014) in the present study.

Different authors (SHAHIDI 1997, OKAKA and OKAKA 2005, OLUSANYA 2008, DURU et al. 2013ab, AMADI et al. 2013, AMADI et al. 2018) have reported the importance of minerals such as Ca, Mg, Zn, and Fe found in food materials to the body. URGA and NARASIMHA (1998) noted that the nutritional value of diet in terms of macro and micro minerals is dependent on the amount of mineral that is bioavailable for physiological processes in the organism much more than the content in the diet. The ability of dietary phyate and oxalate to complex important minerals such as Ca, Mg, Zn, and Fe and prevent them from being available to the body has been reported (GRIFFITH and THOMAS 1981, ONWUKA 2005, IGWE et al. 2013, GEMEDE et al. 2016). The observed minerals and antinutrients were significantly ($p < 0.05$) affected in "akidiagworagwo" when compared to those of "nwagbaraoti". To predict the bioavailability of minerals such as calcium, iron and zinc; antinutrients to mineral molar ratios were calculated and presented in Table 2. The ratios were lower than their individual critical values signifying mineral elements bioavailability in the studied food samples to the body.

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Conclusion

"Akidiagworagwo" and "nwagbaraoti" traditional foods possessed nine of the ten essential amino acids and eight of the ten non-essential amino acids with less than one TBAA to TAAA ratio, which means that they can serve as acids at physiological pH. They fall short of amino acid scores for isoleucine, threonine, and valine. The antinuteint-mineral molar ratios for the two foods revealed availability of nutrients. This study has shown the amino acid profiles, anutinutrients, concentrations of minerals and antinutrient-mineral molar ratios of "akidiagworagwo" and "nwagbaraoti" traditional foods.

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EFFECT OF PROCESSING ON SELECTED QUALITY PARAMETERS OF OAT BEVERAGES

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Key words: oat grain products, color, viscosity, density, consumer evaluation.

Abstract

This study analyzed the impact of basic raw material processing, including the level of addition of oats and flavoring additives as well as the extraction, homogenization and pasteurization conditions on selected quality parameters of oat beverages produced on a laboratory scale. Ordinary and instant flakes were used as the raw material for the research (whole and ground), along with whole meal from naked oat grain. The results were compared with the standards of oat beverages available on the market. Grinding degree was determined for the raw material. while density, viscosity, color and a consumer evaluation were measured in the beverages. It was found that the raw material grinding degree and extraction conditions had the greatest impact on product quality. Beverages from oat grain and ground oat flakes were characterized by high density and viscosity. Its color showed high L* and b* values. Products with sea salt, sugar and sea buckthorn oleosome were the most acceptable for consumers.

Introduction

Oat grain is the main component of cereal blends. Because this raw material is rich in many nutrients, interest in it has been increasing. The most valuable is naked oat, whose crops are much smaller than those of the husked varieties (PRAŻAK and ROMANOWICZ 2014). However, it is characterized by a high content of protein and fat as well as minerals in the grain, thanks to which it has found application in food processing (ZARZECKA et al. 2018). Oat grain is also added to muesli mixes. Since this raw material is rich in dietary fiber, preparations made of it contain a high

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amount of β-glucans (30%) (GIBIŃSKI et al. 2005). Currently, oat grain is used for the production of flakes, bran, flour, groats, cereal-fruit mixes, malt and oat beverages (JURGA 2011a). Oat grain on a large scale is also used in animal nutrition; over 90% of the world cereal harvest is intended for animal feed (JURGA 2011b). People have known and prepared plant beverages for ages. In various cultures, their consumption has a hundred-year tradition (e.g. coconut "milk", Spanish *Horchata*). These products are now a valuable dietary product for consumers who are allergic, intolerant to milk components or vegans. For the production of vegan milk substitutes, raw materials such as soybeans, hazelnuts, sesame seeds, oat, spelt and rice grains are used (SETHI et al. 2016). The offer of raw materials is constantly increasing to include, e.g. almonds, walnuts, peanuts, cashews, hemp seeds and cereal grains such as barley, amaranthus, millet, buckwheat (MÄKINEN et al. 2016). Functional beverages (including plant beverages) supplied to the human organism in appropriate quantity and quality are a good source of nutrients (including dietary fiber, proteins, minerals, vitamins, essential unsaturated fatty acids) (*Wartość odżywcza…* 2015). The final product composition is determined by the content of the basic raw material (from 3% to 16% depending on its type), the degree of its processing (ground grain, flour, flakes) and the extraction process. On an industrial scale, increased pH, elevated temperature and amylolytic enzymes are used to increase extraction efficiency. The leading countries in the production of plant beverages include the USA, Brazil and South Africa as well as European countries such as Great Britain, Spain, France, Italy, Germany and Belgium (ANONYM 2014, *Usda Food Composition Databases...* 2019).

Cereal beverages can be enriched with various additives (e.g. fiber, vitamins, flavors, oleosomes), which not only improve the flavor and aroma of the product, but also increase its nutritional value. One of these additives is sea buckthorn oleosome. Sea buckthorn (*Hippophae rhamnoides* L.) contains about 6% fat, which is found in the fruit pulp in the form of oleosomal globules. Sea buckthorn lipids are composed of omega-3, -6 and -7 acids. They also contain tocopherols, phytosterols and fat-soluble vitamins (A, D, E and K). Additives such as sea buckthorn oleosome make the product functional and innovative (PIŁAT and ZADERNOWSKI 2016).

The current study assessed the impact of the level of basic raw material processing (the level of its addition and the addition of flavoring additives as well as the extraction, homogenization and pasteurization conditions) on selected quality parameters of oat beverages produced on a laboratory scale.

Materials and Methods

The basic raw material for the production of various oat beverage variants were ordinary and instant flakes (Kupiec, Poland) (used in a whole and ground form) as well as the naked oat grain (*Amant* variety) (used as whole meal). Flakes and naked oat grain were ground in a WZ-1 laboratory mill (Baking Industry Research Department Sp. z o. o., Poland). The reference standards were three commercial oat beverages from foreign producers: Berief (Germany), enerBIO (Germany) and Natur Green (Spain). In the first stage of the experiment, 24 variants of oat beverages without flavor additives were produced on a laboratory scale. The variants differed by the type of raw material, the degree of its grinding, the level of its addition (10–20%) and the temperature and time of extraction with water. Depending on the production method, the variants were divided into four series (Table 1).

Table 1

The parameters of the production process used in series I–IV

Series	Production parameters
I	using water at room temperature; homogenization 7 min. and pasteurization 10 min., 90ºC
II	using water at room temperature and 30 minutes extraction; homogenization 7 min. and pasteurization 10 min., 90ºC
III	using water at 30°C; homogenization 7 min. and pasteurization 10 min., 90ºC
IV	using water at 30°C and 30 minutes extraction; homogenization 7 min. and pasteurization 10 min., 90ºC

Oat beverages were produced in a Thermomix® Vorwerk (Germany). The degree of raw material grinding was determined according to Polish Standard *Przetwory zbożowe…* PN-A-74015. In the obtained oat beverages and the controls, the following parameters were determined:

– density, using a Reischauer glass Pycnometer (according to *Wina i miody…* PN-90/A-79120/03),

– color, by the reflection method using the MiniScan XE Plus Hunter-Lab spectrophotometer in the CIE L* a* b* color system with an attachment for liquid products,

– viscosity, using a Brookfield RV DV-II + Pro Extra oscillation rheometer, connected to a computer with Rheocalc V3.2 Build 47-0 software.

The first consumer evaluation of oat beverages was carried out by a 20-person panel, using a point scale from 0 to 10 (Gawęcka and Jędryka 2001). Following the first stage research, the best quality variant of oat beve-

rage was selected. The second stage of research included the preparation (with the selected variant from the first stage of research) of four types of oat flavored beverages using the additives such as sea salt, sugar, sunflower oil, inulin, stevia and sea buckthorn oleosome. Four oat beverages (flavor variants) and three commercial beverages (standards) were subjected to consumer evaluation by a 20-person panel (GAWĘCKA and JĘDRYKA 2001).

All analyses were performed in triplicate, except for color measurement (n = 6). The results were analyzed statistically using Statistica 12.0 PL software (StatSoft, Kraków, Poland). The differences between the means were determined using analysis of variance (ANOVA) with Duncan's test at a $P < 0.05$ significance level.

Results and Discussion

Determination of grinding degree

The study showed that whole flakes were dominated by 3.15 mm particles, while the ground flakes and flour from naked oat grain were characterized by 1.00 mm particles. Whole meal had thicker granulation because the final product contained all-grain fragments (JURGA 2011b).

Determination of oat beverage density

The results of the measurements of oat beverage density are presented in Table 2. A significant relationship was found between the percentage contents of raw material in a beverage and its density. The beverage density increased with the increase in the raw material content. In addition, higher densities were observed in products using 30-minute extraction. Significant differences were observed between beverages produced from whole and ground oat flakes. Beverages obtained from ordinary and instant ground flakes showed a higher density. This means that both the amount of the additive, the degree of its grinding and the method of production have a significant impact on the density of the final product. In addition, products made of raw material ground to a higher degree have a greater ability to absorb water (PIESIEWICZ and ZIELIŃSKI 2006). The commercial oat beverage densities were 1.028, 1.026 and 1.029 g cm^{-3}, respectively for Berief, enerBio and Natur Green. These products contained approximately 11% of an oat additive. Similar values were found for the beverages from whole ordinary flakes with the addition of 14% of raw material from series II and IV and with an 18% addition from series I, and for beverages from whole ground flakes (14% addition – series I, III and 12% addition – series II and IV).

Table 2

Determination of oat beverage density [g cm^{-3}]

Beverage	Raw material addition level [%]											
	10				12				14			
	Series											
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Whole oat flakes	1.005^{dB}	1.011^{dB}	1.006^{dB}	1.019^{cdA}	1.011^{dB}	1.016^{dB}	1.018^{dB}	1.023^{cB}	1.020^{cC}	1.026^{cB}	1.021^{bB}	1.029^{bBC}
Ground oat flakes	1.020^{cdA}	1.020^{cdA}	1.019^{dB}	1.016^{dAB}	1.023^{cA}	1.027^{cA}	1.023^{cA}	1.026^{cA}	1.026^{cB}	1.030^{bA}	1.029^{bA}	1.033^{bB}
Whole instant oat flakes	1.009^{aB}	1.011^{cB}	1.007^{cB}	1.011^{cB}	1.011^{cB}	$1.015b^{cB}$	1.008^{cC}	$1.015b^{cC}$	1.012^{cD}	1.016^{bcC}	1.013^{cA}	1.015^{bcC}
Ground instant oat flakes	1.020^{bA}	1.022^{aA}	1.019^{aA}	1.024^{aA}	1.024^{aA}	1.027^{bA}	1.025^{bA}	1.027^{aA}	1.031^{bA}	1.031^{bA}	1.031^{cA}	1.037^{bA}
Whole meal from naked oat grain	1.010^{cB}	1.016^{bcB}	1.014^{cA}	1.020^{bA}	1.006^{cB}	1.018^{bB}	1.019^{bB}	1.023^{bB}	1.008^{cD}	1.022^{bB}	1.022^{bB}	1.029^{bB}

Beverage	Raw material addition level [%]											
	16				18				20			
	Series											
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Whole oat flakes	1.023^{cB}	1.031^{bB}	1.024^{cB}	1.034^{bB}	1.028^{bB}	1.040^{acB}	1.032^{cB}	1.043^{aB}	1.031^{bC}	1.045^{abB}	1.038^{bcB}	1.050^{aA}
Ground oat flakes	1.035^{bA}	1.040^{abA}	1.036^{bA}	1.041^{aA}	1.038^{bA}	1.045^{aA}	1.043^{aA}	1.047^{aA}	1.050^{aA}	1.049^{aA}	1.049^{aA}	1.054^{aA}
Whole instant oat flakes	1.014^{bcC}	1.021^{bC}	1.017^{bC}	1.020^{bC}	1.017^{bC}	1.028^{aC}	1.018^{bC}	1.026^{aC}	1.020^{bD}	1.034^{aC}	1.022^{aC}	1.035^{aC}
Ground instant oat flakes	1.035^{bA}	1.040^{cA}	1.041^{dA}	1.041^{bA}	1.041^{cA}	1.047^{dA}	1.047^{eA}	1.048^{cA}	1.043^{eB}	1.053^{eA}	1.050^{fA}	1.054^{cA}
Whole meal from naked oat grain	1.009^{cD}	1.028^{bB}	1.025^{bB}	1.033^{bB}	1.012^{cC}	1.036^{abB}	1.032^{dB}	1.040^{aB}	1.016^{bcD}	1.042^{aB}	1.038^{aB}	1.046^{aB}

a, *b*, *c*, … mean values in the rows marked with letter indexes differ significantly at $p < 0.05$.
A, *B*, *C*, … average values in columns marked with letter indexes differ significantly at $p < 0.05$.

Table 3

Determination of oat beverage viscosity [cP]

Beverage	Raw material addition level [%]											
	10				12				14			
	Series											
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Whole oat flakes	19.7^{eB}	20.4^{eB}	21.3^{eB}	30.4^{dB}	20.8^{eB}	24.7^{eB}	24.8^{eC}	33.5^{deB}	25.4eB	37.5^{dC}	29.2^{dA}	49.5^{bB}
Ground oat flakes	26.4^{fA}	33.6^{efA}	25.7^{fA}	37.0^{eA}	31.9^{fA}	43.1^{eA}	35.3^{eA}	44.4^{eA}	35.5eA	57.0^{dA}	50.1^{dA}	56.1^{dA}
Whole instant oat flakes	16.9^{dC}	17.3^{dC}	16.3^{dD}	19.6^{cC}	17.6^{dC}	19.8^{cC}	16.6^{aD}	20.9^{bC}	17.7dC	19.5^{cD}	18.6^{cdC}	20.1^{dC}
Ground instant oat flakes	26.3^{gA}	31.3^{fA}	25.9^{gA}	32.2^{fB}	30.7^{fA}	41.9^{fA}	31.6^{fB}	38.1^{fB}	36.6fA	47.8^{eB}	42.1^{fF}	56.6^{eA}
Whole meal from naked oat grain	17.2^{cC}	18.8bC	18.1^{bC}	19.8^{bC}	$^{16.9}cC$	18.6^{bC}	19.2^{bD}	20.0^{abC}	16.3bC	19.7^{bD}	19.9^{bC}	21.9^{aC}

Beverage	Raw material addition level [%]											
	16				18				20			
	Series											
	I	II	III	IV	I	II	III	IV	I	II	III	IV
Whole oat flakes	28.7^{dB}	42.9^{bC}	30.7^{bC}	57.8^{cC}	34.7^{dB}	69.1^{cC}	44.3^{dC}	83.6^{bB}	37.3^{dB}	82.8^{bC}	54.7^{cC}	99.3^{aB}
Ground oat flakes	43.9^{cA}	81.6^{cA}	65.4^{dA}	80.5^{cA}	51.3^{dA}	98.8^{cA}	81.8^{cA}	118.1^{bA}	91.1^{cA}	118.2^{bB}	89.0^{cA}	146.8^{aA}
Whole instant oat flakes	19.3^{dC}	21.5^{dD}	20.2^{dD}	24.6^{cD}	18.3^{dC}	30.3^{bD}	19.7^{cD}	27.5^{dC}	20.4^{cC}	32.5^{bD}	21.5^{cD}	41.8^{aC}
Ground instant oat flakes	44.0^{efA}	67.9^{dB}	54.2^{eB}	70.4^{dB}	65.7^{dA}	79.4^{cB}	63.4^{dB}	108.4^{bA}	84.0^{cA}	147.4^{aA}	68.1^{dB}	153.4^{aA}
Whole meal from naked oat grain	17.6^{bcC}	21.2^{aD}	21.0^{aD}	24.2^{aD}	17.8^{cC}	23.7^{aD}	22.7^{aD}	26.3^{aC}	18.7^{bC}	25.6^{aD}	24.6^{aD}	29.3^{aD}

a, *b*, c, … mean values in the rows marked with letter indexes differ significantly at $p < 0.05$
A, *B*, *C*, … average values in columns marked with letter indexes differ significantly at $p < 0.05$

Determination of oat beverage viscosity

The viscosity is mainly influenced by the temperature and degree of material grinding and concentration (ZARZYCKI et al. 2011). An analysis of the viscosity measurements presented in Table 3 indicate that (similar to the parameters of density measurement) viscosity also increased with the contents of the raw material added. Fiber is partly responsible for the viscosity in oat beverages, as it has the ability to form viscous solutions (ZARZYCKI et al. 2011). Significantly higher viscosities were determined in beverages made from ground raw materials and previously subjected to 30-minute extraction. Oat grain and its products contain a significant amount of β-glucans, which are mostly responsible for high product viscosity (JURGA 2012). β-glucans and pentosans absorb water during dissolution, creating a dense substance (WACHOWIAK and KIRYLUK 2006). It was found that the degree of extraction of β-glucans is faster in fractions with small particles compared to the fraction with larger particles (WACHOWIAK and KIRYLUK 2005). Commercial beverages were characterized by the following viscosity results: Berief – 20.7 cP, enerBio – 18.6 cP, Natur Green – 22.3 cP. Beverages from ground flakes (10–12% raw material additive) from all production series had the closest viscosity to beverages available on the market.

Measuring oat beverage color

Selected color parameters of the tested oat beverages are presented in Table 3. The most significant results of the determination have been summarized. It was noted that the highest values of the L* parameter (which indicates that brightest color) in all variants were characteristic of products from series IV. An increase in the value of the L* parameter was noted with the increase in the percentage of raw material in the beverages. It was observed that the products from the ground raw material have a more favorable color (higher L* parameters), which significantly affects its attractiveness and acceptability by consumers. The color of beverages made from cereal raw materials is creamy-beige, which is lighter than beverages produced from raw materials such as nuts (HOFFMANN and KOSTYRA 2015).

According to research, color often depends on the content of dry matter in dairy products, which can be related to cereal beverages being a substitute for milk. An increase in the value of the L* parameter and a higher yellowness (parameter b*) of dairy products containing a larger amount of fat was also noted (*Współczesne trendy*… 2016). Oat grain is characterized by a high fat content (JURGA 2012) and it was found that fat contained in this oat grain was responsible for high b* values (Table 4).

Table 4

Selected parameters of color measurement in oat beverages

Beverage	Raw material addition level [%]	Series	Coefficient [-]		
			L*	a*	b*
Whole oat flakes	10	IV	79.41^{BC}	0.21^{C}	11.18^{C}
	12	IV	78.61^{C}	0.27^{F}	11.87^{BC}
	14	II	80.62^{B}	0.20^{F}	12.15^{B}
	16	II	81.77^{AB}	0.40^{E}	12.82^{AB}
	18	II	82.37^{A}	0.67^{C}	13.37^{A}
	20	II	81.48^{B}	0.81^{B}	13.52^{A}
Ground oat flakes	10	III	82.06^{B}	0.19^{F}	12.53^{A}
	12	III	83.53^{A}	0.30^{D}	13.06^{A}
	14	IV	83.64^{A}	0.62^{CD}	13.55^{A}
	16	IV	84.10^{A}	0.82^{B}	13.94^{A}
	18	IV	83.13^{A}	1.03^{A}	14.07^{A}
	20	III	84.28^{B}	0.95^{AB}	14.17^{A}
Whole instant flakes	10	II	76.82^{C}	0.76^{C}	8.37^{D}
	12	II	76.64^{C}	0.13^{F}	9.60^{D}
	14	IV	77.36^{C}	0.48^{DE}	10.36^{C}
	16	IV	78.63^{C}	0.13^{F}	11.39^{C}
	18	IV	80.57^{B}	0.31^{EF}	12.15^{B}
	20	IV	81.74^{B}	0.71^{C}	12.73^{B}
Ground instant flakes	10	I	82.03^{B}	0.13^{F}	11.96^{B}
	12	II	83.87^{A}	0.35^{E}	12.46^{B}
	14	III	82.77^{A}	0.44^{E}	12.83^{A}
	16	IV	82.76^{A}	0.71^{C}	13.28^{A}
	18	IV	83.37^{A}	1.03^{A}	13.88^{F}
	20	IV	84.07^{A}	1.21^{A}	14.04^{A}
Whole meal from naked oat grain	10	II	80.85^{B}	0.24^{B}	11.90^{B}
	12	IV	82.33^{B}	0.54^{D}	12.62^{B}
	14	IV	81.37^{B}	0.67^{C}	13.09^{A}
	16	II	82.42^{B}	0.69^{C}	13.04^{F}
	18	II	81.01^{B}	0.88^{B}	13.80^{A}
	20	III	80.67^{B}	0.93^{B}	13.49^{A}

A, *B*, *C*, … mean values in columns marked with letter indexes differ significantly at $p < 0.05$.

Consumer evaluation of base oat beverages

According to the evaluators, the highest score for flavor and aroma was awarded to the variant with a 14% addition of instant flakes (ground) from series II (using water at room temperature and 30 minutes extraction). The highest score for the color was granted to the oat beverages obtained from ground instant flakes. Oat beverages were lighter in color than beverages made from nuts, which is why they are more appreciated by consumers (HOFFMAN and KOSTYRA 2015). The consistency of oat beverages with 18% and 20% addition of the raw material best suited the evaluators. Oat beverages produced according to series II (water at room temperature and 30-minute extraction) had the highest scores. The best score was awarded to the variant with a 14% addition of the base material (ground instant flakes). The characteristic bitterness and mealy taste was noticeable in beverages made from the whole meal of naked oat. Mealy taste is characteristic of non-fermented cereal beverages made from grain (KITABATAKE et al. 2009). Among the commercial beverages, Berief (all distinguishing features) was awarded the highest score, while Natur Green the lowest.

For the production of beverages with flavoring additives, variant II was used (with 14% addition of the raw material) because it received the highest score for flavor, color and aroma.

Consumer evaluation of oat beverages with flavoring additives

The results of consumer evaluation (beverages with flavoring additives) are presented in Table 5. The lowest rated for the flavor and color was the base beverage (water + 14% of ground instant flakes). Among the commercial beverages, the highest scores were obtained by Berief and enerBio.

Table 5

Consumer evaluation of oat beverages with flavoring additives

Differentiator	Basic beverage (II series. 14% addition of raw material)	Flavoured beverages				Comercial beverages		
		1	*2*	*3*	*4*	berief	ener BIO	Natur Green
Flavor	3.7±0.5	7.2±1.7	7.1±0.5	7.0±2.0	5.8±3.1	7.4±0.9	7.4±1.5	5.6±1.1
Color	6.6±0.4	7.5±1.8	7.3±1.1	8.1±1.9	7.1±1.2	7.5±1.1	7.4±1.6	6.7±1.7
Aroma	6.5±1.1	7.4±1.9	7.8±2.5	7.2±2.4	6.4±1.7	7.2±1.3	7.0±2.1	6.4±1.5
Consistency	6.8±1.6	7.5±1.7	7.1±1.6	7.4±2.1	7.1±2.0	7.8±1.9	7.3±2.2	6.7±1.1

1 – sea salt + sugar; *2* – sea salt + sugar + sunflower oil; *3* – sea salt + sugar + oleosome; *4* – sea salt + stevia + inulin + sunflower oil

Similar scores were awarded to the following beverages: with the addition of sea salt and sugar, with sea salt, sugar and sunflower oil, as well as with sea salt, sugar and oleosome. According to the evaluators, a beverage with the addition of sea salt, sugar and oleosome had the most preferred color. Intensive flavor was characteristic of a beverage with an addition of sea salt, sugar and sunflower oil. The flavor of the variant with sea salt, sugar and oleosome appeared to be the most attractive to the evaluator. However, a beverage with sea salt, stevia, inulin and sunflower oil received the lowest scores for this parameter, along with Natur Green, representing the commercial beverages. Stevia may have a slightly bitter aftertaste, which is why it does not suit all consumers (GOYAL et al. 2010). Berief and enerBio beverages and products with the addition of sea salt and sugar and sea salt, sugar and oleosome had the best consistency, according to the evaluators. The main flavor addition to this type of products is mainly sugar, but fruit extracts and vanilla or cinnamon are also used (RIOS et al. 2017).

Conclusions

The production method has a significant impact on the composition and appearance of the final product. The grinding degree of the raw material and the extraction time are very important technological factors. Higher density and viscosity were observed in beverages from series II and IV (with 30 minutes extraction). The beverages obtained from the ground raw material were characterized by the highest densities and the highest brightness (L* parameter). Beverages obtained from whole meal (from naked oat grain) had a noticeable, characteristic bitterness. The beverage obtained with 14% ground instant flakes, using extraction with water at room temperature for 30 minutes, was characterized as the best quality product. Based on the consumer evaluation, it was found that the quality parameters most similar to the best commercial oat beverage (Berief) were characteristic of the products made with the addition of sea salt, sugar and sunflower oil and with the addition of sea salt, sugar and oleosome.

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THE EFFECT OF STOCKING DENSITY ON THE SURVIVAL AND GROWTH OF SILVER RASBORA (*RASBORA ARGYROTAENIA*) LARVAE

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Key words: biomass gain, length gain, larviculture, new aquaculture species.

Abstract

The purpose of this study was to assess the effect of different stocking densities on the survival and growth of silver rasbora (*Rasbora argyrotaenia*) larvae as new aquaculture species. A total 3,000 larvae aged 2 days after hatching (mean body weight = 1.08 ± 0.06 mg; mean total length = 3.14 ± 0.17 mm) were assigned into five stocking density treatments (*A* – 10, *B* – 20, *C* – 30, *D* – 40, *E* – 50 individuals L^{-1}; four replicates per treatment) and reared for 21 days. The stocking density affected both survival and growth of silver rasbora larvae ($P < 0.05$). The highest survival rate and individual growth occurred at 10 fish L^{-1} treatment. Based on the highest biomass gain, the optimum stocking density treatment was 20 fish L^{-1}. We suggest using 20 fish L^{-1} stocking density in silver rasbora larviculture because estimated more efficient and profitable economically.

Introduction

Silver rasbora (*Rasbora argyrotaenia*) is a tropical freshwater bentopelagic fish that can be found in the Asian region including Mekong, Chao Phraya and Mae Khlong basins, Malay Peninsula to Borneo, Java and Sumatra in Indonesia; occurs mainly in rivers and enters flooded fields (CAPULI and BAILLY 2019). Silver rasbora is a newly cultivated species that have economic values as consumption and ornamental fish in Indonesia and surrounding countries (ADAWIYAH et al. 2019, HERAWATI et al. 2017). The captive production of this fish is limited, so the fulfillments of

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the demands are mainly depending on wild capture (ROSADI et al. 2014). On the other hand, the silver rasbora farming is still developing, remains largely incomplete, and requires a lot of basic information about its cultivation especially for the young life-stages to increase the production to overcome over exploitation on wild.

The fry availability is a limiting factor for fish farmers and affects the reliability of silver rasbora farming and other fish also. Thus, during the larval phase, increasing survival and growth must be one of the research priorities for many species (SLATER et al. 2018). Nevertheless, to the best of our knowledge, there is no literature on the rearing of silver rasbora larvae under the intensive conditions. At present, larvae production is usually under suboptimal conditions. For this reason, commonly silver rasbora juveniles are produced in low quantity and quality.

Rearing practices, feeding strategies, and environmental conditions affect the success of larval production (COWAN al. 2000, KESTEMONT et al. 2003). Stocking density of larvae is known to influence larval rearing performance. Stocking density effects in young stages have been studied in many species of freshwater aquaculture (GOMES et al. 2000, SAHOO et al. 2004, SZKUDLAREK and ZAKĘŚ 2007). However, the stocking density effects on survival and growth may vary (MERINO et al. 2007, NIAZIE et al. 2013), depending on conditions of rearing, fish age, and species (SAOUD et al. 2008). In that sense, some researches have shown that survival rate and growth are negatively influenced by stocking density increase (EL-SAYED 2002, KEER et al. 2018, SAHOO et al. 2004); other studies have found no effects of stocking density on survival or growth rate (KAISER et al. 1995, NIAZIE et al. 2013) while some of them found that low stocking density negatively affects growth (MILLAN-CUBILLO et al. 2016). These statements show that more detailed characterization of the effects of stocking density on survival and growth in silver rasbora larvae is crucial. The aim of this study was to assess the effect of different stocking densities on the survival and growth of silver rasbora larvae.

Materials and Methods

This research was conducted from January to March 2019 in the aquaculture facilities and laboratory at the Airlangga University, Banyuwangi campus (East Java, Indonesia). The study was conducted in compliance with the Law of the Republic of Indonesia Number 18 of 2002 concerning the National System of Research, Development and Application of Science and Technology under the oversight and approved by the

Faculty of Marine Science Universitas Airlangga (based on the letter of assignment from the Dean of Faculty of Marine Science Universitas Airlangga, 1751/UN3.1.16/PPd/2018).

Origin of larvae

Silver rasbora brood fish (approximately 1 year old) were obtained from Technical Implementation of Development Unit of Freshwater Aquaculture of Umbulan (Pasuruan, East Java, Indonesia). A total of two females (5.20 ± 0.14 g body weight and 8.60 ± 0.56 cm total length; average ± SD) and 6 males (4.62 ± 0.07 g of body weight and 7.50 ± 0.40 cm of total length; average ± SD) were mated and spawned naturally using palm tree fibers as spawning substrate in a glass aquarium (40 x 50 x 50 cm^3). The fecundity, fertilization rate, and hatching rate observed were 439.20 ± 22.89 egg per g fish body weight, 94.40 ± 1.80%, and 93.30 ± 0.41%, respectively. After spawning, the brood fish were removed from the aquarium and the eggs attached to the substrate left to hatch for around 2 days. The water quality parameters that were measured during the incubation (2 times per day at 07.00 AM and 04.00 PM) can be seen in Table 1.

Table 1

The water quality parameters measured during the incubation of silver rasbora egg

Parameters	Mean ± SD	Range
Dissolved Oxygen [ppm]	6.82 ± 0.38	6.2–7.5
Temperature [ºC]	24.94 ± 0.60	24–27
pH	7.24 ± 0.32	6.9–7.5
Total ammonia nitrogen [ppm]	0.012 ± 0.001	0.010–0.013

Experimental design and rearing

The experiment was conducted semi outdoor (in a room that is not fully enclosed, some parts of the room roofless), no photoperiods manipulation in fish rearing was set. A total of 3,000 larvae aged 4 days post fertilization (DPF) were taken and measured (n = 100, 1.08 ± 0.06 mg body weight, 3.14 ± 0.17 mm total length; average ± SD) and then assigned to 20 cylinder plastic tank (10 L capacity; 30 cm diameter) with 5 L volume of water with gentle aeration to maintain dissolved oxygen level. A total of 50% water was exchanged every 3 days to maintain the water quality at 09.30 AM. Water for rearing was obtained from the well and was pumped to the reservoir prior to supply to the rearing containers. The water quality in each tank was measured in the morning at 09.00 AM before water exchange and is presented in Table 2.

Table 2
Water quality parameters of silver rasbora larvae rearing at different stocking densities (*A*–*E*; see Table 3)

Paramaters	*A*	*B*	*C*	*D*	*E*
Dissolved oxygen [ppm]	6.49 ± 0.36	6.52 ± 0.38	6.56 ± 0.39	6.56 ± 0.36	6.44 ± 0.40
pH	7.46 ± 0.30	7.37 ± 0.34	7.41 ± 0.36	7.44 ± 0.34	7.33 ± 0.33
Total ammonia nitrogen [ppm]	0.013 ± 0.005	0.013 ± 0.006	0.019 ± 0.004	0.018 ± 0.005	0.013 ± 0.004

The 21-day experiment was started at 4 days post fertilization (DPF further on), a few days after mouth opening and when ability of larvae to feed on artemia nauplii was confirmed. To observe effects of stocking density on the survival and growth, larvae were reared at five different stocking densities as summarized in Table 3. A completely randomized design with four replicates was conducted in the experiment. The stocking density treatments were based on Indonesian National Standards (SNI 7733:2011) on stocking densities of goldfish larvae (*Carassius auratus*). A total 20 artemia nauplius per day per fish larvae was given as feed with the frequency of feeding 4 times a day which is 06.00 AM, 12.00 AM, 05.00 PM, and 09.00 pm.

Table 3
Stocking densities of silver rasbora larvae in five experimental treatments

Experimental treatment	Stocking density [lavae L^{-1}]	Total number of larvae per tank
A	10	50
B	20	100
C	30	150
D	40	200
E	50	250

Observation and measurements of larvae

Larvae sampling in experimental treatments was done at 4 DPF, 11 DPF, 18 DPF, and 25 DPF. At least 10% of the total number of larvae at each stocking density (n = 5–25) was selected as the size of the sample and frequency of sampling while considering the time required and technical limitations of sampling procedure as well as larvae handling caused stress. Sampled larvae were anesthetized with rapid cooling anesthetic method using ice slush exposure at 0°C (CHEN et al. 2013) and total body length [TL, mm] of larvae was measured with a micrometer under a stereo-microscope (based on fish size and magnification, precision varying

from 0.05 to 0.1 mm). A digital scale with a precision of 0.1 mg used for body weight [BW, mg] measuring. Fish were returned to their respective tank after individual measurements. After sampling, no mortality was observed. Mortalities were observed in the end of treatment at 25 DPF by counting the number of initial larvae reduced by surviving larvae.

Observed parameters

The effects of stocking density on growth and survival were determined by calculating the following parameters.

Survival rates (SR), expressed as a percentage, and was calculated based on formula:

$$SR\ [\%] = [(N_f / N_i) \cdot 100]$$

where:

N_i and N_f – the initial and the final number of larvae, respectively.

Fish biomass gain per liter [BG, g L^{-1}] or was calculated following the formula:

$$BG = [(N_f\, BW_f - N_i\, BW_i) / V]$$

where:

N_i and N_f – the initial number and the final number of larvae

BW_i and BW_f – the initial average body weight [mg] and the final average body weight [mg]

V – the tank volume in L.

Length gain [LG, mm] was calculated based on formula:

$$LG = TL_f - TL_i$$

where:

TL_i and TL_f – initial and final average total length [mm].

The specific growth in body weight [SGR_{BW}, % per day] was calculated following formula:

$$SGR_{BW} = [(\ln BW_f - \ln BW_i) / D) \cdot 100]$$

where:

BW_i and BW_f – the initial and final body weights of fish

D – the experiment duration in days.

The specific growth in total length [SGR_{TL}, % per day] was calculated using the same approach, as $SGR_{TL} = [(\ln LT_f - \ln LT_i) /21] \cdot 100$

where:

LT_i and LT_f – the initial and final body lengths of fish.

Data analysis

Data were analyzed statistically by ANOVA test with 95% confidence level (the each analyzed variable data confirmed normal distribution and homogeneity of variances) and continued with Duncan Multiple Range Test (DMRT) using SPSS 17.0 software.

Results

The growth and survival of silver rasbora larvae reared at five different densities is showed in Table 4. Based on the results, the stocking density has significant effect ($P < 0.05$) on all parameters observed.

Table 4
Effects of stocking density (*A*–*E*; see Table 3) on survival and growth of silver rasbora larvae from 4 to 25 days post fertilization

Parameters*	*A*	*B*	*C*	*D*	*E*
SR [%]	97.08 ± 3.44^{a}	86.04 ± 7.18^{b}	87.22 ± 8.30^{ab}	75.63 ± 15.63^{b}	87.00 ± 2.48^{b}
BW_i [mg]	1.08 ± 0.06	1.08 ± 0.06	1.08 ± 0.06	1.08 ± 0.06	1.08 ± 0.06
BW_f [mg]	31.45 ± 1.21^{a}	28.69 ± 0.95^{b}	19.13 ± 0.63^{c}	13.56 ± 0.75^{d}	10.97 ± 0.32^{e}
BG [g L^{-1}]	0.29 ± 0.02^{b}	0.47 ± 0.05^{a}	0.47 ± 0.05^{a}	0.37 ± 0.09^{a}	0.43 ± 0.02^{a}
TL_i [mm]	3.14 ± 0.17	3.14 ± 0.17	3.14 ± 0.17	3.14 ± 0.17	3.14 ± 0.17
TL_f [mm]	15.44 ± 0.53^{a}	13.37 ± 0.24^{b}	12.80 ± 0.21^{c}	11.75 ± 0.36^{d}	10.48 ± 0.29^{e}
LG [mm]	11.92 ± 0.42^{a}	9.99 ± 0.37^{b}	9.40 ± 0.32^{b}	8.33 ± 0.46^{c}	7.16 ± 0.37^{d}
SGR_{BW} [% day^{-1}]	15.87 ± 0.32^{a}	15.44 ± 0.21^{a}	13.70 ± 0.14^{b}	12.21 ± 0.27^{c}	11.23 ± 0.09^{d}
SGR_{TL} [% day^{-1}]	7.04 ± 0.30^{a}	6.56 ± 0.29^{ab}	6.31 ± 0.27^{bc}	5.87 ± 0.33^{cd}	5.47 ± 0.27^{d}

*SR – survival rate ($n = 4$); BW_i – initial body weight ($n = 100$); BW_f – final body weight ($n = 5$–25); BG – biomass gain ($n = 4$); TL_i – initial total length ($n = 100$); TL_f – final total length ($n = 5$–25); LG – length gain ($n = 4$); SGR_{BW} – specific growth rate for body weight ($n = 4$); SGR_{TL} – specific growth rate for total length ($n = 4$). Values are means ± SD. Superscript letters denote significant differences ($P < 0.05$) between treatments

Based on the data overall, the survival and growth of silver rasbora larvae decreased with stocking density increasing, where the lowest survival and growth was observed on the highest density of the larval rearing, except in biomass gain (BG) (see Table 4 and Figure 1) Biomass gain in lowest stocking density treatment was significantly lower than in all other treatments (Table 4).

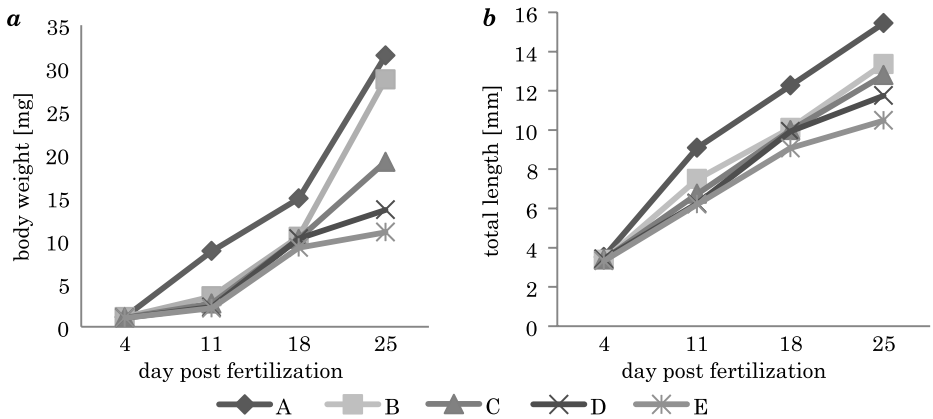

Fig. 1. Body weight (*a*) and total length (*b*) of silver rasbora larvae (n = 5–25) at 4, 11 and 25 days post fertilization in the five stocking density treatments (*A*–*E*; see Table 3)

Discussion

Overall in the present study, stocking density increase had negative effects on the individual fish growth of silver rasbora larvae at the end of study. In contrast, the yield of fish production, shown in current study as fish biomass gain (BG), tended to increase with higher stocking densities. These results are similar to previous study on pikeperch (*Sander lucioperca*) (Szkudlarek and Zakęś 2007) and giant gourami (*Osphronemus goramy*) (Arifin et al. 2019), where high stocking densities led to lower growth but positively influenced biomass gain. On the other hand, stocking density had no effects on growth of African catfish (*Clarias gariepinus*) (Kaiser et al. 1995) and goldfish (*Carasius auratus*) (Niazie et al. 2013); and conversely in meagre (*Argyrosomus regius*) (Millán-Cubillo et al. 2016), increasing stocking density positively effects on growth. The results in other studies indicated that the effects of stocking density on the fish larvae growth are different.

Based on the present study, we found that the stocking density had an effect on the survival of larvae although no cannibalism and no aggressive behavior was observed throughout the experiment and also no difference in water quality in each treatment (Table 2). The highest survival rate was obtained on the lowest stocking density treatment. Survival rate tended to decrease in other treatments, meaning that mortality increases with increasing stocking density above 10 fish L^{-1}. Lower survival rates also observed in Reba carp (*Cirrhinus reba*) fry at high stocking densities were related to stronger food and space competition and also increased stress (Keer et al. 2018). The relationship between stocking density and stress in relation to

mortality in silver rasbora needs to be investigated further in order to find clear regulations in this regard.

The highest survival rate occurred on the lowest stocking density treatment, mortality increased with increasing stocking density above 10 fish L^{-1}, and stocking density increase had negative effects on the growth of silver rasbora larvae ($P < 0.05$). Although the higher stocking density led to the lower growth and survival, the increase of biomass gain in higher stocking density is considered to determine the optimum density for production efficiency. At 20 fish L^{-1} stocking density treatments obtained the biomass gain did not differ to other treatment; however the individual growth parameters were higher compared to the higher stocking densities. It showed that using of 20 fish L^{-1} stocking density in silver rasbora larviculture estimated more efficient and profitable economically. So we recommend using 20 fish L^{-1} stocking density for application on the silver rasbora hatchery.

Conclusions

The stocking density affected survival and growth of silver rasbora larvae ($P < 0.05$). The highest survival rate and individual growth occurred at 10 fish L^{-1} treatment. However, considering the highest biomass gain, the optimum stocking density treatment was 20 fish L^{-1}.

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ALGERIAN POMEGRANATE PEEL DECREASES LEAD CONCENTRATION IN BRAIN AND IMPROVES NEUROLOGICAL DISORDERS

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Key words: pomegranate peel, polyphenols, neurotoxicity, lead, histopathology.

Abstract

The purpose of this study was to determine the neuroprotective potential of pomegranate peel methanolic extract (500 mg kg^{-1}) on lead acetate (1000 ppm) induced neurotoxicity. After 12 weeks, the mice were subjected to behavioral tests. Brain injuries were determined with hematoxylin and eosin staining and lead accumulation was measured by graphite furnace atomic absorption with Zeeman correction. Lead exposure induced neurobehavioral alterations, reduced body growth, lead deposits in brain and histological change in the lead-treated group. Furthermore, co-administration of pomegranate extract with lead decreased locomotion, anxiety and depression in lead-exposed mice as indicated by the number of cells crossed by mice, the residence time in the dark compartment and the immobility time in forced swimming. Also, pomegranate extract improved weight loss and histological change of cortex cerebral and hippocampus by reducing the lead concentration in these sites. Pomegranate methanolic extract has neuroprotective effects against lead-induced neurological disorders probably by its various phytochemical components.

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Introduction

A widely known environmental toxicant, lead (Pb), adversely affects the majority organ systems and extremely impaired fetal brain development (MOUSA et al. 2015). SALEH et al. 2018) found that lead produces hazardous toxic effects including cerebellar damage especially in fetus and female pregnant rats. According to GURER et al. (2000) lead induces oxidative stress, reduces the antioxidative defense system, interfere with certain essential metals which are required for antioxidant enzyme activities, and/or modifying the integrity of the membrane and the fatty acid composition by increasing the cell sensibility to reactive oxygen species.

The beneficial role of antioxidant nutrients within the prevention of lead toxicity has investigated by several researchers however the mechanisms of antioxidant nutrients to restore effective via rebalancing the impaired prooxidant/antioxidant ratio are not completely clear (HSU and GUO 2002). Polyphenols are the most abundant antioxidants found fruits, vegetables, beverages, plants and some herbs. These natural extracts are able to protect neuronal cells through different biological processes like prevented apoptotic neural death, ROS scavenging, regulate of the kinase signal cascade and modulation of Ubiquitin-Proteasome pathway (CAMPOS-ESPARZA and TORRES-RAMOS 2010). Pomegranate is a high potential antioxidant and has several benefits health; antiatherogenic, antihypertensive, and anti-inflammatory properties which leads it's used in the prevention and treatment of diverse diseases. This fruit is rich with several classes of chemical components such as: flavonoids, anthocyanins, punicic acid, ellagitannins, alkaloids, some of carbohydrate, simple organic acids, and other components (ZARFESHANY et al. 2014).

In this regard, the current study is designed experimentally in female mice in order to investigate the beneficial role of pomegranate extract in the protection from brain damage that produced by administration of 1000 ppm lead for 12 weeks.

Materials and Methods

Extract preparation

Punica granatum were collected in the western region of Algeria. They were air-dried for one month and ground into fine powder by using a blender. The powder was kept in airtight container until use. 200 g of the dried powder was soaked in 2 L of methanol and stirred for 24 h using the method of DIALLO et al. (2004) after using 2 L of petroleum ether to remove

the chlorophyll. The operation was repeated on three successive days for the two solvents. The extract obtained after elimination of solvent with the aid of rotary evaporator was stored until at -20°C until used.

Polyphenols

The determination of the total polyphenols in the methanolic extract of *Punica granatum* peel was determined according to the Folin-Ciocalteu method (RAAFAT et al. 2014, MAVI et al. 2004). The concentration of the total polyphenols was deduced from a calibration range established with gallic acid (0–1 mg mL^{-1}). The results are expressed in mg of gallic acid equivalent/g of extract (mg EAG g^{-1} of dry weight).

The total flavonoid content in the methanolic extract from *Punica granatum* peel was determined using a method based on the formation of a flavonoid-aluminum complex having the maximum absorbance at 430 nm (HMID et al. 2017, LAMAISON and CARNAT 1990). Concentrations of flavonoids were deduced from the calibration range established with quercetin (0–40 mg mL^{-1}) and the flavonoid content was expressed in mg of quercetin equivalent per g of extract (mg EQ g^{-1} of dry weight).

The total tannin content was determined by the methods of POLSHETTIWAR et al. (2007). Tannin concentrations are deduced from the calibration range established with tannic acid (0–40 μg mL^{-1}) and the tannin content was expressed in mg of equivalent tannic acid per g of extract (mg ET g^{-1} of dry weight).

The determination of tannins condensed by the vanillin test was carried out according to the method of JULKUNEN-TITTO et al. (1985). The concentration of condensed tannins (proanthocyanidins) is deduced from the calibration range established with catechin (0–0.4 mg mL^{-1}) and is expressed in milligrams of catechin equivalent per gram of extract (mg ECat g^{-1} of dry weight).

Experimental animals

Twenty one healthy mice weighing from 20±2.52 g were taken from Pasteur Institute of Algiers. The animals were maintained room temperature 24 ± 5°C and standard 12 h day/night cycle. The animals were fed on a standard diet and fresh drinking water. The experimental protocol is in accordance to the Guide for the Care and Use of Laboratory Animals (8-th edition, 2011) and approved by the scientific committee of the university. Mice are divided in 3 groups, each group contains 7 mice. Group 1: control mice (*C*), animals receiving drinking water for 90 days. Group 2 (Pb): a dose of 1000 ppm of lead acetate was administered to mice orally for

12 weeks (DJEBLI et al. 2005). Group 3 (Pb-E): mice were treated with 500 mg kg^{-1} of methanol extract of *Punica granatum* peel for 4 h/day followed by acetate of lead at a dose of 1000 ppm orally for 20 h/day for 90 days. Weight gain and water intake were measured daily and weekly for 12 weeks.

Behavioral tests

The locomotor activity of mice was assessed by the number of crossing squares noted as scores per time of 5 min for 20 min investigated. The apparatus (a cage) contains a platform divided into 16 equal squares (SÁENZ et al. 2006).

The anxiety was established by the white/dark box test. A test by which the anxious animal presents conflict between the tendency to explore and the initial tendency to avoid the unfamiliar (CRAWLEY and GOODWIN 1980).

The depressive state was measured by Porsolt test since some aspects of human depression matches with the behavioral immobility of rats during forced swimming (PORSOLT et al. 1977). The mice were observed for 5 min and the immobility time was recorded.

Lead brain concentration

After sacrifice, the brain of each mice was digested in 5 : 1 nitric acid: perchloric acid, as described by GUPTA and GILL (2000). The concentration of lead in the brain was determined by graphite furnace atomic absorption with Zeeman correction (Agilent 240 ZAA/GTA 120) based on the atomization (HOLCOMBE 2010). The operating parameters were: wavelength: 283.3 nm, the slit: 0.5 nm, lamp current: 10.0, standard solution: 1000 mg L^{-1}.

Histological study

After fixation of the brain in 10% formalin, brain has been subsequently dehydrated by a series of alcohol dilution. Dehydrated cerebral tissue was included in paraffin. Sections of 5 μm thicknesses were stained with hematoxylin and eosin (H&E).

Statistical study

All data were expressed as the mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by student *t*-test. *p* values less than 0.05 were considered as statistically significant.

Results

The methanolic extract of pomegranate peel showed the following concentrations: 183.67 ± 1.91 mg GAE g^{-1} of dry weight for total polyphenol, 53.59 ± 0.53 mg EQ g^{-1} of dw for total flavonoid, 143.81 ± 10.53 mg EAT g^{-1} of dw for total tannin and 113.18 ± 21.38 mg EC g^{-1} dw for condensed tannin (Table 1).

Table 1

Total content of polyphenols, flavonoids and total tannins and condensed tannins in methanolic extract of pomegranate peel

Total phenol	Total flavonoid	Total tannin	Condensed tannin
183.67 ± 1.91 mg GAE g^{-1} of dw	53.59 ± 0.53 mg EQ g^{-1} of dw	143.81± 10.53 mg EAT g^{-1} of dw	113.18 ± 21.38 mg EC g^{-1} of dw

Table 2 shows the body weight of the mice and water intake during 12 weeks of experimentation. Mice intoxicated with lead acetate showed a decrease in body weight compared to control mice ($P > 0.05$). The mice receiving the methanolic extract from the pomegranate peel have body weights comparable to those of the controls ($P > 0.05$).

Table 2

Effect of Lead acetate on water intake and body weight in mice after 12 weeks of experiment

Group	Body weight [g]			Water intake [ml]		
	C	Pb	Pb-E	*C*	Pb	Pb-E
Week 1	20.78 ± 1.31	18.21±1.36	21.78±0.91	212.77± 48.65	126.61±42.56*	186.57±31.7
Week 4	30.32 ± 2.74	27.78±0.52	30.42±1.36	–	–	–
Week 8	34.54 ± 2.87	32.6±1.58	35.33±3.07	–	–	–
Week 12	36.18 ± 3.09	32.17±0.42	36.78±2.46	–	–	–

C – control without any treatment (© Lavoisier: Phytothérapie, GADOUCHE et al. (2018). Pb – lead-exposed mice (500 mg kg^{-1}) and (Pb-E) intoxicated treated. Pb vs. control, Pb-E vs. control. * $P < 0.05$.

The locomotor activity of the mice was evaluated by the score which is the number of cells visited by the mice in 20 minutes divided into four phases. Exposure to lead caused significant locomotor hyperactivity in the lead group compared to the control group in the 4 phases. However, methanol extract from pomegranate peel significantly decreased locomotor activity in the third phase, whereas it increased significantly in the last two phases $P < 0.05$ (Figure 1*a*).

The mice that received the lead acetate for 90 days spend significantly more time in the black compartment as compared with the control and the treated group. The light compartment is more aversive and anxious for

intoxicated mice group by lead ($P < 0.05$). However, the treated poisoning group (pomegranate peel) behaves similarly to that of the control group, but not significantly ($P > 0.05$) except in the last phase the methanol extract significantly decreases the residence time in compartment caused by lead (Figure 1*b*).

The state of depression of the mice was evaluated by the duration of immobility time after swimming forced. Exposure to lead caused significant increase of the immobility time in the lead intoxicated group compared to the control group and the treated group ($P < 0.05$). Mice treated with methanol extract of pomegranate peel experienced intermediate immobility between controls and mice that received lead acetate $P > 0.05$ (Figure 1*c*).

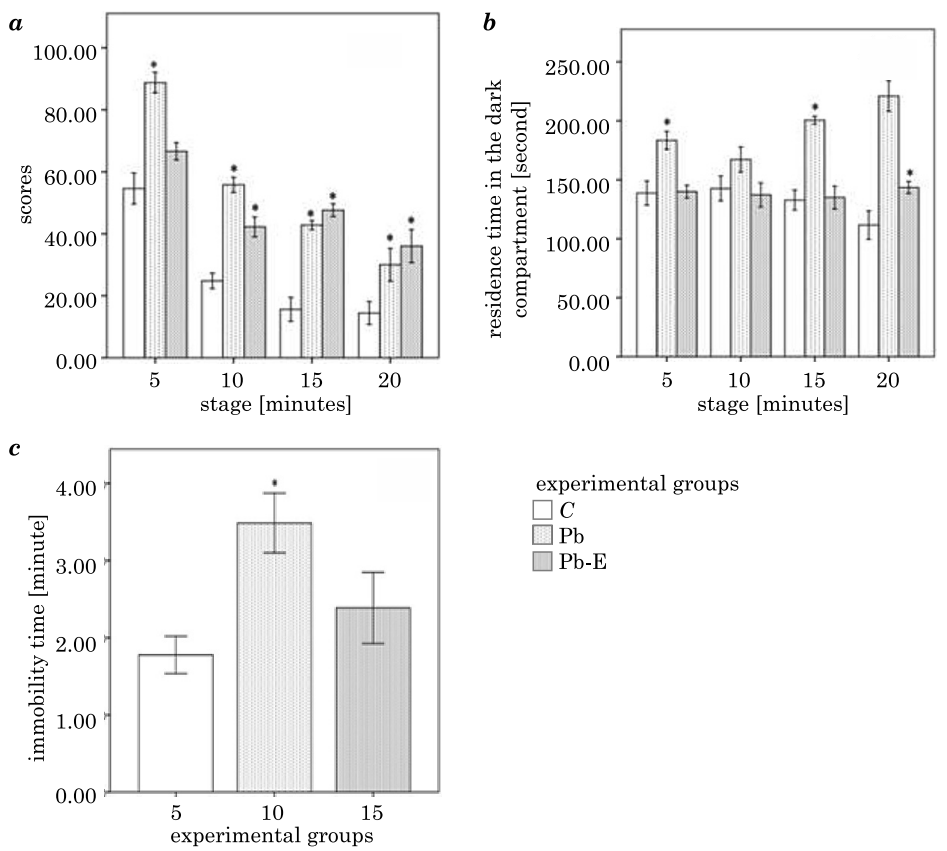

Fig. 1. Tests: *a* – locomotor activity test; *b* – black and white box test; *c* – swimming test. *C* – control group (© Lavoisier: Phytothérapie, GADOUCHE et al. 2018); Pb – mice intoxicated by lead acetate (1000 ppm); Pb-E – mice intoxicated treated with 500 mg kg^{-1} of methanol extract of *Punica granatum* peel. Values represent the means of 5 experiments ± SEM; * $P < 0.05$

The determination of the lead by atomic spectrophotometry is given in Figure 2. The results show a high lead level 7.87 ± 7.9 µg L in the brain, confirming its passage through the blood brain membrane and that the brain constitutes a site for the fixation of heavy metals. Treatment with methanol extract significantly reduced lead levels in the brain (1.07 ± 0.3 µg L^{-1}).

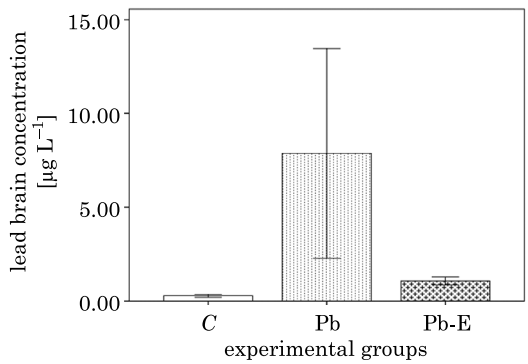

Fig. 2. Lead concentrations [µg L^{-1}] in brain of mice after 12 weeks

The cerebral cortex of the control mice showed normal cell structure. Within the histological sections of the brain of mice which has received lead acetate, we have observed microscopical lesions in many areas of the cerebral cortex and hippocampus, including neuronal degeneration, vacuolization, blood vessel congestion and inflammatory infiltrate with decrease in cell density in both cortex cerebral and hippocampus compared to the control group. Mice treated with the methanolic extract *Punica granatum* peel (500 mg kg^{-1}) showed less neurodegeneration and vacuolization than control and intoxicated mice.

Discussion

Due to its wide environmental distribution, lead alters several organ systems, including the nervous, renal, reproductive and hematological systems (AYKIN-BURNS and ERCAL 2006). Lead induced a significant decrease in body weight and water consumption in mice receiving lead acetate compared to the control group, which clearly shows that lead develop disorder of feed therefore this heavy metal is anorectic. These results are consistent with the work established by SEDDIKI et al. (2010) who observed reduced feed intake in rats with lead diluted acetate at 250 and 500 mg L^{-1} for 90 days.

MISSOUM et al. (2010) found that rats exposed to 1000 ppm of lead acetate had a slow body weight increase over control rats for 8 weeks and that water intake decreased in rats treated with lead during the experiment.

Lead increased the locomotor activity; this results agree with those obtained by SEDDIK et al. (2010), HASSAN and JASSMIN (2010) and KHAROUBI et al. (2011). Lead exposure may lead cognitive and motor impairment, with behavioral alterations as long-term (GARZA et al. 2006). These findings suggested that lead might interfere with catecholaminergic and particularly dopaminergic neurotransmission (DJEBLI et al. 2005).

In the light/dark choice assay, the duration of time spent in both compartments informs us about the degree of anxiety thus an increase in white compartment activity should reflect an anxiolytic effect, whereas a rise in dark compartment activity should reflect anxious behavior (MAXIMINO et al. 2010). In this context; lead intoxicated group spent more time in the dark compartment while mice intoxicated with lead and treated with the methanolic extract from the pomegranate peel have a longer residence time in the light compartment which implies an anxiolytic effect.

Our finding agrees with those obtained by KAHLOULA et al. (2013), who reported that lead increases significantly immobility time in the forced swimming test (FST). MANTOVANI et al. (1999) showed that in the forced swimming test, lead may exert their action repressing directly or indirectly of the *N*-methyl-D-aspartate receptor receptor complex. Within this test, antidepressant activity of drugs is related to reduction in immobility time (WOLAK et al. 2013). Pomegranate peel extract maybe improved the immobility time by modulation the NMDA receptor complex.

The quantification of lead by atomic spectrophotometer shows a high lead level in the brain which reveals that it crosses the blood-brain barrier and that the brain constitutes a fixation site of this metal. Lead damages the prefrontal cerebral cortex, the hippocampus and the cerebellum, which causes several neurological disorders following its ability to replace calcium ions which facilitate its passage through the blood-brain barrier (SANDERS et al. 2009).

STRUŻYŃSKA et al. (1997) reported that the functional state of the blood-brain barrier was altered in prolonged exposure of lead at the low doses. Indeed, these damages are typical for "leaky" microvessels confirmed by both light microscopy and by electron microscopic studies. Methanolic extract from pomegranate peel has decreased lead in the brain and can in part explain its protective effect. The chelating capacity of this metal is due to the flavonoids, tannins and phenolic compounds that potentiate the clearance of the lead of the body. The tannins quantified in the pomegra-

nate bark are polyphenols with excellent binding capacity with metals. PEKDEMIR et al. (2000) have shown that tannic acid under in vitro conditions is a very effective chelator for lead and cadmium.

Lead toxicity affects the normal histological structure of the brain and causes disturbances in the normal functions of the brain. According to BARKUR and BAIRY (2015) histological data indicate that lead exposure caused significant damage to neurons of hippocampus, amygdala and cerebellum regions.

Intake of pomegranate extracts protect brain by inhibition of cholinesterase, the stimulation of antioxidant capacity and decrease of oxidant stress markers; evidenced by the decrease of malondialdehyde and Protein carbonylation levels (AMRI et al. 2017). AHMED et al. (2014) showed that pre-administration of pomegranate extract to rats, can offer a neuroprotective activity against brain injury and DNA damage via decrease of inflammatory and oxidative stress markers, and ATP-replenishing effects. According, to these same authors, many researchers consider natural extracts as novel therapies for neurodegenerative disorders.

Conclusion

The results of the present report demonstrated that lead causes neurological, biochemical and histological alterations of the brain which are restored by daily supplementation of methanolic extract of *Punica granatum* peel. This extract showed anxiolytic and antidepressant activities as well as improved locomotor activity attributed to the high content of diversified bioactive molecules found in this extract polyphenols, flavonoids, and Tannins. These beneficial effects show that the grenade could have a neuroprotector effect.

Conflict of interest

The authors declare no conflict of interest.

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